Microenvironment and bacterial community structure in the gut of wood- and litter-feeding cockroaches

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Ich treibe ein Metier, das man, um es zu lieben, nur leidenschaftlich treiben kann.
(Alexander von Humboldt)

Summary

While the gut microbiota of termites and its role in symbiotic digestion have been studied for decades, little is known about the bacteria colonizing the intestinal tract of detritivorous cockroaches.

To improve the phylogenetic classification of short-read libraries, we first created a curated reference database of the bacterial 16S rRNA gene, based on the SILVA database and 1048 additional full-length 16S rRNA gene sequences from the intestinal tracts of 24 dictyopteran insects (chapter 2). The performance of the database in the classification of short-read libraries from termites and cockroaches was highly superior to that of the current SILVA and RDP databases.

We then investigated the bacterial gut communities in the crop, midgut and hindgut of two xylophagous (chapter 3) and three litter-feeding (chapter 4) cockroaches by Illumina sequencing, and compared them to those in omnivorous cockroaches and termites, focusing on two main questions: First, if host diet determines the gut microbiota in cockroaches, and second, what role environmental variables play in different gut compartments. We found that the gut microbiotas of cockroaches share rare lineages and the phenomenon of gut compartment-specific communities with those of termites, but differ in community structure and show only little diet-specific distinction. In order to identify other potential drivers of microbial community structure in cockroach guts, we determined the intestinal physicochemical parameters pH, redox potential, and oxygen and hydrogen partial pressure. Surprisingly, the localization of intestinal hydrogen accumulation in the crop of two cockroach species differed from that in the posterior midgut observed previously for omnivorous species. Intestinal pH, in addition to other, yet unidentified factors, was a strong determinant of bacterial community structure, posing a strong selection pressure particularly in the hindgut compartment.

For a better understanding of the digestion of lignocellulose by cockroaches in nature, I fed two cockroach species on oak leaf litter, and determined the degradation efficiency and metabolization rates of lignocellulosic fractions and carbohydrate monomers through controlled mass balances (chapter 5). I found that xylan rather than cellulose was degraded in the gut, suggesting that litter-feeding cockroaches preferentially degrade the easily solubilizable diet fractions like hemicelluloses.

Zusammenfassung

Während die Darmmikrobiota von Termiten und ihre Schlüsselrolle beim Abbau von Lignozellulose seit Jahrzehnten intensiv erforscht wurden, ist über die den Darm von detritivoren Schaben kolonisierenden Bakterien vergleichsweise wenig bekannt.

Zur verbesserten phylogenetischen Klassifizierung von Next-Generation-Sequencing(NGS)-Datensätzen erstellten wir zunächst eine kurierte Referenzdatenbank des 16S-rRNA-Gens, basierend auf der SILVA-Datenbank sowie 1048 zusätzlichen vollständigen 16S-rRNA-Gensequenzen aus dem Darmtrakt von 24 Insekten der Superordnung Dictyoptera (Kapitel 2). Die Klassifizierung von NGS-Bibliotheken wurde mithilfe der erweiterten Datenbank gegenüber den Standarddatenbanken SILVA und RDP stark verbessert.

Im Anschluss untersuchten wir die bakteriellen Gemeinschaften in den Darmkompartimenten Kropf, Mitteldarm und Kolon von zwei holzfressenden (Kapitel 3) und drei Detritus fressenden (Kapitel 4) Schabenarten mittels Illuminasequenzierung und verglichen sie mit denen in Termiten und omnivoren Schaben. Dies diente der Beantwortung folgender Fragen: Bestimmt die Ernährungsweise von Schaben die Zusammensetzung ihrer Darmmikrobiota? Welche Rolle spielen Umweltparameter in den Mikrohabitaten des Darms? Die Darmmikrobiotas der untersuchten Schaben teilten einige seltene bakterielle Taxa niedriger Abundanz mit denen der Termiten, unterschieden sich insgeamt aber deutlich von letzteren in ihrer Zusammensetzung. Anders als bei Termiten hatten die unterschiedlichen Ernährungsweisen der Schaben keine nennenswerte Auswirkung auf die Zusammensetzung ihrer Darmmikrobiota. Um andere potentiell entscheidende Faktoren für die Zusammensetzung der Darmmikrobiota von Schaben zu identifizieren, bestimmten wir die physikochemischen Parameter pH, Redoxpotential und Sauer- und Wasserstoffpartialdruck im Darm der fünf Spezies. Überraschenderweise akkumulierte Wasserstoff in zwei der Spezies nicht im hinteren Mitteldarm wie in omnivoren Schaben, sondern im Kropf. Der intestinale pH erwies sich insgesamt als der stärkste bestimmende Faktor der mikrobiellen Gemeinschaft im Kolon.

Für ein besseres Verständnis der Verdauung von Lignozellulose durch Schaben führte ich Fütterungsexperimente mit zwei Spezies auf trockener Eichenblattstreu durch (Kapitel 5). Hierbei überprüfte ich zunächst die einzelnen Lignozellulosefraktionen auf Zersetzung und bestimmte die Metabolisierungsraten der aufgeschlossenen Kohlenhydratmonomere mittels Massebilanzen zwischen aufgenommener Blattstreu und ausgeschiedener Fäzes. Die Zellulosefraktion der aufgenommenen Nahrung wurde nicht maßgeblich abgebaut. Stattdessen

war ein deutlicher Umsatz von vermutlich aus in der Blattstreu enthaltenen Xylans freigesetzter Xylose messbar. Meine Ergebnisse lassen darauf schließen, dass Blattstreu fressende Schaben statt Zellulose eher leichter verfügbare Bestandteile, unter anderem Hemizellulosen (z. B. Xylan), verdauen.

Table of contents

1 General introduction

Niclas Lampert

1.1 Cockroaches

1.1.1 General

Cockroaches constitute a group of terrestrial hemimetabolous insects that are ubiquitous in their distribution, with the exception of the polar regions, and are most abundant in the tropics (Roth and Willis, 1960; Gurney, 1969). They make up the basal lineages of the order Blattodea that also comprises their eusocial descendants, the termites (Inward, Beccaloni and Eggleton, 2007). The morphology of cockroaches is characterized by "an expanded, hard-edged pronotum, inflexed head, slick, flattened, rather light body, and moderately strong, spined legs" (Bell, Roth and Nalepa, 2007). Unlike early "roachoids", all modern cockroaches lack external ovipositors. Out of ca. 4600 recognized cockroach species worldwide (Beccaloni, 2014), only 30 are commonly associated with human habitations, four of which are well known as pests: *Blatta orientalis*, *Blattella germanica*, *Periplaneta americana*, and *Periplaneta australasiae*. These species can be seen as atypical of the order (Gullan and Cranston, 2014), since most cockroach species are not synanthropic.

1.1.2 Behavior

Cockroaches are mostly gregarious, with the exception of several subsocial lineages. The latter comprise all Cryptocercidae, the sister group of the eusocial termites, but also a few species in the Panesthiinae and Zetoborinae subfamilies of the family Blaberidae (Pellens, Grandcolas and Silva-Neto 2002). Several other cockroach species perform brood care, e.g., by providing leaf litter as food to their offspring (Slaytor, 1992), or by using antifungal properties of their feces to suppress fungal growth in the nest (Rosengaus *et al.*, 2013). Coprophagy is common, particularly among the first instars. Self-organized collective decision making, e.g., for choice of shelter and nutrient sources, has been demonstrated for *Blattella germanica* (Amé *et al.*, 2006) and *Periplaneta americana* (Canonge *et al.*, 2009), and likely applies to most cockroach species.

1.1.3 Impact on global carbon cycle

Cockroaches impact the global carbon and nutrient cycle in several ways. As detritivores, they play a crucial role in the decomposition of organic matter through shredding of particles (Mullins and Cochran, 1972; Anderson and Sedell, 1979; Bignell, 1981), thus multiplying the surface area accessible to microbes for degradation (Fenchel, 1970). Additionally, they

constitute prey for small invertebrate and vertebrate predators. Together with termites, diplopods and scarab beetle larvae, cockroaches are among the few arthropods that emit methane (Hackstein, Alen and Rosenberg, 1994). While termites alone are estimated to produce 3 % of global methane emissions (Kvenvolden and Rogers, 2005; Kirschke *et al.*, 2013), the contribution of cockroaches to the global methane budget has not yet been quantified.

1.2 The gut microbiota of cockroaches

An insect's gut microbiota may contribute to host biology in several fundamental ways, such as aiding in digestion efficiency, development, and protection against pathogens (Dillon and Dillon, 2004). Both termites and cockroaches harbor dense microbial communities in their guts (Leidy, 1881; Schultz and Breznak, 1978; Bracke, Cruden and Markovetz, 1979; Köhler *et al.*, 2012; Schauer, Thompson and Brune, 2012). In cockroaches, the gut microbiota comprises bacteria, archaeal, and eukaryotic microbes, in particular anaerobic ciliates (van Hoek *et al.*, 1998). Of all gut compartments, the colon is the one with the highest density of bacteria, ranging from 1.6×10^{10} to 10^{11} cells ml^{-1}, resulting in community sizes of 3.6×10^{8} cells in *Shelfordella lateralis* and 3.7×10^{8} cells in *Periplaneta americana* (Cruden and Markovetz, 1987; Cazemier *et al.*, 1997; Schauer, Thompson and Brune, 2012). In comparison, bacterial cell densities in crop and midgut are about one order of magnitude lower (1.0 to 5.9×10^{9} and 3.6 to 9.2×10^{9} cells ml^{-1} in *Shelfordella lateralis* and *Periplaneta americana*, respectively).

1.3 What do we know about the cockroach gut microbiota?

The most recent family of "eusocial cockroaches" within the order Blattodea are the termites (Inward, Beccaloni and Eggleton, 2007). Due to their unique role as ecosystem engineers (Jones, Lawton and Shachak, 1994; Bignell and Eggleton, 2000) but also as pests in agriculture (Rouland-Lefèvre, 2011) and construction (Su and Scheffrahn, 2000), a lot of research has focused on termites and their gut microbiota, especially from the perspective of lignocellulose degradation (Brune, 2014). However, considerably less effort has been put into the exploration of their more primitive relatives, the "true" cockroaches. Recently, a comparison of bacterial hindgut communities of hosts from all major lineages of the superorder Dictyoptera (comprising mantises, cockroaches, and termites) has revealed sharp contrasts between cockroaches and termites in terms of community structure, but also some shared bacterial lineages (Dietrich, Köhler and Brune, 2014). This has sparked further interest in microbial

lineages that are either of functional relevance within a specific gut system or putatively derived from a common ancestor of termites and cockroaches.

1.4 What makes the gut microbiota of cockroaches interesting?

There are strong arguments why the gut microbiota of cockroaches can serve as a model to understand the evolution of that of termites. Firstly, modern cockroaches consist of several insect families that evolutionarily basal to the termites. Secondly, all cockroaches are detritivores, with a tendency towards more specialization (some wood-dwelling Blaberidae; examples of cellulose digestion in Blattidae; wood-feeding strategy in Cryptocercidae), making them a good system to contrast against and compare to the high dietary specialization in higher termites. Lastly, several cockroach species can be raised gnotobiotically (germ-free), which to date is not possible with termites. Gnotobiotic specimen can reveal the impact of the gut microbiota on host development, nutritional benefits, and immune function. Additionally, mechanisms of gut community assembly can be investigated by assembling artificial gut communities. Interestingly, there are also parallels between the gut microbiota of cockroaches and that of humans. The gut microbiome of both cockroaches and humans consists mainly of *Bacteroidetes* and *Firmicutes* (Turnbaugh *et al.*, 2009; Schauer, Thompson and Brune, 2012; The Human Microbiome Project Consortium, 2012).

1.5 Diet

1.5.1 Dietary strategies

Cockroaches comprise mostly omnivorous and detritivorous species. The synanthropic species are typically considered omnivores, probably because human-built structures are likely to be associated with more diverse food sources than natural habitats. Nonetheless, some omnivorous pest species (e.g., *Periplaneta americana*) can degrade more recalcitrant substrates like cellulose and hemicellulose with little to moderate efficiency (Bignell, 1977a). Surprisingly, there is little evidence for cellulose degradation in detritivores and foliage-feeders, although many cockroach species inhabit rotting plant material (Roth and Willis, 1960; Nalepa and Bandi, 2000), including wood (Bell, Roth and Nalepa 2007, table 3.2). Species like *Pycnoscelus surinamensis* are thought to feed mostly on dead organic matter, but have also been witnessed to gnaw off the bark, young buds, and shoots of rose plants (Doucette and

Smith, 1926), and the roots of pineapples (Illingworth, 1929), yet there is no evidence of cellulose degradation in this species.

However, there are trends towards dietary specialization on lignocellulose from plant material in several lineages of cockroaches. A common ancestor of termites and cryptocercid cockroaches adopted, along with hypermastigid and oxymonadid flagellates (Cleveland, 1924), a diet of cellulose from sound wood (Martin, Jones and Bernays, 1991), with further dietary diversification in the higher termites. Independently, *Parasphaeria boleiriana* (family *Blaberidae*, subfamily *Zetoborinae*), evolved to dwell in and feed on rotting logs (Pellens, Grandcolas and da Silva-Neto, 2002). All known species of the genera *Panesthia* and *Salganea* (family *Blaberidae*, subfamily *Panesthiinae*) are found to dwell in and feed on rotting logs. In addition to the abundant observations on natural associations and feeding behavior, laboratory experiments have shown that *Panesthia cribrata* can survive for several months on a diet of both crystalline cellulose and starch (Scrivener, Slaytor and Rose, 1989).

1.5.2 Gut structure and physiology

As typical for insects, the intestinal tract of cockroaches has three primary regions: stomatodeum (foregut), mesenteron (midgut), and proctodeum (hindgut) (Gullan and Cranston, 2005), which themselves are slightly compartmentalized (Figure 1.1). The foregut, comprising close to 50 % of the total gut volume (Bignell, 1977b) includes the buccal cavity, pharynx, esophagus, and the crop, a dilated compartment that acts as a temporary food storage and allows for a more even flow through and shredding of ingested material by the teeth of the proventriculum (gizzard) into the tubular midgut, where solubilized sugars and amino acids are resorbed by the endothelium. The Malpighian tubules serve as excretory organs that transport waste products from the body to the posterior midgut and anterior hindgut. In the hindgut, water, salts, fatty acids and amino acids are absorbed (Bracke, Cruden and Markovetz, 1979; Zurek and Keddie, 1996). It consists of the dilated colon, a dilated paunch with the highest density of microorganisms along the gut axis (Schauer, Thompson and Brune, 2012; Bauer *et al.*, 2015), and the rectum.

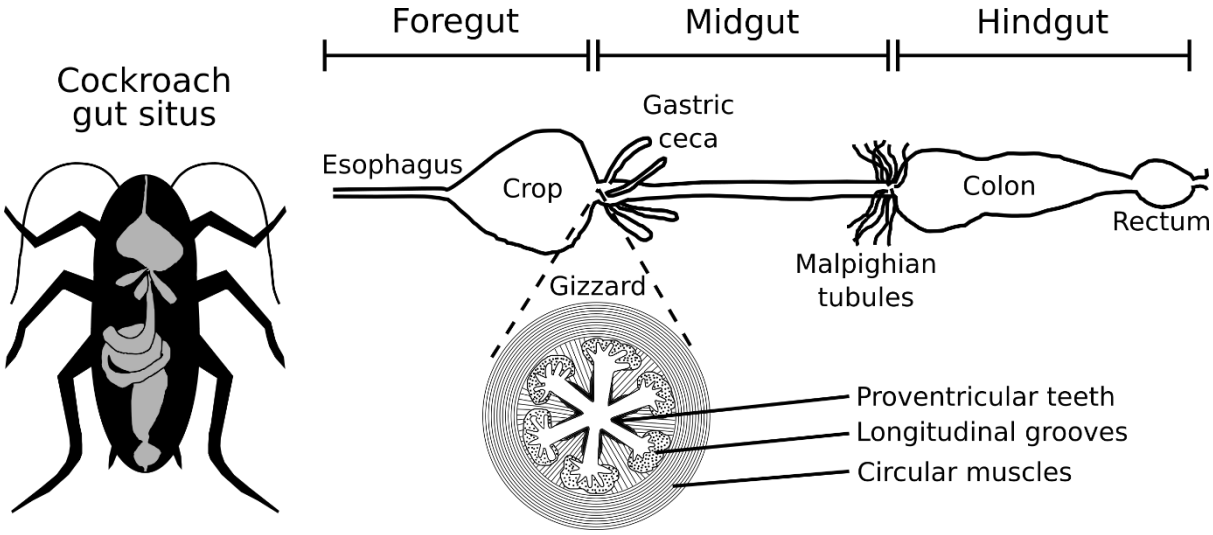

Figure 1.1| Intestinal tract of a cockroach in its natural orientation (left), stretched out longitudinally (top right), and cross-section of the gizzard (lower right). The gizzard's proventricular teeth, whose motion is controlled by the longitudinal grooves and circular muscles, shred food particles before they enter the midgut.

The different gut compartments each present distinct combinations of environmental factors, e.g., physicochemical properties like pH, oxygen, or hydrogen partial pressure, and different concentrations of various metabolites. All of these factors define specific niches for microbes in the respective habitat. Intestinal pH is typically slightly acidic (5–6) in the crop, increases slightly along the gut, and reaches approximately neutral levels (6–8) in the hindgut (Greenberg, Kowalski and Karpus, 1970; Cruden and Markovetz, 1984; Schauer, Thompson and Brune, 2012). Acidity of the foregut has been connected to putative fermentation of ingested sugars (Wigglesworth, 1927). In the hindgut, in contrast, excretory fluid of the Malpighian tubules containing nitrogenous organic compounds is expected to provide buffering capacity (Mullins and Cochran, 1973). Anoxia prevails at the gut center of the enlarged compartments in all adult cockroach specimen analyzed to date. Oxygen is likely consumed during gut microbial respiration, as well as through the presence of glutathione in the Malpighian tubules (Metcalf, 1943). Intestinal redox potential typically decreases along the gut, from oxidizing conditions in crop and midgut (Bignell, 1984; Vinokurov *et al.*, 2007) to reducing conditions of −250 mV to 150 mV in the hindgut (Warhurst, 1964; Bignell, 1981; Schauer, Thompson and Brune, 2012). Hydrogen has been shown to accumulate in the posterior midgut of two species (Lemke *et al.*, 2001; Schauer, Thompson and Brune, 2012), and the close intertwining of midgut and hindgut compartments *in situ* suggests exchange of molecules between these two compartments. Indeed, cross-epithelial hydrogen transport from the midgut to the hindgut has been demonstrated to drive methanogenesis in the hindgut of *Blaberus* sp. (Lemke *et al.*, 2001).

1.5.3 Enzymatic arsenal of cockroaches

In adaptation to their generalist feeding strategy, cockroaches possess a diverse array of digestive enzymes. A recent study demonstrated activity of proteinases and amylase in six cockroach species in three families (Vinokurov *et al.*, 2007). Here, proteinase activity always increased from crop to posterior midgut, and was highest in the blattid species, while amylase activity showed no consistent pattern from crop to midgut. Proteinase activity in the midgut of *Rhyparobia maderae* is proportional to the amount of specific proteins, such as casein, fibrin, elastin, and gluterin, passing from the crop (Engelmann, 1969). Therefore, synthesis and activity of digestive enzymes is most likely regulated through direct chemical stimulation rather than endocrine or mechanical processes (Bignell, 1981). Significant lipolytic activity has been detected in the foregut (Eisner, 1955; Bollade, Paris and Moulins, 1970), however, since no lipase occurs in salivary glands or foregut wall, it may be derived from the midgut by regurgitation (Bignell, 1981). The chitinase activity found throughout the gut (Waterhouse, Hackman and McKellar, 1961) cannot be fully explained by the moulting fluid in the cuticle-secreting epithelia of foregut and colon, and must serve digestion of possibly consumed cast skin or cannibalism (Bignell, 1981), or fungal biomass ingested especially by detritivores.

Carbohydrate-digesting enzymes present in saliva and/or midgut include amylase, invertase, and maltase (Wigglesworth, 1927; Day and Powning, 1949). At least one family of glycosyl hydrolases (GHF9), derived from a common ancestor, is present in species from at least eleven classes of Metazoa, including termites and cockroaches (Davison and Blaxter, 2005). All Blattodea examined to date possess endo-β-1,4-glucanases (EC 3.2.1.4) (Wharton and Wharton, 1965). Endogenous endoglucanases and β-glucosidases (EC 3.2.1.21) in the salivary glands and midgut of cockroaches (Martin, 1983; Slaytor, 1992; Scrivener and Slaytor, 1994) are generally not considered as a complete cellulolytic system due to the lack of an exo-β-1,4-glucanase (EC 3.2.1.91) (Watanabe and Tokuda, 2010). Given the high numbers of bacteria in the hindgut, the colon has been considered the most likely site of plant polysaccharide degradation and fermentation (Bignell, 1977a), which is supported by the considerable amount of short-chain fatty acids accumulating in this compartment (Schauer, Thompson and Brune, 2012).

1.5.4 Putative microbial contribution to plant polymer degradation

It has been speculated that intestinal microbes contribute to plant polymer degradation in cockroaches, but to date, there is no proof of this hypothesis. The dual cellulolytic system of

termites (Ni and Tokuda, 2013), in which the host degrades cellulose with the assistance of flagellates (wood-feeding lower termites), or bacteria (Scrivener, Slaytor and Rose, 1989), implies that at least part of the cellulolytic potential stems from the host. However, cellulase activity in the hindgut of *Periplaneta americana* correlates with the number of *Nyctotherus ovalis* (Gijzen *et al.*, 1994), suggesting that these ciliates, whose archaeal endosymbionts produce methane (Gijzen *et al.*, 1991; van Hoek *et al.*, 2000), contribute to cellulose degradation. High cellulase activity in the feces of adult cockroaches (Wharton, Wharton and Lola, 1965) and the overall low efficiency of cellulose digestion over the gut passage (Bignell, 1981) suggest that cellulose degradation continues after feces deposition.

1.6 What shapes the microbial community in the gut of cockroaches?

Host diet has a major impact on the gut microbiota in mammals (Turnbaugh *et al.*, 2009; De Filippo *et al.*, 2010). Bacterial hindgut communities of higher termites from different subfamilies display dissimilarity primarily by host diet, and those in xylophagous cockroaches are somewhat distinct from those of omnivorous species (Dietrich, Köhler and Brune, 2014; Mikaelyan, Dietrich, *et al.*, 2015). While there is evidence for an effect of diet on the hindgut microbiota based on 16S rRNA gene clone libraries from *Periplaneta americana* under different dietary regimen (Bertino-Grimaldi *et al.*, 2013), effects of diet on microbial community structure were masked by individual variation in *Shelfordella lateralis* (Schauer, Thompson and Brune, 2014).

It has been shown in hominids and ants that host phylogeny may correlate with the composition of the gut microbiota (Sanders *et al.*, 2014). Earlier studies on the termite hindgut microbiota diversity have already established that it reflects host phylogeny to some extent (Hongoh *et al.*, 2005; Noda *et al.*, 2009), and recent high-throughput sequencing has revealed that in cockroaches and termites, it reflects major evolutionary events (Dietrich, Köhler and Brune, 2014). However, high inter-species similarity and large individual variation of hindgut bacterial community structure within cockroaches (Schauer, Thompson and Brune, 2014) suggests that the impact of host phylogeny on the gut microbiota is less important in cockroaches.

Combinations of environmental factors, like pH, temperature, concentrations of various nutrients and metabolites, or physical and structural properties, provide specific niches that shape the microbial community in any habitat. Gut microbial communities may face

considerably different micro-environmental conditions – both in different host species and in different intestinal compartments of the same host (Engel *et al.*, 2013; Brune, 2014). Even within one gut compartment, gradients of oxygen and hydrogen partial pressure (Köhler *et al.*, 2012), the availability of colonisable surface area of flagellates (Stingl *et al.*, 2004) or free wood fibers (Mikaelyan *et al.*, 2014) vs. luminal fluid or the gut wall, provide very different microhabitats in close proximity. The wood fibers in the gut lumen of xylophagous higher termites presents such a particular microhabitat densely colonized by *Fibrobacteres*, a bacterial lineage that was recently also detected in the hindgut of cockroaches (Dietrich, Köhler and Brune, 2014; Schauer, Thompson and Brune, 2014). The phylogenetic relation of *Fibrobacteres* in cockroaches relative to those in termites, as well as which microhabitat they colonize in the cockroach gut, e.g., the surface of ingested leaf litter fibers, remains unknown. It remains unclear in what way the factors mentioned above – host diet, phylogeny, or habitat structure and microenvironmental conditions – determine assembly and structure of microbial gut communities in cockroaches. Future studies need to be designed in a way that allows to investigate these factors in isolation.

1.7 Objectives of this work

The first goal of this thesis was to improve an existing reference database for the classification of short reads of the 16S rRNA gene from cockroach and termite gut intestinal microbiota. This was achieved by generating Sanger libraries of the full-length bacterial 16S rRNA gene from DNA extracted from a selection of cockroach and termite species, creating subtrees and provisionary names for new lineages, and integrating them into the phylogenetic framework of the database. The updated phylogenetic taxonomy was then used to investigate the bacterial diversity in the intestinal microbiota of cockroaches and termites by next generation sequencing.

Secondly, axial profiles of physicochemical conditions in the gut of one xylophagous and three detritivorous cockroach species were determined using microsensors. Furthermore, the bacterial communities in different gut compartments of two xylophagous and three detritivorous cockroach species were investigated via next generation sequencing, and the potential relationships between host diet, gut physicochemical conditions and bacterial community structure were evaluated using different dimension reduction methods, hierarchical clustering, and correspondence analysis methods.

Lastly, the nature of putative lignocellulose degradation by detritivorous cockroaches was addressed by investigating which lignocellulosic components were depleted in the feces during feeding experiments on leaf litter, using the cockroach species *Byrsotria fumigata* and *Ergaula capucina* as model organisms. Mass balances of ingested substrate and excreted feces were combined with basic elemental analysis, separation of acid-detergent fiber and lignin fractions, and quantification via HPLC of carbohydrate monomers released from polymers by complete hydrolysis. Absolute consumption and turnover rates of cellulose, lignin, and soluble fraction were calculated, and major dietary targets were identified.

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2 Classifying the bacterial gut microbiota of termites and cockroaches: A curated phylogenetic reference database (DictDb)

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AM conceived the study, performed the analysis, and wrote the paper. TK conceived the study, and performed experiments and part of the analysis. **NL performed experiments and contributed to the manuscript.** JR, HB, and KM performed experiments. AB conceived the study and secured funding.

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2.1 Abstract

Recent developments in sequencing technology have given rise to a large number of studies that assess bacterial diversity and community structure in termite and cockroach guts based on large amplicon libraries of 16S rRNA genes. Although these studies have revealed important ecological and evolutionary patterns in the gut microbiota, classification of the short sequence reads is limited by the taxonomic depth and resolution of the reference databases used in the respective studies. Here, we present a curated reference database for accurate taxonomic analysis of the bacterial gut microbiota of dictyopteran insects. The Dictyopteran gut microbiota reference Database (DictDb) is based on the Silva database but was significantly expanded by the addition of clones from 11 mostly unexplored termite and cockroach groups, which increased the inventory of bacterial sequences from dictyopteran guts by 26%. The taxonomic depth and resolution of DictDb was significantly improved by a general revision of the taxonomic guide tree for all important lineages, including a detailed phylogenetic analysis of the *Treponema* and *Alistipes* complexes, the *Fibrobacteres*, and the *TG3* phylum. The performance of this first documented version of DictDb (v. 3.0) using the revised taxonomic guide tree in the classification of short-read libraries obtained from termites and cockroaches was highly superior to that of the current Silva and RDP databases. DictDb uses an informative nomenclature that is consistent with the literature also for clades of uncultured bacteria and provides an invaluable tool for anyone exploring the gut community structure of termites and cockroaches.

2.2 Introduction

Termites and their closest phylogenetic relatives, the cock-roaches, represent the majority of species in the insect order Dictyoptera (Bell, Roth and Nalepa, 2007; Engel, Grimaldi and Krishna, 2009) and are ideal models for studying factors that shape microbial community structure in intestinal ecosystems (Brune and Dietrich, 2015). During more than 200 million years of evolution, they have diversified to efficiently utilize a wide range of diets and now comprise numerous omnivorous, detritivorous, xylophagous, and humivorous lineages (Eggleton and Tayasu, 2001). Previous studies have identified both dietary and phylogenetic patterns in the intestinal community structure of termites and cockroaches (Dietrich, Köhler and Brune, 2014). However, understanding the evolution of symbiotic digestion in dictyopteran insects requires a highly resolved analysis of their gut microbiota.

Most studies of bacterial diversity in the guts of termites and cockroaches have employed traditional capillary dideoxy (Sanger) sequencing of cloned 16S rRNA gene amplicons. They provided a wealth of information on the diversity of the gut microbiota and identified numerous novel lineages that are specific for this habitat (e.g., *Elusimicrobia* (Herlemann, Geissinger and Brune, 2007), *Fibrobacteres* subphylum 2 (Hongoh, Deevong, *et al.*, 2006), termite gut spirochetes (Lilburn, Schmidt and Breznak, 1999; Ohkuma, Iida and Kudo, 1999), and Termite Group 3 (Hongoh, Deevong, *et al.*, 2006)). However, cost and effort involved in this approach limit the number of host taxa that can be included in an analysis and the depth to which each community can be sampled.

The development of next-generation sequencing technologies allowed efficient and economical sequencing of multiple 16S rRNA gene libraries with sufficient sampling depth to compare the bacterial communities across a wide host range (Sogin *et al.*, 2006; Degnan and Ochman, 2012). However, the relatively short length of the sequence reads generated by the most commonly employed Roche 454 and Illumina/Solexa platforms (Van_Dijk *et al.*, 2014) limits the amount of information available for phylogenetic analysis. Therefore, it has become common practice to infer the structure and taxonomic composition of microbial communities by assigning the reads using a pre-defined classification scheme and the Naïve Bayesian Classifier (Wang *et al.*, 2007) developed by the Ribosomal Database Project (RDP), which has been implemented in popular workbenches for community analysis (Schloss *et al.*, 2009; Caporaso *et al.*, 2010). Obviously, the quality of such a classification depends strongly on the composition of the reference database and the depth and resolution of its taxonomic framework. The reference taxonomies most commonly used for the classification of short reads are provided by the Silva (Yilmaz *et al.*, 2014) and RDP (Cole *et al.*, 2014) databases, which extend the taxonomic outline for cultured organisms (Garrity, Bell and Lilburn, 2004) by including also phylogenetically coherent groups without cultured representatives.

