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# Influence of lignin in *Reticulitermes santonensis*: symbiotic interactions investigated through proteomics

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**Abstract** The gut of lower termites is populated by numerous microbial species belonging to prokaryotes, fungi, yeasts and protists. These micro-organisms are organized in a complex symbiotic system, interacting together and with the insect host. Their likely ability to degrade lignocellulosic compounds could lead to improvements in second generation biofuels production. Lignin elimination represents a critical point as this polymer significantly interferes with industrial process of cellulose. Although host produces its own lignin-degrading enzymes, some symbionts may participate in digestion of lignin and its degradation products in termite gut. Here, we compared gut proteomes from *R. santonensis* after rearing on artificial diets composed of cellulose with and without lignin. The effect of lignin in artificial diets on different parts of the digestive tract was compared through liquid chromatography associated with tandem mass spectrometry (LC-MS/MS) experiments. Enzymatic assays were performed to characterize activities present in *R. santonensis* digestive tract after feeding on artificial diets. Microscopic observations of microbial communities provided some information on population balances after feeding experiment.

**Keywords** Termites · Lignin · Proteomics

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## 1 Introduction

Cellulose is one of the most abundant and pertinent biopolymer on earth in regards with renewable biofuels, such as second generation bioethanol. Yet, industrial processes still not efficient enough to enable an important extend of this renewable energy. Although crystalline cellulose itself is considered as a recalcitrant material, many issues encountered in its transformation concern other lignocellulosic compounds, such as hemicelluloses and lignin (Dyer 2004). Indeed, lignin and hemicelluloses are known to protect cellulose from digestion. Though some Metazoan species possess their own enzymes active against lignocellulosic material (Tartar et al. 2009), its breakdown generally involve microbial species. Studies over symbiotic systems degrading lignocelluloses suggest that tasks may be specifically distributed among populations. Our insect model is the subterranean wood feeding lower termite *Reticulitermes santonensis* (Feytaud). Microbial community encountered in its gut includes mainly flagellates and

prokaryotes, but also filamentous fungi and yeasts (Inoue et al. 1997). Some of these micro-organisms are known or highly supposed to be digestive symbionts playing particular roles in degradation of one or several compounds of lignocelluloses (Breznak and Brune 1994). Lignin is, after cellulose, another abundant biopolymer on Earth (Ke et al. 2011), and improvements in its industrial breakdown would not only allow more efficient cellulose digestion but also provide a substantial energy source. Some lignin degradation products are toxic, and probably have negative effects on some symbiotic populations. We investigated the influence of a lignin-containing artificial diet along the digestive tract of *R. santonensis* using enzymatic activies assays. Metaproteomic approach was used to analyze the hindgut content of our termites.

## 2 Material and methods

### 2.1 Artificial feeding

Termites reared on pine tree and poplar were placed in tubes containing disks of sterile artificial diets (20 % carbon source; 1,5 % agar; 0,06 % β-sisterol and water). Carbon sources used in this study were microcrystalline cellulose (MCC or avicel, Sigma-Aldrich) and a mix of 3:1 (w/w) avicel and lignin (lignin alkali, Sigma-Aldrich). These artificial diets will be further named diets "A" and "A+L" respectively, when containing only MCC or MCC and lignin. In each tube, 80 workers, 10 nymphs, 10 soldiers and a few young larvae were placed to move closer to the natural conditions of the colony. Workers were used after 30 days of rearing in the dark.

### 2.2 Samples preparation

Termites were chilled on ice before being dissected. Digestive tracts of 10 termites were separated in three parts: foregut and salivary glands (G), midgut (M), and hindgut (H). Samples were all placed in 100 µl of 0,2 M sodium acetate buffer (pH 5,5) containing proteases inhibitors cocktail (Mini-Complete, Roche) and kept on ice during dissections. Hindgut samples were slightly torn and vortexed, and other samples were crushed with a manual pestle. After a short sedimentation of major fragments, supernatant of hindgut samples was removed to form hindgut content samples (Hc) in contrast with hindgut wall samples (Hw), before being both crushed. All samples for enzymatic assays were diluted with 200 µl of acetate buffer and sonicated. These diluted samples were used for enzymatic tests. To eliminate signal from reducing sugars present in samples, part of each sample was boiled 5 min to be used as blank.

