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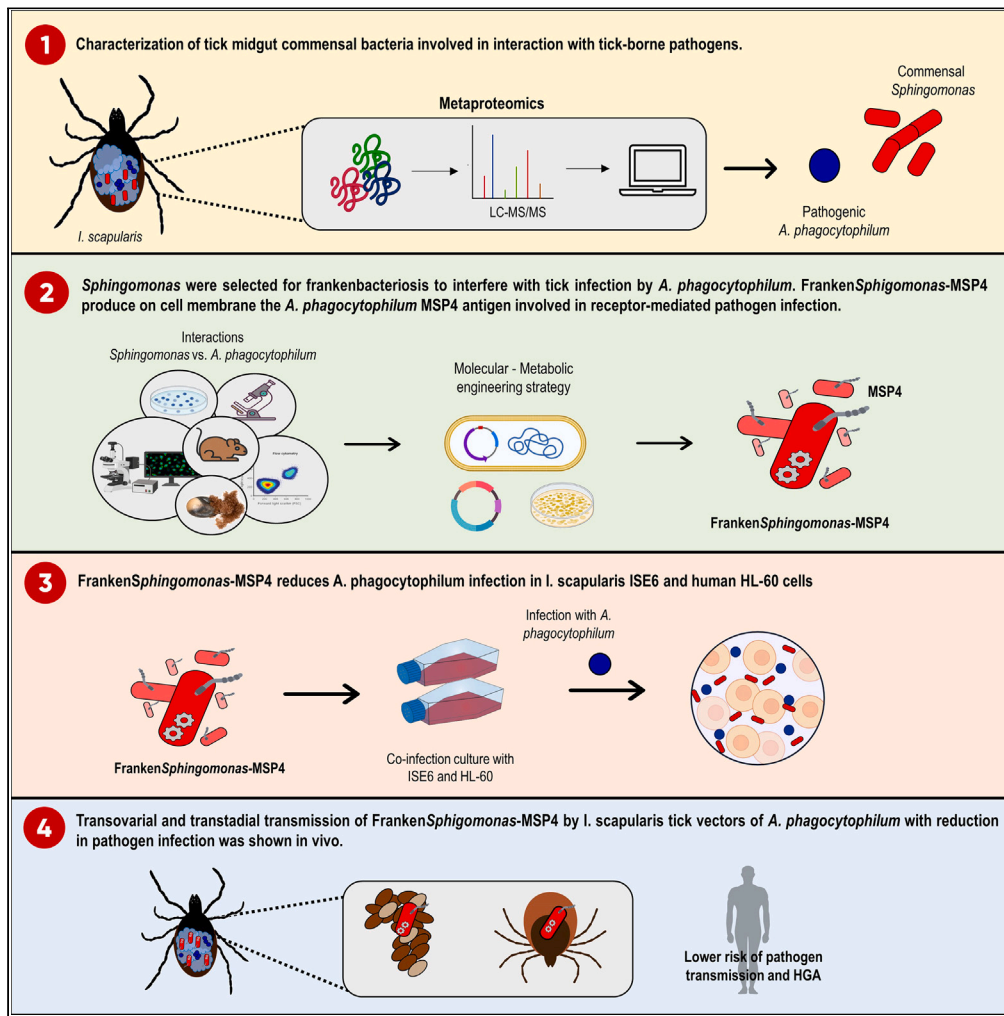
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Article

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Highlights

Frankenbacteriosis paratransgenesis of tick commensal bacteria to control pathogens

Anaplasma phagocytophilum and symbiotic *Spingomonas* compete in tick vector, *I. scapularis*

Franken*Spingomonas*-MSP4/HSP70 mimics pathogen and reduce infection in ticks

Franken*Spingomonas*-MSP4/HSP70 reduces human granulocytic anaplasmosis disease risks

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Article

Frankenbacteriosis targeting interactions between pathogen and symbiont to control infection in the tick vector

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SUMMARY

Tick microbiota can be targeted for the control of tick-borne diseases such as human granulocytic anaplasmosis (HGA) caused by model pathogen, *Anaplasma phagocytophilum*. Frankenbacteriosis is inspired by Frankenstein and defined here as paratransgenesis of tick symbiotic/commensal bacteria to mimic and compete with tick-borne pathogens. Interactions between *A. phagocytophilum* and symbiotic *Sphingomonas* identified by metaproteomics analysis in *Ixodes scapularis* midgut showed competition between both bacteria. Consequently, *Sphingomonas* was selected for frankenbacteriosis for the control of *A. phagocytophilum* infection and transmission. The results showed that Franken *Sphingomonas* producing *A. phagocytophilum* major surface protein 4 (MSP4) mimic pathogen and reduce infection in ticks by competition and interaction with cell receptor components of infection. Franken *Sphingomonas*-MSP4 trans-ovarial and trans-stadial transmission suggests that tick larvae with genetically modified Franken *Sphingomonas*-MSP4 could be produced in the laboratory and released in the field to compete and replace the wildtype populations with associated reduction in pathogen infection/transmission and HGA disease risks.

INTRODUCTION

Ticks (Acari: Ixodidae) are among the most important vectors of pathogens affecting human and animal health, and the increase in human activities and climate change among other factors are favoring their expansion worldwide.^{1,2} Ticks acquire and transmit microorganisms during the blood meal. In the United States, *Ixodes scapularis* or deer tick is the vector of infectious pathogens that cause Lyme disease (*Borrelia burgdorferi*), human granulocytic anaplasmosis (HGA; *Anaplasma phagocytophilum*), human babesiosis (*Babesia microti*) and Powassan encephalitis (Powassan virus).³ Therefore, controlling tick infestations and pathogen infection and transmission is necessary for the control of tick-borne diseases.^{3,4}

Recently, the *I. scapularis* molecular response to infection by the obligate intracellular bacterium *A. phagocytophilum* (Rickettsiales: Anaplasmataceae), a model pathogen affecting human and animal health was characterized using different omics approaches.^{5–8} Despite these advances in the characterization of *I. scapularis*-*A. phagocytophilum* molecular interactions, limited information is available about the impact of interactions with tick midgut microbiota on *A. phagocytophilum* pathogen infection and transmission.⁹

Tick microbiota includes pathogenic, commensal and symbiotic microorganisms that can affect tick biology and vector competence modulating the infection, multiplication and transmission of pathogens.^{9–11} The composition of the tick microbiota is affected by different factors such as tick species, life stage, sex, geographic location, parasitized host, feeding status, pathogen infection, and the presence of commensal bacteria and symbionts.^{12,13} In addition, direct or indirect interactions between bacteria in vector microbiota and vector-borne pathogens can affect pathogen infection and transmission.¹⁴ Therefore, tick midgut microbiota has been proposed as a target to develop novel interventions for the control of tick-borne diseases.^{8,15–17}

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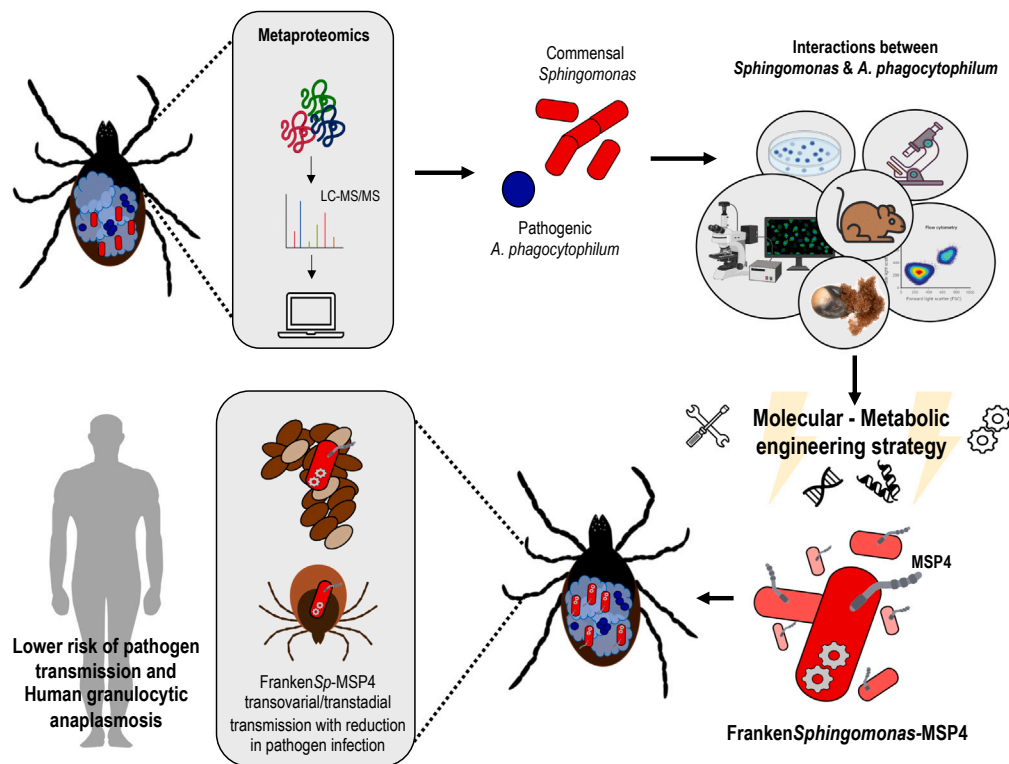


Figure 1. Experimental approach for frankenbacteriosis

The experimental approach combines metaproteomics with validation analyses in tick and human cells and in blood-feeding ticks for the identification and characterization of tick midgut commensal bacteria involved in interactions with tick-borne pathogens. *Sphingomonas* were selected for frankenbacteriosis to interfere with tick infection by *A. phagocytophilum*, the causative agent of HGA. Molecular and metabolic engineering produce FrankenSphigomonas-MSP4 producing on cell membrane the *A. phagocytophilum* MSP4 antigen involved in receptor-mediated pathogen infection. Transovarial and transstadial transmission of FrankenSphigomonas-MSP4 by *I. scapularis* tick vectors of *A. phagocytophilum* with reduction in pathogen infection was shown *in vivo*. The results may translate into a lower risk of pathogen transmission and caused disease HGA.

Vaccines with tick and pathogen derived antigens are considered an effective approach for the control and prevention of tick-borne diseases.⁴ Paratransgenesis through the genetic manipulation of symbiotic or commensal microorganisms has been also proposed as an effective and environmentally sound approach for the control of vector-borne diseases.^{8,15,18,19} Paratransgenic ticks with symbiotic bacteria producing antimicrobial compounds have been achieved and could offer a safe and effective way to reduce pathogen transmission by ticks.^{4,8,15,20,21} However, frankenbacteriosis, inspired by Frankenstein and defined here as paratransgenesis of tick midgut symbiotic/commensal bacteria to mimic and compete with pathogens, has not been addressed before (Figure 1). Tick midgut bacteria that compete with a pathogen²² may be good candidates for frankenbacteriosis.

To gain additional information on *A. phagocytophilum*-tick midgut microbiota interactions and identify putative targets for frankenbacteriosis, in this study we investigated the effect of pathogen infection on symbiotic, gram-negative, strictly aerobic, obligate intracellular *Sphingomonas* spp. identified by metaproteomics analysis in *I. scapularis* midgut (Figure 1). The results showed that *Sphingomonas* spp. compete with *A. phagocytophilum* in ticks. Franken *Sphingomonas* producing *A. phagocytophilum* major surface protein 4 (MSP4) antigens reduce pathogen infection in ticks by competition and interaction with cell receptor components of infection. Characterizing the interactions between *A. phagocytophilum* and *I. scapularis* tick midgut microbiota increases our knowledge about the mechanisms affecting pathogen infection and transmission and provided new targets and putative interventions for the control of HGA. This principle can be applied to other tick-borne diseases.

A *I. scapularis* MG

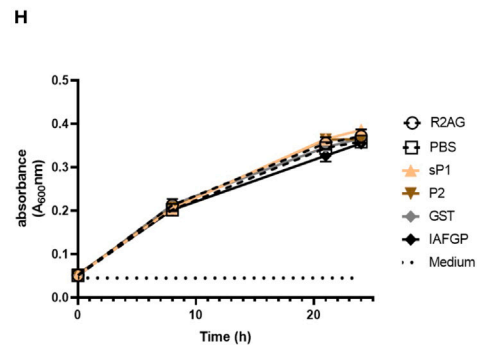
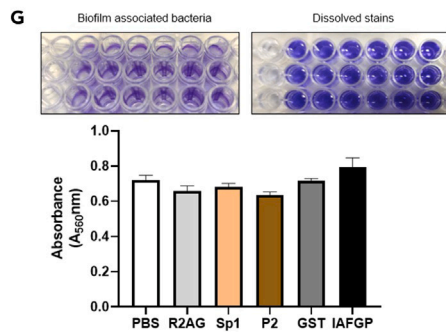
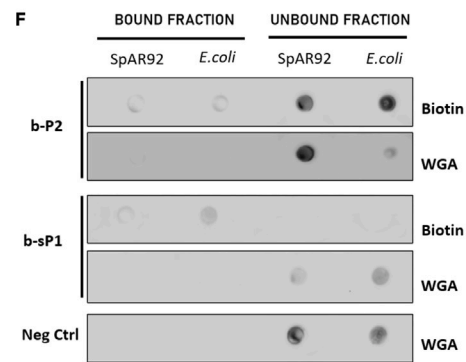
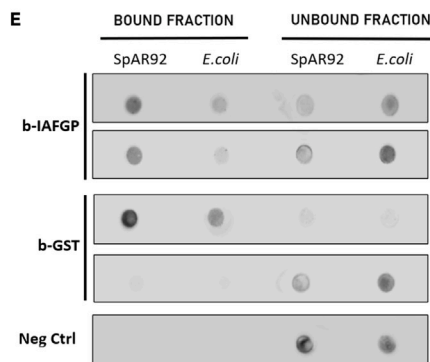
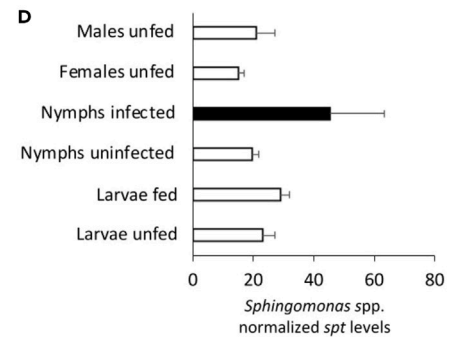
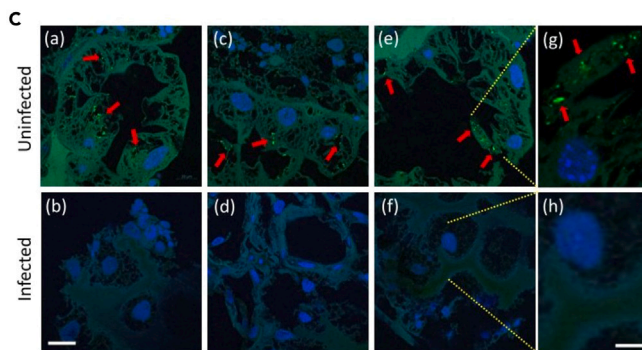
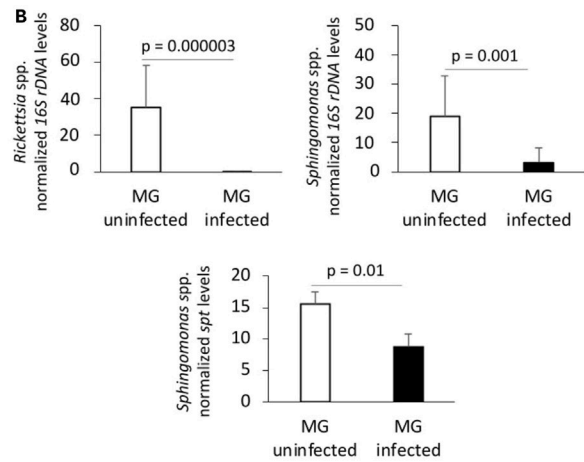
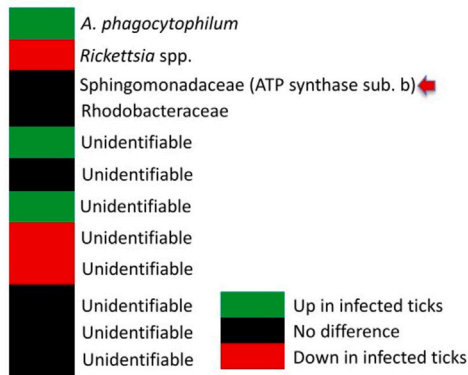