However, general-purpose reference databases have serious shortcomings when it comes to studying microbial diversity in insect guts (Newton and Roeselers, 2012), particularly in termites and cockroaches (Köhler *et al.*, 2012; Werner *et al.*, 2012). One shortcoming is the frequent lack of taxonomic depth in the classification schemes, i.e., the absence of circumscribed taxa particularly at lower taxonomic levels. This is symptomatic for bacterial lineages that are endemic to termites and only rarely encountered in other environments, such as Fibrobacteres or the TG3 phylum (Hongoh, Deevong, *et al.*, 2006; Dietrich, Köhler and Brune, 2014). Another problem is a lack of taxonomic resolution in many genus-level

complexes, which comprise highly divergent 16S rRNA gene sequences that are lumped into inflated taxa (e.g., *Treponema* (Lilburn, Schmidt and Breznak, 1999; Breznak, 2002)) that may even be polyphyletic (e.g., *Ruminococcus* (Ezaki, Li and Kawamura, 2006)). Finally, a lack of representative bacterial phylotypes from insect guts in general-purpose reference databases seriously affects the taxonomic assignment of short reads using the RDP classifier (Newton and Roeselers, 2012; Werner *et al.*, 2012).

To overcome the challenges, we constructed a customized rRNA reference database for an accurate taxonomic analysis of the gut microbiota of termites and cockroaches. The Dictyopteran gut microbiota reference Database (DictDb) is based on the skeleton structure of the Silva database (Yilmaz *et al.*, 2014) and on the collation of published rRNA sequences obtained from termites and cockroaches and rigorous phylogenetic curation of the existing taxonomic framework. Initial, so far undocumented versions of DictDb were successfully used to improve the analysis of bacterial communities in termite guts (version 1.0; (Köhler *et al.*, 2012; Reid *et al.*, 2014)) and subsequently both in termites and cockroaches (versions 2.3 and 2.4; (Thompson *et al.*, 2012; Huang *et al.*, 2013; Dietrich, Köhler and Brune, 2014; Mikaelyan *et al.*, 2014; Otani *et al.*, 2014; Schauer, Thompson and Brune, 2014)).

Here, we document for the first time the general architecture of DictDb and present the latest version (DictDb v. 3.0). This substantially expanded version includes more than 1000 novel phylotypes that were obtained from 11 host species in the context of this study. They represent severely under-sampled host groups among cockroaches (Blaberidae, Polyphagidae), lower termites (Mastotermitidae, Kalotermitidae), and higher termites (Termitidae), including representatives with fundamentally different diets. An improved taxonomic framework based on thorough phylogenetic analyses provided an unprecedented depth and resolution in termite-specific taxa, particularly among *Fibrobacteres* and candidate phylum *TG3*, and hitherto unresolved taxonomic complexes, such as the genera *Treponema* and *Alistipes*. The performance of the taxonomic framework of DictDb in the genus-level classification of deep-sequenced rRNA gene libraries of bacterial communities in termites and cockroaches is compared to that of the SILVA and RDP reference databases.

2.3 Materials and methods

2.3.1 Sample preparation

Termites used in this study were taken from laboratory colonies or were collected in the field. Only worker termites or pseudergates were used for this study. Cockroaches were purchased from a commercial breeder and maintained on leaf litter for several months. Only female cockroaches were used. The origin and other details of the samples are summarized in Table 2.1.

The guts of termites (10–20 individuals) and cockroaches (3 individuals) were dissected with sterilized fine-tipped forceps. Pools of guts, hindguts, or hindgut compartments (see Table 2.1 for sample details) were suspended in 750 µl sodium phosphate buffer (120 mM; pH 8.0) in 2-ml tubes and homogenized. DNA was extracted and purified using a bead-beating protocol as previously described (Paul *et al.*, 2012).

2.3.2 Clone libraries

16S rRNA genes were amplified using the universal bacterial primers 27f and 1492r (Lane *et al.*, 1985). PCR products were purified and cloned as described by Thompson et al. (Thompson *et al.*, 2012). Clones were tested for correct insert size, and inserts were sequenced bidirectionally with M13 vector primers using automated Sanger sequencing (GATC Biotech, Konstanz, Germany). In the case of *Cubitermes ugandensis* and *Ophiotermes* sp., the clone libraries were pre-screened by partial sequencing, and only novel phylotypes (<98% sequence identity to previously published full-length sequences) were sequenced in both directions. Partial sequences from the same clones were assembled using *Seqman* (version 4.05; DNA Star, Madison, WI, USA). Chimeric sequences were identified using the *mothur* (Schloss *et al.*, 2009) implementation of UCHIME (Edgar *et al.*, 2011) and confirmed by fractional treeing (Ludwig *et al.*, 1997).

2.3.3 Construction of the reference database

Quality-checked sequences from the new clone libraries were aligned using the *mothur* aligner against the Silva reference alignment available on the *mothur* website (http://www.mothur.org/wiki/Silva_reference_alignment/) and imported into the Silva database (release 119; http://www.arb-silva.de/documentation/release-119/) using the ARB

software package (Ludwig *et al.*, 2004). When necessary, alignments were manually refined using the ARB alignment editor.

Table 2.1 | Details of the nature and origin of cockroach and termite species used for the construction of clone libraries of bacterial 16S rRNA genes in this and selected previous studies. Host species are sorted by (sub)family; library numbers are the same as in the figures. Libraries were prepared using DNA extracted from entire guts, hindguts, or specific proctodeal compartments (P1–4).

No.	Host species	Clones	Diet	Source
Cockroaches				
Polyphagidae				
1	*Ergaula capucina*	25	Dead leaves	Hindgut[a,h]
Blaberidae				
2	*Byrsotria fumigata*	121	Dead leaves	Hindgut[a,h]
3	*Pycnoscelus surinamensis*	102	Dead leaves, fruit	Hindgut[a,h]
4	*Panesthia angustipennis*	125	Wood	Hindgut [2]
Blattidae				
5	*Shelfordella lateralis*	132	Chicken feed	Hindgut [51]
Lower termites				
Mastotermitidae				
6	*Mastotermes darwiniensis*	31	Wood	Whole gut[b,h]
Hodotermitidae				
7	*Hodotermes mossambicus*	70	Grass	Whole gut[c,h]
Kalotermitidae				
8	*Kalotermes flavicollis*	58	Wood	Whole gut[b,h]
Rhinotermitidae				
9	*Coptotermes formosanus*	213	Wood	Whole gut [31]
10	*Reticulitermes chinensis*	113	Wood	Whole gut[g]
11	*Reticulitermes speratus*	214	Wood	Whole gut [28]
Higher termites (*Termitidae*)				
Macrotermitinae				
12	*Macrotermes gilvus*	72	Wood/Fungus	Whole gut [27]
13	*Macrotermes michaelseni*	77	Wood/Fungus	Whole gut[f,h]
14	*Microtermes* sp.	92	Wood/Fungus	Whole gut [39]
15	*Odontotermes formosanus*	56	Wood/Fungus	Whole gut [54]
16	*Odontotermes somaliensis*	93	Wood/Fungus	Whole gut [39]
Termitinae				
17	*Cubitermes ugandensis*	92	Humus	Whole gut[d,h]
18	*Ophiotermes* sp.	70	Humus	Whole gut[e,h]
19	*Amitermes* sp.	96	Decayed wood	P1, P3, P4[f]
20	*Microcerotermes* sp. 1	166	Wood	Whole gut [26]
21	*Microcerotermes* sp. 2	143	Wood	Whole gut [26]
Nasutitermitinae				
22	*Nasutitermes takasagoensis*	51	Wood	Whole gut [26]
23	*Nasutitermes corniger*	1703	Wood	P3 [61]
24	*Trinervitermes* sp.	157	Grass	P3[f]

[a] Commercial breeder (J. Bernhardt, Helbigsdorf, Germany; http://www.schaben-spinnen.de).
[b] Laboratory colony (Federal Institute for Materials Research and Testing, Berlin, Germany).
[c] Laboratory colony [41].
[d] Laboratory colony [11].
[e] Laboratory colony [43].
[f] Collected from Kakamega Forest, Kenya (by J. Nonoh).
[g] Sequences submitted to GenBank (JQ864374–JQ864487; Chen, W., Li, X., Wang, B., Yang, H. and Liu, S.-J.).
[h] Clones from the current study.

Because of the inconsistent and varied usage of the fields “isolation source” and “host” in the sequence-associated information in the INSDC databases (EMBL, DDBJ, and GenBank), we introduced the fields “DictDb source” and “DictDb specific host” in DictDb v.3.0. “DictDb specific host” indicates the insect host from which a given 16S rRNA sequence was derived. “DictDb source” clarifies the preparation from which it was derived (e.g., a pool of flagellates, a particular gut compartment, or a capillary-picked bacterial filament). Additionally, we introduced a field “DictDb diet” to describe the diet of the insect host from which the rRNA sequence was obtained.

The taxonomic framework of DictDb is based on the phylogenetic taxonomy described by the guide tree in the Silva database. All bacterial clades in the Silva database that contained a substantial fraction of sequences derived from the guts of termites and cockroaches were

phylogenetically analyzed to redefine or further resolve the node-based taxonomy. Conservative column filters were applied to the alignments to eliminate highly variable positions in the alignment. Filtered alignments comprising approximately 1200 valid columns were exported for tree calculations using the maximum-likelihood (ML) method as implemented in PhyML (version 3.0.1; [23]) and a general time-reversible (GTR) model of sequence evolution. ML trees were inferred by subtree pruning and regrafting (SPR) of five random starting trees, and node support was estimated using the Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT) (Anisimova *et al.*, 2011).

The topologies, branch lengths, and node supports from the calculated maximum-likelihood trees were grafted onto the main guide tree. The hierarchy of well-supported nested clusters obtained in the analyses was then used to enhance the taxonomic skeleton of the Silva database. This phylogenetic framework was used to define a ranked taxonomy for each sequence in the database, which was stored as a semicolon-separated taxonomic string in the field "DictDb 3 tax". The expanded guide tree describing the phylogenetic taxonomy of DictDb and all sequence-associated information are included in the ARB database of DictDb v. 3.0 included in the supplementary information (File S1).

Redundancy in the 16S rRNA gene sequences was reduced using UCLUST (Edgar, 2010) with a similarity threshold of 98%; only sequences representing the centroid of each cluster were retained. The dereplicated subset of sequences in the ARB database (around 55,000 sequences) was used to generate the two files required by the RDP classifier implemented in *mothur*: a fasta file with the sequence information, and a tab-delimited file with the taxonomic assignments for each sequence. The files are available in the supplementary information (File S2).

For selected clades, we scrutinized tree topology by additional maximum-parsimony (MP) analysis using the DNAPARS program [18] implemented in ARB. For ML trees, additional tests of node support included both parametric (aBAYES and χ^2) and non-parametric [Felsenstein bootstrap (1000 replicates)] measures. Consensus trees were constructed to summarize the results obtained with both treeing methods. Multifurcations were introduced manually into nodes that were not observed in both MP and ML analyses.

2.3.4 Classification of short-read data

The performance of DictDb in the classification on short-read libraries was compared to that of both the original Silva database (release 119) and the commonly used 16S rRNA gene database from RDP (training set 9). Test datasets were three amplicon libraries obtained by pyrosequencing of the bacterial gut microbiota of a cockroach (*Shelfordella lateralis* (Schauer, Thompson and Brune, 2014)), a lower termite (*Reticulitermes santonensis* (Dietrich, Köhler and Brune, 2014)), and a higher termite (*Nasutitermes corniger* (Dietrich, Köhler and Brune, 2014)). The test datasets were quality filtered as described in Dietrich et al. (Dietrich, Köhler and Brune, 2014) and classified using the RDP Naïve Bayesian Classifier (Wang *et al.*, 2007) implemented in the *mothur* software package (Schloss *et al.*, 2009) with a confidence threshold of 80%. The taxonomic overlap among the three test datasets was visualized using *BioVenn* (Hulsen, de Vlieg and Alkema, 2008).

2.4 Results and discussion

2.4.1 New clone libraries

We constructed clone libraries of bacterial 16S rRNA genes from the hindguts of 11 hitherto unstudied termite and cockroach species (Table 2.1). A total of 1048 clones were sequenced. After elimination of 42 chimeric sequences, the remaining sequences were incorporated into the alignment of the Silva database, which increased the total complement of 16S rRNA sequences from termites and cockroaches to 4869 (Fig. S1). As in previous studies of dictyopteran gut microbiota, the majority of the clones in the libraries belonged to the *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, and *Proteobacteria*. A more resolved taxonomic break-up at the genus level revealed distinct patterns (Fig. 1) that matched differences in community structure previously observed among the major host groups (Dietrich, Köhler and Brune, 2014). The exact taxonomic composition of the clone libraries can be found in an interactive spreadsheet in the supplementary material (Tables S1 and S2).

2.4.2 The general architecture and taxonomic framework of DictDb

In order to define the taxonomic framework of DictDb, we calculated phylogenetic trees for all bacterial clades typically encountered in the guts of termites and cockroaches. Additionally, we incorporated phylogenetic frameworks for several bacterial lineages provided in previous studies from our lab (Herlemann, Geissinger and Brune, 2007; Strassert *et al.*, 2012; Thompson

et al., 2012; Bauer *et al.*, 2015). These subtrees generated in these analyses were incorporated into the original guide tree provided with the Silva database, and formed the basis of the phylogenetic taxonomy of DictDb. The entire database and the flat files required by the RDP classifier are provided in the supplementary material (Files S1 and S2).

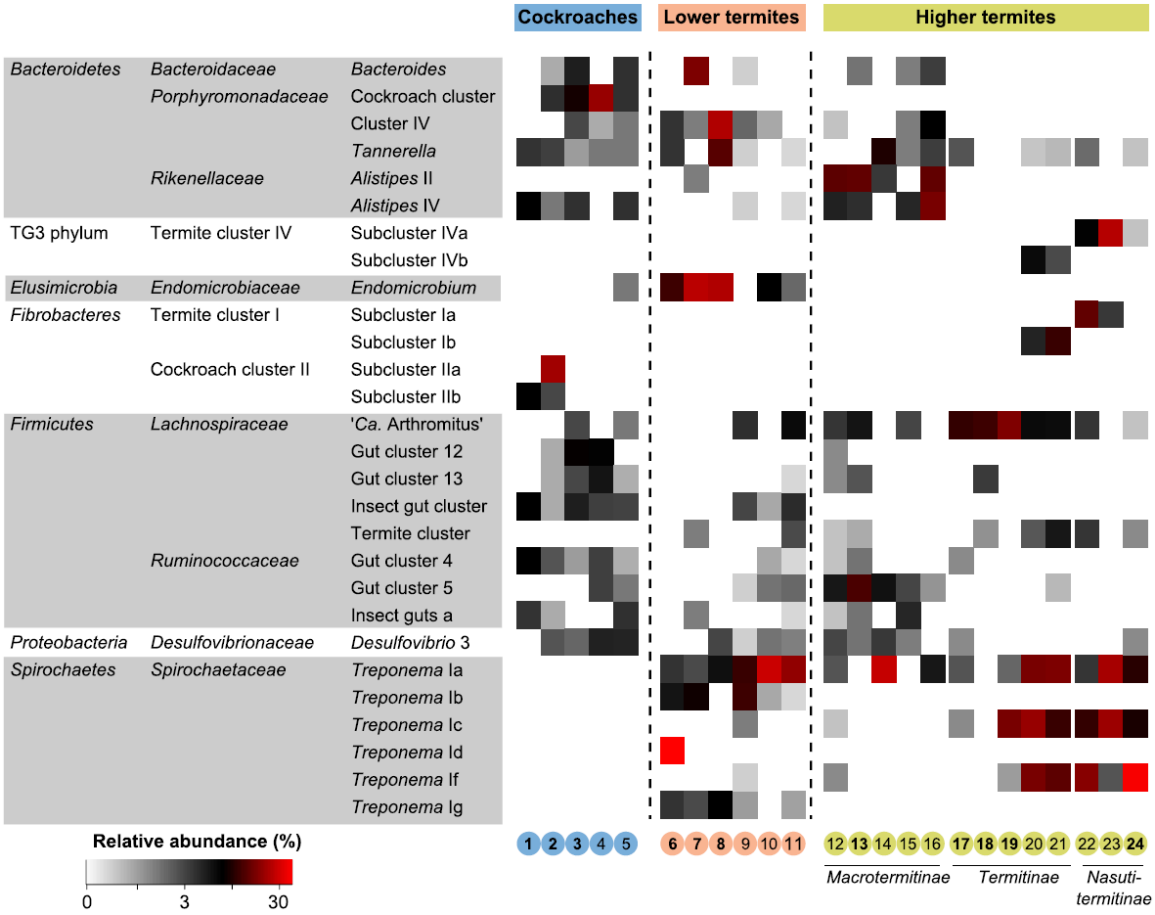

Figure 2.1 | Relative abundance of major genus-level bacterial lineages in clone libraries from cockroaches and lower and higher termites obtained in this and selected previous studies. Clone library numbers shown in bold indicate datasets that were obtained in the present study. For species names and other details, see Table 2.1.

Improvements to the taxonomic framework impacted taxonomic depth and resolution of the reference database particularly at the family and genus levels (Fig. 2). We found that the proportion of sequences from termites and cockroaches that were contained in designated taxa were higher in DictDb than in the original Silva database, particularly at the genus level, and were much higher than in the commonly used RDP database (Fig. 2A). Also in terms of taxonomic resolution, DictDb provided a considerably larger number of taxa containing termite- and cockroach-derived sequences than the two other databases, at both the genus and family level (Fig. 2B).

Another improvement that sets DictDb apart from other databases is the designation of new taxa to accommodate existing sequences that were so far either unassigned or lumped into polyphyletic bins. In the case of RDP, the taxonomy of many sequences from termites and cockroaches was simply "unassigned", often already at the family level. The taxonomic depth of the Silva database was higher, but a large proportion of the sequences from termites and cockroaches assigned to genus-level taxa were designated with the uninformative label "uncultured", which represents a 'garbage bin' of polyphyletic clades, thereby lowering the

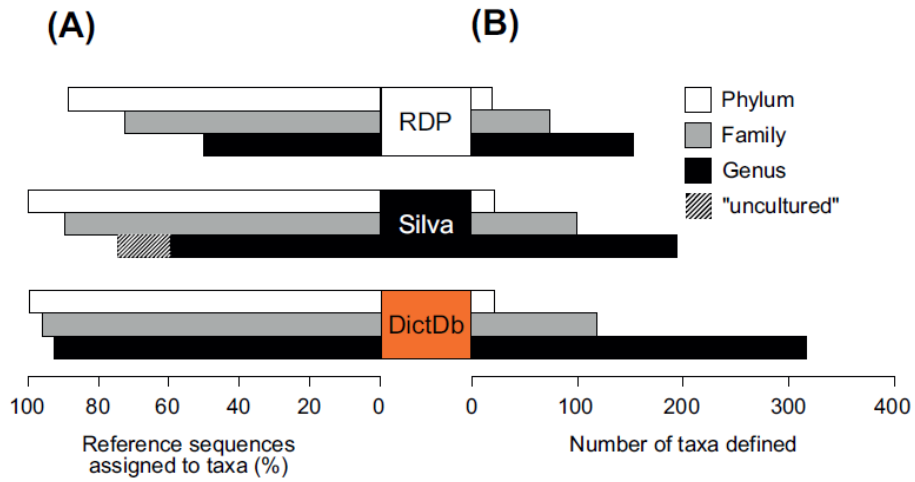

Figure 2.2 | (A) Number of termite- and cockroach-derived sequences assigned to taxonomic ranks in DictDb compared to general-purpose databases. (B) Number of taxa defined by DictDb compared to the number defined by general-purpose databases.

effective classification rate at the genus level to 65%. Special attention was given to resolving paraphyletic and polyphyletic taxa into smaller monophyletic groups that were corroborated by phylogenetic analyses. This not only abolished the nomenclatural problem of the "uncultured" groups, but also allowed resolution of inflated gen-era, such as *Treponema* or *Alistipes*, that represent complexes of monophyletic but highly divergent 16S rRNA gene sequences.

Because of the relative importance of certain bacterial clades in termites and cockroaches, we extended our initial analysis to investigate the phylogenies of the *Treponema* genus complex, the *Fibrobacteres*/*TG3* clade, and the *Alistipes* genus complex in further detail.

2.4.3 The Treponema complex

The uncultured spirochetes in the guts of termites fall into two major clusters, which are most closely related to cultivated members of the genus *Treponema* isolated from other environments. Since these clusters were first outlined (Lilburn, Schmidt and Breznak, 1999;

Ohkuma, Iida and Kudo, 1999), they have grown considerably with clones from subsequent analyses (Yang *et al.*, 2005; Hongoh, Deevong, *et al.*, 2006; Hongoh, Ekpornprasit, *et al.*, 2006; Warnecke *et al.*, 2007; Strassert *et al.*, 2012). Particularly *Treponema* I, the larger of the two clusters, is extremely diverse (Figure 2.3). It comprises numerous deep-branching lineages (*Treponema* Ia–h) with up to 25% sequence dissimilarity, which often co-occur in termites of the same genus or species – an observation consistent with the morphological and physiological diversity of spirochetes within a single termite gut (Breznak, 2002; Breznak and Leadbetter, 2006).

The most diverse subcluster is *Treponema* Ia, with sequence dissimilarities as high as 20%. Members of this subcluster occur in all termite species investigated so far (Figure 2.1) and include *Treponema primitia* (Graber and Breznak, 2004), *Treponema azotonutricium* (Graber, Leadbetter and Breznak, 2004), and *Treponema isoptericolens* (Dröge *et al.*, 2008) – the only isolates of the entire cluster. Other representatives of *Treponema* Ia are phylotypes obtained from capillary-picked flagellates (Ohkuma, Iida and Kudo, 1999; Noda *et al.*, 2003; Strassert *et al.*, 2012). Like their cultivated relatives (Graber, Leadbetter and Breznak, 2004; Dröge *et al.*, 2008)(Dröge *et al.*, 2008), these phylotypes may also grow by reductive acetogenesis from $H_2 + CO_2$ and opportunistically associate with flagellates to make use of them as a source of H_2. Apart from lower termites, the subcluster *Treponema* Ia is also abundantly represented in wood-feeding and grass-feeding higher termites (Dietrich, Köhler and Brune, 2014). Although none of its cultivated members appear to be cellulolytic, *Treponema primitia* is able to catabolize catechol under microoxic conditions, which suggests a possible role of *Treponema* Ia members in the breakdown of aromatic compounds released from the lignin fraction of lignocellulose (Lucey and Leadbetter, 2014).

Unlike *Treponema* Ia, members of *Treponema* Ic and If seem to be specific to higher termites. They are especially abundant in wood-feeding and grass-feeding species (Figure 2.1) and have been shown to colonize wood particles in the hindgut of *Nasutitermes* spp. (Mikaelyan *et al.*, 2014), which indicates that the diverse lineages of treponemes that co-occur in the same gut have distinct ecological niches. The deep-branching subcluster *Treponema* Id consists exclusively of clones from the gut of *Mastotermes darwiniensis*, the most basal of all termite lineages, whereas subcluster *Treponema* Ie contains only clones from fungus-cultivating termites (Figure 2.1).

The second cluster, *Treponema* II, is much smaller than *Treponema* I and also much less diverse. It contains only sequences from lower termites. Many of the clones were obtained

from the gut wall (Yang *et al.*, 2005) or were associated with bacterial filaments (Thompson *et al.*, 2012) or flagellate cells (Iida, 2000) attached to the gut wall, which indicates an affinity of this lineage to surface microhabitats. A third cluster, *Treponema* III, consists exclusively of clones from fungus-cultivating termites. Both clusters represent deep-branching lineages of independent phylogenetic origin and are embedded among *Treponema* species isolated from other environments (Figure 2.3).

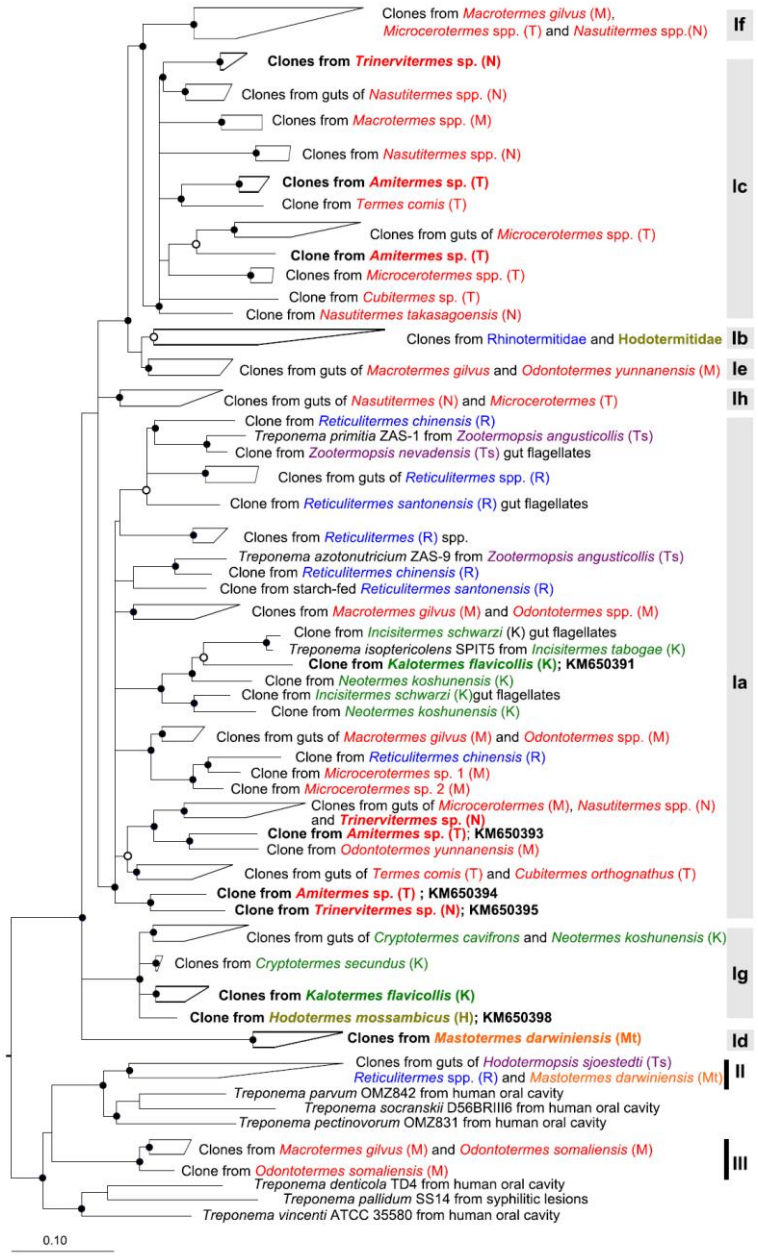

Figure 2.3 | Consensus phylogenetic tree based on maximum-parsimony (MP) and maximum-likelihood (ML) analyses depicting the relationship among the clones affiliated with the Treponema I, II, and III clades in termites and cockroaches. Clones from the current study are in boldface. Nodes marked with circles indicate monophyletic clades in the ML tree that were well supported (○, ≥70%; •, ≥90%) by at least one parametric (aBAYES or $\chi 2$) and one non-parametric test (Felsenstein bootstrap or SH-aLRT; see Fig. S2 for details). Nodes not supported in both MP and ML topologies are shown as multifurcations. Host families and subfamilies are distinguishable by color and indicated in parentheses (Mt, Mastotermitidae; H, Hodotermitidae; Ts, Termopsidae; K, Kalotermitidae; R, Rhinotermitidae; M, Macrotermitinae; T, Termitinae; N, Nasutitermitinae).

Figure 2.4 | Consensus phylogenetic tree based on maximum-parsimony (MP) and maximum-likelihood (ML) analyses depicting the relationship among the clones affiliated with the Fibrobacteres/TG3 clade in termites and cockroaches. Nodes marked with circles indicate monophyletic clades in the ML tree that were well supported (○, ≥70%; •, ≥90%) by at least one parametric (aBAYES or $\chi 2$) and one non-parametric test (Felsenstein bootstrap or SH-aLRT; see Fig. S3 for details). Nodes not supported in both MP and ML topologies are shown as multifurcations. Host families and subfamilies are distinguishable by color and indicated in parentheses (P, Polyphagidae; B, Blaberidae; M, Macrotermitinae; T, Termitinae; N, Nasutitermitinae).

2.4.4 The Fibrobacteres/TG3 clade

Phylogenetic analyses of all full-length sequences available in the radiation of *Fibrobacteres* and the *TG3* phylum confirmed that the two lineages are highly supported sister clades (Hongoh, Deevong, *et al.*, 2006; Sorokin *et al.*, 2014). The clones obtained from termites and cockroaches formed several monophyletic clusters that again separated into subclusters containing clones originating from distinct host groups (Figure 2.4). Clusters I, III, and IV are termite clusters that each contain subclusters of clones derived exclusively from members of the subfamily Nasutitermitinae or Termitinae, corroborating previous observations of host specificity of bacterial lineages affiliated to *Fibrobacteres* and *TG3* in the clone libraries from higher termites (Hongoh, Deevong, *et al.*, 2006). The topology of these clusters is in agreement with a hypothetical cospeciation of these lineages with their hosts.

The clones in Cluster II, the next relatives of Termite cluster I, are exclusively from cockroaches, extending the host specificity of the clones in Fibrobacteres "subphylum 2" (Hongoh, Deevong, *et al.*, 2006) to other dictyopteran groups. They form a sister group of *Fibrobacteres* "subphylum 1", which is found exclusively in mammalian guts and includes also the only cultured representatives of the phylum. The next relatives of the termite-specific clusters in the radiation of the *TG3* phylum are clones from diverse anoxic habitats, including *Chitinivibrioalkaliphilus*, the only isolate of the phylum.

