# Disruption of *Coptotermes formosanus* (Isoptera: Rhinotermitidae) Gut Microbiota Reproduction and its Consequences for the Cellulolytic System[1]

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**Abstract** In this study, we eliminated the microbiota of the gut of *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) via starvation. We then compared the initial responses of the cellulase system and symbiotic reproduction in termites fed filter paper treated with either antimicrobials or distilled water. Three key findings were as follows: (a) an antibiotic mixture of ampicillin and kanamycin significantly depressed reproduction of *Holomastigotoides mirabile* Koidzumi and *Spirotrichonympha leidyi* Koidzumi but promoted reproduction of *Pseudotrichonympha grassii* Koidzumi during 12 to 48 h of feeding, (b) the enzyme activity levels of three cellulases did not correspond to the respective protozoan levels and did not vary directly with the numeric increase of protozoa in either treatment, and (c) in the antibiotics treatment, β-glucosidase (BG, 0–48 h) and cellobiohydrolase (CBH, 0–12 h) activity levels between endogenous (salivary gland/foregut and midgut) and exogenous (hindgut) origin were negatively correlated. Both the activity level (6–48 h) and expression amounts (6–12 h) of endogenous endo-β-1, 4-glucanase was significantly higher in antibiotics treatment. Before initiation of feeding (0 h), BG and CBH activities had relative maximum levels when the protozoa population was at its minimum. These findings suggest that *C. formosanus* possesses an endogenous compensation mechanism which functions when the symbiotic cellulolytic system is impaired.

**Key Words** antibiotics, cellulose, lower termite, protozoa, starvation

The lower wood-feeding termite *Coptotermes formosanus* Shiraki (Rhinotermitidae) is a notorious pest that is economically important worldwide. It has large colonies, the capacity to penetrate a variety of materials, and the ability to consume a variety of woods (Shinzato et al. 2005, Vargo et al. 2003).

*Coptotermes formosanus* has an extremely effective cellulose hydrolysis system (Tokuda et al. 2004) because of two lignocellulolitic systems: one is located in the foregut/salivary glands and midgut where cellulose is digested by endogenous cellulases, and the other is located in the hindgut where symbiotic fauna provide exogenous cellulases (Nakashima et al. 2002, Xie et al. 2012). In a narrow sense, the typical cellulases of *C. formosanus* are endo-β-1, 4-glucanase (EG; EC. 3. 2. 1.4 ); exo-β-1, 4-cellobiohydrolase (CBH; EC. 3.2.1.91); and β-glucosidase (BG;

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EC. 3.2.1.21) (Arakawa et al. 2009, Willis et al. 2010). Although termites themselves can produce a battery of cellulases, hemicellulases, and lignases that contribute significantly to lignocellulose digestion (Scharf et al. 2011, Tartar et al. 2009, Zhou et al. 2007), the large numbers of flagellated protists densely packed in their hindguts are considered to play an irreplaceable role in cellulose digestion by lower termites (Ohkuma et al. 2000).

Three species of protists identified in *C. formosanus* are *Pseudotrichonympha grassii* Koidzumi, *Holomastigotoides mirabile* Koidzumi, and *Spirotrichonympha leidyi* Koidzumi (Ohkuma 2003, Tanaka et al. 2006,). In addition to these eukaryotic symbiota, numerous prokaryotic microbes (bacteria and archaea) either attach to the surfaces of ectosymbionts or live within the protist cells as endosymbionts in an intimate relationship with the protist. Symbiosis in termite guts is not simple tripartite (termite, protest, and prokaryote) but more complicated, involving multiple relationships comprising a variety of associations between protists and prokaryotes (Ohkuma 2008, Scharf et al. 2011).

