Universidade de Lisboa

Faculdade de Ciências

Biologia Animal

How does the spider mite *Tetranychus urticae* respond to bacteria infection?

Gonçalo Santos Matos

Dissertação

Mestrado em Biologia Evolutiva e do Desenvolvimento

2013

Universidade de Lisboa

Faculdade de Ciências

Biologia Animal

How does the spider mite *Tetranychus urticae* respond to bacteria infection?

Gonçalo Santos Matos

Dissertação

Mestrado em Biologia Evolutiva e do Desenvolvimento

Orientadores: Professora Doutora Sara Magalhães e Professor Doutor Élio Sucena

2013

Agradecimentos

Em primeiro lugar, gostaria de agradecer à Professora Doutora Sara Magalhães e ao Professor Doutor Élio Sucena por me terem dado a oportunidade de trabalhar neste projecto, ao longo do último ano e meio. A liberdade que ambos me deram para expor e discutir as minhas ideias, o interesse que demonstraram no meu trabalho e a orientação que providenciaram foram de grande importância neste período da minha vida. Os seus exemplos foram e são uma fonte de inspiração e motivação para mim. Espero ter estado à altura das suas exigências.

Seguidamente, gostaria de agradecer ao Nélson Martins. O apoio que o Nelson me deu, os ensinamentos que me transmitiu, a ajuda que me providenciou e a amizade com que me tratou foram indispensáveis para que pudesse terminar com sucesso a minha tese de Mestrado. Espero no futuro conseguir transmitir a alguém o que me foi transmitido a mim. Muito obrigado por tudo Nélson!

Ao Professor Doutor John Vontas, ao Professor Doutor Thomas van Leeuwen, à Maria Riga e ao Nicky Wybouw, um muito obrigado pelas vossas colaborações. Quero igualmente agradecer ao Professor Doutor Francisco Dionísio por me ter permitido manter as minhas bactérias no seu laboratório.

À Leonor, à Salomé, ao Diogo, à Rita, à Telma, à Inês e à Flore, um muito obrigado pelo vosso apoio, pelas discussões, pela vossa ajuda e pelo incentivo que sempre me deram! Até breve!

Ao pessoal do grupo de Evo-Devo do IGC, aos meninos das moscas, aos meninos das bactérias e a todos do laboratório de Entomologia, obrigado pelo vosso apoio, pela vossa companhia e pela vossa simpatia.

A todos os meus colegas de Mestrado, obrigado pelos bons momentos que me proporcionaram, pelas trocas de ideias e conversas interessantes. Boa sorte a todos!

Aos meus familiares, agradeço por tudo o que me proporcionaram. A pessoa que sou, a vós vos devo. Agradeço-vos do fundo do coração pelo apoio e crença que em mim depositaram. Espero ter estado à altura das espectativas.

Aos meus amigos, biólogos e não biólogos, muito obrigado por tudo! Espero que tenham aprendido que existem mais ácaros para além dos do pó. A vossa amizade significa bastante para mim e trouxe-me força e alegria ao longo deste ano. Foram vocês que me deram força para aguentar os feriados, fins-de-semana e longos dias no laboratório. Espero que tudo vos corra bem no futuro e que eu esteja ao vosso lado sempre que necessitarem.

Apesar de todos terem tido o seu papel, alguns têm de ser destacados: Jorge, Miguel, Tomé, Magui, Barbacena, Márcia, vocês sempre estiveram lá e a vós vos devo muito. Foram vocês que me puxaram para cima quando estive mais em baixo e isso nunca será esquecido. À Filipa Grilo, muito obrigado pelo apoio, pela paciência, pela ajuda, pelo carinho que me deu ao longo deste ano e meio, sem ela tudo teria sido muito mais complicado; as vidas seguem caminhos separados mas há coisas que ficarão para sempre.

Resumo

As bactérias encontram-se distribuídas por todo o planeta, possuindo grande diversidade, o que lhes permite explorar uma vasta gama de habitats e recursos. Durante a vida de um animal, é provável que entre em contacto com estes microrganismos, algumas podendo penetrar as suas defesas, provocando infecção sistémica. Sabe-se que diferentes bactérias possuem diferente patogenicidade e que podem utilizar diferentes vias para infectar os seus hospedeiros. As vias mais comuns de infecção são a ingestão e a penetração por brechas nas barreiras protectoras dos organismos (por feridas, por exemplo). Sabe-se ainda que infecção por diferentes vias leva a diferentes respostas nos hospedeiros. Por exemplo, a infecção por via oral despoleta as defesas ao nível do intestino e do sistema imunitário; por seu turno, uma bactéria que penetre o organismo por uma ferida apenas activa o sistema imune.

Contudo, independentemente da via, a infecção bacteriana exerce uma forte pressão selectiva nos hospedeiros, podendo levar à evolução de mecanismos de defesa contra bactérias. Para combater a infecção, o hospedeiro pode utilizar diferentes estratégias defensivas: 1) evitar ou diminuir o contacto com bactérias, 2) usar as barreiras físicas/químicas do seu corpo para impedir a penetração das bactérias, ou se as bactérias ultrapassarem as duas primeiras estratégias defensivas 3) activar o sistema imunitário, montando uma resposta contra a infecção.

As defesas dos artrópodes contra bactérias têm sido estudadas, recorrendo a diversos organismos. A importância económica deste grupo na agricultura, o facto de terem impacto na saúde humana e as semelhanças que as defesas destes possuem com algumas das defesas dos vertebrados foram alguns dos motivos que levaram à realização destes estudos. Contudo, a maioria dos trabalhos tem-se focado em estudar *Drosophila melanogaster,* que observando a árvore filogenética dos artrópodes, surge como um dos representantes mais derivados, pertencendo à classe Insecta e ordem Diptera. Por ser um organismo tão derivado, as defesas de *D. melanogaster* possuem características que poderão não ser encontradas em organismos mais basais. O estudo de classes mais basais, como os Chelicerata, poderá inferir acerca das características mais ancestrais das defesas dos artrópodes face à infecção bacteriana.

Como representante dos Chelicerata, neste trabalho foram estudadas as defesas do ácaro-aranha *Tetranychus urticae*. A análise do genoma de *T. urticae* revelou que este ácaro não possui inúmeros genes que em *D. melanogaster* são responsáveis pela resposta imunitária contra bactérias. As vias de genes Toll e IMD, responsáveis pela defesa contra bactérias Gram positivas e Gram negativas respectivamente, estão incompletas no ácaro. Para além disso, não foram encontradas AMPs, as proteínas efectoras das duas redes. Várias hipóteses, não exclusivas, foram propostas para explicar estes resultados: 1) *T. urticae* possui mecanismos/vias de genes diferentes de *D. melanogaster*; 2) no seu ambiente natural, a infecção bacteriana não é frequente e portanto não existe uma pressão selectiva selecionando imunidade contra bactérias; 3) os artrópodes mais basais não possuem sistema imune.

A principal questão deste trabalho foi tentar identificar quais as estratégias defensivas que *T. urticae* usa para se proteger contra bactérias, de forma a poder validar algumas destas hipóteses. Para responder a estas questões, diferentes vias de infecção – ingestão, injecção e pulverização - foram testadas com o intuito de perceber se a via de infeção afecta a resposta do ácaro. Diferentes bactérias – *Escherichia coli, Bacillus megaterium, Pseudomonas putida* e *Enterococcus faecalis* - foram usadas para perceber se diferentes desafios levam a diferentes respostas. Usando microarrays, estudou-se a regulação da expressão génica após infecção por injecção; o que permitiu estudar se *T. urticae* possui um mecanismo de regulação da expressão génica diferente de *Drosophila*, ou se não possui nenhum mecanismo. Ainda, estudou-se se *T. urticae* comportamentalmente evita a ingestão de bactérias e se estímulos olfatórios despoletam esses comportamentos. Finalmente, recorrendo a outra espécie de ácaro (*Sancassania berlesei*) com uma ecologia diferente de *T. urticae,* inferiu-se se as características das defesas de *T. urticae* são representativas dos ácaros ou se são exclusivas ao ácaro-aranha.

Primariamente, testou-se como a infecção por diferentes vias (ingestão, pulverização e injecção) afectava a sobrevivência de *T. urticae.*

Para estudar o efeito de infecção por ingestão, por experiência 320 fêmeas adultas de *T. urticae* foram divididas por 4 tratamentos (alimentando-se de LB (controlo negativo) ou de bactérias à densidade óptica de 1, 10 ou 25) e colocados em

arenas com bolhas de parafilm com o alimento contido no seu interior. O período de alimentação durava 48 horas e mais tarde, o número de ácaros vivos era contado, assim como o número de ácaros que ingeriram comida. Esta informação foi utlizada para estudar se *T. urticae* evitava ingerir alimentos contaminados com bactérias. Para além disso, os ácaros que ingeriram a comida contaminada, foram transferidos para folhas de feijão e a sua sobrevivência foi medida durante 4 dias. *E. coli* e a *P. putida* foram as bactérias testadas e para cada bactéria foram feitas 5 repetições da experiencia. Os resultados referentes à ingestão de alimentos contaminados, demonstraram que menos ácaros ingeriam comida contaminada com bactérias, que comida com LB. Esta observação verificou-se quer para *E. coli* , quer para *P. putida.* Em seguida analizou-se a sobrevivência dos ácaros que ingeriram a comida oferecida e observou-se que a sobrevivência dos ácaros era afectada, quer pela ingestão de *E. coli*, quer de *P. putida.*

Considerando a infecção por pulverização, por experiencia, 200 ácaros foram colocados em caixas de petri com folhas de feijoeiro (um ácaro por folha), e foram pulverizados ou com LB ou com bactérias à densidade óptica de 1, 10 ou 25 (50 ácaros por tratamento). A sobrevivência dos ácaros após infecção foi medida durante 4 dias. As bactérias testadas foram *E. coli, P. putida e E. faecalis*. Para cada bactéria, foram feitas 3 repetições da experiencia. A pulverização de bactérias levou a uma grande redução da sobrevivência dos ácaros infectados, para todas as bactérias estudadas. Estes resultados sugerem que *E. coli*, *P. putida* e *E. faecalis* são bactérias patogénicas de *T. urticae*.

Considerando as experiencias de injecção, por tratamento (LB, bactéria à densidade óptica de 0.1, 1 e 10) 100 ácaros foram injectados, transferidos para folhas de feijoeiro e a sua sobrevivência foi contada durante 48 horas. As bactérias testadas foram *E. coli* e *B. megaterium*. A infecção por injecção de bactérias levou a uma redução da sobrevivência dos ácaros infectados. A infecção com *E. coli* levou a uma redução da sobrevivência a valores próximos de 0% em apenas 48 horas. Considerando a experiencia com *B. megaterium*, houve igualmente uma redução da sobrevivência, observando-se diferenças consoante da concentração testada.

Considerando as três vias de infecção testadas, a infecção por injecção levou a uma maior redução da sobrevivência de *T. urticae*.

Após estudar o efeito da infecção por diferentes vias, o estudo da expressão génica de *T. urticae* após injecção foi testado usando microarrays. Analisou-se a expressão génica 3, 6 e 12 horas após injecção. As bactérias injectadas foram *E. coli* e *B. megaterium* à densidade óptica de 1, comparando-se os resultados com os de ácaros injectados com LB. Analizando os resultados dos microarrays, poucos genes sofreram alterações na sua expressão, para infecção quer com *E. coli*, quer para com *B. megaterium.* Não foi possível observar um padrão comum na função dos genes com diferente expressão e ainda, nenhum destes tem um papel conhecido na resposta imunitária, noutras espécies. Estes dados sugerem que *T. urticae* não possui uma resposta imune contra infecção, pelo menos contra *E. coli* e *B. megaterium*.

Com o intuito de estudar o papel do olfacto no comportamento de evitamento de bactérias, recorreu-se a um olfactómetro conectado a uma bomba de vácuo. Estudou-se a influencia dos estímulos olfatórios no comportamento de evitamento de *E. coli*. Os ácaros testados na experiencia do olfactómetro não evitaram *E. coli*, sugerindo que os estímulos olfatórios não despoletam este comportamento.

Visto que as defesas de *T. urticae* aparentam ser pouco eficazes, testou-se se estas seriam representativas dos artrópodes mais basais. Para tal, recorreu-se a outra espécie de ácaro, o *Sancassania berlesei*, e infectou-se esta espécie por pulverização e injecção de bactérias. A sobrevivência de *S. berlesei* não foi reduzida quando as bactérias foram pulverizadas sobre os ácaros e a injecção bacteriana apenas levou a uma redução da sobrevivência quando em concentrações elevadas.

Em geral, os nossos resultados sugerem que *T. urticae* possui poucas defesas contra bactérias, recorrendo principalmente à capacidade de comportamentalmente evitar bactérias e de prevenir a penetração de bactérias graças às suas barreiras físicas/químicas. O habitat de *T. urticae* poderá explicar o porquê destas deficiências nas defesas, visto que as folhas, onde estes ácaros vivem e se alimentam, parecem possuir uma quantidade reduzida de bactérias, diminuindo a probabilidade de infecção. As experiencias com *S. berlesei* parecem apoiar esta conclusão de que a ecologia de *T. urticae* levou, durante a evolução da espécie, a uma degradação das defesas do ácaro-aranha. O facto dos afídios, que partilham o habitat de *T. urticae*, possuírem igualmente defesas fracas reforça a ideia de que a ecologia parece ter sido

determinante. É ainda possível que *T. urticae* possua outras estratégias defensivas contra bactérias, como um aumento da oviposição após infecção, protecção conferida por endossimbiontes, ou ainda que a sua teia possua propriedades antimicrobianas.

