Sociobiology 71(3): e10453 (September, 2024)
DOI: 10.13102/sociobiology.v71i3.10453

Sociobiology
An international journal on social insects

RESEARCH ARTICLE - TERMITES

Intestinal Bacteria Aid Termites in Response to Entomopathogen Infection According to the Metagenome and Meta-transcriptome

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Article History

Edited by
Manuela Ramalho Sanches, WCUPA, USA
Received 12 January 2024
Initial acceptance 06 February 2024
Final acceptance 13 April 2024
Publication date 30 July 2024

Keywords
Symbionts; Termite; Innate immune; Metagenome; Meta-transcriptome; Glutathione.

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Abstract

Intestinal symbionts live in insects' digestive systems and help with food digestion, nitrogen cycling, and energy use. Numerous symbionts, including bacteria, fungi, and protozoa, colonize the posterior gut of termites. Intestinal microbiota affects the Toll or Imd pathways during viral or entomopathogenic fungus infection in mosquitoes, but the role of intestinal symbionts in individual termite immunity is unclear. In the present research, *Odontotermes formosanus* and *Metarhizium robertsii* were selected to investigate the potential mechanism of the intestinal bacteria influencing termite innate immune while encountering an entomopathogen using metagenome sequencing and meta-transcriptome sequencing. We found that termite mortality declined within 48 hours under high spore infections and reached semi-lethal status within 72 hours. We sequenced the control and infestation termite and found that the dominant phyla changed below low-abundance flora during infestation. According to function annotation, the functions of *spirochetes* are significantly different from those of the rest of the phyla, which are mainly enriched in metabolism and cellular processes. There are 261 upregulated and 459 down-regulated DEGs derived from bacteria after infestation. We identified the glutamate-cysteine ligase gene, *gshA*, which is significantly upregulated in the infected group, and the GSH content in the regular infected group was higher than the 5% kanamycin-infected group. We speculated on a potential mechanism that one or more bacteria belonging to *Spirochetes* that upregulated *gshA* gene expression and increased GSH content in the hindgut to integrate with metarhizium toxins and then excrete out of the intestine.

Introduction

Termites are typical social insects with various castes in their community, most of which are workers. The history of termites can be traced back to 250 million years ago, and over 3000 species are distributed worldwide. According to statistics, subterranean termite damage and control costs are estimated to approach $2 billion annually in the United States alone. During termite control, people have found that both conventional and chemical control have their limits. Therefore, the control of termites using new means becomes more important. Over their lifetime, workers are frequently exposed to pathogens through nesting and foraging, and pathogens are taken back to the nest. However, innate immune and social behavior can remove these foreign pathogens from the community. During resistance to pathogens, termite gut microbes can help maintain body homeostasis. Understanding the role of gut microbes in insect immunity becomes essential

ISSN: 0361-6525

in order to achieve control of termite populations. The role of intestinal microorganisms in insect immunity has gradually become clear.

Many microbes live in the gut of insects and play many vital roles in their behavior, metabolism, diet, nutrition, and immunity . The vital activities of insects can only be discussed by considering intestinal symbiosis. Because of the dietary preferences, the composition of the intestinal symbiotes also differs. Due to the different composition of intestinal microorganisms, termites are divided into non-Termitidae termites and Termitidae, formerly called lower termites and higher termites. However, most research regarding termites and intestinal symbionts has focused on how the latter helps the former degrade lignocellulose quickly and effectively . Some intestinal microorganisms have been reported to participate in immunity among other insects. The pathogenic fungus *Beauveria bassiana* Vuillemin interacts with the gut microbiota *Serratia marcescens* Bizio to accelerate mosquito death. In addition, *Serratia* can secrete an enzyme to help mosquitoes fight malaria parasites. Intestinal flora directly mediated the Toll pathway to the anti-dengue virus.

Recently, the meta-transcriptome and metagenome have been used separately or both to investigate the role of uncultivated microorganisms in the gut or other organs of many species, including invertebrates and vertebrates . Research on the termite meta-transcriptome revealed the role of intestinal symbiotes and the expression of the host genes in cellulosic digestion or innate immunity in non-Termitidae termites. However, there are few reports about Termitidae immunity and intestinal flora.

In the current research, we infected the fungus-cultivated Termitidae *Odontotermes formosanus* Shiraki using entomopathogen *Metarhizium robertsii* St Leger & Roberts to resolve the potential function of intestinal bacteria in innate immune by metagenome and meta-transcriptome analysis. We reveal that intestinal flora potentially promotes GSH synthesis by upregulating *gshA* expression, thereby assisting termites in responding to entomopathogen infection. This finding holds significant theoretical value for deciphering termite immune response mechanisms and provides potential guidance for termite management and control strategies.

Materials and Methods

Termite collection and rearing

Workers of *O. formosanus* were chosen as the experimental subjects and gathered in Lion Mountain, the Huazhong Agricultural University, Hongshan District, Wuhan City, Hubei Province. The termites were raised in a Petri dish using 9 cm filter paper. The culture media was kept in a constant-temperature incubator at 25 °C and 85% humidity in the dark to preserve community stability. The filter paper was changed every two days.

Culture of fungal pathogen

The Shanghai Institute of Botany provided the *M. robertsii* (strain #2575) used in this experiment. *M. robertsii* spores were grown for 2-4 weeks on potato dextrose agar (PDA) and mixed with 0.1% Tween 80 to create a conidial solution stored at four °C. The spores were diluted to the desired concentration before the survival experiment to determine the time and concentration of infection.

Each infection and control group contained three biological replicates, each containing thirty termites in good health. The two treatment groups were in the dark for 48 hours in Petri dishes with a 9-cm diameter spread with filter paper and sprayed with either 1 mL of a 1×10^8/mL spore suspension or 1 mL of sterile water. After treatment, the filter paper was removed, and the termite samples were dissected.

Termite gut dissection

Before dissection, the termites and forceps were cleaned with 75% ethanol to remove bacteria. 1X PBS was used when dissecting the samples. The hindguts of termites were chilled in an ice bath. The termite guts were put into 1.5 mL Eppendorf tubes and stored at -80 °C to prevent RNA degradation.