For LC-MSMS analysis, Hc samples of 10 termites in 100 µl sodium acetate buffer were diluted with 700 µl UT buffer (6M urea, 2M thiourea, CHAPS 1 %) prior to sonication. Proteins were precipitated using trichloracetic acid (TCA) 20 % final concentration. After 30 min of incubation on ice, samples were centrifuged 5 min at 14,000×g and pellets were washed in acetone. After 4 sequences of 30 s of vortexing and 10 min incubation at −20 °C, samples were centrifuged as previously. Proteins were re-suspended in UT buffer and quantified using RCDC Protein assay kit (Bio-Rad).

### 2.3 Enzymatic assays

#### *2.3.1 Reducing sugars assay*

Samples (25 µl, in triplicate, 2 experimental replications) and blanks (25 µl) were added in tubes containing 1 mg of substrate, namely carboxymethyl cellulose (CMC, Sigma-Aldrich), avicel or xylan (xylan from beechwood, Sigma-Aldrich). Each tube was complemented by 200 µl of 0,2 M acetate buffer pH 5,5 and incubated for 2 h under agitation at 22 °C. Incubation was stopped by adding 300 µl of 3,5-dinitrosalicylic acid solution (Wood and Bhat 1988). After 5 min of boiling in water, samples and blanks were diluted with 1 ml of distilled water. Absorbance was read at 550 nm. Glucose standards were used for endo- and exocellulase activity measurement and xylose standards were used for xylanase activity measurement.

#### *2.3.2 Glucosidases assay*

Samples (10 µl, in triplicate, 2 experimental replications) and blanks (10 µl) were added in wells containing 90 µl of 5 mM p-nitrophenyl-α-glucopyranoside or p-nitrophenyl-β-glucopyranoside in 50 mM phosphate buffer pH 6,5 to measure respectively α- and β-glucosidase activities. After addition of 100 µl 50 mM phosphate buffer, microplates were incubated 60 min before reading absorbance values at 405 nm.

### 2.4 Microscopic observations

Hindguts of termites fed for 6 weeks on artificial diets were dissected, torn in a drop of solution U (Trager 1934), and observed under microscope until 400X magnification. For each diet of the 2 experimental replication, 6 termites were observed.

### 2.5 LC-MS/MS experiment

Sample was digested by trypsin as follows: sample volumes corresponding to 50 µg of proteins were precipitated as previously and re-suspended in 100 µl of $NH_4CO_3$ 50 mM. Reduction was carried out by adding 5 µl of dithiothreitol (DTT) 200 mM, $NH_4CO_3$ 100 mM and heating at 100 °C for 10 min. Then, 4 µl of alkylation solution (iodoacetamide 1M, $NH_4CO_3$ 100 mM) were added and sample was incubated 1 h in darkness. Alkylation reaction was stopped by adding 20 µl of reduction solution.

