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RESEARCH ARTICLE

Recent Insights into RNAi in Insect Biology and Pest Management

The salivary gland transcriptome of *Varroa destructor* reveals suitable targets for RNAi-based mite control

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Abstract

The mite *Varroa destructor* Anderson and Trueman (Mesostigmata: Varroidae) has a dramatic impact on beekeeping and is one of the main causes of honey bee colony losses. This ectoparasite feeds on honey bees' liquid tissues, through a wound created on the host integument, determining weight loss and a reduction of lifespan, as well as the transmission of viral pathogens. However, despite its importance, the mite feeding strategy and the host regulation role by the salivary secretions have been poorly explored. Here, we contribute to fill this gap by identifying the salivary components of *V. destructor*, to study their functional importance for mite feeding and survival. The differential expression analysis identified 30 salivary gland genes encoding putatively secreted proteins, among which only 15 were found to be functionally annotated. These latter include proteins with putative anti-bacterial, anti-fungal, cytolytic, digestive and immunosuppressive function. The three most highly transcribed genes, coding for a chitin-binding domain protein, a Kazal domain serine protease inhibitor and a papain-like cysteine protease were selected to study their functional importance by reverse genetics. Knockdown (90%–99%) by RNA interference (RNAi) of the transcript of a chitin-binding domain protein, likely interfering with the immune reaction to facilitate mite feeding, was associated with a 40%–50% decrease of mite survival. This work expands our knowledge of the host regulation and nutritional exploitation strategies adopted by ectoparasites of arthropods and allows the identification of potential targets for RNAi, paving the way towards the development of new strategies for *Varroa* mite control.

KEYWORDS

arthropod saliva, chitin-binding, gene silencing, honey bee, RNA sequencing

INTRODUCTION

Parasites and pathogens strongly contribute to honey bee (*Apis mellifera*) colony losses, widely reported in different regions of the world (Steinhauer et al., 2018; Stokstad, 2007), which are frequently associated with heavy infestations by *Varroa destructor* Anderson and Trueman and high loads of vectored viral pathogens (Kielmanowicz et al., 2015). *V. destructor* is an obligate ectoparasite of honey bees, which feeds on liquid tissues through a wound made on the integument of the host (Han et al., 2024; Ramsey et al., 2019). This parasite

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has a direct negative impact on the host, both at colony and individual level (Noël et al., 2020; Rosenkranz et al., 2010), reducing its life span (De Jong et al., 1983) and weight at emergence (Bowen-Walker & Gunn, 2001). However, indirect damages are even more dramatic. Indeed, *V. destructor* acts as a vector of several viral pathogens (Grozinger & Flenniken, 2019), including the widespread deformed wing virus (DWV), a mutualistic symbiont of the mite (di Prisco et al., 2016). The reproductive phase of the mite occurs inside capped cells, where adult females make a hole on the integument of honey bee pupae, through which they feed along with their offspring (Donzé & Guerin, 1994). The feeding strategy, likely interfering with or evading the immune reaction of the host (Yang & Cox-Foster, 2005), also preventing the infection of the open wound by opportunistic pathogens (Kanbar & Engels, 2003), is still poorly understood, even though the available evidence indicates that the saliva can play an important role (Becchimanzi, Tatè, et al., 2020; Zhang & Han, 2018). However, despite the importance of the saliva in the modulation of host–parasite interactions (Kotál et al., 2015; Rodriguez & Hernández-Hernández, 2004; Shi et al., 2022; Villarroel et al., 2016), only few studies investigated, so far, the composition and function of *Varroa* salivary secretions. To date, only one *omics* study has been published on the subject, a proteomic analysis of the mite saliva, collected with an in vitro feeding system, which allowed the identification of components likely acting as virulence factors, anti-microbials or performing anti-oxidant and detoxification functions (Zhang & Han, 2019).

One of the first functional studies on *V. destructor* saliva reported that it can interfere with the cellular immune response of *Lacanobia oleracea* larvae (Richards et al., 2011). Furthermore, a recombinant salivary protein of *V. destructor* resulted toxic for *Apis cerana* and promoted DWV replication in *A. mellifera* (Zhang & Han, 2018). More recently, it was shown a negative impact on metabolic activity of honey bees for a salivary cystatin-L2-like of *V. destructor*, associated with an abnormal pupal development (Zhou et al., 2023).

Through literature mining and a homology-based approach, we recently identified secreted proteins of *V. destructor* with a putative role in parasite–host interaction, focusing our attention on a chitinase (Vd-CHIsal) specifically expressed in the salivary glands (Sg) of the mite (Becchimanzi, Tatè, et al., 2020). The ~97% knockdown of *Vd-CHIsal*, obtained by soaking the mites in a saline solution of dsRNA, was associated with a 60% reduction of their survival, highlighting the importance of this salivary protein for *Varroa* feeding success (Becchimanzi, Tatè, et al., 2020). This result corroborated the hypothesis that the study of molecular interactions between *Varroa* mite and honey bees can provide the background knowledge on which to develop new strategies for mite control mimicking natural suppression mechanisms (Garbian et al., 2012; Leonard et al., 2020; Muntaabski et al., 2022). Indeed, biopesticides based on RNA interference (RNAi) for mite control have been already proved to be effective in field trials (McGruddy et al., 2024), and the identification of new targets and formulations, such as nano-encapsulation, is urgently needed (Ma et al., 2024).

Here, to further contribute to this research area, we characterised the salivary gland transcriptome of *V. destructor*, revealing that mite saliva is a quite complex cocktail which includes several proteins with putative anti-bacterial, anti-fungal, cytolytic, digestive and immunosuppressive function. This provides a molecular atlas of host regulation factors that can be selectively targeted to disrupt the host–parasite interaction. To exploit this potential, we selected the three most abundant transcripts in the mite Sg and assessed the impact of single and multiple dsRNA treatments on gene knockdown and mite survival.

RESULTS

To obtain the transcriptome of *V. destructor* Sg (Figure 1a,b), we carried out a differential expression analysis between Sg and the rest of the mite's body (Rb). The Principal component analysis (PCA) showed a clear separation of Sg from Rb samples (Figure 1c), supporting an obvious transcriptional specificity. Indeed, comparing the Sg with Rb samples, 142 differentially expressed genes (FDR ≤0.05) were detected, of which 54 were overexpressed (FC ≥1.5) and 88 underexpressed (FC ≤1.5) in the Sg. To characterise the salivary gland transcripts, we focused on the overexpressed genes, highlighting that, of the 54 identified, 44 encode proteins and 10 are classified as long non-coding RNAs (Figure 1d).

