Role of Lignin in Nutritional Physiology of a Lower Termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae)

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General Introduction

Termites represent one of the most abundant and efficient lignocellulose decomposers on earth. Since they are able to degrade lignocellulose, termites have an important place in the carbon cycle in nature (Brune, 2014). They have the ability to degrade the cellulose (74%–99%) and hemicellulose (65%–78%) constituents of lignocellulose with the aid of symbiotic microbiota in their hindgut (Ohkuma 2003; Hongoh, 2011; Ni and Tokuda, 2013). More than 2600 species of termites have been described worldwide, and these can be generally classified into seven families: i.e., Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae Serritermitidae, and Termitidae (Ni and Tokuda, 2013). The Mastotermitidae are the oldest family and include only one wood-feeding species from Australia. Termites of Kalotermitidae consume material from dry wood, and Termopsidae nest in and feed on wet dead logs. Hodotermitidae are grass harvesters while Rhinotermitidae consume wood, primarily in temperate zones. Serritermitidae comprise few species and are only found in South America (Yoshimura, 1995; Eggleton, 2011; Ni and Tokuda, 2013).

Termites can be classified based on the presence or absence of symbiotic protists in their hindgut: namely, higher termites lack them while lower termites harbor them. Termites of Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae and Serritermitidae possess symbiotic protists in their hindgut and are described as lower termites, while Termitidae species lack symbiotic protists in the hindgut and are described as higher termites (Yoshimura, 1995; Lo and Eggleton, 2011). *Coptotermes formosanus* Shiraki is one of Rhinotermitidae species, is considered important pests infesting wooden structures worldwide.

Earlier studies reported that approximately 10^3-10^5 protist cells inhabit the hindgut of a lower termite *C. formosanus* Shiraki (Yoshimura, 1995; Inoue et al., 1997). Three symbiotic protists in the hindgut of the lower termite *C. formosanus* have been reported: *Pseudotrichonympha grassii*, *Holomastigotoides hartmanni*, and *Spirotrichonympha leidyi* (Koidzumi, 1921). They have special roles in the digestion of molecular weight (MW) cellulose; *P. grassii* use high-MW cellulose, whereas *H. hartmanni* and *S. leidyi* used only low-MW cellulose (Yoshimura, 1995; Tanaka et al., 2006). *Pseudotrichonympha grassii*, the largest in size, is spindle-shaped, approx. 150–250 μm long and 50–100 μm wide. *H. hartmanni*, the mid-sized protist, is oval or elliptical shape, approx. 50–150 μm long and 30–100 μm wide, and *S. leidyi* is the smallest in size, approx. 20–50 μm long and 10–30 μm wide with a cone shape (Koidzumi, 1921;Yoshimura, 1995).

The lower termites including *C. formosanus* possess bacteria as well as protists in their gut. Bacteria communities in the hindgut of termite are complex and remarkably diverse (Radek, 1999). The density of prokaryotic cells in the termite gut was estimated at around 10^6–10^7 cells per μl (Schultz and Breznak, 1978; Bignell et al., 1980). The phylogenetic diversity of the bacterial community in the gut of the lower termite *C. formosanus* has been surveyed by Shinzato et al. (2005). They recorded several bacteria orders, i.e., Bacteroidales, Spirochaetales, Bacillales, Lactobacillales and a few clones that originated from Planctomycetes, Actinobacteria, and Proteobacteria. A more recent study investigating the hindgut of the lower termite *Coptotermes gestroi* reported 12 bacterial orders, Spirochaetales, Lactobacillales, Bacteroidales, Clostridiales, Enterobacteriales, Pseudomonades, Synergistales, Desulfovibrionales, Xanthomonadales, Burkholderiales, Bacillales, and Actinomycetales, and an estimated 1460 species (Do et

al., 2014). Spirochaetales was the most abundant order found in the gut of *C. gestroi*, a close relative of *C. formosanus*. The most abundant and predominant genus was Treponema, followed by Lactococcus, Pseudomonas, Enterobacter, Dysgonomonas, Clostridium, and Bacteroides (Do et al., 2014).

It has been well known that the lower termites masticate lignocellulose materials with their mandibles and grind them into minute pieces using a sclerotized cuticular armature at the posterior terminus of the foregut (gizzard) (Hongoh, 2011). It has been reported that the foregut and midgut of the lower termite *C. formosanus* contain lignocellulose particles approximately 10–20 μm in size (Fujita et al., 2010). Cellulase enzymes are produced by the host's salivary glands and/or midgut to decompose amorphous cellulose matrix and hydrolyze cellobiose to glucose. These enzymes have been well documented as endoglucanases (endo-β-1,4-glucanases) and β-glucosidases. In addition, other enzymes are required to decompose the crystalline cellulose matrix since endoglucanases cannot decompose this matrix. These enzymes are supplied by the symbiotic microbiota in the termite hindgut and have been well documented as exoglucanases (Watanabe and Tokuda, 2001; Nakashima, 2002; Watanabe and Tokuda, 2010). Since hemicellulose is generally bonded with cellulose and prevents access of cellulolytic enzymes to cellulose, enzymatic hydrolysis of hemicellulose is beneficial for cellulose digestion. Hemicellulolytic enzymes are mainly secreted by protists in the hindgut of lower termites (Arakawa et al., 2009); however, some studies have reported that symbiotic prokaryotic communities in the lower termite gut provide hemicellulolytic enzymes based on metatranscriptomic analyses (Tartar et al., 2009; Xie et al., 2012).

The presence of symbiotic microbiota in the guts is highly crucial for lower termites. The symbiotic microbiota play an important role in lignocellulose digestion, termite

nutrition, and gas production by the termites, and this mutualism is regarded as a useful model of symbiotic association between animals and microorganisms (Shinzato et al., 2005). An earlier study clearly described the role of symbiotic protists for the nutritional physiology of *C. formosanus* (Yoshimura, 1995). As discussed above, lower termites have a dual decomposing system to convert cellulose into acetates and other nutrients that the host further utilizes as energy and carbon sources: namely, the host's endogenous cellulases and a variety of cellulolytic enzymes secreted by protists (Ohkuma, 2003). In addition, symbiotic protists in the hindguts of lower termites are predominant producers of hydrogen (H_2), where H_2 is emitted by symbiotic microbiota as a by-product of lignocellulose decomposition for energy and carbon sources (Inoue et al., 2007; Cao et al., 2012). It has been well recognized that lignocellulose is notoriously low in nitrogen and contains only negligible amounts of amino acids and vitamins (Brune, 2014). The host's nitrogen waste is excreted as uric acid into the gut, where it is degraded by the gut symbionts (Hongoh, 2011). In fact, the bacterial community efficiently recycles the nitrogenous waste products of the termite, assimilates ammonia into nutritionally valuable microbial biomass and amends the nitrogen budget by dinitrogen fixation; in addition, the symbiotic microbiota have roles in acetogenesis and methanogenesis processes (Brune, 2014).

Lignocellulose, as the major nutrient source for termites, is composed mainly of cellulose, hemicelluloses and lignin, and represents the most abundant biomass on earth. Cellulose, a linear polysaccharide composed of β-D-glucopyranose units linked by (1-4) glycosidic bonds, is the major component of lignocellulose and composed of only glucose (O'Sullivan, 1997; Czaja et al., 2004). Hemicellulose is the second major component of lignocellulose, and its main chains are composed of xylose, or glucose and mannose,

which is often acetylated or shortly branched with arabinose, galactose, or other acidic sugars. Chemical compositions of hemicelluloses vary across plant species: hardwood hemicelluloses contain mostly xylans whereas softwood hemicelluloses contain mostly glucomannans (Saha, 2003; Ni and Tokuda, 2013). Lignin, a heterogeneous phenylpropanoid polymer derived primarily from oxidative couplings of *p*-hydroxycinnamyl alcohols (monolignols) and their derivatives, is the key component of the lignocellulose produced in the secondary cell walls of vascular plants, where it serves to encrust cell wall polysaccharides (i.e., cellulose and hemicelluloses) and provide them with increased mechanical strength, imperviousness, and resistance to pathogens (Boerjan et al., 2003; Chen and Dixon, 2007; Jackson et al., 2008; Tobimatsu et al., 2012). Plants have different types of lignins, depending primarily on their taxa. Softwood lignins are composed mainly of guaiacyl (G) units while hardwood lignins consist of G and syringyl (S) units; both softwood and hardwood lignins also contain a small amount of *p*-hydroxyphenyl (H) units. Like hardwood lignins, lignins from grasses incorporate mostly G and S units at comparable levels and often more H units than softwood and hardwood lignins (Boerjan et al., 2003; Bonawitz and Chapple, 2010). In addition, relatively recent studies have revealed that typical grass lignins are highly acylated by *p*-coumarate esters (Ralph, 2010; Petrik et al., 2014) and that they also contain a substantial amount of tricin flavonoid units (Lan et al., 2015; 2016; Li et al., 2016). Due to its chemical complexity and general lack of vulnerable linkages, lignin confers lignocellulose with high resistance to most forms of microbial attack. Lignin biodegradation is thus a key step for the lignocellulose-oriented carbon recycling in terrestrial ecosystems. The process has long been a major focus among researchers motivated by potential biotechnological applications in plant biomass utilization. In this context, lignin biodegradation via wood-

decaying fungi, known as white-rot and brown-rot decay, has been extensively studied (Martinez, 2002; Perez et al., 2002; Guillén et al., 2005; Thevenot et al., 2010; Beckham et al., 2016). However, lignin degradation processes in more complex ecosystems such as those undertaken by wood-feeding insects remain largely elusive.

The mechanisms of the decomposition of polysaccharides by termites have been well-documented (Ohkuma, 2003; Hongoh, 2011; Ni and Tokuda, 2013; Brune, 2014). However, there is almost no information regarding the role of lignin in the nutritional physiology of wood-feeding insects. Studies on the role of lignin in the nutritional physiology of wood-feeding insects would improve our understanding of natural lignocellulose-decomposition mechanisms, and contribute to the development of sustainable technology to convert lignocellulosic biomass to valuable fuels and materials. Various studies have been carried out to elucidate the fate of lignin in the termite digestive system. Several reports have documented that gut flora of wood-feeding lower termites exhibited *in vitro* and/or *in vivo* abilities to modify monomeric and dimeric lignin-associated aromatic molecules (Kuhnigk et al., 1994; Brune et al., 1995; Kuhnigk and König, 1997; Harazono et al., 2003). Kyou et al. (1996) reported that protists in the hindguts of *C. formosanus* workers could be involved in the modification of lignin polymers. Earlier structural studies on polymeric lignins using conventional wet-chemical and spectroscopic approaches detected no conclusive evidence for chemical modification of the polymers upon their passage through the gut of lower termites (Hyodo et al., 1999; Katsumata et al., 2007). However, more recent studies, especially those using advanced thermochemolysis, e.g., pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) with or without *in situ* derivatization with tetramethylammonium hydroxide (TMAH), have provided data supportive of chemical modifications of lignin polymers in the gut

digestive system of lower termites; Py-GC/MS of digested lignocelluloses detected characteristic pyrolytic fragments conceivably derived from degraded lignin polymers with aromatic ring modifications and/or side-chain oxidations (Geib et al., 2008; Ke et *al*., 2011a; 2011b; 2013). As such, thermochemolysis is indeed a versatile tool for characterizing lignins with high sensitivity (Ralph and Hatfield, 1991; Clifford et al., 1995; del Rio et al., 1996; Izumi and Kuroda, 1997; Rodrigues et al., 1999). However, since the method is not fully quantitative, it is yet unclear how abundantly such lignin processing occurs in the termite digestive system.

In this study, the lower termite *C. formosanus* was chosen as a test species due to the easy maintenance of its laboratory colonies (Yoshimura, 1995). This study is divided into 5 chapters. Chapter 1 deals with the lignocellulose deconstruction by the lower termite, *C. formosanus*, with emphasis on the modification of lignin polymers. Softwood, hardwood and grass samples were fed to *C. formosanus* workers, and the fecal materials were subjected to detailed structural analyses using high-resolution 2D NMR as well as a series of wet-chemical analyses. In Chapter 2, the chemical composition in each lignocellulose, and the physiological responses of the lower termite *C. formosanus* fed various lignocelluloses and purified lignins (milled-wood lignins, MWLs) from Japanese cedar (softwood), Japanese beech (hardwood), and rice (grass) were investigated to determine the physiological effects of different lignocellulose sources and lignins on *C. formosanus* such as termite survival, body mass, and the changes of the symbiotic protists in the hindgut of workers. Chapter 3 further investigates the effects of lignin on the nutritional physiology of *C. formosanus*. In this chapter, hydrogen and methane emissions from *C. formosanus* workers fed various lignocellulose, and lignin (MWLs), and artificial diets composed of mixtures of isolated lignins and polysaccharides (holocellulose or

cellulose) were measured. In Chapter 4, the effects of lignins as diet components on the physiological activities of a lower termite, *C. formosanus* were investigated to determine the exact role of lignin in termites. In this chapter, artificial diets composed of polysaccharides (holocellulose or α-cellulose) with and without purified lignins (milled-wood lignins) from Japanese cedar (softwood), Japanese beech (hardwood), and rice (grass) were fed to *C. formosanus* workers. The survival and body mass of the workers and the presence of three symbiotic protists in the hindguts of the workers were observed. In the final chapter, Chapter 5, the effects of dietary lignin on bacterial community profiles in the hindgut of a lower termite *C. formosanus* were discussed. In the experiments for this chapter, diet samples were prepared only from Japanese cedar, and the bacterial community was profiled using ARISA (Automated Ribosomal Intergenic Spacer Analysis).

Chapter 1. NMR studies on lignocellulose deconstructions in the digestive system of a lower termite, *Coptotermes formosanus* Shiraki

1.1. Introduction

Lignin deconstruction is a critical step in lignocellulose digestion as it first enables dissociation of the recalcitrant lignin polymers from the cellulose and hemicelluloses in which they are embedded, making the polysaccharides available for assimilation and energy. Although the degradation of cell-wall polysaccharides in the termite digestive system has been relatively well documented in the literature, the mechanism by which termites overcome the recalcitrant lignin barrier to gain access to embedded polysaccharides for assimilation and energy remains largely unknown. While numerous research attempts have been conducted to assess structural changes of lignin polymers during their passage through the gut of lower termites, it is yet unclear whether or not and, if any, how abundantly lignin modifications/degradations occur in the termite digestive system.

Recently, solution-state multi-dimensional NMR spectroscopy has proven to be highly effective in characterizing the chemical structures of various lignocellulosic materials. Such NMR techniques have been increasingly applied to understanding lignocellulose biodegradation processes such as those undertaken by white-rot and brown-rot fungi (Yelle et al., 2008; Martinez et al., 2011; Yelle et al., 2011; 2014) as well as by a fungus-cultivating higher termite, *Odontotermes formosanus* Shiraki (Li et al., 2017), for whom the detailed chemical changes in the complex polysaccharide and lignin polymer structures were successfully tracked. This study applied these NMR techniques to study lignocellulose decomposition in the gut digestive system of a lower termite

Coptotermes formosanus Shiraki, one of the most destructive and economically important wood-feeding termites in the world (Yoshimura, 1995). To investigate the actions of the *C. formosanus* digestive system on different types of lignocellulose substrates, *C. formosanus* workers were fed three different lignocellulose diets prepared from softwood (Japanese cedar), hardwood (Japanese beech), and grass (rice straw). Structural differences between the original diets and the resulting feces were closely examined by solution-state $^{1}H–^{13}C$ short-range correlation (HSQC) NMR as well as by complementary wet-chemical methods. The obtained structural data were discussed with special emphasis on the fate of lignin polymers in the termite gut digestive system.

1.2. Materials and methods

1.2.1. Termite feeding and sample collection

One thousand two hundred workers of *C. formosanus* were starved for 2 days and then allowed to feed in a glass petri dish containing sapwood blocks [20 (R) × 20 (T) × 10 (L) mm] of Japanese cedar (*Cryptomeria japonica*) or Japanese beech (*Fagus crenata*), or 35-mm-long culm straws of rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare). Termite feces attached to the surface of each cup were carefully collected every 2 days and pooled over 8 weeks to provide enough materials (ca. 500 mg) for subsequent analysis. The obtained feces samples and pulverized original lignocellulose materials (control) were washed successively with water and 80% ethanol, and then lyophilized to give cell wall residue (CWR) samples used for chemical analysis and NMR spectroscopy. In parallel, fifty workers and five soldiers of *C. formosanus* were subjected to the feeding experiments as described above but on smaller sapwood blocks of Japanese cedar and

Japanese beech [10 (R) × 10 (T) × 10 (L) mm] or dried culm straws (25-mm-long). The number of live workers was recorded weekly for 3 weeks. After 3 weeks of feeding, lignocellulose residues were oven-dried and weighed to determine the mass loss during the feeding. Experiments were performed in triplicate. The differences in the survival rate of termites and mass loss of lignocellulose were tested by a IBM SPSS software. Significant differences ($P < 0.05$) between means were calculated by Tukey's post-hoc test.

1.2.2. Wet chemistry

Thioglycolic acid lignin assay was performed as described previously (Suzuki et al., 2009); milled wood lignins prepared from each original lignocellulose (Tarmadi et al., 2017a) were used to construct the standard curves. Analytical thioacidolysis was performed according to Yamamura et al. (2012) and the released lignin monomers were derivatized with *N*,*O*-bis(trimethylsilyl)acetamide and quantified by gas chromatography/mass spectrometry (GC/MS) using 4,4′-ethylidenebisphenol as an internal standard (Yue et al., 2012). Carbohydrate content was determined according to Albersheim et al. (1997) and Foster et al. (2010) with a slight modification. Briefly, matrix polysaccharides in CWR samples were first hydrolyzed with 2 M trifluoroacetic acid (100°C, 5 hr), and the resulting monomeric sugars were derivatized by the alditol-acetate method (Hayashi, 1989) and quantified by GC/MS using myo-inositol as an internal standard. The trifluoroacetic acid-insoluble pellets containing crystalline cellulose were washed by Updegraff reagent (Updegraff, 1969) and then completely hydrolyzed with 72% sulfuric acid (Yamamura et al., 2013). The glucose concentrations in the obtained solutions were measured using Glucose C-II Test Wako reagent (Wako Pure Chemical)

according to the manufacturer's instructions.

1.2.3. NMR spectroscopy

For NMR analysis, the CWR samples (ca. 250 mg) prepared as described above were subjected to fine ball-milling as described previously (Mansfield et al., 2012; Tobimatsu et al., 2013). Aliquots of the ball-milled CWRs (ca. 60 mg) were directly swelled in dimethylsulfoxide (DMSO)-d_6/pyridine-d_5 [4:1 (v/v), 600 µl] for whole-cell-wall NMR analysis (Kim and Ralph, 2010; Mansfield et al., 2012). The remaining ball-milled CWRs (ca. 190 mg) were further digested with crude cellulases (Cellulysin, Calbiochem) and completely acetylated in a DMSO/*N*-methylimidazole/acetic anhydride system as described previously (Tobimatsu et al., 2013). The obtained acetylated lignin-enriched CWRs were dissolved in 600 µl of chloroform-*d* and subjected to NMR analysis. NMR spectra were acquired on a Bruker Biospin Avance III 800US spectrometer fitted with a cryogenically cooled 5-mm TCI gradient probe. Adiabatic HSQC experiments ("hsqcetgpsp.3") were performed using the parameters described by (Mansfield et al., 2012). Data processing used Bruker TopSpin software and the central solvent peaks (DMSO-d_6: δ_C/δ_H, 39.5/2.49 ppm; chloroform-*d*: δ_C/δ_H, 77.0/7.26 ppm) were used as an internal reference. HSQC plots were obtained with typical matched Gaussian apodization in F2 and squared cosine-bell apodization and one level of linear prediction (16 coefficients) in F1. For constructing differential HSQC spectra and acquiring volume integrations, linear prediction was turned off. For comparison between cell-wall NMR spectra of original and digested lignocelluloses (Fig 1.2), major lignin aromatic and polysaccharide anomeric signals (listed in Fig. 1.2b) were integrated and normalized based on the sum of the integrated signals. For analysis of lignin aromatic composition

(Fig. 1.3), C_2–H_2 correlations from $\mathbf{L_G}$, C_2–H_2/C_6–H_6 correlations from $\mathbf{L_S}$ and **P**, and $C_{2'}$–$H_{2'}$/$C_{6'}$–$H_{6'}$ correlations from **T** were integrated, and the $\mathbf{L_S}$, **P**, and **T** integrals were logically halved. For analysis of lignin inter-monomeric linkage distributions (Fig. 1.7), well-resolved C_α–H_α contours from **I**, **II**, **III**, **III´**, and **V**, and C_β–H_β contours from **I´** and **VI** were integrated, and **III** and **III´** integrals were logically halved. The relative contour intensities listed in Figures 1.6 and 1.7 are expressed on $\mathbf{L_S}$ + $\mathbf{L_G}$ = 100 and **I** + **I´** + **II** + **III** + **III´**+ **IV** + **V** = 100 bases, respectively.

1.3. Results

1.3.1. Termite survival and mass loss of lignocellulose

At the onset of this study, the termite survival and dietary mass consumed for *C. formosanus* workers fed lignocellulose diets were observed. The survival rates of workers fed J. cedar (softwood) and J. beech (hardwood) diets after 3 weeks of feeding were similar (~90%) and significantly higher than those recorded for workers fed rice straw (grass) diet (~74%) and starvation controls (~70%) (Fig. 1.1a). The statistical analysis failed to detect any significant difference between the workers fed on rice and the starvation control. Furthermore, it was suggested that *C. formosanus* workers consumed significantly less of the rice grass diets than the J. cedar and J. beech wood diets (Fig. 1.1b). These data collectively suggest that the rice grass diet was less nutritious for *C. formosanus* workers compared to the softwood and hardwood diets. The result is in line with the observation that lower termites including *C. formosanus* generally prefer wood to grass for feeding in nature (Brune, 2014).

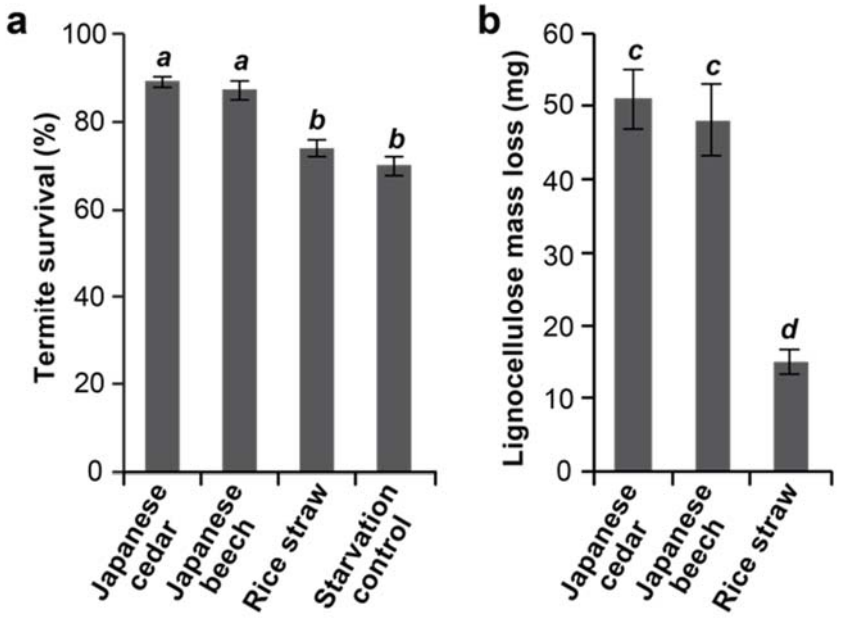

Fig 1.1. Termite survival (a) and lignocellulose mass loss (b) after 3-week feeding by *C. formosanus* termite workers on Japanese cedar (softwood), Japanese beech (hardwood), and rice straw (grass) lignocellulose diets. Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$; n = 3) following one-way ANOVA. Error bars represent standard deviations.

