Abstract:
The antimicrobial roles of Toll-like receptors have been mainly identified in mammalian models and *Drosophila*. However, its immunological function in other insects has yet to be fully clarified. Here, we determined the innate immune response involvement of *TmToll*-2 encountering Gram-negative, Gram-positive, and fungal infection. Our data revealed that *TmToll*-2 expression could be induced by *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* infections in the fat bodies, gut, Malpighian tubules, and hemolymph of *Tenebrio molitor* young larvae. However, *TmToll*-2 silencing via RNAi technology revealed that sole *E. coli* systemic infection caused mortality in the double-strand RNA *TmToll*-2-injected group compared with that in the control group. Further investigation indicated that in the absence of *TmToll*-2, the final effector of Toll signaling pathway, antimicrobial peptide (AMP) genes and relevant transcription factors were significantly downregulated, mainly *E. coli* post-insult. We showed that the expression of all AMP genes was suppressed in the main immune organ of insects, namely, fat bodies, in silenced individuals, while the relevant expressions were not affected after fungal infection. Thus, our research revealed the immunological roles of *TmToll*-2 in different organs of *T. molitor* in response to pathogenic insults.

Keywords: Toll-2; *Tenebrio molitor*; microbial infection; antimicrobial peptides; RNAi technology

1. Introduction
Toll and Toll-like receptors (TLRs) are a conserved family of pattern recognition receptors (PRRs) initially identified for their role in dorso-ventral axis formation in *Drosophila* embryos [1]. The TLR/nuclear factor-kappaB (NF-kB) pathway activates immune responses in *Drosophila* [2,3]. Since the first Toll protein (*Toll1*) and eight other Toll receptors (18-Wheeler and Toll2-9) were characterized in *Drosophila*, 10 TLRs in human (TLR1-10) and 13 TLRs in mouse (TLR1-13) have been identified [4]. TLR activation and function can vary between vertebrates and invertebrates. In vertebrates, the Toll pathway is activated by direct interaction with pathogen-associated molecular patterns (PAMPs) [5]. Whereas, in invertebrates, Toll signaling is initiated by binding to the cytokine-like molecule Spätzle (Spz) [4]. Thus, it is proposed as evolutionary conversion in Toll/TLR immune functions [6]. Although the Toll pathway in invertebrates has immunity and developmental activities, mammalian TLRs are simply involved in immunity and have no developmental roles [7].

The structural properties of insect and mammalian TLRs share analogies and differences [8]. Insect Toll and vertebrate TLRs are characterized by leucine-rich repeat (LRR) domains containing cysteine-rich motifs in the extracellular domain, a single-pass transmembrane domain, and a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain [9]. Although the TIR domain has similarities to that of the IL-1 receptor family, unlike the intracellular domain of Tolls, IL-1 receptors consist of an immunoglobulin-like domain [10]. Conversely, mammalian TLRs contain a single cysteine-rich cluster at the C-terminal end of LRRs, except Toll9; other *Drosophila* Tolls hold cysteine-rich clusters at either, or both, the N-
and C-terminal ends of LRRs [11]. Likewise, *Drosophila* Toll9 was shown to be the closest TLR to vertebrate counterparts, suggesting functional similarities among these receptors in different clades [9].

Similar to its receptor structure, the Toll signaling pathway in insects is divided into three steps: (1) extracellular recognition of pathogenic antigens, including but not limited to Lys-type peptidoglycan (Lys-PGN) of Gram-positive bacterial cell walls, β-glucans of fungi, and virulence factors, by PGN recognition proteins (PGRPs) and glucan-binding proteins (GNBPs); (2) activation of a distinct proteolytic cascade from the developmental activation of Toll signaling, leading to the cleavage and activation of the zymogen Spätzle and binding of a cleaved ligand to the extracellular domain of TLRs; and (3) dimerization of the receptor cytosolic domain, TIR, activation of a phosphorylation cascade of an intracellular signaling cascade, and expression of effector molecules [12,13]. After the activation of PGRP-SA/GNBP1 and GNBP3 by Lys-PGN of Gram-positive bacteria cell walls and β-1,3-glucans of yeast and some fungi, respectively, a cascade of CLIP-domainzymogens mediate signal amplification [12]. In addition to the serine protease cascade, modular serine protease (ModSP) and Grass in turn activate the Spätzle-processing enzyme (SPE) [14,15]. The protease Persephone is likely involved in this proteolytic cascade activation [13]. However, the precise mechanism by which protease directly cleaves and activates SPE is yet to be clarified [16]. The interaction of the mature active C-terminal C-106 domain of Spz leads to the dimerization of intracytoplasmic TIR domains, which in turn, interact with distinct death-domain-containing proteins [2]. Upon the binding of processed TIR to the death-domain-containing adaptor molecules, Myeloid differentiation primary response 88 (MyD88), it is associated with the death domain of Tube [17]. Through a bifunctional death domain, Tube recruits the Ser/Thr kinase Pelle [18]. Subsequently, NF-κB transcription factors are released from their inhibitors through the K48 ubiquitination and degradation of Cactus. Nevertheless, the Cactus kinase is not identified [12]. Freed Rel transcriptional factors, Dorsal-related immunity factor (Dif), and Dorsal are then translocated into the nucleus, bind to κB-response elements, and induce the transactivation of antimicrobial peptides (AMPs), which are the hallmark of insect innate immune responses [12,15,19].

Studies on immune responses of arthropods have focused on dipterans [16]. Moreover, a relatively large insect model should be established for comprehensive and accurate biochemical studies (sufficient hemolymph sampling) by using species belonging to different taxonomic groups [6]. To this extent, Toll signaling pathway and their components in *T. molitor* were investigated in response to various pathogenic sources, including Gram-positive and Gram-negative bacteria and fungi. Seven *Toll* genes (*Tm*Toll-2, -3, -6, -7, -8, -9, and -10) were identified in *T. molitor*. However, the functional importance of these isoforms, except *Tm*Toll-2, is poorly understood [20]. In the present study, we explored the immunological importance of *Tm*Toll-2 against microbial infection. Our study revealed that *Tm*TLR2 is important for immune-mediating responses to Gram-negative *E. coli* (Figure S1). Our findings might provide a basis for developing possible therapeutic strategies against human pathogens.

2. Results

2.1. Sequence Analysis of *Tm*Toll-2

In this study, a Toll-2 homolog from *T. molitor* (*Tm*Toll-2, Accession number: OP566501) was identified by an EST and RNA-seq search by using the *T. castaneum* protein sequence as a query. A 2510 bp open reading frame (ORF) encoded a protein of 834 amino acids. Phylogenetic analysis based on the full-length amino acid sequences of *Tm*Toll-2 and other insect Toll receptors indicated that it clustered with Toll-2 proteins from *T. castaneum*, *Sitophilus oryzae*, *Manduca sexta*, *Vanessa cardui*, *Bicyclus anynana*, *Galleria mellonella*, *Neodiprion fabricii*, *Nilaparvata lugens*, *Homalodisca vitripennis*, TLR4 from *Bombyx mori*, Toll-9, isoform C from *Drosophila melanogaster*, and TLR Tollo from *Anopheles arabiensis*; and *Mus musculus* TLR2, the isoform X1, was used as the outgroup (Figure 1). Phylogenetic analysis showed that
TmToll-2 in the order Coleoptera formed a group with other isoforms of Toll-2 from T. castaneum and S. oryzae.

