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Inter-strain interactions of a vector-borne parasite over its life cycle

Borrelia afzelii – Mus musculus – Ixodes ricinus model

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Abstract

Many pathogens consist of genetically distinct strains. When hosts are simultaneously infected with multiple strains the phenomenon is known as a mixed infection or a co-infection. In mixed infections, strains can interact with each other and these interactions between strains can have important consequences for their transmission and frequency in the pathogen population. Vector-borne pathogens have a complex life cycle that includes both a vertebrate host and an arthropod vector. As a result of this complexity, interactions between strains can occur in both the host and the vector. Interactions between strains in the vertebrate host are expected to influence transmission from the co-infected host to uninfected vectors. Conversely, interactions between strains in the arthropod vector are expected to influence transmission from the co-infected vector to the uninfected host. This thesis used the tick-borne bacterium, *Borrelia afzelii*, as a model system to investigate how co-infection and interactions between strains influence their transmission and lifetime fitness over the course of the tick-borne life cycle. *B. afzelii* is a common cause of Lyme disease in Europe, it is transmitted by the castor bean tick (*Ixodes ricinus*) and it uses small mammals (e.g. rodents) as a reservoir host. An experimental approach with two genetically distinct strains of *B. afzelii* (one Swiss stain, one Finnish strain) was used to investigate the effects of co-infection in both the host and the vector.

In Chapter 1, lab mice were experimentally infected via tick bite with either 1 or 2 strains of *B. afzelii*. The infected mice were then fed upon by *I. ricinus* ticks from a laboratory colony to quantify host-to-tick transmission. qPCR was used to determine the presence and abundance of each strain in the ticks. Chapter 1 found that co-infection in the mice reduced the host-to-tick transmission success of the strains. This chapter also found that co-infection reduced the abundance of each strain in the tick. This is one of the first studies to show that co-infection is important for determining the abundance of the pathogen strains in the vector.

In the lifecycle of *B. afzelii*, the bacterium is acquired by larval ticks that blood feed on an infected host. These larvae subsequently moult into nymphs that are responsible for transmitting the bacterium to the next generation of hosts. The bacterium has to persist inside the midgut of the nymph for a long time (8 – 12 months). Chapter 2 investigated whether nymphal ageing (1-month-old vs 4-month-old nymphs) under different environmental conditions (summer vs winter) influenced the interactions between strains in co-infected ticks. The spirochete abundance inside the nymph decreased with nymphal age, but there was no effect of the environmental conditions investigated.

In Chapter 3, the presence and abundance of the two strains of *B. afzelii* were quantified in the tissues of 6 different organs (bladder, left ear, right ear, heart, ankle joint, and dorsal skin) that were harvested from the co-infected and singly infected mice. This study showed that co-infection in the mouse host reduced the prevalence of the Finnish strain in the host tissues (but the Swiss strain was not affected by co-infection). Chapter 3 found a positive relationship between the prevalence (or abundance) of each strain in the mouse tissues and the host-to-tick transmission of each strain. External tissues (e.g. ears) were more important for host-to-tick transmission than internal organs (e.g. bladder). Chapter 3 enhances our understanding of the biology of mixed infections by showing the causal links between co-infection in the host, the distribution and abundance of the strains in host tissues and the subsequent host-to-tick transmission success of the strains.

Chapter 4 investigated how co-infection in the arthropod vector influences vector-to-host transmission success. A second infection experiment was performed, where naïve mice were exposed to nymphs that were either co-infected or infected with one of the two strains (i.e., using the nymphs generated in Chapters 1 and 2). The infection status of the mice was then tested using the same qPCR-based methods. Importantly, Chapter 4 confirmed that the negative effect of co-infection in the mouse on host-to-tick transmission (observed in Chapters 1, 2, and 3) had real fitness consequences for subsequent tick-to-host transmission. Ticks that had fed on co-infected mice were much less likely to transmit their strains to the host because these strains were less common inside these co-infected ticks. Chapter 4 did not find evidence that co-infection in the nymph influenced the nymph-to-host transmission success of each strain. This Chapter did find that there was a two-fold difference in nymph-to-host transmission success between the two strains.

This work provides evidence for the idea that vector-borne pathogen strains can exhibit trade-offs across the different steps of their complex life cycles. In the co-infected mice, the Swiss strain had higher host-to-tick transmission success than the Finnish strain. Conversely, the Finnish strains had higher spirochete loads in the tick vector and had tick-to-host transmission success. Thus, the Swiss and Finnish strains are specialized on the host versus the vector, respectively.

KEYWORDS: *Borrelia afzelii*, co-infection, inter-strain competition, Lyme borreliosis, *Ixodes ricinus*, transmission, vector-borne pathogen

Résumé

Plusieurs espèces de pathogènes sont constituées de souches génétiquement différentes. Lorsque des hôtes sont simultanément infectés par plusieurs souches, l'infection est alors nommée mixte ou co-infection. Lors des infections mixtes, les souches présentes peuvent interagir et cela peut influencer leur succès de transmission et par conséquent, la fréquence de chaque souche dans la population du pathogène. Les pathogènes à transmission vectorielle ont un cycle de vie complexe, qui inclut à la fois un hôte vertébré et un vecteur arthropode. Ainsi, les interactions inter-souches peuvent se produire à la fois dans l'hôte et dans le vecteur. Les interactions dans l'hôte pourraient donc influencer la transmission des souches vers le vecteur et inversement, les interactions dans le vecteur pourraient influencer la transmission des souches vers un hôte naïf. Dans cette thèse, la bactérie à transmission vectorielle *Borrelia afzelii* fut utilisée comme modèle, pour investiguer les interactions inter-souches et comment celles-ci influencent la transmission des souches et leur fitness au travers de leur cycle de vie. *B. afzelii* est l'une des bactéries responsables de la maladie de Lyme en Europe. Cette bactérie est transmise par la tique du chien (*Ixodes ricinus*) et utilise les petits mammifères (par ex. les rongeurs) comme hôtes réservoirs. Deux souches génétiquement distinctes de *B. afzelii* (suisse et finlandaise) furent utilisées afin d'examiner les effets de la co-infection dans l'hôte et le vecteur.

Dans le chapitre 1, des souris de laboratoire furent expérimentalement infectées, via la piqûre de tiques, avec une souche ou deux souches de *B. afzelii*. Pour établir l'efficacité de la transmission hôte-vecteur des souches, des tiques *I. ricinus* de notre colonie de laboratoire furent nourries sur les souris infectées. Celles-ci furent ensuite testées pour déterminer quantifier la présence des souches. Le chapitre 1 révéla que les co-infections dans les souris réduisirent le succès de transmission hôte-vecteur des souches ainsi que leur abondance dans les tiques. Ce chapitre est l'une des premières études à démontrer que la co-infection est un facteur important pour déterminer l'abondance des souches dans le vecteur.

Dans le cycle de vie *I. ricinus*, les larves acquièrent la bactérie lorsqu'elles se nourrissent sur un hôte infecté. Ces larves muent ensuite en nymphes, qui sont responsables de la transmission de la bactérie à la nouvelle génération d'hôtes l'année suivante. La bactérie doit donc survivre dans l'intestin des nymphes durant une longue période (8 – 12 mois). Le chapitre 2 testa l'influence de l'âge des nymphes (1 mois vs 4 mois) et de différentes conditions environnementales (été vs hiver), sur l'interaction des souches présentes dans les nymphes. Ce chapitre révéla que l'abondance des souches diminua avec l'âge des nymphes, mais que les conditions environnementales testées n'eurent pas d'impact.

