Two Roles for the *Tenebrio molitor* Relish in the Regulation of Antimicrobial Peptides and Autophagy-Related Genes in Response to *Listeria monocytogenes*

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**Abstract:** Relish is a key NF-κB transcription factor of the immune-deficiency (Imd) pathway that combats infection by regulating antimicrobial peptides (AMPs). Understanding of the fundamental role of *Tenebrio molitor* Relish (*TmRelish*) in controlling of *Listeria monocytogenes* virulence through the regulation of both AMPs and autophagy-related (ATG) genes is unclear. Here, we show that *TmRelish* transcripts were highly abundant in the larval fat body and hemocytes compared to the gut upon *L. monocytogenes* infection. Furthermore, significant mortality was observed in *TmRelish*-silenced larvae after intracellular insult. To investigate the cause of this lethality, we measured the induction of AMPs and ATG genes in the *TmRelish* dsRNA-treated *T. molitor* larvae. The expression of *TmTenecin*-1, *TmTenecin*-4, *TmColeptericin*-1, *TmAttacin*-2, and *TmCecropin*-2 were suppressed in the fat body and hemocytes of ds*TmRelish*-injected larvae during *L. monocytogenes* infection. In addition, *TmRelish* knockdown led to a noticeable downregulation of *TmATG1* (a serine-threonine protein kinase) in the fat body and hemocytes of young larvae 6 h post-infection (pi). The notable increase of autophagy genes in the early stage of infection (6 h pi), suggesting autophagy response is crucial for *Listeria* clearance. Taken together, these results suggest that *TmRelish* plays pivotal roles in not only regulation of AMP genes but also induction of autophagy genes in response to *L. monocytogenes* challenge in fat body and hemocytes of *T. molitor* larvae. Furthermore, negative regulation of several AMPs by *TmRelish* in the fat body, hemocytes, and gut leaves open the possibility of a crosstalk between Toll and Imd pathway.

**Keywords:** *Tenebrio molitor*; Relish; NF-κB; antimicrobial peptides; autophagy; *Listeria monocytogenes*

1. **Introduction**

The innate immune system is an evolutionarily conserved defense mechanism that combats infectious pathogens. Once pathogens breach the first line of defense, the sensors, peptidoglycan-recognition proteins (PGRPs), recognize their respective bacterial peptidoglycans (PGNs) [1]. The signal generated from the PGRP/PGN interaction involves other intracellular components of the Toll and immune deficiency (Imd) signaling pathways, and it triggers nuclear translocation of nuclear factor kappa-light-chain-enhancer (NF-κB) transcription factors of those pathways, such as Dorsal and Relish, respectively [2,3]. Ultimately, this signal transduction leads to the transcriptional expression of immune-related effectors, such as antimicrobial peptides (AMPs) [4].
The main molecular components of Gram-negative bacteria and some Gram-positive bacteria (Bacillus and Listeria spp.) consist of meso-diaminopimelic acid DAP-type PGN, which binds to intracellular PGRP-LE and plasma membrane PGRP-LC, resulting in the activation of autophagy and the Imd pathway [5,6]. Moreover, PGRP-LE is not only capable of activation of Imd pathway, but also prophenoloxidase (proPO) cascade [7].

Listeria monocytogenes, a facultative intracellular gram-positive bacterium is able to invade and replicate in a broad range of host cells, including phagocytic and non-phagocytic cells [8]. Over several decades, this human pathogen became a host-pathogen interaction paradigm in vertebrates [9] and invertebrates [6,10]. Previous studies in Drosophila have demonstrated that PGRP-LE mediates the detection of L. monocytogenes invasion and is also responsible for initiating the autophagy pathway to suppress bacterial growth. Conversely, products of Relish, Dorsal, Dif (Dorsal-related immunity factor), and Atg5 were not required for PGRP-LE-mediated suppression of L. monocytogenes growth [6].

Autophagy is a conserved process of self-dysfunctional cell degradation [11]. Autophagy genes are also incontrovertibly required for removing intracellular bacteria and parasites [12–14]. The autophagy-related (ATG) genes, which are involved in the formation of the autophagosome (Core autophagy machinery), consist of four main ATG protein complexes. The autophagosome initiation is firstly regulated by Unc-51 Like Autophagy Activating Kinase 1 (ULK1)/ATG1-ATG13 protein kinase complex, then Beclin 1 (BECN1)/ATG6-Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Epsilon (PIK3CE)/class III PI3K Vacuolar protein sorting 34 (Vps34) complex is responsible for autophagosome nucleation. Finally, two ubiquitin-like conjugation systems, which constitute ATG12-ATG5-ATG16L1 and ATG8-II, are required for expansion and completion [11]. Several autophagy-related genes have been identified in Tenebrio molitor, including TmATG13 [15], TmATG3 and -5 [14], and TmATG8 [13].