1.3.2. Chemical compositional analysis of lignocelluloses digested by C. formosanus

Digested lignocellulose samples (feces) from *C. formosanus* workers were first characterized by lignocellulose compositional analysis using wet chemical methods. Sugar and lignin content analyses determined an approximately 50% proportional decrease in cellulosic crystalline glucan and a 90% proportional increase in lignin content in the digested softwood J. cedar lignocellulose (Table 1.1). While mannan was moderately (22%) depleted, the other major hemicellulosic sugars, i.e., xylan, arabinan, and galactan, were significantly enriched along with lignin in the digested J. cedar lignocellulose. The result suggests the selective digestion of cellulose over hemicelluloses and lignins in the *C. formosanus* digestive system. In the digestion of J. beech hardwood,

an approximately 50% proportional decrease and 70% proportional increase in cellulose and lignin content, respectively, were likewise recorded (Table 1.1). Unlike in the digestion of J. cedar, however, this study observed significant decreases in amorphous glucan (~60%), xylan (~74%), and mannan (~89%) in the digestion of J. beech lignocellulose. In the digestion of grass rice straw, this study observed relatively moderate changes in both cellulose (~22% decrease) and lignin (~37% increase) content (Table 1.1). As with J. beech, in the rice lignocellulose digestion, this study observed significant decreases in the major hemicellulosic sugars such as amorphous glucan (~18%), xylan (~49%), and arabinan (89%), but enrichments in mannan and galactan. Overall, the data are in line with previous studies reporting preferential conversions of cell-wall polysaccharides over lignins in the termite digestive system. It is most likely that polysaccharide conversions in the *C. formosanus* digestive system are highly selective for cellulose degradation, especially in softwood lignocellulose, but expand to include hemicellulosic sugar conversions in hardwood and grass lignocelluloses.

Table 1.1. Chemical analysis of lignocellulose samples digested by *C. formosanus* termite workers

Chemical components	Japanese cedar		Japanese beech		Rice straw	
	Original	Digested	Original	Digested	Original	Digested
Lignin content (mg/g CWR)	239.2 ± 2.0	**463.0 ± 22.2***	196.0 ± 3.7	**334.9 ± 2.8***	150.2 ± 4.7	**205.3 ± 3.0***
Lignin composition						
Syringyl units, S (%)	N.D.	N.D.	75.2 ± 0.1	**70.2 ± 1.3***	43.6 ± 1.5	**54.7 ± 0.3***
Guaiacyl units, G (%)	99.5 ± 0.1	99.8 ± 0.1	24.8 ± 0.1	**29.7 ± 1.3***	52.3 ± 1.4	**41.9 ± 0.6***
p-Hydroxyphenyl units, H (%)	0.5 ± 0.1	0.2 ± 0.1	trace	0.2 ± 0.1	4.2 ± 0.5	3.5 ± 0.4
S/G ratio	0.00 ± 0.00	0.00 ± 0.00	3.05 ± 0.01	**2.37 ± 0.14***	0.84 ± 0.05	**1.31 ± 0.03***
Carbohydrate content (mg/g CWR)						
Glucan (crystalline)	442.1± 8.7	**210.7 ± 10.5***	428.2 ± 40.9	**235.5 ± 23.4***	454.4 ± 18.5	**354.1 ± 52.0***
Glucan (Amorphous)	27.2 ± 1.2	27.4 ± 4.2	23.9 ± 2.6	**9.6 ±1.3***	22.6 ± 1.2	**18.7 ± 0.6***
Xylan	64.4 ± 3.2	**108.7 ± 9.5***	305.82 ± 12.6	**80.2 ±3.2***	81.0 ± 5.5	**41.3 ± 1.1***
Mannan	66.4 ± 2.8	**52.0 ± 4.9***	8.67 ± 0.6	**1.85 ± 0.7***	3.4 ± 0.7	**8.3 ± 0.6***
Arabinan	15.3 ± 0.1	**26.2 ± 2.6***	7.6 ± 0.3	7.83 ± 1.9	29.8 ± 0.6	**19.5 ± 0.2***
Galactan	16.1 ± 1.1	**22.3 ± 1.9***	8.8 ± 0.8	10.9 ±1.5	12.9 ± 1.4	**15.9 ± 0.6***

Lignin composition was determined by analytical thioacidolysis. Values are means ± SD ($n = 3$) and asterisks (*) indicate significant difference between original and digested lignocellulose diets ($P < 0.05$). N.D.: not detected.

1.3.3. Cell-wall NMRs of lignocelluloses digested by C. formosanus

The present study then employed 2D NMR techniques to obtain further detailed chemical information of the lignocellulose diets digested by *C. formosanus* workers. One of the biggest advantages of current plant cell wall NMR techniques is the ability to analyze an entire cell wall fraction via direct dissolution/swelling methods (Lu and Ralph, 2003; Kim and Ralph, 2010; Mansfield et al., 2012). In the present study, NMR analysis was conducted on whole cell wall fractions of the original and digested lignocelluloses by simply swelling them in a DMSO-d_6/pyridine-d_5 (4:1, v/v) NMR solvent system after fine ball-milling. This approach provides a global picture of the chemical composition and structure of cell-wall polysaccharides as well as lignins, although highly crystalline cellulose contents tend to be underestimated due to its incomplete gelation (Kim and Ralph, 2010; Mansfield et al., 2012).

The short-range ^{13}C–^{1}H correlative (HSQC) NMR spectra of cell walls in the original lignocellulose diets revealed prominent structural differences between the three major types of lignocellulose in nature. As revealed by the aromatic signals in the HSQC spectra (δ_C/δ_H, 150-100/8.5-6.0 ppm), lignins in the J. cedar lignocellulose diet follow the pattern of typical softwood lignin, composed of only G units (Fig. 1.2), whereas lignins in the hardwood J. beech and grass rice straw diets are mixtures of G and S units (Figs. 1.3 and 1.4). As is typical for grass lignocellulose, the spectrum of rice cell walls additionally displayed intense signals from *p*-coumarate (Ralph, 2010) and tricin residues (Lan et al., 2015; Lam et al., 2017) attached mainly on lignins, as well as signals from ferulates mainly on arabinoxylan hemicellulose residues (Ralph, 2010). In the polysaccharide

anomeric regions (δ_C/δ_H, 110–90/6.0–3.5 ppm), anomeric signals from abundant hemicellulosic polysaccharides, such as glucomannans, glucuronoxylans, and glucuronoarabinoxylans, are visible in J. cedar, J. beech, and rice straw cell wall spectra, respectively (Figs. 1.2-4, Table 1.2). The aliphatic-oxygenated regions (δ_C/δ_H, 90–45/6.0–3.5 ppm) are overwhelmed by intense and overlapping polysaccharide contours but they also display well-resolved contours from some of the major lignin side-chains as well as those from acetylated hemicelluloses (Figs. 1.2-4, Table 1.2).

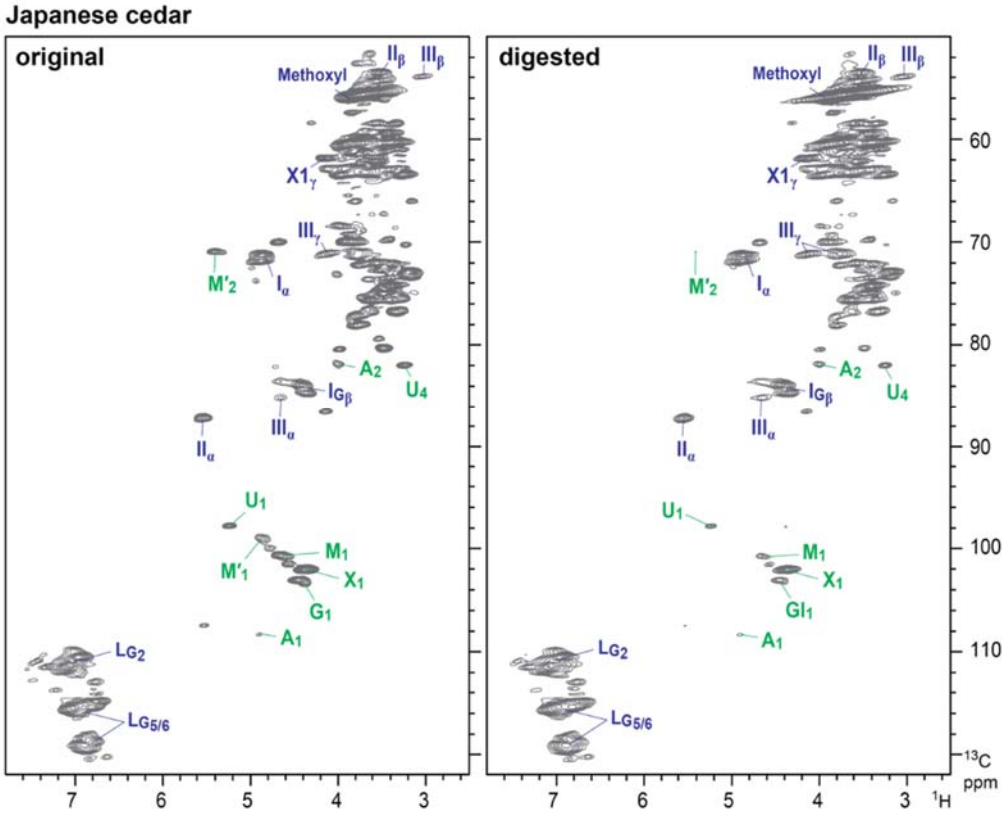

Fig 1.2. 2D ^{1}H–^{13}C correlation (HSQC) spectra of whole-cell-wall gels from original and digested Japanese beech (hardwood) lignocellulose diets fed to *C. formosanus* termite workers. For signal assignments and structure abbreviations, see Table 1.2.

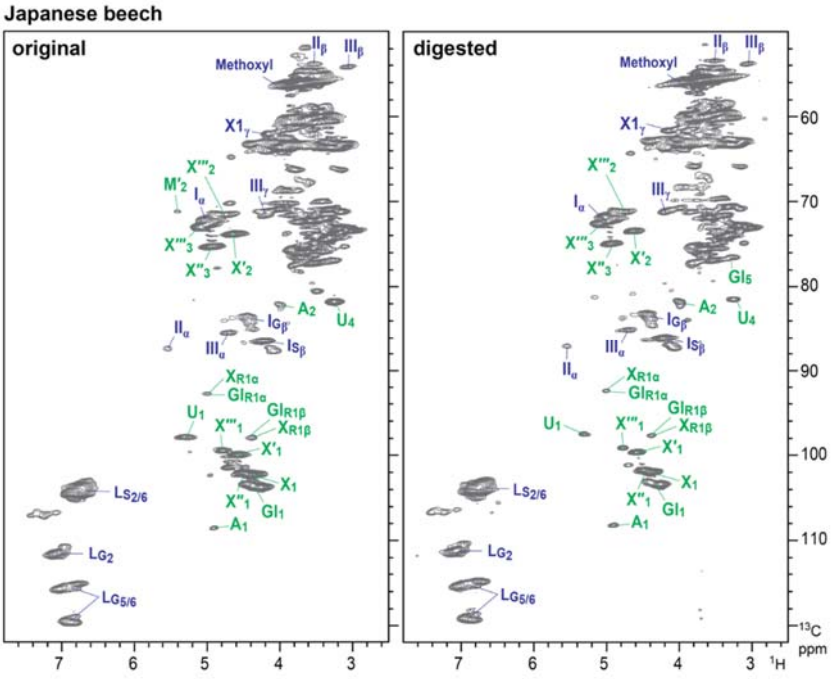

Fig 1.3. 2D ^{1}H–^{13}C correlation (HSQC) spectra of whole cell wall gels from original and digested Japanese beech (hardwood) lignocellulose diets fed to *C. formosanus* workers. For signal assignments and structure abbreviations, see Table 1.2.

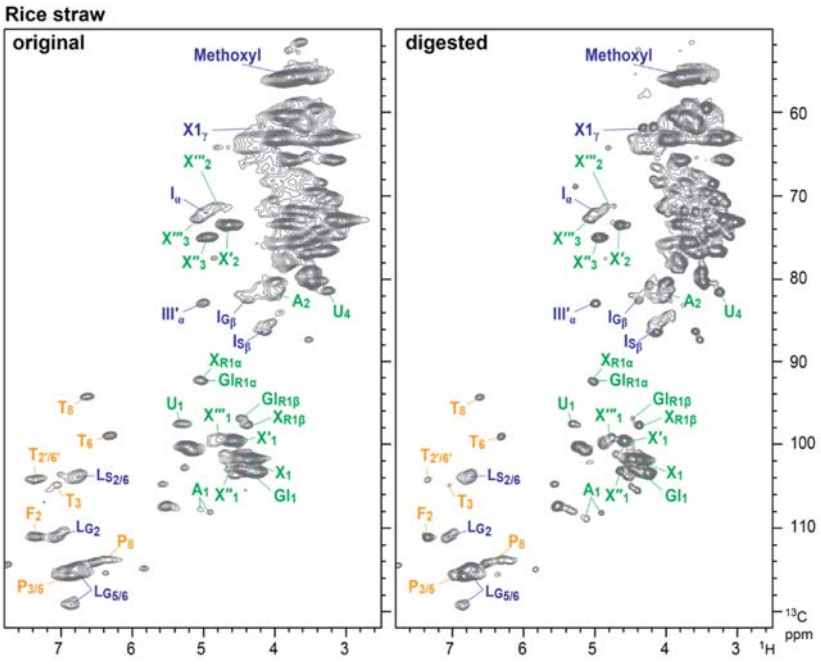

Fig 1.4. 2D ^{1}H–^{13}C correlation (HSQC) spectra of whole cell wall gels from original and digested Rice straw (grass) lignocellulose diets fed to *C. formosanus* workers. For signal assignments and structure abbreviations, see Table 1.2.

Details on the differences between the original and digested lignocellulose structures were deduced by comparing the original and digested cell wall HSQC spectra using differential spectra (original – digested) (Fig. 1.5a) and also by comparing relative signal intensities determined for the major lignin and polysaccharide signals based on their volume integrations (Fig. 5b). Overall, regardless of the lignocellulose diets tested, lignin signals were generally augmented over most of the polysaccharide signals in the digested lignocellulose spectra, which is in line with the chemical analysis data (Table 1.1) and affirms that polysaccharides were preferentially digested over lignins in the *C. formosanus* digestive system. In the digested J. cedar spectrum, signals from xylan (**X**) and arabinan (**A**) were relatively retained, whereas the signals from glucan (**Gl**), mannans (**M** and **M′**) and glucuronan (**U**) were largely depleted, compared to those in the original J. cedar spectrum. The present study also found relatively low depletions or even augmentations in the signals from arabinan (**A**) and 2,3 di-*O*-acetyl xylan (**X′′′**) in both digested J. beech and rice straw lignocellulose spectra, whereas glucan (**Gl**), non-acetylated (**X**) and mono-acetylated (**X′** and **X′′**) xylan, and glucuronan (**U**) signals were clearly depleted. Therefore, apparently, arabinan and highly acetylated xylan residues are relatively well tolerated in the *C. formosanus* digestive system. In the rice straw grass spectra, *p*-coumarate signals (**P**) were remarkably augmented, while in contrast, ferulate signals (**F**) were clearly depleted. Given that *p*-coumarates and ferulates in grass cell walls are mainly attached to lignin and arabinoxylan hemicellulosic backbones, respectively (Ralph, 2010), these data are in a good agreement with the present study`s observations of lignin augmentation and hemicellulose depletion in the digested rice straw lignocellulose. Interestingly, however, lignin-bound tricin signals (**T**) were clearly depleted in contrast to the augmented major lignin backbone and lignin-associated *p*-

coumarate signals. The result suggests preferential removal of tricin-appended lignin residues in grass lignins, as further demonstrated below.

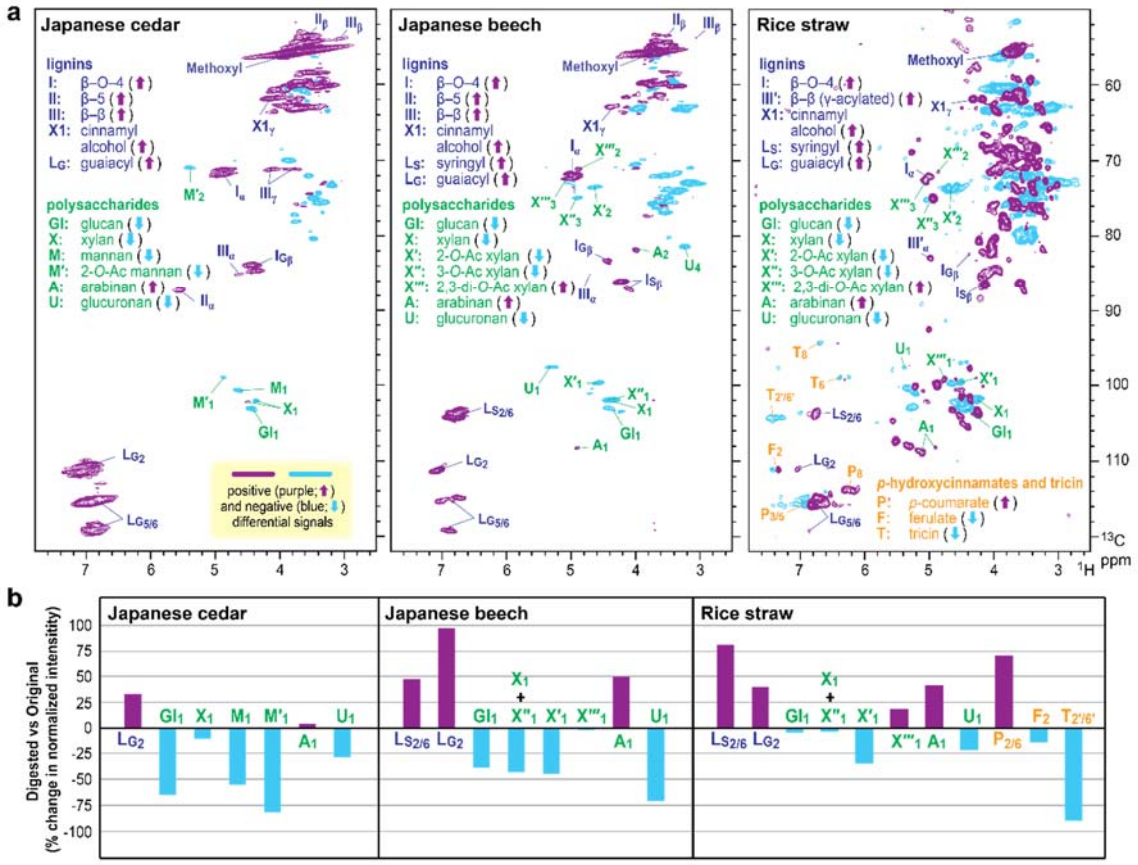

Fig 1.5. Whole cell wall NMR analysis on original and digested Japanese cedar (softwood), Japanese beech (hardwood), and rice straw (grass) lignocellulose diets fed to *C. formosanus* termite workers. (a) Differential 2D ^{1}H–^{13}C correlation (HSQC) spectra for digested versus original cell walls. (b) Changes in relative signal abundances determined for major lignin aromatic and polysaccharide anomeric signals appearing in the original and digested cell wall spectra. For signal assignments and structure abbreviations, also see Table 1.2.

Table 1.2. Assignment of lignin and polysaccharide signals in HSQC spectra of whole cell wall samples.

Labels	δ_C/δ_H (ppm)	Assignment
Lignin signals		
$L_{S2/6}$	104.2/6.77	C_2-H_2 and C_6-H_6 in syringyl units
L_{G2}	111.2/7.06	C_2-H_2 in guaiacyl units
$L_{G5/6}$	114.9/6.78, 115.3/7.02,119.3/6.88	C_5-H_5 and C_6-H_6 in guaiacyl units
I_α	71.5/4.87	C_α-H_α in β-O-4 substructures
$I_{S\beta}$	86.2/4.22	C_β-H_β in β-O-4 substructures linked to syringyl units
$I_{G\beta}$	83.9/4.38	C_β-H_β in β-O-4 substructures linked to guaiacyl units
II_α	87.2/5.50	C_α-H_α in β-5 substructures
II_β	53.6/3.49	C_β-H_β in β-5 substructures
III_α	84.8/4.67	C_α-H_α in resinol-type β-β substructures
III_β	53.7/3.01	C_β-H_β in resinol-type β-β substructures
III_γ	71.1/4.16, 71.1/3.83	C_γ-H_γ in resinol-type β-β substructures
III'_α	82.9/4.98	C_α-H_α in tetrahydrofuran-type β-β substructures
$X1_\gamma$	61.8/4.14	C_γ-H_γ in cinnamyl alcohol end-groups
Methoxyl	3.74/55.7	C-H in aromatic methoxyl groups
Polysaccharide signals		
Gl_1	103.5/4.44	C_1-H_1 in (1→4)-β-D-glucopyranosyl units
$Gl_{R1\beta}$	96.9/4.46	C_1-H_1 in (1→4)-β-D-glucopyranosyl units (reducing end)
$Gl_{R1\alpha}$	92.4/5.07	C_1-H_1 in (1→4)-α-D-glucopyranosyl units (reducing end)
X_1	102.1/4.28	C_1-H_1 in (1→4)-β-D-xylopyranosyl units
$X_{R1\beta}$	97.8/4.38	C_1-H_1 in (1→4)-β-D-xylopyranosyl units (reducing end)
$X_{R1\alpha}$	92.5/5.00	C_1-H_1 in (1→4)-α-D-xylopyranosyl units (reducing end)
X'_1	99.9/4.58	C_1-H_1 in 2-*O*-acetyl-β-D-xylopyranosyl units
X'_2	73.6/4.63	C_2-H_2 in 2-*O*-acetyl-β-D-xylopyranosyl units
X''_1	101.8/4.40	C_1-H_1 in 3-*O*-acetyl-β-D-xylopyranosyl units
X''_3	75.1/4.95	C_3-H_3 in 3-*O*-acetyl-β-D-xylopyranosyl units
X'''_1	99.1/4.78	C_1-H_1 in 2,3-di-*O*-acetyl-β-D-xylopyranosyl units
X'''_2	71.2/4.72	C_2-H_2 in 2,3-di-*O*-acetyl-β-D-xylopyranosyl units
X'''_3	74.8/4.94	C_3-H_3 in 2,3-di-*O*-acetyl-β-D-xylopyranosyl units
M_1	100.7/4.64	C_1-H_1 in (1→4)-β-D-mannopyranosyl units
M'_1	99.0/4.86	C_1-H_1 in 2-*O*-acetyl-β-D-mannopyranosyl units
M'_2	70.5/5.30	C_2-H_2 in 2-*O*-acetyl-β-D-mannopyranosyl units
A_1	107.7/5.05, 108.2/4.90	C_1-H_1 in α-L-arabinofuranosyl units
A_2	81.8/3.98	C_2-H_2 in α-L-arabinofuranosyl units
U_1	97.7/5.24	C_1-H_1 in 4-*O*-methyl-α-D-glucuronopyranosyl units
U_4	81.5/3.24	C_4-H_4 in 4-*O*-methyl-α-D-glucuronopyranosyl units
p-Hydroxycinnamate and tricin signals		
$P_{2/6}$	130.1/7.50	C_2-H_2 and C_6-H_6 in *p*-coumarate residues
$P_{3/5}$	115.6/6.85	C_3-H_3 and C_5-H_5 in *p*-coumarate residues
P_7	145.3/7.64	C_7-H_7 in *p*-coumarate residues
P_8	113.8/6.36	C_8-H_8 in *p*-coumarate residues
F_2	111.0/7.36	C_2-H_2 in ferurate residues
F_6	123.2/7.12	C_6-H_6 in ferurate residues
T_3	104.9/7.06	C_3-H_3 in tricin residues
T_6	98.9/6.31	C_6-H_6 in tricin residues
T_8	94.2/6.63	C_8-H_8 in tricin residues
$T_{2'/6'}$	104.3/7.36	$C_{2'}$-$H_{2'}$ and $C_{6'}$-$H_{6'}$ in tricin residues

Measured in DMSO-d_6/Py-d_5 (4:1, v/v). Signal assigment was based on comparison with literature data (Kim and Ralph, 2010; Rencoret et al., 2011; Brennan et al., 2012; Mansfield et al., 2012).