Dans le chapitre 3, la présence et l'abondance des deux souches furent quantifiées dans 6 organes (la vessie, l'oreille droite, l'oreille gauche, le cœur, l'articulation de la cheville et la peau du dos) de souris infectées avec l'une des souches ou les deux. Cette expérience montra que la co-infection dans les souris réduisit la prévalence de la souche finlandaise dans les organes testés. À l'inverse, la souche suisse ne fut pas affectée par la co-infection. Une relation positive entre la prévalence (ou l'abondance) de chaque souche dans les tissus des souris et le succès de transmission hôte-vecteur de chaque souche fut trouvée. De plus, il fut établi que les tissus externes (par ex. les oreilles) sont plus importants pour définir le succès de transmission hôte-vecteur des souches, que les organes internes (par ex. la vessie). Le chapitre 3 contribua à améliorer notre compréhension de la biologie des infections mixtes, en démontrant les liens de causalité entre les coinfections dans l'hôte, la distribution et abondance des souches dans les tissus de l'hôte et, par conséquent, le succès de transmission hôte-vecteur des souches.

Le chapitre 4 investigua l'influence des infections mixtes dans le vecteur sur le succès de transmission vecteur-hôte des souches. Des nymphes coinfectées ou infectées avec une souche uniquement, se nourrirent sur des souris naïves, puis le statut d'infection de celles-ci fut déterminé. Ce chapitre confirma que l'impact négatif des co-infections dans l'hôte sur le succès de transmission hôte-vecteur des souches (observé dans les chapitres 1,2 et 3) influençait le succès de la transmission suivante (vecteur-hôte) et donc le fitness des souches. La probabilité de succès de la transmission vecteur-hôte des tiques, qui se nourrirent sur les souris coinfectées, était plus faible, car ces souches étaient moins abondantes dans les tiques coinfectées. Le chapitre 4 ne trouva, cependant, pas d'évidence que la co-infection dans les nymphes influence le succès de transmission vecteur-hôte des souches. Ce chapitre montra également une différence de succès de transmission nymphe-hôte des souches de l'ordre de 2.

Cette thèse renforce l'idée que les souches de pathogènes à transmission vectorielle peuvent subir des trade-offs dans différentes étapes de leurs cycles de vie complexes. Dans les souris coinfectées, la souche suisse avait une meilleure transmission hôte-vecteur que la souche finlandaise. À l'inverse, la souche finlandaise était plus abondante dans la tique et sa transmission tique-vecteurs était supérieure. Ainsi, les souches suisse et finlandaise se sont mieux adaptées à l'hôte et au vecteur respectivement.

MOTS-CLES : *Borrelia afzelii*, co-infection, compétition inter-souche, maladie de Lyme, *Ixodes ricinus*, transmission, pathogène à transmission vectorielle.

Table of contents

1. General introduction

1.1. Mixed infections

Many pathogen populations consist of genetically distinct strains. A consequence of strain diversity is that pathogens often establish mixed or multiple-strain infections in their host [2-4]. Advances in molecular biology have greatly contributed to the study of mixed infections, which has important consequences for different fields, such as public health and evolutionary biology. Mixed infections complicate the genetic identification and analysis of pathogens [2] and the development of vaccines [2, 5-8]. Genetic exchange (recombination) between strains can create new pathogen genotypes [2], which has led to the rapid selection for antimicrobial-resistant strains [2, 5, 9]. Compared to single strain infections, disease severity in co-infections can be more or less harmful [3, 4]. Therefore, the presence of mixed infections, strain interactions, and their consequences should be included in the development of control measures [8, 10].

Mixed infections are common in the host [2, 4, 11] and the presence of multiple strains sets the stage for interactions. Strains from the same pathogen species can vary in different life history traits such as virulence, infectivity, and growth rate [12-17] which can influence their performance when alone or in mixed infections. The performance of a strain in a single infection is not necessarily predictive of its performance in a mixed infection [4, 18]; in particular, strains that do well alone might not do well in a mixed infection. In multiple strain infections, the outcome of the inter-strain interaction can either be facilitation [19, 20] or competition [12, 21-23]. Under a neutral or null expectation, the performance of both strains in a co-infection is identical to the sum of their performances when each strain is alone in a single strain infection. In facilitation (or cooperation), the performance of the strains in the co-infection is enhanced relative to their single strain performance. In competition (or inhibition), the performance of the strains in the co-infection is reduced compared to their single strain performance. The outcome of these interactions can vary greatly depending on the identity of the pathogen species or pathogen strains [2, 4]. Interactions between strains can influence critical pathogen life history traits like ability to establish infection in the host and transmission to new hosts [3]. Thus, studying interactions between strains and their effects on strain fitness is critical for understanding the strain composition of pathogen populations in nature.

Genotypes that exploit the same environment are expected to undergo stronger competition [24]. Mechanisms that underlie competitive interactions can be classified into three different categories [2, 10, 24]. (1) Direct interference competition through the production of harmful substances. For example, the interaction between two species of respiratory bacteria, *Streptococcus pneumoniae* and *Staphylococcus aureus* [25], is mediated by the production of hydrogen peroxide (H_2O_2) by *S. pneumoniae*, which is lethal to *S. aureus* [26]. (2) Exploitation competition over shared limited resources such as nutrients or space. Different strains of the malaria parasite *Plasmodium chabaudi* compete over the red blood cells of their rodent host for multiplication and for the production of their sexual stages [27]. (3) Apparent competition mediated by the immune system of the host. Apparent competition occurs whenever one strain stimulates an immune response that reduces the performance of another strain. A study on mixed infections of *P. chabaudi* found that strains suffered less from competitive exclusion in immunodeficient mice compared to immunocompetent mice [28]. However, identifying the underlying mechanism remains difficult and some systems may by characterized by multiple mechanisms [10].

The outcome of interactions between pathogen species or strains can depend on the mechanism of competition [4, 10]. Exploitation competition tends to favour aggressive fast-growing strains that can monopolize limited resources [29]. For example, a study of mixed infections of the malaria parasite *P. chabaudi* in lab mice showed that strains that were most abundant in the blood were more likely to be transmitted to feeding mosquito vectors compared to strains that were less abundant in the blood [22]. In this system, co-infection and competition selected for fast-growing strains that caused more pathology (e.g. weight loss, red blood cell loss) in the rodent host. However, when infection success depends on the production of costly public goods, co-infection can favour avirulent strains. For example, the bacterial pathogen *Pseudomonas aeruginosa* consists of virulent strains that produce iron-binding molecules (siderophores) that are necessary to establish infection and other avirulent 'cheater' strains that do not produce these costly molecules [30]. Mixed infections favour the avirulent cheaters because they avoid the cost of producing siderophores by stealing them from the virulent producer strains, which ultimately reduces the pathology of the mixed infection to the insect host [30]. Competition can influence a wide variety of traits of the interacting strains, such as parasite abundance in host tissues [12, 21], parasite transmission success [22], and the presence/absence of parasite strains in host tissues [13]. In extreme cases of competition, parasite strains can be segregated into different tissues, or the presence of one parasite strain can prevent another strain from establishing infection in the host, a phenomenon known as competitive exclusion [12, 21].