Recent investigations of Drosophila innate immunity have indicated that (stimulator of interferon genes (dmSTING) silencing, following infection with L. monocytogenes, leads to a reduction of immune-related gene expression and consequently, increased mortality. Consistent with these findings, dmSTING has been shown to function through the activation of Relish (Imd pathway), leading to the induction of AMPs, to reduce L. monocytogenes load in Drosophila [16]. Relish is a fundamental downstream component of the Drosophila Imd pathway, however, previous studies have revealed that it plays an essential role in the onset of cell death and neurodegeneration via transcriptional upregulation of innate immune response genes, such as AMPs [17]. Moreover, it has been recently shown that Relish positively controls the programmed cell death and autophagy-related gene, ATG1, as a key activator of the autophagy pathway in the fly salivary gland [18].

The multifunctional pattern recognition molecule, PGRP-LE, acts upstream of Imd pathway and proPO cascade extracellularly while it activates Imd pathway and autophagy intracellularly. Consequently, it is reasonable to hypothesize that there might be a cross-talk between autophagy and Imd pathway [19]. Although it has been reported that listeria-induced autophagy is independent of Imd pathway in Drosophila [20], however, the association between autophagy and Relish-immune responses has been elucidated by earlier study in Drosophila models [18]. Similar results as reported for Drosophila PGRP-LE have been observed in the PGRP-LE homologue from the mealworm, T. molitor (Coleoptera: Tenebrionidae). TmPGRP-LE controls the activation of autophagy, which is crucial for host survival against intracellular L. monocytogenes infection [10]. We wondered whether TmRelish, as a downstream transcription factor of PGRP-LE/Imd pathway, could directly or indirectly regulate both AMP genes expression and ATG genes level after L. monocytogenes challenge. To test our hypothesis, here, we utilized RNA interference (RNAi) methods. Our data present that TmRelish is necessary for the regulation of AMP genes and autophagy-related genes in the fat body and hemocytes of T. molitor larvae.
2. Materials and Methods

2.1. T. molitor Rearing

*T. molitor* was maintained under standard conditions (at 27 ± 1 °C and 60 ± 5% RH) in the dark because they are nocturnal [21,22]. The insects were fed on an artificial diet consisting of 170 g of wheat flour, 0.5 g of chloramphenicol, 20 g of roasted soy flour, 0.5 g of sorbic acid, 0.5 mL of propionic acid, 10 g of soy protein, and 100 g of wheat bran in 200 mL of distilled water. The dietary preparation was autoclaved at 121 °C for 20 min and kept in an insectary. Only healthy 10th to 12th instar larvae (~2.4 cm) were used for all experiments.

2.2. Sample Collection after Immune Challenge with *L. monocytogenes*

*L. monocytogenes* strain American Type Culture Collection (ATCC) 7644 was grown in Brain-heart infusion (BHI) medium. In order to prepare the *L. monocytogenes* culture for microbial challenge studies, a single colony was chosen from *L. monocytogenes* BHI medium using a sterile wire loop and it was cultured overnight in 5 mL of fresh BHI broth at 37 °C at 200 revolutions per minute (rpm) in an orbital shaker. To prevent the death phase and to change the stationary phase to the exponential (log) phase, the overnight culture was subcultured in 5 mL of fresh BHI at 37 °C for 3 h. The new subcultured microorganism was washed three times with 1× PBS (phosphate-buffered saline, pH 7.0) at 3500 rpm for 5 min. The optical density of washed *L. monocytogenes* was measured at 600 nm and the microorganism was resuspended in PBS at a concentration of 1 × 10^6 cells/µL.

1 µL of *L. monocytogenes* (1 × 10^6 cells/µL) was injected into young *T. molitor* larvae in three independent experimental sets (n = 20). Challenged larvae were kept under insectary conditions and fed an artificial diet. Tissue samples including fat body, hemocytes, and gut were dissected from each infected individual and also from PBS-injected controls, at 3, 6, 9, 12, and 24 h post-injection (pi). First, a clean needle was inserted into the prothorax near the head of *T. molitor* larvae and pipette the larval hemolymph using a micropipette in 500 µL PBS buffer and centrifuged at 500 rpm for 15 min. The supernatant was discarded and the pellet (hemocyte) was washed with PBS buffer. To isolate the gut, cut the last abdominal segment and washed the dissected gut in PBS buffer using two forceps. To prevent the contamination of the fat body with Malpighian Tubules, the fat body was washed in the PBS buffer. Collected tissues were added to guanidine thiocyanate RNA lysis buffer (20 mM EDTA, 20 mM MES buffer, 3M guanidine thiocyanate, 200 mM sodium chloride, 40 µM phenol red, 0.05% Tween-80, 0.5% glacial acetic acid [for pH 5.5], and 1% isoamyl alcohol) and homogenized at 8500 rpm for 20 s using a homogenizer (Bertin Technologies, Bretonneux, France). The homogenized samples were then stored at −80 °C for total RNA extraction, cDNA synthesis, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

2.3. RNA Extraction, cDNA Synthesis and qRT-PCR

Total RNAs were extracted from collected samples using the modified LogSpin RNA isolation method [23]. Homogenized samples were incubated at room temperature for 1 min and then centrifuged at 13,000 rpm at 4 °C for 5 min. Next, 100 µL of supernatant was transferred to 200 µL of RNA lysis buffer and the sample was then added to 300 µL of 99.9% ethanol. After gently inverting samples, they were transferred to a silica spin column (Bioneer, Daejeon, Korea; KA-0133-1) and centrifuged at 13,000 rpm for 30 s at 4 °C. The resulting aqueous phase was discarded. To digest genomic DNA, the silica spin column was treated with DNase (Promega, Madison, WI, USA; M6101) and incubated at 37 °C for 15 min. The silica spin column was then washed with 450 mL of 3 M sodium acetate buffer and then with 500 mL of 80 % ethanol and centrifuged as previously. After drying the spin column for 2 min, total RNA was eluted in 30 µL of distilled water.
For cDNA synthesis, 2 µg of total RNA was incubated with an oligo-(dT)_{12–18} primer for 5 min and then incubated in an AccuPower® RT PreMix solution (Bioneer).