The termite-specific clusters in the Fibrobacteres/TG3 clade are predominant members of the bacterial communities in wood-feeding higher termites (Hongoh, Deevong, *et al.*, 2006; Dietrich, Köhler and Brune, 2014). A recent study reported that both phyla form a large proportion of the fiber-associated, cellulolytic community in *Nasutitermes* spp. (Mikaelyan *et al.*, 2014). Also, metagenomic studies of the hindgut of *Nasutitermes corniger* reported several genes encoding glycosyl hydrolases and cellulose-binding domains that could be taxonomically binned to the phylum *Fibrobacteres* (Warnecke *et al.*, 2007; He *et al.*, 2013). Our detection of Fibrobacteres also in the guts of litter-feeding cockroaches corroborates their importance in the digestion of lignocellulose. However, it remains unclear whether apparent host specificity of individual lineages indicates coevolution with the dictyopteran host line or an adaptation to a particular ecological niche.

2.4.5 The Alistipes complex

The sequences in the *Alistipes* genus complex defined in the Silva database are almost as diverse as the sequences assigned to the *Treponema* complex, with sequence dissimilarities amounting to as much as 21%. Phylogenetic analysis of *Alistipes* resulted in four well-supported clusters, three of which are entirely comprised of clones from insect guts, particularly from termites and cockroaches (Figure 2.5). To increase the taxonomic resolution of DictDb, we defined them as separate, genus-level lineages, with Cluster I containing all isolates of the genus *Alistipes* and clones obtained primarily from mammalian guts.

In contrast to the situation in *Fibrobacteres*, the TG3 phylum, and the Treponema complex, there is no strict host pattern in the distribution of termite-specific lineages in the *Alistipes* clusters. Clones from higher termites are randomly interspersed with clones from lower termites, cockroaches, and other insects. The predominance of clones from fungus-cultivating termites is in agreement with their high abundance in all Macrotermitinae (Otani *et al.*, 2014), which contributes to the remarkable similarity of the gut microbiota in fungus-cultivating termites and cockroaches (Dietrich, Köhler and Brune, 2014; Otani *et al.*, 2014). It has been speculated that similarities in diet between cockroaches and fungus-cultivating termites have driven the development of similar communities in these phylogenetically distant host groups (Dietrich, Köhler and Brune, 2014; Otani *et al.*, 2014). Also the similar gut anatomy and physicochemical environment may contribute to the convergence of their communities. Like the hindguts of cockroaches and lower termites, the hindgut of Macrotermitinae is relatively undifferentiated and lacks the compartmentation and extreme variations in intestinal pH found in other higher termites (Brune, 2014). Therefore, the abundance of phylogenetically related bacterial lineages in the guts of cockroaches and fungus-cultivating termites could be the result of habitat selection.

Figure 2.5 | Consensus phylogenetic tree based on maximum-parsimony (MP) and maximum-likelihood (ML) analyses depicting the relationship among the clones affiliated with Alistipes I, Alistipes II, and Alistipes III in termites and cockroaches. Nodes marked with circles indicate monophyletic clades in the ML tree that were well supported (○, ≥70%; •, ≥90%) by at least one parametric (aBAYES or $\chi 2$) and one non-parametric test (Felsenstein bootstrap or SH-aLRT; see Fig. S4 for details). Nodes not supported in both MP and ML topologies are shown as multifurcations. Host families and subfamilies are distinguishable by color and indicated in parentheses (P, Polyphagidae; B, Blaberidae; Bt, Blattidae; M, Macrotermitinae; T, Termitinae; N, Nasutitermitinae).

2.4.6 Classification performance

To evaluate the performance of DictDb in the classification of rRNA amplicon reads, we classified three 16S rRNA amplicon libraries generated by pyrosequencing and compared the quality of taxonomic assignments with those made using the Silva and RDP databases.

When we evaluated the performance of DictDb in the classification of rRNA amplicon reads obtained from the bacterial gut microbiota of termites and cockroaches, we found that DictDb surpassed the Silva and RDP databases in both taxonomic depth and resolution. The average improvements in classification success were noticeable already at the phylum level, at which DictDb outperformed Silva and RDP by 3% and 15%, respectively (Figure 2.6A). However, the boost in performance improved even more at lower taxonomic ranks; at the genus level, DictDb could classify 21% more sequences than Silva and 49% more sequences than RDP. Also the number of taxa identified in the respective communities increased at all taxonomic ranks. At the genus level, the average number of genera with assigned reads increased from 19 (RDP) to 29 (Silva) to 63 (DictDb) (Figure 2.6B).

The improved performance of DictDb over the other two databases has several reasons. The primary factor responsible for the reliable assignment of short reads to designated taxa is the increased taxonomic depth and resolution of DictDb. The second factor responsible for classification success is the proportion of closely matching reference sequences in databases has been previously shown to influence the confidence of taxonomic assignments with the RDP classifier (Newton and Roeselers, 2012; Werner *et al.*, 2012). Yet another important property of DictDb that distinguishes it from the other two databases is that the delineation of all bacterial taxa is strictly based on the criterion of monophyly. This phylogenetic delineation of taxa used in DictDb is in line with the proposition of Yarza et al. (2014), who suggested to split large, taxonomically cumbersome lineages of environmental sequences into candidate taxonomic units (CTUs). The presence of polyphyletic taxa in reference databases is considered to affect the classification success with the RDP classifier, which relies on compositional information averaged over each taxon (Wang *et al.*, 2007). Polyphyletic taxa are problematic also for nearest-neighbor classifiers (e.g., Seq-Match (Cole *et al.*, 2014)), which are more sensitive to the taxonomic designation of individual reference sequences (Wang *et al.*, 2007).

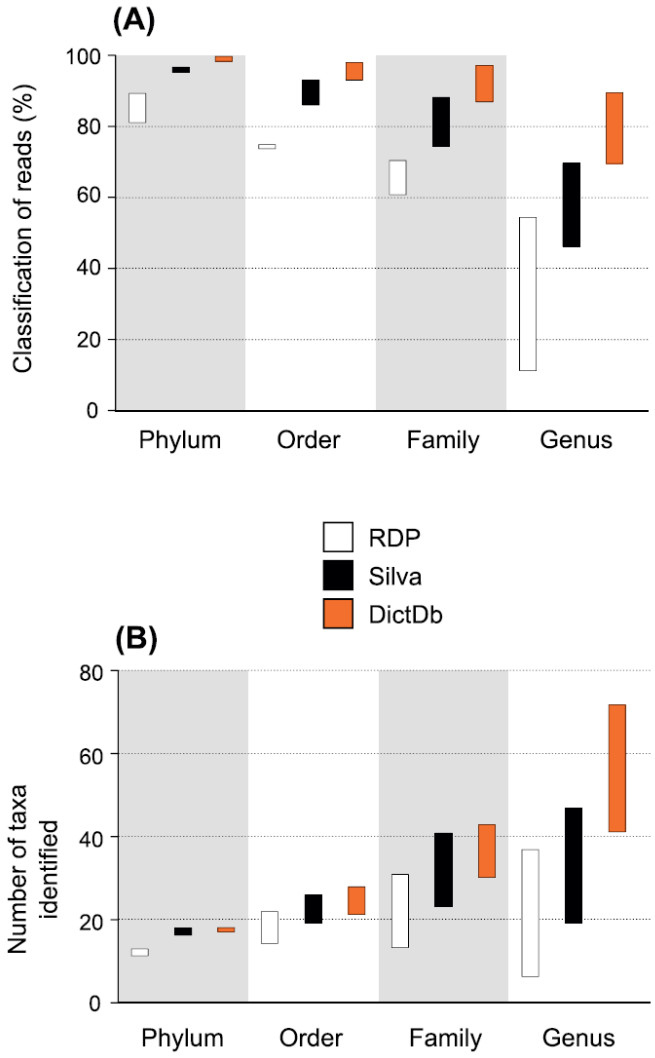

Figure 2.6 | (A) Classification success and (B) number of taxa identified in amplicon sequencing datasets of bacterial gut microbiota of selected dictyopteran species using the RDP classifier with DictDb, Silva, or RDP as reference database.

The consequence of the improved classification becomes apparent in a comparison of the classification results obtained for the three test communities with the three reference databases (Fig.S5). Both the number of genus-level taxa in the three test communities and the compositional overlap between these communities increases from RDP to Silva to DictDb, underlining that the number of taxa shared is severely underestimated when commonly used databases are applied to the intestinal communities of termites and cockroaches. Moreover, even some of the few shared taxa identified by Silva and RDP were due to misclassifications. For example, classification using the Silva database identified the genus *Treponema* as a core taxon present in all three datasets, whereas manual addition of the short reads in the *Shelfordella* dataset to the guidetree of Silva placed the sequences into the radiation of the genus *Spirochaeta*. However, when DictDb was used as reference database, the short reads

were correctly assigned to the genus *Spirochaeta* (not shown). It is likely that such misclassifications result from the forced training of the classifier on highly divergent sequences (with distinct k-mer frequencies) assigned to a single polyphyletic taxon, as observed already by Wang et al. (Wang *et al.*, 2007), underlining once more the importance of curation to achieve a phylogenetic taxonomy.

2.5 Conclusions and perspectives

The large size of databases such as Silva and RDP does not allow achieving the level of curation required for a highly resolved classification of amplicon libraries. Therefore, it is not surprising that several groups have produced specialized reference databases for the classification of host-associated microbiota in the oral cavity (Griffen *et al.*, 2011), rumen (Seedorf *et al.*, 2014), and honey bee gut (Newton and Roeselers, 2012). Previous (undocumented) versions of DictDb have already been adopted by a growing community of gut microbiologists interested in termite and cockroach guts as models for intestinal ecosystems to characterize bacterial community structure in these guts (Köhler *et al.*, 2012; Huang *et al.*, 2013; Dietrich, Köhler and Brune, 2014; Mikaelyan *et al.*, 2014; Otani *et al.*, 2014; Schauer, Thompson and Brune, 2014; Santana *et al.*, 2015). In addition to the expanded inventory of novel reference sequences and a substantially improved taxonomic framework, DictDb v. 3.0 has been enhanced by the inclusion of metadata that provide information relevant to digestive symbiosis (host species and their diet, specific microhabitats). These fields can easily be adopted by other researchers working with insect symbionts as a means of providing minimum information for cataloging microbial diversity in insects.

2.6 Data accessibility

The 16S rRNA gene sequences are deposited under the Genbank accession numbers: KP090460–KP090608 and KM650363–KM651220.

2.7 Acknowledgements

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2.8 Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2015.07.004

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3 Physicochemical conditions, metabolites and community structure of the bacterial microbiota in the gut of wood-feeding cockroaches (Blaberidae: Panesthiinae)

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EB conceived the study, performed experiments, analyzed data, and wrote a first draft of the manuscript. **NL performed experiments, analyzed the data, and wrote the final version of the paper.** AM and TK provided scientific advice and contributed ideas to the manuscript. KM conceived the study. AB conceived the study and secured funding.

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3.1 Abstract

While the gut microbiota of termites and its role in symbiotic digestion have been studied for decades, little is known about the bacteria colonizing the intestinal tract of the distantly related wood-feeding cockroaches (Blaberidae: Panesthiinae). Here, we show that physicochemical gut conditions and microbial fermentation products in the gut of Panesthia angustipennis resemble that of other cockroaches. Microsensor measurements confirmed that all gut compartments were anoxic at the center and had a slightly acidic to neutral pH and a negative redox potential. While acetate dominated in all compartments, lactate and hydrogen accumulated only in the crop. The high, hydrogen-limited rates of methane emission from living cockroaches were in agreement with the restriction of F420-fluorescent methanogens to the hindgut. The gut microbiota of both P. angustipennis and Salganea esakii differed strongly between compartments, with the highest density and diversity in the hindgut, but similarities between homologous compartments of both cockroaches indicated a specificity of the microbiota for their respective habitats. While some lineages were most closely related to the gut microbiota of omnivorous cockroaches and wood- or litter-feeding termites, others have been encountered also in vertebrates, reinforcing the hypothesis that strong environmental selection drives community structure in the cockroach gut.

3.2 Introduction

A wood-feeding lifestyle is encountered in several lineages of dictyopteran insects. In termites and their evolutionary sister group, the cockroach family Cryptocercidae, wood is efficiently digested with the help of microbial symbionts housed in their enlarged hindgut compartments (Brune and Ohkuma, 2011; Brune, 2014). The key role in cellulose and hemicellulose degradation is attributed either to flagellate symbionts (in phylogenetically lower termites and Cryptocercidae) or to a diverse assemblage of cellulolytic prokaryotes (in the flagellate-free higher termites).

By contrast, the strategy of lignocellulose digestion in the cockroach subfamily Panesthiinae (family Blaberidae), which is phylogenetically distinct from termites and Cryptocercidae, is far from clear. Most panesthia cockroaches feed on decaying wood (Maekawa, Matsumoto and Nalepa, 2008). Although their hindgut harbors a characteristic community of protists dominated by morphologically conspicuous ciliates (Kidder, 1937; Yamasaki M, 1939), the latter are not associated with the digestion of wood but are close relatives of the genus

Nyctotherus (Lynn and Wright, 2013), which is commonly encountered also in omnivorous cockroach species (Gijzen *et al.*, 1991; Hackstein, 1997).

Also the importance of the endoglucanases secreted in the salivary glands of panesthia cockroaches (Scrivener, Slaytor and Rose, 1989; Maekawa, Matsumoto and Nalepa, 2008) in the digestive process is not firmly established (Martin, Jones and Bernays, 1991; Slaytor, 1992). Although the activity in *Panesthia cribrata* would suffice to explain the respiratory demand of the insect (Scrivener and Slaytor, 1994), it is entirely unclear to which extent the ingested wood is degraded by microbial symbionts in the hindgut and how much of the nutritional requirement is derived from other food components, e.g. the biomass of fungi and bacteria colonizing the ingested wood. It has been reported that hindgut content of *P. cribrata* contains hardly any cellulolytic activity (Scrivener, Slaytor and Rose, 1989), but studies on higher termites revealed that a substantial proportion of the cellulolytic activity in the hindgut is contributed by fiber-associated bacteria and can be detected only after solubilization of the enzymes with detergents (Tokuda, Lo and Watanabe, 2005; Mikaelyan *et al.*, 2014). Although recent studies have revealed the presence of many termite-specific bacterial lineages in omnivorous cockroaches (Schauer, Thompson and Brune, 2012; Dietrich, Köhler and Brune, 2014), it remains open whether any of the lineages implicated in fiber digestion occur in wood-feeding cockroaches.

Most knowledge of the bacterial gut microbiota of cockroaches is based on cultivation-dependent approaches, which are severely biased when compared to sequence-based methods (Schauer, Thompson and Brune, 2012). The application of high-throughput sequencing techniques removed also the undersampling problem inherent to highly diverse habitats and revealed the full extent of the bacterial diversity in cockroach guts (Schauer, Thompson and Brune, 2012; Dietrich, Köhler and Brune, 2014; Sabree and Moran, 2014), including the presence of a core microbiota in individuals of the same species (Schauer, Thompson and Brune, 2014). However, detailed information on bacterial diversity and community structure in xylophagous Panesthiinae is still lacking.

In this study, we investigated the bacterial microbiota in different gut compartments of the wood-feeding Panesthiinae species *Panesthia angustipennis* and *Salganea esakii*. The archaeal microbiota of these species has already been studied by other authors (van Hoek *et al.*, 2000; Hara *et al.*, 2002). The effect of hydrogen produced by the bacteria on methanogenesis was evaluated by monitoring methane emissions *in vivo*. Since physicochemical conditions, fermentation products and microbial cell densities differ significantly between the gut

compartments of the omnivorous cockroaches (Lemke *et al.*, 2001; Schauer, Thompson and Brune, 2012), we determined those parameters also for the wood-feeding *P. angustipennis*. Similarities between the communities of homologous gut compartments of *P. angustipennis* and *S. esakii* were investigated using both full-length clone libraries and pyrotag sequencing of their 16S rRNA genes. The sequences of *Blattabacterium cuenoti*, the intracellular fat body symbiont of cockroaches (Lo *et al.*, 2003; Tokuda *et al.*, 2013) and a regular contaminant of cockroach gut preparations, were removed from all datasets.

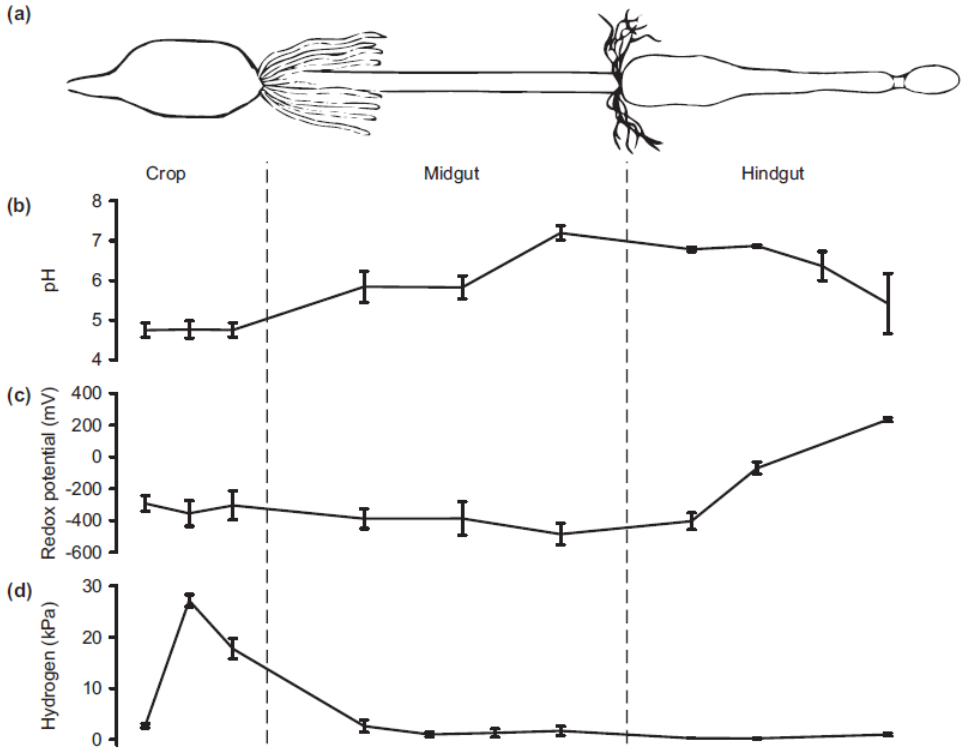

Figure 3.1 | Intestinal tract of P. angustipennis. (a) Typical lengths of the gut compartments: crop, 14 mm; midgut, 44 mm (longer than it appears in the figure); and hindgut, 37 mm. Mc, midgut caeca; Mt, malpighian tubules. Axial profiles determined with microelectrodes: (b) pH, (c) redox potential and (d) hydrogen partial pressure. Values are means ± SD (n = 3).

3.3 Materials and Methods

3.3.1 Cockroaches and dissection

Panesthia angustipennis (subsp. *spadica*) and *Salganea esakii* were collected near the Keta Shrine in Ishikawa Prefecture (Japan, 2 September 2010), and Mt. Yasumandake in Nagasaki Prefecture (Japan, 8 June 2010), respectively. All specimens of *P. angustipennis* were adults; the few individuals of *S. esakii* available for this study were sub-adults. Cockroaches were maintained for several months at room temperature in plastic boxes filled with decaying wood

and a layer of wet tissue paper. The gut was dissected, and the fat body was removed. For analysis of individual compartments, the gut was divided into three sections: crop, midgut and hindgut, which consisted of colon and rectum (Figure 3.1a). After the weight of each compartment was recorded, sodium phosphate buffer (12 mM) was added (1.6 ml per g fresh weight), and the compartments were homogenized in an Eppendorf tube using a small pestle.

3.3.2 HPLC analysis

After centrifugation at 20 000 × g for 5 min, the supernatant was mixed with sodium hydroxide (10 mM final concentration) and filtered through a 0.2 µm cellulose acetate membrane (ReZist, Whatman). Microbial fermentation products were quantified by ion-exclusion chromatography on a high-pressure liquid chromatography (HPLC) system equipped with a Grom resin IEX column (8 µm pore size, 250 mm × 4.6 mm inside diameter; Grom, Rottenburg, Germany) and a refractive index detector (RID-10A; Shimadzu) with a mobile phase of 5 mM sulfuric acid (flow rate 0.8 ml min^{-1}) and a column temperature of 60°C. Peaks were identified using external standards.

3.3.3 GC analysis

Methane production by living animals was determined in rubber-stoppered vials (100–200 ml), which were chosen according to the size of the insects. Headspace gas (300 µl) was sampled at regular time intervals (30 min) and replaced with the same amount of ambient air. The methane concentration in the samples was determined with a gas chromatograph equipped with a packed column. Injector, column and detector temperatures were 110, 40, and 120°C, respectively (for details, see Pester and Brune 2007). After 2.5 h, 20% of the headspace gas was replaced with pure hydrogen, and the headspace gas was sampled two more times. The animals were not harmed by the treatment and remained inactive throughout the procedure.

3.3.4 Microsensor measurements

Intestinal oxygen and hydrogen concentrations, pH and redox potential were measured with microsensors (Unisense, Aarhus, Denmark). Oxygen (10 µm tip diameter) and hydrogen (50 µm tip diameter) microsensors were calibrated in Ringer's solution as described previously (Brune, Emerson and Breznak, 1995) using synthetic air or a H_2/N_2 mixture (5/95, v/v). The pH microelectrode (50 µm tip diameter) was calibrated with commercial pH standard solutions of pH 4.0, 7.0 and 10.0. The redox microelectrode (10 µm tip diameter) was calibrated with

saturated solutions of quinhydrone in pH standards of pH 4.0 and 7.0. For pH and redox microsensors, the electric potential was measured against a custom-built Ag-AgCl reference electrode. For the measurements, the guts were placed in glass-faced microchambers and irrigated with air-saturated Ringer's solution using the same setup as described previously (Charrier and Brune, 2003).

3.3.5 Clone libraries

DNA was extracted from homogenized gut compartments using a bead-beating protocol combined with phenol–chloroform extraction and ethanol precipitation as described previously (Ikeda-Ohtsubo *et al.*, 2007) and stored in 10 mM Tris buffer (pH 8) at −20°C. For clone libraries, hindgut DNA samples were pooled (eight individuals of *P. angustipennis* and four individuals of *S. esakii*), and 16S rRNA genes were amplified using the bacterial primers 27f and 1492r (Lane, 1991). The PCR products were purified and cloned as described previously (Strassert *et al.*, 2010). After screening the clones for the correct insert size, the inserts were sequenced using vector primers (GATC Biotech, Konstanz, Germany). The sequences were aligned using the *mothur* software suite (Schloss *et al.*, 2009) and imported into a manually curated reference database using *arb* 5.5 (Westram *et al.*, 2011). Potential chimeras were identified using *uchime* (Edgar *et al.*, 2011) and verified by partial treeing. Phylogenetic trees were calculated using the phyML algorithm (Guindon and Gascuel, 2003) implemented in *arb*. Sequences were submitted to GenBank (accession numbers: KM650207–KM650361).

3.3.6 Pyrotag sequencing

For pyrotag analysis of the 16S rRNA genes, DNA extracted from individual gut compartments was amplified and sequenced as described previously (Dietrich, Köhler and Brune, 2014). Briefly, the V3–V4 region was amplified with bacteria-specific primers and analyzed on a Roche GS FLX Titanium Technology instrument (GATC Biotech, Konstanz, Germany). The sequence reads (length ca. 440 bp, approximately 10 000 quality-checked reads per sample) were analyzed using *mothur* (Schloss *et al.*, 2009), and submitted to the SRA archive of NCBI (BioProject ID: PRJNA264790). For taxonomic classification with the rdp classifier (Wang *et al.*, 2007), we used a manually curated reference database (Dietrich, Köhler and Brune, 2014), which was further improved in resolution by including the sequences from the clone libraries obtained in this study. Sequences identified as *Blattabacteria* were removed from the dataset. Sampling coverage was determined according to Good (1953); species richness, diversity, and

evenness were calculated using the Chao 1 estimator (Chao, 1984), non-parametric Shannon index (Chao and Shen, 2003), and evenness index (Legendre and Legendre, 1998), respectively (Table S1, Supporting Information). Heat maps were generated with the *R* software (R Core Development Team, 2012) with the package *heatmap.plus* (Day, 2012). For comparison of community structure at the sequence level, we used a weighted UniFrac analysis (Lozupone *et al.*, 2011); cluster analysis was based on Euclidian distances calculated with the Ward method, using the R package *pvclust* (Suzuki and Shimodaira, 2006).

3.4 Results

3.4.1 Physicochemical conditions and fermentation products

Axial profiles of the intestinal pH in *Panesthia angustipennis* revealed acidic conditions (pH < 5) in the crop. The pH increased along the midgut, remained around neutral in the anterior hindgut and decreased again towards the rectum (Figure 3.1b). All gut compartments were entirely anoxic along the center, with steep radial gradients of oxygen in the periphery (details not shown). Reducing conditions prevailed at the center of all compartments except the posterior hindgut, where the redox potential increased to slightly positive values (Figure 3.1c).

The anoxic conditions were in agreement with the presence of microbial fermentation products in all compartments, which differed notably between crop and posterior gut (Figure 3.2). While acetate was the major product in all compartments, the crop also contained high concentrations of lactate. By contrast, midgut and hindgut contained only little lactate but small amounts of succinate and traces of butyrate. Hydrogen accumulated strongly in the crop (up to 28 kPa), decreased in the midgut and was below the detection limit in the hindgut (Figure 3.1d).

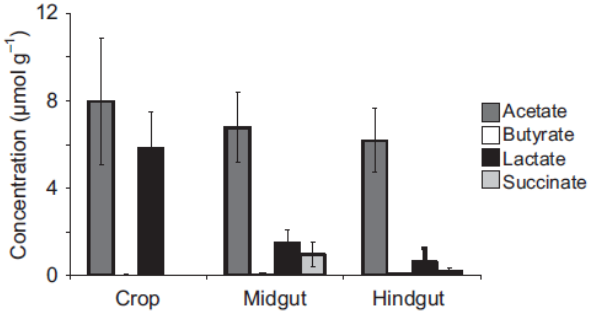

Figure 3.2 | Microbial fermentation products in the intestinal tract of P. angustipennis. Values are based on fresh weight. Error bars indicate standard error of the mean (n = 3).

Epifluorescence microscopy of gut contents of *P. angustipennis* revealed that cells with the characteristic autofluorescence of cofactor F_{420} were restricted to the hindgut, which is in agreement with the general location of methanogens in cockroaches and other insects (Hackstein and Stumm, 1994; Hackstein, van Alen and Rosenberg, 2006; Brune, 2010). In both *P. angustipennis* and *S. esakii*, the *in vivo* rates of methane emission increased 3- to 5-fold after the addition of hydrogen to the headspace (Table 3.1). The restriction of methanogens to the hindgut of *P. angustipennis* and the absence of hydrogen accumulation in this compartment agree with the apparent hydrogen limitation of methanogenesis observed with both cockroaches.

Table 3.1 | In vivo methane emission from wood-feeding *P. angustipennis* and *S. esakii*, before and after addition of hydrogen to the headspace. Values are means ± standard deviation.

Species	Methane emission[a] [nmol (g fresh wt.)$^{-1}$ h^{-1}]			Stimulation factor
	Air	Air + 20% H_2	*n*	
P. angustipennis	101 ± 35	355 ± 169	5	3.4 ± 0.6*
S. esakii	245 ± 58	1305 ± 52	2	5.5 ± 1.1

[a] Rates were determined by linear regression of six data points before and three data points after hydrogen addition.
* Significant increase after hydrogen addition (ANOVA test, $P < 0.001$).

3.4.2 Phylogenetic analysis of bacterial communities

Microbial cell counts in *P. angustipennis* revealed large numbers of microorganisms in all gut compartments, with highest cell densities in the hindgut (Table 3.2). Pyrotag sequencing of the bacterial 16S rRNA genes showed that the bacterial communities in all gut compartments of both *P. angustipennis* and *S. esakii* were highly diverse (Table S1, Supporting Information). The strong differences in community structure between the compartments were apparent already at the phylum level (Figure 3.3). In both species, the crop was dominated by *Proteobacteria*, while *Firmicutes* constituted the most abundant phylum in midgut and hindgut. *Bacteroidetes* was most abundant in the hindgut. Likewise, *Synergistetes* were present in significant numbers only in the hindgut. Cluster analysis targeting the phylotypes in the microbial communities confirmed the clear separation according to the gut compartment (Figure 3.4). The differences in community structure between the homologous compartments of the two host species were less pronounced. In the case of the hindgut, the community structure in individuals of *P. angustipennis* differed as much from each other as from that in individuals of *S. esakii* (Figure 3.4).

Table 3.2 | Gut weight and microbial cell density in gut compartments of P. angustipennis. Values are means ± standard deviation (n = 3).

