Transformation of nitrogenous soil components by humivorous beetle larvae

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Marburg, September, 2007

Teile der Ergebnisse der vorliegenden Arbeit sind in folgenden Artikeln veröffentlicht bzw. zur Veröffentlichung vorgesehen:

Peptidic soil components are a major dietary resource for the humivorous larvae of *Pachnoda* spp. (Coleoptera: Scarabaeidae) J. Andert, O. Geissinger, and A. Brune (in press)

Highly active but not incorporating ^{13}C into nucleic acids: Factors influencing RNA-stable isotope probing in insect intestinal tract microbiota J. Andert, J. Rieckmann, M. Friedrich, and A. Brune (in preparation)

Inter- and intraspecific differences of the bacterial community in the gut of two humivorous scarab beetle larvae and effects of the soil (in preparation)

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1 General Introduction

1.1 Soil Organic Matter

Soil organic matter consists of nonhumic and humic substances. Nonhumic substances are compounds with a still recognizable structure, e.g. carbohydrates (10%), N-containing compounds (10%) alkanes, fatty acids, etc. (10%). Humic substances have lost their chemical characteristics by transformation processes and make up the bulk of soil organic matter (70%; Schulten, 2005).

The primary source of soil organic matter is decaying plant material. The amount and the composition of plant litter are important parameters for the control of organic matter formation (Swift et al., 1979). Major components of this plant material are cellulose (15 – 60%), hemicellulose (10 – 30%), lignin (5 – 8%) and protein (1 – 15%).

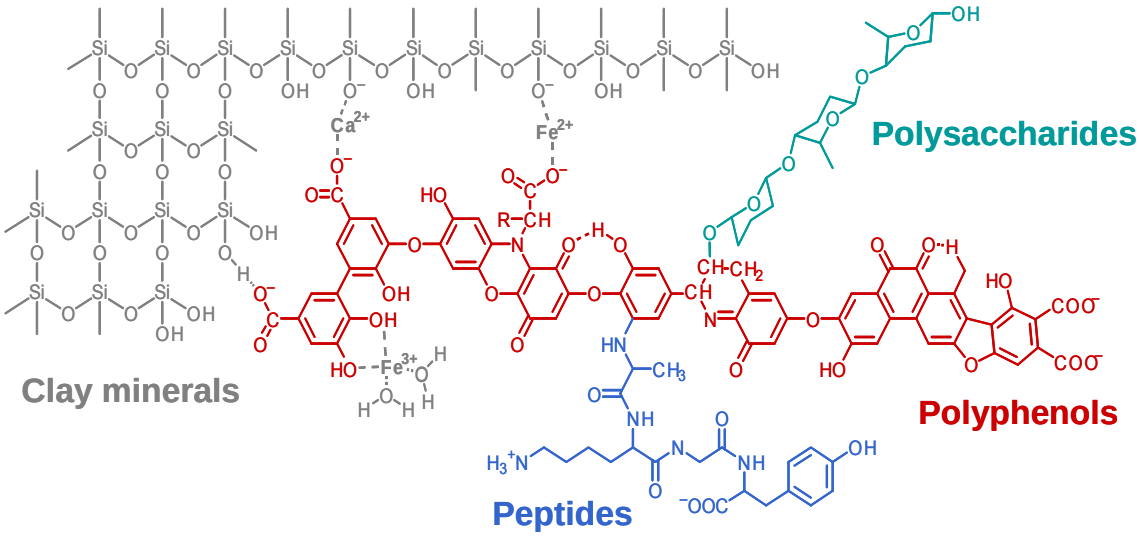

Fig. 1.1 Model structure of humic substances with the characteristic polyphenolic backbone and peptidic and polysaccharidic residues stabilized by the adsorption to clay minerals and the interaction with metal ions [modified after Stevenson (1994) by Kappler et al.].

However, microorganisms (bacteria, archaea, fungi) represent a small fraction of the soil biomass, but are rapidly turned over. Microbial residues are important precursors for soil organic matter (Haider, 1992). Fungal cell walls consist mainly of polymeric amino sugars, while in bacterial cell walls amino acids are further major components.

Although polysaccharides and peptides are easy to decompose by the soil microorganisms, considerable amounts can be found stabilized in humic substances (Stevenson, 1994; Fig. 1.1). For example, carbohydrates account for up to 15% of the total carbon in soil organic matter (Baldock and Nelson, 2000).

Over 90% of the total soil nitrogen exists in organic form. Further, 30 to 45% of the soil organic nitrogen are released as amino acids originating from soil peptides, while only 5 to 10% are resulting from amino sugars (Stevenson, 1994). Nucleic acids contribute to a minor extent to the organic nitrogen pool (0.3%; Cortez and Schnitzer, 1979).

Several mechanisms were suggested for the formation of humic substances from its diverse precursors. The first step is the decomposition of polymeric biomass compounds including lignin to soluble molecules ready for the uptake into the cell. These compounds are in the following subjects of microbial metabolism. At this point the theories are diverging. The lignin theory supposes lignin, modified by demethylation and oxidized to the quinone form, to be the main precursor of humic substances, condensed with protein or other amino compounds for the formation of a Schiff base (Waksman, 1932). According to the more recent Polyphenol theory, lignin is degraded oxidatively to monomeric units and side chain oxidation and demethylation is resulting in polyphenols, whereas also microorganisms can produce polyphenols (Kononova, 1966). After the conversion of the polyphenols to quinones by polyphenoloxidases, the reaction with nitrogen compounds leads to the formation of dark-colored polymers. A third mechanism postulates the condensation of reducing sugars and amines (Maillard reaction) to form highly reactive compounds that polymerize to brown-colored products of unknown composition.

Most probably all pathways are occurring in soil, but not to the same extent or the same order of importance (Stevenson, 1994).

However, it is universally acknowledged that stabilization of soil organic matter is increased by the sorption to clay minerals (Sollins et al., 1996; Sposito, 1989; Stevenson, 1994) and the formation of complexes with metal ions (Stevenson, 1994; Jenny, 1980).

Although soil organic matter is hard to mineralize and highly recalcitrant, it is subject of further, e.g. microbial, decomposition processes (Tate, 1987; Chefetz et al.,

2002). A number of microorganisms are capable to degrade humic substances, e.g. Actinomycetes and white-rot fungi (Saiz-Jimenez, 1996). A very important site for decomposition of humic substances is the gut of soil-feeding, humivorous or detritivorous soil macroinvertebrates (Brussaard and Juma, 1996).

1.2 Soil feeding macroinvertebrates

The soil fauna contributes very much to the transformation and degradation of humic substances (Wolters, 2000). Soil macroinvertebrates carry out about 90% of the conversion processes in soil (Lavelle et al., 1997). There are a variety of possibilities how soil animals can affect the transformation of soil organic matter. Soil animals are able to change the soil physically to a great extent by formation of nests, mounds, burrows, casts and galleries. These structures allow to spatially regulate the environment to a certain extent and therefore to occupy many different habitats (Wood, 1988; Curry, 1994). For this reason, for earthworms, termites and ants the term "ecosystem engineers" was used (Lavelle et al., 1997), which refers to soil organisms that directly or indirectly alter the availability of resources to other species by causing physical changes in biotic or abiotic material (Jones et al., 1994).

Earthworms have a great influence on soil structure, litter degradation and conversion (Brown et al., 2000), which was already recognized and described by Darwin, 1881. They can be differentiated by three feeding categories. Epigeic earthworms feed on plant litter and litter inhabiting organisms and are rarely ingesting soil, whereas anecic earthworms live on particulate organic matter mixed with soil particles. Only the diet of the tropical endogeic earthworms consists mainly of soil organic matter (Brown et al., 2000).

Also the feeding activity of soil-feeding insects itself has great impact on soil structure. The gut can act as a hot spot for decomposition processes (Brussaard and Juma, 1996). But the structure of the food soil and the nutrient content is variable. Soil-feeding phylogenetically higher termites are able to thrive exclusively on mineral soil deriving their nutrition probably from low levels of plant material within the soil (Wood and Johnson, 1986; Donovan et al., 2001). In contrast, humus rich in soil organic matter and decaying plant litter is the food for many species of saprophagous scarab beetle larvae (Werner, 1926; Wiedemann, 1930, Schlottke, 1945, Rössler, 1961).

The ecological importance for of humivorous beetle larvae remains to be studied, but is undoubted for soil-feeding termites and earthworms. Especially, soil-feeding termites are playing an important role in the transformation and mineralization of soil organic matter, due to their high abundance in the tropical rainforest and the consumption of large quantities of soil (Wood, 1978a; Wood and Sands, 1978).

1.3 Processes in the gut of soil-feeding invertebrates

During gut passage, the soil is subject to many different kinds of transformation processes. The gut of soil-feeding termites is highly compartmentalized compared to wood-feeding relatives (Fig 1.2). A particular feature of soil or humus-feeding insects is that at least part of the gut is extraordinary alkaline. In the P1 section of the hindgut of soil-feeding higher termites the pH is highly elevated (Bignell and Eggleton, 1995; Brune and Kühl, 1996, Fig. 1.2), which is regarded as a prerequisite for soil-feeding (Bignell and Eggleton, 1995). Also the midgut of humivorous scarab beetle larvae exhibits generally a highly alkaline pH (Grayson, 1958; Wiedemann, 1930; Werner, 1926).

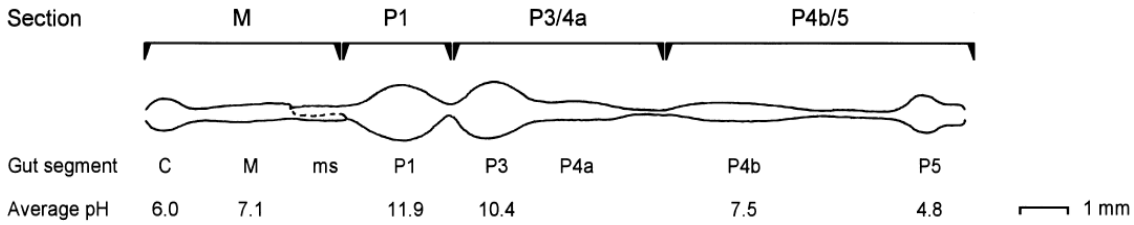

Fig.1.2 Gut morphology of the soil-feeding termite *Cubitermes* sp. with the gut segments crop (C), midgut (M), the mixed segment (ms), first proctodeal segment (P1), proctodeal segment 3 and 4a (P3, P4a), proctodeal segment 4b and 5 (P4b, P5). Under the gut scheme, the pH in the respective gut section is displayed [Taken from Kappler and Brune (1999); the pH was determined by Brune and Kühl (1996)].

With the help of this alkaline pH, the cleavage of bonds between organic material and inorganic soil components in humic substances is achieved (Stevenson, 1994). Additionally, the molecular weight of the humic substances is decreased, whereas the solubility of the humic substances is increased (Kappler and Brune, 1999). Further, it has been proposed that the alkalinity is an evolutionary adaptation to a diet rich in polyphenolic substances because it enhances the solubility of dietary protein and

prevents precipitation of the digestive enzymes (Berenbaum, 1980, Sharma et al., 1984, Martin et al., 1987).

Also the solubilization and digestion of bacteria was improved (Rössler, 1961), so that it is imaginable that intestinal bacteria are used as carbon, nitrogen and energy source. As a result of the high pH, compounds stabilized in humic substances are mobilized by alkaline extraction, supported by feeding studies with soil-feeding termites as well as humivorous beetle larvae. With the help of humic acid stabilized ^{14}C-labelled model compounds, evidence could be provided, that stabilized residues are mobilized during gut passage (Ji and Brune, 2005; Li and Brune, 2005b).

The anterior region of the earthworm gut (Mishra and Dash, 1980) and the midgut of insects are known to be the sites of enzymatic digestion processes (Crowson, 1981; Terra and Ferreira, 1994). For tropical geophagous earthworms proteinase, cellulase, and a whole variety of other glycosidic enzymes could be detected (Lattaud et al., 1998; Mishra and Dash, 1980; Mora et al., 2005; Tillinghast et al., 2001).

For saprophagous beetle larvae proteolytic, cellulolytic and amylolytic activities could be evidenced (Soo Hoo and Dudzinski, 1967; Cazemier et al., 1997; Zhang and Brune, 2004; Schlottke, 1945; Bayon and Mathelin, 1980). A similar spectrum of enzyme activities could be found in soil-feeding termites (Fujita and Abe, 2002; Mishra and Sen-Sarma, 1985; Mora et al., 2005), besides xylanase, phosphatase and chitinase (Brennan et al., 2004, Mora et al., 2005; Tracey and Youatt, 1958). The enzymatic equipment gives indications for the potential substrate spectrum. Further, for the humivorous beetle larva *Pachnoda ephippiata* and for the soil-feeding termite *Cubitermes orthognathus* could be shown that ^{14}C-labelled cellulose, peptidoglycan and peptide are mineralized during gut passage (Li and Brune, 2005; Ji, 2001).

The origin of the enzymatic activities is not always the insect host. Microorganisms (bacteria, archaea, fungi) have almost unlimited abilities to mineralize any substrate of natural origin and the very abundant gut microbiota offers a wide enzyme spectrum. Whereas for the midgut comparably low numbers of microbial cells could be detected, in the hindgut of humivorous beetle larvae and soil feeding termites up to 10^{12} microorganisms (ml gut content)$^{-1}$ were counted (Cazemier et al., 1997b, Lemke et al., 2003, Rössler, 1961, Schmitt-Wagner et al., 2003, Bignell, 2000). However, the macroorganisms are the major regulators of microbial activity (Lavelle et al., 1995).

Apparently, interactions between soil animals and microorganisms are very important due to contribution of the microbiota to the mineralization in the soil. In the gut, microorganisms are in close contact with the soil organic matter (Brussaard and Juma, 1996). The composition of the microbial communities is specific for the gut and different to the ingested soil in the soil-feeding termite *Cubitermes* spp. and the humivorous beetle larva *Pachnoda ephippiata* (Schmitt-Wagner et al., 2003b; Egert et al., 2003). On the contrary, the microbial community in earthworm guts is very similar to the microbiota in the ingested food soil Therefore an abundant indigenous gut community is very unlikely (Egert et al., 2004, Horn et al., 2003, Furlong et al., 2002). Generally, in the hindgut reducing conditions are predominating, and oxygen entering the gut is most probably readily metabolized (Kane, 1997). These conditions are the prerequisites for microbial fermentation processes in the hindgut. Resulting fermentation products, predominantly acetate, are consequently oxidized and used for the catabolism by the host organism.

Degradation of the labile components and stabilization of the residual organic matter during gut passage are regarded as connected processes (Wagner and Wolf, 1998; Wolters, 2000). Also selective feeding on less recalcitrant soil organic matter leads to a higher stability of the residual organic matter (Wolters, 2000), so that feeding activity and digestion processes are leading to a higher stabilization grade of the soil.

1.4 Humivorous scarab beetle larvae

Beetles (Coleopotera) represent the biggest order in the class of insects. In the adults as well as in the larval stages several forms of nutrition can be distinguished. In Scarab beetle larvae herbivorous, saprophagous and humivorous lifestyles are observed (Crowson, 1981). Already early studies dealt with the nutrition and digestion of scarab beetle larvae. Mainly larvae, inhabiting regions with temperate climate living in soils rich in rotting plant material, were examined. An alkaline pH in the midgut is likely to be a prerequisite for the humivorous lifestyle (Bignell and Eggleton, 1995), since it is often found in insects feeding on decaying organic matter (Grayson, 1958, Lemke et al., 2003, Werner, 1926, Rössler, 1961, Schlottke, 1945) and leads to alkaline extraction of the recalcitrant soil organic matter (Stevenson, 1994). In addition, in the midgut most likely host associated enzymatic activities are localized (Crowson, 1981, Fig. 1.3).

The most important nutrient sources might be cellulose and hemicellulose, which are the major components of plants. Feeding studies with *Potosia cuprea* (Cetoiinae) revealed that the larvae are able to thrive exclusively on cellulose (Werner, 1926). These results are supported by Rössler (1961), who fed *Oryctes nasicornes* with filter paper for 6 weeks. Also Wiedemann (1930) supposed that an essential part of the diet of *Oryctes nasicornes* consists of cellulose. Surprisingly, the origin of the cellulose digesting enzymes seemed not be the insect but the microbiota in the gut.

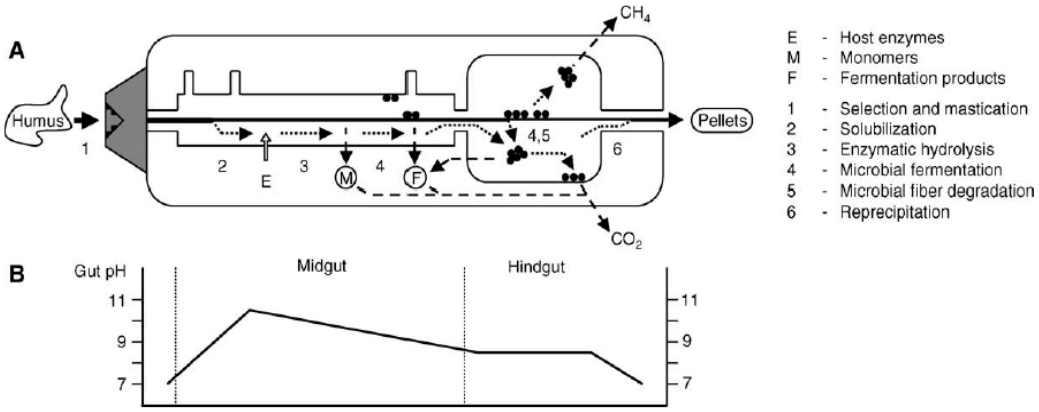

Fig 1.3 Scheme of the fate of ingested humus during gut passage in humivorous beetle larvae (A; Li and Brune, 2005); solid lines represent insoluble material, dotted lines symbolize solubilized organic matter, filled circles represent microorganisms. The protuberances from the midgut stand for the three crowns of midgut ceca, of which the exact function is not known. pH profile in the gut (B; Lemke et al., 2003).

This assumption is in agreement with studies with *Oryctes nasicornes*, where on the one hand no carboxymethylcellulase activity could be detected in the gut (Bayon, 1980a) and consequently cellulolytic activity was attributed to the symbiotic bacteria (Bayon and Mathelin, 1980b). It could be shown that the gut of scarab beetle larvae harbors a high number of microorganisms; the hindgut is much more densely inhabited than the midgut (Werner, 1926, Wiedemann, 1930, Rössler, 1961, Fig. 1.3). Werner (1926) reported that it was possible to solubilize filter paper with the help of the hindgut content of *Potosia cuprea*. The midgut content revealed similar characteristics, although the reaction was considerably slower. With the gut content of larvae of *Oryctes nasicornes* and *Osmoderma eremita* the degradation of plant cell walls was possible (Wiedemann, 1930). Since cellulose is devoid of nitrogen, the question of the nitrogen source has to be answered. For several scarab beetle larvae proteolytic activity was be

demonatrated (Wiedemann, 1930, Schlottke, 1945), which was postulated to be of insect origin. For the pruinose scarab *Sericesthis geminata* the proteinase activity was mainly located in the midgut, where as cellulolytic activity could be found in midgut and hindgut, although the activity in the midgut was inhibited by the highly alkaline pH (Soo Hoo and Dudzinski, 1967).

More recent studies concentrated on African rose chafers from the genus *Pachnoda*, since the generation time is much shorter in comparison to domestic species, making them much more convenient model organisms for laboratory studies.

Also for the larvae of *Pachnoda marginata* cellulose and hemicellulose were postulated to be the main nutrient source (Cazemier et al., 1997). Although only low cellulolytic activity could be measured, high cellulolytic potential is accredited to the numerous intestinal bacteria in the hindgut of *Pachnoda marginata* (Cazemier et al., 1997).

Feeding trials with the larvae of the closely related *Pachnoda ephippiata* showed that through gut passage the mineralization of ^{14}C-labeled cellulose was highly stimulated (Li and Brune, 2005). Also the degradation of polysaccharides from fungal and bacterial cell walls to CO_2 was considerably enhanced. With less recalcitrant material like protein and fungal biomass the stimulation was less pronounced. The results of this study suggest that polysaccharides as well as protein are a possible nutrient source for the larva and its gut microbiota. The stimulation was even more obvious when the polysaccharides and peptides were stabilized as residues of humic acid model compounds (Li and Brune, 2005b). This provides evidence that not only the organic material freely available in the food soil is mineralized but also humic-acid stabilized polysaccharides and peptides are digested.

The exact role of the gut microbiota for the digestion process still remains to be elucidated. Both in midgut and hindgut compartment of *Pachnoda ephippiata*, high concentration of fermentation products such as lactate, acetate and propionate were detected, supported by cultivation-based enumeration of high yields of bacteria producing acetate, lactate and propionate (Lemke et al., 2003). These groups could also be detected by cultivation independent techniques (Egert et al., 2003). Most probably the fermentation products are in the end resorbed by the larvae and mineralized to CO_2

(Fig. 1.3). Additionally, the microorganisms themselves might serve as carbon and nitrogen source.

1.5 Objectives of the study

In previous studies with the larvae of the scarab beetle *Pachnoda ephippiata* the release of humic-acid stabilized peptides during gut passage associated with a high ammonia concentration in the gut was monitored (Lemke et al., 2003; Li and Brune, 2005b). It was suggested that peptides might be a major dietary resource. In the present thesis, we aimed at investigating the fate of humic-acid-stabilized peptides in the intestinal tract. The proteolytic activities in midgut and hindgut contents were estimated. Amino acid mineralization and fermentation were quantified to estimate the contribution of these processes to the total respiration rate of the larvae. Nitrogen species other than amino acids were determined. On the basis of the results, a comprehensive scheme describing the processes affecting peptide carbon and nitrogen during gut passage was developed.

The scarab beetle *Pachnoda ephippiata* and *Pachnoda marginata* are allopatric species, which share the same lifestyle including the same food. Also their larvae occupy the same ecological niche and both are able to thrive exclusively on soil organic matter. In previous studies, the microbial community in the gut of *P. ephippiata* larvae was described using cultivation-independent methods (Egert et al., 2003). It is not known, whether the larvae of the two closely related beetle species harbor similar microbial gut communities due to the similar life style or whether the spatial separation leads to differences.

In the present thesis, the bacterial communities of midgut and hindgut of the larvae were characterized using ecological indices. Further, with the help of T-RFLP fingerprinting and subsequent correspondence analysis, intra- and interspecific differences in the composition of the bacterial communities in the midgut and hindgut of *Pachnoda ephippiata* and *Pachnoda marginata* larvae were statistically analyzed. Also the change of the gut microbiota in larvae of *Pachnoda marginata* in connection with the feeding on different food soils was examined.