The prepared cDNA samples were diluted 1:20 prior to PCR analysis. To analyze the tissue distribution of TmRelish, qRT-PCR was performed using the cycling conditions recommended for the AccuPower® 2X GreenStar qPCR Master Mix (Bioneer) (denaturation of 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s) by adding TmRelish-specific primers. The T. molitor ribosomal protein gene (TmL27a) was used as a reference. Primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and their sequences are listed in Table 1. qRT-PCR data were analyzed using the comparative C_{T} method (2^{-\Delta\Delta C_{T}} method; [24]). Statistical analysis of fold change values was performed using a Student’s t-test (p < 0.05).

Table 1. Sequences of the primers used in this study.

| Primer                      | Sequence                              |
|-----------------------------|---------------------------------------|
| TmRelish_qPCR_Fw            | 5'-AGCGTCAAGTTGGAGCAGAT-3'            |
| TmRelish_qPCR_Rv            | 5'-GTCGGACCTCAATAGTGTG-3'             |
| TmRelish_Temp_Fw            | 5'-TGGGGAATAGTAGGAGGAAA-3'            |
| TmRelish_Temp_Rv            | 5'-CAAATTGGCCACGACTCTCT-3'            |
| dsTmRelish_Rv               | 5'-TTAATACGACTCTATAAGG-3'             |
| dsEGFP_Fw                   | 5'-TTAATACGACTCTACTAGGT-3'            |
| dsEGFP_Rv                   | 5'-TTAATACGACTCTACTAGGT-3'            |
| TmTenecein-1_Fw             | 5'-CAGGCTGAAGATCGAAGAAGG-3'           |
| TmTenecein-1_Rv             | 5'-CAGACCCCTTTCTTGATCAGT-3'           |
| TmTenecein-2_Fw             | 5'-CGTTGAAATCCTGTACCTTCTCG-3'         |
| TmTenecein-3_Fw             | 5'-GATTGTGGATCTCTGTATGTCG-3'          |
| TmTenecein-3_Rv             | 5'-CTGATGCGCCTCTCAAAGTCTC-3'          |
| TmTenecein-4_Fw             | 5'-CGTGAGATCCAGATGCAAGAG-3'           |
| TmTenecein-4_Rv             | 5'-CGTGATCTCTTATGAGACTG-3'            |
| TmDefensin-1_Fw             | 5'-AAATCGGAAAGACGCAACAC-3'            |
| TmDefensin-1_Rv             | 5'-GCAATAATGCAAGACCTTCTTC-3'          |
| TmDefensin-2_Fw             | 5'-GGGATCCATCAAGATGAGT-3'             |
| TmDefensin-2_Rv             | 5'-CCAATGCAAACACATTTAGCT-3'           |
| TmColeopterisin-1_Fw        | 5'-GCGAATACAATGAGATTGCTC-3'           |
| TmColeopterisin-1_Rv        | 5'-CTCAGCAATCTATTGCTGCAGAC-3'         |
| TmColeopterisin-2_Fw        | 5'-GAGCGGAGTTCGCGATTGATG-3'           |
| TmColeopterisin-2_Rv        | 5'-AGCTGTCTTCTTTGTTGTCGTC-3'          |
| TmAttacin-1a_Fw             | 5'-GAAAGGAATAAGGAAGTGGA-3'            |
| TmAttacin-1a_Rv             | 5'-TGCTTGGCAACATACAG-3'               |
| TmAttacin-1b_Fw             | 5'-GAGCTGTGAATGCAGAAAC-3'             |
| TmAttacin-1b_Rv             | 5'-CCCCTTGATGAAACCTCAAC-3'            |
| TmAttacin-2_Fw              | 5'-AACTGGGATATCCGAGAC-3'              |
| TmAttacin-2_Rv              | 5'-CCCTCCGAAATGTCTGGATG-3'            |
| TmCecropin-2_Fw             | 5'-TACAGAGCAGACCCCAACACT-3'           |
| TmCecropin-2_Rv             | 5'-CTGGAACATAGCGGAGAA-3'              |
| TmThaumatin-like protein-1_Fw | 5'-CTCAAAAGCAACCCGAGACT-3'            |
| TmThaumatin-like protein-1_Rv | 5'-ACTTTGAGCTCTGCGGAC-3'              |
| TmThaumatin-like protein-2_Fw | 5'-CGTGCTGTAGAGTTCGTGC-3'             |
| TmThaumatin-like protein-2_Rv | 5'-ACTCTCTAGCTCGTATCAAC-3'            |
| TmL27a_qPCR_Fw              | 5'-TCATCTGAAGGCAAAGGCTCAGG-3'         |
| TmL27a_qPCR_Rv              | 5'-AGTTGGTATAGCGCAGCCACCTTTA-3'       |

Note: Underline indicates T7 promoter sequences.