Compartment	Fresh weight (mg)	Cell density (10^9 cells per g)
Crop	156 ± 69	4.3 ± 1.1
Midgut	137 ± 54	11.9 ± 5.4
Hindgut	204 ± 103	30.7 ± 6.3

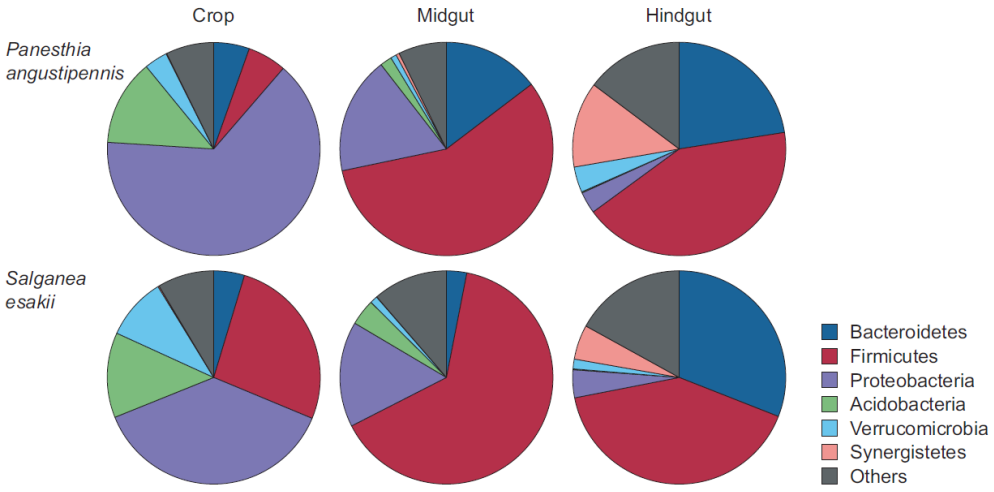

Figure 3.3 | Phylum-level affiliation of bacterial 16S rRNA genes amplified from the different gut compartments of *P. angustipennis* and *S. esakii*, as determined by pyrotag sequencing. Values are means of results obtained independently from two individuals of each species, except for the crop, where one individual was sampled per species.

Classification of the pyrotag reads with the reference database used in a previous study (Köhler *et al.*, 2012) gave poor results at the lower taxonomic levels (family and below; details not shown). Inclusion of the sequences from the hindgut clone libraries obtained in this study (see below) and a study by Dietrich et al. (2014) considerably improved the classification success (Table 3.3; Dietrich, Köhler and Brune 2014). Nevertheless, a substantial fraction of reads remained unclassified, particularly in crop samples of both species, which indicated the presence of additional genus-level groups.

Table 3.3 | Classification success of pyrotag reads at different taxonomic levels. Values are reported as the fraction (%) of classified sequences in crop, midgut, and hindgut of *P. angustipennis* and *S. esakii*.

Taxonomic level	*P. angustipennis*			*S. esakii*		
	Crop	Midgut	Hindgut	Crop	Midgut	Hindgut
Phylum	99.8	100.0	99.7	99.9	100.0	99.8
Class	98.7	99.0	96.7	99.6	95.5	89.8
Order	88.9	97.5	95.2	96.2	93.5	88.7
Family	86.3	90.7	86.6	78.3	88.6	83.1
Genus	53.4	62.8	57.9	45.0	72.4	61.1

The majority of bacterial lineages showed specificity for certain gut compartments. Almost all *Bacteroidetes* lineages were most abundant in the hindgut, with the exception of *Chitinophagaceae*, which also formed substantial populations in the crop (Figure 3.4). In addition, there were obvious differences in the abundance of specific bacterial lineages in the two host species. For instance, *Lactococcus* spp. were highly abundant in the crop and midgut of *P. angustipennis* but absent in *S. esakii*, while *Lactovum* spp. were abundant in the crop and midgut of *S. esakii*, but lacking in *P. angustipennis*. Also, members of the genus *Alistipes* and two clusters of *Enterobacteriales* were more abundant in *P. angustipennis* than *in S. esakii*, but the latter only in the crop.

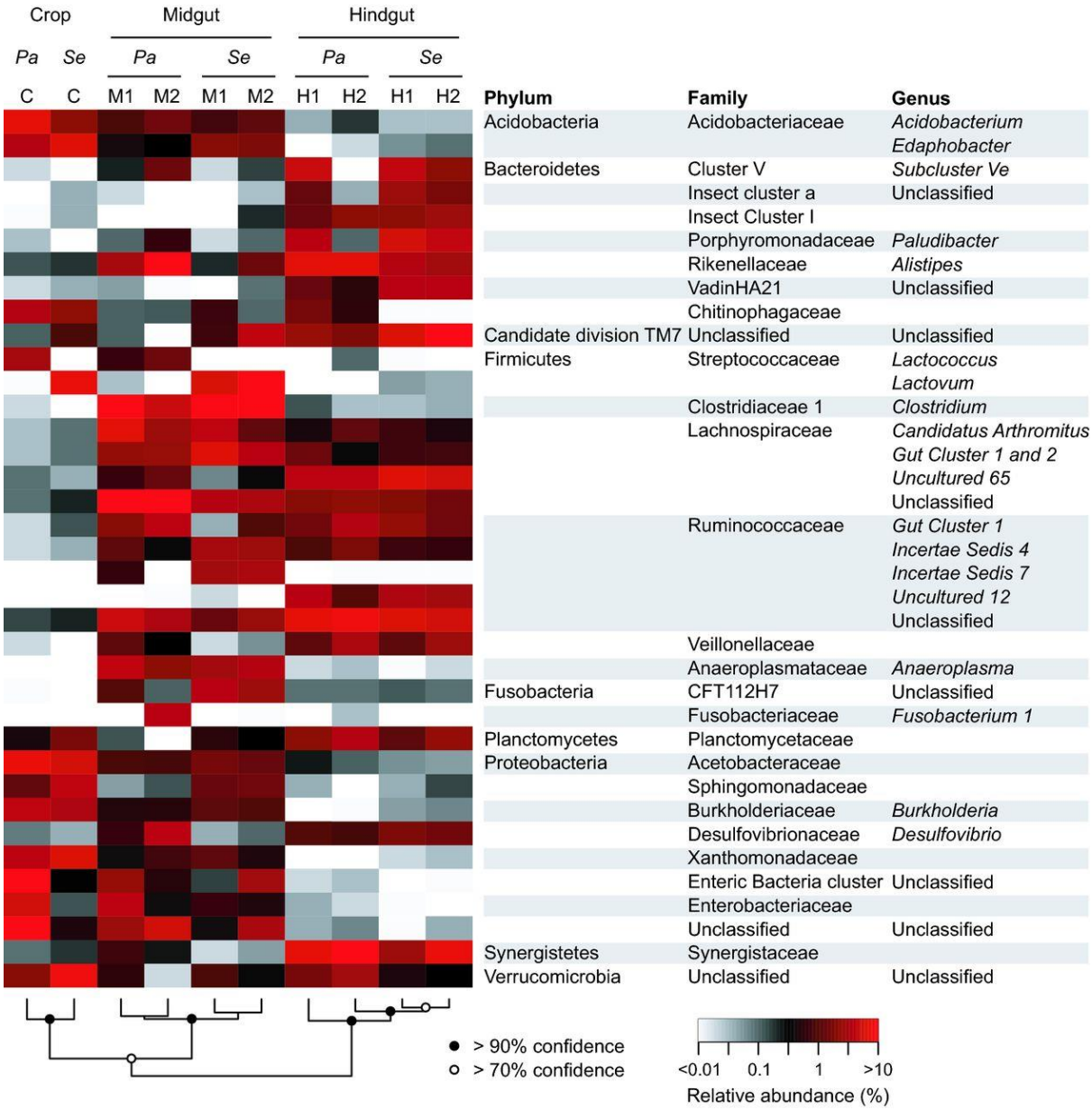

Figure 3.4 | Relative abundance of the most important bacterial taxa in the different gut compartments of *Panesthia angustipennis* (Pa) and *Salganea esakii* (Se). Midgut and hindgut samples were obtained independently from two individuals of each species (M1, M2 and H1, H2). Taxa that represent less than 2.5% of the reads in the entire dataset were omitted (see Table S1, Supporting Information). The dendrogram at the bottom of the figure shows the results of a hierarchical cluster analysis based on the UniFrac metric. Confidence levels of nodes are given as approximately unbiased (AU) P-values.

3.4.3 Clone libraries

To study the detailed phylogenetic relationship of the intestinal symbionts, we constructed clone libraries of the bacterial 16S rRNA genes in the hindgut of *P. angustipennis* and *S. esakii*. Among the sequences obtained from *P. angustipennis* (135 clones), the phyla *Bacteroidetes* (56 clones), *Firmicutes* (53 clones) and *Proteobacteria* (15 clones) made up approximately 90% of the bacterial hindgut community. *Planctomycetes* (five clones) and *Actinobacteria* (four clones) were less abundant, and only one clone of *Synergistetes* was recovered. Although the number of sequences obtained from *S. esakii* (20 clones) was considerably smaller, the major phyla were represented in similar relative abundance. An exception was a single clone from the candidate phylum TM7, which was not represented in *P. angustipennis*.

More than half of the clones assigned to *Bacteroidetes* fell into 'Cluster V' (Figure 3.5), which consists exclusively of uncultured bacteria from termite and cockroach guts (Hongoh, Deevong, *et al.*, 2006; Schauer, Thompson and Brune, 2012). Clones from *P. angustipennis* and *S. esakii* assigned to the *Rikenellaceae* typically clustered with sequences from termites and omnivorous cockroaches. In other cases, the closest neighbors included representatives from the gut of other insects or vertebrates, such as 'Gut group A' (scarab beetle larva), *Porphyromonadaceae* (human gut) and *Marinilabiaceae* (fish gut).

A similar situation was encountered among the *Firmicutes*, where half of the clones were affiliated with the *Ruminococcaceae* (Figure 3.S1, Supporting Information). Also here, many of the clones from *P. angustipennis* and *S. esakii* were closely related to clones from omnivorous cockroaches and scarab beetle larvae. For instance, clones in the gut cluster 'Incertae Sedis 8' consisted exclusively of sequences from wood-feeding cockroach and termite species, whereas 'Gut Cluster 2' comprised both sequences originating from insect and mammalian guts. Clones in 'Gut Cluster' 3 and 4 are restricted to termites and cockroaches, whereas *Papillibacter*-related clones are common to both insect and mammalian hosts.

Likewise, most of the *Lachnospiraceae* clones from *P. angustipennis* and *S. esakii* did not cluster with clones from other cockroaches but with gut microbiota of termites, other insects or mammals (Figure 3.6). Firmicutes clones from 'family XIII' clustered with clones from *Reticulitermes santonensis* (KM650337, KM650341; AB198494, DQ307720); those from *Paenibacillaceae* with *Cohnella* clones from the human gut (KM650359; GQ115556, EF368008, not shown).

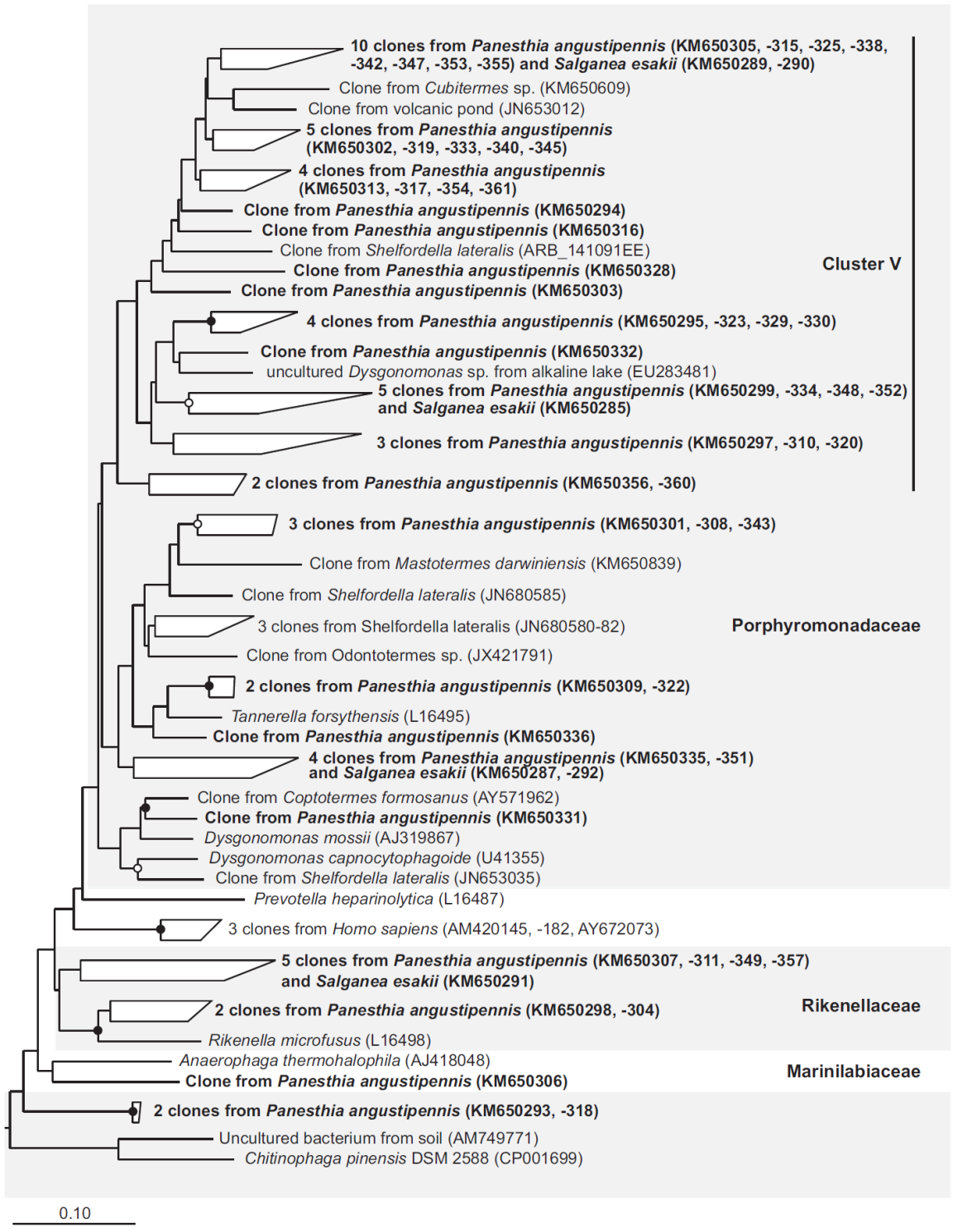

Figure 3.5 | Phylogenetic tree illustrating the position of the *Bacteroidetes*-related 16S rRNA gene sequences from *P. angustipennis* and *S. esakii* relative to other members of the *Bacteroidetes*. The maximum-likelihood tree was reconstructed using phyML algorithm. Sequences from this study are in bold. Scale bar indicates rate of substitution per site; closed and open circles represent bootstraps >90 and >70%, respectively (bootstrap analysis, 1000 repetitions).

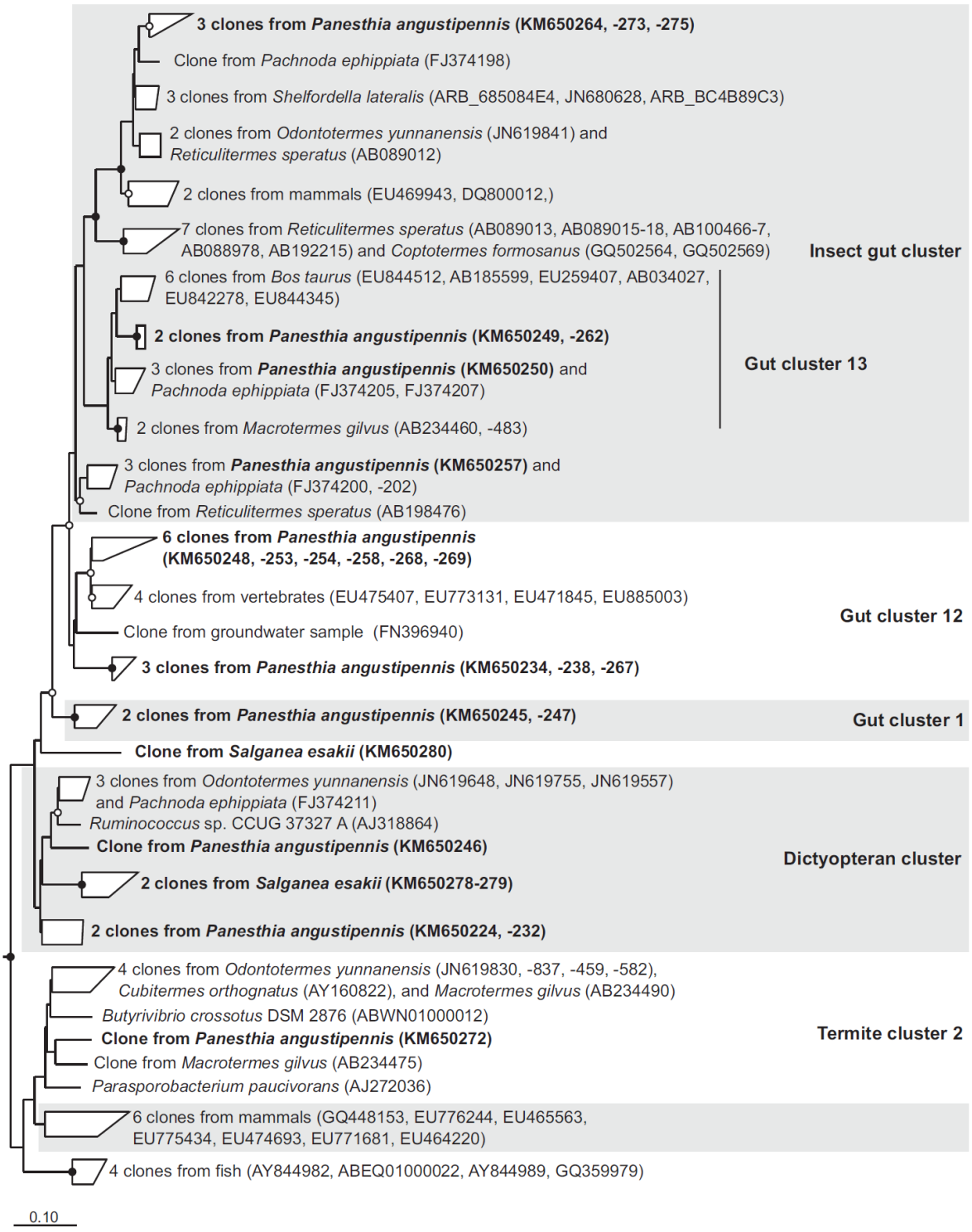

Figure 3.6 | Phylogenetic tree illustrating the position of the *Lachnospiraceae*-related 16S rRNA gene sequences from *P. angustipennis* and *S. esakii* relative to other members of the *Lachnospiraceae*. The maximum-likelihood tree was reconstructed using *phyML* algorithm. Sequences from this study are in bold. Scale bar indicates rate of substitution per site; closed and open circles represent bootstraps >90 and >70%, respectively (bootstrap analysis, 1000 repetitions).

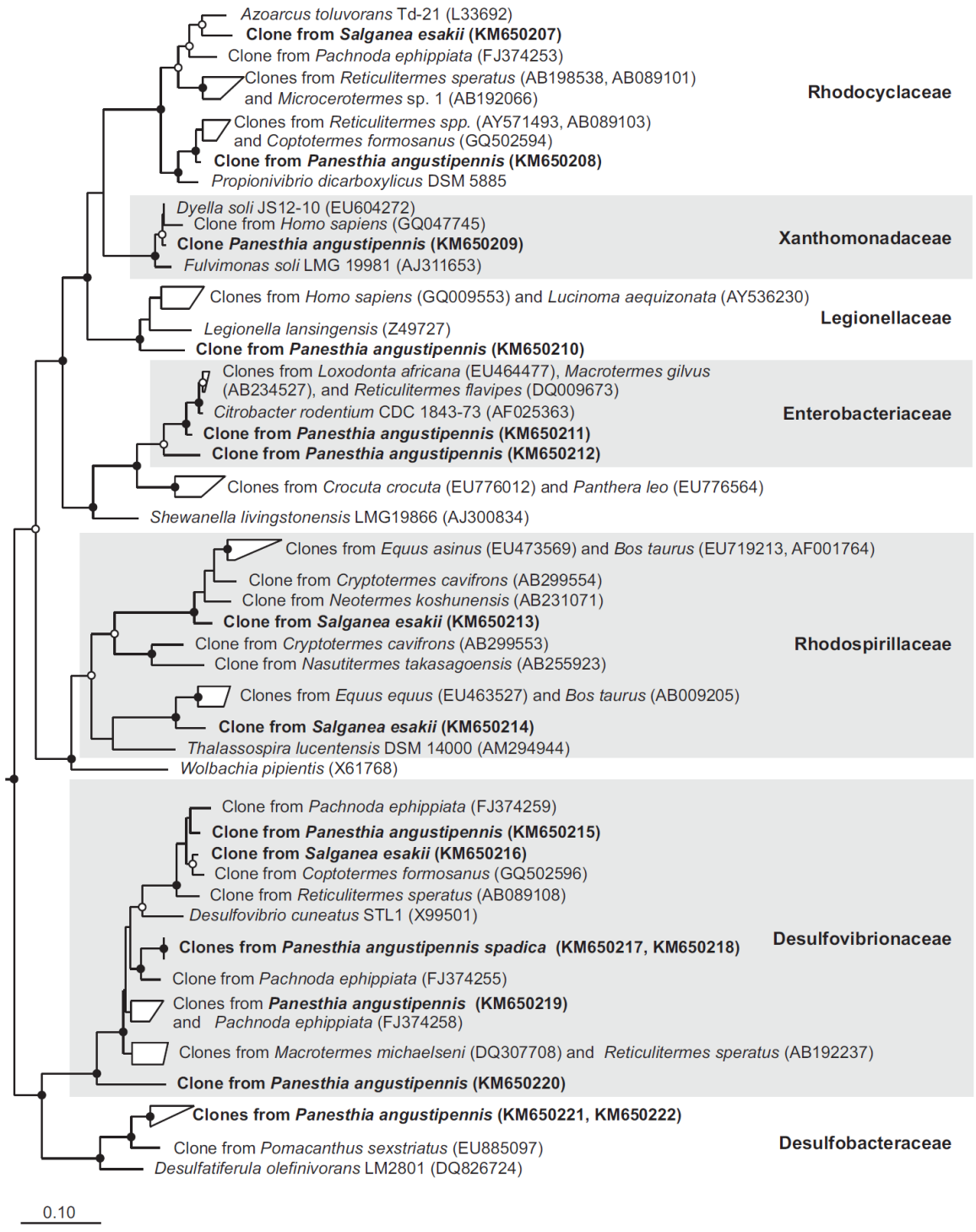

Figure 3.7 | Phylogenetic tree illustrating the position of the *Proteobacteria*-related 16S rRNA gene sequences from *P. angustipennis* and *S. esakii* relative to other members of the *Proteobacteria*. The maximum-likelihood tree was reconstructed using phyML algorithm. Sequences from this study are in bold. Scale bar indicates rate of substitution per site; closed and open circles represent bootstraps >90 and >70%, respectively (bootstrap analysis, 1000 repetitions).

In the case of *Proteobacteria*, most of the clones were closely related to *Desulfovibrio* spp. obtained from the intestinal tract of termites and cockroaches (Figure 3.7). Others fell into *Enterobacteriaceae*, *Rhodospirillaceae* and the candidate family B38, with clones from insect

and mammalian guts as closest relatives. Clones affiliated with *Xanthomonadaceae* and *Legionellaceae* were most similar to clones from human guts.

Also clones from the remaining phyla were affiliated with the gut microbiota of various hosts. Some clones fell into lineages commonly encountered in many termites (*Synergistetes* from *P. angustipennis*, KM650242; candidate phylum TM7, KM650288; *Coriobacteriaceae*, Figure 3.S3, Supporting Information), other insects (other *Planctomycetes*; Figure 3.S2, Supporting Information) or even mammals (*Synergistetes* from S. esakii, KM650281; *Propionibacteriaceae*, *Microbacteriaceae*; Figure 3.S3, Supporting Information), while others seem to be restricted to wood- and soil-feeding species (*Planctomycetes* of the Termite cluster and the CP1-a4-group; Figure 3.S2, Supporting Information).

3.5 Discussion

This is the first in-depth study of the bacterial microbiota in the hindgut of wood-feeding cockroaches of the blaberid subfamily Panesthiinae, which are only distantly related to termites and their sister group, the wood-feeding cockroaches in the genus *Cryptocercus*. Both species live in very similar conditions, with *Panesthia angustipennis* being gregarious without parental interaction (Nalepa *et al.*, 2008), and *Salganea esakii* displaying stomodeal trophallaxis from parents to offspring (Shimada and Maekawa, 2011). Our results show that both *P. angustipennis* and *S. esakii* harbor a diverse gut microbiota, mostly characterized by lineages of obligately anaerobic bacteria commonly encountered in intestinal tracts. The presence of representatives from the microbiota of other host groups (termites, cockroaches, other insects, mammals and fish) strongly suggests that the respective clusters comprise bacteria adapted to intestinal habitats. Together with the high similarity in community structure between homologous gut compartments from two different species and the large variation between individuals from the same species, our findings strengthen the hypothesis that the gut microbiota of cockroaches is shaped by the selection of lineages that are functionally adapted to specific niches (Schauer, Thompson and Brune, 2014). The selection of these lineages most likely occurs via coprophagy, as suggested previously (Nalepa, Bignell and Bandi, 2001). Although the results of our study indicate that the gut community of Panesthiinae is not strongly affected by the differences in sociality, it would be of interest to investigate whether stomodeal feeding in *S. esakii* affects the community composition of the gut microbiota in the younger instars.

3.5.1 Microbial fermentations

The gut of *P. angustipennis* resembles that of other cockroaches in its general morphology and physicochemical conditions (Bracke, Cruden and Markovetz, 1979; Zhang *et al.*, 1993; Lemke *et al.*, 2001; Schauer, Thompson and Brune, 2012). Like omnivorous cockroaches (Lemke *et al.*, 2001; Schauer, Thompson and Brune, 2012), also the panesthiines have an enlarged crop with an acidic pH. The production of soluble sugars by the high endoglucanase activity documented for the crop of several panesthiines (Scrivener, Slaytor and Rose, 1989; Zhang *et al.*, 1993)s apparently stimulates microbial fermentations, which is in agreement with the anoxic, reducing conditions in the crop of *P. angustipennis* (Figure 3.1c) and the accumulation of microbial metabolites in this compartment (Figure 3.2).

The production of lactate, the most prominent fermentation product in the crop, may be partly due to the high abundance of lactic acid bacteria in this compartment. The same phenomenon has been observed already with omnivorous cockroaches (Kane and Breznak, 1991; Schauer, Thompson and Brune, 2012). However, the strong accumulation of hydrogen in the crop (Figure 3.1) is unusual and has not been observed in any other species of cockroaches (Schauer, Thompson and Brune, 2012) or termites (Schmitt-Wagner and Brune, 1999; Pester and Brune, 2007; Köhler *et al.*, 2012) investigated to date. The formation of hydrogen, lactate and acetate — the typical products of a mixed-acid fermentation — is in agreement with the high abundance of *Enterobacteriaceae* in the crop of *P. angustipennis* (Figure 3.4). Unfortunately, the small number of individuals of *S. esakii* available for this study did not allow us to test whether the absence of *Enterobacteriaceae* from its crop was in agreement with the hydrogen concentrations in this compartment.

The circumneutral conditions in midgut and hindgut are similar to those reported for omnivorous cockroaches (Schauer, Thompson and Brune, 2012) and lower termites (Brune, Emerson and Breznak, 1995; Ebert and Brune, 1997). Acetate, the most prominent metabolite in both compartments, is most likely formed by fermentative and homoacetogenic bacteria. An efficient epithelial transport of short-chain fatty acids in the hindgut of *Panesthia cribrata* has been documented already (Hogan, Slaytor and O'Brien, 1985). It is not clear whether the low concentrations of lactate in midgut and hindgut are due to an absorption equilibrium between host and symbionts, as postulated for *Periplaneta americana* (Bracke and Markovetz, 1980), or to a high turnover of the lactate pool, as in lower termites (Tholen and Brune, 2000; Pester and Brune, 2007). Traces of additional fermentation products suggest a higher metabolic diversity of the microbiota in the hindgut compartment. As in omnivorous blaberid (Lemke *et*

al., 2001) and blattid species (Schauer, Thompson and Brune, 2012), hydrogen concentrations in the hindgut compartment were below the detection limit, indicating that the hindgut represents a strong hydrogen sink also in the wood-feeding blaberids.

3.5.2 Methanogenesis

Cross-compartmental transport of hydrogen from the hydrogen-producing midgut to the hydrogen-consuming hindgut compartment has been documented previously in a *Blaberus* sp. (Lemke *et al.*, 2001). This transport was enabled by the juxtaposition of midgut and hindgut in the abdominal cavity of blaberid cockroaches, which explained the high rates of methanogenesis and its strong stimulation by external hydrogen. Also, methanogenesis in *P. angustipennis* and *S. esakii* is strongly stimulated by external hydrogen, which indicates either a limiting production of hydrogen by the microbial fermentations or a competition for hydrogen with other processes, as in the case of certain termites, where a major part of the methanogenic community is situated at the hindgut wall (for details, see Brune 2010). Interestingly, microscopic inspection of the hindgut contents of *P. angustipennis* revealed that a large fraction of the cells with the characteristic autofluorescence of cofactor F_{420} are associated with ciliate protists. The ciliates of *Panesthia* spp. belong to the genus *Nyctotherus* and the morphologically distinct *Clevelandia* and *Paraclevelandia* spp. (Kidder 1937; described also as '*Emmaninius*' spp. by Yamasaki M 1939), which are closely related (Lynn and Wright, 2013) and commonly encountered in many cockroach species (Hackstein, van Alen and Rosenberg, 2006). The cytoplasm of *Nyctotherus* spp. and relatives is densely colonized by *Methanobrevibacter* spp. (Gijzen *et al.*, 1991; van Hoek *et al.*, 2000). Although they are located close to the hydrogenosomes of the host cell (Akhmanova *et al.*, 1998), the absence of hydrogen accumulation in the hindgut and the strong stimulation of methanogenesis by external H_2 indicate that hydrogen production limits methanogenesis.

It is noteworthy that the archaeal communities of *P. angustipennis* and *S. esakii* comprise a high relative abundance of *Methanosarcinales* (Hara *et al.*, 2002), similar to fungus-cultivating and soil-feeding termite species (Ohkuma and Brune, 2011), and a lineage distantly related to *Thermoplasmatales*. The latter were recently identified as representatives of the seventh order of methanogens ('*Methanoplasmatales*'; Paul *et al.* 2012), which are now referred to as *Methanomassiliicoccales* after the first isolate of the order (Dridi *et al.*, 2012) and possess a unique, obligately hydrogen-dependent methylotrophic metabolism (Lang *et al.*, 2014).

