DIGESTIVE SYSTEM OF WORKER TERMITE *Coptotermes curvignathus* HOLMGREN AND ITS CHEMICAL AND CELLULOLYTIC MICROBIAL PROPERTIES

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ABSTRACT

Termites are able to feed and digest wood efficiently through orchestration of host and microbial enzymes. This study was carried out to characterize the anatomy of the *Coptotermes curvignathus* digestive system as well as its chemistry and cellulolytic microbial properties. *Coptotermes curvignathus* has three main regions in their digestive system namely the foregut, midgut and hindgut. The length of foregut was the shortest and the hindgut was the longest compartment. There were seven Malpighian tubules attached at the junction between the midgut and the anterior hindgut. Based on gut metabolites analysis, uric acid was found to be the most concentrated compound in all gut compartments. Some cellulolytic microbes isolated from the *C. curvignathus* digestive system displayed ability to produce uric acid. The second most abundant compound was propionic acid. Other organic acids, antibiotic and antioxidant compounds such as salicylic acid, butyric acid, vancomycin hydrochloride, pyrocatechol, and quercetin were also found in the fluid of *C. curvignathus*. From antioxidant tests, hindgut fluid of *C. curvignathus* exhibited the highest antioxidant activities, followed by midgut and the foregut fluids had the lowest antioxidant activity. The result agrees with the metabolites analysis, where ascorbic acid was found mainly in hindgut. The isolation of uric acid producing microbes from the gut of *C. curvignathus* is quite encouraging for further cultivation-based investigations, which are important to improve our understanding of the functional interactions of the symbiotic microbes involved in the digestion of wood matter in termites.

Keywords: *Coptotermes curvignathus*, gut, chemical compounds, cellulolytic microbes.

ABSTRAK

Anai-anai berkebolehan memakan dan mencerna kayu dengan cekap melalui penggabungan enzim perumah dan mikrob. Kajian ini dijalankan untuk mencirikan anatomi sistem penghadaman *Coptotermes curvignathus* serta sifat kimia dan selulosa mikrob penghadam selulosanya. Spesies ini mempunyai tiga bahagian utama dalam sistem pencernaan iaitu usus depan, usus tengah dan usus belakang. Usus depan adalah paling pendek, manakala usus belakang adalah paling panjang. Terdapat tujuh tiub Malpighian terlekat di persimpangan antara usus tengah dan usus belakang. Berdasarkan analisis metabolit usus, asid urik merupakan sebatian yang berkepekatan paling tinggi di dalam semua bahagian usus. Terdapat beberapa bakteria yang tinggal di dalam usus belakang *C. curvignathus* yang berkemampuan menghasilkan asid urik. Sebatian kedua dengan kepekatan paling tinggi adalah asid propionik. Asid organik lain, sebatian antibiotik dan antioksidan seperti asid salisilat, asid butrik, vankomisin hidroklorida, pirokatekol dan quersetin juga terdapat dalam cecair usus *C. curvignathus*. Melalui ujian antioksidan, usus belakang *C. curvignathus* menunjukkan aktiviti antioksidan yang tertinggi, diikuti oleh usus tengah dan usus depan mempunyai aktiviti antioksidan yang paling rendah. Keputusan ini menepati analisis metabolit, di mana sebatian antioksidan asid askorbik banyak didapati terutamanya di usus belakang. Pemencilan mikrob penghasil asid urik dari usus *C. curvignathus* memerlukan kajian berasaskan teknik pengkulturan yang lebih lanjut, di mana ia penting untuk meningkatkan kefahaman terhadap fungsi interaksi antara mikrob simbiotik yang terlibat dalam pencernaan bahan kayu dalam anai-anai.

Kata kunci: *Coptotermes curvignathus*, usus, sebatian kimia, mikrob selulosa.

INTRODUCTION

Coptotermes curvignathus is a subterranean termite belonging to the family Rhinotermitidae that is found in Malaysian forests (Thapa 1982; Tho 1992). *Coptotermes curvignathus* is unique among termitesas it is able to attack and feed actively on healthy living woody plants (Hoe et al. 2019).

Termite gut is known to be a structured microenvironment with physicochemically diverse micro habitants spanning from the foregut to hindgut. The distinctive micro environmental conditions across each gut compartment enables lignocellulolytic activity to be carried out effectively in the termite digestive system (Brune et al. 1995). Due to the small size of termite guts, the physicochemical gradients across different gut compartments would influence the spatial distribution of microbial populations and their metabolic functions (Brune 2013; Brune & Friedrich 2000). In a recent discovery of gut microbial community in *C. curvignathus* (King et al. 2014), their identities had extended the findings of conversion of chemical compounds from lignin degradation across different gut regions. The symbiotic interaction between microbes and the termite host generates various metabolic pathways which also benefit the host's immune system (Kwong et al. 2017) that implicates in the extension of life span. The understanding of the metabolite profile would provide future research direction to understand the mechanisms of interaction between gut microbiota and the host.

The objectives of this study were to characterize the general gut anatomy and metabolites in *C. curvignathu*s and isolate some microbes that contribute to the production of these metabolites. Specifically, this study aims: 1) to characterize the physical properties of the gut structure; 2) to quantify the gut fluid compounds; 3) to determine the antioxidant level of

each gut compartment; 4) to evaluate the cellulase activity of each gut compartment; and 5) to isolate key metabolite producing gut microbes.

MATERIALS AND METHODS

Sampling for Termites

Termites *C. curvignathus* were baited from Woodman Oil Palm Plantation at Block 16 (N03°01′00.6″, E112°52′51.7″), Semanok Tatau, Bintulu, Sarawak. For termite baiting, fresh rubber woods (*Hevea brasiliensis*) were installed below the ground close to termite infested palm trees. After two to three months, the wood baits were collected and stored under dark conditions in the laboratory prior to gut fluid extraction.