**Figure 1.** Molecular phylogenetic analysis of TmToll-2, T. molitor Toll-2 (OP566501). The phylogenetic tree was constructed using MEGA7 with the maximum likelihood method and 1000 bootstrap replicates (where numbers at nodes indicate bootstrap support). The percentage of trees in which the associated taxa clustered together is shown next to the branches. A neighbor-joining (NJ) tree was constructed on the basis of the protein sequences of TcToll-2, Tribolium castaneum toll-like receptor 2 (XP_015837871.1); SoToll-2, Sitophilus oryzae toll-like receptor 2 (XP_030759691.1); AaTollo, Anopheles arabiensis toll-like receptor Tollo (XP_040168234.1); DmToll-9XC, Drosophila melanogaster Toll-9C, isoform C (NP_001246846.1); MsToll-2, Manduca sexta toll-like receptor 2 (XP_030022594.2); VcToll-2, Vanessa cardui toll-like receptor 2 (XP_046969474.1); BtToll-2, Bicyclus anynana toll-like receptor 2 (XP_023948156.1); GmToll-2, Galleria mellonella toll-like receptor 2 (XP_031764691.1); BmToll-4, Bombyx mori toll-like receptor 4 (XP_012546905.2); NfToll-2X1, Neodiprion fabricii toll-like receptor 2 isoform X1 (XP_046422597.1); NToll-2-T2-X2, Nilaparvata lugens toll-like receptor 2 type-2 isoform X1 (XP_039281792.1); HvToll-2-T2, Homalodisca vitripennis toll-like receptor 2 type-2 (XP_046658785.1); and MmToll-2X1, Mus musculus toll-like receptor 2 isoform X1 (XP_006501523.1), which was used as the outgroup.

2.2. Developmental and Tissue Expression of TmToll-2

The mRNA expression of TmToll-2 was evaluated through qRT-PCR at different developmental stages (Figure 2A) and in different tissues of late-instar larvae (Figure 2B) and adults (Figure 2C). Our data illustrated the developmental and innate immunity accepts of TLR receptors and revealed that the highest expression pattern of TmToll-2 occurred in 1-day-old adults and the embryonic stage in a descending order. In young larvae and adults of T. molitor, the gene expression of TmToll-2 was the highest in gut tissues but negligible in other tissues (less than 0.1-fold change).
Figure 2. Developmental stage- and tissue-specific expression patterns of TmToll-2 measured by qRT-PCR. (A) Relative mRNA expression levels of TmToll-2 in eggs, young larvae, late-instar larvae (LL), pre-pupae, 1-to-7-day-old pupae (P1–P7), and 1-to-5-day-old adults (A1–A5) were presented. Expression levels were the highest in the eggs and 1-day-old adults. The mRNA expression decreased at the larval stage and were the lowest at the young larval stage. TmToll-2 tissue expression patterns in late instar larvae (B) and adults (C) were also examined. Total RNA was extracted from different tissues, including the integument (IT), Malpighian tubules (MT), gut (GT), hemocytes, and fat bodies (FB) of late instar larvae and the IT, MT, GT, hemocytes, FB, ovary, and testis (TE) of 5-day-old adults. Total RNA was isolated from 20 mealworms, and T. molitor 60S ribosomal protein 27a (TmL27a) primers were used as internal control (N = 3). One-way ANOVA and Tukey’s multiple-range test were used for comparisons. Bars with the same letter are not significantly different by Tukey’s multiple-range test (p < 0.05).

2.3. Temporal Expression of TmToll-2 Post-Systemic Infection

The mRNA expression level in T. molitor young larvae was measured and examined post-systemic infection with E. coli, S. aureus, and C. albicans in the MTs (Figure 3A), gut (Figure 3B), hemolymph (Figure 3C), and fat bodies (Figure 3D) 3, 6, 9, 12, and 24 h after injection to determine the immunological roles of TmToll-2 in deflecting pathogenic attacks. PBS was used as the control. The results showed that TmToll-2 mRNA expression was upregulated in response to all infectious sources and varied in tissue- and time-dependent manner. The upregulation of the TmToll-2 gene expression was the highest in the hemolymph and MTs in a descending order against C. albicans 9 h post infection (up to 350- and 90-fold, respectively). Moreover, the expression of TmToll-2 was induced by 150- and 100-fold against S. aureus and E. coli, respectively, because of the involvement of the Toll signaling pathway in the recognition of Gram-positive and Gram-negative invasion. However, the minor gut expression of TmToll-2 was induced mainly after E. coli infection by approximately 3-fold and S. aureus by approximately 2-fold but not after C. albicans infection. Likewise, TmToll-2 expression was relatively low in fat bodies in response to all microorganisms.
We did not report either significant or meaningful mortality rate between 10 days after non-entomopathogen infections. Therefore, a high survivability rate was reported after non-entomopathogen infections.

Matin like protein-1 (TmColeoptericin-A) and 60S ribosomal protein 27a (TmL27a) were used as internal control, and T. molitor 60S ribosomal protein 27a (TmL27a) primers were used as control group. Further, 4 days after dsTmToll-2 RNAi injection, TmToll-2 mRNA levels were decreased by 77% (Figure 4A), confirming the efficiency of RNAi. Subsequently, T. molitor larvae were monitored during 10 days after E. coli (Figure 4B), S. aureus (Figure 4C), and C. albicans (Figure 4D) injection. We did not report either significant or meaningful mortality rate between S. aureus- and C. albicans-infected dsTmToll-2- and dsTmVer RNAi-treated groups, except E. coli-infected larvae. Therefore, a high survivability rate was reported after non-entomopathogen infections.

Figure 3. mRNA expression patterns of TmToll-2 in immune-challenged T. molitor larvae. mRNA levels of TmToll-2 in the Malpighian tubules (A), gut (B), hemocytes (C), and fat bodies (D) were examined by qRT-PCR 3, 6, 9, 12, and 24 h after infection with E. coli (10⁶ cells/µL), S. aureus (10⁶ cells/µL), and C. albicans (5 × 10⁴ cells/µL). TmToll-2 mRNA expression was upregulated in response to all infectious sources and varied in tissue- and time-dependent manners. The upregulation of TmToll-2 gene expression was the highest in the hemolymph and MTs against C. albicans. PBS was used as an injection control, and T. molitor 60S ribosomal protein 27a (TmL27a) primers were used as internal control (n = 3). Asterisks indicate significant differences between infected and PBS-injected larval groups by Student’s t-test (p < 0.05). Vertical bars indicate means ± SD (n = 20).

2.4. Effect of TmToll-2 RNAi on T. molitor Larval Survival

After we observed the TmToll-2 mRNA induction post systemic infection, we further investigated the innate immune responses in T. molitor larvae treated with dsTmToll-2 RNAi. Initially, we checked the survival rates of the infected TmToll-2-silenced larvae compared with those of dsTmVer-treated larvae as the control group. Furthermore, 4 days after dsTmToll-2 RNAi injection, TmToll-2 mRNA levels were decreased by 77% (Figure 4A), confirming the efficiency of RNAi. Subsequently, T. molitor larvae were monitored during 10 days after E. coli (Figure 4B), S. aureus (Figure 4C), and C. albicans (Figure 4D) injection. We did not report either significant or meaningful mortality rate between S. aureus- and C. albicans-infected dsTmToll-2- and dsTmVer RNAi-treated groups, except E. coli-infected larvae. Therefore, a high survivability rate was reported after non-entomopathogen infections.
Figure 4. Effect of TmToll-2 gene silencing on T. molitor larval survival. The RNAi efficiency of dsTmToll-2 RNAi was measured by qRT-PCR 4 days after injection (A). TmToll-2-silenced larvae were injected with E. coli (B), S. aureus (C), and C. albicans (D), and survival rates were studied for 10 days post-pathogen injection (n = 10 per group). Larval survival rates at 10 days post-microbial injection were 80% after E. coli injection and 100% after S. aureus and C. albicans injection compared with the levels in the dsTmVer-injected control group. Data were reported as averages of three biologically independent replicates. Asterisks indicate significant differences between dsTmToll-2- and dsTmVer RNAi-injected groups. Survival analysis was performed using Kaplan–Meier plots (log-rank chi-squared test; * p < 0.05).

