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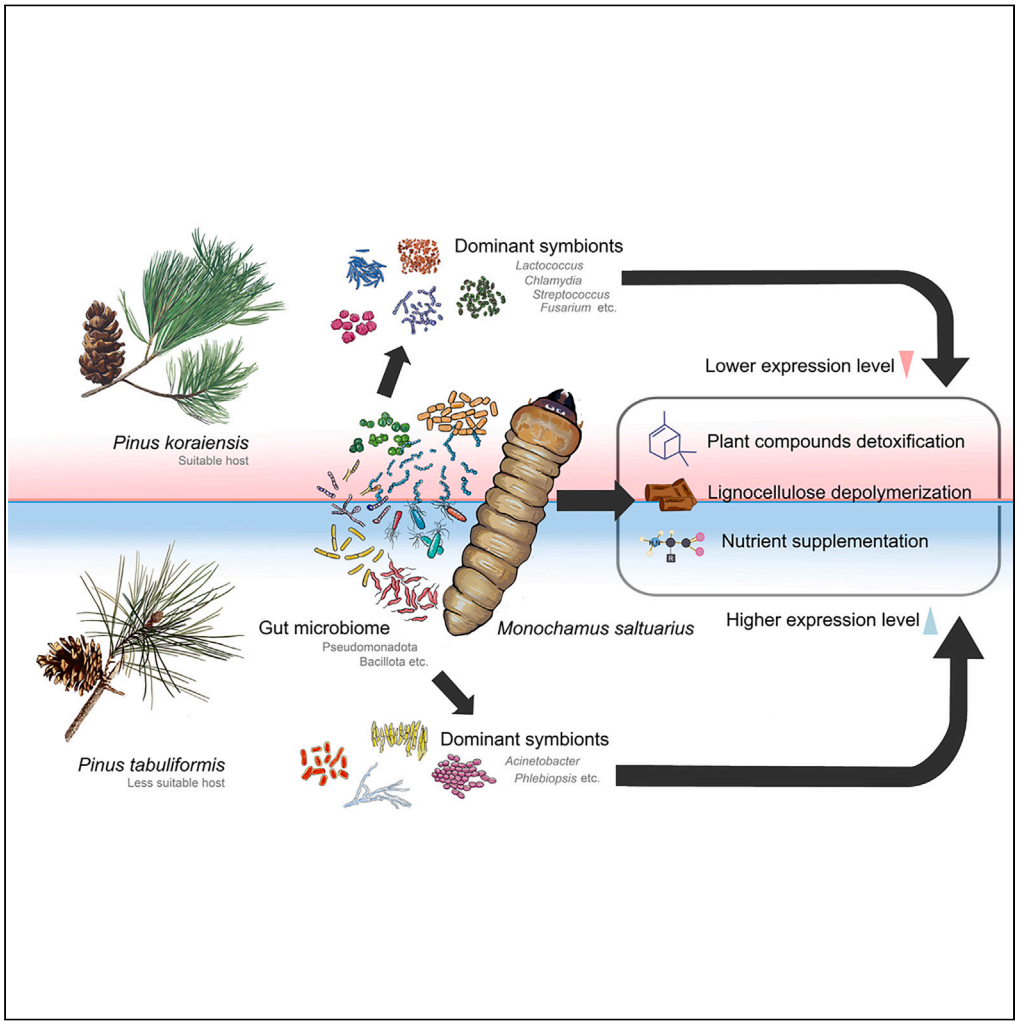

Si-Xun Ge, Tian-Feng Li, Li-Li Ren, Shi-Xiang Zong

lily_ren@bjfu.edu.cn (L.-L.R.)
zongshixiang@bjfu.edu.cn (S.-X.Z.)

Highlights

Parallel metatranscriptome analysis of *M. saltuarius* larvae and its gut microbiome

Active gut microbiota of *M. saltuarius* is limited and facultative

M. saltuarius and its gut microbiome collaborate in host plant adaptation

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Host-plant adaptation in xylophagous insect-microbiome systems: Contributionsof longicorns and gut symbionts revealed by parallel metatranscriptome

Si-Xun Ge,[1] Tian-Feng Li,[1] Li-Li Ren,[1,2,3,*] and Shi-Xiang Zong[1,2,*]

SUMMARY

Adaptation to host plants is of great significance in the ecology of xylophagous insects. The specific adaptation to woody tissues is made possible through microbial symbionts. We investigated the potential roles of detoxification, lignocellulose degradation, and nutrient supplementation of *Monochamus saltuarius* and its gut symbionts in host plant adaptation using metatranscriptome. The gut microbial community structure of *M. saltuarius* that fed on the two plant species were found to be different. Plant compound detoxification and lignocellulose degradation genes have been identified in both beetles and gut symbionts. Most differentially expressed genes associated with host plant adaptations were up-regulated in larvae fed on the less suitable host (*Pinus tabuliformis*) compared to larvae fed on the suitable host (*Pinus koraiensis*). Our findings indicated that *M. saltuarius* and its gut microbes respond to plant secondary substances through systematic transcriptome responses, allowing them to adapt to unsuitable host plants.

INTRODUCTION

Phytophagous insects inhabit various ecological niches with considerable species diversity.[1–3] Adaptation to diverse host plants may be partly responsible for their success and diversity.[4–6] Within these herbivores, wood boring beetles are a unique group which play important roles in forest ecosystems, but also cause massive economic losses. These wood borers exploit a highly specific niche, but have to cope with the challenge of feeding on woody tissues with poor nutrition and abundant secondary compounds.[7–9] These insects have evolved a number of mechanisms as part of host adaptation, to facilitate the degradation of plant secondary chemicals.[10–12] Physicochemical properties of the digestive tract of wood-feeding beetles, such as kinetics and thermodynamics facilitate metabolic processes such as lignocellulose oxidation, hydrolysis, fermentation and acetogenesis.[13,14]

Insects and their microbiome are involved in complex interactions and which have been receiving extensive research attention recently. Many studies have shown that the relationships are variable, sometimes species-specific. Studies investigating the gut microbiota of xylophagous beetles have also indicated that the microbial communities may either be variable or conserved according to species.[15,16] Some have formed stronger mutualistic associations, whereby these insects supplement their nitrogen content and acquire sterols from their microbial associates, to promote their development and survival.[17–19] Conversely, another group of insects have not formed intimate associations with their symbionts, but maintain a consistent microbiota.[20] Advances in amplification sequencing technology has enabled the description of many xylophagous insect microbiomes. The contributions of insect-associated microbes to the host's protection, nutrition, and detoxification have been repeatedly highlighted.[21,22] However, there is a strong need to conduct more in-depth research on these associates to determine whether and how they benefit the insect host. Also, few studies have simultaneously compared the insect's endogenous detoxification and digestion mechanisms versus those attributed to their gut microbiota, to determine their potential contributions to the insect's host plant adaptation.[23,24]

The Japanese pine sawyer, *Monochamus saltuarius* (Coleoptera: Cerambycidae) is considered a serious forest pest in Eurasia because of its catastrophic impact on conifers. This beetle is an oligophagous insect that feeds on a range of conifer species and also serve as a vector for an invasive phytopathogenic

[1]Beijing Key Laboratory for Forest Pest Control, Beijing Forestry University, Beijing 100083, P. R.China

[2]IFOPE, Sino-French Joint Laboratory for Invasive Forest Pests in Eurasia, Beijing Forestry University and INRAE URZF, Beijing 100083, P. R.China

[3]Lead contact

*Correspondence: lily_ren@bjfu.edu.cn (L.-L.R.), zongshixiang@bjfu.edu.cn (S.-X.Z.)