Of the 44 predicted proteins, 30 are putatively secreted proteins (i.e., showing a signal peptide), among which only 15 were found to be functionally annotated in GenBank (Table 1). The predicted salivary components have several putative functions such as, anti-bacterial, anti-fungal, cytolytic, digestive and immunosuppressive function (Table 1).

Among the genes having a functional annotation, we selected the three most highly transcribed in Sg, based on their fold-change (FC) values (Table 1), excluding the endochitinase Vd-CHIsal we already studied (Becchimanzi, Tatè, et al., 2020). The selected target genes encode a chitin-binding domain protein (*chitin-binding*), a Kazal domain serine protease inhibitor (*kazal*) and a papain-like cysteine protease (*papain*).

To assess the gene silencing efficacy of the concatenated dsRNA molecule (1389 bp), as a strategy to target the three genes simultaneously (dsCONC), and to characterise their transcriptional profile, we carried out a time course analysis at 48, 72 and 96 h after mite soaking in dsCONC and dsGFP (negative controls). Two-way analysis of variance (ANOVA) showed a significant effect of treatment factor on gene transcription (*chitin-binding*: $F_{(1,24)} = 19.47$, $p = 0.0002$; *kazal*: $F_{(1,24)} = 56.47$, $p < 0.0001$; *papain*: $F_{(1,24)} = 38.70$; $p < 0.0001$), with a significantly reduced level of the three targets for mites soaked in dsCONC, at 96 h from the treatment (Tukey's post hoc, Table S1). A significant knockdown was also observed at 48 and 72 h for *papain* and *kazal*, respectively (Figure 2). Although not statistically significant (Table S1), we observed a marked transcription reduction of the targeted genes at the other time points considered. Moreover, two-way ANOVA revealed a significant effect of time factor on gene transcription (*chitin-binding*: $F_{(2,24)} = 11.34$, $p = 0.0003$; *kazal*: $F_{(2,24)} = 3.511$, $p = 0.0460$; *papain*: $F_{(2,24)} = 6.637$, $p = 0.0051$). The *chitin-binding*

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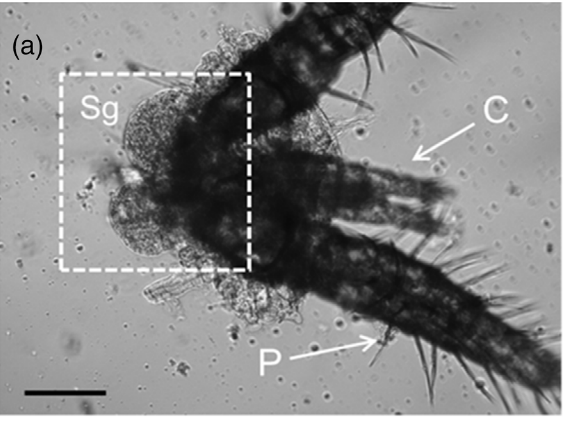

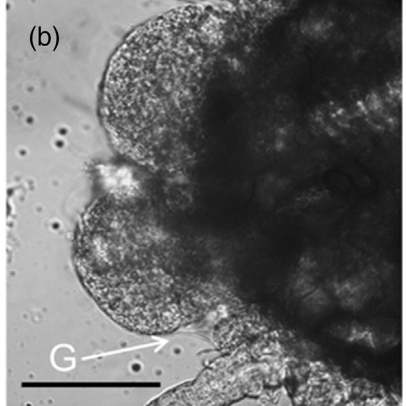

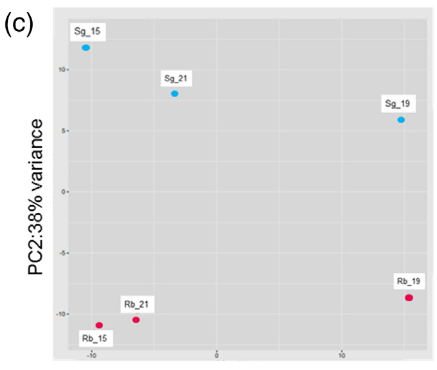

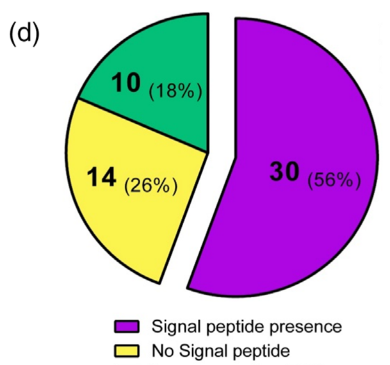

FIGURE 1 Transcriptome of *Varroa destructor* salivary glands (Sg). (a) The Sg are paired, oval and closely connected to the gnathosoma, composed of hypostoma, chelicerae (c) and pedipalps (P). (b) The glands are in contact with each other and are sheathed (g). Scale bar: 50 μm. (c) Principal component analysis (PCA) of normalised RNAseq data. FPKM (fragments per kilobase per million mapped fragments) values were used to perform a PCA. Each coloured dot represents a pool of 45 individuals. Salivary glands and carcass (rest of the body after dissection) are indicated by blue and red circles, respectively. (d) Classification of the transcripts overexpressed in salivary glands. Of the 54 overexpressed transcripts, 30 encode putatively secreted proteins with a signal peptide, 14 encode proteins without signal peptide and 10 are annotated as long non-coding RNAs.

and *kazal* transcription in dsGFP soaked mites was significantly higher at 96 h compared with 48 h (Figure 2). The general trend observed is a transcriptional increase over time of the selected salivary genes during mite feeding on honey bee pupae.

To score the effect of salivary genes knockdown on *Varroa* mites' survival, we performed an artificial infestation of honey bee pupae after soaking the mites in a solution of dsRNA targeting a single salivary gene or all the selected targets simultaneously. The multi-target silencing was achieved by using an equimolar mix of the three dsRNAs (dsMIX) or dsCONC, to compare the efficacy of two different delivery strategies. To check for target knockdown in treatment groups, mites were sampled at 72 h after soaking and processed for RNA extraction and qRT-PCR. One-way ANOVA showed a statistically significant impact of the treatments on the transcription, except for *kazal* (*chitin-binding*: $F_{(3,8)} = 23.50$, $p = 0{,}0003$; *kazal*: $F_{(3,8)} = 3.321$, $p = 0.08$; *papain*: $F_{(3,8)} = 8.33$; $p = 0.0076$).