Stable isotope probing has been frequently used to link structure and function of microbial communities in different environments (e.g. Lueders et al., 2004; Ginige et al., 2004). In previous studies, it was not possible to apply this method successfully to

insect guts. In the present thesis, these results were confirmed. Stable isotope probing in the insect gut environment was carried out, to detect the homoacetogenic bacteria in the gut of the wood-feeding termite *Reticulitermes santonensis* and homoacetogenic and glucose-mineralizing bacteria in the humivorous beetle larva of *Pachnoda ephippiata*. Further, the extent carbon incorporation into RNA from the respective substrates was estimated using ^{14}C as tracer. Factors potentially influencing the carbon incorporation success into RNA in the insect gut environment were studied.

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2 Peptidic soil components are a major dietary resource for the humivorous larvae of *Pachnoda* spp. (Coleoptera: Scarabaeidae)

Janet Andert, Oliver Geissinger and Andreas Brune

2.1 Abstract

Humivorous scarab beetle larvae can thrive exclusively on soil organic matter. Feeding experiments have revealed that the larva of *Pachnoda ephippiata* mineralizes all major humus components except the polyphenolic fraction. High proteolytic activity in the alkaline midgut fluid and an enormous ammonia production during gut passage suggested that peptidic soil components are an important dietary resource for the larva. By comparing acid-hydrolyzable amino acids in food soil and feces, we showed that a significant fraction of the peptides in soil are removed during gut passage. This agrees well with the high concentrations of free amino acids found the midgut section. Incubation experiments revealed the presence of substantial particle-associated proteolytic activity also in the hindgut, most probably due to microbial activity. High rates of ammonia formation in hindgut homogenates and the conversion of radiolabeled amino acids to acetate and propionate indicated that microbial fermentations of soil peptides play an important role in the hindgut. This was corroborated by viable counts of amino-acid-fermenting bacteria, which formed a substantial fraction of the hindgut microbiota. A complete inventory of organic and inorganic nitrogen species before, during, and after gut passage revealed the formation of nitrite and nitrate in midgut and hindgut, and a substantial nitrogen deficit in the feces, suggesting that part of the ammonia formed by mineralization is subjected to oxidation and subsequent denitrification to N_2. Together, the results strongly support the hypothesis that peptidic soil components form a major energy and nutrient source for humivorous insects, supplying the animal with microbial fermentation products and essential amino acids.

Keywords: beetle larvae; insect gut; humivory; peptide digestion; gut microbiology

2.2 Introduction

Although the soil fauna is considered to contribute to the conversion of humic substances, directly by comminution and digestion and indirectly by their enhancing effects on microbial activity (Brussaard and Juma, 1996; Lavelle et al., 1997), the specific nature of the substrates exploited by humivorous insects is unclear.

The major building blocks of soil humus are litter-derived components, mainly cellulose, hemicelluloses, and lignin-derived polyphenols, but also nitrogen-rich components of microbial origin, such as peptides, amino acids, and amino sugars (Sposito, 1989; Fründ et al., 1994; Haider, 1994; Kögel-Knabner, 2002). Although native polysaccharides and peptides are easily degraded by the soil microbiota, they are protected from enzymatic hydrolysis by interaction with humic substances and adsorption to clay minerals (Stevenson, 1994; Martin and Haider, 1986).

The intestinal tracts of soil macroinvertebrates are considered hot spots for the turnover of soil organic matter (Brussaard and Juma, 1996). The comminution during and after ingestion, intestinal peristalsis, and a higher water potential in the gut compared to the food material stimulate degradation processes by increasing surface areas and intimately mixing food particles with digestive enzymes. Moreover, digestion of plant fiber and humic substances in soil- and litter-feeding insects is not accomplished by the animal alone, but is aided by a specific gut microbiota (Bignell, 1994; Insam, 1996; Brune, 2003). It is assumed that the host benefits from its intestinal symbionts by using not only the products of microbial fermentations, mainly short-chain fatty acids, but also the microbial biomass as nutritional resources (Kane, 1997; Brune, 2003).

Humivorous species are found among several insect orders. The most prominent example is the soil-feeding termites (Isoptera; Wood and Johnson, 1986; Brauman et al., 2000). Also many dipteran and coleopteran larvae have a humivorous or detritivorous lifestyle (Crowson, 1981; Bärlocher, 1985; Frouz et al., 2003). The larvae of Scarabaeidae are almost exclusively herbivorous or saprophagous (Crowson, 1981), and it has been pointed out that the increase in gut alkalinity correlates with the degree of decay of the diet (Grayson, 1958). The highest pH values have been encountered among the larvae of rose chafers (Cetoniinae; Lemke et al., 2003; and references therein), many

of which live in soils rich in organic matter and feed on decaying plant material in different stages of humification.

To date, most studies have focused on the ability of rose chafer larvae to thrive exclusively on plant fiber (e.g., Werner, 1926; Wiedemann, 1930; Rössler, 1961). The African rose chafers of the genus *Pachnoda*, which harbors several allopatric species with identical lifestyle, emerged as model organisms for laboratory studies because they are easy to breed and have much shorter generation times than the species from temperate regions. Like other scarab beetle larvae, they possess cellulose- and hemicellulose-degrading activities that have been attributed to the microbiota housed in their enlarged hindgut paunch (Werner, 1926; Cazemier et al., 1997).

Fiber-degrading activities, however, are absent from the midgut of Scarabaeidae (Wiedemann, 1930; Schlottke, 1945). The midgut is the major site of peptide digestion, as shown for *Melolontha melolontha* (Wagner et al., 2002). The high pH and the presence of alkali-stable and humic-acid-tolerant proteolytic activities in the midgut fluid of *Pachnoda ephippiata* were a first indication that dietary peptides have to be considered as an important dietary component (Lemke et al., 2003; Zhang and Brune, 2004).

Peptides are by far the most abundant nitrogenous compounds in soil organic matter (Sowden et al., 1976; Schnitzer, 1985). Feeding experiments with ^{14}C-labeled humic model compounds have provided evidence the peptidic component of humic substances is preferentially solubilized and mineralized during gut passage (Li and Brune, 2005). This is in agreement with the high ammonium concentrations in the hindgut (Lemke et al., 2003; Li and Brune, unpublished results) of *P. ephippiata*, indicating amino acid fermentation after proteolysis and supporting the hypothesis that humic-acid-stabilized peptides might be a major dietary resource (Li and Brune, 2005).

To test this hypothesis, we monitored the fate of soil peptides during gut passage, determined the different nitrogen species, and quantified the proteolytic activities in midgut and hindgut contents. From the rates of amino acid mineralization and the fermentative activities in the hindgut contents, we estimated the relative contribution of amino acid fermentation to the total respiratory activity of the larvae.

2.3 Materials and Methods

2.3.1 Larvae and soil

Most experiments were conducted with third instar larvae of *Pachnoda marginata*, which were purchased from a commercial breeder (b.t.b.e. Insektenzucht, Schnürpflingen, Germany). For proteolytic activities and microbial cell counts, we used the closely related *Pachnoda ephippiata*, obtained from the zoological teaching collection at the University of Konstanz (Germany).

Larvae were kept on a peat-based and clay-enriched potting soil (Compo, Münster, Germany), at 25 °C for at least two weeks prior to all experiments (for details, see Lemke et al., 2003). Larvae were dissected, and the intestinal tract was divided into midgut and hindgut compartments as previously described (Lemke et al., 2003). Gut volumes were estimated by geometric approximation (Lemke et al., 2003). After removal of the gut wall, the content of each gut compartment was suspended in the appropriate buffer (see below). All homogenates used for microbial activity measurements were prepared under a nitrogen atmosphere in an anoxic glovebox. Samples for the analysis of organic and inorganic nitrogen species were frozen until further analysis.

2.3.2 Proteolytic activity in gut contents

Midgut and hindgut contents were suspended in TRIS/CAPS buffer (50 mM; pH 10 for the midgut and pH 8 for the hindgut) to a final concentration of 180 mg dry weight gut ml^{-1}. After brief ultrasonication (60 W, 3 s), using an ultrasonic probe with a microtip (UP 50H, Dr. Hielscher, Stuttgart, Germany), homogenates were centrifuged for 20 min at 20,000 × g and 4 °C. The supernatant (soluble fraction) was removed and kept on ice until further use. The particulate fraction was washed three times to remove residual water-soluble protein; the resulting supernatants were discarded. After the last centrifugation step, the particulate fraction was resuspended in 500 µl of the respective buffer. Aliquots of the soluble and the particulate fractions were heat inactivated for 30 min at 100 °C and served as controls. ^{14}C-Labeled bacterial protein was prepared from *Bacillus megaterium* (DSM 32) as previously described (Ji and Brune, 2001). The proteolytic activity was assayed in triplicate at 25 °C, following the procedure described

by Ji and Brune (2005). The specific radioactivity of the protein preparation was 3.61 kBq mg^{-1}, and the final concentration in the assay was 1.6 mg ml^{-1}.

2.3.3 Ammonia formation in gut homogenates

Since the majority of the gut microbiota seems to consist of obligate or facultative anaerobes (Lemke et al., 2003), homogenates of midgut and hindgut contents were prepared under strictly anoxic conditions using bicarbonate buffer (100 mM, pH 10 for the midgut, and pH 8 for the hindgut) reduced with 1 mM dithiothreitol (DTT); the final concentration was 45 mg dry weight midgut or hindgut per ml. Homogenates were incubated for 48 h at 25 °C. Samples were withdrawn at constant time intervals and frozen at –20 °C until further analysis. Limitation by endogenous amino acid formation was tested by adding a mixture of amino acids (final concentration 1.1 mg ml^{-1}), which was prepared from *Bacillus megaterium* protein (Ji and Brune, 2001) by hydrolysis in 6 M HCl for 24 h at 110 °C and subsequent lyophilization.

2.3.4 Fermentation of ^{14}C-labeled amino acids in hindgut homogenates

The contents of two hindguts were suspended in bicarbonate buffer (100 mM, pH 8) containing DTT (1 mM) and mineral solution (AM-5, Boga and Brune, 2003) to a final concentration of 13 mg dry weight gut ml^{-1}. ^{14}C-Labeled amino acids, prepared from *Bacillus megaterium* protein (see above) with a specific radioactivity of 62.9 kBq mg^{-1}, were added to the homogenates to a final concentration of 0.2 mg ml^{-1}. The homogenates were incubated at 25 °C and sampled at regular time intervals.

2.3.5 Total N and inorganic nitrogen species

For total N, samples were dried overnight at 105 °C and pulverized with a mortar and pestle. CHN analysis was carried out by the Service Facility of the Department of Analytical Chemistry, University of Marburg. Inorganic nitrogen species were extracted with 2 M KCl (sample-to-solution-ratio 1:5) for 1 h. Since the pH in the midgut homogenate samples was alkaline, it was assured that no NH_3 was lost during the extraction procedure. The samples were centrifuged for 10 min at 4 °C and 20,000 ×g, and the supernatant was analyzed. After appropriate dilution with 1 M KCl, total ammonia (Kandeler and Gerber, 1988), nitrate (Schlichting and Blume, 1966), and nitrite (Keeney and Nelson, 1982) were determined colorimetrically as previously described.

2.3.6 Amino acid analysis

For total amino acids, samples were acid hydrolyzed with 6 M HCl for 24 h. All samples were diluted 1:2 with 100 mM H_2SO_4 and centrifuged for 10 min at 4 °C to pellet gut content and precipitated humic acids. The supernatant was filtered (pore size 0.2 µm), and amino acids were quantified according to Godel et al. (1984), except that 0.8% tetrahydrofurane was added to gradient buffer A (12.5 mM sodium phosphate, pH 7.2) and 35% methanol to gradient buffer B (50% acetonitrile in 12.5 mM sodium phosphate, pH 7.2). The HPLC system was equipped with an autoinjector for precolumn derivatization and a Grom-SIL OPA-3 column (3 µm, 125 × 4 mm; Alltech Grom, Rottenburg, Germany) connected to both a fluorescence detector and a UV detector.

2.3.7 Analysis of fermentation products

The humic acids were precipitated (see amino acid analysis), and the supernatant was analyzed by ion-exclusion chromatography using a Resin ZH column (250 mm × 8 mm, Alltech Grom, Rottenburg, Germany) with a mobile phase of 5 mM H_2SO_4 and a column temperature of 60 °C. The HPLC system was equipped with a UV detector and an on-line flow scintillation analyzer (Ramona 2000, Raytest, Straubenhardt, Germany) with a cell volume of 1.2 ml, allowing detection of radioactive products. The scintillation cocktail (Quicksafe Flow 2, Zinsser Analytic, Eschborn, Germany) was used at a buffer/cocktail ratio of 1:3.

2.3.8 Enumeration of bacteria

Midguts and hindguts of five larvae were homogenized separately in buffered saline solution (Tholen et al., 1997) reduced with DTT (1 mM), pooled, and serially diluted (1:10) in DTT-reduced AM-5 medium under an N_2/CO_2 (80/20, vol/vol) atmosphere (Boga and Brune, 2003). The medium contained all coding amino acids (1 mM each, except for glycine at 5 mM and cysteine at 0.1 mM), and 0.02% (w/v) yeast extract. Enrichment cultures were incubated at 25 °C. After 35 days, the concentrations of amino acids were determined by HPLC. Most probable numbers (MPNs) of bacteria fermenting individual amino acids were calculated according to Hurley and Roscoe (1983), scoring as positive the dilution tubes where the concentration of the respective amino acid had decreased by 50%.

Denitrifying bacteria were enumerated in non-reduced AM5 medium containing nitrite (8 mM), acetate (0.6 mM), *N*-acetyl-glucosamine (0.4 mM), yeast extract (0.02%), and casamino acids (0.02%), incubated under an N_2/CO_2 atmosphere. Nitrite consumption was determined semiquantitatively with Quantofix® test sticks (Macherey-Nagel, Düren, Germany); each enrichment culture contained a Durham tube to detect gas production.

2.4 Results

2.4.1 Nitrogen transformation during gut passage

Total nitrogen and different nitrogen species were determined in food soil, midgut and hindgut contents, and fecal pellets (Tab. 2.1). The total nitrogen in the midgut content was twofold higher than in the food soil, indicating selective feeding on humus-rich soil components. This was corroborated by the total organic carbon of the midgut content [34.1 mmol (g dry weight)$^{-1}$], which was 1.5 times higher than in the food soil. Total N decreased from midgut to hindgut to fecal pellets, although the differences between the gut compartments were not statistically significant.

Tab. 2.1 Nitrogen balance. Total N, inorganic and organic N species, and C/N ratio of organic matter in food soil, gut compartments, and fecal pellets of *Pachnoda marginata*. Values are given in µmol N (g dry weight)$^{-1}$. All values are means (± standard deviation) of three independent experiments. Values within a row that carry the same index letter are not significantly different (one-way ANOVA with Tukey test; p = 0.05).

Nitrogen species	Food soil	Midgut[1]	Hindgut[2]	Fecal pellets[3]
Total N	555 ± 118^{a}	1310 ± 184^{c}	1000 ± 156^{b,c}	831 ± 73^{b}
Hydrolyzed amino-acid-N	291 ± 32^{b,c}	273 ± 28^{b}	460 ± 146^{c}	128 ± 9^{a}
Free amino acid-N	0.9 ± 0.1^{a}	55.3 ± 4.1^{b}	19.9 ± 2.4^{c}	8.1 ± 0.3^{a}
Ammonia-N	2.54 ± 0.12^{a}	24.3 ± 3.1^{b}	244 ± 61.9^{c}	104 ± 18^{d}
Nitrite-N	0.4 ± 0^{a}	32.9 ± 4.5^{b}	5.8 ± 2.2^{c}	1.5 ± 0.5^{a}
Nitrate-N	7.5 ± 1.4^{a}	60.0 ± 6.5^{b}	41.9 ± 22.4^{b}	50.3 ± 10.6^{b}
C/N	78.7 ± 24.0^{a}	113 ± 23^{a}	80.7 ± 23.3^{a}	277 ± 7^{b}

[1] Average volume and water content of midgut were 500 µl and 80%, respectively.

[2] Average volume and water content of hindgut were 400 µl and 85%, respectively.

[3] Collected 24 h after the larvae were placed on fresh food soil.

About half of the N in the food soil was released as amino acids upon acidic hydrolysis (Tab. 2.1), indicating that — as in other soils — the majority of N is bound in peptides. The proportion of peptide-N decreased from 52 to 21% of total N between food soil and midgut (Tab. 2.1), accompanied by a concomitant increase in free amino acids and inorganic N species (Fig. 2.1). The fraction of inorganic N increased continuously during gut passage and reached 30% of total N in the hindgut (Fig. 2.1). Especially the ammonia concentration increased greatly from 0.5% of total N in the food soil to 22% in the hindgut.

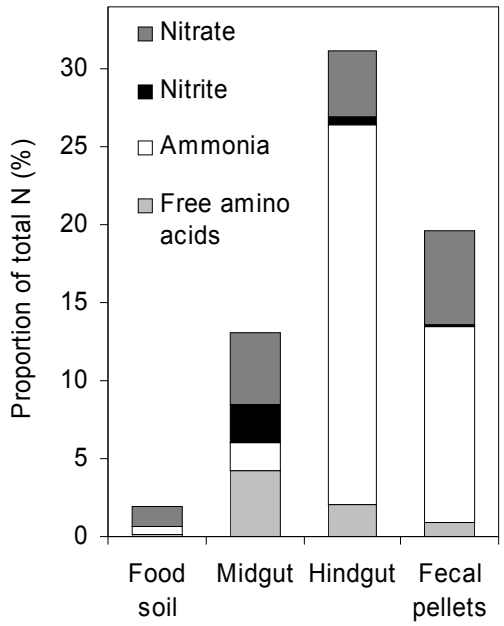

Fig. 2.1 Contribution to total N of free amino acids and inorganic N species in food soil, midgut and hindgut contents, and fecal pellets of *Pachnoda marginata* larvae. Data calculated from Tab. 2.1.

Also nitrate and nitrite were considerably higher in gut contents and fecal pellets than in the food soil, with a high accumulation of nitrite (6 mM) in the midgut, indicating that ammonium is subject to nitrification during gut passage. The decrease in inorganic nitrogen between hindgut and fecal pellets (from 29 to 19%; Fig. 1) suggests loss of nitrogen by denitrification, which agrees well with the concomitant decrease in total

nitrogen and peptide-nitrogen, and is supported by the increasing ratio of carbon and nitrogen in the organic matter (Tab. 2.1).

2.4.2 Proteolytic activities in midgut and hindgut

Although the concentration of free amino acids increased more than 50-fold between soil and midgut (Tab. 2.1), their contribution to total N remained relatively low in both gut compartments (Fig. 2.1). This suggested that the amino acids are either resorbed by the animal or metabolized by the gut microbiota, with the hydrolysis of soil peptides forming the rate-limiting step.

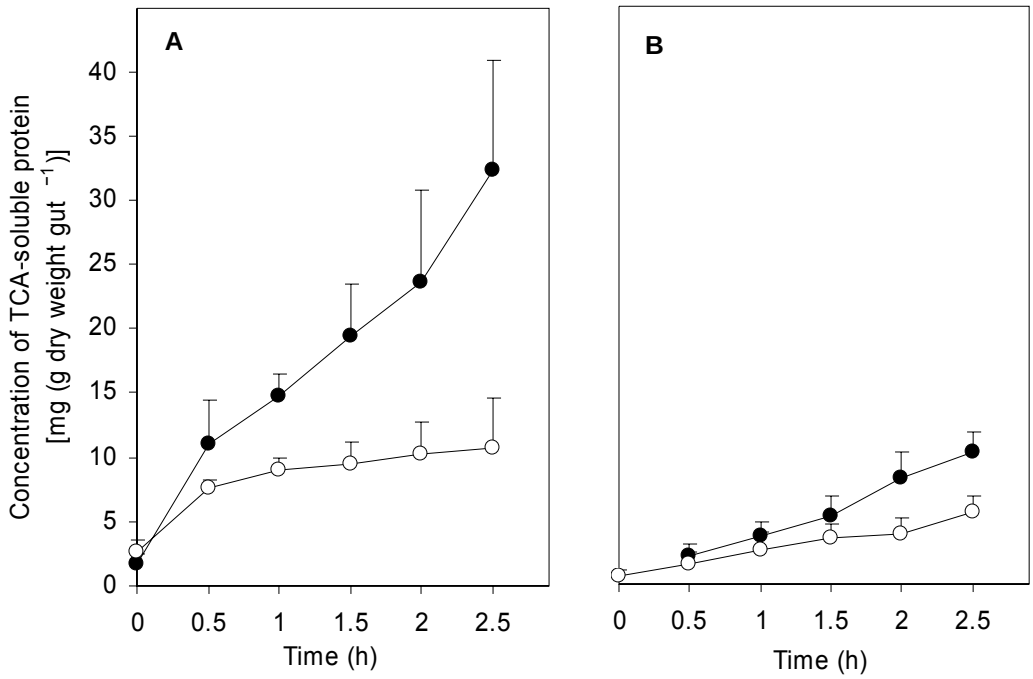

Fig. 2.2 Time course of protein solubilization during incubation of ^{14}C-labeled peptides with soluble (○) and particulate (●) fractions of midgut (A) and hindgut (B) content of *Pachnoda ephippiata* larvae at physiological pH. Error bars represent the standard deviation of three independent experiments.

Kinetic analysis documented that the peptide-solubilizing activity in midgut and hindgut was higher in the particulate fraction than in the soluble fraction (Fig. 2.2). Total initial solubilization rates (soluble and particulate fraction) in the midgut content decreased from 22.2 to 13.8 mg TCA-soluble protein (h × g dry weight)$^{-1}$ owing to a

rapid loss of activity in the soluble fraction. Total initial rates in the midgut were more than three times higher than in the hindgut [6.0 mg TCA-soluble protein (h ×g dry weight)$^{-1}$], with the activity in the particulate fraction being about twice as high as in the soluble fraction.

2.4.3 Hydrolysis and mineralization of soil peptides in gut homogenates

To determine the rate of amino acid mineralization by the gut microbiota in the respective compartments, we followed the time course of free amino acids and ammonia formation in midgut and hindgut content, incubated at their respective physiological pH (Fig. 2.3). In the midgut incubations, ammonia concentration increased only slightly (Fig. 2.3A). The initial rate corresponds to 1.15 µmol (h × g dry weight)$^{-1}$ and decreased even further during the incubation, suggesting that amino acid mineralization is negligible in this compartment. This is in agreement with the continuous increase of the total amino acid concentration over the whole incubation period (Fig. 2.3A).