1.3.4. 2D NMRs on residual lignins in lignocelluloses digested by C. formosanus

To more closely investigate possible lignin polymer modifications/decomposition in the termite digestive system, the present study prepared lignin-enriched cell wall fractions from the original and digested lignocellulose materials via cellulase treatment, which leaves all the lignins and residual polysaccharides (Ralph et al., 2006; Tobimatsu et al., 2013). The obtained lignin-enriched cell wall fractions were then acetylated to be solubilized in chloroform-*d* for further comprehensive analysis by NMR (Lu et al., 2003; Ralph et al., 2006; Tobimatsu et al., 2013). HSQC spectra indicated successful removal of cell wall polysaccharides and concurrent enrichment of lignin polymers and displayed well-resolved signals from major aromatic units as well as inter-monomeric linkage types present in the lignin polymers (Figs. 1.6 and 1.7, and Table 1.3).

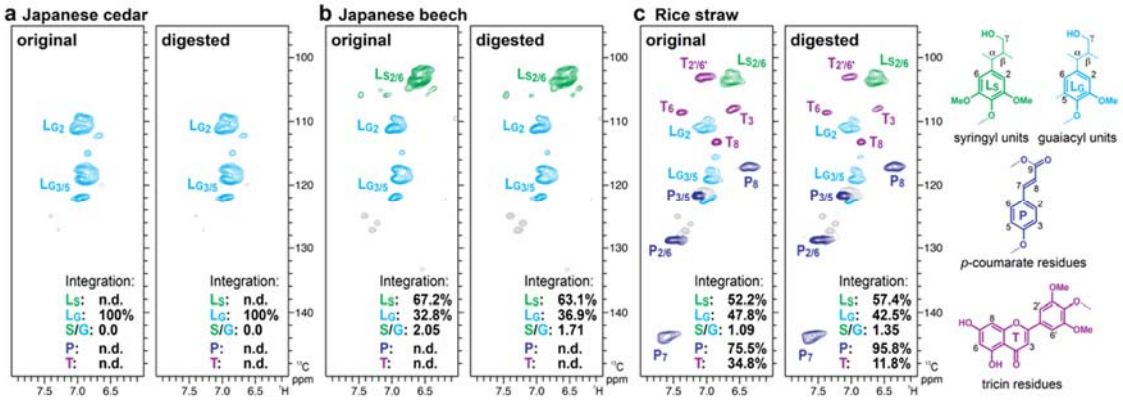

Fig 1.6. 2D ^{1}H–^{13}C correlation (HSQC) spectra of acetylated samples of lignin-enriched cell walls from original and digested lignocellulose diets fed to *C. formosanus* termite workers. Lignin aromatic sub-regions are shown for (a) Japanese cedar (softwood), (b) Japanese beech (hardwood), and (c) rice straw (grass) diets. Volume integrals are given for the major lignin aromatic units that are color-coded to match their assignments in the spectrum. The percentages noted in each spectrum are integrals relative to the sum of the syringyl and guaiacyl lignin unit signals ($\mathbf{L_S}$ + $\mathbf{L_G}$ = 100%). For signal assignments and structure abbreviations, also see Table 1.3. n.d., not detected.

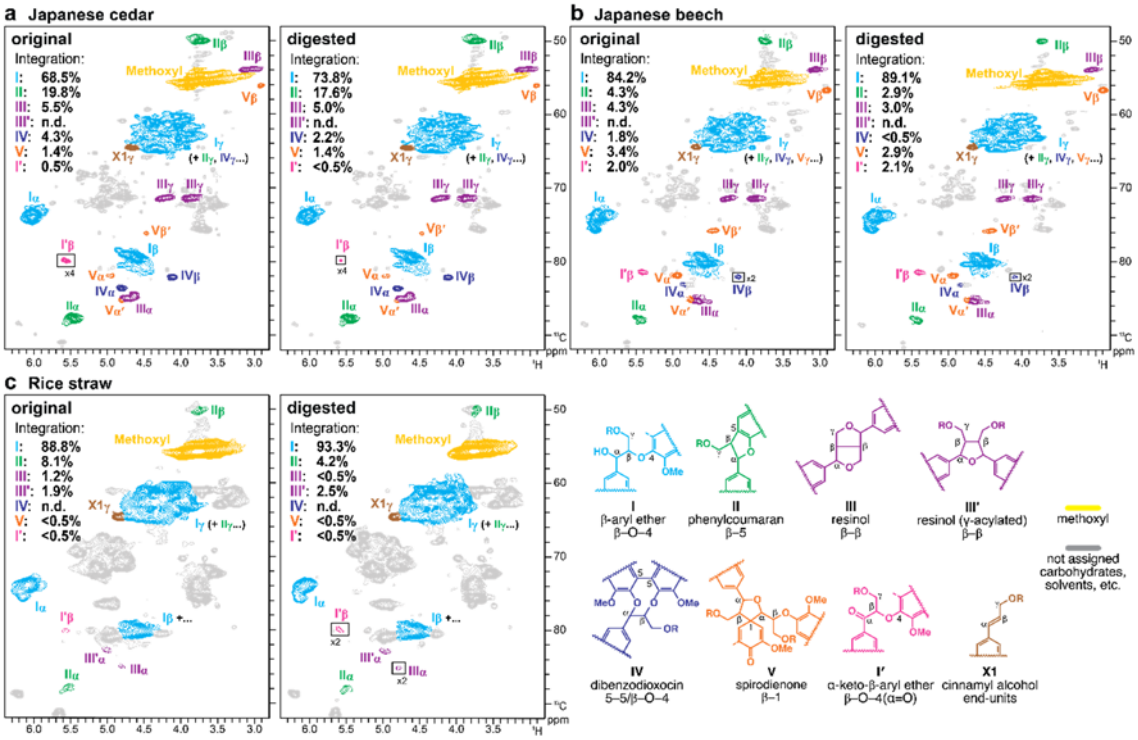

Fig 1.7. 2D ^{1}H–^{13}C correlation (HSQC) spectra of acetylated samples of lignin-enriched cell walls from original and digested lignocellulose diets fed to *C. formosanus* termite workers. Lignin aliphatic-oxygenated sub-regions are shown for (a) Japanese cedar (softwood), (b) Japanese beech (hardwood), and (c) rice straw (grass) diets. Volume integrals are given for the major lignin side-chain structures that are color-coded to match their assignments in the spectrum. The percentages noted in each spectrum are integrals relative to the sum of the annotated lignin side-chain signals (**I** + **I′** + **II** + **III** + **III′** + **IV** + **V**= 100%). For signal assignments and structure abbreviations, also see Table 1.3. n.d., not detected.

For the digested J. cedar lignins, the aromatic spectra regions displaying only G unit signals were practically no different from the original lignin spectrum (Fig. 1.6a). However, the aromatic spectra regions of J. beech and rice straw lignins displayed differences from the corresponding original lignin spectra, and volume integration analysis allowed us to estimate deviations in the aromatic unit distribution patterns

between the original and digested lignins (Fig. 1.6b,c). In the digested J. beech spectrum, syringyl aromatic signals ($\mathbf{L_S}$) were slightly depleted along with a concurrent increment in the guaiacyl aromatic signals ($\mathbf{L_G}$) compared to those observed in the original lignin spectrum (Fig. 1.6b). On the other hand, interestingly, the syringyl signals ($\mathbf{L_S}$) were conversely augmented with a depletion in guaiacyl signals ($\mathbf{L_G}$) in the digested rice straw lignins (Fig. 1.6c). Syringyl-to-guaiacyl (S/G) ratios estimated by 2D NMR ($\mathbf{L_S/L_G}$) were 2.05 and 1.71 in the original and digested J. beech lignins, respectively, and 1.09 and 1.35 in the original and digested rice straw lignins, respectively. Such changes of lignin S/G ratios by the termite digestive system were further corroborated by analytical thioacidolysis (Lapierre et al., 1986; Yamamura et al., 2012; Yue et al., 2012) to determine S/G ratios based on the lignin monomers released by the chemical cleavage of β–*O*–4 lignin substructures; thioacidolysis-derived S/G ratios were 3.05 and 2.37 in the original and digested J. beech lignins, respectively, and 0.84 and 1.31 in the original and digested rice straw lignins, respectively (Table 1.1). In addition to the change in lignin S/G composition, the NMR data suggested that rice straw lignins were considerably enriched in *p*-coumarate residues, with ~27% proportional increment in the $\mathbf{P}/(\mathbf{L_S}+\mathbf{L_G})$ signal ratio, but rather remarkably depleted in tricin residues with ~66% proportional decrease in $\mathbf{T}/(\mathbf{L_S}+\mathbf{L_G})$ signal ratio (Fig. 1.6c). The enrichment in *p*-coumarate residues might be associated with the enrichment of S units in the digested rice straw lignins, since *p*-coumarates in typical grass lignins are attached mainly to S units rather than G units (Ralph, 2010). Taken together, the data provide evidence of partial lignin decomposition in the *C. formosanus* digestive system, where S units in the hardwood J. beech lignins and non-acylated G units and tricin residues in the grass rice straw lignins were preferentially removed.

Table 1.3. Assignment of lignin signals in HSQC spectra of acetylated lignin-enriched cell wall samples.

Labels	δ_C/δ_H (ppm)	Assignment
Aromatic signals		
$\mathbf{L_{S2/6}}$	103.7/6.59	C_2-H_2 and C_6-H_6 in syringyl units
$\mathbf{L_{G2}}$	109.8/6.88, 111.2/6.98	C_2-H_2 in guaiacyl units
$\mathbf{L_{G5/6}}$	117.6/6.91,119.1/6.88, 122.0/6.95	C_5-H_5 and C_6-H_6 in guaiacyl units
$\mathbf{P_{2/6}}$	128.7/7.50	C_2-H_2 and C_6-H_6 in *p*-coumarate residues
$\mathbf{P_{3/5}}$	121.7/7.11	C_3-H_3 and C_5-H_5 in *p*-coumarate residues
$\mathbf{P_7}$	143.8/7.59	C_7-H_7 in *p*-coumarate residues
$\mathbf{P_8}$	117.0/6.35	C_8-H_8 in *p*-coumarate residues
$\mathbf{T_3}$	108.0/6.62	C_3-H_3 in tricin residues
$\mathbf{T_6}$	108.8/7.37	C_6-H_6 in tricin residues
$\mathbf{T_8}$	113.2/6.85	C_8-H_8 in tricin residues
$\mathbf{T_{2'/6'}}$	103.0/7.05	$C_{2'}$-$H_{2'}$ and $C_{6'}$-$H_{6'}$ in tricin residues
Aliphatic signals		
$\mathbf{I_\alpha}$	73.4/5.97	C_α-H_α in β-O-4 substructures
$\mathbf{I_\beta}$	79.7/4.60	C_β-H_β in β-O-4 substructures
$\mathbf{I'_\beta}$	79.8/5.57, 81.4/5.42	C_β-H_β in α-keto-β-O-4 substructures
$\mathbf{II_\alpha}$	87.7/5.45	C_α-H_α in β-5 substructures
$\mathbf{II_\beta}$	50.1/3.72	C_β-H_β in β-5 substructures
$\mathbf{III_\alpha}$	85.2/4.69	C_α-H_α in resinol-type β-β substructures
$\mathbf{III_\beta}$	53.8/3.02	C_β-H_β in resinol-type β-β substructures
$\mathbf{III_\gamma}$	71.6/4.27, 71.6/3.91	C_γ-H_γ in resinol-type β-β substructures
$\mathbf{III'_\alpha}$	82.8/5.00	C_α-H_α in tetrahydrofuran-type β-β substructures
$\mathbf{IV_\alpha}$	83.5/4.82	C_α-H_α in 5-5/β-O-4 (dibenzodioxocin) substructures
$\mathbf{IV_\beta}$	82.2/4.09	C_β-H_β in 5-5/β-O-4 (dibenzodioxocin) substructures
$\mathbf{V_\alpha}$	81.9/4.95	C_α-H_α in β-1 (spirodienone) substructures
$\mathbf{V_\beta}$	56.6/2.91	C_β-H_β in β-1 (spirodienone) substructures
$\mathbf{V_{\beta'}}$	75.8/4.46	$C_{\beta'}$-$H_{\beta'}$ in β-1 (spirodienone) substructures
$\mathbf{X1_\gamma}$	64.4/4.69	C_γ-H_γ in cinnamyl alcohol end-groups
Methoxyl	55.5/3.76	C-H in aromatic methoxyl groups

Measured in chloroform-*d*. Signal assignment was based on comparison with literature data (Ralph et al., 2004; Stewart et al., 2009; Lan et al., 2015

The aliphatic-oxygenated sub-regions of the HSQC spectra provide information of the major inter-monomeric linkage types in the lignin polymers (Fig. 1.7, Table 1.3). Typical lignin linkage signals from β–*O*–4 (**I** and **I′**), β–5 (**II**), β–β (**III** and **III′**), β–*O*–4/5-5 (dibenzodioxocin, **IV**) as well as β–1 (spirodienone, **V**) units were clearly visible in both the original and digested lignin spectra clearly with better resolutions compared to those observed in the whole cell wall spectra (Figs 1.2-1.5). Volume integration analysis was conducted to clarify differences in the distributions of these inter-monomeric linkage types between the original and digested lignins. Regardless of the three different lignin types tested, the inter-monomeric linkage distribution patterns were found to be overall similar between the original and digested lignins, suggesting that the action of the *C. formosanus* digestive system on these major inter-monomeric linkages is not drastic. However, in all the digested lignin spectra, the typical β–*O*–4 unit signals (**I**) were augmented by 3-8% relative to the normalized intensities in the original lignin spectra, mainly at the expense of β–5 (**II**) and resinol-type β–β (**III**) signals. The signals from dibenzodioxin units (**IV**) only seen in the J. cedar and J. beech lignin spectra were also depleted in the digested lignin spectra compared to those observed in the original lignin spectra. Apparently, no clear signal changes were seen for oxidized α-keto-β-aryl ether units (**I′**) and spirodienone (**V**) in J. cedar and J. beech lignins, also for γ-acylated tetrahydrofuran-type β–β (**III′**) in rice straw lignins. Overall, as further discussed below, the data suggest that lignin modification/degradation modes in the termite digestive system vary for different lignin substrates with different chemical structures.

1.4. Discussion

The present data support the view that lignin polymers are at least partially decomposed during their passage through the gut digestive system of lower termites, although, as has been noted by earlier studies (Hyodo et al., 1999; Katsumata et al., 2007; Ke et al., 2011a), polysaccharide decomposition clearly exceeds lignin decomposition, and large parts of lignin polymers remain intact in the digestive residues (Table 1.1, Figure 1.5). High-resolution structural data obtained by solution-state 2D HSQC NMR revealed the actions of the *C. formosanus* digestive system on the three major lignin types in nature, i.e., softwood, hardwood, and grass lignins.

The aromatic unit composition analysis based on both NMR and thioacidolysis determined that S units are degraded preferentially over G units in the digestion of hardwood J. beech lignins while G units are preferentially degraded over S units in grass rice straw lignins (Fig. 1.6). These results suggest that lignin modification modes in the termite digestive system may somehow vary for different lignin substrates with different chemical structures. In a finding that supported this, Ke et al. (2013) reported notably different Py-GC/MS profiles for hardwood (poplar) and grass (barley straw) lignins digested by *C. formosanus*. Both hardwood and grass lignins consist mainly of G and S units, but one of their major structural differences is that the latter is highly γ-acylated by *p*-coumarates, especially on S units (Ralph, 2010). Given that the present study observed notably augmented *p*-coumarate residues (Fig. 1.6) as well as relatively unchanged γ-acylated tetrahydrofuran-type β–β linkages (Fig. 1.7) after the digestion of rice straw lignins, it is conceivable that γ-acylated S units, which are unique and abundant in grass lignins, are relatively tolerable to the lignin processing in the *C. fomosanus* digestive

system. Another intriguing observation made for the digestion of grass lignins here was that tricin flavonoid units were largely depleted after digestion (Fig. 1.7). Tricin was recently identified as an authentic lignin monomer that is incorporated into lignin polymers via copolymerization with traditional lignin monomers, i.e., monolignols and their derivatives, upon lignification in grasses including rice (Lan et al., 2015; Lam et al., 2017). The present study's finding implies that the termite and/or its gut symbionts may possess flavonoid-processing enzymes that preferentially decompose tricin residues in grass lignins. Alternatively, because tricin typically occurs at one terminus of a lignin polymer chain as an end unit (Lan et al., 2015), it is also conceivable that tricin-containing oligomeric lignin fragments that were released upon lignin degradation might be preferentially solubilized and removed in the termite digestive system, leaving tricin-less internal lignin polymer units in the insoluble digestive residues.

Previous studies on lignin degradation by lower termites using sensitive Py-GC/MS methods reported pyrograms displaying several phenolic compounds annotated as markers for lignin side-chain oxidation (Geib et al., 2008; Ke et al., 2011a; 2011b; 2013). However, NMR analysis in this study failed to detect any conclusive evidence for such direct oxidation of lignin polymers in the *C. formosanus* digestive system: no clear signal increments were observed in the low-field HSQC aromatic regions where signals from oxidized lignin aromatic nuclei typically arise (Martinez et al., 2011; Tsuji et al., 2015), nor were there no clear changes in the relative abundance of oxidized α-keto-β-aryl ether units (**I′**) (Martinez et al., 2011; Tsuji et al., 2015) after termite digestion (Figs. 1.6 and 1.7). These results suggest that the proposed lignin degradation pathways involving side-chain oxidations could be substantially minor in the *C. formosanus* digestive system at least under the tested conditions. Meanwhile, for all the three lignocellulose diets tested,

the present study observed slight increments in the relative abundance of β–*O*–4 and decreased β–5 and resinol-type β–β linkages in the digested lignin polymers (Fig. 1.7). Li et al. (2017) recently reported 2D NMR data obtained for hardwood poplar lignins digested by a fungus-cultivating higher termite, *Odontotermes formosanus*, where they likewise observed relatively enriched β–*O*–4 over β–5 and β–β units in the digested lignins. Collectively, both lower and higher termites and/or their gut symbionts may favor degradations of C–C-bonded lignin inter-monomeric units, e.g., β–5 and β–β, over degradation of ether-bonded β–*O*–4, which somehow contrasts with what has been observed in typical lignin-biodegradation processes undertaken by wood-decaying fungi (Yelle et al., 2008; Martinez et al., 2011; Yelle et al., 2011; 2014).

1.5. Chapter summary

In this chapter, softwood, hardwood, and grass lignocellulose diets were fed to *C. formosanus* workers, and structural differences between the original lignocellulose diets and the resulting feces were examined by 2D NMR techniques as well as by complementary wet-chemical methods. Overall, the data support the view that lignin polymers are at least partially modified/decomposed during their passage through the termite gut digestive system, although polysaccharide decomposition clearly dominates the overall lignocellulose deconstruction process and the majority of lignin polymers remain intact in the digestive residues. High-resolution NMR structural data suggested preferential removal of syringyl aromatic units in hardwood lignins, but non-acylated guaiacyl units as well as tricin end-units in grass lignins. In addition, it was suggested that termites and/or their gut symbionts may favor degradation of C–C-bonded β–β and β–5 lignin inter-monomeric units over degradation of ether-bonded β–*O*–4 units, which is in

contrast to what has been observed in typical lignin biodegradation undertaken by wood-decaying fungi.

Chapter 2. The effects of various lignocelluloses and lignins on physiological responses of a lower termite, *Coptotermes formosanus* Shiraki

2.1. Introduction

In chapter 1, the lignocelluloses deconstructions by a lower termite, *Coptotermes formosanus* Shiraki with emphasize the decomposition of lignin polymers were reported. *C. formosanus* workers indeed has an ability to remove partially lignin polymers with different behaviors, where the results showed that syringyl lignin hardwood depleted over guaiacyl during termite digestion. Meanwhile, in the grass cell walls spectra, guaiacyl lignin signals were depleted over syringyl lignin, and tricin flavonoid units were considerably depleted after digested by *C. formosanus*.

Although several investigations have described the physiological responses of termites fed on woods (Fei and Henderson, 2002; Nakayama et al., 2005; Hapukotuwa and Grace, 2011), little information is available regarding the effects of lignins on the physiological activities of wood-feeding insects. Also, there is almost no information regarding the effects of different lignin polymer types from different lignocelluloses on wood-feeding termites. An earlier study by Kyou et al. (1996) tested survival of *C. formosanus* workers fed on an isolated lignin from *Pinus densiflora* (softwood). In this chapter, Chapter 2, was further closely investigate the influence of three major types of lignin polymers isolated from softwood (Japanese cedar), hardwood (Japanese beech), and grass (rice), on the physiological responses of *C. formosanus* workers as well as their symbiotic protists.

2.2. Materials and methods

2.2.1. Lignocellulose components analysis

Sapwood blocks of Japanese cedar, and Japanese beech as well as rice straw were ground into a powder using a ring flaker machine (Wiley mill, Yoshida Seisakusho, Tokyo) and a blender (Vita-Mix Blender, Osaka Chemical, Osaka, Japan), and the particles that passed through a 40-mesh sieve and retained by a 60-mesh sieve were obtained using a high-speed vibrating sample mill (Iida sieve shaker, Iida Seisakusho, Japan).

The sample meals were soxhlet-extracted with a benzene-ethanol (2:1, v/v) solution for 24 h (Waite and Gorrod, 1959). The holocellulose and lignin contents were measured by using the methods of Wise and a thioglycolic acid lignin assay (Suzuki et al., 2009), respectively. The alpha-cellulose was isolated by extracting the holocellulose with a solution of 17.5% NaOH. The hemicellulose content was then determined by subtracting the cellulose content from the holocellulose content (Kusumah et al., 2016). The chemical component analyses were performed in triplicate.

2.2.2. Preparation of milled-wood lignins (MWLs)

The isolation of MWLs was conducted as described by Balakshin et al. (2011). Briefly, the extractive-free sample meals were ball-milled using a planetary micro-mill (Pulverisette 7, Fritsch Industrialist, Idar-Oberstein, Germany) with ZrO_2 vessels containing ZrO_2 ball bearings at 600 rpm for 15 h (with a 5-min break for every 10 min of ball-milling) and then extracted by 96% dioxane, affording crude MWLs. The crude MWLs were dissolved in 90% AcOH (10 ml/g sample) and precipitated in water, filtered,

washed with water, and then freeze-dried. The resultant powder was again dissolved in a dichloroethane-ethanol (2:1, v/v), precipitated in diethyl ether, filtered, washed with diethyl ether, and then dried in vacuo to obtain purified MWLs.

2.2.3. Neutral sugar analysis of MWLs

Neutral sugar contents in the MWLs were determined by the methods as described in Chapter 1.

2.2.4. Nuclear magnetic resonance (NMR) analysis of MWLs

The purified MWLs (approx. 15 mg) were dissolved in 600 μl of dimethylsulfoxide (DMSO)-d_6 and subjected to a NMR analysis on an Avance III 800US system equipped with a cryogenically cooled 5-mm TCI gradient probe (800 MHz, Bruker Biospin, Billerica, MA, USA). The central DMSO solvent peaks (δ_C/δ_H: 39.5/2.49 ppm) were used as an internal reference. Adiabatic heteronuclear single-quantum coherence (HSQC) NMR experiments were carried out using standard implementation ("hsqcgcep.3") with parameters described in the literature (Mansfield et al., 2012). Data processing and analysis used Bruker TopSpin 3.2 software (Bruker Biospin). HSQC plots were obtained with typical matched Gaussian apodization in F2 and squared cosine-bell apodization in F1. For the integration of the lignin aromatic signals (Fig. 1a), C_2–H_2 correlations from G units (**G**) and C_2–H_2/C_6–H_6 correlations from S units (**S**), H units (**H**), and *p*-coumarate units (***p*CA**), and $C_{2'}$–$H_{2'}$/$C_{6'}$–$H_{6'}$ correlations from tricin units (**T**), were used, and the **S**, **H**, ***p*CA**, and **T** integrals were logically halved (Lam et al., 2017). For the integrations of the lignin inter-monomeric linkages (Fig. 1b), well-resolved C_α–H_α contours from **I**, **I'**, **II**, **II'**, **III**, **III'**, **IV** and **V** units were integrated and the **III** and **III'** integrals were logically

halved (Mansfield et al., 2012; Lam et al., 2017).