All else being equal, fast-growing strains that establish higher abundance in the tissues of their hosts are expected to have higher transmission and thus outcompete slower growing strains that establish lower abundance in host tissues. This phenomenon should lead to the dominance of fast-growing strains and the loss of strain diversity over time. In nature, however, pathogen strain diversity is often maintained over time and space [31-34]. In the face of competition, less competitive strains resort to different mechanisms to avoid extinction, which involve life history trade-offs [35]. The competition-colonization trade-off is an example, where inferior competitors in the host have better transmission success than the superior competitors. This trade-off was shown in two species of parasitic lice and their pigeon hosts; the species of louse that was the inferior competitor in co-infections was better at establishing infections in new hosts (i.e. superior colonization ability) [36, 37]. Under exploitation competition, divergence in the use of host resources by the pathogen strains is expected [10, 38], and can result in the colonization of different organs in the same host [39] or in the colonization of different host species [40, 41]. A study on two strains of *Trypanosoma brucei brucei* in tsetse fly found that one strain was found more frequently in the proventriculus, whereas the other strain was found more frequently in the posterior midgut [39]. Another study on two sympatric trematode species, *Diplostomum spathaceum* and *Diplostomum gasterostei* found that they had diverged and used different species of snails as intermediate host [40]. This niche shift will diminish the cost of competition, and can even result in the facilitation of mixed infections [39]. Studying competitive interactions in mixed infections can therefore help us understand the mechanisms underlying the maintenance of strain diversity in nature [24].

1.1.1. Mixed infections in vector-borne parasites

Vector-borne pathogens have a life cycle that includes a vertebrate host and an arthropod vector. The vector-borne life cycle contains two very different transmission steps: transmission from infected hosts to feeding arthropod vectors (i.e. host-to-vector transmission), and transmission from infected vectors to naïve hosts (i.e. vector-to-host transmission). Populations of vector-borne pathogens often consist of multiple strains and mixed infections can occur in both the vertebrate host and the arthropod vector. We expect that co-infection and interactions between strains can potentially influence each of the two transmission steps. Thus, co-infection and interactions between strains in the vertebrate host will influence the subsequent host-to-vector transmission success of the strains. Conversely, co-infection and interactions between strains in the arthropod vector will influence the vector-to-host transmission success of the strains. Studies on mixed infections of vector-borne pathogens have typically focussed on the vertebrate hosts where acquired immunity plays a critical role in mediating interactions between strains [12, 14, 22, 42-46]. In contrast, the role of arthropod vectors has been largely ignored because they have traditionally been viewed as needles that simply transmit what they acquired from the vertebrate host [47]. As a result, few studies have investigated mixed infections in the arthropod vector [13, 39, 48]. However, interactions between strains inside the arthropod vector are likely to be both common and important for strain fitness. Populations of vector-borne pathogens grow and develop inside their arthropod vectors and these dynamic changes are influenced by vector traits as well as abiotic factors. Recent studies have shown that interactions between pathogens strains can occur in arthropod vectors and outcomes include both facilitation and competition [39, 48-50]. A study on a rodent malaria parasite (*P. chabaudi*) and their mosquito vector (*Anopheles stephensi*), found that the presence of a resident strain in the mosquito midgut enhanced the probability of infection by a subsequent strain [48]. Conversely, two studies on different tick-borne pathogens have found that tick vectors can act as bottlenecks, restricting the number of strain that are acquired from the host and transmitted to the host [49, 50]. In summary, co-infection and interactions between strains in the arthropod vector will influence their transmission and hence their frequency in the pathogen population.

1.2. Lyme Borreliosis (LB)

1.2.1. Lyme disease

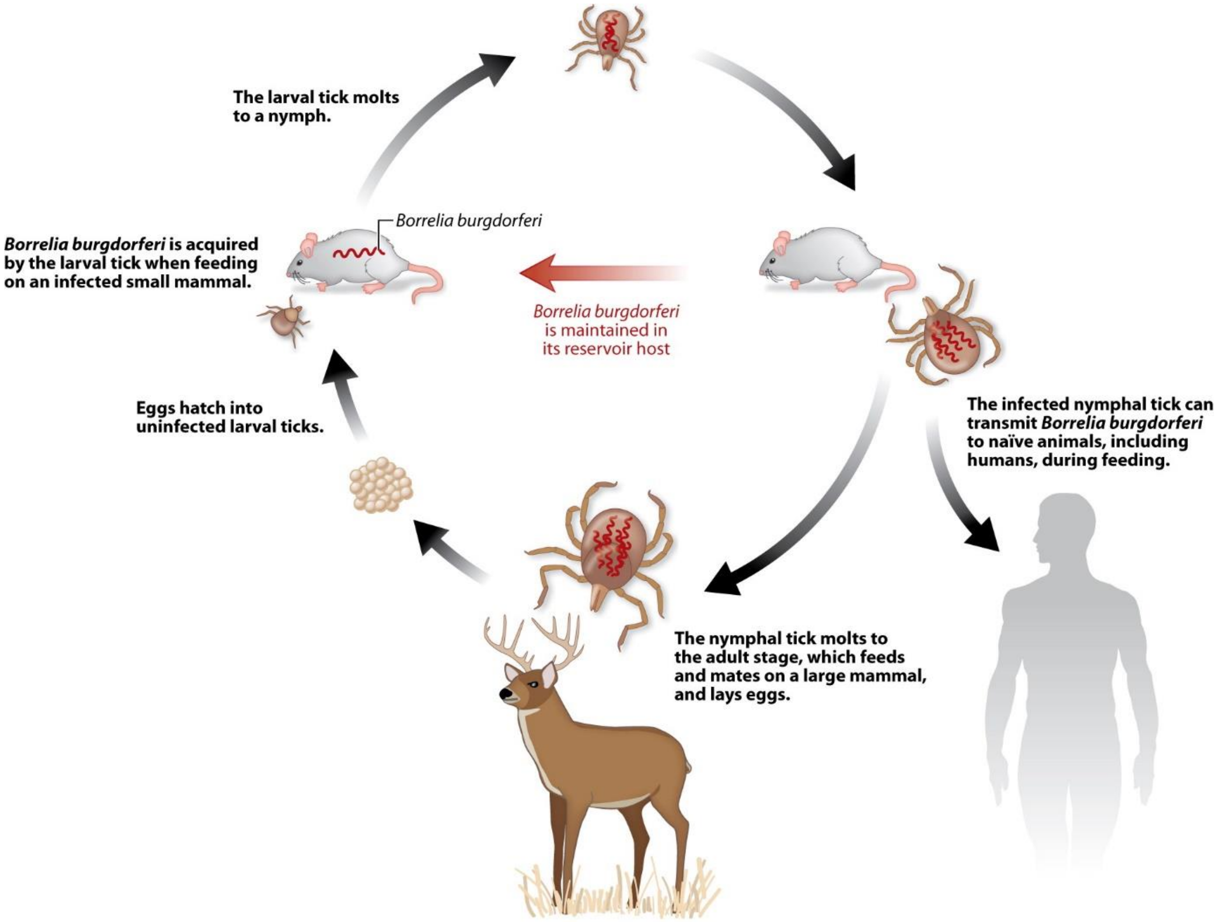
Lyme Borreliosis is the most common vector-borne disease in North America and Eurasia [51-53]. The etiological agents of LB are tick-borne spirochete bacteria that belong to the *Borrelia burgdorferi* sensu lato (sl) genospecies complex. The disease derives its name from the city of Old Lyme, Connecticut, USA [54], where it was first identified. The pathogen was named after the Swiss microbiologist Willy Burgdorfer, who was the first to culture spirochetes from blacklegged ticks [55]. LB is a chronic inflammatory disease that affects many different organ systems [56]. The first symptom, which is highly diagnostic, is an erythema migrans; a ring-shaped lesion on the skin around the site of the tick bite that expands in circumference over time. From the site of the tick bite, the bacteria use the skin and blood to disseminate to different organs such as the heart, spinal cord, joints, and skin where they cause cardiac, neurological, rheumatological, and dermatological disorders, respectively [57]. If the infection is left untreated, it can develop into a chronic infection that persists for years [56]. Symptoms in humans vary according to the genospecies of *B. burgdorferi* sensu lato involved [56].

