RESEARCH ARTICLE

Pre- and post-association barriers to host switching in sympatric mutualists

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Abstract

Coevolution between mutualists can lead to reciprocal specialization, potentially causing barriers to host switching. Here, we conducted assays to identify pre- and post-association barriers to host switching by endosymbiotic bacteria, both within and between two sympatric nematode clades. In nature, *Steinernema* nematodes and *Xenorhabdus* bacteria form an obligate mutualism. Free-living juvenile nematodes carry *Xenorhabdus* in a specialized intestinal receptacle. When nematodes enter an insect, they release the bacteria into the insect hemocoel. The bacteria aid in killing the insect and facilitate nematode reproduction. Prior to dispersing from the insect, juvenile nematodes must form an association with their symbionts; the bacteria must adhere to the intestinal receptacle. We tested for pre-association barriers by comparing the effects of bacterial strains on native versus non-native nematodes via their virulence towards, nutritional support of, and ability to associate with different nematode species. We then assessed post-association barriers by measuring the relative fitness of nematodes carrying each strain of bacteria. We found evidence for both pre- and post-association barriers between nematode clades. Specifically, some bacteria were highly virulent to non-native hosts, and some nematode hosts carried fewer cells of non-native bacteria, creating pre-association barriers. In addition, reduced infection success and lower nematode reproduction were identified as post-association barriers. No barriers to symbiont switching were detected between nematode species within the same clade. Overall, our study suggests a framework that could be used to generate predictions for the evolution of barriers to host switching in this and other systems.

KEYWORDS
constraints, evolution of co-operation, microbes

1 | INTRODUCTION

Coevolution between a host and its symbiont can lead mutualist partners to specialize on each other, which may lead to the evolution of barriers to associating with novel partners. Barriers to partner switching may be pre-association or post-association, analogous to pre- and post-zygotic barriers in the formation of hybrids between incipient species (Coyne & Orr, 1997). This analogy is especially applicable for horizontally transmitted endosymbionts in which pre-association barriers might result from impediments to successful

colonization, and post-association barriers might result from re-duced fitness following colonization. Here, we test whether barriers exist that prevent partner switching in closely related, sympatric species of entomopathogenic *Steinernema* nematodes, which form a mutualism with *Xenorhabdus* bacteria.
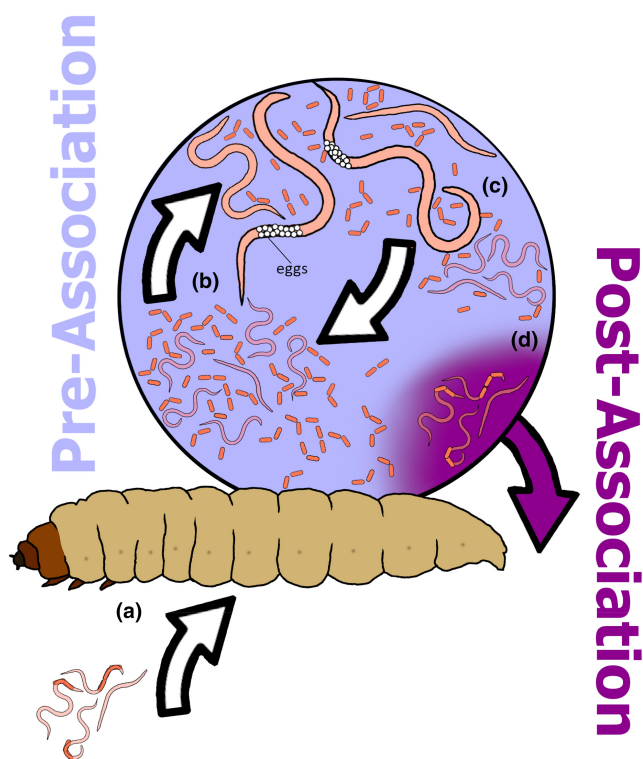
The potential for one or more barriers to host switching stems from the complicated life cycle of this mutualism. *Steinernema* nematodes and *Xenorhabdus* bacteria form an obligate entomopathogenic mutualism in nature (Figure 1). Both the bacteria and nematode produce toxins against insects, which along with their growth quickly kills the insect (Brivio et al., 2002; Forst & Nealson, 1996; Stock, 2019). Some strains of bacteria are also pathogenic towards non-native nematodes, causing dramatic reductions in nematode survival (Murfin et al., 2018; Sicard et al., 2004). If the bacteria kill non-native nematodes, it will create a pre-association barrier. The bacteria also produce many enzymes that digest the insect into a nutrient soup, facilitating nematode reproduction (Richards & Goodrich-Blair, 2009; Thaler et al., 1998). Genotypic variation indicates that different nutrients might be produced by different bacterial strains (Murfin et al., 2015), thereby creating a pre-association

FIGURE 1 Life cycle of *Steinernema* nematodes and *Xenorhabdus* bacteria, (a) the free-living infective juvenile (IJ) nematodes carrying their bacteria enter the insect and (b) release them inside the insect hemocoel, wherein (c) both nematodes and bacteria contribute to killing the insect and reproduce inside the insect cadaver, converting the insect biomass to new nematodes and bacteria, (d) offspring nematodes acquire the bacteria from their environment—Forming the association that dictates the future success of the mutualism—just prior to leaving the insect cadaver (Herbert & Goodrich-Blair, 2007; Sicard et al., 2003). Images within the circle take place within the insect hemocoel. *Note*: Sizes not to scale

barrier for non-native nematodes that require specific nutrients for reproduction. Before dispersing from the insect, newly produced juvenile nematodes pick up bacteria and house them in a special receptacle in their intestine (Figure 1d; Snyder et al., 2007). We have defined this stage as the association step—if the bacteria do not adhere to the nematodes' intestinal receptacle, there is a pre-association barrier. Any fitness reduction after bacteria adhesion is a post-association barrier. Note that the same mechanisms causing pre-association barriers might also act post-association if the association is not prevented entirely.