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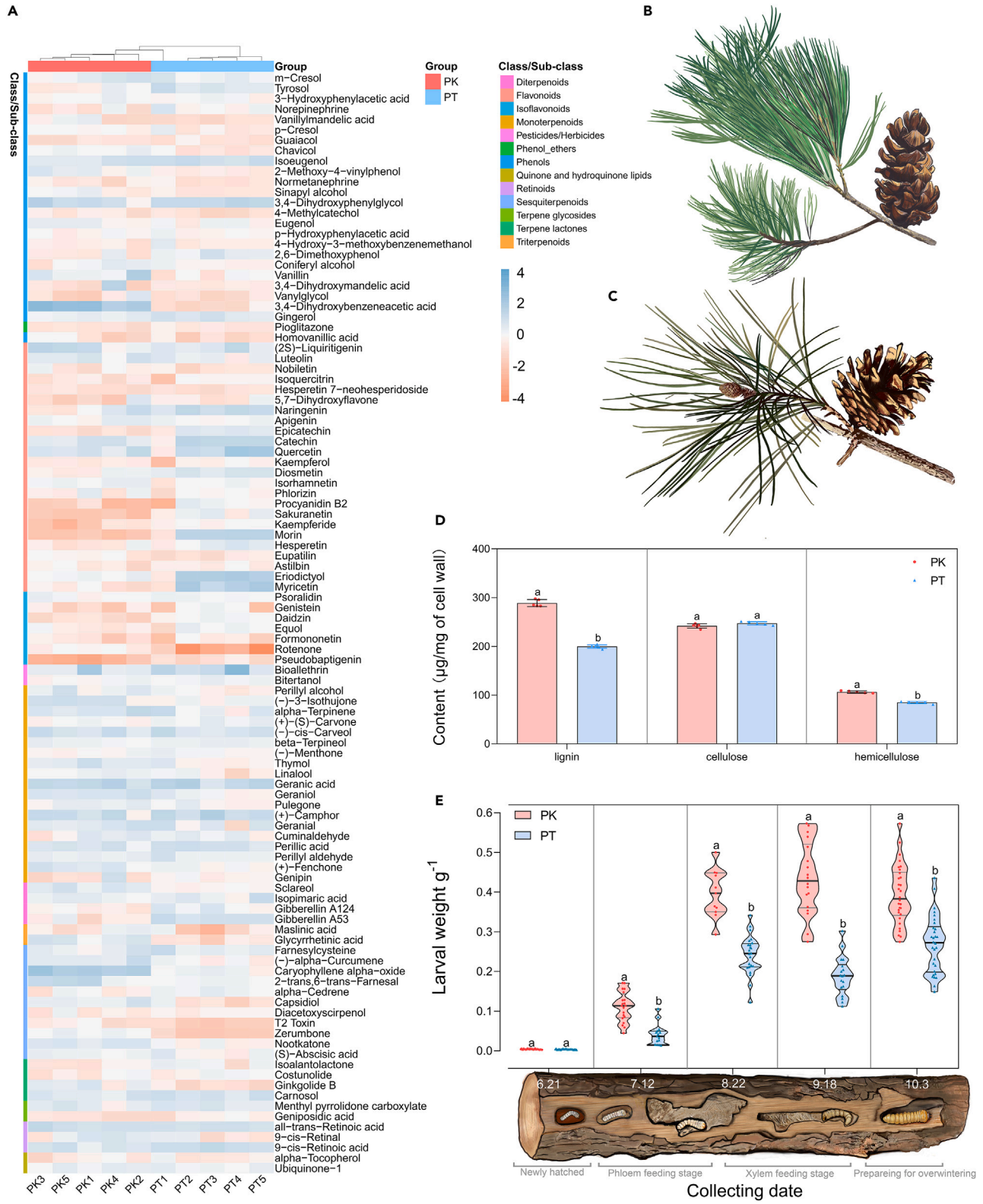

Figure 1. Two host tree species with diverse compounds. *M. saltuarius* larvae showed poor weight gain on *P. tabuliformis*

(A) Heatmap of relative metabolite abundance in phloem of different host tree species. Hierarchical clustering based on complete-linkage (PK: *P. koraiensis* phloem; PT: *P. tabuliformis* phloem).

Figure 1. *Continued*
(B) The host plant *P. koraiensis*.
(C) The host plant *P. tabuliformis*.
(D) Difference in lignocellulose content in phloem of two tree species. Different lowercase letters indicate significant differences between groups (Multiple t test, $p < 0.05$).
(E) Comparison of weight of larvae that fed on the two hosts. Different lowercase letters indicate significant differences between groups (Multiple t test, $p < 0.05$). (PK: *P. koraiensis* feeding larvae; PT: *P. tabuliformis* feeding larvae).

nematode, *Bursaphelenchus xylophilus*, causing the pine wilt disease.[25–28] Intriguingly, *M. saltuarius* has shown a significantly biased preference for *Pinus koraiensis*, on which it performs better, but worse on the less-suitable host, *Pinus tabuliformis*.[12,21,29] Our previous work based on 16S rRNA gene amplicon sequencing and metabolomic analysis showed that *P. tabuliformis* contained more diverse secondary compounds, with higher concentrations and, a more negative correlation between larvae with established gut bacteria which feeds on this plant and plant defense compounds.[21] However, as an important aspect of host plant adaptation, transcriptomic responses of *M. saltuarius* feeding on different host plants and whether gut microbes benefit *M. saltuarius* are still unknown.

In this study, we used metatranscriptome analysis to explore both insect and gut microbe-derived genes expressions, in the degradation of plant secondary chemicals and lignocellulose decomposition. We also compared the transcriptomic responses of insect-microbiome systems of larvae that fed on the two host plants. We used parallel metatranscriptomic sequencing of whole guts of *M. saltuarius* larvae that fed on the phloem of *P. tabuliformis* and *P. koraiensis*, to assess how diet impacted gene expression of both beetles and their gut microbes. We aimed to reveal: (1) the transcriptomic responses of *M. saltuarius* feeding on different host plants, (2) the effect of diet on the gut microbiota of *M. saltuarius* (3) predict the contribution of gut-associated microbes to the decomposition of lignocellulose and detoxification of plant secondary metabolites.

RESULTS

M. saltuarius gained poor weight from feeding on *P. tabuliformis* than on *P. koraiensis*

A total of 8,785 metabolites were captured in all phloem samples with 793 annotated (Table S1). We identified five classes associated with substances potentially toxic to insects (Figure 1A; Table S2): phenols and phenol ethers (26 substances); flavonoids (24 substances); isoflavonoids (7 substances); pesticides/herbicides (2 substances) and prenol lipids which included numerous terpenoids (47 substances). The relative abundance of metabolites in the above-mentioned classes was clustered according to tree species. Potential toxic compounds of the two tree species (Figures 1B and 1C) had different metabolic patterns, except the sample PT1 was relatively close to the PK group. (Figure 1A). Both lignin and hemicellulose in the phloem of *P. koraiensis* were significantly higher than those of *P. tabuliformis* (Figure 1D; multiple t test, $p < 0.0001$; Table S3), with no significant difference in cellulose content (Figure 1D; multiple t test, $p = 0.066$; Table S3). For larvae that fed on different host tree species (Figures 1B and 1C), no significant difference was observed in body weight, at the newly hatched stage (Figure 1E; Multiple t test, $p = 0.973$; Table S4), but body weight of *P. koraiensis* foragers were significantly heavier in all follow up stages (Figure 1E; Multiple t test, $P < 0.05$; Table S4).

