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TESE DE DOUTORADO

EFEITOS DE LECTINAS DE *Myracrodruon urundeuva* NO TRATO INTESTINAL DE *Nasutitermes corniger*: ALTERAÇÕES ESTRUTURAIS, MODULAÇÃO DE ATIVIDADES ENZIMÁTICAS E IDENTIFICAÇÃO DE ALVOS DE LIGAÇÃO

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Tese apresentada para o cumprimento parcial das exigências para obtenção de título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

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A Diogo Vasconcellos por trazer a paz e transbordar de amor a minha vida..

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(Isaac Newton)

RESUMO

Lectinas, proteínas que interagem com carboidratos, isoladas da entrecasca (MuBL), cerne (MuHL) e folha (MuLL) de *Myracrodruon urundeuva* apresentaram atividade termiticida contra cupins operários da espécie *Nasutitermes corniger*. O presente trabalho teve como objetivo investigar mecanismos da ação termiticida dessas lectinas. Inicialmente, foi realizado um estudo de caracterização das atividades enzimáticas encontradas no trato digestivo de operários, de forma comparativa com os soldados da mesma espécie. Extratos de intestinos dos cupins foram avaliados quanto à presença de celulases (endoglucanases, exoglucanase e β-glicosidades), hemicelulases (β-xilosidases, α-L-arabinofuranosidase e β-D-xilanase), amilases e proteases (proteases totais, tripsina, quimotripsina e queratinase). A primeira avaliação dos mecanismos de ação correspondeu à determinação do efeito de MuBL e MuLL sobre as atividades enzimáticas detectadas no intestino dos operários. Na segunda etapa, foram investigadas possíveis proteínas que sejam alvos de ligação das lectinas no intestino dos operários. Para tanto, extratos de intestino foram submetidos à cromatografia em matrizes MuBL-Sepharose e MuLL-Sepharose. As proteínas que adsorveram à matriz foram submetidas à eletroforese e espectrometria de massas para separação e identificação. Na última etapa, operários foram submetidos por 48 h a dietas artificiais compostas por matriz de celulose e suplementadas ou não com MuBL, MuHL ou MuLL (em suas respectivas CL_{50}); em seguida, os insetos tiveram seu intestino médio dissecados, fixados e submetidos à análise histológica, contagem de células digestivas e regenerativas e visualização de células em proliferação, células enteroendócrinas, células em apoptose e da matriz peritrófica. Todas as atividades enzimáticas avaliadas foram detectadas nos extratos de operários e soldados, sendo endoglucanase e β-D-xilanase as principais atividades detectadas. As atividades enzimáticas de operários e soldados foram detectadas em níveis diferentes e apresentaram diferentes respostas a variações de temperatura e pH indicando que as castas possuem aparatos digestivos distintos. MuBL e MuLL foram capazes de modular diferentemente as atividades enzimáticas do trato digestivo dos operários. A atividade de exoglucanase foi neutralizada por MuBL enquanto MuLL promoveu aumento dessa atividade. Atividade de α-L-arabinofuranosidase foi inibida por MuLL e não afetada por MuBL. Ambas as lectinas estimularam a atividade de α-amilase e inibiram as atividades de proteases. As matrizes MuBL-Sepharose e MuLL-Sepharose ligaram a proteína apolipoforina. MuBL-Sepharose também ligou proteína com homologia a transportadores do tipo ABC (*ATP-binding cassette*) e tripsina. MuBL, MuHL e MuLL causaram forte desorganização no epitélio do intestino médio dos operários, reduzindo o número de células digestivas, regenerativas e enteroendócrinas. A ingestão das lectinas resultou em morte das células intestinais por apoptose. A matriz peritrófica foi visualizada nos tratamentos controle e com as lectinas, contudo a marcação foi menos intensa em intestinos de cupins que ingeriram MuBL. Os resultados mostram que as lectinas foram capazes de cruzar essa matriz peritrófica e causar danos às células do epitélio intestinal. Em conclusão, podemos afirmar que: 1) operários e soldados de *N. corniger* apresentam aparatos digestivos diferenciados, com elevada eficiência para digerir biomassa celulósica; 2) a ação termiticida das lectinas MuBL e MuLL pode estar relacionada com a modulação de processos digestivos e absortivos no trato intestinal de operários, tendo como alvos enzimas digestivas, a proteína transportadora apolipoforina e transportadores ABC; 3) MuBL, MuHL e MuLL atuam promovendo desestruturação do epitélio intestinal de operários, causando morte de células digestivas, regenerativas e enteroendócrinas. Esses resultados constituem os primeiros relatos sobre alvos moleculares e celulares de lectinas termiticidas.

Palavras-chave: cupins. apolipoforina. intestino médio. enzimas digestivas. inseticida.

ABSTRACT

Lectins, proteins that interact with carbohydrates, isolated from the bark (MuBL), heartwood (MuHL) and leaf (MuLL) of *Myracrodruon urundeuva* showed termiticidal activity against workers of *Nasutitermes corniger* termite. This study aimed to investigate mechanisms of the termiticidal action of these lectins. Initially, a study was conducted in order to characterize the enzyme activities found in the digestive tract of workers, in comparison with the soldiers of the same species. Termite gut extracts were assessed for the presence of cellulases (endoglucanase, exoglucanase, and β-glucosidase), hemicellulases (β-xylosidase, α-L-arabinofuranosidase and β-D-xylanase), amylases and proteases (total protease, trypsin, chymotrypsin and keratinase). The first evaluation of the action mechanisms corresponded to the determination of MuBL and MuLL effects on the enzyme activities detected in the gut of the workers. In the second step, we investigated possible proteins that would be lectin binding targets in the gut of workers. Therefore, worker gut extracts were subjected to chromatographies on MuBL-Sepharose and MuLL-Sepharose matrices. Proteins that adsorbed to the matrices were submitted to electrophoresis and mass spectrometry for separation and identification. In the last step, workers were submitted for 48 h to artificial diets composed of cellulose matrix and supplemented or not with MuBL, MuHL or MuLL (at their respective LC_{50}); then, the insects had their midguts dissected, fixed and evaluated by histological analysis, counting of digestive and regenerative cells, and visualization of proliferative, enteroendocrine and apoptotic cells, as well as of peritrophic matrix. All the enzyme activities evaluated were detected in worker and soldier extracts, being endoglucanase and β-D-xylanase the main activities detected. The enzyme activities of workers and soldiers were detected at different levels and showed different responses to temperature and pH, indicating that these castes have different digestive apparatuses. MuBL and MuLL were able to modulate differently the enzyme activities from the digestive tract of workers. MuBL neutralized the exoglucanase activity while MuLL promoted increase in this activity. α-L-arabinofuranosidase activity was inhibited by MuLL and was not affected by MuBL. Both lectins stimulated the α-amylase activity and inhibit the activity of proteases. The MuBL-Sepharose and MuLL-Sepharose matrices bound the protein apolipophorin. MuBL-Sepharose also bound proteins with homology to ABC (ATP-binding cassette) transporters and trypsin. MuBL, MuHL and MuLL caused strong disorganization in the midgut epithelium of workers, reducing the number of digestive, regenerative and enteroendocrine cells. Ingestion of lectins resulted in death of intestinal cells by apoptosis. The peritrophic matrix was visualized in the control and lectin treatments but the labeling was less intense in gut of termites that ingested MuBL. These results show that the lectins were able to cross this peritrophic matrix and cause damage to the cells of the midgut epithelium. In conclusion, we can state that: 1) *N. corniger* workers and soldiers have different digestive apparatus, with high efficiency to digest cellulosic biomass; 2) the termiticidal action of MuBL and MuLL can be linked to modulation of digestive and absorptive processes in the intestinal tract of workers, targeting digestive enzymes, the transporter protein apolipophorin and ABC transporters; 3) MuBL, MuHL and MuLL promoted disruption of the midgut epithelium of workers, causing death of digestive, regenerative and enteroendocrine cells. These results are the first reports on molecular and cellular targets of termiticidal lectins.

Keywords: termites. apolipophorin. midgut. digestive enzymes. inseticide.

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1. INTRODUÇÃO

Lectinas são proteínas que têm a capacidade de interagir com glicoconjugados, inclusive aqueles ligados a superfícies das células (SANTOS *et al*., 2013). O potencial biotecnológico das lectinas como inseticidas naturais tem sido estudado e os efeitos entomotóxicos dessas proteínas têm sido reportados contra vários insetos-praga e vetores de doenças das ordens Coleoptera, Diptera, Homoptera, Lepidoptera e Isoptera, dentre outras. A capacidade de interferir na absorção dos alimentos, desenvolvimento e sobrevivência de insetos e os mecanismos de ação envolvidos nesses efeitos estão sob investigação (PAIVA *et al*., 2011, 2013).

Tem sido descrito que a estabilidade das lectinas inseticidas no ambiente hostil do intestino dos insetos, sendo resistentes à proteólise, é considerada um pré-requisito para o efeito inseticida. As lectinas podem interagir com a matriz peritrófica e glicoconjugados do trato intestinal, afetanto a absorção dos nutrientes e vias de sinalização e transporte (FITCHES *et al*., 1998; VANDENBORRE *et al*., 2011; WALSKI *et al*., 2014; OLIVEIRA *et al*., 2015). O metabolismo do inseto também pode ser desestabilizado pela interação das lectinas com enzimas digestivas e proteínas transportadoras (ALBUQUERQUE *et al*., 2012; AGRA-NETO *et al*., 2014; OLIVEIRA *et al*., 2015). Alguns estudos descreveram os efeitos da ingestão de lectinas na organização do intestino médio, na expressão de genes associados com as proteínas do citoesqueleto, metabolismo da quitina, reações de detoxificação e metabolismo energético (LI *et al*., 2009, VANDENBORRE *et al*., 2011, CACCIA *et al*., 2012).

Lectinas de plantas têm sido reportadas com agentes termiticidas contra *Nasutitermes corniger* (SÁ *et al*., 2008; PAIVA *et al*., 2011b; SOUZA *et al*., 2011; ALBUQUERQUE *et*

al., 2012; ARAÚJO *et al*., 2012). As lectinas ligadoras de quitina isoladas da casca (MuBL), cerne (MuHL) e folha (MuLL) de *Myracroduon urundeuva*, uma árvore com madeira-de-lei, foram capazes de induzir a mortalidade de operários e soldados de *Nasutitermes corniger* (SÁ *et al*., 2008; NAPOLEÃO *et al*., 2011). Estas lectinas foram resistentes à incubação com as proteases intestinais de *N. corniger* e são capazes ainda de interferir no crescimento e na sobrevivência de bactérias simbiontes do intestino desse inseto (NAPOLEÃO *et al*., 2011).

O presente trabalho teve como objetivo ampliar o conhecimento sobre os mecanismos da ação termiticida das lectinas de *M. urundeuva* contra *N. corniger*, através de estudos que visaram analisar os possíveis efeitos das lectinas no epitélio do intestino médio dos operários, na atividade das enzimas digestivas, assim como apontar alvos de ligação dessas lectinas no lúmen intestinal. Uma vez que não existem informações sobre as características do aparato enzimático dessa espécie de cupins, foi também realizado um estudo de caracterização das atividades enzimáticas encontradas no trato digestivo de operários e soldados.

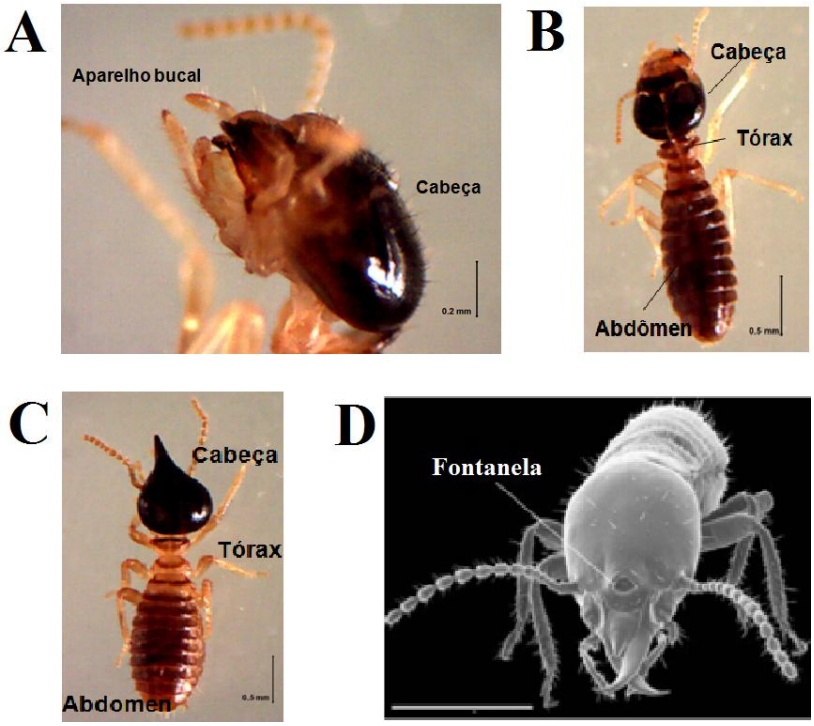
2. FUNDAMENTAÇÃO TEÓRICA

2.1. Cupins ou térmitas

A classe Insecta é a mais bem sucedida do Reino Animal, com mais de 1 milhão de espécies descritas, distribuídas em 30 ordens (GULLAN, 2007). Na Ordem Isoptera, encontram-se insetos conhecidos como cupins ou térmitas, que possuem um aparelho bucal bem desenvolvido do tipo mastigador (Figura 1A) e cabeça livre com formas e tamanhos variáveis (Figura 1B e 1C). Olhos compostos estão presentes nas formas aladas e nos cupins superiores. Na cabeça, há uma depressão chamada fontanela (Figura 1D), que possui um poro central no qual se abre uma glândula cefálica que secreta um líquido com função de defesa. O tórax é achatado, com o protórax destacado. O órgão auditivo está situado nas pernas, na tíbia anterior. O abdome é volumoso, séssil e apresenta 10 segmentos (Figura 1B e 1C). Os cupins possuem dois pares de asas membranosas (GRASSÉ, 1949; GALLO *et al.*, 1988).

Evidências obtidas a partir da análise de marcadores moleculares e morfológicos sugerem que os cupins estão aninhados dentro do grupo Blattodea, sendo provavelmente um grupo irmão do Cryptocercidae, que inclui baratas especializadas em se alimentar de madeira. Acredita-se que os cupins são os insetos eusociais mais antigos na escala evolutiva, pois suas complexas sociedades remontam ao Cretáceo (130 milhoes de anos atrás). O fóssil de cupins mais antigo mostra claramente que eles já eram insetos eusociais incrivelmente similares às espécies modernas desde o Jurássico superior (LO *et al.*, 2000; THORNE *et al.*, 2000; GRIMALDI & ENGEL, 2005; MEYER, 2005).

Figura 1 - Aspectos morfológicos dos cupins. As imagens mostram o aparelho bucal do tipo mastigador (A), uma visão dorsal de operário (B) e soldado (C) da espécie *Nasutitermes corniger* e a fontanela de um *Coptotermes formosanus* (D).



As barras correspondem a 0,2 mm em (A), 0,5 mm (B e C) ou 1 mm (D).

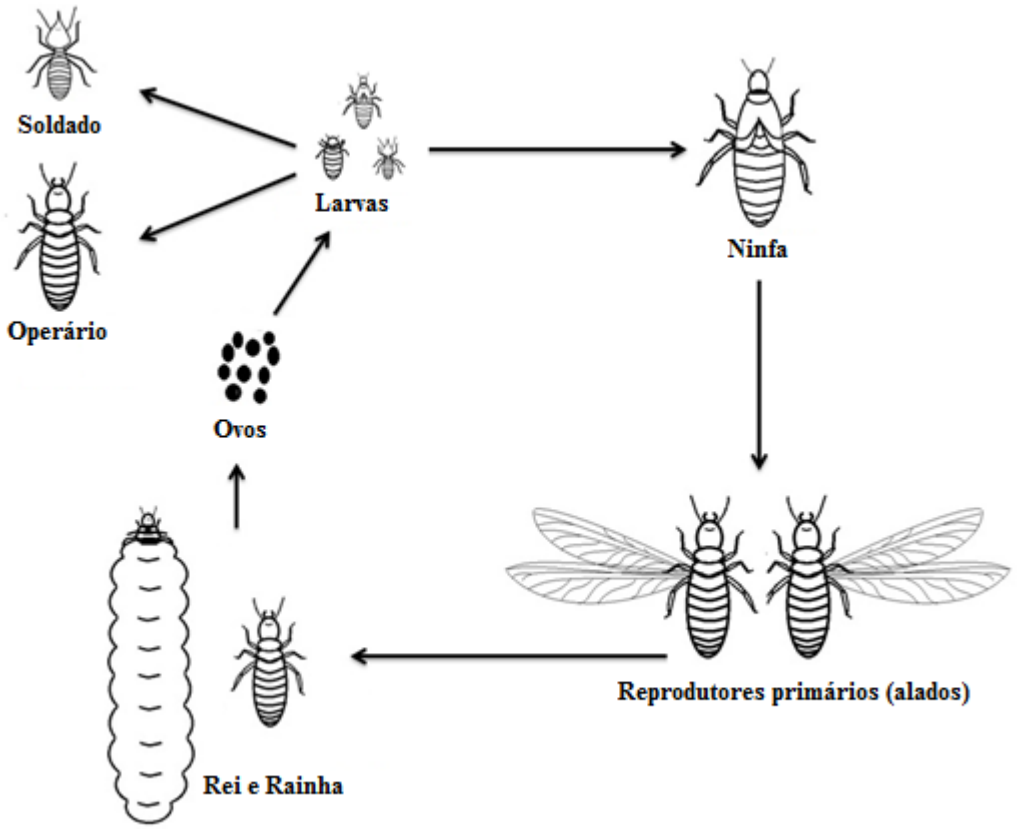
Fonte: A, B, C: fotos da autora. D: Rudolf H. Scheffrahn, University of Florida

A infraordem Isoptera compreende sete famílias: Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae, Serritermitidae e Termitidae, as quais estão subdivididas em quatorze subfamílias. As três primeiras famílias são geralmente chamadas cupins inferiores ou primitivos e as últimas quatro famílias são referidas como cupins superiores (GRASSÉ, 1986; KAMBHAMPATI & EGGLETON, 2000). Engel *et al.* (2009) também listaram as famílias Cratomastotermitidae, Archotermopsidae, Stolotermitidae, Archeorhinotermitidae e Stylotermitidae, em adição às famílias mencionadas acima. Esses autores separaram os cupins em dois grupos: Euisoptera, incluindo as famílias mais próximas

às baratas que se alimentam de madeira, e Neoisoptera, no qual incluem as famílias Rhinotermitidae, Serritermitidae, Archeorhinotermitidae, Stylotermitidae e Termitidae.

Os cupins são divididos em castas morfofisiologicamente distintas: reprodutores alados, operários, soldados e formas imaturas (larvas). As larvas podem substituir soldados, operários e ninfas; estas últimas irão originar os reprodutores alados. Os cupins são diplóides e ambos os sexos estão presentes nas castas estéreis. Eles exibem desenvolvimento hemimetabólico, o qual inclui três fases distintas: o ovo, a ninfa, e o estágio adulto, envolvendo alterações graduais, semum estágio de pupa (Figura 2). As formas reprodutivas representam a casta fértil e podem ser subdivididas em reprodutores primários ou complementares. Os reprodutores primários têm asas bem desenvolvidas e são responsáveis pela dispersão através de vôos nupciais. Após o vôo, o casal perde suas asas e passam a ser rei e rainha, os quais são pigmentados e são responsáveis pela produção de ovos. Em muitas colônias, só existe um casal de reprodutores primários. Se os reprodutores primários morrem, eles serão substituídos por um casal de reprodutores complementares, os quais em geral são mais pigmentados do que os operários. As castas estéreis (operários e soldados) não possuem asas e olhos. Os operários são responsáveis pelo forrageio e construção dos túneis e galerias do ninho; também são responsáveis por alimentar outros membros da colônia. Os soldados se destacam por possuírem a cabeça preta, alargada, com quase metade do comprimento do corpo (Figura 1B) e com o maxilar bem perceptível. São responsáveis pela guarda da colônia e proteção de seus ocupantes (KRISHNA & WEESNER, 1969; THOMPSON, 2000; PHILIP, 2004; MYLES, 2005).

Figura 2. Ciclo de vida dos cupins e diferentes castas encontradas em um cupinzeiro.



Fonte: Lima *et al.* (2015)

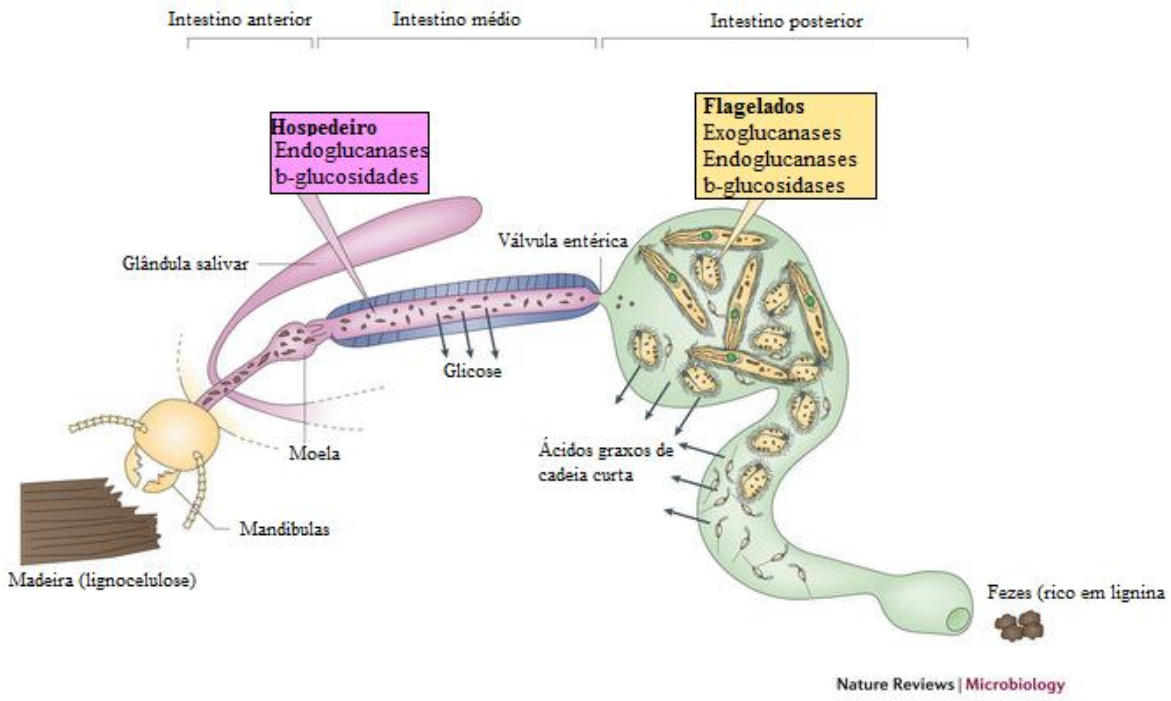
Os cupins são chamados xilófagos porque, geralmente, são consumidores de madeira, viva ou morta. Porém, uma grande diversidade de material orgânico, em vários estágios de decomposição, pode servir de alimento para esses insetos, incluindo gramíneas, plantas herbáceas, serrapilheira, fungos, ninhos construídos por outras espécies de cupins, excrementos e carcaças de animais, liquens e até mesmo material orgânico presente no solo (LIMA & COSTA-LEONARDO, 2007).

A chave evolutiva e os elementos ecológicos da biologia dos cupins, tais como o tamanho da colônia, a história de vida, o desenvolvimento de castas e os habitats (ninhos), estão associados à disponibilidade de recursos alimentares (ABE, 1987; LENZ, 1994). Se

comparados com os consumidores secundários e terciários, os cupins exploram alimentos com baixo valor nutricional, tendo desenvolvido mecanismos digestivos apropriados para extrair o máximo de nutrientes que são necessários a sua sobrevivência. Os cupins trituram os materiais celulósicos ou lignocelulósicos, mas a maior parte da digestão da celulose ocorre pela ação de enzimas que podem ser produzidas pelo próprio inseto ou por microrganismos simbiontes presentes no intestino posterior (WALLER & LA FAGE, 1986). Alguns membros de Nasutiterminae (Termitidae) são especializados na alimentação a base de húmus, carboidratos, micróbios do solo, peptídeos hidrolisados, compostos polifenólicos, e hexosaminas derivadas de restos de artrópodes e fungos mortos (COLLINS, 1983; ANDERSON & WOOD, 1984; BIGNELL & EGGLETON, 2000; JI & BRUNE, 2005).

O sistema digestivo dos isópteros é considerado desenvolvido e geralmente ocupa uma ampla parte de seu abdome (KRISHNA & WEESNER, 1969), sendo composto por três partes: intestino anterior (estomodeum), intestino médio (mesenteron) e intestino posterior (proctodeum) (Figura 3). Os indivíduos imaturos, os soldados e todos os reprodutores são incapazes de se alimentar sozinhos e recebem alimentação estomodeal ou proctodeal dos operários. A alimentação estomodeal pode ser saliva, que é a única fonte de alimento dos reprodutores primários (rei e rainha), ou alimento regurgitado. Os soldados são, em grande parte, nutridos com alimento regurgitado, mas em algumas espécies da Família Termitidae eles têm uma dieta exclusivamente líquida (saliva). A alimentação proctodeal foi descrita para os cupins inferiores e consiste de excreções líquidas, ricas em simbiontes, provenientes do intestino posterior e que são eliminadas em resposta a estímulos táteis de outros cupins (LIMA & COSTA-LEONARDO, 2007).

Figura 3 – Representação esquemática da divisão do trato digestivo de cupins e de eventos que ocorrem em cada região.



Fonte: Brune (2014).

Os protozoários flagelados e as bactérias simbiontes presentes no intestino posterior (Figura 3) colaboram na digestão da celulose através da produção deenzimas (BRESNAK & BRUNE, 1994). As celulases encontradas no trato intestinal de cupins são endoglucanases, exoglucanases e β-glicosidases. As hemicelulases são produzidas pelos cupins com o objetivo de digerir a fração de hemicelulose do seu alimento e a secreção de proteases no intestino médio permite que os aminoácidos das proteínas provenientes da alimentação sejam absorvidos (BRUNE, 2014). A microbiota do intestino do cupim permite a conversão dos carboidratos das fibras da madeira em ácidos graxos de cadeia curta (Figura 3). A digestão da lignina pelos cupins ainda não é clara, mas existem evidências da ação de algumas enzimas.

Após a digestão, resíduos de lignina são eliminados nas fezes, mas também podem ser passados para os outros integrantes do ninho através da alimentação proctodeal.

2.2. Enzimas digestivas dos cupins

2.2.1. Celulases

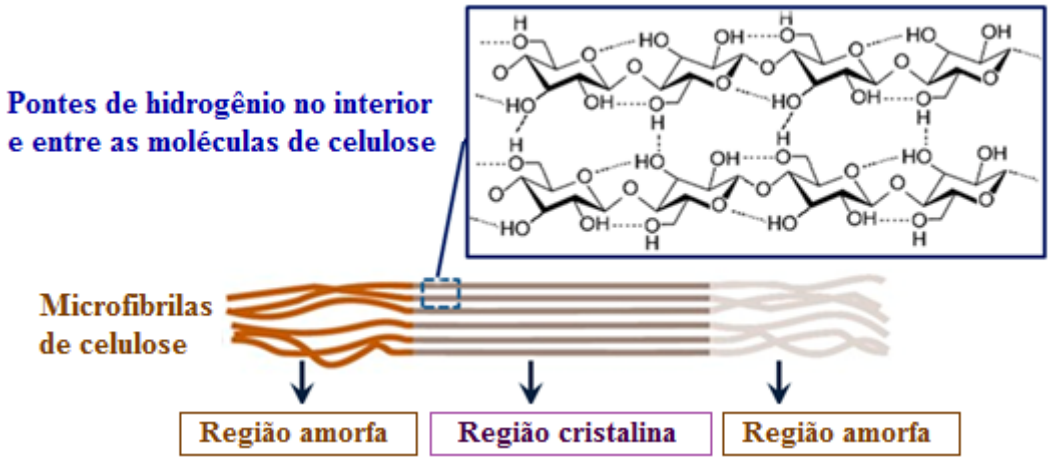
Os carboidratos são nutrientes essenciais que fornecem a energia necessária tanto para o ótimo crescimento larval como para a manutenção da longevidade dos adultos na maioria dos insetos. Porém, o valor nutritivo dos carboidratos depende da disponibilidade de enzimas capazes de digerir carboidratos complexos a monômeros adequados para sua posterior absorção no intestino (DADD, 1985; TERRA *et al.*, 1996).

A celulose é um polímero linear formado exclusivamente por moléculas de D-glicopiranose unidas por ligações glicosídicas β1→4 (DE AZEVEDO & ESPÓSITO, 2004). As moléculas de celulose são orientadas ao acaso e têm a tendência de formar ligações de hidrogênio inter e intramoleculares (ROWELL *et al*., 2005). Os modelos atuais da organização microfibrilar sugerem que ela tem uma subestrutura constituída de domínios altamente cristalinos unidos por ligações amorfas (Figura 4). Regiões cristalinas são formadas a partir do aumento da densidade do empacotamento (TAIZ & ZEIGER, 2004).