Mite soaking in single dsRNA solution induced a statistically significant gene knockdown (98%–99% reduction) 72 h after the treatment for *chitin-binding* and *papain*, while *kazal* expression was reduced but not significantly (Figure 3a and Table S2). A similar effect (95%–93% reduction) was obtained by soaking the mites in dsMIX. Mite soaking in dsCONC induced a statistically significant reduction of *chitin-binding* and *papain* transcription only, with 99.9% and 95% mean reduction, respectively. The transcription of *kazal* was reduced by soaking in dsCONC, but not significantly, showing a slightly less average reduction (80%–90% reduction) compared to the other target genes (Figure 3a).

A significantly lower survival rate was observed for mites soaked in saline solution supplemented with dsMIX and dsCONC (log rank test: $\chi^2 = 6.90$; $p = 0.0136$) compared with mites soaked in control GFP dsRNA (Figure 3b). A similar effect (50%–60% survival at 5 days after treatment) was obtained by targeting chitin-binding protein alone. The specific knockdown of the other single targets (dsKAZAL and dsPAPA) did not significantly reduce mite survival compared with controls (Table S3).

This result prompted us to analyse *chitin-binding* aminoacidic sequence and to perform a phylogeny reconstruction, to obtain more information on its putative function. The *chitin-binding* gene encodes a short protein (111 AA) with a type-2 chitin-binding domain (Vd-CHIBIN), characterised by a six-cysteine motif and several

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TABLE 1 List of transcripts overexpressed in salivary glands encoding putatively secreted proteins.

Annotation (GenBank, Interpro)	Putative function	Adjusted *p* value	Fold-change	Transcript accession (GenBank)	Protein accession (GenBank)
No match		3.20E-04	28.82	XM_022814795.1	XP_022670530.1
Cystein protease papain-like (zingipain-2-like)[a]	Protein digestion/ Immunosuppression	3.74E-04	22.50	XM_022802192.1	XP_022657927.1
lncRNA		1.33E-02	21.74	XR_002671133.1	
lncRNA		2.12E-02	20.73	XR_002673565.1	
No match		1.99E-44	20.60	XM_022792435.1	XP_022648170.1
Endochitinase (Vd-CHIsal)[b]	Chitin digestion	1.43E-38	15.68	XM_022817406.1	XP_022673141.1
No match		2.56E-36	15.39	XM_022797839.1	XP_022653574.1
Chitin-binding (peritrophin-A)[a]	Chitin-binding	4.23E-34	14.90	XM_022792159.1	XP_022647894.1
No match		5.41E-32	13.95	XM_022816118.1	XP_022671853.1
Kazal domain serine protease inhibitor[a]	Anti-clotting	8.51E-38	13.69	XM_022798157.1	XP_022653892.1
lncRNA		3.19E-13	13.44	XR_002674195.1	
No match		3.11E-04	13.08	XM_022797923.1	XP_022653658.1
No match		1.33E-12	12.78	XM_022788266.1	XP_022644001.1
No match		1.73E-08	12.65	XM_022789934.1	XP_022645669.1
lncRNA		1.84E-05	12.38	XR_002675009.1	
Neuropeptide-like	Anti-bacterial	2.41E-31	10.61	XM_022817089.1	XP_022672824.1
Chalycin. lipocalin	Anti-clotting	1.05E-05	10.39	XM_022797609.1	XP_022653344.1
DeoxyribonucleaseII	DNA digestion	4.93E-06	9.85	XM_022807242.1	XP_022662977.1
No match		4.36E-48	9.62	XM_022797558.1	XP_022653293.1
Epididymal secretory protein E1-like (sterol transport NPC2-like)	Chemoreception/Lipid binding	2.22E-03	9.49	XM_022798861.1	XP_022654596.1
No match		4.51E-07	9.42	XM_022810311.1	XP_022666046.1
No match		5.74E-03	8.62	XM_022797512.1	XP_022653247.1
lncRNA		7.89E-48	8.30	XR_002673627.1	
Apolipoprotein D-like	Immunomodulation/Lipids transfer	6.04E-20	7.98	XM_022808251.1	XP_022663986.1
No match		2.27E-08	7.92	XM_022793746.1	XP_022649481.1
No match		5.10E-17	7.76	XM_022809579.1	XP_022665314.1
lncRNA		1.23E-03	7.29	XR_002675351.1	
lncRNA		5.58E-20	7.14	XR_002673421.1	
Adult-specific rigid cuticular protein 15.7-like		4.50E-14	6.91	XM_022794578.1	XP_022650313.1
No match		5.58E-07	6.77	XM_022815881.1	XP_022671616.1
lncRNA		8.86E-03	6.73	XR_002672221.1	
lncRNA		1.12E-02	5.62	XR_002674023.1	
No match		2.81E-05	4.83	XM_022811069.1	XP_022666804.1
Lysozyme-like protein 1/2	Anti-bacterial/Chitin digestion	1.18E-05	4.63	XM_022797535.1	XP_022653270.1
Cuticle protein-like (NCBI)		2.24E-08	3.43	XM_022815110.1	XP_022670845.1
Insect cuticle protein		1.74E-09	3.31	XM_022798156.1	XP_022653891.1
MCFD2 homologue	Anti-clotting	5.30E-04	3.15	XM_022792139.1	XP_022647874.1
Phospholipase-A2	Fatty acids release/ Immunosuppression	2.61E-10	2.98	XM_022796000.1	XP_022651735.1
No match		4.04E-03	2.91	XM_022809177.1	XP_022664912.1
lncRNA		1.90E-02	2.45	XR_002670881.1	

Note: The 12 proteins with a possible role in host–parasite interaction are bold.
Abbreviations: lncRNA, long non-coding RNA; NCBI, National Center for Biotechnology Information; MCFD, Multiple Coagulation Factor Deficiency; NPC, Niemann-Pick intracellular cholesterol transporter.
[a]Target selected for functional characterisation.
[b]Already studied by Becchimanzi, Tatè, et al., 2020.

