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Engineered gut symbiont inhibits microsporidian parasite and improves honey bee survival

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Honey bees (*Apis mellifera*) are critical agricultural pollinators as well as model organisms for research on development, behavior, memory, and learning. The parasite *Nosema ceranae*, a common cause of honey bee colony collapse, has developed resistance to small-molecule therapeutics. An alternative long-term strategy to combat *Nosema* infection is therefore urgently needed, with synthetic biology offering a potential solution. Honey bees harbor specialized bacterial gut symbionts that are transmitted within hives. Previously, these have been engineered to inhibit ectoparasitic mites by expressing double-stranded RNA (dsRNA) targeting essential mite genes, via activation of the mite RNA interference (RNAi) pathway. In this study, we engineered a honey bee gut symbiont to express dsRNA targeting essential genes of *N. ceranae* via the parasite's own RNAi machinery. The engineered symbiont sharply reduced *Nosema* proliferation and improved bee survival following the parasite challenge. This protection was observed in both newly emerged and older forager bees. Furthermore, engineered symbionts were transmitted among cohoused bees, suggesting that introducing engineered symbionts to hives could result in colony-level protection.

honey bee | symbiont | parasite control | microsporidian | *Snodgrassella alvi*

Significance

A major cause of colony failure in honey bees is *Nosema*, a parasitic relative of fungi that causes winter mortality. A strain of a specialized honey bee gut symbiont was genetically engineered to activate the RNA interference system of *Nosema* to specifically suppress parasite genes essential to the infection. The engineered symbionts colonized bee guts, inhibited *Nosema* proliferation, and increased the survivorship of bees challenged with parasite infection. Engineered symbionts were successfully established in bees of different ages, including those with established gut microbiota, and were transmitted from colonized bees to previously uncolonized neighboring bees. Genetically engineered gut symbionts therefore have the potential to provide long-term colony-level protection against one or several parasite species.

Pollination is a central ecosystem service, substantially increasing the proportion of flowers developing into fruit and seeds (1, 2). The honey bee is the most abundant commercial pollinator and is essential for the food supply and the agricultural economy (3, 4). Honey bee colony losses have provoked severe concern for food security (5, 6). During warm months, old or weak honey bees leave the hive, thereby limiting transmission of pathogens to healthy younger honey bees and maintaining the colony's strength (7). However, in winter, the queen stops laying eggs, and resident bees cluster together to keep warm in the hive. This behavior enables pathogens to proliferate, often resulting in the collapse of colonies. Winter losses account for most colony failures (8). However, winter also provides an ideal timeframe to eliminate the pathogens without interfering with larvae and pupae, provided an effective treatment is available.

The primary drivers of colony failure are the ectoparasitic mite *Varroa destructor* and the intracellular parasite *Nosema ceranae* (9, 10). *N. ceranae* is a single-cell microsporidian parasite infecting honey bee midgut epithelial cells. Infections start from ingesting spore-contaminated food (11). The spores germinate and extrude polar tubes, transporting the sporoplasm into gut epithelial cells to initiate proliferation (12). Proliferation lasts approximately 4 d, when infected cells burst, releasing progeny spores into the gut lumen (13). *N. ceranae* infection suppresses the honey bee's immune response, impairs flight navigation, and shortens life span (14–17).

N. ceranae control is challenging because it has developed resistance to fumagillin, the only registered antimicrobial to treat nosemosis (18). Synthetic biology offers a potential alternative solution through RNA interference (RNAi). Accordingly, *N. ceranae*'s native RNAi machinery can be hijacked to prevent the expression of virulence genes. Previous experiments using RNAi to knock down the expression of targeted genes have shown that genes involved in ATP transport, microRNA maturation, and attachment to the host cells are essential to the success of the infection (19–21). Polar tube proteins (PTPs), for example, are essential for transporting the sporoplasm into host cells, and *N. ceranae* has five *PTP* genes (*PTP1* ~ *PTP5*) (22, 23). Another protein essential for successful microsporidian infection is sporoplasm surface protein (SSP), which attracts the host mitochondria needed to provide a supply of ATP during parasite proliferation. Disrupting SSP inhibits the production of progeny spores in another microsporidian, *Encephalitozoon hellem* (24).