In the hindgut homogenate incubation (Fig. 2.3B), ammonia was formed continuously. Initial rates, corresponding to 7.2 µmol ammonia (h × g dry weight)$^{-1}$, were considerably higher than in the midgut. Also in the hindgut, the ammonium formation rate decreased with time, suggesting that proteolysis became the rate-limiting step. This was corroborated by the addition of free amino acids to hindgut homogenates, which increased the initial rates of ammonia formation about threefold over the endogenous rates.

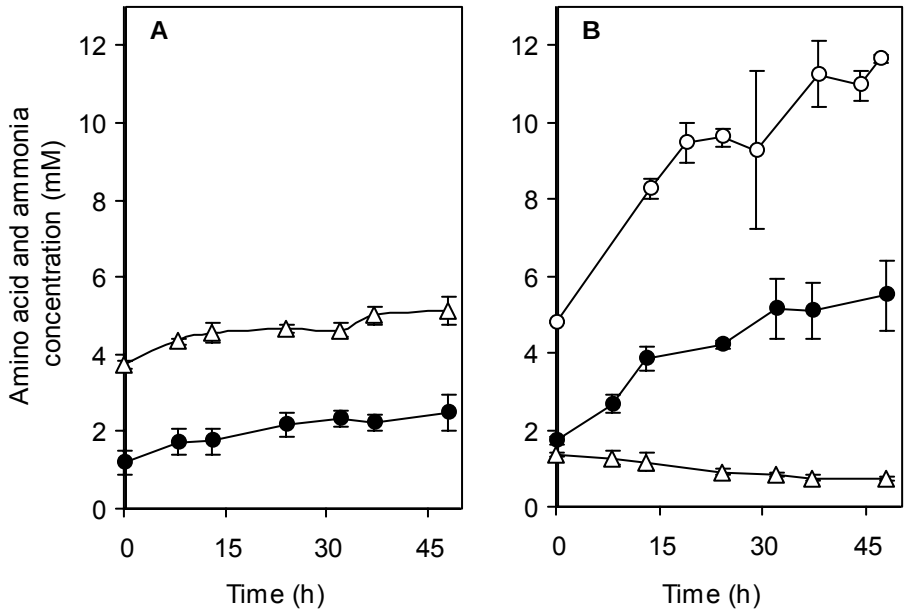

Fig. 2.3 Time course of free amino acid concentration (Δ) and ammonium accumulation (●) in midgut (A) and hindgut (B) homogenates of *Pachnoda marginata* incubated under anoxic conditions at pH 10 and pH 8, respectively. Limitation of ammonium formation in hindgut homogenates (○) was tested by the addition of a mixture of amino acids prepared from *Bacillus megaterium* protein. Bars represent standard deviation of three independent experiments.

To follow the fate of the carbon skeleton of amino acids during N mineralization, hindgut homogenate was incubated with a mixture of ^{14}C-labeled amino acids prepared from *Bacillus megaterium* protein. Radioactivity was detected only in acetate and propionate, with acetate being the most abundant product (Fig. 2.4). The formation rates of acetate and propionate per larva were 0.18 and 0.1 μmol (h × g fresh weight)$^{-1}$, respectively. After 44 h, small amounts of ^{14}C-isobutyrate were formed (not shown).

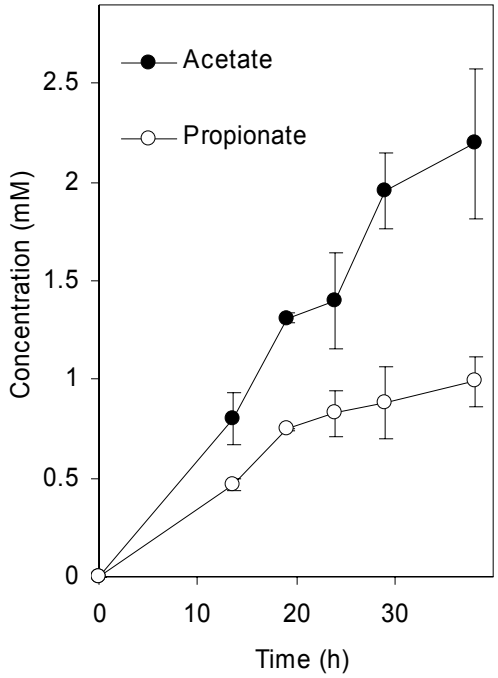

Fig. 2.4 Formation of fermentation products from radiolabeled amino acids in hindgut homogenates of *Pachnoda marginata* incubated under anoxic conditions in reduced bicarbonate buffer (pH 8). ^{14}C-labeled amino acids were prepared from *Bacillus megaterium* protein. Bars represent mean deviation of two independent experiments.

2.4.4 Enumeration of amino-acid-fermenting and denitrifying bacteria

Serial dilution of gut contents yielded most probable numbers (MPNs) of amino-acid-fermenting bacteria that were considerably lower for the midgut than for the hindgut of *P. ephippiata* (4.3×10^7 ml^{-1} and 1.4×10^9 ml^{-1}, respectively). Individual amino acids were utilized to a different extent: in both midgut and hindgut enrichments, polar amino acids were consumed in higher dilutions than non-polar amino acids, indicating that the bacteria responsible for these activities were more abundant (Fig. 2.5). The spectrum of amino acids consumed by the most abundant bacteria was more diverse in the hindgut than in the midgut.

In enrichment cultures for denitrifying bacteria, conducted with hindgut homogenates of *P. marginata*, nitrite consumption was always accompanied by vigorous gas production and considerably more turbidity than in the tubes lacking nitrite reduction. The MPN of denitrifying bacteria was 9.2×10^8 ml^{-1} gut content (three parallel dilutions).

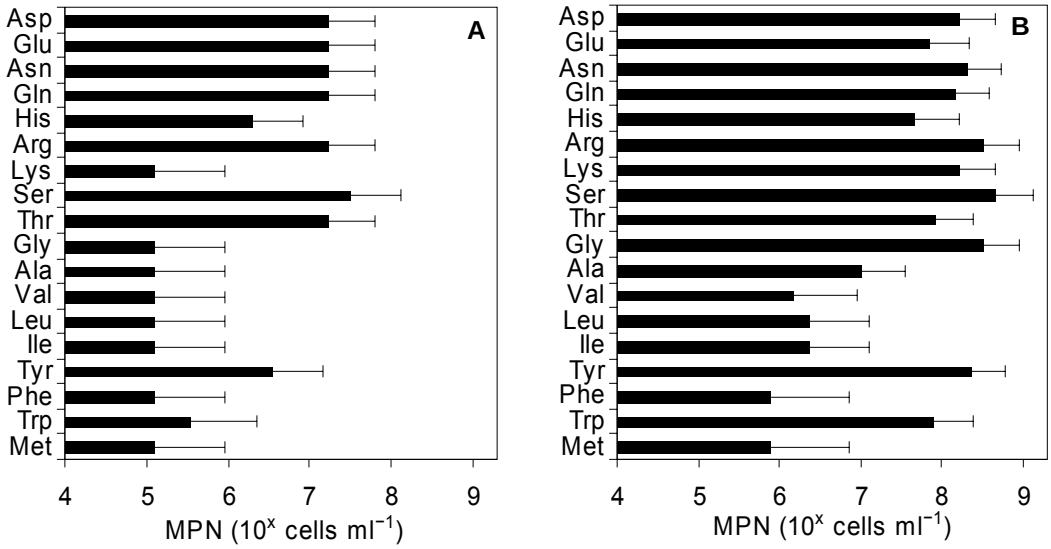

Fig. 2.5 Most probable numbers (MPN) of bacteria fermenting individual amino acids in serial dilutions of midgut (A) and hindgut (B) content of *Pachnoda ephippiata*, incubated for 35 d in reduced growth medium containing a mixture of amino acids. Error bars indicate the standard deviation for *n* parallel dilutions (midgut $n = 3$, hindgut $n = 5$).

2.5 Discussion

Soil-feeding macroinvertebrates play a major role in the transformation of soil organic matter. Although there is evidence that different components of humus are mineralized during gut passage (Li and Brune, 2005), their relative importance in the digestive process remained unclear. In this study, we provide evidence that amino acids resulting from the hydrolysis of humic-acid-stabilized peptides are major dietary resource for the larva of *Pachnoda* spp.

2.5.1 Proteolytic activities

In *Pachnoda* spp., the major part of the proteolytic activity is localized in the midgut, the major site for the secretion of digestive enzymes in insects (Crowson, 1981, Terra and Ferreira, 1994). Here, ingested humic-acid stabilized peptides are solubilized and hydrolyzed to amino acids, which accumulate to concentrations of 2–24 mM in *P. ephippiata* and 2.8–9.8 mM in *P. marginata* (Li and Brune, 2005; this study).

The proteolytic activity in the midgut of *Pachnoda* spp. is most likely caused by host proteinases since the contribution of microbial proteolytic activity is likely minor due to

the low microbial cell numbers (Lemke et al., 2003). Nevertheless, the majority of the proteolytic activity was detected in the particular fraction, indicating that the host enzymes are adsorbed to clay particles in the food soil, as shown by the model experiments on proteinase–clay interactions of Kelleher et al. (2003).

The distribution of proteolytic activities suggests that the midgut is the main site for the hydrolysis of peptides in *Pachnoda* spp. Also in soil-feeding termites (Fujita and Abe, 2002; Ji and Brune, 2005), proteinase activity was maximal in the midgut fluid. The proteinase activity in the midgut of the herbivorous larva of *Costelytra zealandica* (Coleoptera: Scarabaeidae) was seven times higher than that in the hindgut (Biggs and McGregor, 1996), but was much lower than in the humivorous *P. ephippiata* (this study). The protein solubilization rates in the midguts of the black field cricket *Teleogryllus commodus* (Walker) (Christeller et al., 1990) and of three keratinolytic lepidopteran larvae (Christeller et al., 1994) are similar to the proteolytic activity in the midgut of *Pachnoda* species.

However, also the proteolytic activity in the hindgut, which is only 2 to 3.5 times lower than that in the midgut (Fig. 2.2B), contributes to the total proteolytic activity in the gut of *P. ephippiata* larvae. The fact that the majority of the activity was in the particular fraction suggests that the gut microbiota plays a major role in hindgut proteolysis, which is supported by the high density of bacteria in the hindgut (Lemke et al., 2003). Tokuda et al. (2005) made similar observations for the wood-feeding termite *Nasutitermes takasagoensis*, where the particulate fraction of the hindgut contents contained a considerably higher cellulolytic activity than the soluble fraction. Also in the cow rumen, the removal of bacterial cells from the rumen fluid leads to a highly reduced proteolysis rate (Wallace, 1985).

2.5.2 Amino acid fermentation

Accumulation of amino acids in the midgut homogenate incubation (Fig. 2.3A) suggests that amino acid fermentation is the rate-limiting step in the midgut homogenate incubations, which is in agreement with the higher efficiency of proteolysis and the lower ammonia formation rates compared to the hindgut (Fig. 2.2A). In vivo, the amino acids will not accumulate infinitely. Amino acid concentration will depend on the rate of amino acid resorption by the insect host since the number of amino-acid-fermenting bacteria compared to the hindgut is low.

Any amino acids in the midgut fluid that are not resorbed will be eventually transferred to the hindgut, where fermentation processes are responsible for the formation of copious amounts of ammonia. The difference between the amino acid degradation and ammonia formation rates in hindgut homogenates indicates that additional amino acids are released from soil peptides by proteolysis during incubation and mineralized consecutively. The concentration of free amino acids was considerably lower than in the midgut, and the stimulation of ammonia formation after addition of external amino acids suggests that proteolysis remains the rate-limiting step. Also in a continuous culture obtained from the rumen, proteolysis has been identified as rate-limiting step in amino acid fermentation (Griswold et al., 1996). As in the case of the rumen, the proteolytic activity is most likely of bacterial origin, since host enzymes are unlikely to be secreted in the hindgut (Crowson, 1981; Terra and Ferreira, 1994).

The high density of bacterial cells in the hindgut of *Pachnoda* spp. (Cazemier et al., 1997; Lemke et al., 2003) and the high ammonium formation rates suggest that the hindgut paunch is the main site of microbial fermentations. This is in agreement with the MPNs of amino-acid-fermenting bacteria (this study), which indicated that the latter represent about 7% of the total bacterial cell counts in hindgut.

2.5.3 The fate of soil peptides during gut passage

The strong increase in total nitrogen increased between food soil and gut compartments indicates that *Pachnoda* species preferentially ingest nitrogen-rich food particles. Such selective feeding has been reported also for the humivorous larva of *Adoryphorus couloni* (Coleoptera: Scarabaeidae; McQuillan and Webb, 1994). In the midgut, amino acids are released by alkaline extraction and subsequent proteolysis, leading to the accumulation of free amino acids. Free amino acids in the hindgut, originating from the midgut or formed by proteolytic activities of the gut microbiota, are fermented by the microbiota, giving rise to an enormous accumulation of ammonia. The presence of ammonia also in the midgut fluid indicates that amino acid fermentation commences already in the midgut. The significant decrease in peptide contents between hindgut and fecal pellets indicate further mineralization of peptides, this time possibly stemming from microbial biomass formed in the hindgut.

The accumulation of nitrite and nitrate documents the presence of ammonia-oxidizing and nitrite-oxidizing bacteria in the midgut and hindgut (Bedard and Knowles,

1989). Since nitrate is the most preferred electron acceptor in anoxic environments, it is easily reduced by the gut microbiota, as evidenced by the high numbers of denitrifying bacteria in the hindgut of *P. marginata.* This is supported by the strong loss of nitrogen between hindgut and fecal pellets, which cannot be explained by ammonium emission from fecal pellets (Li and Brune, unpublished results) but only by a microbial conversion of nitrate and nitrite to N_2 (denitrification) in gut and feces.

Using the C/N ratio of 3.62 determined for the amino acids released from food soil after acidic hydrolysis for the conversion of nitrogen-based to carbon-based rates, we estimated that the endogenous rates of ammonia formation in midgut and hindgut homogenates contribute about 10% to the total respiratory rate of the larva [10 μmol CO_2 (h × fresh weight larva)$^{-1}$; Lemke et al., 2003]. The contribution of the hindgut alone (8%) agrees well with the value (7%) that can be deduced from the formation rates of radioactive fermentation products from ^{14}C-labeled amino acids in hindgut homogenates.

2.5.4 Conclusion

Soil peptides stabilized in the humic substances are a major nutrient source for humivorous scarab beetle larvae. Amino acids formed by peptide hydrolysis in the midgut can be directly resorbed by the insect, providing the animal with essential amino acids (Dadd, 1973). In addition, the products of microbial amino acid fermentation in the hindgut contribute substantially to the energy metabolism of the larvae. Part of the ammonia released during mineralization is microbially oxidized to nitrate, which stimulates denitrification and causes nitrogen loss from the soil.

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3 Highly active but not incorporating ^{13}C-label into nucleic acids: Factors influencing RNA-stable isotope probing in insect intestinal tract microbiota

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3.1 Abstract

RNA-stable isotope probing is a valuable method to link structure and function of microbial communities. It was applied successfully for a variety of metabolic processes in different environments. However, attempts to identify the microorganisms responsible for the glucose conversion and for acetogenesis to from H_2 and HCO_3^- in the hindgut of the humivorous beetle larva *Pachnoda ephippiata* remained unsuccessful. Also, incubations of whole guts of *Reticulitermes santonensis* with $H^{13}CO_3^-$ and H_2 did not reveal the microorganisms performing homoacetogenesis. In this study, several possibilities for the low incorporation of substrate carbon into RNA of the insect gut microbiota are discussed. Since free nucleotides accumulate in the hindgut of *Pachnoda marginata* to high concentrations, we suggest that salvage pathways, known for many microorganisms as alternative for nucleotide biosynthesis from preformed nucleobases, reduce the *de novo* formation of nucleic acids from substrate carbon. This hypothesis is supported by a considerable inhibition of the incorporation of substrate carbon into RNA in incubations with *Bacillus subtilis* and *Pseudomonas putida* in the presence of nucleotides.

However, the most probable explanation is the uncoupling of catabolism and anabolism, since obviously the substrate carbon is not used for *de novo* synthesis of RNA.

3.2 Introduction

The cultivation of microorganisms was for a long time the only way to study the composition of different environments. However, only a small fraction of microorganisms is cultivable (Amann et al., 1995). Therefore, PCR-based cultivation-independent techniques, like denaturing gradient gel electrophoresis (DGGE, Muyzer et al., 1993) and terminal restriction length polymorphism (T-RFPL, Liu et al., 1997) have been developed. Besides, soils and sediments, the intestinal microbial community of many insects has been already characterized by these means (termites, Hongoh et al., 2003; Schmitt-Wagner et al., 2003; Shinzato et al., 2005; bees, Mohr and Tebbe, 2006; beetle larvae, Egert et al., 2003; Egert et al., 2005). However, the phylogenetic function of the respective microbial population does not reveal the function of the respective organisms in the ecosystem. To link structure and function in microbial communities two methods, stable isotope probing (SIP, Boschker et al., 1998; Radajewski et al., 2003) and bromodeoxyuridine (BrdU; thymidine analog) probing (Borneman, 1999; Urbach et al., 1999), have been established for the detection and identification of microorganisms that consume certain compounds as carbon or nitrogen source.

Stable isotope probing has been used several times successfully to assign distinct metabolic activities to microorganisms with the help of stable-isotope-labeled substrates in soils and sediments (e.g. Ginige et al., 2004; Lueders et al., 2004; McDonald et al., 2005). As phylogenetic biomarkers for stable isotope probing polar-lipid fatty acids (PLFA) DNA and rRNA can be addressed. Using PLFA as phylogenetic marker, the extent of incorporation of carbon from the ^{13}C-labelled substrate was higher compared to the incorporation into rRNA (MacGregor et al., 2006). Nevertheless, the phylogenetic resolution of DNA and RNA is higher compared to PLFA (Radajewski et al., 2000). Therefore, nucleic acid stable isotope probing is preferably used. Stable isotope probing with DNA has been carried out successfully several times (Whitby et al., 2001; Morris et al., 2002). However, since of the long incubation times needed for sufficiently labeled DNA and synthesis of RNA occurs with a faster rate than DNA synthesis, many recent studies use RNA as biomarker, (Manefield et al., 2002). Although stable isotope probing emerged as an important tool in microbial ecology, the lower detection limit for

visible effect of the incorporation of substrate carbon into RNA (between 10 and 20%; Manefield et al., 2002b) is considerably high.

Up to now, stable isotope probing was not carried out in the intestinal environment. The function of individual populations in microbial gut communities could not be assigned, despite the availability of isolates from various cultivation approaches (Bauer et al., 2000, Graber et al., 2004, Kleessen et al., 2000). However, many microorganisms have the potential for several metabolic ways, but are not performing all of them in the respective environment.

In a model system for the human gut, stable isotope probing was applied successfully with glucose (Egert et al., 2007), but only a high substrate concentration (40 mM) lead to sufficient ^{13}C incorporation into RNA. Previous stable isotope probing experiments of the microbiota in the insect gut did not reveal detectable stable isotope probing effects in the RNA, presumably, due to insufficient incorporation of substrate carbon into RNA.

In the present study, these results were confirmed using two insect gut systems and two substrates addressing distinct metabolic groups. Firstly, whole guts of the wood-feeding termite *Reticulitermes santonensis* were incubated with $H^{13}CO_3^-$ in order to identify the microbial homoacetogenic population. Secondly, universally labeled ^{13}C-glucose and $H^{13}CO_3^-$ were used as carbon source in incubations of hindgut homogenate of the humivorous beetle larvae *Pachnoda ephippiata*. Further, the extent of label incorporation into microbial RNA during assimilation of these substrates in the respective gut environment was monitored using ^{14}C, representing a tracer more sensitive than ^{13}C. Additionally, the potential importance of these processes in the respective system was estimated.

3.3 Materials and Methods

3.3.1 Insects and preparation of gut homogenates

Third instar larvae of *Pachnoda ephippiata* and *Pachnoda marginata* were purchased from a commercial breeder (b.t.b.e. Insektenzucht, Schnürpflingen, Germany). Larvae were kept at 25 °C on a peat-based and clay-enriched potting soil (Compo, Münster,

Germany) for at least two weeks prior to all experiments (for details, see (Lemke et al., 2003). Larvae were dissected and the intestinal tract was divided into midgut and hindgut compartments as previously described (Lemke et al., 2003). The hindguts were transferred separately to an anoxic glovebox for homogenization with a glass homogenizer and dilution of the hindgut content with 100 mM HCO_3^- buffer (pH 8) reduced with 1mM Dithiothreitol (DTT) unless indicated differently. The resulting homogenate, generally 0.25 guts ml^{-1}, was used for experimentation.

Reticulitermes santonensis (Feytaud) were collected in the Forêt de la Coubre near Royan (France). It was confirmed that the sequence of subunit II of the cytochrome oxidase was identical to published sequences of *R. santonensis*. The termites were kept in the laboratory in polyethylene boxes with pine wood and water. For experiments, only worker termites were used. Before experimentation, the termites were transferred into an anoxic glove box. With the help of tweezers the whole guts where dissected. Twenty guts were added to 1-ml-glass vials with 800 µl Solution U (pH 7.2; anoxic; 1 mM DTT; Trager, 1934), which were closed air-tightly.

3.3.2 Acetogenesis in *Pachnoda ephippiata* hindgut homogenate and *Reticulitermes santonensis* whole guts from $NaHCO_3$

Hindgut homogenate from *P. ephippiata* (0.7 guts ml^{-1}) was prepared with sterile, anoxic, buffered saline solution (BSS; Tholen et al., 1997) at pH 8.4. The carbon source was 16.3 mM $H^{14}CO_3^-$ with a specific radioactivity of 12.89 kBq μmol^{-1}. The incubation was carried out under H_2 headspace at 25 °C for 6 hours shaking at 160 rpm. Samples of 250 µl were taken in equal intervals and centrifuged for 10 min at 10,000 × g and 4 °C. The supernatants were stored at –20 °C until further analysis.

Further, four times 20 whole guts of *Reticulitermes santonensis* were prepared as described above and the headspace in each vial was exchanged to hydrogen. To start the incubation, 12 µM $H^{14}CO_3^-$ with a specific radioactivity of 6.5 kBq $nmol^{-1}$ were added. After 2, 4, 5 and 7 h at 30 °C, the incubations were stopped and the respective samples were stored until further analysis at –20 °C.