Extraction of Termite Gut

Individual worker termites were sterilized with 70% ethanol and rinsed with sterilized distilled water. Termites were chilled and the whole guts were extracted using fine-tipped sterile forceps by holding the thorax part while grasping the extreme posterior part of the body simultaneously. The whole gut was then separated into the foregut, midgut and hindgut for further analysis of the individual compartments.

Measurements of the Digestive System

Whole guts were obtained from fifteen of the sterilized worker termites. Length and diameter of the foregut, midgut and hindgut were measured using Nikon SMZ1000 stereo zoom microscope. The measurements were carried out with NIS-Elements Viewer 5.21 (Nikon Corporation, Laboratory Imaging, 2017). The length of the foregut (FG) was measured from the anterior to the ending of the foregut/midgut junction; the midgut (MG) was measured from the foregut/midgut junction to the anterior part of midgut/hindgut junction; and the hindgut (HG) was measured from the midgut/hindgut junction towards the end of the abdomen (Figure 1) The diameters of foregut and midgut were measured at evenly distanced points, and the diameter of hindgut was measured at its widest distanced points. Fifteen repetitions were made and the mean and standard deviation were calculated.

Chromatographic Analysis of Gut Metabolites

Metabolites within the different gut compartment fluids were determined by an automated high-performance liquid chromatography (HPLC) system (JASCO, Japan). The system was equipped with an intelligent sampler (AS-2059 Plus), a quaternary gradient pump (PU-2089 Plus), a column thermostat (CO-2060 Plus), and a UV/VIS detector (UV-2075 Plus). The system was operated with EZChrom Elite Version 3.1.6 software. Supernatant fluids of each gut compartments were prepared similar to the method used byto Schauer and co-workers (2012) with some modifications, and were filtered at 0.22 μm MS® PTFE syringe into auto sampler vials. The injection volume was 5μL and the gut fluid samples were detected at 215 nm. Chromatographic separation was performed on a Hypersil Gold C8 column (100 mm × 4.6 mm ID × 5 μm; Thermo Scientific). The mobile phase used consisted of a mixture of 0.015N sulphuric acid in Ultrapure water (solvent A) and acetonitrile (solvent B). The mobile phase was filtered and degassed by sonication before use. The elution program was performed using a 20-min gradient, from initial solvent of 100% solvent A maintained for 4 min, changed over next the 12 min to 50% solvent A: 50% solvent B, and back to 100% solvent A for 4 min. The flow rate was achieved at 0.50 ml/min and the column temperature was 35°C. Presence of different chromatographic peaks were identified by comparing the retention times with the external reference standards.

HPLC assays were carried out in triplicate at least. Data was calculated as mean±standard deviation using Microsoft Office Excel 2007. Analysis of variance and Tukey test were performed using SAS software. A *P*-value < 0.05 indicated significance.

Antioxidant Assays

DPPH radical scavenging activity

The stable DPPH radical was used to determine the free radical scavenging activity of the different gut compartments according to the method of Brand-Williams et al. (1995) and Dorman .et. al (2004) with minor modifications. Ascorbic acid, BHA and α-tocopherol were used as positive controls whereas DPPH solution was used as negative control. An amount of 20 µL gut fluids (50 guts/0.5 mL) was added to 0.1 mL methanolic solution of DPPH (0.05 mg/mL). The mixture was shaken vigorously and incubated in darkness at room temperature. After 30 min, absorbance was measured at 517 nm using an ELISA microplate reader (Sunrise, TECAN). Each sample was done in triplicate. Mean of the three absorption readings were taken to calculate the capability of the different gut compartment fluids in scavenging the DPPH radical using the equation:

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Absorbance negative control} - \text{Absorbance sample}}{\text{Absorbance negative control}} \times 100$$

Determination of total flavonoid content

Total flavonoid content of different gut compartments was measured according to the aluminium chloride method by Ranković and co-workers (2011). 25 µL of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with 25 µL gut fluids (50 guts/0.5 mL). The mixture was shaken vigorously and left to incubate at room temperature for 10 min. The absorbance was measured at 415 nm against a blank sample without $AlCl_3$ using an ELISA microplate reader (Sunrise, TECAN). Quercetin was used as the standard. Quercetin solution was prepared by dissolving 3 mg quercetin in 1 mL methanol and subsequently diluted to various concentrations of 2.4, 1.8, 1.2 and 0.6 mg/mL. Calibration curve of quercetin was plotted by measuring the absorbance of the dilutions at 415 nm with ELISA microplate reader. Total flavonoid contents were expressed as microgram quercetin equivalent by referring to the standard curve of quercetin.

Plate screening for cellulolytic activity

Media of 1.5% agar noble (Difco^{TM}) containing 1% carboxymethylcellulose (CMC) sodium salt (Calbiochem) were prepared according to the manufacturer's instruction and poured into sterile 90 mm Petri dishes. Each isolated gut compartments of 15 individual termites were torn and pooled in 250 µL Ultrapure water separately. The gut fluids were homogenized by vortexing and 5 µL of each gut fluids were pipetted onto a CMC agar plate. A commercial cellulase *Aspergillus niger* was used as positive control and sterilized water as negative control. Three replicates of the CMC agar plates were incubated for 2 hours at 37 °C. The plates were stained with 0.1% Congo red dye for 15 min, followed by distaining with 1.0 M NaCl solutions for 15 min, as described by Teather & Wood (1982). Enzyme activity was evaluated through observations of the clear zone formed.

Isolation and Identification of Cellulolytic Microorganisms

Isolation of cellulolytic microorganisms

Fifteen worker termites were degutted under sterile condition. Different gut compartments, i.e. the foregut, midgut and hindgut, were homogenized in 0.5 mL of Ultrapure water respectively. The suspensions were mixed with 4.5 mL of 1.0% CMC broth media. A serial dilution of the mixture was performed up to 10^{-12} and incubated for 3 days at 37 °C. After incubation, 1.0 mL of the cultures was spread on nutrient agar plates and incubated for 24 hours at 37 °C. Single colonies were obtained by several sub-culturing under sterile conditions for further analysis consisting of DNA extraction and uric acid concentration measurement.