In recent years, antibiotics have been used to compromise and/or eradicate the gut microbiota of termites and study the symbiotic functions related to nutrition, lignocellulose decomposition, reproduction, survival, hydrogen production, and fecundity (Cao et al. 2012, Peterson et al. 2015, Rosengaus et al. 2011, Tokuda and Watanabe 2007). However, previous studies have seldom involved responses of the cellulolytic system to the resumption of feeding by starved termites. Based on our previous studies, we were able to eliminate more than 90% of microbiota in *C. formosanus* via natural starvation. Then we compared the initial responses of the termite cellulase system to symbiont reproduction in antimicrobial-treated and control groups.

## Materials and Methods

**Termites and antibiotic treatments.** *Coptotermes formosanus* workers used in this study were taken from three laboratory-maintained colonies. Ampicillin and kanamycin were antimicrobials selected for use in the study because they are broad-spectrum compounds active against a variety of gram-positive and gram-negative alimentary canal organisms, respectively (Acred et al. 1962, Cao et al. 2012, Hu et al. 2005, Peterson et al. 2015). Approximately 400 termites were supplied with only distilled water and moist vermiculite; they were starved for 3 d at 28°C and 60 ± 5% relative humidity to allow for natural elimination of more than 90% of the gut microbiota. The starved termites were separated into two treatments: one treatment was fed on filter paper soaked with ampicillin (2 mg/ml) and kanamycin (1 mg/ml) and the other fed on filter paper soaked in distilled water ($dH_2O$). For each of the two treatments, 40 termites were placed into separate 9-cm-diameter petri dishes, which were maintained in incubators for 6, 12, 24, and 48 h at 28°C and 60 ± 5% relative humidity, respectively. There were three replicates per treatment.

**Counting and microscopic examination of protozoa.** To estimate protozoa abundance, from each replicate of the two treatments, eight hindguts of worker termites were pulled from the posterior ends of the workers and torn into pieces by forceps. The contents were suspended in 20 μl of 20 mM phosphate buffer (pH 7.4)

per termite and macerated gently to facilitate the release of the protozoa. Then, protozoa cells were counted and identified using a light microscope (Nikon Elipse 80I, Tokyo, Japan).

**Cellulase activity assays.** Extracts of crude cellulases from termite intestines were prepared according to Nakashima et al. (2002) with minor modifications. Briefly, 20 termites collected randomly from each set were immobilized on ice; dissected to separate salivary gland, foregut, and midgut from the hindgut; homogenized in ice-cold sodium acetate buffer (0.1 M sodium acetate, pH 5.6), and then centrifuged at 12,000 $\times$ *g* at 4°C for 15 min. The resulting supernatants were transferred and used as crude enzyme for measuring cellulase activity. EG and BG activity were assayed by measuring the release of reducing sugars from 1% carboxymethyl cellulose sodium (CMC) and 1% salicine, for the respective enzymes, using the dinitrosalicylic acid method. Glucose production was detected colorimetrically with a Victor 3 Multi-label Microplate Reader (Perkin Elmer, Singapore) at 540 nm using glucose as the standard (Eveleigh et al. 2009, Inoue et al. 1997). CBH activity was assayed by measuring the release of *p*-nitrophenol (pNP) from 1 mM *p*-nitrophenyl-β-D-cellobioside (pNPC) and the formation of pNP was detected colorimetrically at 405 nm (Zhou et al. 2007). The reaction mixture consisted of 12 μl of crude enzyme and 120 μl of 1% CMC, 1% salicine, or 1% pNPC were incubated at 37°C for 60 min.

Protein concentrations were determined by the Coomassie brilliant blue G-250 method (Lott et al. 1983) using bovine serum albumin as a standard. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 mg reducing sugar/μmol pNP per minute under the experimental conditions described above. Each replicate was performed in triplicate.