Honey bees maintain core gut symbionts colonizing distinct niches within the bee hindgut (25, 26). Recently, an engineered strain of the bee symbiont *Snodgrassella alvi*, named *S. alvi* wkB2:pDS-VAR, was used to deliver double-stranded RNA (dsRNA) that targeted the mite *V. destructor* through the mite's own RNAi pathway, resulting in mite death (27). In this study, we engineered *S. alvi* to produce *S. alvi* wkB2:pDS-*Nosema*,

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Competing interest statement: Moran is one of 3 inventors on US Patent 11382989, which includes the use of engineered bee gut bacteria as a means of controlling bee pathogens or parasites.

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expressing dsRNA targeting the essential genes (*PTP1* and *SSP*) of the parasite *N. ceranae*. This engineered gut symbiont effectively protected both newly emerged bees and older (forager) bees and substantially improved bee survival. Furthermore, the engineered gut symbiont dispersed among cohoused bees. Together, these results suggest a promising strategy for combating honey bee parasites and thereby improving the winter survival of hives.

Results

Engineered Symbionts Reduced Parasite Proliferation and Improved Survival in Newly Emerged Honey Bees. We first evaluated the ability of the engineered gut symbiont *S. alvi* wkB2:pBTK570 to colonize and persist in the honey bee gut. Inoculation with approximately 1×10^6 cells per microliter of sucrose solution allowed stable colonization of the engineered symbionts in newly emerged honey bees. Antibiotic selection substantially boosted symbiont colonization, increasing the numbers of colony-forming units (CFUs) in plated gut homogenate on selective media (*SI Appendix*, Fig. S1).

We then engineered *S. alvi* wkB2 to express dsRNA designed to knock down the expression of two *N. ceranae* genes (*SSP* and *PTP1*) encoding proteins essential for host infection (*SI Appendix*, Figs. S2 and S3). Another engineered strain, *S. alvi* wkB2:pDS-GFP, expressed dsRNA with no homology to *Nosema* or bee genes and was used as a control for nonspecific effects of dsRNA on *N. ceranae* infection (Table 1). We carried out three experiments, using bees from different hives, to test whether the engineered strains have an effect on the ability of *N. ceranae* infection (Fig. 1).

In the first experiment, we inoculated newly emerged bees with the engineered strain, *S. alvi* wkB2:pDS-*Nosema*, and with *S. alvi* wkB2:pDS-GFP (Fig. 1*A*). The third group of bees received sucrose solution only. The sucrose group determined any inhibitory effect of the *S. alvi* wkB2:pDS-*Nosema* on the infection. *S. alvi* wkB2:pDS-GFP further determined that the inhibitory effect originated from the random or designated dsRNA. Five days later, we inoculated with the parasite *N. ceranae*. Survival was not significantly different between wkB2:pDS-*Nosema* and wkB2:pDS-GFP groups (log-rank test, adjusted $P > 0.05$). However, these two groups showed significantly higher survival than that of the honey bees in the sucrose group (log-rank test, adjusted $P < 0.001$, Fig. 2*A*).

Colonization with the engineered symbiont wkB2:pDS-*Nosema* effectively suppressed parasite proliferation as measured by spore counts. Bees in the wkB2:pDS-*Nosema* group showed the least spores, followed by those in the wkB2:pDS-GFP group. Bees without symbionts showed the most spores (Kruskal–Wallis test, adjusted $P < 0.001$, Fig. 2*B*).

We validated colonization by engineered symbionts by quantifying CFUs on selective media. The engineered symbiont *S. alvi* wkB2:pDS-*Nosema* and *S. alvi* wkB2:pDS-GFP colonized equally well, with an inoculation rate of 97.5% (Wilcoxon test, $P = 0.24$, Fig. 2*C*).