DNA extraction

Pure isolates were grown overnight in nutrient broth. The broth cultures were centrifuged at 20,000 rpm for 2 min at 20 °C. Supernatants were discarded and washed with Ultrapure water for two to three times. DNA was extracted according to the protocol of Qiagen DNeasy® Tissue Kit. The DNA concentrations in the extracts were quantified by agarose gel electrophoresis.

Identification by Polymerase Chain Reaction (PCR) and 16S ribosomal RNA gene sequence

The DNA extracts were amplified in a thermocycler (MJ Mini Personal Thermal Cycler, Bio-Rad). Two bacterial universal primers were used for amplifying the 16S rRNA genes, forward 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). Each PCR mixture with a final volume of 50 μL contained the following components: 38.6 μL sterile Ultrapure water, 5.0 μL 10× PCR buffer [500 mM KCl, 100 mM Tris-HCl, pH 8.3], 1.5 μL $MgCl_2$ (50 mM), 1.0 μL of each primer (10 μM), 2.0 μL deoxynucleoside triphosphate (2mM dNTPs), 0.5 μL isolated DNA (20-50 ng), and 0.4 μL *Taq* DNA polymerase (5U/μL).

PCR profile was started by an initial denaturation at 95 °C for 2 min. Denaturing step continued at 95 °C for 30 s, followed by annealing at 50 °C for 1 min and primer extension at 72 °C for 4 min for a total of 18 thermal cycles. The thermal cycles completed with final extension at 72 °C for 10 min.

For analysis of the PCR products, 1 μL aliquots were loaded onto 1.0% agarose gel in 1.0× TAE buffer (pH 8.0) and electrophoresis was carried out for 1 h at 100 V. Gels were stained with ethidium bromide (10 mg/mL), destained with distilled water and observed under UV light. Three replicates of the PCR products were re-amplified and quantified. DNA bands were excised from the gel with sterile scalpels and placed in micro centrifuge tubes for purification according to QIAquick Gel Extraction Kit protocol. Purified PCR products were sent to 1st Base Company for sequencing analysis. The results of 16S rRNA sequences were analyzed by BLASTN program (National Center for Biotechnology Information, NCBI).

Quantification of Uric Acid Contents

Content of uric acid among the cellulolytic isolates were quantified using Uric Acid Assay Kit (Abnova) according to the manufacturer instructions. Blank control and standard (10 mg/dL uric acid) were provided. Working reagents were freshly prepared by mixing 10 volumes of Reagent A, 1 volume Reagent B and 1 volume Reagent C. The mixture was left to equilibrate to room temperature prior to assay. Fresh broth cultures were prepared by growing the cellulolytic isolates in nutrient broths overnight. The broth cultures were centrifuged at 10,000 rpm for 5 min to remove insoluble particles. Each 5 μL of the blank, standard (10 mg/dL uric acid) and the cell culture supernatant were transferred in triplicate wells with clear bottom 96

well plates, mixed with 200 µL working reagent and tapped lightly to mix. Uric acid concentrations were determined after 24, 48 and 72 h incubation at room temperature. After which optical density (OD) was read at 590 nm on ELISA microplate reader (Sunrise, TECAN). Readings were used to calculate the uric acid concentration using formula as below:

$$\frac{\text{OD Sample} - \text{OD Blank}}{\text{OD Standard} - \text{OD Blank}} \times 10 \text{ mg/dL}$$

RESULTS AND DISCUSSION

Physical Characterization of *Coptotermes curvignathus* Gut Structure

The hindgut was the longest segment (7.27±0.69 mm) of the digestive system in *C. curvignathus*, which accounted for approximately 60% of its total length. The midgut was the second longest in length in the system, 4.16±0.52 mm (approximately 34%) and the foregut was the shortest, 0.75±0.08 mm (approximately 6%).The foregut of *C. curvignathus* was relatively shorter than other reported termite species (Table 2). The Kalotermitidae termites have the longest foregut length compared to other termites, especially *Neotermes bosei*, which foregut proportionally accounts for 24% of the total gut length.

The hindgut of *C. curvignathus* was about 60% the total length of its gut. This indicates the hindgut is the major absorption segment in the digestive system. However, the longest hindgut of termite species was reported in wood-feeding higher termites, *Nasutitermes takasagoensis* workers, where the hindgut makes up approximately 70 % of the total gut length, andthe foregut and midgut constitute only 8 and 22% respectively (Tokudaet al. 2001). Two wood-feeder species of the neotropical genus *Tauritermes* also have proportionally longer hindgut regions, about 61% of their total gut length. Longer hindgut may offer benefits such as better nutrient absorption especially for mono diet insects.

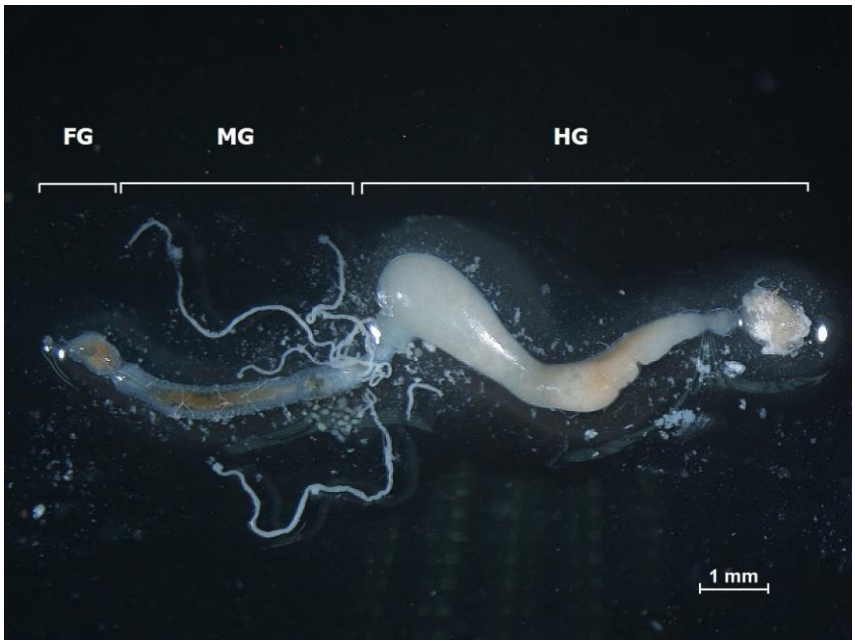

Figure 1. Intestinal tract of worker *Coptotermes curvignathus*. The gut regions are abbreviated as follows: Foregut (FG); Midgut (MG); Hindgut (HG)

