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# Essential amino acid provisioning by termite-associated gut microbiota

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Gut-associated microbes of many insects provide a variety of beneficial nutritive functions to their hosts such as the provisioning of essential amino acids (EAAs) to those that feed on diets limited in assimilable nitrogen (i.e., wood). We investigated this function by the gut microbiota of the eastern subterranean termite (Reticulitermes flavipes) using 13C-stable isotope analysis of EAAs in the diet and termite samples. Evidence of possible microbe input was revealed by 13C-depletion of termite carcass (-27.0 ± 0.43‰, mean ± s.e.), and termite gut filtrate samples (-27.3 ± 0.58‰) relative to their wood diet (-26.0 ± 0.48‰) ($F(2, 63) = 6.2$, $P < 0.004$). An investigation of the identity of non-dietary EAA sources determined that termites predominantly incorporated EAAs derived from bacteria, with minor fungal input. The most likely means of EAA acquisition is through proctodeal trophallaxis (mouth-anus feeding), a well-established feature of termite colony nestmates, and subsequent digestion of the microbial fraction in the transferred food. Our study provides empirical data in support of the gut microbial EAA provisioning function in termites by using 13C-stable isotopes to determine the microbial origins of incorporated EAAs in termite tissues.

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**Title: Essential amino acid provisioning by termite-associated gut microbiota**

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**Abstract**

Gut-associated microbes of many insects provide a variety of beneficial nutritive functions to their hosts such as the provisioning of essential amino acids (EAAs) to those that feed on diets limited in assimilable nitrogen (i.e., wood). We investigated this function by the gut microbiota of the eastern subterranean termite (*Reticulitermes flavipes*) using $^{13}$C-stable isotope analysis of EAAs in the diet and termite samples. Evidence of possible microbe input was revealed by $^{13}$C-depletion of termite carcass (-27.0 ± 0.43‰, mean ± s.e.), and termite gut filtrate samples (-27.3 ± 0.58‰) relative to their wood diet (-26.0 ± 0.48‰) ($F_{(2, 63)} = 6.2$, $P < 0.004$). An investigation of the identity of non-dietary EAA sources determined that termites predominantly incorporated EAAs derived from bacteria, with minor fungal input. The most likely means of EAA acquisition is through proctodeal trophallaxis (mouth-anus feeding), a well-established feature of termite colony nestmates, and subsequent digestion of the microbial fraction in the transferred food. Our study provides empirical data in support of the gut microbial EAA provisioning function in termites by using $^{13}$C-stable isotopes to determine the microbial origins of incorporated EAAs in termite tissues.

**Key words:** $^{13}$C-stable isotope analysis, essential amino acid, gut microbiome, *Reticulitermes flavipes*, and trophallaxis



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## Introduction

Associations between termites and their gut microbes are among the most well studied symbioses. The majority of lower termites (all families except Termitidae) are wood feeders that thrive on these nitrogen-limited diets (Mattson 1980) by relying upon gut microbes that can fix atmospheric nitrogen (Lilburn et al. 2001; Meuti et al. 2010). In the absence of these microbes, wood is an incomplete dietary source since it is incapable of meeting the termites' nutritional demands for nitrogen-rich metabolites such as proteins. Termites also rely on gut microbes to metabolize plant tissues, comprised largely of cellulose, into assimilable carbon. Digestion of cellulose, hemicellulose, and lignocellulose is attributed to a consortium of host and microbe-derived cellulases that ultimately liberate carbon in plant tissues (Scharf 2011; Tartar et al. 2009; Warnecke et al. 2007). Furthermore, evidence of $^{13}$C-metabolite transfer between protists and associated gut bacteria in the desert damp wood termite, *Paraneotermes simplicicornis,* confirms the flow of nutrients in the termite gut following $^{13}$C-cellulose degradation by associated protists (Carpenter et al. 2013). Microbe-specific functions that benefit the termite host include nitrogen fixation (Lilburn et al. 2001) and acetogenesis/carbon dioxide fixation (Breznak and Kane 1990; Pester and Brune 2006), which provide ammonia and acetate, respectively, and these can be used by the host in biosynthetic and energy metabolism processes. Oxygen scavenging and removal of excess hydrogen via methanogenesis are additional microbe-specific functions that are essential to maintaining the physiological and biochemical conditions within the gut microenvironment, ensuring that the aforementioned processes can continue (Brune and Friedrich 2000).



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An important aspect of wood-feeding insects' nutritional ecology is the acquisition of essential amino acid (EAAs) because these cannot be generated by the host *de novo* (Douglas 2013). Proctodeal trophallaxis (mouth-anus transfer of gut contents among nestmates), an essential colony feature, is thought to serve as one of the means by which termites acquire EAAs (Nalepa et al. 2001). Briefly, partially digested and undigested materials (with dead and living microbial fractions) are ingested from the anus of colony members and are used for inoculation or digestion (Osamu and Kitade 2004). The factors mediating inoculation as opposed to digestion are unclear. Inoculation may be more relevant for newly eclosed (hatched) and molted (inter-stadial growth) colony members, and digestion the norm in workers (Osamu and Kitade 2004). Damp wood termites (*Hodotermopsis sjostedti*) that fed on $^{13}$C-cellulose were found to exhibit notably higher normalized intensities of $^{13}$C-labelled EAAs at 24 hrs in the lumen fluids of the midgut relative to the foregut and hindgut, hence suggesting the importance of proctodeal trophallaxis and subsequent digestion of microbial fractions for EAA acquisitioning (Tokuda 2014). It remains to be determined conclusively, however, that termites acquire EAAs from gut microbes following digestion of proctodeal food and incorporation of microbial EAAs, since the study examined EAA intensities in the gut lumen fluids but not actual insect tissues.