**Total RNA isolation, reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR).** An endogenous salivary gland *eg* gene (Genbank: EU853671.1) and an exogenous *P. grassii cbh* gene (GenBank: AB071864) of *C. formosanus* were used to analyze variations of relative transcription levels. Total RNA and total cDNA of the whole termite body were obtained by using a Total Isolation System Kit (Promega Corp., Madison, WI) and the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Shiga, Japan), respectively. Equal quantities of RNA (1.2 μg) were used as a template for cDNA synthesis. qRT-PCR was performed with Luminaris HiGreen Fluorescein qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA), according to manufacturer instructions. Relative expression levels for specific genes were normalized to the reference gene heat shock 70-kDa protein (HSP-70) (Zhou et al. 2006) and determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The suitability of the reference control gene was evaluated with BESTKEEPER version 1.0 (Pfaffl et al. 2004). Primer sets showing 5′ to 3′, were as follows, *eg* (forward, CTGCCATCGCCTACAAGAGTGCT; reverse, GTGTTGTCGTTGGTCGCCCTGTA), *cbh* (forward, AAGGACGGATGC GATTTCAACTCA; reverse, TCGCCTTGAAGTCATTGGTGTCTC), and *hsp-70* (forward, GAAGACAAGGTGAAGGC; reverse, TGCCGTGGATTGACTCTAGC). Each replicate was performed in triplicate.

**Statistical analysis.** The differences in cellulase activities and intestinal distribution ratios, qPCR data, and numbers of protozoa between the two groups were analyzed using the independent-samples *t* test with SPSS 17.0 (SPSS Inc., Chicago, IL) for Windows software.

## Results

**Effect of antimicrobial treatment on reproduction of intestinal protozoa.** The effects of antimicrobial treatment on protozoan reproduction (flagellates *P. grassi*, *H. mirabile*, and *S. leidyi*) were investigated. Numbers of the three species in the hindguts of *C. formosanus* (workers) in the antibiotic or control $dH_2O$ treatments at 0, 6, 12, 24, 48 h are shown in Fig. 1. In lower termites, the total protist count was estimated to be $10^3$–$10^5$cells per gut (Yoshimura et al. 1995). Mean ($\pm$) numbers intestinal protozoan numbers were severely depleted after 3 d of starvation (11 $\pm$ 3, 48 $\pm$ 5, and 117 $\pm$ 18 cells per gut for *P. grassi*, *H. mirabile*, and *S. leidyi*, respectively).

Numbers of the three flagellate species in the $dH_2O$ and antibiotic treatments increased with an increase of feeding time. During 6–12 h of feeding, *H. mirabile* and *S. leidyi* were the major species in both groups. The numbers of *H. mirabile* and *S. leidyi* were greater in the $dH_2O$ treatment than in the antibiotic treatment from 6–48 h. The numbers of *H. mirabile* and *S. leidyi* in the $dH_2O$ treatment were significantly greater than in the antibiotic treatment at 6–24 h, and 6–48 h, respectively. During 12–48 h, the number of *P. grassi* in the $dH_2O$ treatment increased significantly more slowly than in the antibiotic treatment. The number of *P. grassi* in $dH_2O$ was only significantly greater than in the antibiotic treatment at 6 h. These results suggest that the antibiotic treatment inhibited reproduction of *H. mirabile* and *S. leidyi* but enhanced reproduction of *P. grassi*.

**Effect of antimicrobial treatment on cellulase activities and intestinal distribution patterns.** Cellulase activity assays mapped the distribution of endogenous and symbiotic cellulases across the *C. formosanus* digestive tract. The activities of the foregut/salivary gland and the midgut (abbreviated as EG-FM, CBH-FM, and BG-FM), and the hindgut (abbreviated as EG-H, CBH-H, and BG-H) indicated the relative activities of endogenous and symbiont-originated cellulases, respectively. Intestinal distribution ratios [(foregut/salivary gland and midgut)/hindgut)] of activities of cellulases were measured to examine variations in the proportions of endogenous and exogenous cellulases.