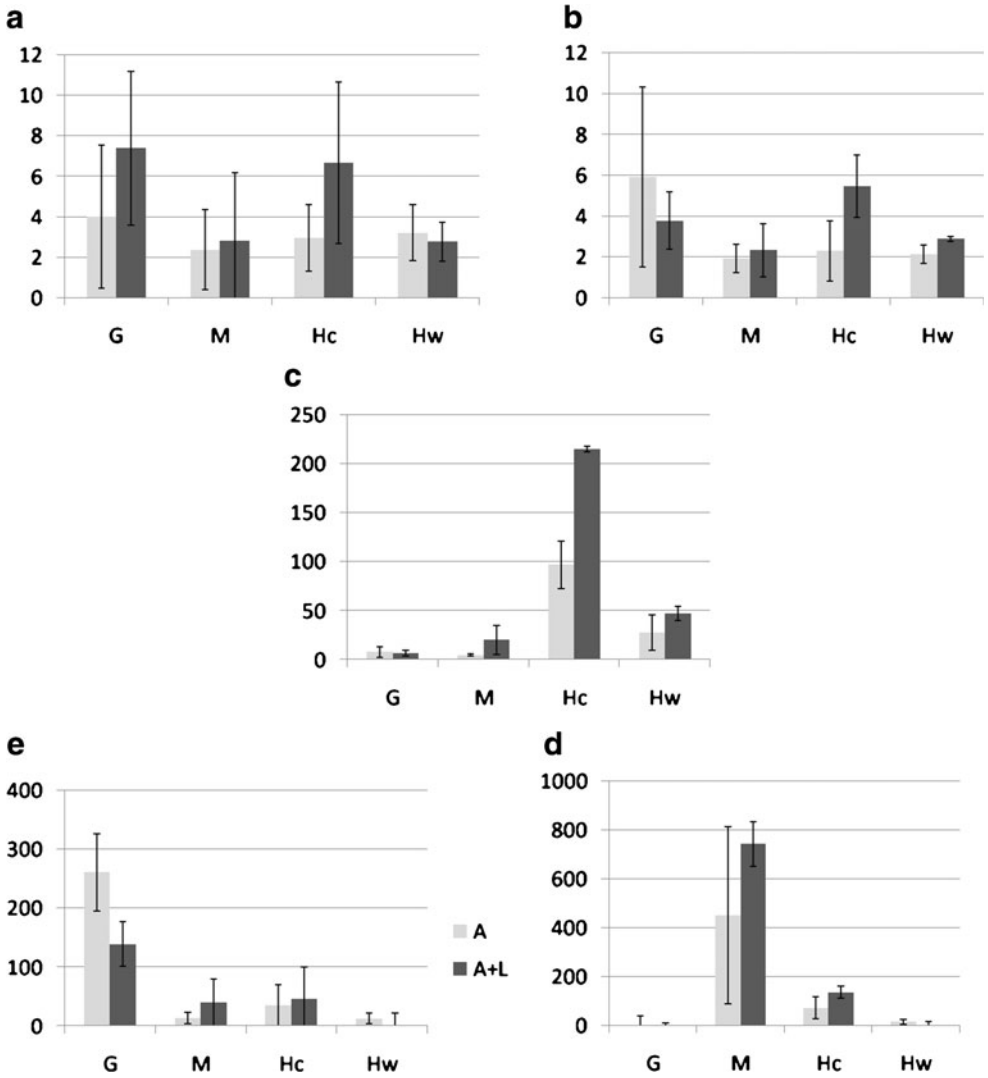


**Fig. 1** Enzymatic activities profiles along the digestive tract (salivary glands+foregut, SG; midgut, M; hindgut content, Hc; hindgut wall, Hw) against MCC (**a**), CMC (**b**), xylan (**c**), p-nitrophenyl-alpha-d-glucopyranoside (**d**) and p-nitrophenylbeta-dglucopyranoside (**e**). Enzymatic activities are represented in nmol of glucose-equivalent produced during 1 hour by μg of protein

Digestion by trypsin was performed for 18 h at 37 °C. Finally, sample was conserved in the fridge after evaporating the liquid from the tubes. Trypsic peptides were analyzed by 2D-LC-MS/MS (Bruker ion trap Esquire HCT) and searched using Mascot in NCBI non-redundant (NR) "all entries" database. Proteins matching at least 2 peptides (scores above 15) including one peptide with score above Mascot identity score were validated. To extract more information, all peptides scoring above 15, including redundant and unassigned ones, were blasted against NCBI NR "all entries" database. Results of theses BLASTs were treated with MEGAN 4 using default algorithm (lowest common ancestor, LCA) parameters, except the minimal support, namely the number of reads needed to consider a taxon being present in sample. This parameter was set at 2 instead of 5. Results were used to construct comparative trees between samples containing lignin or not in the diet.

## 3 Results

### 3.1 Enzymatic assays

Highest enzymatic activity against MCC was observed in G sample of termites feeding on diet A (Fig. 1a). Endocellulase activity was significantly higher in hindgut of termites fed with diet A+L (Fig. 1b). Proportionally, xylanase activity was very low in the first parts of digestive tract compared to exo- and endocellulase (Fig. 1c). The most significant effect of the differential feeding on xylanase endo- and exocellulase was observed for Hc samples. On the other side, alpha-glucosidase activity differed mostly in M samples (Fig. 1d) and beta-glucosidase activity varied more in G (Fig. 1e) following the two feeding treatments. Beta-glucosidase in G is also the only enzymatic activity significantly higher when termites are fed with diet A.