Abstract:

Most organisms contact with bacteria during their lifetime, some of which cross the organism physical barriers, causing systemic infection. To prevent or cope with infection, hosts may use distinct strategies such as avoidance, physical or chemical barriers and deployment of immune defenses. We explored these different levels of defense in the spider mite *Tetranychus urticae*, with the aim to unveil basal features of arthropod immunity, from behavior to physiology and genetics.

For this, we infected *T. urticae* with different bacteria, from different groups and with different degrees of pathogenicity to arthropods, under different infection routes: feeding, spraying and systemic injection. We also determined the impact of olfactory cues on avoidance behavior and the transcriptional response to infection by systemic infection using microarrays.

We found that: i) all bacteria severely reduce the survival of spider mites under all routes of infection ii) avoidance to bacteria is observed, although odor cues do not appear to play a role, iii) no consistent upregulation or downregulation of genes is observed under any of the infection scenarios.

Comparison between *T. urticae* and other mite species with a different ecology, *Sancassania berlesei,* suggests that *T. urticae* defenses may not represent mites' defenses, neither basal arthropods'*. S. berlesei* mites were not as susceptible to bacteria infection as *T. urticae,* when infected by spraying and systemic infection.

Overall, the results from the different infection regimes and microarrays suggest that, independently of the route and bacteria tested, spider mites are unable to respond to infection. *T. urticae* defenses against bacterial infection rely on avoidance behavior and its body phisycal/chemical barriers and we hypothesize that other factors (eg web), may also confer protection.

Index:

1. Introduction

Bacteria can be found in almost any place on Earth, as they present great diversity, allowing them to explore a wide range of habitats and resources[1]. Consequently, all organisms are likely to be in contact with bacteria during their lifetime. Some of these bacteria may break through the organism defenses, possibly promoting systemic infection, with severe fitness losses for hosts[2]. This fitness loss, in turn, exerts a strong selective pressure on the host, which may promote the evolution of its defensive strategies [3].

Bacteria may enter the host using different routes, the most frequent being ingestion and penetration through breaches in the hosts physical/chemical barriers [3–5]. Infection through different routes may lead to different responses in hosts[3]. For example, bacteria ingestion will trigger the gut's defenses and if bacteria can surpass this barriers, the immune system is also activated; however, bacteria that directly penetrate the hosts' organism (ex: by infecting wounded skin) only trigger the immune response [5].

To fight infection, the host may respond using different strategies, which are considered to be hierarchical: 1) it may avoid or diminish physical contact with its parasite (behavioral avoidance), 2) it may rely on its body physical/chemical barriers to prevent the parasite from entering its organism or, when systemically infected, 3) it may rely on its immune system to mount a response against the aggressors [6].

Several studies focus on the arthropods defenses against bacteria. Arthropods are an interesting group as they have relevance as agricultural pests, are vectors of disease and possess similarities with the vertebrates' defenses [2,7–9]. Chelicerata (spiders, scorpions and mites) are the most basal group within arthropods, diverging from the Mandibulata (crustaceans, myriapoda and insects)[10]. It is possible that these different arthropod groups also possess differences in their defensive strategies as groups become more derived. There is information regarding behavioral avoidance, physical barriers and the immune response for several species.

1.1. Arthropod's defenses

1.1.1. Behavioral Avoidance

To avoid being infected, arthropods may actively remove parasites from their environment, for example by grooming, which is used by ants to clean their fungi gardens from other fungi spores and also by honey bee workers removing larvae infected with bacteria from their colonies[11,12].

Arthropods may also avoid habitats where parasites are present. This strategy has been observed in several species such as social lobsters, *Panulirus argus*, which avoid caves containing infected individuals; *Aedes aegypti* females that avoid laying eggs in waters containing parasited mosquito larvae; and predatory mites, *Phytoseiulus persimilis*, which avoid plants containing their prey, *Tetranychus urticae,* if infected with fungi [8,13,14].

Another strategy to avoid being infected is to avoid mating with infected partners. For example, *T. urticae* uninfected females preferred mating with uninfected males when choosing between infected with *Wolbachia* or uninfected virgins; the pill-bug *Armadillidium vulgare* males mount more uninfected females than *Wolbachia* infected females and finally, grain beetle females, *Tenebrio molitor*, show avoidance of males infected with tapeworm[15–17].

Recently, the mechanisms regulating avoidance have been studied in *Drosophila melanogaster*. Avoidance of geosmin, a substance associated with food toxicity, produced by some fungi and different bacteria has been studied. Exposure to geosmin activates sensory neurons with the receptor Or56a that target de DA2 glomerulus. Activation of the DA2 will inhibit positive chemotaxis, feeding and oviposition[18].

1.1.2. Physical/Chemical Barriers

Physical and chemical barriers, such as the arthropod's cuticle, the acidic pH of the digestive gut, or the gut's wall, have a role in preventing parasites from entering the organism. Most of what we know about the role of these barriers comes from studies in *D, melanogaster*. In drosophila, both ROS and AMPs are produced in the gut, to fight bacteria infection[5]. Moreover, the absence of an enzyme, dDuox, present in

the gut mucosa leads to high mortality rates in adult mutant flies, compared to control flies [19]. Additionally, proliferation of stem cells has been observed in response to oral infection with *E. carotovora*, renewing the damaged tissue[5].

There is also the example of the cicada *Psaltoda claripennis* wings, promoting mechanical rupture of bacteria, resulting from the existence of nanopillars that penetrate the bacteria cells, diminishing the probability of systemic infection[20].

1.1.3. Immune system

The immune system has the ability to fight and overcome parasites' infection, and it may rely on an adaptive or an innate response. While humans have both, arthropods can only mount an innate immune response.

The most widely studied immune system of the arthropods is that of *D. melanogaster*.

1.1.3.1. Immune response in D. melanogaster

D. melanogaster presents both cellular and humoral immune responses when infected[9]. The cellular response is mediated by different haemocytes, which clear pathogens and parasites by phagocytosis, melanization and encapsulation. Haemocytes are divided in three categories: plasmatocytes, lamellocytes and crystal cells. Plasmatocytes are the most abundant haemocyte type and are mainly responsible for phagocytosis; lamellocytes play a role in encapsulation; and crystal cells act in the melanization process and production of toxic radicals[21–23].

Concerning the humoral response, *D. melanogaster* has four pathways that protect the organism against pathogens. The RNAi pathway is activated in the presence of viruses, the Jak/Stat pathway is responsible for encapsulation and cross-communication, *t*he Toll pathway responds to fungi and Gram positive bacteria infection and the IMD pathway is activated against Gram negative bacteria[9].

Both the IMD and Toll pathways, are triggered by the PGRBs and the GNBPs and their activation will lead to the production of Anti Microbial Peptides (AMPs) the effector molecules of these pathways[9] [9]. These small proteins are responsible for fighting bacteria and *Drosophila* has several AMP families. AMPs are specific, as for

instance, Attacin, Diptericin and Drosocin are more efficient against Gram negative bacteria, while Defensins are more effective against Gram positive bacteria, and Drosomycin and Metchnikowin against fungi[9,24].

1.1.3.2. Immune response in other arthropods

Insects

Insects possess differences in their immune response. Its complexity seems to increase as groups are more derived. Observing the phylogenetic tree of insects, Dictyoptera are the most basal group here presented, followed by Hemiptera, Hymenoptera, Coleoptera and Lepidoptera. Diptera appear as the most derived group[25].

Analysing other Diptera besides *Drosophila*, mosquitoes seem to have the same set of genes as *Drosophila*, some groups being more diversified but other having lost their function. Mosquitoes in general have less AMP families but have more diversification inside those families[26].

Lepidoptera immune system has been demonstrated to have PGRPs, GNBPs receptors and Lectins. The IMD and Toll pathways are functional and AMPs are upregulated upon infection, having stronger expression in the fat body[27].

For Coleoptera (beetles), the receptors PGRPs are present and the Toll and IMD pathway are complete[28,29].

In Hymenoptera, there is information concerning the bee and a parasitoid wasp, and the 4 pathways described as having a role in immunity in *Drosophila* are present. Bees have 6 different types of AMPs, a smaller diversity than the drosophila's[30]. The wasp has a similar gene repertoire compared to the bee, although having a higher complexity of PGRPs and more AMPs[31].

For hemiptera, the genome annotation of the aphid, *Acyrthosiphon pisum* revealed that the PGRPs are missing, the IMD pathway is incomplete and many of the most common AMPs are absent. Nevertheless, the Toll pathway and some GNBPs are present. Analysis of gene expression showed that the AMPs are not upregulated upon bacteria infection. Regarding cellular mediated response, the aphids seem to recruit some haemocytes, although encapsulation was not found[7]

For Dictyoptera (roaches), individuals immunized with heat killed *Pseudomonas aeruginosa* had higher survival than those immunized with a saline solution when infected with the same bacteria. This suggests that roaches have specific immune responses, which is not seen in other groups of insects[32].

Chelicerates

Apart from our study system, the immune system of two other chelicerates has been studied, that of the horse shoe crab and that of the tick.

Regarding the horseshoe crab's cellular response, one type of haemocytes was found. Concerning its humoral response, it exocytes a potent AMP, Tachyplesin, effective against both Gram negative and positive bacteria. Tachyplesin regulates another AMP, the Defensin, acting against Gram negative bacteria but also affecting Gram positive bacteria, although with less efficiency. Regarding the Toll pathway, genes encoding receptors are present but do not seem to play a role as a receptor and their function was not identified. There is an upregulation of other immune-related factors when Gram positive bacteria are present[33].

Ticks have three types of haemocytes, lectins (responsible for identification of pathogens) and AMPs. Its AMPs are Defensins, Varisin (both effective against Gram positive bacteria), two ADPs (effective against Gram negative and Gram positive) and also Hebraein (effective against bacteria and fungi). Regarding the drosophila immunity related pathways, IMD itself is not present but some downstream elements of the pathway are[34].

In summary, analyzing both the insects' and chelicerates' immunity related genes there seems to be decrease in complexity, as groups become more basal. Hymenoptera is the most basal group where both the IMD and Toll pathway were found, suggesting that these pathways appeared after the Holometabola differentiation. A common point among these species here presented, except for aphids, is the production of AMPs upon infection.

1.2. Our model organism

In this work we focused on the interactions between bacteria and a spider mite species, *Tetranychus. urticae*. Mites are part of the order Acari, within the class Arachnida, belonging to the Chelicerata[35]. They are one of the most diversified group of invertebrates, exploring a wide range of niches. Their feeding habitats vary from predation, to feeding on plants, dead matter or even to live as parasites of both vertebrate and invertebrate animals[35].

T. urticae, commonly referred to as the two-spotted spidermite, is a haplodiploid acariform. Known as an agricultural pest, *T. urticae* is also recognized for its poliphagy, feeding on a great number of plant species; and also for the mites' ability to evolve high resistance to chemical pesticides in short periods of time[35,36].

1.2.1. T urticae's interactions with bacteria

Studies on interactions between bacteria and *T. urticae* as host mainly concern interactions with *Wolbachia*, a vertically-transmitted bacterium[15,37,38]. *Wolbachia* promotes cytoplasmic incompatibility in *T. urticae*[37]. It has been demonstrated that uninfected females prefer mating with uninfected males and that infected females aggregate their siblings[15]. Experiments regarding interaction with horizontally-transmitted bacteria and the mite's defenses to bacterial infection are scarce. Adult spider mite females were infected by spraying a *Pseudomonas putida* strain onto them. A mortality rate of 100% was observed within four days after infection and there was a decrease in fecundity and egg hatching[2]. There is also information on a *P. aeruginosa* infected *T. urticae* population, causing increased mortality[39]. Additionally, *Tetranychus telarius* adults, a species similar to *T. urticae* in several aspects such as niche occupation, were infected with *Bacillus thuringiensis* B-exotoxin and high mortality, an effect on fecundity and on egg hatching were observed[40].

1.2.1.1. Immune system

Comparing the *T. urticae* genome with that of *D. melanogaster*' and focusing on immunity-related genes, it has been shown that a great number of genes present in the *Drosophila* genome were absent from the spider mite genome (Fig. 1). Both the Toll and the IMD pathway were incomplete[35]. For the Toll pathway, genes responsible for Gram positive bacteria and fungi recognition were not identified, as the GNBP are absent, and also were lacking genes as the Tube and Dif [35]. The putative spider mites' IMD pathway was lacking half of the genes present in *D. melanogaster*[35]. Moreover, the AMPs, the effector proteins for both pathways, were not annotated, possibly being absent from the spider mite's genome [35].

Summarizing, *T. urticae* lacks an immune system similar to *D. melanogaster*'. The genes that in *Drosophila* are responsible for the recognition (GNBPs) and elimination (AMPs) of both Gram positive and negative bacteria, and also other components of the Toll and IMD pathways are absent in *T. urticae*. Comparing with other arthropods, the most notorious difference is the absence of the AMPs, present in other arthropods independently of the upstream regulatory mechanisms.

Three hypotheses are proposed to explain these results: 1) *T. urticae* uses different mechanism/pathways than those observed in *D. melanogaster*, to deal with bacterial infection; 2) bacteria are absent or rare in their natural environment, hence there has not been a strong selective pressure to the evolution of immunity against these microorganisms; 3) basal arthropods do not possess an immune response against bacteria. It is important to refer that these hypotheses do not exclude one another.