3.3.3 Fermentation of glucose in *Pachnoda ephippiata* hindgut homogenate

The homogenate, prepared as described above, was filled in 4 ml–vials, closed with butyl rubber stoppers and 5mM ^{14}C glucose with a specific radioactivity of 18.13 kBq µmol^{-1} was added. The vials were incubated in the dark without shaking at 25°C. The incubation was sampled in constant time intervals: For determination of the $^{14}CO_2$ formation, 100 µl were taken from the headspace with a gastight syringe and analyzed by gas chromatography (Shimadzu GC–8A equipped with a FID detector and a Porapak Q packed (180 cm) column (Alltech, Hamburg, Germany) connected to a methanizer and a Ramona radioisotope detector (Raytest, Straubingen, Germany). Furthermore, 50 µl homogenate were taken out and placed into a 2-ml-vial closed tightly with a rubber stopper and filled with 50 µl 100 mM H_2SO_4 to acidify the solution. By these means, the dissolved CO_2 was released from the homogenate. From the headspace of this vial 100 µl were taken and again analyzed with a gas chromatograph. The acidified homogenate was stored at −20°C until further analysis.

3.3.4 Stable isotope *probing* incubations

Hindgut homogenate of *Pachnoda ephippiata* (1 gut ml^{-1}) was prepared as described above, except that sterile and anoxic BSS (Tholen et al., 1997) pH 8.4 was used as buffer. The homogenate was divided, amended with 30 mM $H^{13}CO_3^-$ or 30 mM unlabeled HCO_3^-, respectively, and incubated at 25 °C without shaking in the dark for 6 h.

Further, hindgut homogenate of *Pachnoda ephippiata* (0.6 gut ml^{-1}) was portioned into six 4-ml-vials. To four of these, a mixture of ^{13}C and ^{14}C glucose was added up to final concentration of 5 mM. One control was incubated without glucose, where the volume of the glucose solution was substituted with buffer. In the second control, the ^{13}C glucose was replaced by a mixture of $^{12}/^{14}$C-glucose. In both incubations ($^{12/14}$C, $^{13/14}$C), the specific radioactivity of the glucose solution was 43 Bq (nmol C)$^{-1}$. All samples were incubated at 25 °C without shaking in the dark. After 1, 2, 4 and 6 h, the incubation of the ^{13}C glucose samples was stopped on ice; the two controls were chilled on ice after 6 h.

Lastly, two suspensions of whole guts of *Reticulitermes santonensis* were prepared as described above. $H^{13}CO_3$ or unlabeled HCO_3^- (12 µM both) was added and the vials

were incubated at 30 °C. After 2 and 7 h one ^{12}C and one ^{13}C incubation was stopped, respectively.

All samples were stored at –20 °C until further processing.

3.3.5 Incorporation of carbon from *glucose* into total RNA of *Bacillus subtilis* and *Pseudomonas putida* in the presence of several ribonucleotide concentrations

RNA (Ribonucleic acid sodium salt, Carl Roth GmbH, Karlsruhe, Germany) was hydrolyzed with 5 µg ml^{-1} Ribonuclease (Carl Roth GmbH, Karlsruhe, Germany) in 10 mM Tris (pH 7.5) and 15 mM NaCl to single ribonucleotides at 37 °C for three days. To inhibit microbial growth one drop toluene was added. The digestion success was monitored by gel electrophoresis. Precultures of *Bacillus subtilis* and *Pseudomonas putida* were grown overnight on liquid LB (Bertani, 1951) medium at 30 °C. Furthermore, substrate-free minimal medium MM-4 (Brune et al. 1995) without yeast extract, casamino acids, and naphtoquinone instead of menadione and aromatic fatty acids instead of phenyl fatty acids was prepared. The medium was amended with 10 mM ^{14}C-labelled glucose with a specific radioactivity of 1.06 Bq (nmol glucose)$^{-1}$ and different concentrations of ribonucleotides (0, 1.25, 2.5, 5, 10, 20 mM) before inoculation with 1% of the respective preculture. For incubation with an amino acid mixture, instead of ribonucleotides 0, 1.25, 5, 10 mM of aspartate and glycine were used as potential precursors for nucleotides, respectively. The cells were grown aerobically at 30 °C up to an OD_{600} of 1.6 and harvested in the exponential growth phase by centrifugation at 4 °C and 20,000 ×g for 10 min. The supernatant was discarded and the pellet was stored at –20 °C until further processing.

3.3.6 Estimation of *incorporation* of carbon into total RNA

The concentrations of ^{14}C-labeled RNA extracts were determined with a spectrophotometer (Nanodrop, ND-1000, Wilmington, USA) or using the RiboGreen nucleic acid stain (Quant-iT™ RiboGreen Assay Kit, Invitrogen Molecular Probes, Invitrogen, Karlsruhe, Germany). The radioactivity of the extracts was measured using a Beckman Multi Purpose Scintillation Counter, Beckman Coulter GmbH, Krefeld, Germany) in Pico-Aqua™ liquid scintillation cocktail (Canberra Packard, Dreieich,

Germany). With these data, it was possible to calculate the level of ^{14}C-carbon incorporation into the RNA of the hindgut microorganisms, *Bacillus subtilis* and *Pseudomonas putida*.

3.3.7 Estimation of *carbon* incorporation of from $NaHCO_3$ into 16S rRNA during incubation of *Reticulitermes santonensis* whole guts

The previously extracted total nucleic acids were loaded on an agarose gel to separate DNA, 23S and 16S rRNA from each other. Further on, the concentration of the 16S rRNA band was determined using a gel electrophoresis analysis software (Gel-Pro Analyzer, MediaCybernetics, Silver Spring, USA) and excised from the gel to determine the radioactivity by Liquid scintillation counting in 3.5 ml Cocktail. From these data the specific radioactivity of the extracted RNA was calculated. To obtain the extent of radiolabel incorporation in %, the specfic activity (Bq nmol–1) of the RNA carbon was referred to the specific activity of the substrate carbon (100%).

3.3.8 Analysis of total and ^{14}C labeled fermentation products

Homogenate samples or supernatants from *Pachnoda* sp. hindgut homogenate incubation were acidified with 1 volume of 100 mM H_2SO_4 to precipitate humic acids. Samples from all incubations were centrifuged for 10 min at 20,000 ×g and 4 °C to separate the particulate fraction from the liquid fraction. The liquid fraction was analyzed for short-chain fatty acids by ion exclusion chromatography with an HPLC system equipped with a Resin ZH column (250 mm × 8 mm, Alltech Grom, Rottenburg-Hailfingen, Germany) and a refractive index detector and an UV detector with a mobile phase of 5 mM H_2SO_4 and a column temperature of 60 °C.

Accumulation of radioactive short chain fatty acids was determined with a combination of a UV detector and a subsequent on-line flow scintillation analyzer (Ramona 2000, Raytest, Straubenhardt, Germany) with a cell volume of 1.2 ml. The scintillation cocktail (Quicksafe Flow 2, Zinsser Analytic, Eschborn, Germany) was used at a buffer/cocktail ratio of 1:3.

3.3.9 Analysis of ribonucleotides and ribonucleosides

Ribonucleotides and ribonucleosides were separated isocratically on a reversed phase column (Superspher 100, C18, 200 mm × 4.6 mm × 4 µM; Grom, Germany) with 20 mM triethylammonium phosphate pH 5.1 with 12% methanol as mobile phase (Mesbah et al., 1989) at a flow rate of 1.5 ml min^{-1}. The detection device was a UV detector at 254 nm.

3.3.10 Extraction of nucleic acids

The total nucleic acid extraction of the above described stable isotope incubations of *Pachnoda ephippiata* hindgut and *Reticulitermes santonensis* whole gut was carried out modified after Lueders and Friedrich (2002).For the extraction of RNA, the cell pellets or homogenate samples were resuspended with 1 ml TRIzol® Reagent (Invitrogen) followed by cell disruption, instead. The precipitation of the nucleic acids was performed with 1 volume isopropanol and 0.01 volume 3 M sodium acetate (pH 5.2) at 4 °C overnight. The precipitate was eluted in 100 µl Dnase–free water or 50 µl of elution buffer (Qiaquick PCR Purification Kit; Qiagen, Hilden, Germany). Until further analysis, the extracts were stored at –20 °C.

3.3.11 Gradient density centrifugation of total RNA from Stable isotope incubations of hindgut of *Pachnoda ephippiata* and whole gut of *Reticulitermes santonensis*

DNA was removed from the extract of total nucleic acids by Dnase digestion (0.05 U μl^{-1} ;RQ 1 Rnase-free Dnase, Promega, Mannheim, Germany) for 1.5 h at 37 °C. The RNA was again extracted following the above-described method, and DNA removal was checked by analytical agarose gel (1%) electrophoresis. The RNA concentration was determined fluorimetrically using the RiboGreen nucleic acid stain (Quant-iT™ RiboGreen Assay Kit, Invitrogen Molecular Probes, Invitrogen, Karlsruhe, Germany). 500 ng of RNA were dissolved in 1 ml gradient buffer (0,1 M Tris-HCl (pH 8), 0,1 M KCl, 1mM EDTA) and mixed with 4.5 ml CsTFA (cesium trifluoroacetate, 2 g ml^{-1}, Amersham Biosciences, GE Healthcare, Munich, Germany) and 175 µl formamide (mol. biol. Grade, company). The density was adjusted to 1.79 g ml^{-1} either by addition of CsTFA or gradient buffer and the resulting mixture was centrifuged at 130000 ×*g* for

65 h at 20 °C. After gradient density centrifugation, the gradient was fractionated into 15 fractions of 375 µl each and the density was determined with a refractometer (AR200, Reichert Analytical Instruments). The RNA in these fractions was precipitated by addition of 500 µl isopropanol and 0.01 volumes of sodium acetate (3 M, pH 5.2). The resulting precipitate was washed with 70 % ethanol and eluted subsequently in 30 µl elution buffer (EB, Qiagen GmbH, Hilden Germany).

3.3.12 T-RFLP analysis

The RNA of each gradient fraction was reversely transcribed into crDNA and amplified by PCR (Access RT PCR system, Promega, Mannheim, Germany) according to (Egert et al., 2003). The primers used were 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') (Lane, 1991) and 907r (5'-CCG TCA ATT CCT TTR AGT TT-3') (Lane, 1991) (MWG, Ebersberg, Germany) targeting the 16S rRNA gene of all members of the domain Bacteria. The 27f primer for the RNA sample from the stable isotope experiment with *P. ephippiata* hindgut and $NaHCO_3$ was 5'-labeled with 6-carboxyfluorescein and with IRD700 (pentamethin carbocyanin dye) for the other stable isotope incubations.

The PCR products were checked by gel electrophoresis and purified with the Qiaquick PCR purification kit or the Minelute PCR purification kit (Qiagen, Hilden, Germany) depending on the intensity of the DNA bands on the gel. DNA concentrations of PCR products were determined spectrophotometrically. For digestion, 5 U of *Msp*I (Promega, Mannheim, Germany) were used for ~ 100 ng DNA in a combination of 1 µl of the appropriate 10 × incubation buffer (Promega, Mannheim, Germany) and 0,1 µg acetylated bovine serum albumin filled up to total volume of 10 µl. The incubation was carried out at 37 °C for 3 h.

The fluorescently labeled terminal restriction fragments (T-RFs) resulting were size separated on an ABI 373A automated sequencer (Applied Biosystems) with an internal size standard (GeneScan-2500 ROX, Applied Biosystems). The T-RFLP electropherograms were analyzed with GeneScan 2.1 software (Applied Biosystems). The samples from the stable isotope incubation experiments of *P. ephippiata* hindgut with glucose and *Reticulitermes santonensi*s whole guts with $NaHCO_3$ were separated electrophoretically using a LICOR 4200 automated sequencer (LICOR Biosciences)

resulting in a TIFF file, which was evaluated with the help of the software Gel-Pro Analyser (Version 4.5; Media Cybernetics, Silver Spring, USA).

3.3.13 Reverse transcription quantitative PCR

The concentration of bacterial 16S rRNA in the gradient fractions from the stable isotope incubation approach with glucose in *Pachnoda ephippiata* hindgut homogenate was determined by reverse transcription quantitative PCR using enhanced avian reverse transcriptase and the SYBR Green Jumpstart Taq Ready Mix (both Sigma, Taufkirchen, Germany) according to manufacturers instructions. As standard RNA for the calibration curve RNA of *Bacillus subtilis* was used, grown on MM-4 (Brune et al. 1995) with 5 mM glucose at 30 °C. The amplification reactions were performed in an iCycler (Bio-Rad, Munich, Germany) with primers 519f (Lane, 1991) and 907r (Lane, 1991). The concentrations of the amplicons were calculated with the help of the iCycler software (BioRAD, Munich, Germany).

3.4 Results

3.4.1 Turnover of the compounds used for stable isotope probing

Prior to stable isotope probing experiments, the suitability of the potential labeled substrates as carbon source was tested. The criterion was high turnover rates of the respective compound.

In incubations of hindgut homogenate of *Pachnoda ephippiata*, glucose was quickly turned over (Fig. 3.1) with a rate of 1.2 µmol (h × g fresh weight gut)$^{-1}$. The main products were acetate and CO_2, which were formed with rates corresponding to 1 µmol (h × g fresh weight gut)$^{-1}$ and 0.8 µmol (h × g fresh weight gut)$^{-1}$, respectively. After 14 hours, also butyrate and propionate accumulated, but since no glucose was detectable anymore, the origin was apparently secondary fermentation of the products from glucose fermentation.

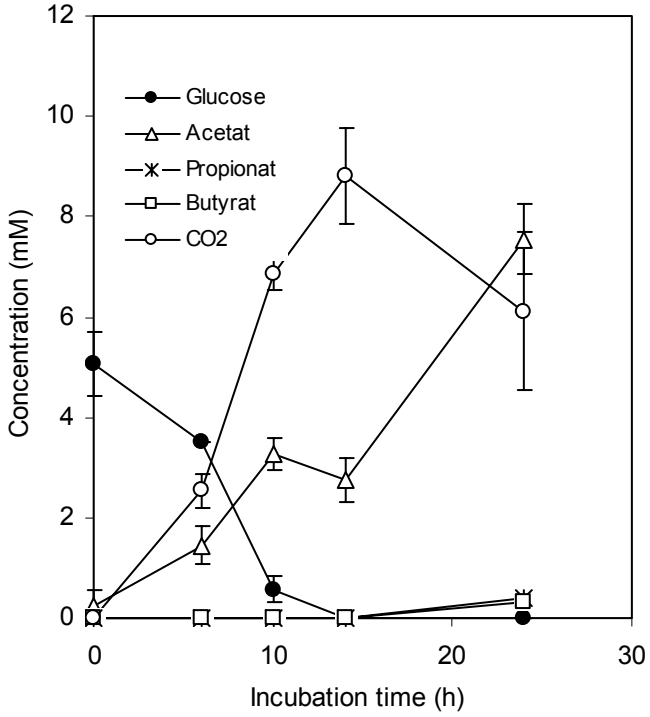

Fig. 3.1 Turnover of ^{14}C labeled glucose and formation of radioactive fermentation products during incubation of hindgut homogenate of the humus-feeding beetle larvae of *Pachnoda ephippiata* at pH 8 under anoxic conditions. Error bars represent mean deviation (n = 2).

To confirm the turnover of $^{14}CO_2$ in incubations with whole guts of *Reticulitermes santonensis* and the hindgut of *Pachnoda ephippiata*, the time course of ^{14}C acetate formation was monitored (Fig. 3.2). In both incubations, acetate accumulated immediately and the concentration increased consistently during the time monitored. The acetate formation rate in the incubation of whole gut suspensions of *Reticulitermes santonensis* accounted for 4 µmol (h × g fresh weight termite)$^{-1}$ (Fig. 3.2A), which was considerably higher than the rate in *Pachnoda ephippiata* hindgut homogenate incubations [6.5 nmol (h × g fresh weight larva)$^{-1}$] (Fig. 3.2B).

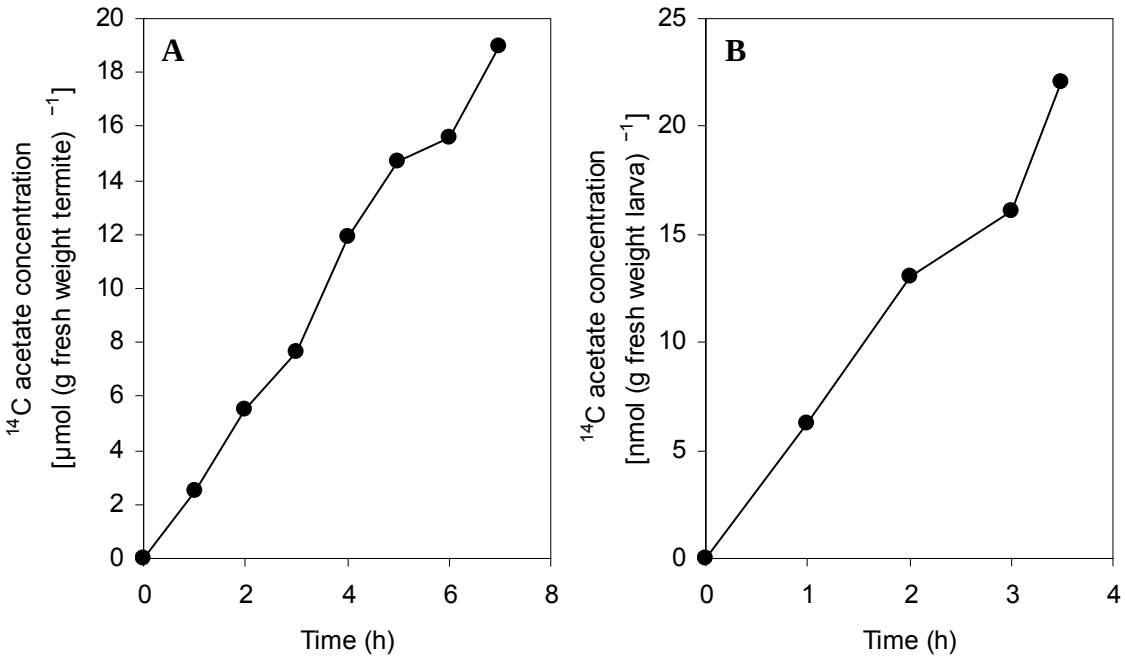

Fig. 3.2 Time course of the formation of ^{14}C labeled acetate from $H^{14}CO_3^-$ under hydrogen atmosphere and anoxic conditions during incubations of whole gut suspension of *Reticulitermes santonensis* (A) and hindgut homogenate of *Pachnoda ephippiata* (B).

3.4.2 Stable isotope experiments with glucose and HCO_3^-

Since glucose was rapidly turned over in hindgut incubations of *Pachnoda ephippiata*, glucose appeared to be a valuable substrate for stable isotope probing. To monitor the success of ^{13}C incorporation from glucose, fractionated 16S rRNA was reversely transcribed and amplified quantitatively, also for the unlabeled control gradient (Fig. 3.3A). A successful stable isotope probing experiment should result in a clearly visible shift of the 16S rRNA from the ^{13}C approach towards fractions with higher buoyant density (i.e. 1.80 g ml^{-1}) in comparison to the gradient fractions of the incubation with unlabeled glucose. To visualize maximum incorporation of ^{13}C-label into rRNA, a pure culture of *Bacillus subtilis* was incubated with $^{13}C_6$-glucose, and extracted RNA was density separated (Fig. 3.3B). Isotopically light, ^{12}C and heavy ^{13}C RNA were separated by three gradient fractions (BD of 1.76 – 1.80 g ml^{-1}), which is in agreement with Lueders et al. (2004. However, RNA from hindgut incubation of *P. ephippiata* with ^{13}C-glucose did not contain detectable amounts of ^{13}C labeled RNA in gradient fractions with a BD characteristic of fully ^{13}C-labeled RNA (Fig. 3.3A).

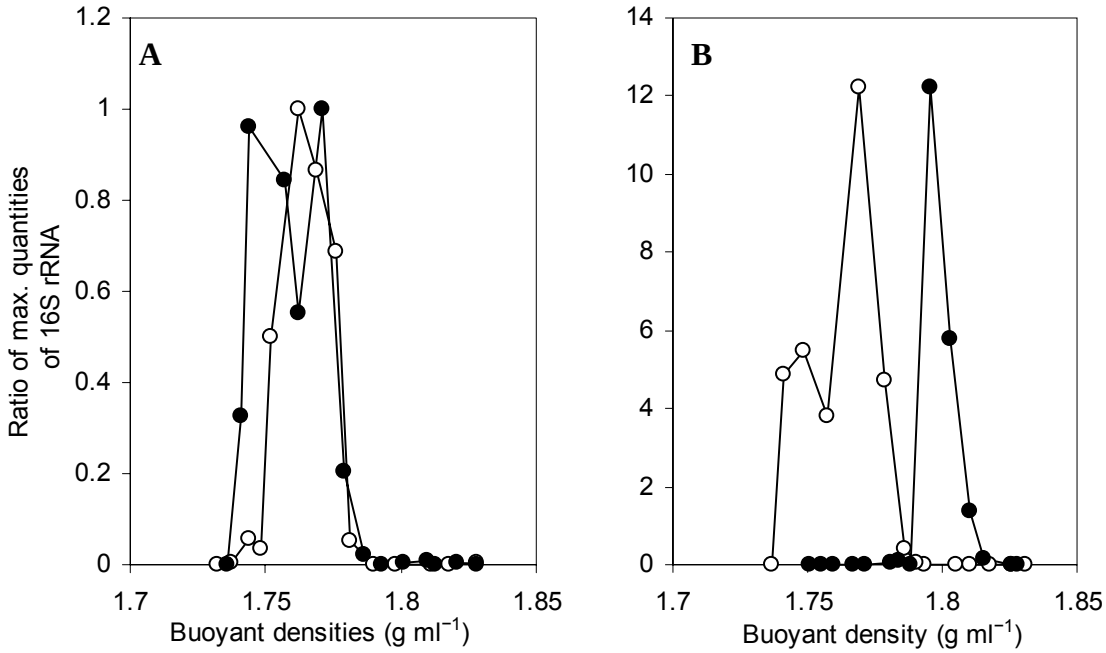

Fig. 3.3 Concentration of bacterial 16S rRNA at different buoyant densities of cesium trifluoroacetate gradients with total RNA recovered from *Pachnoda ephippiata* hindgut homogenates (A) and *Bacillus subtilis* (B) incubated with unlabeled glucose (empty circles) and ^{13}C-labeled glucose (full circles). For an easy comparison, concentrations are given in relative units, i.e. referred to the gradient fraction with the highest number of 16S rRNA copies, i.e fraction number 10 of the unlabeled control (1.48 * 108 copies) and fraction number 8 of the ^{13}C-glucose gradient (2.21*107 copies).