Figure 2. Changes in *I. scapularis* tick microbiota composition in response to *A. phagocytophilum* infection

(A) Representation heatmap profile in response to *A. phagocytophilum* infection of bacteria identified by metaproteomics analysis in the microbiota in the midgut (MG) of uninfected and infected ticks. The peptides with hits matching to specific bacteria were confirmed and assigned to the corresponding species, genus or family (Data S1). The remaining peptides matching to multiple families were assigned to unidentifiable bacteria. Bacterial assignments were grouped and the total number of PSM for each classification category were normalized against the total number of PSM to compare results between midgut from *A. phagocytophilum*-infected and uninfected ticks by Chi2-test ($p < 0.01$; $N = 2$ biological replicates) (Data S1). Sphingomonadaceae were identified with ATP synthase subunit b (red arrow) and selected for further analysis.

(B) *Rickettsia* and *Sphingomonas* spp. DNA levels in *A. phagocytophilum*-infected and uninfected adult female ticks. The *16S rDNA* and *spt DNA* levels were determined by qPCR, normalized against tick *16S rDNA* and *rpS4* genes and normalized Ct values were compared between infected and uninfected ticks by Student's t-test with unequal variance ($p \leq 0.01$; $N = 13$ biological replicates).

(C) Representative confocal microscopy images of MG tissue sections from uninfected and *A. phagocytophilum*-infected *I. scapularis* female ticks. Red arrows point at the localization of *Sphingomonas* spp. (Green). Blue, nuclear DAPI dye. Scale bar (a)-(f), 20 μ m at 40x optical magnification. Scale bar (g)-(h), 60 μ m at 120x optical magnification.

(D) *Sphingomonas* spp. DNA levels in unfed and fed larvae ($N = 5$), *A. phagocytophilum*-infected and uninfected nymphs ($N = 4$) and unfed uninfected adult females and males ($N = 5$). The *spt DNA* levels were determined by qPCR, normalized against tick *16S rDNA* and *rpS4* genes and normalized Ct values were compared between infected and uninfected nymphs by Student's t-test with unequal variance ($p > 0.05$; $N = 4-5$ biological replicates).

(E-H) IAFGP but not P2 peptide binds to SpAR92 peptidoglycan but do not inhibit biofilm formation. Streptavidin-coated magnetic beads were incubated with 0.1 mg/ml of (E) biotinylated proteins (b-IAFGP-GST and b-GST) or (F) biotinylated peptide (b-P2 and scramble peptide b-sP1) and peptidoglycan isolated from SpAR92 and *E. coli* cultures. Bound and unbound fractions were collected and spotted onto nitrocellulose membrane. Biotin was detected using monoclonal anti-biotin antibody. Bacterium peptidoglycan was detected using a polyclonal goat wheat germ agglutinin antibody (WGA). Bacterium peptidoglycan incubated with magnetic beads alone was used as negative control. (G) SpAR92-associated biofilm formation was determined after static incubation at 30 °C for 24 h in media alone (R2AG) or supplemented with 0.1 mg/mL of sP1 control scrambled peptide, P2, GST, GST-tagged IAFGP (IAFGP) or an equal amount of PBS. Representative images of biofilm formation in 96-well plates and dissolved stains for quantitative analysis are shown above the graph. (H) Bacterial growth was measured at 0 h, 8 h, 21 h and 24 h at 600 nm during the static biofilm assay. Medium represents the uninfected control sample. Results were pooled from 3 independent experiments with 3-4 technical replicates each. Data represent mean \pm SEM. Statistical significance was calculate using One-Way Anova followed by Tukey post-hoc test ($p < 0.05$; $N = 3$ biological replicates).

RESULTS AND DISCUSSION

Metaproteomics analysis reveals an effect of *A. phagocytophilum* infection on bacterial midgut microbiota in *I. scapularis*

A metaproteomics approach was used to identify bacterial proteins in the midgut of *I. scapularis* female ticks infected with *A. phagocytophilum*. A total of 1,164 peptide spectrum matches (PSM) of which 78 and 298 were identified in the midgut from uninfected and infected ticks, respectively were initially assigned to different bacteria (Data S1). The increasing number of available microbial genome sequence databases contributes to the technical progress of proteomics and bioinformatics, providing new possibilities for the characterization of bacterial microbiota using high-resolution LC-MS/MS platforms.²³ However, proteomics results may face the challenge of using discriminative peptide fragments that could coincide with several genera or related species, which requires additional analysis with peptide sequences used to identify the proteins to confirm the identifications.^{23,24} After BLAST analysis with peptide sequences, the genera initially identified as *Pannonibacter*, *Hyphomonas*, *Phenylobacterium*, *Ketogulonicigenium*, and some *Rickettsia* spp. were reassigned to *A. phagocytophilum*. Some sequences initially identified as *Rickettsia* remained as *Rickettsia* spp., and the low number of peptides previously associated with *Labrenzia* and *Zymomonas* were confirmed at the family level as Rhodobacteraceae and Sphingomonadaceae, respectively (Data S1). The analysis finally identified *A. phagocytophilum* (218 PSM), *Rickettsia* spp. (28 PSM), and families Rhodobacteraceae (4 PSM) and Sphingomonadaceae (3 PSM). The rest of bacterial protein assignments were confirmed as unidentifiable (126 PSM) (Figure 2A).

As expected, the analysis of normalized protein PSM showed that *A. phagocytophilum* levels were significantly higher in infected than uninfected ticks (Figure 2A). In contrast, normalized protein PSM of *Rickettsia* spp. were significantly lower in *A. phagocytophilum*-infected ticks when compared to uninfected controls (Figure 2A). The normalized PSM of Rhodobacteraceae and Sphingomonadaceae did not show significant differences between infected and uninfected ticks (Figure 2A), but the identification of these bacteria was done with less than 3 PSM per sample.

The results of metaproteomics analysis suggested that although *Rickettsia* spp. are highly represented in tick midgut microbiota, Rhodobacteraceae and Sphingomonadaceae are present at low levels. To validate the results of metaproteomics analysis at the DNA level, qPCR and sequencing were used for highly and lowly represented *Rickettsia* spp. and Sphingomonadaceae, respectively. The results confirmed that

Rickettsia spp. levels decreased in response to infection (Figure 2B). The analysis of *Rickettsia* spp. 16S rDNA sequences showed a 99–100% identity to *Rickettsia buchneri* strain ISO7 (NR_134842.1). For Sphingomonadaceae and considering that the ATP synthase subunit b amino acid sequence used to identify these bacteria is highly conserved among different bacteria, two DNA targets, 16S rDNA and *spt*, were used to validate metaproteomics analysis at the DNA level. The results confirmed the presence of these bacteria in tick midgut and showed that its DNA levels decrease in infected ticks when compared to uninfected controls (Figure 2B). Furthermore, the results of sequence analysis suggested that the identified Sphingomonadaceae were *Sphingomonas* spp. (Figures S1 and S2). These results were confirmed at the protein level by IFA with antibodies with high affinity for *Sphingomonas* (Figures S3A and S3B) in uninfected and *A. phagocytophilum*-infected *I. scapularis* female ticks (Figure 2C). To gain additional information, the presence of *Sphingomonas* spp. DNA was characterized in *I. scapularis* unfed and fed larvae, uninfected and *A. phagocytophilum*-infected fed nymphs and in unfed uninfected female and male ticks (Figure 2D). Although differences were not observed between infected and uninfected nymphs, the results confirmed the presence of *Sphingomonas* spp. in all *I. scapularis* developmental stages.

The metaproteomics approach allowed the identification of ticks co-infected with *A. phagocytophilum* and *Rickettsia* spp. This finding has been reported before using metagenomics approaches.⁹ Although the pathogenicity of these *Rickettsia* spp. is unknown, some of them may be pathogenic. Co-infections with two or more tick-borne pathogens have been reported before with important implications for human and animal health because of the risk of pathogen co-transmission.^{25,26}

The genus *Sphingomonas* was first described in 1990 by Yabuuchi et al.²⁷ as a group of bacteria isolated from human clinical specimens and hospital environments, but since then these bacteria have been also isolated from a variety of anthropogeneously contaminated environments such as terrestrial subsurface, rhizosphere and contaminated soils as degraders of polycyclic aromatic hydrocarbons, river and subsurface sediments, and aquatic wastewater, groundwater, freshwater, and marine water habitats.²⁸ Sphingomonadaceae have been previously described in various tick species such as *I. scapularis*, *Ixodes ventralloii*, *Ixodes persulcatus* and *Ixodes ricinus*.^{9,12,13,23,29,30} Environmental bacteria such as Sphingomonadaceae may colonize tick midgut from contact with their environment either before attachment³¹ or during contact with host fur and skin during feeding.³² Metaproteomics has been previously shown to effectively identify bacteria potentially metabolically active in the tick microbiota.²³ In support to these findings, the protein identified in tick midgut and assigned to *Sphingomonas* spp. is an ATP synthase subunit b, a component of the F₀ channel, which forms part of the peripheral stalk, linking F₁ to F₀ to produce ATP from ADP in the presence of a proton or sodium gradient ($\text{ATP} + 4\text{H}^+ + \text{H}_2\text{O} = \text{ADP} + 5\text{H}^+ + \text{phosphate}$).^{33,34}

These facts together with the presence of bacteria in all tick developmental stages suggested that *Sphingomonas* spp. are symbiotic bacteria in *I. scapularis* midgut microbiota.³⁵ The results suggested that at least in *I. scapularis* female midgut, the infection with *A. phagocytophilum* affects microbiota composition by decreasing the levels of *Sphingomonas* spp.

Tick IAFGP binds to *Sphingomonas* spp. peptidoglycan, but do not inhibit biofilm formation

The composition of the tick midgut microbiota changes to take advantage of the changing milieu.¹⁰ Recently, Abraham et al.⁹ showed that *A. phagocytophilum* manipulates the *I. scapularis* tick midgut microbiota by inducing the vector to produce the antifreeze glycoprotein IAFGP that directly inhibits bacterial biofilm formation and favors pathogen infection.⁹ In this study, the authors identified various Gram-positive (e.g., *Staphylococcus* spp.) and Gram-negative (e.g., *Rickettsia* spp.) bacteria in tick midgut and noted that their levels decreased in *A. phagocytophilum*-infected ticks. The levels of *Rickettsia* spp. were affected by the IAFGP-mediated biofilm formation.⁹ However, the IAFGP-mediated mechanism was not effective against other Gram-negative bacteria.⁹ Herein, both identified bacteria which levels decreased in infected ticks, *Rickettsia* and *Sphingomonas* spp. (Figures 2A and 2B) are Gram-negative. Although *Rickettsia* spp. decreased in *A. phagocytophilum*-infected ticks by previously described IAFGP-mediated antivirulence effect,⁹ the mechanism affecting *Sphingomonas* spp. is unknown.

Tick-mediated mechanisms through production of IAFGP altering biofilm formation may result in lower *Sphingomonas* spp. levels in ticks infected with *A. phagocytophilum*. To test this hypothesis, we examined the mechanism by which IAFGP and/or its derivative peptide (P2) interact with *Sphingomonas* sp. reference strain AR92 (thereafter SpAR92) bacterial peptidoglycan and alters biofilm development through an *in vitro*

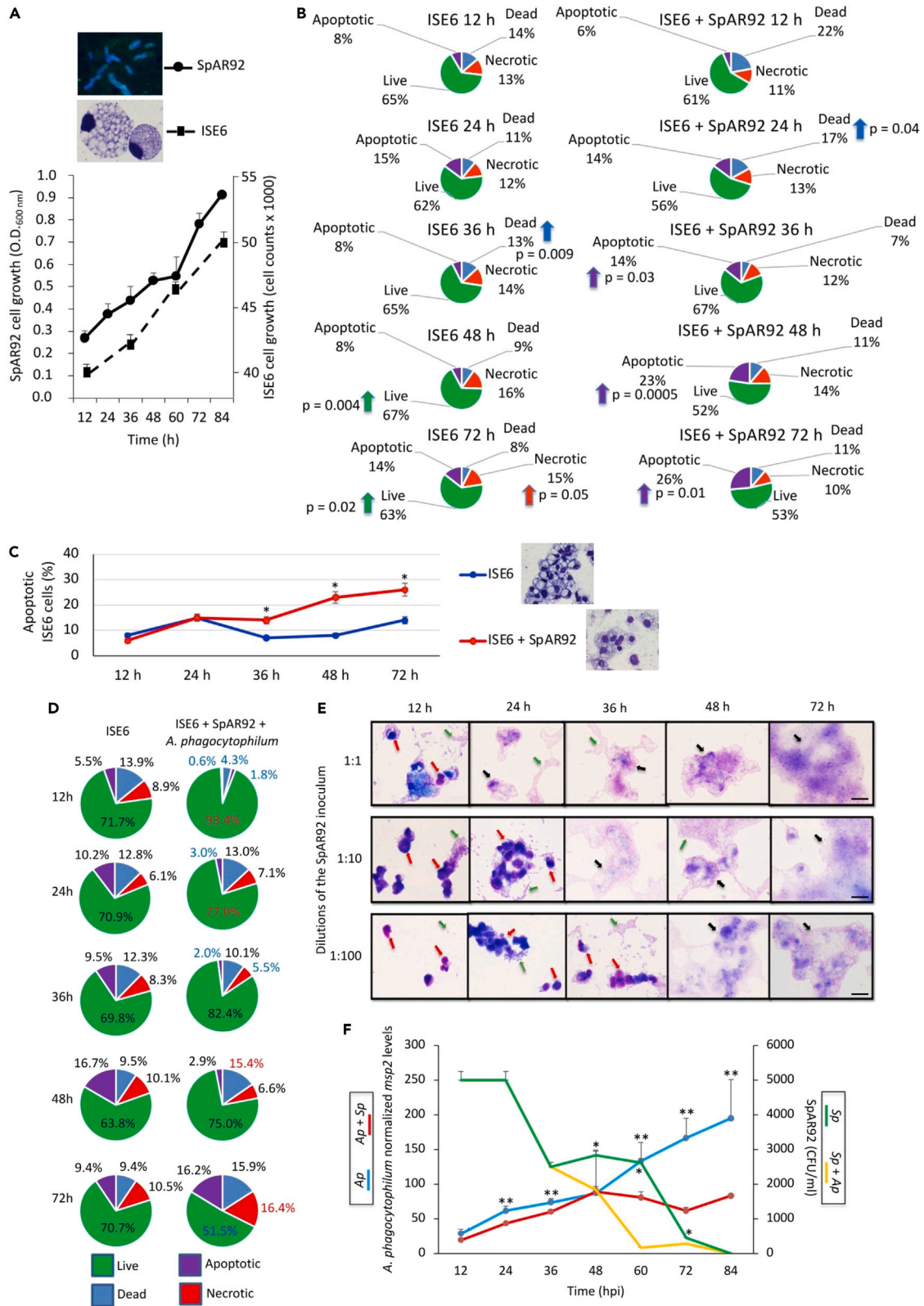


Figure 3. Effect of SpAR92 on the viability of uninfected and *A. phagocytophilum*-infected ISE6 tick cells

Experiments were conducted with ISE6 tick cells uninfected and infected with *A. phagocytophilum* NY18 isolate and cultured in L-15B300:R2A (1:1) medium with SpAR92. Uninfected ISE6 cells were cultured in L-15B300:R2A (1:1) medium with and without SpAR92 and samples collected at different time points.