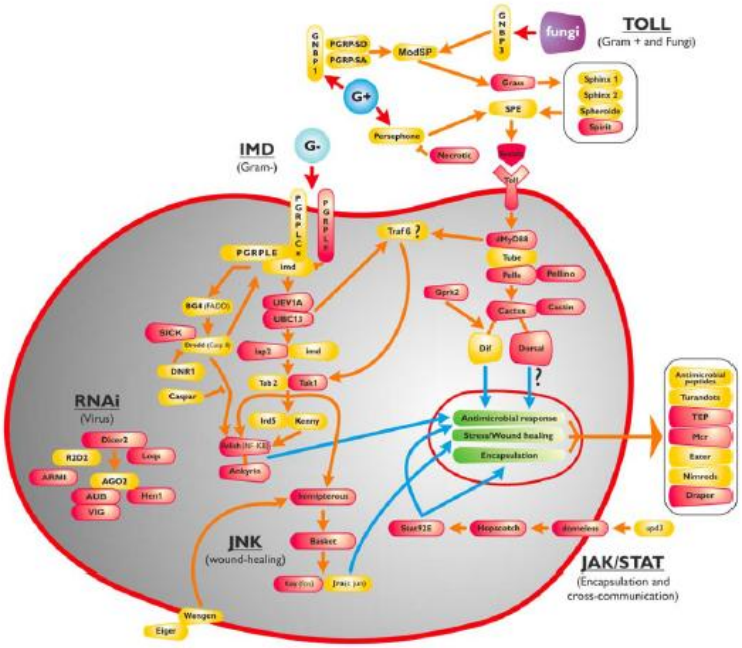

Figure 1: The four main immunity pathways in *D. melanogaster* and their counterparts in *T. urticae*. The IMD, Toll, JNK and JAK/STAT pathways are responsible for immunity against different parasites in *D. melanogaster*. In red, genes found in both *Drosophila* and *T. urticae* genomes. In yellow, genes absent from the *T. urticae* genome.

2. Aims

The major question of this work is to identify the defensive strategies *T. urticae* uses against bacteria, mainly focusing on the role of avoidance and the immune system. We also aim to understand if *T. urticae*'s defenses are representative of the basal arthropods'.

Firstly we will infect mites using different infection routes (ingestion, spraying and injection). It is possible that the route of infection affects the spider mites survival differently and these experiments will allow understanding that. We will also test these routes with different bacteria that possess different levels of pathogenicity in *D. melanogaster*, to study if the defenses respond differently to different bacteria infecting by the same route.

To understand if *T. urticae* avoids bacteria, we will use two different approaches. We will observe if mites reduce food ingestion in the presence of food

contaminated with bacteria and we will test if olfactory cues trigger avoidance, using a Y-olfactometer.

To study if *T. urticae* mounts an immune response when infected, the transcriptional response after infection by injection will be analyzed using microarrays. This data will allow us testing if *T. urticae* possesses an immune response, different than that of *D. melanogaster*, or if a response is absent.

Finally, to test if *T. urticae* defenses may represent the basal arthropods, and specially the mites' defenses, we will compare results obtained for *T. urticae* with another mite species, *Sancassania berlesei*. I will infect *S. berlesei* mites by spraying and injection and will observe how bacteria affects these mite's survival and compare these results with those obtained for *T. urticae*.

3. Materials and Methods

3.1. Spider mites (Tetranychus urticae)

Bean plants, *Phaseolus vulgaris*, were used in all experiments regarding *T. urticae* infection. Plants were kept at a greenhouse at Faculdade de Ciências da Universidade de Lisboa (FCUL) and only the primary leaves were used in our experiments**.** Leaves used were collected from plants with 14-21 days.

All individuals tested were collected from a population of London strain spider mites maintained in our laboratory at FCUL since 2010. The London strain of *T. urticae* was originally collected from fields in the Vineland region, Ontario, Canada. This strain was used in the Genome Sequencing project and is an isogenic line[35].

The population at FCUL was kept in a room with controlled photo period and temperature (16:8 photoperiod and 25^{o}C). Under these conditions London strain spider mites have a generation time of 10 days. For all experiments with spider mites, only adult females were tested and their age was controlled.

3.2. Sancassania berlesei

Sancassania berlesei mites were tested as other representative of the Acari sub-class. Comparison of the results from *T. urticae* and *S. berlesei* experiments may help discriminating if the *T. urticae* defenses are specific to this spider mite species or if they can be generalized to other mites.

S. berlesei is a mite species belonging to the superorder Acariformes, order Sarcoptifiormes and family Acaridae. This species is described as polyphagous, feeding on deteriorated plants or animals, occurring in habitats with high humidity or in darn yards and poultry litter. Other habitats have also been described for this species, exploring dead matter as its food source[41].

S. berlesei life cycle consists of an egg, three immature stages, three quiescent stages and an adult stage. Females are bigger than males and have a large bulbous abdomen whereas males have two different forms, differing in the third pair of legs[41]. Simmilar to *T. urticae*, *S. berlesei* reproduction happens promptly after the final quiescent stage. Regarding fecundity, females have high fecundity being able to lay more than 100 eggs during their lifetime. In laboratory conditions with a controlled

environment and controlled diet, mites developed from egg to adult in less than 8 days (180 hours)[41,42].

All individuals that founded our population were kindly provided by Professor Dr. Jacek Radwan from the Institute of Environmental Sciences, Jaggelonian University, Poland. Mites have been maintained in Petri dishes with fly food since the founding of our population (May, 2013) and were kept in a room with controlled conditions. Under the described conditions, mites have a generation time of 8 days. For all experiments, only adult females were tested.

3.3. Bacteria

Bacteria tested were *Escherichia coli* (Gram negative), *Pseudomonas putida* (Gram negative), *Enterococcus faecalis* (Gram positive) and *Bacillus megaterium* (Gram positive).

Bacterial stocks were kept both at -80^{o}C and 5^{o}C, and bacteria were collected from the 5^{o}C stock and plated on Petri dishes with LB, every 15 days. Per experiment, one colony was picked from the Petri dishes, transferred to liquid LB and grown overnight.

The *E. coli* strain tested in these experiments was DH5α. It had a plasmid, pSCM21, containing GFP and resistance to kanamycin. *E. coli* were grown at 37^{o}C in these experiments, as it is the optimal temperature for bacteria growth. This *E. coli* strain is not pathogenic to *D. melanogaster*.

The *P. putida* strain tested possessed the same plasmid as *E. coli*, pSCM21, containing GFP and resistance to kanamycin and had an optimal growth at 30^{o}C. *P putida* is a spider mites' pathogen, leading to mite's death by spraying[2].

The E. faecalis strain tested has an optimal growth at 37^{o}C and has been shown to be pathogenic to *D. melanogaster* by pricking[4].

B. megaterium tested in these experiments had resistance to lyncomycin and optimal growth at 30^{o}C. *B. megaterium* is not pathogenic to *D. melanogaster*

3.4. Experiments with T. urticae

3.4.1. Infection by Spraying

T. urticae females were individually placed on pieces of bean leaf (1.5cm length and 1cm width) placed on top of cotton wool inside a large Petri dish (29cm diameter), each containing 25 leaf discs. Spraying was performed using a sprinkler, at a height of 30cm, each Petri dish being sprayed three times and each spatter having an approximate volume of 0.33 ml. For each bacteria tested, the experiment was repeated 3 times.

Subsequently, spider mites were kept in a controlled environment (16:8 photoperiod and 25^{o}C) for 96 hours and survival was measured each 24 hours. Spraying experiments were performed at FCUL.

200 mites per replicate were tested (50 sprayed with Luria Broth (LB), and 50 per each bacteria concentration). LB was the medium used to culture all the bacteria used in this study. Bacteria tested were *E. coli*, *Pseudomonas putida* and *Enterococcus faecalis* at an optical density (OD) of 1, 10 and 25. *P. putida* and *E. faecalis* are *D. melanogaster*'s pathogens. *E. coli* was selected as a possible negative control, as it is not pathogenic to *Drosophila melanogaster*[4].

3.4.2. Infection by Ingestion

Females were placed on a circular arena with a 2 cm diameter; and 2 parafilm bubbles filled with food contaminated with bacteria as their only food source. Mites fed for a period of 48 hours and then individually transferred to small pieces of bean leaf (1.5cm length and 1cm width) placed on top of a cotton wool inside a large Petri dish (29cm diameter). Spider mites transferred were kept in a controlled environment (16:8 photoperiod and 25^{o}C) for 96 hours and survival was measured each 24 hours. For each bacteria tested, the experiment was repeated 5 times.

Per replicate 320 mites were feeding on the parafilm bubbles (80 on LB and 80 per each bacteria concentration). Bacteria tested were *E. coli* and *P. putida* at an optical density (OD) of 1, 10 and 25. *P. putida* is a *D. melanogaster*'s pathogens. *E. coli* was selected as a possible negative control, as it is not pathogenic to *Drosophila melanogaster*[4].

Parafilm bubbles were made using a vacuum manifold attached to a vacuum pump. Each parafilm bubble was filled with approximately 30 µl of food and bubbles were closed using adhesive tape. Bubbles filled with food were placed at the centre of the tested arenas, and per arena, both bubbles were filled with the same food mixture. The tested food consisted of Schneider's medium and LB with bacteria at a 1:1 proportion. Subsequently, a colouring dye, sybr green, was added to the mixture at a 1:4 proportion. Sybr green allowed distinguishing between spider mites that fed on the bubbles from those that did not, as mites fed on the bubbles had their intestine coloured with the dye. Only mites with colouring dye in their intestine were transferred to bean leaves. 48 hours after the beginning of the experiment, the total number of spider mites alive in the arenas and the number of spider mites containing dye in their stomach were counted. This data was used to test if there was a decrease in percentage of spider mites feeding on bubbles infected with bacteria, which may be considered as an avoidance behavior.

3.4.3. Avoidance Experiments with an Olfactometer

To test if olfactory cues trigger an avoidance behavior in *T. urticae,* we used a Y-maze olfactometer. The Y-maze, connected to a vacuum pump, was kept in a room with controlled temperature, humidity and uniform light. Avoidance of *E. coli* at a concentration of 25 OD was tested with an air flow of 0.4-0.5 m/s. Spider mite females with 1 day old as an adult were collected from bean leaves and were placed at the end of the Y-maze. The preference for bacteria or clean LB was measured. Cotton filled with LB was placed inside a syringe connected to one of the Y-maze's arm; the same procedure was performed for cotton filled with bacteria. Spider mites were tested one at a time and choice was considered valid when the mite entered the last 1/3 of one of the olfactometer arm. To avoid confounding effects, every 5 valid tests, bacteria were changed from one arm of the olfactometer to the other. Per experiment, 20 valid tests were performed and four experiments were done.

3.4.4. Infection by Systemic injection and Microarrays

3.4.4.1. Infection by Systemic Injection

Systemic injections were performed at the University of Crete, Greece, in collaboration with John Vontas and Thomas van Leeuwen groups. London strain spider mite females, with one day old as adult, were injected (bacteria were injected inside the spider mites abdomen) using an automatic injector connected to a microscope. 100 spider mites were injected per treatment - LB, OD0.1, OD1 and OD10 - and per treatment were transferred to a single bean leaf (10 cm long and 7 cm width) placed on top of a cotton wool in a Petri dish (29cm diameter). Mites' survival was followed for 96 hours, and survival was counted each 24 hours.

Bacteria tested were *E. coli* and *B. megaterium*, at 0.1, 1 and 10 ODs concentration. We chose to test these bacteria as they are not pathogenic to *D. melanogaster* when infected by pricking and we wanted to test if *T. urticae* was able to deal with systemic infection.

3.4.4.2. Microarrays

We did microarray analysis to study if there was a genetic response after bacterial infection by systemic injection.

Microarray analysis was performed at the University of Ghent in collaboration with Thomas van Leewen lab. Spider mites were injected with *E. coli* and *B. megaterium* at 1 OD concentration and with LB as a negative control. We chose to test these bacteria as they are known not to be pathogenic to *D. melanogaster*, but activate the fly's defenses.

Spider mites gene expression was analysed for three timepoints: 3, 6 and 12 hours after injection. For each timepoint 300 mites were collected, frozen using liquid nitrogen and subsequently sent to Ghent. The comparison between mites injected with bacteria and mites injected with LB was performed for all time points. Additionally, for each bacteria, genes differentially expressed at different time points were also compared.

Microarray analysis was performed as followed: RNA was extracted using the RNeasy Minikit (Qiagen) and subsequently treated with DNase (Turbo DNA-*free* kit,

Ambion). The Low Input Quick Amp Labeling Kit (Agilent Technologies) was followed to produce Cy5- or Cy3-labelled cRNA.

cRNA samples were pooled and hybridized to a custom Sureprint G3 8x60K array (Agilent Technologies) using the Gene Expression Hybridization Kit (Agilent Technologies). The array custom design is accessible under the GEO-platform GPL16890. Slides were washed with the Gene Expression Wash Buffer Kit (Agilent Technologies), prior to being scanned by an Agilent Microarray High Resolution Scanner (Agilent Technologies).

Agilent Feature Extraction software (Protocol GE2_107_Sep09) was used to retrieve the output files. The image output files are accessible under the GEO-data set GSEXXXXX. Further statistical analysis of these files was performed with limma. The data was pre-processed by a background correction using method "normexp" with an offset of 50, followed by a global loess and an Aquantile normalization (used for data normalization between arrays). Probes that bound to multiple genes in the *T. urticae* genome were excluded from further analysis. Differentially expressed genes were detected with $\log_2 |FC|$ (FC standing for Fold Changes in expression), cutoffs at 0.585 with a FDR adjusted p-value cut off (Benjamin-Hochberg corrected) at 0.075. These cutoffs are the least strict one can apply.