In the second experiment, we also tested for a protective effect of wild-type *S. alvi* on bees, by including a fourth treatment group, nonengineered *S. alvi* wkB2 (Fig. 1*B*). In this experiment, bees in the *S. alvi* wkB2:pDS-*Nosema* group achieved the highest survival, and the sucrose group showed the lowest survival. The bees in the sucrose group showed lower survival than that of bees in the wkB2:pDS-*Nosema* (log-rank test, adjusted $P < 0.001$), wkB2:pDS-GFP (log-rank test, adjusted $P < 0.001$), and wkB2 (log-rank test, adjusted $P < 0.001$) groups (Fig. 3*A*). No significant difference in survival was found among the wkB2:pDS-*Nosema*, wkB2:pDS-GFP, and wkB2 groups. Additionally, spore load differed among the four groups (Kruskal–Wallis test, adjusted $P < 0.01$, Fig. 3*B*). Bees in the *S. alvi* wkB2:pDS-*Nosema* group showed fewer spores than those in the *S. alvi* wkB2:pDS-GFP (Wilcoxon rank-sum test, adjusted $P < 0.001$), *S. alvi* wkB2 (Wilcoxon rank-sum test, adjusted $P < 0.001$), and the sugar groups (Wilcoxon rank-sum test, adjusted $P < 0.001$). Spore loads were not significantly different between the *S. alvi* wkB2:pDS-GFP and *S. alvi* wkB2 groups or between *S. alvi* wkB2 and sugar groups. However, bees in the wkB2:pDS-GFP group showed significantly fewer spores than those in the sugar group (Wilcoxon rank-sum test, adjusted $P < 0.05$).

Engineered Symbionts Protect Forager Honey Bees and Are Transmitted to Cohoused Bees. Once established in individual workers, engineered symbionts may colonize and protect older bees, potentially conferring colony-level protection. First, we tested whether engineered symbionts could colonize older bees that already have an established gut microbiota. Following the inoculation of foragers with the engineered symbionts, the proportion of colonization was 50% on day 4 and 62.5% on day 8. However, antibiotic selection increased the colonization rate to 100% and 87.5% on day 4 and day 8 (*SI Appendix*, Table S3). There was a trend of higher CFU levels with antibiotic selection, but the difference was not statistically significant (*SI Appendix*, Fig. S4).

We also inoculated older honey bees, already possessing an established microbiome, with the engineered *S. alvi* wkB2:pDS-*Nosema* or with *S. alvi* wkB2:pDS-GFP, followed by exposure to *N. ceranae* spores. *S. alvi* wkB2:pDS-*Nosema* treatment substantially suppressed the spore load compared with the *S. alvi* wkB2:pDS-GFP group or the sucrose group, and the foragers without symbionts showed the highest spore loads (Kruskal–Wallis test, adjusted $P < 0.05$, Fig. 4*A*).

To explore whether the engineered symbiont might be able to spread within a bee colony, we tested whether transmission could occur among bees cohoused in the same rearing cups. When bees inoculated with the engineered symbiont and bees lacking symbionts were together, the engineered symbionts were transmitted to the uninoculated bees. After 3 d of cohousing, the symbionts had colonized 60% of previously uninoculated bees, and similar CFU values were observed for inoculated honey bees and those colonized through bee-to-bee transfer (Wilcoxon test, $P = 0.204$, Fig. 4*B*).

Table 1. Three plasmids introduced into honey bee symbiont *Snodgrassella alvi*

Symbiont	Plasmid	Function	Target gene	Accession ID	Reference
S. alvi wkB2	pBTK570	Resistant to spectinomycin	NA	110615	(28)
S. alvi wkB2	pDS-GFP	Resistant to spectinomycin, and expressing dsRNA	GFP	183129	(27)
S. alvi wkB2	pDS-*Nosema*	Resistant to spectinomycin, and expressing dsRNA	*SSP* (G9O61_00g011170) *PTP1* (G9O61_00g021140)	NA	This study

All the three plasmids were spectinomycin resistant. pDS-GFP delivers dsRNA targeting GFP, which serves as a foreign dsRNA control. pDS-*Nosema* provides dsRNA targeting the *N. ceranae* genes. pBTK570 does not deliver dsRNA.