Metagenome library preparation, sequencing, assembly, and annotation

Three biological samples were combined and sequenced. DNA was extracted using The E.Z.N.A.® Stool DNA Kit (Omega Bio-tek Inc., Norcross, USA) according to the manufacturer's recommendations for bacterial DNA extraction, which excluded lysis stages and was stored at -20 °C for future analysis. Using the Covaris M200 and the NEXTFLEX® Rapid DNA-Seq Kit, crude DNA was sheared at a length of 400 bp. The library was prepared with NovaSeq Reagent Kits (Illumina, San Diego, USA) and sequenced with paired 150bp reads.

Reads of the two libraries, including the control and infected group, were obtained from the sequencer. Low-quality reads were removed using Fastp v0.12.4. Contamination of the host genome (*Coptotermes formosanus*, Taxonomy ID: 36987) was also removed using KneadData (https://github.com/biobakery/kneaddata) with the default parameters. Cleaned reads were assembled by Megahit v1.2.9 with the default Kmer value, and assembly effectiveness was assessed with Quast 5.0.2. Prodigal v2.6.3 was used for ORF prediction with the option "-m -p meta" for predicting bacteria genes with no gaps. The Prodigal (-meta) results were processed using cd-hit v4.8.1 to eliminate the predicted gene redundancy. To obtain a solid taxonomic classification of species, reads were classified at multiple levels – phylum, class, order, family, genus, and species – by Kraken2 and Bracken [Citing: Kraken 2 (31779668), Bracken (36171387)]. Additionally, Contigs from different phyla were retrieved using TBtools for further species annotation. Diamond aligned predicted genes to the eggnog 5.0 bacteria database under the e-value of 1e-5 while annotating the alignment result using the COG, KO, and CAZy databases.

Meta-transcriptome library preparation, sequencing, assembly, and annotation

Trizol was used to extract the total RNA. The Ribo-Zero rRNA Removal Kit was used to remove the rRNA from the total RNA (bacteria). Utilizing the TruSeqTM RNA Sample Prep Kit, cDNA was generated and used to create the meta-transcriptome libraries (Illumina, San Diego, USA). The NovaSeq Reagent Kit was used to sequence RNA fragments (Illumina, San Diego, USA). The library was prepared with NovaSeq Reagent Kit (Illumina, San Diego, USA) and sequenced with 150 bp paired reads.

The SAMSA2 pipeline was used to perform the conducted meta-transcriptome analysis. Quality control was undertaken using the default parameters of Trimmomatic v0.39 and FastQC v0.11.9. Cleaned reads were merged and used by PEAR to produce raw counts. Merged reads were then exported to SortMeRNA v2.1.4 to remove ribosomal reads. MEGAHIT was used to assemble the cleaned reads, and the contig quality was evaluated using Quast 5.0.2. Kraken2 was used to annotate the contigs, and bracken was used for hierarchical categorization. For functional annotation, contigs from several phyla were retrieved using TBtools. For ORF prediction, Prodigal v2.6.3 was used with the option "-m -p meta" to predict genes without gaps. Additionally, the Prodigal (-meta) result was used to remove redundant genes and to convert the RNA to protein sequences by cd-hit (Citing: cdhit: 23060610). The protein sequence was annotated using KOBAS 3.0 software.

Glutathione content measurement

In order to demonstrate the positive role of intestinal bacteria in glutathione synthesis in the hindgut of termites, we first treated termites with 5% kanamycin (dissolve 1 g of kanamycin powder in 20 mL of distilled water) for 48 hours and then with *M. robertsii* for 48 hours. Each group consisted of 10 termites with three biological replicates. The posterior gut of the termite is dissected for GSH content detection. Refer to the instructions of the glutathione detection kit (Nanjing Jiancheng Bioengineering Institute, China) for the detection method.

Results

Metarhizium robertsii infection effectively increases termite mortality level

Figure 1b shows a typical *O. formosanus* termite. The termites were infected with various concentrations of *M. robertsii*, and the termite survival rates were calculated at various time points, as per our prior study, to calculate the mortality rate. The termite survival at different spore concentrations is shown in Figure 1d. Before 48 hours, the mortality level in the four groups was higher than 85% but not significantly so. The survival rate of termites treated with suspended spores at 1×10 ^{8}/mL decreased sharply at 48 hours and halved after 72 hours.

Additionally, it differs significantly from the CT group. Based on the aforementioned findings, the 48-hour time point was chosen as the best option for subsequent experiments. The growth of fungal spores on the termite surface and their transformation from white to green after two weeks of high-concentration *M. robertsii* infection demonstrated that infection was the cause of termite mortality (Fig 1c).