Transcriptomic responses of *M. saltuarius* larvae that fed on different host plants

General profile and annotated genes in metatranscriptome analysis

For metatranscriptome analysis, about 6.72 (6.09–7.51) gigabases (Gb) of sequence data in 1.22×10^8 (1.07×10^8-1.38×10^8) reads were obtained from each pooled *M. saltuarius* gut sample. Across the 9 pooled samples, we obtained an average of 4.03×10^7 reads from beetles and an average of 1.3×10^7 reads from gut microbiota (Table S5). In total, 11,658 genes were predicted in *M. saltuarius*, with an average of 9,161.4 (9,131-9,222) in the PK group and 11,517.2 (11,363-11,658) in the PT samples. (Table S6).

Differentially expressed genes in M. saltuarius guts

A total of 2,618 DEGs of *M. saltuarius* were annotated in at least one of the nine databases. (PT versus PK 134 down-regulated, 2,484 up-regulated. Figure 2A, Table S7). We identified genes associated with lignocellulose depolymerization and detoxification enzymes: 311 genes coding glycoside hydrolases involved in the degradation of cellulose and xylan were predicted in all samples (84 DEGs, PT versus PK: 9 down-regulated; 75 up-regulated, Table S8). Three genes involved in lignin catabolic process were identified (1 DEG,

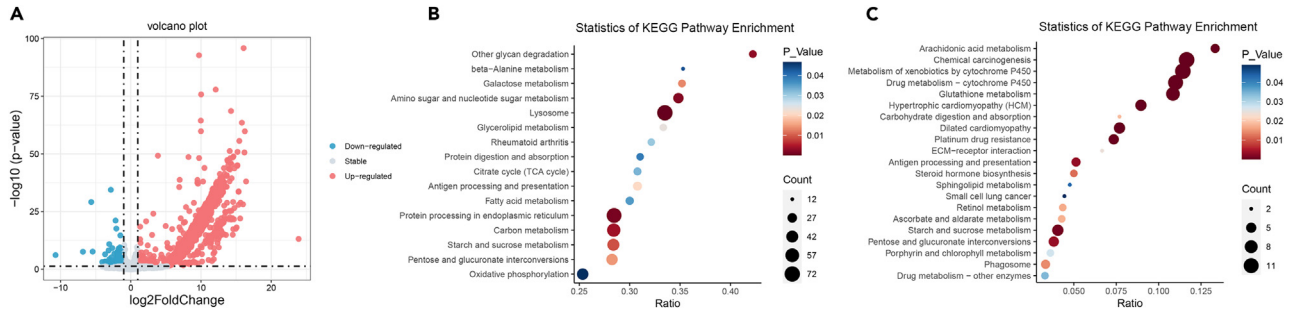

Figure 2. Transcriptomic responses of *M. saltuarius* gut from feeding on different host plants

(A) Volcano plot of insect-derived DEGs between PT versus PK (|log2FC|>1 and q value ≤0.05).

(B) PT versus PK: Enriched up-regulated genes in KEGG pathways.

(C) Enriched down-regulated genes in KEGG pathways. The color of the point represents p value; smaller p value means the enrichment was more significant.

PT versus PK: up-regulated, Table S9). For multigene families associated with detoxification enzymes, there were 88 cytochrome P450s (P450s) (33 DEGs, PT versus PK: 5 down-regulated; 28 up-regulated, Table S10), 40 carboxyl/cholinesterases (CCEs) (16 DEGs, PT versus PK: 3 down-regulated; 13 up-regulated, Table S11), 32 glutathione S-transferases (GSTs) (16 DEGs, PT versus PK: 8 down-regulated; 8 up-regulated, Table S12), 19 UDP-glycosyltransferases (UGTs) (7 DEGs, PT versus PK: 2 down-regulated; 5 up-regulated, Table S13) and 21 ATP binding cassette transporters (ABCs) (5 DEGs, PT versus PK: all up-regulated, Table S14) were annotated totally.

Differentially expressed genes are enriched to different functions according to different hosts

1,919 DEGs were annotated using the GO database, from the data obtained from *M. saltuarius* that fed on two host plants (Figure S1; PT versus PK: 78 downregulated, 1,841 upregulated). The enriched GO terms were mainly associated with catalytic activity, cellular processes, metabolic processes, binding, cell part and membrane. In the parallel analysis based on the KEGG database, we found 1,548 DEGs of guts between *M. saltuarius* that fed on different hosts (PT versus PK: 62 down-regulated, 1,486 up-regulated). When Compared PT versus PK diet, up-regulated DEGs were mainly enriched other glycan degradation, signal transduction and carbohydrate metabolism pathways, with 16 terms of significance identified (Figures 2B and S2A). Accordingly, the down-regulated DEGs on PT diet were mainly enriched xenobiotics biodegradation, metabolism and carbohydrate metabolism pathways (Figure S2B), with 21 significantly enriched terms identified (Figure 2C).

Correlations between M. saltuarius gut DEGs and host plant differential metabolites

Correlation analysis between insect and microbe-derived DEGs and differential secondary metabolites of host trees were performed. The down-regulated DEGs of both insect-derived and microbe-derived were positively correlated with the down-regulated differential metabolites, for each host plant species. Similar correlations were also observed for up-regulated DEGs and metabolites (Figure S3; Table S15).

Effects of hosts on *M. saltuarius* gut microbiota

Composition and abundance of gut microbiota in M. saltuarius fed on different host

Among the non-redundant microbial genes, 91.54% sequences were from bacteria (94.93% of PK; 87.29% of PT), 2.51% were from fungi (1.89% of PK; 3.28% of PT), and 5.95% were from viruses (3.18% of PK; 9.42% of PT) (Table 1).

The metatranscriptome results indicated that a limited number of transcriptional active microbe genera occurs in *M. saltuarius* gut. However, the results showed rather diverse gut microbes of *M. saltuarius*, according to different host trees (Figures 3 and S4; Table S16). Among all groups identified, bacteria dominated all samples. There were 3 bacterial phyla in the guts of *M. saltuarius*, with the most abundant phyla being Pseudomonadota (99.11% in total; 98.87% of PK; 99.41% of PT; Mann-Whitney test, $p = 0.286$), followed by Bacillota (0.74% in total; 0.89% of PK; 0.55% of PT; Mann-Whitney test, $p = 0.413$) and Chlamydiota (0.15% in total; 0.24% of PK; 0.04% of PT; Mann-Whitney test, $p = 0.016$). Another diverse kingdom was fungi, with 2 phyla identified, including Ascomycota (4.77% in total; 88.06% of PK; 14.65% of

Table 1. Comparison of the relative abundance of gut microbes per sample (PK: gut of *P. koraiensis* foragers; PT: gut of *P. tabuliformis* foragers)

		Percentage (%)		
Group	Sample ID	Fungi	Bacteria	Virus
PK	PK1	1.76	95.60	2.64
	PK2	1.62	91.28	7.09
	PK3	1.70	95.17	3.13
	PK4	1.77	96.76	1.48
	PK5	2.60	95.86	1.54
PT	PT1	2.95	86.29	10.76
	PT2	2.17	72.12	25.71
	PT3	5.93	93.94	0.13
	PT5	2.08	96.84	1.08

PT; Mann-Whitney test, p = 0.032) and Basidiomycota (95.23% in total; 11.94% of PK; 85.35% of PT; Mann-Whitney test, p = 0.032). There were 2 phyla of viruses, with one unclassified (97.55% in total; 98.17% of PK; 96.77% of PT; Mann-Whitney test, p = 0.318) and another Negarnaviricota (2.45% in total; 1.83% of PK; 3.23% of PT; Mann-Whitney test, p = 0.318).