In this study, we investigated gut microbial EAA provisioning in the eastern subterranean termite, *Reticulitermes flavipes,* using naturally occurring variations in the $^{13}$C/$^{12}$C ratios of EAAs from bacteria, fungi, and plants. The premise of this approach is two-fold. First, because insects are incapable of *de novo* EAA biosynthesis and must rely solely on dietary sources, the $^{13}$C-signature (determined via isotope ratio mass



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spectrometry) of an EAA in an insect consumer ($\delta^{13}C_{Consumer\ EAA}$) is expected to approximate that of its the diet ($\delta^{13}C_{Dietary\ EAA}$), with little change in the ratio of $^{13}C/^{12}C$ stable isotopes in the carbon skeleton of that particular EAA(Caut 2008; McMahon et al. 2010; Newsome 2011). The isotopic difference between consumer $\delta^{13}C_{EAA}$ and dietary $\delta^{13}C_{EAA}$ (given by the delta notation; $\Delta\delta^{13}C = \delta^{13}C_{Consumer\ EAA} - \delta^{13}C_{Dietary\ EAA}$) is estimated to within 1‰ ppm (parts per million) of the dietary $\delta^{13}C_{EAA}$. Any significant deviation from the expected $\Delta\delta^{13}C$ of 1 suggests the possibility of alternate or additional sources of EAAs(Newsome 2011; Tieszen et al. 1983).

The second premise relies on the fact that plants, bacteria and fungi are the only organisms capable of synthesizing EAAs and non-essential amino acids *de novo*. Additionally, bacteria, fungi, and plants have unique and distinct EAA signatures as a result of different biosynthetic pathways and processes that eventually lead to different $^{13}C/^{12}C$ stable isotopes ratios. The distinct $\delta^{13}C_{EAA}$ signatures across these groups have been empirically demonstrated (Larsen et. al. 2009) and used in several ecological studies (Larsen et al. 2011; Larsen et al. 2013; Vokhshoori, McCarthy & Larsen 2014).

Thus, according to the first premise, a determined discrimination/offset factor ($\Delta\delta^{13}C$) greater than 1 between the $\delta^{13}C_{EAA}$ of a consumer and its diet, suggests the possibility of an additional/alternate source of EAAs for the consumer. The second premise enables the identification of the possible contributing source, within a predictive model framework, based on the unique $\delta^{13}C_{EAA}$ signatures of plants, bacteria, and fungi. In this study, we investigated the $^{13}C$-offset between termites and their dietary substrates and assessed biosynthetic contributions from plant, bacteria and fungi to termite $\delta^{13}C_{EAA}$ signature.



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Actual insect tissues in addition to gut lumen fluids were examined in order to conclusively determine the incorporation of microbial EAAs into the termite body.

## Materials and Methods

***Insects.*** *R. flavipes* originated from Orient, OH (39°46'19.99"N, 83°09'22.30"W) and were maintained in a laboratory colony in a plastic container fitted with a lid and provisioned with wood (a mixture of hardwood and softwood mulch and pine (*Pinus* spp.) that was moistened periodically with distilled water. The termite colony originated from a single inbred colony that had been established during May 2010 by pairing a single male and female de-alate, and was maintained at room temperature (~22ºC ± 2ºC) in the dark, under ambient laboratory conditions.

***Sample collection and preparation***. After 8 weeks of feeding, 50 individual workers were removed from the colony and surface sterilized by rinsing once in 10x Coverage Plus (Steris, Mentor, OH, USA) and twice in sterile distilled water. A total of 5 termite samples (n= 5, each made up of 10 pooled workers) were obtained. The entire alimentary system was removed from each worker and placed in a 1x phosphate buffered saline solution (PBS). The remaining termite carcass was place in a 1.5 ml Eppendorf tube (Eppendorf, Hauppauge, NY, USA). Hence, each termite sample was subdivided into the termite carcass (n= 5) and its gut fraction (n= 5). Pooled termite guts were homogenized in PBS and filtered through a 0.45 µm membrane filter (EMD Millipore, Bellerica, MA, USA) to eliminate insect debris. Gut filtrates were stored at -80°C for 48 hrs prior to lyophilization. Wood samples (n=4) from the termite colony were also ground into a coarse powder in a coffee mill and frozen at -80°C for 48 hrs before lyophilization.



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Termite and wood samples then were sent to the Stable Isotope Facility (SIF) at UC Davis, Davis, CA, USA, for $^{13}$C-stable isotope analysis.

***EAA stable isotope analysis.*** Freeze-dried termite and wood samples were acid hydrolyzed and derivatized resulting in the addition of a known carbon residue to the analytes of interest (Walsh et al. 2014). Non-analyte carbon correction was subsequently performed to correct for the addition of carbon during the derivatization process (Doherty 2001; Walsh et al. 2014). Approximately 0.2-0.5 µl aliquots of derivatized samples were injected into a splitless liner at 250 °C with a helium flow rate of 2.8 mL/min. Compound-specific isotope $^{13}$C-amino acid analysis (CSI- $^{13}C_{AA}$) was performed using the TRACE GC Ultra gas chromatograph (GC; Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Delta V Advantage isotope ratio mass spectrometer via the GC Combustion Interface III (Thermo Electron, Bremen, Germany) using the high polarity VF-23ms capillary column (Agilent Technologies). Combustion and reduction furnace temperatures were 950 °C and 650 °C, respectively. $\delta^{13}$C isotopic abundances are reported as $\delta^{13}$C values relative to the standard Vienna Pee Dee Belemnite (V-PDB) scale. For all samples, distinct peaks, with no overlaps were obtained from the GC capillary column for the five selected EAAs (isoleucine, leucine, valine, phenylalanine, and lysine). These selected EAAs were quantified from termite carcasses, termite gut filtrates, and wood samples, following a non-analyte correction relative to internal amino acid standards, and used in the statistical analyses.