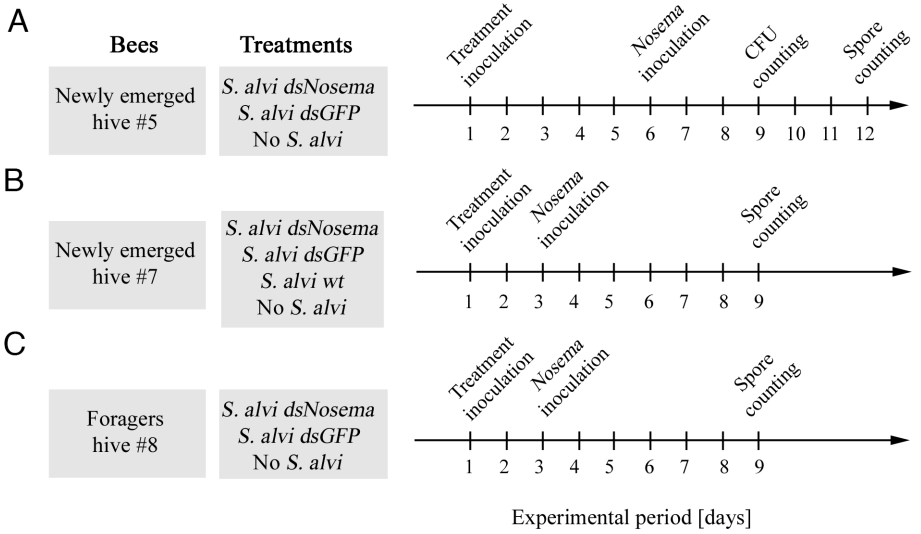

Fig. 1. Experimental schemes for testing the effects of engineered gut symbionts (*Snodgrassella alvi*) on the susceptibility of honey bees to infection by *Nosema ceranae*. In each case, *S. alvi* treatments were on day 1. (*A*) *N. ceranae* inoculation was on day 6; samples for evaluating engineered symbiont colonization were taken on day 9, and samples for *N. ceranae* proliferation were taken on day 12. (*B*) Similar to *A*, but a nonengineered *S. alvi* treatment was included, *N. ceranae* inoculation was on day 3, and samples for *N. ceranae* proliferation were taken on day 9. (*C*) Similar to *B*, except older forager bees were used, the nonengineered *S. alvi* treatment was not included. Each experiment used bees from a different hive.

Discussion

The exploitation of RNAi machinery as a strategy to cause pests to self-destruct has shown promise in several systems with agricultural relevance (29–32). Such an approach circumvents the ecological destruction associated with broad-range chemical pesticides. For insect pests of crops, dsRNA targeting essential pest genes has been shown to be feasible in beetles, moths, stinkbugs, aphids, and other groups (33–36). In honey bees, orally delivered dsRNA can result in *Varroa* mite death, and limit mortality of bees exposed to virus, suggesting that the dsRNA can enter and circulate in the bee hemolymph (37–39). The central challenge for RNAi-based strategies is the instability of RNA in the environment and the expense of continually providing exogenous sources of dsRNA. For plant-feeding insects or other eukaryotic parasites, a potential, though costly, solution is to engineer the crop species to constitutively produce dsRNA. An alternative approach is to engineer a symbiotic bacterium to produce dsRNA targeted toward the pest species (40, 41), and this approach has shown promise in controlling harmful viruses and mites (27). We developed this symbiont-mediated RNAi approach for the microsporidian *N. ceranae*, a major eukaryotic parasite of honey bees.