(A) Typical growth of ApAR92 and ISE6 cells cultured in L-15B300:R2A (1:1) medium at 31°C.

(B) Cell viability (proportion of live/viable, necrotic, dead/late apoptotic and apoptotic cells) was measured by flow cytometry using the FITC apoptosis detection kit. The percentage of apoptotic, dead, necrotic and live cells was compared between ISE6 and ISE6 + SpAR92 cells at each time point by Student's t-test with unequal variance ($p = 0.05$; $N = 3$ biological replicates).

(C) The percent of apoptotic ISE6 cells was compared between groups by two-way ANOVA test ($*p < 0.03$; $N = 3$ biological replicates). Representative images of Giemsa-stained cells at 72 h are shown.

(D) Tick cell viability after incubation with SpAR92 at different time points. Cell viability was measured by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit. The percentage of apoptotic, dead/late apoptotic, necrotic and viable/live cells was compared between infected and uninfected cells by Student's t-test with unequal variance ($p < 0.05$; $N = 3$ biological replicates). Values in red and blue are significantly higher and lower when compared to uninfected ISE6 cells, respectively.

(E) Selected samples of ISE6 tick cells infected with *A. phagocytophilum* NY18 isolate and cultured in L-15B300:R2A (1:1) medium with SpAR92 were examined by microscopy (Zeiss, 10× objective) at 12, 24, 36, 48 and 72 h after treatment in cultures with 1:1, 1:10 and 1:100 dilutions of the SpAR92 inoculum. Red arrows point at the localization of ISE6 cell nuclei. Green arrows point at SpAR92. Black arrows point at lysing ISE6 cells mixed with SpAR92. Bars, 10 μm.

(F) Competition between *A. phagocytophilum* and SpAR92 in ISE6 tick cells. Bacterial growth profile of *A. phagocytophilum* (Ap) and SpAR92 (Sp) cultured alone (Ap and Sp) or combined (Ap + Sp or Sp + Ap). The ISE6 tick cells were incubated at 31°C with 100 μL/mL of SpAR92 suspension or culture medium alone in 24-well plates for 12 h before infection with 100 μL of semi-purified *A. phagocytophilum* or culture medium alone and incubated for additional 84 h. Samples (3 wells per time point) were collected every 12 h after *A. phagocytophilum* infection. *A. phagocytophilum* DNA levels were determined by *msp2* qPCR and normalized against tick *16S rDNA* and *rpS4* genes. Normalized *A. phagocytophilum msp2* Ct-values and SpAR92 CFU/ml were compared by two-way ANOVA test between Ap/Ap + Sp (blue and red lines; $**p < 0.05$; $N = 3$ biological replicates) and Sp/Sp + Ap (green and yellow lines; $*p < 0.05$; $N = 3$ biological replicates).

assay, similar to as previously described.⁹ The IAFGP but not P2 peptide binds to SpAR92 peptidoglycan (Figures 2E and 2F). Nevertheless, this binding does not affect the static biofilm formation or bacterial culture growth during their development (Figures 2G and 2H), suggesting that IAFGP might not manipulate tick microbiota through SpAR92 associated biofilms and might induce other alternative mechanisms to manipulate it. As expected, and previously reported by Abraham et al. (2017), IAGFP and P2 do not bind to *Escherichia coli* gram-negative peptidoglycan (Figures 2E and 2F).

Sphingomonas* spp. affects viability of tick cells and compete with *A. phagocytophilum* in *I. scapularis

The *I. scapularis* midgut cells activate several mechanisms to control pathogen infection and multiplication.^{3,5,6,36,37} In contrast, *A. phagocytophilum* manipulates tick regulatory and metabolic pathways and alters bacterial biofilm formation to reduce tick response to infection and the composition of the midgut microbiota to facilitate pathogen infection.^{5,6,8,9,36,37} Direct competition is one of the mechanisms that may affect interactions between pathogens and vector microbiota.²² Among these mechanisms, the type VI secretion system (T4SS) that delivers to target cells toxic effector proteins³⁸ and epigenetic factors are present in *A. phagocytophilum*³⁹ and *Sphingomonas* spp.⁴⁰ The presence of the T4SS interbacterial systems may be used to evaluate the possibility of bacterial direct competition mechanisms.^{13,41} Therefore, bacterial competition or interference may constitute a mechanism by which *A. phagocytophilum* infection interferes with and reduces the infection of other microorganisms such as *Rickettsia* and *Sphingomonas* spp. present in the tick midgut microbiota.^{8,13}

The effect of *Sphingomonas* on the viability of ISE6 tick cells was evaluated using the *Sphingomonas* reference strain SpAR92, related to *S. piscinae* (Figure S2) as a model. First, both SpAR92 and ISE6 cells were shown to grow in the combined L-15B300:R2A (1:1) culture medium at 31°C (Figure 3A). However, the results showed that co-culture of SpAR92 with ISE6 cells resulted in a significant increase in the percentage of apoptotic cells and the reduction of live tick cells over time (Figures 3B and 3C). These results suggested that *Sphingomonas* spp. affect tick cell viability (Figures 3C and S4).

To evaluate the possible competition between *Sphingomonas* spp. (strain SpAR92) and *A. phagocytophilum* during tick infection, *in vitro* experiments were conducted in *I. scapularis* ISE6 cells and by tick injection, capillary feeding, and artificial feeding. When SpAR92 was co-cultured with *A. phagocytophilum*-infected ISE6 cells, a reduction in tick cell viability was observed at 72 h (Figure 3D). The effect of SpAR92 on ISE6 cells resulted in lysis of tick cells proportional to bacterial growth in time (Figure 3E). Then, an experiment was conducted to evaluate the effect of SpAR92-*A. phagocytophilum* co-infection in ISE6 cells (Figure 3F). The results showed that the effect of SpAR92 on tick cells affect *A. phagocytophilum* infection as shown by

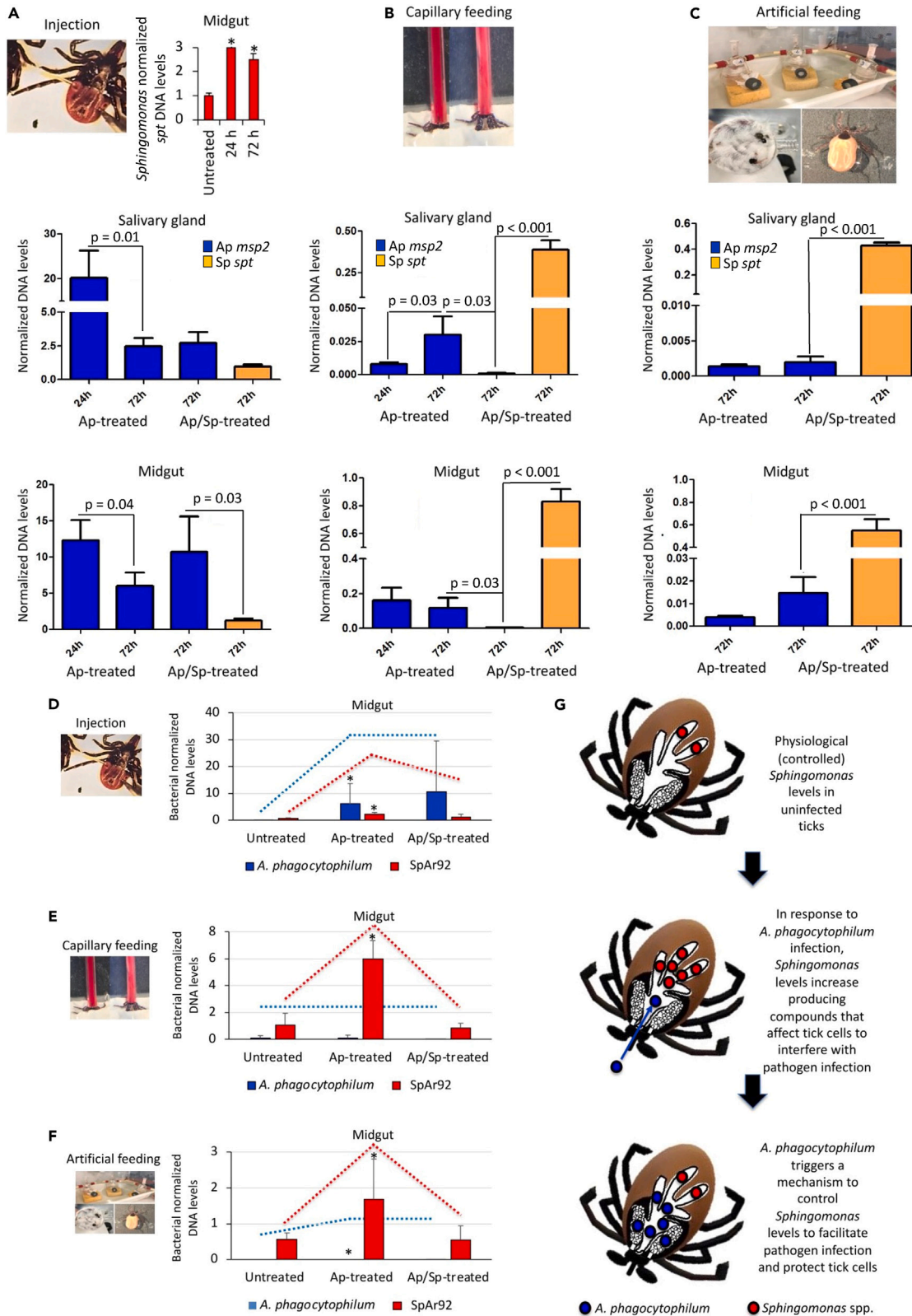


Figure 4. Infection of *I. scapularis* female ticks with *A. phagocytophilum* and SpAR92 and competition between both bacteria

(A) Tick injection.

(B) Tick capillary feeding.

(C) Tick artificial feeding. Experiments were conducted with *A. phagocytophilum* Norway isolate and SpAR92 in *I. scapularis* female ticks. Groups of 10 ticks each (5 ticks only for artificial feeding) were untreated or treated with *A. phagocytophilum* (Ap) and Ap + SpAR92 (Sp). All ticks survived after each treatment. Groups of 5 ticks were collected and dissected at 24 and 72 h post treatment (72 h only for artificial feeding). An independent experiment was conducted with tick injection containing SpAR92 alone to evaluate bacterial levels in the midgut of untreated ticks at 24 and 72 h after treatment. Salivary glands and/or midgut were extracted from dissected ticks. *A. phagocytophilum* and SpAR92/indigenous *Sphingomonas* spp. DNA levels were determined by qPCR targeting *msh2* and *spt*, respectively and normalized against tick *16S rDNA* and *rpS4* genes. Normalized Ct-values were compared by Student's t-test with unequal variance ($p < 0.05$; $N = 5$ biological replicates) for *Sphingomonas* between untreated and treated groups (upper graph with red bars in tick injection), for *A. phagocytophilum* between Ap-treated groups at 24 and 72 h and between Ap-treated and Ap/Sp-treated groups at 72 h, and between *Sphingomonas* and *A. phagocytophilum* in Ap/Sp-treated group at 72 h.

(D) Tick injection.

(E) Tick capillary feeding.

(F) Tick artificial feeding. Tendency lines with significant differences between untreated and treated groups at 72 h are shown in blue for *A. phagocytophilum* (Ap) and in red for SpAR92. *A. phagocytophilum* and SpAR92 DNA levels were determined by qPCR targeting *msh2* and *spt*, respectively and normalized against tick *16S rDNA* and *rpS4* genes. Normalized Ct-values were compared between 24 and 72 h or between untreated and treated groups by Student's t-test with unequal variance ($*p < 0.05$; $N = 5$ biological replicates).

(G) Proposed mechanism for competition between *Sphingomonas* spp. in *I. scapularis* tick microbiota and *A. phagocytophilum*.

stable bacterial levels at 48–84 hpi (Figure 3F, red line versus blue line). However, competition between *A. phagocytophilum* and SpAR92 resulted in lower *Sphingomonas* levels when compared to SpAR92 alone (Figure 3F, yellow line versus green line).