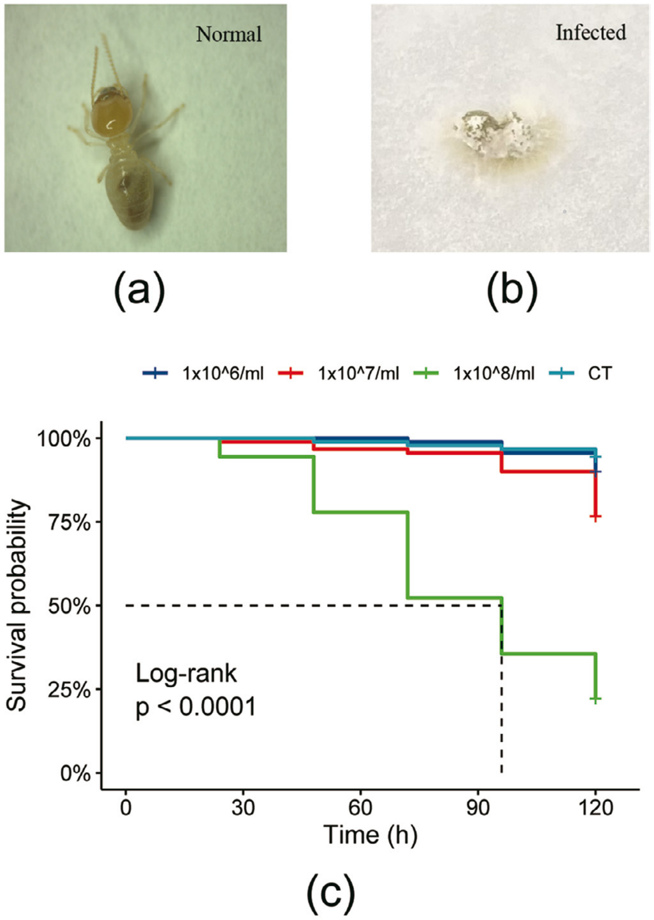


Fig 1. The background of this experiment. (a): Normal termite *Odontotermes formosanus* in the filter paper, captured with iPhone 11. (b): Two weeks later, the termite died of fungal infection. (c): Survival of termites (n = 30) fed different spore concentrations per mL (AT). (CT termites were fed 1 mL sterile water. The experiments were performed in three biological replicates. 1x10^8/mL spore suspension significantly increased termite death compared with other groups at 72 h [log-rank (Mantel-Cox) test, $p < 0.0001$]. The experiments were performed in three biological replicates. The log-rank test was used to assess the significance of differences between two survival curves using GraphPad Prism8 software.).

Summary of metagenome and meta-transcriptome data

The intestinal flora at the gene and transcription levels were analyzed, respectively, to clarify the variety of intestinal flora after infection (Table 1). Overall, there were

25,192,940 and 24,551,605 raw reads in the sequencing of metagenomes and 27,045,469 and 26,520,565 raw reads in the sequencing of meta-transcriptomes. The former number is controlled, and the latter is processed. In the two sequencing strategies, the total base of the raw data was 3804118840, 3682740750, 4083865819, and 4004605315. More than 98.20% of the raw data passed the filter, and the Q30 values of 93.66% demonstrated the high quality of the sequencing data. The meta-transcriptome was assembled independently in 313,422 and 290,833 contigs with 50.01% and 51.40% GC content in the control and infected treatment, respectively. The metagenome data were assembled separately in 291,977 and 282,923 contigs with 49.36% and 50.82% GC content in the control and infected treatment, respectively. N50 was 1475, 1396, 1795, and 1710 in the four libraries. Following contig assembly, most sequences were between 300 and 600 bp in length (Fig S1). As mentioned earlier, the findings showed that the constructed contigs were trustworthy and high-quality.

Table 1. Summary statistics for sequencing data from the Termitidae hindgut gut metagenome and meta-transcriptome.

Parameters	Metagenome/MG		Meta-transcriptome/MT	
Treatments	Control	Infected	Control	Infected
Sequence strategy	Paired-end	Paired-end	Paired-end	Paired-end
No. of reads	25,192,840	24,551,605	27,045,469	26,520,565
[1]Q30 (%)	95.92	95.24	94.38	93.66
The total length of reads, bp	3,804,118,840	3,682,740,750	4,083,865,819	4,004,605,315
The average length of reads, bp	151	151	151	151
Reads passed filters (%)	99.99	99.99	98.49	98.20
Numbers of Contig	291,977	282,923	313,422	290,833
N50	1475	1396	1795	1710
N75	784	759	843	829
Largest contig	273378	346846	809968	532306
GC content (%)	49.36	50.82	50.01	51.40

Note: [1] Q30 means the percentage of bases with Phred ≥ 30.

The change range of high-abundance flora under the infection of ***M. robertsii*** *is smaller than that of low-abundance flora*

After proving the reliability of the data, the species of the control group and the experimental group were annotated in the metagenome and meta-transcriptome data and then compared at three taxonomic levels (Fig 2).

In the metagenome, either before or after the infection of *M. robertsii*, the most dominant bacteria are *Proteobacteria*, Firmicutes, *Bacteroides*, and *Actinobacteria* (Fig 2a and 2b). After the infection, the relative abundance of the phyla except the Firmicutes increased slightly, while the Firmicutes decreased to a certain extent. The dominant flora in the meta-transcriptome has analogous changes. *Proteobacteria*, after the infection of pathogenic fungi, the relative abundance in the gut has increased by nearly 0.5-fold. However, the relative abundance of other phylum has decreased to different degrees except for Proteobacteria, among which the relative abundance of *Spirochaetes* has decreased by nearly 70%, and *Actinobacteria* has also decreased by 60% (Fig 2b).

When the level of annotation is Genus or species, the bacteria existing in the meta-genome become low abundance in the meta-transcriptome, including *Escherichia coli*, *Citrobacter*, and *Lactococcus* (Fig 2c). There is no significant abundance in the meta-genome, but there is a relatively significant increase in the meta-transcriptome of bacteria, including *Kosakonia*, *Enterobacter*, *Streptococcus*, and *Bacteroides*. However, the activity of *Streptomyces*, *Clostridium*, and *Bacillus* decreased after the invasion of *M. robertsii*. The trend at the species level is similar to that at the genus level (Fig 2d).

The function of Spirochetes changed significantly after infection by ***M. robertsii***

The abundance of intestinal microflora has changed significantly after the infestation in the previous section, but its function in the immune response is still unknown. Next, we split the sequence in the meta-transcriptome at the phylum level and analyzed the functional changes of the top five dominant bacteria in the termite gut before and after the infection of *M. robertsii* to termites in detail.

Figure 3 shows that the functional changes of *Proteobacteria* and *Firmicutes* before and after the infection were minimal or even constant. In contrast, the functional changes of *Bacteroides*, *Actinobacteria*, and *Spirochaetes* were more significant in termite resistance to *M. robertsii*.