As celulases são enzimas responsáveis pela degradação de celulose ou outros celo-oligossacarídeos até glicose. São conhecidos três tipos: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) e β-glucosidases (EC 3.2.1.21). A habilidade de digerir a celulose, através da ação de enzimas celulolíticas, confere aos cupins um importante papel ecológico na degradação e reciclagem de nutrientes. Porém, esta mesma habilidade está ligada

ao fato de que algumas espécies de cupins são pragas que podem causar danos aos mais diversos tipos de materiais e construções.

Figura 4 – Organização das moléculas de celulose nas microfibrilas.



Fonte: Zhou & Wu (2012).

A digestão da celulose começa pela ação de endoglucanases secretadas pelas glândulas salivares ou pelo intestino médio. Protozoários flagelados estão presentes principalmente em cupins inferiores epertencem às classes Parabasalia (gêneros *Trichonympha*, *Calonympha*, e *Tricercomitus*) e Preaxostyla (gênero *Pyrsonympha*). As bactérias simbiontes são principalmente espiroquetas (filo Spirochaetes), as quais têm uma marcante mobilidade e estão presentes em alta abundância e diversidade no intestino posterior da maioria dos cupins que se alimentam de madeira. A maioria dos cupins emite substâncias de metano produzidas por arqueobactérias simbiontes (BRUNE, 2014). Análises metagenômica e funcional da microbiota associada com o intestino de uma espécie Termitidae mostraram que existe uma gama muito diversificada de bactérias, tais como espiroquetas e fibrobactérias, que possuem

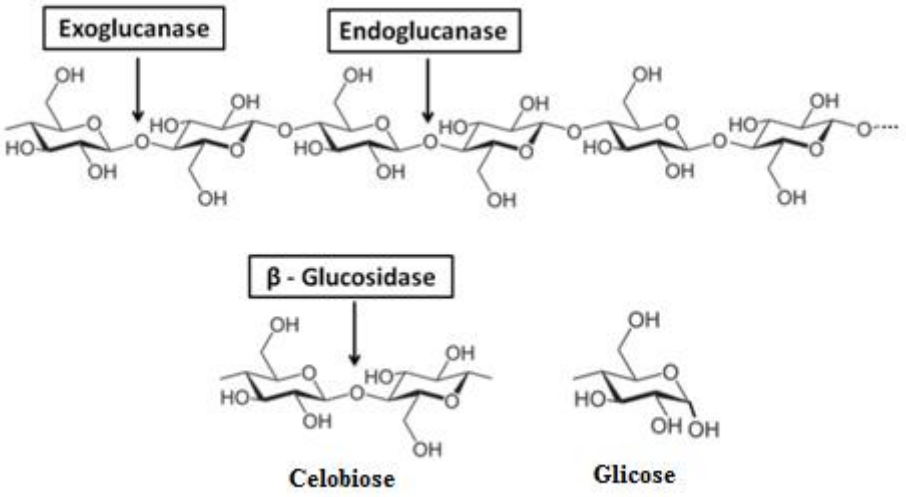
genes relacionados com a degradação da lignocelulose e envolvidos na hidrólise da celulose e xilana (WARNECKE *et al.*, 2007).

Estudos relataram que cupins inferiores *Neotermes koshunensis* produzem celulase em suas glândulas salivares (TOKUDA *et al.*, 2002). Em cupins superiores, atividade celulolítica tem sido detectada no intestino médio e glândulas salivares (ROULAND *et al.*, 1989; SLAYTOR, 2000). *Nasutitermes exitiosus* é capaz de secretar suas próprias celulases, não sendo dependente da sua flora intestinal para digerir celulose (O'BRIEN *et al*., 1979). Entretanto, estudos têm demonstrado que o aparato celulolítico de outras espécies de *Nasutitermes* é formado por celulases endógenas e também de origem simbiótica. A atividade celulolítica no intestino posterior de *Nasutitermes takasagoensis* foi reduzida significativamente após tratamento com antibiótico, sugerindo produção de celulases pelas bactérias simbiontes (TOKUDA & WATANABE, 2007). O envolvimento da atividade microbiana na degradação de celulose em *N. takasagoensis* pode ser atribuído à presença de complexos celulolíticos denominados "celulossomos", que se encontram ancorados à parede celular bacteriana (TOKUDA *et al*., 2005). Estudos anteriores detectaram a expressão de RNAs mensageiros de β-glicosidases no epitélio do intestino médio de *N. takasagoensis* (TOKUDA *et al*., 1999). Assim, o aparato celulolítico das espécies de *Nasutitermes* é formado por enzimas de origem endógena e exógena.

As endoglucanases clivam a cadeia de celulose no interior da molécula de celulose (Figura 5), de forma aleatória, criando novas extremidades (SÁNCHEZ, 2009). Estas enzimas hidrolisam a celulose amorfa e celuloses modificadas quimicamente (solúveis), como carboximetilcelulose (CMC) e hidroxietilcelulose (HEC). As regiões amorfas, com menor organização estrutural, são mais facilmente atacadas, pois suas cadeias não possuem ligações intermoleculares de hidrogênio tão fortes quanto as que ocorrem nas regiões cristalinas.

Assim, o ataque das endoglucanases leva a uma maior exposição das ligações glicosídicas mais internas das cadeias de celulose. A celulose cristalina e o algodão, substratos com elevado grau de cristalinidade, são menos hidrolisados devido ao maior grau de organização molecular que apresentam (MEDVE, 1997; ZANDONÁ FILHO, 2001).

Figura 5. Representação esquemática dos sítios de clivagem na molécula de celulose pelas endoglucanases, exoglucanases e β-glucosidases.



Fonte: Lima *et al*. (2015).

Exoglucanases atuam nas extremidades da cadeia de celulose (Figura 5), produzindo majoritariamente celobiose (dissacarídeo composto por duas moléculas de glicose), além de glicose e celotriose (ZANDONÁ-FILHO, 2001). O sítio ativo das exoglucanases possui a forma de um túnel por onde a cadeia de celulose penetra e sofre hidrólise de suas ligações glicosídicas terminais (HUI *et al.*, 2002). As exoglucanases atuam sobre celulose cristalina (Avicel), produzindo uma redução lenta e gradual do seu grau de polimerização.

As β-glicosidases, também denominadas celobiases, possuem a função de hidrolisar a celobiose gerada pelas exoglucanases e endoglucanases em glicose, completando a hidrólise da celulose (Figura 5). As β-glicosidases são extremamente importantes para a eficiência da hidrólise da celulose por removerem do meio reacional a celobiose, que é um potente inibidor competitivo das exoglucanases (MEDVE, 1997; MUÑOZ *et al*., 2001).

2.2.2. Hemicelulases

A parede celular dos vegetais é composta de microfibrilas de celulose que estão inseridas em uma matriz de polissacarídeos composta de hemiceluloses, pectina e lignina (TAIZ & ZEIGER, 2004; SÁNCHEZ & CARDONA, 2008). As hemiceluloses são uma mistura de polímeros de pentoses e hexoses (como xilose, arabinose, glicose, manose e galactose), bem como ácidos urônicos, que fazem pontes de hidrogênio com a celulose envolvendo-a em uma rede, juntamente com a pectina. Esse recobrimento impede que as fibras paralelas de celulose colapsem entre si (BUCKERIDGE *et al.*, 2008; ROWELL, *et al*., 2005).

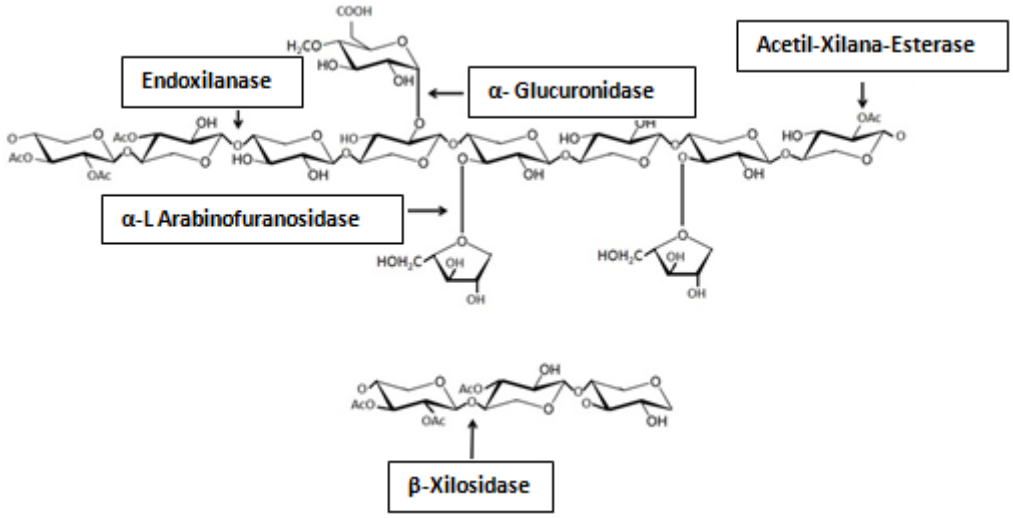
Além da celulose, os cupins são capazes de digerir a fração hemicelulósica presente em seus alimentos. As hemicelulases são enzimas que hidrolisam a hemicelulose de maneira aleatória, produzindo oligossacarídeos lineares e ramificados. Devido à grande complexidade e heterogeneidade da rede dehemicelulose, a sua hidrólise completa requer a atuação de várias enzimas (Figura 6) que atuam cooperativamente (SAHA, 2000; MOREIRA & FILHO, 2008):

- As endo-β-1,4-D-xilanases rompem ligações glicosídicas ao acaso produzindo grandes quantidades de xilo-oligossacarídeos substituídos e não-substituídos de diversos tamanhos.

- As exo-β-1,4-D-xilanases rompem somente unidades de xilose a partir das extremidades da cadeia de xilana.
- As β-xilosidases hidrolisam sacarídeos como xilobiose e xilo-oligossacarídeos maiores até xilose.
- As α-L-arabinofuranosidases hidrolisam terminais não-redutores de polissacarídeos contendo resíduos de L-arabinosil de cadeias laterais ou de arabinoxilanas, arabinana, goma arábica e arabinogalactana.
- As manosidases, mananases e outras enzimas degradam polímeros como arabinogalactana e galactoglicomanana.
- As α-D-glicuronidases hidrolisam resíduos laterais de ácido glicurônico.
- As esterases de acetilxilana hidrolisam grupos acetil e as esterases de ácido ferúlico que hidrolisam resíduos de ácido ferúlico.

Rouland *et al.* (1988) isolaram duas β-D-xilanases de cupins operários da espécie *Macrotermes mülleri* (Termitidae, Macrotermitinae), uma a partir de extrato bruto do intestino do inseto e outra a partir de extrato bruto de somente seu microorganismo simbionte, o fungo *Termitomyces sp.*

Figura 6. Representação esquemática da molécula de hemicelulose e os respectivos sítios de clivagem das hemicelulases.



Fonte: Lima *et al*. (2015).

2.2.3. α-Amilases

As α-amilases ou α-1,4-glucano-4-glucanohidrolases são um grupo de enzimas monoméricas com a função de catalisar a hidrólise de ligações glicosídicas α1→4 presentes no amido, glicogênio e outros carboidratos. As amilases são essenciais para o crescimento e o desenvolvimento de muitos insetos; várias isoformas dessas enzimas são encontradas especialmente em insetos-praga que vivem em estoques de sementes e grãos ricos em amido.

As α-amilases são metaloproteínas que contém pelo menos um átomo de cálcio por molécula e precisam deste metal para manter sua atividade e estabilidade (FISCHER & STEIN, 1960). Muitas delas são proteínas glicosiladas, mas a proporção de glicosilação em sua estrutura varia consideravelmente (PLANCHOT & COLONNA, 1994). A presença de α-amilases tem sido demonstrada no sistema digestivo de muitos insetos, incluindo membros

das ordens Orthoptera, Hymenoptera, Diptera, Lepidoptera e Coleoptera (TERRA & FERREIRA, 1994). Porém, existem poucos estudos de atividade amilolítica em cupins. Park et al. (2014) identificaram uma sequência completa de amilase em *Reticulitermes speratus* através da análise de RNAm. Tarayre *et al.* (2014) isolaram uma cepa de *Streptomyces* sp. do intestino de *Reticulitermes santonensis* a qual tem a habilidade de produzir amilase.

2.2.4. Proteases

As proteases são enzimas que catalisam a hidrólise de ligações peptídicas e podem ser classificadas de acordo com a região da molécula sobre a qual atuam, sendo denominadas exopeptidases quando hidrolisam ligações peptídicas nas regiões N-terminal (aminopeptidases) ou C-terminal (carboxipeptidases), ou endopeptidases (ou proteinases) quando catalisam a hidrólise de ligações peptídicas internas (FRANCO *et al.*, 1999; SILVA JÚNIOR & DE SIMONE, 2001; DEVLIN, 2002). As proteases podem ser classificadas, de acordo com a União Internacional de Bioquímica, em quatro grandes classes: serinoproteases, cisteinoproteases, asparticoproteases e metaloproteases. As três primeiras classes são definidas de acordo com o resíduo nucleofílico presente no sítio ativo que hidrolisa a ligação peptídica do substrato (HEDSTROM, 2002). Já as metaloproteases são assim denominadas porque seu mecanismo catalítico envolve a participação de um íon metálico (KLEINER & STETLER-STEVENSON, 1993).

Para crescerem e se desenvolverem, os insetos requerem os mesmos aminoácidos essenciais que os mamíferos. Para obtê-los, eles se alimentam dos mais diversos compostos orgânicos, tais como madeira, folhas, flores, tubérculos, néctar, sementes, animais vivos ou mortos, sangue, fungos e bactérias (MURDOCK & SHADE, 2002; FORTUNATO *et al.*,

1995). Essa plasticidade é conferida pela diversidade de enzimas digestivas, incluindo as proteases, que podem ser encontradas no intestino médio desse grupo de organismos (TERRA *et al.,* 1996). Em cupins, as enzimas proteolíticas estão envolvidas na digestão de proteínas e peptídeos presentes nas substâncias húmicas do solo (JI & BRUNE, 2005). Sethi *et al.*(2011) estudaram a presença de atividade proteolítica no intestino anterior, médio e posterior de *Coptotermes formosanus*, bem como avaliaram a produção dessas enzimas por protozoários flagellados isolados do intestino posterior de operários dessa espécie. Eles relataram que o intestino médio mostrou uma alta atividade proteolítica proveniente de extratos de protozoários e as proteases mais encontradas foram serino proteases, sendo as principais tripsina e quimotripsina.

2.2.5. Degradação da Lignina

É bem aceito que alguns cupins são capazes de despolimerizar a lignina e tolerar seus subprodutos tóxicos, enquanto que outras espécies podem promover modificações menores na estrutura da ligninaou até mesmo eliminar a lignina nas fezes sem alterações. Assim, este é o aspecto mais obscuro relacionado aos mecanismos fisiológicos de digestão dos cupins. Cupins da subfamília Macrotermitinae (Termitidae) estão associados com fungos simbióticos capazes de degradar a lignina (HYODO *et al.,* 2003). Sethi *et al.*(2013) encontraram transcriptomas de lignases (enzimas capazes de despolimerizar a lignina) e fenoloxidases expressas por *Reticulitermes flavipes* e seus simbiontes. Os autores também apresentaramevidência da participação de aldo-ceto redutases e catalases como enzimas envolvidas na detoxificação de bioprodutos tóxicos derivados da despolimerização da lignina.

2.3. Papel ecológico dos cupins

O aparato digestivo dos cupins permite que eles tenham hábitos alimentares que os habilitam a desempenharem vários serviços ambientais. Os cupins agem como consumidores primários e decompositores (herbívoros e detritívoros), sendo seu principal papel ecológico a reciclagem de nutrientes através da trituração, decomposição, produção de húmus e mineralização de recursos celulósicos e suas variantes, com cerca de 74-99% da celulose e 65-87% da hemicelulose ingerida sendo hidrolisada e consumida por eles (PRINS & KREULEN, 1991; TAYASU *et al.,* 1997; COSTA-LEONARDO, 2002; BRUNE, 2014).

Nos trópicos, os cupins são considerados os mais importantes engenheiros do ecossistema do solo (BIGNELL, 2006). Em climas áridos e semi-áridos, eles são os principais macroinvertebrados decompositores e exercem um impacto adicional através da criação de uma bioestrutura (cupinzeiros, túneis e galerias) com propriedades físicas e químicas diferentes do solo que a circunda. Eles influenciam na distribuição dos recursos naturais como água e nutrientes e, consequentemente, na diversidade da microbiota do solo, plantas e animais do ambiente em que se encontram. Um dos maiores efeitos dos cupins no solo é seu papel na redução da densidade do solo através de um processo chamado de bioturbação (*bioturbation*), que corresponde ao transporte do solo no sentido vertical e horizontal de diversas profundidades para a construção dos túneis e galerias do cupinzeiro e a subsequente erosão de suas construções (JOUQUET *et al.,* 2011). Nas savanas tropicais, os cupins são praticamente o único grupo de invertebrados detritívoros que sobrevivem na estação seca, devido sua habilidade de manter constante a temperatura e umidade dos ninhos (COLLINS, 1981, 1983; WHITFORD *et al.,* 1992; GRANT & SCHOLES, 2006; JOUQUET *et al.,* 2011).

A trituração mecânica e a digestão enzimática da biomassa das plantas por cupins aumentam a superfície de contato acessível aos microorganismos do solo quando esta biomassa retorna ao solo, via fezes (JOUQUET *et al.,* 2011). Em florestas tropicais, os cupins contribuem significantemente na mineralização da serrapilheira através da digestão de grandes quantidades de biomassa (BIGNELL & EGGLETON, 2000; YAMADA *et al.,* 2005; FREYMANN *et al.,* 2010). Quando consomem a serrapilheira e matéria orgânica, os cupins reduzem os insumos que estariam disponíveis para outras vias de decomposição, e desta forma, eles acumulam recursos dentro do ninho (JOUQUET *et al.,* 2011). A matéria orgânica consumida pelos cupins ficará aprisionada nas estruturas do ninho, em agregados biogênicos estáveis, e nos corpos dos térmitas. A volta desses nutrientes e minerais ao solo ocorre através das fezes, secreções salivares, corpos em decomposição, predação e erosão do cupinzeiro, de forma que a região no solo onde se encontra o cupinzeiro terá maiores níveis de nitrogênio, uma maior capacidade de trocas catiônicas e mais nutrientes e minerais (Ca^{2+}, Mg^{2+}, K^{+}) que o solo que o circunda (JI & BRUNE, 2006; BRÜMER *et al.,* 2009; NGUGI *et al.,* 2011).

2.4. Aparato digestivo de cupins como modelo para sistemas de produção de biocombustíveis e alvo para controle de espécies-praga.

Atualmente, os biocombustíveis gerados a partir de biomassa lignocelulósica têm recebido bastante atenção da indústria e de comunidades acadêmicas de todo o mundo, em consonância com o objetivo atual de promover economias sustentáveis e a preservação do meio ambiente. Assim, a utilização desses biocombustíveis é uma alternativa para reduzir a dependência por combustíveis fósseis. Contudo, as indústrias têm encontrado um grande desafio na conversão de biomassa em combustível, ainda de alto custo e pouco eficiente para

produção em larga escala. Nesse sentido, tem crescido a busca por novas tecnologias viáveis e de baixo custo (XIE *et al.*, 2014)**.**

Apesar de décadas de pesquisa e inovações em biocombustíveis, torná-los acessíveis e sustentáveis ainda é um desafio. Esse desafio encontra-se na resistente e insolúvel parede celular das plantas, formadas pela mistura complexa e estável de celulose, hemicelulose e lignina. Dessa forma, um pré-tratamento químico envolvento condições extremas de pH, temperatura e outras condições são necessários para quebrar a estrutura da parede celular tornando a celulose e hemicelulose acessível para a degradação (DING *et al.*, 2012). Os avanços tecnólogicos que visam tornar os biocombustíveis sustentáveis e rentáveis priorizam diminuir o custo do pré-tratamento, desenvolver biocatalizadores eficientes e alcançar a utilização de todos os componentes da parede celular, incluindo hemicelulose e lignina. Os sistemas lignocelulolíticos naturais, como, por exemplo, o de cupins e outros insetos que se alimentam de madeira, apresentam alta eficiência e são altamente especializados no processamento de biomassa lignocelulolósica, sendo considerados verdadeiros biorreatores naturais. Esses sistemas têm sido considerados importantes modelos a serem estudados para aplicação na tecnologia de produção de biocombustíveis, permitindo o desenvolvimento de biocatalisadores eficientes e de baixo custo (SUN & SCHARF, 2010; XIE *et al.*, 2014).

O intestino do cupim pode degradar até 99% da biomassa lignocelulósica em 24 horas (SUN & SCHARF, 2010). Uma análise metagenômica comparativa destacou a relevância do tipo de alimentação do inseto e sua capacidade de decompor a biomassa lignocelulósica; esse estudo destacou que o intestino dos cupins contém as enzimas mais eficazes na degradação celulolítica dentre três espécies de insetos comparados (SHI *et al.*, 2013). Em cupins, todo o processo da degradação da lignocelulose acontece via um processo contínuo e integrado. A biomassa ingerida é primeiramente processada no intestino anterior, e levada ao intestino

médio onde será degradada (KE *et al.*, 2010). Por fim, a biomassa já processada passará pelos processos de sacarificação e fermentação no intestino posterior (TARTAR *et al.*, 2009). Estudos estabeleceram que o intestino anterior e médio são os principais locais de despolimerização da lignina. Já o intestino posterior é rico em celulases e hemicelulases. Mais de 700 genes de glicosil-hidrolases foram identificados, correspondendo a 45 diferentes famílias de enzimas envolvidas na degradação da celulose e hemicelulose (WARNECKE *et al.*, 2007; TARTAR *et al.*, 2009). Com os avanços em biologia molecular, bem como em genômica e proteômica, essas enzimas podem ser isoladas e sequenciadas, permitindo a clonagem e expressão em sistemas heterólogos para uso em larga escala.

Enzimas digestivas de cupins também são alvos de estudos focados na descoberta de novos compostos para uso no controle de cupins-praga. Cupins-praga são conhecidos pelos danos que eles causam nos centros urbanos. As espécies praga são a minoria, correspondendo cerca de 7% do total (VERMA *et al.*, 2009). Os danos causados pelos cupins em construções causam uma grande perda econômica e a infestação é usualmente evidente apenas quando a estrutura atacada está em um estágio avançado de infestação, tornando o processo de controle mais difícil. Millano & Fontes (2002) estimam que cerca de 1-10 bilhões de doláres são gastos anualmente no controle e reparo dos danos. *Coptotermes gestroi* (Rhinotermitidae), *Heterotermes tenius* (Hagen) e *Heterotermes longiceps* (Snyder) (Rhinotermitidae), *Nasutitermes corniger* (Motschulsky) (Termitidae), e *Coptotermes brevis* (Kalotermitidae) são considerados as principais espécies praga dos centros urbanos. O sucesso da espécie *N. corniger* na colonização de áreas urbanas tem sido atribuído a sua versatilidade biológica, estratégias de colonização e hábitos alimentares (FONTES, 1995; VASCONCELLOS, 1999; ELEOTÉRIO & BERTI-FILHO, 2000; VASCONCELLOS *et al.*, 2002; OLIVEIRA *et al.*, 2006; OLIVEIRA & BEZERRA-GUSMÃO, 2010).

2.5. Mecanismos de ação de lectinas inseticidas

As plantas, diferentemente dos animais, não possuem sistemas imunológicos para enfrentar certas situações adversas. Esse fato, associado à sua imobilidade, fez com que elas desenvolvessem, ao longo do tempo, estratégias de defesa tanto pré-formadas quanto induzidas (HAMMOND-KOSACK & JONES, 2000). Taninos, flavonóides, lectinas e inibidores de enzimas de plantas são componentes da defesa química das plantas que têm a habilidade de interferir nos processos digestivos de insetos.

As lectinas são definidas como proteínas ou glicoproteínas de origem não imunológica que apresentam um ou mais domínios ligantes a~pp carboidratos, os quais podem interagir específica e reversivelmente com monossacarídeos e glicoconjugados através de pontes de hidrogênio e ligações de van der Walls (PAIVA *et al.*, 2012). Em geral, as lectinas podem ser encontradas e isoladas dos mais diversos tecidos de uma planta, tais como folhas (NAPOLEÃO *et al.*, 2011), raízes (SOUZA *et al.*, 2011), rizomas (SANTANA *et al.*, 2012), entrecascas (SÁ *et al.*, 2009; VAZ *et al.*, 2010; ARAÚJO *et al.*, 2012), flores (SANTOS *et al*., 2009) e sementes (CARVALHO *et al.*, 2015). Nas plantas, as lectinas têm um papel fisiológico que tem atraído a atenção de muitos pesquisadores. Hoje já se conhece alguns desses papéis como: (1) ativação de enzimas (KESTWAL *et al*., 2007), (2) participação no mecanismo de nodulação em leguminosas (LIMPENS & BESSELING 2003) e (3) defesa contra microorganismos e insetos (PEUMANS & VAN DAMME, 1995).

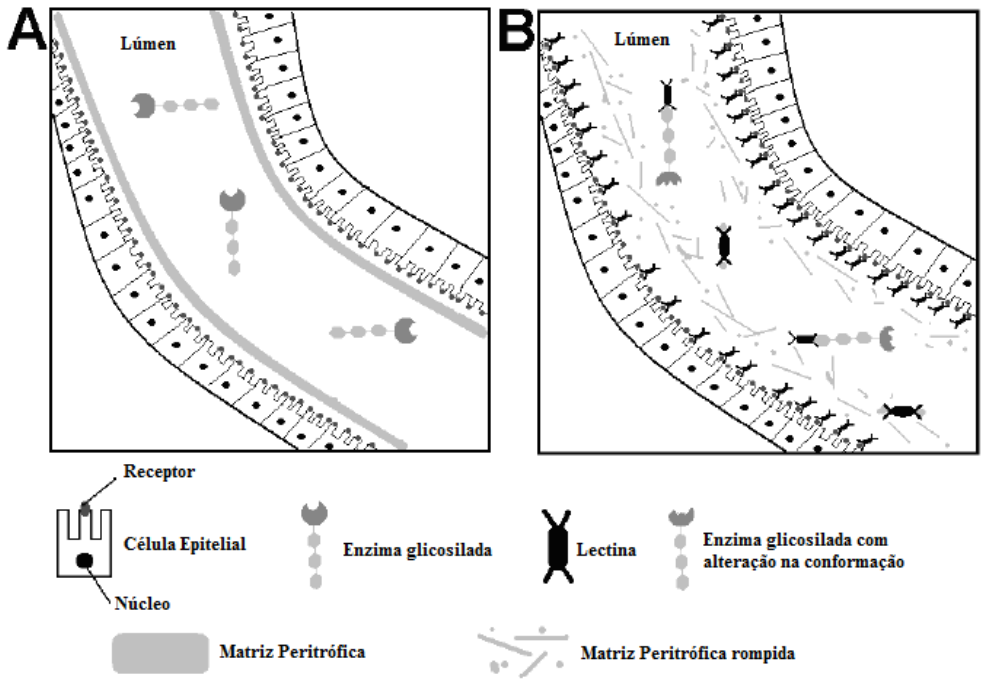
Nas últimas décadas, muitas lectinas de plantas têm mostrado efeito tóxico para várias espécies de insetos-praga de diferentes ordens, tais como Coleoptera, Diptera, Homoptera, Lepidoptera e Isoptera (PAIVA *et al.*, 2013). Esta propriedade estimula a avaliação do uso das lectinas de plantas como agentes inseticidas naturais contra pestes que causam prejuízos à

produção agrícola e danos à saúde humana (MACEDO *et al*., 2007; COELHO *et al*., 2009; SÁ *et al.* 2009; OLIVEIRA *et al.*, 2011; NAPOLEÃO *et al.*, 2013a).

O mecanismo de ação inseticida das lectinas de plantas não está completamente elucidado. A resistência ao meio ambiente hostil do trato digestivo do inseto é um dos pré-requisitos para que possam agir de diferentes formas no corpo do inseto. As lectinas podem agir de três formas principais: (1) ligando-se a polissacarídeos e glicoconjugados da superfície de estruturas do intestino do inseto; (2) através da interação com moléculas, glicosiladas ou não, como enzimas e proteínas de transporte e assimilação; (3) atravessando a barreira intestinal e alcançando a hemolinfa, causando diversos efeitos sistêmicos (PAIVA *et al.*, 2013), conforme representado na Figura 7.

O lado luminal do intestino médio da maioria dos insetos é contornado pela matriz peritrófica (MP), a qual é secretada pelas células digestivas (HEGEDUS *et al.*, 2009). A MP é um finíssimo envelope membranoso que recobre o bolo alimentar no intestino da maioria dos insetos. Ela está organizada num arranjo de quitina (4-13%) em uma matriz de proteína (21-51%) e carboidratos (RICHARDS & RICHARDS, 1997). As proteínas estruturais da MP estão principalmente associadas com as fibras de quitina por ligações não covalentes via domínios de ligação à quitina. A presença de múltiplos domínios ligantes de quitina nas proteínas da MP torna possível a formação de uma estrutura membranosa semelhante a um gel sustentado pelas fibras de quitina. Os domínios ligantes de quitina são ricos em cisteína e a presença de ligações dissulfeto contribui para a estabilidade (WANG *et al*., 2001). Dentre as principais funções atribuídas a esta estrutura estão a proteção mecânica contra injúrias; a formação de uma barreira física contra microorganismos; o funcionamento como uma barreira de seletividade para as enzimas digestivas e produtos da digestão; e a atuação no mecanismo e conservação das enzimas digestivas (TERRA, 2001).