2.2.5. Termite survival and body mass

Sapwood blocks of Japanese cedar and Japanese beech [10 (R) × 10 (T) × 10 (L) mm] or 25-mm-long rice straws were put into an acrylic cylinder with a plaster bottom. Approximately 100 mg of purified MWL was placed in a plastic cup (20 mm dia., 10 mm deep), and this container was set on the center of an acrylic cylinder with a plaster bottom (65 mm dia., 60 mm high). Fifty workers and five soldiers of *C. formosanus* were obtained from a laboratory colony at the Deterioration Organisms Laboratory (DOL), Research Institute for Sustainable Humanosphere (RISH), Kyoto University, were fed on the various diets. The number of live workers was recorded and balanced weekly for 4 weeks. The termite survival and body mass observations were performed in triplicate.

2.2.6. Changes of protist fauna in the hindguts of C. formosanus workers

In this manner, 130 workers and 13 soldiers of *C. formosanus* were fed on the lignocellulose and MWL diets as described above. Every week, 10 workers were randomly selected from each container, and their hindguts were pulled out from the posterior ends and disintegrated into pieces by using fine forceps. The contents were then suspended in 0.4% NaCl. The hindgut pieces were macerated gently in the solution to facilitate the release of protozoa. The species of protozoa were identified by using a digital microscope (VHX-5000, Keyence, Osaka, Japan) (Yoshimura, 1995; Tanaka et al., 2006). These observations were performed in triplicate.

2.2.7. Statistical analysis

Differences in the termites' survival and body mass and the presence of protists in the hindgut of the workers fed on various lignocelluloses and MWLs were analyzed using SPSS ver. 23 software (IBM, Armonk, NY). Significant differences between means were identified by Tukey's post-hoc test. Significance levels were set at $P < 0.05$.

2.3. Results

2.3.1. Characterization of lignocellulose and MWL dietary samples

The present study first characterized the lignocellulose and lignin samples used as diets for termites. The composition of the three major lignocellulose components, i.e., cellulose, hemicelluloses, and lignin in Japanese cedar (softwood), Japanese beech (hardwood), and rice (grass) samples is summarized in Table 2.1. Polysaccharides, i.e., cellulose and hemicelluloses, accounted for 67%–77% of the three lignocellulose samples. The lignin content in the rice (approx. 15%) was somewhat lower than those in the two wood samples (20–24%) as reported in Chapter 1 (Table 1.1). Purified lignin samples were isolated as MWLs by typical procedures (Balakshin et al., 2011), and their purity and structure were checked by sugar analysis and 2D-NMR. The sugar analysis data indicated that the purities of all three MWLs were reasonably high (>85%) (Table 2.2). All of the MWLs contained approx. 10% residual cellulosic glucans. The rice MWL additionally contained approx. 5% hemicellulosic sugars, mainly composed of xylose, whereas the MWLs from Japanese cedar and Japanese beech had almost negligible amounts of hemicellulosic sugars.

Table 2.1. Cellulose, hemicellulose and lignin composition in Japanese cedar, Japanese beech, and rice lignocellulose diet samples

Samples	Lignocellulose composition (%)[a]		
	Cellulose	Hemicelluloses	Lignin*
Japanese cedar sapwood	44.14 ± 0.46	30.16 ± 0.62	23.92 ± 2.00
Japanese beech sapwood	41.48 ± 0.72	35.77 ± 0.67	19.60 ± 0.37
Rice straw	39.52 ± 0.97	26.94 ± 2.27	15.02 ± 0.47

[a]Values are means ± SD (n = 3). SD, standard deviation. Asterisk (*) indicates the data based on previous results in Chapter 1.

The HSQC NMR data firmly established that the three MWLs respectively display the characteristics of the three major lignin types in nature. As revealed by the aromatic signals in the HSQC spectra (Fig. 2.1a), the MWL from Japanese cedar is a typical softwood lignin composed of only G units, whereas the MWLs from the hardwood Japanese beech and grass rice are mixtures of G and S units. The volume integration of the contour signals allowed reasonable quantifications of the S/G unit ratios 1.38 and 0.66 in the Japanese beech and rice MWLs, respectively.

Table 2.2. Carbohydrate contents and composition in MWL diet samples prepared from Japanese cedar, Japanese beech, and rice

Carbohydrates	Content (%)[a]		
	Japanese cedar	Japanese beech	Rice
Hemicellulosic sugars[b]			
Arabinan	0.03 ± 0.00	0.05 ± 0.00	0.75 ± 0.07
Xylan	0.04 ± 0.00	0.95 ± 0.03	4.39 ± 0.50
Mannan	0.16 ± 0.01	0.16 ± 0.01	0.21 ± 0.01
Galactan	0.11 ± 0.00	0.11 ± 0.01	0.18 ± 0.01
Glucan	0.07 ± 0.01	0.31 ± 0.01	1.31 ± 0.13
Cellulosic glucan	7.99 ± 1.47	9.50 ± 2.99	9.14 ± 1.49

[a]Values are means ± SD (n = 3). [b]Determined as trifluoroacetic acid-hydrolyzable sugars.
SD, standard deviation.

As a typical lignin in grasses, the spectrum of rice MWL displayed more intense signals from H units (**H**) than those in the spectra of the Japanese cedar and Japanese beech MWLs, and the rice MWL also displayed clear signals from *p*-coumarate (***p*CA**) and tricin (**T**) units, which were totally absent in the Japanese cedar and Japanese beech MWL spectra. The aliphatic regions of the HSQC spectra (Fig. 2.1b) resolve most of the correlations for the various lignin interunit linkage types. In all of the MWL spectra, the most predominant lignin linkage type was β–*O*–4 units (**I** and **I'**) with lesser amounts of β–5 (**II** and **II'**), β–β (**III** and **III'**), 5–5/β–*O*–4 (**IV**), and β–1 (**V**) units. Compared to the Japanese cedar MWL, the proportions of β–*O*–4 units were apparently higher and those of β–5 and 5–5/β–*O*–4 units were conversely lower in the Japanese beech and rice MWLs.

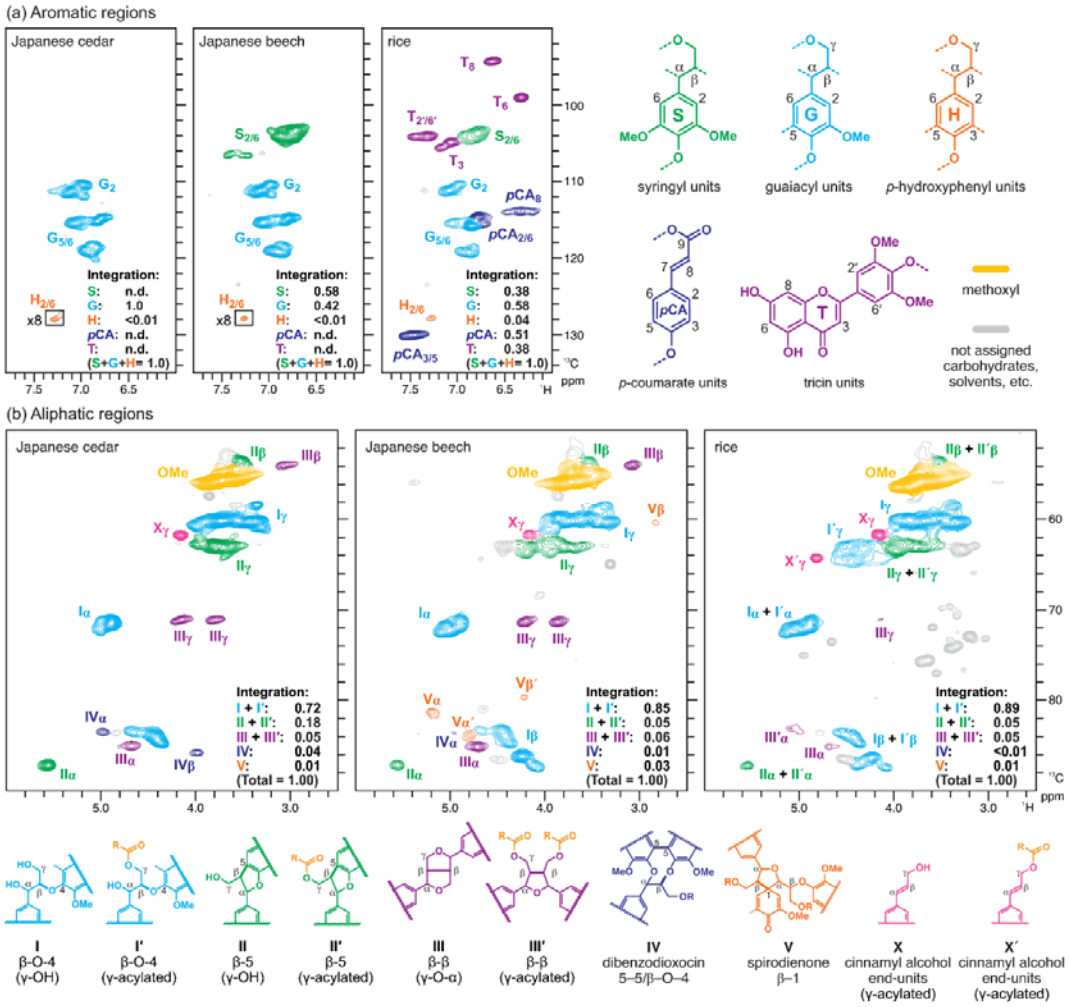

Fig 2.1. Aromatic (a) and aliphatic (b) sub-regions of short range ^{1}H–^{13}C correlation (HSQC) NMR spectra of milled-wood lignins isolated from Japanese cedar, Japanese beech, and rice lignocellulose samples. Volume integrals are given for the major lignin aromatic and side-chain structures that are color-coded to match their assignments in the spectrum. Boxes labeled x8 indicate regions that are vertically scaled 8-fold.

2.3.2. Termite survival and body mass

The periodic changes in termite survival are summarized in Fig. 2.2. The survival of termites fed on lignocelluloses from Japanese cedar, Japanese beech, and rice showed no significant differences at the 1st-week observation ($F = 7$, $P = 0.27$) at 100%, 99%, and 98%, respectively, or at 2 weeks ($F = 3$, $P = 0.131$) at 99%, 95%, and 91%, respectively. Interestingly, at 3 weeks, the survival of the termites fed on rice was significantly lower than that of the termites fed on Japanese cedar and Japanese beech ($F = 76$, $P < 0.05$), and there was no significant difference in survival between the Japanese cedar and Japanese beech groups ($F = 1$, $P = 0.374$) at 92%, 91%, and 76%, respectively. The results at the end of the experiment (4 weeks) were similar to those at 3 weeks, in that the survival rate of the termites fed on rice was significantly lower than those of the termites fed on Japanese cedar and Japanese beech ($F = 116$, $P < 0.05$), with no significant difference between the Japanese cedar and Japanese beech groups ($F = 2$, $P = 0.230$) at 87%, 85%, and 70%, for Japanese cedar, Japanese beech, and rice, respectively. These results clearly suggest that wood was more nutritious than rice.

As shown in Fig. 2.2, the average of the workers' survival rate in the three lignocellulose groups (Japanese cedar, Japanese beech, and rice) compared to starvation were significantly different at the 1st-week ($F = 28.3$, $P < 0.05$) and 2nd-week ($F = 17.5$, $P = 0.001$) observations at 92% for the lignocellulose groups and 80% for starvation. Surprisingly, at the 3rd-week, the survival rate was not significantly different between rice and starvation, whereas the survival of the starvation group was significantly lower than those of the termites fed on Japanese cedar and Japanese beech ($F = 29$, $P < 0.05$) at 69% for starvation. Finally, at the 4-week, the present study observed that the survival rate of the starvation group was significantly lower than those of the groups fed on

Japanese cedar, Japanese beech, and rice ($F = 47$, $P < 0.05$) at 51% for starvation. These results suggest that the lower termite *C. formosanus* can still utilize some of carbohydrate components in rice straw.

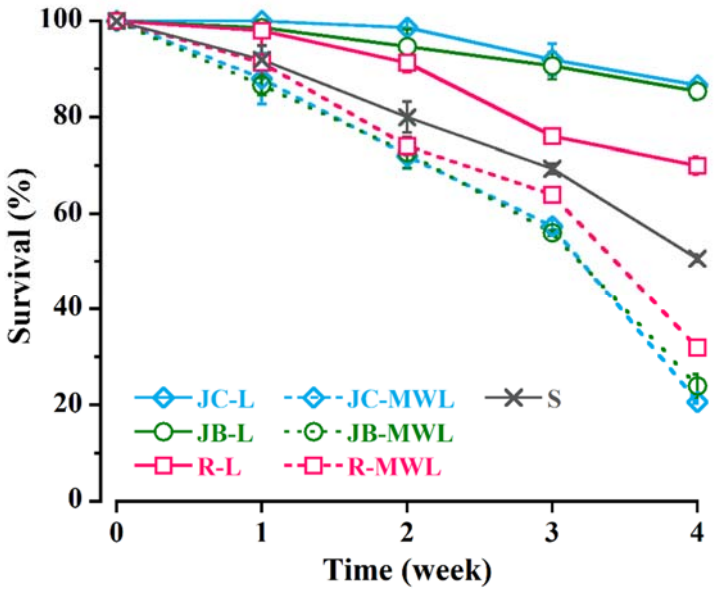

Fig 2.2. Changes in the survival rate of *C. formosanus* workers fed on various lignocelluloses and isolated lignins (MWLs). Error bars represent standard deviation (n=3). JC-L, Japanese cedar lignocellulose; JB-L, Japanese beech lignocellulose; R-L, rice lignocellulose; JC-MWL, Japanese cedar MWL; JB-MWL, Japanese beech MWL; R-MWL, rice MWL; S, starvation control group.

Fig. 2.2 also shows the survival rates of workers fed on the three MWLs from Japanese cedar, Japanese beech, and rice. The statistical analysis revealed that at 1, 2, 3, and 4 weeks, there were no significant differences in survival among the three MWLs from Japanese cedar, Japanese beech, and rice. Subsequently, compared to starvation, the survival rates for the three MWL groups were also not significantly different at 1 week ($F = 1.6$, $P = 0.271$), 2 weeks ($F = 1.6$, $P = 0.269$), and 3 weeks ($F = 3.3$, $P = 0.078$). However, at the end of the observation at 4 weeks, the statistical analysis showed that the survival rates of the three MWL groups were significantly lower than that of the starvation group ($F = 14$, $P = 0.002$). These results revealed that the three MWLs from Japanese

cedar, Japanese beech, and rice were not nutritious for *C. formosanus*, and rather were somewhat detrimental when the termites were fed on each MWL solely.

Figure 2.3 shows the periodic changes in the body mass of the workers fed on the lignocelluloses and MWLs. There was no significant difference in the body mass of the workers among the three lignocellulose groups at 1 week ($F = 0.8$, $P = 0.500$), 2 weeks ($F = 3.5$, $P = 0.100$), and 3 weeks ($F = 2.4$, $P = 0.169$). Interestingly, the components in rice apparently affect the body mass of termites, as at 4 weeks the body mass values of the termites were significantly lower than those of the termites fed on Japanese cedar and Japanese beech sapwoods ($F = 46.3$, $P < 0.05$), while no significant difference was observed between the Japanese cedar and Japanese beech groups ($F = 0.1$, $P = 0.831$), at 86.9%, 86.5%, and 76.3% of the relative body mass for Japanese cedar, Japanese beech, and rice, respectively. Compared to the starvation group, the body mass values of the termites in the three lignocellulose groups were not significantly different at 1, 2, and 3 weeks. At 4 weeks, the body mass values of the termites fed on rice were significantly lower than those of the starvation-group termites ($F = 214$, $P < 0.05$), whereas no significant difference was observed between both sapwood groups (Japanese cedar and Japanese beech) and the starvation group ($F = 1.4$, $P = 0.321$). These results suggest that *C. formosanus* use part of the carbohydrates in rice as nutrients as discussed above, but this nutrient supply was insufficient for termites, resulting in the decreased body mass.

The body mass values of the termites fed on the three MWLs from Japanese cedar, Japanese beech, and rice (Fig. 2.3) were not significantly different at 1, 2, 3 or 4 weeks. In addition, all three MWL groups and the starvation group did not show any significant differences at 1, 2, 3 or 4 weeks. These results further support the possibility that termites hardly use lignins as a nutrient source. Given that the termite survival rates were

significantly lower in the MWL groups compared to starvation (Fig. 2.2), cannibalism (Cook and Scott, 1933) may help termites maintain their body mass when being fed on non-nutritious food and even possibly toxic lignin polymers. As described above, even with no significant differences between the workers tested with the three different MWL diets, both the survival rate and body mass of the workers fed with rice grass lignocellulose were significantly lower than those with Japanese cedar and Japanese beech wood lignocelluloses. This suggests that some components other than lignin in grass lignocellulose are negatively affecting the physiology of *C. formosanus* workers.

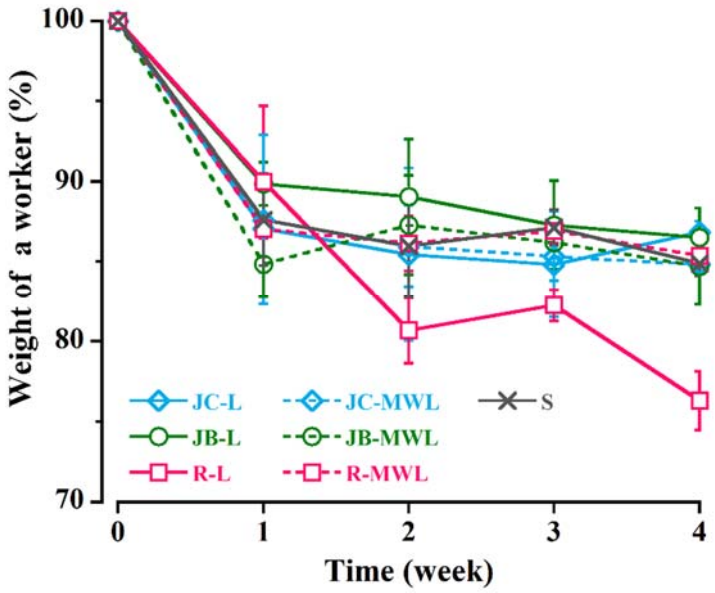

Fig 2.3. Changes in the body mass of *C. formosanus* workers fed on various lignocelluloses and isolated lignins (MWLs). Error bars represent standard deviation (*n*=3). JC-L, Japanese cedar lignocellulose; JB-L, Japanese beech lignocellulose; R-L, rice lignocellulose; JC-MWL, Japanese cedar MWL; JB-MWL, Japanese beech MWL; R-MWL, rice MWL; S, starvation control group.

2.3.3. Intestinal protist profiles

Lastly, the present study investigated the changes in the protist profiles in the hindguts of the worker termites fed on the lignocelluloses and MWLs (Tables 2.3-2.5).

The results showed that all the three protists (i.e. *P. grassii*, *H. hartmanni*, and *S. leidyi*) remained in the hindguts of all of the workers fed on the three lignocelluloses at the 1st-week observation. However, feeding on rice resulted in a loss of *P. grassii* in 17% of the workers and 47% of the workers at the 2nd- and 3rd-week observations, respectively, whereas *H. hartmanni* and *S. leidyi* remained in all of the workers at these time points.

Table 2.3. Presence rates of *P. grassii* in the hindguts of *C. formosanus* workers fed on various lignocellulose and lignin diets.

Samples	Presence (%)			
	Observation time (week)			
	1	2	3	4
Lignocelluloses				
Japanese cedar	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}
Japanese beech	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}
Rice	100 ± 0^{a}	83.3 ± 11.5^{b}	53.3 ± 5.8^{b}	26.7 ± 5.8^{b}
MWLs				
Japanese cedar	0^{b}	0^{c}	0^{c}	0^{c}
Japanese beech	0^{b}	0^{c}	0^{c}	0^{c}
Rice	0^{b}	0^{c}	0^{c}	0^{c}
Starvation	0^{b}	0^{c}	0^{c}	0^{c}

Values are means ± SD ($n = 3$). Values with the same letter are not significantly different (Tukey HSD test; $p < 0.05$) following one-way ANOVA.

At the end of the observation (4 weeks), the rice feeding had resulted in a loss of *P. grassii* in 73% of the workers, *H. hartmanni* in 17% of the workers, and *S. leidyi* in 7% of the workers (Tables 2.3-2.5). In contrast, all three protists remained in all of the workers fed on Japanese cedar and Japanese beech even at 4 weeks. There were significant differences between both sapwoods and rice for *P. grassii* ($F = 484$, $P < 0.05$), and *H. hartmanni* ($F = 25$, $P = 0.001$), but not for *S. leidyi* ($F = 1$, $P = 0.422$). These results are in line with the previous observation of termite survival (Fig. 2.2) and body mass (Fig. 2.3), and with the fact that lower termites including *C. formosanus* prefer woods compared to grasses as diets; as discussed above, it is plausible that minerals rich in grass lignocellulose could be negatively affecting the survival of the two major protists. Nevertheless, the results demonstrated that the presence of all three of these protists in workers fed on rice was significantly higher than those in the starved workers (Tables 2.3-2.5), suggesting that the protists still can utilize carbohydrates in rice as a nutrient source.

On the other hand, after termites were fed on the three MWLs from Japanese cedar, Japanese beech, and rice for 1 week, *P. grassii* disappeared from all of the workers, whereas *H. hartmanni* and *S. leidyi* remained in all workers. There was no significant difference among the three MWLs for all protists at 2 and 3 weeks. At the end of the 4th-week observation, 67%–77% of the workers had lost *H. hartmanni* and 40%–50% of the workers had lost *S. leidyi* (Tables 2.3-2.5). No significant difference regarding the survival of protists fed on the three MWLs was observed at the end of the observation for *P. grassii* (lost in all workers), *H. hartmanni* ($F = 3$, $P = 0.125$), and *S. leidyi* ($F = 1.8$, $P = 0.252$). In addition, there was no significant difference between the three MWLs and starvation for all of the protists at the end of the observation: *P. grassii* (lost in all workers), *H. hartmanni* ($F = 2$, $P = 0.193$), and *S. leidyi* ($F = 1.3$, $P=0.349$). Collectively, the data

suggest that all the three types of lignin polymers, regardless of their structural differences, had no effect on the survival of protists in the hindgut of workers of *C. formosanus*.

Table 2.4. Presence rates of *H. hartmanni* in the hindguts of *C. formosanus* workers fed on various lignocellulose and lignin diets.

Samples	Presence (%)			
	Observation time (week)			
	1	2	3	4
Lignocelluloses				
Japanese cedar	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]
Japanese beech	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]
Rice straw	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	83.3 ± 5.8[b]
MWLs				
Japanese cedar	100 ± 0[a]	66.7 ± 20.8[b]	53.3 ± 10[b]	23.3 ± 5.8[c]
Japanese beech	100 ± 0[a]	63.3 ± 11.6[b]	50 ± 10[b]	23.3 ± 10[c]
Rice straw	100 ± 0[a]	76.7 ± 5.8[b]	70 ± 15.3[b]	33.3 ± 5.8[c]
Starvation	100 ± 0[a]	100 ± 0[a]	66.7 ± 11.6[b]	26.7 ± 10[c]

Values are means ± SD (n = 3). Values with the same letter are not significantly different (Tukey HSD test; $p < 0.05$) following one-way ANOVA.

Table 2.5. Presence rates of *S. leidyi* in the hindguts of *C. formosanus* workers fed on various lignocellulose and lignin diets.

Samples	Presence (%)			
	Observation time (week)			
	1	2	3	4
Lignocelluloses				
Japanese cedar	100±0[a]	100±0[a]	100±0[a]	100±0[a]
Japanese beech	100±0[a]	100±0[a]	100±0[a]	100±0[a]
Rice	100±0[a]	100±0[a]	100±0[a]	93.3±11.6[a]
MWLs				
Japanese cedar	100±0[a]	100±0[a]	96.7±5.8[ab]	53±5.8[b]
Japanese beech	100±0[a]	100±0[a]	93.3±11.6[ab]	50±0[b]
Rice	100±0[a]	100±0[a]	96.7±5.8[ab]	60±10[b]
Starvation	100±0[a]	100±0[a]	80±0[b]	53.3±5.8[b]

Values are means ± SD (n = 3). Values with the same letter are not significantly different (Tukey HSD test; $p < 0.05$) following one-way ANOVA.