3.5.3 Bacterial community structure

The hindgut of *P. angustipennis* contains the most dense and diverse microbiota of all compartments (Table 3.2; Table S1, Supporting Information), which is in accordance with previous studies on other cockroaches (Bignell, 1977b; Cruden and Markovetz, 1984; Schauer, Thompson and Brune, 2012) and termites (Bignell, Oskarsson and Anderson, 1980; Schmitt-Wagner *et al.*, 2003; Köhler *et al.*, 2012). The dominance of lineages of obligate anaerobes from the phyla *Bacteroidetes* and the *Firmicutes* in the hindgut of *P. angustipennis* and *S. esakii* resembles the hindgut microbiota of omnivorous cockroaches (Schauer, Thompson and Brune, 2012; Bertino-Grimaldi *et al.*, 2013) and fungus-cultivating termites (Hongoh, Ekpornprasit, *et al.*, 2006; Shinzato *et al.*, 2007; Otani *et al.*, 2014) but differ fundamentally from the microbial assemblages in wood-feeding higher (Hongoh *et al.*, 2005; Köhler *et al.*, 2012).

Based on the common evolutionary origin of cockroaches and termites (Inward, Beccaloni and Eggleton, 2007), it has been speculated that many elements of the termite gut microbiota originated already from an ancestral cockroach (Nalepa, Bignell and Bandi, 2001; Schauer, Thompson and Brune, 2012; Dietrich, Köhler and Brune, 2014). This notion is supported by the large number of monophyletic lineages of bacteria that consist exclusively of members from the gut of cockroaches and termites. In the present study, such clusters were mostly encountered among the *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, but the conspicuous presence of cockroach sequences in apparently termite-specific clusters in these and other phyla, e.g. *Planctomycetes* and candidate division TM7, had been pointed out already in a previous study of the cockroach *Shelfordella lateralis* (Schauer, Thompson and Brune, 2012).

If the presence of such termite–cockroach clusters were indeed based on vertical transmission of the symbionts, the internal phylogeny of each cluster should reflect that of their respective hosts. However, clear evidence for cospeciation has been provided only in a few cases restricted to the bacterial symbionts of flagellate protists in lower termites (Noda *et al.*, 2007; Ikeda-Ohtsubo and Brune, 2009). In all other cases, the datasets suffer from poor taxon sampling that precludes rigorous testing of cocladogenesis. In other words, none of the clusters contain a sufficient number of bacterial phylotypes from different host lineages.

In a recent high-throughput sequencing analysis of 34 termite and cockroach species, Dietrich *et al.* (2014) documented that the bacterial core microbiota in termites and cockroaches is comprised of a relatively small number of genus-level lineages that are shared among the majority of the members of all major host groups. Here, the hindgut communities of *P.*

angustipennis and *S. esakii* were more similar to each other than to any other cockroach species investigated. Although the amplified 16S rRNA gene fragments were too short to fully resolve the phylogeny in each genus-level bin, the internal branches in the respective trees often contained identical or closely related phylotypes that were derived from different host groups. The authors speculated that the members of these lineages are not derived from a common evolutionary ancestor but must have been acquired independently from the environment.

Also, the present study provides evidence that is in agreement with an environmental selection of specific bacterial lineages. Particularly in the *Bacteroidetes*, it is striking that the *Alistipes*-related clones from cockroach guts are most closely related to clusters consisting exclusively of clones from fungus-cultivating termites (Figure 3.S5, Supporting Information). It cannot be excluded that the missing phylotypes from lower termites (which would bridge the phylogenetic gap between the two host groups) have not been discovered in any clone libraries because of their low abundance. However, the phylogenetic analysis of the reads in the genus level bin of *Alistipes* in the study of Dietrich *et al.* (2014; Figure S4, Supporting Information) rather supports a repeated uptake of *Alistipes* species from an environmental seedbank. Members of the genus *Alistipes* are known to be proteolytic (Song *et al.*, 2006), which may explain their selective colonization of the gut in those host groups that have a protein-rich diet (fungus gardens in the Macrotermitinae and decaying wood infested with fungi in the wood-feeding cockroaches). The same argument would also apply to the presence of *Chitinophagaceae* in the crop of both *S. esakii* and *P. angustipennis*, which are related to saprophytic species capable of degrading chitinous fungal cell walls (Kämpfer, Lodders and Falsen, 2011).

A selection of bacterial lineages with similar functions by particular environmental conditions may also explain some obvious differences in the microbiota of homologous gut compartments. Although both *P. angustipennis* and *S. esakii* harbored large populations of *Streptococcaceae* in their crop, these were represented by phylogenetically distinct lineages (Figure 3.4). It is possible that *Lactococcus* spp. in *P. angustipennis* and *Lactovum* spp. in *S. esakii* occupy the same niche in their respective host.

3.5.4 Cellulose degradation

The independent evolution of wood feeding in Panesthiinae and termites (Klass, Nalepa and Lo, 2008) explains the differences in the digestive strategies between the two groups. While *Panesthia* species have a strongly enlarged crop with high activity of endogenous cellulases

(Scrivener, Slaytor and Rose, 1989), they lack the cellulolytic flagellates characteristic of the hindgut of lower termites (Ohkuma and Brune, 2011; Brune, 2014). In higher termites, which lost all gut flagellates, a community of fiber-associated bacteria apparently took over the niche vacated by the flagellates (Mikaelyan *et al.*, 2014). Since also the hindgut of cockroaches is enlarged and a site of high microbial activity (Schauer, Thompson and Brune 2012; this study), it is possible that also the bacterial microbiota of panesthiines is involved in fiber digestion.

The results of our study document that the wood fiber-associated bacterial lineages in wood-feeding *Nasutitermes* spp. that are also represented in other wood-feeding Termitinae (Hongoh, Deevong, *et al.*, 2006; Warnecke *et al.*, 2007; He *et al.*, 2013; Dietrich, Köhler and Brune, 2014) are absent or not very abundant in panesthiine cockroaches. However, it is possible that the diverse members of *Clostridiales* and *Bacteroidetes* present in *P. angustipennis* and *S. esakii* comprise lineages with fiber-digesting capacities. Such speculations are nourished by the predominance of these phyla also in dung-feeding and humus-feeding Termitinae (Dietrich, Köhler and Brune, 2014) and the differences in the spectrum of carbohydrate-active enzymes in the metagenomes of *Nasutitermes* and *Amitermes* spp. (Warnecke *et al.*, 2007; He *et al.*, 2013). Particularly, the increased abundance of genes related to hemicellulose digestion and clostridial cellulosomes suggest that the fiber-digesting strategies may differ also among higher termites. Therefore, it is possible that also the *Ruminococcaceae* and *Lachnospiraceae* in the hindgut microbiota in wood-feeding (and also omnivorous) cockroaches possess enzymes acting on structural polysaccharides of wood and other cellulosic substrates.

Overall, the similarities in the bacterial hindgut microbiota of the two wood-feeding panesthiines to that of omnivorous cockroaches and fungus-cultivating termites suggest that the structure of the intestinal community is not shaped by vertical inheritance of the individual lineages but by a selection of lineages with an ecological amplitude that matches the conditions in the respective microenvironments. This is in agreement with the observation that the hindgut microbiota of omnivorous cockroaches comprises numerous core taxa at family level but only very few shared phylotypes, even between individuals from the same batch (Schauer, Thompson and Brune, 2012), and would also explain similarities of the microbiota in homologous gut compartments of *P. angustipennis* and *S. esakii*. It is likely that at least in cockroaches, selection by the host environment plays a more important role in shaping the intestinal communities than vertical transmission of particular bacterial lineages.

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4 Diet does not drive bacterial community structure in the gut of litter-feeding cockroaches

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4.1 Abstract

While the gut microbiota of termites and its role in symbiotic digestion have been studied for decades, little is known about the bacteria colonizing the intestinal tract of cockroaches that degrade lignocellulosic matter. Here, we investigate the gut microbiota of three cockroach species that were fed on dried leaf litter, *Ergaula capucina*, *Byrsotria* sp., and *Pycnoscelus surinamensis*, and compare them to that of the previously investigated wood-feeding cockroaches *Panesthia angustipennis* and *Salganea esakii*. Using microsensors, we found that the physicochemical conditions in the gut of the litter-feeding species differed only slightly from those reported previously for other cockroaches. All gut compartments were anoxic at the center and had a slightly acidic to neutral pH and variable, but slightly reducing conditions. By contrast, the localization of hydrogen accumulation in the crop as previously shown in *P. angustipennis* was here reproduced in *Byrsotria* sp., and strongly differs from data on omnivorous species. In all analyzed species, the microenvironmental conditions in the hindgut compartment were the strongest determinant of bacterial community structure. An extended comparison including other cockroaches and termites of diverse feeding groups revealed that most of the core taxa in the hindgut bacterial communities of cockroaches with a lignocellulosic diet were shared with other, omnivorous species but differed strongly from those in wood-feeding higher termites.

4.2 Introduction

Cockroaches and termites are closely related (Cleveland *et al.*, 1934; McKittrick, 1965; Lo *et al.*, 2000; Inward, Beccaloni and Eggleton, 2007; Legendre *et al.*, 2015). Both insect groups are consistently associated with microorganisms (bacteria, archaea, and protists), which densely colonize their intestinal tract (Brune, 2014; Brune and Dietrich, 2015). While the dense gut microbiota of termites and its role in symbiotic digestion have been intensively studied (Brune, 2014), much less is known about the bacteria colonizing the intestinal tracts of the diverse cockroach lineages.

All termites feed on wood or other lignocellulosic materials in different stages of humification. A key role of the gut microbiota is the digestion of cellulose and hemicelluloses. In the evolutionarily “lower” termites, this is done mainly by oxymonadid and hypermastigid flagellates (Honigberg, 1970; Breznak and Brune, 1994), in higher termites (family Termitidae) by specialized fiber-associated prokaryotic communities (Tokuda and Watanabe, 2007; Mikaelyan *et al.*, 2014). The diet of cockroaches is much more diverse (Bell, Roth and Nalepa,

2007). While most cockroaches are omnivorous generalists and feed on a variety of food sources, certain detritivorous lineages have specialized on plant litter or rotting wood, and play critical roles in the turnover of organic matter in forest ecosystems. *Parasphaeria boleiriana* (family *Blaberidae*, subfamily *Zetoborinae*), and all known species of the genera *Panesthia* and *Salganea* (family *Blaberidae*, subfamily *Panesthiinae*) dwell in and feed on rotting logs (Kidder, 1937; Roth, 1979; Pellens, Grandcolas and da Silva-Neto, 2002). This instance of xylophagy most likely evolved independently from that in the Cryptocercidae + Isoptera clade (Pellens, Grandcolas and da Silva-Neto, 2002). Many other detritivorous cockroach species do not feed on degrading wood but other lignocellulosic substrates, such as leaf litter, one of the most abundant fractions of dead plant organic matter (Vitousek, 1982). While the survival of xylophagous Panesthiinae on cellulose, as well as putative contributions of endogenous and gut microbiota, have been investigated previously (Kidder, 1937; Hogan, Slaytor and O'Brien, 1985; Scrivener, Slaytor and Rose, 1989; Zhang *et al.*, 1993; Scrivener and Slaytor, 1994; Scrivener, Watanabe and Noda, 1998), insights into cellulose digestion, as well as the factors determining microbial gut community structure, are lacking for litter-feeding cockroaches.

Niche heterogeneity determines community structure in general (Hutchinson, 1961) and specifically bacterial community structure in the guts of termites (Yang *et al.*, 2005). Among termites, there appears to be an increase in alkalinity of the anterior hindgut with the transition from wood- to humus- and soil-feeders (Brune, Emerson and Breznak, 1995; Brune and Kühl, 1996), and gut compartment-specific microbial communities are associated with distinct physicochemical conditions in both higher termites and wood-feeding panesthiine cockroaches (Bauer *et al.*, 2015; Mikaelyan, Meuser and Brune, 2016). In all cockroaches investigated to date, the gut is slightly acidic to neutral and displays a negative redox potential (Schauer, Thompson and Brune, 2012; Bauer *et al.*, 2015). However, hydrogen accumulates in the posterior midgut in omnivorous *Blaberus* sp. and *Shelfordella lateralis* (Lemke *et al.*, 2001; Schauer, Thompson and Brune, 2012), versus the crop in wood-feeding *Panesthia angustipennis* (Bauer *et al.*, 2015). While the center of all gut compartments is anoxic in adult cockroaches, oxygen present in the early larval stages has been shown to impact microbial community assembly during host development (Tegtmeier *et al.*, 2015). Moreover, in experiments with germ-free cockroaches that were inoculated with gut communities from various hosts, have similar microbial lineages were selected by the gut environment, irrespective of the inoculum (Mikaelyan, Thompson, *et al.*, 2015), suggesting a strong selection pressure by the microenvironmental conditions and the functional niches available in the gut.

While host diet has been identified as the primary determinant of bacterial community structure in the intestinal tract of higher termites (Mikaelyan, Dietrich, *et al.*, 2015), conclusive data from cockroaches is still lacking. In the omnivorous cockroach *Blattella germanica*, diets of different protein content lead to significant changes in community structure and composition (Perez-Cobas *et al.*, 2015), however, the core gut microbiota in *Periplaneta americana* has been shown to be resilient to dietary changes (Tinker and Ottesen, 2016). Likewise, in the omnivorous *Shelfordella lateralis*, potential effects of high-protein and high-fiber diets were masked by individual variation (Schauer, Thompson and Brune, 2014). A comparison of cockroaches and termites from different feeding groups revealed that the bacterial hindgut communities in wood-dwelling Panesthiinae differ slightly from those of omnivorous cockroaches (Dietrich, Köhler and Brune, 2014). However, taxon sampling was insufficient and testing the effect of host diet on hindgut community structure in cockroaches still requires to include additional host species that represent different feeding groups.

To determine the factors shaping bacterial community structure and composition in cockroaches, we characterized the bacterial gut microbiota of three cockroach species fed on leaf litter, *Byrsotria* sp., *Pycnoscelus surinamensis* (both Blaberidae), and *Ergaula capucina* (Corydiidae) using deep-sequencing, and compared them to previously published datasets from other diet groups. We taxonomically analyzed the communities using a phylogenetically curated reference database (DictDb), tailor-made for the accurate identification of bacterial lineages specific to termite and cockroach guts (Mikaelyan, Köhler, *et al.*, 2015). To test the influence of microhabitats on the bacterial community in the guts of these cockroaches, we determined intestinal pH, redox potential, oxygen, and hydrogen partial pressure with microsensors. Here, we hypothesize that the physicochemical environment in each gut compartment defines a distinct microhabitat that selects for a compartment-specific bacterial community. In order to determine if host diet drives bacterial community structure in cockroaches, we compared the core bacterial families in cockroaches with a lignocellulosic diet to those in omnivorous cockroaches and xylophagous higher termites. Within the cockroach species on a lignocellulosic diet, we identified those bacterial families that comprised the core communities of homologous gut compartments.

4.3 Materials and methods

4.3.1 Sampling and dissection

Cockroaches of the species *Ergaula capucina* (Corydiidae – formerly Polyphagidae (Beccaloni and Eggleton, 2013), Corydiinae), *Byrsotria fumigata*, *Byrsotria rothi* (Blaberidae, Blaberinae), and *Pycnoscelus surinamensis* (Blaberidae, Pycnoscelinae) were purchased from a commercial breeder (J. Bernhardt, Halsbrücke, Germany, http://www.schaben-spinnen.de). Colonies were maintained in the laboratory and kept in plastic containers in the dark at room temperature on dried oak leaf litter and water for at least two months.

4.3.2 Microsensor measurements

Intestinal oxygen and hydrogen concentrations, pH, and redox potential were measured with microelectrodes (50-µm tip diameter; Unisense, Aarhus, Denmark). Oxygen and hydrogen microsensors were calibrated as described previously (Brune, Emerson and Breznak, 1995) using synthetic and a H_2/N_2 mixture (5/95, v/v), respectively. The pH microelectrode was calibrated with commercial pH standard solutions of pH 4.0, 7.0, and 10.0. The redox microelectrode was calibrated with saturated solutions of quinhydrone in pH standards of pH 4.0 and 7.0. For pH and redox microelectrodes, the electric potential was measured against a custom-built Ag/AgCl reference electrode. For the measurements, the guts were placed in Plexiglas-faced micro chambers and covered with air-saturated Insect Ringer's solution (7.5 g of NaCl, 0.35 g of KCl, and 0.21 g of $CaCl_2$ per liter).

4.3.3 Library construction

Cockroaches were dissected, and the guts were separated into crop, midgut, and hindgut compartments. The samples obtained from three individuals of each species were pooled in 2-ml tubes containing 750 µl sodium phosphate buffer (120 mM; pH 8.0), and homogenized. DNA was extracted and purified using a bead-beating protocol (Paul *et al.*, 2012). The V3-V4 region of the 16S rRNA genes in each sample was amplified using the universal bacterial primers 343Fmod and 784Rmod (Köhler *et al.*, 2012). In a second amplification, the fragments were tagged with sample-specific hexameric barcodes. Purified PCR products were mixed in equimolar amounts and commercially sequenced (2 × 350 nt paired-end sequencing) on an Illumina MiSeq platform (iTag libraries, GATC Biotech, Konstanz, Germany). The iTag libraries obtained in this study and previously published datasets obtained from termites

(Dietrich, Köhler and Brune, 2014; Mikaelyan, Dietrich, *et al.*, 2015) were processed as previously described (Mikaelyan, Köhler, *et al.*, 2015). Briefly, reads with a minimum length of 250 bp and a maximum expected error of 0.5 were selected, and the sequence pairs were merged when applicable. The resulting contigs were quality-trimmed (minimum length of 200 nucleotides, no homopolymers > 10 nucleotides, no ambiguities, average phred score > 25 on a moving window of five nucleotides), and the barcode and primer sequences removed using *mothur* (Schloss *et al.*, 2009). Sequences in each sample were clustered at a threshold of 99% similarity using *dnaclust* (Ghodsi, Liu and Pop, 2011). Next, sequences were de-replicated and aligned using the *mothur* aligner.

4.3.4 Comparison of community structure

Aligned sequences were screened, degapped, and clustered into operational taxonomic units (OTUs) at 97% sequence similarity. The OTUs were assigned to taxonomic groups using the naïve Bayesian classifier implemented in *mothur* with a confidence threshold of 80% in combination with a manually curated reference database DictDb v.3.0 (Mikaelyan, Köhler, *et al.*, 2015). The libraries were subsampled to the size of the smallest sample (53,896 reads per sample for the comparison between the nine samples in this study; 1,643 for the comparison between all hindgut communities from 28 hosts). Community structure was compared using the taxonomy-dependent Bray-Curtis metric (based on the classification results), a statistic used to quantify the compositional dissimilarity between two different samples, based on counts in each sample (Bray and Curtis, 1957), and using the phylogeny-dependent weighted UniFrac algorithm (Lozupone and Knight, 2005) embedded in *mothur*. The high dimensionality of the pairwise dissimilarity scores was then compressed to two dimensions via non-metric multidimensional scaling (NMDS) using the *vegan* package in R (Oksanen *et al.*, 2016). Covariance between community structure, gut compartment, and physicochemical parameters was determined by permutational multivariate analysis of variance (PERMANOVA) and visualized using canonical correspondence analysis (CCA), both implemented in the *vegan* package.

4.3.5 Analysis of core microbial taxa

To identify core microbial lineages, all unclassified reads and all reads in taxa represented by fewer than 10 reads were removed from the dataset. Bacterial genera that were present in at least 70% of all samples from a group of insect hosts or from a specific compartment were

considered core genera of this group. For bacterial families, this threshold was set to 100%. The similarity on family level between the gut communities of the five cockroach species with a lignocellulosic diet was determined using the *Morisita Horn* index (Morisita, 1959; Horn, 1966), and visualized using an arc diagram, implemented in the *vegan* and *arcdiagram* (Sanchez, 2014) packages in *R*, respectively.

4.4 Results

4.4.1 Physicochemical conditions in different gut compartments

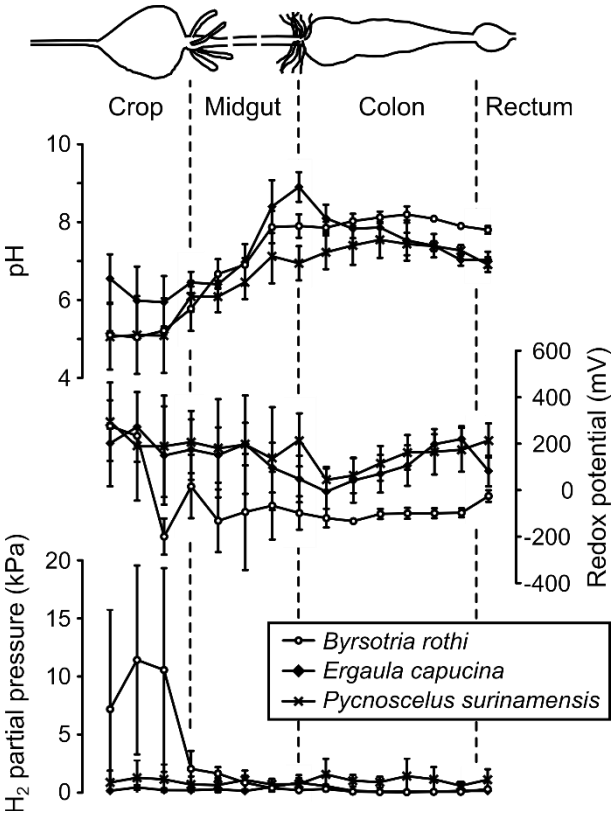

Figure 4.1 | Axial profiles of intestinal pH, redox potential, and hydrogen partial pressure in the gut of the litter-feeding cockroaches *Byrsotria rothi*, *Ergaula capucina*, and *Pycnoscelus surinamensis* determined with microsensors. All measurements were made at the gut center; symbols indicate means with standard error of three guts.

We obtained axial profiles of pH, redox potential and hydrogen partial pressure in the intestinal tracts of *Byrsotria rothi*, *Ergaula capucina*, and *Pycnoscelus surinamensis* (Figure 4.1). All gut compartments were anoxic at the center. In all species, the pH was slightly acidic in the crop and increased steadily along the midgut to neutral or slightly alkaline values in the hindgut. Only in *E. capucina*, the pH showed a distinct maximum at the midgut/hindgut junction, but slowly decreased again to neutral in the posterior hindgut. Redox potential was highly variable in foregut and midgut but consistent in the hindgut compartment of all species.

Although all compartments were anoxic at the gut center (not shown), negative redox potentials (–100 to –200 mV) were observed only in *B. rothi*. In the other species, the values ranged from +100 to +200 mV even in the dilated hindgut. Hydrogen partial pressures in all gut compartments were either low (0.3–3.5 kPa) or below the detection limit (< 0.1 kPa in the hindguts of *B. rothi*). Only *B. rothi* showed a moderate accumulation of hydrogen in the crop (6–21 kPa).

4.4.2 Community structure of homologous gut compartments

The V3-V4 region of the 16S rRNA genes in the three gut compartments of the three cockroach species was amplified with universal, barcoded primers. We obtained an average of 1.2×10^5 high-quality sequence reads per sample, and identified a total of 4,297 OTUs (at 97% sequence similarity), with an average of 1,154 OTUs per sample (Table 4.1). Rarefaction curves (Good, 1953) of the sampling depth of OTUs showed that 99.3–99.7% of all expected OTUs per sample were recovered. The highest numbers of OTUs were found in the hindgut communities. Accordingly, the hindgut communities displayed also the highest richness, diversity and evenness. Classification success was >97% at class level, and >72% at family level in all samples. However, classification success dropped to 42–77% at genus level, indicating that many sequence reads still lacked representatives in the reference database.

Table 4.1 | Properties of the iTag libraries from the gut compartments crop (C), midgut (M), and hindgut (H) of *Ergaula capucina*, *Byrsotria fumigata*, and *Pycnoscelus surinamensis* fed on oak leaf litter. Coverage (Good, 1953) of OTUs was 99.3–99.7% in all samples.

				Diversity indices[a]			Classification success (%) at the level of			
Host species	Sample	Total no. of reads	No. of OTUs (97% sim.)	Richness	Diversity	Evenness	Class	Order	Family	Genus
Ergaula capucina	C	169,596	1,116	1,520	4.10	0.584	99.3	97.0	78.5	47.5
	M	114,698	1,166	1,503	3.64	0.516	98.1	95.2	77.8	47.2
	H	53,896	1,515	1,583	5.78	0.789	97.4	95.4	89.0	64.4
Byrsotria fumigata	C	193,791	905	1,422	3.45	0.506	99.5	98.4	72.3	41.7
	M	68,113	810	1,012	3.66	0.547	99.0	96.9	76.5	50.1
	H	58,848	1,437	1,521	5.31	0.730	98.6	95.7	84.2	64.4
Pycnoscelus surinamensis	C	170,089	1,080	1,375	3.28	0.469	98.8	98.1	86.6	56.8
	M	100,215	1,076	1,405	3.05	0.437	99.3	97.8	89.9	77.0
	H	151,774	1,284	1,669	4.88	0.681	99.6	98.1	92.3	73.2

[a] Based on OTUs. Richness, Chao1 estimator (Chao, 1984); diversity, nonparametric Shannon index (Chao and Shen, 2003); evenness index (Legendre and Legendre, 1998).

In total, 99.3% of the reads could be assigned to 28 phyla in the DictDb taxonomy. The bacterial communities in all samples were dominated (on average) by *Firmicutes* (43%), *Bacteroidetes* (24%), *Proteobacteria* (17%), and *Actinobacteria* (8%) (Figure 4.2). *Actinobacteria* and *Firmicutes* decreased in abundance from crop to midgut to hindgut, whereas *Bacteroidetes* increased. The mid- and hindgut of *E. capucina* also contained *Fibrobacteres* (1%). The crop communities were dominated (on average) by lineages from the *Bifidobacteriaceae*, *Lactobacillaceae*, *Lachnospiraceae* (all *Firmicutes*), and *Pseudomonadaceae* (*Proteobacteria*), which together represented more than a third of the reads. By contrast, hindgut communities were dominated by *Porphyromonadaceae* and *Rikenellaceae* (both *Bacteroidetes*), *Lachnospiraceae* and *Ruminococcaceae* (both *Firmicutes*), accounting (on average) for roughly two thirds of the reads.

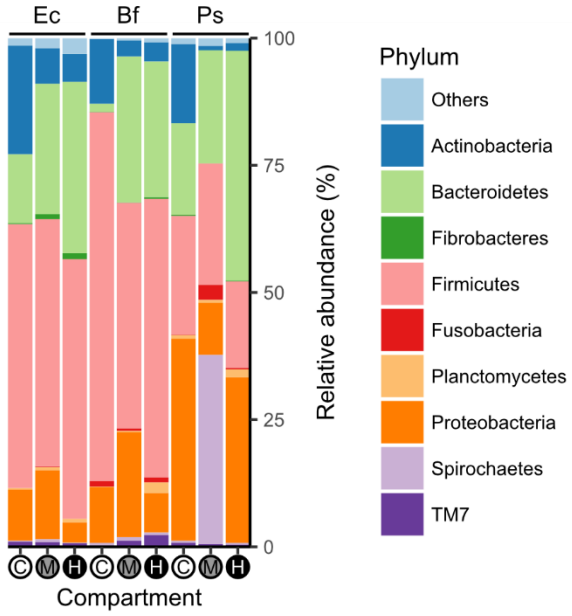

Figure 4.2 | Relative abundance of bacterial phyla in the crop (C, white), midgut (M, grey), and hindgut (H, black) of *Ergaula capucina* (Ec), *Byrsotria fumigata* (Bf), and *Pycnoscelus surinamensis* (Ps) fed on oak leaf litter.