Another way to illustrate the incorporation success during stable isotope probing is by T-RFLP analysis of the gradient fractions. Effective incorporation of substrate carbon is visible in a shift from a more diverse bacterial community in the 16S rRNA fractions of lower buoyant density (light) to a lower microbial diversity in the fractions of high density (heavy). This indicates enrichment of ^{13}C from the substrate in the rRNA in the represented phylogenetic groups.

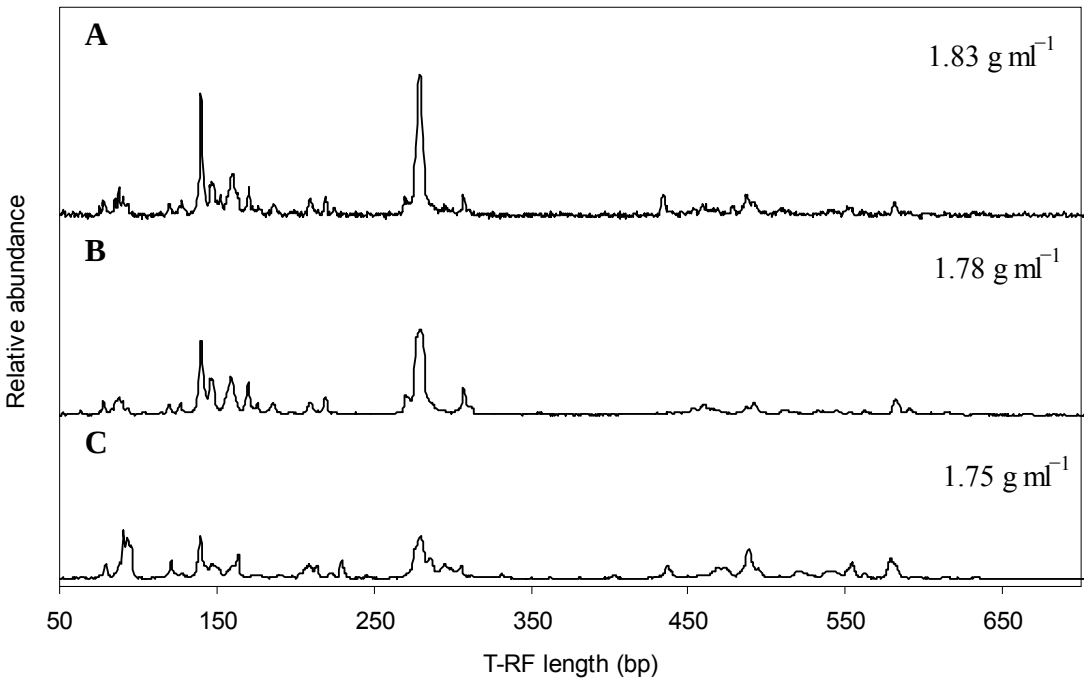

Fig. 3.4 Terminal restriction fragment length patterns from 16S rRNA after separation according to density by gradient centrifugation in cesium trifluoroacetate of the gut microbiota of *Reticulitermes santonensis* after incubation of guts with $H^{13}CO_3^-$. Communities of representative fractions with high (A), middle (B) and low (C) buoyant density are displayed, respectively.

In the 16S rRNA bacterial community fingerprints of *Reticulitermes santonens*is whole guts, no change in the diversity of the lower density fractions compared to high density fractions was visible, suggesting no or only low substrate carbon incorporation into 16S rRNA (Fig. 3.4). Also in the bacterial hindgut community of *Pachnoda ephippiata* no carbon incorporation from $H^{13}CO_3^-$ into the 16S rRNA was detected according to the TRFLP fingerprints (Fig. 3.5). The visible bacterial diversity remained similar along the density gradient.

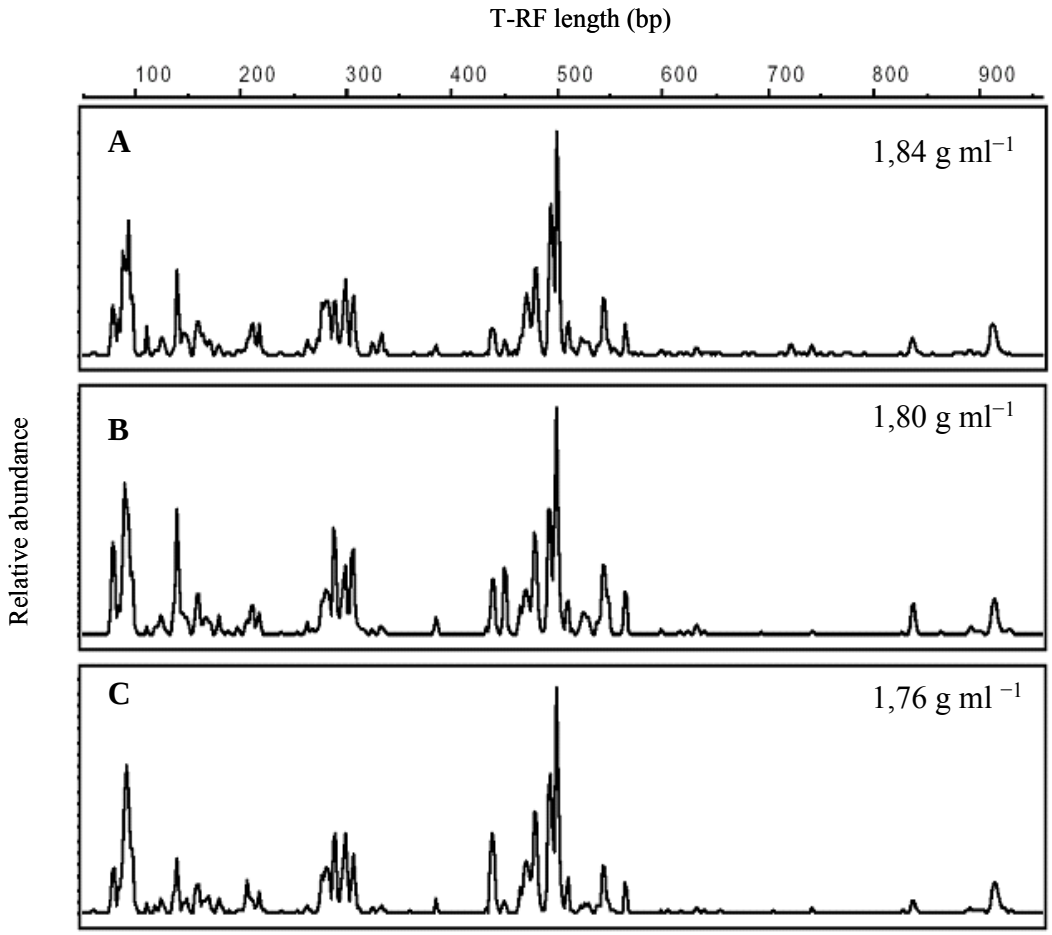

Fig. 3.5 Three selected terminal restriction fragment length polymorphism profiles from 16S rRNA of the hindgut microbiota of *Pachnoda ephippiata* after incubation of guts with $H^{13}CO_3^-$. Separation of RNA of different buoyant density was achieved by gradient centrifugation in cesium trifluoroacetate. Communities of representative fractions with high (A), middle (B) and low (C) buoyant density are displayed, respectively.

3.4.3 Incorporation of ^{14}C substrate carbon into RNA

In previous gut incubations with ^{13}C-labeled compounds it was shown that obviously not enough substrate carbon was incorporated into RNA to separate ^{12}C and ^{13}C labeled RNA. To estimate the extent of incorporation of substrate carbon into RNA compounds labeled with ^{14}C are more appropriate, since ^{14}C is generally easier to detect than ^{13}C. The incorporation of ^{14}C into the 16S rRNA of the bacterial community of whole guts of *Reticulitermes santonensis* during incubation with $H^{14}CO_3^-$ did not exceed 8% (Fig. 3.6A). Already after 2 hours, the incorporation reached a steady state and did not change considerably until the end of sampling. In incubations with *Pachnoda*

ephippiata hindgut homogenate amended with a ^{14}C-labeled amino acid mixture the incorporation did not reach 1 % (Fig. 3.6B).

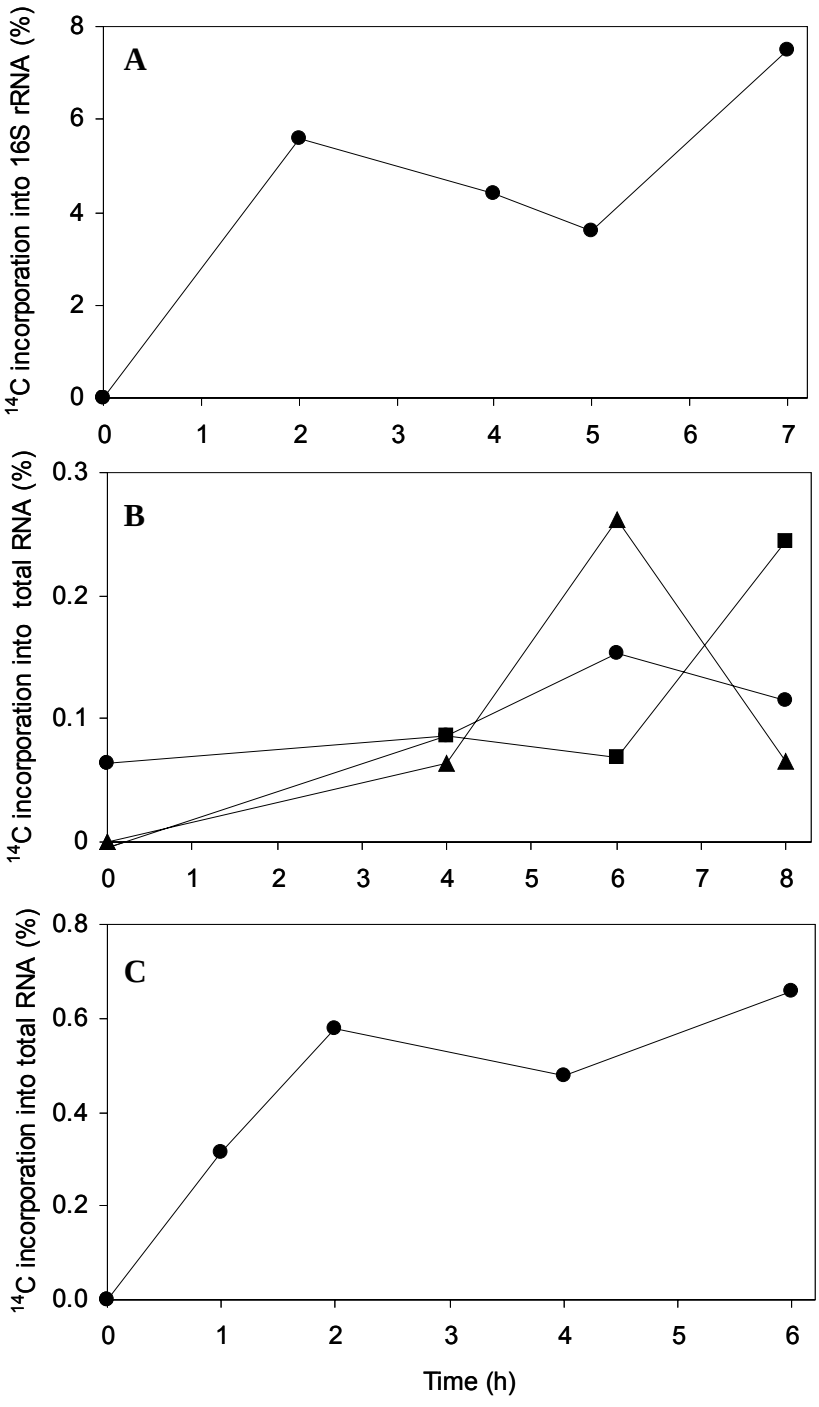

Fig. 3.6 Incorporation of ^{14}C into RNA during the incubation of (A) *Reticulitermes santonensis* with $H^{14}CO_3^-$ in anoxic Solution U buffered at pH 8.4, and *Pachnoda ephippiata* hindgut homogenate in anoxic and reduced $NaHCO_3^-$ pH 8 with a mixture of ^{14}C labeled amino acids obtained from *Bacillus megaterium* (B) and ^{14}C labeled glucose (C).

The course of incorporation was unstable, indicating that the measurements occurred along the detection limit for radioactivity. The same was true for incubation of hindgut homogenate with ^{14}C labeled glucose (Fig. 3.6C). The maximum incorporation (0.6%) of ^{14}C was achieved after 2 h and did not change significantly until the end of incubation.

3.4.4 Substrate incorporation into RNA by *Bacillus subtilis* and *Pseudomonas putida* in the presence of ribonucleotides or amino acids

The incubations with ^{14}C labeled substrates confirmed the results of the stable isotope approaches, where the incorporation of substrate carbon remained undetectable. In the hindgut of *Pachnoda ephippiata* a free nucleotide concentration of 14 µmol (g dry weight gut)$^{-1}$ ± 2 corresponding to 2.1 mM was detected. Since the intestinal tract is an environment densely populated by microorganisms, the gut fluid could be enriched with residuals of dying microbial cells, such as nucleotides.

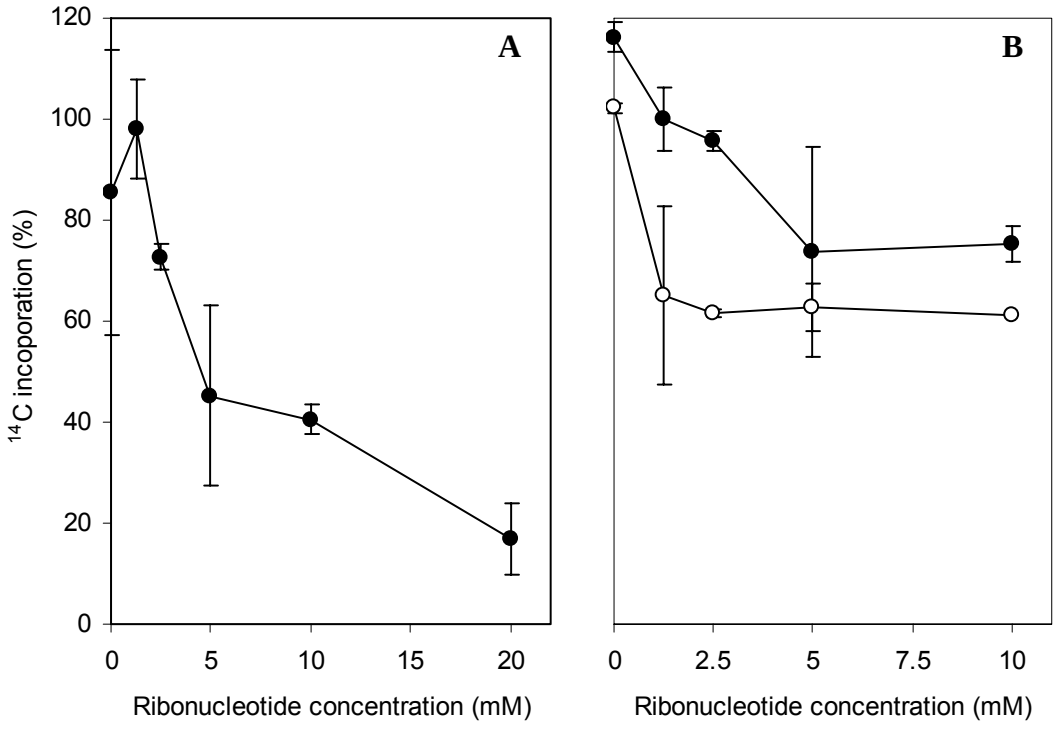

Fig. 3.7 Effect of the presence of increasing concentrations of ribonucleotides (full circles) and amino acids (empty circles) on the incorporation of ^{14}C from radiolabeled glucose into RNA of *Bacillus subtilis* (A) and *Pseudomonas putida* (B). The error bars represent the standard deviation of three independent experiments.

These nucleotides could be used by the intestinal microbiota in salvage pathways for *de novo* synthesis of nucleic acid.

Thus, the incorporation of carbon from the actual substrate might be low. To test this, titration experiments were carried out with glucose as carbon source with pure cultures of *Bacillus subtilis* and *Pseudomonas putida*, to monitor the effects of the presence of nucleotides or glycine and aspartate, as their carbon precursors, on incorporation of substrate carbon into RNA (Fig. 3.7).

For *B. subtilis*, the presence of amino acids did not have a clear influence on the carbon incorporation (not shown). However, in presence of ribonucleotides it was possible to reduce the percentage of substrate carbon incorporation continuously to 17% (Fig. 3.7A). For *P. putida*, the effects of ribonucleotides in the medium were less striking, since the highest concentration of ribonucleotides (10mM) still allowed a carbon incorporation of 75 % (Fig. 3.7B). Furthermore, the presence of glycine and aspartate only decreased the incorporation to 60 %. In both cases, the course of incorporation indicated that higher concentrations of ribonucleotides or amino acids would not result in lower carbon incorporation.

3.5 Discussion

Intestinal tracts are very complex environments and *in situ* experiments revealed the potential for a variety of metabolic processes carried out by the gut microbiota. With the help of stable isotope probing it is possible to assign a specific function to the respective microorganisms. In this study, the first time stable isotope probing was applied to the gut microbiota of insects.

The rate of glucose consumption in hindgut homogenates of the humivorous beetle larvae *Pachnoda ephippiata* is high and accounted for 18% of the total respiratory activity of the larva referred to the total carbon flux of 10 µmol (h × g fresh weight larvae)$^{-1}$ (Lemke et al., 2003). By contrast, the potential contribution of acetogenesis from $^{13}HCO_3^-$ to the total carbon flux is with 0.1% very low. In the wood-feeding termite *Reticulitermes santonensis* the respective rate is considerably higher (12%). Hence, glucose in *P. ephippiata* and HCO_3^- in *R. santonensis* are actively converted to short-chain fatty acids and should therefore be valuable substrates for stable isotope

probing. However, incubations with ^{13}C-labeled glucose or $H^{13}CO_3^-$ did not reveal the microorganisms actively mineralizing these compounds due to no detectable isotope effects. Incubations with ^{14}C-labeled compounds confirmed that the incorporation of substrate carbon did not exceed 1% in *P. ephippiata* (glucose, amino acids) and 8 % in *R. santonensis* (HCO_3^-). Since the detection limit for a successful rRNA stable isotope experiment was postulated to be 10 – 20% (Manefield et al., 2002b), it is not surprising that no ^{13}C-incorporation effects are visible.

Other than soils or sediments, the gut represents in general an environment rich in a variety of freely available substrates. The presence of monomeric compounds resulting from enzymatic hydrolysis like sugars (Itakura et al., 1997; Egert et al., 2005) or amino acids (Andert et al., in press), as well as, fermentation products (Lemke et al., 2003) makes the intestinal tract of insects to a convenient habitat for the gut microbiota. Consequently, microorganisms accumulate to high numbers in intestinal tracts (Lemke et al., 2003; Tholen et al., 1997). Due to dying microorganisms, nucleotides and nucleosides are released from decaying cells. In salvage pathways nucleotides are degraded to nucleobases, which are forming the precursors for *de novo* nucleotide synthesis. These processes can be found in almost all microorganisms (Hammer-Jespersen, 1983, Nygaard, 1983; Nygaard, 1993). Since *de novo* synthesis is very energy consuming, salvage of nucleotides or amino acids for the biosynthesis of nucleic acids is very beneficial for the microbial cells.

In the hindgut of the larvae of *P. ephippiata*, nucleotides accumulate up to 2 mM (this study). Also the use of glycine or aspartate as precursors for nucleotide biosynthesis is possible, since the concentration of these amino acids in the hindgut of *Pachnoda ephippiata* is 3 mM and 2 mM, respectively (Andert et al., unpublished). In incubations of pure cultures of *Bacillus subtilis* and *Pseudomonas putida* with ^{14}C-glucose the presence of ribonucleotides results in a lowered incorporation of substrate carbon into RNA, providing evidence for the *de novo* synthesis of RNA from free ribonucleotides (this study, Nygaard, 1993). The nucleotides and the amino acids present in the insect gut fluid may inhibit the formation of microbial RNA from substrate carbon during labeling experiments.

Another possible explanation for the low incorporation of substrate carbon into microbial RNA might be that the fraction of the population, which is actively forming

RNA from the labeled substrate carbon, is too small and the label was diluted by the major part of community RNA. A further reason could be that the majority of the population incorporated only a minor part of the substrate carbon into the RNA. Therefore, in both cases, the incorporation is probably not detectable and hides behind the background noise.

Furthermore, the low levels of carbon incorporation into microbial RNA in gut incubations could be explained by the absence of *de novo* formation of RNA during the incubations, associated with no cell division (Kramer and Morris, 1990). These observations have been made by Nold and Ward (1996) with microbial mat communities, which revealed very high rates of photosynthesis from $^{14}CO_2$, but the ^{14}C incorporation into RNA was below detection limit. Instead, the ^{14}C was incorporated into polyglucose, which represents a storage polymer. Accumulation of polyglucose occurs as a result of limited growth condition in the presence of an excess source of carbon (Preiss, 1984). The carbon balance in the incubation of *Pachnoda ephippiata* with ^{14}C-glucose was not closed (not shown); 40% of the radiolabeled carbon was missing. The formation of polyglucose from ^{14}C might be a possible explanation. The used glucose concentration of 5 mM is by far higher, than the physiological concentration in the gut (< 0.1 mM, Egert et al., unpublished) and, therefore, represents an excess carbon source, supporting this hypothesis.

A stable isotope approach in an *in vitro* model for the human intestine resulted in sufficient label incorporation (Egert et al., 2007). There, three different glucose concentrations were used (2, 20 and 40 mM). Only in the experiment using the highest glucose concentration resulted in a detectable incorporation of ^{13}C into microbial RNA. It is possible, that under these conditions stable isotope probing could work in the insect gut also. However, these concentrations are unphysiological for insect guts (Itakura et al., 1997; 15 µM, Tholen and Brune, 2000; 3 mM Egert et al., 2005) and might possibly bias the incorporation success.

3.5.1 Conclusions

In this study, stable isotope probing was carried out unsuccessfully in the insect gut of a wood-feeding termite and a humivorous beetle larvae. Therefore, the microorganisms using glucose or CO_2 as carbon-and energy source were not identified.

It was not revealed, why stable isotope probing is not working in the insect intestinal environment. One possibility to overcome the low incorporation efficiency into nucleic acids might be the use of un-physiologically high substrate concentrations. However, the chance that microorganisms are incorporating label, which are under natural conditions not involved in the respective metabolic process, is high. Obviously, in the insect gut systems tested in this study catabolism and anabolism are not coupled. The substrates were transformed during incubations, but were rather used as energy source than as carbon source.