Generic diversity was comparatively high. In total, 14 bacterial genera were identified among all samples (Figure 3C), with the majority of sequences (>2% of all bacterial sequences) being from a few genera: *Acinetobacter* (55.91% in total; 23.26% of PK; 96.72% of PT; Mann-Whitney test, p = 0.016), *Lactococcus* (16.62% in total; 28.8% of PK; 1.4% of PT; Mann-Whitney test, p = 0.016), *Chlamydia* (8.45% in total; 15.09% of PK; 0.14% of PT; Mann-Whitney test, p = 0.016), *Streptococcus* (6.15% in total; 10.73% of PK; 0.43% of PT; Mann-Whitney test, p = 0.016), *Escherichia* (4.39% in total; 7.51% of PK; 0.5% of PT; Mann-Whitney test, p = 0.016), *Shigella* (2.81% in total; 4.89% of PK; 0.2% of PT; Mann-Whitney test, p = 0.016), and *Klebsiella* (2.17% in total; 3.82% of PK; 0.11% of PT; Mann-Whitney test, p = 0.016). We found a total of 8 genera of fungi (Figure 3C), consisting of (>2% of all fungal sequences) *Fusarium* (6.32% in total; 30.6% of PK; 2.64% of PT; Mann-Whitney test, p = 0.016), *Phlebiopsis* (69.49% in total; 0 of PK; 80.02% of PT; Mann-Whitney test, p = 0.008), *Colletotrichum* (2.02% in total; 4.92% of PK; 1.58% of PT; Mann-Whitney test, $P > 0.05$), *Erysiphe* (20.44% in total; 62.2% of PK; 14.11% of PT; Mann-Whitney test, p = 0.556). The viruses belonged to 6 genera (Figure 3C), mainly including (>2% of all the Virus sequences) *unclassified_k_viruses* (85.93% in total; 94.7% of PK; 83.07% of PT; Mann-Whitney test, p = 0.905), *unclassified_f__*Leviviridae (5.72% in total; 0.11% of PK; 12.73% of PT; Mann-Whitney test, p = 0.064), and Alphainfluenzavirus (2.32% in total; 1.59% of PK; 3.23% of PT; Mann-Whitney test, p = 0.603).

Within-habitat diversity and community structure of gut microbes of M. saltuarius that fed on different host trees

The significant differences among all three α-diversity indicators (Shannon, Simpson and Inv-simpson) were only observed in bacteria among the different diet treatments (Figure 3A). For the fungal and viruses dataset, no significant difference was observed in α-diversity among the treatments for all indicators.

The PCoA on the microbiota data (bacteria, fungi and viruses) at the genus level showed that the two diet treatments were distributed in diverse quadrants (Figure 3B). ANOSIM analysis identified significant differences among bacterial genera (ANOSIM, R = 1, p = 0.007), fungal genera (ANOSIM, R = 0.556, p = 0.008) and viral genera (ANOSIM, R = 0.35, p = 0.036).

Functional annotation of gut microbiota in host plant adaptation

In total, 3,424 non-redundant microbial genes were identified in all samples. The PK diet group had an average of 2,476.8 (2433–2507) genes and PT diet group, 2,989.5 (2942–3036) genes. Of these genes, 2,865 were annotated to the nr database and 2,041 genes to the eggNOG database (Table S17).

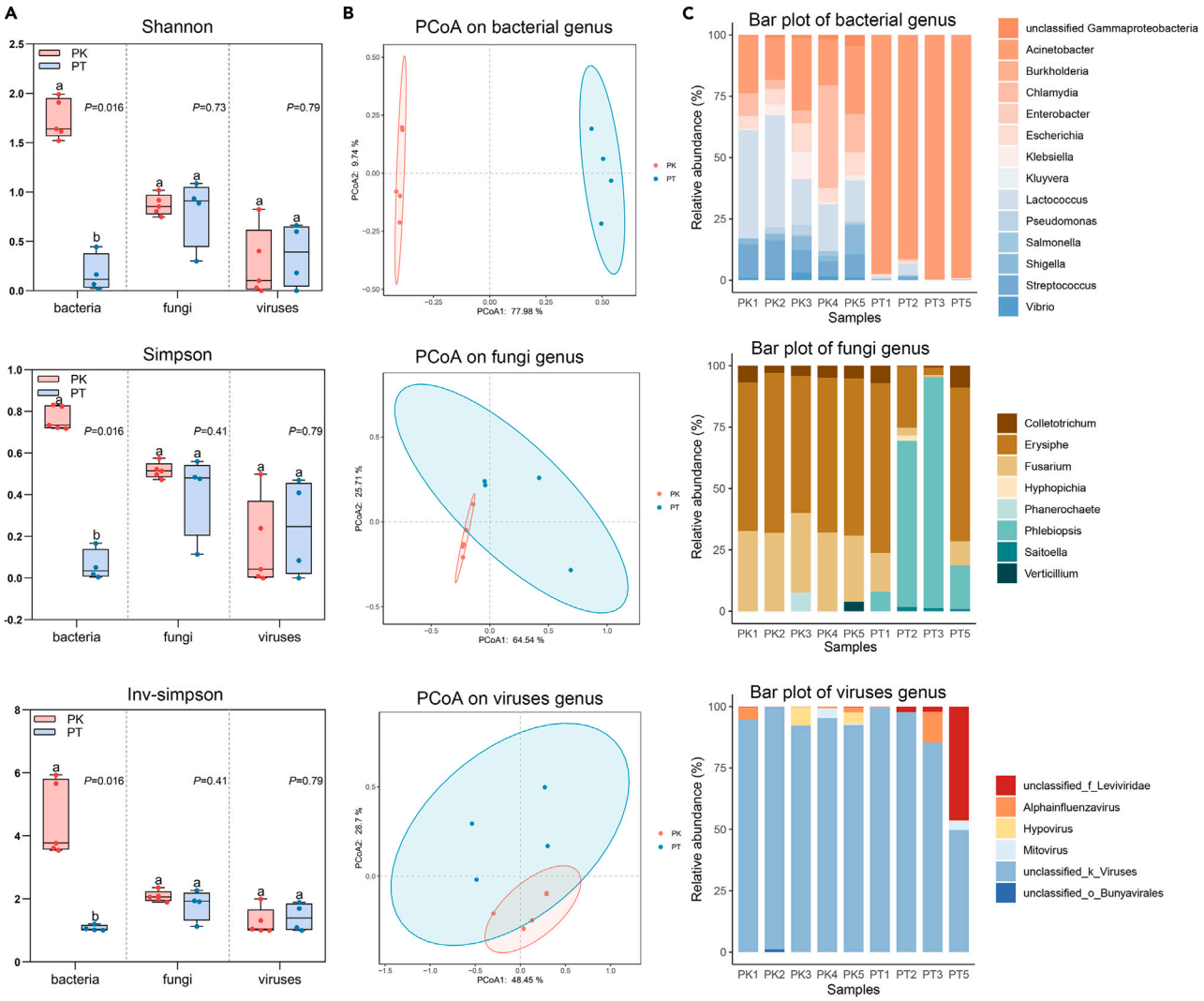

Figure 3. Effects of host plant species on gut microbiota of *M. saltuarius*

(A) comparisons of α-diversity based on Shannon, Simpson and Inv-simpson indexes between gut microbes of beetles that fed on different hosts. Different lowercase letters indicate significant differences between groups (Mann–Whitney test, $p < 0.05$).