Throughout the total feeding interval, EG activities in both treatment groups were mainly contributed by hindgut symbionts. The treatments showed the same trend of EG intestinal distribution ratios (Fig. 2C). EG-FM and EG-H in the antibiotic treatment were both higher than in the $dH_2O$ treatment in general. EG-FM levels in the antibiotic treatment were significantly greater than in the $dH_2O$ after 6–48 h of feeding. EG-H levels of the antibiotic treatment were significantly higher than those of $dH_2O$ after 6–24 h of feeding. The increasing amplitude of EG-FM was larger than that of EG-H at most of sampling times, which led to significant fluctuation in intestinal distribution ratios of EG activity after 6–24 h of feeding (Fig. 2).

Most BG activity in both treatments was contributed by the foregut/salivary gland and midgut. The treatments had similar trends in the intestinal distribution ratios of EG (Fig. 3C). The divergence in BG-FM between the two treatments was much smaller than that shown by EG-H. Statistical differences between treatments for BG-FM were only seen at 12 h. For BG-H, differences between the treatments were significant at all sample times. BG-FM varied inversely with BG-H in both treatments

in general; this relationship was more obvious in the antibiotic treatment due to greater BG-H fluctuations (Fig. 3).

CBH activity, in both treatments at all sample times, occurred mainly in the hindgut. The intestinal distribution ratios of CBH, between the treatments, varied inversely and the differences were highly significant at all sample times (Fig. 4C). CBH-FM in the $dH_2O$ treatment was significantly higher and lower than in the antibiotic treatment at 6 and 12 h, respectively. CBH-H in the $dH_2O$ treatment was significantly lower than in antibiotic treatment at 6 and 24 h. CBH-FM varied inversely with CBH-H in both treatments during 0–12 h, and this relationship was more obvious in the antibiotic treatment (Fig. 4).

**Effect of antimicrobial treatment on expression of the cellulase genes.** We correlated two different cellulase expressions from different organisms with specific cellulolitic activities using the relative transcript levels of two genes (endogenous salivary glande *eg* and symbiotic *P. grassi cbh*) measured by qRT-PCR. Termites from the $dH_2O$ treatment were used as controls to gene expression. The housekeeping gene *hsp-70* was evaluated with BESTKEEPER version 1.0 and was adequately stable to use as reference control gene in this study (SD [± crossing point] = 0.55, coefficient of variation [% crossing point] = 2.67).

Endogenous *eg* in the antibiotic treatment had a significantly greater transcriptional amount than the control group at 6 ($t = 7.58$, $P = 1.63E-03$) and 12 h ($t = 11.78$, $P = 2.97E-04$) and a significantly lower transcriptional amount than the control group at 24 ($t = 47.72$, $P = 1.15E-06$ ) and 48 h ($t = 21.92$, $P = 2.56E-05$). Exogenous *P. grassi cbh* in the antibiotic treatment exhibited a significantly lower transcriptional amount than the control group at 6 ($t = 27.28$, $P = 9.98E-06$), 12 ($t = 30.41$, $P = 6.96E-06$), and 24 h ($t = 32.19$, $P = 5.56E-06$), and no significant difference after 48 h of antibiotic treatment ($t = 1.4$, $P = 0.23$) (Fig. 5).

## Discussion

Cellulose digestion in lower termites occurs through the synergistic combination of the separate endogenous and symbiotic cellulolytic systems (Nakashima et al. 2002). Intestinal symbionts play important roles in cellulose digestion and host nutrition (Ohkuma 2008). In this study, we eliminated the gut microbiota of *C. formosanus* by starvation. Then, we fed starved termites on either antibiotic-treated cellulose or $dH_2O$-treated filter paper and characterized effects on protozoan resumption and dual cellulolitic activity.