2.4. Double-Stranded RNA TmRelish Synthesis

To prepare RNA interference (RNAi)-directed silencing of TmRelish, specific forward and reverse primers containing T7 promoter sequences at their 5’ ends were designed using SnapDragon-Long software (https://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl). Amplicons of 851 bp were generated using AccuPower® Pfu PCR PreMix, cDNA templates from T. molitor larvae, and specific primers (Table 1), under the following PCR conditions: 95 °C for 2 min, followed by 30 cycles of denaturation.
at 95 °C for 20 s, annealing at 56 °C for 30 s, and extension at 72 °C for 5 min. The generated products were purified using an AccuPrep® PCR Purification Kit (Bioneer) and were then used to synthesize TmRelish RNAi in vitro with the EZ™ T7 High Yield In Vitro Transcription Kit (Enzymomics, Daejeon, Korea), as per the manufacturer’s instructions. After incubating the mixture at 37 °C for 3 h and then 25 °C for 1 h, the synthesized dsRNA was gently mixed with one volume of 5 M ammonium acetate and incubated on ice for a further 15 min. The generated dsTmRelish was centrifuged at 13,000 rpm at 4 °C for 10 min and then washed three times with 70%, 80%, and 99.9% ethanol. After the final drying stage, the pellet was suspended in 30 µL of distilled water (Sigma, St Louis, MO, USA; W4502-1L). A dsRNA targeting enhanced green fluorescent protein (EGFP) was subsequently synthesized to serve as a negative control.

2.5. Mortality Assay of TmRelish-Knockdown T. molitor Larvae Upon L. monocytogenes Infection

To understand the immunological role of TmRelish in response to L. monocytogenes, 60 healthy T. molitor larvae were divided into two groups. The first group (n = 30) was injected with double-stranded EGFP RNA, dsEGFP, as a negative control and the other group (n = 30) was injected with dsTmRelish. Three days after TmRelish RNAi injection, knockdown efficiency was verified (73%) and both groups were then challenged with L. monocytogenes (1 x 10⁶ cells/µL). Same sets (n = 60) were prepared for PBS-injected larvae as a control larval mortality was evaluated and T. molitor cadavers were removed from experimental plates daily for 10 days. The experiments were performed in triplicate.

2.6. Effect of TmRelish Gene Silencing on AMP Expression After Bacterial Insult

To assess the participation of TmRelish in humoral immunity and to determine whether high susceptibility of TmRelish-silenced larvae is related to AMP gene regulation, the mRNA expression levels of 14 identified AMPs, comprising TmTenein family (TmTenein-1, TmTenein-2, TmTenein-3, TmTenein-4) [25–28], TmAttacin family (TmAttacin-1a, TmAttacin-1b, TmAttacin-2) [29], TmDefensin-1, TmDefensin-2, TmColeptericin-1, TmColeptericin-2, TmCecropin-2, TmThaumatin like protein family (TmThaumatin like protein-1, and TmThaumatin like protein-2) [30,31] were measured by qRT-PCR in fat body, hemocytes, and gut of dsTmRelish-injected larvae compared with dsEGFP-injected larvae after microbial infection. At 24 h post-L. monocytogenes infection, immune defense tissues were dissected in cold PBS solution, total RNAs were extracted and cDNA was synthesized as described above. AMP-specific primers (Table 1) were used for qRT-PCR.

2.7. TmRelish RNAi and Regulation of Autophagy-related Genes in Response to L. monocytogenes

To further understand the possible role of TmRelish in cellular immune responses, we assessed the expression levels of autophagy-related genes involved in the initiation, vesicle nucleation, and vesicle expansion and completion stages of autophagy. The relative mRNA levels of TmATG1 (Atg9 cycling system), TmVps34 which is a components of Phosphoinositide 3-kinase (PI3K) complex, TmATG9 (Atg9 cycling system), TmATG5 (Ubl system) [14], and TmATG8 (Ubl system) [13] were analyzed using qRT-PCR in hemocytes, fat body, and gut of dsTmRelish-injected larvae compared with dsEGFP-injected larvae at 3, 6, and 9 h post-L. monocytogenes infection. The autophagy-related gene-specific primers were also designed using Primer3Plus and the primer sequences are shown in Table 2. PBS-injected and dsEGFP-treated larvae were used as the mock and negative controls, respectively.