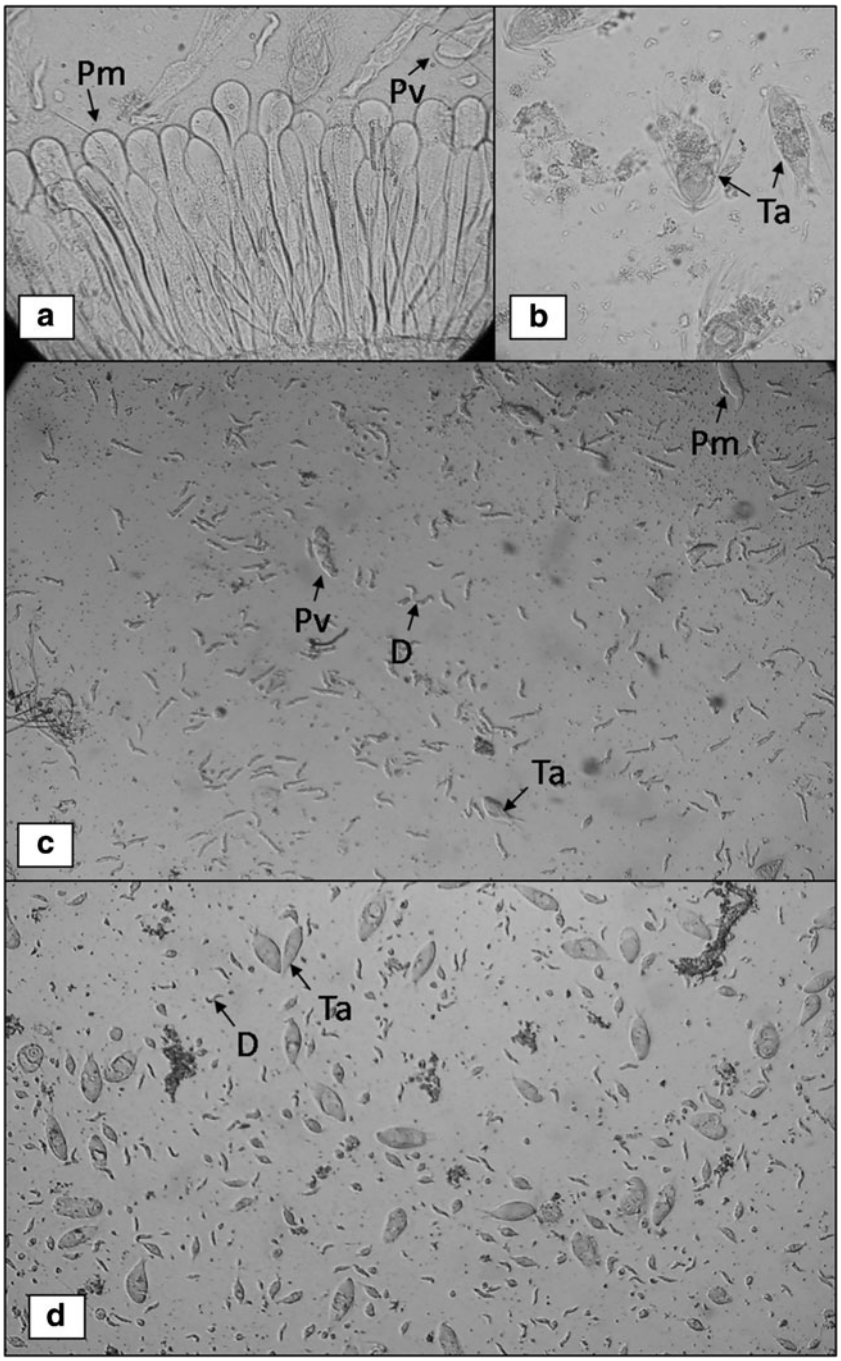


**Fig. 2** Microscopic observations of flagellates' community balances after artificial feeding experiment. *Arrows* indicate discussed populations: large Oxymonads, Pyrsonympha major (Pm) and P. vertens (Pv), middle-sized Oxymonads, Dinenympha fimbriata and D. gracilis (D), and large Parabasalids, mainly Trichonympa agilis (Ta). **a** large Oxymonads densely colonizing hindgut wall. **b** Trichonympa agilis. **c** representative micrograph of A-fed termite hindgut content. **d** representative micrograph of A+L-fed termite hindgut content