***Statistical analyses.*** Mixed model analysis and mean separations (Tukey's HSD) were carried out on EAA $\delta^{13}$C data using JMP 10 (SAS Inc., NC, USA). Overall $^{13}$C-offset between termite ($\delta^{13}C_{Termite}$) and wood ($\delta^{13}C_{Wood}$) samples was determined as $\Delta$



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176 $\delta^{13}C = (\delta\ ^{13}C_{Termite} - \delta\ ^{13}C_{Wood})$. Individual patterns of $^{13}C$-offset across the five EAAs
177 between termite and wood samples was determined as $\Delta\ \delta^{13}C_{EAA} = (\delta\ ^{13}C_{Termite\ EAA} - \delta$
178 $^{13}C_{Wood\ EAA})$.

179 ***Calibration and model validation.*** An inter-lab calibration was performed to
180 minimize instrumental error and/or variability between $\delta\ ^{13}C_{EAA}$ data from our study and
181 from (Larsen et al. 2013) for representative fungi (n= 9), bacteria (n= 11), and plants (n=
182 12). Leucine was omitted from the predictive modeling because $\delta\ ^{13}C_{EAA}$ data were
183 unavailable for three termite gut filtrate samples (Table S1). The predictive model was
184 validated using the reference bacteria, fungi and plant samples as classifiers to ascertain
185 distinctness of each group. This was followed by a supervised discriminant analysis to
186 determine group membership of termite samples (carcass and gut filtrate) and wood
187 samples to the respective classifier groups (Larsen et al. 2013). Linear discriminant
188 function analysis (LDA) was carried out using the R package MASS (Venables 2002).
189 We considered wood samples as predictors in the predictive modeling and not as
190 classifiers.

191 **Ethics statement**

192 No animal rights were violated in the execution of this study and conditions were
193 within the guidelines of the Ohio State University's Office of Responsible Research
194 Practices.

195

196 **Results**

197 ***$^{13}C$-offset ($\Delta\ \delta^{13}C_{EAA}$) between termite sample and wood diet indicates alternative***
198 ***(non-dietary) EAA sources.*** Relative to the wood diet, termite carcass and termite gut



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filtrate samples were both $^{13}C$-depleted across the EAAs measured in this study. There was a significant difference ($F_{(2, 63)} = 6.2$, $P < 0.004$) between termite gut filtrate samples (-27.3 ± 0.6‰)(mean ± s.e) and wood diet (-26.0 ± 0.5‰), but not between termite carcass samples (-27.0 ± 0.4‰) and wood diet (Table 1). Termite carcass and termite gut filtrate samples were respectively $^{13}C$-depleted relative to wood diet (-1 ± 0.4‰)(Table 1). The pattern of $^{13}C$-offset of the five-measured EAAs in the termite samples relative to the wood diet is presented in Fig. 1. Briefly, phenylalanine, lysine and valine from the termite samples (carcass and gut filtrate) and wood diet were not significantly different from the each other; even though lysine in the termite samples was $^{13}C$-enriched relative to the lysine in the wood-diet, and phenylalanine and valine in termite samples were $^{13}C$-depleted, relative to the wood diet (Fig. 1). Isoleucine and leucine from termite gut filtrate samples was significantly $^{13}C$-depleted relative to termite carcass and wood diet. Despite the individual $^{13}C$-offsets ($\Delta\ \delta^{13}C_{EAA}$) between termite carcass EAAs (Fig. 1), none were significantly different from the wood diet $\delta^{13}C_{EAA}$ in the present study.

***Validation of predictive model and classification of termite sample EAAs.*** Linear discriminant function analysis (LDA) was used to assess the distinct $^{13}C$-signature of classifiers groups (bacteria, fungi and plants) using their $\delta^{13}C_{EAA}$ values (training data). Classification of the training data samples was performed using the jackknifed (i.e., leave one out) predictions. In the LDA plots, the 95% confidence limits decision regions for each group/classifier are depicted as ellipses around the classfiers



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and the decision boundaries between the groups/classifiers as lines. After establishing the discrimination model, we then predicted posterior probabilities, i.e. the probability that a particular sample belonged to one or another of the three groups. The greater the distance of a particular consumer from the centroid of a classification group, i.e. potential EAA source, the greater the probability mixing of EAA sources occurred. Given the distinct discrimination scores between the classification groups, we interpreted discriminant scores of consumers falling outside the 95% confidence limits of their food sources as strong indications of symbiotic EAA provisioning.

The predictive model was validated, based on the correct classification of bacteria (n = 11), fungi (n = 9) and plants (n = 12) to their respective groups ($F_{(8,54)} = 25$, $P < 0.0001$; Wilk's lambda= 0.04, a test of appropriateness of classifiers in predicting group membership of predictors)(Fig. 2). Gut microbial (predominantly bacterial, with minor fungal input) EAA provisioning in wood-feeding termites was evidenced from the predictive model analyses. Plant, bacteria, and fungi classifiers used in training the model (Table S1) were correctly classified by the model and showed distinct $\delta^{13}C$ clustering from each other in the discriminant plot (Fig. 2). Wood was not used as a classifier in the analysis, because we were interested whether it would be correctly classified with the plant classifier.