Several abundant bacterial genus-level groups were shared between consecutive gut compartments. For instance, *Bacteroides* (0.1–8.6%) and *Dysgonomonas* (0.1–18.3%) species were present in all gut compartments of the three host (Figure 4.3). The relative abundances of the 30 genus-level groups that contributed most to differences between samples, according to PCA loading factors, are shown as a heat map. While several *Lactobacillus* and one *Enterococcus* species were consistently found in high abundance in the crop and midgut, the

hindgut harbored mainly bacteria belonging *Bacteroidaceae*, *Porphyromonadaceae*, and *Lachnospiraceae*, many of which remained unclassified at the genus level. *Pycnoscelus surinamensis* presented an exception to this trend, where individual bacterial lineages like

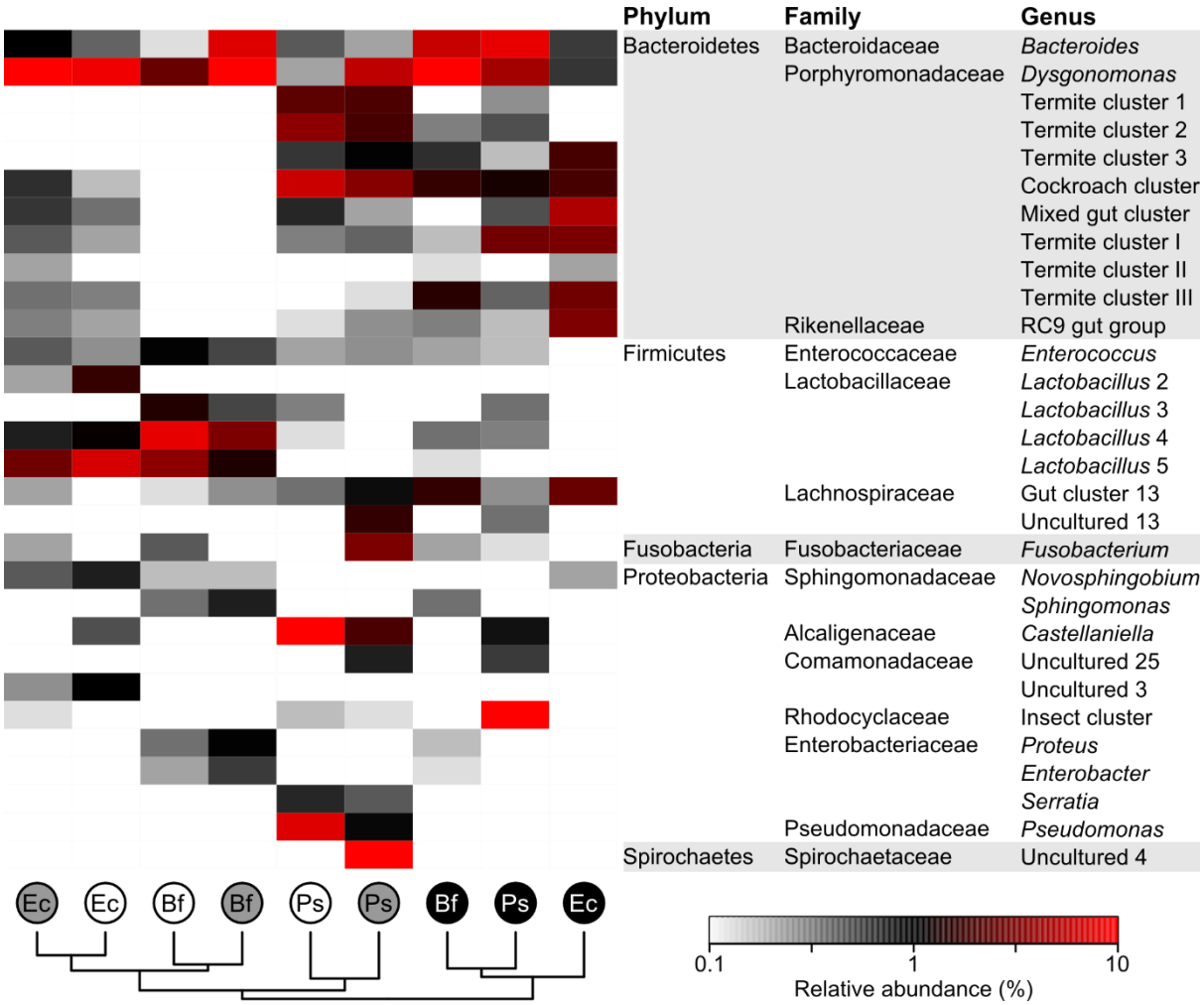

Figure 4.3 | Heat map of the 30 most abundant genus-level groups in the crop (white), midgut (grey), and hindgut (black) of *Ergaula capucina* (Ec), *Byrsotria fumigata* (Bf), and *Pycnoscelus surinamensis* (Ps) fed on oak leaf litter. Phylogram indicates hierarchical cluster analysis of all classified reads (*hclust*, euclidian distances).

Castellaniella and *Pseudomonas* in the crop, uncultured *Spirochaetaceae* in the midgut, and uncultured *Rhodocyclaceae* in the hindgut made up a major part of the bacterial community.

Canonical correspondence analysis (CCA) between bacterial community structure and environmental variables such as physicochemical conditions, compartment, and host species, constrained 92.4% of the variance in bacterial community structure, and revealed the variables with the highest impact (Figure 4.4). Intestinal pH and the hindgut compartment both corresponded significantly with changes in gut community composition (Table S2). Several bacterial lineages, most notably *Ruminococcaceae*, *Rikenellaceae*, and *Porphyromonadaceae*, were typically associated with the hindgut compartment, high pH, and low hydrogen partial pressure. Contrastingly, lineages like *Lactobacillaceae* and *Enterobacteriaceae* corresponded

with lower pH and higher hydrogen partial pressure. The crop and midgut of *P. surinamensis* presented outliers to the other samples, partially due to high redox potential in these samples.

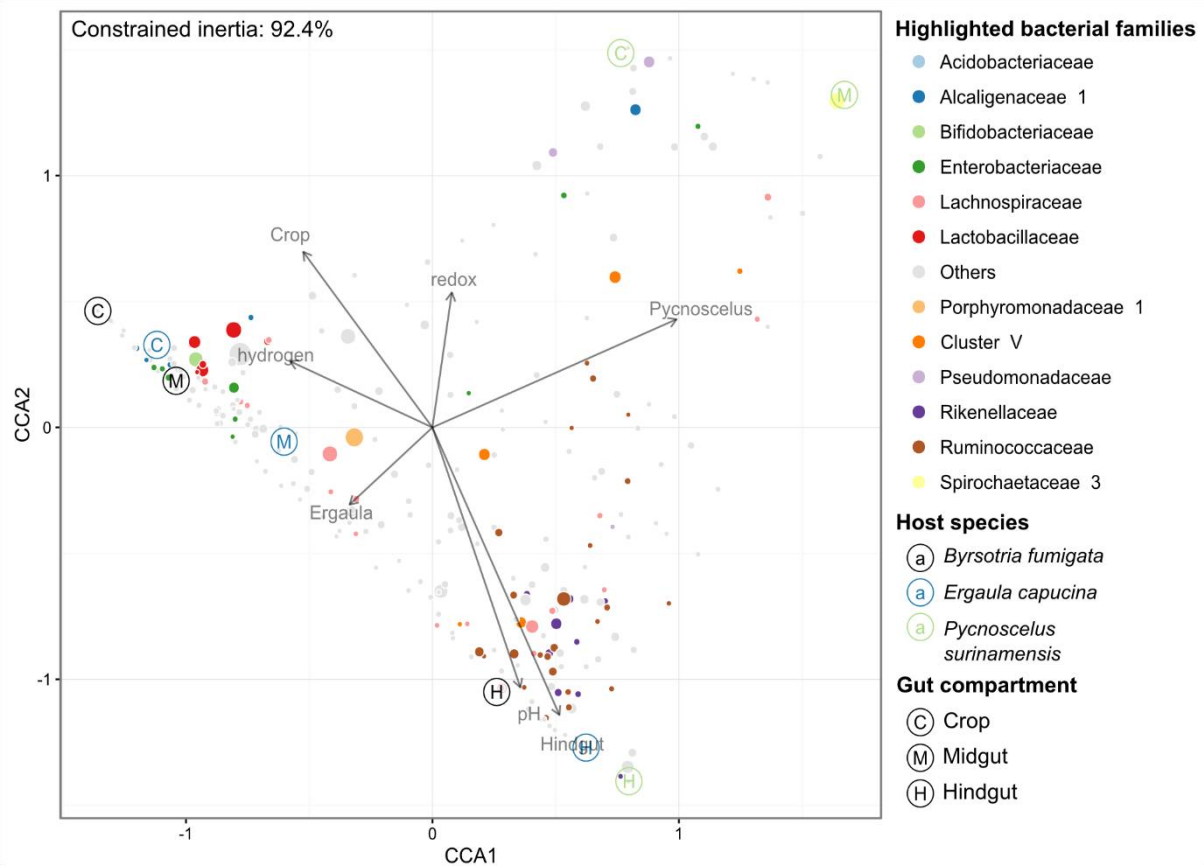

Figure 4.4 | Canonical correspondence analysis (CCA) of bacterial genera and environmental variables in gut compartments of litter-feeding cockroaches. Each of the 435 bacterial genus-level groups was tested for covariance with the environmental variables pH, hydrogen partial pressure, and redox potential, as well as with insect host (*Ergaula capucina*, *Byrsotria fumigata*, *Pycnoscelus surinamensis*,) and gut compartment (crop, midgut, hindgut). Each dot represents one bacterial genus-level group, size corresponds to mean relative abundance. Averages of the whole communities are given by circled letters. The position of a genus or community along the axis of an environmental variable indicates the level of correspondence between the genus or sample and the environmental variable. Constrained inertia is equivalent to the total variance constrained by all environmental variables combined.

Core bacterial families were identified for each of the three homologous gut compartments of the five lignocellulose-feeding cockroaches. We found a strong similarity between the communities from the hindgut compartments of different species, and little similarity between those from different gut compartments of the same species (Figure 4.5). However, the communities from *Pycnoscelus surinamensis* deviated sharply from this trend, showing only little similarity to those of any other cockroach. The average contribution of the core families to the overall abundance increased from crop to midgut and hindgut (37, 66, and 81% of the bacterial community, respectively). In the hindgut, the 18 core bacterial families (Tab. S3) accounted for 64 - 93% of the bacterial community. This was mostly due to the comparably low abundance of lineages that were typically highly abundant in all the other lignocellulose-

feeding species (e.g., *Lachnospiraceae* and *Ruminococcaceae* in the hindgut) or high abundance of lineages that were not so abundant in other lignocellulose-feeding cockroaches (e.g., *Rhodocyclaceae*, uncultured *Spirochaetaceae*; Fig. 2). Several core bacterial families made up for a major part of the bacterial communities, especially in the hindgut. The different lineages of the polyphyletic *Porphyromonadaceae* together comprised the most abundant bacterial family in both midgut and hindgut of lignocellulose-feeding cockroaches, covering on average 22 and 23% of the bacterial community, respectively. However, *Porphyromonadaceae*_1 were more abundant in the midgut, while *Porphyromonadaceae*_2, as well as previously undescribed members binned to *Porphyromonadaceae*, "Cluster V" and "Gut group", were more abundant in the hindgut. More lineages that accounted for 22% of the bacterial hindgut community fell within the *Ruminococcaceae*, most of which had no cultured representatives (e.g., "Insect cluster", "gut cluster" and "uncultured"). Members of the genus *Ruminococcus* were abundantly represented in the hindgut of *Ergaula capucina* and *Byrsotria fumigata*, while *Papillibacter* was present in all hindguts except *Pycnoscelus surinamensis*. *Lachnospiraceae* made up on average 12 and 13% of the bacterial community in the crop and midgut of the lignocellulose feeders and were represented by several major uncultivated

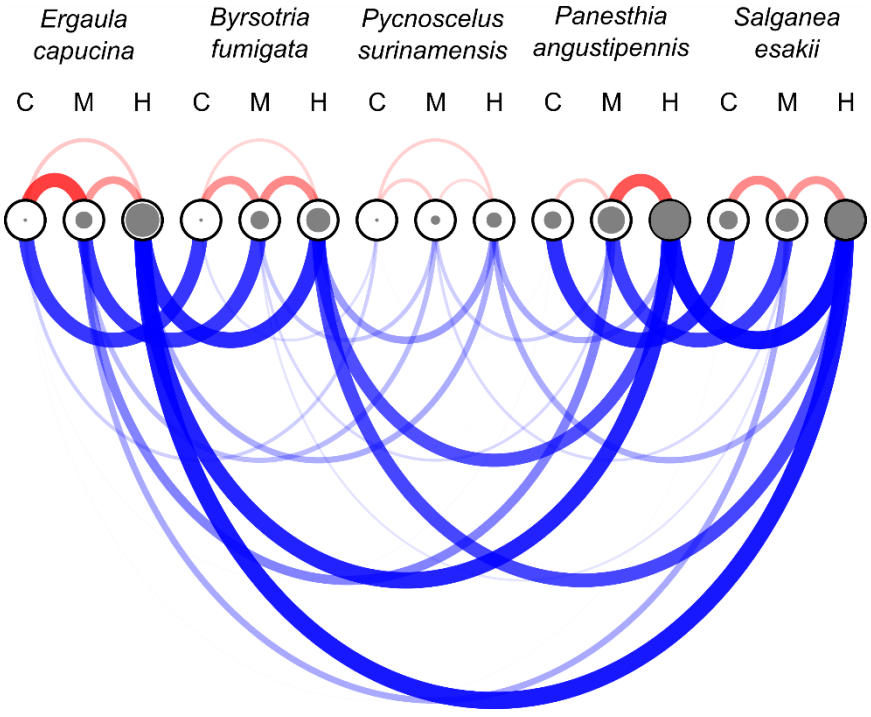

Figure 4.5 | Arc diagram showing similarity of gut bacterial communities on family level (arcs) and abundance of compartment-specific core bacterial families (size of grey circles) in five lignocellulose-feeding cockroaches. Each edge connects two bacterial communities indicated by the vertices, either between (C) crop, (M) midgut, and (H) hindgut of the same species (red, above), or between homologous gut compartments of different species (blue, below). Arc width and opacity represent the Morisita Horn similarity index (Morisita, 1959) based on family-level classification between the respective communities. The size of the grey dots in the nodes represents the relative abundance of gut compartment-specific core bacterial families.

lineages in the hindgut, like “gut cluster 13” or “Incertae sedis 30”. *Ca*. Arthromitus made up 17% and 10% of the midgut community of *Panesthia angustipennis* and *Salganea esakii*, respectively. *Endomicrobiaceae* were found in very low abundance (≤0.8%) in the hindgut communities of all lignocellulose feeders.

4.4.3 Effect of diet on gut community structure

To evaluate the impact of host diet, we checked if the hindgut communities in cockroaches with a lignocellulosic diet shared core bacterial genera and families with those in wood-feeding termites. The gut microbiota of wood- and litter-feeding cockroaches was screened for bacterial taxa that comprised the core bacterial community in wood-feeding higher termites. The genus level groups that were present in at least 70% of all cockroaches made up on average 72% of the bacterial community in cockroaches, but only 8% in wood-feeding higher termites, indicating that only few lineages were shared between cockroaches and wood-feeding higher termites (Figure 4.6A). To rule out the possibility that co-evolving bacterial lineages were phylogenetically too far apart to be captured at the genus level, and assuming that members of the same bacterial family often carry out similar metabolic processes, we also determined core taxa at the family level. A high number of bacterial families were shared between wood- and litter-feeding cockroaches (Figure 4.6B). These lineages made up more than 90% of the relative bacterial abundance in the wood-feeding Panesthiinae, and, on average, 60% in the litter-feeding species (Figure 4.6B). Contrastingly, while the proportion of taxa from these core families in termites feeding on humus, litter, or fungus was comparable to that in wood-feeding termites, the relative abundance of these bacterial groups in humus-, litter-, and fungus-feeding termites was around 60% as opposed to 25% of the bacterial community in wood-feeding termites. 13 out of the 18 core families in lignocellulose-feeding cockroaches were present in litter-feeding termites, more than in any other termite feeding group. However, the relative abundance of the lignocellulose-feeding cockroach core families was, among the termites, highest in fungus- or soil-feeding termite species. Litter-feeding cockroaches and termites were also similar in terms of total number of bacterial families (58 and 66, respectively). The relative abundance of the lignocellulose-feeding cockroach core families across all cockroaches ranged from 63 to 96 %, and within the cockroaches was lowest in the litter-feeding cockroaches. Between six and nine of the eleven bacterial families that made up the core community in wood-feeding termites were also present in the cockroach hindgut community, but made up a much smaller part of the total diversity and relative abundance (Figure 4.6C). Overall, the

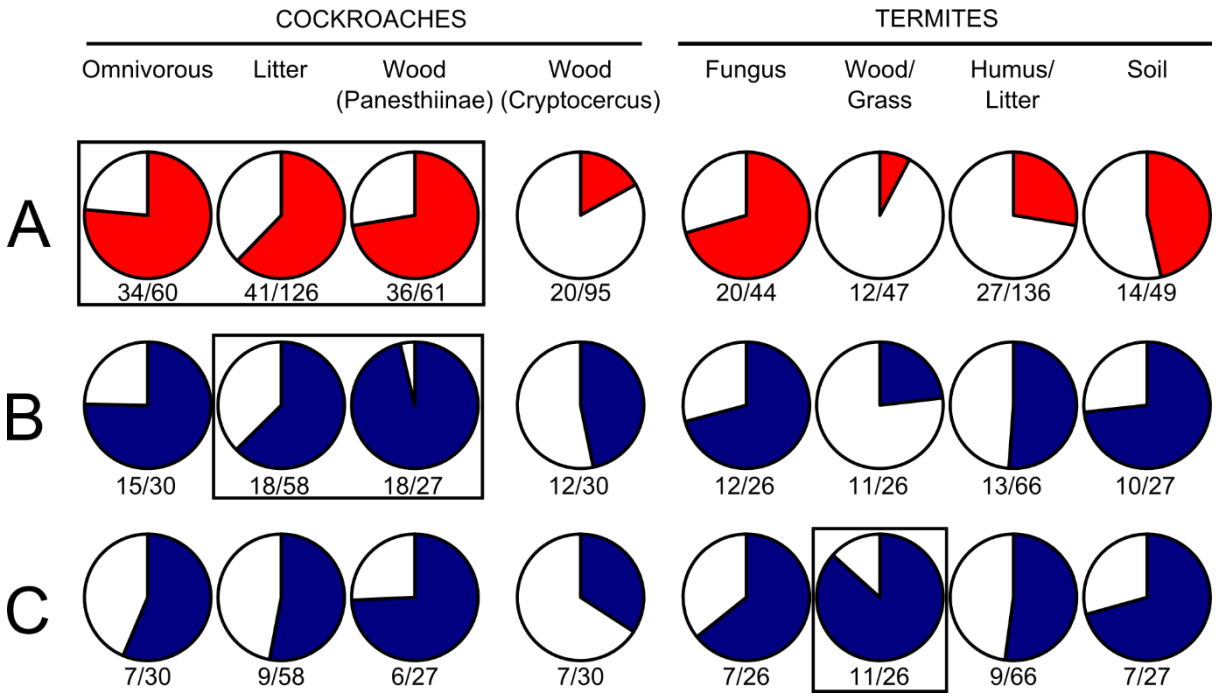

Figure 4.6 | Core bacterial taxa in the hindgut of different feeding guilds of cockroaches and higher termites. The pie charts represent the average proportion of reads from core bacterial genera (red) and families (blue) relative to the entire bacterial community. The rectangles indicate the host group for which the core bacterial groups were calculated: (A) all cockroaches except *Cryptocercus punctulatus*, (B) wood and litter-feeding cockroaches, and (C) wood-feeding higher termites. Bacterial genera present in >70% (A) and bacterial families present in all (B, C) of the hosts grouped by a rectangle were considered core linages for that particular host group. Numbers below the charts provide the average proportion of core taxa over the total number of taxa. Those bacterial lineages that were part of the core community in wood and litter-feeding cockroaches (B) were very low in abundance in wood-feeding termites.

hindgut bacterial communities of cockroaches with a lignocellulosic diet featured core bacterial taxa different from those of wood-feeding termites.

In some cases, similar core patterns on the family level between the different host feeding groups were due to the abundance of different genus-level lineages within the same family. For instance, *Lachnospiraceae* contributed, on average, 13 and 25 % of the bacterial community in lignocellulose-feeding cockroaches and soil-feeding termites, respectively. However, while the undescribed "Gut cluster 13" within this family was among the most dominant genus-level group in both host groups, soil-feeding termites additionally featured *Ca.* Arthromitus in high relative abundance (Table S4). The *Rikenellaceae* were represented by *Alistipes* II in fungus-feeding termites, *Alistipes* IV in lignocellulose-feeding cockroaches, and *Alistipes* III and IV in omnivorous cockroaches. Notably, the *Acholeplasmataceae*, represented in most cockroaches by the genus *Acholeplasma* with up to 1.4 % of the bacterial community, was completely absent in all higher termites and *Cryptocercus punctulatus*.

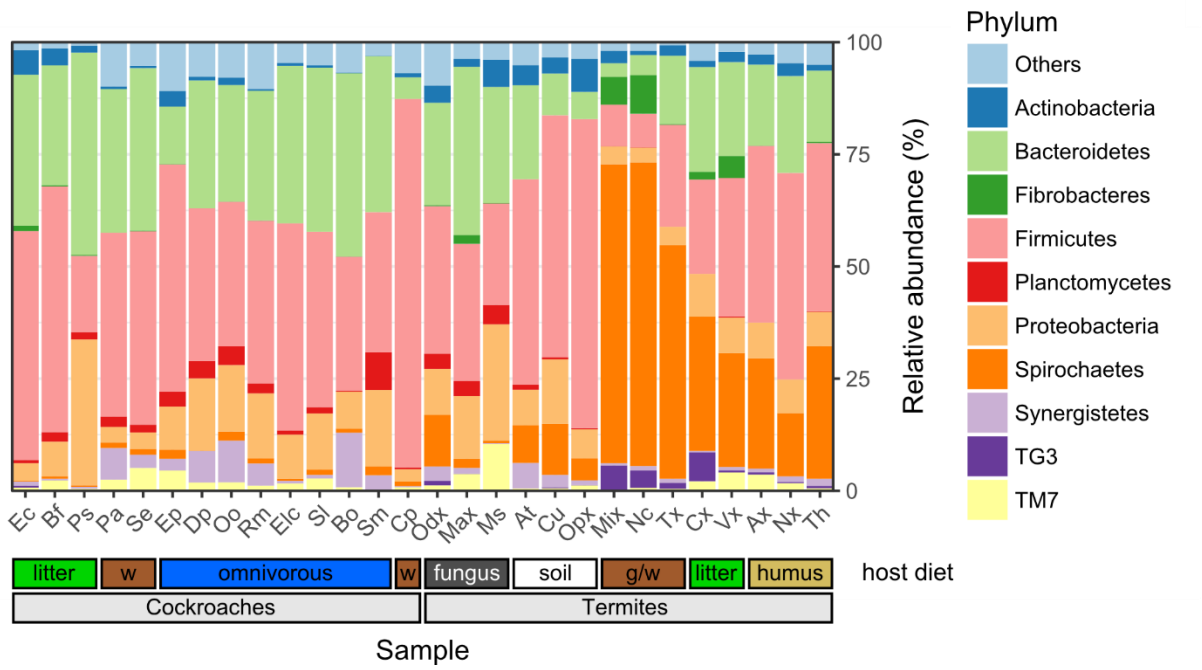

Figure 4.7 | We compared the bacterial hindgut community of cockroaches and termites of different feeding groups. Bacterial phyla with a mean relative abundance < 0.7% were summarized as "Others". Host species include *Ergaula capucina* (Ec), *Byrsotria fumigata* (Bf), *Pycnoscelus surinamensis* (Ps), *Panesthia angustipennis* (Pa), *Salganea esakii* (Se), *Eublaberus posticus* (Ep), *Diploptera punctate* (Dp), *Opisthoplatia orientalis* (Oo), *Rhyparobia maderae* (Rm), *Elliptorhina chopardi* (Elc), *Shelfordella lateralis* (Sl), *Blatta orientalis* (Bo), *Symploce macroptera* (Sm), *Cryptocercus punctulatus* (Cp), *Odontotermes* sp. (Odx), *Macrotermes* sp. (Max), *Macrotermes subhyalinus* (Ms), *Alyscotermes trestus* (At), *Cubitermes ugandensis* (Cu), *Ophiotermes* sp. (Opx), *Microcerotermes* sp. (Mix), *Nasutitermes corniger* (Nc), *Trinervitermes* sp. (Tx), *Cornitermes* sp. (Cx), *Velocitermes* sp. (Vx), *Atlantitermes* sp. (Ax), *Neocapritermes* sp. (Nx), *Termes hospes* (Th).

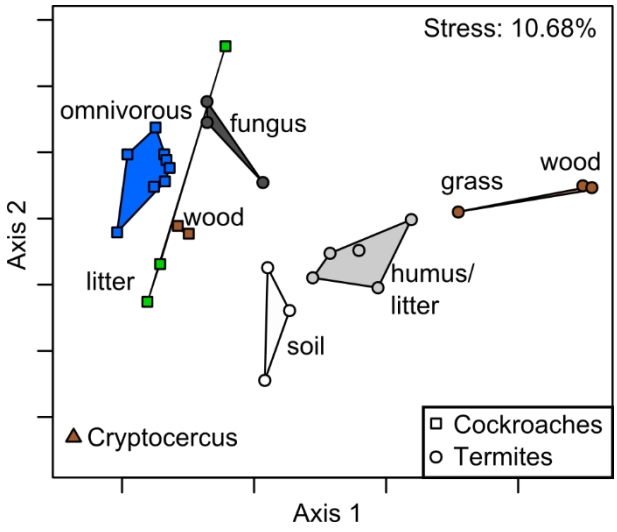

Figure 4.8 | Similarity between the hindgut microbiota of cockroaches and higher termites visualised by non-metric multidimensional scaling (NMDS) of the weighted UniFrac metric. Polygons circumscribe samples from cockroaches and higher termites (by subfamily). Symbols indicate host diet. Wood feeding cockroaches included *Panesthia angustipennis* and *Salganea esakii*. Litter feeders from this study: *Ergaula capucina*, *Byrsotria fumigata*, and *Pycnoscelus surinamensis*. For details on the other species, see Supplementary Table S5.

We compared the hindgut community composition to the hindguts of other cockroaches and higher termites from previous studies (Dietrich, Köhler and Brune 2014; Mikaelyan *et al.* 2015a). Many gross differences observed among the hosts were apparent already at the phylum level (Figure 4.7). Overall, the hindgut communities of cockroaches were clearly distinct in community structure from those of higher termites, and the hindgut communities of the wood-feeding *Panesthia angustipennis* and *Salganea esakii* were distinct from those of all other cockroaches. A majority of the reads in cockroaches were assigned to *Firmicutes* and *Bacteroidetes*.

A comparison of the bacterial community structure based on the weighted UniFrac metric (Figure 4.8) showed that hindgut communities of litter-feeding cockroaches were most similar to those in omnivorous species, but displayed also more variation.

A closer look at just the cockroach hindgut communities featured many bacterial genus-level groups – particularly among *Bacteroidetes* and *Firmicutes* – that were shared between all cockroach species (Figure 4.9). Shared lineages included genera within the *Porphyromonadaceae*, e.g., from the genera *Dysgonomonas*, *Butyricimonas*, *Paludibacter*, and *Tannerella*. The most universally lineages with the highest mean relative abundance across all

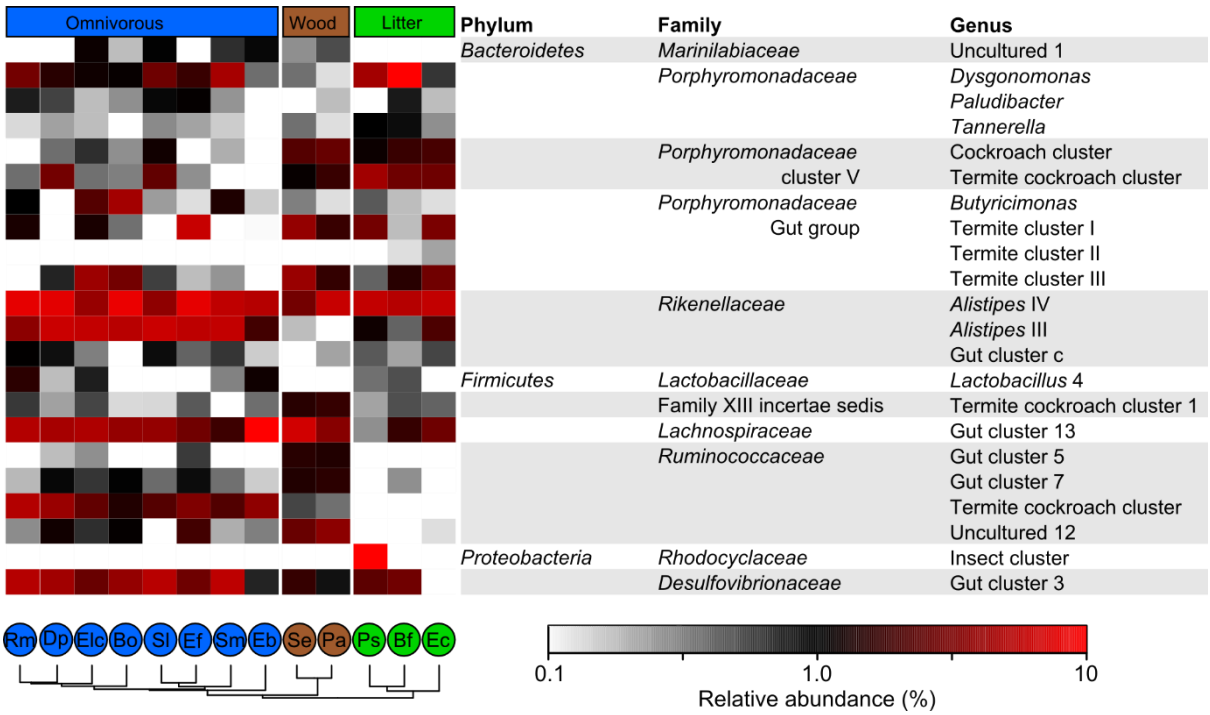

Figure 4.9 | Heat map of the 22 most abundant bacterial genus-level groups in the hindgut of omnivorous (blue), wood (brown)- and litter-feeding (green) cockroaches. Hosts are *Rhyparobia maderae* (Rm), *Diploptera punctate* (Dp), *Elliptorhina chopardi* (Elc), *Blatta orientalis* (Bo), *Shelfordella lateralis* (Sl), *Eurycotis floridana* (Ef), *Symploce macroptera* (Sm), *Eublaberus posticus* (Eb), *Salganea esakii* (Se), *Panesthia angustipennis* (Pa), *Pycnoscelus surinamensis* (Ps), *Byrsotria fumigata* (Bf), and *Ergaula capucina* (Ec).

cockroach hindguts were found in the genus *Alistipes* (*Rikenellaceae*). While the individual *Alistipes* lineages I and II never exceeded 1.3 and 2.4% in relative abundance, group III and IV reached mean/maximum relative abundance of up to 3.5/7.6%, and 5.7/11.7%, respectively. More shared lineages between all cockroaches were presented by the so far uncultured "Gut cluster 13" (*Lachnospiraceae*) However, a few lineages were enriched specifically in the guts of cockroaches with a lignocellulosic diet, e.g., two unclassified lineages of *Porphyromonadaceae* "Cluster V" (i.e., "Cockroach cluster" and "Termite cockroach cluster").

4.5 Discussion

Our high-resolution taxonomic analysis of the gut microbiota, combined with the determination of key physicochemical parameters (pH, redox potential, and hydrogen partial pressure), in the different gut compartments of litter-feeding cockroaches reveal the importance of the gut habitat as a major driver of bacterial community structure. The homologous gut compartments of five lignocellulose-feeding cockroaches share a characteristic distribution and abundance of bacterial families. Members of these core families typically represented the majority of the microbial community in the hindgut. We will discuss how environmental factors like pH, redox potential, and hydrogen partial pressure may select the same microbial lineages, and thus drive community structure in the guts of lignocellulose-feeding cockroaches.