3.5. Experiments with S. berlesei

3.5.1. Infection by Spraying

S. berlesei adult females were placed in Petri dishes (7cm diameter) filled with agar and a top layer of yeast, each dish with 10 mites. Spraying was performed using a sprinkler, at a height of 30 cm, each Petri dish being sprayed three times and each spatter having an approximate volume of 0.33 ml. For each bacteria tested, the experiment was repeated 3 times.

After spraying, mites were kept in a controlled environment (16:8 photoperiod and 25^{o}C) for 96 hours and survival was measured each 24 hours. Experiments were performed at Instituto Gulbenkian de Ciência. (IGC).

Per experiment, 120 mites were tested (30 sprayed with LB, and 30 per each bacteria concentration). Bacteria tested were *Escherichia coli* and *Enterococcus faecalis* and

were tested at a concentration of 1, 10 and 25 ODs. LB was also sprayed upon mites as a negative control. *E. faecalis* is a *D. melanogaster*'s pathogens. *E. coli* was selected as a possible negative control, as it is not pathogenic to *Drosophila melanogaster*..

3.5.2. Infection by Systemic Injection

S. berlesei adult females were injected by piercing the female's abdomen. The automatic injector used in these experiments was attached to an inverted microscope. After injection, mites were transferred to Petri dishes (7cm diameter) filled with agar and a top layer of yeast, each dish containing 10 mites. Survival was observed for 96 hours and measured every 24 hours. Per bacteria, 3 experiments were performed and experiments were performed at the IGC.

Per experiment, 120 mites were tested (30 injected with LB, and 30 per each bacteria concentration). Bacteria tested were *Escherichia coli* and *Bacillus megaterium* and were tested at a concentration of 0.1, 1 and 10 ODs. *P. putida* is a *D. melanogaster*'s pathogen. *E. coli* was selected as a possible negative control, as it is not pathogenic to *Drosophila melanogaster*.

3.6. Statistical Analysis

All statistical analyses were performed using the software R i386 3.0.1 (2013).

3.6.1 .Infection by Spraying, Feeding experiments and Injection Experiments:

To analyze the effect of the treatment on mites' survival, the function coxme was employed.

We started by testing a model considering the interaction between Treatment and different replicates (coxme(Surv(day,census)~trat*exp+(1|rep)) against a model without the interaction (coxme(Surv(day,census)~trat+exp+(1|rep)), using ANOVA. If the effect of the interaction was not significant, we used the model without the interaction to test the effect of the treatment.

If an interaction between treatment and experiment was found, we created a new variable, concatenating the experiment and treatement (exptrat) and we altered our model (coxme(Surv(day,census)~trat+(1|exptrat/rep),data=mites)) and tested the effect of the treatment, again using ANOVA.

To test differences among treatments we employed a Tukey Contrasts analysis (glht(model,mcp(trat="Tukey"))).

To test the effect of different treatments on the percentage of mites feeding on food contaminated with bacteria, a generalized linear model was employed (glm(cbind(fed,unfed)~trat*exp,family="binomial",data=data.frame) and tested with ANOVA. If residuals were overly dispersed, we changed the model's distribution from binomial, to quasibinomial.

Tukey Contrast analysis was employed to test if different treatments had different effects.

3.6.2. Avoidance

To analyze spider mites' avoidance to *E. coli*, a Pearson's Chi-square test was applied.

3.7. Graphs:

All graphs were obtained using the software GraphPad Prism 6 Demo.

4. Results

4.1. T. urticae

4.1.1 Infection by Spraying

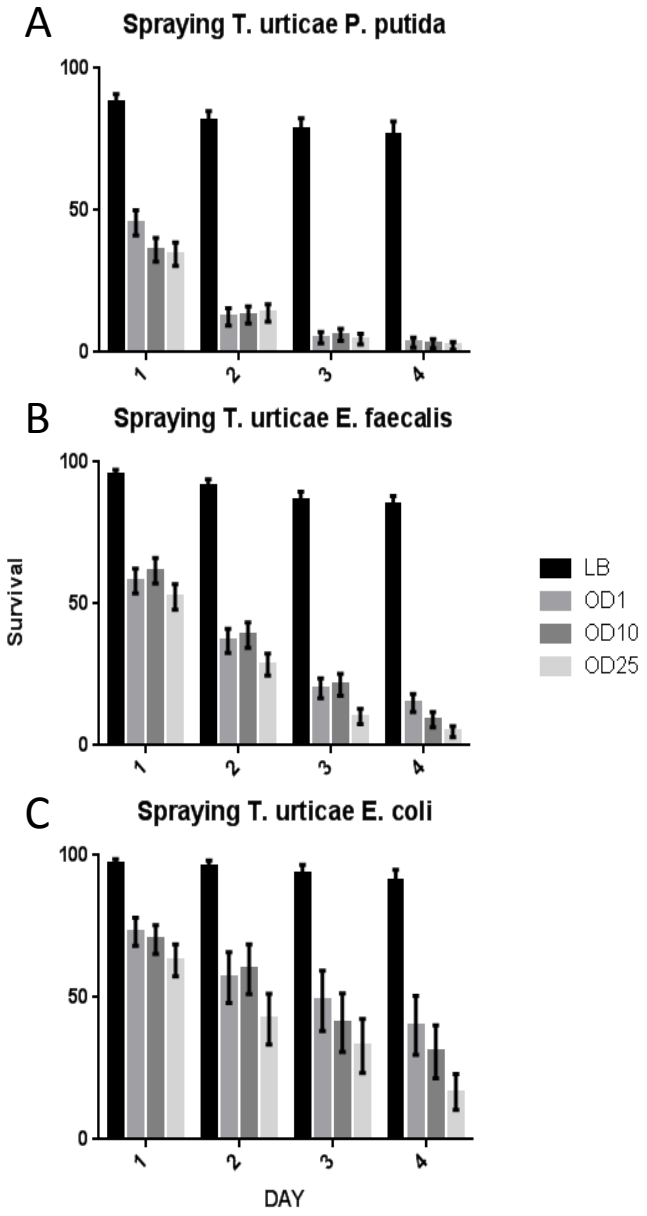

Figure 2 – Survival of *T. urticae* individuals infected by spraying. A- with *P. putida*; B- with *E. faecalis;* C- with *E. coli*

There was a significant effect of the treatment on spider mites' survival sprayed with *P. putida* (X^2_3=35.612, P<0.001) (Fig.2-A). Tukey comparisons revealed that when spider mites were sprayed with any concentration of *P. putida*, their survival is reduced compared to spider mites sprayed with LB (for all comparisons, Z>14 and P<0.001). If comparing the effect of different bacteria concentrations on spider mites survival, differences were not found (for these comparisons, Z<2.3 and P>0.09). These results indicate that *P. putida* is pathogenic to *T. urticae* if infected by spraying, as infection led to low survival rates.For *E. faecalis*, there was a significant effect of the treatment on spider mites survival (X^2_3=31.854, P<0.001) (Fig.2-B). Spider mites sprayed with *E. faecalis* had their survival reduced, compared with those sprayed with LB (for all comparisons, Z>11 and P<0.001). However, if comparing the effect of different bacteria concentrations on spider mites survival, differences were not found (for these comparisons, Z<2.7 and P>0.07). Infection by spraying with *E. faecalis* led to high mortality rates in *T. urticae*, suggesting that *E. faecalis,* is pathogenic if sprayed upon *T. urticae*.

As for *P. putida* and *E. faecalis*, for *E. coli* there was a significant effect of the treatment on spider mites' survival (X^2_3= 24.481, P<0.001) (Fig.2-C). Infection by spraying with *E. coli* led to reduced survival when compared with spider mites sprayed with LB (for all comparisons, Z>7 and P<0.001). However, different bacteria concentrations did not affect survival differently (for these comparisons, Z<1.5 and P>0.4). *E. coli* seems to be pathogenic to *T. urticae*, suggesting that these spider mites do not have mechanisms to protect them against bacteria infection.

4.1.2. Infection by Ingestion

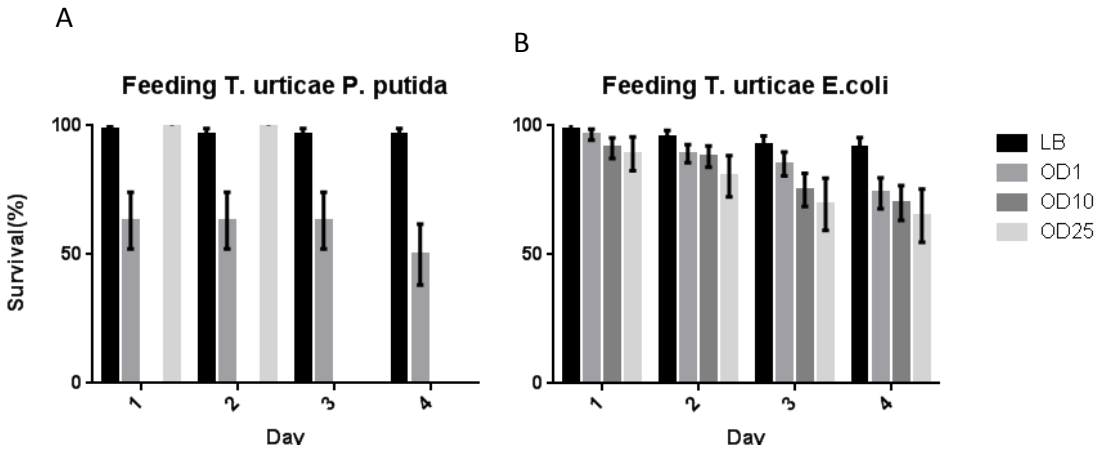

Figure 3 – Survival of *T. urticae* individuals infected by ingestion. A- with *P. putida*; B- with *E. coli*

Feeding was tested as an alternative infection route, as bacteria ingestion may be frequent.

There was an effect of the treatment on spider mites' survival, after *P. putida* ingestion (X^2_3=17.937, P<0.001) (Fig. 3-A). Spider mites feeding on bubbles with *P. putida*, at any concentration, had their survival reduced compared to spider mites feeding on LB (for all comparisons, Z>3 and P<0.02). However, different bacteria concentrations did not affect survival differently (for these comparisons, Z<1.2 and P>0.6). Overall, this data suggests that when ingested, *P. putida* is pathogenic to *T. urticae*.

Analyzing spider mites survival after *E. coli* ingestion there was an effect of the treatment (X^2_3=10.736, P= 0.01324) (Fig. 3-B). Spider mites feeding on bubbes filled with *E. coli* at OD10 and OD25 had their survival reduced, compared to mites feeding on bubbles with LB (for these comparisons, Z>2 and P<0.02; for the comparison between LB and OD1, Z=2.122 and P=0.14516).

4.1.3. Infection by Systemic Injection Experiments and Microarrays

Results obtained from the feeding and spraying experiments suggest that *T. urticae* is not able to deal with bacteria infection as all the bacteria tested, infecting by different routes, reduced spider mites' survival. We wanted to test if spider mites died

when injected with bacteria and also if they were able to mount a genetically regulated immune response when infected.

4.1.3.1. Systemic Infection by Injection

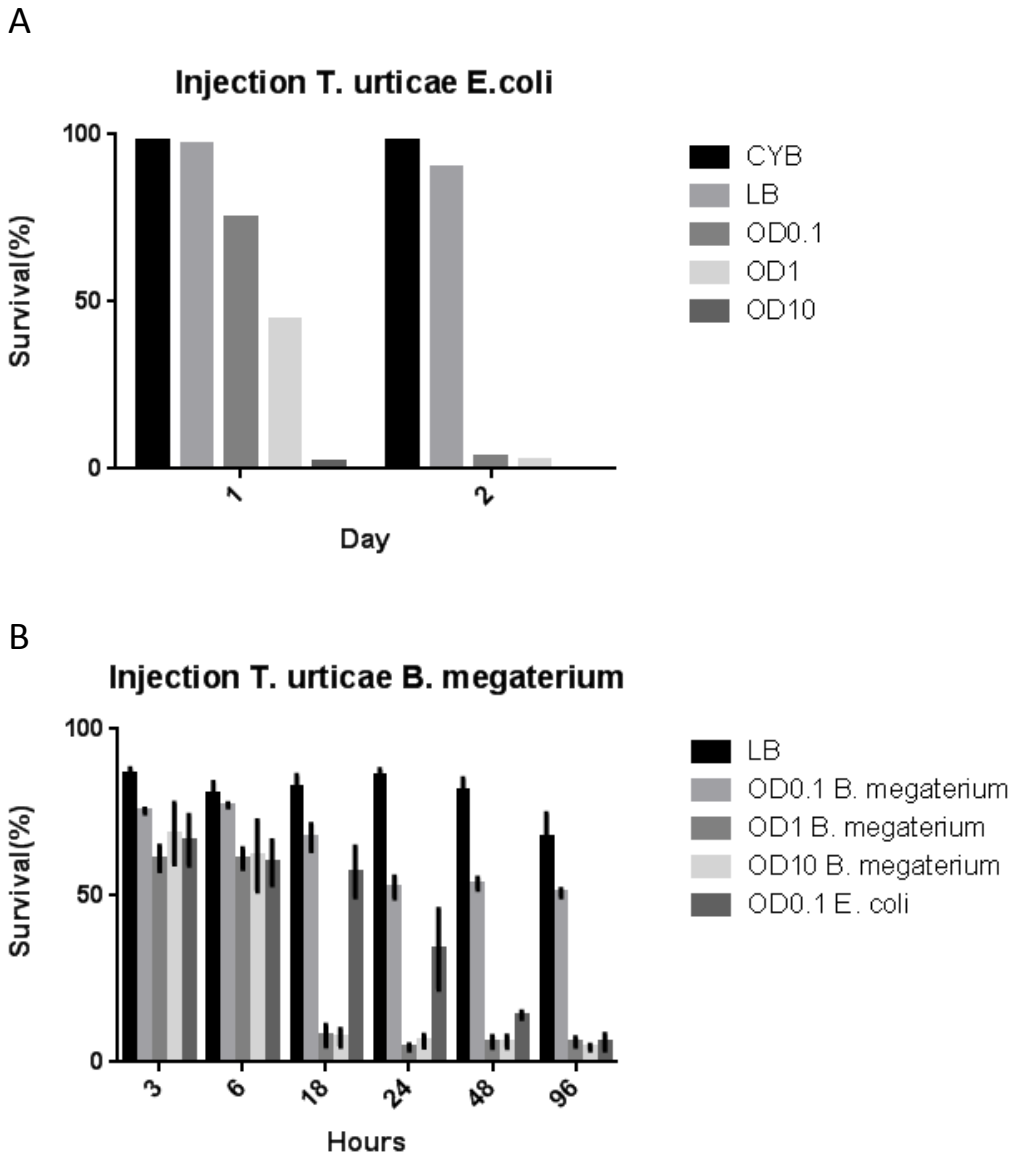

Figure 4 – *T. urticae* survival upon systemic injection with bacteria.
A- Spider mites injected with *E. Coli* died within 2 days after injection, LB was tested as a positive control. B- Spider mites survival upon injection with *B. Megaterium*. Differences were found analysing different concentrations.