Figura 7. Mecanismos de ação de lectinas inseticidas a nível intestinal. **(**A) Representação do intestino médio de um inseto saudável com as células epiteliais e a matriz peritrófica intactas e no lúmen intestinal as enzimas digestivas ativas. (B) Representação do intestino médio sob ação de lectinas inseticidas, que podem agir rompendo a integridade da matriz peritrófica atingindo as células epiteliais e também interagir com enzimas glicosiladas no lúmen do intestino.



Fonte: Paiva *et al.* (2013).

Lectinas ligadoras de quitina têm sido estudadas como agentes inseticidas, e essa atividade tem sido atribuída à ligação destas à MP (FITCHES & GATEHOUSE, 1998). Lectinas que se ligam à quitina podem afetar indiretamente o mecanismo regulatório das enzimas por perturbarem a síntese e integridade da MP (Figura 7B). Estudos têm mostrado anormalidades na formação e função de matriz peritrófica de insetos que foram alimentados com lectinas, assim como mostraram o rompimento das microvilosidades (VANDENBORRE *et al*., 2011). Após romper a MP e microvilosidades, as lectinas podem penetrar através do

epitélio intestinal e se acumular na hemolinfa, túbulos de Malpighi, ovários e corpo gorduroso (FICTHES *et al*., 2001; POWELL, 1998; VANDENBORRE *et al*., 2011).

Li *et al.* (2009) revelaram que 61 transcriptomas foram expressos diferentemente depois que larvas de *Drosophila* foram alimentadas com uma dieta rica em lectina de *Triticum vulgaris* (WGA). Esses genes estavam associados à organização do citoesqueleto, metabolismo da quitina, enzimas digestivas, reações de detoxificação e metabolismo energético. A expressão diferenciada desses genes está de acordo com as mudanças morfológicas já mencionadas como: desorganização de microvilosidades; danos na matriz peritrófica causando hiper expressão de quitina para o reparo; e ativação de enzimas.

Muitas das proteínas de insetos são glicosiladas, o que as tormam potenciais alvos de lectinas. Dentre elas, encontram-se enzimas glicosiladas, proteínas de transporte secretadas no intestino médio ou proteínas de membrana do epitélio intestinal. Estudos mostram a ferritina, aminopeptidases e α-amilases como sendo alvos de ligação de lectinas (DU *et al.*, 2000; SADEGHI *et al.*, 2008; CRISTOFOLETTI *et al.*, 2006; MACEDO et al., 2007).

Lectina do rizoma de *Microgramma vacciniifolia* mostrou forte efeito termiticida contra cupins operários e soldados da espécie *N. corniger* (CL_{50} de 0,130 e 0,085 mg/mL, respectivamente) e foi capaz de inibir a atividade de tripsina de operários, a atividade de β-glucosidase de operários e soldados e atividade de endoglucanase de soldados.Também, a lectina estimulou a atividade de fosfatase ácida e endoglucanase de operários (ALBUQUERQUE *et al*., 2012).

2.5.1. Lectinas de *Myracrodruon urundeuva*

A aroeira-do-sertão (*Myracrodruon urundeuva*), que está inclusa na família Anacardiaceae, é uma espécie arbórea tropical de ampla distribuição geográfica, ocorrendo desde o Brasil até a Argentina, sendo também encontrada na Bolívia e no Paraguai. Do ponto de vista medicinal, todas as partes da planta aparecem com várias indicações. A infusão da casca do caule é um dos remédios vegetais mais utilizados na medicina popular, com indicações para febres, hemoptises, bem como processos inflamatórios e infecciosos (úlceras, gastrites e diarréias). Os metabólitos secundários do cerne da madeira de *M. urundeuva* apresentam propriedades antioxidante, antifúngica, e repelente de térmitas.Por causa de suas diversas qualidades, é muito explorada e tornou-se escassa em todas as áreas de ocorrência (LEITE, 2002; SÁ *et al*., 2008).

As lectinas isoladas de entrecasca, cerne e folha de *M. urundeuva* (MuBL, MuHL e MuLL, respectivamente) são lectinas com afinidade por N-acetilglicosamina e ligadoras de quitina. Em SDS-PAGE, MuBL, MuHL e MuLL se apresentaram massas moleculares como polipeptídeos de 14,0 kDa, 14,4 kDa, 14,2 kDa, respectivamente. As três lectinas apresentam diferentes características físico-químicas, tais como diferentes valores de pH ótimo para sua atividade hemaglutinante, mas são todas estáveis frente ao aquecimento a 100 °C. Ainda, interagem diferencialmente com glicoproteínas e monossacarídeos. Todas apresentaram caráter básico e glicoprotéico, porém com diferentes teores de carboidratos: 33% para MuLL, 16,6% para MuBL e 21% para MuHL (SÁ *et al.*, 2009; NAPOLEÃO *et al.*, 2011).

As lectinas de *M. urundeuva* também são diferentes quanto à potencialidade de suas atividades biológicas. MuBL, MuHL e MuLL apresentaram atividade larvicida contra larvas de *Aedes aegypti* no quarto estágio, sendo as concentrações letais necessárias para matar 50%

das larvas de 0,125, 0,04 e 0,202 mg/ml, respectivamente (SÁ *et al.*, 2009; NAPOLEÃO *et al.*, 2012).

Quanto à atividade termiticida contra *N. corniger*, MuLL induziu 100% de mortalidade de operários e soldados em 7 e 9 dias, com CL_{50} para 4 dias de 0,374 e 0,432 mg/mL, respectivamente. MuBL, a uma concentração de 0,8 mg/mL, promoveu a mortalidade de 100% de operários e soldados em 8 e 9 diascom CL_{50} (4 dias) de 0,974 e 0,787 mg/ml, respectivamente. Para MuHL, a CL_{50} foi de 0,248 mg/mL para operários e de 0,199 mg/mL para soldados, após 4 dias. Ensaios conduzidos para avaliar a resistência das lectinas de *M. urundeuva* ao ambiente proteolítico do intestino dos cupinsdemonstraram que as atividades hemaglutinantes de MuBL, MuHL e MuLL não foram afetadas após incubação com extratos de intestino de *N. corniger* contendo atividade de tripsina; adicionalmente, as lectinas de *M. urundeuva* apresentaram efeitos bacteriostático e bactericida sobre simbiontes do intestino desses térmitas (NAPOLEÃO *et al.*, 2011).

2.6. Imobilização de lectinas eaplicações biotecnológicas

A característica de ligação a carboidratos torna as lectinas proteínas relevantes para a confecção de matrizes cromatográficas, por meio da imobilizaçãodessas em suporte insolúvel, sendo uma pontencial ferramenta no isolamento e separação de glicoconjugados, polissacarídeos, componentes celulares e até mesmo células (AMBUDKAR *et al*., 1998; SHARMA & MAHENDROO *et al*., 1980). Para que uma lectina possa ser imobilizada e utilizada eficientemente para esses fins, ela deve: 1) ser resistente às soluções usadas durante o processo de imobilização, de forma que sua estrutura tridimensional não seja afetada; 2) possuir grupos funcionais que liguem ao suporte e que esses grupos funcionais não sejam

essenciais para sua função biológica, ou seja, não afete sua propriedade de ligação. Os grupos funcionais podem ser: amino (-NH_2), carboxila (-COOH), aldeído (-CHO), tiol (-SH) e hidroxila (-OH) (NAPOLEÃO *et al*., 2013b).

Existem várias técnicas de imobilização de lectinas, que podem ser através de ligações covalentes e não-covalentes. A técnica mais conhecida de imobilização não-covalente é a da ligação reversível da Con A (Concanavalina A) com a polimixina B (um decapeptídio) através de interações hidrofóbicas. Nesta técnica, a polimixina B é covalentemente imobilizada através dos seus grupamentos amino numa placa de sensor de ressonância de plásmons de superfície (SPR- Surface Plasmon Ressonance) e, logo após, a Con A é aplicada e adsorvida espontaneamente por fortes interações hidrofóbicas. Esse sistema de imobilização se tornou uma importante ferramenta para a confecção de biosensores e bioreatores (MASÁROVÁ, *et al*., 2004; MONZO, 2007). Outra técnica de imobilização não-covalente é a adsorção indireta de uma lectina glicosilada (no caso, a de *Griffonia simplicifolia*) a Con A imobilizada covalentemente em sepharose, através de ligações envolvidas no reconhecimento da porção glicosilada pela Con A (WANG, 1988).

As técnicas de imobilização covalente de lectinas são mais utilizadas. Os suportes mais comuns são: agarose, Sepharose 4B ativada com brometo de cianogênio e álcool polivinílico (PVA). A imobilização em suporte de Sepharose 4B ativada com brometo de cianogênio se dá pela reação do grupo amino da lectina e o resíduo imidocarbonato da sepharose. As matrizes de lectinas imobilizadas são utilizadas para diversos fins, como: purificação de enzimas de interesse industrial (AHMAD *et al*., 2001); purificação de carboidratos e glicoproteínas e posterior estudos em glicômica e glicoproteômica (ALBUQUERQUE *et al*., 2016, ALVES *et al*., 2015); purificação de proteínas bioativas (SILVA *et al*., 2011). Aliar as técnicas de imobilização de lectinas a métodos modernos de espectrometria de massas em proteômica e

glicômica torna-se uma ferramenta estratégica para identificar as moléculas alvos, tanto para fins biotecnológicos como de diagnóstico.

As lectinas também podem ser imobilizadas covalentemente em biosensores, a fim de serem utilizados como meio diagnóstico para detectar a constituição diferenciada de proteínas plasmáticas de indivíduos normais e infectados com os diversos sorotipos da dengue (AVELINO *et al*., 2014, LUNA *et al*., 2014).

3. OBJETIVOS

3.1. Geral

Investigar os efeitos de lectinas termiticidas de *Myracrodruon urundeuva* na estrutura histológica e nos diferentes tipos celulares e atividades enzimáticas presentes no trato intestinal de cupins da espécie *Nasutitermes corniger*, bem como identificar alvos de ligação das lectinas.

3.2. Específicos

- Identificar e caracterizar as atividades enzimáticas presentes no trato digestivo de cupins operários e soldados da espécie *N. corniger*.
- Determinar os efeitos das lectinas de entrecasca (MuBL) e folha (MuLL) de *M. urundeuva* sobre as atividades enzimáticas do trato digestivo de operários.
- Obter matrizes cromatográficas contendo MuBL e MuLL imobilizadas em Sepharose CL-4B.
- Avaliar a interação de proteínas presentes em extratos de intestino de operários através de cromatografia nas matrizes MuBL-Sepharose e MuLL-Sepharose.
- Identificar as proteínas que se ligaram às matrizes MuBL-Sepharose e MuLL-Sepharose contendo as lectinas por meio de espectrometria de massas.
- Avaliar danos histológicos no intestino médio de operários submetidos à dieta artificial contendo MuBL, MuLL ou a lectina do cerne de *M. urundeuva* (MuHL).

- Investigar o intestino médio de operários que ingeriram as lectinas quanto à presença de células digestivas, regenerativas, proliferativas e enteroendócrinas.
- Investigar a ocorrência de apoptose em células do epitélio intestinal de operários que ingeriram as lectinas.
- Visualizar o efeito das lectinas sobre a integridade da matriz peritrófica no intestino médio dos operários.

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5. ARTIGO 1

Digestive enzymes from workers and soldiers of termite *Nasutitermes corniger*

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Digestive enzymes from workers and soldiers of termite *Nasutitermes corniger*

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ABSTRACT

The digestive apparatus of termites may have several biotechnological applications, as well as being a target for pest control. This report discusses the detection of cellulases (endoglucanase, exoglucanase, and β-glucosidase), hemicellulases (β-xylosidase, α-L-arabinofuranosidase, and β-D-xylanase), α-amylase, and proteases (trypsin-like, chymotrypsin-like, and keratinase-type) in gut extracts from *Nasutitermes corniger* workers and soldiers. Additionally, the effects of pH (3.0–11.0) and temperature (30–100°C) on enzyme activities were evaluated. All enzymes investigated were detected in the gut extracts of worker and soldier termites. Endoglucanase and β-xylanase were the main cellulase and hemicellulase, respectively. Zymography for proteases of worker extracts revealed polypeptides of 22, 30, and 43 kDa that hydrolyzed casein, and assays using protease inhibitors showed that serine proteases were the main proteases in worker and soldier guts. The determined enzyme activities and their response to different pH and temperature values revealed that workers and soldiers contained a distinct digestive apparatus. The ability of these termites to efficiently digest the main components of lignocellulosic materials stimulates the purification of gut enzymes and further investigation into their biotechnological potential.

Keywords: *Nasutitermes corniger*; amylase; cellulase; serine proteases; trypsin-like; hemicellulase.

1. Introduction

Termites (order Isoptera) are eusocial insects that can be classified into morpho-physiologically distinct castes called queens, kings, alate forms (reproductive members), workers, soldiers, and immature forms. Termites participate in nutrient recycling by grinding, decomposition, humification, and mineralization of cellulosic resources and their variants, because they have appropriate digestive mechanisms capable of metabolizing different biopolymers found in wood, fruits, tubers, crops, and soil components (Tayasu et al., 1997; Hartke and Baer, 2011).

These lignocellulosic materials are degraded by cellulases and hemicellulases, while starch and peptide components are hydrolyzed by amylases and proteases, respectively (Waller and La Fage, 1986; Ji and Brune, 2005). In higher termites (family Termitidae), digestive enzymes are encoded by the termite genome or produced by gut symbiotic bacteria, such as spirochetes and fibrobacters (Warnecke et al., 2007). The high efficiency of the lignocellulolytic systems found in the termite gut makes them important models to be studied for use in the processing of lignocellulosic biomass for biofuel production, for example (Sun and Scharf, 2010; Scharf et al., 2011; Mathew et al., 2013). Amino acid sequencing, cloning, and expression of enzymes in heterologous systems allow the production of termite enzymes on a large scale through genetic engineering techniques (Olempska-Beer et al., 2006).

Apart from the biotechnological potential, the characterization of digestive enzymes from termites can contribute to the development of new insecticides. Natural insecticides derived from plants, such as enzyme inhibitors, lectins, and secondary metabolites, are known to interfere in digestive enzyme activities. One study showed that *Microgramma vaccinifolia* rhizome lectin possessed termiticidal activity, and was able to promote imbalances in the activities of trypsin-like protease, acid phosphatase, and cellulase from the gut of termites (Albuquerque et al., 2012).

Nasutitermes corniger (Termitidae) is a soil-feeding and wood-damaging termite species. It is considered a threat to urban centers and has been favored by environmental imbalance (Figueiredo, 2004). However, reports on digestive enzymes from this species are scarce. Napoleão et al. (2011) and Albuquerque et al. (2012) have reported the detection of

trypsin, amylase, cellulase, and phosphatase activities in the gut of *N. corniger*, but to our knowledge, there is no other information about the characteristics of these and other digestive enzymes. Tokuda et al. (2012) studied the wood digestion of other species from this genus, *Nasutitermes takasagoensis*, and reported that wood fragments are firstly broken by mastication and salivary β-glucosidases, followed by the digestive action of endoglucanases and β-glucosidases in the midgut luminal space.

This paper describes the assessment and partial characterization of cellulolytic (endoglucanase, exoglucanase, and β-glucosidase), hemicellulolytic (β-xylosidase, α-l-arabinofuranosidase, and β-D-xylanase), amylolytic (α-amylase), and proteolytic (trypsin-like, chymotrypsin-like, and keratinase-type proteases) activities in gut extracts of *N. corniger* workers and soldiers.

2. Materials and methods

2.1. Insects

Colonies of *N. corniger* were collected at an Atlantic Forest fragment located at the campus of the *Universidade Federal Rural de Pernambuco*. The authors have authorization from the *Instituto Chico Mendes de Conservação da Biodiversidade* from Brazilian Ministry of the Environment for termite collection (number 36301-2). Termite colony was selected according to overall integrity criteria and the nest was carefully removed from the trunk of a tree using a machete and transferred to laboratory packaged into a black plastic bags. The colony was maintained at 28 ± 2 ºC (70 ± 5% relative humidity) in the dark during 6 h. In this period, the workers and soldiers were collected and separated for use in preparation of termite gut extracts.

2.2. Chemicals

Acrylamide, Avicel, azocasein, *N*-benzoyl-DL-arginyl-ρ-nitroanilide (BApNA), bovine serum albumin, carboxymethylcellulose (CMC), casein from bovine milk, citric acid, Coomassie Brilliant Blue R-250, 3,5-dinitrosalicylic acid (DNS), ethylene-diaminetetraacetic acid (EDTA), D(+)-glucose, glutaraldehyde, *N*,*N*′-methylenebis(acrylamide), ρ-nitrophenyl-α-L-arabinofuranoside (ρNPAraf), ρ-nitrophenyl-β-D-glucopyranoside (ρNPG), ρ-nitrophenyl-β-D-xylopyranoside (ρNPX), ρ-nitrophenol, pepstatin A, phenylmethylsulfonyl fluoride

(PMSF), sodium bicarbonate, sodium dodecyl sulphate (SDS), N-succinyl-alanyl-alanyl-alanyl-ρ-nitroanilide (Suc-Ala-Ala-Ala-pNA), N-succinyl-L-phenylalanine-ρ-nitroanilide (Suc-Phe-pNA), trans-Epoxysuccinyl-L-leucyl-amido (4-guanidino)butane (E-64), Trishydroximethylaminomethane (Tris), xylan and D(+)-xylose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, chloridric acid, dibasic sodium phosphate, monobasic sodium phosphate, sodium chloride, and trichloroacetic acid were purchased from Vetec (Rio de Janeiro, Brazil). Ammonium persulphate, calcium chloride, sodium acetate, sodium hydroxide, soluble starch, *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), and Triton X-100 were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.3. Termite gut extracts

Groups of workers or soldiers of *N. corniger* were immobilized by placing them in a freezer at -20 ºC for 10–15 min. Each termite was decapitated using an 8-mm-long, 0.3-mm needle (BD Ultra-Fine II from Becton, Dickinson and Company, NJ, USA) and had its gut removed intact by pulling of the last abdominal segments. Next, guts were stored on ice in 0.1 M sodium acetate pH 5.5 or 0.1 M Tris-HCl pH 8.0, both containing 0.15 M NaCl.

N. corniger gut extracts were obtained according to Napoleão et al. (2011). A group of 100 guts from workers or soldiers was placed in a 2-mL glass tissue grinder and manually homogenized with 1 mL of the buffer solution used in dissection. The homogenates were then centrifuged at 9,000 *g* at 4 °C during 15 min. The collected supernatants (worker or soldier gut extracts) were used for evaluation of enzyme activities. The extracts in sodium acetate buffer were used in assays for cellulase, hemicellulase and α-amylase activities while extracts in Tris buffer were used in the assays for protease activities. Protein concentration of extracts was determined according to Lowry et al. (1951) using bovine serum albumin (31.25–500 µg/mL) as standard.

2.4. Cellulase activities

Assays for endoglucanase and exoglucanase activities were carried out according to adaptations of the methods described by Li et al. (2009) and Wood and Bhat (1988), respectively. The reactions started by incubating (50 °C, 10 min) gut extract from workers

(100 µL, 330 µg of protein) or soldiers (100 µL, 270 µg of protein) with 400 µL of 1% (w/v) CMC (for endoglucanase activity) or 1% (w/v) Avicel (for exoglucanase activity) in 0.1 M sodium acetate pH 5.5 containing 0.15 M NaCl. After incubation, 500 µL of DNS were added to stop the reaction and the mixtures were heated (100 ºC, 6 min) and immediately cooled in ice (15 min). Then, the absorbance at 540 nm was measured. The amount of reducing sugars was determined using glucose as standard (Y=0.1261X–0.0157; Y is the absorbance at 540 nm; X is the glucose concentration in mg/mL). One unit of enzyme activity was defined as the amount of enzyme required to generate 1 µmol of glucose per minute. Blanks were performed submitting worker and soldiers gut extracts to the same reaction steps in absence of substrate. Also, blanks of the substrate in absence of gut extracts were also achieved.

An adaptation of the method described by Tan et al. (1987) was used to assess β-glucosidase activity. Gut extract from workers (100 µL, 330 µg of protein) or soldiers (100 µL, 270 µg of protein) was incubated (50 °C; 10 min) with 400 µL of 0.1 % (w/v) ρNPG in sodium acetate pH 5.5 containing 0.15 M NaCl. After incubation, 500 µL of 10% (w/v) sodium bicarbonate were added to stop the reaction and the absorbance at 410 nm was measured. The amount of ρ-nitrophenol released by hydrolysis of ρNPG was determined using a calibration curve (Y=32.224X+0.0783; Y is the absorbance at 410 nm; X is the ρ-nitrophenol concentration in mg/mL). One unit of activity was defined as the amount of enzyme required to generate 1 µmol of ρ-nitrophenol per minute. Reaction blanks were performed without ρNPG to eliminate colorimetric interference of extract as well as using the substrate in absence of gut extract to correct non-enzymatic turnover.

2.5. Hemicellulase activities

Assays for β-xylosidase and α-L-arabinofuranosidase activities were carried out according to Tan et al. (1987). Gut extract from workers (100 µL, 330 µg of protein) or soldiers (100 µL, 270 µg of protein) was incubated at 50 °C during 10 min with 400 µL of 0.1% (w/v) ρNPX (for β-xylosidase) or ρNPAraf (for α-L-arabinofuranosidase) in 0.1 M sodium acetate pH 5.5 containing 0.15 M NaCl and, after incubation period, the reaction was stopped with 500 µL of 10% (w/v) sodium bicarbonate. The absorbance at 410 nm was measured and the amount of ρ-nitrophenol released was determined as described in section 2.4. One unit of activity was defined as the amount of enzyme required to generate 1 µmol of

ρ-nitrophenol per minute. Reaction blanks were performed in absence of substrates as well as in absence of gut extracts.

Assay for β-D-xylanase activity was performed according to Wood and Bhat (1988). Initially, gut extract from worker (100 µL, 330 µg of protein) or soldier (100 µL, 270 µg of protein) was incubated (50 °C, 10 min) with 400 µL of 1% (w/v) xylan in 0.1 M sodium acetate pH 5.5 containing 0.15 M NaCl. Next, 500 µL of DNS were added to stop the reaction and the mixtures were heated at 100 °C during 6 min and immediately cooled in ice (15 min). The absorbance at 540 nm was measured and the amount of reducing sugars was determined using xylose as standard (Y=0.1183X–0.0704; Y is the absorbance at 540 nm; X is the xylose concentration in mg/mL). One unit of xylanase activity was defined as the amount of enzyme required to generate 1 µmol of xylose per minute. Reaction blanks were performed without xylan or without gut extracts.

2.6. α-Amylase activity

The assay was carried out based on the method described by Bernfeld (1955). Gut extract from worker (100 µL, 330 µg of protein) or soldier (100 µL, 270 µg of protein) was incubated at 50 °C for 10 min with 400 µl of 1% (w/v) soluble starch in 0.1 M sodium acetate pH 5.5 containing 0.02 M $CaCl_2$ and 0.15 M NaCl. The reaction was stopped by adding 500 µl of DNS. Next, the assays were heated at 100 ºC in boiling water for 6 min and immediately cooled on ice for 15 min. Then, absorbance was measured at 540 nm. The amount of reducing sugars was determined using the calibration curve of glucose described in section 2.4. One unit of amylase activity was defined as the amount of enzyme required to generate 1 µmol of glucose per minute. Reaction blanks were performed without starch or without extracts.

2.7. Protease assays

Total protease activity was determined using azocasein as substrate according to Azeez et al. (2007). Worker (100 µL, 360 µg of protein) or soldier (100 µL, 245 µg of protein) gut extract was mixed with 300 µL of 0.1 M sodium phosphate pH 7.5 containing 50 µL of 0.6% (w/v) azocasein. The mixture was supplemented with 100 µL of 0.1% (v/v) Triton X-100 and incubated at 37 ºC for 3 h. The reaction was stopped by adding 200 µL of 10% (v/v) trichloroacetic acid and the mixture was incubated at 4 ºC for 30 min. Next, it was

centrifuged at 9,000 *g* for 10 min and the absorbance of the supernatant at 366 nm was determined. One unit of protease activity was defined as the amount of enzyme that gave an increase of 0.01 in absorbance. Reaction blanks were performed by adding 10% trichloroacetic acid before the addition of substrate.

The effect of the protease inhibitors pepstatin A (8 mM), E-64 (8 mM), PMSF (8 mM) and EDTA (8 mM) was investigated. Each inhibitor was separately added (1:1, v/v, ratio) to *N. corniger* gut extract and the mixtures were incubated at 37 °C for 30 min. Next, protease activity assay was performed. Residual protease activity was determined in comparison with control without inhibitors (100% of activity). Blanks containing the substrate without addition of gut extracts were also performed.

Zymography for protease activity was carried out according the method described by Garcia-Carreño et al. (1993). Sample of worker or soldier gut extract (25 µg of protein) in Tris buffer was submitted to polaycrylamide gel electrophoresis containing SDS using a 12 % (w/v) gel prepared according Laemmli (1970). After electrophoresis at 4°C, the gel was immersed in 2.5% Triton X-100 in 0.1 M Tris-HCl pH 8.0 to remove SDS and incubated with 3% casein (w/v) in 0.1 M Tris-HCl pH 8.0 for 30 min at 4ºC. The temperature was raised to 37ºC and kept for 90 min to allow the digestion of casein by peptides with protease activity. Finally, the gel was stained for protein using 0.02% (v/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid and the gel was washed with distaining solution (40% methanol, 10% acetic acid, and 50% distilled water). Light bands against the dark background indicated protease activity. Zymography was repeated three times.

Trypsin-like, chymotrypsin-like, and keratinase-type enzymes were evaluated using the synthetic substrates BApNA (8 mM), Suc-Phe-pNA (11 mM), and Suc-Ala-Ala-Ala-pNA (11 mM), respectively. Worker or soldier gut extract (150 µL, 50 µg of protein) was incubated for 60 min at 37 °C with Tris buffer (35 µL) and substrate (15 µL). The substrate hydrolysis was followed by measurement of absorbance at 405 nm. One unit of activity was defined as the amount of enzyme that hydrolyzes 1 µmol of substrate per minute.

2.8. Effect of temperature and pH on enzyme activities

Enzyme activities from *N. corniger* gut extracts were evaluated after heating for 30 min at 30–100 ºC. The effect of pH on activities was evaluated by incubation (24 h; 4 ºC) of *N. corniger* gut extracts in different solutions (0.1 M citrate phosphate buffer, pH 3.0 to 6.0;

0.1 M sodium phosphate buffer pH 7.0; 0.1 M Tris-HCl buffer pH 8.0 and 9.0; NaOH solution pH 10.0 and 11.0) before determination of enzyme activities.

2.9. Statistical analysis

Three independent experiments in triplicate were performed for all enzyme, temperature and pH assays. Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA) and data were expressed as a mean of replicates ± SD. Significant differences between treatments were analysed by Student's t-test (significance at $p<0.05$) using the Origin 6.0 program.

3. Results and discussion

3.1. Cellulase and hemicellulase activities

Endoglucanases were the most active celluloytic enzymes in the gut extracts from *N. corniger* workers and soldiers, followed by exoglucanases, and finally β-glucosidase, in which a very low level of activity was seen (Table 1). Endoglucanase activity in workers was more than twice as high as that detected in soldiers. Similarly, endoglucanase activity in the midgut of *N. takasagoensis* was high in workers and low in soldiers (Fujita et al., 2008), and endoglucanase levels were much higher (approximately 18 fold) than β-glucosidase levels (Tokuda et al., 2012). Nevertheless, exoglucanase and β-glucosidase activity from *N. corniger* were highest in soldiers (Table 1).

Workers and soldiers have distinct eating habits, and the results obtained here concerning cellulase activity may reflect the biological differences between castes. According to Grassé (1949), immature and alate forms, as well as soldiers, are unable to feed themselves and receive stomodeal or proctodeal nourishment from workers, such as regurgitated food, saliva, or liquid excreta rich in symbionts. Once the soldiers are nourished by partially or completely digested food, the levels of endoglucanase seen in workers, as mentioned above, would not be required in a soldier's gut. Otherwise, the enzymes exoglucanase and β-glucosidase, which are responsible for the final digestion of cellulose components, would be present at higher concentrations in soldiers than in workers, aiming to optimize cellulose digestion, since the soldiers do not actively feed.

The effect of heating on the activity of cellulases is shown in Fig. 1. Endoglucanase and β-glucosidase activity from worker and soldier gut extracts were sensitive to high temperatures. Endoglucanase activity from workers declined 20% and 88% after heating at 50 and 60°C, respectively, while soldiers' endoglucanase activity was more sensitive to heating, declining 46% after treatment at 50°C, and being neutralized at 60°C (Fig. 1A, *a*). Exoglucanase activity from workers increased six times after heating at 100°C and the activity from soldiers increased about five fold after heating at 90°C, although it was neutralized at 100°C (Fig. 1A, *b*). The workers' β-glucosidase activity was reduced by 82% after heating at 50°C, and soldiers' β-glucosidase activity was reduced by 90% at 40°C (Fig. 1A, *c*).

The effect of temperature on cellulase activity from other termite species has been reported in the literature, and a moderate stability of enzymes toward heating is frequently observed. Similar to our results, β-glucosidase from *Neotermes koshunenis* resisted the effects of heating; though only at temperatures lower than 50°C (Tokuda et al., 2002). In addition, an endoglucanase from *Coptotermes formosanus* (Isoptera), heterologously expressed in *Escherichia coli*, was active between 20 and 70°C (Inoue et al., 2005), the same temperature range at which the endoglucanase from *N. corniger* workers was active.