2.4. Discussion

Lignocelluloses as diet source for termites including a lower termite, *C. formosanus* have differences of the chemical composition. The present study showed that lignin content in rice lignocellulose was lower than those in wood lignocellulos samples. This finding is in line with previous reports (Ehara et al., 2005; Zhu et al., 2006). It is conceivable that rice straw sample used in this study also contained amounts of minerals

such as silica (Jenkins et al., 1998; Binod et al., 2010). Later, the present study observed that the rice MWL additionally contained approx. 5% hemicellulosic sugars, mainly composed of xylose, whereas the MWLs from Japanese cedar and Japanese beech had almost negligible amounts of hemicellulosic sugars; the difference might be due to that lignin and hemicelluloses are cross-linked more highly in grass lignocellulose than in softwood and hardwood lignocelluloses (Ralph, 2010). In addition, based on 2D NMR analysis, the differences of the lignin types in softwood, hardwood, and grass were clearly detected. As mentioned above the MWL from Japanese cedar is a typical softwood lignin composed of only G units, whereas the MWLs from the hardwood Japanese beech and grass rice are mixtures of G and S units. In rice MWL also displayed clear signals from *p*-coumarate (***p*CA**) and tricin (**T**) units (Lam, et al., 2017), which were totally absent in the Japanese cedar and Japanese beech MWL spectra. Such shifts of lignin linkage distributions are typical when lignin incorporates more S units than G units (Stewart et al., 2009; Wagner et al., 2015). The appearance of characteristic signals from acylated lignin units (**I'**, **II'**, and **III'**) in the rice MWL spectrum confirmed that rice MWL, as a typical grass lignin, is highly acylated, presumably by *p*-coumarates (Ralph, 2010; Petrik et al., 2014; Lam et al., 2017).

Termites use the carbon source from woods as a nutrient to maintain their survival (Morales-Ramos and Rojas, 2001; Tanaka et al., 2006). Termite workers break down wood with their mandibles and foregut gizzard. Thus, endogenous cellulases and a variety of cellulolytic enzymes secreted by the symbiotic microorganisms community in the gut coordinately digest carbohydrates and efficiently convert them into acetates and other nutrients as the main energy and carbon source for the host (Hongoh, 2011, Ni and Tokuda, 2013). The results suggested the survival rates and body mass of *C. formosanus* workers

as well as the survival rates of two protists, i.e., *P. grassii* and *H. hartmanni* in the hindguts of workers fed on wood samples were much higher than those fed on rice straw powder sample. Some components other than lignins (possibly minerals in grass lignocellulose) may negatively affect the physiological responses of *C. formosanus* and its major symbiotic protists, since, as mentioned in Chapter 1 that unlike wood lignocelluloses, grass lignocellulose typically contains substantial amounts of minerals such as silica (Jenkins et al., 1998; Binod et al., 2010). In addition, as mentioned in general introduction, termites are divided into higher and lower termites based on symbiotic protists in the hindgut. The lower termites possess symbiotic protists; higher termites lack symbiotic protists (Yoshimura, 1995; Lo and Eggleton, 2011). The lower termites are well known as wood-feeding insects (Su and Tamashiro, 1986; Morales-Ramos and Rojas, 2001). In nature, grasses can also be decomposed by the higher termites (grass-feeding termites) (Ohiagu, 1979; Jouquet et al., 2011). On the other hand, the present study noted that the three MWL diets were much lower in termite survival compared to starvation, although these diets showed no significant effects on body mass or the protist profiles, suggesting that *C. formosanus* workers are hardly utilized lignins as a nutrient source.

2.5. Chapter summary

Overall, the results suggest that different types of lignins from softwood, hardwood, and grass have no effect on the physiological responses of *C. formosanus* and its symbiotic protists. The results of this study established that lignins are hardly utilized as a nutrient source by *C. formosanus* workers and even are rather somewhat detrimental to termites when fed on solely. The present study also observed that some components other

than lignins (possibly minerals in grass lignocellulose) negatively affect the physiological responses of *C. formosanus* and its major symbiotic protists. These new findings contribute to the basic knowledge on the mechanism of lignocellulose decomposition by wood-feeding insects. Further study is needed to investigate the nutritional effects of combinations of carbohydrates and lignins on the physiological activities of *C. formosanus* and its protists to identify the exact role of lignin in termites.

Chapter 3. Hydrogen and methane emissions by a lower termite, *Coptotermes formosanus* Shiraki on various lignocellulose and lignin diets

3.1. Introduction

As a by-product of lignocellulose decomposition, the gut symbiotic micro-organism community produces H_2 (Sugimoto et al., 1998; Inoue et al., 2007), suggesting that amount of H_2 emitted by termite expresses the micro-organisms profiles in the termite`s gut. It has been known that H_2 emissions of lower termites are generally higher than those of higher termites (Brauman et al., 1992). Subsequent research identified symbiotic protists in the hindgut of lower termite as the main agents responsible for H_2 emissions (Inoue et al., 2007; Cao et al., 2012), and two iron hydrogenase genes from *P. grassii* in the hindgut of *C. formosanus* were isolated as the main metabolic source of the hydrogen gas (Inoue et al., 2007). In addition to H_2, termites also emit CH_4 plausibly from H_2 and CO_2 by methanogenic bacteria present in the hindgut (Tsunoda et al., 1993a; 1993b). Lower termites including *C. formosanus* seem to be heavily colonized by *Methanobrevibacter* attached to the gut wall or associated with flagellates (Brune, 2014). The production of CH_4 by methanogenic termites is estimated to comprise as much as 5% of the total CH_4 emissions on earth (Rasmussen & Khalil, 1981; 1984).

It was reported that H_2 and CH_4 emissions by termites could be affected by diet components (Sugimoto et al., 1998; Cao et al., 2010). Although several studies have investigated H_2 and CH_4 emissions by termites fed on various woods and cellulose substrates (Tsunoda et al., 1993a; 1993b; Kawaguchi et al., 2006; Cao et al., 2010; Yanase et al., 2013), little is known about the effects of lignins on these emissions. Therefore, the

present chapter, Chapter 3, investigated the dietary effects on *C. formosanus* worker termites' H_2 and CH_4 emissions of (1) various lignocellulose samples i.e., softwood, hardwood, and grass; (2) isolated lignins (milled-wood lignins, MWLs) from different lignocelluloses, and (3) isolated lignins when combined with polysaccharides (holocelluloses or celluloses) in an artificial diet.

3.2. Materials and Methods

3.2.1. Preparation of lignocellulose and artificial diet samples

Sapwood blocks of Japanese cedar and Japanese beech, and dried culm straw of rice were ground into a powder using a ring flaker machine (Wiley mill, Yoshida Seisakusho, Tokyo, Japan) and a blender (Vita-Mix Blender, Osaka Chemical, Osaka, Japan). The resultant particles that passed through a 40-mesh sieve but were retained by a 60-mesh sieve using a high-speed vibrating sample mill (Iida sieve shaker, Iida Seisakusho, Japan) were then extracted by refluxing in a benzene: ethanol (2:1/v:v) mixture for 24 h (Waite & Gorrod, 1959). The isolation of milled-wood lignin (MWLs) was conducted as described earlier (Tarmadi et al., 2017a). Holocellulose samples were prepared using the standard Wise method, and α-cellulose samples were isolated by extracting the holocellulose samples with a solution of 17.5% NaOH (Kusumah et al., 2016). Six diet samples per each lignocellulose were prepared, i.e., wood powder (WP) or rice straw powder (RSP), holocellulose (H), cellulose (C), lignin (MWL), holocellulose mixed with lignin (H+MWL), and cellulose mixed with lignin (C+MWL) (Table 3.1). For preparations of H+MWL and C+MWL diets, MWL and H or C samples were ground and mixed homogeneously using a grinder. The formulation ratios, i.e., polysaccharide and lignin contents, were determined based on the chemical composition analysis data as

reported in Chapter 2.

Table 3.1. Lignocellulose diet samples used in this study.

Diet sources	Diet components
Japanese cedar (softwood)	Wood powder (WP)
	Holocellulose (H)
	Cellulose (C)
	Lignin (MWL)
	Holocellulose + MWL (H+MWL), 76:24, wt/wt
	Cellulose + MWL (C+MWL), 65:35, wt/wt
Japanese beech (hardwood)	Wood powder (WP)
	Holocellulose (H)
	Cellulose (C)
	Lignin (MWL)
	Holocellulose + MWL (H+MWL), 79:21, wt/wt
	Cellulose + MWL (C+MWL), 68:32, wt/wt
Rice straw (grass)	Rice straw powder (RSP)
	Holocellulose (H)
	Cellulose (C)
	Lignin (MWL)
	Holocellulose + MWL (H+MWL), 82:18, wt/wt
	Cellulose + MWL (C+MWL), 69:31, wt/wt

3.2.2. Measurement of H_2 and CH_4 emissions

Approximately 200 mg of diet samples were put into a 20-mm-diameter, 10-mm-deep plastic cup and set on the center of a 66-mm-diameter, 60-mm-deep acrylic cylinder with a plaster bottom. One hundred workers and ten soldiers of *C. formosanus* obtained from a laboratory colony of the Deterioration Organisms Laboratory (DOL), Research

Institute for Sustainable Humanosphere (RISH), Kyoto University, were fed each of the various diets. Every week for 4 weeks, twenty to fifty live workers were randomly selected from each container and placed in a glass vial (50 ml) with a rubber septum. After 1 hour, a 1.0-ml gas sample was collected by syringe and injected into a gas analyzer (New Cosmos Electrical Co., Ltd) equipped with a semiconductor gas sensor designed for H_2 and CH_4 measurement (Yanase et al., 2013). Gas concentrations in the vials (ppm) were converted into emission rates as nmol/termite/h. These measurements were performed in triplicate. Differences in H_2 and CH_4 emission rates were tested by SPSS ver. 23 (IBM Corporation, NY, USA). Significant differences between means were calculated by Tukey's HSD and significance levels were set at $P < 0.05$.

3.3. Results

3.3.1. H_2 and CH_4 emission from C. formosanus workers fed on lignocellulose diets

First, the study tested H_2 and CH_4 emissions from *C. formosanus* workers fed pulverized lignocellulose diets prepared from Japanese cedar (softwood) and Japanese beech (hardwood) sapwoods, and rice (grass) culm straws. H_2 emission was detected for all three lignocellulose diets and also from the starvation control (Fig. 3.1 and Table 3.2). The H_2 emission rate by starved workers was significantly lower than that by workers fed on lignocellulose diets, affirming that H_2 is mainly produced by the digestion of lignocellulose. H_2 emission rates from the three lignocellulose samples through the 3rd week of measurement were roughly similar, at 1.28-1.78 nmol/termite/h. However, at the 4th week, the H_2 emission rate by workers fed rice lignocellulose was significantly lower than that by workers fed the two wood lignocellulose samples ($F = 37$, $P < 0.05$), at 0.95 nmol/termite/h, compared to 1.82–1.88 nmol/termite/h for wood lignocellulose samples.

In contrast, no significant difference was observed between the softwood and hardwood diets throughout the test period ($F = 0.1$, $P = 0.772$). The results suggest that some specific components in rice lignocellulose affect the production of H_2 by the gut symbiotic micro-organism community. The present study also detected CH_4 emissions from all tested termite groups (Fig. 3.2 and Table 3.3). Compared to H_2 emissions, however, CH_4 emission rates were generally low (< 0.3 nmol/termite/h). The CH_4 emission rates among the three lignocellulose diet groups and the starvation control group were similar throughout the test period.

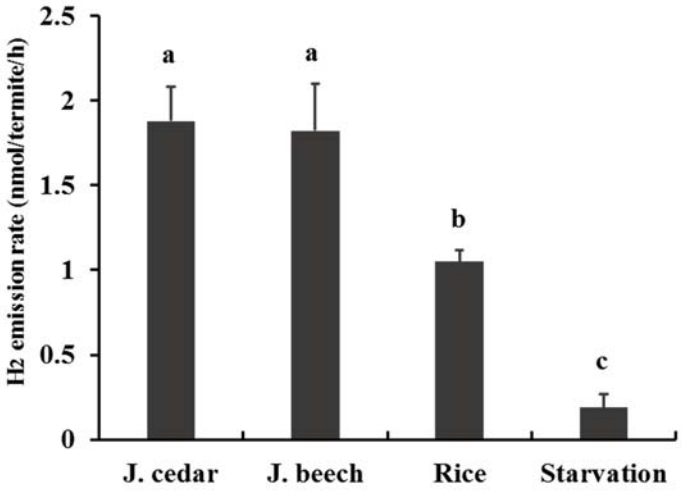

Fig 3.1. Periodical H_2 emission rates by *C. formosanus* workers fed on lignocellulose samples at 4th week observation. Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$) following one-way ANOVA. Error bars represent standard deviations.

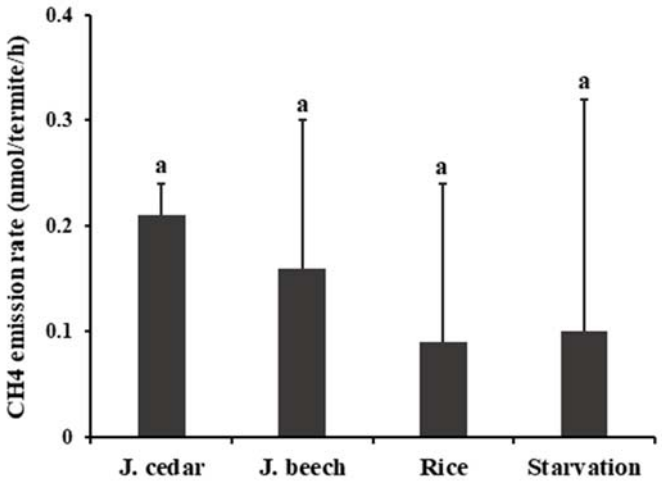

Fig 3.2. Periodical CH_4 emission rates by *C. formosanus* workers fed on lignocellulose samples at 4th week observation. Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$) following one-way ANOVA. Error bars represent standard deviations.

3.3.2. H_2 and CH_4 emissions from C. formosanus workers fed on artificial diets

Next, to further investigate the effect of lignins on H_2 and CH_4 emissions by termites, *C. formosanus* workers were fed to diets composed of mixtures of isolated lignin and polysaccharides. Purified lignin polymers (MWLs), holocellulose (H) and cellulose (C) were isolated from Japanese cedar (softwood) and Japanese beech (hardwood) sapwoods, and rice (grass) culm straws, and mixed with varied formulations for feeding to *C. formosanus* workers. The formulation ratios, i.e., polysaccharide and lignin contents, were decided based on the chemical composition analysis data on each original plant material (Tarmadi et al., 2017a).

H_2 and CH_4 emission rates by workers fed diets composed of only MWLs were similar among all diet groups throughout the test period (Figs. 3.3 and 3.4). In addition, H_2 and CH_4 emissions from the termites fed MWLs were similar to those from the starved control group. Thus, lignin alone is unlikely to contribute to the productions of H_2 and

CH_4 in the termite digestive system.

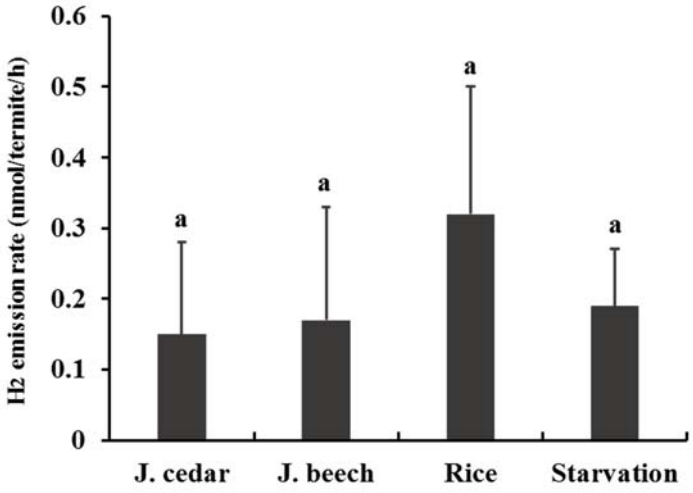

Fig 3.3. Periodical H_2 emission rates by *C. formosanus* workers fed on isolated lignins (MWLs) at 4th week observation. Values with the same letter is not significantly different (Tukey HSD test; $P < 0.05$) following one-way ANOVA. Error bars represent standard deviations.

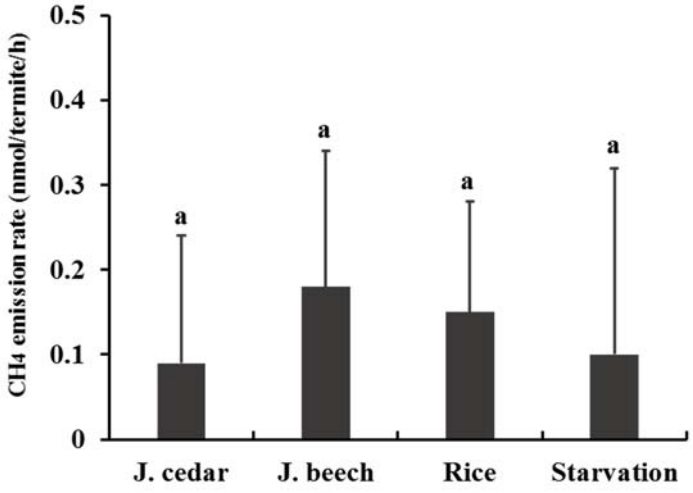

Fig 3.4. Periodical CH_4 emission rates by *C. formosanus* workers fed on isolated lignins (MWLs) at 4th week observation. Values with the same letter is not significantly different (Tukey HSD test; $P < 0.05$) following one-way ANOVA. Error bars represent standard deviations.

Compared to the workers fed only lignins, workers fed polysaccharide diets, i.e., holocellulose (H) and cellulose (C) diets, showed significantly increased H_2 emission rates (Fig. 3.5 and Table 3.2). In the case of artificial diets prepared from Japanese cedar and Japanese beech, the workers fed holocellulose (H) diets displayed similar H_2 emission rates as those fed the original lignocellulose (WP) at the 4th week of observation. On the other hand, workers fed holocellulose (H) from rice straw showed significantly higher H_2 emission rates than those fed the original rice straw lignocellulose (RSP) (Fig. 3.5 and Table 3.2). This suggests that some non-lignocellulosic components of rice straw negatively affect the digestion of polysaccharides and the production of H_2 from rice lignocellulose as further discussed below. For all lignocellulose sources, there was a tendency that workers fed only cellulose (C) showed higher H_2 emission rates than workers fed holocellulose (H), which contains both cellulose and hemicelluloses from the original lignocellulose (Fig. 3.3 and Table 3.2). It is therefore plausible that H_2 emissions from *C. formosanus* workers are mainly derived from the digestion of cellulose rather than hemicelluloses. CH_4 emission rates from all polysaccharide diets were similar throughout the test period (Table 3.3).

The present Chapter lastly tested polysaccharide diets mixed with lignins. The results found that no significant difference in terms of H_2 and CH_4 emission rates between the workers fed only holocellulose (H) and those fed holocellulose plus lignin (H+MWL) as well as between those fed only cellulose (C) and those fed cellulose plus lignin (C+MWL) throughout the test period (4 weeks) (Fig. 3.3 and Tables 3.2, 3.3). Overall, the data suggest that lignin has little effect on H_2 and CH_4 emissions from the digestion of cell wall polysaccharides by *C. formosanus* workers. Interestingly, while workers fed the holocellulose plus lignin (H+MWL) diets from Japanese cedar and Japanese beech

showed similar H_2 emissions compared to the workers fed the original lignocellulose (WP), workers fed H+MWL from rice displayed significantly higher H_2 emission rates compared to the workers fed the original rice straw lignocellulose (RSP) (Fig. 3.3 and Table 3.2). This suggests that lignins are unlikely the major factor limiting the H_2 production from grass lignocellulose.

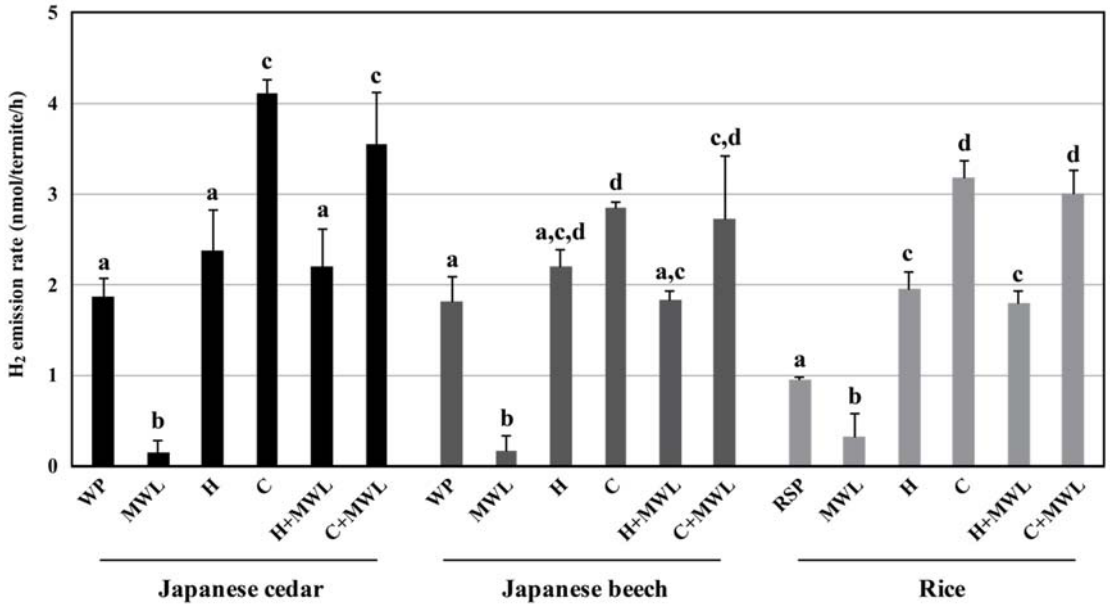

Fig 3.5. H_2 emission rates by *C. formosanus* workers fed diet samples prepared from Japanese cedar, Japanese beech, and rice at 4th week observation. Statistical analyses were determined per each lignocellulose sample. Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$) following one-way ANOVA. Error bars represent standard deviations. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; MWL, milled wood lignin.

Table 3.2. H_2 emission rates by workers fed on various diets prepared from Japanese cedar (softwood), Japanese beech (hardwood), and rice (grass)

Diet sources	Diet components	Emission rates (nmol/termite/h)			
		1st week	2nd week	3rd week	4th week
Japanese cedar	WP	1.17 ± 0.16^{a}	1.42 ± 0.10^{a}	1.78 ± 0.58^{a}	1.88 ± 0.20^{a}
	MWL	0.55 ± 0.04^{b}	0.83 ± 0.07^{b}	0.52 ± 0.03^{b}	0.15 ± 0.13^{b}
	H	1.27 ± 0.13^{a}	1.81 ± 0.23^{ac}	1.87 ± 0.10^{a}	2.39 ± 0.43^{a}
	C	1.22 ± 0.09^{a}	2.27 ± 0.26^{cd}	4.06 ± 0.08^{c}	4.11 ± 0.15^{c}
	H+MWL	1.14 ± 0.05^{a}	1.70 ± 0.29^{a}	1.85 ± 0.41^{a}	2.21 ± 0.40^{a}
	C+MWL	1.43 ± 0.23^{a}	2.44 ± 0.08^{d}	3.43 ± 0.41^{c}	3.55 ± 0.57^{c}
Japanese beech	WP	1.44 ± 0.18^{a}	1.36 ± 0.16^{a}	1.54 ± 0.25^{a}	1.82 ± 0.28^{a}
	MWL	0.52 ± 0.12^{b}	0.74 ± 0.03^{b}	0.36 ± 0.03^{b}	0.17 ± 0.16^{b}
	H	1.44 ± 0.13^{a}	2.17 ± 0.33^{ac}	1.87 ± 0.29^{a}	2.12 ± 0.18^{acd}
	C	1.36 ± 0.04^{a}	2.38 ± 0.32^{c}	3.78 ± 0.42^{c}	2.86 ± 0.05^{d}
	H+MWL	1.43 ± 0.12^{a}	1.70 ± 0.50^{ac}	1.83 ± 0.07^{a}	1.84 ± 0.10^{ac}
	C+MWL	1.41 ± 0.05^{a}	1.77 ± 0.17^{ac}	2.97 ± 0.44^{c}	2.73 ± 0.70^{cd}
Rice	RSP	1.15 ± 0.06^{a}	1.21 ± 0.10^{a}	1.28 ± 0.05^{a}	0.95 ± 0.03^{a}
	MWL	0.63 ± 0.20^{b}	0.79 ± 0.05^{b}	0.53 ± 0.13^{b}	0.32 ± 0.28^{b}
	H	1.41 ± 0.07^{a}	1.91 ± 0.15^{cd}	2.25 ± 0.14^{c}	1.97 ± 0.18^{c}
	C	1.46 ± 0.11^{a}	2.31 ± 0.25^{d}	3.22 ± 0.04^{d}	3.18 ± 0.19^{d}
	H+MWL	1.12 ± 0.31^{a}	1.56 ± 0.25^{cd}	2.23 ± 0.25^{c}	1.80 ± 0.14^{c}
	C+MWL	1.42 ± 0.11^{a}	1.93 ± 0.26^{cd}	3.17 ± 0.33^{d}	3.01 ± 0.26^{d}
Starvation	S	0.64 ± 0.11^{b}	0.80 ± 0.08^{b}	0.42 ± 0.06^{b}	0.19 ± 0.08^{b}

Values are means ± SD (n = 3). Statistical analyses were determined per each column and lignocellulose sample. Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$) by one-way ANOVA. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; MWL, milled wood lignin.