Four termite carcass samples and two termite gut filtrate samples had discriminant scores within the 95% confidence limit decision region of the bacteria classifier, indicative of bacterial EAA input (Fig. 2). One termite carcass sample was with the 95% confidence limit decision region of the fungal classifier, suggestive of fungal EAA input in that sample (Fig. 2). Three termite gut filtrate samples were outside of the 95%



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confidence limit decision region of the bacterial classifier, but were within the decision boundary of the bacterial classifier and thus indicative of bacterial EAA input (Fig. 2). The displacement of these termite gut filtrate samples is attributed to their $^{13}C$-depleted isoleucine values (Table S1). All wood samples fell within the 95% confidence limit decision region of the fungal classifier. The posterior probabilities associated with the model classifications are summarized for both the model classifiers and the termite and wood samples in Table S2.

## Discussion

The diverse gut microbiota of wood-feeding termites like *R. flavipes,* play several key important roles related to their nutritional ecology. Essential amino acid provisioning by these gut microbes have been proposed, but remain to be empirically determined. In this study, we sought to determine gut microbial EAA provisioning to termite hosts, by taking advantage of the natural variations in the $\delta^{13}C_{EAA}$ stable isotope signatures between plants, bacteria, and fungi. One premise of this approach is that termite $\delta^{13}C_{EAA}$ would closely resemble dietary $\delta^{13}C_{EAA}$ (wood) in the absence of microbial provisioning. While we did not determine a significant difference between termite carcass and dietary $\delta^{13}C_{EAA}$ ($\Delta\ \delta^{13}C = 1$) (Table 1), there were notable variations in the patterns of individual EAAs between termites and their diet (Fig. 1). The predictive model subsequently classified termite samples as bacterial (Fig. 2) and diet (wood) samples as mainly fungal, accounting for the individual differences in $\Delta\ \delta^{13}C_{EAA}$ discrimination. Frameworks for interpreting these results are provided below.



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Most wood-feeding lower termites are notably $^{13}C$-depletd compared to fungus feeding and soil-feeding termites (Tayasu 1998). Degradation of cellulose and lignocellulose by host and microbe-derived cellulolytic processes, liberates carbon from wood in the termite gut (Watanabe 2010). However, there are multiple microbial processes taking place in the termite gut besides cellulose degradation, including reductive acetogenesis (Brune and Friedrich 2000), which affects the $^{13}C$-signatures of carbon liberated due to cellulose degradation and the carbon finally incorporated into insect host tissues. The higher incidences of microbial acetogenesis relative to methanogenesis in wood-feeding lower termites, followed by absorption of the newly formed and $^{13}C$-depleted acetate is proposed to be responsible for the determined negative $^{13}C$-discrimination between wood-feeding lower termites and their woody diets (Bignell et al. 1995; Tayasu 1998). While the $\Delta\ \delta^{13}C_{EAA}$ between termite carcass and diet was not greater than the posited 1‰, the observed $\delta^{13}C_{EAA}$ depletion (-1‰) is similar to previously reported negative bulk ($\delta^{13}C$) $^{13}C$-offsets between termite and their wood diet based on bulk $\delta^{13}C$ data (Bignell et al. 1995; Tayasu 1998). Thus for termites, based on the diverse composition and functionalities of the gut microbiota, perhaps a $^{13}C$-offset of 1‰ may be sufficient to indicate non-dietary EAA input. Patterns of $^{13}C$-offsets varied between the wood diet and termite carcass and gut filtrate samples for the 5 EAAs quantified in this study, further providing evidence in support non-dietary input (Fig. 1).

Results from the predictive model validate the assertion of microbial EAA input to the termite host (Fig. 2). The most likely route of this microbial input is via proctodeal food transfer, a well-established feature of termite colonies (Nalepa et al. 2001). Proctodeal trophallaxis is an important component of the nutritional ecology of the



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termite colony, and is responsible for the close evolutionary and phylogenetic relationship between termites and their gut microbiota (Osamu and Kitade 2004). This is reflected in queen's reliance solely on proctodeal food transfers for nutrition, coupled with the loss of their gut-associated protists and lower gut microbial diversity (Shimada et al. 2013) and the rapid turnover/detection of labeled $^{13}$C-EAAs and $^{13}$C-metabolites in gut lumen of nestmates within 24 hrs of being fed $^{13}$C-cellulose diet (Tokuda 2014). Digestion of the microbial mass following proctodeal transfer is considered the most likely scenario, as opposed to the selective digestion and replacement of endogenous microbes in the midgut by the insect. Little is known about the presence, if any, of a resident midgut microbial community in lower termites that is adapted to withstand the digestive processes occurring in the midgut or is selectively/preferentially maintained by their host insects (Brune 2014).

Gut microbial EAA provisioning has been similarly observed in the gregarious omnivorous cockroach, *Periplaneta americana* (Ayayee PA, unpublished data), as well as a solitary wood-feeding cerambycid, *Anoplophora glabripennis* (Ayayee PA, unpublished data). In the absence of a eusocial structure in these two insects, coprophagy (the re-ingestion/consumption of previously deposited fecal matter) is the mode of delivery of microbes for digestion (Zimmer and Topp 2002). Re-ingestion and digestion of partially digested fecal material by nymphs may account for the observed microbial EAA input in *P. americana*, even though they were reared individually and not in groups (Ayayee PA, unpublished data). Similarly in *A. glabripennis,* coprophagy resulting from the ingestion of previously deposited frass (by larvae themselves or others) might account for the significant microbial input (Ayayee PA, unpublished data).