2.8. Statistical Analysis

All experiments were carried out in triplicate and data were subjected to one-way analysis of variance (ANOVA). In order to evaluate the difference between groups (p < 0.05), Tukey’s multiple range tests were performed. The results for the mortality assay were analyzed using Kaplan–Meier plot (log-rank Chi-square test) by Excel (http://www.real-statistics.com/survival-analysis/kaplan-meier-procedure/real-statistics-kaplan-meier/).
Table 2. Sequences of the primers used in this study.

| Autophagosome Protein Complex | Autophagy-Related Genes | Sequence |
|------------------------------|-------------------------|----------|
| Initiation                   | TmATG1-qPCR-Fw           | 5′-TTGCGATTATCTCAACGC-3′ |
|                              | TmATG1-qPCR-Rv           | 5′-TTCATGGCCAGCTAAATTC-3′ |
| Nucleation                   | TmVps34-qPCR-Fw          | 5′-AGCAACAGATATCGAGGAT-3′ |
|                              | TmVps34-qPCR-Rv          | 5′-ATGTTGCCTGTCGTGTC-3′ |
| Recycling                    | TmATG9-qPCR-Fw           | 5′-ATGCGAAGACGCAAACCTG-3′ |
|                              | TmATG9-qPCR-Rv           | 5′-ATGCCTCTGATTTCTC-3′ |
| Elongation                   | TmATG5-qPCR-Fw           | 5′-GGGCTTGAAATCGAAGTTG-3′ |
|                              | TmATG5-qPCR-Rv           | 5′-GTTTTCGGGTCATCTATC-3′ |
| Completion and extension     | TmATG8-qPCR-Fw           | 5′-AAGATCCCGCGAAGTGATCC-3′ |
|                              | TmATG8-qPCR-Rv           | 5′-AACTGCGCAGCTGCAAATC-3′ |

3. Results

3.1. Expression and Induction of TmRelish Transcripts Upon L. monocytogenes Challenge

Previous studies of the immune function of TmRelish have shown that this transcription factor can be detected in all examined tissues, with high levels of expression in the gut, fat body, and hemocytes, and relatively low levels of expression in Malpighian tubules and integument [32]. Since the highest levels of TmRelish expression are in the most important immune-related tissues in T. molitor, these tissues were chosen to study the time-course of TmRelish expression using qRT-PCR. Following L. monocytogenes challenge, the mRNA levels of TmRelish in the fat body of challenged larvae were significantly upregulated from 3 h to 24 h, with the first peak at 3 h and the second peak at 9 h pi (Figure 1A). In hemocytes of L. monocytogenes-challenged larvae, the transcriptional levels of TmRelish reached their maximum at 6 h and then gradually decreased (Figure 1B). The expression levels of TmRelish in the gut of T. molitor larvae increased to 1.75-fold at 6 h, followed by 2.01-fold at 9 h, and then ultimately decreased to 0.5-fold at 24 h (Figure 1C).

Figure 1. TmRelish mRNA expression levels in the fat body (A), hemocytes (B), and gut (C) upon L. monocytogenes infection. Total RNAs were isolated from young larvae (10th–12th instar larvae) at 3, 6, 9, 12, and 24 h post-injection. T. molitor 60S ribosomal protein L27a (TmL27a) was used as an endogenous control. TmRelish expression in PBS-treated larvae was normalized to 1. * shows significant differences (p < 0.05).
3.2. Effect of TmRelish Gene Knockdown on T. molitor Larval Mortality after L. monocytogenes Infection

Given that TmRelish expression was modulated by L. monocytogenes infection, TmRelish is likely to play a key role in the immune responses against L. monocytogenes. Accordingly, T. molitor larvae may be more susceptible to L. monocytogenes after the RNAi-mediated depletion of TmRelish. To test this hypothesis regarding the functional role of TmRelish, survival was assessed in 10th–12th instar T. molitor larvae injected with TmRelish dsRNA and then challenged with L. monocytogenes (1 × 10^6 cells/insects). Larvae injected with dsRNA targeting TmRelish mRNA exhibited a knockdown efficiency of 73% at 3 days post-dsTmRelish treatment (Figure 2A). After 10 days, the final survival curve significantly differed in larvae injected with dsEGFP (90%) compared to those injected with dsTmRelish (57%) (Figure 2B).

Figure 2. Effect of TmRelish silencing on the survival of T. molitor larvae after challenging with L. monocytogenes for 10 days. Knockdown efficiency of TmRelish mRNA, extracted from dsTmRelish-injected larvae in comparison dsEGFP injected group was measured 3-day post-injection by qRT-PCR (A). Survival results for dsTmRelish-injected larvae after L. monocytogenes challenge are presented as the average of three biological replicates (B). The dsEGFP-injected groups followed by same microbial infection were used as negative controls. * indicates significant differences between dsTmRelish and dsEGFP-treated groups (p < 0.05).

3.3. Effect of TmRelish RNAi on AMP Genes Expression in Response to L. monocytogenes

Our mortality data showed that TmRelish gene knockdown rendered T. molitor significantly more susceptible to L. monocytogenes infection, suggesting that TmRelish is a pivotal protein to combat infection with intracellular gram-positive bacteria. Next, to investigate the genes regulated by TmRelish, particularly those involved in the production of AMPs, we performed a TmRelish RNAi experiment and the transcript abundance of 14 AMP genes were subsequently measured by qRT-PCR.

In the fat body of dsEGFP-injected T. molitor larvae, we observed clear upregulation of 10 AMPs: TmTene1 and TmTene4; TmDef1 and TmDef2; TmCole1 and TmCole2; TmAtt1a, TmAtt1b, and TmAtt2; and TmCec2 (Figure 3A). Meanwhile, the expression levels of these AMPs significantly decreased in the context of infection and TmRelish depletion. Conversely, the expression of antifungal AMPs such as TmTLP1 and TmTLP2 was notably higher in TmRelish-silenced larvae compared to dsEGFP-treated controls (Figure 3A).