Cellulase activity was also determined after incubation of the gut extracts at different pH values, and these enzymes from workers and soldiers were active after incubation at a wide range of acidic (3.0–6.0) and basic (9.0–11.0) pH. The highest activity of endoglucanase (8,100 mU/mg), exoglucanase (470 mU/mg), and β-glucosidase (140 mU/mg) from worker gut extracts was detected at pH 4.0, 3.0, and 11.0, respectively. For soldiers, the maximum activity of endoglucanase (5,400 mU/mg), exoglucanase (620 mU/mg), and β-glucosidase (140 mU/mg) was found after incubation of the gut extracts at pH 4.0, 7.0, and 4.0, respectively. These results indicate that barring endoglucanase, exoglucanase and β-glucosidase from workers have a more distinct physicochemical behavior than soldiers' enzymes.

The detection of activity at different pH values has been reported for termite cellulases, and the pH in which they have maximum activity varies between different species. Similar to the endoglucanase activity of *N. corniger*, cellulases from *Macrotermes mülleri* showed the highest activity at pH 4.4, and a *C. formosanus* endoglucanase showed a maximum activity at pH 5.8–6.0 (Rouland et al., 1989; Inoue et al., 2005). The optimum pH

for β-glucosidase from *Nasutitermes exitiosus* ranged from 2.0–2.8 in the foregut, 2.8–3.8 in the rectum, and 6.8–7.5 in the midgut and mixed segment (McEwen et al., 1980).

Enzymes usually have their activity affected by pH, since the ionization of side chains of amino acids in both the active site and whole enzymes influences protein shape and charge, as well the positioning of the substrate (Purich, 2010). Crystallography of endoglucanase isolated from the higher termite *N. takasagoensis* revealed a common folding pattern for glycosyl hydrolases and the overall structure of the enzyme at pH 6.5 is similar to that at pH 5.6, which corresponds to the optimum pH (Khademi et al., 2002). The authors also observed a conformational change at the active site in side chain displacement of the Glu412 residue, which may decrease the enzyme activity at a pH higher than 5.6.

The three hemicellulases evaluated in this work were detected in gut extracts from workers and soldiers (Table 1). β-D-xylanase was the most active hemicellulase, and the level of α-L-arabinofuranosidase was the lowest. Xylans are among the main components of lignocellulosic structures, and the high level of β-D-xylanase indicated the high adaptability of *N. corniger* to feed on these materials. The level of α-L-arabinofuranosidase is less pronounced than that of other hemicellulases in the termite gut, probably because these enzymes act specifically by hydrolyzing non-reducing terminals containing L-arabinosil residues, which are present only in some moieties of the polysaccharide molecules. The β-xylosidase and α-L-arabinofuranosidase activities showed similar levels in workers and soldiers, but β-D-xylanase activity was higher in workers than soldiers, probably due to the same reason stated above for endoglucanases.

The effect of heating on the activity of hemicellulases is shown in Fig. 1. The β-xylosidase activities from workers and soldiers were sensitive to heating, and the enzymes were inactivated at 40°C (Fig. 1B, *a*). The α-L-arabinofuranosidase and β-D-xylanase activities also decreased with heating, but the α-L-arabinofuranosidase activities from workers and soldiers were neutralized only at 100°C (Fig. 1B, *b* and *c*) and β-D-xylanase activity from workers was not abolished, even after heating at 100°C (Fig. 1B, *c*). The β-D-xylanase activity from soldiers was neutralized after heating at 80°C (Fig. 1B, *c*).

Hemicellulase activity was also determined after incubation of the extracts at different pH values. Hemicellulases from workers and soldiers were active after incubation at a wide range of acidic (3.0–6.0) and basic (9.0–11.0) pH, with the exception of α-L-arabinofuranosidase from soldiers, for which activity was not detected at pH 4.0, 5.0, 10.0,

and 11.0. The highest activity of β-xylosidase (8.0 mU/mg), α-L-arabinofuranosidase (0.7 mU/mg), and β-D-xylanase (2,700 mU/mg) from worker gut extracts was detected after incubation at pH 11.0, 6.0 and 7.0, respectively. For soldier gut extracts, the highest activity of β-xylosidase (7.0 mU/mg), α-L-arabinofuranosidase (0.4 mU/mg), and β-D-xylanase (1,240 mU/mg) was determined at pH 11.0, 7.0, and 8.0, respectively.

The detection of high levels of cellulase and hemicellulase activity in the *N. corniger* gut, and the relative heat-stability of endoglucanase, exoglucanase, and β-D-xylanase activities, are interesting characteristics that stimulates the investigation of biotechnological applications. Cellulases may also be used in the production of pharmaceuticals and detergents, wastewater treatment, and the processing of fruits and vegetables (Mamma et al., 2009).

3.2. α-Amylase activity

Gut extracts of *N. corniger* workers and soldiers were able to promote starch hydrolysis (Table 1). The α-amylase activity in workers was higher than that detected for soldiers, and this activity from both castes was sensitive to heating. The activity was reduced by 81% after heating at 40°C for workers and reduced by 61.5% after heating at 50°C for soldiers (Fig. 1C, *a*). The activity of worker and soldier amylases was neutralized after heating at 70 and 60°C, respectively. The results are similar to those reported for amylase activity from the gut of coleopterans *Prostephanus truncates* and *Morimus funereus*¸ which was abolished at 60 and 70°C, respectively (Mendiola-Olaya et al., 2000; Dojnov et al., 2008).

Worker and soldier gut amylases were active after incubation at a wide pH range (3.0–11.0), and the activities were highest at pH 6.0 (24.9 and 5.4 U/mg, respectively). The pH at which other insect amylases showed the best activity is also variable. The highest activities of coleopteran and lepidopteran α-amylases have been described at pH 5.2 (*Morimus funereus*), 6.0 (*Prostephanus truncatus*), 7.0 (*Rhyzopertha dominica*), and 9.0 (*Chilo suppressalis* and *Glyphodes pyloalis*) (Mendiola-Olaya et al., 2000; Dojnov et al., 2008; Zibaee et al., 2008; Priya et al., 2010; Yezdani et al., 2010).

The presence of α-amylase has been reported in the digestive systems of insects from the orders Orthoptera, Hymenoptera, Diptera, Lepidoptera, and Coleoptera (Terra and Ferreira, 1994), and these enzymes are important for optimal larval growth and adult

longevity. However, amylases from termites have been poorly studied. Hogan et al. (1988) reported the presence of amylase activity in *Nasutitermes walkeri* salivary glands (18%) and midgut (73%), with traces also found in other gut sections. However, in *Mastotermes darwiniensis* (lower termite), amylase activity was detected mainly in salivary glands, constituting 81% of the total amylase activity (Veivers et al., 1982). Amylases found in termites may also have a microbial origin, such as the amylases produced by symbiotic bacteria found in the gut of *Reticulitermes santonensis* (Mattéotti et al., 2012).

Insect α-amylases have been described as targets of enzyme inhibitors involved in defense from plants (Mendiola-Olaya et al., 2000). The α-amylase inhibitors are highly specific for their target enzymes, and their use for insect control is dependent on a clear understanding of the expression of different α-amylase isoforms in the insect digestive tract (Franco et al., 2002).

3.3. Protease activity

Protease activity was detected in workers, but was particularly low in soldiers (Table 1). Protease activity has been detected in the gut of other termite species such as *Odontotermes formosanus*, *Pericaproitermes nitobei*, *Termes comis*, *N. takasagoensis*, and *Macrotermes annandalei* (Fujita and Abe, 2002). Ji and Brune (2005) showed that the combined action of extreme alkalinity in the hindgut, autooxidative processes, and proteolytic activity make soil-feeding termites able to use peptidic components of humic acids as a source of carbon and energy. Soil is composed of a large proportion of hydrolysable peptides, and up to 20% of the total organic carbon adsorbed from soil particles may be from peptidic carbon (Kelley and Stevenson, 1996; Knicker et al., 2000).

N. corniger worker proteases showed thermal stability, and azocasein hydrolysis was still detected in extracts heated up to 90°C; the highest activity (312 U/mg) was detected after heating at 60°C, and this was neutralized only by heating at 100°C (Fig. 1C, *b*). An increase in enzyme activity can be promoted by molecular rearrangement of proteins, or may lead to a higher number of collisions and subsequent binding of enzymes to substrates (Campbell et al., 2008). Proteases in soldiers were more sensitive to heating than those in workers, and soldier protease activity was neutralized after heating at 70°C (Fig. 1C, *b*). Worker and soldier proteases were active after incubation at a wide pH range (3.0–11.0); the highest activities

were detected after incubation at pH 4.0 and 8.0 for workers (248 U/mg) and soldiers (40.0 U/mg), respectively.

Zymography of worker gut extract revealed proteolytic activity for polypeptide bands with 22, 30, and 43 kDa, and for a polypeptide of high molecular mass (≥120 kDa) observed on the top of the gel (Figure 2). Proteolytic activity from soldier gut extract was not detected by zymography, probably due to a low concentration of enzymes in the extract. The molecular masses of the *N. corniger* worker proteases detected on gel are similar to those found for other insect proteases. Polypeptide bands of 23–24 kDa were visualized on casein zymography of midgut preparations from *Rhyzopertha dominica*, and mass spectrometry analysis revealed trypsins from *Spodoptera littoralis* with 24.3 and 24.4 kDa (Marchetti et al., 1998; Zhu and Baker, 1999). SDS-PAGE revealed molecular masses of trypsins from *Manduca sexta* (Lepidoptera) larvae of 24 kDa, *Aedes aegypti* (Diptera) adults ranging from 28.5 and 32.0 kDa, and *Locusta migratoria* (Orthoptera) adults of 23, 27, and 29 kDa (Miller et al., 1974; Graf and Briegel, 1985; Lam et al., 2000). Proteases from gut of *Coptotermes formosanus* workers showed molecular mass of 65, 86 and 102 kDa (Sethi et al., 2011), higher than those detected by us. However, similarly to our results, these authors also found proteases with molecular mass higher than 120 kDa (123 and 142 kDa, specifically).

The protease activity from workers was reduced after incubation with pepstatin A, EDTA, E-64 and PMSF being this last the best inhibitor (Table 2). These results suggest that worker guts contain serine, aspartate, metallo, and cysteine proteases. Soldier protease activity was only inhibited by PMSF but in a high level (83%), indicating that protease activity of soldiers was is predominantly linked to serine proteases. Sethi et al. (2011) reported that *C. formosanus* workers posses mainly serine proteases at their gut, similarly to *N. corniger*.

Aiming to characterize the serine protease activity from *N. corniger* workers and soldiers, substrates specific for trypsin-like, chymotrypsin-like, and keratinase-type proteases were used. Trypsin-like protease activity in workers was higher than that for soldiers, while chymotrypsin level was low in both castes (Table 1). The activity of keratinase-type enzymes in *N. corniger* worker and soldier gut extracts was very low (Table 1), indicating that keratinized materials are not a very relevant source of nutrients for these termites, or at least to those collected in the region investigated in this work. Some soils are rich in keratin or keratinized materials, mainly from decomposing animal materials, and insects synthesize keratinases to digest the keratin present in their diet (Lin et al., 1982).

The effects of temperature and pH on the activity of the main serine protease detected were evaluated. Worker gut extract showed increased trypsin-like activity after heating from 40–70°C, and enzyme activity was neutralized by heating at 100°C (Figure 4C). Soldier trypsin-like activity was more sensitive to heating than worker enzymes, since it was neutralized after heating at 50°C (Figure 4C). *N. corniger* worker trypsin was more stable than trypsins described for other insects. Two trypsins found in *S. littoralis* lost their activity at 50–55°C (Marchetti et al., 1998), and the activity of *Ostrinia nubilalis* larval trypsin was reduced more than 70% at 60°C (Bernardi et al., 1996).

Trypsin-like protease activity was detected after incubation at pH 3.0–11.0, with the highest activity at pH 8.0 (workers) and 9.0 (soldiers). The results suggest that pH changes did not interfere drastically on the structure of the serine proteases from *N. corniger* workers and soldiers, and consequently on their activity. The *R. dominica* (Coleoptera) trypsin was also active from pH range from 7.0–9.5, but trypsin-like protease from *Vespa orientalis* and *Vespa cabro* (Hymenoptera) was inactive at pH 8.0, and unstable below pH 5.0 (Jany et al., 1978; Zhu and Baker, 1999). Trypsins from *Choristoneura fumiferana* (Lepidoptera) and *Lygus lineolaris* (Heteroptera) showed the highest activity at pH 10–12 (Zeng et al., 2002; Wang et al., 2003).

Napoleão et al. (2011) similarly detected a higher trypsin-like activity in the worker gut than the soldier gut. However, to our knowledge, there are no reports involving the isolation and characterization of termite trypsins. The stability of termite proteases stimulates their isolation and evaluation of potential biotechnological applications. Proteases may have several applications, for example, in leather processing, the production of biodegradable plastics, milk-clotting, meat maturation, and enzymatic synthesis of sweeteners (Naveena et al., 2004; Ogino et al., 2008; Haddar et al., 2009; Merheb-Dini et al., 2009).

4. Conclusion

The gut of *N. corniger* contains a high level of enzymes able to digest the main components of lignocellulosic materials, which is probably linked to the fact that these termites are considered a voracious pest. Enzyme activities differed between *N. corniger* workers and soldiers, revealing the presence of distinct digestive apparatuses in these castes, which may be linked to their differential feeding behavior. This study is a first step in the

characterization of digestive enzymes from *N. corniger*, and stimulates the purification and evaluation of their biotechnological potential.

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Figure captions

Figure 1. Effect of heating on digestive enzyme activities in gut extracts from *N. corniger* workers (♦) and soldiers (□): (A) cellulases [endoglucanase (a), exoglucanase (b) and β-glucosidase (c)], (B) hemicellulases [β-xylosidase (a), α-L-arabinofuranosidase (b), and β-xylanase (c)], (C) α-amylase (a), protease (b) and trypsin-like activities (c).

Figure 2. Zymography for proteases from *N. corniger* worker's gut extract on SDS-PAGE containing casein. Arrows indicated polypeptide bands with proteolytic activity.

Table 1. Enzyme activities from gut extracts of *N. corniger* workers and soldiers.

Enzyme	**Workers**	**Soldiers**
	Specific activity [a]	Specific activity [a]
Cellulases (mU/mg)		
Endoglucanase	4600 ± 300	2100 ± 500 *
Exoglucanase	140 ± 10	300 ± 25 *
β-glucosidase	10 ± 0.0	80 ± 1 *
Hemicellulases (mU/mg)		
β-xylosidase	5.8 ± 1.0	6.1 ± 0.8
α-L-arabinofuranosidade	0.72 ± 0.3	0.63 ± 0.1
β-D-xylanase	960 ± 170	130 ± 20 **
α-Amylase (U/mg)	24.7 ± 2.5	5.2 ± 1.5 **
Proteases		
Total protease (U/mg)	215 ± 12.2	18.3 ± 5.3 **
Trypsin-like (mU/mg)	6.0 ± 0.41	2.4 ± 0.06 *
Chymotrypsin-like (mU/mg)	0.53 ± 0.2	0.13 ± 0.09 *
Keratinase-type (mU/mg)	0.40 ± 0.08	0.15 ± 0.1 *

[a] Specific enzyme activities were calculated by the ratio between the number of enzyme units and the total amount (mg) of protein used in the assays. All data are presented as mean ± SD of three independent experiments in triplicate. Statistical analysis using Student *t*-test indicates that enzyme activity from soldier gut extract was significantly different from that of worker gut extract at $p < 0.05$ (*) or $p < 0.01$ (**).

Table 2. Effect of inhibitors on protease activity from worker and soldier gut extracts.

Inhibitor	Worker		Soldier	
	Activity (U)	Residual activity (%)	Activity (U)	Residual activity (%)
Pepstatin A	5.5 ± 0.16 a	85.0 ± 2.4 a	2.1 ± 0.13 a	100 a
EDTA	5.0 ± 0.24 b	77.2 ± 3.6 b	1.94 ± 0.23 a	100 a
E-64	5.6 ± 0.11 a	86.1 ± 1.8 a	2.0 ± 0.34 a	100 a
PMSF	3.6 ± 0.27 c	55.2 ± 4.1 c	0.35 ± 0.05 b	17.4 ± 2.6 b
Control	6.5 ± 0.37 d	100 d	2.0 ± 0.11 a	100 a

One unit of protease activity was defined as the amount of enzyme that gave an increase of 0.01 in absorbance at 366 nm. Different lowercase letters indicate significant differences at $p<0.05$. Control treatments correspond to extract activity in absence of inhibitors.

Figure 1

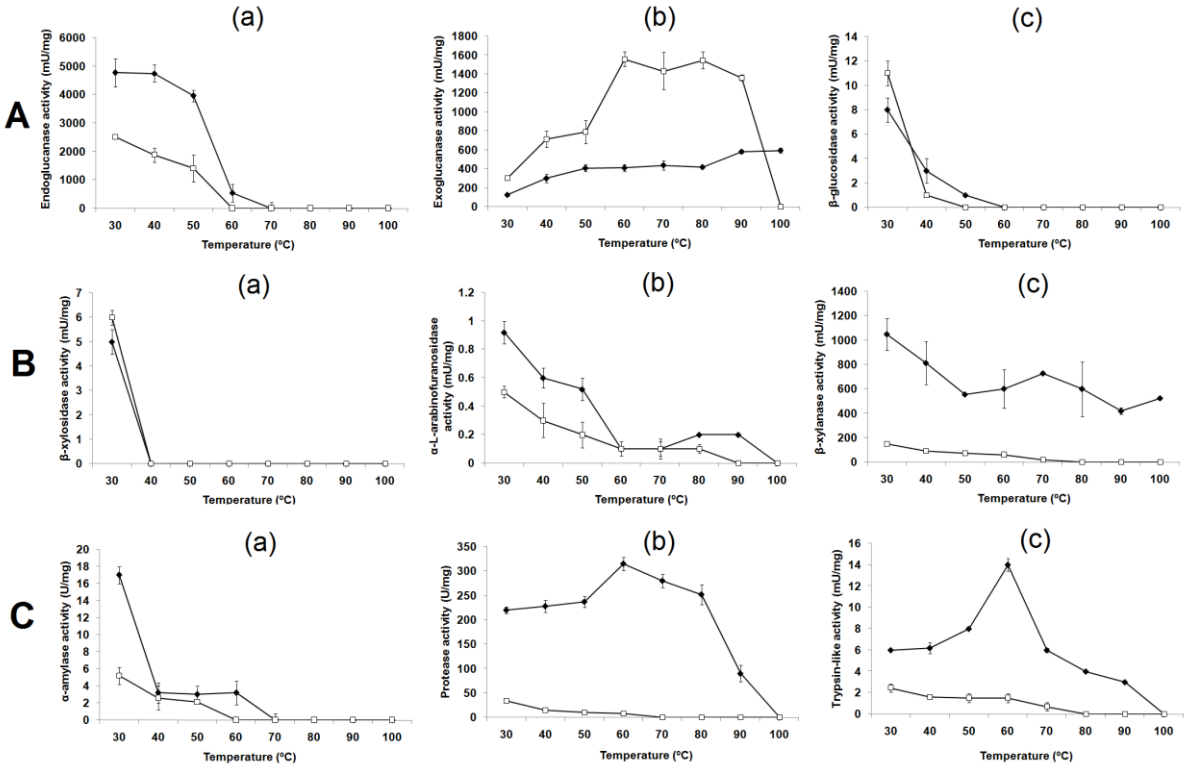
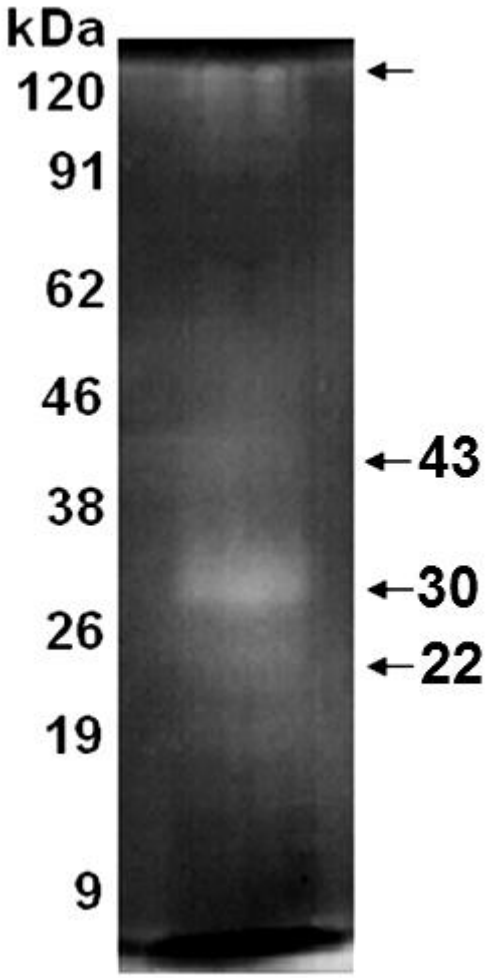

A
(a)
Endoglucanase activity (mU/mg)
Temperature (°C)
(b)
Exoglucanase activity (mU/mg)
Temperature (°C)
(c)
β-glucosidase activity (mU/mg)
Temperature (°C)
B
(a)
β-xylosidase activity (mU/mg)
Temperature (°C)
(b)
α-L-arabinofuranosidase activity (mU/mg)
Temperature (°C)
(c)
β-xylanase activity (mU/mg)
Temperature (°C)
C
(a)
α-amylase activity (U/mg)
Temperature (°C)
(b)
Protease activity (U/mg)
Temperature (°C)
(c)
Trypsin-like activity (mU/mg)
Temperature (°C)

Figure 2



x

x

6. ARTIGO 2

Binding targets of termiticidal lectins from bark and leaf of *Myracroduon urundeuva* at gut of *Nasutitermes corniger* workers

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Binding targets of termiticidal lectins from bark and leaf of *Myracroduon urundeuva* at gut of *Nasutitermes corniger* workers

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Abstract

Lectins, carbohydrate-binding proteins, from bark (MuBL) and leaf (MuLL) of *Myracrodruon urundeuva*are termiticidal agents on *Nasutitermes corniger*, but their action mechanisms of them remain to be elucidated. In this work, it was investigated binding targets of MuBL and MuLL at the gut of *N. corniger* workers by determination of the effect of these lectins on activity of digestive enzymes and identification of peptides from proteins that adsorbed to MuBL-Sepharose and MuLL-Sepharose by mass spectrometry and comparison of the sequences with others present in NCBInr database. Exoglucanase activity from workers was neutralized by MuBL while MuLL promoted an increase of this activity. The α-L-arabinofuranosidase activity was inhibited by MuLL and not affected by MuBL. The lectins stimulated α-amylase activity and inhibited protease and trypsin-like activities. MuBL-Sepharose bound proteins with homology to apolipophorin, trypsin-like enzyme and substrate-binding protein from an ABC transporter. Apolipophorin peptides were also detected in eluate from MuLL-Sepharose. This study revealed that digestive enzymes and transport proteins can be binding targets of *M. urundeuva* lectins at the gut of *N. corniger* workers. Thus, the termiticidal mechanism would involve alteration of the process of digestion and absorption of nutrients.

Keywords: insecticidal activity; affinity chromatography; apolipophorin; lectin; digestive enzymes.

1. Introduction

Nasutitermes corniger is a soil-feeding and wood-damaging termite species being considered a pest in urban areas (Figueiredo, 2004). Termites damage seed crops, building structures and other materials because they are able to grind the lignocellulosic materials use the chewing-type mouthparts andto digest their components through the action of cellulases and hemicellulases that may be produced by symbiotic microorganisms or by own termites (Lima et al., 2015). Lima et al. (2014) reported the activity of cellulases (endoglucanase, exoglucanase, and β-glucosidase), hemicellulases (β-xylosidase, α-L-arabinofuranosidase, and β-D-xylanase), α-amylase, and proteases (trypsin-like, chymotrypsin-like, and keratinase-type) in gut of *N. corniger* workers and soldiers.

Lectins are carbohydrate-binding proteins able to interact with glycoconjugates, including those attached to cell surfaces (Santos et al., 2013). Some plant lectins are able to interfere with feeding, development, and survival of insects and the mechanisms of action proposed include: interaction with cell membrane glycoconjugates along the digestive tract affecting signaling pathways and transport processes; binding to chitin and *N*-acetylglucosamine residues in the peritrophic membrane affecting digestion and absorption of nutrients; and destabilization of insect metabolism by interfering with enzymatic functions (Carlini and Grossi-de-Sá, 2002; Fitches et al., 1998; Albuquerque et al., 2012; Agra-Neto et al., 2014; Walski et al., 2014; Oliveira et al., 2015).

Lectins can be immobilized in insoluble supports and then applied to separate glycoconjugates present in complex mixtures (Napoleão et al., 2013). The combination of lectin affinity chromatography with proteome techniques constitutes a powerful strategy to identify biomolecules that interact with lectins and thus may contribute to the understanding

of the mechanisms involved in the biological activities of these proteins. Macedo et al. (2007) used lectin affinity chromatography to show that *Bauhinia monandra* leaf lectin is able to bind proteins present at lumen and membranes of the digestive tract of *Callosobruchus maculatus* larvae.

Lectins have been reported as termiticidal agents against *N. corniger* workers and soldiers (Sá et al., 2008; Paiva et al., 2011; Souza et al., 2011; Albuquerque et al., 2012; Araújo et al., 2012). Among these, there are the chitin-binding lectins isolated from bark (MuBL) and leaf (MuLL) of *Myracroduon urundeuva*. These proteins were able to kill *N. corniger* workers when ingested, with LC_{50} values (4 days) ranging from 0.374 and 0.974 mg/mL, respectively (Napoleão et al., 2011).

The action mechanism involved in termiticidal activity of *M. urundeuva* lectins is not fully elucidated. In an initial effort, it was demonstrated that MuBL and MuLL exhibited bacteriostatic and bactericidal effects on gut symbionts from *N. corniger* workers; also, MuBL and MuLL retained their carbohydrate-binding properties after incubation with worker gut proteases (Napoleão et al., 2011).

In this work, it was investigated the interaction between MuBL and MuLL and molecules present at the gut of *N. corniger* workers as an important step in the study of action mechanism. This study reports the effect of these lectins on the activity of digestive enzymes from workers gut and proteins from gut that bind to matrices containing immobilized MuBL or MuLL.

2. Materials and methods

2.1. Chemicals

Acetonitrile, acrylamide, Avicel, ammonium bicarbonate, ammonium sulphate, azocasein, *N*-benzoyl-DL-arginyl-ρ-nitroanilide, bovine serum albumin, carboxymethylcellulose, chitin from shrimp shells (powder), Coomassie Brilliant Blue, cyanogen bromide-activated Sepharose CL-4B, 3,5-dinitrosalicylic acid, dithiotreitol, ethanolamine, D(+)-glucose, glutaraldehyde, iodoacetamide, *N*,*N*′-methylenebis(acrylamide), ρ-nitrophenyl-α-L-arabinofuranoside, ρ-nitrophenyl-β-D-glucopyranoside, ρ-nitrophenyl-β-D-xylopyranoside, ρ-nitrophenol, sodium bicarbonate, sodium dodecyl sulphate (SDS), Trishydroximethylaminomethane (Tris), xylan and D(+)-xylose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, chloridric acid, dibasic sodium phosphate, monobasic sodium phosphate, sodium chloride, and trichloroacetic acid were purchased from Vetec (Rio de Janeiro, Brazil). Ammonium persulphate, calcium chloride, sodium acetate, sodium hydroxide, soluble starch, *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), and Triton X-100 were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.2. Insects

Colonies of *N.corniger* were collected at an Atlantic Forest fragment (8º00'45" S, 34º56'57" W) located at the campus of the *Universidade Federal Rural de Pernambuco*, Recife, Brazil. The collection was authorized by the *Instituto Chico Mendes de Conservação*

da Biodiversidade (ICMBio) from Brazilian Ministry of the Environment (authorization 36301). The nests were selected according to integrity and then transferred to laboratory, where they were maintained at 28 ± 2ºC at darkness.

2.3. Plant material

Bark and leaves of *M. urundeuva* were collected in Caxias city, state of Maranhão, northeastern Brazil, with authorization (38690) of the ICMBio. A voucher specimen is archived under number 054 at the herbarium Aluisio Bittencourt, *Centro de Estudos Superiores de Caxias*, *Universidade Estadual do Maranhão*, Brazil. The tissues were air dried, powdered (40 mesh) and stored at 28 ºC.

2.4. Termite gut extracts

N. corniger workers were immobilized by placing them at -20ºC for 10–15 min. Termite guts were removed intact using a needle (8 mm in length; 0.3 mm caliber) by pulling on the last abdominal segments. During the dissection procedure, the guts were stored on ice in 0.1 M sodium acetate pH 5.5, 0.1 M Tris-HCl pH 8.0 or 0.15 M NaCl. Next, groups of 100 guts were placed in a 2 mL glass tissue grinder manually homogenized with 1 mL of acetate buffer, Tris buffer or 0.15 M NaCl. The homogenates were then centrifuged at 9,000 *g* at 4 °C during 15 min and the collected supernatants corresponded to the gut extracts. The extracts were evaluated for protein concentration according to Lowry et al. (1951) using bovine serum albumin (31.25–500 µg/mL) as standard.