In *C. curvignathus*, the hindgut was proportionally the longest region compared to the foregut and midgut. In addition, the hindgut was relatively wider in diameter compared to the foregut and midgut. At its widest diameter, it was measured up to 0.70±0.14mm whereas the uniform diameter of the midgut was measured at 0.50±0.07mm and the foregut was at 0.50±0.09 mm (Table 1). A wider diameter would allow more anaerobes to reside in the hindgut, thus indicating that fermentation or anaerobic metabolism is the major activity in the hindgut. On the other hand, less anaerobes are found in the foregut and midgut, because the oxygen will penetrate through the gut membrane and due to the smaller diameter, the anaerobic zone in the lumen would be very limited or lacking. The highly oxygenated condition would promote lignin oxidation (Ke et al. 2010; Li et al. 2012) and allow lignocelluloses to be degraded further in the hindgut.

Table 1. Gut measurement (Mean ± standard deviation) in length and diameter (mm) of *C. curvignathus* workers (*n* = 15)

Gut Compartments	Length	Diameter
Foregut (FG)	**0.75±0.08**	**0.50±0.09**
Midgut (MG)	4.16±0.52	0.50±0.07
Hindgut (HG)	7.27±0.69	0.70±0.14

Table 2. Comparison of *C. curvignathus* gut length measurements (mm) and number of Malpighian tubules with other termite families

Species	Foregut	Midgut	Hindgut	Numbers of Malpighian tubules
Rhinotermitidae				
Coptotermes curvignathus(*n* = 15)	0.75±0.08	4.16±0.52	7.27±0.69	7
Kalotermitidae				
Tauritermes taurocephalus (*n* = 30)	1.80±0.16	2.40±0.30	6.60±0.59	8
Tauritermes sp. (*n* = 30)	1.80±0.13	2.30±0.27	6.60±0.55	8
Neotermes bosei (*n* = 10)	3.32±0.57	3.86±0.61	6.56±0.91	Not reported
Nasutitermitidae				
Nasutitermes takasagoensis (*n* = 5)	Approx. 0.90	Approx. 2.50	Approx. 8.10	Not reported

Adapted from Hariprabowo et al. (2006), Godoy (2004) and Tokuda et al. (2001)

There was an average of seven Malpighian tubules attached between the midgut and the anterior hindgut region of *C. curvignathus*. The number was slightly lower than *Tauritermes* spp., which have eight Malpighian tubules (Godoy 2004). Malpighian tubules are the main osmoregulatory and excretory organs that responsible for carrying metabolites and toxic compounds from hemolymph into the hindgut (Nocelli et al. 2016). Variation in number of Malpighian tubules could reflect diet differences. *Tauritermes* is known as drywood termite, while as *C. curvignathus* feeds on living tree.

Chromatographic Determination of Gut Metabolites

HPLC was used to determine the chemical compounds present in the *C. curvignathus* digestive system. Compounds that were detectable throughout the digestive tract of *C. curvignathus*are shown in Table 3. Uric acid was found to be the most concentrated compound in all of the gut compartments. Uric acid was detected right from the beginning of the digestive tract. This indicates *C. curvignathus* have an extraordinary ability in extracting and storing uric acid. Concentration of uric acid further increased approximately 15-fold in the midgut. Uric acid is probably stored in the fat body of the termite midgut as a significant nitrogen reservoir (Coasta-Leonardo et al. 2013). Retaining uric acid rather than voiding it into feces has been reported in other termite species such as *Reticulitermes flavipes* (Potrikus & Breznak 1981) and *Mastotermes darwiniensis* (Coasta-Leonardo et al. 2013). Malpighian tubules are attached at the foremost part of the hindgut and are known to actively transporturic acid to the hindgut, which is believed to be the main reason that caused the continuous increment of uric acid in the hindgut of *C. curvignathus*. The concentration of uric acid was the highest in hindgut among all the gut segments. This indicates the hindgut could be the major uric acid storage site. The uric acid would be further recycled by uricolytic bacteria into carbon, nitrogen and energy (Potrikus & Breznak 1980a; 1980b; 1981). Since the diet of termites is usually in short supply of nitrogen, recycling of uric acid nitrogen in termites is a strategy for nutrient conservation.

Table 3. Concentration of chemical compounds detected in the main gut compartments of *C. curvignathus* workers

Compounds	**Estimated Concentration (mM)**		
	Foregut	**Midgut**	**Hindgut**
Ascorbic acid	–	–	0.194±0.008^{a}
Butyric acid	–	18.114±2.473^{a}	16.650±6.270^{a}
Propionic acid	2.733±0.086^{c}	28.612±4.086^{b}	48.496±4.468^{a}
Pyrocatechol	0.014±0.001^{c}	0.528±0.084^{a}	0.386±0.045^{b}
Quercetin	0.360±0.085^{a}	0.686±0.108^{a}	0.377±0.306^{a}
Salicylic acid	0.010±0^{b}	0.094±0.014^{a}	0.076±0.005^{a}
Uric acid	36.135±1.071^{c}	511.210±78.865^{b}	752.872±56.110^{a}
Vancomycin HCl	0.001±0^{b}	0.010±0.002^{a}	0.008±0.002^{a}