Furthermore, hemocytes of TmRelish-silenced T. molitor larvae displayed similar expression patterns for TmTene1, TmTene4, TmAtt2, TmCole1, and TmCec2 (Figure 3B). Notably, seven AMPs such as TmTene2, TmTene3, TmAtt1a, TmAtt1b, TmCole2, TmTLP1 and TmTLP2 were not induced in the hemocytes of dsEGFP-injected groups while all aforementioned AMPs were barely detected in dsTmRelish-injected larvae (Figure 3B). Strikingly, TmRelish knockdown did not suppress the mRNA expression of TmDef2 (Figure 3B). Collectively, the antimicrobial response of the larval fat body to L. monocytogenes infection was considerably higher compared to the larval hemocytes.
Figure 3. Analysis of AMP genes in the fat body (A), hemocytes (B), and gut (C) of *T. molitor* larvae after *TmRelish* silencing followed by microbial challenge with *L. monocytogenes*. The mRNA levels of 14 AMP genes comprising *TmTene1*, *TmTene2*, *TmTene3*, *TmTene4*, *TmDef1*, *TmDef2*, *TmCole1*, *TmCole2*, *TmAtt1a*, *TmAtt1b*, *TmAtt2*, *TmCec2*, *TmTLP1*, and *TmTLP2* were measured by qRT-PCR. Statistical significance of the fold change in AMP gene expression in *TmRelish*-knockdown larvae compared with negative control and double stranded EGFP-treated larvae is indicated by asterisks (*p* < 0.05) and ns = not significant. The number above the bars indicates the AMP transcript level. Error bars indicate the SEM of three biological experiments.

Interestingly, the mRNA levels of eleven AMPs including *TmTene1*, *TmTene2*, *TmTene4*, *TmDef1*, *TmDef2*, *TmAtt1a*, *TmAtt1b*, *TmAtt2*, *TmCole1*, *TmCole2*, and *TmCec2* showed a significant increase in the larval gut when *TmRelish* was silenced (Figure 3C). However, similar to hemocytes, there were no considerable changes in the transcriptional levels of antifungal AMPs namely *TmTene3*, *TmTLP1*, and *TmTLP2* in the gut (Figure 3C). Taken together, our results suggest that *TmRelish* negatively regulates AMP expression in the gut of *T. molitor* whereas it acts as a positive regulator in the fat body in response to *L. monocytogenes* infection.

3.4. *TmRelish* Gene Contribution to the Regulation of Autophagy-Related Genes in Response to *L. monocytogenes*

The significant decrease in survival of ds*TmRelish* larvae upon *L. monocytogenes* infection led us to assess autophagy genes. To understand whether autophagy-related genes are affected when the transcription factor, *TmRelish*, is silenced, we performed RNAi experiments in the fat body, hemocytes, and gut of young larvae 3, 6, and 9 h pi.
In the fat body of TmRelish-knockdown larvae 3 h post-L. monocytogenes infection, TmVps34 and TmATG5 expression levels were considerably decreased compared with dsEGFP-treated controls. In contrast, the mRNA levels of TmATG1, TmATG9, and TmATG8 were upregulated in TmRelish-silenced larvae (Figure 4A). In a similar pattern to what we found in the fat body of TmRelish-silenced larvae, TmATG1 was induced at lower levels in the hemocytes and gut at 6 h pi (Figure 4). Silencing of TmRelish did not have an effect on TmVps34, TmATG5, and TmATG8 expression in the fat body at 6 h pi (Figure 4A). However, intriguingly, the transcriptional levels of TmATG1, TmVps34, TmATG9, TmATG5, and TmATG8 were markedly downregulated in the hemocytes of dsTmRelish larvae in comparison to dsEGFP-injected larvae at 6 h pi (Figure 4B). Additionally, in the hemocytes of TmRelish-silenced larvae, all examined autophagy-related gene expression levels were moderately upregulated, regardless of TmATG9 at 3 h pi (Figure 4B). In contrast with hemocytes at 9 h pi, the expression levels of two autophagy-related genes comprising TmATG1 and TmVps34 were downregulated in the fat body of TmRelish-knockdown larvae (Figure 4A,B). While expression analysis showed a notable upregulation of TmATG1, TmVps34, and TmATG5 expression in hemocytes of dsTmRelish-injected larvae at 9 h pi, also we observed no significant fold change in TmATG9 and TmATG8 expression in the same groups of larvae (Figure 4B).

Figure 4. Effect of TmRelish gene silencing on the expression level of T. molitor autophagy-related genes (TmATG) in the fat body (A), hemocytes (B), and gut (C) of larvae at 3, 6, and 9 h after L. monocytogenes challenge. Transcriptional levels of TmATG1, TmVps34, TmATG9, TmATG5, and TmATG8 were quantified by qRT-PCR. All experiments were performed on three independent sets. * indicates statistically significant differences between the dsTmRelish- and dsEGFP-treated groups using Student's t-tests (p < 0.05) and ns = not significant.
Similar to the fat body and hemocytes, TmATG1 transcript was significantly increased in TmRelish-silenced groups at 3 h pi (Figure 4). Conversely, the transcript levels of TmATG5 and TmATG8 were slightly decreased in the gut of TmRelish dsRNA-treated T. molitor larvae at 3 h pi (Figure 4C). Of note, all the autophagy genes evaluated showed a significant upregulation in the larval gut against L. monocytogenes at 9 h pi (Figure 4C).