(B) Comparison of β-diversity based on bray-curits distance between bacterial, fungi and viruses communities of beetles that fed on different hosts.

(C) Relative abundance of bacterial, fungi and viruses genera of beetles that fed on different hosts. The relative percent abundance of microbial genera is represented by different colors.

β-diversity of M. saltuarius gut microbes function

Based on the KEGG Orthology (KO) dataset, a PCA revealed clear overlapping of the predicted bacterial function according to different plant diet groups. The first principal component explained 57.88% of the total variance and the second principal component explained 15.79% of the variance (Figure 4A). In terms of fungal functions, the ellipses were clearly separated according to host diet, with the first principal component explaining 78.78% of the total variance, and the second principal component explaining 10.49% of the variance (Figure 4B). ANOSIM analysis indicated a significant difference between different host diet groups for fungi (ANOSIM, $R = 0.4625$, $p = 0.007$), but no significance difference for bacteria (ANOSIM, $R = 0.00625$, $p = 0.359$).

Differentially expressed M. saltuarius gut microbial genes

A total of 630 DEGs were identified in all annotated microbial genes (PT versus PK, 44 down-regulated; 586 up-regulated; Figure 4C). Among them, we identified several gene groups that may be involved in

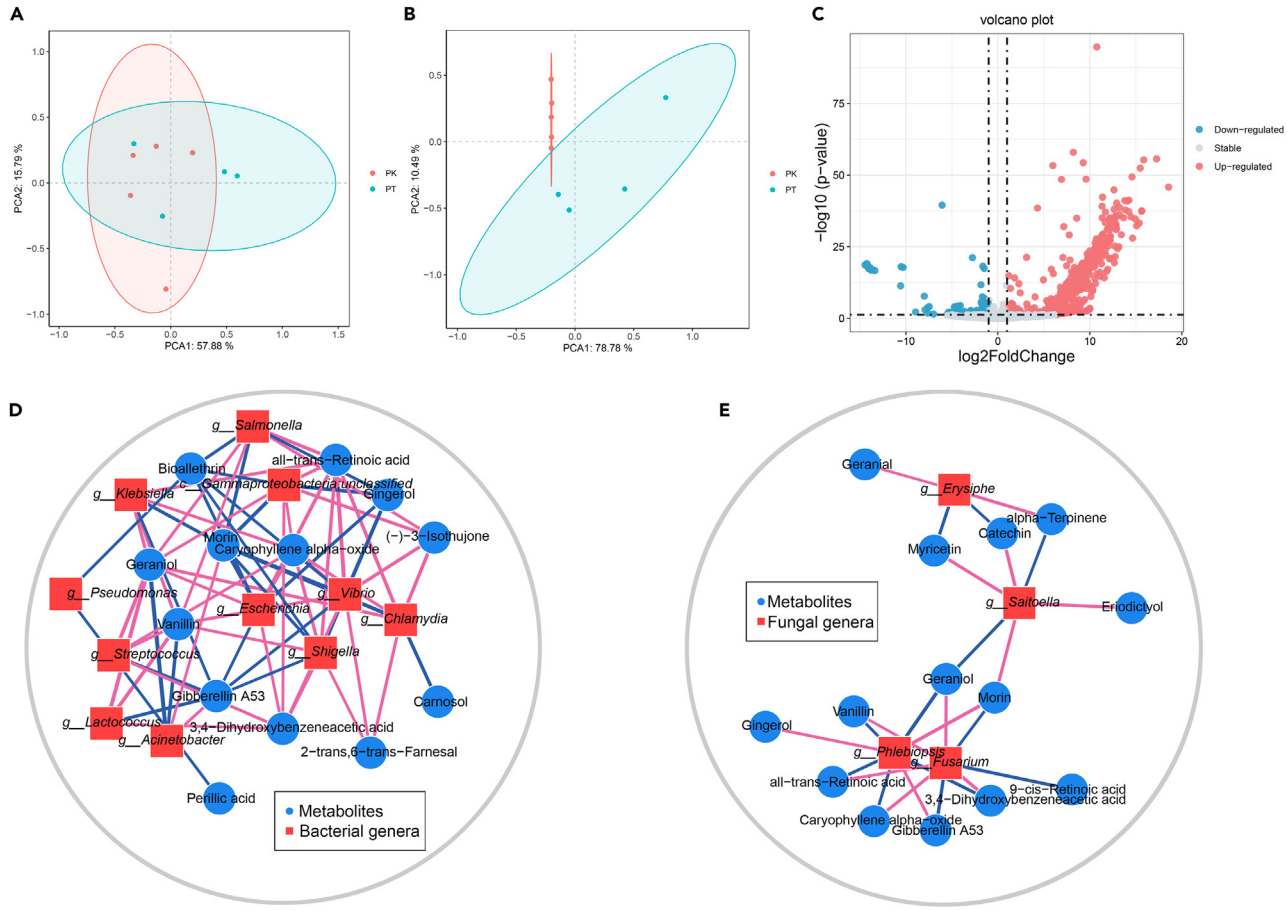

Figure 4. Potential function of *M. salturaius* gut microbiota in host plant adaptation

(A) Comparisons of β-diversity (Hellinger distance) predicted bacterial functions of beetles that fed on different hosts.

(B) Comparisons of β-diversity (Hellinger distance) based on predicted fungal functions in beetles that fed on different hosts.

(C) Volcano plot of microbial-derived DEGs compared between PT versus PK (log2FC|>1 and q value ≤0.05).

(D) Correlation network between bacterial genera and plant secondary metabolites, with only significant correlations presented (adjusted p value ≤0.05).

(E) Correlation network between fungal genera and plant secondary metabolites, with only significant correlations presented (adjusted p value ≤0.05).

detoxification, digestion and nutrition supplementation in their insect hosts. Comparison of PT versus PK, identified 14 DEGs within 45 genes coding glycoside hydrolases (1 down-regulated, 13 up-regulated Table S18). No DEG was found for the 9 genes involved in the benzoate degradation-related pathways (Table S19). 5 DEGs were identified as up-regulated within 68 genes that were associated with nitrogen, amino acid, carbohydrate and lipid metabolisms (Table S20).

Functional enrichment of M. saltuarius gut microbes

A total of 2,107 and 1,686 genes were identified in GO and KEGG databases, respectively, with 256 KEGG pathways annotated (Table S17). Comparison between PT and PK showed that a few pathways were enriched with down-regulated genes (Figure S5A). However, many pathways that were enriched with up-regulated genes were associated with microbial infections (Figure S5B). The enriched GO terms were mainly associated with binding, catalytic activity, cellular processes and metabolic processes (Figure S6).

Relationship between microbial DEGs and host plant differential metabolites

Spearman's correlations between microbial-derived DEGs (up-regulated and down-regulated) and differential secondary metabolites of host tree species were calculated. DEGs and differential metabolites showed many positive correlations between groups (Figure S7; Table S21).