E. coli

Spider mites were injected with *E. coli* at different concentrations (0.1 OD, 1 OD and 10 OD) (Fig. 4-A). Most spider mites injected with *E. coli* died within 2 days

independently of the bacteria concentration tested, whereas mites injected with LB had a high survival rate.

Additionally, an effect of the dose tested was found as, for the first day after injection, a concentration of 0.1 OD led to higher survival than 1 OD and 10 OD. As for spraying and feeding experiments, *E. coli* reduced spider mites' survival when injected, suggesting that *E. coli* is pathogenic to *T. urticae*, independently of the route of infection.

B. megaterium

Concerning injection with *B. megaterium* there was an effect of the treatment on spider mites' survival (X^2_3=37.96, P<0.001) (Fig. 4-B).

There were found differences in *T. urticae*'s survival comparing spider mites injected with LB or with bacteria at different concentrations (for all comparisons, Z>6 and P<0.001). Additionally, different bacteria concentrations affected *T. urticae*'s survival differently, as OD1 and OD10 led to reduced survival compared with OD0.1 (OD0.1-OD1: Z=10.556, P<0.001; OD0.1-OD10: Z=10.038, P<0.001).

B. megaterium injection led to a great reduction on survival, demonstrating that this bacteria is pathogenic to *T. urticae*.

4.1.3.2. Microarrays analysis

E.coli vs LB

Comparing the results obtained from spider mites injected with *E. coli* against injected with LB, differences in gene expression only appeared 6 hours after injection. At this timepoint, 35 genes were differentially expressed and twelve hours after injection, the number of genes was raised to 37 (Table S.I.1 – Table S.I.2).

Four genes were found to be present at more than one timepoint: tetur03g08300 (unknown protein function), tetur04g01580 (unknown protein function) and tetur05g05060 and tetur05g05030, both coding for a glycosyl transferase. Of the total of differentially regulated genes, time-dependent overexpression was not found. Tetur05g05060 and tetur05g05030 were the only genes found to have time dependent downregulation.

Analysing the function of those differentially expressed genes, it was not possible to detect a pattern among them. None of the *D. melanogaster* immunity related genes was differentially expressed and several of the genes observed to be differentlally expressed do not have a known function.

Results show that *T. urticae* does not mount an induced immune response upon systemic injection with *E. coli.*

B. megaterium vs LB

Concerning results from the *B. megaterium* microarray, differences in genes' expression only appear at the last time point studied, 12 hours after injection. At this time point, 17 genes were differentially expressed (Table S.I.3).

Similarly, when analyzing results for the *E. coli*' microarray, we could not identify a pattern in genes differentially regulated. Analysing those genes, several have unknown functions and genes known for its role in immunity, were not found to be differentially expressed.

These results suggest that *T. urticae* is unable to mount an induced immune response upon systemic injection with *B. megaterium*.

4.1.4. Avoidance of Bacteria

4.1.4.1. Reduction of Food Ingestion in the Presence of Bacteria

The protocol used in our ingestion experiments allowed us not only to analyze spider mites' survival after bacteria ingestion but also check if spider mites reduce ingestion when exposed to contaminated food, comparing the rate of spider mites that fed on the food bubbles between different treatments.

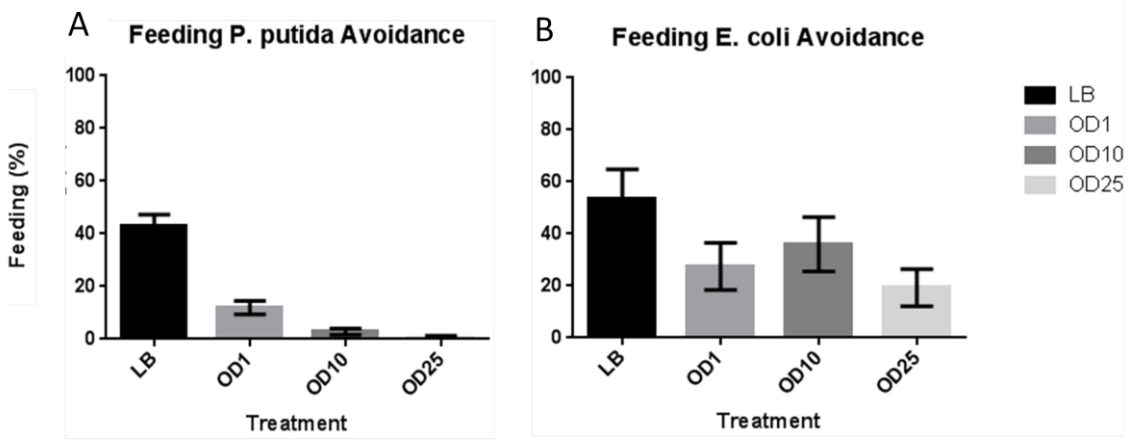

Figure 5 – Results from the ingestion experiments. A- Percentage of mites feeding on food contaminated with *P. putida*; B – Percentage of mites feeding on food contaminated with *E.coli*

Comparing the percentage of spider mites feeding on bubbles contaminated with *P. putida*, there was an effect of the treatments tested (X^2_3=5.810, P<0.001) (Fig.5-A). Differences were found in the percentage of spider mites feeding on bubbles filled with LB and mites feeding on bubbles with food contaminated with *P. putida* at any concentration (for all comparisons, Z<-4 and P<0.001). Results suggest that spider mites avoid food infected with *P. putida.*

Analyzing the percentage of spider mites feeding on bubbles contaminated with *E. coli*, there was an effect of the treatments tested (X^2_3=38.988, P=0.003) (Fig.5-B). Differences were found in the percentage of mites feeding on bubbles filled with LB and spider mites feeding on bubbles with food contaminated with bacteria at 1OD (Z=-2.733; P=0.0318) and 25OD (Z=-3.816; P<0.001). Result suggests that *T. urticae* avoids *E. coli*, as *T. urticae* individuals seem to reduce their feeding habits when food is infected.

4.1.4.2. Avoidance of E. coli using olfactory cues

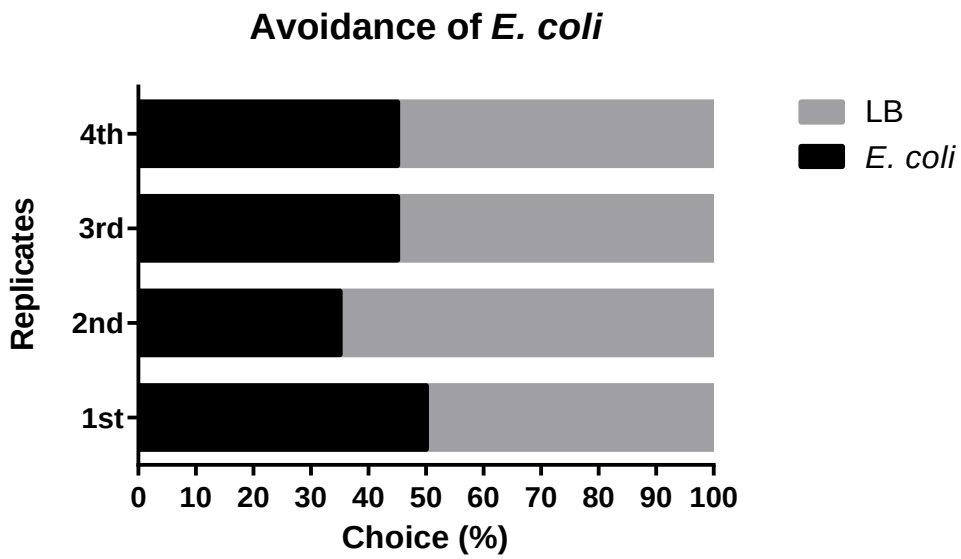

Figure 6 – *T. urticae* avoidance of *E. coli* at a concentration of 25 OD. Four replicates were performed and, per replicate, 20 valid tests were done. Spider mites did not avoid *E. coli*, using olfactory cues.

Results from the ingestion experiments suggest that *T. urticae* avoids bacteria (Fig.6). To test if spider mites avoided *E. coli* using olfactory cues, we used a Y-olfactometer. Treatments tested did not have a significant effect (X^2_1=1.25, P= 0.2636), suggesting that olfactory cues do not trigger avoidance to *E. coli* (Fig.4).

4.2. Sancassania berlesei

4.2.1. Infection by Spraying

S. berlesei mites were sprayed with either *E. coli* or *E. faecalis*.

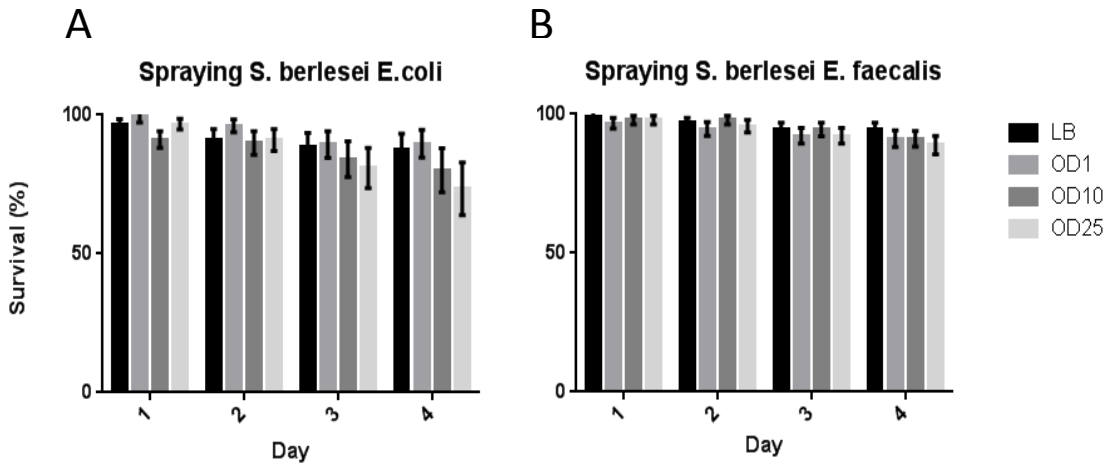

Figure 7– *Sancassania berlesei* survival upon bacteria infection by spraying. A- infection with *E. coli* did not affect mites' survival, within 4 days after infection. B- infection with *E. faecali* did not reduced mites' survival.

E. coli sprayed onto *S. berlesei* did not affect the mites' survival (X^2_3=4.4219, P=0.2194) (Fig. 7-A). Regarding *S. berlesei*'s survival after spraying with *E. faecalis*, mites' survival was not reduced after exposure to bacteria(X^2_3=1.8429, P=0.6056) (Fig. 7-B).

4.2.2. Systemic Infection by Injection

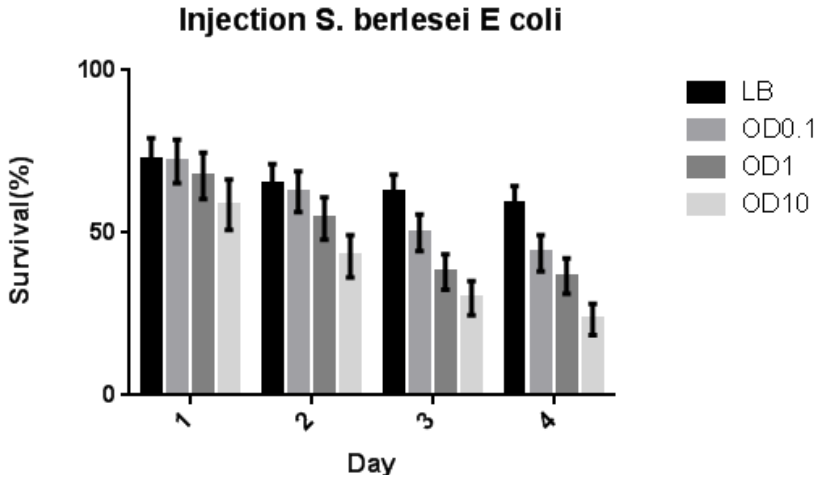

Figure 8 – *Sancassania berlesei* survival upon systemic injection with *E. coli*. Reduced survival was observed, compared to the negative control (mites injected with LB), but only at OD10

E .coli

There was an effect of the treatment on *S. berlesei*'s survival (X^2_3=7.7751, P=0.0509) (Fig. 8). Additionally, it was observed that mites injected with LB had higher survival than those injected with *E. coli* at OD10 (Z=3.751, P<0.001). Mites injected with *E. coli* at OD0.1 (Z=1.177 and P=0.6412) and OD1 (Z=2.406 and P=0.0760) did not had their survival reduced compared to mites injected with LB.