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The classification of wood samples as fungal in the predictive model (Fig. 2, Table S2) is most likely the result of fungal infestation and growth on the wood in the termite colony. Fungal growth quickly overtakes Jones' lab colonies when termite numbers are low or stressed. This is however noteworthy, since none of the termite samples were classified as fungal. The significance of this however potentially lies in the reliance on gut bacteria as opposed to fungi for nutritional supplementation in the lower wood-feeding termite *R. flavipes* (Brune 2014; Tokuda et al. 2014). It is however also likely that fungi growing on the wood may begin the cellulolytic process prior to termite ingestion, thus enhancing cellulose degradation (Hyodo et al. 2003).

## Conclusions

Overall, this study provides evidence in support of EAA provisioning by termite-associated gut bacteria. Our results additionally corroborate previous assertions of the importance of proctodeal trophallaxis as a vehicle for the acquisition of EAAs by termite colony nestmates. Our study provides a framework for understanding how gut microbial EAA provisioning proceeds mechanistically, and it illustrates the utility of the $\delta^{13}C$ fingerprinting approach in investigating mutualist/symbiotic systems.

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446 **Table 1.** Mean $\delta^{13}C_{EAA}$ and $\Delta\ \delta^{13}C$-offsets of between termite samples (carcass and gut
447 filtrate) and wood diet. Shown are mean values for 5 replicates per termite sample, 4
448 replicates for wood sample. Different letters represent a significant difference between
449 each group ($F_{(2, 63)} = 6.2$, $P < 0.004$).
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| Sample | $\delta^{13}C$ data (‰) | $\Delta\ \delta^{13}C$ |
|---|---|---|
| Termite carcass | -27.0 ± 0.43 (B) | -1.0 |
| Termite gut filtrate | -27.3 ± 0.58 (A) | -1.3 |
| Wood | -26.0 ± 0.48 (B) | 0 |

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**Figure 1.** $\delta^{13}$C-offset ($\Delta\ \delta^{13}C_{EAA}$) (enrichment or depletion) of 5 five essential amino acids (EAAs) in termite carcass (termites) and termite gut filtrate samples relative to the wood diet EAAs; $\Delta\ \delta^{13}C_{EAA}$ (‰) = ($\delta^{13}C_{Termite\ EAA} - \delta^{13}C_{Wood\ EAA}$). ($F_{(2, 63)} = 6.2$, $P <$ 0.004). Shown are mean values for 5 replicates per termite sample (termite and termite gut filtrate) and 4 replicates for wood diet. The EAAs were isoleucine (Ile), lysine (Lys), phenylalanine (Phe), and valine (Val). Error bars represent standard errors of the mean.