The antibiotic mixture significantly depressed the reproduction of *H. hartmanni* and *S. leidy* in the 6–48-h feeding treatments in general but stimulated reproduction of *P. grassi* during 12–48-h feeding (Figs. 1). Protist-associated bacteria can support the growth and activity of the host protists by supplying essential nutrients such as amino acids and cofactors (Hongoh et al. 2008, Ohkuma 2008). Ampicillin/kanamycin-treated diets can negatively affect the protist ectosymbiotic spirochetes of *C. formosanus* and *Reticulitermes flavipes* Kollar and significantly reduce their populations (Cao et al. 2012, Peterson et al. 2015).This may explain why reproduction of *H. hartmanni* and *S. leidy* was significantly reduced in the antibiotic

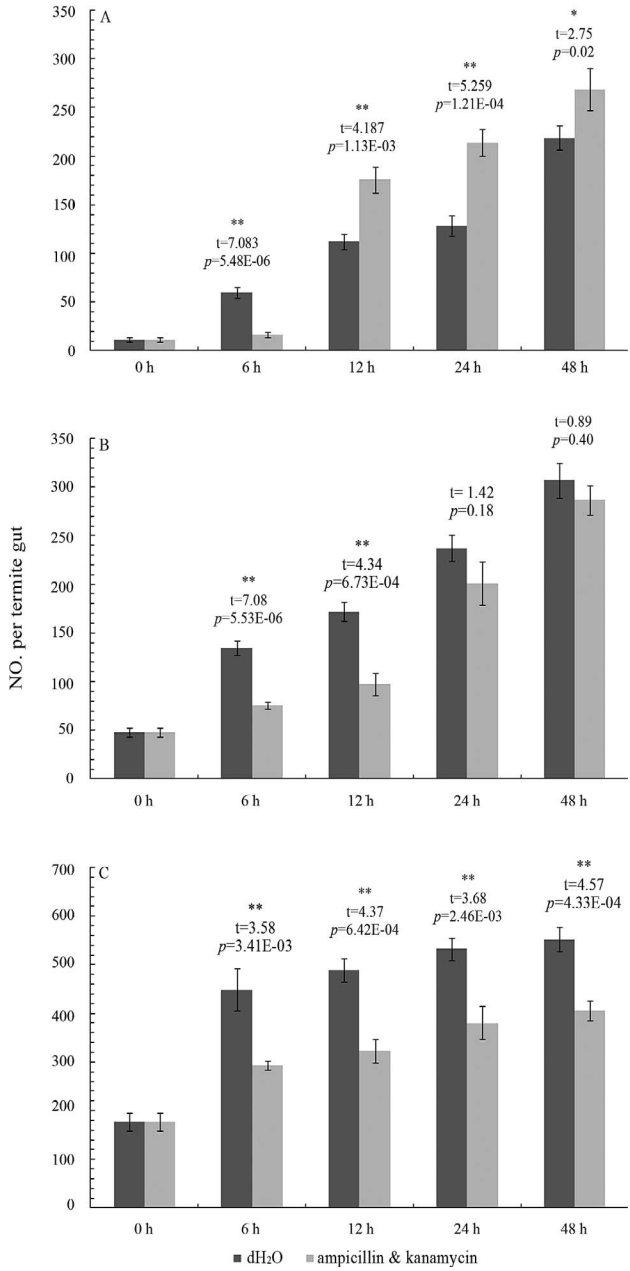


**Fig. 1. The number of *Pseudotrichonympha. grassii* (A), *Holomastigotoides mirabile* (B) and *Spirotrichonympha leidyi* (C) count per termite gut of Coptotermes formosanus workers after feeding on control diet treated with distilled water or diet treated with ampicillin & kanamycin**

treatment. However, 70–80% of the endosymbiotic bacteria in *C. formosanus* occur within cells of *P. grassii* and belong to the order Bacteroidales (Noda et al. 2005, Ohkuma 2008). The Bacteroidales inhabiting *P. grassii* could survive the ampicillin and tetracycline treatments (Cao et al. 2012). In *R. flavipes*, kanamycin can increase the abundance of Bacteroidetes clones (Peterson et al. 2015). In the present study, kanamycin may have increased Bacteroidetes populations in the antibiotic treatment, which was beneficial to *P. grassii* reproduction.