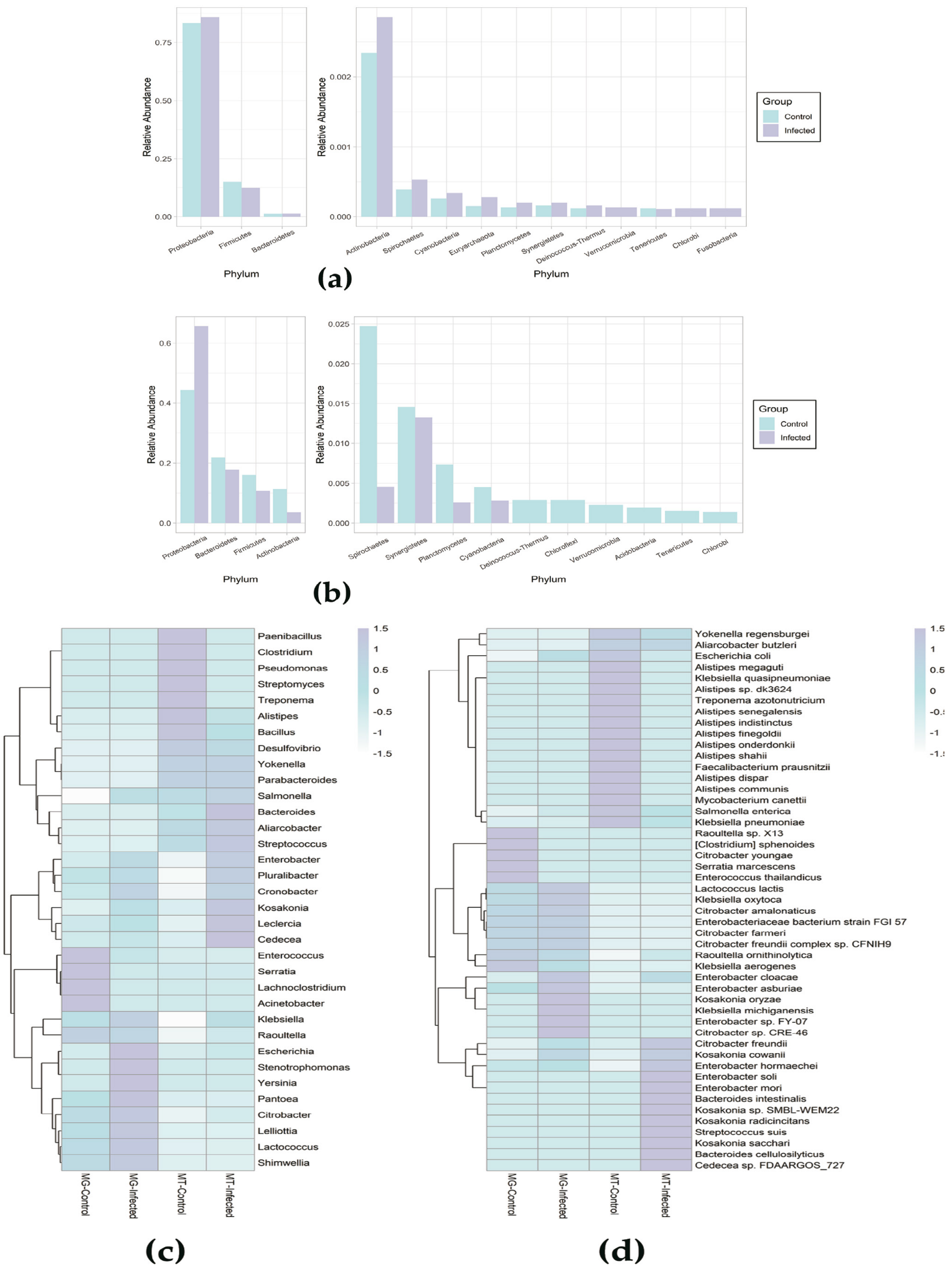


Fig 2. Comparison of relative abundance of microflora in the metagenome and meta-transcriptome sequencing. (a): an abundance of phylum in the metagenome. (b): an abundance of phylum in meta-transcriptome. (c): comparative heatmap of the abundance change of genus in MG/MT after infestation. (d): comparative heatmap of the abundance change of species in MG/MT after infestation. MG_control: control sample in metagenome sequencing; MG_infected: infected sample in metagenome sequencing; MT_control: control sample in meta-transcriptome sequencing; MT_infected: infected sample in meta-transcriptome sequencing.

First, after termites were infected by *M. robertsii*, the transcriptional activity of *Bacteroides* in amino acid metabolism increased by about 8%. However, the transcriptional activity of the metabolism-related protein family decreased by about 5%, which indicates that the infection of *M. robertsii* promoted the amino acid metabolism of this phylum but led to a decline in the transcription of the metabolism-related protein. Secondly, Actinomycetes' carbohydrate and energy metabolism increased to a certain extent after the infection of *M. robertsii*, while the cofactor and vitamin metabolism decreased. There was no significant change in the functional items above the four phyla.

The transcription activity of *spirochetes* has changed significantly compared with the first four phyla. The infestation inhibited the genetic information process, energy, amino acid metabolism, folding, sorting, and degradation while significantly increasing the transcriptional activity of the genes, including signal and cellular processes, carbohydrate metabolism, and membrane transport processes. These functions were related to protein synthesis and secretion.

*Bacteria have the potential to participate in termite resistance to infestation by upregulating the glutathione synthesis rate-limiting enzyme gene **gshA***

Figure 4 shows the significant differentially expressed genes, allowing us to evaluate and understand the potential function of bacteria in immunity at a deeper level.

The results of differentially expressed genes showed that many genes were significantly upregulated or down-regulated after the infection of *M. robertsii* (Table 2, Fig 4a). To understand the specific functions of these genes, we carried out a KEGG enrichment analysis. Most are related proteins in coding genetic information, including signal and cellular processes (Fig S3). In particular, the gene *gshA*, glutamate-cysteine ligase, participated in synthesizing glutathione (Fig S4),

Table 2. The differential genes expressed in meta-transcriptome.