2.5. Effect of TmToll-2 Gene Silencing on the Expression of Antimicrobial Peptide and NF-κB Genes

An interesting result of TmToll-2 gene silencing indicated that the absence of this gene did not affect the survivability of T. molitor larvae following Gram-positive and fungal infections. However, the TmToll-2 mRNA expression significantly upregulated in hemolymph and Malpighian tubules in response to bacterial and fungal infections. Therefore, to verify these observations, we checked the hallmark of T. molitor innate immunity and the regulation of 15 AMP genes: TmTenein-1, -2, -3, and -4 (TmTene1, 2, 3, and 4); TmAttacin-1a, -1b, and -2 (TmAtt1a, 1b, and 2); TmDefensin (TmDef); TmDefensin-like (TmDef-like); TmCecropin-1, -2, and -4 (TmCec1, 2, and 4); TmTene2; TmAttacin, and C. albicans. We also confirmed the TmToll-2 knockdown efficiency. In fat bodies, which are the main immune organ of insects, 13 out of 15 AMP genes were downregulated in response to E. coli, and 14 out of 15 AMP genes were downregulated in response to S. aureus (Figure 5 A–O). In accordance with previous reports of AMP production in T. molitor [20–22], we showed that TmDef, TmDef-like, and TmCec2 are involved in immune responses against Gram-positive, Gram-negative, and fungal infections (Figure 5E,F). Likewise, the downregulation of TmTLP2 following C. albicans infection in TmToll-2-silenced larvae presented the analogy of previous findings (Figure 5O). In the gut (Figure 6A–O), eight AMP genes were downregulated in TmToll-2-silenced larvae after E. coli, S. aureus, and C. albicans infections. However, the main effect of TmToll-2 gene silencing in the gut could be observed after E. coli infection, particularly TmTene2 (5000-fold), TmTene4 (2000-fold), TmCole A (2000-fold), and TmCole C (3500-fold), compared with that in dsTmVer-treated larvae (Figure 6B,D,H,J). Similarly, in Malpighian tubules (Figure 7A–O), the superior negative regulation in most AMP genes, including TmTene1 (Figure 7A), TmTene4 (Figure 7D),
TmDef-like (Figure 7F), TmCec2 (Figure 7G), TmColeA (Figure 7H), TmColeB (Figure 7I), TmColeC (Figure 7J), TmAtt1a (Figure 7K), and TmAtt1b (Figure 7L), was observed after *E. coli* infection. Hence, *S. aureus* injection in dsTmToll-2 RNAi-treated larvae remarkably downregulated the expression levels of TmTene1 (Figure 7A), TmTene2 (Figure 7B), TmTene3 (Figure 7C), TmTene4 (Figure 7D), TmDef-like (Figure 7F), TmColeA (Figure 7H), TmColeB (Figure 7I), TmColeC (Figure 7J), TmAtt1a (Figure 7K), and TmAtt1b (Figure 7L). Additionally, the AMP genes were marginally negatively regulated after *C. albicans* in the TmToll-2-silenced group. Interestingly, dsTmToll-2 RNAi increased the mRNA levels of some AMPs in response to pathogens in the whole body samples (Figure 8A–O), gut, and Malpighian tubules, precisely the levels of Cecropin, Coleoptericin, Attacin, Thaumatin-like protein, and Tencin families (Figure 6A,E–G,I–M and Figure 7B,E,M–O). Unexpectedly, seven AMP genes in the whole body samples were mostly upregulated in response to all infections, explaining the high survivability rate in response to invasions (Figure 8A–O).

Figure 5. Induction of 15 AMP genes in the fat bodies of TmToll-2-treated *T. molitor* larvae infected with *E. coli* (Ec), *S. aureus* (Sa), and *C. albicans* (Ca) by using PBS as a control. At 24 h post microbial injection, AMP genes, including TmTene1 (A), TmTene2 (B), TmTene3 (C), TmTene4 (D), TmDef (E), TmDef-like (F), TmCec2 (G), TmColeA (H), TmColeB (I), TmColeC (J), TmAtt1a (K), TmAtt1b (L), TmAtt2 (M), TmTLP1 (N), and TmTLP2 (O) were examined via qPCR by using dsTmVer as a knockdown control and *T. molitor* ribosomal protein (*TmL27a*) as an internal control. All experiments were performed in triplicate. Asterisks indicate significant differences between dsTmToll-2- and dsTmVer RNAi-treated groups determined by Student’s *t*-test (*p* < 0.05).
Figure 6. Effect of TmToll-2 RNAi on the induction of 15 AMP genes in the gut of T. molitor larvae infected with E. coli (Ec), S. aureus (Sa), and C. albicans (Ca) by using PBS as the control. At 24 h post microbial injection, AMP genes, including TmTene1 (A), TmTene2 (B), TmTene3 (C), TmTene4 (D), TmDef (E), TmDef-like (F), TmCec2 (G), TmColeA (H), TmColeB (I), TmColeC (J), TmAtt1a (K), TmAtt1b (L), TmAtt2 (M), TmTLP1 (N), and TmTLP2 (O) were examined via qPCR by using dsTmVer as a knockdown control and T. molitor ribosomal protein (TmL27a) as an internal control. All experiments were performed in triplicate. Asterisks indicate significant differences between dsTmToll-2- and dsTmVer RNAi-treated groups determined by Student’s t-test (p < 0.05).
Figure 7. Effect of TmToll-2 gene silencing on the induction of 15 AMP genes in the Malpighian tubules of T. molitor larvae infected with E. coli (Ec), S. aureus (Sa), and C. albicans (Ca) by using PBS as the control. At 24 h post microbial injection, AMP genes, including TmTene1 (A), TmTene2 (B), TmTene3 (C), TmTene4 (D), TmDef (E), TmDef-like (F), TmCec2 (G), TmColeA (H), TmColeB (I), TmColeC (J), TmAtt1a (K), TmAtt1b (L), TmAtt2 (M), TmTLP1 (N), and TmTLP2 (O) were examined via qPCR by using dsTmVer as a knockdown control and T. molitor ribosomal protein (TmL27a) as an internal control. All experiments were performed in triplicate. Asterisks indicate significant differences between dsTmToll-2- and dsTmVer RNAi-treated groups determined by Student’s t-test (p < 0.05).
Figure 8. Induction of 15 AMP genes in the whole body of TmToll-2-silenced T. molitor larvae infected with E. coli (Ec), S. aureus (Sa), and C. albicans (Ca) by using PBS as control. At 24 h post microbial injection, AMP genes, including TmTene1 (A), TmTene2 (B), TmTene3 (C), TmTene4 (D), TmDef (E), TmDef-like (F), TmCec2 (G), TmColeA (H), TmColeB (I), TmColeC (J), TmAtt1a (K), TmAtt1b (L), TmAtt2 (M), TmTLP1 (N), and TmTLP2 (O) were examined via qPCR by using dsTmVer as a knockdown control and T. molitor ribosomal protein (TmL27a) as an internal control. All experiments were performed in triplicate. Asterisks indicate significant differences between dsTmToll-2- and dsTmVer RNAi-treated groups determined by Student’s t-test (p < 0.05).

In accordance with the same protocol used to evaluate the expression of AMP genes after TmToll-2 knockdown, we further examined the NF-κB pathway genes TmDorX1, TmDorX2, and TmRelish in the whole body, fat bodies, gut, and Malpighian tubules (Figure 9A–D) to determine the exact transcription factors downstream of the main signaling pathways responsible for AMP production. Consistent with previous findings and AMP expression levels, the results showed that the expression level of TmDorx2, downstream of the Toll signaling pathway, decreased following E. coli and S. aureus infection in all the examined tissues, whereas the level of TmRelish, downstream of Imd signaling, was not strongly affected [23–26]. Additionally, the comparison of the expression pattern
of TmDorX1 in different tissues explained the main negative and positive regulation of AMP gene expression; in the whole body, TmDorX1 was positively regulated (Figure 9A). Conversely, in the fat bodies and gut, the NF-kB gene expression was negatively regulated (Figure 9B,C).

![Figure 9](image-url)

Figure 9. Effect of TmToll-2 gene silencing on NF-kB gene expression. dsTmToll-2 RNAi-treated T. molitor larvae were infected with E. coli, S. aureus, and C. albicans; at 24 h post pathogen injection, the mRNA levels of the NF-kB pathway genes TmDorX1, TmDorX2, and TmRelish in the whole body (A), fat bodies (B), gut (C), and Malpighian tubules (D) were measured via RT-qPCR. The expression level of TmDorX2 was suppressed following E. coli and S. aureus infection in all the examined tissues, while the level of TmRelish was not affected. In the fat bodies and gut, the NF-kB genes expression was negatively regulated. TmVer dsRNA was assessed as a negative control, and T. molitor ribosomal protein (TmL27a) was used as an internal control. All experiments were performed in triplicate. Asterisks indicate significant differences between dsTmToll-2- and dsTmVer RNAi-treated groups determined using Student’s t-test (p < 0.05).