2.5. Isolation of M. urundeuva lectins

MuBL and MuLL were isolated according to Sá et al. (2009) and Napoleão et al. (2011), respectively. Powdered bark or leaves (10 g) were suspended in 0.15 M NaCl (100 mL). Clear supernatants (crude extracts) were obtained after homogenization in a magnetic stirrer (16 h at 4°C) followed by filtration through gauze and centrifugation (3,000 *g*, 15 min). Soluble proteins in crude extracts were fractionated with ammonium sulphate according to Green and Hughes (1955). The 40% supernatant fraction from bark extract and the 60-80% from leaf extract were collected. The fractions were dialyzed (3,500 Da cut-off membrane, 4 ºC) against distilled water (4 h) followed by 0.15 M NaCl (4 h) and separately loaded onto chitin columns (7.5 × 1.5 cm) equilibrated with 0.15 M NaCl (100 mL) at flow rate of 20 mL/h. The unabsorbed proteins were eluted with equilibrating solution until the absorbance at 280 nm was negligible. Then the adsorbed proteins (MuBL or MuLL) were eluted with1.0 M acetic acid and exhaustively dialyzed against 0.15 M NaCl (1 L) for eluent elimination.

2.6. Hemagglutinating activity

Carbohydrate-binding ability of the lectins was evaluated by hemagglutinating activity assay. The tests were carried out according to Paiva and Coelho (1992) using a suspension (2.5% v/v) of rabbit erythrocytes treated with glutaraldehyde (Bing et al., 1967). A lectin sample (50 µl) was serially two-fold diluted in 0.15 M NaCl in a 96-well microplate row and then 50 µl of the erythrocyte suspension were added to each well. A control without lectin was also performed. The number of hemagglutinating activity units was determined as the

reciprocal of the highest dilution of the sample that promoted full agglutination of erythrocytes. The specific HA was defined as the ratio between the units and the protein concentration (mg/mL).

2.7. Effect of lectins on digestive enzyme activities

Worker gut extracts in acetate (350 µg of protein) or Tris (350 µg of protein) buffers were incubated (30 min at 27 ºC) with MuBL or MuLL (35 µg in 0.15 M NaCl) before determination of enzymatic activities. In all assays, the following controls were performed: reaction blank (absence of substrate), substrate blank (absence of gut extract and lectin), 100% activity (absence of lectin), and lectin blank (absence of gut extract). In each case, it was added an equivalent volume of the solution in which the missing component was diluted. The enzyme assays are described below.

2.7.1. Endoglucanase, exoglucanase and α-amylase activities

Endoglucanase, exoglucanase and α-amylase activities were determined according to adaptations of the methods described by Li et al. (2009), Wood and Bhat (1988) and Bernfeld (1955), respectively. The sample (100 µL) was incubated for 10 min at 50ºC with 400 µL of solutions (1%, w/v) of the substrates carboxymethylcellulose (endoglucanase activity), Avicel (exoglucanase activity) or soluble starch (amylase activity) in sodium acetate pH 5.5 containing 0.15 M NaCl. After incubation, 500 µL of 3,5-dinitrosalicylic acid (DNS) were added and the assay was heated (100ºC, 6 min) and immediately cooled in ice (15 min). Next, the absorbance at 540 nm was measured. The amount of reducing sugars was determined

using a standard curve of glucose (Y=0.1261X–0.0157; Y is the absorbance at 540 nm; X is the glucose concentration in mg/mL). One unit of enzyme activity was defined as the amount of enzyme that produces 1 µmol of glucose per minute.

2.7.2. β-Glucosidase, β-xylosidase and α-L-arabinofuranosidase activities

These activities were determined according to an adaptation of the method described by Tan et al. (1987). The sample (100 µL) was incubated (50°C, 10 min) with 400 µL of solutions (0.1%, w/v) of the substrates ρ-nitrophenyl-β-D-glucopyranoside (β-glucosidase activity), ρ-nitrophenyl-β-D-xylopyranoside (β-xylosidase activity) or ρ-nitrophenyl-α-L-arabinofuranoside (α-L-arabinofuranosidase activity) in sodium acetate pH 5.5 containing 0.15 M NaCl. After incubation, 500 µL of 10% (w/v) sodium bicarbonate were added and the absorbance at 410 nm was measured. The amount of ρ-nitrophenol (ρNP) released by hydrolysis of the substrate was determined using a standard curve of ρNP (Y=32.224X+0.0783; Y is the absorbance at 410 nm; X is the ρNP concentration in mg/mL). One unit of activity was defined as the amount of enzyme required to generate 1 µmol of ρNP per minute.

2.7.3. β-D-Xylanase activity

The β-D-xylanase activity was evaluated according to an adaptation of method described by Wood & Bhat (1988). The sample (100 µL) was incubated (50°C, 10 min) with 400 µL of a 1% (w/v) xylan solution in sodium acetate pH 5.5 containing 0.15 M NaCl. Next, 500 µL of DNS were added and the mixtures were heated (100°C, 6 min) and immediately

cooled in ice (15 min). The absorbance at 540 nm was measured and the amount of reducing sugars was determined using a standard curve of xylose (Y=0.1183X–0.0704; Y is the absorbance at 540 nm; X is the glucose concentration in mg/mL). One unit of enzyme was defined as the amount of enzyme that generates 1 µmol of xylose per minute.

2.7.4. Protease activities

Protease activity was determined according to Azeez et al. (2007). The sample (100 µL) was incubated at 37ºC for 3 h with 300 µL of 0.1 M sodium phosphate pH 7.5, 50 µL of 0.6% (w/v) azocasein and 100 µL of 0.1% (v/v) Triton X-100. The reaction was stopped by adding 200 µL of 10% (v/v) trichloroacetic acid and the assay was incubated at 4ºC for 30 min. Next, it was centrifuged at 9,000 *g* for 10 min and the absorbance of the supernatant at 366 nm was determined. One unit of protease activity was defined as the amount of enzyme that gave an increase of 0.01 in absorbance.

Trypsin-like activity was evaluated by incubating the sample (50 µL) with 15 µL of the substrate *N*-α-benzoyl-DL-arginyl-ρ-nitroanilide (8 mM) and 35 µL of 0.1 M Tris-HCl pH 8.0 for 60 min at 37°C. The substrate hydrolysis was followed by measurement of absorbance at 405 nm. One unit of trypsin-like activity was defined as the amount of enzyme that hydrolyzes 1 µmol of substrate per minute.

2.8. Immobilization of lectins on Sepharose CL-4B

Cyanogen bromide-activated Sepharose CL-4B (2.0 g) was washed with 1 mM HCl (500 mL) and then incubated with 300 mL of coupling buffer (0.1 M sodium bicarbonate pH

8.3 containing 0.5 M NaCl and 1.0 mM HCl). Next, MuBL or MuLL (10 mg) was added and the coupling reaction was performed by incubation for 2 h at 24ºC under agitation followed by 16 h at 4ºC without agitation. The non-coupled lectin molecules were removed by filtration through a sintered glass and the gel was incubated for 2 h with a solution of 1.0 M ethanolamine pH 8.0. Then, the matrices MuBL-Sepharose and MuLL-Sepharose were washed with 0.1 M sodium bicarbonate pH 8.2, followed by 0.1 M Tris-HCl pH 8.0, 0.1 M sodium acetate pH 4.0 containing 0.5 M NaCl, and finally 0.1 M Tris-HCl pH 8.0 containing 0.5 M NaCl. The matrices were then washed and stored in 0.15 M NaCl at 4°C.

2.9. Chromatographies on MuBL-Sepharose and MuLL-Sepharose

Worker gut extract in 0.15 M NaCl (1.0 mL, 11 mg of protein) was loaded onto columns (4.0 × 1.5 cm) containing 3 mL of MuBL-Sepharose or MuLL-Sepharose previously equilibrated with 0.15 M NaCl. The chromatography process were performed at 1.0 mL/min flow rate and fractions of 6.0 mL were collected. The non-adsorbed proteins were eliminated by washing the column with equilibrating solution (72 mL) until absorbance was lower than 0.02. Then, the adsorbed proteins were eluted with 1.0 M NaCl (96 mL). The adsorbed proteins were dialyzed against distilled water (4 h) and evaluated for protein concentration according to Lowry et al. (1951).

2.10. Polyacrylamide gel electrophoresis and mass spectrometry

The pool of adsorbed proteins eluted from MuBL-Sepharose and MuLL-Sepharose columns were submitted to polyacrylamide gel electrophoresis in presence of sodium dodecyl

sulphate (SDS-PAGE) in 12% gels prepared according to Laemmli (1970). The gels were stained with 0.02% Coomassie Brilliant Blue in 10% acetic acid and 40% ethanol. A molecular mass standard (molecular masses ranging from 10 to 225 kDa) was also submitted to electrophoresis in the same gel. The experiments were repeated five times.

The polypeptide bands were excised from the gel and submitted to discoloration by three washing steps (each 15 min) with 400 µL of a 1:1 (v/v) solution of 50% (w/v) acetonitrile and 25 mM ammonium bicarbonate pH 8.0, under agitation. After the last washing, the gel was covered with 100% acetonitrile and submitted to dehydration using a vacuum concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Next, it was added 10 µL of 10 mM dithiotreitol prepared in 100 mM ammonium bicarbonate and the material was incubated for 1 h at 56ºC. After washing with 100 mM ammonium bicarbonate, it was added 10 µL of 50 mM iodoacetamide prepared in 100 mM ammonium bicarbonate and the assay was incubated at 28ºC in darkness. The iodoacetamide was then removed and the gel was washed with 100 mM ammonium bicarbonate and dehydrated again with 100% acetonitrile. The polypeptides were then digested during 16 h at 37ºC with 5 µL of a 20 mg/mL trypsin (mass spectrometry grade, Promega Corporation, USA) solution prepared in 50 mM ammonium bicarbonate. The solution was then transferred to a sterilen tube to which was added 30 µL of 1% (w/v) trifluoroacetic acid in 30% (w/v) acetonitrile for extraction of peptides. The resulting supernatant was removed, stored and the extraction procedure was repeated twice. The supernatants were then pooled and dried under vacuum concentrator. The peptides were analyzed using a Q-TOF spectrometer (Waters, Milford, USA) and the spectra obtained were compared with those present in NCBInr database using the MASCOT software search engine.

2.11. Statistical analysis

The enzyme assays were performed in triplicate and three independent experiments were performed using different gut extracts. Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) and data were expressed as a mean of replicates ± SD. Significant differences between treatments were analyzed by Student's *t*-test (significance at $p < 0.05$) using the Origin 6.0 program.

3. Results and discussion

MuBL and MuLL were isolated according procedures previously established and showed specific hemagglutinating activities of 2,455 and 3,890, respectively. This assures that the carbohydrate-binding property of the lectins was working and thus we used these preparations in the following assays.

Digestive enzymes may have their activity modulated by plant lectins with insecticidal activity due to interaction of these proteins with glycosylated moieties or other regions of enzyme molecules. The lectins may exert inhibitory or stimulatory effects on insect enzymes, deregulating the digestion process (Paiva et al., 2013). The effects of MuBL and MuLL on cellulolytic, hemicellulolytic and proteolytic activities from gut of *N. corniger* workers were determined since these are enzymes important for the nutrition of this species, which is a wood-damaging and soil-feeding termite (Lima et al., 2014). Table 1 shows that MuBL and MuLL were able to modulate the activity of enzymes present in the gut of *N. corniger* workers.

Endoglucanase and β-glucosidase activities from *N. corniger* workers were not significantly affected ($p > 0.05$) after incubation of gut extract with *M. urundeuva* lectins, in comparison with 100% activity controls. On the other hand, exoglucanase activity was neutralized by MuBL while MuLL promoted an increase of about two times in this activity. The three hemicellulase activities were also distinctly affected by lectins. The β-D-xylanase and β-xylosidase activities were not significantly ($p > 0.05$) affected while the α-L-arabinofuranosidase activity was inhibited in 66% by MuLL and not affected by MuBL.

These results show that *M. urundeuva* lectins did not affect the activity of the major cellulase (endoglucanase) and hemicellulase (β-D-xylanase) activities found at *N. corniger* worker gut. However, the different types of cellulases and hemicellulases usually act together in a balanced manner and then deregulation of one activity can lead to impairment of the whole process. The exoglucanase activity, which was affected by both lectins, has a central role in the cooperation between cellulases since there are synergisms between it and endoglucanase and β-glucosidase activities as well as between the own exoglucanase molecules that are acting on reducing and nonreducing ends of cellulose chain (Olsson et al., 2004). The *M. urundeuva* lectins had distinct effects on the cellulases but both stimulation and inhibition may interfere with the synergism in the cellulose digestion.

Cellulases from *N. corniger* workers were also affected by a termiticidal lectin from *Microgramma vacciniifolia* rhizome (MvRL) but the effects of this lectin were opposed to those of *M. urundeuva* lectins since MvRL stimulated exoglucanase activity, inhibited β-glucosidase and had no effect on exoglucanase (Albuquerque et al., 2012). The results from that work and the different effects described here for MuBL and MuHL show that the action mechanisms of termiticidal lectins may involve interference with cellulases but the types of modulation are variable. Cellulase modulation by peptide molecules was also reported by

Fayyaz-ur-Rehman et al. (2009), who reported that protein fractions from aqueous extracts of *Azadirachta indica* and *Buxus sempervirens* leaves showed inhibitory effect on cellulases from the bug *Planococcus citri*.

MuBL and MuLL stimulated (28% and 48%, respectively) α-amylase activity from gut extract. A stimulatory effect on α-amylase may lead to increased release of glucose from ingested starch and this may affect the balance of cellulose digestion since the glucose is an inhibitor of cellulases. The starch can be found in soil, derived from plant materials, but usually in very lower amounts than cellulose (Greenland and Oades, 1975). In this sense, the relevance of α-amylase activity for *N. corniger* workers, and consequently the repercussion of its modulation, will depend on the composition of the soil where the insects are living. Similarly to the results reported here, MuLL also stimulated the α-amylase activity from *A. aegypti* larvae and a lectin from *Bauhinia monandra* seeds stimulated α-amylase from *Callosobruchus maculatus* (Macedo et al., 2007; Napoleão et al., 2012).

The protease activity from worker gut extract was inhibited by MuBL and MuLL in 40.4 and 27.0% while the activity of trypsin-like enzymes was inhibited by MuBL (43%) and was neutralized by MuLL. The proteases are essential for bioavailability of amino acids for biosynthesis of proteins required to growth, development and survival of insects (Carlini and Grossi-de-Sá, 2002). The impairment of protease activities at *N. corniger* gut may reduce the utilization of amino acids derived from peptidic components of soil and affect negatively several processes that depend of protein renewal. The *M. vacciniifolia* rhizome lectin also exerted an inhibitory effect on trypsin-like activity from gut of *N. corniger* workers (Albuquerque et al., 2012) and MuLL also inhibited the trypsin-like activity from *A. aegypti* larvae (Napoleão et al., 2012).

In summary, the results reveal that digestive enzymes from gut of *N. corniger* workers are modulated by MuBL and MuLL and thus it is possible that these enzymes can be binding targets of termiticidal lectins. MuBL and MuLL affected distinctly the enzyme activities, which may be a factor responsible for their differential toxicity level, as evidenced by the LC_{50} values previously reported for them (Napoleão et al., 2011).

MuBL and MuLL were efficiently immobilized to Sepharose 4B and a mean of 5.2 mg of lectin attached to the inert support, corresponding to 52% of the protein amount used in coupling reaction. Gut extract from *N. corniger* workers was chromatographed MuBL and MuLL-Sepharose aiming to evaluate if these matrices would bind to proteins from gut. In lectin affinity chromatography, the interactions between support and ligand may be bioselective or not i.e. may involve or not the carbohydrate-binding site of the lectin and thus, even non-glycosylated molecules can also bind to the matrices (Coelho et al., 2012).

MuBL-Sepharose and MuLL-Sepharose matrices were able to bind worker gut proteins, which were eluted with 1.0 M NaCl (Figure 1A). The matrices were effective even after ten chromatographic procedures were performed. On average, 0.3 mg of protein was recovered from MuBL-Sepharose and MuLL-Sepharose columns, representing 2.8% of the total amount loaded onto the column. SDS-PAGE profile of the eluates revealed the presence of multiple polypeptide bands with molecular mass ranging from 16.5 to 188 kDa (Figure 1B).

Eight polypeptide bands excised from the gels and digested using trypsin, indicated in Figure 1B, showed homology with proteins included in the NCBInr database (Table 2). The polypeptide bands 1, 2 and 3 from MuLL-Sepharose eluate showed homology with an apolipophorin from the termite *Zootermopsis nevadensis*. Peptides derived from MuBL-Sepharose eluate (bands 5, 6 and 8) also had similarity with this apolipophorin, while the band

4 showed homology with a mammalian cationic trypsin-like protein (from the bat *Myotis brandtii*) and peptides from band 7 were homologous to those from the substrate-binding unit of an ATP-binding cassette (ABC) transporter protein from *Burkholderia* sp.

The apolipophorins are insect glycoproteins with approximately 80 to 250 kDa, which are components of lipophorin, the main lipoprotein found in insect hemolymph and responsible for lipid transport (Pompilho, 2006). Some apolipophorins are also described as able to bind lipopolysaccharides, teichoic acids and β1,3-glucans from bacteria and fungi, acting as pathogen recognition receptors (Zdybicka-Barabas & Cytryńska, 2013). Gupta et al. (2010) described the participation of apolipohorins as defensive agents in the midgut of *Anopheles gambiae* against *Plasmodium berghei*.

The apolipophorins of *Manduca sexta* (Sphingidae, Lepidoptera) are synthesized by the fat bodies, which secrete a lipophorin containing apolipophorins and phospholips that travels to the midgut, where it acquires diacylglycerols (Turunen and Crailsheim, 1996). In termites, the lipophorin is responsible for transport of juvenile hormone and also of hydrocarbons to the cuticular surface, where they are important for water balance and as recognition cues (Fan et al., 2004). It is plausible that the binding of *M. urundeuva* lectins to apolipophorins interferes with the absorption of lipids at *N. corniger* gut and may also affect the other functions of lipophorins if they remain retained in the midgut.

The identification of peptides with homology to a trypsin-like enzyme among the proteins that adsorbed to MuBL-Sepharose is in agreement with the ability of this lectin in inhibit trypsin-like activity from worker gut extract. Insect trypsins are similar to those from vertebrates showing homologous regions in the catalytic site (Kalhok et al., 1993).

The ABC transporters are integral membrane proteins found in organisms ranging from bacteria to humans. They are responsible for the transport of a great diversity of

substances, such as sugars, proteins, lipids, drugs and others, against high concentration gradients, using ATP-derived energy (Dassa and Bouige, 2001; Holland et al., 2003). They also participate in regulation of osmotic pressure (Schinkel and Jonker, 2012). In bacteria, these transporters are linked to absorption of nutrients and rare elements like molybdenum. In eukaryotic cells, their functions also include efflux of insecticides, herbicides, fungicides, antibiotics, and heavy metals. For examples, in insects they have been related to the resistance to pesticides (Buss and Callagan 2008; Heckel 2012) and in aquatic invertebrates, the ABC transporters are involved in the elimination of pollutants from the cytoplasm (Bardy, 2000; Pain and Parant, 2003). The interaction between *M. urundeuva* lectins and ABC transporters may affect the transport processes of several substances across the gut. It can be also considered that these lectins may be transported by these proteins but this only can be affirmed after further studies.

The ABC transporter protein with homology to that present in worker gut extract belongs to a bacterium. Napoleão et al. (2011) showed that MuBL was able to inhibit growth and cause death of symbionts bacteria present in *N. corniger* worker gut. In this sense, it is also possible that the lectins are also binding to proteins from the symbionts.

In conclusion, the present study identified target proteins of termiticidal *M. urundeuva* lectins at the gut of *N. corniger* workers. This work reveals MuBL and MuLL interact with digestive enzymes (carbohydrases and proteases) and transport proteins (apolipophorin and ABC transporters) involved in the digestion and metabolism of proteins, carbohydrates and lipids as well as with the absorption of substances at gut level. The identification of these targets will contribute to the knowledge of temiticidal mechanism of action of lectins from *M. urundeuva*.

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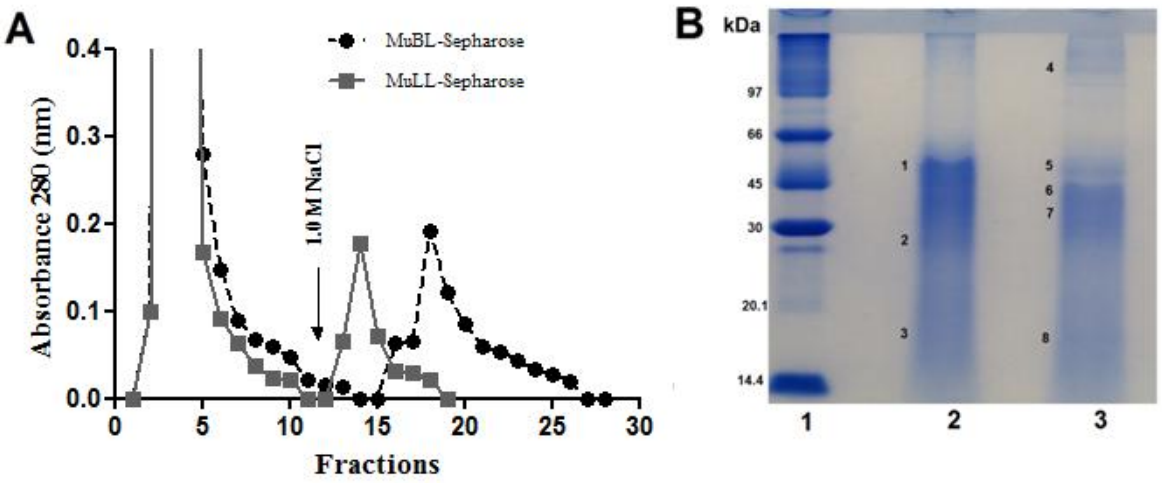

Figure 1. Evaluation of binding of proteins from gut extract of *N. corniger* workers to supports containing immobilized lectins from *M. urundeuva* bark (MuBL) and leaf (MuLL). (A) Chromatographic profiles of gut extract on columns of MuBL-Sepharose and MuLL-Sepharose. The matrices were equilibrated with 0.15 M NaCl. The adsorbed proteins were eluted with 1.0 M NaCl. (B) SDS-PAGE with 200 µg of proteins that were eluates from chromatographies on MuBL-Sepharose and MuLL-Sepharose. Lane 1: molecular mass markers; lane 2: eluate from MuLL-Sepharose; lane 3: eluate from MuBL-Sepharose. The numerated polypeptide bands [molecular masses in kDa: 50.3 (1), 28.3 (2), 16.5 (3), 123.4 (4), 51.6 (5), 45.4 (6), 40.0 (7), 15.9 (8)] showed homology with peptide sequences in NCBInr database (see Table 2).

Table 1. Enzyme activities from gut extracts of *N. corniger* workers previously incubated or not with *M. urundeuva* bark (MuBL) and leaf (MuLL) lectins.

Enzyme activities	**Gut extract**	**Gut extract + MuBL**	**Gut extract + MuLL**
	Specific activity	Specific activity	Specific activity
Cellulases (mU/mg)			
Endoglucanase	4150 ± 230	4244 ± 327	4122 ± 199
Exoglucanase	110.0 ± 15	0.0 ± 0.0 *	214.0 ± 24 *
β-glucosidase	12.0 ± 1.2	11.7 ± 3.0	12.1 ± 1.1
Hemicellulases (mU/mg)			
β-xylosidase	4.5 ± 0.8	4.8 ± 0.7	4.6 ± 0.3
α-L-arabinofuranosidase	0.42 ± 0.2	0.38 ± 0.1	0.14 ± 0.0 *
β-D-xylanase	940 ± 138	951 ± 101	944 ± 68
α-Amylase (U/mg)	30.7 ± 1.5	39.5 ± 0.8 *	45.5 ± 0.7 *
Proteases			
Total protease (U/mg)	230 ± 10.8	137 ± 13.4 *	167.9 ± 17.6 *
Trypsin-like (mU/mg)	4.0 ± 0.21	2.28 ± 0.1 *	0.0 *

[a] Specific enzyme activities were calculated by the ratio between the number of enzyme units and the total amount (mg) of protein used in the assays. All data are presented as mean ± SD of three independent experiments in triplicate. Statistical analysis using Student *t*-test indicates that enzyme activity from lectin treatment was significantly different from that of control at $p < 0.05$ (*).

Table 2. Homology of peptides derived from proteins that adsorbed to MuBL-Sepharose and MuLL-Sepharose matrices.

Band	Peptide sequences	Homologous proteins	Score
1	K.QYSFDGQSK.L K.VNDFGNSWK.I K.NHQLDSELR.I K.LLNDADEYSVK.L	Apolipophorin [*Zootermopsis nevadensis*]	165
2	K.AAFYPLNGPSK.F K.LLNDADEYSVK.L R.ELCTEEKDSEASVNIK.R	Apolipophorin [*Zootermopsis nevadensis*]	150
3	K.LAYLGVGALGGR.Y K.AAFYPLNGPSK.F K.LLNDADEYSVK.L	Apolipophorin [*Zootermopsis nevadensis*]	156
4	R.LGEHNIEVVEGNEQFINAAK.I	Cationic trypsin-3-like [*Myotis brandtii*]	66
5	K.VNDFGNSWK.I K.LAYLGVGALGGR.Y	Apolipophorin [*Zootermopsis nevadensis*]	101
6	K.VNDFGNSWK.I K.LAYLGVGALGGR.Y R.QENLDLIAER.L	Apolipophorin [*Zootermopsis nevadensis*]	139
7	K.LFNTPTR.L R.AEQIAFSSK.L R.AEQIAFSSK.L K.SVGVEQGTIQETYAK.T	ABC transporter substrate-binding protein [*Burkholderia* sp. UYPR1.413]	179
8	R.QENLDLIAER.L K.LLNDADEYSVK.L	Apolipophorin [*Zootermopsis nevadensis*]	105

Polypeptide bands from SDS-PAGE of eluates were numerated according to Figure 1. The bands 1, 2 and 3 were from MuLL-Sepharose eluate and the bands 4 to 8 were from MuBL-Sepharose eluate. Peptides were obtained after trypsinization and the sequences obtained by mass spectrometry were compared with those present in NCBInr database.

7. ARTIGO 3

Midgut damages in *Nasutitermes corniger* workers resulting from the ingestion of termiticidal lectins from *Myracrodruon urundeuva*

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Termiticidal lectins from *Myracrodruon urundeuva* (Anacardiaceae) cause midgut damages when ingested by *Nasutitermes corniger* (Isoptera; Termitidae) workers

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Abstract

Myracrodruon urundeuva is a hardwood tree whose bark, heartwood, and leaf contain lectins (MuBL, MuHL and MuLL, respectively) with termiticidal activity against *Nasutitermes corniger*. In this work, the effects of these lectins on the midgut of *N. corniger* workers were evaluated. The insects were supplied with an artificial diet containing the lectins at their respective LC_{50}, which were determined in a previous study. Forty-eight hours after the treatment, the midguts were dissected and fixed for histopathology analyses. Toluidine blue-stained midguts from lectin-treated workers showed disorganization, with presence of debris in the lumen and absence of the brush border. Fluorescence microscopy revealed that the numbers of digestive and proliferating cells were lower in lectin-treated individuals than in the control, and caspase-3 staining confirmed occurrence of cell apoptosis. Enteroendocrine cells were not seen in the treated individuals. The midguts from treated insects showed greater staining for peroxidase than the control, suggesting that the lectins caused oxidative stress. Staining with wheat germ agglutinin conjugated to FITC revealed that the lectins interfered with the integrity of the peritrophic matrix. This work represents an important step toward understanding the termiticidal activity of lectins extracted from a plant known for its resistance to termite attack.

Keywords: insecticidal activity; midgut epithelium; peritrophic matrix; apoptosis; hardwood tree.

1. Introduction

Lectins are proteins that recognize carbohydrates and glycoconjugates, including those attached to cell surfaces [1]. The biotechnological potential of lectins as natural insecticides and the entomotoxic effects of these proteins on Coleoptera, Diptera, Homoptera, Lepidoptera, and Isoptera have been reported [2].

When ingested by insects, plant lectins are able to interfere with feeding, development, and survival. It has been reported that insecticidal lectins are usually stable against proteolytic activity inside the insect gut environment, which is considered a pre-requisite to the insecticidal effect [3]. The action mechanisms involved in these effects are still under investigation and may differ according to the species affected and the carbohydrate specificity of lectin. Lectins may interact with the peritrophic matrix (PM) and glycoconjugates along the digestive tract, affecting nutrient absorption, signaling pathways, and transport processes [4–8]. The lectins may also bind to digestive enzymes [8–10] and their ingestion can promote midgut disorganization, morphological changes in microvilli, alterations in the expression of genes associated with cytoskeleton proteins, chitin metabolism, digestive enzymes, detoxification reactions, and energy metabolism [6, 11, 12].

Lectins isolated from the bark (MuBL), heartwood (MuHL), and leaves (MuLL) of *Myracrodruon urundeuva*, a hardwood tree native to Brazilian Caatinga, showed insecticidal activity against workers of the termite *Nasutitermes corniger*. It was suggested that MuHL plays a role in the resistance of *M. urundeuva* heartwood to biodeterioration [13–15]. Initial studies on the action mechanisms of these lectins showed that they were resistant to incubation with *N. corniger* worker gut proteases and were able to impair the growth and survival of termite gut symbionts [15]. Besides these three lectins, other plant lectins have

also been reported as termiticidal agents against *N. corniger* [9, 16–18]. It was reported that the termiticidal activity of *Microgramma vacciniifolia* rhizome lectin on *N. corniger* is linked to the chitin-binding and enzyme-modulating properties of this protein [9].