Taxon	Number up-regulated	Number down-regulated	No change
Bacteria	261	459	2011

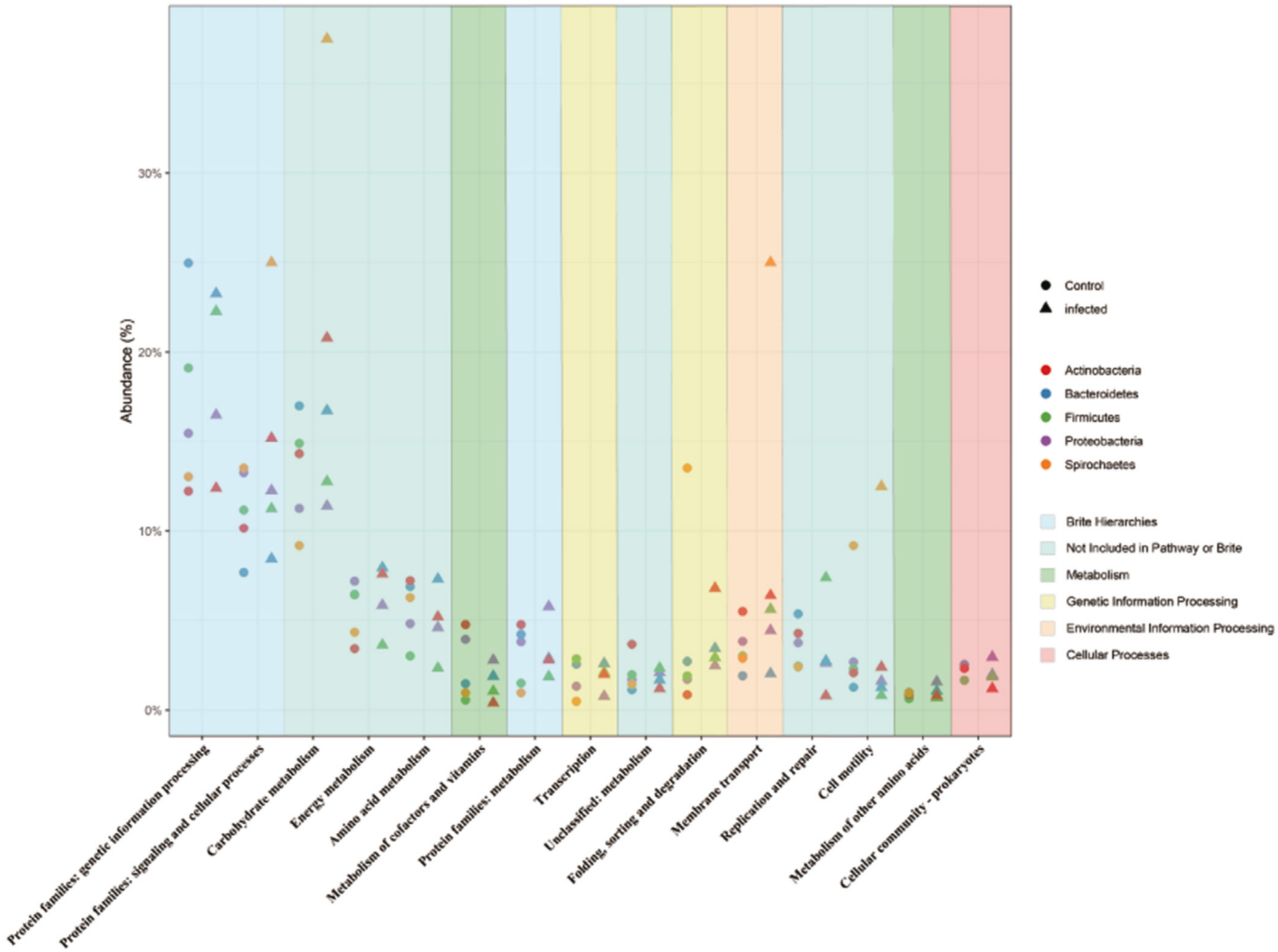
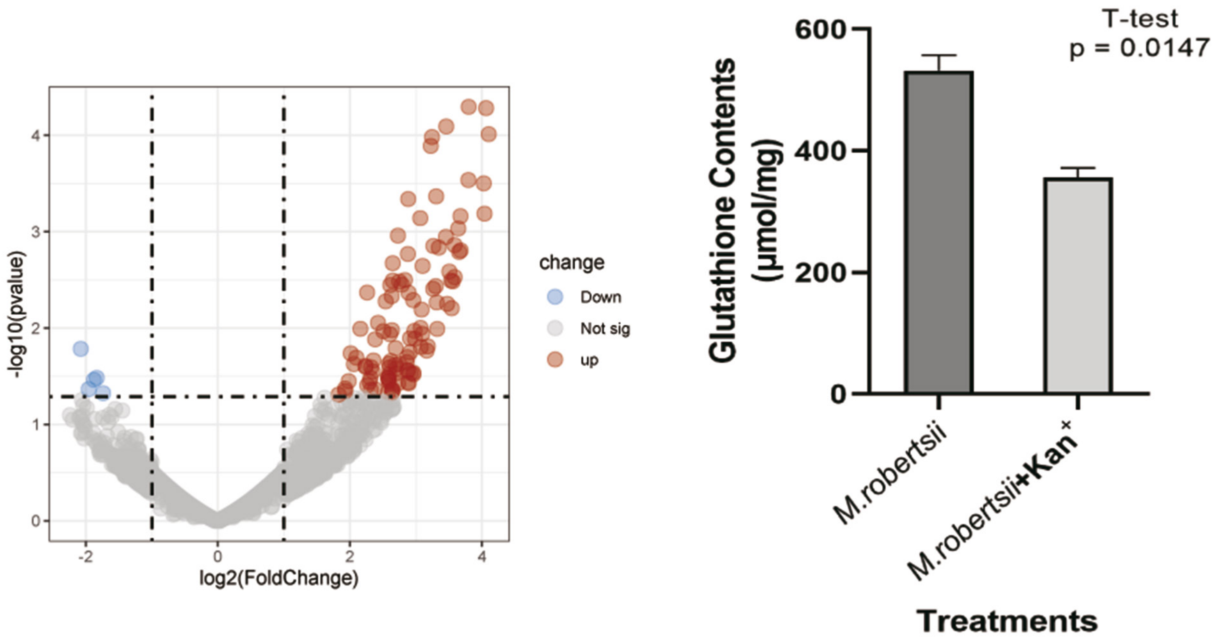


Fig 3. Functional changes of the top five phylum levels after infection. Functional items were based on KEGG pathway level 2, and other features are shown in the legends.