4.5.1 Differences in hydrogen accumulation

Physicochemical conditions in the gut of litter-feeding cockroaches were not fundamentally different from those in omnivorous (Schauer, Thompson and Brune 2012) and wood-feeding (Bauer et al. 2015) species. However, while redox potential was negative in *Byrsotria rothi*, it was mainly positive in both *Pycnoscelus surinamensis* and *Ergaula capucina*. While slight acidity in the crop has been connected to putative fermentation of ingested sugars (Wigglesworth, 1927), the neutral conditions in the hindgut may be connected to the excretory fluid of the Malpighian tubules, whose nitrogenous compounds are expected to provide substantial buffering capacity (Mullins and Cochran, 1973). Interestingly, in two of the cockroaches with a lignocellulosic diet that were examined to date, *Panesthia angustipennis* (Bauer *et al.*, 2015) and *Byrsotria rothi* (this study), hydrogen accumulates in the crop up to a magnitude of 30 and 21 kPa. This may be due to a highly active hydrogen-producing community and the lack of hydrogen consuming processes, such as acetogenesis or

methanogenesis. By contrast, hydrogen accumulation in the omnivorous cockroaches *Blaberus* sp. and *Shelfordella lateralis* is restricted to the midgut or anterior hindgut, reaching magnitudes of 29 and 24 kPa, respectively (Lemke *et al.*, 2001; Schauer, Thompson and Brune, 2012). The complete absence of hydrogen pools in the medial and posterior hindgut of all cockroaches that accumulate hydrogen in the anterior compartments poses the question if hydrogen-consuming processes, like methanogenesis in *Blaberus* sp. or acetogenesis, are typical in the hindgut of all cockroaches (Lemke *et al.* 2001; Schauer, Thompson and Brune 2012; this study).

4.5.2 Microenvironmental conditions as determinant of community structure

All hindguts of wood and litter feeders had a similar bacterial community composition, suggesting that the hindgut provides essentially the same microhabitat with similar niches that select for the same bacterial lineages. Also, the hindgut was the only compartment with a core community that comprised a major proportion of the total bacterial abundance in both litter- and wood-feeding species. Contrarily, crop and midgut bacterial communities were more similar within the respective host species (Figure 4.3, Figure 4.5). We therefore assume that these anterior compartments do not enrich specific microbial lineages to large densities but simply provide new lineages to the hindgut as the main fermentation chamber of the intestinal tract that selects specific bacterial lineages. The occurrence of similar bacterial lineages across different host species may be explained by stochastic uptake from the environment, e.g., through coprophagy, which has been shown to be beneficial for development of the first instar in *Blattella germanica* (Kopanic et al. 2001), and may be common in many cockroach species (Nalepa, Bignell and Bandi 2001; Nalepa 2015). Overall, our finding that specific bacterial lineages correspond strongly with specific physicochemical parameters suggests that the microhabitat of the gut is a strong selecting factor for the microbial community, and is in agreement with recent insights from compartment-specific bacterial communities in higher termites (Mikaelyan, Meuser and Brune, 2016). It also supports the hypothesis that most microbial ecosystems are dominated by specialist taxa (Mariadassou, Pichon and Ebert 2015).

4.5.3 Host diet and putative cellulose digestion

One major hypothesis on the assembly of intestinal communities concerns the diet of the host. Our results confirm previous findings that the gut communities in cockroaches are overall similar, and distinct from those in termites (Dietrich, Köhler and Brune, 2014). The hindgut

communities of the litter-feeding species were overall similar to those of the omnivorous species, but more variable and diverse. The two most dominant phyla in these communities, *Firmicutes* and *Bacteroidetes*, have been reported to characteristically dominate the gut communities of most fungus-feeding termites (Otani *et al.*, 2014). Recent studies have revealed a stark similarity in gut community structure between fungus-feeding higher termites and their primitive relatives, the cockroaches (Dietrich, Köhler and Brune, 2014) — an unexpected similarity that possibly represents a convergent adaptation of the microbiota to a protein-rich diet in cockroaches and macrotermitine termites. Simultaneously, the hindgut community of all cockroaches was very similar, with the exception of two wood-feeding species.

In our analysis, the core families in lignocellulose-feeding cockroach made up on average 63 % or more of the relative abundance across all cockroach feeding groups. Interestingly, these core lineages were more abundant in the omnivorous cockroaches than in the litter-feeding species. We found that most of the hindgut core microbial lineages were not shared between lignocellulose-feeding cockroaches and wood-feeding termites. An exception to this overall trend was presented by *Fibrobacteres* colonizing the midgut and hindgut of *Ergaula capucina*, albeit in low relative abundance (1%). In wood-feeding higher termites, *Fibrobacteres* constitute members of the fiber-associated community contributing to cellulose degradation (Mikaelyan *et al.*, 2014).

While host diet has been shown to change the hindgut community in the omnivorous *Blattella germanica* (Perez-Cobas *et al.*, 2015), no such effect has been observed in *Shelfordella lateralis* (Schauer, Thompson and Brune, 2014), and in *Periplaneta americana* the core gut community appears to be stable and resilient to changes in diet (Tinker and Ottesen, 2016). The three litter-feeding cockroach species examined in this study can be maintained on a lignocellulosic diet consisting of litter and water, but typically feed on the softer leaf material and leave the more recalcitrant leaf parts (stems etc.) behind (observations from lab colonies). While it is known that termites degrade almost all of the cellulose (Breznak and Brune, 1994) but hardly any of the lignin (Griffiths *et al.*, 2013) contained in their diet, comparable data from cockroaches is still lacking. Since the hindgut microbiotas of panesthiine cockroaches dwelling in degrading wood show similarity to those of macrotermitine fungus cultivating termites, it has been proposed that they feed on the wood-degrading fungi rather than the wood itself (Bauer *et al.*, 2015). Moreover, the fact that the gut communities of the litter-feeding species in this study were overall similar to those of omnivorous species, while the wood feeders were clearly separate, raises the question to what extent the litter-feeding species actually differ from

omnivorous species in terms of the digested compounds – rather than cellulose, these may be residual protein, sugars, or hemicelluloses. It remains to be investigated which compounds of the ingested plant material are actually digested by cockroaches with a lignocellulose-rich diet.

4.5.4 Conclusion

We found that microenvironmental conditions, particularly pH, present the major determinants of bacterial community structure in the hindgut of cockroaches. The presence of closely related bacterial lineages in the hindgut of phylogenetically distant cockroaches of different feeding groups, combined with the absence or minute abundance in cockroaches with a lignocellulosic diet of those bacterial lineages that are abundant in wood-feeding termites, strongly suggest that the gut habitat, rather than host diet, plays a critical role in constraining the structure of microbial communities in cockroaches. Future studies will have to describe further mechanisms of selection in the cockroach gut environment, and assign functional roles to individual members of the gut microbial communities.

4.6 Acknowledgements

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5 Selective digestion of lignocellulose in litter-feeding cockroaches

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NL conceived the study, performed the experiments and analyzed the data, and wrote the manuscript. AB contributed scientific advice and secured funding.

Manuscript in preparation

5.1 Abstract

As detritivores, cockroaches contribute to the degradation of dead organic matter in terrestrial ecosystems. All cockroaches possess endoglucanases, and several species can be maintained on a diet consisting exclusively of cellulose or hemicelluloses. However, the degree to which cockroaches digest natural lignocellulosic substrates such as leaf litter is unknown. Here, I performed feeding experiments on two litter-feeding cockroach species, *Byrsotria fumigata* and *Ergaula capucina*, and determined the content of cellulose, lignin, and solubilizable fraction in the ingested leaf litter and the excreted feces. No significant degradation of cellulose or lignin was detected. Extraction of total non-polymeric carbohydrates from leaf litter and feces revealed that xylose turnover exceeded that of glucose. Additionally, the three gut compartments crop, midgut and hindgut yielded decreasing pools of xylose along the gut axis, suggesting that xylan, rather than cellulose, is degraded by cockroaches.

5.2 Introduction

Forests produce 2 to 15 tons of litter per ha and year, more than half of which consists of leaf litter (Bray and Gorham, 1964; Vitousek, 1982). Decomposition of organic matter comprises a crucial step of the nutrient cycle, and, in terrestrial ecosystems, is dominated by bacteria, archaea, fungi, and invertebrate animals (Swift, Heal and Anderson, 1979). Detritivorous invertebrates play a crucial role through shredding of particles (Anderson and Sedell, 1979), multiplying the surface area accessible to microbes (Fenchel, 1970). Important detritivores include, but are not limited to, Diplopoda (millipedes), terrestrial worms, and cockroaches.

Within the Dictyoptera, which comprise mantises, cockroaches, and termites, degradation of cellulose from sound wood is limited to termites and cryptocercid cockroaches (Martin, Jones and Bernays, 1991). However, several other cockroach lineages can be maintained on a lignocellulose-rich diet. In *Periplaneta americana*, varying host diet fiber content results in changes in hindgut microbial community composition (Bertino-Grimaldi *et al.*, 2013; Tinker and Ottesen, 2016). In *Shelfordella lateralis*, putative effects of a fiber-rich diet are masked by high individual variation (Schauer, Thompson and Brune, 2014). At least one family of glycosyl hydrolases (GHF9), derived from an ancient ancestor, is present in species from at least eleven classes of Metazoa, including termites and cockroaches (Davison and Blaxter, 2005). Although the endogenously produced endo-β-1,4-glucanases (EC 3.2.1.4) and beta-glucosidases (EC 3.2.1.21) in the salivary glands and midgut of cockroaches (Martin, 1983;

Slaytor, 1992; Scrivener and Slaytor, 1994) are not considered as a complete cellulolytic system due to the lack of an exo-β-1,4-glucanase (Watanabe and Tokuda, 2010), *Panesthia cribrata* can survive indefinitely on a diet of pure crystalline cellulose in the absence of gut microbes (Scrivener, Slaytor and Rose, 1989). Cellulase activity measured as filter paper digesting activity (FPase) and carboxymethyl cellulose (CMCase) correlates with numbers of *Nyctotherus ovalis* in hindgut extracts of *Periplaneta americana* (Gijzen *et al.*, 1994), suggesting some microbial contribution to cellulose digestion. This is in agreement with the finding that *P. americana* kept on diets of artificial cellulose or hemicellulose degrades both. Despite all records of cellulolytic potential, it remains to be investigated to which extent cockroaches degrade lignocellulose when facing natural, more complex lignocellulosic substrates, such as leaf litter.

Studies on the degradation of lignocellulose in termites have used a wide range of analytical methods, ranging from chemical extraction followed by gravimetric determination (Cohen, 1933; Esenther and Kirk, 1974), tracing of ^{14}C-labelled lignocellulose (Butler and Buckerfield, 1979; Cookson, 1987), and, more recently, column chromatography (Veivers *et al.*, 2006) and 2D-NMR spectroscopy (Li *et al.*, 2017). The different methods for quantification of distinct types of structural plant polymers – essentially cellulose, hemicelluloses, pectin, and lignin – across different sample types are each associated with their own advantages and biases. While gravimetric methods revealed early on that lignocellulose digestion in termites is marked by substantial removal of cellulose, but not lignin (Esenther and Kirk, 1974), 2D-NMR spectroscopy recently revealed major modifications in the structure of lignin that most likely stem from its intraintestinal separation from cellulose and hemicelluloses (Li *et al.*, 2017).

Cellulose and lignin content can be assessed fairly accurate (±1.5% of dry weight) via the extraction and gravimetric determination of acid detergent fiber (ADF) and acid detergent lignin (ADL), as previously described (Van Soest, 1963; Goering and Van Soest, 1970). Additionally, it was desirable to also detect and quantify non-cellulosic neutral and acidic sugars. This has been achieved from samples as complex as soil through extraction with hot fluoroacetic acid (Fengel and Wegener, 1979), with methodological improves later (Amelung, Cheshire and Guggenberger, 1996).

One way to investigate which components of ingested food are degraded is through controlled feeding experiments, during which a mass balance of food intake and feces deposition is created. After identifying and quantifying the major components in both food and feces, one can calculate if specific compounds are being depleted. Assuming that the monitored organism

is in steady-state, i.e., not undergoing moulting, producing oothecae or otherwise gaining or losing biomass, the compounds depleted in the feces should be the ones that are digested and taken up by the organism for its energy metabolism.

The objectives of this study were to determine if cellulose, hemicelluloses, or lignin are degraded or modified through the gut passage of litter-feeding cockroaches. The consumption of leaf litter and the production of feces by the two detritivorous species *Byrsotria fumigata* and *Ergaula capucina* was recorded during feeding experiments, and the content of the elements carbon, hydrogen, nitrogen, and sulphur, as well as the content of acid detergent fiber (ADF) and lignin (ADL) in the food and feces were determined. Additionally, complex carbohydrates were hydrolyzed from leaf litter and feces with trifluoroacetic acid (TFA), and identified via HPLC. In order to trace the fate of complex carbohydrates across the gut passage, the TFA extractions were also performed on the content of the gut compartments crop, midgut, and hindgut of *B. fumigata*.

5.3 Materials and methods

5.3.1 Feeding experiments

Feeding experiments on leaf litter were carried out with *Byrsotria fumigata* and *Ergaula capucina*. Three to ten female individuals per experiment were placed in a plastic container with water supplied, and starved for two days to remove previously ingested material from their intestinal tracts. All feces produced during this period were discarded. On day three, several grams of dried oak leaves were added, and kept in for a duration of five to eleven days. The remaining leaf litter was removed, dried, and weighed, and the insects were starved for two more days. Feces from the feeding period and the second starving period were collected daily, dried in a desiccator, and the total weight of ingested leaf material and produced feces was determined. The samples were homogenized in a ball mill and stored in a desiccator before further analysis. Individual survival and body weight were recorded at the beginning and the end of the feeding experiment. Experiments were replicated for *Byrsotria fumigata* (four replicates) and *Ergaula capucina* (five replicates).

5.3.2 Elemental analysis

Ten mg of leaf litter and feces samples were submitted to, the elemental concentrations of carbon, hydrogen, nitrogen, and sulfur were determined by complete oxidation followed by

temperature programmed desorption (TPD) on a *vario MICRO cube* CHN(S)-Analysator (Elementar Analysensysteme GmbH, Langenselbold, Germany).

5.3.3 Acid detergent fibre and lignin content

Acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined using an internationally standardized extraction procedure as described previously (Möller, 2009), based on the Van Soest method (Goering and Van Soest, 1970). Briefly, empty 50 ml borosilicate glass crucibles with 30–50 µm pore diameter were oven-dried (105 °C, overnight), cooled in a desiccator (15 min), and weighed to an accuracy of 0.1 mg (w_1). 100 ml of 0.5 M H_2SO_4 with 20 g/l cetrimonium bromide (CTAB) were added to 0.1 g (w_2) of homogenized dry sample in a 500 ml glass bottle, with a few drops of n-octanol to prevent foaming, and the bottle was sealed with a butyl rubber stopper. The bottles were placed in a steam pot, and boiled at 105 ± 5°C and 1.5 ± 0.1 bar for 60 ± 5 min. Each sample was transferred to a glass crucible, rinsed three times with 40 ml of hot water, and vacuum-dried. Samples were soaked in 30 ml of acetone for 3-5 min, and vacuum-dried. The last step was repeated until the filtrate was colorless. The remaining sample material in each crucible was oven-dried and cooled as above, and weighed (w_3, acid detergent fiber, ADF). The crucibles were placed in 500 ml Erlenmeyer flasks, filled with 50 ml of 12 M H_2SO_4 (15°C), and kept draining for 3 h with hourly stirring. Next, crucibles were washed thoroughly with hot water to remove all residual acid. Crucibles were oven-dried and cooled, and weighed again (w_4, acid detergent lignin, ADL). Finally, samples were oxidized in a furnace (525°C, 3 h), dried and cooled, and the remaining ashes were weighed (w_5). Two replicates were run per sample. Two out of every twelve crucibles were processed empty, and the mean of their weights at each step was taken as blank (b_1–b_5). Lignin, cellulose soluble, and ash content were calculated as follows (for more information, see Möller 2009):

$$lignin_{H_2SO_4}(\%\, dry\, wt) = ADL \quad = 100 \times \frac{[(w_4 - w_5) - (b_4 - b_5)]}{w_2}$$

$$cellulose\ (\%\, dry\, wt) = 100 \times [(w_3 - w_4) - (b_3 - b_4)]/w_2$$

$$soluble\ (\%\, dry\, wt) = 100 - ADF$$

$$= 100 - 100 \times [(w_3 - w_1) - (w_3 - w_1)]/w_2$$

$$ash\ (\%\, dry\, wt) = 100 \times (w_5 - w_1)/w_2$$

The method was first performed with standardized substrates to verify proper separation of the different fractions (Figure S.5.1). As expected, all xylan was solubilized in the first step, all

cellulose was recovered in the ADF but removed in in the ADL fraction. Cellulose and lignin content in oak wood approximately matched previously published ranges.

5.3.4 Extraction of monomers from carbohydrates

All carbohydrate polymers present in substrate, gut compartment content, and feces were hydrolyzed using trifluoroacetic acid (TFA) as described previously (Amelung, Cheshire and Guggenberger, 1996). Briefly, trifluoroacetic acid (4 mol/l) was added to a glass vial containing the sample in a ratio of 10 ml per 500 mg sample, and extracted at 105°C for 4 h. After cooling, samples were aliquoted into 2 ml Eppendorf tubes, and centrifuged (20,000 × g, 60 min, 25 °C). The supernatant was centrifuged again (20,000 × g, 30 min, 25 °C), and all liquid was evaporated by vacuum centrifugation overnight (30°C). Released monomers were dissolved in 0.5 ml of distilled water, and separated on a Grom H^+ resin column as described below.

5.3.5 HPLC analysis of metabolites

For determination of short-chain fatty acids, the supernatant was collected, acidified with sulfuric acid to a concentration of 10 mM, centrifuged, and 30 µl of the filtered supernatant were run on a Grom H^+ resin IEX column (8 µm pore size, 250 mm × 4.6 mm inside diameter; Grom, Rottenburg, Germany) and a refractive index detector (RID-10A; Shimadzu) with a mobile phase of 5 mM sulfuric acid (flow rate 0.8 ml min^{-1}) and a column temperature of 40 °C. Peaks were identified using external standards.

5.3.6 Respiration rates

Respiration rates of the insects were determined by carbon dioxide (CO_2) production rate over time. The body weight of the insects was recorded before being placed into a glass vial sealed by a butyl rubber stopper. Every 30 minutes, 200 µl of the headspace were replaced by the same volume of ambient air, and injected into a GC equipped with a methanizer, allowing for simultaneous detection of CO_2 and methane (CH_4). Production rates were calculated for both gases based on the part of the incubation period where production was linear (Figure S.5.1).

5.4 Results

The total survival rates of *Byrsotria fumigata* and *Ergaula capucina* over the course of the experiment were 24 out of 27 and 24 out of 26, respectively. All surviving individuals retained more or less their initial body weight (Figure 5.1a/b), allowing for the assumption that most of the individuals were in steady-state. Mean leaf litter consumption was 3.21 ± 0.51 and 22.55 ± 6.12 mg per g body weight and day for *B. fumigata* and *E. capucina*, respectively, with 1.75 ± 0.30 and 16.89 ± 0.51 mg feces per g body weight and day being produced (Figure 5.1c), resulting in a mean mass loss between food and feces of 1.46 ± 0.41 and 5.66 ± 2.15 mg per g body weight and day for *B. fumigata* and *E. capucina*, respectively (Figure 5.2a).

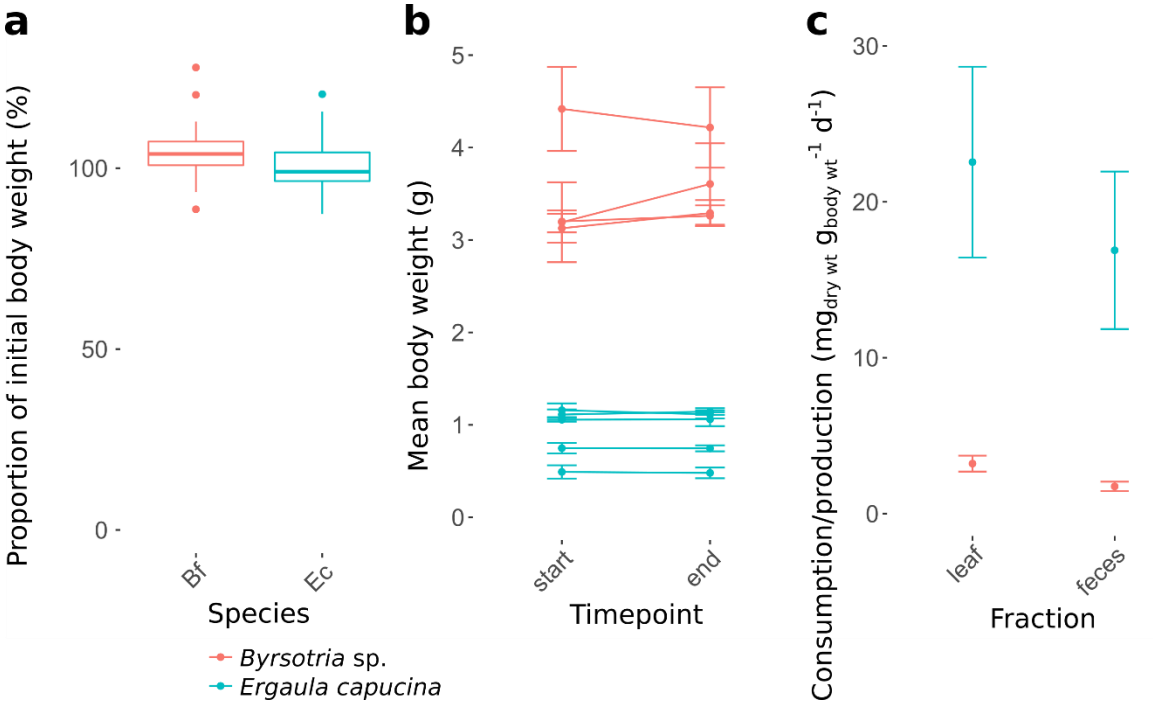

Figure 5.1 | Summary statistics of the feeding experiments, showing (a) the mean proportion of initial body weight retained after the experiment (24 individuals per species), (b) mean body weight in four and five biological replicates of *Byrsotria* sp. and *Ergaula capucina*, respectively. Each biological replicate consisted of three to ten individuals incubated together for four to eleven days. (c) Mean rate of consumption of leaf litter and production of feces. Thick bars in (a) indicate medians. Error bars in (b) and (c) indicate standard error of the mean.

Elemental analysis revealed that the dry leaf material was low in nitrogen content (1.1% of dry weight), whereas the feces of *B. fumigata* and *E. capucina* were relatively nitrogen-enriched (2.0 and 1.5%, respectively). Carbon content did not differ greatly between samples, and sulfur content was always < 0.2% of dry weight. In congruence with these results, the carbon/nitrogen ratios were higher in the substrates than in the feces produced. Turnover rates in *E. capucina* generally exceeded those in *B. fumigata*. Carbon was by far the element with the highest

turnover rate, making up for almost half of the dry weight turnover (Figure 5.2b). In *E. capucina*, turnover rates of the cellulose and lignin fractions were low in comparison to that of the soluble fraction, which contained all soluble and easily solubilizable compounds, such as sugars, soluble protein, hemicelluloses, and other heteropolysaccharides (Figure 5.2c). The extractions with trifluoroacetic acid were in agreement with this finding considering the high turnover rate of xylose, a monomer of the hemicellulose xylan, relative to glucose, the monomer of cellulose (Figure 5.2d).

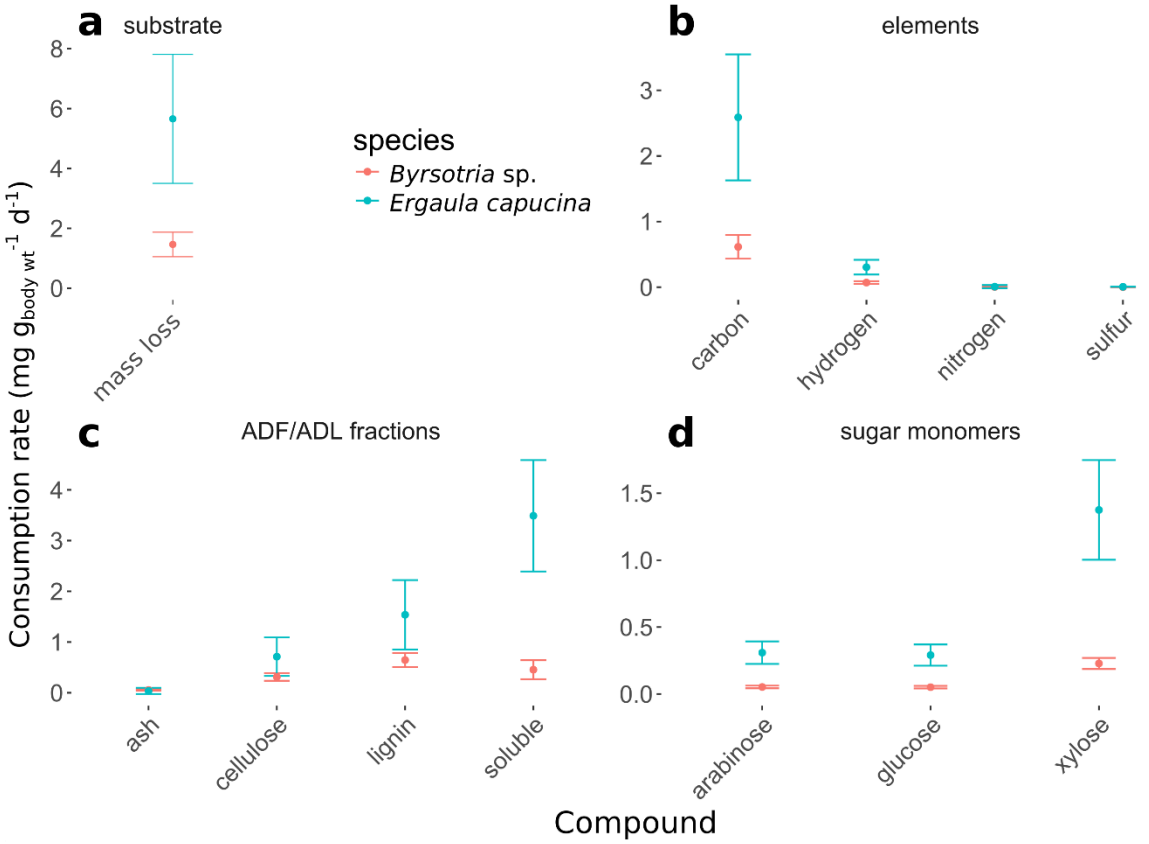

Figure 5.2 | Mean turnover rates of different components of leaf litter by *Byrsotria* sp. (red, n = 4) and *Ergaula capucina* (blue, n = 5), given as absolute mass loss per insect biomass and time between food and feces. Rates are shown for (a) total mass loss, (b) main elements, (c) fractions from acid detergent fiber (ADF) and acid detergent lignin (ADL) extraction, and (d) monomers extracted with trifluoroacetic acid. Points indicate means with standard errors.

On average, between 50 and 75 % of the ingested leaf litter mass was recovered in the feces (Figure 5.3). If all components of the leaf litter were degraded evenly, the relative mass loss of each component (cellulose, lignin, sugars etc.) should be equal to that of the total dry weight. The different fractions of the acid-detergent fiber extraction – cellulose, lignin, ash, and soluble fraction – showed no significant relative depletion in the feces. In contrast, glucose, xylose, and arabinose determined by TFA extraction were consistently underrepresented in the feces of both analyzed species.

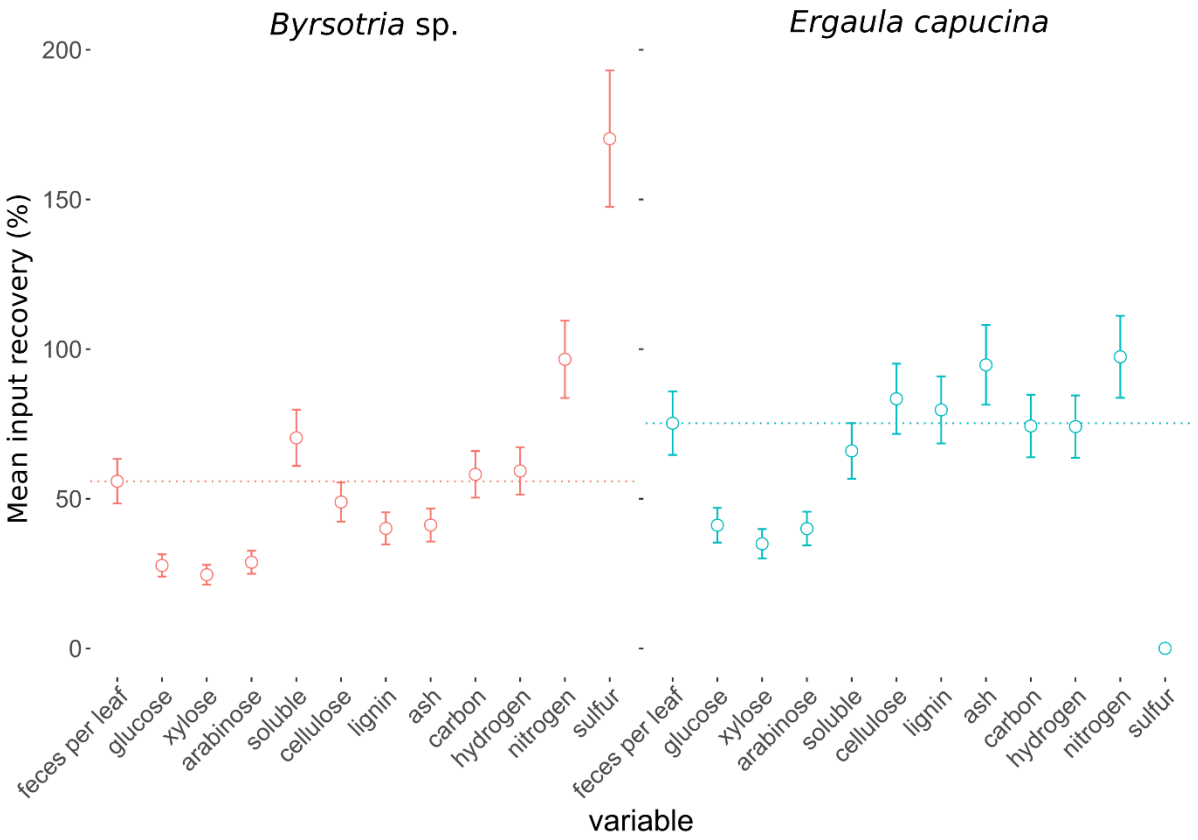

Figure 5.3 | Mean proportions of components remaining in the feces after feeding experiments on leaf litter for *Byrsotria* sp. (left, n = 4) and *Ergaula capucina* (right, n = 5). Dotted horizontal lines indicate the percentage of total leaf litter dry weight recovered in the feces, which would be the expected value for all components if the leaf litter was degraded evenly. Points indicate means with standard error.