Midgut alterations have been caused by insecticidal agents. The exposure of *Aedes aegypti* larvae to the insecticide imidacloprid interfered with the differentiation of stem cells and dramatically reduced the number of digestive and endocrine cells, leading to the malformation of the midgut epithelium of adults [19]. A leaf extract of *Schinus terebinthifolius* (Anacardiaceae) promoted intense disorganization of the *A. aegypti* larval midgut epithelium, affecting digestive, enteroendocrine, stem (regenerative) and proliferative cells causing deformation and hypertrophy, disruption of microvilli, and vacuolization of the cytoplasm [20].

Given the above, we hypothesize that the insecticidal activity of *M. urundeuva* lectins (MuBL, MuHL and MuLL) against *N. corniger* workers may involve damage to the gut structure of the insects. In this work, we studied the effects of *M. urundeuva* lectins (MuBL, MuHL, and MuLL) on the midgut epithelium and on the PM of *N. corniger* workers after intake of an artificial diet supplemented with lectins.

2. Materials and methods

2.1. Insects

N. corniger nests were collected at the campus of the *Universidade Federal Rural de Pernambuco* (Recife, Brazil) in a fragment of the Atlantic Rain Forest (8º00'45" S, 34º56'57" W). The authors had authorization (36301) of the *Instituto Chico Mendes de Conservação da*

Biodiversidade (ICMBio) from the Brazilian Ministry of the Environment. The termite nests, selected based on integrity, were removed from the trunk of a tree, transferred to the laboratory, and maintained at 28 ± 2ºC during the period necessary for separation and transfer of workers to the bioassays.

2.2. Isolation of M. urundeuva *lectins*

The bark, heartwood, and leaves of *M. urundeuva* were collected in Caxias city, state of Maranhão, north-eastern Brazil, under authorization (38690) by ICMBio. A voucher specimen was archived under number 054 at the herbarium Aluisio Bittencourt, *Centro de Estudos Superiores de Caxias*, *Universidade Estadual do Maranhão*, Brazil. The tissues were air dried, powdered (40 mesh), and stored at 28ºC.

MuBL, MuHL and MuLL were isolated according to the procedure reported by Napoleão et al. [15] and Sá et al. [21]. Tissue extracts from powdered bark, heartwood, and leaf (10 g) in 0.15 M NaCl (100 mL) were obtained after homogenization in a magnetic stirrer (16 h, 25°C), followed by filtration through gauze and centrifugation (3,000 *g*, 15 min). Proteins in the crude extracts were fractionated with ammonium sulfate according to Green and Hughes [22]. The 40% supernatant fraction from bark extract, 40-60% fraction from heartwood extract, and 60-80% fraction from leaf extract were dialyzed (3,500 Da cut-off membrane, 4 ºC) against distilled water (4 h) followed by 0.15 M NaCl (4 h) and separately loaded onto chitin columns (7.5 × 1.5 cm) equilibrated with 0.15 M NaCl (100 mL) at a flow rate of 20 mL/h. The unabsorbed proteins were eluted with equilibrating solution until the absorbance at 280 nm was negligible. Then, the adsorbed proteins (MuBL, MuHL, or MuLL) were eluted with 1.0 M acetic acid and dialyzed (8 h, 25°C) against 0.15 M NaCl (2 L, three

changes of solution) for eluent elimination. Protein concentration was estimated according to Lowry et al. [23] using bovine serum albumin (31.25–500 µg/ml) as the standard. Carbohydrate-binding ability of the lectins was analyzed by a hemagglutinating activity assay [15] using rabbit erythrocytes (collection authorized by the Ethics Committee of Animal Experimentation of *Universidade Federal de Pernambuco*; process number 23076.033782/2015-70).

2.3. Termiticidal assays and fixation of midguts

Termiticidal activity was performed in petri dishes, which were maintained in the darkness at 28ºC. The lectins were incorporated into artificial diets according to their previously reported LC_{50} concentrations (4 days) i.e., 0.97, 0.25 and 0.37 mg/mL for MuBL, MuHL and MuLL, respectively [15]. To prepare the artificial diet, a lectin solution was mixed with a 20% (w/v) cellulose matrix (Avicel) solution [24] and the mixture was placed (1 mL) in petri dishes. The dishes were kept in the incubator (56°C) for 24 h to allow drying of the disks. Next, 16 workers were transferred directly from the nest to each petri dish. For the control, an artificial diet without lectins was used. Two independent assays were performed in triplicates. The insects were exposed during 48 h because mortality of workers occurs only from the third day after the beginning of ingestion of lectins [13, 15]. After the treatments, workers were immobilized by placing them at 20ºC for 10–15 min, and the midguts (20 per treatment) were dissected in a physiologic solution for insects (0.1 M NaCl, 20 mM KH_2PO_4, 20 mM Na_2HPO_4). The dissected midguts were fixed in 4% paraformaldehyde for 3 h and maintained in 0.1 M phosphate-buffered saline (PBS), pH 7.4.

2.4. Histology analysis

Ten midguts each from the control, MuHL, MuLL, and MuBL treatments were washed with PBS, dehydrated in a graded series of ethanol (70–100%), and embedded in Historesin (Leica, Solms, Germany). The tissue was cut into 3-μm sections, stained with toluidine blue 1% (w/v), and mounted in Eukitt medium (Fluka, USA). The stained midguts were observed under an optical microscope (Olympus BX60, Olympus America, Inc., NY, USA) and photographed using a digital camera (Olympus Q-Color 3 - Olympus America, Inc., NY, USA).

2.5. Midgut analysis by fluorescence microscopy

For fluorescence microscopy analysis, 25 midguts each from the control, MuHL, MuLL, and MuBL treatments were washed three times for 30 min with 1% PBST (phosphate buffered saline with 1% Tween; Sigma-Aldrich, USA). After washing, the midguts were incubated for 24 h at 4°C with the following primary antibodies, diluted in PBS: (1) anti-FMRFamide (1:500) (Peninsula Lab, UK), (2) anti-phospho-histone H3 (1:100) (Cell Signaling, USA), (3) anti-caspase-3 (1:500) or (4) anti-peroxidase (1:800) (both from Sigma-Aldrich, USA) antibodies. After incubation, the midguts were washed three times with PBS and then incubated for 24 h at 4°C with a solution (1:500) of anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma-Aldrich, USA) diluted in PBS. The midguts labeled for caspase-3 and FMRFamide were washed again, and cell nuclei were stained for 1 h with TO-PRO-3 iodide (Life Technologies, USA); next, these midguts were washed three times, mounted with Mowiol antifading solution (Sigma-Aldrich, USA),

and observed under a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany). Cell nuclei in midguts labeled for phospho-histone H3 and peroxidase were stained with diamidino-2-phenylindole (DAPI) (Biotium, USA), cut into 7 μm-thick histological sections, mounted on slides using Mowiol, and observed under an epifluorescence microscope (Olympus BX53 coupled with an Olympus DP 73 digital camera).

For the detection of the PM, which contains glycoconjugates and polysaccharides with β-1-4 N-acetyl-glucosamine residues, five midgut sections from each treatment were washed with PBS two times for 10 min and incubated for 30 min with WGA-FITC (wheat germ agglutinin conjugated to FITC) (Sigma Aldrich, USA). The sections were washed again, mounted using Mowiol solution, and analyzed under the epifluorescence microscope (see above).

As a negative control, five midguts were used and treated as described above, excluding the primary antibody incubation. As negative controls for WGA-FITC staining, histological sections of midguts were stained with DAPI and mounted with Mowiol solution.

2.6. Morphometric and statistical analyses

The digestive and stem cells were counted using six fields of longitudinal DAPI-stained sections per midgut, which were visualized with a 40× objective lens (total area: 0.414 mm^2) [25]. The morphometric analyses were performed with the image analysis program Image Pro Plus 4.0 for Windows (Media Cybernetics). Standard deviations (SDs) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San

Diego, CA, USA) and data have been expressed as mean of replicates ± SD. Significant differences between treatments were analyzed by ANOVA (significance at $p < 0.05$).

3. Results

Histology analysis revealed remarkable disorganization of the midgut epithelium of *N. corniger* workers that were fed diets supplemented with MuBL, MuHL, or MuLL for 48 h (Figure 1). In the treated midguts, debris and deformed stem cells were observed in the lumen. The brush border was not visualized in the digestive cells of the insects treated with MuHL and MuLL while the digestive cells were not seen in the midgut of termites from MuBL treatment (Figure 1).

The proliferating cells could be observed in the midgut of workers from control and MuHL treatments (Figure 2), while no staining was seen in midguts from insects that ingested MuLL or MuBL. The stainings with DAPI and the anti-PH3 antibody were used to determine the number of digestive and proliferating cells, respectively, in the midgut. The number of digestive cells was 87.0–89.5% lower in lectin treatments in comparison with the control (Figure 3A), while the number of PH3-positive cells was 80.0-83.2% lower in lectin treatments than for the control (Figure 3B). The numbers of digestive or proliferating cells were not significantly different ($p > 0.05$) between the lectin treatments.

FMRF-immunoreactive cells were also noted in the midgut of control insects, but they were not observed in the midgut of lectin-treated termites (Figure 4). On the other hand, the staining for caspase-3 revealed a large number of apoptotic cells in the midgut of termites that ingested the lectins, confirming the induction of cell death (Figure 5).

Staining for peroxidase was observed in the muscles but not in digestive and stem cells of the control midguts; on the other hand, peroxidase positivity was detected in the midgut epithelium after treatments with the lectins (Figure 6). Labeling of PM with WGA-FITC revealed that the PM was observed in the control and MuHL treatments, but not in the midgut of workers that ingested MuLL or MuBL (Figure 7).

4. Discussion

To our knowledge, this work reports by the first time the effects of plant lectins on the midgut of a termite species. The *M. urundeuva* heartwood is resistant to the attack of termites and it has been shown that this resistance can be related to a termiticidal lectin (MuHL) [13, 14]. Further, two other lectins with termiticidal activity were also isolated from the bark (MuBL) and the leaf (MuLL) of this plant [15].

The midgut damage observed in termites that ingested *M. urundeuva* lectins corroborate our hypothesis that these proteins act at the level of the digestive tract. These proteins showed different toxicity levels in accordance with the previously determined LC_{50} values, with MuHL showing the lowest LC_{50} value, followed by MuLL and MuBL [15]. In the present work, the three lectins were assayed at their respective LC_{50} values in order to standardize their degree of effectiveness; however, it was observed that MuBL promoted more drastic structural alterations in the *N. corniger* worker midguts than the other two lectins. The reduction in the number of digestive and stem cells in the midgut of *N. corniger* workers treated with MuBL, MuHL, and MuLL may impair the epithelial renovation, digestion, and absorption processes, compromising the nutrition of insects. In a termite

colony, the impairment of digestion of workers has an additional impact, since the workers provide pre-digested food to other castes in the colony [26].

Enteroendocrine cells were not visualized in the midguts of termites treated with *M. urundeuva* lectins. These cells synthesize and secrete peptides, monoamines, and other substances that exert paracrine effects in stem and digestive cells, influencing the production of enzymes, pheromones, and defensive compounds [27–29]. Thus, the termiticidal activity of *M. urundeuva* lectins may result from damage to enteroendocrine cells and the consequent impairment of digestive and endocrine processes of the insects.

Ingestion of the *Ostrinia nubilalis* lectin by the larvae of the cotton leafworm *Spodoptera littoralis* (Lepidoptera) promoted disruption of the PM scaffold, leading to the hypersecretion of many disorganized layers of PM into the midgut lumen and the presence of many disintegrated microvilli [30, 31]. The results obtained in the present work show that the *M. urundeuva* lectins promoted PM disruption but the effect differed from that for *O. nubilalis* lectin in the lepidopteran since the synthesis of new PM layers did not occur in *N. corniger* workers as a mechanism of compensation; this is probably due to the death of the digestive cells. The disruption of PM could enable the lectins to come into contact with the epithelium and cause damage to epithelial cells from the midgut of *N. corniger* workers. The passage of lectins through the PM has been reported for other lectins that exert toxic effects by interaction with the midgut epithelium [7], leading to abnormalities in the PM and disruption of microvilli [6].

The *M. urundeuva* lectins promoted reduction in the number of cells in the *N. corniger* workers midgut, possibly by the activation of caspase 3, an apoptosis related enzyme, as previously reported for the lectin of the fungus *Sclerotium rolfsi* in the midgut of *Spodoptera litura* larvae (Lepidoptera) [32]. Lectins have been reported to be able to induce cell apoptosis

by binding to glycosylated proteins at the cell surface, thereby activating cell death pathways; similarly, it has been reported that some lectins can be internalized into the cells triggering cell death mechanisms [33]. For instance, lectins isolated from the plant *Sambucus nigra* and from the fungus *Rhizoctonia solani* induced apoptosis of cells isolated from the midgut of the lepidopterans *Spodoptera littoralis* and *Choristoneura fumiferana* via the activation of caspases 3, 7, 8 and 9 [34, 35].

The midgut epithelium of *N. corniger* workers that ingested *M. urundeuva* lectins showed positive staining for peroxidase, which was not observed in control midguts. Herbivorous insects are often exposed to allelochemicals and toxic compounds produced by plants as a defense against herbivory. There is usually an increase in the production of reactive-oxygen species when these substances are ingested, which consequently activates antioxidant defense systems that may include antioxidant enzymes, such as peroxidases [36, 37]. Thus, it is probable that the presence of toxic *M. urundeuva* lectins in the *N. corniger* midgut caused oxidative stress leading to the expression of peroxidase-type enzymes.

5. Conclusion

This study showed that termiticidal lectins from *M. urundeuva* bark, heartwood, and leaf could promote severe injuries as well as induce oxidative stress and cell death by apoptosis in the midgut of *N. corniger* workers, affecting different cell types and PM. These results contribute significantly toward the understanding of termiticidal activity of *M. urundeuva* lectins.

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Figure captions

Figure 1. Toluidine blue-stained histological sections of the midgut of *Nasutitermes corniger* workers from controls and individuals fed a diet supplemented with MuHL, MuLL, or MuBL. Midgut of control workers showed an epithelium (ep) comprised of height digestive cells (n, digestive cell nuclei) with intact brush border (b), and grouped stem (s) cells. l, midgut lumen; m, muscle. Midgut from workers treated with MuHL, MuBL or MuLL showed intense disorganization, including a thin epithelium (ep) without brush border and with deformed stem cells (s). Notice that in treated individuals, clusters of stem cells are not prominent as in the control. Tissue/cell debris is seen in the midgut lumen (l).

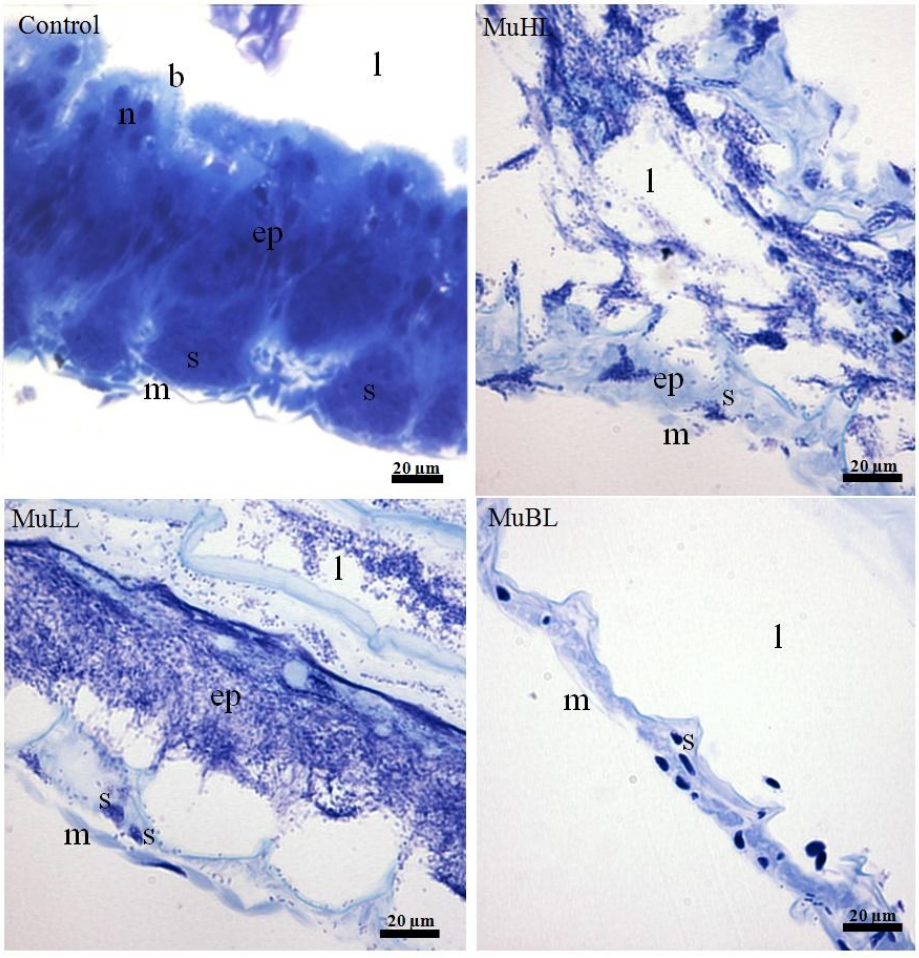


Figure 2. Investigation of proliferating stem cells in the midgut of *Nasutitermes corniger* workers from control and individuals fed on diet supplemented with MuHL, MuLL or MuBL. The nuclei of all epithelium cells were stained with DAPI (blue fluorescence). The nuclei of proliferating stem cells were green stained as phosphohistone H3-positive (PH3).

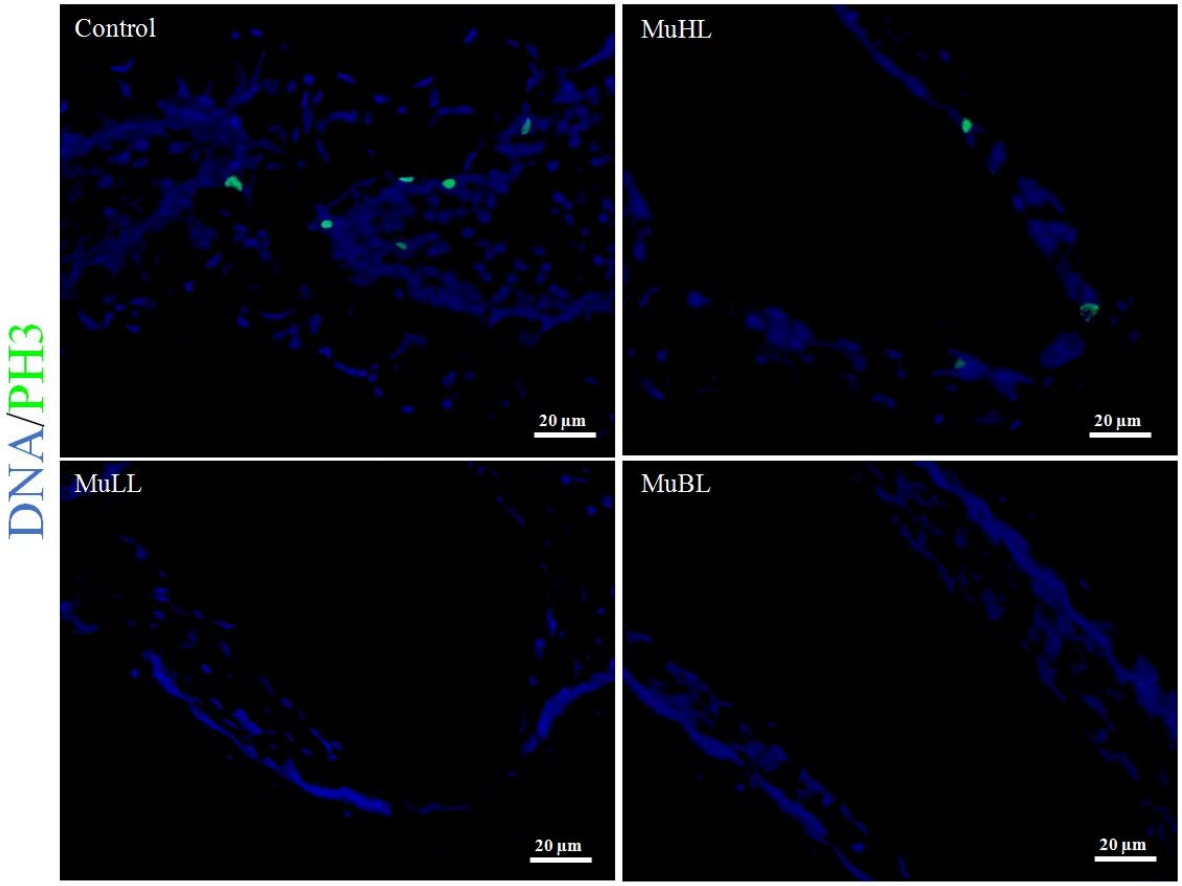


Figure 3. Number of digestive (A) and mitosis/proliferating (B) cells in midguts of *Nasutitermes corniger* workers from control and lectin treatments, determined by DAPI and PH3 stainings. Different letters indicate significant differences ($p < 0.05$) between treatments.

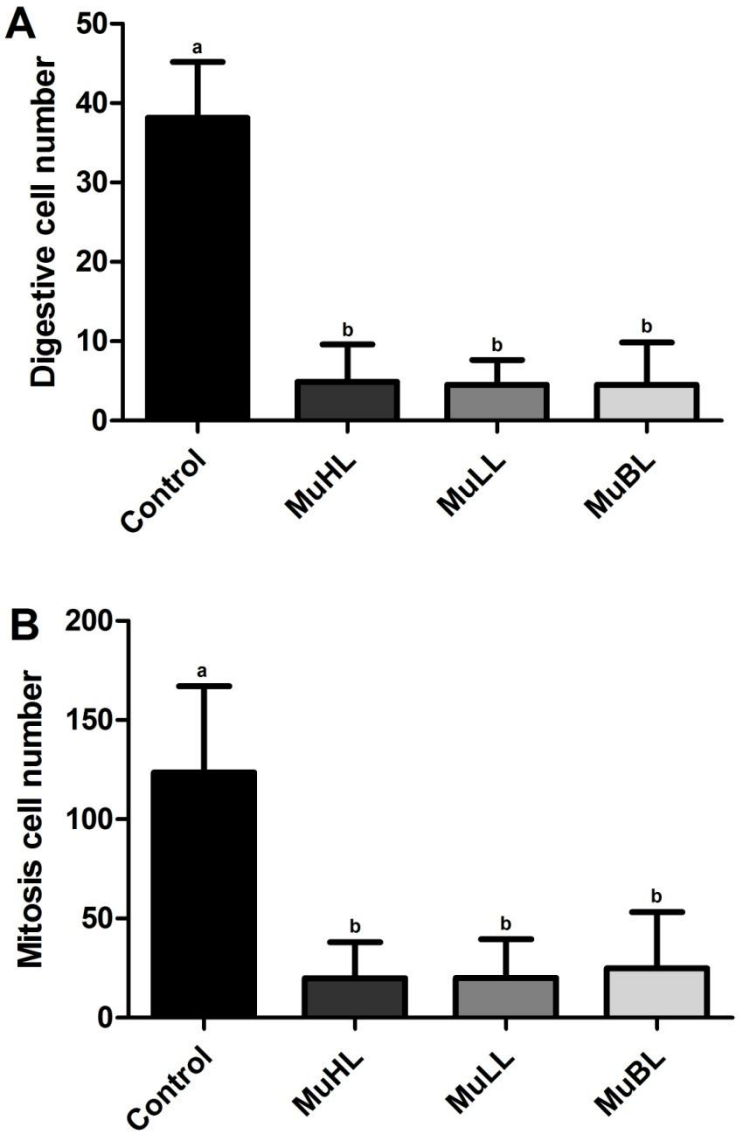


Figure 4. FMRF-immunoreactive cells in the midguts of *Nasutitermes corniger* workers. Control represents the midgut from worker fed with artificial diet without lectins and enteroendocrine cells positive for the FMRF peptide (green) were observed close to stem cell clusters (*). The nuclei of epithelium cells were stained with TO-PRO-3 iodide (red). Enteroendocrine cells were not visualized in the disorganized midgut of insects that ingested the *Myracrodruon urundeuva* lectins.

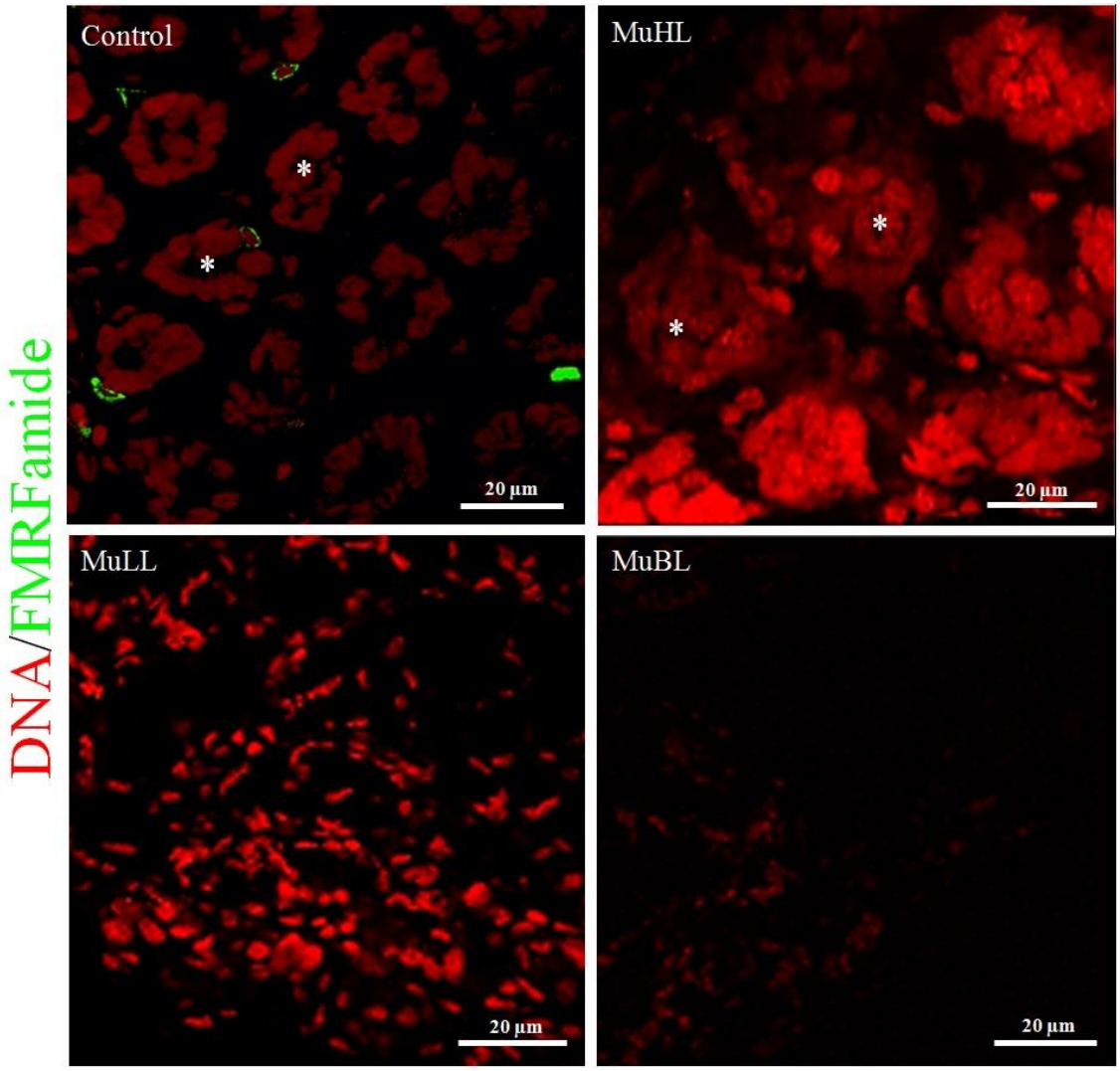


Figure 5. Labeling of caspase-3 (green fluorescence) in the midgut of *Nasutitermes corniger* workers. No labeling was observed in the midgut of workers that fed on artificial diet without lectins (control). Midgut from workers treated with MuHL, MuLL and MuBL showed intense labeling for caspase-3, indicating cell-death via apoptosis. The nuclei of epithelium cells were stained with TO-PRO-3 iodide (red).