3.6 References

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4 Inter- and intraspecific differences of the bacterial community in the gut of two humivorous scarab beetle larvae and effects of the food soil

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4.1 Abstract

Pachnoda ephippiata and *Pachnoda marginata* are closely related but allopatric scarab beetles, sharing the same way of living. However, the studies focussed on the gut microbiota of their larvae, which also can be found in the same habitat feeding on soil rich in organic matter. The bacterial microbiota of the gut of both beetle larvae was compared to answer the following question: Is the microbiota in the gut similar as a result of the occupation of the same ecological niche or lead the spatial separation to differences in the bacterial composition of the gut? The composition of the midgut and the hindgut microbiota of the larvae of *Pachnoda ephippiata* and *Pachnoda marginata* was analyzed by 16S rRNA gene based T-RFLP (terminal restriction length fragment polymorphism). The resulting fingerprints were processed and evaluated statistically using Correspondence Analysis. The analysis revealed that the bacterial gut communities in midgut and hindgut of the two beetle species are significant different. However, the ordination plots and Morisita indices indicate considerably high intraspecific variance in the bacterial community, probably due to differences in the relative abundance of the phylotypes (T-RFs).

Further, it was examined, whether the feeding of *Pachnoda marginata* larvae with different types of soil effects the composition of the bacterial community. No significant changes in the bacterial community of the hindgut were visible, indicating that the gut microbiota is host-related und not derived from the food soil.

4.1 Introduction

In general, the microbial community in intestinal tracts is very abundant and diverse (e.g. Cazemier et al., 1997; Egert et al., 2003). Since only a small fraction of these microorganisms is cultivable, it is inadequate to study the phylogenetic diversity with the cultivation approach. Therefore, the application of cultivation-independent techniques emerged as useful method to explore complex microbial communities. Phylogenetic profiling techniques, mainly of the 16SrRNA gene, like DGGE (Muyzer et al., 1993), SSSC (Schwieger and Tebbe, 1998) and T-RFLP (Liu et al., 1997) have been used several times to study the microbiota in many environments, eg. soils and sediments. Using these methods, the diversity of the microbiota in the intestinal environment of humans (Eckburg et al., 2005), cows (Lin et al., 1997), and several insect species was examined (Schmitt-Wagner et al., 2003, Egert et al., 2003, Mohr and Tebbe, 2006).

The action of the intestinal microbiota is essential for the host. Besides the enzymes secreted by the insect host, also the gut microbiota contributes to enzymatic hydrolysis of the ingested food particles. Cellulolytic activity in the gut of humivorous beetle larvae and xylophagous termites were shown to be of microbial origin (Wiedemann, 1930; Rössler, 1961, Tokuda et al., 2005). Further on, microorganisms are responsible for fermentation processes in the gut (Insam, 1996; Bignell, 1994). The host organisms are using the resulting fermentation products, mainly short-chain fatty acids, as nutritional source (Kane, 1997; Brune, 2003). But also amino acids (Dadd, 1973) and vitamins (Dillon and Dillon, 2004) might be essential compounds for the insect host.

Hence, it is not surprising that it was observed several times that the gut microbiota is specific to its host. Wood-feeding termites revealed a high proportion of termite specific microbial lineages, and the gut microbiota within one genus was very similar supporting the theory that the intestinal microbiota has coevolved with its insect host (Hongoh et al., 2005; Shinzato et al., 2005). Nevertheless, it was possible to differentiate individuals of different colonies of the wood-feeding termite *Hodotermes mossambicus* on the basis of dissimilarities within the bacterial gut community (Minkley et al., 2006). But also dependence of the composition of the gut microbiota on the food source was demonstrated. Brauman et al. (2001) connected differences in the

intestinal microbiota to different feeding habits. However, different termite genera consuming the same diet showed distinct microbial gut communities.

Half of all termite genera feed on soil (Noirot, 1992). The soil harbors a complex microbial community. However, for soil-feeding termites of the genus *Cubitermes*, the microbial community differed significantly from the microbiota in the ingested soil (Schmitt-Wagner et al., 2003), which is supporting the theory of a specific gut community. Also the intestinal microbiota of the humivorous beetle larvae of *Pachnoda ephippiata* proved to be distinct of that of the food soil (Egert et al., 2003). This is a further indication that the microbial gut community is selected and not simply taken up by the host.

The two scarab beetle *Pachnoda ephippiata* and *Pachnoda marginata* are allopatric species. The former can be found in East Africa and the latter in West Africa. They occupy the same ecological niche, feeding on ripe fruits and flowers (Löwenberg, 1999). Also the larvae share the same lifestyle and live both in humus-rich soils and feed on organic matter in different states of decay. Both larvae harbor a very abundant gut microbiota (Egert et al., 2003, Cazemier et al., 1997), whose composition was already studied in detail for *P. ephippiata* (Egert et al., 2003).

Our experiments were carried out to verify either one of the following hypothesis, 1) the bacterial gut microbiota in the two species are similar due to the similar lifestyle as found for soil-feeding termites of the genus Cubitermes sp. (Schmitt-Wagner et al., 2003b) or 2) the spatial separation lead to the development of a species-specific gut microbiota (Hongoh et al., 2005). With the help of T-RFLP profiling, intra-and interspecific differences in the microbial community in the midgut and hindgut of *Pachnoda ephippiata* and *Pachnoda marginata* were monitored. Also the change of the gut microbiota in connection with different food sources was examined.

4.2 Materials and Methods

4.3.1 Larvae and soil

Third instar larvae of *Pachnoda marginata* and *Pachnoda ephippiata*, were purchased from a commercial breeder (b.t.b.e. Insektenzucht, Schnürpflingen,

Germany). Further larvae of *Pachnoda ephippiata* were obtained from the zoological teaching collection at the University of Konstanz (Germany).

Larvae were kept on a peat-based and clay-enriched potting soil (Compo, Münster, Germany), at 25 °C for at least two weeks prior to sampling (for details, see Lemke et al., 2003). The influence of differences resulting from different food soils were exclusively examined on *Pachnoda marginata* larvae fed with the above-described soil (soil I), with a mixture of forest soil (Schnürpflingen, Germany; soil II) and peat and, thirdly, clay-enriched potting soil from a different brand (oekohum, Herbertingen, Germany; soil III). Larvae were dissected, and the intestinal tract was divided into midgut and hindgut compartments as previously described (Lemke et al., 2003).

4.3.2 Nucleic acid extraction and 16S rRNA amplification

DNA was extracted from midgut and hindgut samples using a protocol including bead-beating established by Henckel et al. (1999) modified after Egert et al. (2004).

Bacterial 16S rRNA genes were amplified using 25 cycles with a combination of the 27f primer (5'-GAG-TTT-G(AC)T-CCT-GGC-TCA-G-3', Lane, 1991) with fluorescent label (IRD 700, pentamethine carbocyanin, MWG, Ebersberg, Germany) and 907r (5'-CCC-GTC-AAT-TC(AC)-TTT-GAG-TTT-3', Lane, 1991, MWG, Ebersberg, Germany) according to Egert et al.(2003).

To avoid inhibition by humic substances, extracted DNA was diluted 1:10 and/or 0.4 mg ml^{-1} bovine serum albumin was added to each amplification reaction.

4.3.3 T-RFLP analysis

The PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany). The DNA concentration in the purified PCR products was determined spectrophotometrically (Eppendorf). Preliminary restriction experiments were carried out with seven different enzymes (*Mbo*I, *Msp*I, *Pau*I, *Rsa*I, *Ssp*I, *Hha*I, *Hae*III, all Promega, Mannheim, Germany) to find the appropriate restriction enzyme. The restriction digestion was carried out according to Egert et al. (2004), except that 80 ng DNA were digested.

The T-RFLP analysis was carried out as described in Pester et al. (2004) The calculation of fragment sizes was done according to a molecular size marker (IRDye™,

50-700 bp, LI-COR Biosciences) using the Gel-Pro software (Gel-Pro Analyzer, version 4.5, Media Cybernetics). The community profiles were created in Microsoft Office Excel after smoothing of the T-RF-length dataset with the help of a macro installed in Excel (see http://www.quantdec.com/Excel/smoothing.htm). Great care was taken that independent aliquots of the same sample resulted in virtually the same profile.

Prior to statistical analysis, the dataset resulting from T-RFLP analysis was normalized according to Dunbar et al. (2001) with the following changes. First, the background was subtracted from the values for relative abundance. As background, the maximal fluorescence between the bands in the lanes loaded with molecular size marker was estimated. Values for relative fluorescence in the T-RF datasets, which became negative, were set to zero. Since from the software every T-RF was given 3-5 times the maximum relative abundance for each T-RF was determined. For further analysis only these maximal T-RFs were considered. Only the peak maximum was taken into account and the ascent and descent of each peak was neglected. To confirm that the relative abundance data set for the several T-RFLP profiles was comparable, the sum of all peak heights per profile was set to 100. Only major T-RFs were considered, i.e. the relative abundance of the respective peaks had to account for at least 1 % of the total peak height. Again, the total peak height was set to 100. Since differences were observed in the behaviour of the same T-RFs on distinct acryl amide gels, T-RFs separated by 1 to 2 base pairs were combined in one operational taxonomic unit (OTU).

4.3.4 Determination of ecological indices

The ecological indices were calculated with the help of the free PAST data analysis package (http://folk.uio.no/ohammer/past).

The richness (S) of a community is characterized by the number of different contributing species. Increasing number of species is leading to increasing species richness. The abundance of respective species is not considered.

Simpson's Index (D) is a measure for diversity. Here, the number of species and their abundance are considered. It can be calculated with the help of the formula:

$$D = \frac{\sum n(n-1)}{N(N-1)},$$

where n represents the number of individuals of a particular species and N the total number of individuals of all species. In this study, 1-D is used, indicating the smaller D, the lower is the diversity. The Simpson's index gives more weight to more abundant species in a community.

The evenness (H) is taking the abundance of one species and the richness of a community into account. The distribution of the individuals on the different species is described by the formula:

$$H = \frac{D}{\log(S)}.$$

Another diversity index is the Shannon-Wiener Index (H_S) calculated with the formula:

$$H_S = -\sum_{i=1}^{S} p_i \log p_i ,$$

where S is the species richness and p_i is the relative abundance of species, measuring in the range of zero to one. H_S describes the degree of uncertainty in case of random sampling of a community, which species has been caught, e.g. if there is only one species, the uncertainty is zero. Therefore, it is dependent on the abundance of species and the density of the community.

For estimation of the similarity of two bacterial gut communities on the basis the T-RFLP fingerprints the Morisita Index (I_M) based on the Simpson's index of dominance according to Dollhopf et al. (2001) was used.

$$I_M = \frac{2\sum n_{1i} n_{2i}}{(l_1 + l_2))N_1 N_2},$$

with n_i as the number of individuals of species i, N as the total number of individuals sampled and l as the Simpson's index of dominance

$$l = \frac{\sum_{i-1}^{S} (n_i (n_i - 1))}{N(N-1)}.$$

The values for the Morisita Index range from 0, indicating no similarity, to 1, which means complete identity.

4.3.5 Statistical analysis

Statistics were performed with CANOCO 4.5 and SPSS 12.0 for Windows.

The differences between the bacterial communities of midgut and hindgut of *Pachnoda ephippiata* and *Pachnoda marginata* were determined by correspondence analysis (CA) of the respective TRFLP dataset. The differences resulting from treatments of *Pachnoda marginata* larvae with different food soil were also estimated by CA, after confirmation that canonical correspondence analysis (CCA) using the Monte Carlo permutation test (499 permutations) was not appropriate. If effects were observed, the significance of the differences was confirmed by univariate ANOVA (analysis of variance) with at least $P < 0.05$ as significance threshold.

4.3 Results

4.4.1 The bacterial community of midgut and hindgut of larvae of *Pachnoda* spp.

With the help of T-RFLP fingerprinting in connection with the appropriate restriction enzyme it is possible to visualize the composition of complex microbial communities.

The T-RFLP profiles of the bacterial community of *Pachnoda marginata* and *Pachnoda ephippiata* midgut were considerably different (Fig. 4.1), since only few T-RFs were shared.

Only a few phylogenetic groups could be assigned to the T-RFs on the basis of a previous clone library for *P. ephippiata* (Egert et al., 2003). Bacillales was the only assignable group in the fingerprint of the bacterial community of *P. marginata* midgut (Fig. 4.1A). In the profile of the midgut of *P. ephippiata* besides Bacillales also Actinobacteria and Clostridiales could be assigned (Fig. 4.1B).

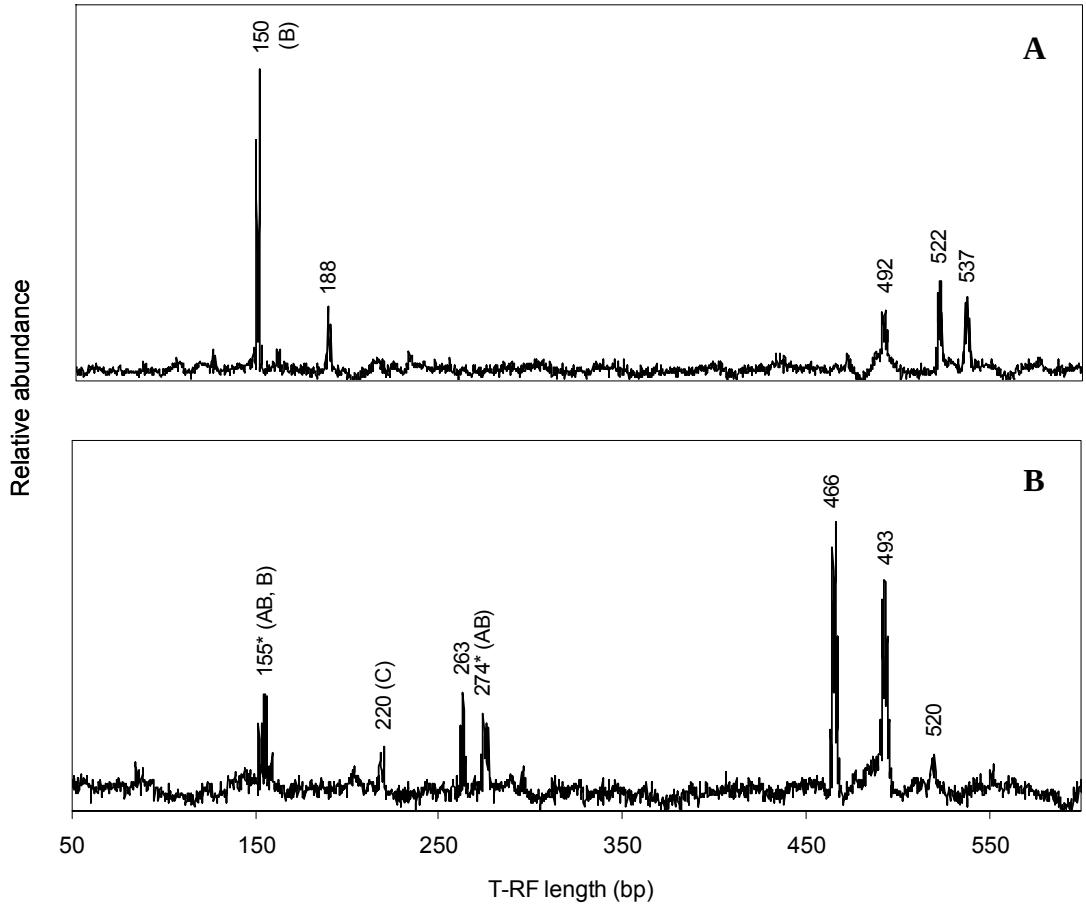

Fig. 4.1 Representative T-RFLP fingerprints of the bacterial midgut community of *Pachnoda marginata* (A) and *Pachnoda ephippiata* (B) after amplification of the 16SrRNA gene with general bacteria primers and restriction digestion with *Msp*I. T-RFs were tentatively assigned based on reported conservation of restriction sites according to Egert et al. (2003) using the following abbreviations: AB, Actinobacteria; B, Bacillales, C, Clostridiales). Asterisks indicate T-RFs probably influenced by pseudo-T-RF formation.

The community profiles for the hindgut of *P. ephippiata* and *P. marginata* are similar on the first glance (Fig. 4.2A and 4.2B). Although, major T-RFs could be found in both profiles, the relative abundance of the respective T-RFs differed considerably. Several T-RFs could be assigned to phylogenetic groups, i.e. members of the CFB phylum, Clostridales, Bacillales and Actinobacteria.

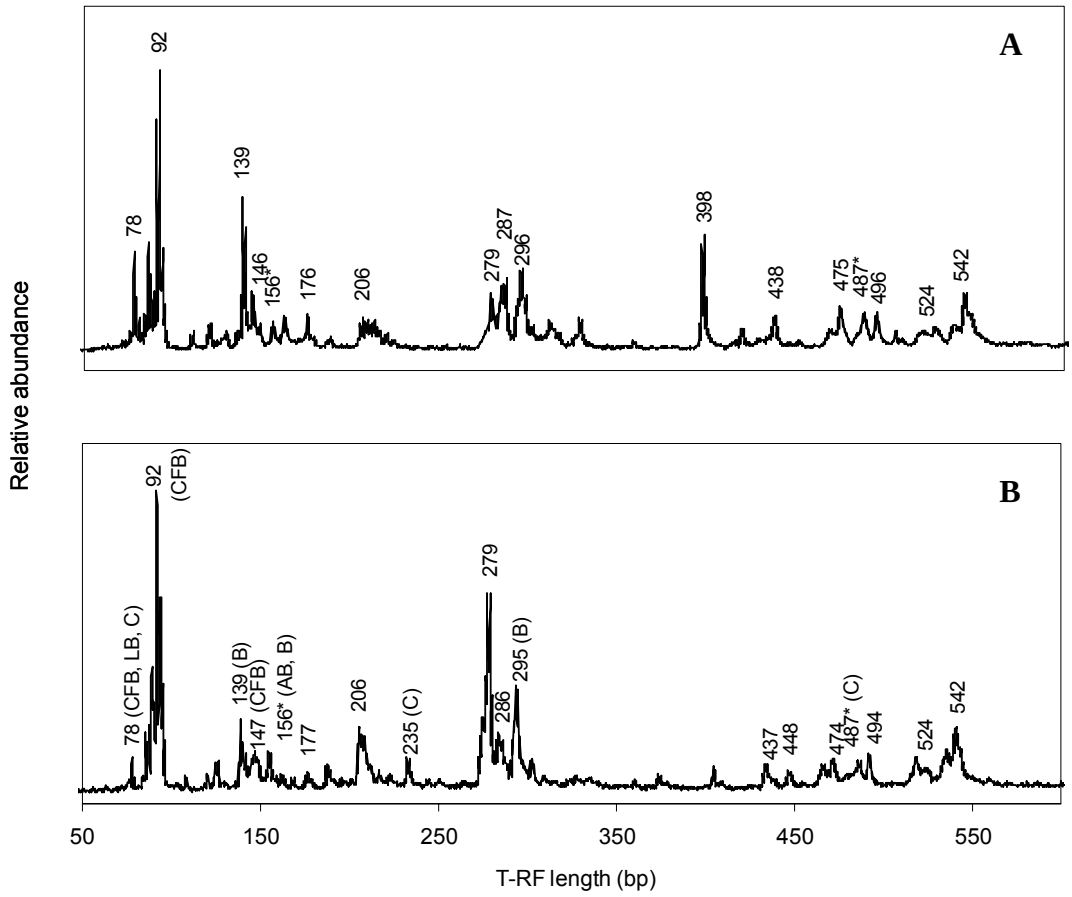

Fig. 4.2 Typical T-RFLP fingerprints of the bacterial community of the hindgut of *Pachnoda marginata* (A) and *Pachnoda ephippiata* (B) after amplification of the 16SrRNA gene with general bacteria primers and restriction digestion with *Msp*I. T-RFs were tentatively assigned based on reported conservation of restriction sites according to Egert et al. (2003) using the following abbreviations: AB, Actinobacteria; B, Bacillales, C, Clostridiales; CFB, CFB phylum, LB, Lactobacillales). Asterisks indicate T-RFs probably influenced by pseudo-T-RF formation.

4.4.2 Diversity and similarity indices of bacterial midgut and hindgut communities

With the help of ecological indices like the Shannon-Wiener index it is possible to characterize the community with respect to the diversity. The diversity indices for the bacterial community of the midgut of *Pachnoda ephippiata* and *Pachnoda marginata* are very similar (Tab. 4.1). In the hindgut, larvae of *P. marginata* revealed a considerably lower bacterial species richness and diversity compared to *P. ephippiata*.

Tab. 1 Diversity indices of the bacterial communities of midgut and hindgut of larvae of *Pachnoda marginata* and *Pachnoda ephippiata* determined by means of indices ± SD for 3 bacterial community profiles of midgut per species and 8 profiles of hindgut per species using T-RFLP dataset after amplification of the 16S rRNA gene with general bacteria primers and the following restriction digestion with *Msp*I.

	Midgut		**Hindgut**	
	P. ephippiata	*P.* marginata	*P.* ephippiata	*P.* marginata
Species Richness (S)	10 ± 3	10 ± 2	25 ± 5	17 ± 5
Simpson's Index (1-D)	0.73 ± 0.13	0.75 ± 0.05	0.91 ± 0.02	0.84 ± 0.05
Shannon-Wiener (H_S)	1.81 ± 0.52	1.84 ± 0.25	3.20 ± 0.35	2.50 ± 0.43
Eveness (H)	0.65 ± 0.10	0.67 ± 0.17	1.02 ± 0.18	0.77 ± 0.13

Community similarity was determined using the Morisita index. The similarity of the pair wise-compared bacterial midgut communities of individuals of *P. ephippiata* is low compared to the hindgut community (Tab. 4.2). In contrast to that, in *P. marginata* the midgut community appeared to be more stable. Comparing *P. ephippiata* and *P. marginata*, for the midgut microbiota very low similarity was detected. For the bacterial t community of the hindgut the similarity accounted for 40% (Tab. 4.2).

Tab. 2 Similarity indices of the bacterial communities of midgut and hindgut of larvae of *Pachnoda marginata* and *Pachnoda ephippiata* compared to each other. The indices are means ± SD of 3 bacterial community profiles for the midgut per species and 8 profiles for the hindgut per species based on the T-RFLP dataset after amplification of the 16S rRNA gene with general bacteria primers and the following restriction digestion with *Msp*I.