3. Discussion

The Toll signaling pathway is important for the development and immunity of insects in their life. However, intensive studies have described the roles of TLRs in infectious diseases and innate immunity; furthermore, the current understanding of these receptors and relevant signaling is chiefly limited to mammalian and some invertebrate models such as Drosophila, mosquitoes, moths, beetles, and shrimp. These receptors are evolutionarily conserved among all living organisms from mammals to plants [6]. Homologies between toll receptors in vertebrates and invertebrates can be characterized by TIR and LRR domains [27]. Except for Toll-1 in D. melanogaster [28], the specific functions, including their immunological roles, of other proteins within this family remain elusive.

With economic importance, T. molitor was developed into a distinctive immune study model. We identified seven Toll genes in T. molitor. TmToll-7 roles in the innate immune response following Gram-negative E. coli infection through AMP production was reported [20]. Here, we attempted to reveal further roles in the TLR family by examining TmToll-2 activity in warding off pathogenic invasions via RNAi technology.

Initially, our phylogenetic analysis indicated that the Toll-2 cluster in insects is distinguished from mammalian TLRs, suggesting distinct evolutionary events and consequently different co-players and functions [29]. In addition to their roles in dorso-ventral
axis formation, TLRs have a dynamic expression pattern throughout the development of *Drosophila* [30]. Moreover, Toll-2, -6, -7, and -8 in *T. castaneum*, which forms a group with *T. molitor*, with 62% amino acid identities in our phylogenetic analysis, share a common characteristic-encoding more LRRs, and *Drosophila* long Tolls function in embryonic development [9]. Accordingly, TmToll-2 expression patterns vary in an insect’s life; in addition to the embryonic stage, the highest expression in 1-day-old adults highlights the TLR significance in the entire development of insects. The early adult stage expression of TmToll-2 can be attributed to compartment boundary restrictions, which are mediated by Toll-1 and -2 as adhesion molecules in *Drosophila* [31]. Moreover, the highest TmToll-2 expression in the gut of *T. molitor* larvae and adults in tissue-specific gene expression experiments is supported by former reports, which show that Imd signaling within an insect’s gut is not the solo player in an innate immunity match; instead, a possible crosstalk exists between Imd and Toll signaling pathways [32,33].

Toll signaling in *T. molitor* can be activated after various microbial challenges, including but not limited to Gram-positive, Gram-negative, and fungal infections [20,25,26]. The DAP-type peptidoglycan (DAP-PGN) of Gram-negative bacteria can be sensed by *Tenebrio* recognition protein PGRP-SA and induces activation [34]. Here, we reported that *S. aureus*, *C. albicans*, and *E. coli* induce TmToll-2 mRNA expression, and the relevant expression was distinguished in the hemolymph of *T. molitor* larvae. Previous studies showed that the Toll-dependent wound healing activity is initiated by the influx of extracellular calcium ions in epidermal cells in the wound site of *Drosophila*, which eventually triggers Spätzle cleavage and activation [35]. Additionally, transglutaminase activity and calcium ions from injury in insects mediates the prophenoloxidase-activating (proPO) system [8]. The same protease cascade leading to the PO activity can mediate Spätzle cleavage and Toll pathway-dependent AMP production in *M. sexta* [13,36]. Consequently, the extensive induction of TmToll-2 in the hemolymph samples of the pathogen-injected groups could be triggered by PO activity, the injection site of epidermal cells with oxidative stress, and proteolytic cascade activation following microbial sensation by Toll-related PRRs. Because of the constant hemolymph flow into Malpighian tubules [37], the expression pattern of TmToll-2 in this tissue was also relatively high.

*Tm*Toll-2-silenced larvae were more susceptible to Gram-negative infection than to the controls. In accordance with the survivability result, AMP expressions showed to be in favor of *T. molitor* larvae after *E. coli* in the *T. molitor* vermilion (*TmVer*) double-strand RNA (dsTmVer) injected group. The negative regulation of all AMPs in the fat bodies of insects following Gram-negative bacterial infection was consistent with our previous report on TmToll-7, in which AMPs downregulated in the silenced larvae following *E. coli* infections [20]. Moreover, we observed that the mRNA expression levels of TmTen2, TmTen4, TmCoeA, TmCoeC, TmCec2, and TmTLP1 were negatively regulated in TmToll-2-silenced individuals. The secretion of cecropin and coleoptericins is dependent on Imd signaling in *Drosophila* and weevil *Sitophilus*, respectively [38,39]. Additionally, the gut multifunction in insect metabolism and immunity is extensively described [40]; conversely, Imd and Toll signaling pathways play major roles in insects’ innate immunity, and the Toll pathway in *Drosophila* does not have a role in gut immunity [41]. Hence, reactive oxygen species (ROS) production and Imd signaling function as a homeostasis regulator of the gut microbiota in *Drosophila* and red palm weevil (RPW) [42,43]. Similar to our former studies in *T. molitor* and RPW [14], the present study exhibited that TmToll-2 could mediate immunity within the gut by regulating the AMP production, confirming that (i) the Imd signaling pathway mainly functions in immunity within the gut [44–46]; (ii) Gram-negative bacteria are recognized by PRRs of Toll signaling [47,48]; (iii) possible cross-talks existed between Toll and Imd signaling pathways [33,49]. Interestingly, in *Drosophila*, AMP expression in fat bodies can be regulated through ROS signaling in the gut, and hemocytes serve as a signal-relaying organ between the gut and fat bodies after oral infection [50]. This finding can explain why *T. molitor* larval fat bodies invest the least energy on the expression of the TmToll-2, because the signaling pathway, its relevant transcription factor, and AMPs as final effectors can be
also activated by other organs after the infections. Surprisingly, the positive regulation of the mRNA expression of AMPs in the whole body samples suggested that major immune organs, such as fat bodies, gut, and Malpighian tubules, affect the total AMP production in *T. molitor* larva; other tissues and relative cells, such as the integument and hemocytes, are vital players in the overall immunity of insects [51]. Furthermore, the mRNA production of AMPs in the whole body can explain a high survival ratio post systemic infection by different pathogens. Studies on NF-κB function under pathological conditions revealed that the mRNA levels of several AMPs are regulated by *TmRelish* (downstream of Imd signaling) and *TmDorX2* (downstream of Toll signaling) pathways in the fat body [45,52]. Accordingly, our NF-κB results were consistent with the mRNA expression of AMPs. This result suggested that the negative relationship between *TmDorX2* and *TmRelish* is responsible for the downregulation of AMP genes in dissected tissues. Moreover, in whole body samples, *TmDorX1* is positively regulated, indicating the upregulation of AMP genes in the relevant samples.

Overall, our molecular analysis provided insights into the innate immunity of *T. molitor*. We illustrated that *TmToll-2* regulates antimicrobial activities in epithelial tissues such as Malpighian tubules and the gut, in addition to fat bodies. *TmToll-2* is activated by Gram-positive and Gram-negative bacteria in *T. molitor*, similar to *B. mori* but in contrast to some insects such as *M. sexta* and *D. melanogaster* [2]. In addition, the activation of AMP genes was nonspecific after *E. coli*, *S. aureus*, and *C. albicans* challenges. Further comprehensive studies of possible ligands and simulators of TLRs and possible cross-talks of this signaling with other pathways should provide a clear perspective of the underlying mechanisms involved in innate immunity.

4. Materials and Methods

4.1. Insect Rearing and Preparation of Microorganisms

*T. molitor* larvae were reared under dark conditions at 26 ± 1 °C and 60% ± 5% relative humidity in an environmental chamber established in our laboratory. Larvae were fed with an artificial diet consisting of 1.1 g sorbic acid, 1.1 mL propionic acid, 20 g bean powder, 10 g brewer’s yeast powder, and 200 g wheat bran in 4400 mL distilled water. The feed was autoclaved at 121 °C for 15 min. All experiments were conducted with healthy 10th–12th instar larvae.