Microsporidia, a group of obligate intracellular animal parasites, are close relatives of fungi (42). Microsporidian infection has been exceptionally damaging in apiculture and in the silk and shrimp industries (10, 43, 44). Protected by their spore wall, these parasites remain infective in harsh environments (45, 46). RNAi knockdown has previously been used to identify genes essential

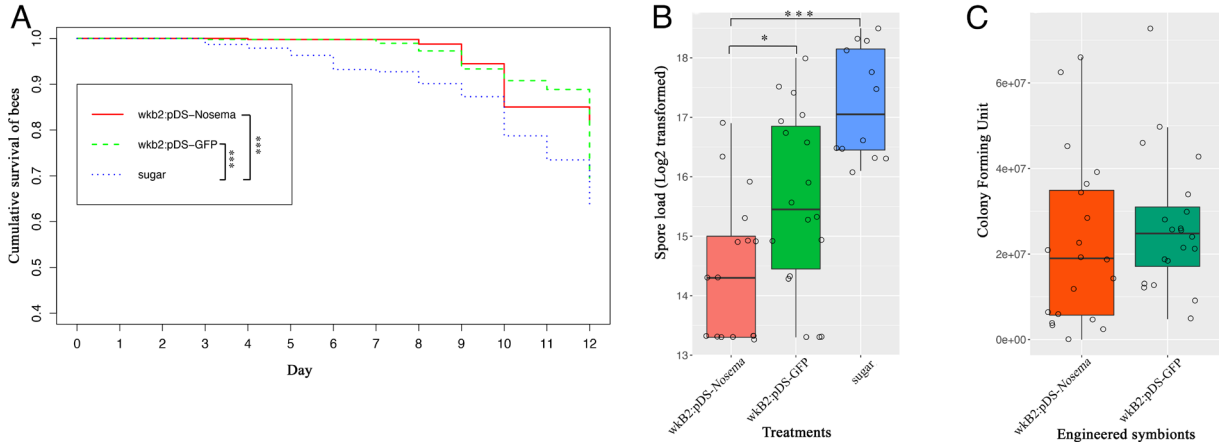

Fig. 2. The impact of the engineered gut symbionts on honey bee survival and *N. ceranae* proliferation. (*A*) Bee survival following challenge with *N. ceranae*. The bees in the wkB2:pDS-*Nosema* ($N = 61$) and wkB2:pDS-GFP ($N = 57$) groups showed higher survival than that of bees in the sugar group ($N = 71$) (log-rank sum test, adjusted $P < 0.05$, Fig. 2). (*B*) Spore loads (log2 transformed) in the three treatment groups. Honey bees inoculated with the engineered symbiont *S. alvi* wkB2:pDS-*Nosema* ($N = 16$) contained fewer spores than those with *S. alvi* wkB2:pDS-GFP ($N = 18$) (Wilcoxon rank-sum test, adjusted $P < 0.05$). Bees without symbionts ($N = 12$) showed significantly more spores than those inoculated with either *S. alvi* wkB2:pDS-*Nosema* (Wilcoxon rank-sum test, adjusted $P < 0.001$) or *S. alvi* wkB2:pDS-GFP (Wilcoxon rank-sum test, adjusted $P < 0.05$). (*C*) Colonization by two engineered *S. alvi* in honey bee gut. The two engineered symbionts colonized honey bee guts at similar rates on day three post-*N. ceranae* inoculation ($N = 20$ bees in each group; Wilcoxon test, $P = 0.24$). Box plots show high, low, and median values, with the lower and upper edges of each box denoting the first and third quartiles, respectively. Multiple comparisons were adjusted with FDR. * Indicates a significant level of 0.05; ** significance level of 0.01; *** significance level of 0.001.