In traditional view, prokaryotic symbionts make no clear contribution to lignocellulose digestion (Cho et al. 2010, Ohkuma 2003, Nakashima et al. 2002, Warnecke et al. 2007). The hindgut activities of three cellulases here did not correspond to the respective protozoan levels and did not correspond to the numeric increase of protozoa in either treatment. Both EG-FM and EG-H in the antibiotic treatment were significantly greater than in the $dH_2O$ treatment from 6 to 48 h. Hindgut BG activity had significantly greater activity in the antibiotic treatment at 6 and 12 h, but activity was greater in the $dH_2O$ treatment at 24 and 48 h. At 6 and 24 h, CBH activity in the hindgut of the antibiotic treatment was significant greater than in the $dH_2O$ treatment. In a recent study, the expression of some candidate bacterial cellulases in the hindgut of *R. flavipes* increased when the spirochetes were eliminated by tetracycline and metronidazole feeding. This suggests that evacuation of the ectosymbiotic spirochetes may be beneficial to the nutrition of antimicrobial-tolerant prokaryotes with similar metabolic capabilities (Peterson et al. 2015). In addition, potential cellulitic roles for prokaryotes have been found in another lower termite, *Coptotermes gestroi* Shiraki (Do et al. 2014). Profiling of the metatranscriptome of lower termites revealed many cellulolitic bacteria hydrolases (Tartar et al. 2009, Xie et al. 2012). The inconsistency observed here between protozoa number and cellulose levels may have been influenced by an uncharacterized cellulolytic-related prokaryote.

In both treatments, BG (0–48 h) and CBH (0–12 h) activity levels between endogenous (salivary gland/foregut and midgut) and exogenous (hindgut) origin were negatively correlated in general. This relationship was more apparent in the antibiotic treatment due to the greater fluctuations in cellulase activity. The relative transcription amounts of endogenous salivary gland *eg* and protozoa-origin *cbh* were consistent with this finding. The *eg* had a significant transcription increase at 6 and 12 h while *cbh* had a significant transcription reduction at 6 and 12 h. The endogenous EG activity levels were also significantly higher in antibiotic treatments, especially at 6 and 12 h. Prior to feeding initiation (0 h), BG and CBH activities were maximized when the protozoa population was lowest. Symbiont-compromised *R. flavipes* also had stable expression of endogenous host cellulases and stable primarily host-derived BG activity (Peterson et al. 2015). Flows of endogenous EG from the midgut to the hindgut and refluxes of EG from the hindgut back into the midgut were not observed in previous studies on *C. formosanus* (Belitz and Waller 1998, Tokuda et al. 2007). We suggest that

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**at different hours. Error bars indicate standard error of the mean (SEM). Sampling time points with the * ($P < 0.05$) indicate the significant between two groups (independent samples *t* test).**