The nematode–bacteria mutualism is taxonomically diverse, with over 50 species of *Steinernema* nematodes and 20 species of *Xenorhabdus* bacteria, which are globally distributed (Stock & Goodrich Blair, 2008; Tailliez et al., 2010). Although each species of nematode is associated with a single species of bacteria (Bird & Akhurst, 1983; Liu et al., 2001), some species of bacteria are able to associate with multiple species of nematode (Stock & Goodrich Blair, 2008; Lee & Stock, 2010; Murfin et al., 2015). For example, *Xenorhabdus bovienii* pairs with at least ten *Steinernema* species across two distinct clades (Nadler et al., 2006; Lee & Stock, 2010).

Several studies have attempted to experimentally associate species pairs of *Steinernema-Xenorhabdus* to assess specificity. Some nematode species do not reproduce when paired with non-native bacterial strains (Sicard et al., 2005; McMullen et al., 2017). Even when reproduction is successful, nematodes may not carry non-native bacteria (Sicard et al., 2004). Non-native pairings can also result in nematode offspring that have decreased survival and lower parasitic success (Chapuis et al., 2009; Murfin et al., 2015). In general, non-native pairings exhibit more reduced fitness with increased phylogenetic distance between the native and non-native partners (Chapuis et al., 2009; Sicard et al., 2004). This phylogenetic signal was found to hold even when the non-native bacterial partner was of the same species as the native partner (Murfin et al., 2015). No successful associations were obtained when nematodes were partnered with an *X. bovienii* strain from a nematode from a distinct clade. Meanwhile, pairings with *X. bovienii* strains associated with nematodes from the same clade were successful, albeit to varying degrees, contingent on phylogenetic distance (Murfin et al., 2015). These experiments strongly suggest the existence of pre- and/ or post- association barriers to pairing with non-native partners. However, as these studies utilized allopatric species pairs, they leave open the question of whether there is the potential for host switching in sympatric populations.

Here, we expand on the pioneering work by Murfin et al. (2015) by testing for barriers between sympatric pairs of species across two *Steinernema* nematode clades associated with *X. bovienii*. We also identify association barriers at three levels: the bacteria strain level, the nematode species level and the nematode clade level. We first asked whether each strain of bacteria kill or inhibit the development of non-native nematodes, which would indicate a pre-association barrier. We then experimentally associated nematodes and bacteria in live caterpillar hosts, and measured components of fitness as infection success, nematode fecundity and bacterial carriage. To

identify pre-association barriers, we asked whether native pairings showed higher fitness than novel pairings. Finally, we identified post-association barriers by comparing the fitness of native and novel pairings when nematodes rely on their carried bacteria. The results showed both pre- and post-association barriers between, but not within, nematode clades.

2 | MATERIALS AND METHODS

2.1 | Nematode isolates and bacterial strains

Nematodes carrying bacteria were isolated from five soil samples collected within 60 m of each other at the Indiana University Research and Teaching Preserve (Moores Creek, IN, USA) in October 2011 (as previously described in Bertoloni & Bashey, 2018). The bacterial strain associated with each nematode isolate was identified as *X. bovienii* via 16S rRNA gene sequencing (Tailliez et al., 2006) and found to be genetically distinct by ERIC sequence fingerprinting (Hawlena et al., 2010). Bacteria were stored as freezer stocks in 20% glycerol at −80°C. Each nematode isolate was identified to the species level using the 28S rRNA gene (Stock et al., 2001). Nematode stocks were maintained by passaging through *Galleria mellonella* larvae (as described below) and stored culture flask at 4C between passages. Six nematode isolates (Table 1) were used to attempt generating aposymbiotic nematodes (methods below). Only nematodes from the two isolates of *S. affine* (clade I) and one isolate of *S. kraussei* (clade III) successfully generated aposymbiotic juveniles (Table 1, bolded). These three nematode isolates were experimentally paired with all six bacterial strains in a fully factorial design to examine association barriers within and between nematode clades. We used fingerprinting and 28S sequence differences to confirm the identity of bacterial strain and nematode species after the experimental associations.

2.2 | *In vitro* development assay—virulence

One possible pre-association barrier involves bacterial virulence to non-native host nematodes. The virulence of each bacterial strain on each nematode isolate was assayed by rearing infective juvenile nematodes (IJs) on lawns of the bacteria on a liver–kidney agar (LKA) plates (following Murfin et al., 2018). LKA plates provide adequate nutrition for IJs; therefore, development should be prevented and juvenile death ensue only if the bacteria are virulent to the nematodes. Bacteria were plated from freezer stocks and then inoculated into LB media (Difco). After overnight growth, 2.0 mL of the bacteria culture was spread onto 60 × 15 mm LKA plates. Lawns of bacteria were grown by keeping these plates at 28°C for 24 h. One hundred unaltered IJs (carrying native symbionts) were placed on each of five replicate plates for each bacterial strain. Given the lawn of bacteria ($>10^6$ cells/mm^2), the small number of native bacteria cells carried by the IJs ($<10^4$ per plate) would have little opportunity to grow and influence the assay results. IJ growth and development at 20°C were monitored for 8 days. Plates were observed under a dissecting scope once daily. At each census, nematodes were assessed to be alive or dead and their developmental stage recorded.

2.3 | *In vitro* development assay—nutrition

We assessed whether the bacteria failed to produce the necessary nutrients for nematode development, which could result in another pre-association barrier. For this assay, we reared infective juvenile nematodes (IJs) on lawns of the bacteria on lipid agar media (following Murfin et al., 2018). Lipid agar is nutritionally deficient for nematodes; they require additional nutrients that their native bacterial strains can supply. This assay allows us to identify whether the bacteria provide sufficient nutrients for the nematodes to grow, develop and reproduce. The nutrition assays were conducted concurrently with the virulence assays, and using the same methods, except using Lipid Agar instead of liver–kidney agar.