1.2.2. *Borrelia burgdorferi* sensu lato

To date, the *B. burgdorferi* sl (Bbsl) genospecies complex contains 19 genospecies [58-60] of which four are known to be pathogenic for humans: *B. burgdorferi* sensu stricto (ss), *B. afzelii*, *B. garinii*, and *B. spielmanii* [59, 61, 62]. Some genospecies can infect a wide variety of hosts, while other are associated with specific reservoir hosts [58, 63]. For example, *B. burgdorferi* ss in North America is considered to be a host generalist that can infect different species of mammals and birds [64, 65]. In contrast, the most common genospecies in Europe, *B. afzelii* and *B. garinii*, use rodents and birds as reservoir hosts, respectively [66, 67]. This host specificity is mediated by the innate immune system of the vertebrate host, more specifically by its complement system [68].

1.2.3. The life cycle of *Ixodes* ticks

The *B. burgdorferi* sl genospecies are transmitted to vertebrate hosts by hard ticks that belong to the genus *Ixodes*. The main vectors of LB in the United States and in Europe are the blacklegged tick (*I. scapularis*) and the castor bean tick or sheep tick (*I. ricinus*), respectively. Both tick species are generalists and feed on a variety of mammal, bird, and lizard species [53, 69]. Their life cycle consists of three blood-seeking stages: larva, nymph and adult. Each stage requires a blood meal either to develop into the next stage (larva and nymph) or to produce eggs (adult female tick); the exception is adult male ticks that do not blood feed (Fig. 1). Newly hatched larvae are not infected with *B. burgdorferi* sl because transovarial transmission of spirochetes is rare [70, 71]. Larval ticks acquire *B. burgdorferi* sl after blood-feeding on a previously infected host; they then develop into infected nymphs that transmit the pathogen back to the reservoir host population the following year. Thus, in between the larval and nymphal blood meals, the spirochete population spends 8-12 months in the midgut of the nymph where it has to survive through different events such as molting, digestion of the blood meal, and winter. To survive the cold winter months, ticks overwinter under the leaf litter [72, 73] where they enter a dormant state called diapause [74-76]. The following spring, the ticks emerge from diapause, they become active, and search for hosts in a behaviour known as questing. The questing activity of *Ixodes* ticks and the epidemiology of *B. burgdorferi* sl are highly seasonal [51]. Nymphs are generally active before larvae, which ensures that nymphs infect the reservoir hosts before they are fed upon by the larvae [51]. This phenology increases the probability that the larvae feed on hosts that were previously infected by nymphs. Adult ticks feed on large vertebrates, such as deer, that are incompetent hosts for *B. burgdorferi* sl pathogens [77-79]. The nymph is therefore the most important stage for the transmission of *B. burgdorferi* sl and is therefore the stage where interactions between strains are most likely to occur.



Brisson D, et al. 2012.
Annu. Rev. Genet. 46:515–36

Figure 1. The enzootic life cycle of *Borrelia burgdorferi* sl [1]

1.2.4. Population dynamics of *B. burgdorferi* sl pathogens during the lifecycle

The population size of *B. burgdorferi* sl spirochetes undergo dramatic fluctuations in the immature ticks. Understanding these spirochete population dynamics is important because they influence transmission success. During the larval blood meal, larval ticks acquire ~100 spirochetes from the infected host [80], and this inoculum expands inside the larval midgut [81]. Acquisition of spirochetes by larval ticks (i.e. host-to-tick transmission success) has been correlated with the abundance of the spirochetes in the host tissues [82, 83], with the duration and volume of the larval blood meal [84, 85]. Following acquisition during the larval blood meal, *B. burgdorferi* sl has to establish infection in the midgut of the larval tick, survive the digestion of the blood meal (this process takes months), the larva-to-nymph moult (~6 weeks after the larval blood meal), and persist for ~12 months until the nymphal blood meal. After the larvae-to-nymph moult, the spirochete population size in the nymph reaches a peak of 2,000 to 32,000 cells [80, 86, 87]; the spirochete population then decreases over time [80] or reaches a plateau [88]. During the nymphal blood meal, spirochetes replicate and migrate from the midgut to the salivary glands through the hemocoel in ~24 hours [89-91]. The spirochete abundance in the tick salivary glands is low [91] and nymphs inoculate ~100 spirochetes into the vertebrate host [92]. The nymph-to-host transmission success depends on the duration of nymphal attachment [90, 93]. The relationship between the spirochete population size in the nymphal midgut and nymph-to-host transmission success has not been investigated. A field study found that *B. afzelii* strains that established a high spirochete load in field-captured *I. ricinus* nymphs had higher frequency in the pathogen population (as measured in *I. ricinus* nymphs in another study from the same location) [32]. This study suggests that *B. afzelii* strains with high spirochete load in the nymphal tick have higher nymph-to-host transmission success [32]. To study how strains interact inside the rodent host and the tick vector, it is important to have a good understanding of the population dynamics of *B. burgdorferi* sl pathogens in both the vertebrate host and the tick vector. Anytime the spirochete population expands dramatically (e.g. following inoculation into the vertebrate host or following acquisition by larval ticks), there is potential for differences in growth rates between strains to change their relative abundance in either the vertebrate host or the tick vector.

1.2.5. Strain typing systems and the role of the OspC protein

In addition to the diversity among the genospecies of the *B. burgdorferi* sl complex, each genospecies is further composed of multiple strains. These strains are genetically distinct entities that differ in phenotypes including bacterial abundance in the tissues of the vertebrate host, pathology in the vertebrate host, host-to-tick transmission, bacterial abundance in the tick vector, and ability to disseminate in human hosts [17, 82, 86, 94-96]. The *ospC* gene is often used to differentiate among strains of *B. burgdorferi* sl [41, 97-100]. The *ospC* gene is highly polymorphic within each *Borrelia* genospecies and the *ospC* alleles can be classified into so-called *ospC* major groups (oMGs) [94, 97, 101]. The percentage of nucleotide divergence between the *ospC* alleles is used to define the oMGs. The members of an oMG are less than 2% divergent in their DNA sequence, and >8% divergent from sequences belonging to another oMG [94, 101]. This nucleotide divergence translates into OspC antigens that are 20% to 60% divergent in amino acid sequence. To date, ~20 oMGs have been found in each of the three genospecies that are known to be pathogenic for humans, *B. burgdorferi* ss, *B. garinii*, and *B. afzelii* [102-104].