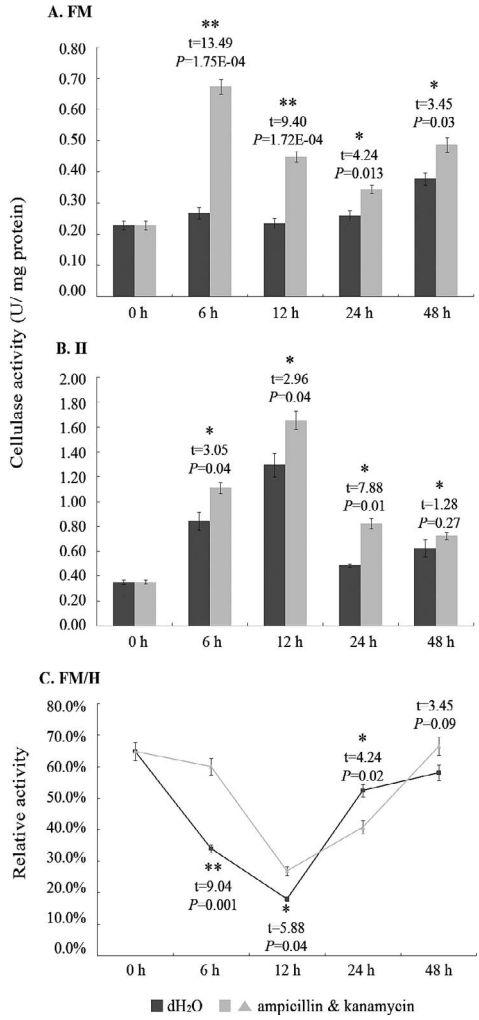


**Fig. 2. Endo-β-1, 4-glucanase activity (EG) in foregut/salivary gland and midgut (A) and hindgut (B) and its activity distribution ratios within the alimentary canal of *Coptotermes formosanus* workers after feeding on diet treated with dH2O and ampicillin & kanamicine at different hours. Error bars represent standard error of the mean (SEM). Sampling time points with the * ($P < 0.05$) and ** ($P < 0.01$) indicate the significance between two groups (independent samples *t* test). Abbreviations: FM, foregut/salivary gland and midgut; H, hindgut; FM/H, intestinal distribution ratio, [(foregut/salivary gland and midgut)/hindgut] of activities of EG.**

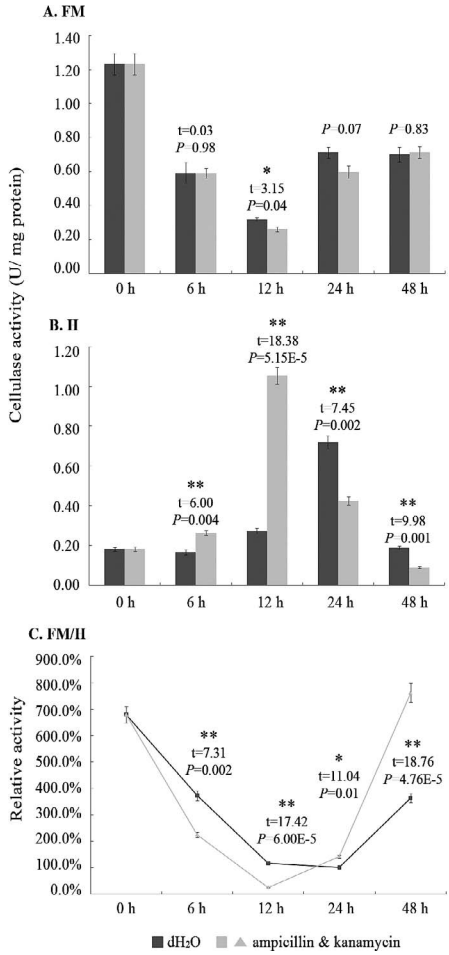


**Fig. 3. Beta-glucosidase activity (BG) in foregut/salivary gland and midgut (A) and hindgut (B) and its activity distribution ratios within the alimentary canal (C) of *Coptotermes formosanus* workers after feeding on diet treated with distilled water and ampicillin and kanamycin at different hours. Error bars represent standard error of the mean (SEM). Sampling time points with the * ($P < 0.05$) and ** ($P < 0.01$) indicate the significance of differences between two groups (independent-sample *t* test). Abbreviations: FM, foregut/ salivary gland and midgut; H, hindgut; FM/H, intestinal distribution ratio, [(foregut/salivary gland and midgut)/hindgut] of activities of BG.**

*C. formosanus* possesses an endogenous compensation mechanism, which occurs when the symbiotic cellulase system is impaired. However, the characteristics of the intestinal distribution ratio of the three cellulases were not altered by the increase in foregut/salivary gland and midgut activity. EG and CBH