2.4 | Rearing aposymbiotic nematodes

To evaluate association barriers, we reared aposymbiotic nematodes, which do not carry symbiotic bacteria, allowing us to create experimental pairings of nematodes and bacteria. *Steinernema* IJs from all 6 nematode isolates were reared on lipid agar plates with lawns of their native bacteria at 20°C until gravid females were apparent

TABLE 1 Nematode isolates and bacteria strains used in this study. All bacteria are strains of *Xenorhabdus bovienii*

Nematode clade	Nematode species	Isolate/strain name	Isolate/strain ID number	*Steinernema* isolate 28S GenBank #	*Xenorhabdus bovienii* strain genome accession
I	***S. affine***	**Sa1**	**235**	**OK319045**	**JAILST000000000**
I	***S. affine***	**Sa2**	**226**	**OK319044**	**JAILSW000000000**
III	***S. kraussei***	**Sk1**	**239**	**OK319049**	**JAILSS000000000**
III	*S. kraussei*	Sk2	266	OK319050	JAILSO000000000
III	*S. texanum*	St1	233	OK319052	JAILSU000000000
III	*S. texanum*	St2	241	OK319051	JAILSR000000000

Note: Each row is a naturally isolated combination. Bold rows indicate focal nematode isolates used in experimental assays and infections. The isolate/strain name is used for ease of reference in this paper, whereas ID number can be used for cross reference across studies.

(Sicard et al., 2003). Females were washed in an alkaline bleach solution (250 mM KOH, 0.5 M NaClO) and inverted in conical tubes for 10 min to dissolve female tissue and to surface sterilize eggs. Because bacteria are not vertically transmitted, surface-sterilized eggs are axenic. Eggs were suspended in 1X PBS and transferred to LKA plates with 100 mg/ml of kanamycin and 100 mg/mL of ampicillin. These LKA plates were kept for up to 2 weeks at 20°C, until IJs became visible (Sicard et al., 2003). IJs were collected by washing from the liver-agar plates and kept in culture flasks at 4°C. A sample of 200 IJs for each nematode stock was surface sterilized and crushed in PBS (following Sicard et al., 2004, substituting PBS for Ringer's solution). This solution was then plated on Nutrient Agar +0.0025% bromothymol blue +0.004% triphenyltetrazolium chloride (NBTA) and grown at 28°C for 48 hours to determine if any *Xenorhabdus* bacteria remained in the IJs (Akhurst & Boemare, 1988). Only aposymbiotic IJs—those without *Xenorhabdus*—were used in experiments. Of the six stocks attempted, only *S. kraussei* isolate 1 (*Sk1*) successfully produced axenic IJs.

We altered the methodology in the second attempt to create axenic nematodes. Due to species differences in dissolution rates, we used a blunt probe to agitate females in solution on a petri dish while viewing under a dissecting scope. We adjusted the speed and frequency of agitation based on how quickly the females were dissolving, thereby preventing egg mortality by overbleaching. Eggs were suspended in LB with 50 μg/ml of kanamycin and 150 μg/ml of ampicillin to improve hatching rates (Murfin et al., 2012). To collect IJs from LKA plates, we used modified white traps and allowed IJs to crawl off the plates, which reduced debris in the collection flask (Murfin et al., 2012). All other methods were as described above for the first attempt. The second attempt yielded axenic IJs for *S. affine* isolate 1 and 2, and *S. kraussei* isolate 1. However, the second yield of *S. kraussei* isolate 1 was much lower; rather than coinfect with several strains of bacteria, we chose to only coinfect with the most successful strain of Clade I bacteria, *Sa2*.

2.5 | *In vivo* association assays

Aposymbiotic *S. kraussei* nematodes were experimentally associated with each of the six bacterial strains by infecting 20 waxworm (*Galleria mellonella*; Vanderhorst Wholesale) caterpillars per bacterial strain as detailed below. The second batch of aposymbiotic *S. kraussei* nematodes was used exclusively to coinfect 60 caterpillars with a single strain of bacteria (Sa2). Aposymbiotic *S. affine* nematodes from each of the two nematode isolates were experimentally associated with each of the six bacterial strains. Enough aposymbiotic nematodes were generated to allow for two replicate blocks, performed within 7 days of each other. Due to expectations of lower infection success for more distant pairings, each replicate of *S. affine* nematodes paired with bacteria from clade III nematodes consisted of 40 caterpillars, whereas each replicate of *S. affine* nematodes paired with bacteria from clade I nematodes consisted of 20 caterpillars. For each of the three axenic nematode isolates (Sa1, Sa2 and Sk1),

a single replicate of 20 caterpillars was infected with aposymbiotic nematodes and injected with sterile PBS as a no-bacteria control. In addition, a single replicate of 20 caterpillars per isolate was infected with unaltered nematodes associated with symbiont bacteria.

Caterpillars were obtained from a wholesale supplier and were selected by weight (between 100 and 320 mg). To infect caterpillars, 100 aposymbiotic IJs in 500 μl of dH$_2$O were pipetted onto the dorsum of each insect. About 24 hours after nematode infection, 10^4 cell were injected each caterpillar by using a 30-gauge needle to deliver 0.02 ml of inoculum (bacteria cultured in LB overnight and diluted with 1× PBS). Each inoculum was further diluted, plated on NBTA and grown at 28°C for 48 h. Colony-forming units (CFUs) were counted and used to estimate actual bacterial dose (Chapuis et al., 2009). These doses (mean = 128, range 1.8–666) were close to the target of 100 CFU/IJ, which was based on the carriage reported from studies of *S. carpocapsae* (Synder et al., 2007). Negative controls (IJs without bacteria) were injected with 0.02 ml of sterile PBS. Unaltered controls (IJs retaining their native bacteria) were not injected. Mortality was assayed within the first week, and dead caterpillars were moved to white traps (White, 1927). Nematode emergence was assayed for a further 3 weeks, and all IJs were collected within 6 weeks. Collections from each caterpillar were kept in separate culture flasks at 4°C. For the first batch of *S. kraussei* aposymbiotic nematodes, due to the poor emergence success from coinfections with bacteria from *S. affine*, all juveniles that emerged were pooled within the bacterial strain. To maintain consistency, coinfections with bacteria from *S. kraussei* or *S. texanum* juveniles were pooled across the five caterpillars with the highest visible nematode emergence within each bacterial strain. Because the collections were pooled, these samples are excluded from statistical analyses and figures. Instead, the metrics were measured for the secondary infections. The number of nematodes that emerged from each caterpillar was estimated by volumetric subsampling. Three 50-μl samples were viewed under a dissecting scope and counted, and the average of these counts was then multiplied by the total volume of the collection. For each pairing, at least one sample of IJs was surface sterilized, crushed in PBS and grown on NBTA plates at 28°C for 48 h to measure bacteria carriage as CFU per IJ.