The *ospC* gene exists as a single copy on circular plasmid 26 [105, 106] and codes for the outer surface protein C (OspC), which is necessary for the establishment of infection in the vertebrate host [107, 108]. OspC production is induced about 36 hours after the beginning of the nymphal blood meal [109, 110], and during the migration of the spirochetes from the nymph mid gut to the nymph salivary glands [110, 111]. OspC is an immunodominant antigen that is strongly targeted by the immune system of the vertebrate host [112, 113]. It is generally believed that selection by the acquired immune system of the vertebrate host has caused the *ospC* gene to diversify into the different oMG alleles [31, 94, 101]. The diversity of the *ospC* gene is used by strains to avoid cross-reactive antibody responses and to facilitate super-infection of previously infected hosts [102, 114, 115]. A host that is infected by a given *ospC* strain develops a protective antibody response against all strains carrying that particular OspC antigen, but it does not protect the host against infection with another *ospC* strain [82, 114, 116, 117]. Another peculiarity of the OspC antibody response is that while protective against strains carrying the same OspC antigen, it does not clear the resident *ospC* strain because *B. burgdorferi* pathogens reduce the expression of the *ospC* gene once they establish infection in the host tissues [118]. The strong protective antibody response explains the strong nucleotide divergence (>8%) and amino acid divergence (20% to 60%) between strains carrying different oMGs and OspC antigens. If the differences between two OspC antigens are too small, the resultant cross-reactive antibody response will make it difficult for the two strains to co-exist in the same local Lyme disease system. Thus, a genetic divergence of >8% (and an associated amino acid divergence of >20%) is necessary to evade the cross-reactive antibody response of the other oMG alleles and their encoded OspC antigens.

Strains of *B. burgdorferi* sl genospecies can also be distinguished using the multi-locus sequence typing (MLST) system, which is based on 8 housekeeping genes that are located on the main bacterial chromosome [119]. The MLST system has been very useful for investigating the phylogenetic relationships between genospecies and between strains [119, 120]. The main disadvantage of the MLST method is that it cannot handle mixed strain infections [121]. The reason is because if a sample contains two strains with two different alleles at each of two loci (e.g. alleles A1 and A2 at locus A and alleles B1 and B2 at locus B), it is not possible to determine the linkage groups (unless these are known 'a priori'). An advantage of the *ospC* gene typing system is its ability identify mixed infections, and this is one of the main reasons why this strain typing system has been used [41, 42, 97, 101, 121]. Another advantage is that because the oMGs are highly divergent, they are easy to recognize, and the *ospC* strain diversity in a local ecosystem is not influenced by small changes in the nucleotide divergence thresholds used to classify ospC sequences into oMGs [97]. A disadvantage of the *ospC* gene typing system is that it is based on a single gene. Due to recombination and strong diversifying selection on the OspC antigen by the immune system of the vertebrate host, the *ospC* gene does not reflect the phylogenetic history of the *B. burgdorferi* sl genospecies or strains within genospecies [120, 122]. Across continental scales, *B. burgdorferi* sl genospecies contain hundreds of MLSTs but always the same limited set of ~20 oMGs. For populations of *B. afzelii* for example, the same oMGs are found in Sweden and Switzerland, but the MLSTs between these two countries will be very different [33, 42, 97, 123]. Thus, a criticism of studies that use *ospC* typing to monitor the frequencies of *ospC* strains over time is that the same oMG could be linked with different MLSTs [33, 123]. However, studies at smaller spatial scales (e.g. hectares) have shown that there is strong linkage disequilibrium between oMG and MLST [124-126]. One explanation for this strong linkage disequilibrium is that cross-reactive antibody responses to the same OspC antigen would make it difficult for two strains with the same oMG but different MLSTs to co-exist in the same local Lyme disease system. These observations suggest that at small spatial scales, the *ospC* gene could be a relatively good guide to the rest of the genome [125]. For the present PhD thesis, the two *B. afzelii* strains that we used, NE4049 and Fin-Jyv-A3, carried oMGs A10 and A3, respectively, and we were therefore able to use the *ospC* typing system for strain identification and quantification.

1.2.6. Mixed infections in the vertebrate host and the tick vector

Mixed strain infections of the same *B. burgdorferi* sl genospecies are commonly found in both vertebrate hosts and immature *Ixodes* ticks [32, 41, 42, 46, 97-99, 101]. Surveys of *B. afzelii* strain diversity in field-captured small mammal hosts in Sweden found that > 50% of the infected hosts carried more than one strain of *B. afzelii* [42, 125]. On average, small mammal hosts were infected with 2.2 strains of *B. afzelii* [46]. Larval ticks that feed on a host that is carrying multiple strains will therefore acquire mixed strain infections. Surveys of field-captured *I. ricinus* nymphs found a high prevalence of mixed infections [32, 97]. Two independent field studies on a local population of *I. ricinus* in Neuchatel, Switzerland found that 77–79% of *B. afzelii*-infected nymphs carried multiple strains and that the mean strain richness was 2.4–2.9 strains per nymph [32, 97]. These field studies show that co-infections of *B. afzelii* strains are common in nature, and that there is substantial potential for interactions between strains in both the vertebrate host and the tick vector. Studies on co-infections of *B. burgdorferi* sl strains in rodent hosts have found evidence for competitive interactions in host tissues [42, 46] and with apparent consequences for the host-to-tick transmission success of strains [44, 45]. In contrast, no studies have investigated whether co-infections of *B. burgdorferi* sl strains in immature *Ixodes* ticks result in interactions between strains and whether these interactions influence the nymph-to-host transmission success of the strains.

1.2.7. The maintenance of strain diversity of *B. burgdorferi* sl pathogens in nature

Studies on wild population of *B. burgdorferi* sl strains have found that strains carrying a dozen different oMGs can co-exist at the spatial scale of a few hectares in the field [31, 32, 41, 97, 101]. Long-term studies of the frequencies of *ospC* strains in *B. afzelii* populations in the tick vector and the vertebrate host have shown that the *ospC* strain composition is relatively constant over time [32, 33], which suggests that the frequencies of these *ospC* strains are maintained by strong selective forces. Numerous researchers have suggested that the diversity of the *ospC* gene is under balancing selection [31, 41, 99, 101]. Two alternative hypotheses have been proposed to explain the maintenance of *ospC* strain diversity.