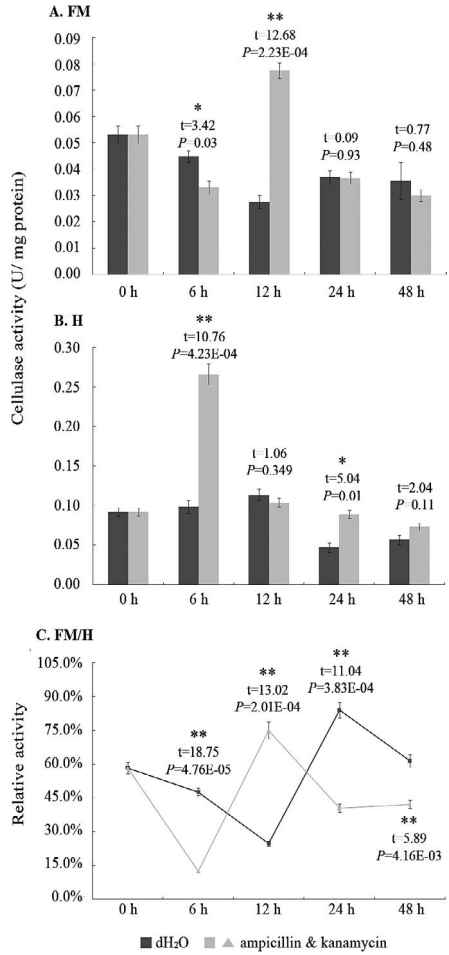


**Fig. 4. Exo-β-1, 4-cellobiohydrolase activity (CBH) in foregut/salivary gland and midgut (A) and hindgut (B) and its activity distribution ratios within the alimentary canal (C) of *Coptotermes formosanus* workers after feeding on diet treated with distilled water and ampicillin and kanamycin at different hours. Error bars represent standard error of the mean (SEM). Sampling time points with the * ($P < 0.05$) and ** ($P < 0.01$) indicate the significant between two groups (independent-samples *t* test). Abbreviations: FM, foregut/salivary gland and midgut; H, hindgut; FM/H, intestinal distribution ratio, [(foregut/salivary gland and midgut)/hindgut] of activities of CBH.**

activities were mainly due to exogenous enzymes from the hindgut. BG activity (except for the 12-h sample) was mostly found in the salivary gland/foregut and midgut. The increased activity of endogenous cellulases would have a metabolic limit, consistent with previous studies showing that the low rate of endogenous

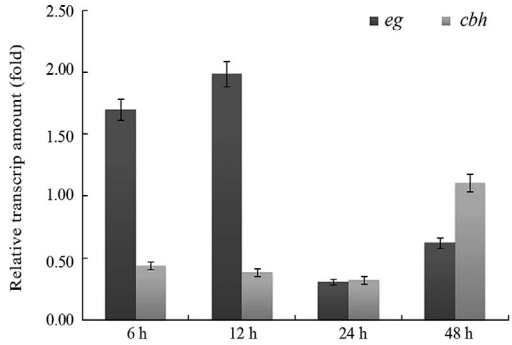


**Fig. 5. Relative transcript abundances of two cellulase genes determined by quantitative real-time polymerase chain reaction. Termites fed with distilled water–soaked filter paper were used as control, and the relative expression levels for specific genes were normalized to the reference gene *hsp-70*. Error bars represent standard error of the mean (SEM). The relative transcription amounts were analyzed using independent-samples *t* test. Abbreviations: *eg*, β-1, 4-glucanase gene; *cbh*, β-1, 4-cellobiohydrolase gene; *hsp-70*.**

glucose production in termites was insufficient to meet their metabolic needs (Nakashima et al. 2002). Additional research is needed to characterize the metabolic pathways associated with the dual cellulolitic systems of lower termites.

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