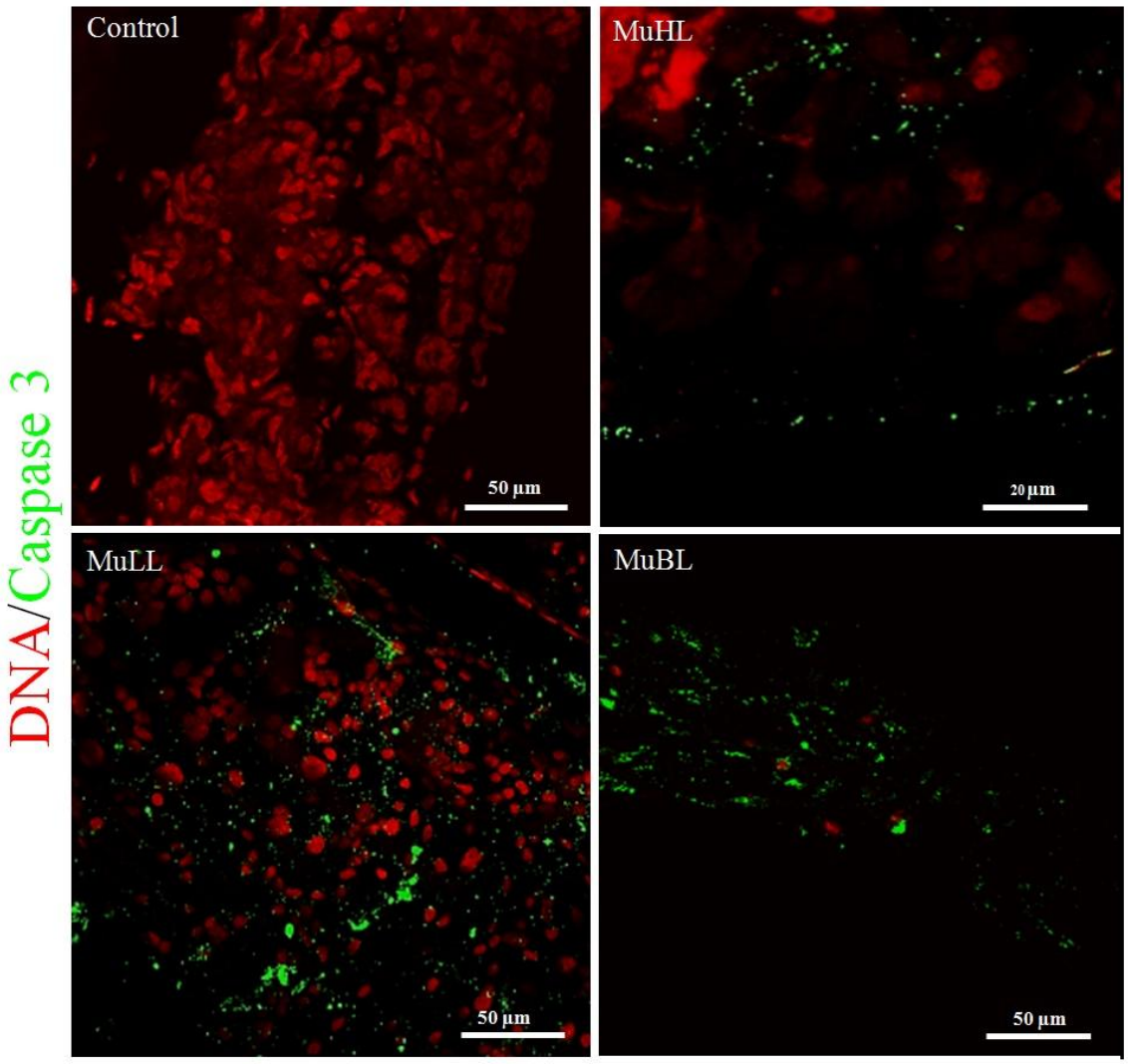


Figure 6. Labeling of peroxidase (green fluorescence) in midgut of *Nasutitermes corniger* workers. Peroxidase staining is seen only in the muscle in control midguts. In midgut from workers treated with MuHL, MuLL, and MuBL, there is an intense staining in the epithelium. The nuclei of epithelium cells were stained with DAPI (blue).

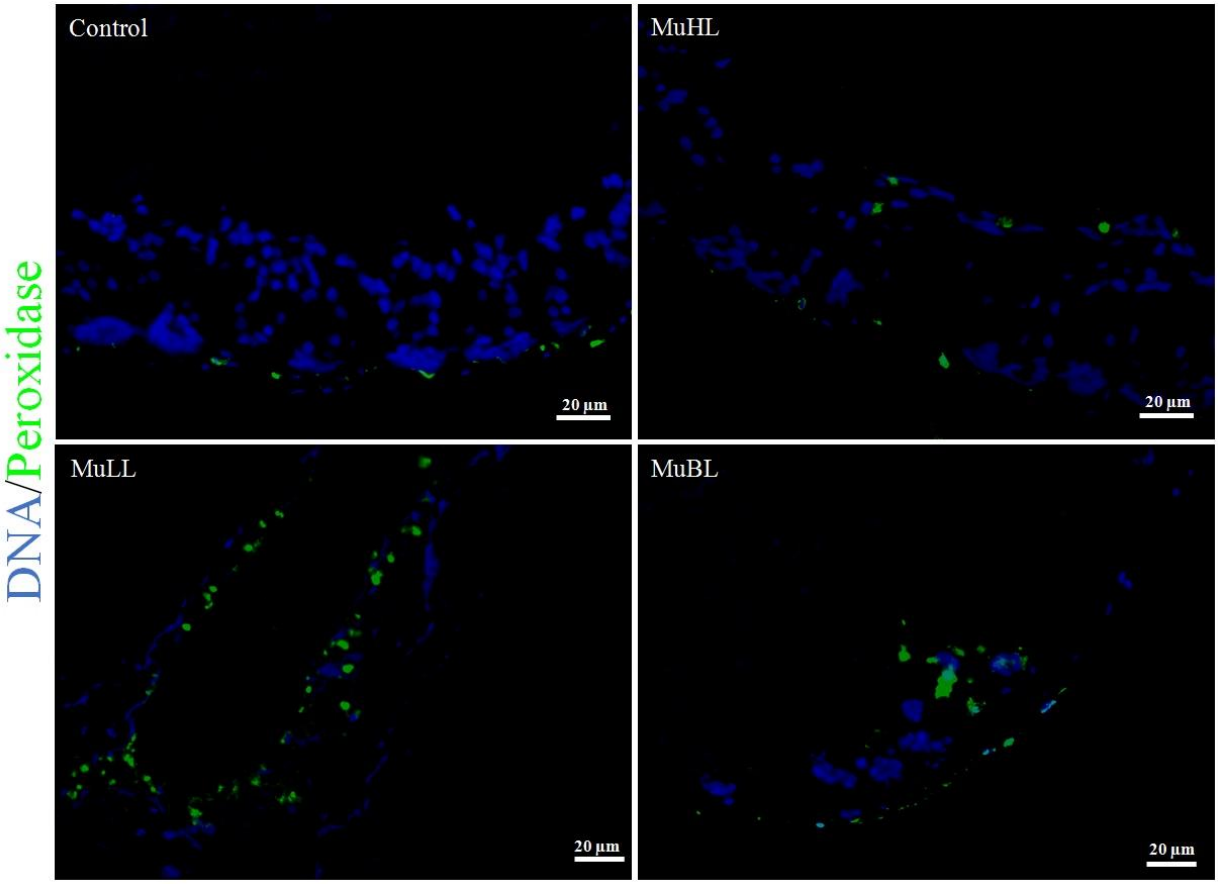
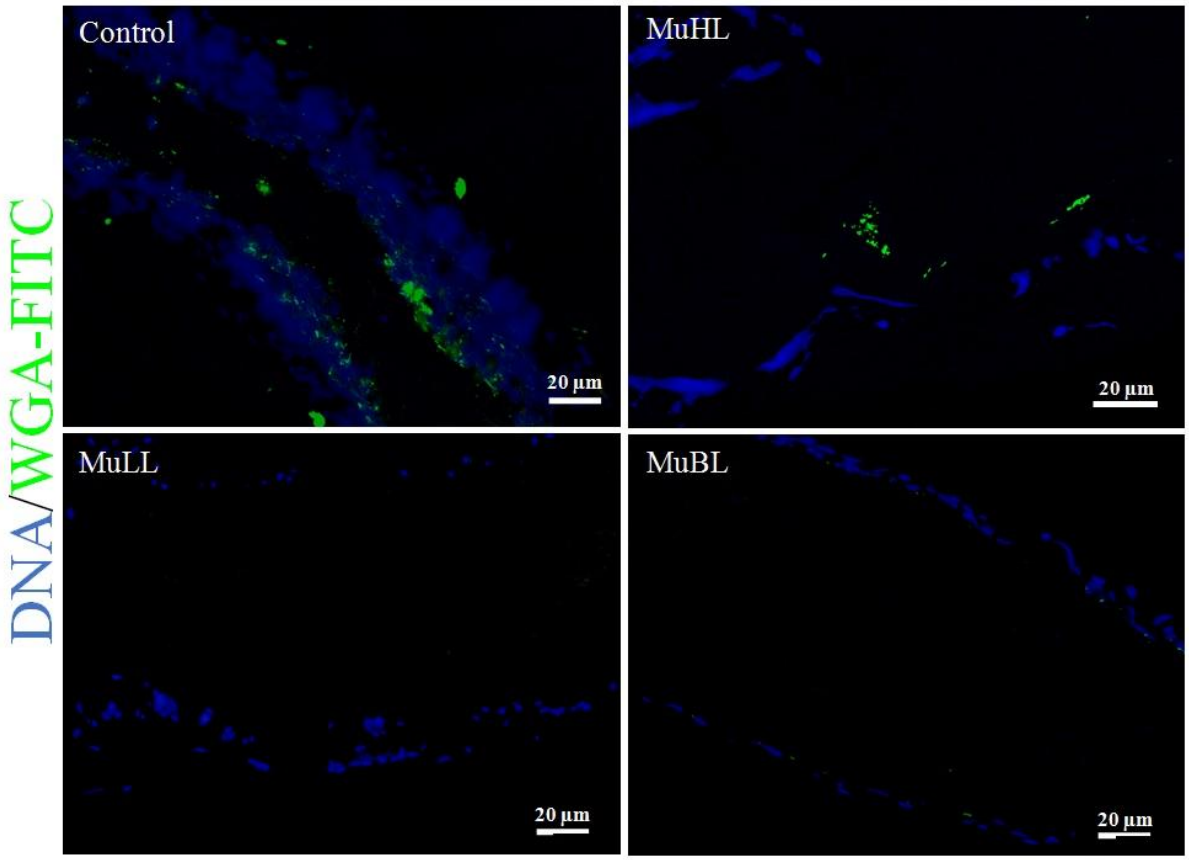


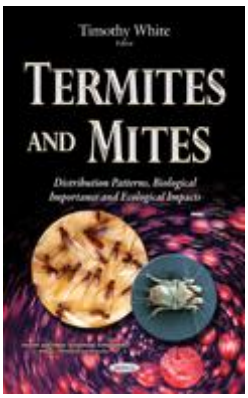
Figure 7. Midguts of *Nasutitermes corniger* workers stained with WGA-FITC, for the peritrophic matrix (PM) labeling. The sparse PM (green) is present in the control and in MuHL-treated midguts. In the midguts of insects treated with MuLL and MuBL, the PM is not seen. The nuclei of epithelium cells were stained with DAPI (blue).



8. CAPÍTULO DE LIVRO

An overview on the interface between feeding habits, ecological role and digestive processes of termites

Capítulo publicado no livro *Termites and Mites: Distribution Patterns, Biological Importance and Ecological Impacts*, Timothy White (editor), Nova Science Publishers, Inc., Nova York, ISBN: 978-1-63484-008-8



Chapter 1

AN OVERVIEW ON THE INTERFACE BETWEEN FEEDING HABITS, ECOLOGICAL ROLE AND DIGESTIVE PROCESSES OF TERMITES

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ABSTRACT

This chapter discuss on the connections between the digestive processes of termites and their feeding habits and ecological role. The termites are believed to be the oldest eusocial insects and their morpho-physiologically distinct castes are called reproductives, workers, soldiers and immature forms. Termites are called xylophages or wood-decaying insects but can also feed on a variety of organic materials, including grasses, litter, humus, and components of the soil, such as hydrolysable peptides and polyphenolic compounds. These specialized feeding habits of termites are linked to their digestive apparatus and allow them to act as primary consumers and decomposers (herbivores and detritivores). The ability of termites to digest lignocellulosic materials is due to the presence in their gut of a set of digestive enzymes, which can be produced by the insect or by symbiotic microorganisms (flagellate protozoa and bacteria). Digestive enzymes found in termite gut are cellulases (endoglucanases, exoglucanases, and β-glucosidases), hemicellulases (endoxylanases, exoxylanases, β-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, mannosidases, and mannanases), α-amylases, and proteases. Due to their high efficiency, the lignocellulolytic natural systems of termites have been considered important models to be studied for use in biofuel production technology. Digestive enzymes of termites are also targets of studies focusing on the discovering of new compounds for use in control of termite pests.

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1. INTRODUCTION

The Class Insecta (Phylum Arthropoda) is considered the most successful of Animal Kingdom. The Infraorder Isoptera of the Order Blattodea comprises insects known as termites, which possess well developed mouthparts of the chewing type (Figure 1A), a free head (Figure 1B and 1C) with variable shape and size. In some species, compound eyes are present usually in the winged forms and workers. In the head, there is a depression called fontanelle, which contains a pore linked to a cephalic gland that secretes a liquid with defensive role. The thorax is flattened and divided in prothorax, mesothorax, and metathorax. The tympanal organ is located in the legs, in the anterior tibia. The abdomen (Figure 1B and 1C) is bulky, sessile and possess ten segments(Grassé, 1949; Gallo et al., 1988).

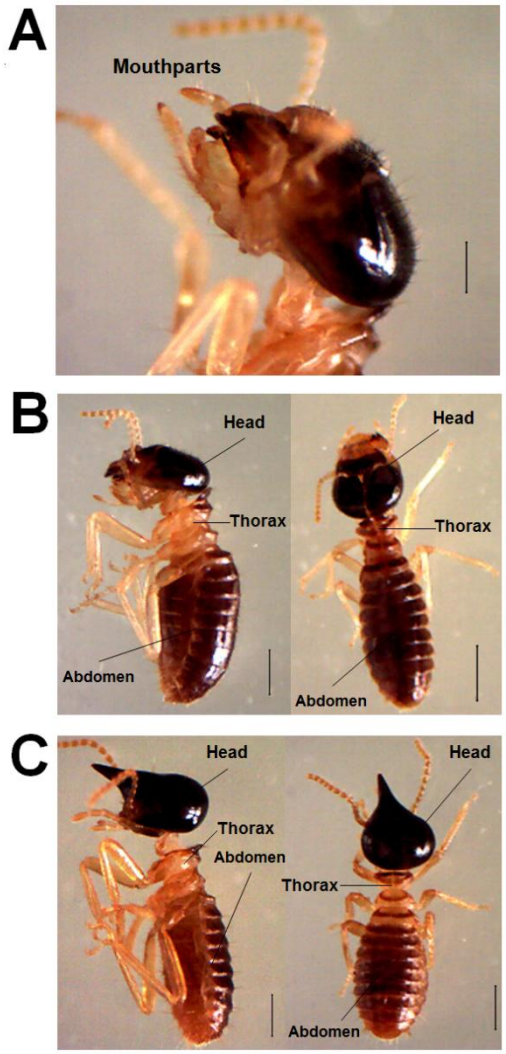

Figure 1. Termites from the species *Nasutitermes corniger*. The images show the mouthparts of the chewing type (A) and lateral and dorsal views of a worker (B) and a soldier (C). The bars correspond to 0.2 mm (A) or 0.5 mm (B and C).

Evidences obtained through analysis of molecular and morphological markers indicates that termites are a sister group of Cryptocercidae (wood roaches or wood cockroaches). It is believed that termites are the oldest eusocial insects because their complex societies dating back from the Cretaceous

(130 million years ago). Termite fossils shows that they were already eusocial insects, suggesting an origin in the Late Jurassic or even prior to the Pangea breakup in the Early Mesozoic (Lo et al., 2000; Thorne et al., 2000; Grimaldi and Engel, 2005; Meyer, 2005; Bordy et al., 2009).

The infraorder Isoptera includes the families Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae, Serritermitidae, and Termitidae (Grassé, 1986; Kambhampati and Eggleton, 2000). Following a phylogenetic classification, the termites can be considered as lower termites (Mastotermitidae, Kalotermitidae, Hodotermitidae) or higher termites (Termopsidae, Rhinotermitidae, Serritermitidae, and Termitidae) (Aanen et al., 2002; Verma et al., 2009). Engel et al. (2009) also listed the families Cratomastotermitidae, Archotermopsidae, Stolotermitidae, Archeorhinotermitidae and Stylotermitidae, in addition to those mentioned above. These authors also separated the termites in two groups: Euisoptera, including the families more closely related to the wood cockroaches, and Neoisoptera, which includes the Rhinotermitidae, Serritermitidae, Archeorhinotermitidae, Stylotermitidae and Termitidae families.

The termites are divided in morpho-physiologically distinct castes: winged reproductives (alates), workers, soldiers and immature forms (larvae). The larvae may evolve for soldiers, workers and nymphs; these last will originate the reproductives. Termites are diploid and individuals from both sexes are present in the sterile castes. Also, they undergo a hemimetabolous metamorphosis (Figure 2) involving gradual changes without pupal stage. The reproductive forms represent the fertile caste and may be subdivided in primary or complementary reproductives. The primary reproductives have well developed wings and are responsible for dispersion through nuptial flights. After the flight, the couple lost their wings and become the king and the queen, which are pigmented and responsible for egg production. In most colonies, there are only a couple of primary reproductives. If the primary reproductives die, they are replaced by a couple of complementary reproductives, which are usually more pigmented than the workers. Sterile castes (workers and soldiers) are wingless and usually did not have eyes. The workers are responsible for foraging and building of the nest tunnels and galleries; also, they have the task of feeding the other colony members. Soldiers differ by having a black and enlarged head, with nearly the half of body length and a well noticeable jaw; they are responsible for the safekeeping of the colony (Krishna and Weesner, 1969; Thompson, 2000; Philip, 2004; Myles, 2005).

2. FEEDING STRATEGIES OF TERMITES

Termites are called xylophages or wood-decaying insects because it is usually assumed that all termites are consumers of woods, alive or dead. However, these insects may feed on a wide variety of organic materials, in various stages of decomposition, such as grasses, herbaceous plants, litter, fungi, other termite nests, dung, carrion, lichens and even organic components of the soil (Lima and Costa-Leonardo, 2007).

The evolutionary key and the ecological elements (colony size, life histories, and habitats) in the biology of termites are associated with the availability of food resources (Abe, 1987; Lenz, 1994). A wood-based diet has some disadvantages, such as low nitrogen content, wood hardness and presence of toxic compounds. However, termites are able to feed on wood and other materials considered of low nutritional value because they developed appropriate digestive mechanisms to extract the maximum of nutrients (Waller and La Fage, 1986). Some members of Nasutitermitinae (Termitidae) are specialized to feed on humus, carbohydrates, soil microbes, hydrolysable peptides, polyphenolic compounds and hexosamines derived from remains of arthropods and fungi (Collins, 1983; Anderson and Wood, 1984; Bignell and Eggleton, 2000; Ji and Brune, 2005).

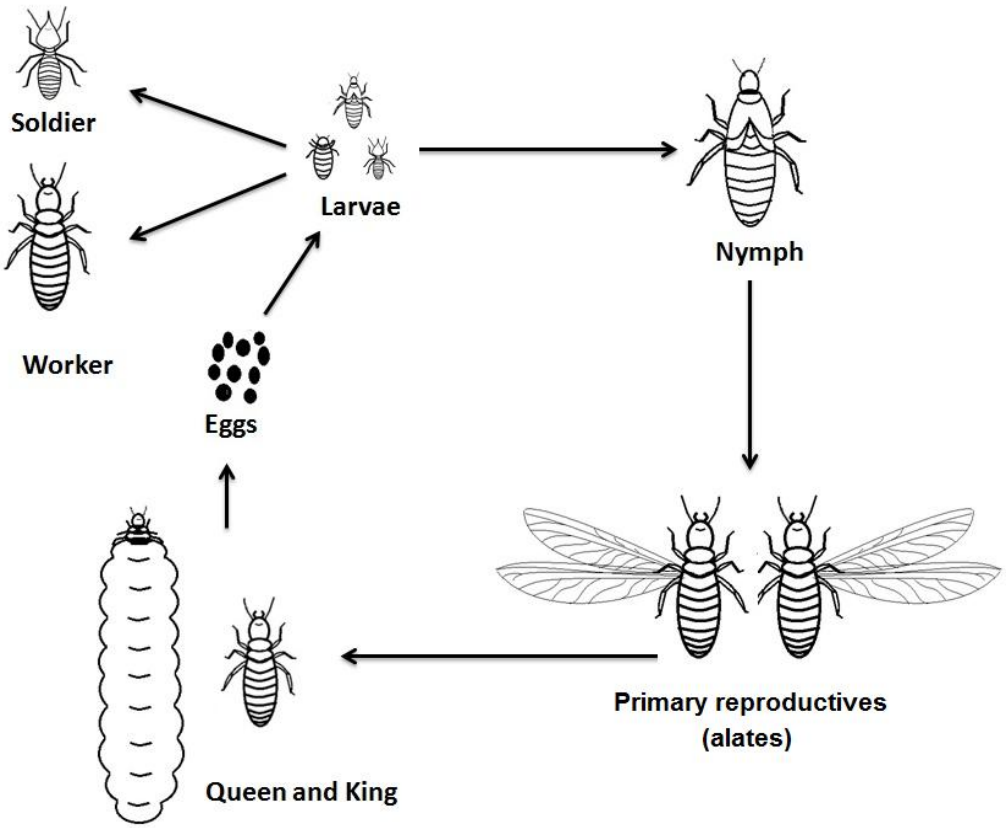

Figure 2. Life cycle of termites and representations of the castes found in a termite nest.

The digestive system of termites usually occupies a large part of their abdomen (Krishna & Weesner, 1969) and is composed of three parts: foregut, midgut and hindgut. The immature individuals, the soldiers and the reproductives are unable to feed themselves and receive from workers undigested or partly digested food. This food can have an estomodeal origin, such as saliva or regurgitated food, or a proctodeal origin, consisting mainly in liquid excretions, usually rich in symbionts, that are disposed in response to tactile stimuli by other termites (Lima and Costa Leonardo, 2007).

The termites are able to grind the cellulosic or lignocellulosic materials and to digest them through the action of enzymes that may be also produced by symbiotic microorganisms present in the hindgut (Brune, 2014). Flagellate protozoa and symbiotic bacteria collaborate with digestion of cellulose by producing endoglucanases, exoglucanases and β-glucosidases (Bresnak and Brune, 1994). The hemicellulases are produced by termites in order to digest the hemicellulose fraction of their food and the secretion of proteases in the midgut allows the release of amino acids from food proteins (Brune, 2014). The microbiota in termite gut is able to convert the carbohydrates from wood fibers to short-chain fatty acids and gut flagellates can also produce lactate. The digestion of lignin by termites remains unclear but there are evidences of the action of some enzymes. Lignin-rich residues after digestion are voided as feces but can also be passed to nestmates by proctodeal feeding (Brune, 2014). More details on the enzymes present in digestive apparatus of termites are described in the next section.

3. Digestive Enzymes of Termites

3.1. Cellulases

Carbohydrates are essential nutrients for insects, providing the necessary energy for the optimal larval growth and to maintain the longevity of adults. However, the nutritional value of materials rich in

polysaccharides depends on the availability of enzymes capable of digesting complex carbohydrates and then become the monomers suitable for absorption in the intestine of insects (Dadd, 1985; Terra et al., 1996).

The cellulose is a linear polymer composed by glucopyranoses linked by β1→4 glycosidic bonds. The cellulose molecules are randomly oriented and have the tendency to form inter and intra molecular hydrogen bonds (Rowell et al., 2005). Models of microfibrillar organization indicate that the cellulose is composed by two types of domains: crystalline and amorphous; the crystalline regions are formed due to an increased packing density (Taiz and Zeiger, 2004). Cellulases are enzymes responsible for degrading cellulose or other cello-oligosaccharides to glucose and can be classified as endoglucanases, exoglucanases and β-glucosidases.

The ability to digest cellulose, due to the action of cellulolytic enzymes, confers to termites important ecological roles that will be discussed later. On the other hand, this same ability are linked to the fact that a little of termite species are pests, being able to cause damages to several types of materials and buildings.

The digestion of cellulose starts by the action of endoglucanases secreted by the salivary glands or by the midgut. Although cellulose is a major food source for termites, the amount of cellulases produced by the own termite is not enough and, therefore, they depend on symbiotic microorganisms which produce cellulolytic enzymes. Flagellate protozoa are present mainly in lower termites and belong to the classes Parabasalia (genera *Trichonympha*, *Calonympha* and *Tricercomitus*) and Preaxostyla (genus *Pyrsonympha*). The bacterial symbionts are mainly spirochetes (phylum Spirochaetes), which have a remarkable motility and are present in high abundance and diversity in the hindgut of most wood-feeding termites. Other symbiotic microorganisms found in termite gut are smaller bacteria and archaea. Most termites emit substantial amounts of methane produced by symbiotic archaea (Brune, 2014). Metagenomic and functional analysis of microbiota associated with the gut of a Termitidae species showed that there is a very diverse range of bacteria, such as spirochetes and fibrobacters, which possess genes related to the lignocellulose degradation and involved in the hydrolysis of xylan and cellulose (Warnecke et al., 2007).

Study reported that the lower termite *Neotermes koshunensis* produces cellulase in their salivary glands (Tokuda et al., 2002). In the higher termites, self-produced cellulolytic activity has been detected mainly in the midgut but also in the salivary glands (Hogan et al., 1988; Rouland et al., 1989; Slaytor, 2000).

Nasutitermes exitiosus is able to secrete its own cellulases and is not strictly dependent on the intestinal microbiota to digest cellulose, since cellulolytic activity was detected along all the gut even after removal of the intestinal microorganisms by feeding the termites with tetracycline (O'Brien et al., 1979). Other study showed that cellulolytic activity in the hindgut of *Nasutitermes takasagoensis* was significantly reduced after treatment with antibiotics, suggesting production of cellulases by bacterial symbionts (Tokuda & Watanabe, 2007). The involvement of microbial activity in cellulose degradation in *N. takasagoensis* was attributed to the presence of cellulolytic complexes called cellulossomes, which are anchored to the bacterial cell wall (Tokuda et al., 2005). Thus, the cellulolytic apparatus of *Nasutitermes* species is formed by enzymes with both endogenous and symbiotic origin.

The endoglucanases cleave the glycosidic bonds present within the cellulose molecule (Figure 3), creating randomly new ends (Sanchez, 2009). These enzymes hydrolyze amorphous regions of cellulose, which have a less structural organization and are more easily attacked because their chains do not have intermolecular hydrogen bonds as strong as those that occur in the crystalline regions. Thus, the attack of endoglucanases leads to a greater exposure of the internal glycosidic linkages of the cellulose chains (Medve, 1997; Zandoná-Filho, 2001).

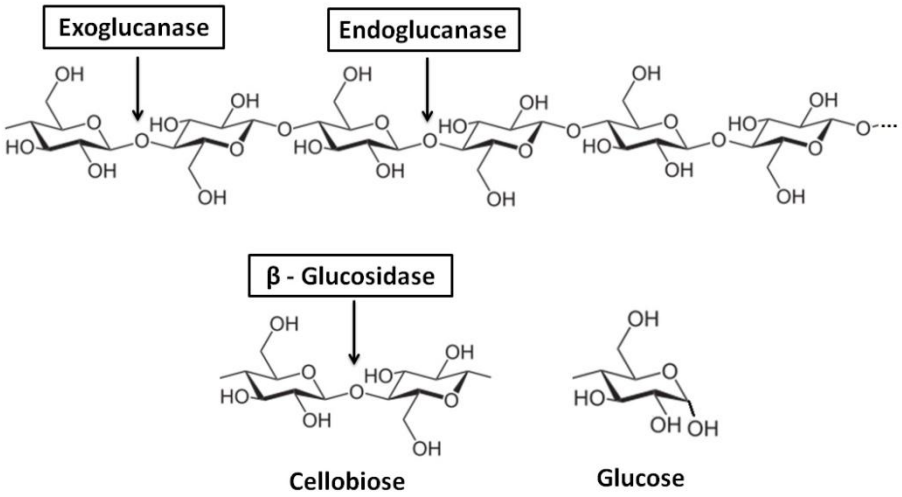

Figure 3. Schematic representation of the cleavage sites in cellulose molecule for endoglucanases, exoglucanases and β-glucosidases.

Exoglucanases act on the ends of cellulose chain (Figure 3), yielding mainly cellobiose but also glucose and cellotriose (Zandoná-Filho, 2001). The active site of exoglucanases has the form of a tunnel through which the cellulose chain penetrates and where the hydrolysis of glycosidic terminals undergoes (Hui et al., 2002). The exoglucanases act on crystalline cellulose, producing a slow and gradual reduction of the polymerization degree (Coughlan, 1992).

The β-glucosidases (or cellobiases) hydrolyze the cellobiose generated by endoglucanases and exoglucanases, completing the hydrolysis of cellulose (Figure 3). The β-glucosidases are extremely important to the efficiency of cellulolytic process because they remove the cellobiose, which is a potent competitive inhibitor of exoglucanases (Medve, 1997; Muñoz et al., 2001). In *Nasutitermes takasagoensis*, expression of messenger RNAs of endogenous β-glucosidases was detected in midgut epithelium (Tokuda et al., 1999).

Lima et al. (2014) reported that the gut of *N. corniger* workers and soldiers contain high levels of endoglucanases and exoglucanases, and a lower level of β-glucosidases (Figure 4A). In workers, the endoglucanase activity was 2.2-times higher than in soldiers, which is probably associated with the differences in feeding habits of these castes, since soldiers feed on material pre-digested by the workers.

3.2. HEMICELLULASES

The plant cell wall is composed of cellulose microfibrils that are embedded in a polysaccharide matrix composed by hemicellulose, pectin and lignin (Taiz and Zeiger, 2004; Sánchez and Cardona, 2008). The hemicellulose is a mixture of polymers composed by pentoses and hexoses (such as xylose, arabinose, glucose, mannose, and galactose) as well as uronic acids. The hemicellulose makes hydrogen bonds with the cellulose wrapping it in a network along with pectin and this coating prevents the collapse of cellulose fibers (Buckeridge et al., 2008; Rowell et al., 2005).