Negative frequency-dependent selection (NFDS), mediated by the immune system of the vertebrate hosts, suggests that rare *ospC* strains have a fitness advantage over common *ospC* strains [99, 101]. Wang et al. (1999) proposed that common *ospC* strains are less likely to establish persistent infection in the vertebrate host, because the host would develop a stronger antibody response against these common strains due to repeated exposure via nymphal tick bite [101]. However, this particular mechanism seems unlikely because *B. burgdorferi* sl pathogens establish chronic infection in the vertebrate host [127]. Once *B. burgdorferi* sl pathogens have established infection in the tissues of the rodent host, they stop expressing the *ospC* gene [118]. Thus, it is difficult to imagine how repeated exposure to the same OspC antigen via tick bite would somehow influence the fate of the same resident *ospC* strain that is no longer expressing this OspC antigen. A recent experimental study showed that female bank voles can transmit maternal antibodies to their offspring, which protects them from infectious challenge [128]. Rodent reproduction occurs during the summer, which coincides with the time when nymphs feeding on and infecting their rodent hosts. If a particular *ospC* strain is common in the nymphal population, the mothers will develop IgG antibodies to this common *ospC* strain and transmit them to their offspring, which will protect the offspring against this common *ospC* strain. Less common *ospC* strains would have the advantage of invading a largely susceptible offspring generation. Thus, maternal transfer of antibodies is a potential source of NFDS by which the immune system of the vertebrate host could maintain the diversity of *ospC* strains in nature.

Multiple niche polymorphism (MNP) hypothesis proposes that the different *ospC* strains are adapted to different host species [1, 41]. Thus, under MNP the prevalence of the *ospC* strains depends on the abundance of their reservoir host. To date, the only evidence for MNP comes from field studies that have found that certain *ospC* strains or certain MLST strains are associated with certain vertebrate hosts [41, 129]. The problem with these field surveys is that it is always possible that the observed host-specificity patterns are driven by spatio-temporal variation in exposure to the different strains rather than intrinsic differences among host species in their susceptibility to different strains. Other studies investigating host-specificity did not find the expected associations between *ospC* strains and host species [33, 65]. The field survey of Brisson and Dykhuizen (2004) that led to the creation of the MNP hypothesis, actually found that most *ospC* strains were found in most host species, but that some strains had much higher host-to-tick transmission success in certain host species compared to other host species. An alternative explanation is that competitive interactions between strains in the vertebrate host were responsible for these patterns. Finally, if there was perfect separation of the *ospC* strains into different hosts (i.e. perfect host specificity), then there would be no selection on the *ospC* gene to evolve a pattern of divergence that minimizes cross-reactive antibody responses between OspC antigens. The fact that the *ospC* alleles have evolved to minimize cross-reactive antibody responses between OspC antigens suggests that *ospC* strains do frequently encounter each other in the same vertebrate host. Thus, studies that explore interactions between strains of *B. burgdorferi* sl in both the host and the vector can provide additional information to our understanding of the maintenance of *B. burgdorferi* sl strain diversity in nature.

2. Aims of the PhD project

This PhD thesis used *B. afzelii* as a model multi-strain vector-borne pathogen for studying how mixed infections influence the fitness of the constituent strains. *B. afzelii* is one of the most common genospecies in Europe [130, 131]. This spirochete bacterium is transmitted by *Ixodes ricinus* ticks and uses small mammals (rodents and insectivores) as reservoir hosts. Across Europe, the genospecies of *B. afzelii* contains ~20 *ospC* strains [97]. Ticks carrying multiple *ospC* strains of *B. afzelii* are common in nature. However, to date there is no experimental study that has investigated the nature of the interactions between strains of the same *B. burgdorferi* sl genospecies in the tick vector. The aim of this PhD thesis is to characterize the interactions between two strains of *B. afzelii* in their tick vector (*I. ricinus*) and a model rodent host (*Mus musculus*) to understand how the spirochete populations of both strains change over the life cycle, and to test whether co-infection can explain the transmission success of the strains.

This PhD thesis consists of two separate studies. In the first study, we used an experimental infection approach to create mice that were either singly infected with one of two strains, Fin-Jyv-A3 and NE4049, or co-infected with both of these strains. These mice were infested with *I. ricinus* larvae to measure whether co-infection in the rodent host influences the host-to-tick transmission success of the strains. In the second study, we used the nymphs from the first study to challenge naïve mice. This study allowed us to investigate whether co-infection in the nymph influences the tick-to-host transmission success. The presence/absence of the strains and their densities were measured using strain-specific qPCR assays. The different chapters of this PhD thesis explore the interactions between two *B. afzelii* strains at different steps of their tick-borne life cycle. Chapters 1, 2, and 3 are based on the first study and chapter 4 is based on the second study. In all four chapters, the effect of co-infection was measured by comparing the performance of the focal strain in single infection to the performance of the focal strain in co-infection.

In chapter 1, we investigated whether co-infection and interactions between two *B. afzelii* strains in their rodent host (*Mus musculus*) influenced the host-to-tick transmission success of the strains. We also investigated whether co-infection and interactions between strains in the tick vector influenced the abundances of the two strains in the tick vector. Mice were infected with either one of the strains or co-infected with both strains. Larval *I. ricinus* were fed on the mice and were allowed to moult into nymphs. We measured the prevalence and densities of the two strains in 1-month-old nymphs.

In chapter 2, we tested the effects of nymphal age and season on the interaction of the two *B. afzelii* strains in *I. ricinus* nymphs. Larval ticks were fed on mice infected with either one strain of *B. afzelii* or co-infected with both strains. Engorged larvae were allowed to moult into nymphs that were subsequently exposed for a duration of 90 days to three seasonal treatments, artificial summer, artificial winter, and natural winter, which differed in temperature and light conditions. We measured the presence and abundance of each strain in the immature ticks. In this chapter, we also described the spirochete population dynamics in immature ticks by quantifying spirochete loads in engorged larvae, 1-month-old nymphs, and 4-month-old nymphs.

In chapter 3, we studied the interactions between the two *B. afzelii* strains in the tissues of the rodent host (*M. musculus*), using mice that were either singly infected or co-infected. We measured the presence and abundance of each strain in 6 organs of the mice. We also tested whether the distribution (presence/absence and abundance) of the two strains in the host tissues influenced the host-to-tick transmission of the strains.

In chapter 4, we investigated the effect of co-infection and interactions between strains in the nymphs on the tick-to-host transmission success of the strains. We used nymphs from the first study, that were either singly infected or co-infected. Each naïve mouse was infested with one challenge nymph (one nymph per mouse). The strain-specific infection status of the engorged nymphs and the mice they infested were determined to examine the nymph-to-host transmission success of the strains.

3. Chapter 1

Competition between strains of Borrelia afzelii inside the rodent host and the tick vector

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The supplementary material of this chapter can be found in the appendix section.

ABSTRACT

Multiple-strain pathogens often establish mixed infections inside the host that result in competition between strains. In vector-borne pathogens, the competitive ability of strains must be measured in both the vertebrate host and the arthropod vector to understand the outcome of competition. Such studies could reveal the existence of trade-offs in competitive ability between different host types. We used the tick-borne bacterium *Borrelia afzelii* to test for competition between strains in the rodent host and the tick vector and to test for a trade-off in competitive ability between these two host types. Mice were infected via tick bite with either one or two strains, and these mice were subsequently used to create ticks with single or mixed infections. Competition in the rodent host reduced strain-specific host-to-tick transmission and competition in the tick vector reduced the abundance of both strains. The strain that was competitively superior in host-to-tick transmission was competitively inferior with respect to bacterial abundance in the tick. This study suggests that in multiple-strain vector-borne pathogens there are trade-offs in competitive ability between the vertebrate host and the arthropod vector. Such trade-offs could play an important role in the coexistence of pathogen strains.