Importantly, the mRNA levels of TmATG1 were significantly decreased in all dissected tissues 6 h pi (Figure 4). These results suggested that TmRelish is crucial for the expression of TmATG1 in the fat body, hemocytes, and gut 6 h pi.

4. Discussion

Insects are the most evolutionarily successful organisms and they can adapt well to the so-called pathosphere. The versatile immune system of insects provides them with a high capacity to eliminate pathogenic infections, which is a key factor in the successful dispersal of insects in the wild [33]. Among all insect models, T. molitor, a heat-tolerant beetle, has emerged as a useful host-pathogen interaction paradigm to investigate human pathogens, such as L. monocytogenes [34].

In recent years, it has been consistently reported that autophagy, as an innate immune response, plays important roles in the defense against intracellular infections [6,35]. Furthermore, the classical Imd pathway recognizes the DAP-type PGN, a major component of the cell walls of gram-negative bacteria and several gram-positive Bacillus and Listeria spp. [36]. Relish, an Imd transcription factor, engages a complex array of ancient defensive molecules (i.e., AMPs) to ward off microbial insults [37].

It is clear from the results presented here that TmRelish plays a significant role in the fat body, hemocytes, and gut of T. molitor larvae after L. monocytogenes challenge. TmRelish transcripts were highly abundant in fat body and hemocytes, suggesting that similar to other invertebrates, the immune response in T. molitor larvae also originates in both of these main immune-related organs [38–40].

Mortality assay was primarily performed to determine whether L. monocytogenes was capable of triggering a lethal infection in dsTmRelish-treated larvae. Our data showed that dsRNA knockdown of TmRelish resulted in an increased susceptibility of the larvae to listeria, suggesting the involvement of TmRelish in the defense against DAP-type bacteria. In congruence with our finding, the result of mortality assay in TmPGRP-LE RNAi-treated T. molitor larvae has revealed a significant death rate of insects in response to L. monocytogenes [10]. This was further supported by lethality of Galleria mellonella larvae after L. monocytogenes infection (1 × 10⁶ cells/µL) during 7 days [41]. Furthermore, a previous study in Drosophila reported a 100% mortality rate 6–8 days after L. monocytogenes infection [42]. Also, the implication of Relish (REL2) in mosquitoes immune responses (Anopheles gambiae and Aedes aegypti) has also been reported against other gram-positive bacteria such as Staphylococcus aureus [43,44]. Thus, understanding the reasons for the significant susceptibility of dsTmRelish-injected larvae to L. monocytogenes infection, is a major concern. Therefore, the abundance of 14 AMP genes transcripts was examined in TmRelish-depleted larvae after L. monocytogenes insult.

In T. molitor fat body, the expression levels of 10 AMPs were upregulated in non-TmRelish-silenced groups after L. monocytogenes infection; however, RNAi TmRelish-injected larvae showed downregulated expression of TmTene1 and -4; TmDef1 and -2; TmCec2 and -2, TmAtt1a, -1b, and -2; and TmCec2. The results of the current study agree with the results of our previous studies on the gram-positive bacteria, S. aureus, in which all listed AMPs were found to be downregulated in the fat body of dsTmRelish-treated larvae following S. aureus challenge [32]. Therefore, TmRelish plays a critical role in response to Gram-positive bacteria, including intracellular bacteria in the larval fat body of T. molitor. However, L. monocytogenes similar to gram-negative bacteria contains DAP-type PGN, which is recognized by PGRP-LE in hemocytes of Drosophila [6]. As it has been confirmed, due to the strong elicitor activity of the DAP-type PGN, AMP expression levels of Bombyx mori are higher than lysine (Lys)-type PGN [45]. Furthermore, the antifungal AMP genes, TmTLP1 and -2 were highly expressed in the fat body of TmRelish-depleted larvae after infection with S. aureus and L. monocytogenes, suggesting that TmRelish negatively regulates antifungal AMPs.
In the fat body, loss of TmRelish decreased expression of 10 AMP genes, noticeably weakening the T. molitor defense from infection with L. monocytogenes. Although we observed the increased levels of antifungal AMPs and TmTene2, suggesting TmRelish plays an important role in regulation of AMPs in the fat body. In the hemocytes of TmRelish knockdown larvae, five AMPs were similarly downregulated while eight AMPs were not affected by TmRelish dsRNA-injection. TmRelish, therefore, acts as a positive regulator for several AMPs in hemocytes. We also found that, in contrast to the fat body, in the gut of TmRelish-knockdown larvae, the expression levels of 13 AMPs were significantly increased by L. monocytogenes. Together, these data indicate that TmRelish is not required for the induction of AMPs in the gut of T. molitor larvae when infected with intracellular bacteria. This is in agreement with recent finding in Manduca sexta that MsRel2 and MsDorsal heterodimers suppressed AMPs activation [46].