	Morisita Indices	
	Midgut	Hindgut
P. ephippiata : P. ephippiata	0.37 ± 0.35	0.66 ± 0.17
P. marginata : P. marginata	0.68	0.41 ± 0.27
P. ephippiata* : *P. marginata	0.19 ± 0.14	0.40 ± 0.22

4.4.3 Specific differences in the bacterial gut community of larvae of *Pachnoda* spp.

The T-RFLP dataset was analyzed with respect to community differences by correspondence analysis (CA), which represents a common ordination technique. The results were monitored in ordination plots. According to the TRFLP-fingerprints (Fig. 4.1), the bacterial community in the midgut of *Pachnoda ephippiata* was very distinct from the community in the midgut of *Pachnoda marginata*. Therefore, the gut communities of the respective species were arranged separately in the ordination plot, whereas the separation was only visible on the first axis after correspondence analysis (Fig. 4.3).

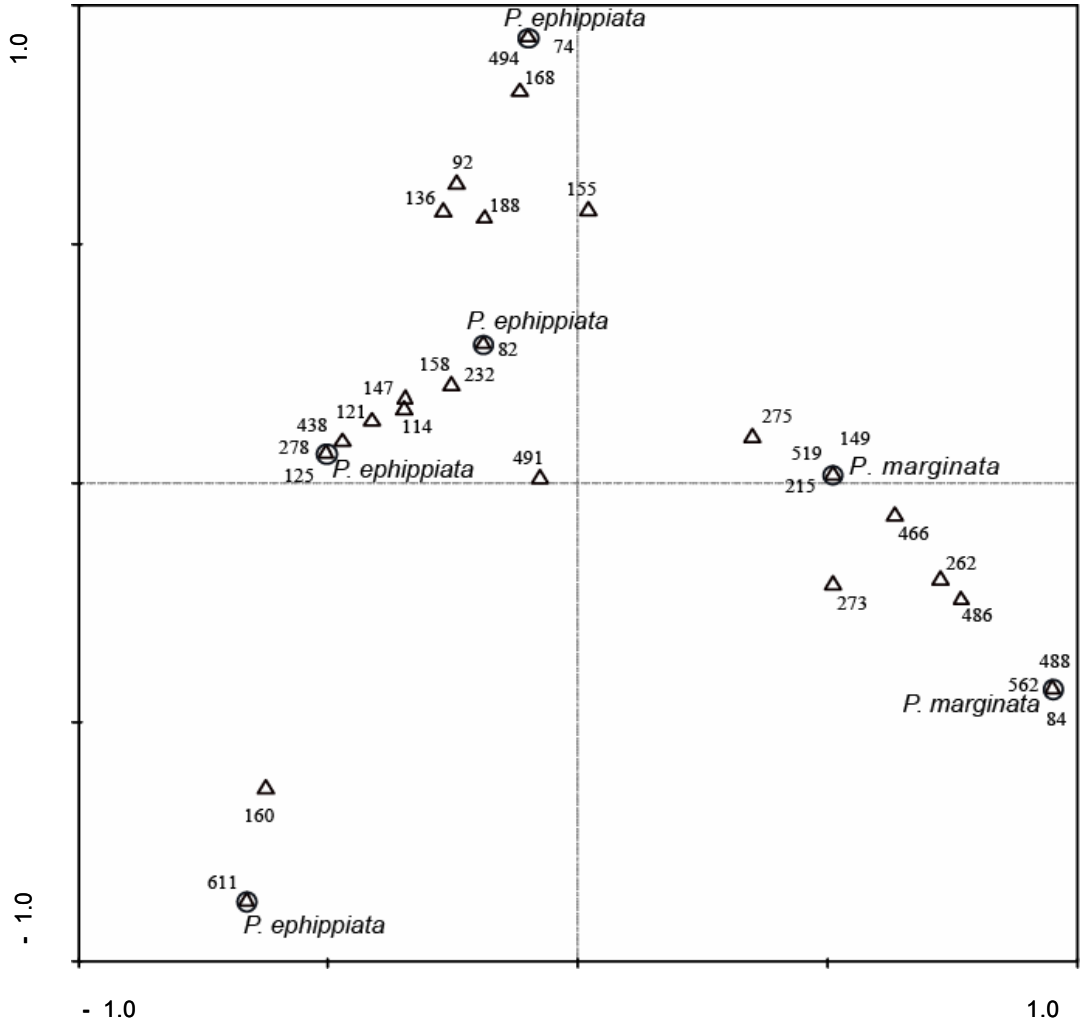

Fig. 4.3 Correspondence analysis ordination plot of the T-RFLP dataset of the bacterial midgut communities of *Pachnoda marginata* vs. *Pachnoda ephippiata*. Open circles represent the respective beetle larvae species and open triangles the associated T-RFs characterizing the differences between the samples. The eigenvalues for the first and second axis are 0.713 and 0.464, respectively.

The differences were confirmed to be significant ($P < 0.01$). However, on the second axis of the ordination plot, large intraspecific differences were observed, since the individual samples spread along the axis and were not concentrated to one distinct spot within one species. T-RFs, scattering around the all samples indicate OTUs that typically occurred in the respective midgut fingerprint.

The composition of the bacterial hindgut communities of *Pachnoda marginata* and *Pachnoda ephippiata* appeared to be more similar in contrast to the midgut communities based on the T-RFLP fingerprints (Fig. 4.2). In the ordination plots, the specific dissimilarity was visible in the spatial separation of the species on the first axis (Fig. 4.4). It was confirmed that for midgut and hindgut, specific differences were highly significant ($P < 0.001$). The differences were to a lesser extent due to the presence or absence of specific T-RFs, but more based on differences in the relative abundance of T-RFs. For instance, the relative abundance of T-RF 92 ranges from 1 to 27% of total relative abundance, but is present in almost all profiles (not shown).

Further, a variety of T-RFs are responsible for the dissimilarities, what became visible from the multiplicity of T-RFs spreading around each sample in the plot (Fig. 4.4). However, the scattering of the all samples along the second axis indicates a remarkably high intraspecific diversity.

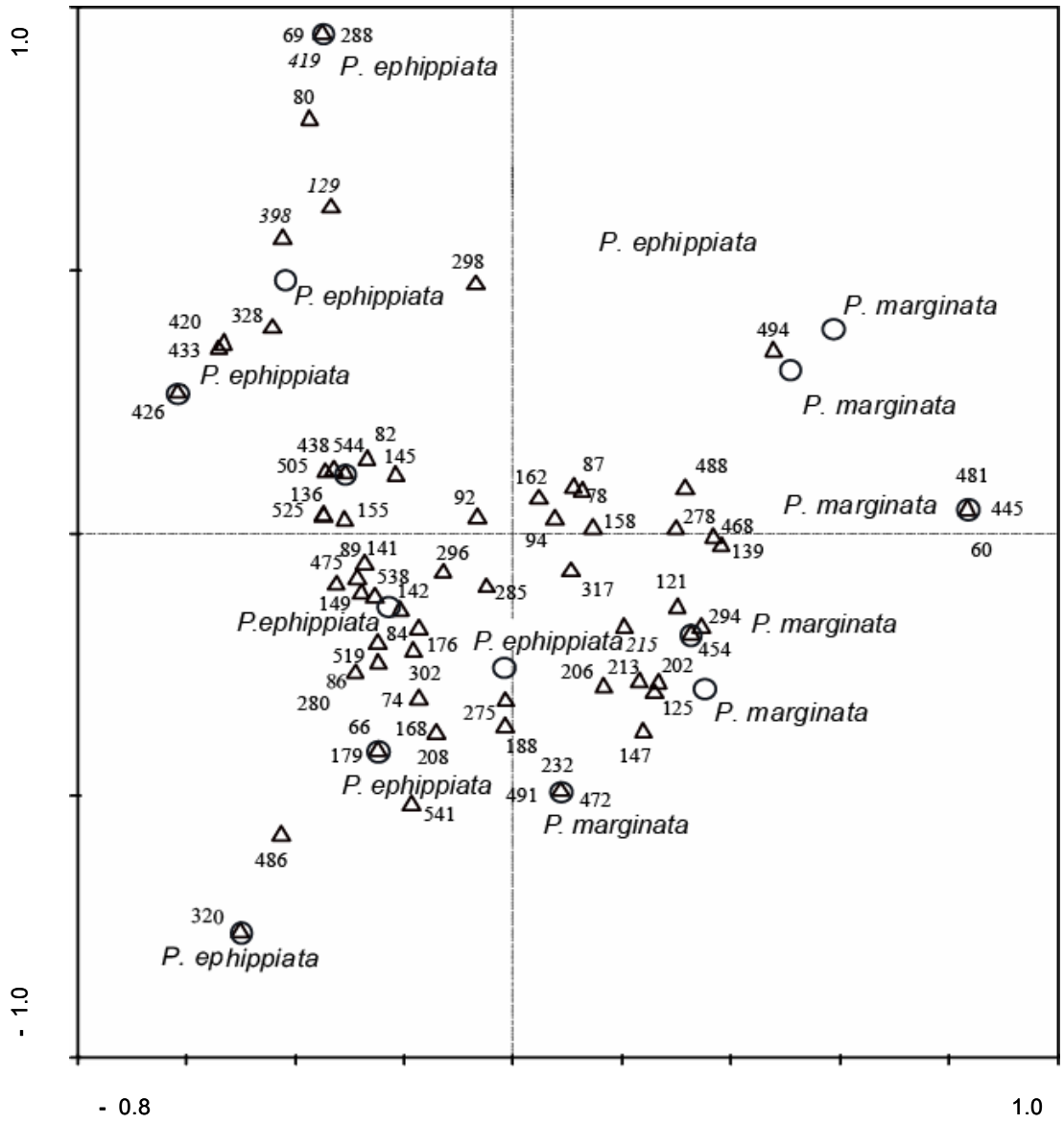

Fig. 4.4 Correspondence analysis ordination plot of the T-RFLP dataset of the bacterial hindgut communities of *Pachnoda marginata* vs. *Pachnoda ephippiata*. Open circles represent the respective beetle larvae species and open triangles the associated T-RFs characterizing the differences between the samples. The eigenvalues for the first and second axis are 0.356 and 0.244, respectively.

4.4.4 Differences in the bacterial hindgut community of *Pachnoda marginata* larvae depending on the food soil

To examine the dependency of the bacterial hindgut community from the food source, *Pachnoda marginata* larvae were fed with three different kinds of soils. The T-RFLP data was analyzed with the help of CA. The community samples of the hindgut of the larvae fed with the same soil did not produce a specific cluster (Fig. 4.5). All samples clustered near to each other, indicating a relatively similar bacterial hindgut

community. According to the ordination plot, the hindgut community of the larvae, treated with soil II, were very different, due to two outliers.

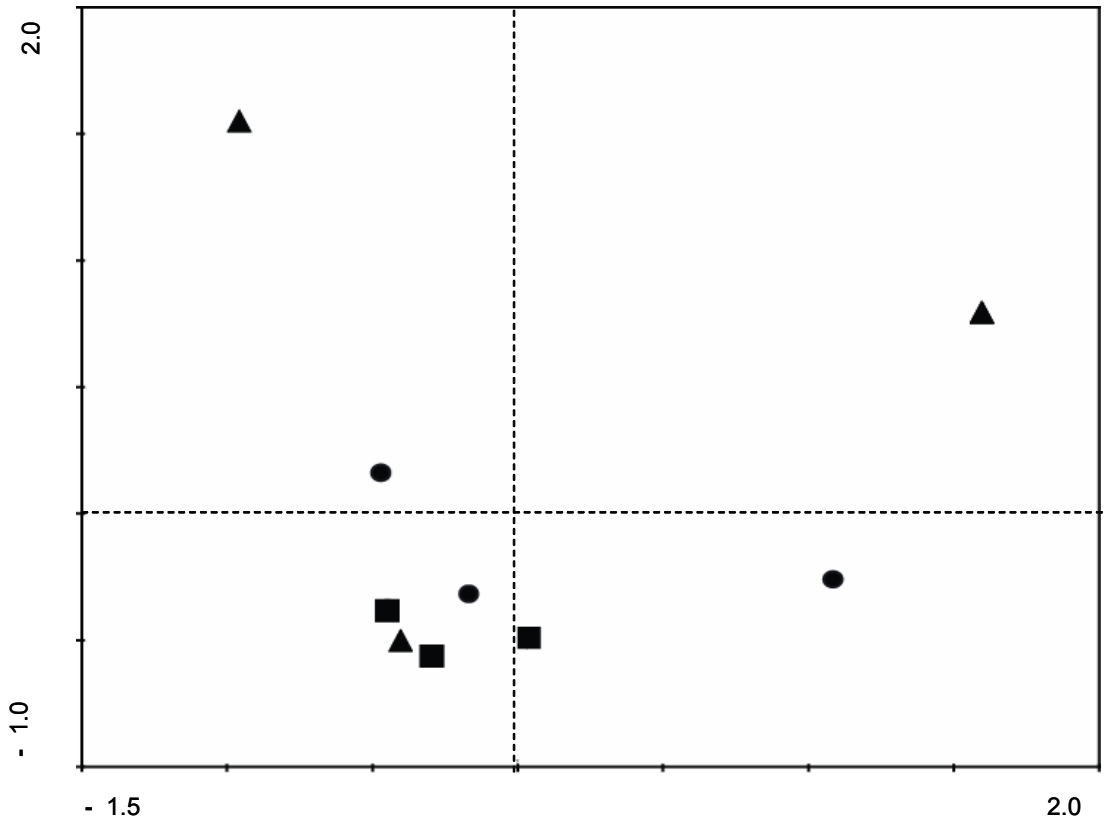

Fig. 4.5 Correspondence analysis ordination plot of the T-RFLP dataset of the bacterial hindgut communities of *Pachnoda marginata* treated with three different food soils. Filled squares represent soil I, filled triangles soil II and filled circles soil III (see method section). O The eigenvalues for the first and second axis are 0.609 and 0.474, respectively.

4.4 Discussion

The two scarab beetle species *Pachnoda ephippiata* and *Pachnoda marginata* as well as their larvae live naturally spatially separated. However, they share the same diet and occupy the same ecological niche. In this study, the microbial communities of the midgut and the hindgut of two humivorous beetle larvae were examined with respect to differences in the bacterial composition and changes due to different food sources.

4.5.1 Diversity and bacterial composition of midgut and hindgut

For both beetle larvae, the species richness based on the number of OTUs in midgut is much lower than in hindgut. These findings support previous results for *Pachnoda ephippiata* (Egert et al., 2003; Lemke et al., 2003) and *Pachnoda marginata* (Cazemier et al., 1997), where the abundance and the diversity in the hindgut were considerably higher. This is understandable, since the midgut is regarded to be the site for enzymatic digestions (Crowson, 1981; Terra and Ferreira, 1994) and the hindgut is mainly the location for microbial fermentation processes, supported by generally high cell-numbers (Cruden and Markovetz, 1984; Santo Domingo et al., 1998; Lemke et al., 2003) and high concentrations of fermentation products (Cruden and Markovetz, 1984; Tholen et al., 1997; Lemke et al., 2003). It is very likely, that the higher phylogenetic diversity in the hindgut reflects a higher metabolic diversity.

Some T-RFs in the midgut and hindgut community fingerprints were assigned to phylogenetic groups, e.g. Lactobacillales, Clostridiales and Bacillales (Brune, 1998; Whitford et al., 1998; Hongoh et al., 2003), commonly found in other intestinal tracts. Most known members of these phyla are characterized by a fermentative metabolism, corroborated by the high concentration of acetate and lactate in both gut compartments of *Pachnoda ephippiata* (Lemke et al., 2003). Further, the high number of acetogenic and lactogenic bacteria is confirmed by MPNs (most probably number) carried out with the midgut and hindgut of *P. ephippiata* (Lemke et al., 2003).

4.5.2 Differences in the bacterial communities

The bacterial communities in the gut of *Pachnoda ephippiata* and *Pachnoda marginata* are significantly different. Differences may be a consequence of spatial separation, which was found also for the harvester termite *Hodotermes mossambicus* (Minkley et al., 2006) and the wood-feeding termite *Reticulitermes speratus* (Matsuura, 2001). However, congeneric species of soil-feeding termites occupying the same ecological niche harbor a highly similar gut microbiota (Schmitt-Wagner et al., 2003b). Similar results were obtained for wood-feeding termites, where sympatric termite species shared considerably more phylotypes than allopatric species (Hongoh et al., 2005)

However, considering the Morisita indices, which proved to be an objective measure of community similarities (Krebs, 1998), already the intraspecific similarity was

comparably low, suggesting fluctuation within the microbial gut community between individuals. The differences are rather due to changes in abundance of the phylotypes than to changes in the composition. In contrast to that, the soil-feeding termite *Cubitermes ugandensis* revealed similarities of up to 94% among individuals also sampled at different sites (Schmitt-Wagner et al., 2003b).

4.5.3 Effects of different food soils on the bacterial community

The intestinal microbiota plays an important role for the insect host. It was postulated that a similar gut microbiota in termites as social insects is very important for nestmate recognition (Matsuura, 2001; Minkley and Kirchner, 2003; Wei et al., 2007). Microorganisms are also involved in the digestion of food, for instance, the cellulose and lignocellulose digestion in many insect is carried out by the microbial gut community (e.g. Schlottke, 1945; Breznak and Brune, 1994). Therefore, changes in the insect diet might effect the composition of the gut microbiota. The change of the types of food soils did not significantly alter the intestinal microbiota in *Pachnoda marginata* larvae. By contrast, in other insect gut environments, e.g. the cricket and the cockroach hindgut (Santo Domingo et al., 1998; Santo-Domingo et al., 1998b; Kane and Breznak, 1991), structure and function of the intestinal microbiota altered according to changes in the diet. These variations might be due to differences in the composition of the food source and a consequential selective enrichment of respective microorganisms.

4.5.4 Conclusions and outlook

The larvae of *Pachnoda* spp. harbor a species-specific gut microbiota. However, the individual differences are relatively high, indicating a comparably unstable bacterial gut community. Obviously, for the hindgut the differences were more related to the relative abundance of individual phylotypes than to the absolute composition of the community.

The intestinal microbiota of insects is in general highly diverse (e.g. Cazemier et al., 1997; Tholen et al., 1997, Lemke et al., 2003). Whereas cloning and sequencing for the comparison of gut communities require much effort and are cost-intensive, molecular fingerprinting techniques like T-RFLP proved several times to be an effective and highly reproducible method to explore complex microbial communities (e.g. Schmitt-Wagner et al., 2003b; Minkley et al., 2006). Since microorganisms of different phylogenetic groups can have the same restriction site, a single peak in a T-RFLP

profile does not necessarily represent a single phylogenetic group. For this reason T-RFLP should always be combined with a clone library (Friedrich et al., 2001), which is already in progress for this study. Although for P. ephippiata a clone library exists, Many T-RFs could not be assigned by the clone library of (Egert et al., 2003), there fore a new clone library is in progress.

4.5 References

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5 General Discussion

5.1 Carbon metabolism in *Pachnoda* spp.

Previous studies have speculated that peptides are carbon and nitrogen source for the larvae and the gut microbiota (Li and Brune, 2005; Li and Brune, 2005b). The tracing of humic-acid stabilized and free peptides during gut passage revealed that peptides are the starting point of several host-induced as well as microbe-mediated processes (Chapter 2, Fig. 5.1). Peptides stabilized in humic-substances are hydrolyzed during gut passage. The main-site for proteolysis is the midgut, where enzymes are secreted by the insect host (Terra and Ferreira, 1994). The proteolytic activity is humic-acid-tolerant and alkali-stable (Zhang and Brune, 2004), which represents adaptation to the alkaline pH and the high concentration in the midgut. In the hindgut the proteolytic activity is mainly particle associated, indicating the contribution of microorganisms to proteolysis. Resulting amino acids are either resorbed by the host (Dadd, 1973) or subject to microbial fermentation processes. Another source of nitrogen and carbon could be peptidoglycan, since Li and Brune (2005b) provided evidence that humic-acid stabilized peptidoglycan is mobilized and mineralized during gut passage. However, peptidoglycan N (1-14%, Baldock and Nelson, 2000) is less abundant than peptide N (~40%; Schulten and Schnitzer, 1998) in soil organic matter and is therefore most likely playing a minor role as nitrogen and carbon source for the insect host and its gut microbiota. Also bacterial biomass is mineralized during gut passage (Rössler, 1961; Li and Brune, 2005) and might therefore represent valuable sources of nitrogen and carbon.

Within this thesis, it could be shown that the larvae are selectively feeding on the nitrogen rich fraction of the food soil (this thesis). Also carbon was selectively enriched during ingestion by the larvae. The conversion of peptides during gut passage contributed with only 10% to the total respiratory rate of the larvae. Most probably polysaccharides are playing a bigger role as carbon source in nutrition of scarab beetle larvae than peptides. Cellulose carbon accounts for up to 30% in soil organic matter, whereas peptide carbon is less abundant is soil organic matter (up to 6%, Oades, 1989). Previous studies already concentrated on cellulose digestion in scarab beetle larvae (e.g.

Wiedemann, 1930; Rössler, 1961; Cazemier et al., 1997). In these studies the enzymatic cellulose digestion was attributed mainly to the gut microbiota (e.g. Werner, 1926; Wiedemann, 1930; Cazemier et al., 1997). Consequently, according to the comparably higher cell numbers, the main site of cellulose digestion might be the hindgut. By comparative analysis of food source and feces it was observed that to up to 65 % of the fibers in the food material are digested in *Pachnoda marginata* (Cazemier et al., 1997). In *Oryctes nasicornes* the cellulose w converted mainly to volatile fatty acids (Bayon and Mathelin, 1980). Also the emission of CO_2 from radiolabeled cellulose was measurable (Li and Brune, 2005). However, this was at least partly due to resorption of fermentation products by the larvae (Kane, 1997) and subsequent oxidation.

5.2 The role of the gut microbiota in nitrogen metabolism

Over 90 % of the nitrogen in soil organic matter occurs in organic form (e.g. Stevenson, 1994). The major part of this nitrogen is represented by peptides (40%, Sowden et al., 1976; Schulten and Schnitzer, 1998). The fate of nitrogen in intestinal tracts of insects is poorly examined. This thesis provides insights in the bacterial nitrogen metabolism in the gut of the humivorous larvae of *Pachnoda* spp..

In midgut and hindgut of *Pachnoda* spp. ammonia is accumulating, providing evidence for amino acid fermentation (Fig. 5.1). Additionally, we demonstrate the formation of acetate and propionate from radiolabeled amino acids. The amino acid fermentation rates in the hindgut are considerably higher than in the midgut (Chapter 2). This, Together with higher bacterial cell numbers compared to the midgut (Lemke et al., 2003), suggests that the hindgut to be main site for amino acid fermentation.