KEYWORDS: *Borrelia afzelii*, co-infection, inter-strain competition, *Ixodes ricinus*, life history trade-off, transmission

INTRODUCTION

Many populations of pathogens consist of genetically distinct strains. As a result of this strain diversity, hosts are often infected with multiple strains, a phenomenon known as co-infections, mixed infections, or multiple-strain infection [1-3]. Co-infection implies that the strains can interact with each other, which can result in cooperation or competition [3-5]. Empirical evidence for inter-strain competition typically shows that the performance (e.g. abundance, transmission) of a given strain in a co-infection is reduced compared to when it occurs alone [6-10]. Pathogen strains with similar ecological niche requirements will experience intense competition, which can result in competitive exclusion and loss of strain diversity [11-13]. Thus, a fundamental task is to understand the mechanisms that can maintain strain diversity in the face of strong within-host competition and one potential explanation is life history trade-offs [5, 14].

A commonly assumed life history trade-off for pathogens is between transmission and virulence [15, 16], which is essentially a trade-off between current and future reproduction [14]. In mixed infections, competition between strains should select for fast-growing virulent strains that can monopolize the limited host resources and thereby outcompete slow-growing avirulent strains [6, 8, 17-20]. At the pathogen population level, a diversity of strains can be maintained because virulent strains outcompete avirulent strains in mixed infections, but the reverse is true in single strain infections [21, 22]. Other life history trade-offs that have been reported for pathogens include trade-offs between multiplication rate (and presumably competitive ability) and persistence in the abiotic environment [23, 24], and between competitiveness and ability to colonize new hosts [25]. Given the variety of pathogen life cycles, we should expect a similar diversity of trade-offs between competitive ability and other life history traits.

Trade-offs in competitive ability might be particularly common in parasites that must infect more than one host type to complete their life cycle [2]. In these complex or vector-borne life cycles, the genes and phenotypes that lead to competitive success in one host type (e.g. vertebrate host) may have little bearing on within-host competition in another host type (e.g. arthropod vector). Despite the plausibility of this idea, there are few studies that have compared the strain-specific competitive ability between different host types [26]. In the case of vector-borne pathogens, numerous studies have investigated interactions between strains in the vertebrate host [6, 10, 12, 27-31] but similar studies in the arthropod vector are rare [32-35], and we are not aware of any studies that have investigated both host types. To investigate these questions, we used *Borrelia afzelii*, a spirochete bacterium that requires both a vertebrate host and an arthropod vector to complete its life cycle as a model system.

Borrelia afzelii belongs to the *Borrelia burgdorferi* sensu lato (sl) genospecies complex, which include the etiologial agents of Lyme borreliosis in North America and Eurasia [36, 37]. In Europe, *Borrelia afzelii* is a common genospecies [36, 38, 39], which is transmitted by the hard tick *Ixodes ricinus* and uses rodents as reservoir hosts [38, 39]. The life cycle of *I. ricinus* consists of three stages: larva, nymph, and adult. The larvae acquire *B. afzelii* from infected rodents during the larval blood meal, and develop into infected nymphs that transmit the pathogen back to the reservoir host population the following year. The engorged larva and the resultant nymph are therefore the two stages where interactions between strains are most ecologically important. Populations of *B. burgdorferi* sl consist of multiple strains and mixed infections are common in both the vertebrate host [10, 29, 40, 41] and the tick vector [41-44]. Competition between strains of *B. burgdorferi* sl in the vertebrate host has been shown in field studies [10, 29] and experimental infections [9, 45, 46]. Field studies on our local *I. ricinus* population found that ~80% of *B. afzelii*-infected nymphs were infected with multiple strains and that the mean strain richness was 2.4–2.9 strains per nymph [42, 44]. In co-infected nymphs, the spirochete load per strain decreased with increasing strain richness, and this result provides indirect evidence for competition [44]. However, to date there is no direct experimental evidence that competition between strains of *B. burgdorferi* sl can occur in the tick vector.

The purpose of this study was to test whether strains of *B. burgdorferi* sl compete inside their rodent host and their tick vector and whether there was a trade-off in strain-specific competitive ability between the two host types. Mice were infected via tick bite with either one or two strains of *B. afzelii*. The infected mice were infested with larval ticks and these were allowed to molt into nymphal ticks that carried either single strain infections or co-infections. The mouse-to-tick transmission success and the spirochete load in the nymph were used as measures of competitive success in the rodent host and the tick vector, respectively. We predicted that inter-strain competition in the mouse and the nymph would reduce the strain-specific mouse-to-tick transmission success and the strain-specific spirochete load, respectively. We also predicted that the strain that was competitively superior in the rodent host would be competitively inferior in the tick vector, and vice versa.

MATERIALS AND METHODS

Mice, ticks, and strains of *B. afzelii*: Forty female, pathogen-free *Mus musculus* BALB/c mice aged 5 weeks were used as the rodent reservoir host. All *I. ricinus* ticks came from the *Borrelia*-free laboratory colony that has been maintained at the University of Neuchâtel since 1978. *B. afzelii* isolates Fin-Jyv-A3 and NE4049 were used in this study, which were obtained from a bank vole (*Myodes glareolus*) in Finland and an *I. ricinus* nymph in Switzerland, respectively. We had originally started the study with two Swiss strains, but one of the strains failed and we used strain Fin-Jyv-A3 as a back-up solution. Fin-Jyv-A3 has *ospC* major group (oMG) A3, multi-locus sequence type (MLST) 676, and strain ID number 1961 in the *Borrelia* MLST database. Isolate NE4049 has oMG A10, MLST 679, and strain ID number 1887 in the *Borrelia* MLST database. The purity of these isolates with respect to the oMG allele has been assessed using 454-sequencing. For isolates Fin-Jyv-A3 and NE4049, 137 and 1313 *ospC* gene sequences were obtained, respectively, and all but one belonged to the correct oMG. We are confident that these isolates are genetically homogeneous and will hereafter refer to them as strains Fin-Jyv-A3 and NE4049.

I. ricinus nymphs infected with either strain Fin-Jyv-A3 or strain NE4049 were created as follows. Female BALB/c mice (n = 5) were infected with one of the two strains via needle inoculation. At 4 weeks post-infection, *Borrelia*-free larval ticks from our laboratory colony of *I. ricinus* were fed on these mice. Engorged larval ticks were placed in individual eppendorf tubes and were allowed to moult into nymphs. At 4 weeks after the larva-to-nymph moult, a random sample of nymphs was selected for each strain and tested for *B. afzelii* infection using qPCR. The percentage of nymphs infected with *B. afzelii* was 70% (7/10) for strain Fin-Jyv-A3 and 71.4% (10/14) for strain NE4049.

Infection of mice via tick bite with one or two strains: The study consisted of experiments 1 and 2 where the focal strains were Fin-Jyv-A3 and NE4049, respectively. In experiment 1, mice were randomly assigned to infection with strain Fin-Jyv-A3 (n = 10 mice) or to co-infection with strains Fin-Jyv-A3 and NE4049 (n = 10 mice). In experiment 2, mice were randomly assigned to infection with strain NE4049 (n = 10 mice) or to co-infection with strains NE4049 and Fin-Jyv-A3 (n = 10 mice). All mice were infected via tick bite. Mice in the co-infection treatments were infested with 5 Fin-Jyv-A3-infected nymphs and 5 NE4049-infected nymphs. Mice in the single-strain infection treatments were infested with 5 Fin-Jyv-A3-infected nymphs or 5 NE4049-infected nymphs. Each mouse in the single-strain infection treatment was also infested with 5 uninfected nymphs. When nymphs take a blood meal, they secrete saliva that contains immunosuppressive molecules [47] and for this reason, each mouse in the study was infested with the same number of nymphs. Details of the tick infestation procedure have been described elsewhere [48].