AMP genes induction in hemocytes of dsEGFP-injected larvae after L. monocytogenes infection was comparatively lower than AMP genes induction in the fat body. Moreover, TmTene1 and -4, TmCec2, TmAtt2, and TmCec2 induction was at a noticeably lower level in hemocytes of TmRelish-silenced larvae, while the expression of TmDef2 was increased. Our study revealed that TmDef2 expression was significantly high in the hemocytes and gut of TmRelish knockdown larvae while the mRNA expression of TmDef1 was strikingly strong in the gut. Early studies showed that L. monocytogenes was strongly inhibited by defensins [47,48]. Therefore, we propose that T. molitor Defensin family might function as a prominent antimicrobial peptide in the hemocytes and gut in response to L. monocytogenes. The presence of AMPs in the larval hemolymph of Galleria mellonella can inhibit the L. monocytogenes growth [41,49]. Similar to the results seen after L. monocytogenes infection, when TmRelish was knocked down, the expression levels of same AMPs were affected during S. aureus challenge [32]. Therefore, TmRelish positively regulates five AMPs in T. molitor hemocytes in response to a Gram-positive bacterial infection. Consequently, these findings indicate that the expression of TmTene1 and -4, TmCec2, TmAtt2, and TmCec2 rely on TmRelish in the fat body and hemocytes of T. molitor larvae in response to a challenge by Gram-positive bacteria (S. aureus and L. monocytogenes) (Figure 5).

In addition, it has been shown that Relish can cause autophagy (or cell death) with various effects, including the death of Drosophila photoreceptor cells in norpA mutant flies [50], the elimination of loser cells in Drosophila wing discs during cell competition [51], and Drosophila neurodegeneration [52]. Intriguingly, a dual role of Relish has recently been described, whereby it positively regulates the induction of Atg1, leading to the activation of autophagy in the developmental stage of Drosophila salivary glands and also regulates immune-related genes after microbial insult [16,18].

In hemocytes 6 h pi, a significant downregulation of autophagy genes was observed in TmRelish-depleted larvae, whereas this downregulation was only seen for TmATG1 in the fat body at the same time point. In fact, our results showed that TmRelish regulated the expression of TmATG1, a core component of the autophagy process in both fat body and hemocytes in response to intracellular infection. Previous studies have demonstrated that Drosophila ATG1 is critical for autophagy in the fat body and its expression is sufficient to affect the overall autophagy mechanisms [53]. Furthermore, the expression levels of TmATG1 was noticeably downregulated in the fat body 9 h pi, but not in hemocytes and gut of TmRelish-silenced larvae. The relatively high expression of TmATG1 in the fat body compared to other autophagy genes indicates the importance of this gene in mounting an autophagic response which is consistent with the previous reports [54,55]. Silencing of TmATG8, as an approved marker of autophagy, impaired the autophagic signal and reduced the resistance of T. molitor larvae against Listeria infection in hemocytes [13]. Similarly, we observed that TmATG8 transcripts of dsTmRelish knockdown larvae slightly decreased in the hemocytes 6 h pi. These data provide compelling evidence that the reduced survival of larvae might be also related to autophagy dysfunction. Moreover, an increase expression of autophagy genes over time with the highest level 6 h pi in the dsEGFP-treated larvae after infection, suggesting that autophagy plays a major role in clearance of L. monocytogenes in the hemocytes. As has been previously reported, exposure of J774 cells to cytosolic L. monocytogenes lead to autophagosome formation 6 h after insult [56]. Furthermore, the rapid autophagic responses in the early phase of infection limited growth of L. monocytogenes.
following vacuole formation [57]. However, *L. monocytogenes* is able to escape autophagic recognition via several surface proteins [58]. Regarding the PGRP family (PGRP-LE and PGRP-SA) and how it recruits both Imd and Toll pathway components to erase the invading and escaped *Listeria*, as well as autophagy [9,10,16]. One intriguing possibility is that *L. monocytogenes* is recognized by *TmPGRP-LE*. This recognition subsequently leads to *TmRelish* translocation which modulates not only AMPs induction via Imd signaling but also ATG genes expression in autophagy.

![Figure 5. Schematic depiction of *TmRelish*-mediated activation of the immune-deficiency (Imd) pathway in the larval fat body (A), hemocytes (B), and gut (C) by *L. monocytogenes*. Upon intracellular infection, the N-terminus domain of *TmRelish*, the Rel homology domain (RHD), translocates from the cytoplasm to the nucleus to induce the expression of antimicrobial peptides (AMPs) in the larval fat body and hemocytes of *T. molitor*. *TmRelish* negatively regulates the induction of several AMPs in the fat body, hemocytes, and gut of *T. molitor* larvae in response to *L. monocytogenes*.](image-url)

### 5. Conclusions

Our report demonstrates the immunological and autphagic function of *TmRelish* in various immune-related tissues of *T. molitor* during *Listeria* infection, through the regulation of AMP and ATG genes. The current investigation delineates the possible connection between different immune response mechanisms, including the Imd signaling pathway and autophagy.

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