Data are shown as mean±standard deviation (n = 3).
Means with different letters within a row are significantly different ($p < 0.05$).
" – ": not detected

Presence of pyrocatechol, quercetin and salicylic acid showed that there are changes in the chemical structure of lignin during passage through the entire gut system, which was initiated in the foregut and continues in the midgut and hindgut. These three compounds were initially detected in the foregut, and found abundantly in the midgut and slightly decreased in the hindgut (Table 3). Mastication in foregut changes the structure of lignin in most of the phylogenetically lower termites (Ke et al. 2012). Many wood-feeding termites including *C. formosanus* (Shiraki) have been reported to have the capability in metabolizing aromatic compounds, such as lignin, efficiently beginning at the foregut and is intensified in the midgut regions (Ke et al. 2011). Decomposition of lignin is continued via enzyme digestion when entering midgut. This explains the 10-fold increase of propionic acid in the midgut. The amount of pyrocatechol, salicylic acid and vancomycin HCI also significantly increased in the midgut and reduced slightly when entering the hindgut. This indicates that lignocellulosic materials were continuously digested in the midgut, and the hindgut was the major nutrients absorption

site. An immunohistochemistry study done on the midgut of *C. formosanus* (Shiraki) showed evidence that there are distributions of endogenous cellulase in the endoperitrophic space and on the microvilli lining (Fujita et al. 2010), in both lower and higher termites (Nakashima et al. 2002; Terra & Ferreira 1994; 2012). These enzymes help in decomposition of wood diets in termites. Digestion products include soluble sugars such as glucose and cellobiose which could pass through and be absorbed through the midgut wall.

Indication of high lignin modification in the midgut of *C. curvignathus* was consistent with the physical characteristic of the midgut. As shown in Table 1, the midgut of *C. curvignathus* was relatively small in diameter compared to the hindgut; indicating that the midgut mainly constitutes anaerobic region. In fact, studies have shown that wood-feeding termites contain higher oxygen concentration in the midgut compared to other gut regions (Ke et al. 2010). This is an important feature as it pertains to its function in digestion. Lignin, which is the main component that protects the lignocellulosic material from decomposition, can only be modified in aerobic conditions. Earlier studies have supported that lignin oxidation is promoted in the midgut (Breznak & Brune 1994; Geib et al. 2008; Katsumata et al. 2007; Scharf &Tartar 2008). Phenol oxidase and esterase act in aerobic conditions to modify lignin and solubilize hemicellulose (Karl & Scharf 2015; Scharf & Tartar 2008; Tartar et al. 2009). In addition, actinobacteria that are known to have the ability in producing wide range of catabolic enzymes such as peroxidases to depolymerize lignin or degrade lignin derivatives (Tian et al. 2014), was found abundantly in the intestinal tract of *C. curvignathus* (King et al. 2014). In Hoe et al. (2019), several laccases (lignin degrading enzyme) produced in the digestive tract of *C. curvignathus* have been characterized. This helps to understand the breakdown of lignin in *C. curvignathus*. In termite *R. flavipes*, lignase genes particularly laccases and peroxidases were identified in the salivary gland and foregut tissues, which implies lignin degradation occurs in the front part of the digestive system (Scharf &Tartar 2008). The protective lignocelluloses structure is disrupted further via mastication in the proventriculus (Geng et al. 2018; Watanabe & Tokuda 2010). This would expose the cellulose fibers that were previously buried in lignin and hemicelluloses, thus making them accessible to the termite endogenous enzymes for further decomposition. This explains the surge in concentration for most of the chemical compounds such as propionic acid, butyric acid, uric acid, pyrocatechol, quercetin, salicylic acid and vancomycin HCl in the midgut region (Table 3).

The presence of vancomycin HCl in the digestive fluids of *C. curvignathus* supports the findings of high percentage of *Streptomyces* in the gut of *C. curvignathus* (King et al. 2014). *Streptomyces*, a member of the Actinomycetes has been reported to produce bioactive secondary metabolites compound; vancomycin hydrochloride (Berdy 2005; Chaudhary et al. 2013; Levine 2006; Moellering 2006). *Streptomyces niverotuber* that was isolated from termite's gut is capable in producing bioactive compounds with a wide spectrum of biological activities against human pathogens (Khucharoenphaisan et al. 2012).

Pyrocatechol (Table 3) was found in the highest concentration in the midgut. Pyrocatechol, also known as catechol, is an important building block in the cuticle of an insect. Catechol strengthens the cuticle layer by cross linking the chitin to other structural proteins. In termites, the cuticle layer lines the top of the epithelial cell layer of the foregut and hindgut (Engel & Moran 2013). This offers protection to the epithelial cell layer of the foregut and hindgut against direct exposure to microorganisms or toxins. These physical barriers between epithelium and lumen are good examples for tolerance mechanisms as the termites do not have to reduce the bacterial load in the gut as most of the bacteria are beneficial to their digestive system, while also reducing the impact of the bacteria on the host.

From Table 3, the midgut can be regarded as the major production site for several metabolites such as butyric acid, pyrocatechol, quercetin, and salicylic acid. However, several metabolites could only be detected or reached their highest concentration in the hindgut, which were the ascorbic acid, propionic acid and uric acid. This means decomposition of a wood diet continues in hindgut. Ascorbic acid or commonly known as vitamin C, was only detectable in the hindgut of *C. curvignathus*. Most intestinal tract of insect are unable to synthesize ascorbic acid internally although a trace amount is still required for growth and development purposes. This requirement perhaps is fulfilled by certain gut microbial symbionts that are able to synthesize it (Kramer & Seib 1982). Ascorbic acid is a highly antioxidative compound that removes free radicals to prevent tissue damages and acts as a cofactor for various enzymatic hydroxylation reactions (Mathews et al. 1997). Actinomycetes are microbes that capable of synthesizing antioxidants, as well as degrading cellulose and lignin (Chaudhary et al. 2013). These microbes have been isolated from the gut of various species of termite including *C. curvignathus* (King et al. 2014; Upadhyay 2011).