2.6 | Subsequent infection after association

100 IJs carrying experimental bacteria, created in the *in vivo Association Assays*, were suspended in 500 μl of dH$_2$O and were pipetted onto caterpillars. Each replicate consisted of 20 caterpillars per within clade pairing, and 40 caterpillars per between clade pairing. Mortality was assayed within the first week, and dead caterpillars were moved to white traps (White, 1927). Emergence was assayed for a further 3 weeks, and all IJs were collected within 6 weeks. Collections from each caterpillar were kept separately in culture flasks at 4°C. For at least 5 collections for each group, a sample of IJs was surface sterilized, crushed in PBS and grown on NBTA plates at 28°C for 48 h to measure bacteria carriage.

2.7 | Statistical tests

To test for differences in the timing of developmental milestones when nematodes were grown on different bacterial strains in the *in vitro* assays, we performed an ordered logistic regression assuming cumulative logits via Proc Logistic in SAS 9.4. For each assay type, bacterial strain, day and nematode isolate (for Clade I nematodes) were used as predictors of developmental stage, which was ordered as shown in Figures 2 and 3. All remaining statistics were performed in R, version 3.6.3 (R Core Team, 2020). For infections with *S. kraussei* nematodes (Clade III), the data were analysed separately for each batch of aposymbiotic nematodes. For each analysis, bacterial strain was assessed as a fixed factor in a generalized linear model. Infection success (i.e. whether nematodes emerged or not from a caterpillar) was analysed as a binomial response. The mean number of juveniles that emerged per successful infection and the mean number of bacterial cells carried per nematode were both $\log_{10}$ transformed and analysed using normal error distributions. Analysis of the *S. affine* nematode (Clade I) associations was analysed similarly, except that nematode isolate and the interaction between nematode isolate and bacterial strain were also included as fixed effects. Additionally, the replicate block was added as a random effect. For each model, we computed the type III test statistic for fixed effects and estimated the marginal means and 95% confidence intervals for each bacterial strain using the emmeans package (Lenth, 2020). We performed planned comparisons to test whether bacteria isolated from each nematode species and clade was significantly different from the controls and whether the two bacterial strains isolated from each nematode species differed from each other.

In the *in vitro* development assays, Sa1 nematodes showed a slight but significant delay in development relative to Sa2. However, in the *in vivo* association assays, no significant differences were observed between the two clade I nematode isolates (Sa1 and Sa2), including how they interacted with each bacterial strain ($p > 0.21$ for all assays). Therefore, for brevity and clarity, we focus the presentation of results on the main effects of bacterial strain.

3 | RESULTS

For each nematode–bacteria combination, we assessed pre-association barriers by *in vitro* development assays to measure bacterial virulence and nutritional support, and *in vivo* association assays to measure reproductive success and bacterial carriage in the insect host. If the *in vivo* association resulted in successful nematode reproduction, we evaluated the possibility of post-association barriers by attempting to passage the newly associated nematode–bacteria pairs through a new batch of caterpillars. We have structured our findings by each nematode species, first examining the results when nematodes were paired with bacterial strains from nematodes of the same clade and then from the different clade.
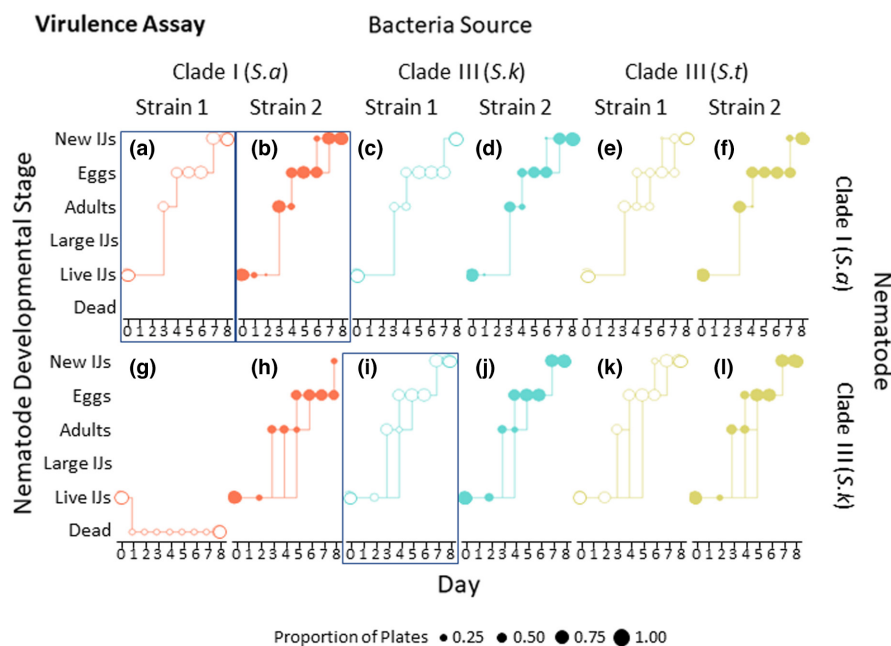
FIGURE 2 Results of the virulence assay, in which nematodes develop normally as long as the bacteria are not virulent towards them. Each row corresponds to nematodes added to the plate, labelled on the right. Each column represents the source of the bacterial lawn grown on the plate. Boxes indicate native nematode–bacteria pairs. The day of the observation is listed on the *x*-axis, and the *y*-axis shows which developmental milestone is seen on the plates. Points represent overlapping results from each of five replicates, with the size of point proportional to the number of plates showing that response. The two isolates of clade I (*Sa*) nematodes are presented together. Lines with no points indicate assumed stages, when no nematodes were visible on the plates