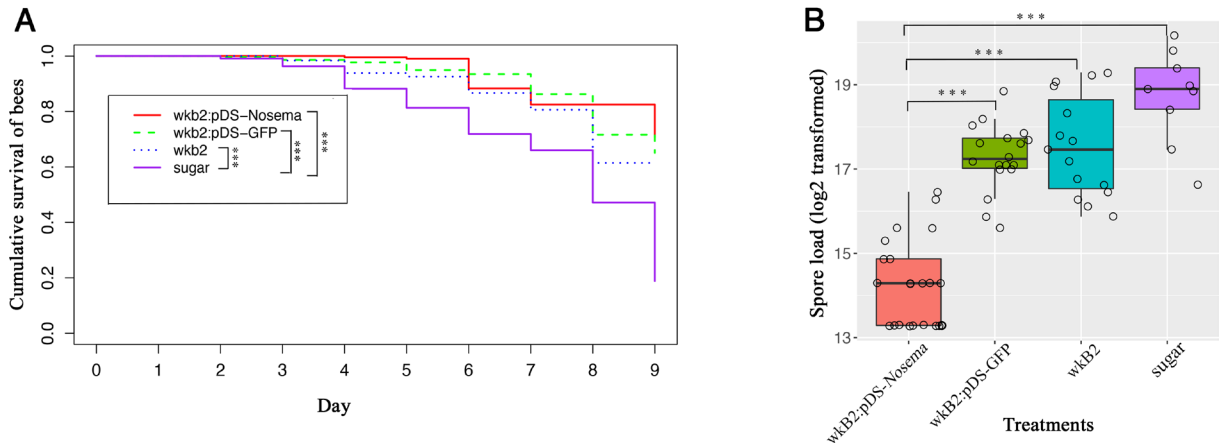

Fig. 3. The second experiment testing engineered gut symbionts protecting newly emerged honey bees. (*A*) Bee survival following challenge with *N. ceranae*. The bees in the wkB2:pDS-*Nosema* ($N = 51$), wkB2:pDS-GFP ($N = 51$), and wkB2 ($N = 52$) groups showed significantly higher survival than those in the sugar group ($N = 48$) (log-rank sum test, adjusted $P < 0.05$, Fig. 3*A*). (*B*) Spore loads (log2 transformed) in the four treatment groups. The honey bees inoculated with the engineered symbiont *S. alvi* wkB2:pDS-*Nosema* ($N = 24$) produced significantly fewer spores than those of the bees in the wkB2:pDS-GFP ($N = 18$), wkB2 ($N = 15$), and the sugar groups ($N = 9$) (Kruskal–Wallis test, adjusted $P < 0.001$). Box plots show high, low, and median values, with the lower and upper edges of each box denoting the first and third quartiles, respectively. Multiple comparisons were adjusted with FDR. * Indicates a significant level of 0.05; ** significance level of 0.01; *** significance level of 0.001.

for microsporidian proliferation in their host (19–21, 24, 47–50). However, a cost-effective approach to delivering interfering RNA to these organisms has not been available.

In this study, we used the bee microbiome toolkit to engineer a plasmid encoding dsRNA targeting essential genes of *N. ceranae* (23, 28). We established this plasmid in *S. alvi* wkB2, producing an engineered strain, *S. alvi* wkB2:pDS-*Nosema*. The plasmid expresses dsRNA targeting two essential *N. ceranae* genes, encoding SSP and polar tube protein 1 (PTP1). The polar tube is a specialized invasion apparatus that interacts with the host cell surface and transports the sporoplasm into the cells, as required for infection success (48, 51, 52). A previous study showed that inhibiting the expression of *PTP3* through dsRNA delivered orally to bees substantially reduces *N. ceranae* spore loads (20). Our current study suggests that PTP1 also contributes to parasite proliferation. As an intracellular parasite, the energy required for proliferation is acquired from the host. SSP localizes on the surface of the sporoplasm, which interacts with PTP and host cell voltage–dependent anion channels, maintaining the proximity associated with the mitochondria (24). *SSP* and *PTP1* are highly expressed early in infection, after which their expression levels decrease (53).