Female *I. scapularis* ticks were injected, capillary fed or artificially fed with *A. phagocytophilum* alone or in combination with SpAR92 (75–125 × 10², 20–30 × 10⁶ and 12–60 × 10⁶ SpAR92/tick after injection, capillary and artificial feeding, respectively). DNA from *A. phagocytophilum* and SpAR92/indigenous *Sphingomonas* spp. were detected in both midgut and salivary glands (Figures 4A–4C). According to each treatment and without correlation to blood meal, normalized *Sphingomonas* DNA levels after 72 h were higher in injected ticks when compared to capillary/artificially fed ticks (Figures 4A–4C). Tissue-specific differences in *A. phagocytophilum* DNA levels were observed between 24 and 72 h after treatment (Figures 4A–4C). In an independent experiment with tick injection, the *Sphingomonas* spp. DNA levels increased in the midgut of treated ticks when compared to untreated controls and remained similar at 24 and 72 h after treatment with SpAR92 alone (Figure 4A, graph with red bars). Nevertheless, *A. phagocytophilum* DNA levels decreased in both salivary gland and midgut of *A. phagocytophilum* vs. combined *A. phagocytophilum* and SpAR92 capillary fed ticks (Figure 4B). However, except in midgut of injected ticks, *Sphingomonas* DNA levels were similar or higher than *A. phagocytophilum* levels at 72 h after treatment with combined *A. phagocytophilum* and SpAR92 (Figures 4A–4C). Considering the *A. phagocytophilum* and *Sphingomonas* DNA profiles in ticks treated with *A. phagocytophilum* alone by different experimental approaches, the *Sphingomonas* levels increased in response to infection (Figures 4D–4F, Ap-treated) but decreased to levels in untreated ticks when treated with the combination of SpAR92 and *A. phagocytophilum* (Figures 4D–4F, Ap/Sp-treated). These results suggested that *Sphingomonas* levels increase in response to *A. phagocytophilum* infection producing compounds that affect tick cells to interfere with pathogen infection but bacterial competition triggers mechanisms that results in a decrease in *Sphingomonas* levels to facilitate pathogen infection and protect tick cells (Figure 4G).

Taken together, these results support that *Sphingomonas* spp. and *A. phagocytophilum* compete in *I. scapularis* thus suggesting that symbiotic *Sphingomonas* are good candidates for frankenbacteriosis to reduce pathogen infection in ticks.

Franken *Sphingomonas* reduces *A. phagocytophilum* infection in *I. scapularis* ISE6 and human HL-60 cells

Sphingomonas sp. SpAR92 was then genetically modified to produce Franken *Sphingomonas* with *A. phagocytophilum* MSP4 or HSP70 that have been shown to be involved in tick-pathogen functional interactions³⁷ (Figures 5A, 5B, S5A, and S5B). Gene expression in Franken *Sphingomonas* was validated at mRNA level (Figure 5A) and production of recombinant MSP4 and HSP70 was shown on the cell membrane of Franken *Sphingomonas* (Figures S5A and S5B) at levels similar to *A. phagocytophilum* MSP4 (Figure 5B). Once recombinant *A. phagocytophilum* MSP4 and HSP70 proteins production was validated, Franken *Sphingomonas* were *in vitro* tested for their effect on pathogen infection in tick and

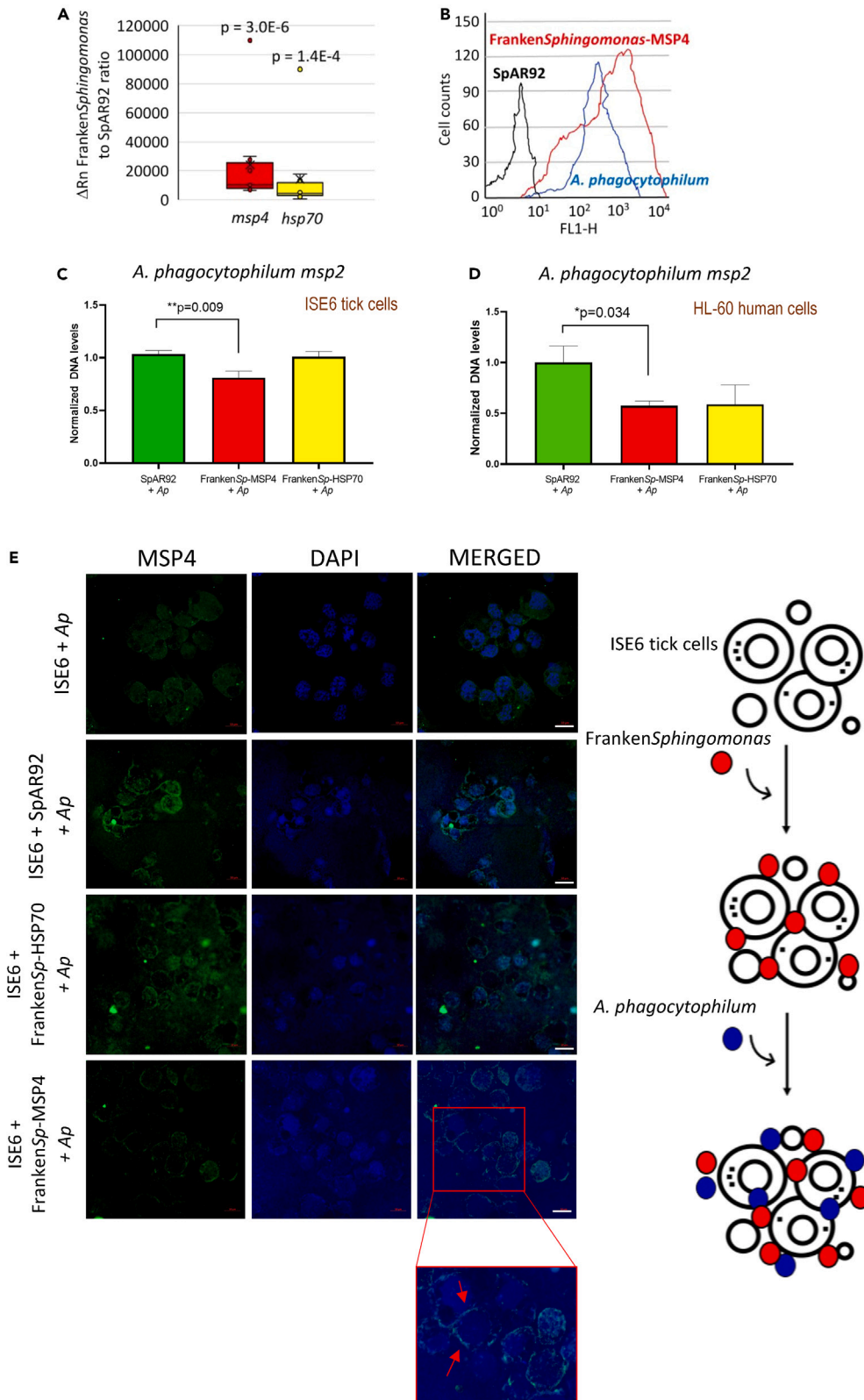


Figure 5. Characterization of Franken *Sphingomonas*

(A) The expression of *msp4* and *hsp70* genes in Franken *Sphingomonas* were characterized by RT-PCR ΔRn at 30th Cq and compared between Franken *Sphingomonas* and control SpAR92 by Student's t-test with unequal variance ($p < 0.001$; N = 10 biological replicates). Results are represented as ΔRn Franken *Sphingomonas* to SpAR92 ratio.

(B) Flow cytometry showing the presence of MSP4 on the surface of Franken*Sphingomonas*-MSP4, SpAR92 and *A. phagocytophilum* (NY18). For flow cytometry, cells were stained with FITC-goat anti-rabbit IgG to visualize MSP4, and the viable cell population was gated according to forward-scatter and side-scatter parameters. The SpAR92 and *A. phagocytophilum* were included as negative and positive MSP4 controls, respectively. The MFI geometric mean determined by flow cytometry was compared between SpAR92 and *A. phagocytophilum* or Franken*Sphingomonas*-MSP4 by Student's ttest with unequal variance ($*p < 0.001$, N = 5 biological replicates).

(C) *A. phagocytophilum msp2* DNA levels at 72 h after co-infection of ISE6 tick cells with *A. phagocytophilum* and SpAR92, FrankenSp-MSP4 or FrankenSp-HSP70. DNA levels were normalized to *rpS4* ($**p < 0.01$, N = 4 biological replicates).

(D) *A. phagocytophilum msp2* DNA levels at 72 h after co-infection of HL60 human cells with *A. phagocytophilum* and SpAR92, FrankenSp-MSP4 or FrankenSp-HSP70. DNA levels were normalized to β -actin ($*p < 0.05$, N = 4 biological replicates).

(E) Representative images of immunofluorescence analysis of MSP4 in ISE6 tick cells at 72 h after infection with *A. phagocytophilum* alone and in combination with SpAR92, FrankenSp-MSP4 or FrankenSp-HSP70. Cytospin preparations were incubated with MSP4 primary antibody (green, conjugated with FITC) and mounted on ProLong Diamond Antifade Mountant with DAPI reagent (blue). The localization of MSP4 protein around tick cells is illustrated for Franken*Sphingomonas*-MSP4 in a red square. Bars, 10 μ m.

human cells (Figures 5C and 5D). Franken*Sphingomonas*-MSP4 (FrankenSp-MSP4) co-infection with *A. phagocytophilum* in ISE6 tick cells reduced pathogen infection (Figure 5C). No differences in pathogen infection levels were found when ISE6 tick cells were co-cultivated with Franken*Sphingomonas*-HSP70 (FrankenSp-HSP70) and infected with *A. phagocytophilum* in comparison to its control (SpAR92) (Figure 5C). Similar results were obtained in human HL-60 cells, in which *A. phagocytophilum* interactions with FrankenSp-MSP4 but not FrankenSp-HSP70 significantly reduced pathogen infection levels (Figure 5D). Immunofluorescence analysis showed that MSP4 is located around tick cells reflecting *A. phagocytophilum*-FrankenSp-MSP4 interactions to interfere with pathogen infection, whereas FrankenSp-HSP70 did not affect intracellular infection with *A. phagocytophilum* (Figure 5E).

Franken *Sphingomonas* increases fitness and reduces *A. phagocytophilum* infection in *I. scapularis* ticks

The Franken *Sphingomonas* producing *A. phagocytophilum* MSP4 or HSP70 were then evaluated *in vivo* for their effect on tick fitness and *A. phagocytophilum* infection in *I. scapularis* (Figures 6A and 6B). Deer ticks normally lay between 2,000 and 3,000 eggs (http://bioweb.uwlax.edu/bio203/s2008/clarin_bria/Reproduction.htm). However, in ticks with both Franken*Sphingomonas*-MSP4 and Franken*Sphingomonas*-HSP70 oviposition was significantly higher ($p < 0.001$; Figure 6A), supporting a positive effect of Franken *Sphingomonas* on tick fitness.^{10,11,30} As with other tick endosymbionts,⁴² the results showed transovarial and trans-stadial transmission of Franken *Sphingomonas* (Figure 6A). Although as shown by artificial tick infection, *A. phagocytophilum* triggers a mechanism to control *Sphingomonas* levels to facilitate pathogen infection and protect tick cells (Figures 6A and 6B), Franken *Sphingomonas* and particularly Franken*Sphingomonas*-MSP4 significantly reduced *in vivo* tick pathogen infection (99.98% and 80.14% by Franken*Sphingomonas*-MSP4 and Franken*Sphingomonas*-HSP70, respectively; $p < 0.01$; Figure 6B). Other tick endosymbionts have also shown to affect pathogen infection and provide targets for RNA interference and paratransgenic control strategies^{43,44} but by mechanisms different from the pathogen-receptor competition elicited by Franken*Sphingomonas*-MSP4.

To provide additional support to results of the study, *I. scapularis* adult female ticks were *in vivo* capillary fed with blood collected from *A. phagocytophilum*-infected mice alone or in combination with SpAR92, FrankenSp-MSP4 or FrankenSp-HSP70 (Figure 6C). The results corroborated findings of *in vivo* naturally fed ticks (Figure 6B) by reduction in pathogen infection levels in ticks treated with FrankenSp-MSP4 (99.97% reduction; $p < 0.01$) or FrankenSp-HSP70 (93.23% reduction; $p < 0.01$) (Figure 6C). However, ticks treated with wild type *Sphingomonas* SpAR92 did not show a significant reduction in *A. phagocytophilum* levels (22.58% reduction; $p > 0.05$) with significant differences when compared to ticks treated with FrankenSp-MSP4 or FrankenSp-HSP70 ($p < 0.05$) (Figure 6C). As expected, *Sphingomonas* levels were similar in all groups (Figure 6C).

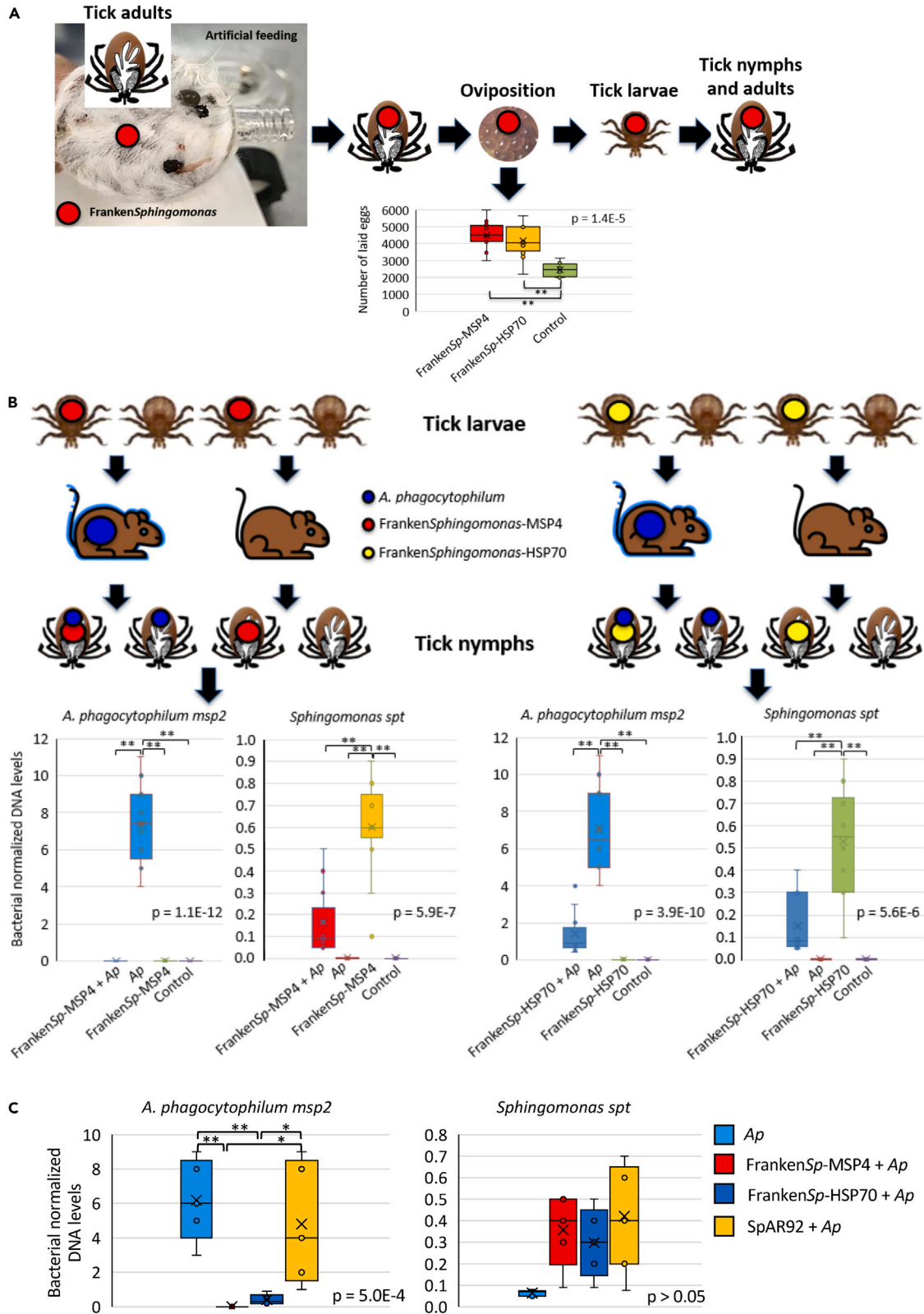


Figure 6. Infection of *I. scapularis* female ticks with Franken *Sphingomonas* and *A. phagocytophilum*

(A) Adult female and male ticks were artificially fed with Franken *Sphingomonas* (producing *A. phagocytophilum* MSP4 or HSP70) or control blood and incubated for oviposition and hatching of larvae. After oviposition, egg masses were counted under microscopy and DNA extracted from individual egg masses and to identify Franken *Sphingomonas* by qPCR targeting *spt* and *mSP4* or *hSP70*. Analyzed eggs (laid by 10 different adults for each gene and control) and 100% of the analyzed larvae (N = 30, 10 larvae from 3 different adults for each gene) from adult ticks only artificially fed with Franken *Sphingomonas* were positive for both *spt* and *mSP4* or *hSP70*. A group of nymphs were incubated for molting to adults and 100% of the analyzed female adults (N = 20) only derived from nymphs with Franken *Sphingomonas* were positive for both *spt* and *mSP4*.