5.5 Discussion

5.5.1 Initial substrate conditions

The elemental proportions of the oak leaf litter samples were in a similar range as those reported previously (Sariyildiz and Anderson, 2005). Previous studies have determined the cellulose and lignin content of oak leaf litter to be in the range of 23 – 45 and 15 – 37 % of dry weight, respectively (Zimmer, 1999; Fioretto *et al.*, 2005; Sariyildiz and Anderson, 2005). While the cellulose concentration of oak leaf litter determined in this study (18 – 21 % of dry weight) were slightly lower than expected, lignin content was within the expected range (28 – 36 % of dry weight), as well as the soluble fraction (41 – 47 compared to 47±4 % of dry weight reported by Sariyildiz and Anderson 2005).

5.5.2 Steady state conditions

The value of lignocellulose as a food source for cockroaches is controversial. In an earlier study with *Periplaneta americana*, access to fiber in addition to an energy-limiting omnivorous diet did not yield additional nutritional value (Bignell, 1976). However, *Panesthia cribrata* can survive more than twelve weeks with crystalline cellulose as the only food source (Scrivener, Slaytor and Rose, 1989), and both species analyzed in this study displayed growth, moulting, and full maturation when fed on oak leaf litter for two months prior to the controlled feeding experiments. While in principle some of the ingested organic material may be converted to insect biomass, the short duration of the feeding experiment (five to eleven days) makes this negligible for adults. The mean food consumption rate of both species in this study was within the range of 3.5 – 40.0 mg per g body weight per day reported previously for *Periplaneta americana* on diets with comparable cellulose content (Bignell, 1978), and mean body weight did not change significantly over the course of the experiment, therefore it was assumed that the feeding regiment presented no immediate stress for the insects, and that the nutritional balance could be treated as steady-state.

5.5.3 Depletion of substrates and metabolic rates

Omnivorous cockroaches like *Periplaneta americana* increase their total food intake under diets enriched in cellulose, most likely to meet their energy requirement that is mainly derived from non-cellulosic compounds (Bignell, 1978). The total rate of leaf litter consumption by *Ergaula capucina* and *Byrsotria fumigata* in this study was within the range reported for

Periplaneta americana under comparable cellulose content; this suggests that their digestive strategy on a lignocellulosic diet is to maximize throughput and degradation of easily solubilizable substrates, rather than dietary specialization on recalcitrant fractions.

5.6 Supplementary material

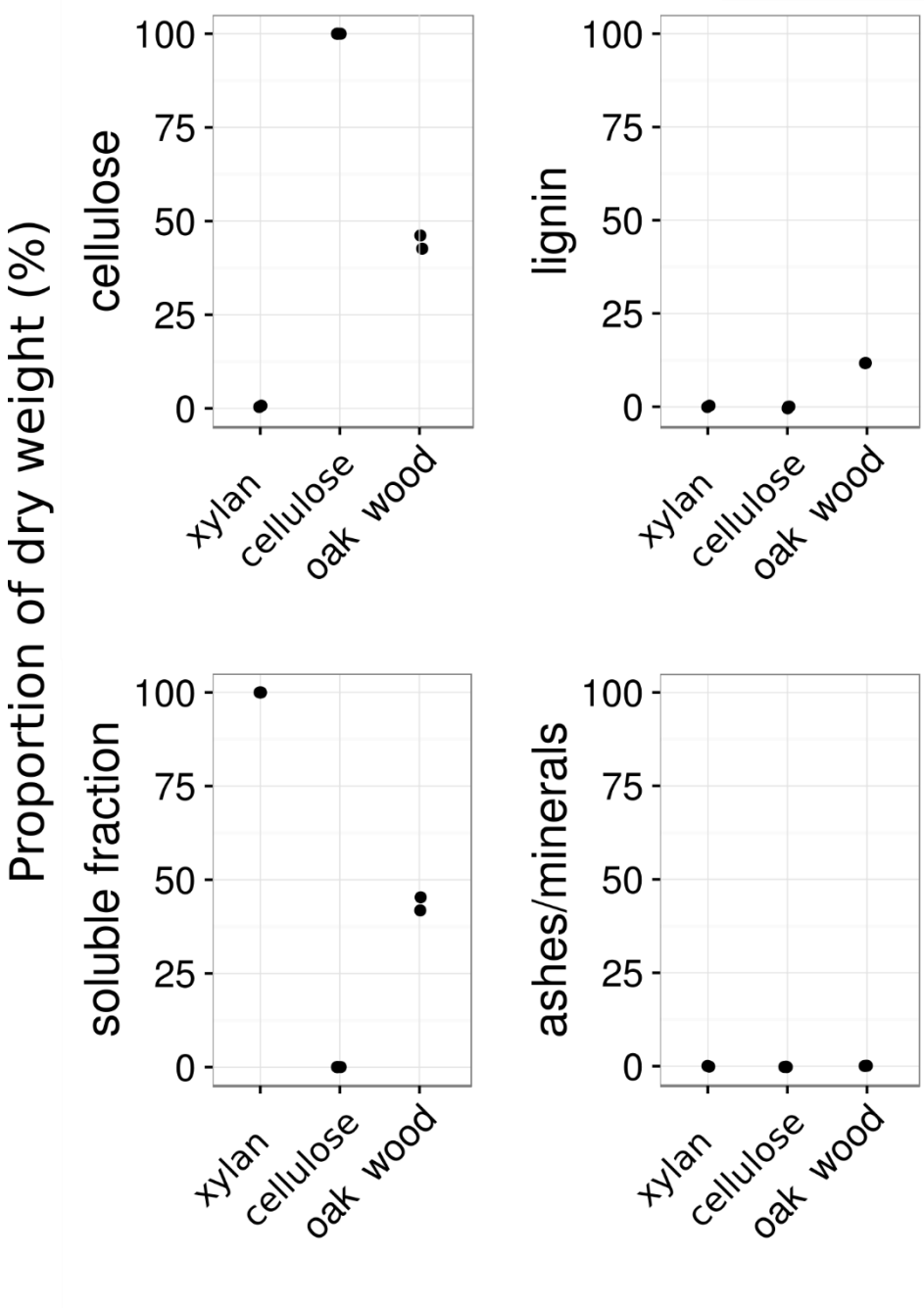

Figure S.5.1 | Test results of the acid detergent fiber (ADF) / acid detergent lignin (ADL) extraction using the Van Soest method according to Möller (2009) with standard substrates. All xylan was solubilized in the first step, all cellulose was recovered in the ADF but removed in in the ADL fraction. Cellulose and lignin content in oak wood approximately matched previously published ranges.

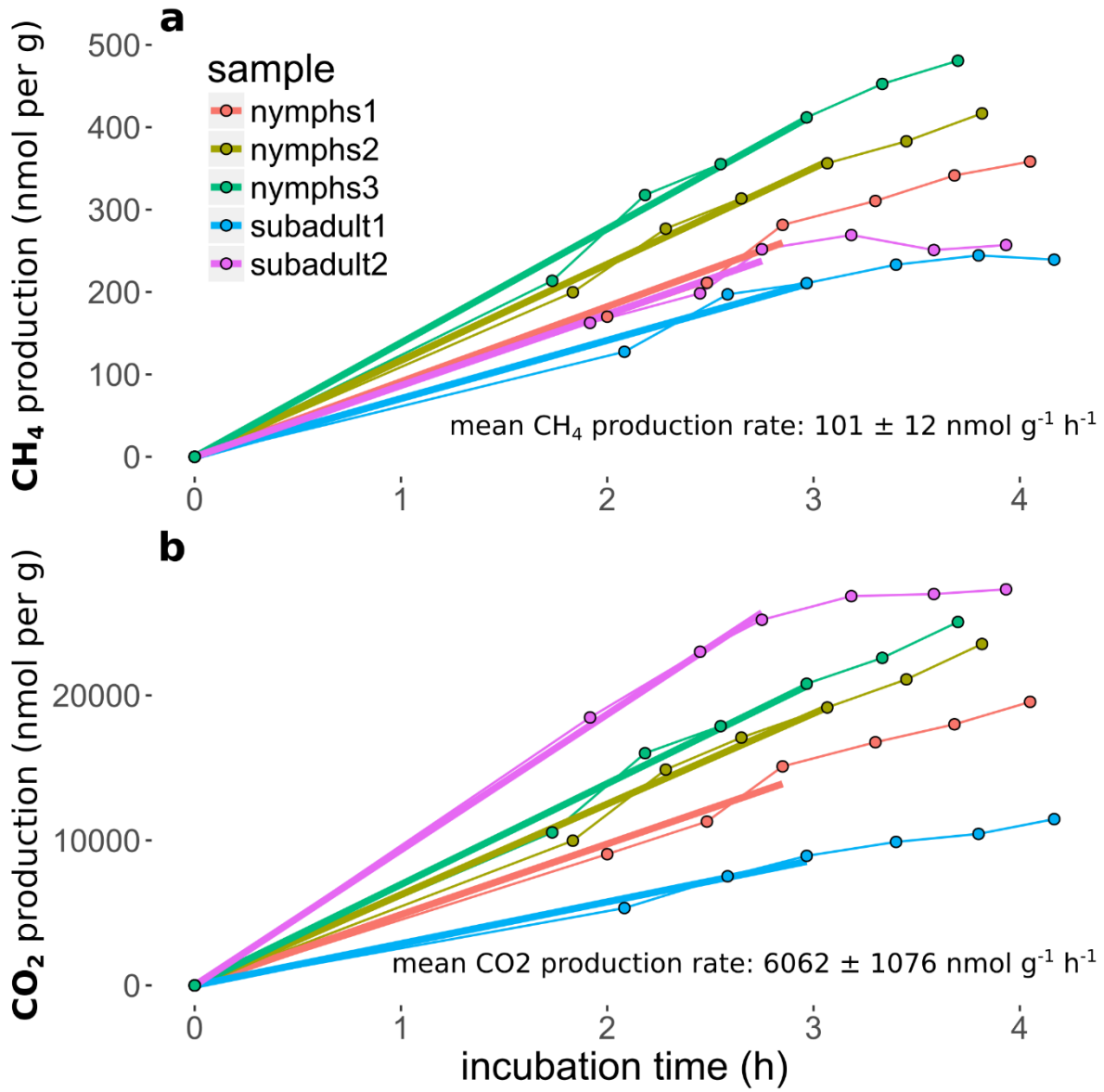

Figure S.5.2 | Production rates of (a) methane (CH_4) and (b) carbon dioxide (CO_2) from living individuals of *Byrsotria fumigata*. Mean CO_2 production rates were used to estimeate respiration rates of individuals in the feeding experiment.

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6 General discussion

Niclas Lampert

6.1 Bacterial lineages in the cockroach gut

The bacterial hindgut microbiota in cockroaches differ substantially in composition and structure from those in termites (Dietrich, Köhler and Brune 2014; this work, chapter 4). While *Spirochaetes*, *Fibrobacteres*, and *Elusimicrobia* are abundant in the hindgut of termites, most cockroaches are dominated by *Bacteroidetes* and *Firmicutes* (Brune, 2014; Dietrich, Köhler and Brune, 2014), two phyla common in arthropods (Colman, Toolson and Takacs-Vesbach, 2012) and highly abundant in omnivorous mammals (Leser *et al.*, 2002; Eckburg *et al.*, 2005; Ley *et al.*, 2008). However, the gut microbiota of termites and cockroaches also comprises bacterial lineages universally recovered in most blattodean insects, even if only in low abundance (Mikaelyan, Köhler, *et al.*, 2015).

The neighboring phylogenetic positioning of the *Fibrobacteres* phylum (Montgomery, Flesher and Stahl, 1988) with its two cultured species, *Fibrobacter succinogenes* (Cato, Moore and Bryant, 1978) and *Fibrobacter intestinalis* (Montgomery and Macy, 1982), and the "Termite Group 3" (TG3) cluster with one isolated organism, *Chitinivibrio alkaliphilus* (Sorokin *et al.*, 2014), has been established previously (Hongoh, Deevong, *et al.*, 2006; Sorokin *et al.*, 2014). Our phylogenetic analysis of the *Fibrobacteres*/TG3 clade (chapter 2) supports this relation, and is in agreement with a recent phylogenomic analysis of cultured representatives and draft genomes which concluded that TG3 should be incorporated as the class *Chitinivibrionia* into the *Fibrobacteres* phylum (Abdul Rahman *et al.*, 2015). A recently cultured species within the phylum, *Chitinispirillum alkaliphilum*, has led to the definition of a third class, *Chitinispirillia* (Sorokin *et al.*, 2016). With our approach of defining new taxonomic clusters based on sequences of the small subunit ribosomal RNA (16S rRNA) gene (Mikaelyan *et al.* 2015b; chapter 2), we support the idea of a unified classification and nomenclature framework for all bacteria and archaea that is compatible with the taxonomy of cultured bacteria and archaea. Unlike other reference databases, the taxonomic framework of DictDb relies strictly on monophyly, as proposed recently (Yarza *et al.*, 2014). Our approach is also in agreement with the concept of Candidate taxonomic units (CTUs) by Yarza et al., which allows reproducible taxonomic binning of so far uncultivated clades.

Fibrobacteres were not known to be part of the bacterial gut community in any cockroach species studied to date (Bracke, Cruden and Markovetz, 1979; Schauer, Thompson and Brune, 2012, 2014; Bertino-Grimaldi *et al.*, 2013; Bauer *et al.*, 2015; Tinker and Ottesen, 2016). However, I found a deep-branching *Fibrobacteria* lineage in 16S rRNA gene clone libraries

from the hindgut of both *Byrsotria fumigata* and *Ergaula capucina* (chapter 2); it was distinct both from sequences in termites, and from the clade comprising *Fibrobacter* that was isolated from the rumen. This new Fibrobacteres lineage made up 1 % of the bacterial community in *E. capucina*, as shown via high throughput sequencing (chapter 4), but was conspicuously absent in the wood-feeding Panesthiinae (chapter 3). The low relative abundance or complete absence of *Fibrobacteria* in cockroaches suggests that they do not play a major role in cockroaches, in contrast to wood-feeding higher termites, where they comprise approximately 10 % of the bacterial community and are considered to contribute to intestinal cellulose digestion (Warnecke *et al.*, 2007; He *et al.*, 2013; Mikaelyan *et al.*, 2014). However, they may be derived from a common ancestor of cockroaches and termites, and become abundant only in wood-feeding higher termites, where the niche of degrading free intestinal wood particles is available (Dietrich, Köhler and Brune, 2014; Mikaelyan *et al.*, 2014).

The bacterial genus *Alistipes* (phylum *Bacteroidetes*, family *Rikenellaceae*) features prominently in the hindgut microbiota of many cockroaches, covering omnivorous, litter-, and wood-feeding species (Lampert, Mikaelyan and Brune, in preparation; Schauer, Thompson and Brune 2012; Bauer *et al.* 2015). In this work, *Alistipes* phylotypes II – IV occurred in all cockroach species except *Cryptocercus punctulatus*, with individual phylotypes comprising up to 12 % of bacterial relative abundance (chapter 3 and 4). The high abundance of *Alistipes* spp. in the hindgut microbiota is a feature that cockroaches share with fungus-feeding higher termites, which is reflected in their overall similarity of hindgut community structure (Dietrich, Köhler and Brune 2014; chapter 4).

6.2 Factors shaping the gut microbiota of cockroaches

In every habitat, environmental constraints define functional niches that can be occupied by appropriately adapted species (Grinnell, 1917; Elton, 1927; Hutchinson, 1978). In intestinal microbial communities, these constraints may be imposed by factors such as host phylogeny, diet, or gut structure. Both host diet and phylogeny were previously identified as significant drivers of gut microbial community structure in insects, based on 16S rRNA gene sequences from the hindgut microbiota of seven insect orders and nine dietary categories (Colman, Toolson and Takacs-Vesbach, 2012). However, covariance between either of the two factors and gut community structure could also be caused by underlying environmental parameters such as physicochemical conditions or microhabitat structure.

6.2.1 Host phylogeny

A recent analysis has confirmed the previously established phylogenetic positions of cockroaches, lower, and higher termites within the order Blattodea, and determined the emergence of Blattodea to be most likely in the Permian 270 Ma ago (Inward, Beccaloni and Eggleton, 2007; Legendre *et al.*, 2015). The phylogenetic relationship between lower and higher termites and cockroaches is reflected in the dissimilarity of their hindgut bacterial community structure (Colman, Toolson and Takacs-Vesbach, 2012; Dietrich, Köhler and Brune, 2014). Within each of the three major groups, however, this phylogenetic signal weakens. Within higher termites, a correlation between host phylogeny and similarity of community structure coincides mostly with host dietary specialization; within both lower termites and cockroaches, there is none (Brune and Dietrich, 2015). A direct impact of phylogenetic relations between different cockroach species on bacterial gut community structure is therefore unlikely. However, individual microbial lineages that are vertically transmitted within protists in different Blattodea can co-evolve with their unicellular host, as shown for the different phylotypes of the bacterial flagellate endosymbiont *Ca.* Endomicrobium trichonymphae in different *Trichonympha* spp. in lower termites (Ikeda-Ohtsubo and Brune, 2009). *Methanobrevibacter* spp., which are endosymbionts of *Nyctotherus* spp. (Gijzen *et al.*, 1991), an anaerobic flagellate commonly found in many cockroach species (Hackstein *et al.*, 2006), represent such an archaeal lineage that may display signs of co-speciation with its ciliate host.

6.2.2 Diet

In insects, gut bacterial community structure shows significant covariance with diet (Colman, Toolson and Takacs-Vesbach, 2012), and in higher termites, diet has previously been identified as the major determinant of gut community structure (Mikaelyan, Dietrich, *et al.*, 2015). An earlier study already suggested a similar phenomenon in cockroaches, where the bacterial hindgut communities in two wood-feeding cockroaches differed slightly from those in omnivorous species (Dietrich, Köhler and Brune, 2014), but lacked sufficient sampling of cockroaches with different diets.

In chapter 4, I expanded the scope of hosts by introducing three detritivorous cockroach species, and compared their bacterial hindgut microbiota to those of wood-feeding (Bauer *et al.* 2015, chapter 2) and omnivorous cockroaches and higher termites (Dietrich, Köhler and Brune, 2014; Mikaelyan, Dietrich, *et al.*, 2015). This comparison reproduced the distinction

between the gut communities of wood-feeding and omnivorous cockroaches, but those of litter-feeding species showed no consistent signal relative to other cockroaches (Figure 4.7). This allows for three possible conclusions, as explained below.

First, host diet may have no impact on hindgut community structure in cockroaches. However, this would require another unknown factor that explains the observed differences between the gut microbiota of wood-feeding and omnivorous cockroaches. Also, increased production of fatty acids and methane under cellulosic diet in *Periplaneta americana* (Kane and Breznak, 1991; Keddie and Zurek, 1998) imply a considerable effect of diet on the gut microbiota.

As a second alternative, different diets in cockroaches may have stochastic rather than deterministic effects on the gut community, causing random shifts in community structure. This relates to the previously observed phenomenon of increased β-diversity in microbial communities under stressed conditions compared those in their normal state, also known as a variant of the Anna Karenina principle, which spins off the first sentence of Leo Tolstoy's novel *Anna Karenina*:

> *Happy families are all alike; every unhappy family is unhappy in its own way. (Tolstoy, 1878)*

The Anna Karenina principle was recently popularized to describe endeavors that can fail for multiple reasons, making the variation between failed outcomes greater than that between successful ones (Diamond, 1997). Examples of increased β-diversity under stress include microbial communities in the cavity and upper respiratory tract of smokers (Charlson *et al.*, 2010; Wu *et al.*, 2016), the intestinal microbiota under type 1 diabetes (Giongo *et al.*, 2011), obesity (Holmes, Harris and Quince, 2012), and alcoholism (Mutlu *et al.*, 2012), and the microbiome of coral reef sponges exposed to acidity (Lesser *et al.*, 2016). In cockroaches, the assembly of the gut microbiota follows both deterministic and stochastic factors (Mikaelyan, Thompson, *et al.*, 2015). This implies that a change in host physiology, such as a dietary change, could be accompanied by strong stochastic effects. For instance, the high individual variation in gut microbial community structure of *Shelfordella lateralis* in response to high-protein and high-fiber diets (Schauer, Thompson and Brune, 2014) may either mask the effect, or constitute the effect itself. However, this hypothesis is in contrast to feeding experiments with the omnivorous cockroaches *Periplaneta americana* and *Blattella germanica*, where diets with differing cellulose and protein content, respectively, resulted in a change in bacterial hindgut community structure and composition (Bertino-Grimaldi *et al.*, 2013; Perez-Cobas *et*

al., 2015). Interestingly, the core hindgut community in *P. americana* appears to be stable and resilient to dietary shifts (Tinker and Ottesen, 2016).

As a third possible conclusion, type and structure of compounds ingested and degraded by litter-feeding cockroaches may not differ significantly from those in omnivorous species, even if their designated dietary strategies differ. If litter-feeding species degrade primarily soluble and easily digestible plant components such as residual protein, sugar, and hemicelluloses, then the similarity of their hindgut microbiota to that of omnivorous species becomes more plausible. In chapter 5, I approached this question by analyzing the degradation processes in cockroaches fed on oak leaf litter. Here, xylose, solubilized from xylan, the second most abundant plant polymer, had the highest turnover rate from ingested substrate to feces, and intestinal xylan pools showed considerable reduction from crop to midgut and hindgut. Therefore, hemicelluloses may constitute an important energy source for litter-feeding cockroaches. This is in line with the activity of endogenous endoxylanases in the crop and midgut of both xylophagous and omnivorous cockroaches (Scrivener, Watanabe and Noda, 1998), and the observation that even omnivorous species are capable of hemicellulose degradation (Bignell, 1977a).

6.2.3 Coprophagy

Feeding experiments on leaf litter in chapter 5 of this thesis mostly excluded coprophagy by preventing access to fecal pellets and removing fresh feces on a regular basis, in order to accurately assess ingestion and degradation rates between substrate and feces. However, coprophagy is common among cockroaches, as it offers several major benefits: It is a source of intestinal symbionts, microbial protein, as well as of enzymes and metabolites of variable origin (Nalepa, Bignell and Bandi, 2001). Intraspecific coprophagy is most prevalent in younger instars, as shown for several synanthropic cockroach species (Shimamura *et al.*, 1994; Wang, Yang and Chow, 1995; Kopanic and Schal, 1997) and earwigs (Körner, Diehl and Meunier, 2016), and is vital for development of first instars of *Blattella germanica* (Kopanic *et al.*, 2001). Additionally, deposition of fecal pellets onto plant litter inoculates it with microbes and initiates its microbial degradation, a phenomenon known as “microbial conditioning” (Swift, Heal and Anderson, 1979), which increases digestibility for detritivorous species. Hence, coprophagy both provisions the insect with more easily digestible substrates and re-inoculates the intestinal tract with microorganisms, which may benefit even adult

specimen. Additionally, varying the extent of coprophagy enables the insect to respond to differences in food quality (Bignell, 1981).

6.2.4 The gut habitat

The intestinal tracts of cockroaches differ greatly in morphology from those of lower and higher termites, the biggest distinction being the enlarged crop and the lack of compartmentalization of the hindgut in cockroaches (Noirot and Kovoor, 1958; Bignell, 1981; Brune and Dietrich, 2015). Along with anatomical features, the gut environment presents specific structural and physicochemical conditions that define the niches that are available for microbes. In termites, the conditions provided in the different compartments within one insect host are associated with completely different microbial communities (Köhler *et al.,* 2012), while homologous compartments in different host species select similar microbial communities (Mikaelyan, Meuser and Brune, 2016), raising the question if micro-environmental conditions have a similar impact on the microbial communities in gut compartments of cockroaches.

The basic compartmentalization of the gut, with crop, midgut, and colon as the three functionally most important compartments, is the same in all cockroaches (Bignell 1981; Schauer, Thompson and Brune 2012; Bauer *et al.* 2015, chapter 3; chapter 4). In xylophagous Panesthiinae, the homologous compartments each select for similar bacterial communities across replicates and different host species (Bauer *et al.* 2015, chapter 3, Figure 4). The selective pressure may stem at least in part from the specific physicochemical conditions provided in each compartment. In the crop of the cockroaches analyzed so far, slight acidity (pH 5 – 6) and accumulation of lactic acid suggest bacterial lactic acid fermentation (Schauer, Thompson and Brune 2012; Bauer *et al.* 2015, chapter 3). The fermenting agents are most likely abundant lineages, e.g., *Enterobacteriaceae*, *Lactobacillaceae* or *Bifidobacteriaceae* (Bauer *et al.* 2015, chapter 3; chapter 5). However, in litter-feeding species, the bacterial communities in the crop are more similar to those of the subsequent midgut compartment of the same host, rather than the homologous compartments in other species (Figure 4.2). This suggests two things concerning crop and midgut of litter-feeding cockroaches: First, that microbial community assembly depends on stochastic processes, even if the resulting physiology, e.g., low pH and lactic acid fermentation, is the same; and second, that the type of microbial lineages carried over from crop to midgut alongside ingested food particles has a strong impact on the resulting microbial community.

The hindgut is the only gut compartment that presents a strong selection pressure in all cockroaches, and hence results in core lineages that make up a major part of the bacterial hindgut community (Figure 4.3). The overall similarity of bacterial hindgut communities in cockroaches of different diets suggests that in cockroaches, the micro-environmental conditions in the gut habitat present a stronger driver of microbial community structure than host diet (Schauer, Thompson and Brune 2014; Bauer *et al.* 2015; chapter 4).

6.3 Impact of the gut microbiota on the host

The contribution of the gut microbiota of cockroaches to digestion has been discussed previously (Bignell, 1981). Endogenous enzymes in crop and midgut, combined with microbial degradation processes in the hindgut, increase the overall digestion efficiency of the cockroach host; the hindgut microbiota then ferments remaining substrates to short-chain fatty acids that can be resorbed by the host (Cruden and Markovetz, 1987). However, the intestinal microbiota impacts the cockroach host in multiple other ways. First instars benefit from consuming feces of mature conspecifics, suggesting that they require degradation products from intestinal microbes, the microbes themselves, or both (Kopanic *et al.*, 2001). Young instars deprived of coprophagy develop more slowly, and germ-free cockroaches do not reach adulthood (Kopanic *et al.*, 2001; Tegtmeier *et al.*, 2015), highlighting the important role of the gut microbiota in host development. Conspecific aggregation is triggered by volatile carboxylic acids in the feces that are produced by intestinal microbes, proving that the gut microbiota of cockroaches can induce a specific behavior in its host (Wada-Katsumata *et al.*, 2015). Further beneficial effects of intestinal microbes in other insects include mediation of insecticide resistance (Kikuchi *et al.*, 2012), and protection against pathogens (Dillon *et al.*, 2005) and parasites (Koch and Schmid-Hempel, 2011). Given the high number of so far undescribed bacteria we detected in the gut of xylophagous (chapter 3) and litter-feeding (chapter 4) cockroaches, many of their functions remain to be investigated.

6.4 Concluding remarks and outlook

The gut microbiotas of cockroaches share rare lineages and the phenomenon of compartment-specific communities with those of termites, but differ in community structure and show less diet-specific distinction. Despite their hardy nature and easy maintenance, the study of the cockroach gut microbiota presents challenges due to high inter-individual variation due to the highly stochastic nature of gut microbial community assembly in these insects. In this work, I

have provided insights into the intestinal physiology of cockroaches with a lignocellulosic diet, characterized the bacterial communities in the major gut compartments, and analyzed the degradation processes that accompany their diet on leaf litter. My results show that, unlike termites, litter-feeding cockroaches retain a generalist approach even under a lignocellulosic diet: They digest primarily easily accessible nutrients like sugars and hemicelluloses as long as they are available. Their intestinal microbiota is complex, comprising both abundant lineages that are characteristic for all cockroaches, but also low-abundant lineages that are of greater functional relevance in higher termites. Given the high number of so far undescribed bacteria we detected in the gut of xylophagous (chapter 3) and litter-feeding (chapter 4) cockroaches, many of their functions remain to be investigated.

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Appendices

Contributions

Chapter 1 | NL wrote the manuscript.

Chapter 2 | AM conceived the study, performed the analysis, and wrote the paper. TK conceived the study, and performed experiments and part of the analysis. **NL performed experiments and contributed to the manuscript.** JR, HB, and KM performed experiments. AB conceived the study and secured funding.

Chapter 3 | EB conceived the study, performed experiments, analyzed data, and wrote a first draft of the manuscript. **NL performed experiments, analyzed the data, and wrote the final version of the paper.** AM and TK provided scientific advice and contributed ideas to the manuscript. KM conceived the study. AB conceived the study and secured funding.

Chapter 4 | NL conceived the study, performed the experiments, analyzed the data, and wrote the manuscript. AM conceived the study and analyzed the data. AB wrote the manuscript and secured funding.

Chapter 5 | NL conceived the study, performed the experiments and analyzed the data, and wrote the manuscript. AB contributed scientific advice and secured funding.

Chapter 6 | NL wrote the manuscript.

Erklärung der Eigenständigkeit

Ich versichere, dass ich die Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, Oktober 2017