Gram-negative *E. coli* (strain K12), Gram-positive *S. aureus* (strain RN4220), and fungus *C. albicans* (strain AUMC 13529) were used as representatives of different pathogenic sources to investigate the roles of *TmTLR2* in immunological challenges. *E. coli* and *S. aureus* were cultured in Luria–Bertani [13] broth, and *C. albicans* was cultured in Sabouraud’s dextrose broth at 37 °C overnight. The microorganisms were harvested, washed twice in 1× phosphate-buffered saline (PBS; 8.0 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄, 0.24 g KH₂PO₄ in 1 L distilled water, pH 7.0), and centrifuged at 3500 rpm for 15 min. Subsequently, the samples were suspended in PBS, and concentrations were measured at 600 nm (OD600) through spectrophotometry (Eppendorf, Hamburg, Germany). *E. coli* and *S. aureus* were diluted to 1 × 10⁶ cells/µL, and *C. albicans* was diluted to 5 × 10⁴ cells/µL for immune challenge studies. The relevant optimization of microbial concentration was adjusted in accordance with our previous studies [20,53,54].

4.2. In Silico Analysis of *TmToll-2*

The *T. castaneum* TLR2 amino acid sequence (accession number: XP_015837871.1) was used as a query to perform Local-tblastn analysis and obtain *TmToll-2* gene sequence (accession number: OP566501) from RNAseq analysis and NCBI Expressed Sequence Tag [28] database [55]. The full-length ORF and deduced amino acid sequences of *TmTLR2* were analyzed using BLASTp (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple sequence alignment of the *TmTLR2* amino acid sequence with representative TLR amino acid sequences from other insects (retrieved from GenBank) was generated using ClustalX 2.1 [56]. A phylogenetic tree was constructed on the basis of amino acid
sequence alignments via the maximum likelihood method (bootstrap trial set to 1000) with several protein sequences, including those of *Tc*Toll-2, *T. castaneum* toll-like receptor 2 (XP_015837871.1); *So*Toll-2, *S. oryzae* toll-like receptor 2 (XP_030759691.1); *Ad*Tollo, *Anopheles arabiensis* toll-like receptor Tollo (XP_040168234.1); *Dm*Toll-9XC, *D. melanogaster* Toll-9, isoform C (NP_001246846.1); *Ms*Toll-2, *M. sexta* toll-like receptor 2 (XP_030022594.2); *Vc*Toll-2, *V. cardui* toll-like receptor 2 (XP_046969474.1); *Ba*Toll-2, *Bicyclus anynana* toll-like receptor 2 (XP_023948156.1); *Gm*Toll-2, *Galleria mellonella* toll-like receptor 2 (XP_031764691.1); *Bm*Toll-4, *B. mori* toll-like receptor 4 (XP_012546905.2); *Nf*Toll-2X1, *Neodiprion fabricii* toll-like receptor 2 isoform X1 (XP_046422597.1); *Nl*Toll-2-T2-X2, *Nilaparvata lugens* toll-like receptor 2 type-2 isoform X1 (XP_039281792.1); *Hv*Toll-2-T2, *Homalodisca vitripennis* toll-like receptor 2 type-2 (XP_046658785.1); and *Mm*Toll-2X1, *M. musculus* toll-like receptor 2 isoform X1 (XP_006501523.1). Phylogenetic analyses were performed using the Tree Explorer view in Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 [57](https://megasoftware.net).

4.3. Expression and Induction Pattern Analysis of *Tm*Toll-2

Total RNA was isolated at different developmental stages, including eggs, young larvae (instars 10–12), late larvae (instars 14–15), prepupae, 1-to-7-day-old pupae, and 1-to-5-day-old adults and tissues (integument (IT), gut, fat bodies (FBs), Malpighian tubules (MTs), hemocytes [28] of last instar larvae and 5-day-old adults, and ovary [17] and testis (TE) of 5-day-old adults) of *T. molitor* to investigate the temporal and spatial expression patterns of *Tm*Toll-2.

Suspensions containing $1 \times 10^6$ cells/µL of *E. coli* and *S. aureus* and $5 \times 10^4$ cells/mL of *C. albicans* were injected into *T. molitor* larvae at 10th–12th instars (*n* = 20) to analyze the induction pattern of *Tm*Toll-2 against microorganisms. PBS-injected *T. molitor* larvae were used as the control group. Samples were collected 3, 6, 9, 12, and 24 h after the microbial challenge.

Total RNA was extracted by using a Clear-S total RNA extraction kit (Invirustech Co., Gwangju, Republic of Korea) in accordance with the manufacturer’s instructions. Then, 2 mg of total RNA was used as the template to synthesize cDNA by using the Oligo (dT)12–18 primers under the following reaction conditions: 72 °C for 5 min, 42 °C for 1 h, and 94 °C for 5 min. MyGenie96 Thermal Block (Bioneer, Daejeon, Republic of Korea) and AccuPower® RT PreMix (Bioneer) were used in accordance with the manufacturer’s instructions. cDNA was stored at −20 °C until further use.

The relative mRNA expression level of *Tm*Toll-2 was investigated via quantitative real-time polymerase chain reaction (qRT-PCR) by using an AccuPower® 2X Greenstar™ qPCR Master Mix (Bioneer, Daejeon, Republic of Korea) with synthesized cDNAs and specific primers (Table 1) at an initial denaturation of 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s, and 60 °C for 30 s. *T. molitor* ribosomal protein (*Tm*L27a) was used as an internal control, and the results were analyzed using the 2$^{-\Delta\Delta Ct}$ method [58]. The results were presented as means ± standard error (SE) of three biological replicates.
Table 1. Primers used in the present study.

| Name                        | Primer Sequences (5'→3')                                    |
|-----------------------------|-------------------------------------------------------------|
| TmToll-2-qPCR-Fw             | TCTAGTAGACGTAGCGGTGA                                         |
| TmToll-2-qPCR-Rev            | AATCGCAAGTGAAATGGGGTTG                                      |
| TmToll-2-T7-Fw               | TAATACGACTCACTATAGGGTTGCGGAAGCACCAAAGAAGT                  |
| TmToll-2-T7-Rev              | TAATACGACTCACTATAGGGTTGCGGAAGCACCAAAGAAGT                  |
| TmL27a-qPCR-Fw               | TCATCTGAAAGCCAAAGCTCCAGT                                   |
| TmL27a-qPCR-Rev              | AGTGGTGTAGGCAGCCACCTTTA                                    |
| TmVer-T7-Fw                  | TAATACGACTCACTATAGGGTTGCGGAAGCACCAAAGAAGT                  |
| TmVer-T7-Rev                 | TAATACGACTCACTATAGGGTTGCGGAAGCACCAAAGAAGT                  |
| TmTenecin-1-Fw               | CAGCTGAAGAACATCGAACAAAG                                   |
| TmTenecin-1-Rev              | CAGACCTCTTTTCCGTTACAGT                                     |
| TmTenecin-2_Fw               | CAGCAAAAAGCAGAGATGGTG                                       |
| TmTenecin-2-Rev              | CGTGGGAATGCTGAGTCTGTC                                       |
| TmTenecin-3-Fw               | GATTTGCTTGTCTGGTGTTG                                       |
| TmTenecin-3-Rev              | CTGATGCGCTCTCTAAATGTCC                                     |
| TmTenecin-4-Fw               | GCAGATTAGAAATGCAAGAAG                                      |
| TmTenecin-4-Rev              | CGGTGTTCTCTTATGAGACTG                                      |
| TmDefensin-Fw                | GAATGCAACAAGGCAGCAAC                                       |
| TmDefensin-Rev               | GCAAATGCAAGCCACCTTTC                                       |
| TmDefensin-like-Fw           | GCGATGCTCTATAGAAAGATG                                      |
| TmDefensin-like-Rev          | CCAAATGCAAAACATTCGTG                                       |
| TmColoptericinA-Fw           | GACAGAAATGCTGATGGTC                                         |
| TmColoptericinA-Rev          | CTCAAACATTCCAGTGAGG                                       |
| TmColoptericinB-Fw           | CAGCTGTTGACCCACAAAGT                                       |
| TmColoptericinB-Rev          | CTCAACGTGTTGCCGTCGGT                                        |
| TmColoptericinC-Fw           | CAGCTGTTGACCCACAAAGT                                       |
| TmColoptericinC-Rev          | CTCAACGTGTTGCCGTCGGT                                        |
| TmAttacin-1a-Fw              | GAAACGGAATAGGAAAGTGGGA                                     |
| TmAttacin-1a-Rev             | TGCTTTCGCAAGAAATACAG                                       |
| TmAttacin-1b-Fw              | CCCCCTGATGAAACACCTCCAA                                     |
| TmAttacin-1b-Rev             | GACGCTGAAATGCAAGAACAA                                     |
| TmAttacin-2-Fw               | AACTGGGATATTCGGAGTGGC                                      |
| TmAttacin-2-Rv               | CCCTCCGGAATTCTTGCGT                                       |
| TmCecropin-2-Fw              | TACTAGACGGGCAAAACC                                       |
| TmCecropin-2-Rev             | CTGGAACATAGGCGCGGGAA                                      |
| TmThaumatin-likeprotein-1-Fw | CTCAAGGCCAGCGCGAGCACT                                      |
| TmThaumatin-like protein-1-Rev| ACTTTGAGCTCTCTTGCGGACA                                     |
| TmThaumatin-like protein-2-Fw| CCGTTCTGCTAGGAGCTTGT                                      |
| TmThaumatin-like protein-2-Rev| ACCTGTCACGTAGGCGGTTACA                                    |
| TmDorsall-qPCR-Fw            | ACGGTTGAGTTGCGTAATG                                      |
| TmDorsall-qPCR-Rev           | TCTTTTGAGACGCGAAACAC                                      |
| TmDorsall-qPCR-Rev           | ACACCCCGAAATCAACAAAC                                      |
| TmDorsall-qPCR-Rev           | TTTCAAGCGCCAGGTGGTT                                        |
| TmRelish-qPCR-Fw             | AGGCTCAGATTTGGAGACGATOLD                                    |
| TmRelish-qPCR-Rev            | GTCGCGACCTCATAAACAG                                        |