FIGURE 3 Results of the nutrition assay, in which nematodes require bacterial nutrient production in order to develop and reproduce. Each row corresponds to nematodes added to the plate, labelled on the right. Each column represents the source of the bacterial lawn grown on the plate. Boxes indicate native nematode–bacteria pairs. The day of the observation is listed on the *x*-axis, and the *y*-axis shows which developmental milestone is seen on the plates. Points represent overlapping results from each of five replicates. The two isolates of clade I (*Sa*) nematodes are presented together. Lines with no points indicate assumed stages, when no nematodes were visible on the plates
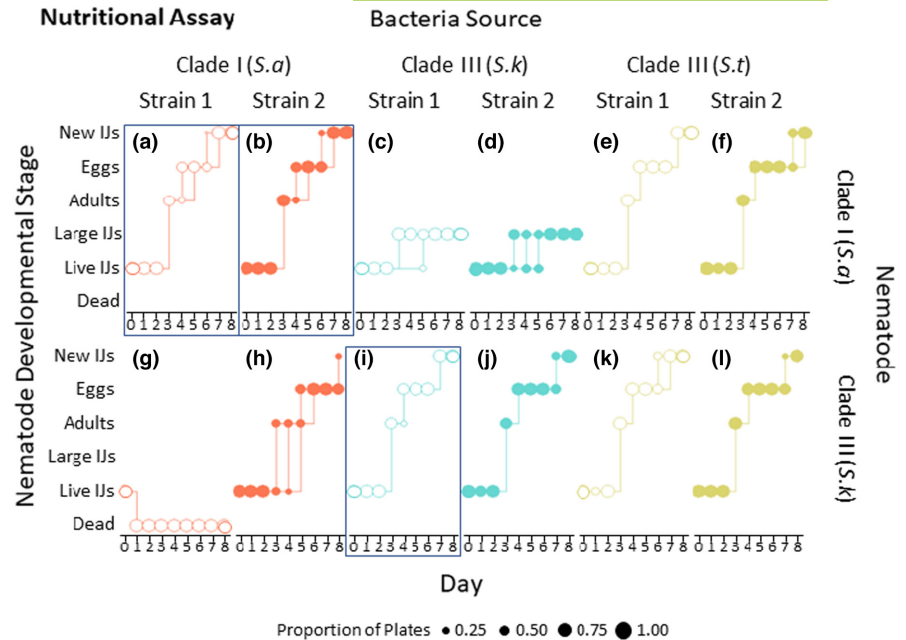
3.1 | Symbiont effects on clade I (Sa) nematodes

3.1.1 | *In vitro* development assays

Clade I (Sa) nematodes developed equally well on lawns of either strain of bacteria from Sa nematodes, regardless of whether the agar was nutritionally complete or deficient. Thus, there was no evidence of virulence (Figure 2a,b) or nutritional deficiency (Figure 3a,b) for bacteria isolated from the same nematode species. Similarly, Sa nematodes developed well on both strains of bacteria from clade III *S. texanum* (St) indicating no virulence (Figure 2e,f) or nutritional deficiency (Figure 3e,f). In contrast, Sa nematodes showed a difference in development in the presence of bacteria from clade III *S. kraussei* (Sk) in the two *in vitro* assays. When grown on the nutritionally complete agar in the presence of either strain of bacteria from Sk, each isolate of Sa nematodes grew normally (Figure 2c,d), indicating no-bacterial virulence (chi-square = 8.34, df = 5, *p* = 0.1386). However, when grown on the nutritionally deficient agar, both isolates of Sa nematodes failed to mature and reproduce (Figure 3c,d) on either strain of bacteria from Sk (chi-square = 227.72, df = 5, *p* < 0.0001). Thus, bacteria from Sk do not nutritionally support Sa nematodes, despite having no direct virulence to these nematodes. In total, from the *in vitro* development assays, the only barrier to host switching is based on nutritive deficiency between clade I (Sa) nematodes and bacteria from one species (Sk) of clade III nematodes.

3.1.2 | *In vivo* association assays

When aposymbiotic clade I (Sa) nematodes were used to infect caterpillars without bacteria, neither isolate was able to successfully reproduce. These results strongly indicate that clade I (Sa) nematodes are dependent on their symbiotic bacteria for infection success *in insecta*. In contrast, when aposymbiotic Sa nematodes were coinfected with their own bacteria (hereafter called reassociation controls), infection success (Figure 4a) was restored and even exceeded that of unaltered controls ($F_{1,505} = 25.790$, $p < 0.001$). This pattern suggests that infection success of Sa nematodes is limited by the growth of their bacteria. Infection success of Sa nematodes when paired with bacteria from clade III nematodes varied with strain (Figure 4a). Infection success with Sk1 or St2 bacteria was not significantly different than the reassociation controls (Sk1: $F_{1,505} = 0.071$, $p = 0.789$; St2: $F_{1,505} = <0.001$, $p = 0.997$). In contrast, Sa nematodes had significantly fewer successful infections with Sk2 or St1 bacteria than when reassociated with their own bacteria (Sk2: $F_{1,505} = 28.970$, $p < 0.001$; St1: $F_{1,505} = 7.296$, $p = 0.007$). These results suggest that strain-level variation leads to differences in the bacteria's ability to support nematode reproduction in these novel cross-clade combinations.

When Sa nematodes were able to successfully infect a caterpillar, the number of emerging progeny (Figure 4b) was not significantly different between the unaltered controls and the reassociation controls ($F_{1,272} = 0.072$, $p = 0.789$). In contrast, significantly fewer nematode progeny emerged from associations with bacteria from clade III nematodes, except Sk1 (Sk1: $F_{1,272} = 3.266$, $p = 0.072$, Sk2: $F_{1,272} = 53.896$, $p < 0.001$, St1: $F_{1,272} = 41.962$, $p < 0.001$, St2: $F_{1,272} = 10.842$, $p = 0.001$). These results demonstrate that Sa nematodes do not reproduce as well with bacteria from clade III nematodes as they do with their own bacteria (Figure 4b).