In addition to cellulose, termites are able to digest the hemicellulose fraction present in their food. The hemicellulases are enzymes that hydrolyze the hemicellulose in a random fashion, producing linear and branched oligosaccharides. Due to the great complexity and heterogeneity of the hemicellulose, the complete hydrolysis requires the action of distinct hemicellulolytic enzymes.

Xylans are usually degraded by a combined action of a set of enzymes (Figure 5). The endo-β-1,4-D-xylanases break glycosidic bonds randomly producing large amounts of xylo-oligosaccharides, substituted and unsubstituted, with various sizes. The exo-β-1,4-D-xylanases are responsible for the release

only of xylose units present at the ends of the xylan chain. The β-xylosidases hydrolyze the xylobiose and larger xylo-oligosaccharides (Battan et al., 2007).

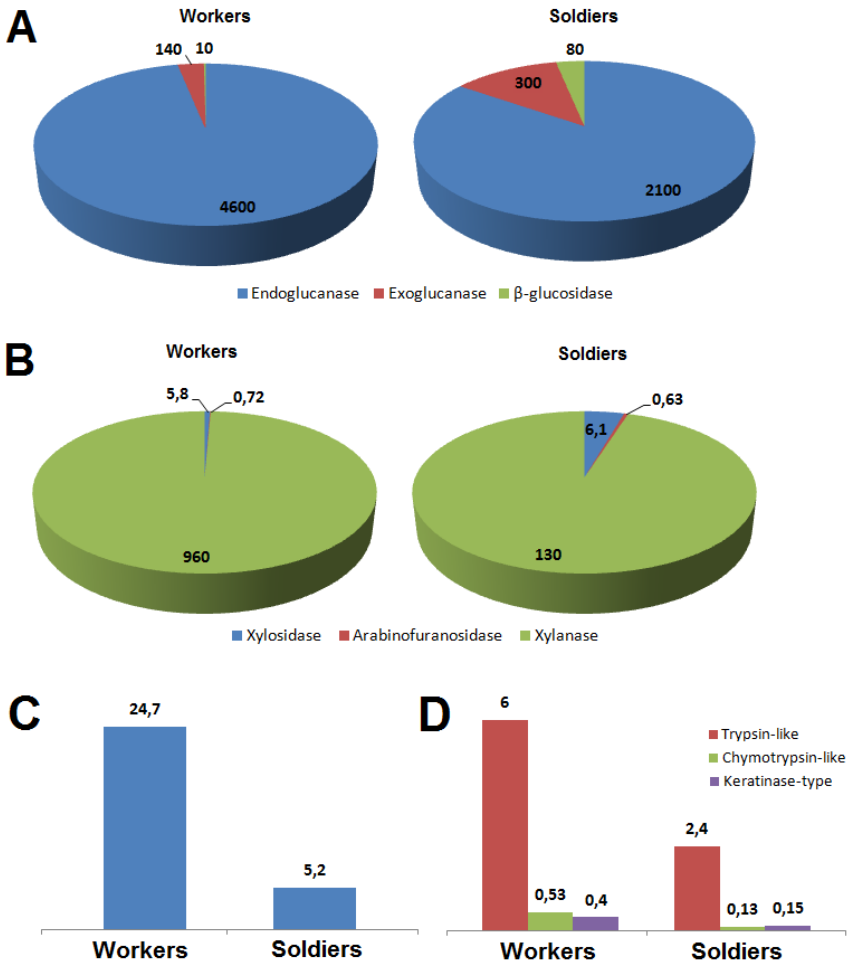

Figure 4. Digestive enzyme activities from gut extracts of *Nasutitermes corniger* workers and soldiers: cellulases (A), hemicellulases (B), α-amylase (C) and proteases (D). The values indicates the specific activities in mU/mg (for cellulases, hemicellulases and proteases) ou U/mg (α-amylase).

The α-arabinofuranosidases hydrolyze the glycosidic bonds in non-reducing terminals of polysaccharides containing L-arabinosyl residues (Figure 5) or L-arabinosyl residues from arabinoxylans, arabinan, arabinogalactan and arabic gum. The α-glucuronidases hydrolyze the bonds of side chains containing glucuronic acid residues (Figure 5). The α-D-glucuronidases act together with mannosidases, mannanases and other enzymes for the degradation of polymers such as arabinogalactan and galactoglucomannan (Saha, 2000; Moreira and Filho, 2008).

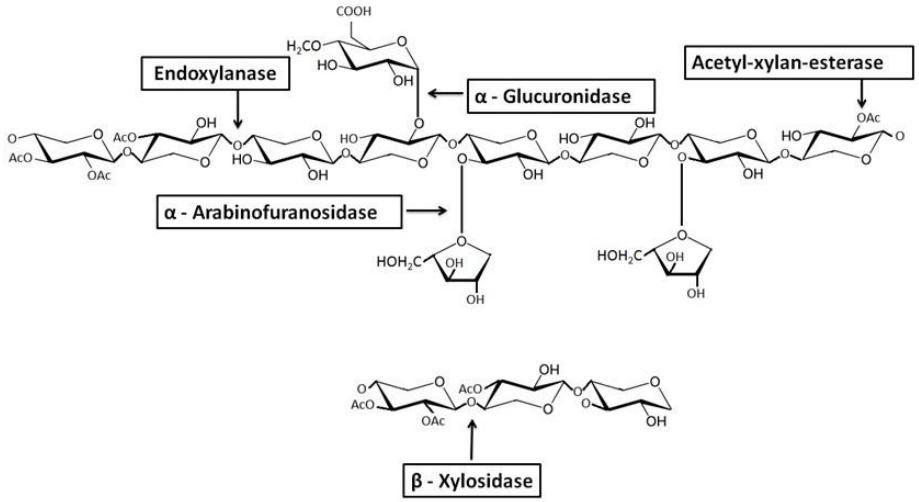

Figure 5. Schematic representation of hemicellulose molecule and the respective cleavage sites of hemicellulases.

Rouland et al. (1988) isolated two β-D-xylanases of *Macrotermes mulleri* (Termitidae, Macrotermitinae) workers, one from insect gut and other from its symbiont, the fungus *Termitomyces* sp. Activities of β-D-xylanases, α-Larabinofuranosidases and β-xylosidases were detected in the gut of *N. corniger* workers and soldiers (Figure 4B); only the xylanase activity was higher in workers than in soldiers while the other enzymes were found in similar levels between these castes (Lima et al., 2014). The xylan is the main component of hemicellulose and this may explain the highest level of this enzyme found by these authors. Also, the highest level of this enzyme in workers may also be due to the fact that this caste is responsible for the initial digestion of food that will be consumed by the other castes.

3.3. AMYLASES

The α-amylases (or α-1,4-glucan-4-glucanohidrolases) are a group of enzymes that catalyze the hydrolysis of glycosidic bonds (α1→4) present on starch, glycogen and other carbohydrates. Amylases are essential for the growth and development of many insects, especially in pests living in stocks of seeds and grains rich in starch.

Studies have been conducted in order to unravel the action mechanisms of α-amylases. The α-amylases are usually metalloproteins, containing at least one calcium atom per molecule and dependent of this metal to maintain its activity and stability (Fischer and Stein, 1960). In general, amylases can be glycosylated, but the proportion of glycosylation varies considerably (Planchot and Colonna, 1994).

The presence of α-amylases has been demonstrated in the digestive system of many insects, including members of the orders Orthoptera, Hymenoptera, Diptera, Lepidoptera and Coleoptera (Terra and Ferreira, 1994). However, there are few reports on amylase activity in termites. Lima et al. (2014) found amylase activity in gut of *N. corniger* workers and soldiers, being the enzyme level 4.75 times higher in workers (Figure 4C). Park et al. (2014) identified a complete amino acid sequence of amylase from *Reticulitermes speratus* through analysis of total mRNA. Tarayre et al. (2014) isolated a *Streptomyces* sp. strain from the gut *of Reticulitermes santonensis* which is able to produce amylase.

3.4. PROTEASES

Proteases are enzymes that catalyze the hydrolysis of peptide bonds and can be classified according to the region of the molecule on which they operate: exopeptidases hydrolyze peptide bonds in the N-terminal (aminopeptidases) or C-terminal (carboxypeptidases) regions while endopeptidases (or

proteinases) catalyze the hydrolysis of internal peptide bonds (Franco et al., 1999; Silva Junior and De Simone, 2001; Devlin, 2002). Also, proteases can be classified according to the International Union of Biochemistry into four major classes: serine proteases, cysteine proteases, aspartyl proteases, and metalloproteases. The first three classes are defined in accordance with the nucleophilic residue which hydrolyzes the peptide bond of the substrate (Hedstrom, 2002) and metalloproteases are so named because their catalytic mechanism involves the participation of a metal ion (Kleiner and Stetler-Stevenson, 1993).

Insects require essential amino acids to grow and develop and they are obtained from the proteins present in the diet. The midgut of insects contain a diversity of proteases, giving them the ability to consume and use efficiently a large variety of food resources, including wood, leaves, flowers, tubers, nectar, seeds, living or dead animals, blood, fungi and bacteria (Terra et al., 1996; Murdock and Shade, 2002; Fortunato et al., 2007).

In termites, proteolytic enzymes are involved in the digestion of proteins and peptide components present in the soil humic substances (Ji and Brune, 2005). Sethi et al. (2011) studied the presence of protease activity in foregut, midgut and hindgut of *Coptotermes formosanus* as well as evaluated the production of these enzymes by protozoa isolated from hindgut of workers of this species. They found that midgut showed the highest protease activity followed by protozoa extracts and the most of the proteases were serine proteases, highlighting trypsin-like and chymotrypsin-like activities. Lima et al. (2014) detected the activity of distinct proteases in the gut of *N. corniger*: trypsin-like, chymotrypsin-like and keratinase-type activities were detected in gut of workers and soldiers (Figure 4D). Protease levels were higher in workers of this species and trypsin-like enzymes were the main serine proteases detected.

3.5. LIGNIN DEGRADATION

It is known that some termites are able to depolymerize lignin and tolerate its toxic byproducts, while other species may promote minor modifications in lignin structure and others only eliminate the lignin in feces without alterations. Indeed, this is the most unclear aspect of the termite's digestive physiology. Termites of the subfamily Macrotermitinae (Termitidae) are associated with a symbiotic fungus able to degrade lignin (Hyodo et al., 2003). Sethi et al. (2013) found transcripts of lignases (enzymes able to depolymerize lignin) and phenoloxidases expressed by *Reticulitermes flavipes* and its symbionts. Also, the authors show evidenced on the participation of aldo-keto reductases and catalases as enzymes involved in detoxification of the toxic byproducts derived from lignin depolymerization.

4. ECOLOGICAL ROLE OF TERMITES

The digestive apparatus of termites allow them to have feeding habits that enable many environmental services. The termites act as primary consumers and decomposers (herbivores and detritivores), having a major ecological role in nutrient recycling through grinding, decomposition, humus production and mineralization of cellulosic resources and their variants, with about 74–99% of the cellulose and 65–87% of the hemicellulose being hydrolyzed and consumed by termites (Prins and Kreulen, 1991; Tayasu et al., 1997; Costa-Leonardo, 2002; Brune, 2014).

In the tropics, termites are considered the most important "engineers" of the soil ecosystem (Bignell, 2006). In arid and semi-arid climates, they are the main macroinvertebrate decomposers and exert an additional impact on the ground by creating biostructures (tunnels and galleries) with physical and chemical properties distinct of the soil that surrounds them. They influence the distribution of water and nutrients in the soil and, consequently, the diversity of microbes, plants and animals. One of the biggest effects of termites in the soil is their role in reducing soil density through a bioturbation process, which occurs when these insects make the vertical and horizontal transport of soil components from several depths for the construction of nests and galleries. In tropical savanna, termites are practically the only group of invertebrate scavengers that survive in dry season because of their ability to maintain constant the

temperature and humidity of their nests (Collins 1981, 1983; Whitford et al., 1992; Grant and Scholes, 2006; Jouquet et al., 2011).

The mechanical grind and enzymatic digestion of plant biomass by termites increase the contact surface for access by soil microorganisms when this biomass returns to the soil via feces (Jouquet et al., 2011). In tropical forests, termites contribute significantly in mineralization of litter through the digestion of large quantities of biomass (Bignell and Eggleton, 2000; Yamada et al., 2005; Freymann et al., 2010). When consuming the litter and soil organic matter, the termites reduce inputs that would be available for other routes of decomposition and, thus, they accumulate resources within their nests (Jouquet et al., 2011). The organic matter consumed by termites remains trapped in the nests, which are then referred as stable biogenic aggregates. The return of these material and minerals to the soil occurs via the feces, salivary secretions, decaying termite bodies, predation and erosion of the nest. The nests have high nitrogen levels, a high cationic exchange capacity and high concentration of nutrients and minerals (Ji and Brune, 2006; Brumer et al., 2009; Ngugi et al., 2011).

5. DIGESTIVE APPARATUSES OF TERMITES: MODELS FOR BIOFUEL PRODUCTION SYSTEMS AND TARGETS FOR PEST CONTROL

Currently, biofuels generated from lignocellulosic biomass have received extensive attention from industry and academic communities around the world, in line with the objective of promoting sustainable economies with preservation of the environment. However, the conversion of biomass in biofuel has been a challenge since it still has a high cost and not reasonably efficient for large-scale production; therefore, it has grown the search for new viable and cost-effective technologies.

The resistant and insoluble plant cell walls constitute the main problem, since they are formed by a complex and stable mixture of cellulose, hemicellulose and lignin. Usually, chemical pretreatment involving extreme conditions of pH and temperature is required to break the cell wall structure making the cellulose and hemicellulose accessible for degradation (Ding et al., 2002). Technological advances prioritize a reduction of the cost of pretreatment, developing efficient biocatalysts, in order to achieve the use of all cell wall components, including hemicellulose and lignin. Natural lignocellulolytic systems, such as those found in digestive tract of termites, are highly specialized in processing lignocellulosic biomass. For this reason, they are referred as true "natural bioreactors". These systems have been considered important models to be studied for use in biofuel production technology (Sun and Scharf, 2010; Xie et al., 2014).

The gut of a termite can degrade about 99% of the ingested lignocellulosic biomass in 24 h (Sun and Scharf, 2010). A comparative metagenomics analysis highlighted the importance of insect feeding type and its ability to decompose lignocellulosic biomass, pointing out that guts of termites contain the most efficient enzymes in cellulolytic degradation (Shi et al., 2013).

In this sense, the hindgut has been the focus of metagenomics and metatranscriptomics studies, which revealed that they are rich in cellulases and hemicellulases. More than 700 genes of glycosyl hydrolases were identified, corresponding to 45 different families of enzymes involved in degradation of cellulose and hemicellulose (Warnecke et al., 2007; Tartar et al., 2009). With the advances in molecular biology techniques as well as in genomics and proteomics, these enzymes can be isolated and sequenced, allowing cloning and expression in heterologous systems for use in large scale in industry.

Digestive enzymes of termites are also targets of studies focusing on the discovering of new compounds for use in control of termite pests. Termite pests are known for damages that they cause to crops, buildings and other constructions. The pest species are the minority among termites, corresponding to about 7% of the total (Verma et al., 2009). The damages caused by termites in buildings cause great economic losses and the infestation is usually evident only when the attacked structure is in an advanced state of impairment and the level of infestation is already quite high, making it difficult to control. The main problems resulting from termite infestations are attacks on furnitures, works of art, libraries, and especially the wood used in

construction. Millano and Fontes (2002) estimated that are about 1–10 billion dollars are spent annually in control and repair measures. However, it is likely that this value must lie underestimated, since studies assessing the economic impact of infestations are scarce.

Coptotermes gestroi (Rhinotermitidae), *Heterotermes tenius* (Hagen) and *Heterotermes longiceps* (Snyder) (Rhinotermitidae), *Nasutitermes corniger* (Motschulsky) (Termitidae), and *Coptotermes brevis* (Kalotermitidae) are considered main pest species in urban centers. The success of the species *N. corniger* in the colonization of urban areas has been attributed to its biological versatility, reproduction strategies and feeding habits (Fontes, 1995; Vasconcellos, 1999; Eleotério and Berti-Filho, 2000; Vasconcellos et al., 2002; Oliveira et al., 2006; Oliveira and Bezerra-Gusmão, 2010).

In order to control the population of insect pests, it has grown the search for new methods that use natural compounds instead of synthetic pesticides that are widely used. Table 1 shows plants that are sources of termiticidal compounds. Plants, unlike animals, do not have immune systems to address certain adverse situations and this fact, associated with immobility, made them develop over the time defense strategies against insects (Hammond-Kosack and Jones, 2000). Tannins, flavonoids, lectins, and enzyme inhibitors from plants are able to interfere with insect digestive process. The toxicity of tannins, flavonoids and enzyme inhibitors is due to their ability to complex with proteins, including enzymes, at the digestive tract of the insect exerting a negative impact on nutrition by enzyme inactivation or formation of complexes with low digestibility (Taiz and Zeiger, 2009).

The lectins are proteins that possess carbohydrate-binding sites, interacting reversibly and specifically with monosaccharides, oligosaccharides, polysaccharides and glycoconjugates through hydrogen bonds and van der Walls interactions (Paiva et al., 2011a). These proteins have several physiological roles in plants participating in activation of enzymes, nodulation mechanisms and defense against microorganism and insects (Peumans and van Damme, 1995; Limpens and Bisseling, 2003; Kestwal et al., 2007). Plant lectins with insecticidal activity may interfere with the activity of insect digestive enzymes by binding to glycosylated moieties or other regions of enzyme molecules as well as by binding to substrates (Macedo et al., 2007). The *Microgramma vaccinifolia* rhizome lectin showed stong termiticidal effect on *N. corniger* workers and soldiers (LC_{50} of 0.130 and 0.085 mg/mL, respectively) and was able to inhibit trypsin-like activity from workers, β-glucosidase activity from workers and soldiers, and endoglucanase activity from soldiers. Also, the lectin stimulated acid phosphatase and endoglucanase activities from workers (Albuquerque et al., 2012). Lectins from *Myracrodruon urundeuva* bark, heartwood and leaves were also toxic to workers (LC_{50} of 0.974, 0.248 and 0.374 mg/mL, respectively) and soldiers (LC_{50} of 0.787, 0.199 and 0.432 mg/mL, respectively) of *N. corniger*; these proteins were resistant to degradation by proteases present at termite gut and showed bactericidal effect on symbionts found in gut of workers and soldiers (Sá et al., 2008; Napoleão et al., 2011).

Termiticidal activity may be evaluated by different methods. Figure 6 shows two assays useful to determine the toxicity of compounds when ingested by termites. Both are performed in petri plates and should be maintained in the dark at 28ºC. In the first (Figure 6A), the sample is incorporated into an artificial diet composed disks of cellulose matrix (Hardy et al., 2013) and the termites (16 workers and 4 soldiers) are transferred directly from the nest to the plate (Kang et al., 1990). In the second (Figure 6B), the sample is impregnated in a disk of filter paper with 4 cm diameter and the same number of insects used in the method described above are transferred to the plate. In both methods, the mortality rate is evaluated daily until the death of all termites. The repellent activity of the sample may be also evaluated using the assay shown in Figure 5C. A petri plate is filled with 2% (w/v) agar and, after solidification, wells are made in agar by the removal of a central cylinder and eight peripherical cylinders. In each peripherical well, it is put a filter paper soaked with the sample. The termites (16 workers and 4 soldiers) are then transferred to the central well and the plates are maintained in the dark at 28ºC. The presence of termites in peripherical wells and the construction of tunnels in agar and the closing of the galleries are analyzed daily during 15 days (Su et al., 1982).

CONCLUSION

Feeding habits and ecological roles of termites are intrinsically linked to their digestive apparatus. From a biotechnological standpoint, the lignocellulolytic natural systems of termites are important models to be studied for use in biofuel production technology. Digestive enzymes of termites are also targets of studies focusing on the discovering of new termiticidal agents, including plant compounds.

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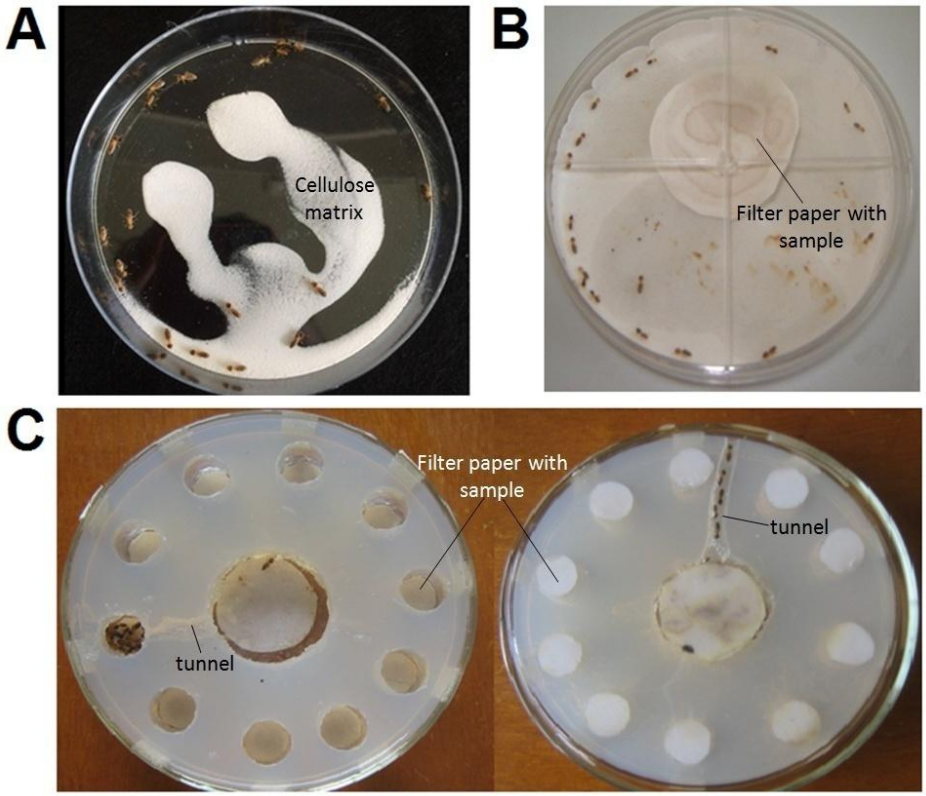

Figure 6. Assays for evaluation of termiticidal and repellent activities of compounds. Toxicity by ingestion is evaluated by assays in which the termites are incubated in a petri plate with a cellulose matrix containing the sample (A) or a filter paper impregnated with the sample (B). Repellent activity is investigating by evaluation of the construction of tunnels by termites in agar (C). The result is positive if the termites close the galleries build in direction to a peripheral well, where a filter paper impregnated with the sample is placed. The image on the left shows a top view and on the right an inferior view.

Table 1. Activity of plant compounds on termites.

Active compound / Preparation	Plant and tissue	Termite species affected	Activity
Terpenes			
Limonoids	*Azadirachta indica* seed	*Reticulitermes speratus*	Antifeedant
Cedrol and α-cadinol	*Taiwania cryptomerioides* Wood	*Coptotermes formosanus*	Toxic
Nootkatone	*Vetiveria zizanoides* root	*C. formosanus*	Feeding deterrent, toxic, repellent
(Z)-ocimene	*Tagetes erecta* leaf	*Odontotermes obesus*	Toxic
Elemene, γ-terpinene and terpinolene	*Melaleuca gelam* and *Melaleuca cajuputi* leaves	*R. speratus*	Toxic
Monoterpenes, sesquiterpenes, hydrocarbons and diterpene	*M. cajuputi* leaf	*R. speratus*	Toxic
Guaiol, α-eudesmol and β-eudesmol, citronellic acid and geranic acid	*Callitris glaucophylla* wood	*C. formosanus*	Repellent
D-limonene	*Citrus* peel	*C. formosanus*	Toxic
Phorbol esters	*Jatropha curcas* seed	*O. obesus*	Toxic
Essential oils and their components			
Cinnamaldehyde	*Cinnamomum osmophloeum* leaf	*C. formosanus*	Toxic
Benzylthiocynate, 3-methoxyphenylacetonitrile and β-ionone	*Lepidium meyenii* leaf	*C. formosanus*	Feeding deterrent
Isomers of nepetalactone	*Nepeta cataria*	*Reticulitermes flavipes* and *Reticulitermes virginicus*	Toxic
T-muurolol	*Calocedrus formosana* leaf	*C. formosanus*	Toxic
Diallyl trisulphide, diallyl disulphide, eugenol, diallyl sulfide and β-caryophyllene	*Allium sativum* and *Eugenia caryophyllata* bud	*R. speratus*	Toxic
Essential oil	*Chamaecyparis obtusa* var. *formosana, Calocedrus macrolepis* var. *formosana* and *Cryptomeria japonica* (heartwood, sapwood and leaf, respectively)	*C. formosanus*	Repellent and toxic
Essential oil	*Mentha arvensis, Carum capticum* and *Cymbopogon citrates*	*O. obesus*	Toxic
trans-pinocarveol, (+)-*α*-pinene, (−)-limonene, (−)-*α*-pinene, *β*-pinene, and *β* phellandrene	*Chamaemelum nobileSantolina chamaecyparissus*, *Ormenis multicaulis*, and *Eriocephalus punctulatus*	*R. speratus*	Fumigant and toxic
Essential oil	*Lippia sidoides* and *Pogostemon cablin*	*Nasutitermes corniger*	Toxic
Essential oil	*Cymbopogon citratus*, *Eucalyptus globulus*, *Syzygium aromaticum*, *Origanum vulgare*, *Rosmarinus officinalis*, *Cinnamomum verum* and *Thymus vulgaris*	*Odontotermes assamensis*	-
Quinones			
Plumbagin, isodiospyrin and microphyllone	*Diospyros sylvatica* root	*O. obesus*	Toxic

Lectin	*Bauhinia monandra* root, *Myracrodruon urundeuva* heartwood, bark and leaf, *Microgramma vaccinifolia*rhizome, *Crataeva tapia* bark, *Opuntia ficus indica* cladodes.	*N. corniger*	Toxic
Extracts			
Chloroform extract	*Lantana camara* var. *aculeata* leaf	*Microcerotermes beeson*	Toxic
Hexane, ethyl acetate and methanol extracts	Leaves of *Aristolochia bracteolata*, *Andrographis paniculata*, *Datura metel*, *Eclipta prostrata*, *Andrographis lineata*, *Tagetes erecta*, *Argemone mexicana* and *Sesbania grandiflora*	*C. formosanus*	Toxic
Alkaloidal, cyclohexane and ethyl acetate extract	*Bowdichia virgilioides* heartwood, *Hymenaea stigonocarpa* heartwood and *Anadenanthera colubrina* wood	*N. corniger*	Repellent and toxic
Ethanol extract	*Juniperus virginiana* needle	*R. flavipes*	Toxic
Flavonoid			
Karanjin	*Pongamia pinnata* seed	*O. obesus*	Toxic
Oil			
Oleic acid	*Cerbera manghas* seed	*Cryptotermes cynocephalus*	Toxic

References: Yatagai et al., 1991; Serit et al., 1992; Shimanouchi et al., 1992; Yoshida et al., 1998; Chang et al., 2001; Maistrello et al., 2001; Zhu et al., 2001; Ganapaty et al., 2004; Maistrello et al., 2003; Nix et al, 2006.; Chang and Cheng, 2002; Singh et al., 2002; Tellez et al., 2002; Kobaisy et al., 2003; Peterson and Ems-Wilson, 2003; Sakasegawa et al., 2003; Cheng et al., 2004; Kim et al., 2005; Park and Shin, 2005; Watanabe et al., 2005; Verma and Verma, 2006; Cheng et al., 2007; Raina et al., 2007; Sá et al., 2008; Eller et al., 2010; Santana et al., 2010; Gupta et al., 2011; Napoleão et al., 2011; Paiva et al., 2011b, Souza et al., 2011; Verma et al., 2011; Albuquerque et al., 2012; Araújo et al., 2012; Elango et al., 2012; Pandey et al., 2012; Lima et al., 2013; Amiralian et al., 2014; Seo et al., 2014; Tarmad et al., 2014.

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9. CONCLUSÕES

- ✓ Intestino de cupins da espécie *Nasutitermes corniger* contém atividades de celulases, hemicelulases, amilase e proteases, com altos níveis das principais enzimas envolvidas na digestão de biomassa celulósica. Os níveis e características das enzimas diferem entre operários e soldados, revelando que eles têm aparatos digestivos diferentes, o que está provavelmente ligado aos diferentes hábitos alimentares.
- ✓ As lectinas de entrecasca (MuBL) e folha (MuLL) de *M. urundeuva* têm como alvos moleculares no intestino de opérarios enzimas digestivas (carbohidrases e proteases) e proteínas transportadoras (apolipoforina e transportadores dotipo ABC). Dessa forma, são capazes de interferir na digestão e absorção de metabolismo das proteínas, carboidratos e lipídeos a nível de intestino.
- ✓ MuBL, MuLL e a lectina do cerne (MuHL) de *M. urundeuva* são capazes de atravessar a matriz peritrófica do intestino médio de operários e, assim, promoveram uma forte desorganização no epitélio intestinal e redução no número de células digestivas, regenerativas e enteroendócrinas, através da indução de morte celular do tipo apoptose dependente de caspases.
- ✓ O presente trabalho é o primeiro a apontar alvos moleculares e celulares de lectinas com ação termiticida.