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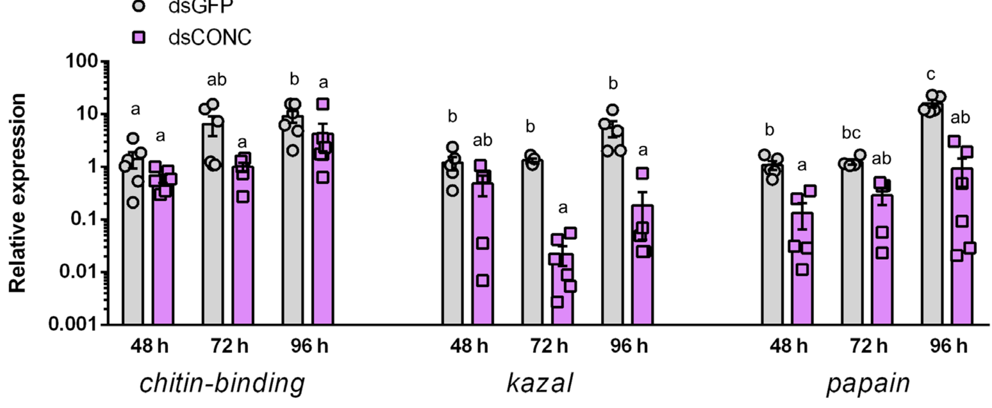


FIGURE 2 Time course analysis of salivary transcripts expression after soaking in dsRNA. Relative expression of *chitin-binding*, *kazal* and *papain* genes in treated groups of *V. destructor* adult females. Mites were soaked in saline solution supplemented with mock dsRNA (dsGFP) or concatenated dsRNA targeting all three selected genes (dsCONC). After soaking, mites were maintained on artificially infested honey bee pupae. qRT-PCR data are presented as mean fold-changes of five independent biological replicates, each consisting in a pool of three mites. Time and treatment effects on gene expression were analysed through two-way ANOVA followed by Tukey's post hoc test using ΔCt data. Each target gene was separately analysed. Relative expression (fold-change) was calculated by ΔΔCt method using controls at 48 h as calibrators. For each gene, mean values denoted with different letters are significantly different. Error bars represent standard error of the mean (SEM). Values on *y*-axis are reported in Log10 scale.

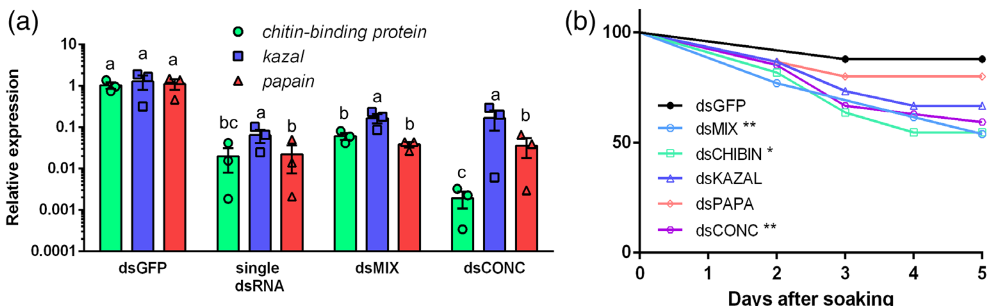
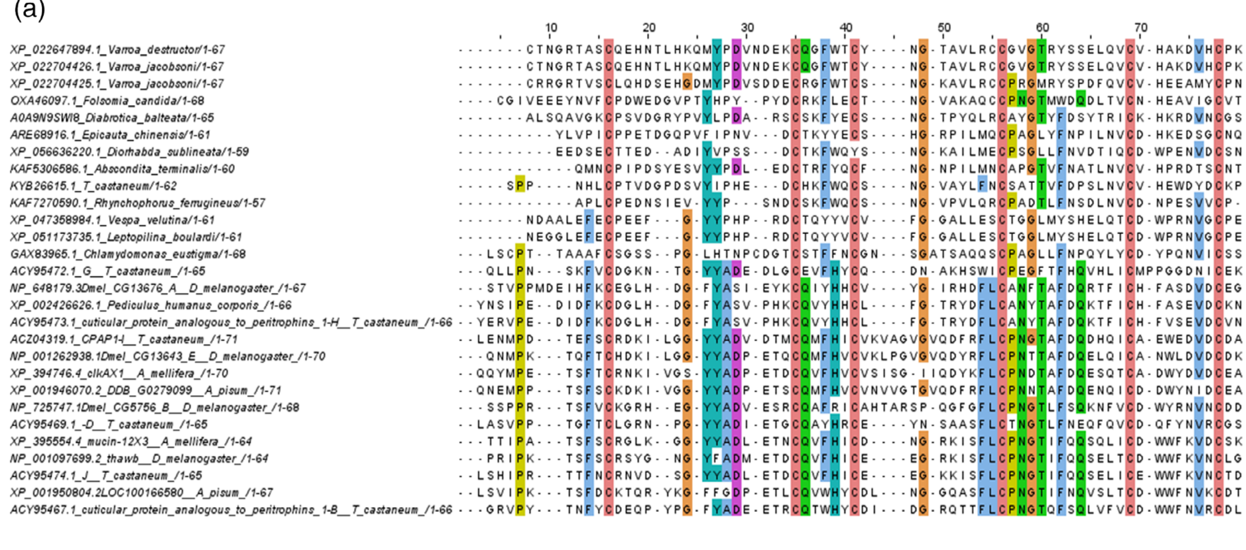


FIGURE 3 Survival of *Varroa destructor* as affected by RNAi-mediated knockdown of genes encoding salivary factors. (a) Relative expression of *chitin-binding*, *kazal* and *papain* genes in treated groups of *V. destructor*. Mites were soaked in saline solution supplemented with mock dsRNA (dsGFP), a single dsRNA targeting one of the three selected genes (single dsRNA), a mix of the dsRNAs (dsMIX) or a concatenated dsRNA (dsCONC) targeting all of them. After soaking, mites were maintained on artificially infested honey bee pupae. qRT-PCR data are presented as mean fold-changes of three independent biological replicates, each consisting in a pool of three mites. Time and treatment effects on gene expression were analysed through one-way ANOVA followed by Tukey's post hoc test using ΔCt data. Each target gene was separately analysed. Relative expression (fold-change) was calculated by ΔΔCt method using controls (dsGFP) as calibrators. For each gene, mean values denoted with different letters are significantly different. Error bars represent standard error of the mean (SEM). Values on *y*-axis are reported in Log10 scale. (b) Kaplan–Meier survival curves of mites soaked in a solution of dsRNA targeting genes highly expressed in salivary glands of *V. destructor*. dsRNA-soaked mites were individually maintained on the same host pupa throughout the whole duration of the assay. Soaking mites in saline solutions supplemented with dsRNA targeting the three selected genes simultaneously (dsMIX and dsCONC) or chitin-binding protein alone (dsCHIBIN) reduced significantly their survival compared to controls (dsGFP). The statistical details of comparison between dsGFP and other survival curves are reported in Table S3. Number of individuals per treatment were 33, 13, 11, 15, 15 and 27 for dsGFP, dsMIX, dsCHIBIN, dsKAZAL, dsPAPA and dsCONC, respectively. Log rank test significance compared to dsGFP: $*p < 0.05$, $**p < 0.01$.