4.4. RNA Interference Analysis

TmToll-2 gene silencing was performed as reported previously [20,37,59]. Briefly, the primers containing the T7 promoter sequence at their 5’ end were designed using SnapDragon-Long dsRNA Design (Table 1) to synthesize the dsRNA of the TmToll-2 gene. The primary PCR for the TmToll-2 gene was performed using an AccuPower PfuPCR PreMix (Bioneer) with cDNA and specific primers of the TmToll-2 gene (Table 1). The second PCR was conducted with primers tailed with T7 promoter sequences and 100× dilution of the second PCR products. According to the developmental expression pattern of TmToll-2, cDNA was synthesized from the whole bodies of the pupae on day 5 and used as a template under the following cycling conditions: an initial denaturation step at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s, with a final extension step at 72 °C for 5 min. The PCR products
were purified using a Clear-STM PCR/Gel DNA fragment purification kit (Invirustech Co., Gwangju, Republic of Korea). The dsRNA was synthesized using an AmpliScribe T7-Flash transcription kit (Epicentre Biotechnologies, Madison, WI, USA) in accordance with the manufacturer’s instructions. After synthesis, the dsRNA was purified by precipitation with 5 M ammonium acetate and 80% ethanol and quantified using an Epoch spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Then, 2 µg of synthesized dsTmToll-2 RNAi was injected into 10th–11th instar larvae for gene silencing, and dsTmVer was used as a control.

4.5. Effect of TmToll-2 Gene Silencing on Larval Mortality against Microbial Challenge
dsTmToll-2 RNAi (2 µg/µL) was first injected into early-instar larvae (instars 10–12; n = 30) by using disposable needles mounted onto a micro-applicator (Picospritzer III Micro Dispense System; Parker Hannifin, Hollis, NH, USA) to measure the effect of TmToll-2 in the T. molitor mortality following a pathogenic invasion. An equal amount of dsTmVer was injected into the larvae at the same stage as the negative control. The efficiency of TmToll-2 knockdown was evaluated through qRT-PCR, and over 95% knockdown was achieved 4 days after injection. TmToll-2-silenced and dsTmVer-injected larval groups were challenged with E. coli (10⁶ cells/µL), S. aureus (10⁶ cells/µL), or C. albicans (5 × 10⁴ cells/µL) in triplicate experiments. The number of the surviving challenged larvae was monitored for 10 days, and the survival rates of TmToll-2-silenced larvae were compared with those of the control larvae. The relevant analysis was performed using Kaplan–Meier plots [60].

4.6. Effect of dsTmToll-2 RNAi on AMP and NF-κB Expression in Response to Microbial Challenge
The TmToll-2 silenced larvae were challenged with E. coli, S. aureus, or C. albicans via RNAi technology to further evaluate the function properties of the TmToll-2 gene in the humoral innate immune response. dsTmVer and PBS were used as the negative and injection controls, respectively. At 24 h post injection, the fat bodies, gut, and MTs were dissected, total RNA was extracted from each tissue, and cDNA was synthesized as described above. Subsequently, qRT-PCR was applied to study the expression levels of 15 AMP genes: TmTenecin-1, -2, -3, and -4 (TmTene1, 2, 3, and 4); TmAttacin-1a, -1b, and -2 (TmAtt1a, 1b, and 2); TmDefensin (TmDef); TmDefensin-like (TmDef-like); TmColeoptericin-A, -B, and -C (TmColeA, B, and C); TmCecropin-2 (TmCec-2); and TmThaumatin like protein-1 and -2 (TmTLPI and 2). Moreover, the expression patterns of NF-κB genes, such as TmDorsal isoform X1 and X2 (TmDorX1 and X2), and TmRelish, were investigated. A relative quantitative PCR was performed as mentioned above by using the AMP- and NF-κB gene-specific primers (Table 1).

4.7. Statistical Analysis
All experiments were carried out in triplicate, and data were subjected to one-way ANOVA. Tukey’s multiple range tests were used to evaluate the difference between groups (p < 0.05). The fold change in gene expression levels compared with the internal control (TmL27a) and external control (PBS) levels was calculated using the 2−∆∆Ct method.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms232214490/s1.

Author Contributions: Y.S.H., Y.H.J. and M.A.M.K. conceived and designed the experiments. M.A.M.K. performed the experiments. H.A.J. and Y.S.L. contributed reagents/materials/analysis tools. M.A.M.K., Y.H.J. and H.A.J. analyzed the data. M.A.M.K. wrote the first draft of the manuscript. M.A.M.K., H.A.J., Y.S.L., Y.H.J. and Y.S.H. revised and edited the manuscript. All authors have read and agreed to the published version of the manuscript.
Funding: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (Grant No. 2022R1A2C1013108) and by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through Agriculture, Food and Rural Affairs Convergence Technologies Program for Educating Creative Global Leader Program (or Project), funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (no.321001-03).