(a) (b)

Fig 4. Changes of bacterial abundance and differential genes in meta-transcriptome. (a): Differential expression genes in bacteria after metarhizium infection. (b): Results of experiments to estimate GSH content in termite's hindgut. The differential expression matrix is calculated by DESeq2 and |Log2Foldchange| >=1 means significant change. *M. robertsii* means eight termites infected by metarhizium for 48 hours, and *M. robertsii* + Kan$^+$ is eight termites infected by metarhizium after treatment with 5% kanamycin for 48 hours.

indicating that the gene with changed expression may participate in innate immunity. In order to evaluate the above speculation, we measured the GSH contents after *M. robertsii* and with or without 5% Kanamycin (Fig 4b). The result found that GSH contents in infestation were significantly higher than in infestation after antibiotic treatment, which means intestinal bacteria played positive roles in GSH synthesis.

Discussion

In this study, metagenome and meta-transcriptome approaches were combined and used to examine the role and mechanism of intestinal bacteria in termite host immunity. It was found that *M. robertsii* infestation significantly changed the gut flora and affected low-abundance flora. This effect is not only reflected in abundance but also affects functional gene transcription, the most obvious of which is *Spirochete*. The genes of microbial origin after infection reveal a possible mechanism that bacteria significantly upregulated the expression of glutamate-cysteine ligase and released it into the intestinal cavity to promote the synthesis of glutathione in the host or bacteria, thus combining with *M. robertsii* toxin to help the host resist *M. robertsii* infection.

It was discovered that intestinal invasion by foreign pathogens could be aided by the host immunity provided by the gut symbiont. The taxonomic analysis and functional annotation in these studies revealed that the abundance of symbiotic bacteria, genus, and expressed transcripts changed to accommodate the presence of metarhizium. In another study on mosquitoes, *Serratia* appeared to interact with invasive pathogens and hasten the death of the mosquitoes. In the present study, termite hindgut pathogens affected intestinal flora's diversity and composition. The relative abundance of *Proteobacteria*, Firmicutes, and Bacteroidetes was not transformed in metagenome level following infection, and low abundance phyla like *Spirochaetes* and *Actinobacteria* raised significantly (Fig 2a). It demonstrated that the imbalance of the microenvironment caused by *M. robertsii* infection is more conducive to the proliferation of non-dominant bacteria in the intestinal flora. At the same time, a different trend appeared in the meta-transcriptome level (Fig 2b). Combined with the above results, we found that the *Actinobacteria* and *Bacteroides* in the dominant bacteria group increased in the metagenome but decreased in the meta-transcriptome, indicating that although the proliferation led to the increase of its abundance, its activity was decreased. After the fungi infection, *Proteobacteria* increased in both omics, indicating that the invasion of *M. robertsii* promoted Proteobacteria's proliferation and metabolic activity.

Finally, the trend of change in Firmicutes is opposite to the former. Therefore, we infer that the bacteria with significantly enhanced metabolic activity may assist termites in resisting *M. robertsii*. Firmicutes and Bacteroidetes, on the other hand, decreased following pathogenic infection in this study. Enzymes that lyse the fungal cell wall appear mostly produced by *Firmicutes* and *Bacteroidetes*. Therefore, metarhizium infection decreased the content of these two bacteria, thereby reducing the threat of intestinal microorganisms to pathogens. *Bacillus* sp., which belongs to *Firmicutes* and is found in termites (*Anacanthotermes*), was discovered to produce a novel beta-1,4-glucanase that degrades fungal cell walls. Except for the above flora, the transcriptional abundance of *Spirochetes* decreased nearly three times after infection by *M. robertsii*. However, the function of change was the most abundant among all phyla (Fig 2 & 3). In our previous study, *Spirochetes* expressed the same trend during infestation from 12 to 72 hours. *Spirochetes* can undergo various metabolic processes, including acetate production, nitrogen fixation, and degradation of lignin phenols. Faced with intestinal imbalance, the ability to process cellulose of *Spirochetes* raised over one-fold and their advantages have been replaced by other antimicrobial tolerance gates with similar metabolic abilities, which are generally believed to enhance the resilience of microbial communities in the face of stress.

Additionally, Peterson et al. discovered that the bacteria in the termite hindgut potentially upregulate an amidohydrolase two gene, working in conjunction with the other symbiont and host endogenous antifungal enzymes to combat invaders during *B. bassiana* infection. Also, the potentially antifungal gene bacteria-derived *gshA* was upregulated 3.8-fold in termite hindgut after *M. robertsii* infestation 48 hours. Glutamate-cysteine ligase is the rate-limiting enzyme in glutathione biosynthesis and occurs in prokaryotes and eukaryotes. It positively affects the synthesis of glutathione and is inhibited by glutathione feedback. In many lower organisms, the GCL enzyme is a single polypeptide, but most eukaryotic GCL enzymes are heterodimeric complexes consisting of two distinct gene products. GSH and glutathione were well known for their protective abilities against the detrimental effects of oxidative stress within the human body and protection against infection by exogenous microbial organisms. When insects encounter a pathogen, organisms release many reactive oxygens (ROS) to kill them directly, including hydrogen peroxide, hydroxyl radical, superoxide anion, and other oxygen-free radicals.

On the one hand, glutathione can combine with the toxin released by the pathogen or insecticide and then be eliminated from the body. On the other hand, it can combine with reactive oxygen species to prevent the body from producing too many reactive oxygen species to damage its cell membrane. In addition, studies have shown that GSH can promote the participation of macrophages in cellular immunity. The GSH contents of the gut infestation group were found to be significantly higher than the group infestation with 5% kanamycin (Fig 4b).