Furthermore, although the mean log number of bacterial cells carried per Sa nematode (Figure 4c) did not differ significantly between the unaltered and reassociation controls ($F_{1,53} = 0.084$, $p = 0.772$), significantly fewer bacteria from clade III nematodes (Sk1: $F_{1,53} = 5.654$, $p = 0.017$, Sk2: $F_{1,53} = 21.996$, $p < 0.001$, St: $F_{1,53} = 25.964$, $p < 0.001$) were carried by clade I (Sa) nematodes
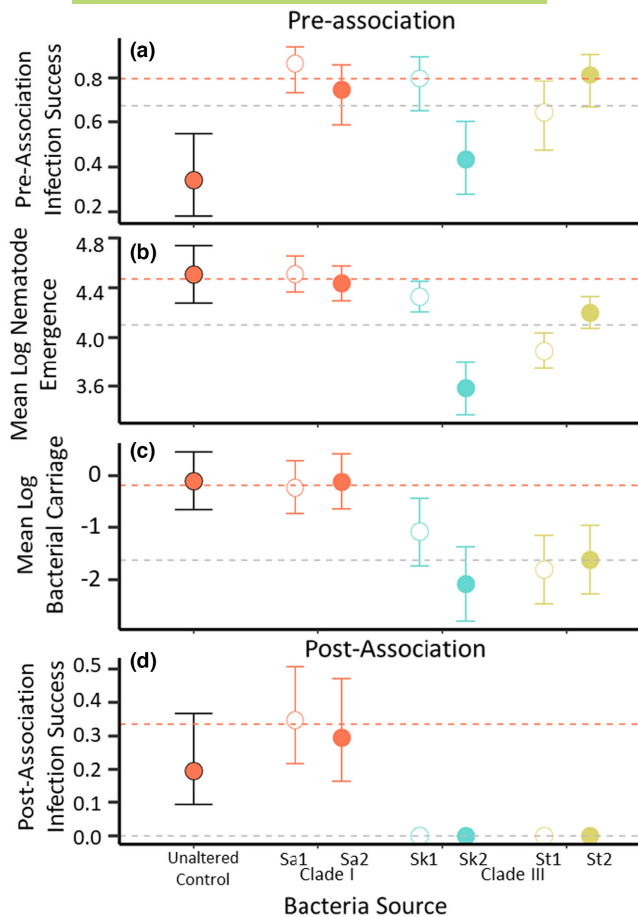
FIGURE 4 Results of experimental associations of clade I (*Steinernema affine*) nematodes with *Xenorhabdus bovienii* bacteria isolated from the same species of nematodes (Sa1, Sa2) or from nematodes of different species and clade (Sk1, Sk2, St1 and St2). When bacteria were injected into caterpillars along with aposymbiotic nematodes, (a) infection success (proportion of caterpillars with emerging nematode progeny) improved over unaltered controls (*S. affine* nematodes carrying their natural symbiont), although less so when the bacteria were from clade III nematodes than from clade I (Sa) nematodes ($F_{1,505} = 6.989$, $p = 0.008$). (b) the $\log_{10}$ mean number of nematodes that emerged from successful infections was also higher when nematodes were exposed to bacteria from their own species than to bacteria from a different clade ($F_{1,272} = 52.916$, $p < 0.001$) and these nematode progeny (c) carried significantly fewer bacterial cells ($F_{1,53} = 33.619$, $p < 0.001$), shown here as $\log_{10}$ mean CFU per nematode. These pre-association barriers precluded host switching as none the clade I (Sa) nematodes carrying bacteria from clade III nematodes were able to successfully infect new caterpillars (d), whereas those carrying their own bacteria were not significantly different from unaltered controls. All symbols show the estimated marginal means with 95% confidence intervals for each bacterial strain. The faint dotted lines indicate the estimated mean for bacteria from each nematode clade

relative the reassociation controls (Figure 4c). The fitness consequence of this difference in bacterial carriage was seen when these nematodes were used to infect new caterpillars (Figure 4d). The infection success of Sa nematodes was not significantly different between

reassociation and unaltered controls ($F_{1,114} = 1.690$, $p = 0.194$). In stark contrast, of the 80 caterpillars infected per clade III bacteria strain, no successful infections were observed (Figure 4d).

In summary, these results indicate that there are no pre- or post-association barriers to host switching within clade I in this population. In contrast, pre-association barriers completely prevented bacteria associated with clade III nematodes from successfully switching onto clade I nematodes.

3.2 | Symbiont effects on clade III (Sk) nematodes

3.2.1 | *In vitro* development assays

Clade III (Sk) nematodes developed equally well when placed on lawns of any strain of bacteria from clade III (Sk, St) nematodes. Normal development occurred on both nutritionally complete agar (chi-square = 0.4943, df = 3, $p = 0.9201$), indicating that no bacteria is virulent to the nematodes (Figure 2i,j,k,l), and on nutritionally deficient agar (chi-square = 1.15, df = 3, $p = 0.765$), indicating that all strains provided sufficient nutrition (Figure 3i,j,k,l). Thus, there is no evidence for a pre-association barrier to host switching within clade III.

In contrast, Sk nematodes died in the presence of strain 1 bacteria from clade I nematodes (Sa1) in both the virulence (Figure 2g) and the nutrition (Figure 3g) assays, indicating that this strain is highly virulent to clade III (Sk) nematodes. In the presence of strain 2 bacteria from clade I (Sa2), some Sk nematodes did develop (Figures 2h, 3h). However, in both the virulence (chi-square = 24.37, df = 1, $p < 0.0001$) and nutrition assays chi-square = 35.926, df = 1, $p < 0.0001$), they reached developmental milestones 1–2 days later than nematodes grown on native bacteria (Figures 2i, 3i). The similarity of the virulence and nutrition assays suggests that Sa2 bacteria from clade I nematodes is partially virulent towards clade III (Sk) nematodes. Additionally, the nematodes grown on Sa2 bacteria reproduced; thus, these bacteria provided sufficient nutrients to the nematodes despite their virulence. The virulence of both strains of bacteria from clade I (Sa) towards clade III (Sk) nematodes indicates a pre-association barrier to host switching.

3.2.2 | *In vivo* association assays

Similarly to clade I, in experimental infections of clade III (Sk) nematodes without bacteria, no nematodes were able to successfully reproduce. This indicates that clade III (Sk) also relies on its symbiont for infection *in insecta*. Interestingly, when paired with any bacteria from clade III (Sk, St) nematodes, high fitness was restored via both infection success and nematode emergence (data not shown). On the contrary, the infection success from experimental associations of Sk nematodes with bacteria from clade I was so poor that we decided to use all the emerging nematodes to test whether these laboratory-created associations could successfully reproduce

on their own. None of the novel pairings with clade I (Sa) bacteria produced any progeny indicating a severe barrier to host switching across nematode clades.