aromatic residues (Figure 4a). Sequences retrieved through Blast searches and those used in a phylogenetic reconstruction of cuticle proteins analogous to perithrophin 1 (CPAP1) (Tetreau et al., 2015) were aligned to infer Vd-CHIBIN phylogeny. Unexpectedly, BlastP search against non-redundant (nr) National Center for Biotechnology Information (NCBI) database returned only eight similar sequences (above the *E*-value threshold of 1e-3), belonging to distantly related taxa, including Coleoptera, Lepidoptera and bivalves. Maximum-likelihood analysis revealed a strong divergence between proteins containing a single chitin-binding domain identified in *Varroa* species (purple) and those classified as CPAP1s (blue), which have a putative role in cuticle formation (Figure 4b).

DISCUSSION

Parasites use several strategies to deliver molecules inside the host, to evade immune response and to allow its nutritional exploitation



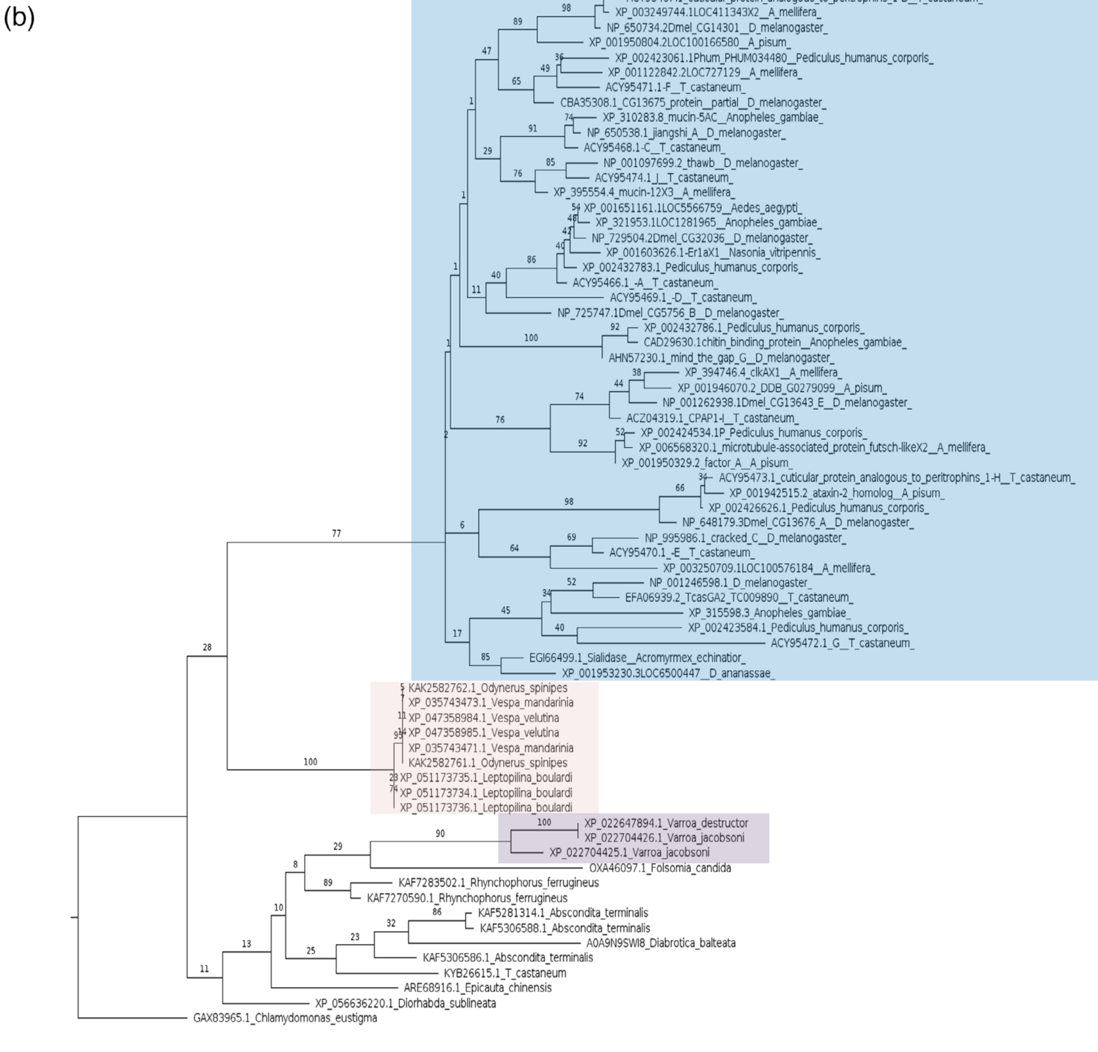


FIGURE 4 Sequence alignment and phylogenetic reconstruction of proteins with a single chitin-binding domain. (a) The protein encoded by *chitin binding* gene of *V. destructor* (Vd-CHIBIN) was aligned with putative homologues identified by BlastP and cuticle proteins analogous to peritrophin 1 (CPAP1). Vd-CHIBIN (XP_022647894.1) has a single type-2 chitin-binding domain, which is characterised by the six cysteine residues highlighted in pink. (b) Phylogenetic tree based on maximum likelihood analysis of chitin-binding domains showed a clear divergence of proteins identified in *Varroa* species (purple), those identified in Hymenoptera species (pink) and those classified as CPAP1s (light blue). Bootstrap support values are indicated at each node. The scale bar indicates the number of amino acid substitutions per site.