### 3.2 Microscopic observations

Populations of hindgut symbionts showed alteration between the two feeding treatments. Large Oxymonads of the genus Pyrsonympha, *P. major* (Pm) and *P. vertens* (Pv), were numerous in hindgut of termites fed only with diet A (Fig. 2c), on the hindgut walls particularly (Fig. 2a). When lignin was present (diet A+L), these symbionts were generally less abundant or even not observed (Fig. 2d). Populations of middle-sized Oxymonads (*Dinenympha fimbriata* and *D. gracilis*) were also less represented in some hindguts of termites fed with diet A+L. By contrast, some populations of Parabasalids, such as *Trichonympha agilis* (Ta, Fig. 2b), were larger when diet A+L was used (Fig. 2d).

### 3.3 LC-MS/MS experiment

Number of queries detected differed between samples, unlike the number of non-redundant (NR) peptides in total protein hits (Table 1).

**Table 1** Number of queries detected, number of total peptides with score above 15, and number of non redundant (NR) peptides for each sample analyzed

| Sample | Queries | Total peptides >15 | NR peptides |
|---|---|---|---|
| A | 2312 | 2599 | 175 |
| A+L | 1771 | 1502 | 175 |

*Hindgut content* In regards of queries detected and even non-redundant peptides, number of validated protein hits (Table 2) was low. Only 121 peptides, including redundant ones, matched validated proteins from the 350 NR peptides from both hindgut samples.

Using the Metagenome Analyzer software MEGAN (Huson et al. 2007), we attempted to extract more information from our datasets (Fig. 3).

## 4 Discussion

Enzymatic activities on MCC and CMC were relatively low. Proportionally higher endocellulase activity in foregut and salivary glands is consistent with previous work (Zhou et al. 2007). Most significant differences of xylanase, exo- and endocellulase activities between termites fed with and without lignin were measured with hindgut content samples. Previous studies on digestive system of termite showed that lignin degradation mainly occurs in foregut and midgut and also that

**Table 2** Description of protein hits, matching at least two peptides scored above 15. Hits are present in avicel-fed termites (A) or avicel+lignin-fed termites (A+L)