Propionate and butyrate were among the fermentable products that were detectable in high concentration in the hindgut region. Both compounds could be derived from lactate fermentation by *Bacteroides* strains (Schultz & Breznak 1979). In the gut of *C. curvignathus, Bacteroides* were reported as the most abundant microbes (King et al. 2014). Many studies have evidenced that Bacteriodetes are the most dominant phyla in natural ligninolytic environments such as termite gut and rumen (Kudo 2009; Wu & He 2013). They display excellent cellulolytic activity with high biomass conversion rate.

Butyric acid was the third major metabolites found in the gut of *C. curvignathus* (Table 3). *Clostridium* species, another common gut microbe in *C. curvignathus* (King et al. 2014) has the ability to utilize glucose and xylose as a carbon source for butyric acid production. *Clostridia* are facultative anaerobes (Rieu-Lesme et al. 1996) and are reported to be associated with the midgut epithelium and colonize on the midgut wall (Tokuda et al. 2000). This explains the high concentration of butyric acid at the midgut and continuous increase in concentration in the hindgut of *C. curvignathus*.

Although *Lactobacillales* is regarded as normal microflora in the gut of *C. curvignathus* (King et al. 2014), there was no lactic acid compound detectable in the gut. This may be because the lactate is present only in trace amounts and is quickly broken down into other metabolites. Previous studies show that the lactate produced by *Streptococcus lactis,* residing in the gut of *R. flavipes* will be fermented by *Bacteroides* into propionate and acetate (Schultz & Breznak 1978; 1979). Thus the lactic acid formed in the gut of *C. curvignathus* could be rapidly broken down by *Bacteroides,* which is the major constituent of *C. curvignathus* gut microflora (King et al. 2014).

Antioxidant across the Digestive System

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The result of the antioxidant study based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method is shown in Figure 2. Purified antioxidants such as ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol were used as standard. The hindgut fluid of *C. curvignathus* exhibited the highest antioxidant activities, followed by the midgut and the foregut fluids had the lowest antioxidant activity. The result is in agreement with Table 3, where ascorbic acid is mainly found in hindgut.

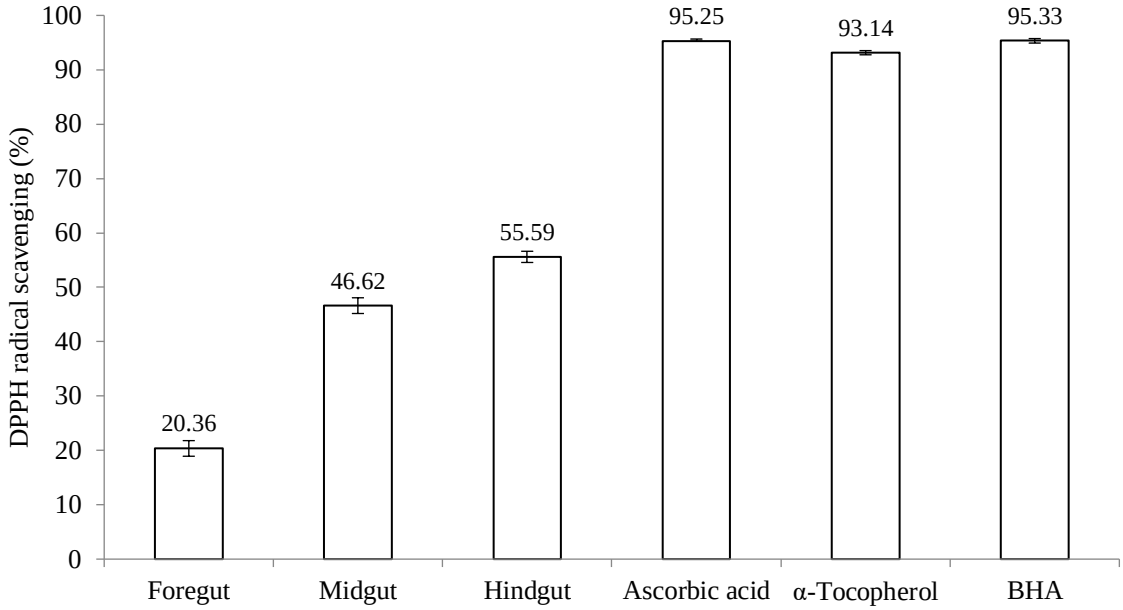

Figure 2. Free radical scavenging activity of gut fluids in different compartment

Determination of Total Flavonoid Content

A standard quercetin graph with $R^2 = 0.9956$ was obtained and its equation was $y = 126.55x + 0.5597$, where y is the reading of absorbance at 415 nm and x represents the total flavonoid content in the gut fluids of *C. curvignathus* (Figure 3). Table 4 shows the mean absorbance of a spectrum concentration of quercetin. The total flavonoid content of the studied gut fluids of *C. curvignathus* is shown in Table 5. The results showed that the hindgut and midgut fluids were 8.1 and 2.1 mM quercetin equivalent respectively. Foregut fluids showed less than 2 mM of quercetin equivalent, which was below the detectable limit of the used method.