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(Pennacchio & Strand, 2006; Schmid-Hempel, 2009; Zhang et al., 2024). Studying the natural processes modelled by the co-evolutionary history of host–parasite interaction can pave the way towards the development of new strategies of pest control (Pennacchio et al., 2012; Pennacchio & Strand, 2006). Here, we analysed the transcriptome of *V. destructor* Sg to identify the major host regulation factors, which represent potential targets for developing RNAi-based strategies of mite control, aiming to disrupt its feeding efficiency. Of the 54 transcripts differentially expressed in Sg, 10 are annotated as long non-coding RNA (lncRNA). It has been suggested that, in ticks' saliva, lncRNAs act as 'sponge' molecules that inhibit miRNA–mRNA interactions in the host, thus affecting host responses to tick feeding (Aounallah et al., 2020). Considering the emerging role of lncRNAs in the vector–host–pathogen triad (Ahmad et al., 2021; Arunima et al., 2023), their involvement in *Varroa*–honey bee interaction is worth of further studies.

Among the 30 salivary gland genes encoding putatively secreted proteins (i.e., showing a signal peptide), only 15 were found to be functionally annotated in GenBank. However, even though the unannotated proteins are not easy to characterise from a functional point of view, they likely include bioactive salivary components very specific, which reveal poor similarities even between phylogenetically related species (Becchimanzi, Avolio, et al., 2020; Jonckheere et al., 2016; Villarroel et al., 2016).

Among annotated salivary components with a signal peptide, which suggests extracellular secretion, we identified proteins with anti-bacterial, anti-fungal, cytolytic, digestive and immunosuppressive functions. The major protein families encoded by salivary gland genes are hydrolytic enzymes, such as cysteine protease, serine protease, endochitinase and phospholipase (PLA2), which are frequently found as components of the glandular secretion of parasites and parasitoids (Fry et al., 2009; Kim et al., 2014; Laurino et al., 2016; Vincent et al., 2010). Notably, some of the protein families identified, such as PLA2 and lysozyme, were also found in a proteomic study of *V. destructor* saliva (Zhang & Han, 2019). Moreover, our data further corroborate the high expression in the Sg of *Vd-CHIsal* we previously reported along with its effect on honey bee immunity and mite survival (Becchimanzi, Tatè, et al., 2020).

Among all the other highly transcribed genes, we selected a chitin-binding domain protein (Vd-CHIBIN), a Kazal domain serine protease inhibitor and a papain-like cysteine protease, based both on differential expression analysis, and on their occurrence and putative functions in other sialomes of parasitic arthropods. For example, proteins containing chitin-binding domains expressed in Sg of ticks participate in mucus formation (mucins), being involved in mouthpart lubrication and entrapment of bacteria (Korayem et al., 2004) and as components of the cement cone structure (Hollmann et al., 2018). Kazal domain serine protease inhibitors are serine protease inhibitors, identified in salivary secretions of mosquitoes (Watanabe et al., 2010) and in triatomine bugs (Friedrich et al., 1993), which function as anti-coagulants. Cysteine proteases have important roles in physiological events that are crucial to the ectoparasitic lifestyle, including digestion of host blood, embryogenesis and vector transmission (Sojka et al., 2011).

To score the impact of salivary factors knockdown on mite survival, we used an RNAi approach (Campbell et al., 2010), targeting single and multiple salivary genes. In a first experiment, we tested the efficacy of a 1389 bp long dsRNA designed by concatenating the three selected sequences (dsCONC). Soaking the mites in dsCONC resulted in a significant multiple knockdowns of the three genes at 4 days after treatment, indicating that this dsRNA delivery strategy can be suitably used for targeting multiple genes. This knockdown was associated with an increase of target gene expression over time in controls, which suggested that the selected salivary factors are involved in the feeding process on honey bee pupae. Indeed, positive modulation of salivary components over time also occurs in ixodid ticks, where salivary gene expression is temporally regulated along feeding, resulting in several changes in saliva composition. It has been suggested that this 'saliva switching' is associated with the evasion of the host immune response (de Castro et al., 2017; Ribeiro & Mans, 2020).

In a second experiment, we used different strategies to target salivary genes by RNAi and scored the impact of gene knockdown on mite survival by performing artificial infestations. As expected, single and mixed (dsMIX) dsRNA administration by soaking reduced the expression of the three salivary genes by 98%–99% and 93%–95%, respectively, at 72 h after treatment, compared with controls. A similar effect was observed after soaking in dsCONC solution, except for *kazal* expression, which was reduced by 80%–90%. Since the dsCONC and dsMIX, used at the same concentration of the other treatments, provide a lower number of a specific dsRNA, this may partly account for different levels of silencing efficiency observed. Overall, the levels of gene knockdown observed are comparable to those previously reported for other salivary genes at 72 h after soaking (Becchimanzi, Tatè, et al., 2020; Zhang & Han, 2018).

Survival decreased to 50%–60% when mites were soaked in dsCONC, dsMIX and dsCHIBIN (i.e. dsRNA targeting chitin-binding protein transcripts) solutions, compared to dsGFP-treated controls. This suggests that most of the impact of multiple knockdowns is due to knockdown of *Vd-CHIBIN*, highlighting the important role of the encoded salivary factor in mite feeding. The targeted transcript encodes for a short protein (111 AA) with a single type-2 chitin-binding domain (CBD2), which is mainly found in metazoans, fungi and baculovirus proteins (Chang & Stergiopoulos, 2015) and is characterised by having a six-cysteine motif and several aromatic residues (Gaines et al., 2003). Proteins with a single CBD2 are considered structural components of cuticle and, indeed, have been denominated 'cuticular proteins analogous to peritophins 1' (CPAP1) (Jasrapuria et al., 2010). Our phylogenetic analysis showed that proteins with a single CBD2 form highly divergent clades, with CHIBIN only distantly related to CPAP1s. Indeed, the complex evolutionary history of this domain is characterised by convergent evolution (Shen & Jacobs-Lorena, 1999) and horizontal gene transfer, which driven episodic lineage-specific expansions and contractions that probably reflect adaptations to specific lifestyles (Chang & Stergiopoulos, 2015). In *Varroa* species, Vd-CHIBIN may interact with chitin of the host integument, which is likely processed by the salivary chitinase Vd-CHIsal

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(Becchimanzi, Tatè, et al., 2020). The chitooligosaccharides produced by chitin degradation may have an immunostimulatory effect on the parasitized honey bee (Frevert et al., 2018; Saltykova et al., 2003), which can be prevented if the elicitor is bound by parasite effectors, as reported for several pathogenic fungi (de Jonge et al., 2010; Li et al., 2024; Liu et al., 2020). This interesting hypothesis is worth of further studies aiming to elucidate if Vd-CHIBIN can prevent the wound induced activation of the honey bee immune system.