Table 3.3. CH_4 emission rates by workers fed on various diets prepared from Japanese cedar (softwood), Japanese beech (hardwood), and rice (grass)

Diet sources	Diet components	Emission rates (nmol/termite/h)			
		1st week	2nd week	3rd week	4th week
Japanese cedar	WP	0.18 ± 0.20[a]	0.19 ± 0.04[a]	0.13 ± 0.12[a]	0.21 ± 0.03[a]
	MWL	0.17 ± 0.10[a]	0.09 ± 0.08[a]	0.12 ± 0.11[a]	0.09 ± 0.15[a]
	H	0.21 ± 0.19[a]	0.18 ± 0.05[a]	0.13 ± 0.12[a]	0.19 ± 0.06[a]
	C	0.19 ± 0.19[a]	0.19 ± 0.05[a]	0.16 ± 0.06[a]	0.10 ± 0.09[a]
	H+MWL	0.23 ± 0.11[a]	0.27 ± 0.04[a]	0.10 ± 0.09[a]	0.10 ± 0.10[a]
	C+MWL	0.17 ± 0.15[a]	0.23 ± 0.10[a]	0.20 ± 0.10[a]	0.22 ± 0.09[a]
Japanese beech	WP	0.27 ± 0.16[a]	0.16 ± 0.00[a]	0.15 ± 0.04[a]	0.16 ± 0.14[a]
	MWL	0.20 ± 0.13[a]	0.13 ± 0.13[a]	0.17 ± 0.15[a]	0.18 ± 0.16[a]
	H	0.31 ± 0.23[a]	0.13 ± 0.11[a]	0.10 ± 0.10[a]	0.19 ± 0.08[a]
	C	0.27 ± 0.10[a]	0.18 ± 0.03[a]	0.18 ± 0.05[a]	0.10 ± 0.10[a]
	H+MWL	0.26 ± 0.08[a]	0.33 ± 0.05[a]	0.20 ± 0.03[a]	0.16 ± 0.01[a]
	C+MWL	0.25 ± 0.15[a]	0.18 ± 0.05[a]	0.25 ± 0.08[a]	0.18 ± 0.03[a]
Rice	RSP	0.29 ± 0.09[a]	0.13 ± 0.02[a]	0.07 ± 0.12a	0.09 ± 0.15[a]
	MWL	0.21 ± 0.11[a]	0.12 ± 0.11[a]	0.11 ± 0.11[a]	0.15 ± 0.13[a]
	H	0.34 ± 0.13[a]	0.10 ± 0.09[a]	0.11 ± 0.10[a]	0.12 ± 0.11[a]
	C	0.26 ± 0.08[a]	0.19 ± 0.04[a]	0.19 ± 0.01[a]	0.11 ± 0.10[a]
	H+MWL	0.26 ± 0.08[a]	0.22 ± 0.06[a]	0.18 ± 0.03[a]	0.11 ± 0.10[a]
	C+MWL	0.29 ± 0.12[a]	0.27 ± 0.09[a]	0.20 ± 0.33[a]	0.12 ± 0.11[a]
Starvation	S	0.15 ± 0.04[a]	0.05 ± 0.09[a]	0.13 ± 0.22[a]	0.10 ± 0.22[a]

Values are means ± SD (n = 3). Statistical analyses were determined per each column and lignocellulose sample. Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$) by one-way ANOVA. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; MWL, milled wood lignin.

3.4. Discussion

Symbiotic micro-organisms, especially flagellated protists in the hindgut of lower termites like *C. formosanus*, play an important role in the production of H_2 as a by-product from the breakdown of polysaccharides in the termite digestive system. The amount of H_2 emitted by termites during lignocellulose digestion may correlate with the community profile of symbiotic micro-organisms in the termite hindgut. The present study found that *C. formosanus* workers released much more H_2 when fed wood lignocellulose (Japanese cedar and Japanese beech) than when fed grass lignocellulose (rice) (Fig. 3.1 and Table 3.2). In line with this, the previous work recorded that the presence of flagellated protists in the hindgut of *C. formosanus* workers fed grass lignocellulose samples were remarkably depleted compared with workers fed wood lignocellulose samples (Tarmadi et al., 2017a). Given that MWL from rice did not affect H_2 production from termites fed artificial diets mixed with polysaccharides (Fig. 3.3 and Table 3.2), lignins are unlikely the major factor limiting H_2 production from grass lignocellulose. Mineral components present in the grass lignocellulose samples might limit the survival of flagellated protists in the hindgut of *C. formosanus* workers (Tarmadi et al., 2017a) and thus decrease H_2 productions; unlike wood lignocelluloses, grass lignocellulose typically contains substantial amounts of minerals such as silica (Jenkins et al., 1998; Binod et al., 2010).

H_2 and CH_4 emission rates by workers fed the three isolated lignins (MWLs) from softwood, hardwood, and grass were similar throughout the test period. Those H_2 emission rates were also similar with those of the starved control group, suggesting that lignin has little effect on H_2 and CH_4 production during the digestion of cell wall polysaccharides in the termite digestive system, at least under the current test conditions. Overall, the data from this study are supportive of the previous observations that lignins

are hardly utilized as a nutrient source by *C. formosanus* (Tarmadi et al., 2017a). However, some earlier studies reported that lignin polymers could be, at least partially, decomposed in the hindgut of wood-feeding insects including *C. formosanus* (Geib et al., 2008; Ke et al., 2010; 2013; Li et al., 2017). The protists and bacteria in the hindgut of *C. formosanus* workers may perform some modification/degradation of lignin barriers to support efficient lignocellulose degradation (Kyou et al., 1996; Harazono et al., 2003), but lignin itself most likely has no nutritional role in termites.

In order to more closely investigate the effect of lignins as diet components, *C. formosanus* workers were fed to artificial diets composed of lignins and polysaccharides (holocellulose or cellulose). The present study noted that H_2 emission rates by workers were generally higher when fed cellulose than when fed holocellulose or wood samples from Japanese cedar and Japanese beech (Fig. 3.1 and Table 3.2). An earlier study reported that similar amounts of protists in the hindgut of *C. formosanus* were detected when termites were provided wood powder and cellulose substrates (Tanaka et al., 2006). It has been well recognized that flagellated protists, especially *P. grassii*, are predominant producers of metabolic hydrogen (Inoue et al., 2007). On the other hand, gut symbiotic prokaryotes such as methanogens and acetogens were reported as major H_2 consumers in the termite hindgut (Brauman et al., 1992; Breznak & Brune, 1994; Cao et al., 2010; 2012). Therefore, there are two hypotheses to explain the significant increase in H_2 emissions of cellulose-fed workers: (1) an increase of cellulose fermentation activity in the protists; and/or (2) a decrease of H_2 consumption by the prokaryotes in the termite hindgut. The present results showed an almost double H_2 emission rate and low and stable CH_4 emission in the cellulose-fed workers (Table 3.3), consistent with the earlier findings of Kawaguchi et al (2006). Therefore, the first option seems the most probable scenario. In

the present study, however, detected no significant difference regarding H_2 emission rates between workers fed only polysaccharides (holocellulose or cellulose) and those fed polysaccharides with lignins. These results suggest that the addition of lignins to polysaccharides results in no drastic changes in micro-organism communities in the hindgut of workers, at least until the 4th week of incubation. Further studies are required to link lignin to the physiological activities and micro-organism community profiles in the hindgut of *C. formosanus* workers.

3.5 Chapter summary

H_2 emission rate by workers fed on rice was remarkable low than that by workers fed on wood samples after 4 weeks. H_2 and CH_4 emission rates by workers fed on the three MWLs were similar with that of starved workers, suggesting that the profiles of micro-organisms community in the starved termite with those fed on three MWLs were similar. The present results well support the previous observation that lignins are hardly utilized as a nutrient source by *C. formosanus* as reported in Chapter 2. Since H_2 emission rates by workers fed on polysaccharides (holocellulose or cellulose) and polysaccharides with lignins until 4 weeks measurement, these results are likely to indicate that lignins when served with polysaccharides result in no drastic changes in micro-organisms communities in the hindgut of workers until 4th week incubation.

Chapter 4. Effects of lignins as diet components on the physiological activities of a lower termite, *Coptotermes formosanus* Shiraki

4.1. Introduction

As reported in the preceding chapters, Chapters 1-3, *C. formosanus* has an ability to remove partially lignin polymers during termite digestion, and lignins are hardly utilized as a nutrient source and are even rather detrimental to *C. formosanus* workers when they are the sole food source. Interestingly, however, it has been reported that *C. formosanus* workers fed on wood showed better survival than those fed on an artificial diet composed of only polysaccharide without lignin (Yoshimura, 1995; Kyou et al., 1996). Besides termites, another wood-feeding insect, *Lyctus africanus,* also preferred wood to α-cellulose as a matrix of artificial diets (Kartika and Yoshimura, 2015). However, little is known regarding the role of lignins on the physiological activities of wood-feeding insects. The present Chapter, Chapter 4 was conducted to provide further insights into the effects of lignins as diet components on physiological activities, such as the survival rate, body mass change, and gut protist profiles of *C. formosanus* workers. Three different types of lignin polymers, i.e., those isolated from softwood (Japanese cedar), hardwood (Japanese beech), and grass (rice) lignocelluloses, were tested for comparison.

4.2.Materials and Methods

4.2.1. Preparation of diet samples

Preparations of artificial diets from Japanese cedar, Japanese beech, and rice straw were conducted by the methods described in Chapter 3. Five diet samples per each

lignocellulose were prepared, i.e., wood powder (WP) or rice straw powder (RSP), holocellulose (H), cellulose (C), holocellulose mixed with lignin (H+MWL), and cellulose mixed with lignin (C+MWL).

4.2.2. Bioassay

Approximately 200 mg of each diet was put into a plastic cup that was 20 mm in diameter and 10 mm in depth, and the cup was set on the center of an acrylic cylinder with a plaster bottom (65 mm in diameter and 60 mm in height). One hundred workers and 10 soldiers of *C. formosanus* were obtained from a laboratory colony from Deterioration Organisms Laboratory (DOL), Research Institute for Sustainable Humanosphere (RISH), Kyoto University, and forced to feed on those diets. The number of live workers was recorded and balanced every two weeks for 12 weeks. The termite survival and body mass observations were performed in triplicate.

4.2.3. Evaluation of protist fauna in the hindguts of C. formosanus workers

One hundred and thirty workers and 13 soldiers of *C. formosanus* were fed on diets with various components as described above. Every two weeks for 8 weeks, 10 workers were randomly selected from each container, and their hindguts were pulled out from the posterior ends and disintegrated into pieces using fine forceps. The contents were then suspended in 0.4% NaCl. The hindgut pieces were macerated gently in the solution to facilitate the release of protozoa. The species of protozoa were identified using a digital microscope (VHX-5000, Keyence, Osaka, Japan) (Yoshimura, 1995; Tanaka et al., 2006; Tarmadi et al., 2017a). These observations were performed in triplicate.

4.2.4. Statistical analysis

The differences in the survival rates and body masses of workers and the rates at which protists were present in the hindguts of workers fed on various diets prepared from Japanese cedar (softwood), Japanese beech (hardwood), and rice (grass) were tested by SPSS ver. 23 (IBM, Armonk, NY, USA). Significant differences between means were calculated by Tukey's post-hoc test. Significance levels were set at $P < 0.05$.

4.3. Results

4.3.1. Preparation of artificial lignocellulose diet samples

To closely investigate the effect of lignins in lignocellulose diets on the physiological activities of termites, a series of artificial lignocellulose diet samples that varied in their lignocellulose composition was prepared. Purified MWLs (lignin), holocellulose (hemicelluloses + cellulose), and cellulose samples were prepared using conventional methods from Japanese cedar (softwood), Japanese beech (hardwood), and rice (grass) lignocellulose samples, and mixed to the formulations listed in Table 3.1. The formulation ratios, i.e., the polysaccharide and lignin contents, were determined based on the chemical composition analysis data for each original plant material (Tarmadi et al., 2017a). The purity (>85%) and structures of the MWL samples used in this study were analyzed by neutral sugar analysis and 2D NMR (Nuclear Magnetic Resonance) as reported in detail elsewhere (Tarmadi et al., 2017a). Using the thioglycolic acid lignin assay (Suzuki et al., 2009), the present study also confirmed the reasonably high purity (less than 3% lignin content) of the holocellulose and cellulose polysaccharide samples used in this study (Table 4.1).

Table 4.1. Lignin content in the polysaccharides isolated from Japanese cedar, Japanece beech, and rice

Lignocelluloses	Polysaccharides	Lignin content (%) ± SD
Japanese cedar	Holocellulose	3.07 ± 0.09
	Cellulose	0.65 ± 0.02
Japanese beech	Holocellulose	2.30 ± 0.12
	Cellulose	0.55 ± 0.03
Rice straw	Holocellulose	0.06 ± 0.02
	Cellulose	0.06 ± 0.02

4.3.2. Effects of lignins on the survival of C. formosanus workers

Figure 4.1 shows the changes in the survival rates of *C. formosanus* workers fed on various lignocellulose diets (wood powder, holocellulose, cellulose, holocellulose + MWL, and cellulose + MWL) prepared from Japanese cedar (softwood). The survival of workers tended to be decreased with increasing feeding time. The survival rates of workers fed on all of the softwood diets were similar for the first 6 weeks. After 8 weeks, the survival of workers fed on diets containing lignin were apparently higher than that of workers fed on only polysaccharides, although there were no significant differences among the diets at the 8- (F = 2.9, P = 0.079), and 10-week (F = 3.4, P = 0.055) observations. At the end of the observation period (12 weeks), a significant difference was observed between the wood powder and both the holocellulose and cellulose samples (F = 25.5, P = 0.001): the survival rate of workers fed on wood powder, at 50%, was significantly higher than those of workers fed on either holocellulose or cellulose, at 33% and 30%, respectively. In addition, the survival for workers fed on holocellulose+MWL was significantly higher than that for those fed only on holocellulose (F =123.1, $P < 0.05$) at the 12-week observation. Similarly, the survival for workers fed on cellulose+MWL

was significantly higher than that for those fed only on cellulose ($F = 26.3$, $P = 0.007$) at the end of the observation. There was no significant difference between the holocellulose+MWL and cellulose+MWL diets ($F = 2.8$, $P = 0.169$), and also between the holocellulose and cellulose diets ($F = 1.6$, $P = 0.272$) in terms of the survival rates of workers at the 12-week observation (Figs. 4.1 and 4.4). These results collectively suggested that softwood lignins have a beneficial effect on the survival of *C. formosanus* workers when served with polysaccharides. As the survival of starved workers was significantly lower than those fed on any of the diets, it is conceivable that workers still utilize polysaccharides as their main carbon sources.

The results of the experiments with Japanese beech (hardwood) diet samples were similar overall to the results for Japanese cedar (softwood) diet samples (Figs. 4.2 and 4.4). At the 12-week observation, the survival of *C. formosanus* workers fed on wood powder was significantly higher than that of workers fed on only polysaccharides ($F = 51.5$, $P < 0.05$), with the rates being 49%, 34%, and 30% for wood powder, holocellulose, and cellulose diet samples, respectively.

In addition, it was clear that the survival rates of workers fed on the diets containing lignin (holocellulose+MWL or cellulose+MWL) were significantly higher than those of workers fed on diet groups without lignin (holocellulose or cellulose) ($F = 28$, $P < 0.05$). Thus, as with softwood lignins, hardwood lignins positively affect the survival of *C. formosanus* workers when served with polysaccharides. As observed in the experiments with softwood diet samples, there were no significant differences among wood powder, holocellulose+MWL, and cellulose+MWL ($F = 3$, $P = 0.125$), nor between holocellulose and cellulose ($F = 3.8$, $P = 0.121$) at the 12-week observation (Figs. 4.3 and 4.4).

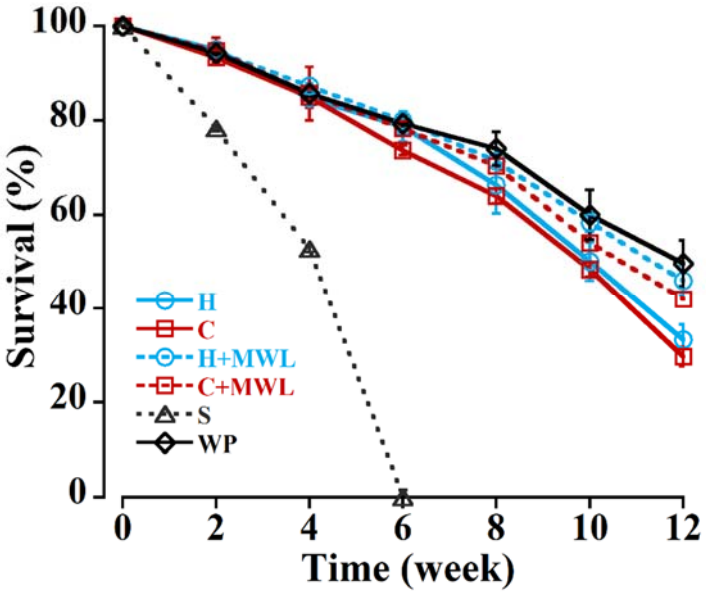

Fig. 4.1. Changes in the survival rates of *C. formosanus* workers fed on various diets prepared from Japanese cedar, Error bars represent standard deviation. WP, wood powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation.

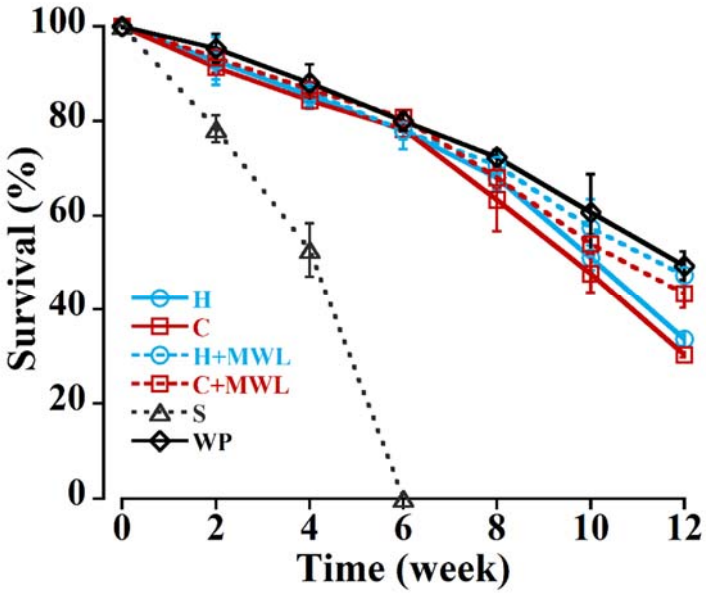

Fig. 4.2. Changes in the survival rates of *C. formosanus* workers fed on various diets prepared from Japanese beech. Error bars represent standard deviation. WP, wood powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation.

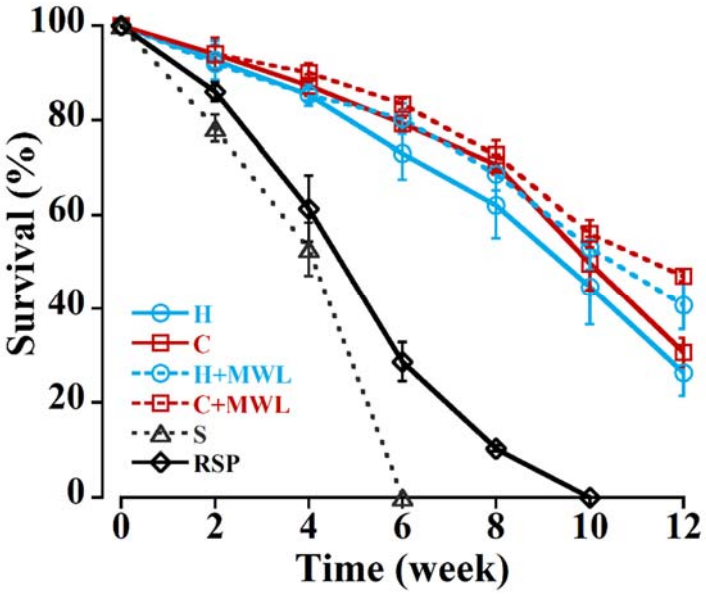

Fig. 4.3. Changes in the survival rates of *C. formosanus* workers fed on various diets prepared from rice straw. Error bars represent standard deviation. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation.

Figure 4.3 shows the changes in the survival rates of *C. formosanus* workers fed on various diets prepared from rice straw (grass). Again, the results clearly observed a positive effect of the addition of lignin to the diets on termite survival. The survival of workers fed on holocellulose+MWL or cellulose+MWL was significantly higher than that of workers fed on holocellulose (F = 12.4, P = 0.024) or cellulose (F =77.5, P = 0.01) without lignins. Interestingly, however, the survival rate of workers fed on the original rice straw powder samples was significantly lower than the survival rates of workers fed on the other diets, and no workers survived at the 10th week of observation, while more than 40% of workers still survived when fed on the other artificial diet samples.

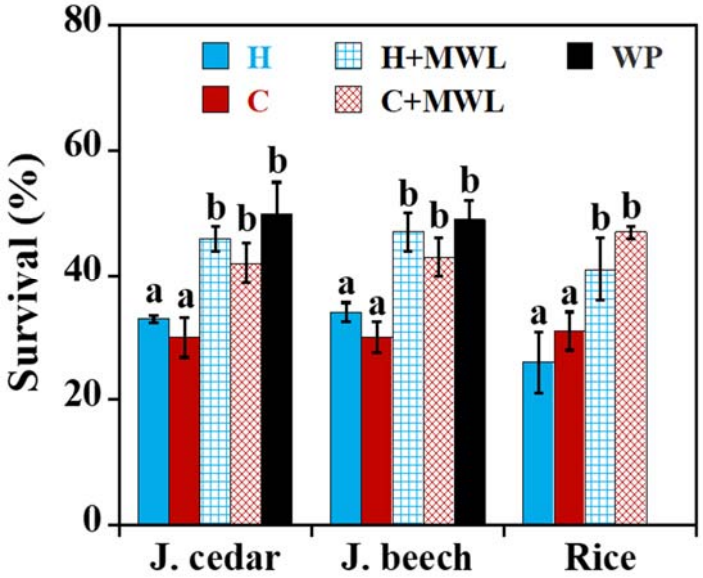

Fig. 4.4. Changes in the survival rates of *C. formosanus* workers fed on various diets prepared from Japanese cedar, Japanese beech, and rice straw at the 12-week observation. Error bars represent standard deviation. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation.