| Accession | Protein | Diet | Score | #Pept | SC [%] | RMS90 [ppm] |
|---|---|---|---|---|---|---|
| Symbiotic community | | | | | | |
| gi\|37572554 | beta-tubulin [Trichonympha agilis] | A | 959.0 | 15 | 48,4 | 56 |
| | | A+L | 641.9 | 12 | 43,5 | 46,87 |
| gi\|128168639 | alpha tubulin [Pseudotrichonympha grassii] | A | 699.2 | 11 | 34,5 | 45,33 |
| | | A+L | 576.4 | 10 | 35 | 39,65 |
| gi\|118766742 | beta-tubulin [Oxymonadida environmental sample] | A | 576.8 | 5 | 32 | 75,44 |
| gi\|1101023 | ubiquitin [Trichomonas vaginalis] | A+L | 137.3 | 2 | 47,1 | 69,31 |
| gi\|37572552 | enolase [Trichonympha agilis] | A | 138.1 | 2 | 5,3 | 39,77 |
| | | A+L | 186.7 | 2 | 6,1 | 44,28 |
| gi\|218117507 | glyceraldehyde-3-phosphate dehydrogenase [Trichonympha sp. HsT29] | A | 98.9 | 2 | 7,4 | 19,1 |
| | | A+L | 161.4 | 3 | 11 | 84,22 |
| gi\|110189773 | glyceraldehyde-3-phosphate dehydrogenase [Monocercomonoides sp. PA203] | A+L | 95.5 | 2 | 7,1 | 50,94 |
| gi\|3510736 | cytosolic heat shock protein 70 [Trichomonas vaginalis] | A+L | 119.5 | 2 | 3,9 | 82,41 |
| gi\|19112410 | actin Act1 [Schizosaccharomyces pombe 972h-] | A | 354.2 | 2 | 23,7 | 78,67 |
| gi\|256769456 | phosphoenolpyruvate carboxykinase [Streptomyces sp. C] | A | 94.8 | 2 | 3,4 | 89,67 |
| Host | | | | | | |
| gi\|155966246 | actin [Lepeophtheirus salmonis] | A | 481.1 | 10 | 35,9 | 71,66 |
| gi\|167683040 | actin [Euagrus chisoseus] | A+L | 319.8 | 2 | 33,6 | 65,42 |
| gi\|50428904 | arginine kinase [Periplaneta americana] | A | 341.3 | 7 | 21,1 | 21,74 |
| | | A+L | 335.4 | 5 | 17,7 | 73,66 |
| gi\|296317309 | tubulin beta chain-like [Saccoglossus kowalevskii] | A | 587.7 | 5 | 29,7 | 69,12 |
| gi\|296434304 | tubulin, beta, 2-like [Saccoglossus kowalevskii] | A+L | 418.4 | 4 | 23 | 51,41 |
| gi\|109290430 | beta tubulin [Culex pipiens pipiens] | A+L | 406.8 | 2 | 21,9 | 48,29 |
| gi\|308322917 | tubulin beta-2 chain [Ictalurus punctatus] | A | 329.2 | 2 | 20 | 52,99 |
| gi\|125659345 | alpha-tubulin [Dicyema japonicum] | A | 409.2 | 2 | 18,8 | 43,43 |
| gi\|113207854 | phosphoenolpyruvate carboxykinase [Crassostrea gigas] | A+L | 82.9 | 2 | 4,7 | 70,54 |
| gi\|307190724 | Myosin heavy chain, muscle [Camponotus floridanus] | A | 87.5 | 2 | 2,4 | 55,49 |
| gi\|242019568 | muscle lim protein, putative [Pediculus humanus corporis] | A+L | 87.9 | 2 | 6,3 | 64,26 |
| gi\|1854621 | 70-kDa heat shock protein [unidentified soil organism] | A | 85.3 | 2 | 14,9 | 34,98 |
| gi\|157118472 | calponin/transgelin [Aedes aegypti] | A+L | 118.0 | 2 | 22,5 | 66,41 |

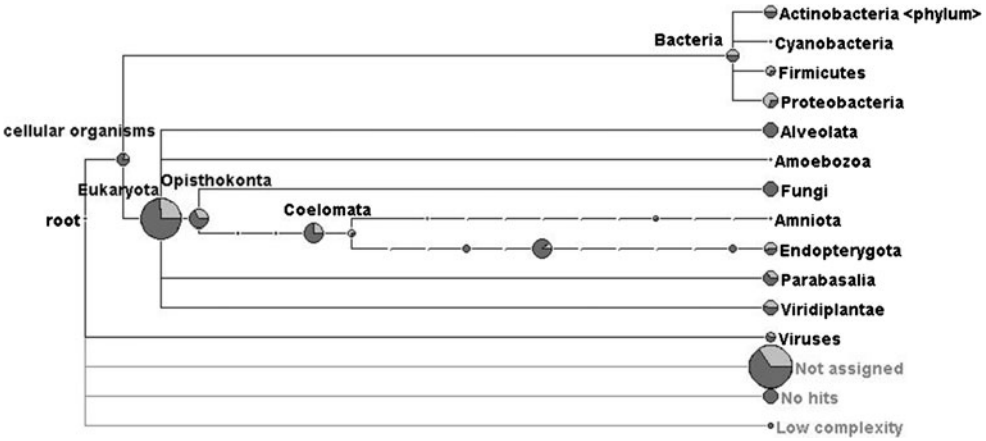


**Fig. 3** Comparative phylogenetic tree for hindgut contents, constructed using BLASTP results in MEGAN. Pie charts: proportion of reads from A-fed (*light grey*) and A+L-fed (*dark grey*) samples. Disk area: total number of reads that could not be assigned to lower taxonomic level (excepted for the Pancrustacea attribution, that represent assignments to Neoptera, but was collapsed for presentation of results)