The previously demonstrated efficacy of symbiont-mediated RNAi against ectoparasitic mites suggested that the dsRNA entered the bee hemolymph and was then ingested by the mites. Our current results for *N. ceranae* support the entry of the activating RNA into *N. ceranae* cells, which themselves are intracellular within bee epithelial cells. Previous studies suggested that orally feeding dsRNA and siRNA both can suppress *Nosema* proliferation (20, 21). However, it remained unclear whether dsRNA was processed to siRNA by the RNAi machinery of the

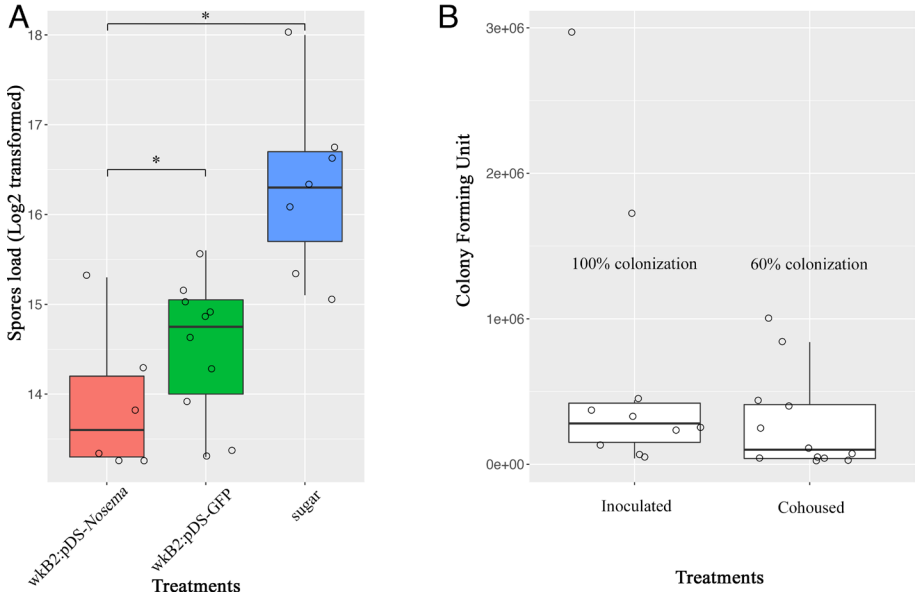

Fig. 4. Spore load and symbiont dispersal in forager bees. (*A*) Numbers of *N. ceranae* spores in bees from the three treatment groups. *S. alvi* wkB2:pDS-*Nosema* ($N = 6$) showed significantly fewer spores than those in the *S. alvi* wkB2:pDS-GFP ($N = 10$) and sugar groups ($N = 7$) (Kruskal–Wallis test, adjusted $P < 0.05$). (*B*) Dispersal of engineered gut symbiont in cohoused bees ($N = 12$) exposed to inoculated bees ($N = 10$). Box plots show high, low, and median values, with the lower and upper edges of each box denoting the first and third quartiles, respectively. Multiple comparisons were adjusted with FDR. * Indicates a significant level of 0.05.

host or the parasite before separating the sporoplasm from the cytoplasm.

The nonengineered *S. alvi* wkB2 also conferred some protection from *N. ceranae* infection, as evidenced by a lower spore load and higher bee survival than those observed for bees in the sugar group. A previous study revealed a negative association between levels of *S. alvi* and *N. ceranae* spore load (54). In our study, bees inoculated with wkB2:pDS-GFP and wkB2 showed similar spore loads and survival, suggesting that the observed protection for these two treatments originates from colonization by *S. alvi*. Bees inoculated with *S. alvi* showed higher survival than those without symbionts, providing evidence that *S. alvi* itself protects bees from *N. ceranae*, a result consistent with previous findings that disrupting the normal microbiota increases *Nosema* susceptibility (55).

In commercial apiaries, antiparasite treatments are usually applied in spring and autumn and can be toxic to larvae and pupae (56–58). Caging the queens may be required depending on the treatment, and the colony suffers from losing newly emerged workers for at least 3 wk (59). In winter, hives should not be opened for treatments, because cold temperatures freeze the bees. In the current study, we found that engineered symbionts could colonize older bees and substantially suppress *N. ceranae* spore load. Additionally, the engineered symbionts dispersed and colonized 60% of cohoused bees within 3 d, suggesting that they could spread within a hive. Together, these results raise the possibility that the introduction of engineered symbionts in late autumn could achieve parasite control over the winter, without interfering with larvae, pupae, and colony strength. Future field experiments, performed with a proper biocontainment strategy, have the potential to reveal the extent to which the engineered symbionts can increase winter survival in outdoor hives.