Although bacteria in the hindgut potentially release GCL protein into epithelial cells to promote GSH total or directly release GSH into the gut lumen to participate immune, the exact bacteria species were not found in this study. Future research should concentrate on finding specific bacteria involved in GCL expression when termites are infected by *M. robertsii*, as well as investigating whether this expression may affect the production of termite antifungal peptides.

Conclusion

This study investigated changes in the quantity, species, and gene expression at the transcriptional level of termite gut microorganisms that encountered foreign pathogens in the host and entered the intestine. Infection with *M. robertsii* significantly changes the intestinal flora of the hindgut of termites, with a more significant impact mainly on the low-abundance flora. This effect is reflected in changes in abundance and the transcription of functional genes, with the most apparent impact being on genes belonging to the *Spirochetes* bacteria. Therefore, we have established a possible mechanism that bacteria significantly upregulate the expression of glutamate-cysteine ligase and release it into the intestinal cavity to promote the synthesis of glutathione in the host or bacteria, thereby binding to the toxin of *M. robertsii* to help the host resist infection.

Acknowledgments

Dr. Cheng-zhu Wang kindly provided the metarhizium strain used in this study from the Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences. This work was supported by the National Natural Science Foundation of China, 32070504 and 31872289.

Disclosure

The authors declare no conflict of interest.

Authors' Contributions

Ya-ling Tang: formal analysis, investigation, methodology, visualization, writing-original draft.
Si Zhou: data curation, validation, writing-review, and editing.
Yun-hui Kong: software; data curation; formal analysis.
Sheng Qin: software; data curation; formal analysis.
Xia Sun: conceptualization; supervision.
Qian Wang: funding acquisition; conceptualization; resources; supervision.
Mu-wang Li: supervision, resources, funding acquisition.

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Supporting Information

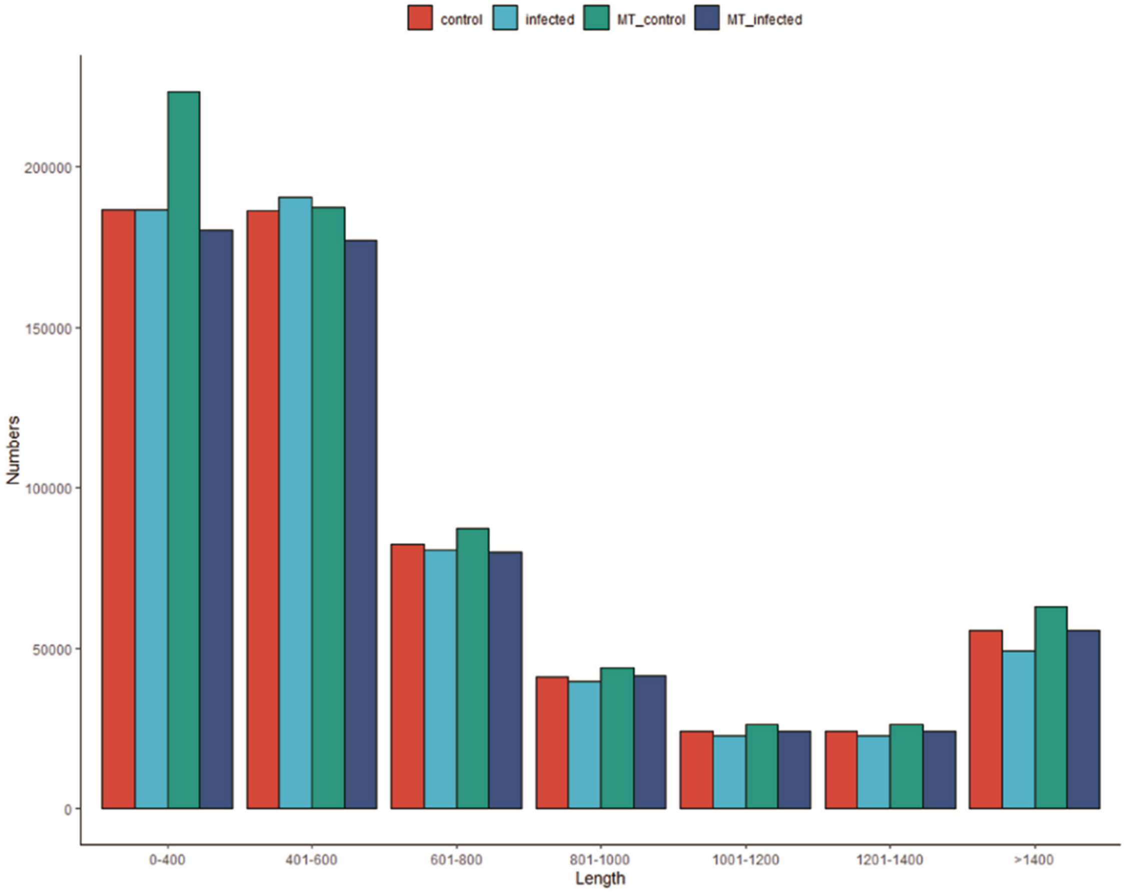

Fig S1. The length distributions of filtered metagenome and meta-transcriptome sequencing reads.