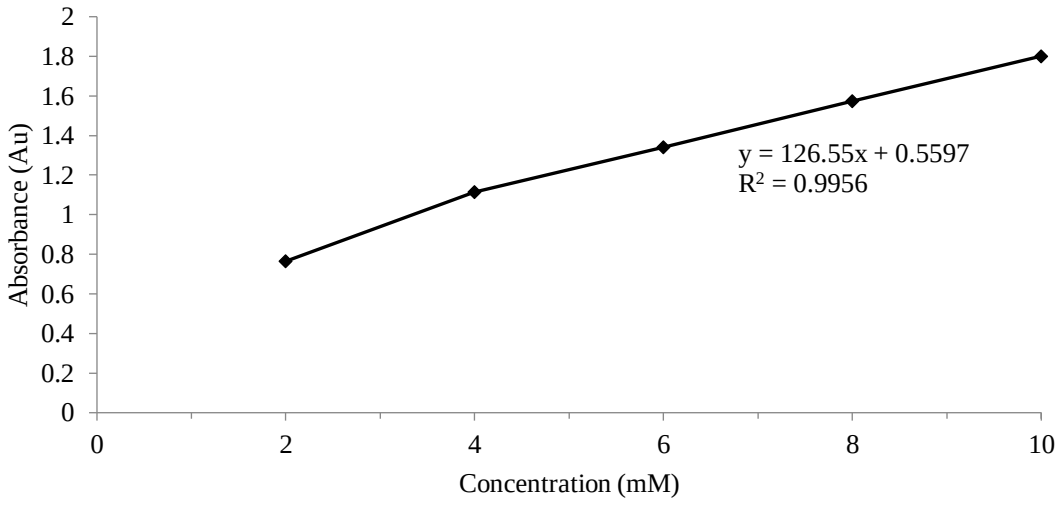

Figure 3. Standard curve of Quercetin

Table 4. Absorbance of standard compound (Quercetin)

Concentration (mM)	**Mean Absorbance (Au) λmax = 415nm**
2	0.765
4	1.115
6	1.340
8	1.574
10	1.801

Table 5. Total flavonoid content in different gut fluids of *C. curvignathus*

Gut Compartments	**Total Flavonoid (mM Quercetin equivalent)**
Foregut	- 1.2*
Midgut	2.1±0.071
Hindgut	8.1±0.045

Each value is expressed as mean ±standard deviation (n = 5).
* Below detection limit (< 2 mM).

The results of DPPH free radical scavenging activities and total flavonoid contents throughout the gut compartments revealed that *C. curvignathus* contains high antioxidant properties. Uric acid which was most prevailing metabolite in the gut of *C. curvignathus*, especially the hindgut, could act as antioxidant. A study using Japanese termite *R. speratus* shows that uric acid could effectively suppress reactive oxygen species (ROS) in the body of *R. speratus* (Tasaki et al. 2017). Moreover, administration of uric acid increases the survival of termites during deficiencies in uric acid production. Uric acid is also known as one of the major antioxidants to have scavenging capacity against oxygen radicals in human and insects (Ames et al. 1981; Matsuo & Ishikawa 1999). Strong antioxidant defense mechanisms might explain the extraordinary long lifespan of highly eusocial insects such as ants, termites, honeybees and wasps, especially the worker and queen castes.

Apart from uric acid, ascorbic acid is also known to possess antioxidant properties. Presence of ascorbic acid in the hindgut of *C. curvignathus* enhances the antioxidant capacities of the gut compartment. Additionally, pyrocatechol which contains phenolic compounds also could have contributed to antioxidant activities in *C. curvignathus*. Pyrocatechol, which belongs to the *ortho* position of phenol hydroxyl group, was found to be the best and the most stable antioxidant properties against superoxide radicals (Veliká & Kron 2013).

Mapping of Cellulase Activity to Gut Segments

All three main gut segments of *C. curvignathus* showed presence of cellulase activity that can be observed as cleared zones on the CMC agar plate stained with congo red (Figure 4). Zhang and colleagues (2011) showed that wood digestion starts in the mouth of termites, where endogenous endoglucanases are secreted from salivary glands in foregut to initiate the reaction. Cellulase activity occurred in all segments and this is in agreement with the metabolites result shown in Table 3.

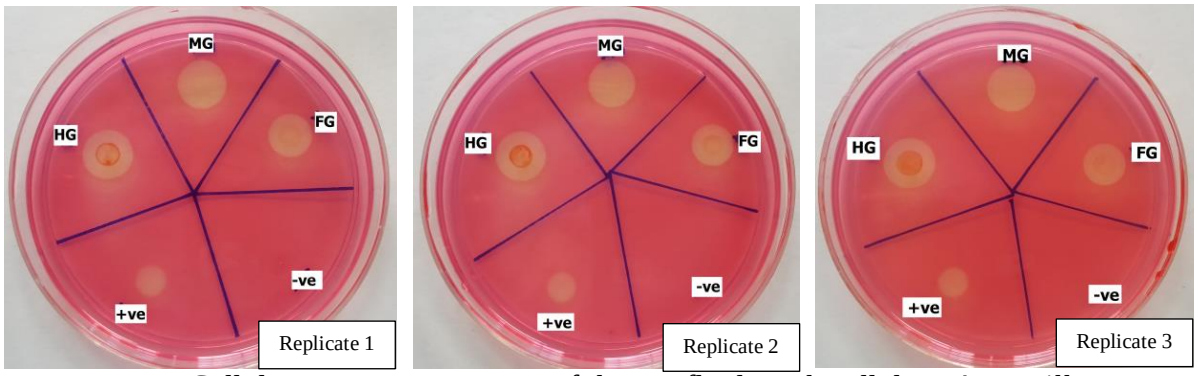

Figure 4. Cellulase activity screening of the gut fluids with cellulase *Aspergillus* serving as positive control

Cellulolytic Microorganisms

There were 21 cellulolytic microorganisms isolated from the foregut, midgut and hindgut compartments. Their DNA were extracted, amplified and sequenced. Based on 16S rRNA sequencing analysis, these isolates were identified mainly as *Bacillus* spp. as shown in Table 6.