In conclusion, our work sheds light on the complex cocktail of salivary effectors adopted by *Varroa* mites to feed on honey bees and provides experimental evidence supporting their functional importance for successful parasitism. Studying the molecular interactions at the interface between *V. destructor* and *A. mellifera* is a key-step for understanding parasite–host co-evolution and for developing novel RNAi-based strategies of mite control.

EXPERIMENTAL PROCEDURES

Biological material

For the transcriptomic analysis, *V. destructor* mites and honey bees were collected from brood combs of *A. mellifera* colonies maintained in the experimental apiary located in Portici (Naples, Italy). Brood frames were collected between June and August and then stored in a dark room for 24–48 h, at 34°C ± 1°C, 70% ± 5% RH. Mites were collected by uncapping the brood combs and then using tweezers and a paintbrush to remove them from the honey bee pupae.

Sg dissection

The adult *V. destructor* females, stored in microcentrifuge tubes at −80°C, were ventrally stuck on double-sided tape placed on a microscopy slide to proceed with the dissection. Using a stereomicroscope, each mite was incised in the posterior and lateral region, making a total of three incisions with a razor blade (Chrome Platinum, BIC). Then each mite was soaked in 30 μL of phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4) solution on another microscope slide and kept on ice. Using fine tweezers and dissecting needles, the carapace of the mite was gently lifted, uncovering the internal organs; then, the Sg were isolated from the other tissues and pooled. Three pools of 45 mites were processed as above to separate the Sg from the Rb and to store both samples in 200 μL of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) at −80°C.

Isolation of RNA

RNA extraction was performed, after freeze-thawing the samples, using TRIzol Reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. The final pellet was re-suspended in 16 μL of nuclease-free H_2O, and the quality and quantity of total RNA isolated were assessed by measuring absorbance with Varioskan Flash (Thermo Fisher Scientific). For mite tissues, extraction methods resulted in the retention of significant amounts of contaminating DNA; therefore, RNA samples were treated with DNase RQ1 (Promega, Madison, WI, USA), following the manufacturer's instructions.

RNAseq analysis

RNAseq analysis was performed by the company Genomix4Life (Baronissi, Italy). A total of six RNA samples were processed, three of them obtained from Sg and three from the Rb (carcass). Approximately 1 μg of total RNA was used to construct the cDNA libraries, using the TruSeq stranded kit (Illumina, San Diego, CA, USA), and the 75-bp paired-end sequencing run was performed on the Illumina NextSeq 550 platform (Illumina). Approximately 10 million reads per sample were generated and then subjected to quality control, using the FastQC tool, while trimming and mapping to the *V. destructor* genome (GCF_002443255.1) was carried out using STAR software (version 2.7.3a) (Dobin et al., 2013), with standard parameters for paired reads.

Using the FeatureCounts (version 2.0.1) algorithm, quantification of expressed transcripts was performed for each sequenced sample. The DESeq2 software package (Bioconductor) was used to normalise the data and perform the analysis of differentially expressed genes (Love et al., 2014), to produce a table showing the expression values in terms of FC and statistical significance values expressed in terms of false discovery rate-adjusted *p* value (FDR) for each gene. Genes with FDR ≤0.05 were considered as differentially expressed genes, those with FC ≥1.5 as overexpressed in Sg, while those with FC ≤1.5 as underexpressed. To assess the overall similarity among samples, a principal component analysis (PCA) was performed among all samples, for each condition considered.

Target selection and cloning

Candidate targets in the salivary gland transcriptome were selected for functional studies based on their FC values and their putative function inferred through NCBI annotation, InterproScan and SignalP 5.0, which predicts the presence of the signal peptide as a marker of secretion.

The amplified regions of the three genes were used as query in BLASTn searches, to assess the potential risk of dsRNA off-target effects towards *A. mellifera*. BLASTn analyses showed no significant similarity between the targeted sequences and honey bee transcripts.

The DNA sequences, serving as templates for dsRNA production, were generated through PCR amplification and subsequent cloning. The RNA extracted from the Sg was retrotranscribed with High-Capacity cDNA RT Kit according to manufacturer's instructions (Thermo Fisher Scientific). The resulting cDNA was used as template for PCR reactions containing 25 μL of 2× DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), the two primers at 500 nM final

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concentration, 2 μL of cDNA template and nuclease-free water, to a total volume of 50 μL. The cycle conditions used were based on manufacturer's instructions (Phusion Flash High-Fidelity PCR Master Mix, Thermo Fisher Scientific). Primers and annealing temperatures are listed in Table S4. The eGFP sequence used as control was amplified from p2GW7 (Karimi et al., 2002). Subsequently, the amplified sequences were incorporated into the L4440 plasmid (Duan & Wang, 2024) using KpnI and SacI restriction sites. This was achieved by including KpnI and SacI restriction sites in the primer sequences.

For the construction of a plasmid housing all three target sequences consecutively (concatenated), the L4440_XM_022802192 plasmid underwent SacI digestion. In parallel, the XM_022792159 and XM_022798157 sequences were re-amplified using primer pairs 5 and 6, respectively (Table S4). The digested plasmids and the re-amplified sequences were gel purified and assembled using the NEBuilder HiFi DNA assembling mix (New England Biolabs).

Following transformation of OneShot TOP10 chemically competent *Escherichia coli* cells (Thermo Fisher Scientific), positive clones were selected by PCR reactions performed on plasmid DNA purified using the Pure Link HQ Mini Plasmid kit (Thermo Fisher Scientific). A comprehensive list of the generated plasmids in this study is provided in Table S5. In all instances, the integrity of the DNA plasmids was confirmed through enzymatic digestion and Sanger sequencing.

dsRNA production

The purified plasmids were used as template (10 ng) in PCR reactions using T7 primer (5′TAATACGACTCACTATAGGG3′), according to the same protocol described above and an annealing temperature of 48°C. Finally, several reactions were assembled to obtain at least 1 μg of the amplicon, the minimum required for subsequent dsRNA synthesis. Five μL of the amplified products were run on a 1% agarose gel to verify that the PCR reaction produced a single band of the expected size. The amplified products were then purified by adding one volume of phenol/chloroform/isoamyl alcohol (25:24:1), centrifuging at 10,000*g* for 15 min. Precipitation of the supernatant with 2.5 volumes of ethanol (100%) and 1/10 volumes of sodium acetate (pH 5.3) was performed at −20° overnight. After centrifugation at 14,000*g* for 30 min, pelleted DNA was resuspended in nuclease-free water and quantified measuring the absorbance with Varioskan Flash (Thermo Fisher Scientific). Following the manufacturer's instructions (MEGAscript RNAi kit, Thermo Fisher Scientific), the transcription reactions were assembled using 1.2 μg of purified PCR product. After DNA and ssRNA digestion, the dsRNA was purified and eluted in 50 μL of a 0.9% NaCl solution. The dsRNA concentration was determined spectrophotometrically, and the quality was checked on a 1% agarose gel.