(B) Larvae of *I. scapularis* ticks with and without Franken *Sphingomonas* were fed on uninfected or *A. phagocytophilum* (NY18)-infected C3H/HeN mice. Eight groups of 5 mice each were inoculated with *A. phagocytophilum*-infected (N = 20) or uninfected (N = 20) HL-60 cultured cells. Franken *Sphingomonas* were identified in tick larvae by qPCR targeting *spt* and *mSP4* genes. Mice were infested with 30 *I. scapularis* larvae per mouse. The engorged larvae were held in a humidity chamber for 34 days until molting into nymphs. DNA was extracted from 10 nymphs from each experimental group. Individual nymphs were analyzed by *mSP2* qPCR for infection with *A. phagocytophilum* and by *spt* qPCR for detection of Franken *Sphingomonas* and indigenous *Sphingomonas* spp. DNA levels were normalized against tick *16S rDNA* and *rpS4* genes. Normalized Ct-values were compared between groups by one-way ANOVA test with post-hoc Tukey HSD (https://astatsa.com/OneWay_Anova_with_TukeyHSD/); **p < 0.01; N = 10 biological replicates).

(C) Adult female *I. scapularis* ticks were *in vivo* capillary fed with blood collected from *A. phagocytophilum*-infected mice (N = 10) alone or in combination with SpAR92, FrankenSp-MSP4 or FrankenSp-HSP70. For each treatment, 5 ticks were collected and dissected at 72 h post-feeding and individually analyzed in combined internal organs for *A. phagocytophilum* (*mSP2* qPCR) and *Sphingomonas*/Franken *Sphingomonas* (*spt* qPCR) as for *in vivo* fed ticks. DNA levels were normalized against tick *16S rDNA* and *rpS4* genes.^{45,46} Normalized Ct-values were compared between groups by one-way ANOVA test with post-hoc Tukey HSD (https://astatsa.com/OneWay_Anova_with_TukeyHSD/); **p < 0.01, *p < 0.05; N = 5 biological replicates).

Conclusions

The conclusions of the study are summarized in Figure 7. *Sphingomonas* spp. levels in tick midgut microbiota are reduced by *A. phagocytophilum* whereas *Sphingomonas* affect cell viability and compete with *A. phagocytophilum* in *I. scapularis*. Glycolipids protect bacteria from antibacterial molecules and this stable structure makes these bacteria strong candidates for paratransgenesis. Midgut microbiota commensal *Sphingomonas* levels increase in response to *A. phagocytophilum* infection producing compounds causing metabolic damage to tick cells and inactive IAFGP-mediated inhibition of bacterial biofilm formation to affect pathogen infection. However, bacterial competition triggers mechanisms that results in a decrease in *Sphingomonas* levels to facilitate pathogen infection and protect tick cells. Accordingly, wild type and Franken *Sphingomonas* levels increase in response to *A. phagocytophilum* infection producing compounds that affect tick cells and pathogen infection.

Franken *Sphingomonas* producing *A. phagocytophilum* MSP4 or HSP70 mimic pathogen to add an additional component to compete for interactions with cellular biomolecules for infection of tick and human cells, thus reducing pathogen infection. These results will translate into reduced *A. phagocytophilum* pathogen infection and transmission. Based on Franken *Sphingomonas* transovarial and trans-stadial transmission, frankenbacteriosis could be applied to reduce risks associated with HGA. Tick larvae with genetically modified Franken *Sphingomonas*, and particularly Franken *Sphingomonas*-MSP4, could be produced in the laboratory and released in the field to compete and replace the wild-type populations with associated reduction in pathogen infection/transmission and disease risks. Future studies could evaluate the possibility that interactions between Franken *Sphingomonas*-MSP4 and host cell receptor may also activate immune-mediated protective mechanisms. This approach for genetic manipulation of bacteria to allow the production of a relevant protein involved in interactions between symbiotic/commensal bacteria and pathogens could be applied to the control of other infectious diseases.

Limitations of the study

In this study, we elaborated frankenbacteriosis, a paratransgenic approach to target tick-pathogen interactions. The findings demonstrate that genetically modified Franken *Sphingomonas*-MSP4 and Franken *Sphingomonas*-HSP70 could be used to compete with *A. phagocytophilum* interaction with cell receptor resulting in reduction of pathogen infection in both tick and human cells. Although the results were validated *in vivo* using the mouse model for *I. scapularis*-*A. phagocytophilum*, further experiments are required to confirm results such as stable colonization of ticks by Franken *Sphingomonas* and during completion of tick live cycle for multiple generations on different hosts. The results demonstrated that Franken *Sphingomonas* are more effective in controlling pathogen infection when compared to wildtype *Sphingomonas*. Nevertheless, regulatory requirements would have to be addressed when applying paratransgenesis under field conditions.

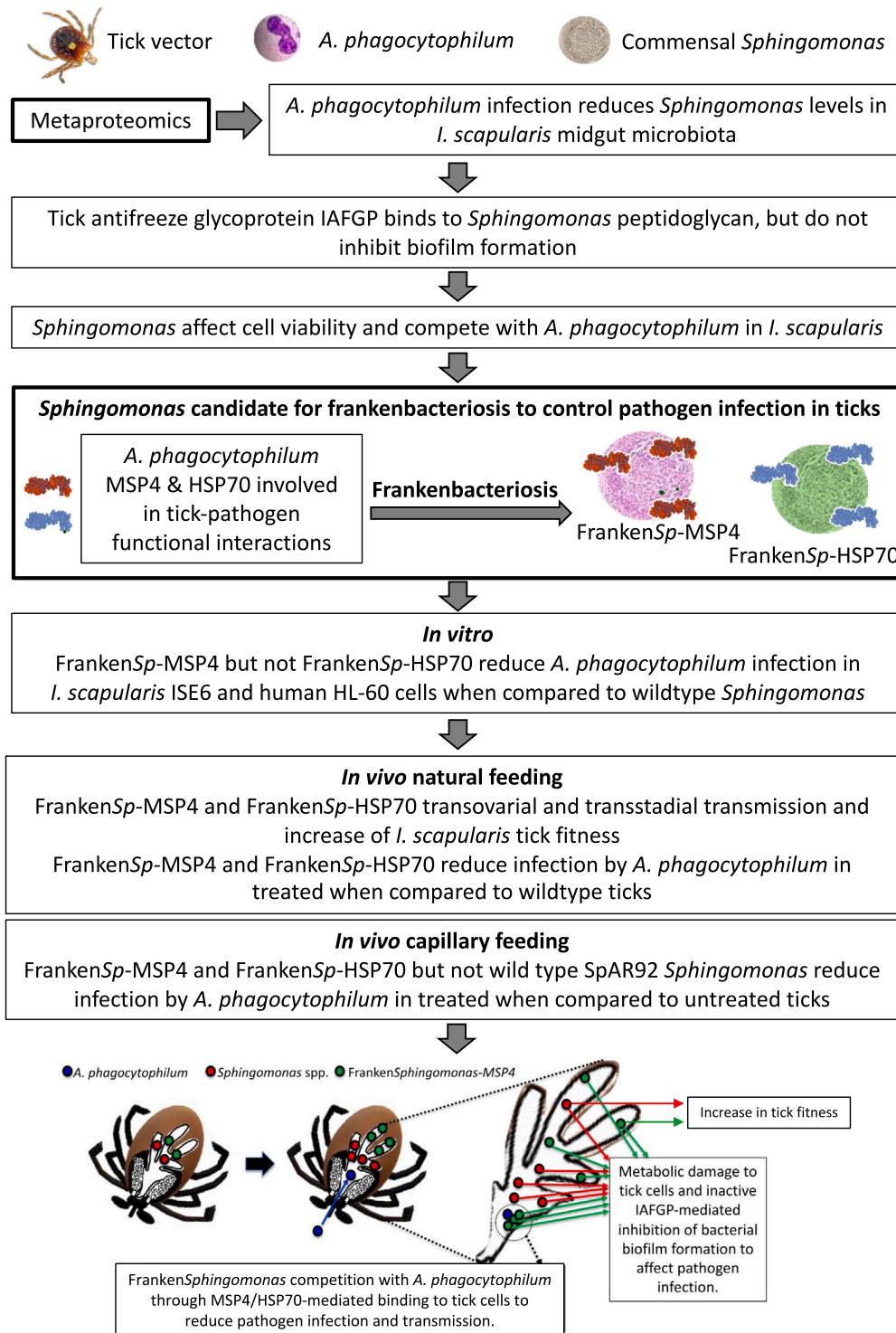


Figure 7. Results workflow and conclusions

Summary of the results and mechanisms affected by the interactions between *A. phagocytophilum* and commensal *Spingomonas* or Franken *Spingomonas* in tick midgut.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2023.106697>.

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AUTHOR CONTRIBUTIONS

J.F., P.A., S.D-S., L.M., A.C-C., and M.V. conceived the study and designed the experiments. L.M., P.A., A. H-J., M.V., L.S., A.G-G., and M.C. performed the experiments. L.M., P.A., A. H-J., M.V., A. C-C., and J.F. performed data analysis. G.N., S.M., S.I.B., and E.F provided samples and participated in the discussion of results. J.F., L.M., A. H-J., P.A., S. D-S., and A. C-C. wrote the manuscript. All authors approved and contributed to the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal IgG against <i>Sphingomonas</i>	This paper	N/A
Anti-rabbit IgG-horseradish peroxidase (HRP) conjugate	Sigma Aldrich	Cat# RABHRP1
Anti-rabbit IgG FITC conjugated antibody	Sigma Aldrich	Cat# AP106F
Biotin antibody	Thermo Fisher Scientific	Cat# 31852
Wheat Germ Agglutinin antibody	Vector Laboratories	Cat# AS-2024
Fluorescein isothiocyanate (FITC) goat anti-rabbit IgG	Abcam	Cat# ab6717
Goat anti-rabbit IgG FITC	Sigma Aldrich	Cat# F0382
Monoclonal anti-HSP70 primary antibody	Sigma Aldrich	Cat# H5147
Goat-anti mouse IgG FITC	Sigma Aldrich	Cat# F2012
Bacterial and virus strains		
<i>A.phagocytophilum</i> NY18	NY18 human isolate	N/A
<i>Sphingomonas</i> sp. AR92 (SpAR92)	Colección Española deCultivos Tipo (Paterna, Valencia, Spain)	CECT 7178
<i>Escherichia coli</i>	NCTC	NCTC 086
Chemicals, peptides, and recombinant proteins		
R2A medium	Himedia	Cat# M1687
TriReagent	Sigma Aldrich	Cat# 93289
12% SDS-polyacrylamide pre-cast gel	Bio-Rad	Cat# 4561043
Bovine serum albumin	Sigma Aldrich	Cat# A9418
TMB (3,3', 5,5'- tetramethylbenzidine)	Promega	Cat# W4121
Proteinase K	Dako	Cat# S3020
Proteinase K	Sigma Aldrich	Cat# 70663
ProLong Antifade reagent containing DAPI	Thermo Fisher Scientific	Cat# P36962
Sheep blood	BioMérieux	N/A
Streptomycin-Penicillin	Thermo Fisher Scientific	Cat# 15140122
4-hydroxy-3-methoxybenzoic acid	Sigma Aldrich	Cat# 659282
Critical commercial assays		
AllPrep DNA/RNA/Protein Mini Kit	Qiagen	Cat# 80004
Kapa One-Step with SYBR Green	Sigma Aldrich	Cat# KR0393_S-v2.17
Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit	Immunostep	Cat# ANXVKF7
Pierce BCA Protein Assay Kit;	Thermo Fisher Scientific	Cat# 23225
ProSep-A affinity chromatography media	Merck Millipore	Cat# C175805
Dynabeads MyOne Streptavidin T1	Invitrogen	Cat# 65602
DynaMagTM spin	Invitrogen	Cat# 12320D
Genomic DNA from Tissue kit	Maecherey-Nagel	Cat# 740952.50
TOPO Cloning kit	Invitrogen	Cat# 450071
Gateway™ LR Clonase™ II kit	Thermo Fisher Scientific	Cat# 11791020
SYBR Green Quantitative RT-qPCR Kit	Sigma-Aldrich	Cat# QR0100

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNeasy Mini Kit	Qiagen	Cat# 74004
Intracell Solution	Immunostep	Cat# INTRA-V500T
Experimental models: Cell lines		
HL-60 cell line	ATCC	CCL-240
ISE6 tick cell line	Provided by U. G. Munderlo (University of Minnesota)	N/A
Experimental models: Organisms/strains		
<i>I. Scapularis</i> ticks	Oklahoma State University Tick Rearing Facility	N/A
Mice C3H/HeN	Jackson Laboratories	N/A
Recombinant DNA		
Plasmid: pVHD vector	Addgene (from Julia Vorholt)	#61303 http://n2t.net/addgene:61303 ; RRID:Addgene_61303
Software and algorithms		
Uniprot	Uniprot	http://www.uniprot.org
SEQUEST algorithm	Thermo Fisher Scientific	Proteome Discoverer version 1.4.0.29
BLAST	NCBI	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Molecular Evolutionary Genetics Analysis (MEGA)	MEGA	https://www.megasoftware.net/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, José de la Fuente (jose_delafuente@yahoo.com).

Material availability

There are no restrictions to the availability of newly generated materials in this study.