These results suggest *S. berlesei* may deal with *E. coli* systemic infection, at low concentrations. Nevertheless, at concentrations equal or higher than OD10, *E. coli* is pathogenic.

5. Discussion

Spider mites infected with bacteria via different infection routes (feeding, spraying and injection) had lower survival than uninfected individuals. Morever, microarray analysis suggested that *T. urticae* does not mount an immune response upon systemic infection, as no consistent upregulation or downregulation of genes was observed under any of the infection scenarios. This absence of changes in gene expression associated with an immune response explains the low survival rates found after infection as it seems that spider mites do not detect bacteria and do not fight its proliferation. However, we show that spider mites avoid feeding on food containing bacteria. This avoidance is not triggered by olfactory cues.Our results suggest that different infection routes affect *T. urticae* survival differently. Spider mites are less susceptible to infection by bacteria ingestion than by spraying, either because they may prevent bacteria that are in contact with their body from entering its organism or by tolerating infection. Bacteria injection was the most harmful route tested. Overall, these results demonstrate the importance of studying different routes of infection to characterize an organism's defenses.

Concerning the ingestion and spraying experiments, it is important to take into consideration that we cannot infer how many spider mites were systemically infected (i.e. if bacteria were able to penetrate the spider mites' physical/chemical barriers), in these experiments. Both for feeding and spraying assays, we cannot tell if mites that survived were systemically infected at any time, and therefore we cannot infer if mites that survive were tolerant to bacterial infection. Spider mites that ingested bacteria had high survival rates for some of the different concentrations tested. We know that spider mites ingested contaminated food but we do not know which the amount of food ingested was. It is possible that mites control the amount of food ingested and thereby diminish the probability of systemic infection, by ingesting smaller quantities.

Individuals injected with *B. megaterium* at a concentration of 0.1 OD had survival rates above 50%. Results suggest that *T. urticae* tolerates the presence of these Gram positive bacteria at low concentrations. *B. megaterium* has an optimal growth temperature around 30^{o}C and spider mites were kept at 25^{o}C during experiments. This difference in temperature probably led to a reduced number of cell

divisions and to a slow bacteria proliferation, which may have allowed spider mites to tolerate infection. If this theory is accurate, given more time, spider mites would die as they would not be able to fight bacteria proliferation and infection.

Considering that both the IMD and Toll pathways were demonstrated to be incomplete we did not expect to find an immune response in *T. urticae* simmilar to *D. Melanogaster*[35]. Nevertheless, we would expect to see an alternative upregulation pattern, given that the immune response is an important mechanism ensuring host's protection.

We hypothesized that *T. urticae* defenses against bacteria may be representative of the basal arthropods' defenses. Comparison between *T. urticae* and *S. berlesei* revealed that *T. urticae* is more susceptible to bacteria infection both by spraying and by injection. *S. berlesei*'s survival was not affected when bacteria were sprayed upon them and a reduction was observed, but only when *E. coli* was injected at high concentrations. This increased protection against bacteria found in *S. berselei* may be due to possessing less permeable physical/chemical barriers, to a higher tolerance or to a more efficient immune response against infection than *T. urticae* mites. We cannot infer which are the mechanisms/pathways that *S. berlesei* uses to fight infection. Future studies should try to identify if *S. berlesei* mounts an immune response, by studying the gene expression of some immune related genes using, for example, quantitative PCR.

This comparison suggests that *T. urticae* defenses are not a good representative of the basal arthropods' as the spider mite weak defenses were not found in other mite species. We propose that these differences are explained by differences in species ecology.

In theory, if an organism lives in a habitat where bacteria are present, but the probability of infection by pathogens is scarce or if there is other selective pressure stronger than the pressure caused by bacterial infection, the cost associated with the maintenance of the immune response may be higher than the benefits. If in a population there are individuals that reduce the investment in the immune response and invest that energy in other traits, this allocation may lead to a higher fitness and therefore selection may favor individuals redirecting resources, leading to a

deterioration of the immune system[43]. If selection acts for a long period of time it is possible that the immune system loses function. This theory may explain why *T. urticae* had high mortality rates when infected, why we did not find any pattern in the function of differentially expressed genes when analyzing the results from the microarrays and why *S. berlesei* mites seem to possess stronger defenses compared to *T. urticae*.

Indeed, *S. berlesei* mites are detritivores, commonly found in habitats with damp conditions. Their feeding habits are polyphagous as they eat both deteriorating animals and plants, yeast, and during these experiments cannibalism was observed. *S. berlesei* environment is thus prone to bacteria's growth and the probability of bacteria ingestion and/or contact with bacteria seems to be high, or higher than the *T. urticae*'s environment, the plant leaves.

In contrast, *T. urticae* lives on plant leaves and feed on leaf cells. Leaves have been described as a hostile environment to bacteria as they have a cuticle layer which significantly reduces the moist in its surface, they are exposed to high temperature fluctuations, as sunlight heats the leaf during the day to temperatures higher than the surrounding air and temperatures decrease at night; and also to UV radiation[44]. Additionally, leaves' surface has several structures as stomates or trichomes, creating an environment described by Hirano as "a jumbled matrix of peaks, valleys, caves and plains for bacteria colonization". Therefore, leaves may be seen as extreme environments to bacteria colonization, due to the heterogeneity of their environment, changing considerably in few hours[45]. Observing the distribution of pathogenic bacteria *Pseudomonas syringae* inside the leaves, these bacteria are found in the mesenchima and also on the epidermal surface, but they were not found inside the plant cells[45]. Interestingly, different parts of the leaf seem to offer different conditions to bacteria colonization[46]. Monier, in 2004, demonstrated that *Pseudomonas syringae* cannot colonize stomates after inoculation, and preferentially colonizes trichomes and veins. In the same study he analysed the leaf area covered by bacteria before and after inoculation. 8 days after inoculation, under conditions favoring the bacteria's growth, bacteria were occupying 12% of the leaf's area; however, before inoculation, which may mimic conditions spider mites find in nature, the area covered was 0,75%[46]. These results suggest that bacteria are found in specific areas of the leaf and at low

concentrations in spider mites natural environment. If spider mites avoid these infected plants areas, or rely on their body physical and chemical barriers to prevent bacteria penetration, the probability of infection by pathogenic bacteria may be small and the immune system may not be necessary.

Summarizing, comparison between *S. berlesei* and *T. urticae* suggests that bacteria exert a stronger selective pressure in *S. berlesei*'s environment than *T. urticae*'s, selecting *S. berlesei*'s defenses against bacteria. These results demonstrate that ecology had an important role in the evolution of the spider mites defenses, specially compared to phylogeny.

Recently, the aphids response to bacteria infection has been studied[47]. Aphids share the spider mite's habitat as they live, reproduce and feed on plants. These insects were challenged by wounding with a needle infected with *E. coli*. The aphids haemolymph was collected after bacterial infection and was applied to culture plates with *E. coli* or *Micrococcus luteus* and no inhibition of the bacteria's growth was found. Additionally, analyzing the cDNA obtained from individuals infected, no known AMPs were identified. Two hypothesis were proposed to explain these results. As aphids eat phloem sap, which has been described as sterile it was suggested that the absence of pathogenic bacteria from the aphids food may explain why the immunity genes are missing. If infection by bacteria ingestion is not frequent, it is probable that the selective pressure on the aphids defenses is low, explaining the weak defenses found. The other hypothesis proposed is that endossymbionts may provide protection against Gram negative bacteria in aphids, replacing the IMD's role in immunity.

These two hypothesis may be extrapolated to *T. urticae.* As spider mites feed on plant cells, which have been demonstrated to have low bacteria concentrations, the probability of bacteria ingestion seems to be low, specially compared to other species with different ecology, such as *D. melanogaster* or *S. berlesei*. Moreover, the endossymbionts *Wolbachia*, *Rickettsia* and *Cardinium* have been found in *T. urticae* populations and remarkably, some of these symbiont are known to confer protection against parasites[38,48]. *Wolbachia* has been described to confer resistence to viruses and in some cases, to bacteria[49–51]. In particular, in mosquitos (*Aedes Aegypti*), *Wolbachia*, conferred protection against *Erwinia carotovora*, a Gram negative bacteria,

but protection against Gram positive bacteria was not found[50]. *Rickettsia* and *Ricketsiella* have been proven to significantly increase resistance to fungal pathogens, *Pandora neoaphidis*, in aphids, but there is no evidence that these symbionts protect against bacteria[52]. Lukasik et al, in the same work also demonstrate that aphids carrying *Spiroplasma* and *Regiella* have partial protection to the same funghi. In tse-tse flies, individuals without the *Wigglesworthia* endosymbiont died of infection by an *E. coli* strain, while wild-type flies eliminated the infection, suggesting that this endossymbiont confers protection against bacteria[53]. As *Wigglesworthia* is a primary endossymbiont of tse-tse flies and co-evolution with its host led to specific interactions, I would not expect *Wigglesworthia* to be found and to have a similar role in spider mites. The London strain tested in our experiments did not possess endossymbionts as it had been treated with tetracycline. In the future it will be important to test how spider mites infected with different endossymbionts deal with horizontally transmitted bacteria.

Additionally, Altincicek demonstrated that aphids invest resources in increasing their fecundity when infected, promoting a final burst of egg laying. This may be seen as an alternative strategy to a genetically regulated immune response, increasing the hosts fitness [51]. Although there is no information on spider mites, it is possible that *T. urticae* present the same behavior as aphids. Future studies should address this question, analyzing spider mites fecundity after bacterial infection, possibly testing different infection routes.

Results regarding aphids' defenses against bacteria also suggest that different ecological environments select different defensive strategies.

If mites are able to avoid bacteria in their environment, *T. urticae* could avoid leaves or plants infected with pathogenic bacteria, reducing the probability of infection. Our experiments show that *T. urticae* avoids feeding on food infected with *E. coli* or with *P. putida*. However, these experiments did not provide information on how spider mites identify the bacteria presence. We then tested if spider mites used odor clues to avoid bacteria, but results were not significant. These results were surprising as we know that *T. urticae* is able to identify different stimuli using odor. It has been demonstrated they prefer leaves that had been infested with conspecifics over

uninfested leaves; or they prefer uninfected leafs than with heterospecifics[54–56]. It is possible that our protocols are not adequate to test if spider mites reduce bacteria ingestion or if olfactory cues trigger avoidance. In the future we would like to repeat the ingestion experiments for both bacteria, and also to test if spider mites avoid *P. putida*, using olfactory cues. Additionally, we would like to film the spider mites' behavior when placed on the parafilm bubbles, to confirm our results regarding the reduction of food ingestion.

Besides avoidance, the body's physical and chemical barriers and genetically regulated immune response, *T. urticae* has other trait that may confer protection to infection: the web. Spider mites continuously produce web, described by Grbic et al. in 2011 as being "used to establish a colonial micro-habitat, protect against abiotic agents, shelter from predators, communicate via pheromones and provide a vehicle for dispersion"[35,57]. Despite being a physical barrier to bacteria infection, the web may have antimicrobial properties. Although there are no evidences that the spider mite's silk has antimicrobial properties, there are some results indicating that, in spiders, the web may provide protection to bacteria[58]. Silk produced by *T. domestica* inhibited the growth of *B. subtilis*, a Gram positive bacteria, but did not inhibit *E. coli*'s growth. Additionally it was observed that the egg silk of *P. phrygianus* also seems to have an inhibitory effect on bacteria, for both Gram positive and negative bacteria and that the effect lasted for at least 72 hours[58]. There is also another report demonstrating that a social mite species, *Stigmaeopsis longus*, uses its silk to clean the plant leaves. These mites increase silk production if its habitat its nest is dirty with particles[59]. In the future, the effect of the web as a physical barrier and as a source of antimicrobial compounds should be studied as the silk has a major impact on spider mites life.

6. Conclusions

Our results point out that *T. urticae* defenses against bacteria are weak, mainly relying on avoidance and the body's physical/chemical barriers to prevent infection.

We hypothesized that either *T. urticae* possessed an immune response different than *D. melanogaster* or an immune response was absent. Spider mites, when infected by different routes had their survival reduced, independently of the bacteria tested, suggesting that they lack an immune response. Results from the microarray experiments confirmed that the spider mites do no mount an immune response when infected.

Avoidance could be an important defensive strategy in *T. urticae*. Spider mites reduced ingestion in the presence of contaminated food, which may be considered as avoidance of bacteria. However, when testing the effect of olfactory cues on avoidance, it did not trigger the behavior.