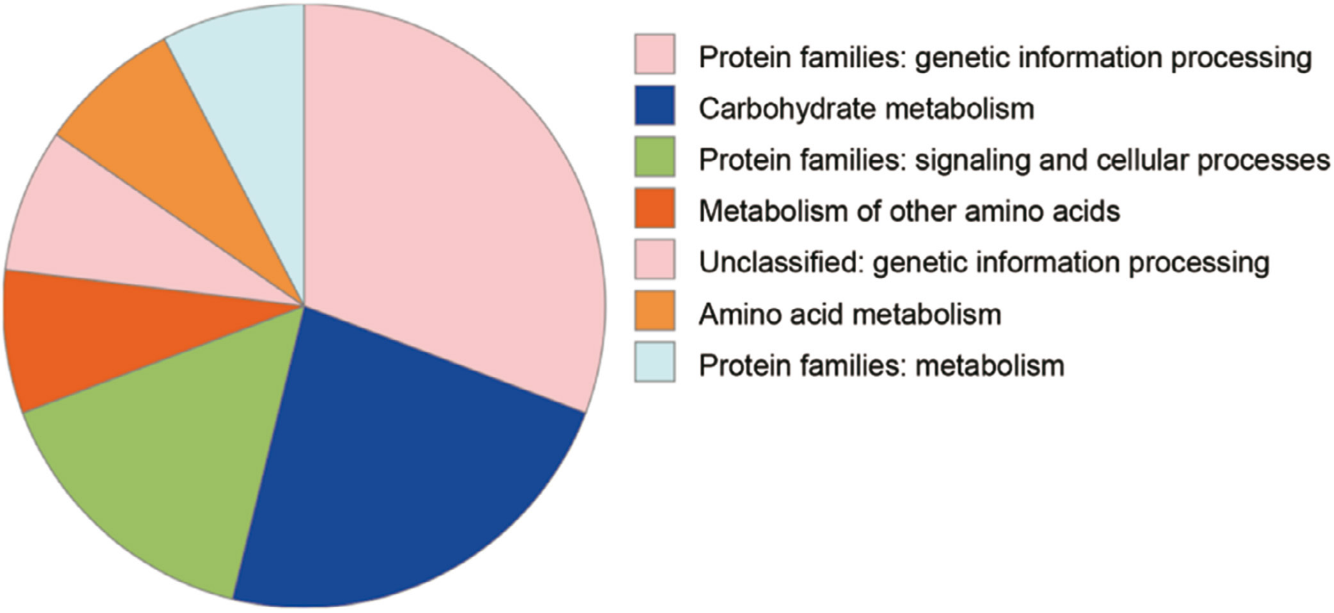

Fig S2. KEGG enrichment analysis.

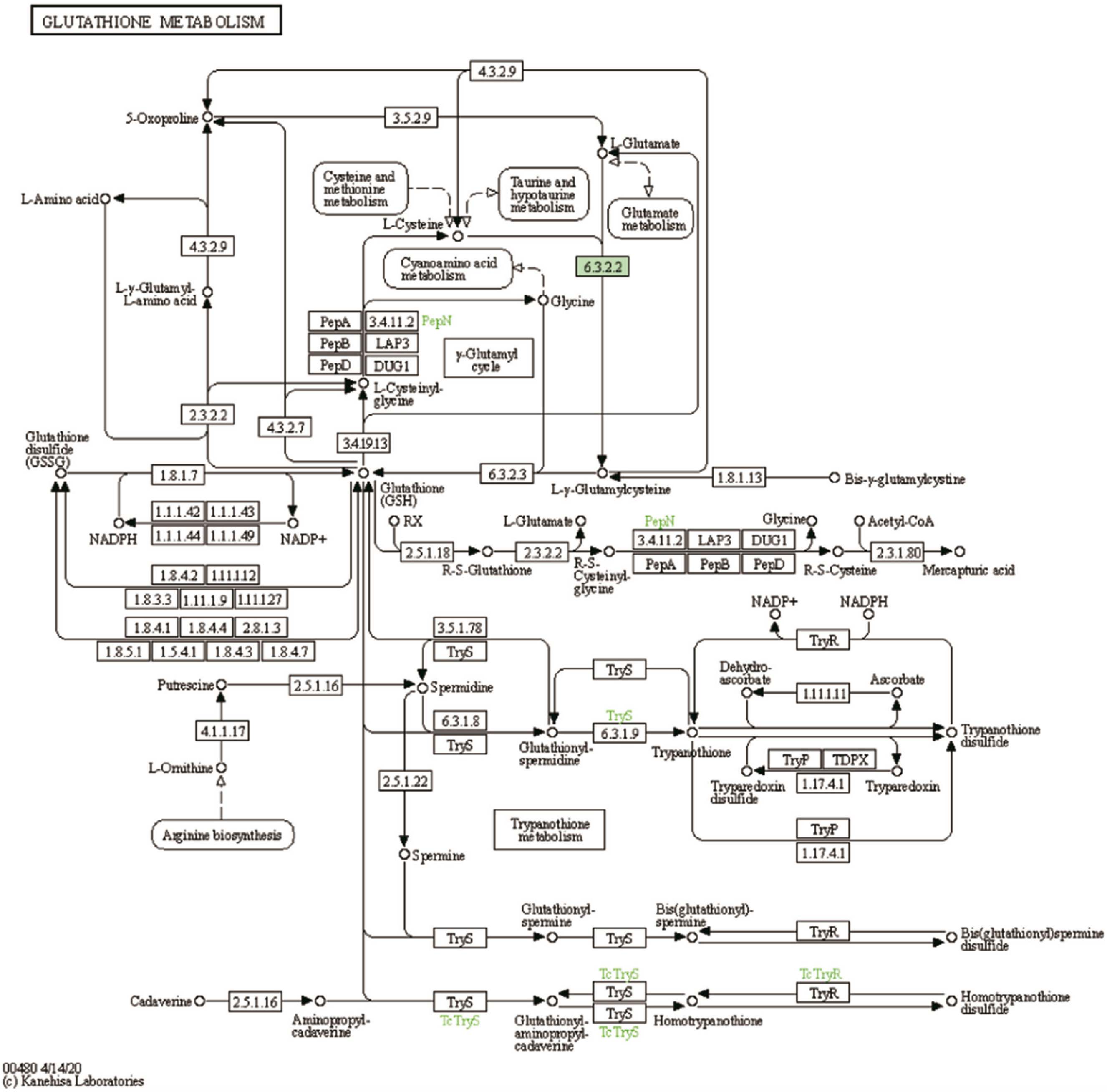

Fig S3. The pathway map of glutathione metabolism.