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Morisato, D.; Anderson, K.V. Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. *Annu. Rev. Genet.* 1995, 29, 371–399. [CrossRef] [PubMed]
2. Yu, B.; Sang, Q.; Fan, G.; Li, C.; Zhou, Z. A toll-spätzle pathway in the immune response of *Bombyx mori*. *Insects* 2020, 11, 586. [CrossRef] [PubMed]
3. Rosetto, M.; Engström, Y.; Baldari, C.T.; Telford, J.L.; Hultmark, D. Signals from the IL-1 receptor homolog, toll, can activate an immune response in a *Drosophila* hemocyte cell line. *Biochem. Biophys. Res. Commun.* 1995, 209, 111–116. [CrossRef]
4. Nee, L.; Cai, S.-Y.; Shao, J.-Z.; Chen, J. Toll-like receptors, associated biological roles, and signaling networks in non-mammals. *Front. Immunol.* 2018, 9, 1523. [CrossRef]
5. Kawasaki, T.; Kawai, T. Toll-like receptor signaling pathways. *Front. Immunol.* 2014, 461. [CrossRef]
6. Ali Mohammadie Kojour, M.; Han, Y.S.; Jo, Y.H. An overview of insect innate immunity. *Entomol. Res.* 2020, 50, 282–291. [CrossRef]
7. Valanne, S.; Wang, J.-H.; Rämet, M. The *Drosophila* toll signaling pathway. *J. Immunol.* 2011, 186, 649–656. [CrossRef]
8. Sheehan, G.; Garvey, A.; Croke, M.; Kavanagh, K. Innate humoral immune defences in mammals and insects: The same, with differences? *Virology* 2018, 9, 1625–1639. [CrossRef]
9. Umetsu, D. Cell mechanics and cell-cell recognition controls by toll-like receptors in tissue morphogenesis and homeostasis. *Fly* 2022, 16, 234–247. [CrossRef]
10. Takeda, K.; Akira, S. Toll-like receptors in innate immunity. *Int. Immunol.* 2005, 17, 1–14. [CrossRef]
11. Leulier, F.; Lemaitre, B. Toll-like receptors—Taking an evolutionary approach. *Nat. Rev. Genet.* 2008, 9, 165–178. [CrossRef] [PubMed]
12. Hetru, C.; Hoffmann, J.A. NF-κB in the immune response of *Drosophila*. *Cold Spring Harb. Perspect. Biol.* 2009, 1, a000232. [CrossRef] [PubMed]
13. Gilbert, L.I. *Insect Molecular Biology and Biochemistry*; Academic Press: Cambridge, MA, USA, 2012.
14. Muhammad, A.; Habineza, P.; Wang, X.; Xiao, R.; Ji, T.; Hou, Y.; Shi, Z. Spätzle homolog-mediated toll-like pathway regulates innate immune responses to maintain the homeostasis of gut microbiota in the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae). *Front. Microbiol.* 2020, 11, 846. [CrossRef]
15. Manniello, M.; Moretta, A.; Salvia, R.; Scieuzo, C.; Lucchetti, D.; Vogel, H.; Sgambato, A.; Falabella, P. Insect antimicrobial peptides: Potential weapons to counteract the antibiotic resistance. *Cell. Mol. Life Sci.* 2021, 78, 4259–4282. [CrossRef] [PubMed]
16. Lima, L.F.; Torres, A.Q.; Jardim, R.; Mesquita, R.D.; Schauma, R. Evolution of toll, spätzle and MyD88 in insects: The problem of the diptera bias. *BMC Genom.* 2021, 22, 562. [CrossRef] [PubMed]
17. Horng, T.; Medzhitov, R. *Drosophila* MyD88 is an adapter in the toll signaling pathway. *Proc. Natl. Acad. Sci. USA* 2001, 98, 12654–12658. [CrossRef] [PubMed]
18. Tauszig, S.; Jouanguy, E.; Hoffmann, J.A.; Imler, J.-L. Toll-related receptors and the control of antimicrobial peptide expression in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 2000, 97, 10520–10525. [CrossRef]
19. Moure, U.A.E.; Tan, T.; Sha, L.; Lu, X.; Shao, Z.; Yang, G.; Wang, Y.; Cui, H. Advances in the immune regulatory role of non-coding RNAs (miRNAs and lncRNAs) in insect-pathogen interactions. *Front. Immunol.* 2022, 13, 856457. [CrossRef]
20. Park, S.; Jo, Y.H.; Park, K.B.; Ko, H.J.; Kim, C.E.; Bae, Y.M.; Kim, B.; Jun, S.A.; Bang, I.S.; Lee, Y.S. TmToll-7 plays a crucial role in innate immune responses against gram-negative bacteria by regulating 5 AMP genes in *Tenebrio molitor*. *Front. Immunol.* 2019, 10, 310. [CrossRef]
21. Ali Mohammadie Kojour, M.; Jang, H.A.; Edosa, T.T.; Keshavarz, M.; Kim, B.B.; Bae, Y.M.; Patnaik, B.B.; Han, Y.S.; Jo, Y.H. Identification, in silico characterization, and expression analysis of *Tenebrio molitor* cecropin-2. *Entomol. Res.* 2021, 51, 74–82. [CrossRef]
22. Jang, H.A.; Park, K.B.; Kim, B.B.; Ali Mohammadie Kojour, M.; Bae, Y.M.; Baliaisringh, S.; Lee, Y.S.; Han, Y.S.; Jo, Y.H. In silico identification and expression analysis of defense genes in the mealworm beetle *Tenebrio molitor* Entomol. Res. 2020, 50, 575–585. [CrossRef]
23. Jang, H.A.; Patnaik, B.B.; Ali Mohammadie Kojour, M.; Kim, B.B.; Bae, Y.M.; Park, K.B.; Lee, Y.S.; Jo, Y.H.; Han, Y.S. TmSpz-like plays a fundamental role in response to *E. coli* but not *S. aureus* or *C. albican* infection in *Tenebrio molitor* via regulation of antimicrobial peptide production. *Int. J. Mol. Sci.* 2021, 22, 10888. [CrossRef] [PubMed]
24. Bae, Y.M.; Jo, Y.H.; Patnaik, B.B.; Kim, B.B.; Park, K.B.; Edosa, T.T.; Keshavarz, M.; Kojour, M.A.M.; Lee, Y.S.; Han, Y.S. Tenebrio molitor spätzle 1b is required to confer antibacterial defense against gram-negative bacteria by regulation of antimicrobial peptides. *Front. Physiol.* 2021, 12, 758859. [CrossRef] [PubMed]

25. Edosa, T.T.; Jo, Y.H.; Keshavarz, M.; Bae, Y.M.; Kim, D.H.; Lee, Y.S.; Han, Y.S. *TmSpz6* is essential for regulating the immune response to *Escherichia coli* and *Staphylococcus aureus* infection in *Tenebrio molitor*. *Insects* 2020, 11, 105. [CrossRef]

26. Edosa, T.T.; Jo, Y.H.; Keshavarz, M.; Bae, Y.M.; Kim, D.H.; Lee, Y.S.; Han, Y.S. *TmSpz4* plays an important role in regulating the production of antimicrobial peptides in response to *Escherichia coli* and *Candida albicans* infections. *Int. J. Mol. Sci.* 2020, 21, 1878. [CrossRef]

27. Zhang, G.; Ghosh, S. Toll-like receptor-mediated NF-κB activation: A phylogenetically conserved paradigm in innate immunity. *J. Clin. Investig.* 2001, 107, 13–19. [CrossRef]

28. Lewis, M.; Arnot, C.J.; Beeston, H.; McCoy, A.; Ashcroft, A.E.; Gay, N.J.; Gangloff, M. Cytokine spätzle binds to the *Drosophila* immunoreceptor toll with a neurotrophin-like specificity and couples receptor activation. *Proc. Natl. Acad. Sci. USA* 2013, 110, 20461–20466. [CrossRef]

29. Zhang, R.; Li, X.; Zhang, J.; Li, Y.; Wang, Y.; Song, Y.; Ren, F.; Yi, H.; Deng, X.; Zhong, Y. Toll9 from *Bombyx mori* functions as a pattern recognition receptor that shares features with toll-like receptor 4 from mammals. *Proc. Natl. Acad. Sci. USA* 2021, 118, e210321118. [CrossRef]

30. Kambris, Z.; Hoffmann, J.A.; Imler, J.-L.; Capovilla, M. Tissue and stage-specific expression of the tolls in *Drosophila* embryos. *Gene Expr. Patterns* 2002, 2, 311–317. [CrossRef]

31. Iijima, N.; Sato, K.; Kuranaga, E.; Umetzu, D. Differential cell adhesion implemented by *Drosophila* toll corrects local distortions of the anterior-posterior compartment boundary. *Nat. Commun.* 2020, 11, 6320. [CrossRef]

32. Zhai, Z.; Boquete, J.-P.; Lemaitre, B. Cell-specific Imd-NF-κB activation system in larval hemolymph. *Front. Immunol.* 2018, 9, 887–910.e897. [CrossRef] [PubMed]

33. Nishide, Y.; Kageyama, D.; Yoko, K.; Jouraku, A.; Tanaka, H.; Futahashi, R.; Fukutsu, T. Functional crosstalk across Imd and Toll pathways: Insight into the evolution of incomplete immune cascades. *Proc. R. Soc. B* 2019, 286, 20182207. [CrossRef] [PubMed]