The accumulation of high concentrations of nitrite and nitrate in the midgut and the hindgut strongly indicates nitrification from ammonia (Fig. 5.1). Further evidence for nitrification is provided by a PCR product with primers specific for nitrifies from the hindgut (Ngugi and Brune, unpublished). As generally known, nitrification is an oxygen-dependent process. Therefore, nitrification is most likely only occurring in the oxic zones in the gut, i.e. the gut periphery (Lemke et al., 2003). Recent literature about nitrification in connection with insects is mainly restricted to the examination of

mounds of soil-feeding termites rather than the nitrifying activity in the intestinal tracts (Lensi et al., 1992; Ndiaye et al., 2004).

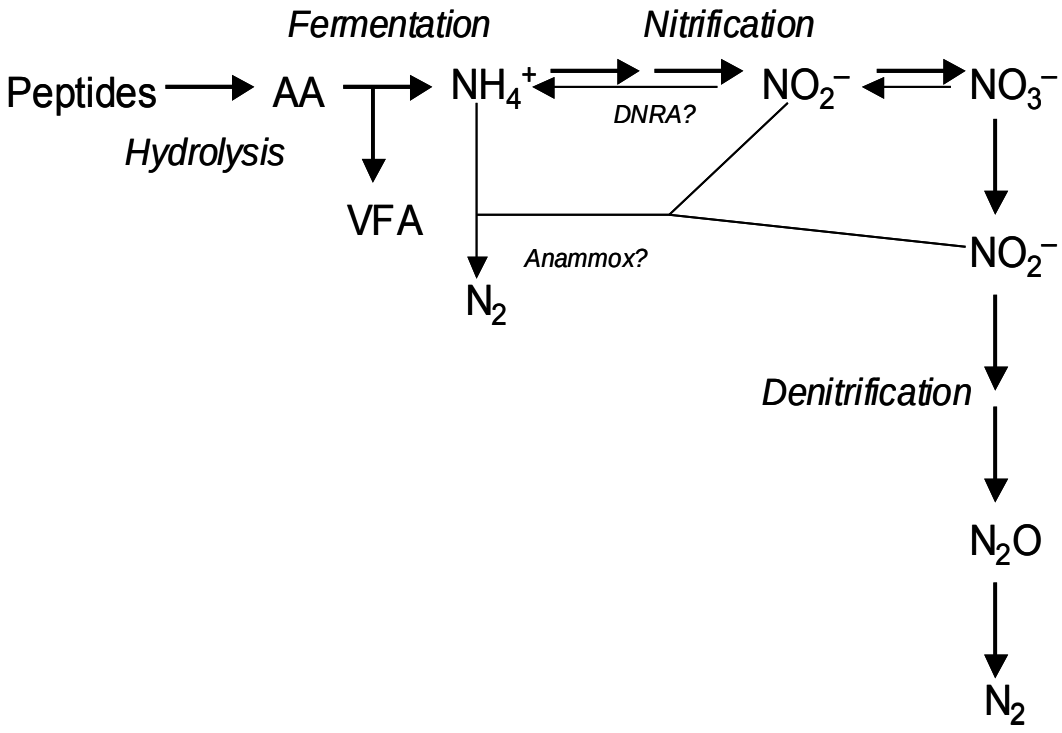

Fig. 5.1 Survey of actual and potential processes in microbial nitrogen metabolism in the gut of *Pachnoda* spp. Only compounds, which actually were detected, are shown. Processes with question marks are not proven to occur, but possible. (AA − amino acids, Anammox − anaerobic ammonia oxidation, DNRA − dissimilatory nitrate reduction to ammonia, VFA – volatile fatty acids).

There is also evidence for denitrification in the gut of *Pachnoda* spp. (Fig. 5.1), which is probably located in the anoxic gut center. In high dilutions of MPN (most probable number) determinations of midgut and hindgut of *Pachnoda marginata* nitrate is reduced in association with N_2 formation. Nitrate reduction was also shown for several isolates from *Pachnoda ephippiata* hindgut (Geissinger and Brune, unpublished). Further, N_2O, as intermediate of denitrification, was emitted from *Pachnoda marginata* with a rate of 143 ± 39 pmol (g fresh weight)$^{-1}$ (Ngugi and Brune, unpublished), probably originating from the action of denitrifying bacteria. Electron donors for denitrification could be short chain fatty acids from intestinal fermentation processes, which are abundant in midgut and hindgut (Lemke et al., 2003). Also humic substances were proposed by Lovley et al. (1996) as electron donors for denitrification.

Since humic substances are ingested with the soil to a great extent, they also represent potential electron donors for this process. So far, the earthworm is the only soil macroinvertebrate, where denitrification has been studied in more detail. N_2O emission by earthworms is also attributed to denitrifying bacteria (Karsten and Drake, 1997; Matthies et al., 1999), which are most likely originating from ingested soil (Horn et al., 2003; Ihssen et al., 2003).

Besides denitrification, N_2O can also be produced a side product of nitrification (Ritchie and Nicholas, 1972; Bollmann and Conrad, 1998) or originate from dissimilatory nitrate reduction to ammonia (DNRA). This process could be demonstrated for the rumen of cows (Kaspar and Tiedje, 1981) and sheep (Lewis, 1951).

In the gut, the concurrent presence of ammonia and nitrite under anoxic conditions also allows anaerobic ammonia oxidation (anammox). All known anammox bacteria belong to the bacterial phylum Planctomycetes (Fuerst, 2005). Therefore, the Planctomycetes found in the clone library from the hindgut of *Pachnoda ephippiata* (Egert et al., 2003) could be responsible for anammox in the gut of *Pachnoda* spp..

In this thesis, it was not examined whether the nitrogen cycle in the gut of *Pachnoda* spp. is closed and nitrogen fixation is an issue. In wood-feeding termites, nitrogen fixation plays a major role in supplementing nitrogen, due to the nitrogen-depleted diet (Benemann, 1973; Breznak et al., 1973; Golichenkov et al., 2002). In contrast, the food soil of *Pachnoda* spp. is comparably rich in nitrogen and the demand for nitrogen fixation is likely to be very small. Furthermore, during gut passage losses of nitrogen from the ingested soil have been detected (Chapter 2), indicating the minor role of nitrogen fixation in the gut of *Pachnoda* spp..

5.3 Stable isotope probing – a powerful tool for linking structure and function in the insect gut?

The physiological potential of some representatives of the gut microbiota of *P. ephippiata* was examined by MPNs (Lemke et al., 2003; Chapter 2) and cultivation (Geissinger and Brune, unpublished). By isolation and characterization of microorganisms from intestinal tracts, the metabolic potentials of the respective isolate

can be found out. However, it is not possible to assign the actual function of the isolate in the gut. Conclusions concerning the metabolism of the gut microorganisms from the phylogeny on 16S rRNA gene basis are also not possible. Further, clone libraries and community fingerprints are in general made on DNA basis and do not necessarily represent the actual active community. However, methods based on nucleic acid labeling of the metabolic active organisms, e.g. stable isotope probing, allow assigning functions to single members of complex microbial communities. The basis of stable isotope probing is the incorporation of ^{13}C labeled substrate carbon into RNA or DNA by the microorganism consuming the respective compound in the microbial community and subsequent phylogenetic analysis of the labeled nucleic acids.

In incubations of whole guts of *Reticulitermes santonensis* with $^{13}HCO_3$ no effects of incorporation of ^{13}C into the RNA of the microbial gut community were detectable. Also, during incubations of hindgut homogenate of *Pachnoda ephippiata* with ^{13}C-glucose no incorporation of substrate carbon into microbial RNA was measurable (Chapter 3). However, in both cases, it was confirmed that the substrate was converted with high rates. Repetitions of these incubations with $H^{14}CO_3$ or ^{14}C-labeled glucose, respectively, revealed that for *Reticulitermes santonensis* whole guts the ^{14}C incorporation into microbial RNA does not exceed 8%. The carbon incorporation from ^{14}C-glucose in *Pachnoda ephippiata* hindgut homogenate was even lower (1%). Firstly, one possible explanation might be that the fraction of the population, which is actively forming RNA from the labeled substrate, was too small and the label was diluted by the major part of community RNA. Secondly, the majority of the population could have incorporated only a minor part of the substrate carbon into their RNA. However, for a successful stable isotope probing experiment the ^{13}C carbon should make up 10 – 20% of total carbon in the microbial RNA (Manefield et al., 2002).

In incubations of photosynthetic microbial mats with $^{14}CO_2$ no incorporation of substrate carbon into RNA was detectable (Nold and Ward, 1996). The lack of incorporation was explained by the absence of *de novo* synthesis of RNA, due to the absence of microbial cell division (Kramer and Morris, 1990), which might also be considered for the insect gut. Lastly, salvage pathways concerning the *de novo* synthesis of RNA from nucleotides in the gut fluid are further possible explanation of the low

incorporation of substrate carbon in insect guts. This aspect is discussed in detail in Chapter 3.

The uncoupling of catabolism and anabolism is the most probable explanation for low incorporation of substrate carbon into RNA. Therefore, stable isotope probing is considered being an inappropriate method for linking structure and function in the insect gut.

5.4 The microbial gut communities of *Pachnoda ephippiata* and *Pachnoda marginata* exhibit species-dependent differences

The composition of the microbial community of the humivorous beetle larvae *Pachnoda ephippiata* has already been studied (Egert et al., 2003). In the present thesis, the gut microbiota was re-examined according to species-specific differences in the composition and the abundance of the gut microbiota. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of the of the microbial gut community from the two species, combined with correspondence analysis was the method of choice.

For both beetle larvae, the bacterial diversity and abundance in the midgut was much lower than in hindgut, which is in agreement with previous studies for *Pachnoda ephippiata* (Egert et al., 2003; Lemke et al., 2003) and *Pachnoda marginata* (Cazemier et al., 1997). Further, we demonstrated that the composition of the bacterial gut communities of midgut and hindgut of *Pachnoda ephippiata* and *Pachnoda marginata* were significantly different. Differences may be a consequence of spatial separation, which was confirmed for the harvester termite *Hodotermes mossambicus* (Minkley et al., 2006) and the wood-feeding termite *Reticulitermes speratus* (Matsuura, 2001). Congeneric species of soil-feeding termites occupying the same ecological niche harbor a highly similar gut microbiota (Schmitt-Wagner et al., 2003b). Comparable results were obtained for wood-feeding termites, where sympatric termite species shared considerably more phylotypes than allopatric species (Hongoh et al., 2005).

Together, these findings suggest that the microbial composition of the gut is influenced by the environment including the individuals sharing the respective ecological niche.

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Summary

The humivorous scarab beetles of the genus *Pachnoda* are indigenous to the African continent. The larvae of these insects preferably live in humic soils and feed on plant material and soil organic matter. However, the nature of the soil components used as carbon and energy sources are so far not understood. Previous studies indicated that peptides stabilized in humic acid model compounds are released and mineralized during gut passage. The present thesis documents extent of transformation and mineralization of peptidic compounds of the food soil. Further, the role of peptides in the nutrition of the larvae was estimated.

The major site of proteolytic activity was located in the midgut. Also in the hindgut minor proteolytic activities were found. We postulate that the former activities are associated with host-secreted proteinases while the latter are mainly of microbial origin. As a consequence of high proteolytic activities in the midgut considerably high concentration of amino acids are accumulating. Relatively low turnover rates of amino acids suggest amino acids fermentation is the rate-limiting step in the midgut. In vivo, the insect host presumably takes up part of these amino acids. The high ammonia concentration in the hindgut is associated with high a turnover rate of amino acids. Addition of amino acids even stimulated the fermentative activity, suggesting that the supply of free amino acids by proteolysis is the rate-limiting step in this process. Thus, the hindgut is the main site of amino acid fermentation in the gut. Further, nitrification was indirectly observed by the accumulation of nitrite and nitrate. In high dilution steps of MPNs nitrite is subject to denitrification associated with gas production, most probably N_2, supported by detectable losses of nitrogen from the food soil during gut passage.

Up to now, it is not known which organisms in the hindgut of *Pachnoda* larvae are responsible for metabolic processes like amino acid fermentation. With the help of stable isotope probing, for several environments and diverse substrates the function of specific microbial populations was elucidated. However, previous experiments addressing the functions of the insect gut microbiota difficulties were observed using this method. In stable isotope probing experiments with whole guts of the wood-feeding termite *Reticulitermes santonensis* and hindgut of the humivorous beetle larvae of

Pachnoda ephippiata using $H^{13}CO_3^-$ or ^{13}C-labeled glucose it was confirmed that no isotope effect in the RNA of the gut microbiota can be detected. Also in incubations with ^{14}C-labeled substrates, generally a more sensitive tracer, very little incorporation of substrate carbon into RNA was detected. We suggest several explanations as to why stable isotope probing in the gut of insects is not feasible. Since free nucleotides accumulate in the hindgut of *Pachnoda marginata* to high concentrations, we hypothesize that salvage pathways, known for many microorganisms as alternative for nucleotide biosynthesis, reduce the *de novo* formation of nucleic acids from substrate carbon. This hypothesis is supported by considerable inhibition of the incorporation of substrate carbon into RNA in incubations with *Bacillus subtilis* and *Pseudomonas putida* in the presence of ribonucleotides. In the insect gut environment, we reached the limits of the otherwise very convenient and successful application of stable isotope probing for the above-mentioned substrates. The actual reason still remains be elucidated.

The allopatric scarab beetle larvae of *Pachnoda ephippiata* and *Pachnoda marginata* are closely related and both feeding on soil organic matter. In the present thesis, the question is answered, whether the microbial community in the gut is similar, due to the same food source, or reveals differences as a consequence of the spatial separation. The composition of the bacterial communities of the midgut and the hindgut was studied with the help of terminal restriction fragment length polymorphism (T-RFLP) analysis, representing a PCR-based cultivation-independent technique. In both larvae, the bacterial midgut community is less diverse than the microbial hindgut community. In the T-RFLP profiles from the midgut of the two species only few T-RFs are shared with the hindgut profiles. By contrast, the community fingerprints of the hindguts appeared to be very similar, although the relative abundance of the single T-RF varied between the two beetle species. However, correspondence analysis of the T-RFLP dataset, we demonstrated significant species-dependent differences in the bacterial communities of both, the midgut and the hindgut, respectively. The feeding of larvae of *Pachnoda marginata* on different food soils did not result in significant differences in the bacterial hindgut community. Together, these results indicate that the differences in the gut microbiota are rather due spatial separation of the species than to differences in the diet.

Zusammenfassung

Die Käfer der Gattung *Pachnoda* aus der Familie der Scarabaeiden sind auf dem afrikanischen Kontinent ansässig. Die Larven dieser Insekten leben bevorzugt in huminstoffreichen Böden und ernähren sich von Pflanzenbestandteilen und organischer Bodensubstanz. Jedoch ist bisher noch nicht bekannt, welche Bestandteile des Bodens als Kohlenstoff- und Energiequelle genutzt werden. Frühere Studien wiesen darauf hin, dass Peptide, stabilisiert in Modellsubstanzen für Huminsäuren, während der Darmpassage frei und mineralisiert werden. Die vorliegende Arbeit untersucht das Ausmaß der Transformation und Mineralisierung der Peptidverbindungen des Futterbodens. Weiterhin, wurde die Rolle von Peptiden in Bezug auf die Ernährung der Larve eingeschätzt.

Während der Hauptteil der proteolytischen Aktivität im Mitteldarm lokalisiert wurde, konnte auch im Enddarm geringe proteolytische Aktivität gefunden werden. Erstere Aktivitäten gehen höchstwahrscheinlich auf vom Insekt selbst sekretierte Proteinasen zurück, während letztere mikrobieller Herkunft sind. Als Folge der hohen proteolytischen Aktivität im Mitteldarm akkumulierten dort hohe Aminosäurekonzentrationen. Relativ niedrige Umsetzungsraten der Aminosäuren weisen darauf hin, dass die Fermentation von Aminosäuren der geschwindigkeits-bestimmende Schritt im Mitteldarm ist. Die hohe Ammoniumkonzentration im Enddarm ist begleitet von einer hohen Aminosäureumsatzrate. Durch Zugabe von Aminosäuren konnte die Fermentationsaktivität noch gesteigert werden, was darauf hindeutet, dass die Zufuhr von freien Aminosäuren durch Proteolyse den geschwindigkeitsbestimmenden Schritt in diesem Prozess darstellt. Daher ist der Enddarm der Hauptort der Aminosäurefermentation. Zudem wurde indirekt über die Detektion von Nitrit und Nitrat Nitrifikation nachgewiesen. In hohen Stufen von MPN-Verdünnungsreihen wurde Nitrit denitrifiziert. Der messbare Verlust von Stickstoff aus dem Futterboden während der Darmpassage deutet ebenfalls auf Denitrifikation von Nitrat und Nitrat zu N_2 hin.

Bislang ist nicht bekannt, welche Mikroorganismen im Enddarm von *Pachnoda*-Larven für Stoffwechselprozesse, wie Aminosäurefermentation, verantwortlich sind.

Mit Hilfe von stabiler Isotopenbeprobung wurde für einige Lebensräume und verschiedene Substrate die Funktion einzelner mikrobieller Populationen beleuchtet.

In früheren Experimenten zur Charakterisierung der Funktion der Insektendarmmikrobiota traten jedoch Schwierigkeiten im Zusammenhang mit dieser Methode auf. In stabilen Isotopenbeprobungsexperimenten mit ganzen Därmen der holzfressenden Termite *Reticulitermes santonensis* und dem Enddarm der humusfressenden Käferlarve von *Pachnoda ephippiata* mit $H^{13}CO_3^-$ oder ^{13}C-Glucose wurde bestätigt, dass kein Isotopeneffekt in der RNA messbar war. Auch in Inkubationen mit ^{14}C-markierten Substraten, im Allgemeinen ein empfindlicher Tracer, konnte nur geringer Einbau von Kohlenstoff aus dem verwendeten Substrat gemessen werden. Wir schlagen mehrere Erklärungen vor, warum stabile Isotopenbeprobung in Insektendärmen nicht durchführbar ist. Da im Enddarm der Larve von *Pachnoda marginata* hohen Konzentrationen an Nukleotiden akkumulieren, stellen wir die Hypothese auf, dass sogenannte „Salvage Pathways“, bekannt für eine Reihe von Mikroorganismen als Alternative zur Nukleotidbiosynthese genutzt werden. Dadurch wird die Neubildung von Nukleotiden aus Susbtratkohlenstoff verringert. Diese Hypothese wird unterstützt durch die erhebliche Hemmung des Einbaus von Substratkohlenstoff in RNA während Inkubationen von *Bacillus subtilis* und *Pseudomonas putida* in Anwesenheit von Ribonukleotiden.

Im Lebensraum des Insektendarms sind wir an die Grenzen der sonst sehr komfortablen und erfolgreichen Anwendung der stabilen Isotopenbeprobung für die oben genannten Substrate gestoßen. Die tatsächliche Ursache gilt es noch herauszufinden.

Die allopatrischen Scarabaeidenlarven *Pachnoda ephippiata* und *Pachnoda marginata* sind eng verwandt und ernähren sich beide von organischer Bodensubstanz. In der vorliegenden Arbeit wurde die Frage beantwortet, ob die mikrobielle Gemeinschaft im Darm ähnlich ist aufgrund der ähnlichen Nahrungsquelle oder ob Unterschiede bestehen aufgrund der räumlichen Trennung des Vorkommens. Die Zusammensetzung der bakteriellen Gemeinschaft des Mitteldarms und Endarms wurde mit Hilfe von terminaler Restriktionsfragmentlängenpolymorphismus-Analyse untersucht, die eine PCR-basierte kultivierungsunabhängige Methode darstellt. In beiden Larven war die bakterielle Gemeinschaft im Mitteldarm weniger vielfältig als im

Endarm. Die T-RFLP-Profile des Mitteldarms der beiden Arten hatten nur wenige terminale Restriktionsfragmente gemeinsam. Im Gegensatz dazu wiesen die Profile der Bakteriengemeinschaft im Enddarm viele Ähnlichkeiten auf, obwohl sich die relativen Abundanzen der einzelnen Restriktionsfragmente innerhalb der beiden Arten unterschieden. Jedoch konnten mit Hilfe einer eine Korrespondenzanalyse der T-RFLP-Datensätze signifikante, artspezifische Unterschiede in den Bakteriengemeinschaften für Mitteldarm und Enddarm nachgewiesen werden. Die Fütterung von Larven der Art *Pachnoda marginata* mit drei verschiedenen Futterböden führte nicht zu signifikanten Abweichungen in der Bakteriengemeinschaft im Enddarm. Zusammenfassend weisen die Ergebnisse daraufhin, dass die Unterschiede in der Darmmikrobiota eher auf die räumliche Trennung der Arten zurückzuführen ist, als auf Unterschiede in der Nahrung.

Lebenslauf

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Ausbildung

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Publikationen

Synthesis and characterization of metal binding pseudotripeptides (Kuenzel S, Pretzel D, **Andert J,** Beck K, Reissmann S; J Peptide Sci, 2003)

Stable isotope fractionation of tetrachloroethene during reductive dechlorination by *Sulfurosprillum multivorans* and *Desulfitobacterium* sp. Strain PCE-S and abiotic reactions with cyanocobalamin (Nijenhuis I,

Andert J, Beck K, Kästner M, Diekert G, Richnow HH; Appl Environ Microbiol, 2005)

Factors controlling the carbon isotope fractionation of tetra- and trichloroethene during reductive dechlorination by *Sulfurospirillum* spp. and *Desulfitobacterium* sp. strain PCE-S (Cichoka D, Siegert M, Imfeld G, **Andert J**, Beck K, Diekert G, Richnow HH, Nijenhuis I; accepted for publication in FEMS Microbiol Ecol)

Peptidic soil components are a major dietary resource for the humivorous larva of *Pachnoda* spp. (Coleoptera: Scarabaeidae) (Andert J, Geissinger O and Brune A; in press in J Insect Physiol)

Beitrag zu Tagungen und Symposien

VAAM Jahrestagung 2005, Jena: **The role of peptides and amino-acid-fermenting bacteria in the gut of a humivorous beetle larva (Poster)**. J. Andert, O. Geissinger, A. Brune

Abgrenzung der Eigenleistung

Soweit nicht anders erwähnt, wurden alle Experimente von mir selbst geplant und durchgeführt, sowie anschließend in Form eines Manuskriptes ausgewertet. Das abschließende Verfassen der Manuskripte erfolgte zusammen mit meinem Betreuer Prof. Dr. A. Brune.

Die MPN-Verdünnungsreihen (Kapitel 2) wurden von Oliver Geißinger durchgeführt.

Die statistische Auswertung der T-RFLP-Profile (Kapitel 4) erfolgte unter Beratung durch Andreas Marten und Prof. Dr. R. Brandl.

Alle Experimente mit *Reticulitermes santonensis* (Kaptiel 3) wurden von Julia Rieckmann durchgeführt.