Correlations between M. saltuarius gut microbes and differential host plant metabolites

We also analyzed correlations between microbial datasets and potentially toxic plant secondary metabolites. Bacterial genera such as *Streptococcus*, *Escherichia*, *Shigella* and *Chlamydia* showed positive correlations with these compounds, whereas *Acinetobacter* and *Pseudomonas* negatively correlated with these compounds (Figure 4D). A total of four fungal genera showed significant correlations with plant secondary compounds: i.e., *Phlebiopsis*, *Saitoella*, *Fusarium*, and *Erysiphe* (Figure 4E).

DISCUSSION

The interactions between insects and microbes form much of the basis for their success in diverse ecosystems. By partnering with symbionts, insects can enhance their host adaptability, by exploiting new resources and habitats. In this study, we employed a parallel metatranscriptomic analysis to evaluate: (1) The impact of host diet on the diversity of the microbiome, (2) the differences in insect and microbe-derived gene expressions involved in detoxification and lignocellulose degradation when the insect fed on different hosts.

Core gut microbiota of *M. saltuarius* is limited and facultative

As a model of great ecological significance, interactions between insects and their microbial symbionts have been widely studied. Some interaction patterns have shown strong reliance, resulting in highly interwoven systems with extreme genome reduction of symbionts.[30–32] However, based on our results and previous studies,[21,33,34] no intracellular symbionts were identified in *M. saltuarius* guts or even the genus *Monochamus*. This indicated that interactions between *Monochamus* and gut microbes are mostly facultative, because these insects have not established intimate associations with their symbionts nor maintain a consistent microbiota.[20] In contrast with our previous findings on xylem feeding larvae,[21] metatranscriptome analysis showed that gut microbes of phloem feeding larvae showed a limited diversity. Several hypotheses might explain the above findings: (1) Starve treatment not only eliminates the contamination of plant tissue, but also removes some transient microbes, which may not play roles in the insect guts. (2) A large number of studies have indicated that the gut microbial communities of phloem-feeding beetles are strikingly species-poor relative to those of xylem-feeding insects.[15,35–37] This indicates the differences existent in insect dietary requirements and the toxic defensive chemicals encountered between conifer phloem and xylem. (3) As most works were based on 16S rRNA gene sequencing analysis, quiescent and dead microbiota (non-transcriptionally active) could not be ruled out from gut microbes.[38]

Host tree species influence *M. saltuarius*'s gut microbiota

The driving effect of host plant species on the gut microbiota of phytophagous insects has been extensively studied (e.g. Berasategui et al.[8]; Santos-Garcia et al.[39]). Similar to previous studies,[21] our results showed that the structure of the microbial community found in the *M. saltuarius* gut was heterogeneous according to host tree species and with higher α-diversity in *P. koraiensis* feeders. In addition, comparison of PT versus PK, identified many insect and microbiota-derived genes which were up-regulated, indicating systematic responses of insect and symbionts to a less suitable host plant. This emphasizes the influence of diet and the gut microbiome, on host adaptation.

Relationship between the taxonomic composition and functions of *M. saltuarius* gut microbes

Results showed that diverse microbial communities contribute to *M. saltuarius* digestion and detoxification of host plants. Many plant compounds identified in this study (e.g. terpenoids, flavonoids, phenolic acids, glycosides and lignocellulose) could be degraded by related microorganisms identified in *M. saltuarius* guts. For example, bacterial genera such as *Enterobacter*, *Pseudomonas* and *Klebsiella*, as well as the fungal genus, *Fusarium,* have been identified to be involved in phenolic acids degradation.[40] The bacterial genera, *Pseudomonas* and *Burkholderia* were identified to be involved in terpenes degradation[15,41]; the genus *Acinetobacter* was reported to have a strong capacity for glycosides degradation.[42] In addition, the wood-decaying fungal genus *Phlebiopsis* and the genus *Fusarium* associated with lignocellulose degradability were also identified as dominant groups in *M. saltuarius* guts.[43,44] Of interest, many of these microbes showed significant correlations with plant compounds, suggesting a functional role of helping insects to cope with host plants. Notably, comparison of taxonomic annotations showed that significant differences in the bacterial datasets existed between PK and PT groups. However, no significant difference between groups was observed when bacterial functions were compared. This indicates that larvae that fed on different diets have taxonomically heterogeneous gut bacteria, but which are functionally homogeneous. It further indicates that larvae that encounter different host plant could recruit different gut bacteria with similar functions to provide some common benefits.

Differences in insect-microbiome system involved in detoxification, lignocellulose degradation and nutrient absorption of plant compounds of different hosts

Many DEGs were identified between beetle-microbiome system on different host plants. Compared with larvae that fed on *P. koraiensis* (the suitable host plant), many DEGs showed up-regulation in *P. tabuliformis* foragers, whether they were insect-derived or microbial-derived. A large amount of positive correlation between differential plant metabolites and insect-microbial systems DEGs were identified. This indicated that both insects and microorganisms were involved in host plant adaptations. Among the insect-derived DEGs, detoxification-related genes were important, including P450s, CCEs, GSTs, UGTs, and ABCs. Many of these DEGs are involved in the detoxification of plant defensive chemicals.[45–47] Comparison between PT versus PK showed that more genes were up-regulated on PT diet, suggesting that insects show specific transcriptional responses to chemicals derived from a less suitable host plant.

Genes involved in the detoxification of plant compounds were also identified from microbial sources, especially genes involved in the benzoate degradation-related pathways. These genes are involved in degradation of insecticidal compounds, such as phenolic and flavonoids.[11] Intriguingly, no DEG was identified within the above-mentioned pathways, indicating that microbes may be involved in the detoxification of chemicals, but may not be specific according to host plant species.

The lignocellulose degradation of wood-boring insects and their gut microbiome has been repeatedly reported, especially in longicorns.[48,49] Similar to the results of previous studies, in our results, genes coding glycoside hydrolases involved in the degradation of cellulose and xylan were annotated in both insect and microbiota data. These glycoside hydrolases included enzymes such as endocellulases (families GH5, 9, and 48), endoxylanases (families GH5, 10, 11, 30 and 43), cellobiohydrolases (families GH5 and 9), cellodextrinases (families GH1), β-glucosidases (families GH1 and 116), β-xylosidases (families GH30, 43 and 116) and lytic polysaccharide monooxygenases (family AA10). Families of glycoside hydrolases involved in the degradation of other wood polysaccharides, such as arabinan, mannan, glucomannan and galactomannan, were also detected, including α-*l*-arabinofuranosidase (GH43), β-mannosidases (GH1, 2 and 5) and β-mannanases (GH5 and 26). Most of the above-mentioned genes were up-regulated on PT than PK. This was highly related to the difference in lignocellulose content of phloem from the two hosts. The significantly lower lignin and hemicellulose contents in *P. tabuliformis*, may have influenced both insects and their associated microbes to enhance their lignocellulose utilization capacity. In addition, only 3 genes coding for laccase involved in lignin degradation were identified, which indicated that potential ectosymbioses such as fungi with lignin degradation capacity exist on the body surfaces of insects or galleries.[22] Differences in DEGs identified between different host plants also reflect the contributions of the insect host and microbes to digestion and nutrient absorption. In terms of insect-derived DEGs, genes involved mainly in arachidonic acid metabolism, carbohydrate digestion and absorption as well as other glycan degradation showed enrichment. Similarly, microbial-derived DEGs were enriched in carbohydrate, amino acid and lipid metabolism. These results indicated that both insects and their gut microbes participated in nutrient metabolism that could help the insect-microbiome system contend with host plant derived stress factors.