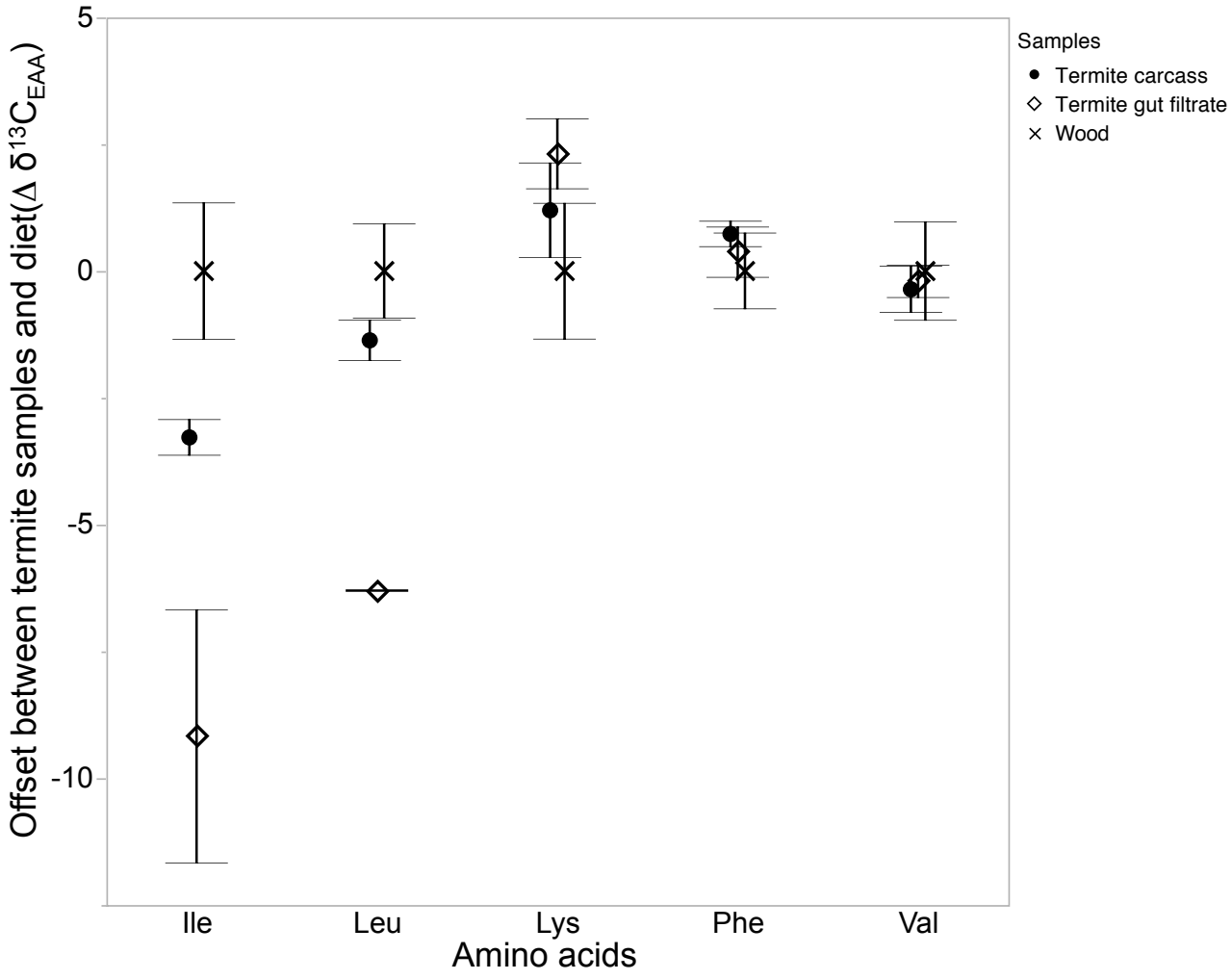


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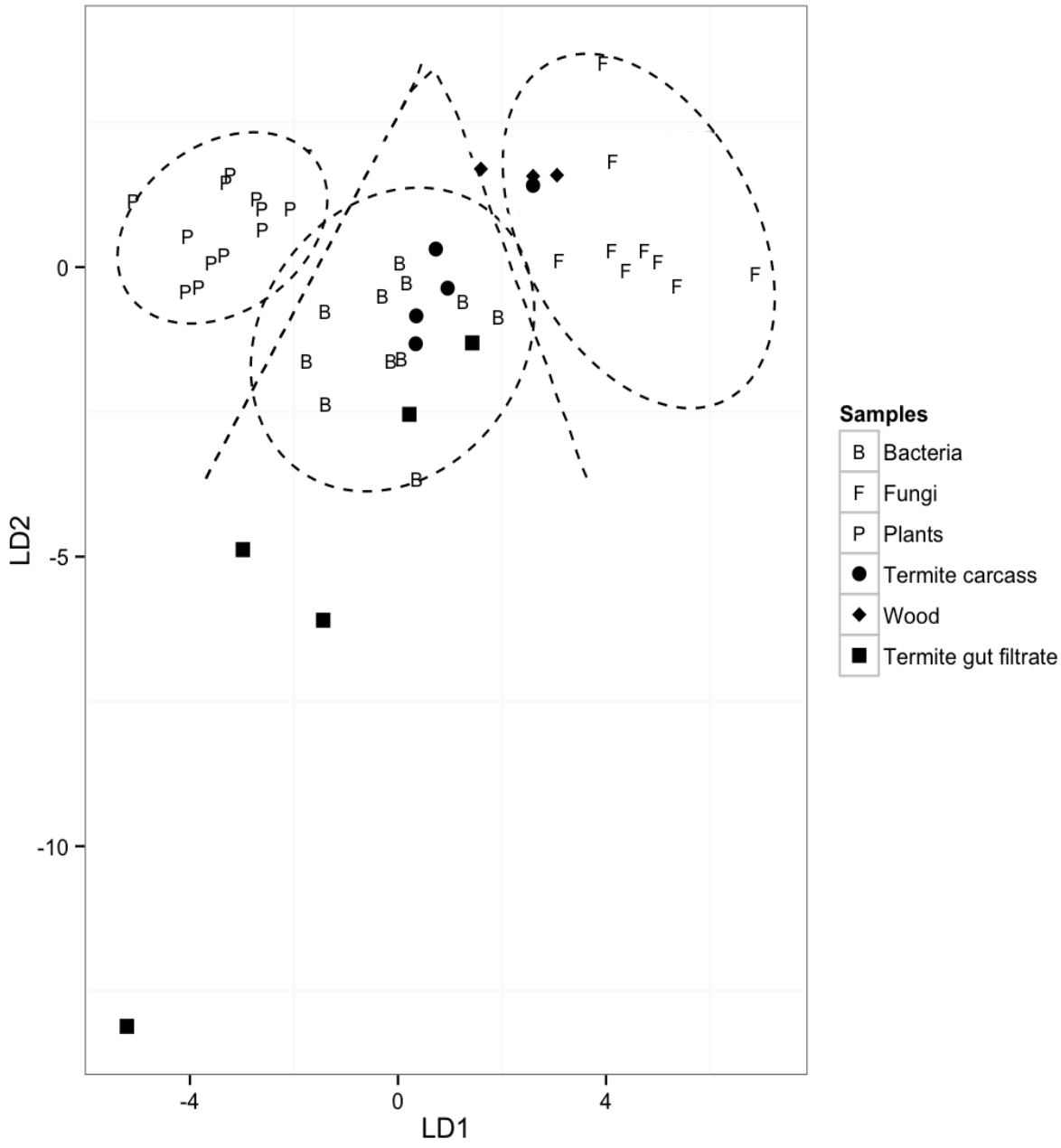
**Figure 2.** Predictive modeling (LDA) using $\delta^{13}C_{EAA}$ data based on three classifier groups [plants (n= 12), fungi (n= 9), and bacteria (n= 11)] and three predictor groups [termite carcass (n= 5), termite gut filtrate (n= 5), and wood diet (n= 3)] using the EAAs isoleucine (Ile), lysine (Lys), phenylalanine (Phe), and valine (Val). Wilks' lambda = 0.09, $P < 0.0001$; LD1 = 92.6%, LD2= 7.4%.