Table 6. Bacterial isolates from different gut compartments

Name of the Isolate	**Phylum/Class**	**Identities of Isolates**
Foregut		
F1	Firmicutes	*Bacillus cereus*
F2	Firmicutes	*Bacillus cereus*
F3	Firmicutes	*Bacillus cereus*
F4	Proteobacteria	*Burkhloderia cepacia*
Midgut		
M1	Firmicutes	*Bacillus cereus*
M2	Proteobacteria	*Salmonella enterica*
M3	Proteobacteria	*Citrobacter koseri*
M4	Firmicutes	*Bacillus cereus*
M5	Firmicutes	*Bacillussubtilis*
M6	Firmicutes	*Bacillus cereus*
M7	Firmicutes	*Bacilluscereus*
M8	Proteobacteria	*Citrobacter koseri*
M9	Proteobacteria	*Dyella japonica*
Hindgut		
H1	Firmicutes	*Bacillus cereus*
H2	Firmicutes	*Bacillus cereus*
H3	Firmicutes	*Bacillus* cereus
H4	Firmicutes	*Bacillus cereus*
H5	Firmicutes	*Bacillus cereus*
H6	Proteobacteria	*Citrobacter koseri*
H7	Proteobacteria	*Citrobacter koseri*
H8	Firmicutes	*Bacillus cereus*

F = foregut; M = midgut; H = hindgut

Overall, the BLAST results showed high degree of similarity, ranging from 99 to 100 %, with E-value equalled to "0" when BLASTN to annotated sequences deposited in the NCBI databases. Most of the isolated cellulolytic microorganisms from each gut compartment were *Bacillus cereus*, followed by *Citrobacter koseri*.

Uric Acid Producing Microorganisms

The isolated cellulolytic microorganisms displayed ability to gradually increase uric acid concentration after 72 hours of incubation in nutrient broth (Table 7). Previous studies show that uric acid gradually accumulated within termite bodies after being captivated in a laboratory setting for a period of time (Potrikus & Breznak 1980b). Accumulation of uric acid in *R. speratus* workers was found to increase the antioxidant activities and suppress the ROS levels in the body of termites after captivity (Tasaki et al. 2017). Tasaki et al. (2017) explained that when termites are outside of the colony with more aerobic conditions, termites might experience elevated oxidative stress that causes reduction of intestinal anaerobes and decelerated uric acid degradation. Thus, uric acid could be produced by facultative anaerobes, such as the isolated cellulytic microbes from the gut of *C. curvignathus* to increase the survival of their host in laboratory condition.

Table 7. Uric acid production (mg/dL) from cellulolytic microorganisms after different incubation periods

Incubation Periods	**24 hours**	**48 hours**	**72 hours**
Foregut			
F1. *Bacillus cereus*	2.182±0.064 ^{b}	2.626±0.121 ^{b}	3.507±0.113 ^{a}
F2. *Bacillus cereus*	1.959±0.080 ^{b}	2.270±0.146 ^{b}	3.452±0.397 ^{a}
F3. *Bacillus cereus*	2.301±0.154 ^{b}	2.326±0.135 ^{b}	3.385±0.165 ^{a}
F4.*Burkhloderia cepacia*	2.346±0.051 ^{ab}	2.293±0.059 ^{b}	2.536±0.026 ^{a}
Midgut			
M1. *Bacillus cereus*	1.881±0.102 ^{b}	1.942±0.053 ^{b}	3.115±0.151 ^{a}
M2. *Salmonella enterica*	1.840±0.045 ^{b}	1.921±0.068 ^{b}	2.904±0.180 ^{a}
M3. *Citrobacter koseri*	1.845±0.004 ^{b}	2.254±0.037 ^{b}	3.053±0.174 ^{a}
M4. *Bacillus cereus*	1.835±0.021 ^{a}	2.336±0.116 ^{a}	2.304±0.298 ^{a}
M5. *Bacillus subtilis*	1.815±0.141 ^{b}	2.090±0.117 ^{b}	4.535±0.133 ^{a}
M6. *Bacillus cereus*	1.815±0.024 ^{c}	2.200±0.091 ^{b}	2.880±0.064 ^{a}
M7. *Bacillus cereus*	1.729±0.035 ^{c}	2.101±0.084 ^{b}	3.151±0.058 ^{a}
M8. *Citrobacter koseri*	2.053±0.164 ^{b}	2.401±0.176 ^{b}	3.583±0.246 ^{a}
M9. *Dyellajaponica*	1.184±0.078 ^{a}	2.168±0.173 ^{a}	2.695±0.592 ^{a}
Hindgut			
H1. *Bacillus cereus*	2.063±0.085 ^{c}	2.438±0.068 ^{b}	3.297±0.024 ^{a}
H2. *Bacillus cereus*	1.979±0.079 ^{c}	2.422±0.050 ^{b}	3.105±0.149 ^{a}
H3. *Bacillus cereus*	2.096±0.095 ^{b}	2.706±0.102 ^{ab}	3.343±0.232 ^{a}
H4. *Bacillus cereus*	1.990±0.023 ^{c}	2.792±0.067 ^{b}	3.285±0.089 ^{a}
H5. *Bacillus cereus*	1.977±0.103 ^{b}	3.108±0.332 ^{b}	5.791±0.551 ^{a}
H6. *Citrobacter koseri*	1.820±0.055 ^{a}	2.280±0.078 ^{a}	3.099±0.525 ^{a}

H7. *Citrobacter koseri*	1.898±0.134 [b]	2.637±0.090 [a]	2.856±0.040 [a]
H8. *Bacillus cereus*	1.852±0.016 [c]	2.744±0.085 [b]	3.373±0.098 [a]

Data are shown as mean ± standard errors (n = 3).
Means with different letters within a row are significantly different ($p < 0.05$).

CONCLUSION

In summary, this study identified the distribution of metabolic compounds in the intestinal tract which could be relate the metabolism in the termite species, *C. curvignathus*. However, the characterization of overall gut fluid compounds in the termite is still inadequate. A more detailed approach of the chemical compounds analysis is required to evidence them. The specificity of certain compounds found in different compartments seems to provide insights into the digestive processes of *C. curvignathus*, involving the presence of distinct fermenting microorganisms and physiocochemical conditions of the gut environment that affect the rate of cellulose hydrolysis.

ACKNOWLEDGEMENTS

This research was partially funded by MOSTI via ScienceFund 02-05-20-SF11118.

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