Data and code availability

- All data reported in this paper will be shared by the [lead contact](#) upon request.
- All original code is available in this paper's supplemental information.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

I. scapularis ticks

Adult *I. scapularis* ticks were acquired from the laboratory colony maintained at the Oklahoma State University Tick Rearing Facility. Larvae and nymphs were fed on rabbits and adults were fed on sheep. Off-host ticks were maintained in a 12 h light: 12 h dark photoperiod at 22–25°C and 95% relative humidity. Adult female *I. scapularis* were infected with *A. phagocytophilum* NY18 isolate as previously described⁶ by feeding on a sheep inoculated intravenously with approximately 1×10^7 *A. phagocytophilum*-infected HL-60 cells (90–100% infected cells). In this model, over 85% of tick midguts become infected with *A. phagocytophilum*. Adult female ticks were removed from the sheep at 7 days post-infestation (dpi), held in a humidity chamber for 4 days and midgut dissected for DNA and protein extraction. Midgut from two pooled samples of 100 ticks each (for metaproteomics analysis) and 13 pools of 5 ticks each (for qPCR analysis) from uninfected and *A. phagocytophilum*-infected female ticks were obtained from the study previously reported by Ayllón et al.⁶ Partially fed *I. scapularis* nymphs were used for DNA extraction from whole internal tissues dissected from individual nymphs (N = 4) uninfected or

A. phagocytophilum (NY18)-infected after feeding on C3H/HeN mice. Larvae, nymphs and adult female and male uninfected ticks were prepared in a similar way but feeding on an uninfected host. Individual uninfected adult female ticks (N = 5–10) were treated with *A. phagocytophilum* Norway isolate⁴⁷ and/or *Sphingomonas* sp. reference strain AR92 (thereafter SpAR92) by injection, capillary or artificial feeding as described below. For infection of adult female ticks, sheep were housed, and experiments conducted with the approval and supervision of the Oklahoma State University Institutional Animal Care and Use Committee (Animal Care and Use Protocol, ACUP No. VM1026). The protocol (permit number: 16–017) used to feed nymphal ticks on mice was approved by the Old Dominion University Institutional Animal Care and Use Committee (Animal Welfare Assurance Number: A3172-01).

Sphingomonas sp. reference strain

Sphingomonas sp. reference strain SpAR92 (CECT 7178) was obtained from Colección Española de Cultivos Tipo (Paterna, Valencia, Spain; <https://www.uv.es/uvweb/spanish-type-culture-collection/en/cect/strains/culture-media-catalogue/strains-search-engine-1285892802374.html>). *Sphingomonas* SpAR92 strain was isolated in 2004 from drinking water in Seville, Spain. Bacteria were grown at 30°C in 250 mL of R2A medium (Sigma-Aldrich, St. Louis, MO, USA) containing 0.5 g/L each of yeast extract, proteose peptone, casein hydrolysate, glucose and soluble starch, 0.3 g/L each of sodium pyruvate and K₂HPO₄ and 0.05 g/L of magnesium sulfate.

METHOD DETAILS

Metaproteomics approach for the identification and quantitation of bacteria in the tick midgut microbiota

Proteins from the midgut of *A. phagocytophilum*-infected and uninfected *I. scapularis* female ticks were extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA, USA) and trypsin digested using the filter aided sample preparation (FASP) protocol as described previously.⁶ The resulting peptides were eluted by centrifugation with 50 mM ammonium bicarbonate (twice) and 0.5 M sodium chloride (once). Trifluoroacetic acid (TFA) was added to a final concentration of 1% and the peptides were finally desalted onto C18 Oasis-HLB cartridges and dried-down and stored at –20°C until mass spectrometry analysis. The desalted protein digests were resuspended in 0.1% formic acid and analyzed by reverse-phase (RP)-liquid chromatography (LC)-mass spectrometry (MS)/MS (RP-LC-MS/MS) using an Easy-nLC II system coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 × 20 mm C18 RP precolumn (Thermo Fisher Scientific), and then separated using a 0.075 × 250 mm C18 RP column (Thermo Fisher Scientific) operating at 0.3 mL/min. Peptides were eluted in a continuous acetonitrile gradient consisting of 0–30% B in 145 min, 30–43% A in 5 min and 43–90% B in 1 min (A = 0.5% formic acid; B = 90% acetonitrile, 0.5% formic acid). A flow rate of ca. 300 nL/min was used to elute peptides from the reverse phase nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on orbital ion trap mass spectrometers (both Orbitrap Elite and QExactive mass spectrometers, Thermo Fisher Scientific). Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by fifteen data dependent MS/MS scans (Top 15), using an isolation width of 2 mass-to-charge ratio units, normalized collision energy of 35%, and dynamic exclusion applied during 30 s periods. The MS/MS raw files were searched against a compiled database containing all sequences from Ixodida, Ruminantia and Alphaproteobacteria (3825786 Uniprot entries in February 2016) (<http://www.uniprot.org>) using the SEQUEST algorithm (Proteome Discoverer version 1.4.0.29, Thermo Fisher Scientific). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 10 ppm for precursor ions and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. A false discovery rate (FDR) < 0.01 was considered as condition for successful peptide assignments and at least two peptides per protein were the necessary condition for protein identification. A metaproteomics approach was applied for the identification of bacterial proteins as previously described by Hernández-Jarguín et al.²³ with an additional verification at peptide sequence level for the bacterial proteins identified by RP-LC-MS/MS. First, to verify the bacteria associated with each protein assignment, peptide sequences were searched against protein and translated nucleotide sequence databases using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)⁴⁸ in April 2017 and updated in February 2022. The peptides with hits matching to specific bacteria were confirmed and assigned to the corresponding species, genus or family. The remaining peptides matching to multiple families were assigned to unidentifiable bacteria. Bacterial assignments were grouped and the total number of peptide spectrum matches (PSM) for each classification

category were normalized against the total number of PSM to compare results between midgut from *A. phagocytophilum*-infected and uninfected ticks by Chi2-test ($p = 0.05$; $N = 2$ biological replicates) to identify significant differences in tick bacterial microbiota composition in response to *A. phagocytophilum* infection. Metaproteomics results were included in [Data S1](#). The analysis for the final assignments to bacterial species, genus or family was included in [Data S1](#) and [Tables S1–S3](#).

A metaproteomics approach was applied for the identification of bacterial proteins as previously described by Hernández-Jarguín et al.²³ with an additional verification at peptide sequence level for the bacterial proteins identified by RP-LC-MS/MS. First, to verify the bacteria associated with each protein assignment, peptide sequences were searched against protein and translated nucleotide sequence databases using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) in April 2017 and updated in February 2022. The peptides with hits matching to specific bacteria were confirmed and assigned to the corresponding species, genus or family. The remaining peptides matching to multiple families were assigned to unidentifiable bacteria. Bacterial assignments were grouped and the total number of PSM for each classification category were normalized against the total number of PSM to compare results between midgut from *A. phagocytophilum*-infected and uninfected ticks by Chi2-test ($p = 0.01$; $N = 2$ biological replicates) to identify significant differences in tick bacterial microbiota composition in response to *A. phagocytophilum* infection. Metaproteomics results were included in [Data S1](#). The analysis for the final assignments to bacterial species, genus or family were included in [Data S1](#).

Characterization of *A. phagocytophilum*, *Rickettsia* spp. and *Sphingomonas* spp. DNA levels by qPCR

Total DNA was extracted from uninfected and *A. phagocytophilum*-infected tick and ISE6 cell samples using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA, USA). DNA samples were used for qPCR with specific primers and annealing conditions (*Ixodidae* 16S rDNA⁴⁵ forward (F): 5'-GACAAGAAGACCCTA-3' and reverse (R): 5'-ATCCAACATCGAGGT-3', annealing 42°C 30 s, amplicon 456 bp; *Ixodes* *rsp4*⁴⁶ F: 5'-GGTGAAGAAGATTGTCAAGCAGAG-3' and R: 5'-TGAAGCCAGCAGGGTAGTTTG-3', annealing 54°C 30 s, amplicon 789 bp; *A. phagocytophilum* *msp2*⁴⁹ F: 5'-ATGGAAGGTAGTGTTGGTTATGGTATT-3' and R: 5'-TTGGTCTTGAAGCGCTCGTA-3', annealing 60°C 30 s, amplicon 77 bp; *A. phagocytophilum* *msp4* F: 5'-CACCATGAATTACAGAGAATTGCTTGTA-3' and R: 5'-CTAATTGAAAGCAAATCTTGCTCCTAT-3', annealing 52°C 60 s, amplicon 846 bp; *A. phagocytophilum* *hsp70* F: 5'-CACCATGGCGGCTGAGCGTA TAATAGGT-3' and R: 5'-CTAAGTATTTCTTCTTCCTCGGCCTT-3', annealing 52°C 60 s, amplicon 1935 bp; *Rickettsia* 16S rDNA⁵⁰ F-FD1: 5'-AGAGTTTGATCCTGGCTCAG-3' and R-Rc16S-452n: 5'-AACGT CATTATCTTCTTGC-3', annealing 54°C 30 s, amplicon 416 bp; *Sphingomonas* 16S rDNA F-ZF1: 5'-AGTT GACGCTGAGGTACGAA-3' and R-ZR1: 5'-GGCAACTAGAGGTGAGGGTT-3', annealing 58°C 45 s, amplicon 386 bp; *Sphingomonas* 16S rDNA F-ZF2: 5'-ACTAGCTGTCCGGGTACATG-3' and R-ZR2: 5'-CAG CACCTGTCTCTGATCCA-3', annealing 58°C 45 s, amplicon 233 bp; *Sphingomonas* *spt*⁵¹ F-694f: 5'-GATCGTCCGCTTCCGC-3' and R-983r: 5'-CCGACCGATTTGGAGAAG-3', annealing 55°C 60 s, amplicon 289 bp; Human HL-60 cells β -actin⁵² F: 5'-TGATATCGCCGCGCTCGTCGTC-3' and R: 5'-GCCGATCCA CACGGAGTACT-3', annealing 54°C 30 s, amplicon 1018 bp). The qPCR targeted *Rickettsia* and *Sphingomonas* spp. 16S rDNA, *Sphingomonas* spp. *serine palmitoyltransferase* (*spt*) or *A. phagocytophilum* *major surface protein 2* (*msp2*) genes. The qPCR was performed using the KAPA SYBR FAST (Merck, Rahway, NJ, USA) and the Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany) following manufacturer's recommendations. DNA levels were normalized against tick 16S rDNA and 40S ribosomal protein S4 (*rpS4*) genes using genNorm method (ddCT) as previously reported.^{45,46} Normalized Ct-values were compared between uninfected and infected tick samples by Student's t-test with unequal variance ($p = 0.05$; $N = 3$ –13 biological replicates).

Phylogenetic analysis of *Sphingomonas* spp.

Four 16S rDNA amplicons (Sp.UNI1, Sp.UNI2, Sp.INF1, Sp.INF2) from *Sphingomonas* spp. identified in the tick microbiota and the reference strain SpAR92 were sent for sequencing to the National Center for Oncological Research (CNIO, Madrid, Spain). The DNA sequences were searched for homology against the NCBI nucleotide sequence database using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).⁴⁸ The phylogenetic tree was constructed with 16S rDNA sequences of different *Sphingomonas* spp. available in the GenBank ([Figures S1](#) and [S2](#)). Sequences were aligned with MAFFT (v7) configured for the highest accuracy using the scoring matrix 200PAM/kD2, alignment strategy MAFFT-FFT-NS-I, gap-opening penalty 1.53 and offset value 0.123. Non-aligned regions were removed using Molecular Evolutionary Genetics Analysis

(MEGA) version 6 software.⁵³ The best-fit model of sequence evolution was selected based on Corrected Akaike Information Criterion (cAIC) and Bayesian Information Criterion (BIC) implemented in MEGA. A total of 139 positions were included in the final dataset. Neighbor joining (NJ) and Maximum likelihood (ML) methods, implemented in MEGA, were used to obtain the best tree topologies. Reliability of internal branches was assessed using the bootstrapping method with 1000 bootstrap replicates.⁵⁴

Cultured *I. scapularis* ISE6 tick cells and *A. phagocytophilum* inoculum

The ISE6 tick cell line, originally derived from *I. scapularis* embryos (provided by U. G. Munderloh, University of Minnesota), was cultured in L-15B300 medium as described previously.⁵⁵ To evaluate ISE6 cells growth, tick cells were detached from the bottom of the wells using a pipette, an aliquot was collected, and the cells counted using a Neubauer chamber under an inverted phase-contrast microscope (Nikon Instruments Europe B.V., Amsterdam, The Netherlands). The ISE6 cells were inoculated with *A. phagocytophilum* NY18 isolate propagated in HL-60 cells and maintained according to the procedures previously described by de la Fuente et al.⁵⁶ Infected cultures were sampled at 8 dpi (65–71% infected cells (Ave \pm SD, 68 \pm 3)). The percentage of cells infected with *A. phagocytophilum* was calculated by examining at least 200 cells using a 100x oil immersion objective. *A. phagocytophilum* inoculum was obtained by semi-purification. Briefly, infected cells were harvested by pipetting and centrifuged at 200 x g for 5 min at room temperature (RT). The cell pellet was resuspended in complete L-15B300 medium and using a syringe the cell suspension was mechanically disrupted five to ten times through a 26-gauge needle. After centrifugation at 1500 x g for 5 min, the supernatant was collected and used for inoculation of ISE6 cells.

Sphingomonas-A. phagocytophilum co-infection of *I. scapularis* ISE6 tick cells

The SpAR92 bacteria were grown in 250 mL of R2A medium (Sigma-Aldrich) at 30°C for 72 h until an optical density at 600 nm (O.D._{600 nm}) of 0.6 was obtained. For the inoculum, 25 mL culture were centrifuged at 5,000 rpm for 5 min and the bacterial pellet resuspended in phosphate-buffered saline (PBS). The ISE6 tick cells were cultured in L-15B300:R2A (1:1) medium and incubated at 31°C with 100 μ L/mL of SpAR92 suspension or culture medium alone in 24-well plates for 12 h prior to infection with 100 μ L of semi-purified *A. phagocytophilum* or culture medium alone and incubated for additional 84 h. Samples (3 wells per time point) were collected every 12 h after *A. phagocytophilum* infection. Total DNA was extracted from ISE6 cells using TriReagent (Sigma-Aldrich) following manufacturers recommendations. *A. phagocytophilum* DNA levels were determined by qPCR targeting *msp2* and normalized against tick *16S rDNA* and *40S ribosomal protein S4 (rpS4)* genes as described above (Table S2). SpAR92 cell growth was determined by O.D._{600 nm} and colony forming units counts per mL (CFU/mL) were counted in R2A agar plates at 10³ dilution. Normalized *A. phagocytophilum msp2* Ct-values and SpAR92 CFU/ml were compared between untreated and treated tick samples by two-way ANOVA test ($p = 0.05$; $N = 3$ biological replicates).