In contrast, reassociations of Sk nematodes with either strain of bacteria from Sk did not differ significantly in infection success (Figure 5a, $F_{1,95} = 0.85$, $p = 0.360$) or number of emerging nematodes (Figure 5b, $F_{1,73} = 0.72$, $p = 0.3996$) from unaltered controls. Similarly, Sk nematodes associated with either bacteria from St nematodes did not differ in infection success (Figure 5a, $F_{1,95} = 0.58$, $p = 0.450$) and number of emerging nematodes (Figure 5b, $F_{1,73} = 0.48$, $p = 0.4924$) relative to reassociations with native bacteria. Thus, Sk1 nematodes could reproduce equally well when associated with any bacteria isolated from other clade III nematodes. Furthermore, no significant difference was observed in bacterial carriage across bacterial strains associated with clade III nematodes (Figure 5c, $F_{1,19} = 1.231$, $p = 0.267$). Taken together, these results indicate that there are no pre- or post-association barriers to host switching for bacteria associated with clade III nematodes in this community.

In our second attempt to experimentally associate aposymbiotic clade III (Sk) nematodes with bacteria from clade I nematodes, we focused on the less virulent Sa2 strain. To our surprise, the emergence success (Figure 5d) in this second attempt was not significantly different from the unaltered controls ($F_{1,78} = 0.254$, $p = 0.616$). Although, the mean number of nematodes ($\log_{10}$ transformed) that emerged from each caterpillar (Figure 5e) was significantly lower for the pairing with Sa2 bacteria compared with the unaltered controls ($F_{1,71} = 5.70$, $p = 0.020$). Nevertheless, the mean number of bacterial cells carried by juvenile nematodes that emerged from infected caterpillars (Figure 5f) was not significantly different from nematodes that emerged from unaltered controls ($F_{1,23} = 1.142$, $p = 0.296$). We do not know what caused the difference in these results—nematode species and bacterial strain identity were confirmed in these experimental associations.

When clade III (Sk) nematodes associated with Sa2 bacteria were used to infect caterpillars, the infection success was significantly lower than the unaltered controls ($F_{1,78} = 10.25$, $p = 0.002$, Figure 5g). In addition, the mean number of juveniles emerged was significantly lower for the experimental pairing than the unaltered controls ($F_{1,19} = 7.74$, $p = 0.012$, Figure 5h). Together, these results indicate that fitness is lower for the nematode after association with Sa2 bacteria, suggesting a post-association barrier. However, there was not a significant difference in the mean number of bacterial cells carried between the two groups ($F_{1,8} = 0.403$, $p = 0.543$, Figure 5i), indicating that the nematodes still do not show bacterial specificity.

In summary, the pre-association barrier of bacteria virulence completely prevented strain 1 bacteria from clade I nematodes (Sa1) from switching to clade III (Sk) nematode hosts. In contrast, pre-association barriers did not prevent an association between clade III (Sk) nematodes with strain 2 bacteria from clade I nematodes (Sa2). In fact, despite lowered reproductive success, the novel bacteria were carried equally as well as native bacteria. Additionally,
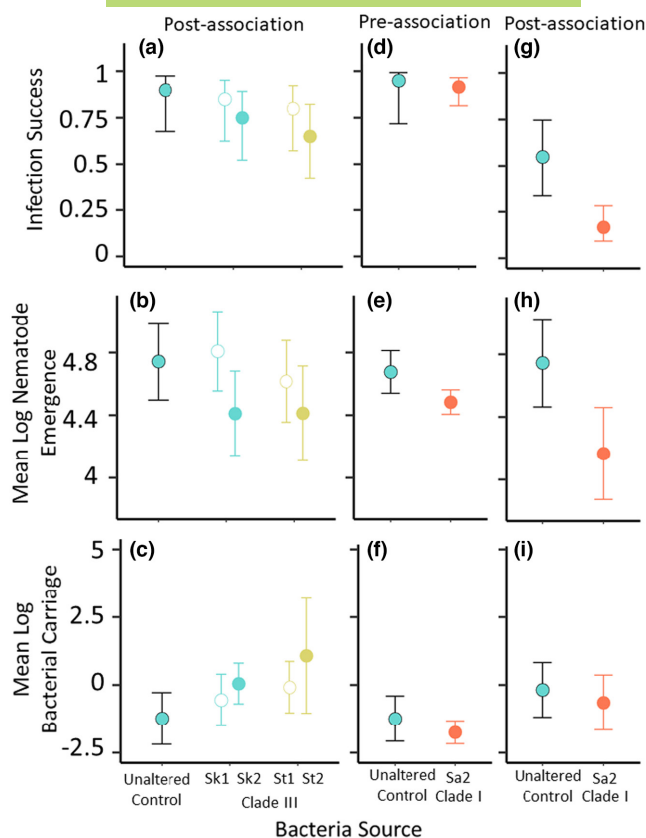
FIGURE 5 Results of experimental associations of clade III (*Steinernema kraussei*) nematodes with *Xenorhabdus bovienii* bacteria isolated from nematodes of the same species (Sk1 and Sk2), different species (St1 and St2) or different clade (Sa2). Pre-association data (infections where bacteria is injected into caterpillars) are not available for coinfections within clade III, so only post-association data is shown. When the resulting progeny used to infect new caterpillars, (a) infection success (proportion of caterpillars with emerging nematode progeny), (b) mean $\log_{10}$ number of nematodes that emerged from successful infections, and (c) mean $\log_{10}$ number of bacterial cells carried per nematode did not significantly differ from each other or the unaltered controls, indicating no barriers to host switching across nematode species in the same clade. In contrast, when Sa2 bacteria (from clade I nematodes) were injected into caterpillars along with aposymbiotic nematodes (d) infection success was high, but (e) $\log_{10}$ mean number of nematodes that emerged from successful infections was significantly lower than the unaltered control, indicating a pre-association barrier to host switching. Nevertheless, (f) mean $\log_{10}$ number of bacterial cells carried per nematode did not differ between these cross nematode–clade associations and the unaltered control, such that these nematodes were able to (g) successfully infect a new caterpillars, albeit at a lower rate and with (h) less reproductive output than the unaltered controls, demonstrating post-association barriers to host switching across clade. Although this barrier is not complete, the emerging nematodes still did not exhibit a difference in bacterial carriage (i). All symbols show the estimated marginal means with 95% confidence intervals

post-association barriers in the form of lower infection success and nematode reproduction were greater than the pre-associations barriers seen for Sa2.