We proposed that *T. urticae* defenses could be representative of the basal arthropods and to test this hypothesis we infected other mite species with a different ecology than *T. urticae*, *S. berlesei*, by spraying and injection and compared the results. Spider mites defenses were weaker than *S. berlesei'*s, suggesting that *T. urticae* may not represent the basal arthropods'.

7. Bibliography

1. Madigan, M. T., Martinko, J. M., Stahl, D. & Clark, D. P. *Brock Biology of Microorganisms*. 1152 (Benjamin Cummings, 2010).

2. Aksoy, H. M., Ozman-Sullivan, S. K., Ocal, H., Celik, N. & Sullivan, G. T. The effects of Pseudomonas putida biotype B on Tetranychus urticae (Acari: Tetranychidae). *Exp. Appl. Acarol.* **46,** 223–30 (2008).

3. Martins, N. E., Faria, V. G., Teixeira, L., Magalhães, S. & Sucena, E. Host Adaptation Is Contingent upon the Infection Route Taken by Pathogens. *PLoS Pathog.* **9,** e1003601 (2013).

4. Shirasu-Hiza, M. M. & Schneider, D. S. Confronting physiology: how do infected flies die? *Cell. Microbiol.* **9,** 2775–83 (2007).

5. Buchon, N., Broderick, N. a, Poidevin, M., Pradervand, S. & Lemaitre, B. Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell Host Microbe* **5,** 200–11 (2009).

6. Schmid-Hempel, P. & Ebert, D. On the evolutionary ecology of specific immune defence. *Trends Ecol. Evol.* **18,** 27–32 (2003).

7. Gerardo, N. M. *et al.* Immunity and other defenses in pea aphids, Acyrthosiphon pisum. *Genome Biol.* **11,** R21 (2010).

8. Zahiri, N. & RAU, M. Oviposition Attraction and Repellency of Aedes aegypti (Diptera: Culicidae) to Waters from Conspecific Larvae Subjected to Crowding, Confinement, Starvation, or Infection. *J. Med. Entomol.* **35,** (1998).

9. Lemaitre, B. & Hoffmann, J. The host defense of Drosophila melanogaster. *Annu. Rev. Immunol.* **25,** 697–743 (2007).

10. Regier, J. C. *et al.* Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. *Nature* **463,** 1079–83 (2010).

11. Little, A. E. F., Murakami, T., Mueller, U. G. & Currie, C. R. Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. *Biol. Lett.* **2,** 12–6 (2006).

12. Spivak, M. & Reuter, G. S. Resistance to American foulbrood disease by honey bee colonies Apis mellifera bred for hygienic behavior. *Apidologie* **32,** 555 – 565 (2001).

13. Behringer, D. C., Butler, M. J. & Shields, J. D. Avoidance of disease by social lobsters. *Nature* **441,** 421 (2006).

14. Seiedy, M., Saboori, A. & Zahedi-Golpayegani, A. Olfactory response of Phytoseiulus persimilis (Acari: Phytoseiidae) to untreated and Beauveria bassiana-treated Tetranychus urticae (Acari: Tetranychidae). *Exp. Appl. Acarol.* **60,** 219–27 (2013).

15. Vala, F., Egas, M., Breeuwer, J. a J. & Sabelis, M. W. Wolbachia affects oviposition and mating behaviour of its spider mite host. *J. Evol. Biol.* **17,** 692–700 (2004).

16. J. Moreau, A. Bertin, Y. Caubet, T. R. Sexual selection in an isopod with Wolbachia-induced sex reversal: males prefer real females. *J. Evol. Biol.* **14,** 388–394 (2001).

17. Worden, B., Parker, P. & Pappas, P. Parasites reduce attractiveness and reproductive success in male grain beetles. *Anim. Behav.* **59,** 543–550 (2000).

18. Stensmyr, M. C. *et al.* A conserved dedicated olfactory circuit for detecting harmful microbes in Drosophila. *Cell* **151,** 1345–57 (2012).

19. Ha, E.-M., Oh, C.-T., Bae, Y. S. & Lee, W.-J. A Direct Role for Dual Oxidase in Drosophila Gut Immunity. *Science (80-.).* **310,** 847–850 (2005).

20. Ivanova, E. P. *et al.* Natural bactericidal surfaces: mechanical rupture of Pseudomonas aeruginosa cells by cicada wings. *Small* **8,** 2489–94 (2012).

21. Meister, M. & Lagueux, M. Drosophila blood cells. *Cell. Microbiol.* **5,** 573–580 (2003).

22. Labrosse, C., Eslin, P., Doury, G., Drezen, J. M. & Poirié, M. Haemocyte changes in D. Melanogaster in response to long gland components of the parasitoid wasp Leptopilina boulardi: a Rho-GAP protein as an important factor. *J. Insect Physiol.* **51,** 161–70 (2005).

23. Matova, N. & Anderson, K. V. Rel/NF-kappaB double mutants reveal that cellular immunity is central to Drosophila host defense. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 16424–16429 (2006).

24. Imler, J.-L. & Bulet, P. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. *Chem. Immunol. Allergy* **86,** 1–21 (2005).

25. Trautwein, M. D., Wiegmann, B. M., Beutel, R., Kjer, K. M. & Yeates, D. K. Advances in insect phylogeny at the dawn of the postgenomic era. *Annu. Rev. Entomol.* **57,** 449–68 (2012).

26. Waterhouse, R. M. *et al.* Evolutionary Dynamics of Immune-Related Genes and Pathways in Disease-Vector Mosquitoes. *Science (80-.).* **316,** 1738–1743 (2007).

27. Tanaka, H. *et al.* A genome-wide analysis of genes and gene families involved in innate immunity of Bombyx mori. *Insect Biochem. Mol. Biol.* **38,** 1087–110 (2008).

28. Altincicek, B., Knorr, E. & Vilcinskas, A. Beetle immunity: Identification of immune-inducible genes from the model insect Tribolium castaneum. *Dev. Comp. Immunol.* **32,** 585–95 (2008).

29. Zou, Z. *et al.* Comparative genomic analysis of the Tribolium immune system. *Genome Biol.* **8,** R177 (2007).

30. Weinstock, G. M. *et al.* Insights into social insects from the genome of the honeybee Apis mellifera. *Nature* **443,** 931–949 (2006).

31. Tian, C., Gao, B., Fang, Q., Ye, G. & Zhu, S. Antimicrobial peptide-like genes in Nasonia vitripennis: a genomic perspective. *BMC Genomics* **11,** 187 (2010).

32. Faulhaber, L. M. & Karp, R. D. A diphasic immune response against bacteria in the American cockroach. *Immunology* **75,** 378–81 (1992).

33. Iwanaga, S. The molecular basis of innate immunity in the horseshoe crab. *Curr. Opin. Immunol.* **14,** 87–95 (2002).

34. Pagel Van Zee, J. *et al.* Tick genomics: the Ixodes genome project and beyond. *Int. J. Parasitol.* **37,** 1297–305 (2007).

35. Grbić, M. *et al.* The genome of Tetranychus urticae reveals herbivorous pest adaptations. *Nature* **479,** 487–92 (2011).

36. Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W. & Tirry, L. Acaricide resistance mechanisms in the two-spotted spider mite Tetranychus urticae and other important Acari: a review. *Insect Biochem. Mol. Biol.* **40,** 563–72 (2010).

37. Breeuwer, J. A. J. Wolbachia and cytoplasmic incompatibility in the spider mites Tetranychus urticae and T . turkestani. *Heredity (Edinb).* **79,** 41–47 (1997).

38. Tsagkarakou, A., Guillemaud, T., Rousset, F. & Navajas, M. Molecular identification of a Wolbachia endosymbiont in a Tetranychus urticae strain (Acari: Tetranychidae). *Insect Mol. Biol.* **5,** 217–21 (1996).

39. Poinar, G. J. Parasites and Pathogens of Mites. *Annu. Rev. Entomol.* **43,** 449–69 (1998).

40. Hall, I. M., Hunter, D. K. & Arakawa, K. Y. The Effect of the on the Fraction Citrus of Mite Bacillus thuringiensis. *J. Invertebr. Pathol.* **18,** 359–362 (1971).

41. Timms, S., Ferro, D. N. & Emberson, R. M. General biology and nomenclature of Sancassania berlesei (Michael). *Acarologia* **22,** 385–390 (1982).

42. Radwan, J., Unrug, J. & Tomkins, J. L. Status-dependence and morphological trade-offs in the expression of a sexually selected character in the mite, Sancassania berlesei. *J. Evol. Biol.* **15,** (2002).

43. Rigby, M. C. & Jokela, J. Predator avoidance and immune defence: costs and trade-offs in snails. *Proc. Biol. Sci.* **267,** 171–6 (2000).

44. Monier, J.-M. & Lindow, S. E. Aggregates of resident bacteria facilitate survival of immigrant bacteria on leaf surfaces. *Microb. Ecol.* **49,** 343–52 (2005).

45. Hirano, S. S. & Upper, C. D. Bacteria in the Leaf Ecosystem with Emphasis on Pseudomonas syringae − a Bacteria in the Leaf Ecosystem with Emphasis on Pseudomonas syringae — a Pathogen , Ice Nucleus , and Epiphyte. *Microbiol. Mol. Biol. Rev.* **64,** 624–653 (2000).

46. Monier, J.-M. & Lindow, S. E. Frequency , Size , and Localization of Bacterial Aggregates on Bean Leaf Surfaces Frequency , Size , and Localization of Bacterial Aggregates on Bean Leaf Surfaces. *Appl. Environ. Microbiol.* **70,** (2004).

47. Altincicek, B., Gross, J. & Vilcinskas, a. Wounding-mediated gene expression and accelerated viviparous reproduction of the pea aphid Acyrthosiphon pisum. *Insect Mol. Biol.* **17,** 711–6 (2008).

48. Gotoh, T., Noda, H. & Ito, S. Cardinium symbionts cause cytoplasmic incompatibility in spider mites. *Heredity (Edinb).* **98,** 13–20 (2007).

49. Teixeira, L., Ferreira, A. & Ashburner, M. The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. *PLoS Biol.* **6,** e2 (2008).

50. Kambris, Z., Cook, P. E., Phuc, H. K. & Sinkins, S. P. Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. *Science* **326,** 134–6 (2009).

51. Glaser, R. L. & Meola, M. a. The native Wolbachia endosymbionts of Drosophila melanogaster and Culex quinquefasciatus increase host resistance to West Nile virus infection. *PLoS One* **5,** e11977 (2010).

52. Łukasik, P., van Asch, M., Guo, H., Ferrari, J. & Godfray, H. C. J. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecol. Lett.* **16,** 214–8 (2013).

53. Weiss, B. L., Maltz, M. & Aksoy, S. Obligate symbionts activate immune system development in the tsetse fly. *J. Immunol.* **188,** 3395–403 (2012).

54. Pallini, A., Janssen, A. & Sabelis, M. W. Odour-mediated responses of phytophagous mites to conspecific and heterospecific competitors. *Oecologia* **110,** 179–185 (1997).

55. Oriuchi, J. H. *et al.* Lima bean leaves exposed to herbivore-induced conspecific plant volatiles attract herbivores in addition to carnivores. *Appl. Entomol. Zool.* **38,** 365–368 (2003).

56. Dicke, M. Volatile spider-mite pheromone and host-plant kairomone, involved in spaced-out gregariousness in the spider mite Tetranychus urticae. *Physiol. Entomol.* **11,** 251–262 (1986).

57. Helle, W. & Sabelis, M. W. *Spider Mites: Their Biology, Natural Enemies, and Control*. 458 (Elsevier Inc., 1985).

58. Wright, S. & Goodacre, S. L. Evidence for antimicrobial activity associated with common house spider silk. *BMC Res. Notes* **5,** (2012).

59. Kanazawa, M., Sahara, K. & Saito, Y. Silk threads function as an “adhesive cleaner” for nest space in a social spider mite. *Proc. Biol. Sci.* **278,** 1653–60 (2011).

8. Supplementary Information

Table S.I.1 - Comparison between Genes Differentially expressed 6 hours after injection with *E. coli* vs injected with LB.