Materials and Methods

Parasite Infection Survey and Cultivation. Honey bee (*Apis mellifera*) hives were maintained at the University of Texas at Austin on a building rooftop. For each hive, the infection level was surveyed twice before the experiment (*SI Appendix*, Table S1). Sucrose solutions (2 μL) containing 1×10^5 spores were fed to each newly emerged honey bee. At 7 d postinfection, all infected honey bees were dissected to harvest spores, which were purified and refrigerated for the following inoculation assay. Detailed procedures are described in *SI Appendix*.

Honey Bee Gut Symbiont Engineering. The gut symbiont *S. alvi*, strain wkB2, was previously isolated and cultivated on Columbia Blood Agar Base with 5% sheep blood (60). The online tool SnapDragon (https://www.flyrnai.org/snapdragon) was used to design dsRNA targeting the genes encoding SSP and PTP1 (*SI Appendix*, Figs. S2 and S3). Golden Gate Assembly was used to insert the synthesized gene fragment into the dsRNA expression plasmids (27, 61). Detailed procedures and dsRNA sequence are described in *SI Appendix*.

Suppressing Parasite Proliferation in Newly Emerged Honey Bees. The newly emerged honey bees were divided into three treatment groups, with 30 honey bees per cup and two cups per treatment group. The bees were inoculated with engineered *S. alvi* wkB2:pDS-*Nosema*, *S. alvi* wkB2:pDS-GFP, and sucrose solution with 60 μg/mL spectinomycin. Then, the bees were individually inoculated with 1×10^5 *N. ceranae* spores in 2 μL sucrose. The hindgut was dissected to quantify the CFU of *S. alvi* at 3 d postparasite inoculation. The midgut was dissected to count spores at 6 d post-parasite inoculation. The experiment was repeated using honey bees from a different colony (Fig. 1). Detailed procedures are described in *SI Appendix*.

Suppressing Parasite Proliferation in Older Honey Bees. Colonization of the engineered symbionts on adult bees was quantified with antibiotic selection (*SI Appendix*, Tables S2 and S3). Approximately 180 flying foragers were collected near the hive entrance using an insect net. Bees were inoculated with engineered *S. alvi* wkB2:pDS-*Nosema*, *S. alvi* wkB2:pDS-GFP, and sucrose solution using the shaking method (27). The bees were inoculated with 1×10^5 *N. ceranae* spores in 2 μL sucrose solutions. On day 9, midguts were dissected to count the spore load in each bee (Fig. 1). When the spore load was very low, it could not be precisely estimated. Detailed procedures are described in *SI Appendix*.

Dispersal of the Engineered Gut Symbiont Transfer among Nestmates. Eighty newly emerged honey bees were collected and divided into four cups. Forty bees were marked with fluorescent color on the thorax and inoculated with the engineered symbiont. The remaining forty bees were fed with sucrose solution. At 2 d post engineered symbiont inoculation, bees were anesthetized with CO_2. Ten bees with engineered symbionts and ten sucrose-fed bees were assigned to a new cup. At 5 dpi, all bees were dissected to measure CFU. Detailed procedures are described in *SI Appendix*.

Statistics

The persistence of plasmid in newly emerged honey bees was analyzed with the Kruskal–Wallis test implemented in R (version 3.6.3) (62). The post-inoculation day and antibiotic selection were treated as independent variables, and the CFUs were the dependent variable. The treatment groups were set as the independent variable, and the rearing cups were set as a random variable. The variance of the spore load among the treatment groups was analyzed with paired Wilcoxon rank-sum test, and multiple comparisons were adjusted for FDR. Survival was analyzed using Kaplan–Meier estimate in the survival package, adjusted for multiple comparisons with FDR, R (63).

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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