4 | DISCUSSION

The purpose of the present study was to identify pre- and post-association barriers to host switching between sympatric species of the *Steinernema-Xenorhabdus* mutualism (Figure 1). All experimental pairings moving *X. bovienii* bacteria between nematodes of different clades (I vs III) exhibited barriers to the association. Clade I (Sa) nematodes paired with bacteria from clade III (Sk and St) nematodes had no success after association due to low bacterial carriage. In addition, Clade III (Sk) nematodes faced increased mortality or delayed development in the presence of bacteria from clade I (Sa1 and Sa2, respectively) nematodes. This pre-association barrier completely prevented host switching between the clades for one strain (Sa1), and it led to reduced measures of fitness post-association for the other (Sa2). By contrast, we did not find barriers in any pairing within clades. Thus, despite being a considered a specialized, coevolved mutualism, we found that Sk nematodes could partner with bacteria from a different nematode species within the same clade with no fitness costs. Taken together, these results indicate that pre-association barriers limit symbiont switching between nematode clades, but that symbionts can switch between closely related nematode hosts.

Associations between clade I (Sa) nematodes and bacteria from clade III nematodes were prevented by low bacterial carriage (Figure 4c), indicating a pre-association barrier. This result is consistent with the idea that partner choice can prevent costs to the association (Sachs et al., 2004), as low infection success and poor nematode reproduction were observed in most of these pairings. This finding was further supported by the *in vitro* assay results, which indicated that bacteria from clade III (Sk) nematodes may not nutritionally support clade I (Sa) nematodes (Figure 3c,d). It is possible that a signal related to nutrition is correlated with attachment in the nematode receptacle. This could prevent cheater bacteria within the association from being transmitted. It would also fortuitously prevent associations with more distant bacterial strains, which do not produce the same compound.

It seems reasonable to suggest that pre-association barriers such as partner choice could evolve to prevent low fitness of mismatched pairs. However, one pairing shows a contradictory pattern. Coinfections of clade I (Sa) nematodes with bacteria (Sk1) from clade III nematodes had low bacterial carriage, a strong pre-association barrier which prevented the association; although this pairing was not significantly different in infection success or mean nematode emergence compared with reassociation controls (Figure 4a,b). Even further, this experimental pair had *improved* infection success compared with the unaltered controls. This shows that, rather than a reproductive cost, these experimental pairs could actually benefit the new partners if they associated. Why then do we see such strong pre-association barriers? The symbiont specificity of clade I (Sa) nematodes, combined with the virulence of its bacteria towards competitors, could indicate close coevolution. Remember that native symbionts of clade I (Sa) are virulent to clade III (Sk) nematodes (Figure 2g,h). In addition, the only natural circumstance in which

these nematodes would encounter non-native bacteria would be competitive coinfections. Thus, Clade I (Sa) nematodes may have faced selective pressure to only associate with bacteria that can kill nematode competitors (Hillman & Goodrich-Blair, 2016).

In contrast, clade III (Sk) nematodes seem not to be choosy. Despite the costs to associating with Sa2 bacteria from clade I nematodes (Figures 2h and 5e), clade III (Sk) nematodes carry this bacteria and their native bacteria (Figure 5f). This lack of specificity could allow for host switching. However, the post-association barriers observed limit the success of these novel pairings (Figure 5g,h). Thus, in the absence of partner choice, the partner-fidelity feedbacks seen post-association favour bacteria associations among clade III nematodes (Figure 5a,b) and serve to prevent Sa2 bacteria from switching to Sk1 hosts in nature (Sachs et al., 2004; Foster & Wenseleers, 2006; see also Figure 5 Murfin et al., 2015).

We did not find barriers to association at the nematode species level within clade III—*S. kraussei* nematodes associated equally well with bacteria from *S. texanum* nematodes as they did with their own bacteria. This result is perhaps surprising, because Murfin et al. (2015) found both pre- and post-association barriers within some clade III pairings. Additionally, one might predict that in sympatry these barriers might be reinforced to reduced fitness costs associated with host switching. However, as Murfin et al. did not detect barriers to host switching in all of the clade III pairings, it could be that our nematode species are more recently diverged than the species that showed barriers in their study. To test this, we compared the genetic difference between the nematode species at the 28S locus (656 bp). Murfin et al. found barriers to host switching for nematode species pairs showing >1.37% sequence divergence, whereas our two clade III species show more divergence (2.59%). Thus, it is not lack of general genetic divergence between the nematode hosts that explains lack of barriers to host switching we observed. Alternatively, perhaps, the frequent contact of sympatric pairs has selected for the ability to switch partners. In soil cores containing any of the nematode species used in this experiment, co-occurrence was found in 18% of samples from an adjacent study site (A. Ramesh, unpublished data).

The barriers explored in this study are not an exhaustive set of possibilities, even for our system. Future experiments on host switching in this system could explore additional barriers to association, such as increased juvenile mortality, decreased symbiont carriage over time or bacteria–bacteria antagonism (Chapuis et al., 2009; Flores-Lara et al., 2007; Hawlena et al., 2012). These experiments would not only explore the extent that barriers prevent host switching in sympatric species of this nematode–bacteria mutualism, but they would also provide insights into how coevolutionary trajectories can differ within a mutualism. In addition to these system-specific future directions, considering mutualisms in light of barriers to host switching could allow us to create more general predictions. These predictions might mirror theoretical predictions for barriers to speciation (Rundle & Nosil, 2005). For example, in general, pre-association barriers might be expected to build up more rapidly in sympatry than allopatry to prevent costs of unfavourable

host switches. Ultimately, this framework could be used to test hypotheses about the prevalence and consequences of host switches in mutualist systems.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.0rxwdbs2f.

PEER REVIEW

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