4.3.3. Effects of lignins on body mass of C. formosanus workers

The periodic changes in body mass of workers fed on the lignocellulose diets with and without lignins are presented in Figs. 4.5-4.8. The body mass of all the tested workers including the starvation control showed a similar decrease during the initial two weeks of observation and, except for workers fed with rice straw powders (discussed below), became stable after that period. A similar phenomenon has been observed in other lab studies of termites (Yoshimura, 1995; Tarmadi et al., 2017a), and such initial decrease in body mass of termites might be attributed to a gradual decrease in the water content of termite body by being exposed to relatively less humid test conditions after retrieved from the nest colony. Overall, there were no significant differences between the artificial

polysaccharide diets with and without lignins from Japanese cedar (softwood), Japanese beech (hardwood), and rice (grass) lignocellulose samples (Figs. 4.5-4.8), suggesting that all three major types of lignins are unlikely to have any effect on the body mass of *C. formosanus* workers when served with polysaccharides. Meanwhile, it was found that the body mass of workers fed on rice straw powder was significantly lower than that of workers fed on the other four diets ($F = 39.8$, $P < 0.05$) and was even lower than that of the starvation controls ($F = 354$, $P < 0.05$) after 4 weeks of observation (Fig. 4.7). Along with the data on termite survival described above (Fig. 4.3), the data strongly suggested that compounds other than major cell wall polysaccharides and lignin in rice lignocellulose negatively affected the physiological activities of *C. formosanus* workers.

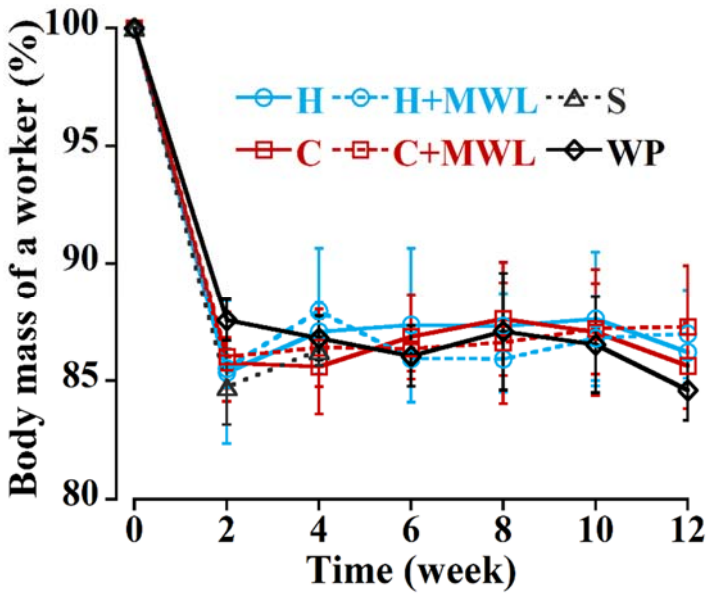

Fig. 4.5. Changes in body mass of *C. formosanus* workers fed on various diets prepared from Japanese cedar. Error bars represent standard deviation. WP, wood powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation.

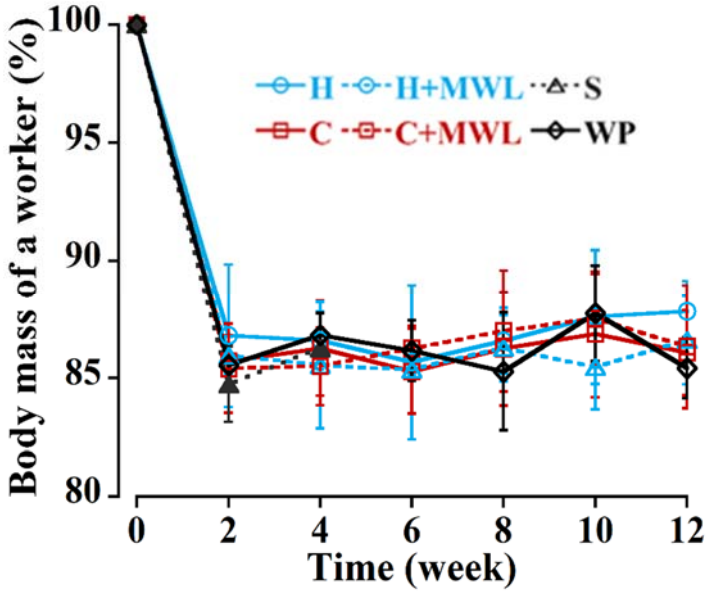

Fig. 4.6. Changes in body mass of *C. formosanus* workers fed on various diets prepared from Japanese beech. Error bars represent standard deviation. WP, wood powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation.

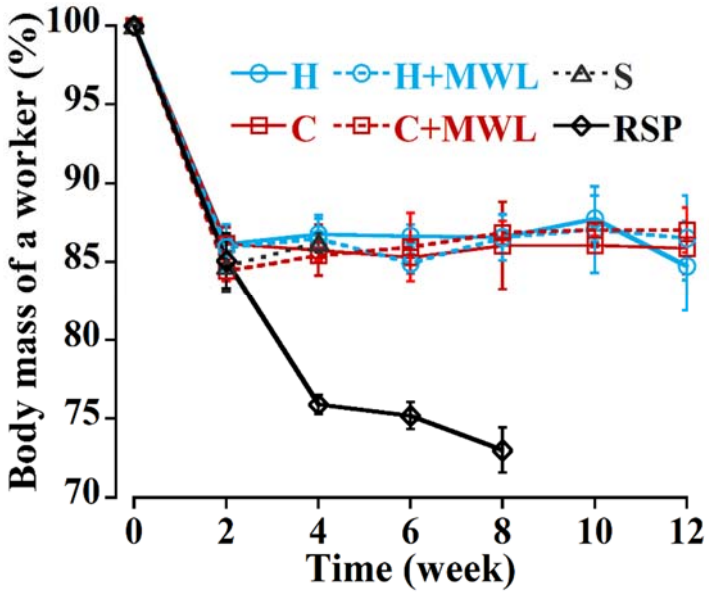

Fig. 4.7. Changes in body mass of *C. formosanus* workers fed on various diets prepared from rice straw. Error bars represent standard deviation. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation.

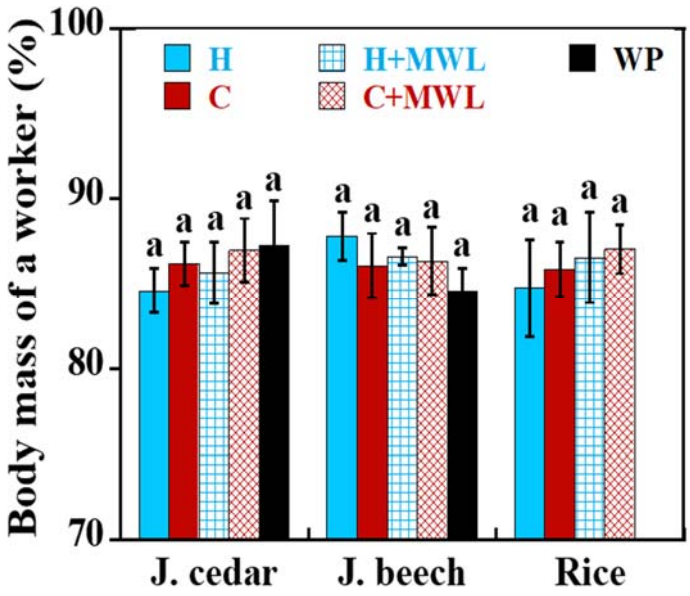

Fig. 4.8. Changes in body mass of *C. formosanus* workers fed on various diets prepared from Japanese cedar, Japanese beech, and rice straw at the 12-week observation (d). Error bars represent standard deviation. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation.

4.3.4. Flagellated protist profiles

Next, the present study recorded the presence of three protists in the hindguts of the workers fed on the lignocellulose diets with and without lignins (Tables 4.2-4.4). A microscopy approach (Yoshimura, 1995; Tanaka et al., 2006) was used to obtain the profiles of the three protists present in the hindguts of *C. formosanus* workers, i.e., *P. grassii*, *H. hartmanni*, and *S. leidyi*. The present study did not observe significant differences in the presence rates of protists among the artificial diet samples, excluding the original wood and rice straw powders, until the 6-week observation. However, at the 8-week observation, it was clearly observed that, regardless of the lignocellulose diet sources (softwood, hardwood, or grass), feeding on polysaccharides with lignins

(holocellulose+MWL and cellulose+MWL) resulted in higher presence rates of protists in the hindguts of workers than did feeding on only polysaccharides (holocellulose and cellulose). With artificial diets prepared from Japanese cedar (softwood), the presence rates of *P. grassii* ($F = 43$, $P < 0.05$) and *H. hartmanni* ($F = 43$, $P < 0.05$) in the hindguts of workers fed on polysaccharides with lignins were significantly higher than those of workers fed on only polysaccharides, as 20-27% workers fed on only polysaccharides lost *P. grassii* and *H. hartmanni* (Tables 4.2 and 4.3). In contrast, only 3-7% workers fed on polysaccharides with lignin lost *P. grassii* and *H. hartmanni*. Likewise, lignins from Japanese beech (hardwood) and rice (grass), when served with polysaccharides, indeed improved the presence rates of *P. grassii* and *H. hartmanni* at the 8-week observation. The present study did not observe any significant differences in the presence rate of *S. leidyi* in the hindguts of workers among all the artificial diets (Table 4.4). Collectively, the data demonstrated that lignins show positive effects on the survival rates of the two major protists, *P. grassii* and *H. hartmanni,* in the hindguts of *C. formosanus* workers. Given that the present study did not observe significant differences among the lignin sources, i.e., softwood, hardwood, or grass, it is likely that the structural differences in lignin polymers do not affect the presence rates of the three protists in the hindguts of workers evaluated in this study.

In parallel, the present study also collected the protist profiles for unseparated lignocellulose diets (wood and rice straw powders). All three protists remained in the hindguts of all workers fed on Japanese cedar and Japanese beech wood powders until the end of the observation. In contrast, feeding on rice straw powder resulted in drastic losses of all three protists in the hindguts of workers: approximately 90%, 53%, and 13% of workers had lost *P. grassii*, *H. hartmanni*, and *S. leidyi*, respectively, at the end of the

observation (8 weeks). Given that the present study did not observe such drastic losses of the protists with the other artificial diets composed of lignin and polysaccharides, it is likely that non-lignocellulose components in rice straw powder may negatively affecting the survival of the protists in the hindguts of workers.

Table 4.2. Presence rates of *P. grassii* in the hindguts of *C. formosanus* workers fed on various lignocellulose diets.

Diet sources	Diet components	*P. grassii* (%)			
		Observation time (week)			
		2	4	6	8
Japanese cedar	WP	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}
	H	100 ± 0^{a}	100 ± 0^{a}	93.3 ± 5.8^{a}	76.5 ± 5.8^{b}
	C	100 ± 0^{a}	100 ± 0^{a}	93.3 ± 5.8^{a}	80.0 ± 0^{b}
	H+MWL	100 ± 0^{a}	100 ± 0^{a}	96.7 ± 5.8^{a}	93.3 ± 5.8^{a}
	C+MWL	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	93.3 ± 5.8^{a}
Japanese beech	WP	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}
	H	100 ± 0^{a}	100 ± 0^{a}	93.3 ± 5.8^{a}	80.0 ± 0^{b}
	C	100 ± 0^{a}	100 ± 0^{a}	90.0 ± 10^{a}	73.3 ± 11.5^{b}
	H+MWL	100 ± 0^{a}	100 ± 0^{a}	93.3 ± 5.8^{a}	96.7 ± 5.8^{a}
	C+MWL	100 ± 0^{a}	100 ± 0^{a}	96.7 ± 5.8^{a}	93.3 ± 5.8^{a}
Rice straw	RSP	90 ± 10^{a}	33.3 ± 5.8^{b}	16.7 ± 5.8^{b}	10.0 ± 0^{c}
	H	100 ± 0^{a}	100 ± 0^{a}	90.0 ± 10^{a}	73.3 ± 5.8^{b}
	C	100 ± 0^{a}	100 ± 0^{a}	93.3 ± 5.8^{a}	80.0 ± 0^{b}
	H+MWL	100 ± 0^{a}	100 ± 0^{a}	93.3 ± 5.8^{a}	93.3 ± 5.8^{a}
	C+MWL	100 ± 0^{a}	100 ± 0^{a}	96.7 ± 5.8^{a}	96.7 ± 5.8^{a}
Starvation	S	0 ± 0^{b}	0 ± 0^{c}	-	-

Values are means ± SD (n = 3). Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$) following one-way ANOVA. Error bars represent standard deviation. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation; - indicates that all workers were dead.

Table 4.3. Presence rates of *H. hartmanni* in the hindguts of *C. formosanus* workers fed on various lignocellulose diets.

Diet sources	Diet components	*H. hartmanni* (%)			
		Observation time (week)			
		2	4	6	8
Japanese cedar	WP	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]
	H	100 ± 0[a]	100 ± 0[a]	93.3 ± 5.8[a]	80.0 ± 0[b]
	C	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	76.7 ± 5.8[b]
	H+MWL	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	93.3 ± 5.8[a]
	C+MWL	100 ± 0[a]	100 ± 0[a]	93.3 ± 0[a]	93.3 ± 5.8[a]
Japanese beech	WP	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]
	H	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	80.0 ± 0[b]
	C	100 ± 0[a]	100 ± 0[a]	93.3 ± 5.8[a]	76.7 ± 5.8[b]
	H+MWL	100 ± 0[a]	100 ± 0[a]	93.3 ± 5.8[a]	93.3 ± 5.8[a]
	C+MWL	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	96.7 ± 5.8[a]
Rice straw	RSP	100 ± 0[a]	86.7 ± 5.8[b]	73.3 ± 5.8[b]	46.7 ± 5.8[c]
	H	100 ± 0[a]	100 ± 0[a]	90.0 ± 10[a]	76.7 ± 5.8[b]
	C	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	80.0 ± 0[b]
	H+MWL	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]
	C+MWL	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]
Starvation	S	100 ± 0[a]	30 ± 10[c]	-	-

Values are means ± SD (n = 3). Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$) following one-way ANOVA. Error bars represent standard deviation. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation; - indicates that all workers were dead.

Table 4.4. Presence rates of *S. leidyi* in the hindguts of *C. formosanus* workers fed on various lignocellulose diets.

Diet sources	Diet components	*S. leidyi* (%)			
		Observation time (week)			
		2	4	6	8
Japanese cedar	WP	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]
	H	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	93.3 ± 5.8[a]
	C	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	90 ± 10[a]
	H+MWL	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]
	C+MWL	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]
Japanese beech	WP	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]
	H	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	90 ± 10[a]
	C	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	90 ± 10[a]
	H+MWL	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]
	C+MWL	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]
Rice straw	RSP	100 ± 0[a]	96.7 ± 5.8[a]	93.3 ± 5.8[a]	86.7 ± 11.5[a]
	H	100 ± 0[a]	100 ± 0[a]	93.3 ± 5.8[a]	86.7 ± 11.5[a]
	C	100 ± 0[a]	100 ± 0[a]	93.3 ± 5.8[a]	93.3 ± 5.8[a]
	H+MWL	100 ± 0[a]	100 ± 0[a]	96.7 ± 5.8[a]	93.3 ± 5.8[a]
	C+MWL	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]
Starvation	S	100 ± 0[a]	63.3 ± 5.8[b]	-	-

Values are means ± SD (n = 3). Values with the same letter are not significantly different (Tukey HSD test; $P < 0.05$) following one-way ANOVA. Error bars represent standard deviation. WP, wood powder; RSP, rice straw powder; H, holocellulose; C, cellulose; H+MWL, holocellulose+MWL; C+MWL, cellulose+MWL; S, starvation; - indicates that all workers were dead.

4.4. Discussion

As mentioned earlier, the mechanisms of the decomposition of polysaccharides by termites have been well-documented (Ohkuma, 2003; Hongoh, 2011; Ni and Tokuda, 2013; Brune, 2014). The lower termite *C. formosanus* makes use of a dual decomposing system, comprising the termite's endogenous cellulases as well as a variety of cellulolytic enzymes secreted by its gut protists, to efficiently convert polysaccharides into acetates and other nutrients that the host further utilizes as energy and carbon sources (Ohkuma, 2003). Therefore, the presence of protists in the hindguts of workers is very important for the nutritional physiology of *C. formosanus* (Yoshimura, 1995). Some studies have been carried out to elucidate the fate of lignin in the termite digestive system. It has been shown that, especially in winter, *C. formosanus* workers digest nest materials consisting mainly of digestive residues rich in lignin (Yoshimura, 1995). Kyou et al. (1996) reported that protists in the hindguts of *C. formosanus* workers could be involved in the modification of lignin polymers. In addition, it was reported that the gut flora of wood-feeding termites has the ability to degrade and modify monomeric and dimeric lignin model compounds (Kuhnigk et al., 1994; Brune et al., 1995; Kuhnigk and König, 1997). Two species of bacteria, *Burkholderia* sp. and *Citrobacter* sp., isolated from the gut of *C. formosanus* display the ability to decompose aromatic lignin model compounds (Harazono et al., 2003). Furthermore, some recent studies using advanced analytical methods to characterize the lignocellulose structure have suggested that high-molecular-weight lignin polymers could be, at least partially, decomposed in the hindgut of wood-feeding insects including *C. formosanus* (Geib et al., 2008; Ke et al., 2010, 2011a, 2013). Meanwhile, however, no potential lignin degradation enzymes have yet been found in the symbiotic protistan community in the hindgut of *C. formosanus* (Xie et al., 2012).

Although it has been generally accepted that termites hardly utilize lignin as a main nutrient source (Tarmadi et al., 2017a), lignin may play an important role in maintaining the physiological activities of termites during their lignocellulose decomposition. An earlier study by Kyou et al. (1996) found that the survival of *C. formosanus* workers fed on wood powder was higher than that of workers fed on an artificial diet consisting of holocellulose without lignin. The present study more closely investigated the effect of lignins on the physiological activities of *C. formosanus* and the hindgut protist profiles by testing a series of artificial lignocellulose diet samples varying in their lignin/polysaccharide composition. The data clearly demonstrated that lignins from softwood, hardwood, and rice all have marked positive effects on the survival of workers (Fig. 1) as well as on the maintenance of two major protists in the hindguts of workers, i.e., *P. grassii* and *H. hartmanni* (Tables 4.2 and 4.3). Earlier study suggested that these protists play important roles in the digestion and utilization of high-molecular-weight polysaccharides (Tanaka et al., 2006). It is therefore plausible that lignins or lignin-derived low-molecular-weight aromatic compounds might have the beneficial effect for the symbiotic protists in the hindguts of workers when served with polysaccharides as diet components, and consequently support the survival of workers. Also, lignins or lignin-derived aromatic compounds may trigger positive changes in the bacteria community in the hindguts of *C. formosanus* workers. In a finding that supported this, it was reported that changes in the bacterial community are closely correlated with the protist profile (Tanaka et al., 2006). Further detailed profiling of the bacterial community in the hindguts of *C. formosanus* workers is required to clarify the link between the hindgut bacterial community and the feeding of lignins. In contrast to the pronounced effects of lignins on the survival of workers as well as on that of the protists in their

hindguts, body mass of *C. formosanus* workers fed on all the artificial diets with and without lignins were overall similar (Figs. 4.5-4.8). The result suggests that lignin itself has at least no negative effect on the physiology of *C. formosanus* workers when fed with polysaccharides and the polysaccharide diets provided sufficient basic nutrient to the workers for maintaining their body mass.

Plants have different types of lignins, depending primarily on the plants' taxa. Softwood lignins are composed mainly of guaiacyl (G) units, whereas hardwood and grass lignins consist of G and additional syringyl (S) units. Typical grass lignins are also highly acylated by *p*-coumarate esters (Ralph, 2010; Petrik et al., 2014), and they also contain a substantial amount of tricin flavonoid units integrated into the polymer chain (Lan et al., 2015; 2016). The previous study using 2D NMR analysis, confirmed that the presence of such structural deviations among the MWL samples used in this study (Tarmadi et al., 2017a). The results presented here, however, suggested that such differences among lignin types, i.e., among lignins from softwood, hardwood, and grass, have no effect on the physiological activities of *C. formosanus* as well as its major symbiotic protists. On the other hand, the present study noted that the survival rates and body mass of workers as well as the survival rates of major protists in the hindguts of workers fed on rice straw powder were remarkably lower than those fed on all the other diets, suggesting that some components other than major cell wall polysaccharides and lignin in rice lignocellulose negatively affected the physiological activities of *C. formosanus*. The mineral components could be responsible for this phenomenon, as, unlike wood lignocelluloses, grass lignocellulose typically contains substantial amounts of minerals such as silica (Jenkins et al., 1998; Binod et al., 2010). In support of this hypothesis, an earlier study reported that silica had a negative effect on the body mass of

Eldana saccharina Walker (Keeping and Meyer, 2002). Such minerals might have physical/chemical effects on the protists that ingest lignocellulose particles by phagocytosis (Yoshimura et al., 1996), and silica compounds have also been evaluated for their effectiveness in insect control (Ebeling, 1971; Keeping and Meyer, 2002). In addition, non-structural carbohydrates such as starch and oligosaccharides in grass vegetative tissues (Okahisa et al., 2006; Slewinski, 2012) might be partly responsible for the negative impacts observed for rice straw diets. In fact, several earlier studies reported detrimental effects of starch and soluble oligosaccharides on lower termites (Kanai et al., 1982; Veivers et al., 1983; Tanaka et al., 2006). In either scenario, the data are in line with the fact that the lower termites including *C. formosanus* prefer wood to grass for feeding in nature. Given that the grass-feeding higher termites have no symbiotic protists in their guts (Hongoh, 2011; Brune, 2014), the negative effects of grass lignocellulose on the lower termites' protists, as observed in this study, could be a key factor limiting the consumption of grass lignocellulose by lower termites.

Overall, lignins, regardless of their source (softwood, hardwood, and grass), have marked positive effects on the survival of *C. formosanus* workers as well as the maintenance of the major protists in their hindguts when fed with polysaccharide diets. The results strongly suggest that presence of lignins in lignocelluloses is crucial to maintaining the physiological activities of *C. formosanus* workers. An analysis of the bacterial community in the hindguts of *C. formosanus* workers fed on various lignocellulose and artificial lignin diets is required to provide further insights into the mechanism of lignocellulose biodegradation by wood-feeding insects, which may also contribute to the development of sustainable technology to convert lignocellulosic biomass to valuable fuels and materials.

4.5. Chapter summary

Overall, lignins, regardless of their source (softwood, hardwood, and grass), have marked positive effects on the survival of *C. formosanus* workers as well as the maintenance of the major protists in their hindguts when fed with polysaccharide diets. The results strongly suggest that presence of lignins in lignocelluloses is crucial to maintaining the physiological activities of *C. formosanus* workers. Further detailed profiling of the bacterial community in the hindguts of *C. formosanus* workers is required to clarify the link between the hindgut bacterial community and the feeding of lignins.

Chapter 5. The effects of dietary lignin on bacterial community in the hindgut of a lower termite, *Coptotermes formosanus* Shiraki

5.1. Introduction

In the previous chapter, Chapter 4, it was demonstrated that dietary lignins when served with polysaccharides have markedly positive effects on the survival of *C. formosanus* workers as well as on the maintenance of the major protists in their hindguts. While eukaryotic protists are considered to be the major contributors to polysaccharide digestion in the digestive system of lower termites, numerous prokaryotic microbes existing on the surface or within the protist cells are also suggested to be involved in a variety of physiological and biochemical associations with the protists (Gast et al., 2009; Hongoh, 2011; Ni and Tokuda, 2013; Brune, 2014). In fact, an earlier study suggested that the protist profiles in the hindgut of *C. formosanus* workers could be correlated with the bacterial community profiles (Tanaka et al., 2006). In this chapter, therefore, the effects of dietary lignins on the bacterial community profiles in the hindgut of *C. formosanus* workers are investigated. Toward this aim, a DNA fingerprinting tool, automated ribosomal intergenic spacer analysis (ARISA) was used; ARISA detects fragment-length variations in the intergenic spacer region between the 16S rRNA and the 23S rRNA genes, a hypervariable region that varies among bacterial species/strains, and has been used to characterize and compare bacterial communities in various biological systems (Fisher and Triplett, 1999; Kovacs et al., 2010).