TeturID	logFC	AveExpr	t	P.Value	adj.P.Val	B	Description
tetur03g08300	1,119929	7,901475	4,798199	0,000866	0,060409	-0,2682	HCP2:Hypothetical Cuticular Protein (No hits found)
tetur07g04400	0,959654	10,17604	8,285327	1,29E-05	0,011654	3,575351	N/A:Hypothetical protein (No hits found)
tetur30g01850	0,906173	16,5496	10,60471	1,56E-06	0,011379	5,281003	N/A:PREDICTED: hypothetical protein (PREDICTED: hypothetical protein LOC100575965)
tetur04g01580	0,785382	9,365419	4,319859	0,001751	0,068584	-0,94453	HCP1:Hypothetical Cuticular Protein (No hits found)
tetur03g08010	0,777771	12,7323	8,683883	8,66E-06	0,011379	3,90829	TuPap-26:Cathepsin B (cathepsin B)
tetur01g02660	0,762611	16,52019	4,531308	0,001278	0,063299	-0,64107	MDL6:Conserved secreted protein with MD-2-related lipid recognition domain. ALL2 Grou
tetur05g01730	0,753391	16,98527	4,142387	0,002293	0,071284	-1,2045	N/A:Hypothetical protein (No hits found)
tetur22g02530	0,748759	14,44429	5,238141	0,000467	0,053482	0,320955	PLAT6:PLAT single domain protein (No hits found)
tetur23g01430	0,741636	13,17798	5,736284	0,00024	0,044873	0,949424	N/A:PREDICTED: hypothetical protein (PREDICTED: hypothetical protein)
tetur20g01390	0,728173	11,01574	8,650056	8,95E-06	0,011379	3,88074	N/A:Hypothetical protein (No hits found)
tetur11g05700	0,713635	9,445766	4,096117	0,002462	0,071984	-1,27304	N/A:Hypothetical protein (No hits found)
tetur22g02120	0,712675	11,60704	5,128871	0,000543	0,055355	0,177614	PLAT2:PLAT single domain protein (No hits found)
tetur09g03620	0,667643	16,2317	5,224669	0,000475	0,053482	0,303389	TuCPI-3:Cystatin (Cystatin precursor)
tetur03g03480	0,665536	10,52675	4,143476	0,002289	0,071284	-1,20289	N/A:26S proteasome non-ATPase regulatory subunit 9 (hypothetical protein)
tetur47g00120	0,660262	9,182855	9,145276	5,58E-06	0,011379	4,271531	N/A:Hypothetical protein (No hits found)
tetur30g01810	0,63815	11,65557	4,487223	0,001364	0,064794	-0,70376	N/A:PREDICTED: hypothetical protein (PREDICTED: hypothetical protein isoform 1)
tetur30g01880	0,636756	11,84548	9,517015	3,97E-06	0,011379	4,547919	N/A:PREDICTED: hypothetical protein (PREDICTED: hypothetical protein isoform 1)
tetur04g07960	0,636489	12,80595	4,468687	0,001402	0,064794	-0,73021	N/A:40S ribosomal protein S29 (PREDICTED: 40S ribosomal protein S29-like)
tetur24g01950	0,624904	13,03155	4,59681	0,00116	0,061987	-0,5485	N/A:DnaJ (DnaJ)
tetur06g01060	0,602835	15,27863	4,079149	0,002527	0,072187	-1,29825	TuCPI-22:Cystatin (cystatin precursor)
tetur09g04270	0,596931	10,1163	4,442095	0,001458	0,066045	-0,76825	N/A:copper chaperone (copper transport protein)
tetur26g01540	0,588238	9,101386	4,229945	0,002006	0,069835	-1,07565	N/A:PREDICTED: hypothetical protein (PREDICTED: protein FAM36A-like)
tetur20g01400	0,585086	9,297012	7,711141	2,33E-05	0,018037	3,062133	N/A:Hypothetical protein (No hits found)

tetur27g02510	-0,59678	13,95423	-6,51534	9,03E-05	0,038016	1,852134	N/A:phosphoenolpyruvate carboxykinase, cytosolic (phosphoenolpyruvate carboxykinase
tetur05g05060	-0,59885	10,61346	-4,43907	0,001465	0,066046	-0,77259	N/A:Glycosyltransferase, MGT (glycosyltransferase, MGT family)
tetur12g00580	-0,63829	7,241497	-9,59067	3,72E-06	0,011379	4,601039	N/A:Hypothetical protein (No hits found)
tetur08g06340	-0,65374	7,416383	-5,98943	0,000173	0,042328	1,253275	N/A:sialin (Putative inorganic phosphate cotransporter)
tetur11g05740	-0,71713	11,35327	-6,01757	0,000167	0,042328	1,286419	PLAT9:PLAT single domain protein (No hits found)
tetur17g00080	-0,72118	9,683857	-4,55483	0,001234	0,062964	-0,60775	TuCCE-38:Carboxyl/cholinesterase (esterase TCE2)
tetur08g06330	-0,78035	8,772396	-5,10113	0,000564	0,055776	0,140909	N/A:sialin (Sialin)
tetur05g05030	-0,78081	9,036201	-4,63834	0,001092	0,0614	-0,49016	N/A:Glycosyltransferase, MGT (glycosyltransferase, MGT family)
tetur12g00590	-0,82021	8,592884	-13,8185	1,49E-07	0,002646	6,924442	N/A:C-factor (C-factor)
tetur11g05720	-0,84308	14,86109	-5,97554	0,000176	0,042328	1,236877	PLAT10:PLAT single domain protein (No hits found)
tetur04g02440	-0,86427	7,145307	-6,07448	0,000155	0,042328	1,353056	N/A:PREDICTED: similar to adenylate cyclase (GK23339)
tetur11g05730	-1,31761	13,69016	-8,72814	8,30E-06	0,011379	3,944138	PLAT11:PLAT single domain protein (No hits found)

Table S.I.2 - Comparison Between Genes Differentially Expressed 12 hours after injection with *E. coli* vs injected with LB

TeturID	logFC	AveExpr	t	P.Value	adj.P.Val	B	Description
tetur16g03410	1,374846	11,23552	5,750954	0,00024	0,047874	0,94792	ApoD11:Apolipoprotein D precursor (apolipoprotein D precursor)
tetur06g02780	1,245876	7,616549	7,105713	4,67E-05	0,029712	2,442313	N/A:Hypothetical protein (No hits found)
tetur06g03070	1,134382	8,195967	5,231559	0,00048	0,051504	0,297851	ApoDR5:Apolipoprotein D related protein, beta lactoglobuline homologue (No hits found)
tetur11g05230	1,053894	9,216218	7,261058	3,93E-05	0,027345	2,596324	ApoD1:Apolipoprotein D (apolipoprotein D)
tetur06g03350	1,034549	7,668296	7,467433	3,13E-05	0,027345	2,795796	ApoD7:Apolipoprotein D (apolipoprotein D)
tetur05g01720	1,003672	8,141165	4,735011	0,000965	0,062345	-0,36544	N/A:Hypothetical protein (No hits found)
tetur11g05210	0,956239	9,261133	5,868741	0,000207	0,04743	1,089189	ApoD2:Apolipoprotein D (apolipoprotein D)
tetur06g03440	0,871887	8,448165	4,565264	0,001234	0,06577	-0,60149	ApoD4:Apolipoprotein D (apolipoprotein D precursor)
tetur06g01610	0,864448	8,94604	4,588721	0,001193	0,065605	-0,56859	ApoDR9:Apolipoprotein D related protein (No hits found)
tetur06g03380	0,845115	10,00694	4,366116	0,001657	0,072835	-0,88431	N/A:Hypothetical protein (No hits found)
tetur10g00740	0,840131	8,564694	4,840015	0,00083	0,059551	-0,22177	N/A:PREDICTED: similar to lipase 1 (hypothetical protein SELMODRAFT_152478)
tetur06g03360	0,715452	7,728203	8,221202	1,42E-05	0,023021	3,477123	ApoD16:Apolipoprotein D precursor (No hits found)
tetur16g03450	0,694697	7,234372	5,545706	0,000315	0,047874	0,696349	ApoD10:Apolipoprotein D precursor (apolipoprotein D precursor)
tetur06g03550	0,640527	6,515675	7,212278	4,15E-05	0,027345	2,548324	ApoD18:Apolipoprotein D precursor (Lipocalin family protein)
tetur05g05640	0,615124	9,577384	4,564404	0,001236	0,06577	-0,60269	HESP3:Highly Expressed Secreted Protein Family (No hits found)
tetur01g13320	0,590708	11,14276	6,152699	0,000144	0,039528	1,420598	SSPA1:Small Secreted Protein, family A (No hits found)
tetur12g00610	-0,59108	10,22609	-5,28621	0,000446	0,050909	0,368349	N/A:short-chain dehydrogenase/reductase SDR (C-factor)
tetur14g03190	-0,62686	10,76867	-5,35515	0,000406	0,050468	0,456584	N/A:hypothetical protein IscW_ISCW022785 (hypothetical protein IscW_ISCW022785)
tetur30g00140	-0,63331	14,80804	-4,48393	0,001391	0,068584	-0,71624	N/A:Hypothetical protein (No hits found)
tetur19g00760	-0,64801	7,618279	-10,0553	2,59E-06	0,011509	4,864593	CPR G:Putative cuticle protein (AGAP000345-PA)
tetur11g04820	-0,66143	8,756759	-5,11526	0,000564	0,052408	0,146169	N/A:phytoene dehydrogenase (phytoene desaturase)
tetur25g01840	-0,74387	6,742263	-6,49214	9,54E-05	0,036084	1,800152	N/A:Hypothetical protein (No hits found)
tetur06g03930	-0,74481	10,98564	-4,44709	0,001469	0,069907	-0,76855	N/A:hypothetical protein (hypothetical protein IscW_ISCW004702)
tetur08g04180	-0,80916	7,421645	-6,45853	9,93E-05	0,036084	1,763355	N/A:Hypothetical protein (No hits found)
tetur01g00130	-0,81603	12,35351	-7,53783	2,90E-05	0,027345	2,862524	CPR 30:cuticle protein (cuticle protein, putative)

tetur30g02200	-0,8183	12,5794	-6,67782	7,65E-05	0,034877	2,00032	N/A:PREDICTED: hypothetical protein (metal dependent phosphohydrolase)
tetur11g04810	-0,94053	8,472092	-7,62884	2,63E-05	0,027345	2,947817	N/A:phytoene dehydrogenase (phytoene dehydrogenase)
tetur09g06230	-0,98126	7,44213	-6,76035	6,94E-05	0,034877	2,087649	CPR 22:cuticle protein (cuticle protein, putative)
tetur06g04510	-1,00897	7,650443	-6,78841	6,72E-05	0,034877	2,117105	N/A:Hypothetical protein (No hits found)
tetur05g05030	-1,02617	10,19742	-5,93331	0,00019	0,045746	1,165683	N/A:Glycosyltransferase, MGT (glycosyltransferase, MGT family)
tetur05g05050	-1,1071	10,16935	-7,57665	2,78E-05	0,027345	2,899037	N/A:Glycosyltransferase, MGT (glycosyltransferase, MGT family)
tetur04g01580	-1,11068	9,415302	-6,49876	9,46E-05	0,036084	1,807376	HCP1:Hypothetical Cuticular Protein (No hits found)
tetur04g01610	-1,19203	11,06353	-7,25767	3,94E-05	0,027345	2,592996	CPR 51:Cuticle (secreted) protein, putative (No hits found)
tetur05g05020	-1,19653	9,908645	-9,76385	3,33E-06	0,011856	4,666845	N/A:Glycosyltransferase, MGT (glycosyltransferase, MGT family)
tetur20g00200	-1,23781	12,39541	-6,12601	0,000149	0,039643	1,389991	SERP:Secreted Protein with 8aa repeat structure (No hits found)
tetur05g05060	-1,335	10,84969	-7,51983	2,96E-05	0,027345	2,845528	N/A:Glycosyltransferase, MGT (glycosyltransferase, MGT family)
tetur03g08300	-1,42409	7,179511	-5,70001	0,000257	0,047874	0,886122	HCP2:Hypothetical Cuticular Protein (No hits found)

Table S.I.3- Comparison Between Genes Differentially Expressed 6 hours after injection with *B. megaterium* vs injected with LB

TeturID	logFC	AveExpr	t	P.Value	adj.P.Val	B	Description
tetur02g14420	1,138845	11,41107	11,40152	8,68E-07	0,007723	5,524947	N/A:Hypothetical protein (No hits found)
tetur02g14470	0,937284	11,37633	6,568657	8,70E-05	0,042302	1,852396	N/A:Hypothetical protein (No hits found)
tetur05g05050	0,861657	10,16935	5,896905	0,000199	0,059303	1,108058	N/A:Glycosyltransferase, MGT (glycosyltransferase, MGT family)
tetur19g03360	0,704863	14,11264	5,566756	0,000306	0,068059	0,715564	N/A:intradiol ring-cleavage dioxygenase (intradiol ring-cleavage dioxygenase)
tetur19g02300	0,664403	13,9403	5,875481	0,000205	0,059303	1,083132	N/A:intradiol ring-cleavage dioxygenase (intradiol ring-cleavage dioxygenase)
tetur09g00680	0,652362	9,467448	12,02063	5,45E-07	0,007723	5,839101	N/A:PREDICTED: similar to Chromodomain-helicase-DNA-binding protein 2
tetur03g10063	0,62286	13,70455	6,297364	0,000121	0,049987	1,560348	N/A
tetur02g14460	0,621473	9,892088	7,323059	3,67E-05	0,042302	2,606833	N/A:Hypothetical protein (No hits found)
tetur06g03360	-0,5992	7,728203	-6,88537	6,01E-05	0,042302	2,179207	ApoD16:Apolipoprotein D precursor (No hits found)
tetur30g01760	-0,60697	7,8451	-5,32823	0,000421	0,074922	0,420708	N/A:sensory box protein/histidinol phosphate phosphatase family protein (No hits found)
tetur03g04460	-0,625	9,953757	-5,87398	0,000205	0,059303	1,081384	N/A:Hypothetical protein (No hits found)
tetur16g03450	-0,67721	7,234372	-5,4061	0,000379	0,072577	0,518011	ApoD10:Apolipoprotein D precursor (apolipoprotein D precursor)
tetur21g01420	-0,69552	8,144211	-7,2049	4,18E-05	0,042302	2,494061	N/A:Hypothetical protein (No hits found)
tetur43g00560	-0,78224	9,175325	-5,9417	0,000188	0,05876	1,159931	N/A:Hypothetical protein (No hits found)
tetur11g05010	-0,88344	8,709589	-6,10808	0,000153	0,053245	1,349766	N/A:Hypothetical protein (No hits found)
tetur09g04730	-0,91948	8,584367	-7,75104	2,31E-05	0,042302	2,999452	ApoD20:Apolipoprotein D precursor (apolipoprotein D)
tetur06g03350	-0,92365	7,668296	-6,66697	7,75E-05	0,042302	1,955452	ApoD7:Apolipoprotein D (apolipoprotein D)