Besides, the detection of viral genes in both host plants fed larvae may show the potential relationships between viruses and insects. Various hypotheses might explain the above findings. For example, the up-regulated DEGs of microbial-derived (PT versus PK) were enriched in several pathways associated with microbial infections. The abundance of viruses detected in the guts of *P. tabuliformis* foragers was much higher than that of *P. koraiensis* foragers. This implied that larvae may face a higher risk of entomopathogenic infections on unsuitable host plants. Another explanation for the findings is that these genes were originated from viruses that lacks a complete genome and cannot replicate or highlighted the possibility of horizontal gene transfer between viruses and *M. saltuarius*. The occurrence of antagonistic conflict among members of the insect-microbiome systems highlighted a divergence of selective interests among host-microbiome communities.[50] Thus, we employed the concept of "insect-microbiome systems" to describe the versatile host-microbiome communities, rather than holobionts.

Limitations of the study

1. The results based on metatranscriptome could highlight the potential role of insects and associated microbiomes at a specific period of time. It is still necessary to verify whether a group of insect genes or a microbial species can exact perform specific functions.

2. We consolidated our conjecture through correlation analysis, however, correlation did not directly represent causality. Functional verification tests for certain genes and important bacteria strains are also under way and will be published in the future.
3. The insect microbiome could be rather variable, our results only represent a certain stage of interactions between wood-feeding beetles and their microbes. More generalized results of wood feeding beetle-microbiome interactions require more in-depth and comprehensive research.

Conclusions

This study employed a metabolomic analysis of host plant compounds and a parallel metatranscriptome to evaluate an insect-microbiome system in the adaptation to an unfavorable host. Our results suggested that host plant species influenced insect gut microbial community. Both insects and microbes responded to the unsuitable plant by differential gene expressions, especially in terms of detoxification of plant secondary compounds, lignocellulose degradation and nutrient supplementation. Our results supported the hypothesis that *M. saltuarius* cope with plant secondary metabolites and lignocellulose by showing plasticity in gene expression responses. It also emphasized the role of microbes in the digestion and detoxification of plant tissues in their insect hosts. Through joint analysis on both insects and microorganisms, we illuminated how the host-microbe systems play roles according to diverse plant diets. The cooperation between insects and microorganisms enhances the overall adaptability of the insect-microbiome system.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106680.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.S.X., R.L.L., and Z.S.X.; Methodology, G.S.X. and R.L.L.; Software, G.S.X.; Formal Analysis, G.S.X.; Investigation, G.S.X. and L.T.F.; Resources, G.S.X., L.T.F., and Z.S.X.; Writing – Original

Draft, G.S.X.; Writing – Review and Editing, G.S.X., R.L.L., and Z.S.X.; Visualization, G.S.X. and R.L.L.; Supervision, R.L.L. and Z.S.X.; Funding Acquisition, R.L.L. and Z.S.X.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
M. saltuarius larvae feed on *P. koraiensis*	This paper	N/A
M. saltuarius larvae feed on *P. tabuliformis*	This paper	N/A
Phloem tissues of *P. koraiensis*	This paper	N/A
Phloem tissues of *P. tabuliformis*	This paper	N/A
Critical commercial assays		
E.Z.N.A.® Soil RNA Midi Kit	Omega Bio-tek, Norcross, GA, U.S.	Cat# R6825
Ribo-zero Magnetic kit	Epicentre, an Illumina® company	Cat# MRZSR116
TruSeq™ RNA sample prep kit	Illumina	Cat# RS-122-2001
Deposited data		
Raw data of metatranscriptome	NCBI database	PRJNA907471
LC-MS metabolomics data	This paper	Tables S1 and S2
Software and algorithms		
ProteoWizard software package	Smith et al.[51]	v3.0.8789
ImageGP	Chen et al.[52]	N/A
Fastp	Chen et al.[53]	v0.20.0
MEGAHIT	Li et al.[54]	N/A
MetaGene	Noguchi et al.[55]	N/A
CD-HIT	Fu et al.[56]	v4.6.1
SOAPaligner	Li et al.[57]	N/A
DIAMOND	https://github.com/bbuchfink/diamond	v0.9.19
RSEM	Li et al.[57]	N/A
DESeq2	Love et al.[58]	v1.6.3
Goatools	https://github.com/tanghaibao/Goatools	N/A
KOBAS	Xie et al.[59]	N/A
pheatmap	Kolde[60]	N/A
Igraph	Kolde[60]	N/A
GraphPad Prism 8	San Diego, CA, United States	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Li-Li Ren (lily_ren@bjfu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Metatranscriptome data has been deposited at NCBI and are publicly available as of the date of publication. Accession number is listed in the key resources table.
- All relevant data supporting the findings of this study are available from the lead contact upon request.

- The published article and supplemental information include all data generated and analyzed during this study. This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In this study, we wild collected *M. saltuarius* larvae from two host plants: *P. koraiensis* (PK) and *P. tabuliformis* (PT). In the comparison of larval weight, we randomly selected logs from two host plants at five time points, with all larvae in a log detected. Third instar phloem-feeding stage larvae were selected for metatranscriptomic sequencing. After collected, these larvae were starved for 24 h (completely dark, 25°C, 60% relative humidity) to avoid contamination from diet. The instar of larvae were determined according to the width of head capsule reported in Li et al.[61] Phloem tissues were collected from *M. saltuarius* infested *P. koraiensis* and *P. tabuliformis* logs. All plant tissues were stored at −80°C until use.

METHOD DETAILS

Sample collection and preparation

Larvae of *M. saltuarius* were collected from *P. koraiensis* (PK) and *P. tabuliformis* (PT) in Dahuofang forest farm (Fushun, Liaoning, China). Larvae for weight comparison were collected on different dates in 2021: Jun. 21st (PK: n = 30, PT: n = 25), Jul. 12th (PK: n = 20, PT: n = 15), Aug. 22nd (PK: n = 11, PT: n = 24), Sep. 18th (PK: n = 18, PT: n = 18) and Oct. 3rd (PK: n = 30, PT: n = 30). Larvae for metatranscriptomic sequencing were collected in July, with 25 healthy 3rd instar phloem-feeding stage larvae selected from each host plant (5 pools of 5 larvae each were created to generate 5 biological replicates.) The instar of larvae was determined according to the width of head capsule reported in Li et al.[61] To detect the relative abundance of secondary metabolites, phloem tissues were collected from *M. saltuarius* infested *P. koraiensis* and *P. tabuliformis,* using sterile fine-tipped forceps, with each sample accurately weighed as 50 mg. Five replicate samples were collected for each tree species.

Before dissection, *M. saltuarius* larvae were starved for 24 h to avoid contamination from diet. To obtain the gut, larvae were surface-sterilized with 70% ethanol and 2% bleach for 1 min and rinsed twice with sterile water before dissection. Larvae were dissected under aseptic conditions with sterilized dissection scissors and forceps, and guts from 5 larvae were pooled into one replicate. Each host plant treatment was performed with five biological replicates of 5 pooled larval guts, for a total of 25 larvae per treatment group. (the sample PT4 was excluded from metatranscriptome sequencing because of slight contamination).