Viability assays in *I. scapularis* ISE6 tick cells

Approximately 5×10^5 ISE6 tick cells were collected after incubation with SpAR9 in L-15B300:R2A (1:1) culture medium for 12, 24, 36, 48 and 72 h. Cell viability (proportion of live/viable, necrotic, dead/late apoptotic and apoptotic cells) was measured by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Immuno-step, Salamanca, Spain) as previously described.⁴⁸ The percentage of apoptotic, dead, necrotic and live cells was compared between ISE6 and ISE6 + SpAR95 cells at each time point by Student's t-test with unequal variance ($p = 0.05$; $N = 3$ biological replicates). (B) The percent of apoptotic ISE6 cells was compared between groups by two-way ANOVA test ($p = 0.03$; $N = 3$ biological replicates). Selected samples were examined using a phase contrast microscope (Carl Zeiss, 10x objective) and Giemsa staining.

Characterization of *A. phagocytophilum* and SpAR92 bacterial growth

Bacteria SpAR92 were cultivated at 30°C in 250 mL of R2A medium (Sigma-Aldrich) or with uninfected and *A. phagocytophilum*-infected ISE6 cells cultured in L-15B300:R2A (1:1) medium. Every 12 h for SpAR92 and 24 h for *A. phagocytophilum* from 12 to 84 h of cultivation, O.D._{600 nm} and cell counts were determined to characterize bacterial growth. Medium samples containing bacteria were collected and centrifuged at 5,000 rpm, 4°C for 10 min. The supernatant was discarded, and the bacterial pellet resuspended in 1 mL PBS and 800 μ L of bacterial suspension was used to measure the O.D._{600 nm} in a spectrophotometer (Bio-Rad, Hercules, CA, USA). The remaining 200 μ L were used for bacterial cell or plate counts. From them, 100 μ L were directly seeded and 100 μ L were diluted 1:10 and then seeded in R2A agar plates incubated at 30°C for 96 h.

Rabbit polyclonal IgG antibodies against *sphingomonas*

Antibodies were produced against SpAR92 membrane proteins. Cultured bacteria were lysed in lysis buffer, sonicated and centrifuged at 11,000 x g for 5 min at 4°C to separate the membrane protein fraction as previously described.⁵⁶ The membrane fraction was washed in PBS and protein concentration determined using bicinchoninic acid (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific). Two New Zealand white rabbits (*Oryctolagus cuniculus*) were subcutaneously injected at weeks 0, 4, and 6 with 50 µg proteins in 0.4 mL Montanide ISA 50 V adjuvant (Seppic, Paris, France). Blood was collected before injection and 2 weeks after the last immunization to prepare pre-immune and immune sera, respectively. The IgG antibodies were purified from serum samples using the Montage antibody purification kit and spin columns with Pro-Sep-A affinity chromatography media (Millipore, Burlington, MA, USA) following manufacturer's recommendations. To improve the specificity of anti-*Sphingomonas* IgGs, antibodies were subjected to the following treatment. *Escherichia coli* 086 were cultivated for 14 hat 37°C, and then centrifuged at 9,000 rpm, 4°C for 10 min. The cell pellets were homogenized in a 1 mL syringe with 5 mL lysis buffer and protease inhibitors. Cell extracts were washed twice with PBS after centrifugation at 11,000 rpm, 4°C for 5 min. The pellet was resuspended in 2 mL of anti-*Sphingomonas* IgGs and incubated overnight at 4°C with shaking. Then, the suspension was centrifuged at 12,000 rpm, 4°C for 5 min. The supernatant was stored at 4°C for immediate use or at -20°C for long term storage. The reactivity of the IgG antibodies was characterized by Western blot and immunofluorescence. Total proteins were extracted with AllPrep DNA/RNA/Protein Mini Kit (Qiagen) from SpAR92, *Zymomonas mobilis* subsp. *mobilis* NRRL B-806, *Pseudomonas* sp., and *E. coli* 086 following manufacturer recommendations. Ten µg of total proteins from each bacterium were loaded onto a 12% SDS-polyacrylamide pre-cast gel (Bio-Rad) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich) for 2 hat RT and washed four times with TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween 20). Purified rabbit IgGs were used at a 1:200 dilution in TBS, and the membrane was incubated overnight at 4°C and then washed three times with TBS. The membrane was incubated with an anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) diluted 1:1,000 in TBS with 3% BSA. The membrane was washed five times with PBS and finally developed with TMB (3,3', 5,5'- tetramethylbenzidine) stabilized substrate for HRP (Promega, Madrid, Spain) according to manufacturer recommendations.

Immunofluorescence assay (IFA) in *I. scapularis* ISE6 and SpAR92 cells and adult female ticks

Adult *I. scapularis* females uninfected and infected with *A. phagocytophilum* (NY18) were fixed, embedded in paraffin, and sections (4 µm) prepared and mounted on glass slides as previously described.⁵⁷ The paraffin was removed from the tissues using xylene. Tissues were hydrated by successive 2 min washes with a graded series of 100, 75 and 50% ethanol, and then were permeabilized adding proteinase K (Dako, Barcelona, Spain) for 7 min. Tissues were washed with PBS and incubated with 3% BSA (Sigma-Aldrich) in PBS for 1 hat RT. Anti-*Sphingomonas* SpAR92 rabbit IgGs were used as primary antibodies diluted 1:100 in 3% BSA/PBS. Tissues were incubated with the primary antibodies for 14 hat 4°C. After the incubation, the tissues were washed three times in PBS and then incubated for 1 h with the secondary anti-rabbit IgG FITC conjugated antibody (Sigma-Aldrich) diluted 1:100 in 3% BSA/PBS. Tissues were washed twice with PBS and mounted in ProLong Antifade reagent (Thermo Fisher Scientific). The sections were examined using a Zeiss LSM 800 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) with oil immersion objectives. Sections of uninfected tick tissues were used as controls. Tick cells and bacteria were washed twice in PBS (4000 g, 5 min), resuspended in PBS and then incubated with 3% Bovine Serum Albumin (BSA, Sigma-Aldrich) in PBS for 1 hat RT. Anti-*Sphingomonas* SpAR92 rabbit IgGs were used as primary antibodies diluted 1:100 in 3% BSA/PBS. Cells were incubated with the primary antibodies for 14 hat 4°C. After the incubation, they were washed three times in PBS and then incubated for 1 h with the secondary anti-rabbit IgG FITC conjugated antibody (Sigma-Aldrich) diluted 1:100 in 3% BSA/PBS. Aliquots of stained samples were used for immunofluorescence assays after air-drying and mounting in ProLong Antifade reagent containing DAPI (Molecular Probes, Eugene, OR, USA). The sections were examined using a Zeiss LSM 800 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) with oil immersion objectives (x63).

Muropeptide binding assay

The experiment was conducted with SpAR92 and *Escherichia coli* (Gram-negative control strain NCTC 086). Extraction of peptidoglycan, muramidase digestion, muropeptide binding assay and static biofilm assay were conducted as described by Abraham et al.⁹ using Biotin-GST, Biotin-IAFGP, IAFGP and peptides Biotin-P1, Biotin-sP1, P1 and sP1 provided by E. Fikrig laboratory (Yale University School of Medicine,

New Haven, CT 208022, USA). For peptidoglycan extraction, 500 mL cultures of *E. coli* and SpAR92 were grown in LB or R2A media, respectively, until grow cultures reached an optical density for O.D. 600 nm (overnight at 37 °C for *E. coli* and 3 days at 30°C for SpAR92). Bacteria were harvested by centrifugation and resuspended in PBS. Peptidoglycan extraction were isolated following previously methods reported.^{9,58} Briefly, an equal volume of SDS 10% (w/v) was added to the samples, boiled for 3 h and left stirring overnight at RT. The following day, sacculi was repeatedly washed with 100 mM Tris-HCl pH 8 until fractions were free from SDS by ultracentrifugation (150,000 x g, 15 min, 25 °C). Samples were treated with 20 mg/mL of Proteinase K (Sigma-Aldrich) at 37 °C for 1 h to remove Braun's lipoprotein or other PG-associated proteins. The reaction was stopped with SDS 10% and heat-inactivated for 5 min. Peptidoglycan fraction was further washed by ultracentrifugation as described above. Finally, purified peptidoglycan was resuspended in 50 mM phosphate buffer and stored at 4 °C to avoid degradation with freeze/thaw process. Then, muropeptide binding assay was performed using magnetic, dynabeads MyOne Streptavidin T1 (Invitrogen, Waltham, MA, USA) at 10 mg/mL washed for 3 times with PBS pH 7.4 and separated using a magnetic DynaMag™ spin (Invitrogen). The solution was incubated with 1 mg/mL of biotinylated recombinant IAFGP-GST, GST proteins or 1 mg/mL of biotinylated peptide P2 (b-P2) or scramble peptide sP1 (b-sP1). One h after incubation at room temperature with gentle agitation, samples were separated, and supernatant (unbound biotinylated protein or peptide) was discarded. Magnetically bound fraction was washed and resuspended in PBS. Beads were blocked with 1 µg biotin for 30 min to avoid non-specific signals. 100 µL of peptidoglycan fractions isolated from bacteria was added to the magnetically bound-protein bead solution at 1:1 ratio. After incubation at RT for 2 h with gentle agitation, supernatant (unbound fraction) was collected. Beads were washed three times with PBS+0.1% BSA and resuspended. Finally, samples were eluted with Laemli sample buffer (5% β-mercaptoethanol) and heated for 5 min at 65 °C to dissociate the streptavidin beads from the biotinylated muropeptide or proteins. Supernatants (bound fraction) were collected. Bound and unbound fraction were plotted on a nitrocellulose membrane (Life Sciences, Sigma-Aldrich) and bio-dot microfiltration was used to detect different proteins following manufacturer instructions (Bio-dot microfiltration apparatus, Bio-Rad). Biotin was detected using monoclonal biotin antibody (ThermoFisher Scientific, Waltham, MA, USA); Endogenous peptidoglycan was detected using a polyclonal goat Wheat Germ Agglutinin antibody (Vector Laboratories, Burlingame, CA, USA).

Static biofilm assay

Static biofilm assays were performed as previously described.^{9,59} Planktonic cultures of SpAR92 and *E. coli*-086 were diluted in glucose-supplemented R2A broth medium (1% glucose; R2AG) or Luria-Bertani broth (1% glucose, LBG) respectively, alone or media supplemented with 0.1 mg/mL of GST (GST), recombinant GST-tagged IAFGP (IAFGP), sP1 control scramble peptide or peptide P2. 150 µL of each bacterial condition were distributed in replicates into 96-well plates (Thermo Fisher Scientific) and incubated for 24 h at 30 °C for *Sphingomonas* and 37 °C for *E. coli*. Bacterial growth in each well was confirmed at 0, 8, 21 and 24 h after treatment application by measurement of O.D. 600 nm using a Multiskan spectrophotometer (Thermo Fisher Scientific). Then, the supernatant with non-adherent bacteria was discarded and the wells were washed twice with water. Biofilm associated bacteria to the well surface were dried at room temperature and stained with crystal violet dye (0.5% in 20% methanol). Dye excess was removed, and the wells were washed twice again with water. Biofilm were imaged and dye was then dissolved in 33% acetic acid. Absorbance was quantified at 560 nm using a Multiskan spectrophotometer (Thermo Fisher Scientific).

Injection of *I. scapularis* adult female ticks with *A. phagocytophilum* and/or SpAR92

The ISE6 tick cells were inoculated with *A. phagocytophilum* Norway isolate⁴⁸ using the conditions described above. For this assay, 500 µL of ISE6 tick cells infected with *A. phagocytophilum* were sampled at 8 dpi (60–70% of infected cells). ISE6 cells were centrifuged at 2,500 rpm for 10 min and the supernatant discarded. The cell pellet was resuspended in 10 µL of L-15B300 medium and processed to obtain *A. phagocytophilum* inoculum as described above in "Cultured *I. scapularis* ISE6 tick cells and *A. phagocytophilum* inoculum". *Sphingomonas* inoculum was prepared from 10 mL of culture of SpAR92 with an O.D._{600 nm} of 0.3 corresponding to approximately 3-5x10⁹ cells/ml. The bacterial culture was centrifuged at 3,500 rpm for 10 min and the supernatant discarded. The bacterial pellet was washed twice with PBS, resuspended in 1 mL PBS and diluted to an O.D._{600 nm} of 0.03, which corresponds to approximately 3-5 x 10⁷ cells/ml in a final volume of 800 µL of PBS. *Ixodes* females were injected by glass capillary connected to the nanoliter injector head (Drummond) driven by Micro4 Micro-Syringe Pump Controller (World Precision Instruments, Berlin, Germany). Ticks were placed upside down on double-sided sticky tape and ventral abdomen cuticle was used for injection. Groups of 10 ticks each were injected with 250 nL PBS (control), or

250 nL of bacterial inoculum of *A. phagocytophilum* or SpAR92 alone and *A. phagocytophilum*-*Sphingomonas* combination. Ticks were separated by groups in 25 cm² cell culture sterile flasks with filter caps and stored for 72 h after injection in a 12 h light: 12 h dark photoperiod at 22–25°C in a desiccator with 95% relative humidity. Groups of 5 ticks were collected and dissected at 24 and 72 h post-injection.

Capillary feeding of *I. scapularis* adult female ticks with *A. phagocytophilum* and/or SpAR92

One mL of ISE6 tick cells infected with *A. phagocytophilum* (Norway isolate) was prepared as described above for injection but resuspending cell pellet in 300 µL of sheep blood acquired from BioMérieux (Lyon, France) for cell disruption and *A. phagocytophilum* collection. The inoculum of SpAR92 was prepared from 15 mL culture following the procedure described above but the bacterial pellet was resuspended in a final volume of 140 µL of blood containing *A. phagocytophilum*. Groups of 10 ticks each were fixed with double-sided adhesive tape and attached to a slide that was placed on a Petri dish rim. Microhematocrit capillary tubes (3.5 mm) filled with 20 µL of blood (untreated blood for the control group, blood containing *A. phagocytophilum* alone or combined with SpAR92) were placed on the tick mouthparts for feeding in a humidity chamber. The capillaries were replaced every 3 h during 15 h. After feeding, ticks were collected from the double-sided tape, separated by groups in sterile flasks with filter caps and stored for 72 h before being processed for analysis. Groups of 5 ticks were collected and dissected at 24 and 72 h post-feeding.