5.2. Materials and methods

5.5.1. Feeding of diet samples to C. formosanus workers

Preparations of artificial diets from Japanese cedar (*Cryptomeria japonica*) sapwood were conducted by the methods described in Chapter 3 (Table 3.1), while feeding experiments with *C. formosanus* workers were conducted by the methods described in Chapter 4.

5.2.2. DNA Extraction from termite gut

After 2, 4, and 8 weeks of feeding, five workers were randomly selected, and their guts were manually pulled out from the posterior end using fine forceps (Shinzato, et al., 2005) and pooled in a bead tube. DNA extraction was conducted using Extrap Soil DNA Kit Plus Ver. 2 (Tokyo Future Style, Inc) according to the manufacturer's instructions.

5.2.3. PCR Amplification of the 16S-23S rRNA intergenic spacer region

PCR was performed using a bacterial ARISA primer set, ITSF (5′-GTCGTAACAAGG TAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) fluorescently labeled with the phosphoramidite dye 6-FAM (6-carboxyfluorescein). PCR reactions were performed using 20–50 ng of extracted DNA in 25-µl volumes containing 12.5 µl Kapa HiFi Hotstart Ready Mix and 0.3 µM of each primer. Amplification was carried out at 95°C denaturation for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min, with a final extension at 72°C for 5 min. The amplified DNA fragments were run on a 1.5% agarose gel to check amplification success, and purified with QIAquick PCR purification kit (QIAGEN) according to the manufacturer's

instructions. Fragment lengths were resolved on a 3130xl Genetic Analyzer (Applied Biosystems, New York,USA) using Liz-1200 internal size standard (Applied Biosystems) *at FASMAC* Co., Ltd. (Kanagawa, Japan).

5.2.4. Analysis of ARISA Profiles

Changes in microbial community profiles in the hindgut of *C. formosanus* workers fed different diet components were analyzed by calculating fragment length and peak height in ARISA profiles (200–1200 bps). Three independent analyses using 5 workers per run were conducted, and peak heights were averaged after normalization based on the fluorescence intensity obtained for the internal standard. Pearson correlation analysis was used to establish the relationship between ARISA profiles. Principal coordinate analysis (PCoA) and agglomerative hierarchical clustering (AHC) were carried out using XLSTAT 2017 (Addinsoft, New York, USA).

5.3. Results

Coptotermes formosanus workers were fed various lignocellulose diet samples prepared from softwood J. cedar (Table 4.1) as described in Chapter 4. The bacterial community profiles in the hindgut of *C. formosanus* workers were investigated using ARISA fingerprinting based on the electrophoretic separation of the amplified 16S-23S rRNA intergenic spacer region of bacteria DNA (Fisher and Triplett, 1999; Geib et al., 2009; Kovacs et al., 2010). The analysis generates complex profiles of PCR-amplified DNA fragments ranging from 200 bp to 1200 bp (Fig 5.1). Although rigorous sequencing analysis is needed to identify the taxonomical origins of each fragment, fractional analyses, i.e., PCoA (Fig 5.2) and AHC (Fig 5.3), of the fragment profiles enabled

comparative evaluation of the genetic structures of bacterial communities in the hindguts of *C. formosanus* workers fed different lignocellulose diets. Unconstrained PCoA and AHC analyses were first performed on all the profiles obtained for *C. formosanus* workers fed different lignocellulose diets for different feeding periods (Figs 5.2a and 5.3a). Overall, the obtained fractional data suggested that the bacterial community profiles in the hindgut of *C. formosanus* workers changed markedly depending on both the composition of the lignocellulose diets and the feeding period.

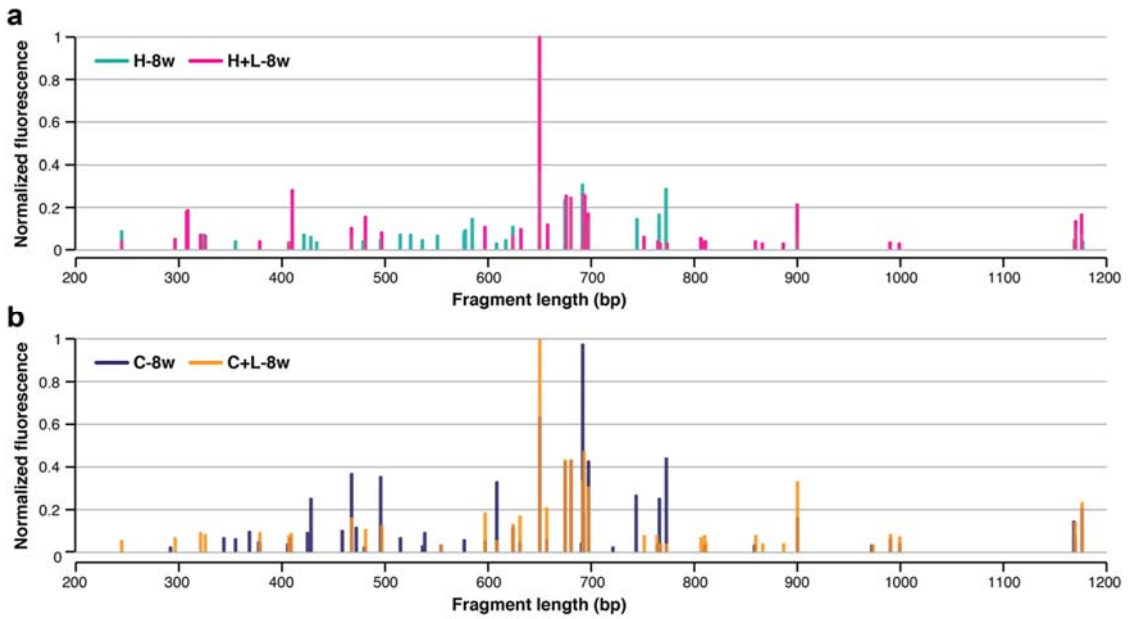

Fig. 5.1. Representative ARISA profiles of the bacterial communities in the hindgut of *C. formosanus* workers fed different lignocellulose diets. Profiles obtained for the workers fed cellulose only (C-8w) and cellulose + lignin (C+L-8w) (a), and workers fed holocellulose only (H-8w) and holocellulose + lignin (H+L-8w) (b) at 8 weeks of feeding. Reported fluorescence intensities of each fragment are means of three independent analyses using 5 workers per run.

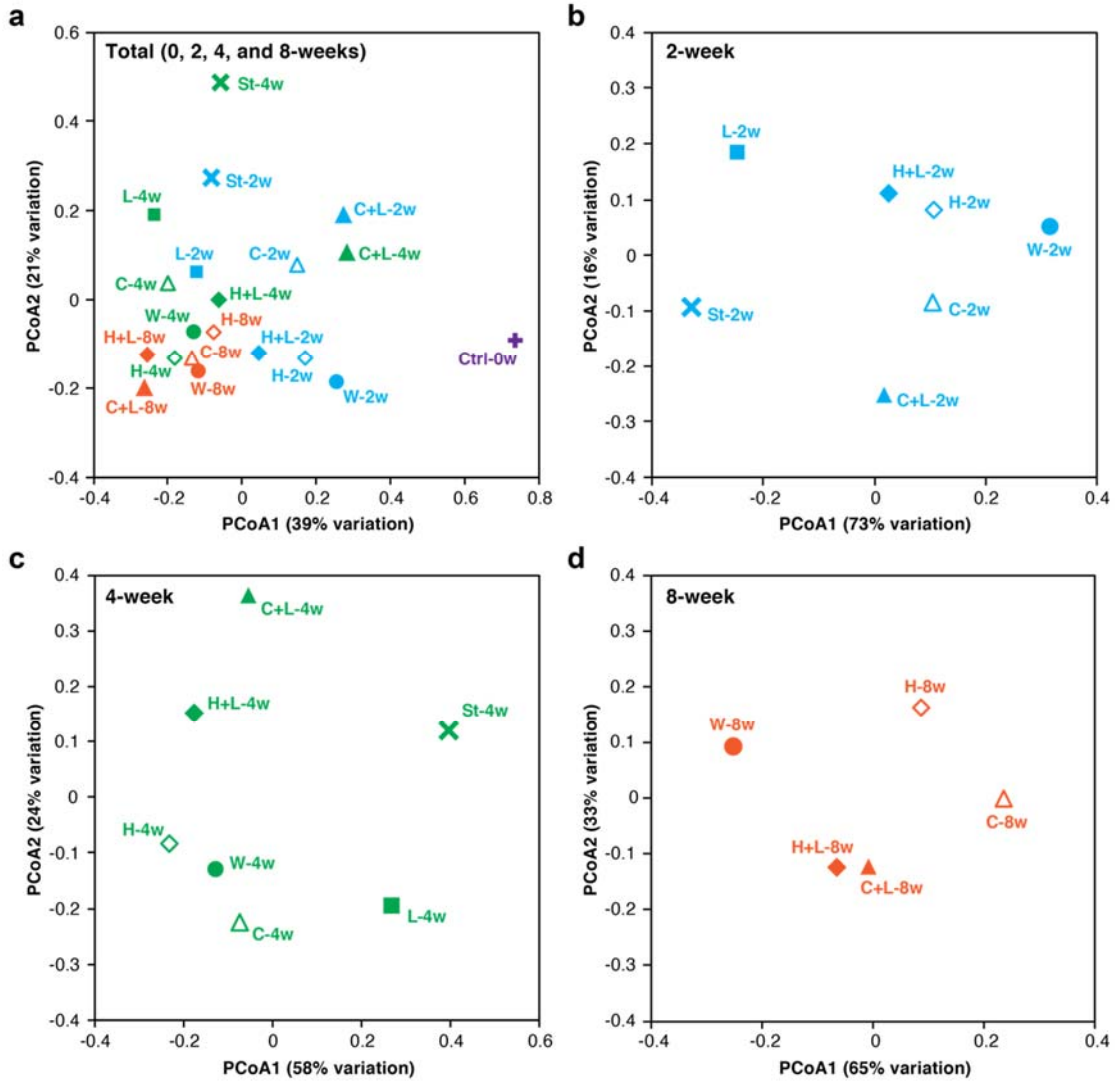

Fig. 5.2. Principal coordinate analysis (PCoA) of ARISA profiles of the bacterial communities in the hindgut of *C. formosanus* workers fed different lignocellulose diets. (a) Comparison between all the workers after 2, 4, and 8 weeks of feeding and control workers before feeding (0 week). (b-d) Comparisons only among the workers at each feeding period: 2 (b), 4 (c), and 8 (d) weeks. W, wood powder; H, holocellulose; C, cellulose; H+L, holocellulose + lignin; C+L, cellulose + lignin; L, lignin; St, starvation control; Ctrl, control workers before feeding. Gut DNA samples were not available for starvation controls (St) and workers fed on only lignin (L) at 8 weeks because no workers had survived.

To determine the differences between the hindgut bacterial pools of workers fed different lignocellulose diets, PCoA and AHC analyses were performed independently at each feeding period (Figs 5.2b-d and 5.3b-d). At 2 and 4 weeks of feeding, the bacterial community profiles of the workers fed on lignin-only (L) and starvation control (St) diets formed a distinct cluster apart from the profiles of all the other workers fed polysaccharide-containing diets, i.e., natural wood powder (W), and artificial holocellulose (H), holocellulose with lignin (H+L), cellulose (C), and cellulose with lignin (C+L) diets (Figs 5.2b,c and 5.3b,c). By PCoA, the bacterial profiles of L and St diet conditions were clearly separated from the profiles of all the other polysaccharide-containing diet conditions on the first coordinates (PCoA1), which accounted for the relatively large portions of the data variance at both 2 weeks (73%) and 4 weeks (58%) of feeding (Fig 5.2 b and c). Among the workers fed diets containing polysaccharides, the profiles of workers fed with lignins (H+L and C+L) were separated from the profiles of workers fed without lignins (H and C) mainly on the second coordinates (PCoA2), accounting for relatively small portions of the data variance (16% and 24% at 2 and 4 weeks, respectively). These results collectively suggest that lignin has less impact on the hindgut bacterial pools compared to the other polysaccharide components in the lignocellulose diets, at least for 4 weeks of feeding.

At 8 weeks of feeding, as observed in the previous feeding experiments described in Chapters 2 and 4, no worker survived under L and St diet conditions. Meanwhile, the fractional analyses on the ARISA profiles obtained for workers fed artificial polysaccharide diets with and without lignin detected notable differences between the diets with and without lignin (Figs 5.2d and 5.3d). The PCoA analysis clearly separates the profiles of diets with lignin (H+L and C+L) from those of the diets without lignin (H

and L) on both the first PCoA1 and PCoA2 coordinates, collectively accounting for 98% of data variance (65% and 33% on PCoA1 and PCoA2, respectively). In accordance with PCoA data, AHC analysis determined that the ARISA profiles obtained for workers fed H+L and C+L diets formed a distinct cluster apart from the cluster of workers fed H and C diets. It was also notable that the profiles of H+L and C+L diets further form a secondary cluster with the profile of wood powder diet (W) yet apart from the cluster of H and C diet profiles. These ARISA data suggest that the presence of lignin in lignocellulose diets has a pronounced effect on the hindgut bacterial pools of *C. formosanus* at 8 weeks of feeding.

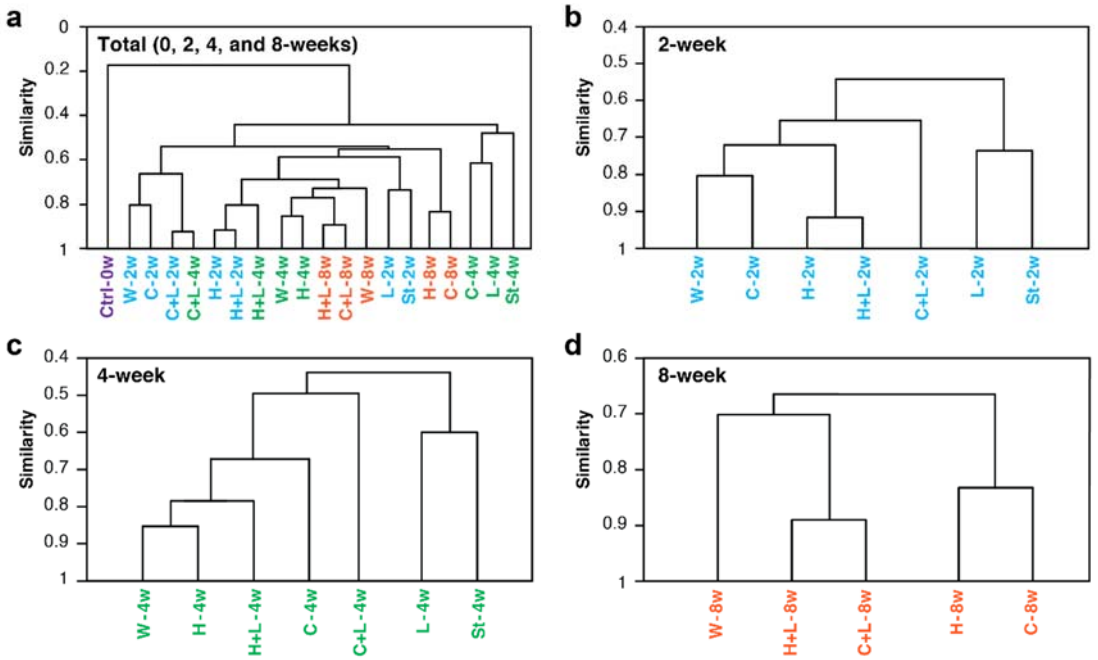

Fig. 5.3. Agglomerative hierarchical clustering (AHC) of ARISA profiles of the bacterial communities in the hindgut of *C. formosanus* workers fed different lignocellulose diets. (a) Comparison between all the workers after 2, 4, and 8 weeks of feeding and control workers before feeding (0 week). (b-d) Comparisons only among the workers at each feeding time: 2 (b), 4 (c), and 8 (d) weeks. W, wood powder; H, holocellulose; C, cellulose; H+L, holocellulose + lignin; C+L, cellulose + lignin; L, lignin; St, starvation control; Ctrl, control workers before feeding. Gut DNA samples were not available for starvation controls (St) and workers fed on only lignin (L) at 8 weeks because no workers had survived.

5.4. Discussion

Previously, Tanaka et al. (2006) applied a PCR-denaturing gradient gel electrophoresis (DGGE) approach to detect changes in the gut bacterial community of *C. formosanus* workers upon feeding with various artificial carbohydrate diets. This study was the first to attempt to determine the effects of lignin in lignocellulose diet on the bacterial communities in the termite gut digestive systems using the ARISA microbial community profiling technique. Although the ARISA pattern generally cannot be used to detect the taxonomic composition of the microbial community, as large numbers of unrelated populations overlap in each detected fragment (Ranjard et al., 2001), the method has been shown to be highly sensitive and robust while being relatively repeatable and unbiased compared to other polymorphism techniques based on PCR-derived DNA fragments (Cardinale, et al., 2004; Danovaro, et al., 2006; Geib, et al., 2009).

The ARISA community profiling data obtained in this study suggested that lignocellulose diet components as well as feeding period considerably affect the bacterial pools in the hindgut of *C. formosanus* workers. The community profiles obtained for workers fed only lignin displayed apparently high similarities to the profiles of starvation control workers (Figs 5.2a-c and 5.3a-c), suggesting that lignin has little effect on the gut bacterial pools when served as a sole food. The result is in line with the author's previous observations that lignin when served alone has no nutritional effects upon *C. formosanus* workers (Chapter 2). However, the fractional analysis on ARISA profiling data obtained for workers fed polysaccharide diets with and without lignins detected notable differences between the samples: addition of lignin to both holocellulose and cellulose diets induced similar profile changes in the gut bacterial pools at 8 weeks of feeding, while such effects of lignin were not so conspicuous at 2 and 4 weeks of feeding (Figs 5.2b-d and 5.3b-d).

Furthermore, it was suggested that ARISA gut bacterial profiling data obtained for workers fed polysaccharide diets with lignin were more similar to those obtained for workers fed natural wood diets rather than to those for workers fed only polysaccharide diets without lignin. These results are also in a good agreement with the author's previous observations of gut protists, in which the presence of two major protists, i.e., *P. grassii* and *H. hartmanni*, were significantly higher in the hindguts of *C. formosanus* workers fed both artificial lignin-containing polysaccharide diets and natural lignocellulose diets than those in the hindguts of workers fed only polysaccharide diets without lignins (Tharmadi et al., 2017b). Numerous prokaryotic microbes are known to inhabit the surface or the interior of protist cells (Gast et al., 2009; Hongoh, 2011; Ni and Tokuda, 2013; Brune, 2014). Given that the bacterial pools in the workers fed only lignin and starved workers were apparently similar, the bacterial profile changes upon lignin feeding observed in this study might be associated mainly with prevention of the loss of the major gut protists without lignin, although lignin may somehow directly affect the populations of the gut bacterial community. Importantly, the author has demonstrated that addition of lignin to polysaccharide diets promotes the physiological activities of *C. formosanus* workers (Chapter 4). Taken together, lignin may play an important role in lignocellulose digestion in the termite digestive system by maintaining the gut microbial communities that critically support polysaccharide digestion for assimilation and energy.

5.5. Chapter Summary

The ARISA community fingerprinting method has been successfully applied to detect changes in bacterial community profiles in the hindgut of *C. formosanus* workers fed different diet components. The results suggested that lignin when served as a sole

food had apparently little effect on the bacterial pools in the hindgut of *C. formosanus* workers. However, fractional analysis on ARISA profiling data obtained for workers fed polysaccharide diets with and without lignins suggested that lignin when served with polysaccharides has a marked effect on the bacterial pools in the hindgut of *C. formosanus* workers. Taken together with the author's previous findings, the results obtained in this chapter support the view that lignin plays an important role in maintaining gut microbial communities during lignocellulose digestion in lower termites' digestive systems.

General Conclusions

The role of lignins in termite physiology has been successfully explained in this dissertation. Chapter 1 investigated lignocelluloses deconstructions by the lower termite *C. formosanus* Shiraki, with emphasis on the deconstruction of lignin polymers. The results support the view that lignin polymers are at least partially decomposed during their passage through the termite gut digestive system, although polysaccharide decomposition clearly dominates the overall lignocellulose deconstruction process and the majority of lignin polymers remain intact in the digestive residues. High-resolution NMR structural data suggested preferential removal of syringyl aromatic units in hardwood lignins, but non-acylated guaiacyl units as well as tricin end-units in grass lignins. In addition, the data suggest that termites and/or their gut symbionts may favor degradation of C–C-bonded β–β and β–5 lignin inter-monomeric units over degradation of ether-bonded β–*O*–4 units.

Results from Chapter 2 showed that based on NMR spectroscopy, the three kinds of lignin polymers (milled-wood lignins, MWLs) display the characteristics of the three major lignin types in nature. The MWL from Japanese cedar is a typical softwood lignin composed of only G units, whereas the MWLs from the hardwood Japanese beech and grass rice are mixtures of G and S units. As a typical lignin in grasses, the spectrum of rice MWL displayed more intense signals from H units (**H**) than those in the spectra of the Japanese cedar and Japanese beech MWLs, and the rice MWL also displayed clear signals from *p*-coumarate (***p*CA**) and tricin (**T**) units.

The three purified MWLs, regardless of their structural differences, have no negative effect on termites' survival or body mass or the survival of all the three protists

(*P. grassii*, *H. hartmanni*, and *S. leidyi*) in their hindguts. In addition, the three MWL diets resulted in much lower termite survival compared to starvation conditions. Those results suggested that lignins are hardly utilized as a nutrient source by *C. formosanus* workers and are even rather detrimental to termites when used as a sole food source.

In Chapter 3, the effects of various lignocellulose and lignins on termites' production of hydrogen (H_2) and methane (CH_4) were investigated. H_2 and CH_4 emission rates by workers fed the three MWLs were similar overall to those of starved workers, suggesting that the microbe community profiles in the starved termites and termites fed the three MWLs were similar. The results well support the previous observation that lignins are hardly utilized as a nutrient source by *C. formosanus* as reported in Chapter 2.

In Chapter 4, the exact role of lignin in termites was examined. Lignins, regardless of their source (softwood, hardwood, and grass), have markedly positive effects on the survival of *C. formosanus* workers as well as the maintenance of the major protists *P. grassii* and *H. hartmanni* in their hindguts when fed with polysaccharide diets. The results strongly suggested that the presence of lignins in lignocelluloses is crucial to maintaining the physiological activities of *C. formosanus* workers. The data also suggested that some components in grass lignocellulose, possibly minerals and/or non-structural carbohydrates, negatively affect the survival of *C. formosanus* workers as well as the present rate of the symbiotic protists in their hindguts.

In Chapter 5, the effect of lignin on bacterial community profiles was observed. The results suggested that lignin when served as a sole food had apparently little effect on the bacterial pools in the hindgut of *C. formosanus* workers. However, fractional analysis on ARISA profiling data obtained for workers fed on polysaccharide diets with and without lignins suggested that lignin when served with polysaccharides give marked effect on the

bacterial pools in the hindgut of *C. formosanus* workers. Taken together with the author's previous findings, the results obtained in this chapter support the view that lignin takes an important role to maintain the gut microbial communities during the lignocellulose digestions in lower termites' digestive system.

Overall, the results support the view that lignin polymers are partially decomposed during their passage through the termite gut digestive system. It was also found that lignins give marked positive effects on the survival of *C. formosanus* workers as well as on their maintenance of hindgut protists when served with polysaccharide diets. Furthermore, it was suggested that the gut bacterial community profiles are also affected by dietary lignins. This study has provided evidence that the presence of lignin is crucial to maintaining the physiological activities and a wholesome hindgut digestive system of *C. formosanus* workers for their efficient lignocellulose decomposition.

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List of publications

Original articles

1. Didi Tarmadi, Yuki Tobimatsu, Masaomi Yamamura, Takuji Miyamoto, Yasuyuki Miyagawa, Toshiaki Umezawa, Tsuyoshi Yoshimura. NMR studies on lignocellulose deconstructions in the digestive system of the lower termite *Coptotermes formosanus* Shiraki. Sci Rep 8: 1290.
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