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**Supplemental material; Table S1**. Sample source and mean inter-lab calibrated essential amino acid values ($\delta^{13}C_{EAA}$) used in this study. Means are based on 2 technical replicates for each biological sample and 3 technical replicates for fungi, bacteria, and plant reference samples (Larsen et al. 2013).

| ID | Sample | Species | Category | LDA | Ile | Leu | Lys | Phe | Val |
|---|---|---|---|---|---|---|---|---|---|
| F1 | Fungi | *Ascomycota* | Fungi | Training set | -24 | -30.60 | -22.6 | -26.9 | -25.6 |
| F2 | Fungi | *Aureobasidium pullulans* | Fungi | Training set | -22.8 | -27.80 | -21 | -26.1 | -22.2 |
| F3 | Fungi | *Bionectria orhroleuca* | Fungi | Training set | -25.4 | -31.70 | -22.2 | -29.7 | -26.6 |
| F4 | Fungi | *Nectria vilior* | Fungi | Training set | -23.2 | -30.10 | -23.1 | -27.5 | -27.3 |
| F5 | Fungi | Unidentified | Fungi | Training set | -9.1 | -15.20 | -7.2 | -14.1 | -10.9 |
| F6 | Fungi | Unidentified | Fungi | Training set | -8.7 | -14.40 | -9 | -14.8 | -8.8 |
| F7 | Fungi | Unidentified | Fungi | Training set | -10 | -17.80 | -8.7 | -15 | -10.6 |
| F8 | Fungi | Mortierella alpi | Fungi | Training set | -6.6 | -13.00 | -5.5 | -12.9 | -9.2 |
| F9 | Fungi | Unidentified | Fungi | Training set | -9.8 | -17.30 | -7.9 | -14.8 | -12.1 |
| B1 | Bacteria | *Burkholderia xenovorans* | Bacteria | Training set | -12.5 | -13.40 | -4.9 | -18.3 | -14.1 |
| B10 | Bacteria | Bacteria G | Bacteria | Training set | -19.2 | -18.00 | -12.3 | -20.4 | -22 |
| B11 | Bacteria | Bacteria H | Bacteria | Training set | -19.1 | -19.60 | -13.1 | -21.5 | -22.7 |
| B12 | Bacteria | Bacteria J | Bacteria | Training set | -24.6 | -24.80 | -19.3 | -28.3 | -26.5 |
| B3 | Bacteria | *Klebsiella* sp. | Bacteria | Training set | -19.5 | -20.40 | -14.8 | -24.1 | -21.4 |
| B4 | Bacteria | *Rhodococcus* sp. | Bacteria | Training set | -24.3 | -25.70 | -15.7 | -28.5 | -26.9 |
| B5 | Bacteria | Bacteria B | Bacteria | Training set | -16.6 | -18.30 | -9.3 | -17.9 | -18.3 |
| B6 | Bacteria | Bacteria C | Bacteria | Training set | -17.6 | -19.70 | -9.8 | -19.7 | -21.6 |
| B7 | Bacteria | Bacteria D | Bacteria | Training set | -16.1 | -18.00 | -7.4 | -18 | -19.6 |
| B8 | Bacteria | Bacteria E | Bacteria | Training set | -16.6 | -16.40 | -9 | -17.3 | -18.2 |
| B9 | Bacteria | Bacteria F | Bacteria | Training set | -21.7 | -24.70 | -16 | -26.6 | -25.6 |
| T1 | Plants | *Quercus robur* | Plants | Training set | -29.7 | -38.90 | -20.1 | -32.1 | -37.7 |
| T10 | Plants | *Salix reticulata* | Plants | Training set | -25.7 | -35.10 | -17.7 | -26 | -31 |