enzymatic activities in hindgut are considered to be independent from the upstream production of endogenous enzymes (Nakashima et al. 2002; Fujita et al. 2010). However, other recent studies also pointed out synergistic effects between enzymes expressed by the host, a prokaryote and a flagellate (Scharf et al. 2011). Thus, the effect of lignin on the enzymatic activities exhibited by hindgut fluids should be deeper investigated to determine if this synergy is responsible of the enzymatic activities increase or if these are influenced by the symbiotic populations' balances.

Alpha-glucosidase activity, which was expected to be low or null in the gut of a wood-feeding insect, was very high in midgut of termites reared on A+L diet. This is probably due to an endogenous and non-specific enzyme activity, as major pretreatment occurs in the anterior parts of digestive tract (Ke et al. 2010). Enzymatic activities varied generally more between the two experimental replications than between the triplicates of the same experiment. This is explain by the disturbance of termites when separated in small colonies on artificial diets.

Microscopic observations mainly concerned large species as their easier visualization and identification renders population balances more evident. Thought these observations were not quantitative, we observed a strong decrease of Oxymonads with the addition of lignin. This result raises an issue as termites are considered healthier when lignin is present in their diet. Indeed except for salivary glands activities and hindgut β-glucosidase activity, measured enzymatic activities were higher for termites fed with MCC and lignin.

Number of queries detected represents the whole raw information, workable or not, contained in sample. Number of non-redundant peptides represents the total qualitative information that could be extracted from all unique matches with sufficient score. The relatively weak number of NR peptides could be explained by the abundance of unsequenced proteins or the high protein diversity.

Shotgun proteomics on the hindgut of lower termites should be achieved after splitting up the proteome, to decrease the proportion of abundant unsequenced proteins from symbiotic flagellates. This could be achieved by adding steps in microorganisms (Yang et al. 2005) or peptides separation (Rudney et al. 2010).

Unculturability of most termite flagellates (Ohkuma 2008), their particular metabolism and thus the few genetic data available tend to let these symbionts hidden within the unassigned reads. Indeed, some targeted studies on tubulins (Moriya et al. 2001), actins, enolases or glycosyl hydrolases family 7 (Todaka et al. 2007; Tartar et al. 2009) were realized but there's still a important need in genetic information on symbiotic flagellates.

Following Rudney et al. (2010) minimal support in a metaproteomic study may be lowered from 5 reads to a single one. However, to minimize false-positive occurrence, we decided to set at 2 the minimal number of reads attributed to taxonomic level validating its identification in our sample. In accord with microscopic observations, only small Oxymonads, from the genus Monocercomonoides, were identified in termites fed with A+L diet. From termites fed with diet A, peptides assigned to Oxymonads included Monocercomonoides and Oxymonadida environmental sample, corresponding to a symbiont of *Cryptocercus punctulatus*. Only one peptide (sequence: DGCDFASYR; score: 64,74) scoring above identity threshold was assigned to a cellulose-degrading enzyme (gi|67942391), from hindgut of termites fed with diet A+L. This exoglucanase-type cellulase was related to *Reticulitermes flavipes* gut symbiont cell-3 which belongs to Parabasalians (Zhou et al. 2007). As this analysis mainly identifies abundant proteins, this result may suggest that lignin increased the relative abundance of this enzyme.

## 5 Conclusion and perspectives

Results support previous works according to which termites are healthier when lignin is present in their diet. Data suggest that lignin enhance enzymatic activities against cellulose and hemicelluloses in hindgut fluid. Microscopic observations of hindguts also suggest that populations of the genus Pyrsonympha decrease when lignin is added in

artificial diets contrarily to some Parabasalian populations, which seem to increase. It is not clearly defined whether the reason of this decrease is the toxicity of lignin degradation products on Oxymonads or their prokaryotic symbionts. The link between enzymatic and populations' balances has to be investigated, to determine if detected enzymes are globally more expressed or if a synergy between diverse enzymes is responsible of the observed effect.

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