LC-MS metabolomics processing

To compare differences in secondary compounds content between host plants, we carried out LC-MS metabolomics for phloem tissues. Phloem samples (50 mg) were accurately weighed, with 600 μL MeOH (stored at −20°C) (containing 2-amino-3-(2-chloro-phenyl)-propionic acid (4 ppm) added, then vortexed for 30 s. After that, 100 mg glass bead were added to the mixture and placed in a tissue grinder for 90 sat 60 Hz, followed by ultrasonication for 15 minat room temperature. After centrifugation at 12,000g and 4°C for 10 min, the supernatants were carefully transferred to sample vials for LC-MS/MS analysis. The LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA). Chromatography was carried out with an ACQUITY UPLC ® HSS T3 (150 × 2.1 mm, 1.8 μm) (Waters, Milford, MA, USA). The column temperature was maintained at 40°C, with the flow rate and injection volume set at 0.25 mL/min and 2 μL, respectively. For LC-ESI (+)-MS analysis, the mobile phases consisted of (B2) 0.1% formic acid in acetonitrile (v/v) and (A2) 0.1% formic acid in water (v/v). Separation was conducted as the following gradient: 0∼1 min, 2% B2; 1∼9 min, 2%∼50% B2; 9∼12 min, 50%∼98% B2; 12∼13.5 min, 98% B2; 13.5∼14 min, 98%∼2% B2; 14∼20 min, 2% B2. For LC-ESI (-)-MS analysis, the analyses were carried out with (B3) acetonitrile and (A3) ammonium formate (5 mM). Separation was conducted as the following gradient: 0∼1 min, 2% B3; 1∼9 min, 2%∼50% B3; 9∼12 min, 50%∼98% B3; 12∼13.5 min, 98% B3; 13.5∼14 min, 98%∼2% B3; 14∼17 min, 2% B3.[62]

Mass spectrometric data were collected using Orbitrap Exploris 120 (Thermo Fisher Scientific, USA) with ESI ion source. Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition was used, with parameters as sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and −2.50 kV for ESI(+) and ESI(-), respectively; capillary temperature, 325°C; MS1 range, m/z 100–1000; MS1 resolving power, 60000 FWHM; number of data dependant scans per cycle, 4; MS/MS resolving power, 15000 FWHM; normalized collision energy, 30%; dynamic exclusion time, automatic.[63]

The raw data were first converted to mzXML format, using MSConvert in the ProteoWizard software package (v3.0.8789)[51] and processed using XCMS[64] for feature detection, retention time correction and alignment. The metabolites were identified by accuracy mass (<30 ppm) and MS/MS datawhich were matched with HMDB[65] (http://www.hmdb.ca), massbank[66] (http://www.massbank.jp/), LipidMaps[67] (http://www.lipidmaps.org), mzcloud[68] (https://www.mzcloud.org) and KEGG[69] (http://www.genome.jp/kegg/). The robust LOESS signal correction (QC-RLSC)[70] was applied for data normalization to correct for any systematic bias. After normalization, only ion peaks with relative standard deviations (RSDs) under 30% in QC were kept to ensure proper metabolite identification.

A multivariate statistical analysis was performed using the ropls4 (version 1.6.2) R package. Identified metabolites between groups were categorized into annotated class and sub-class as: phenols, phenol ethers, flavonoids, isoflavonoids, pesticides/herbicides, and prenol lipids. Heat maps of the metabolites were generated with ImageGP.[52]

Lignocellulose content measurement

We measured lignin, cellulose and hemicellulose contents in phloem samples by ultraviolet spectrophotometry, to compare the differences between host tree phloem.

RNA extraction and metatranscriptome sequencing

Total RNA was extracted from the pooled *M. saltuarius* gut samples using the E.Z.N.A.® Soil RNA Midi Kit (Omega Bio-tek, Norcross, GA, U.S.), following the manufacturer's protocol. The total RNA of samples was subjected to an rRNA removal procedure using the Ribo-zero Magnetic kit according to the manufacturer's instruction (Epicentre, an Illumina® company). cDNA libraries were constructed using the TruSeq™ RNA sample prep kit (Illumina) and sequenced on the Illumina Hiseq 2500 platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). All the raw metatransgenomics datasets have been deposited into NCBI Sequence Read Achieve database PRJNA907471.

Sequence quality control and genome assembly

Raw metatranscriptomic reads were first processed for QC using fastp v0.20.0[53] with default parameters (reads with N bases, minimum length threshold of 50bp and phred quality score <20). The clean reads obtained were then assembled into contigs using MEGAHIT[54] (parameters: kmer_min = 47 , kmer_max = 97 , step = 10) (https://github.com/voutcn/megahit, version 1.1.2), which makes use of succinct deBruijn graphs. Contigs with length 300 bp or higher were selected as the final assembling result.

Gene prediction, taxonomy, and functional annotation

We used MetaGene[55] (http://metagene.cb.k.u-tokyo.ac.jp/) for open reading frames (ORFs) identification. Predicted ORFs with length $\geq$100 bp were retrieved and translated into amino acid sequences using the NCBI translation table. We constructed the non-redundant gene catalog using CD-HIT[56] (http://www.bioinformatics.org/cd-hit/, version 4.6.1) with 90% sequence identity and 90% coverage. Reads after quality control were mapped to the non-redundant gene catalog with 95% identity using SOAPaligner[57] (http://soap.genomics.org.cn/, version 2.21), with evaluation of gene abundance in each sample.

The taxonomy and functional annotation of predicted non-redundant gene catalog were conducted by aligning them to the following databases using DIAMOND v0.9.19 (https://github.com/bbuchfink/diamond): NCBI non-redundant protein sequences (nr); gene ontology (GO); clusters of orthologous groups of proteins (COG); Kyoto Encyclopedia of Genes and Genomes (KEGG); protein family (Pfam); A manually annotated and reviewed protein sequence database (Swiss-Prot) and non-supervised orthologous groups (eggNOG), with an e-value cutoff of $1e^{-5}$.

Differential expression analysis and functional enrichment

To identify both insect and microbe-derived differentially expressed genes (DEGs) between two host plants diet samples, the expression level of each gene was calculated using the transcripts per million reads (TPM) method. We used RSEM to quantify expression levels[57] and performed a differential expression analysis using the R package, DESeq2 (v1.6.3).[58] We considered genes with $|log2FC|>1$ and q value ≤ 0.05 to be differentially expressed. GO functional enrichment and KEGG pathway analysis were carried out using Goatools (https://github.com/tanghaibao/Goatools) and KOBAS.[59]

Microbial community composition and relative abundance analysis

Species composition and relative abundance of microbes were determined by comparing non-redundant gene dataset with taxonomic information. Three α-diversity estimators were employed, (Shannon, Simpson and Inv-simpson). We used Mann–Whitney test to assess the significance of differences between groups for each of these estimators.

To test whether the microbial community composition differed between groups, β-diversity was estimated using a principal coordinates analysis (PCoA) based on Bray–Curtis distances, with significance tested by ANOSIM.

Correlation analysis between DEGs and plant secondary metabolites

Correlations between different metabolites and DEGs were assessed by Spearman's correlation analysis using the pheatmap package in R[60]; insect-derived DEGs and microbial-derived DEGs were calculated respectively, and up-regulated and down-regulated genes were also considered as separated.

Correlation analysis between *M. saltuarius* gut microbial community and secondary plant metabolites

The correlation between different metabolites and microbial communities was assessed by Spearman's correlation analysis using the igraph package in R[60]; Bacterial dataset and fungi dataset (relative abundance of bacterial genus and fungal genus) were calculated respectively, with the top 30 abundant toxic plant compounds selected.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Statistical analyses on larvae weight and lignocellulose content were performed using Multiple t test in GraphPad Prism 8 (GraphPad Software, San Diego, CA, United States). Analyses on metatranscriptomic data, LC-MS Metabolomics data and correlations were performed in R (ver 4.1.2).