Artificial feeding of *I. scapularis* adult female ticks with *A. phagocytophilum* and/or SpAR92

Rabbit skins were obtained from animals previously used for rearing tick colonies in the laboratory. The pieces of animal skin were obtained by skinning slaughtered animals. The skin pieces were treated and used as described by Bonnet et al.¹¹ After 4 days of feeding, the experimental infection began. The 2.5 mL of ISE6 tick cells infected with *A. phagocytophilum* (Norway isolate) were prepared as described above for injection but resuspending cell pellet in 6 mL of sheep blood (BioMérieux, Lyon, France) previously treated with amphotericin B and heparin for cell disruption and *A. phagocytophilum* collection. The SpAR92 inoculum was prepared from 25 mL bacterial culture centrifuged at 3,500 rpm for 10 min. The supernatant was discarded, and the pellet was washed twice with PBS and resuspended in 3 mL blood containing *A. phagocytophilum*. For feeding of groups of 5 ticks each, 3 mL of untreated and treated blood was placed inside a glass feeder and kept for 3 days with two blood changes per day. The skins were washed with sterilized PBS supplemented with 100 units/ml penicillin and 100 µg/mL streptomycin (Life Technologies Corporation, Carlsbad, CA) before each blood change. After feeding for 72 h, ticks were detached from the skin and processed for analysis.

Analysis of bacterial DNA levels in *I. scapularis* ticks subjected to injection, capillary, or artificial feeding

Before dissection, ticks were washed in PBS, then in 70% ethanol and again in PBS. For dissection, a scalpel was used per group and between individuals the scalpel was submerged in 70% ethanol. Ticks were dissected and the salivary glands and midgut extracted. Tick tissues were stored individually and frozen at –20°C for the subsequent extraction of DNA using a Genomic DNA from Tissue kit (NucleoSpin Tissue; Macherey-Nagel GmbH, Düren, Germany) following manufacturer recommendations. *A. phagocytophilum* and SpAR92 DNA levels were determined by qPCR targeting *msh2* and *spt*, respectively as described above (Table S2). DNA levels were normalized against tick *16S rDNA* and *rpS4* genes.^{45,46} Normalized Ct-values were compared between groups by Student's t-test with unequal variance ($p = 0.05$; $N = 5$ biological replicates).

FrankenSphingomonas: Genetic modification of SpAR92

The *hsp70* and *msh4* genes were amplified by PCR. Resulting PCR products were cloned using a pENTR Directional TOPO Cloning kit (Thermo Fisher Scientific) following manufacturers protocols. An expression construct was generated by performing an LR recombination reaction (Thermo Fisher Scientific) between the entry clone and the Gateway destination vector pVHD, a Vanillate-inducible gene expression plasmid for *Sphingomonas* (pVHD was a gift from Julia Vorholt; Addgene plasmid # 61303; <http://n2t.net/addgene:61303>; RRID:Addgene_61303). Competent *E. coli* cells were transformed, and the appropriate antibiotic-resistant expression clones were selected. The expression construct was introduced into *Sphingomonas* SpAR92 by electroporation as previously described.⁶⁰ Briefly, cells from 50 mL cultures of *Sphingomonas* (optical density at 600 nm = 0.7 to 0.8) were collected by centrifugation and washed twice with 10 mL of chilled 10% glycerol. The cells were resuspended in the same buffer to a final volume of 100 µL, mixed with plasmid DNA (1 µg), and put into 0.2-cm cuvettes. Electroporation was performed using

a Gene Pulser (Bio-Rad, Hercules, CA, USA) with a single pulse at 25 μ F and 2.5 kV. The cells were allowed to grow in R2A medium for 2 h and then were spread on selective plates containing chloramphenicol for selection. Franken*Sphingomonas* carrying pVHD-*msp4* or pVHD-*hsp70* were grown to mid-exponential phase and gene expression was induced by the addition of 250 μ M Vanillate (4-hydroxy-3-methoxybenzoic acid; Sigma-Aldrich).

Characterization of Franken*Sphingomonas*

Gene expression by RT-qPCR

The expression of *msp4* and *hsp70* genes in Franken*Sphingomonas* were characterized by RT-PCR using the SYBR Green Quantitative RT-qPCR Kit (Sigma-Aldrich). Total RNA was extracted using the RNeasy Mini Kit (Qiagen Ltd., Crawley, UK). Delta normalized reporter values (Δ Rn) at 30th quantitation cycle (Cq) was compared between Franken*Sphingomonas* and control SpAR92 by Student's t-test with unequal variance and results represented as Δ Rn Franken*Sphingomonas* to SpAR92 ratio ($p = 0.05$; $N = 10$ biological replicates).

Protein production by flow cytometry

The flow cytometry analysis of bacterial MSP4 content was conducted as previously described for alpha-Gal.⁶¹ Franken*Sphingomonas*-MSP4, SpAR92 and *A. phagocytophilum* (NY18 from HL60 cells) were incubated for 14 h at 4°C with anti-*A. phagocytophilum* MSP4 rabbit polyclonal antibodies⁶² diluted 1:50 in 3% human serum albumin (HSA)/PBS. Fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (Abcam, Cambridge, UK)-labeled antibody diluted 1:200 in 3% HSA/PBS was used as a secondary antibody and incubated for 1 h at RT. The SpAR92 and *A. phagocytophilum* were included as negative and positive MSP4 controls, respectively. Samples were analyzed on a FACScalibur flow cytometer equipped with CellQuest Pro software (BD BioSciences, Madrid, Spain). The viable cell population was gated according to forward-scatter and side-scatter parameters. The mean fluorescence intensity (MFI) was determined by flow cytometry, and the geometric mean compared between SpAR92 and *A. phagocytophilum* or Franken*Sphingomonas*-MSP4 by Student's ttest with unequal variance ($p = 0.05$, $N = 5$ biological replicates). Additionally, the production of MSP4 and HSP70 membrane proteins in Franken*Sphingomonas* was confirmed by flow cytometry. Cells from 50 mL cultures of Franken*Sphingomonas* in which gene expression was induced by the addition of 250 μ M Vanillate (4-hydroxy-3-methoxybenzoic acid; Sigma-Aldrich) were grown until an optimum optical density was obtained (O.D. 600 nm = 0.6–0.7). A volume of 1 mL was used for sample replicates. In parallel, cells were permeabilized for an internal protein study control. Bacterial permeabilization was performed using Intracell Solution (Immuno-Step, Salamanca, Spain) following manufacturer staining protocol. Cultures were washed twice with PBS plus 5% FBS followed by centrifugation at 7000 \times g. For MSP4 protein analysis, rabbit anti-MSP4 primary antibodies were used at 1:50 dilution (0.4 mg/mL). Bacterial culture was incubated for 2 h at RT. Then, cells were washed as described above and incubated with secondary goat anti-rabbit IgG FITC (F0382; Sigma-Aldrich) at 1:100 dilution for 1 h at RT. HSP70 protein analysis was performed as described for MSP4 but using a monoclonal anti-HSP70 primary antibody (H5147; Sigma-Aldrich) was used at 1:100 dilution and incubated with a secondary goat-anti mouse IgG FITC (F2012; Sigma-Aldrich) at 1:100 dilution. All samples were analyzed on a FAC-Scalibur flow cytometer equipped with CellQuest ProSoftware (BD Bio-Sciences, Madrid, Spain).

Protein localization by confocal microscopy

Franken*Sphingomonas* 50 mL cultures in which gene expression was induced by addition of 250 μ M Vanillate (4-hydroxy-3-methoxybenzoic acid, Sigma-Aldrich) were grown until an optimum optical density was obtained (O.D. 600 nm = 0.6–0.7). Then, bacteria culture was centrifuged at 5,000 rpm for 5 min and the bacterial pellet resuspended in PBS. Cytospin preparation was performed and cells were fixed using 4% paraformaldehyde for 10 min at RT. After washing 3 times, samples were permeabilized with 0.15% Triton X-100 (Sigma-Aldrich) and washed again. Slides were blocked with 2% BSA (Sigma-Aldrich) in PBS-Tween 20 during 1 h at RT. The slides were then incubated overnight at 4°C with antibodies and protocol used for flow cytometry. After two washes with PBS the slides were mounted on ProLong Diamond Antifade Mountant with DAPI reagent (Thermo Scientific, Madrid, Spain). The sections were examined using a Zeiss LSM 800 with Airyscan (Carl Zeiss, Oberkochen, Germany).

Franken*Sphingomonas*-*A. phagocytophilum* co-infection of *I. scapularis* ISE6 tick cells

Co-infections were performed as previously described but using Franken*Sphingomonas* of transformed SpAR92 with *msp4* or *hsp70* and wild type SpAR92 as a control. Bacterial 25 mL culture were centrifuged

at 5,000 rpm for 5 min and the bacterial pellet resuspended in PBS. The ISE6 tick cells were cultured in L-15B300:R2A (1:1) medium and incubated at 31°C with 100 µL/mL of SpAR92 (transformed and wild-type) suspension or culture medium alone in 24-well plates for 12 h prior to infection with 100 µL of semi-purified *A. phagocytophilum* or culture medium alone and incubated for additional 72 h (3 wells per treatment). Total DNA was extracted from ISE6 cells using TriReagent (Sigma-Aldrich) following manufacturers recommendations. *A. phagocytophilum* DNA levels were determined by qPCR targeting *msp2* and normalized against tick *40S ribosomal protein S4 (rpS4)* gene.^{45,46} Normalized *A. phagocytophilum msp2* Ct-values were compared between tick cell samples by Student's t-test with unequal variance ($p = 0.05$; $N = 3$ biological replicates). The confocal microscopy analysis of *A. phagocytophilum* and MSP4 proteins in ISE6 tick cells infected with *A. phagocytophilum* or co-infected with *FrankenSphingomonas* and *A. phagocytophilum* was performed as described above for protein localization in *FrankenSphingomonas*. Briefly, cytospin preparations were fixed using 4% paraformaldehyde, permeabilized with Triton X-100 and blocked with 1% BSA-PBST-20. The slides were incubated overnight at 4 °C with rabbit anti-MSP4 primary antibodies at 1:100 dilution in 1% BSA-PBST. On the following day, samples were incubated for 1 h at RT in the dark with secondary goat anti-rabbit IgG (conjugated with FITC) at 1:50 dilution. Finally, after 3 washes in PBS of 5 min each, the slides were mounted on ProLong Diamond Antifade Mountant with DAPI reagent. Slides were examined using a Zeiss LSM 800 with Airyscan (Carl Zeiss, Oberkochen, Germany).

FrankenSphingomonas-A. phagocytophilum co-infection of human HL-60 cells

Co-infections in human HL-60 cells were performed as described above for tick ISE6 cells using *FrankenSphingomonas* of transformed SpAR92 with *msp4* or *hsp70* and wild type SpAR92 as a control. Briefly, 25 mL culture were centrifuged at 5,000 rpm for 5 min and the bacterial pellet resuspended in PBS. The HL-60 cells were cultured in RPMI:R2A (1:1) medium and incubated at 37°C with 100 µL/mL of *FrankenSphingomonas* or SpAR92 suspension or culture medium alone in 24-well plates for 12 h prior to infection with 100 µL of semi-purified *A. phagocytophilum* or culture medium alone and incubated for additional 72 h (4 wells per treatment). Total DNA was extracted from HL-60 cells using TriReagent (Sigma-Aldrich) following manufacturer recommendations. Levels of *A. phagocytophilum* DNA were determined by qPCR targeting *msp2* and normalized against β -actin gene. Normalized *A. phagocytophilum msp2* Ct-values were compared between human cell samples by Student's t-test with unequal variance ($p = 0.05$; $N = 4$ biological replicates).

FrankenSphingomonas-A. phagocytophilum co-infection of I. scapularis ticks

Larvae of *I. scapularis* ticks with and without *FrankenSphingomonas* were fed on uninfected or *A. phagocytophilum* (NY18)-infected C3H/HeN mice (Blas-Machado et al., 2007). Eight groups of 5 mice each were inoculated with *A. phagocytophilum*-infected ($N = 20$) or uninfected ($N = 20$) HL-60 cultured cells. Cultures collected on the day of inoculation were centrifuged and resuspended in serum-free RPMI 1640 medium to the concentration of 2×10^6 cells/ml. Mice were injected intraperitoneally with 0.5 mL (1×10^6 cells) of the infected or uninfected cell cultures as previously described.⁶³ Infection of mice was confirmed by *msp2* qPCR as described above, isolation and propagation in HL-60 cell cultures and by light microscopy (LM) identification of morulae in neutrophils from stained blood smears.⁶⁴ Adult female and male ticks were artificially fed with *FrankenSphingomonas* (producing *A. phagocytophilum* MSP4 or HSP70) or control blood as described above and incubated for oviposition and hatching of larvae.⁶⁵ Live ticks were held in a desiccator at 21 °C and 90–95% humidity until egg masses were produced. After oviposition, egg masses were counted under microscopy and DNA extracted from individual egg masses and larvae using a Genomic DNA from Tissue kit (NucleoSpin Tissue; Macherey-Nagel GmbH) following manufacturer recommendations.⁶⁶ *FrankenSphingomonas* were identified in tick eggs and larvae by qPCR targeting *spt* and *msp4* or *hsp70*. Analyzed eggs (laid by 10 different adults for each gene and control) and 100% of the analyzed larvae ($N = 30$, 10 larvae from 3 different adults for each gene) from adult ticks only artificially fed with *FrankenSphingomonas* were positive for both *spt* and *msp4* or *hsp70*. Mice were infested with 30 *I. scapularis* larvae per mouse 5 days after being inoculated with infected or uninfected cell cultures as described previously.^{63,65} Unattached larvae were removed 12 h after infestation and engorged larvae were collected for 7 days from each mouse. The engorged larvae were held in a humidity chamber for 34 days until molting into nymphs. DNA was extracted from 10 nymphs from each experimental group. Individual nymphs were analyzed by *msp2* qPCR for infection with *A. phagocytophilum* and by *spt* qPCR for detection of *FrankenSphingomonas*. DNA levels were normalized against tick *16S rDNA* and *rpS4* genes.^{45,46} Normalized Ct-values were compared between groups by one-way ANOVA test with post-hoc Tukey HSD (https://astatsa.com/OneWay_Anova_with_TukeyHSD/;

$p = 0.05$; $N = 10$ biological replicates). A group of nymphs were incubated for molting to adults and 100% of the analyzed female adults ($N = 20$) only derived from nymphs with Franken*Sphingomonas* were positive for both *spt* and *mSP4*.

Using the procedure described above for capillary feeding of *I. scapularis* with *A. phagocytophilum* and/or SpAR92, adult female ticks were fed with blood collected from *A. phagocytophilum*-infected mice ($N = 10$) alone or in combination with SpAR92, FrankenSp-MSP4 or FrankenSp-HSP70 ($3-4 \times 10^7$ *Sphingomonas* per tick). For each treatment, 5 ticks were collected and dissected at 72 h post-feeding and individually analyzed in combined internal organs for *A. phagocytophilum* (*mSP2* qPCR) and *Sphingomonas*/Franken-*Sphingomonas* (*spt* qPCR) as for *in vivo* fed ticks. DNA levels were normalized against tick *16S rDNA* and *rpS4* genes.^{45,46} Normalized Ct-values were compared between groups by one-way ANOVA test with post-hoc Tukey HSD (https://astatsa.com/OneWay_Anova_with_TukeyHSD/; $p = 0.05$; $N = 5$ biological replicates).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details of the experiments can be found in the figure legends and corresponding sections of [method details](#).