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|---|---|---|---|---|---|---|---|---|---|
| T11 | Plants | *Eriophorum angustifolium* | Plants | Training set | -23.8 | -33.50 | -16.3 | -26.5 | -30.5 |
| T12 | Plants | *Rumex arcticus* | Plants | Training set | -28.2 | -37.70 | -20.1 | -29.5 | -34.7 |
| T2 | Plants | *Alnus glutinosa* | Plants | Training set | -31.3 | -40.20 | -22.4 | -33.4 | -36.6 |
| T3 | Plants | *Salix* sp. | Plants | Training set | -24.8 | -35.00 | -17 | -26.6 | -31.9 |
| T4 | Plants | *Polygonum viviparum* | Plants | Training set | -27.9 | -35.90 | -18.4 | -28.4 | -33.8 |
| T5 | Plants | *Carex aquatilis* | Plants | Training set | -27.3 | -34.10 | -17 | -27.2 | -32.1 |
| T6 | Plants | *Calamagrostis cadensis* | Plants | Training set | -27.7 | -35.00 | -19.5 | -28.4 | -32.6 |
| T7 | Plants | *Menganthes trifoliata* | Plants | Training set | -28 | -34.20 | -18 | -28.2 | -32.7 |
| T8 | Plants | *Betula* | Plants | Training set | -25.7 | -35.70 | -18.4 | -27.4 | -31.1 |
| T9 | Plants | *Carex utriculata* | Plants | Training set | -28.3 | -36.00 | -18.8 | -28.5 | -33.2 |
| T1 | Termite | Termite carcass | Termite carcass | Termite carcass | -26.59 | -29.69 | -19.7 | -26.84 | -27.4 |
| T2 | Termite | Termite carcass | Termite carcass | Termite carcass | -26.56 | -29.85 | -19.28 | -26.54 | -26.79 |
| T3 | Termite | Termite carcass | Termite carcass | Termite carcass | -27.62 | -31.20 | -21.72 | -27.25 | -28.92 |
| T4 | Termite | Termite carcass | Termite carcass | Termite carcass | -27.97 | -31.51 | -24.37 | -27.66 | -28.77 |
| T5 | Termite | Termite carcass | Termite carcass | Termite carcass | -26.11 | -29.72 | -20.04 | -26.25 | -26.89 |
| TG1 | Termite gut | Termite gut filtrate | Termite gut filtrate | Termite gut filtrate | -42.35 | *N/A | -18.93 | -25.37 | -27.08 |
| TG2 | Termite gut | Termite gut filtrate | Termite gut filtrate | Termite gut filtrate | -29.26 | *N/A | -20.8 | -28.16 | -27.68 |
| TG3 | Termite gut | Termite gut filtrate | Termite gut filtrate | Termite gut filtrate | -31.3 | -35.33 | -18.05 | -27.47 | -28.79 |
| TG4 | Termite gut | Termite gut filtrate | Termite gut filtrate | Termite gut filtrate | -32.96 | *N/A | -19.78 | -27.4 | -27.05 |
| TG5 | Termite gut | Termite gut filtrate | Termite gut filtrate | Termite gut filtrate | -28.49 | -32.16 | -21.98 | -27.92 | -27.41 |
| Wood 1A | Wood | Wood | Wood | Wood | -21.54 | -26.27 | -19.49 | -26.35 | -24.83 |
| Wood 1B | Wood | Wood | Wood | Wood | -27.45 | -30.26 | -24.46 | -29.78 | -29.53 |
| Wood 2B | Wood | Wood | Wood | Wood | -23.78 | -29.66 | -20.36 | -27.44 | -27.74 |

* N/A = not available

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484 **Supplemental material; Table S2.** Posterior probabilities of the classifier samples

485 (fungi, bacteria, and Plants) and experimental group samples used in the predictive model

486 plot in Fig. 2. (Wilks' lambda = 0.09, $P < 0.0001$).

| Samples | Actual | Bacteria | Fungi | Plants |
|---|---|---|---|---|
| Fungi | Fungi | 0 | 100 | 0 |
| Fungi | Fungi | 0 | 100 | 0 |
| Fungi | Fungi | 0.8 | 99.2 | 0 |
| Fungi | Fungi | 0 | 100 | 0 |
| Fungi | Fungi | 0 | 100 | 0 |
| Fungi | Fungi | 0 | 100 | 0 |
| Fungi | Fungi | 0 | 100 | 0 |
| Fungi | Fungi | 0 | 100 | 0 |
| Fungi | Fungi | 0.01 | 99.9 | 0 |
| Bacteria | Bacteria | 100 | 0 | 0 |
| Bacteria | Bacteria | 99.4 | 0 | 0.6 |
| Bacteria | Bacteria | 99.4 | 0 | 0.6 |
| Bacteria | Bacteria | 99.5 | 0.5 | 0 |
| Bacteria | Bacteria | 93.2 | 6.8 | 0 |
| Bacteria | Bacteria | 99.4 | 0 | 0.6 |
| Bacteria | Bacteria | 100 | 0 | 0 |
| Bacteria | Bacteria | 88.4 | 0 | 11.6 |
| Bacteria | Bacteria | 93 | 0 | 7 |
| Bacteria | Bacteria | 100 | 0 | 0 |
| Bacteria | Bacteria | 100 | 0 | 0 |
| Plants | Plants | 0.5 | 0 | 99.5 |
| Plants | Plants | 0.2 | 0 | 99.8 |
| Plants | Plants | 0 | 0 | 100 |
| Plants | Plants | 0.2 | 0 | 99.8 |
| Plants | Plants | 0 | 0 | 100 |
| Plants | Plants | 0 | 0 | 100 |
| Plants | Plants | 0 | 0 | 100 |
| Plants | Plants | 0 | 0 | 100 |
| Plants | Plants | 0.9 | 0 | 99.1 |
| Plants | Plants | 0.2 | 0 | 99.8 |
| Plants | Plants | 2.7 | 0 | 97.3 |
| Plants | Plants | 0.2 | 0 | 99.8 |
| **Samples** | **Predicted** | **Bacteria** | **Fungi** | **Plants** |
| Termite carcass | Bacteria | 100 | 0 | 0 |
| Termite carcass | Bacteria | 100 | 0 | 0 |
| Termite carcass | Bacteria | 99.7 | 0.3 | 0 |



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| | | | | |
|---|---|---|---|---|
| Termite carcass | Fungi | 0.8 | 99.2 | 0 |
| Termite carcass | Bacteria | 99.8 | 0.2 | 0 |
| Termite gut filtrate | Bacteria | 100 | 0 | 0 |
| Termite gut filtrate | Bacteria | 100 | 0 | 0 |
| Termite gut filtrate | Bacteria | 99.2 | 0 | 0.8 |
| Termite gut filtrate | Bacteria | 100 | 0 | 0 |
| Termite gut filtrate | Bacteria | 99.7 | 0.3 | 0 |
| Wood | Fungi | 0.06 | 99.94 | 0 |
| Wood | Fungi | 0.5 | 95 | 0 |
| Wood | Fungi | 35 | 65 | 0 |

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