34. Yu, Y.; Park, J.-W.; Kwon, H.-M.; Hwang, H.-O.; Jang, I.-H.; Masuda, A.; Kurokawa, K.; Nakayama, H.; Lee, W.-J.; Dohmae, N. Diversity of innate immune recognition mechanism for bacterial polymeric meso-diaminopimelic acid-type peptidoglycan in insects. *J. Biol. Chem.* 2010, 285, 32937–32945. [CrossRef] [PubMed]

35. Capilla, A.; Karachentsev, D.; Patterson, R.A.; Hermann, A.; Juarez, M.T.; McGinnis, W. Toll pathway is required for wound-induced expression of barrier repair genes in the *Drosophila* epidermis. *Proc. Natl. Acad. Sci. USA* 2017, 114, E2682–E2688. [CrossRef] [PubMed]

36. Yang, F.; Wang, Y.; Sumathipala, N.; Cao, X.; Kanost, M.R.; Jiangu, H. Manduca sexta serpin-12 controls the prophenoloxidase activation system in larval hemolymph. *Insect Biochem. Mol. Biol.* 2018, 99, 27–36. [CrossRef]

37. Kojour, M.A.M.; Edosa, T.T.; Am Jang, H.; Keshavarz, M.; Jo, Y.H.; Han, Y.S. Critical roles of spätzle5 in antimicrobial peptide production against *Escherichia coli* in *Tenebrio molitor* malpighian tubules. *Front. Immunol.* 2021, 12, 60475. [CrossRef]

38. Tingvall, T.O.; Roos, E.; Engström, Y. The imd gene is required for local necroptosis in *drosophila* barrier epithelia. *EMBO Rep.* 2001, 2, 239–243. [CrossRef] [PubMed]

39. Maire, J.; Vincent-Monégat, C.; Masson, F.; Zaidman-Reymy, A.; Heddi, A. An imd-like pathway mediates both endosymbiotic control and host immunity in the cereal weevil *Sitophilus* spp. *Microbiome* 2018, 6, 1–10. [CrossRef]

40. Ma, M.; Tu, C.; Luo, J.; Lu, M.; Zhang, S.; Xu, L. Metabolic and immunological effects of gut microbiota in leaf beetles at the local and systemic levels. *Integr. Zool.* 2021, 16, 313–323. [CrossRef]

41. Davis, M.M.; Engström, Y. Immune response in the barrier epithelia: Lessons from the fruit fly *drosophila melanogaster*. *J. Innate Immun.* 2012, 4, 273–283. [CrossRef] [PubMed]

42. Lee, J.-H.; Lee, K.-A.; Lee, W.-J. Microbiota, gut physiology, and insect immunity. In *Advances in Insect Physiology;* Elsevier: Amsterdam, The Netherlands, 2017; Volume 52, pp. 111–138.

43. Dawadi, B.; Wang, X.; Xiao, R.; Muhammad, A.; Hou, Y.; Shi, Z. PGRP-LB homolog acts as a negative modulator of immunity in maintaining the gut-microbe symbiosis of red palm weevil, *Rhynchophorus ferrugineus* olivier. *Dev. Comp. Immunol.* 2018, 86, 65–77. [CrossRef]

44. Myllymmäki, H.; Valanne, S.; Rämet, M. The drosophila imd signaling pathway. *J. Immunol.* 2014, 192, 3455–3462. [CrossRef] [PubMed]

45. Keshavarz, M.; Jo, Y.H.; Patnaik, B.B.; Park, K.B.; Ko, H.J.; Kim, C.E.; Edosa, T.T.; Lee, Y.S.; Han, Y.S. *TmRelish* is required for regulating the antimicrobial responses to *Escherichia coli* and *Staphylococcus aureus* in *Tenebrio molitor*. *Sci. Rep.* 2020, 10, 4258. [CrossRef] [PubMed]

46. Keshavarz, M.; Jo, Y.H.; Edosa, T.T.; Han, Y.S. *TmPGRP-LE* plays a critical role in gut antimicrobial peptide production in response to *Escherichia coli*. *Front. Physiol.* 2020, 11, 320. [CrossRef] [PubMed]

47. Jo, Y.H.; Patnaik, B.B.; Hwang, J.; Park, K.B.; Ko, H.J.; Kim, C.E.; Bae, Y.M.; Jung, W.J.; Lee, Y.S.; Han, Y.S. Regulation of the expression of nine antimicrobial peptide genes by *TmIMD* confers resistance against gram-negative bacteria. *Sci. Rep.* 2019, 9, 10138. [CrossRef] [PubMed]

48. Keshavarz, M.; Jo, Y.H.; Edosa, T.T.; Bae, Y.M.; Han, Y.S. *TmPGRP-SA* regulates antimicrobial response to bacteria and fungi in the fat body and gut of *Tenebrio molitor*. *Int. J. Mol. Sci.* 2020, 21, 2113. [CrossRef] [PubMed]
49. Jang, H.A.; Ali Mohammadie Kojour, M.; Patnaik, B.B.; Han, Y.S.; Jo, Y.H. Current status of immune deficiency pathway in *Tenebrio molitor* innate immunity. *Front. Immunol.* **2022**, 13, 906192. [CrossRef] [PubMed]

50. Wu, S.-C.; Liao, C.-W.; Pan, R.-L.; Juang, J.-L. Infection-induced intestinal oxidative stress triggers organ-to-organ immunological communication in *Drosophila*. *Cell Host Microbe* **2012**, 11, 410–417. [CrossRef] [PubMed]

51. Beckage, N.E. *Insect Immunology*; Academic Press: Cambridge, MA, USA, 2011.

52. Keshavarz, M.; Jo, Y.H.; Park, K.B.; Ko, H.J.; Edosa, T.T.; Lee, Y.S.; Han, Y.S. *TmDorX2* positively regulates antimicrobial peptides in *Tenebrio molitor* gut, fat body, and hemocytes in response to bacterial and fungal infection. *Sci. Rep.* **2019**, 9, 16878. [CrossRef]

53. Chae, J.-H.; Kurokawa, K.; So, Y.-I.; Hwang, H.O.; Kim, M.-S.; Park, J.-W.; Jo, Y.-H.; Lee, Y.S.; Lee, B.L. Purification and characterization of tenecin 4, a new anti-gram-negative bacterial peptide, from the beetle *Tenebrio molitor*. *Dev. Comp. Immunol.* **2012**, 36, 540–546. [CrossRef]

54. Jo, Y.H.; Kim, Y.J.; Park, K.B.; Seong, J.H.; Kim, S.G.; Park, S.; Noh, M.Y.; Lee, Y.S.; Han, Y.S. *TmCactin* plays an important role in gram-negative and-positive bacterial infection by regulating expression of 7 AMP genes in *Tenebrio molitor*. *Sci. Rep.* **2017**, 7, 1–12. [CrossRef] [PubMed]

55. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, 215, 403–410. [CrossRef]

56. Larkin, M.; Blackshields, G.; Brown, N.; Chenna, R.; McGettigan, P.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X Version 2.0. *Bioinformatics* **1990**, 23, 2947–2948. [CrossRef] [PubMed]

57. Kumar, S.; Stecher, G.; Tamura, K. Mega7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, 33, 1870–1874. [CrossRef]

58. Schmittgen, T.L.; Ivak, K.J. Analyzing Real-Time PCR Data by the Comparative *C*<sub>T</sub> Method. *Nat. Protoc.* **2008**, 3, 1101–1108. [CrossRef]

59. Ko, H.J.; Patnaik, B.B.; Park, K.B.; Kim, C.E.; Baliarsingh, S.; Jang, H.A.; Lee, Y.S.; Han, Y.S.; Jo, Y.H. *TmIKKε* is required to confer protection against gram-negative bacteria, *E. coli* by the regulation of antimicrobial peptide production in the *Tenebrio molitor* fat body. *Front. Physiol.* **2022**, 12, 758862. [CrossRef]

60. Goel, M.K.; Khanna, P.; Kishore, J. Understanding survival analysis: Kaplan-meier estimate. *Int. J. Ayurveda Res.* **2010**, 1, 274.