dsRNA administration

The dsRNA administration was performed by introducing groups of 10 mites in separate 1.5 mL Eppendorf tubes containing 20 μL of dsRNA solution (1 μg/μL in 0.9% NaCl) or 20 μL of saline solution for controls (Campbell et al., 2010). The tube was gently shaken, to drop the mites into the solution and kept at room temperature for approximately 6 h. Every 30 min, mites were checked to be sure that they were all submerged in the solution, where they usually remained stuck at the bottom of the tube. At the end of the treatment, mites were recovered using a fine paintbrush and dried on filter paper. *Varroa* mites were soaked in six different dsRNA solutions: separate dsRNAs targeting genes encoding Papain-like cysteine protease (dsPAPA), a Kazal domain serine protease inhibitor (dsKAZAL) and a Chitin-binding domain protein (dsCHIBIN), respectively; an equimolar mix of the three dsRNAs above (dsMIX); the concatenated dsRNA targeting the three selected genes (dsCONC) and the dsGFP as a control.

Artificial infestation

Each mite was individually introduced in a transparent gelatin capsule (6.5 mm) containing a single honey bee worker pupa. The capsules were perforated using a syringe needle, to allow gas exchange and to prevent moisture accumulation, and fixed to the bottom of a petri dish using double-sided sticky tape so that pupae were laying on their dorsum (Nazzi & Milani, 1994). Artificial infestation was performed at 32° ± 1°C, 80 ± 2% relative humidity and in the dark to simulate the hive environment. Mite survival was daily checked. The mites found dead 24 h after soaking were excluded from the assay (1%–10% of each treated group) because they were considered as negatively affected by soaking, rather than by gene knockdown. After 72 h, six survived mites for each treatment were stored in −80° and then processed for RNA extraction to check gene expression, as described below.

Knockdown assessment by qRT-PCR

Differential relative expression of targeted genes was evaluated by one-step qRT-PCR, using the Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. Each sample was prepared in 20 μL total volume containing 10 μL of qRT-PCR 2× reaction mix, forward and reverse primers at 100 nM final concentration, 0.16 μL of 125× RT enzyme mix, DEPC-treated water and 50 ng of DNase-treated total RNA. For the experimental run, the following thermal profile was used: 48°C for 30 min (RT); 95°C for 10 min; 40 cycles at 95°C for 15 s and 1 min at 58°C; a last cycle consisting of 15 s at 95°C, 60s at 58°C and 15 s at 95°C was added for carrying out a dissociation curve. Each sample was analysed in triplicate on a Step One Real Time PCR System (Applied Biosystems). The 18S gene of *V. destructor* (Accession Number: XM_022831401.1) was used as endogenous control for RNA loading (Campbell et al., 2016). Relative gene expression data were analysed using the ΔΔCt method (Livak & Schmittgen, 2001). For validation of the ΔΔCt method, the difference between the Ct values of the target and the 18S transcripts [ΔCt = Ct (target)-Ct (18S)] was plotted versus the log of five-fold serial dilutions (100, 20, 4, 0.8 and 0.16 ng) of the purified RNA samples. The plot of

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log total RNA input versus ΔCt displayed a slope less than 0.1, indicating that the efficiencies of the two amplicons were approximately equal. The results are presented as mean FC of three independent biological replicates. Primers (Table S6) were designed with Primer Express version 1.0 software (Applied Biosystems) to amplify outside the dsRNA fragment used in knockdown experiments.

Sequence analysis and phylogeny reconstruction

Putative homologous protein sequences of Vd-CHIBIN were identified by sequence similarity searches through a BlastP analysis versus the nr NCBI (Bethesda, MD, USA) database. Most representative hits selected below the *E*-value threshold of 1e-3 were aligned, along with CPAP1 (Tetreau et al., 2015), using Muscle v.3.8 (Edgar, 2004) with default settings. Protein alignment was plotted using Jalview v.2 (Waterhouse et al., 2009), and sequences were analysed with ScanProsite (de Castro et al., 2006) and visually inspected, to identify conserved patterns.

To reconstruct phylogeny, alignments were manually trimmed to avoid comparisons of non-conserved regions present only in a subset of the taxa. Best-fit model of amino acid substitution and phylogenetic reconstruction was performed using RAxML v.8.2.12 (Stamatakis, 2006). The maximum-likelihood tree was run for 1000 bootstrap replicates and the tree figure was plotted using FigTree v.1.4.3.

Statistical analysis

To perform time course analysis and to validate knockdown in *Varroa* mites during artificial infestation experiments, we used two- and one-way ANOVA, respectively. Multiple comparison was based on Tukey's test. In all cases statistical significance was set at $p < 0.05$. The log rank (Mantel–Cox test) was used to compare the survival distributions of the observed groups of *Varroa* mites and statistical significance was set at 0.05. All statistical analyses were performed using the software GraphPad Prism 7.

AUTHOR CONTRIBUTIONS

Andrea Becchimanzi: Conceptualization; methodology; data curation; investigation; formal analysis; writing – original draft; writing – review and editing. **Alfonso Cacace:** Methodology; investigation; writing – original draft. **Martina Parziale:** Investigation. **Giovanna De Leva:** Investigation. **Sergio Iacopino:** Conceptualization; investigation; methodology. **Giovanni Jesu:** Investigation. **Ilaria Di Lelio:** Methodology. **Virgilio Stillittano:** Conceptualization. **Emilio Caprio:** Conceptualization. **Francesco Pennacchio:** Conceptualization; project administration; writing – review and editing; supervision; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence data are available at SRA (BioProject ID: PRJNA1097857), while raw data used to produce the figures in this manuscript are available on Zenodo (DOI: 10.5281/zenodo.10979620).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Tukey's multiple comparisons test of time course analysis.

Table S2. Tukey's multiple comparisons test of salivary transcripts expression in knockdown check experiment.

Table S3. Pairwise comparison of survival curves (log rank test) between dsGFP and the other treatments.

Table S4. List of primers used for cloning the selected targets.

Table S5. List of plasmids realised in this work.

Table S6. List of primers used for qRT-PCR.

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