Four weeks after the nymphal challenge, ear tissue biopsies and blood samples were taken from each mouse to confirm their *B. afzelii* infection status [48]. A *Borrelia*-specific qPCR assay was used on the ear tissue samples to determine the presence of *B. afzelii* spirochetes. A SERION® ELISA classic *Borrelia burgdorferi* IgG/IgM immunoassay was used on the blood samples to determine the presence of *Borrelia*-specific IgG antibodies. The two infection phenotypes were 100% congruent.

Host-to-tick transmission: Five weeks after the nymphal challenge, each mouse was challenged with ~100 *Borrelia*-free xenodiagnostic larvae from our laboratory colony of *I. ricinus*. Blood-engorged larvae were kept in individual Eppendorf tubes, and were allowed to moult into xenodiagnostic nymphs under standard laboratory conditions (20–25°C, 12 light: 12h dark). To maintain high humidity, each tube contained a piece of moistened paper towel. Four weeks after the larva-to-nymph moult, 10 live nymphs were randomly selected from each mouse and frozen at –20°C. To test whether the spirochetes were viable, we placed up to 3 xenodiagnostic nymphs in BSK medium for all *B. afzelii*-infected mice that had sufficient numbers of ticks. The cultures were checked using a dark field microscope on a weekly basis for the presence of live spirochetes.

DNA extraction: Four-week-old nymphs were crushed in the TissueLyser II using a previously described protocol [48]. The crushed nymphs were digested with proteinase K at 56°C overnight. The DNA of the nymphs was extracted using QIAGEN DNeasy 96 Blood and Tissue kit well plates and following the QIAGEN protocol. Each plate contained 2 negative DNA extraction controls (*Anopheles gambiae* mosquitoes). DNA from the mouse ear samples was extracted using QIAGEN DNeasy Blood & Tissue mini spin columns and following the QIAGEN protocol. Ear tissue DNA and nymph DNA were eluted into 65 µl of water.

General and strain-specific qPCR assays: Each tick was tested with three independent qPCR assays. A *Borrelia*-diagnostic qPCR assay that targets a 132 bp fragment of the highly conserved *flagellin* gene was used to determine infection and quantify the total spirochete load in each nymph. We developed two strain-specific qPCRs that allowed us to detect and quantify the oMG allele A3 of strain Fin-Jyv-A3 or the oMG allele A10 of strain NE4049. Each strain-specific qPCR used the same primers to amplify a 143 bp fragment of the *ospC* gene, but used a different strain-specific probe to detect the two different oMG alleles (see supplementary material for details). To validate the *ospC* qPCR assays, we created communities that differed in the percentage of the A3 and A10 alleles. This independent validation experiment showed that the *ospC* qPCR assays were highly reliable at estimating the frequencies of each of the two oMG alleles (see supplementary material for details).

The qPCRs were done using a LightCycler® 96 Multiwell Plate white (Roche). All the plates contained negative controls for the DNA extraction (mosquito DNA), negative controls for the qPCR (water), and 4 standards containing 10^2, 10^3, 10^4, and 10^5 gene copies. All the controls (and standards) were run in duplicate (or triplicate) in each plate. The quantification of copy gene numbers in the samples was done using the LightCycler® 96 Software (Roche). For each qPCR assay, a sample of 81 ticks was tested twice to determine the repeatability of the assay. The repeatability of the log10-transformed gene copy number for each of the three independent qPCR assays was very high (see supplementary material). The estimates of the *ospC* gene copy numbers were also highly correlated with the estimates of the *flagellin* gene copy numbers (see supplementary material). These results indicate that our methods of estimating the strain-specific spirochete load in the nymphal ticks are highly reliable.

STATISTICAL ANALYSES

All the statistical analyses were done using R version 3.4.2.

General statistical approach: The two measures of strain-specific fitness include host-to-tick transmission and the spirochete load inside the nymphal tick. The experimental design of the study contains two fixed factors that are orthogonal to each other: focal strain (2 levels: Fin-Jyv-A3, NE4049) and infection treatment (2 levels: single, co-infection). To determine the effect of competition, the performance of the focal strain (host-to-tick transmission and spirochete load in the nymphal tick) was compared between the single strain infection and the co-infection. A significant interaction between the focal strain and the infection treatment indicates that each strain is affected differently by the presence of the co-infecting strain.

Generalized linear mixed effects (GLME) models with binomial errors or linear mixed effects (LME) models with normal errors were used to analyse the response variables. The focal strain (2 levels: Fin-Jyv-A3, NE4049), the infection treatment (2 levels: single, co-infection), and their interaction were fixed factors and mouse identity was included as a random factor. To determine statistical significance, models that differed with respect to the fixed factor of interest were compared using a log-likelihood ratio test (LLR).

Host-to-tick transmission of *Borrelia afzelii*-infected nymphs: Host-to-tick transmission refers to the percentage of nymphs that acquired *B. afzelii* during their larval blood meal. The *flagellin* qPCR was used to determine the infection status of the ticks. Of the 346 ticks, 22 ticks (distributed over 17 different mice) were excluded from the analysis because they had contradictory results between the *flagellin* qPCR and the *ospC* qPCR. The strain-specific *ospC* qPCR was used to determine the presence of the focal strain in the ticks. Tick infection status with the focal strain was modelled as GLME model with binomial errors.

Spirochete loads of the nymphs: The analyses were done on the subset of infected nymphs. The gene copy number estimated from the *flagellin* qPCR assay was adjusted to give an estimate of the total spirochete load for the entire nymph. For nymphs that were co-infected with strains Fin-Jyv-A3 and NE4049, the estimates of the strain-specific spirochete loads from the *ospC* qPCR assays were constrained to sum to the total spirochete load estimated by the *flagellin* qPCR assay (see the supplementary materials for details). The strain-specific spirochete loads were log10-transformed to improve their fit to the normal distribution. The log10-transformed spirochete load of the focal strain was modelled as an LME model with normal errors.

RESULTS

Infection success: Three of the 40 mice in the study were excluded: two mice (S5 and S12) died during the study, and one mouse (S37) did not become infected with *B. afzelii* following the nymphal challenge. In the co-infection treatment, four mice (S17, S20, S25, and S29) were only infected with strain Fin-Jyv-A3. Two of these four mice had been challenged with at least one NE4049-infected nymph. These four mice and their nymphs were excluded from the analyses because the aim of our study was to test whether strains in co-infected mice and co-infected nymphs experienced competition. Including these four mice in the statistical analyses made the results more statistically significant (see the supplementary material). The final analysis therefore included 301 nymphs from 33 *B. afzelii*-infected mice (Table 1). For a subsample of 29 infected mice, we obtained live cultures of *B. afzelii* from one or more nymphs; this result indicates that the mice transmitted live spirochetes to the nymphs.

Comparison of fitness between strains Fin-Jyv-A3 and NE4049: For the mice infected with one strain, the host-to-tick transmission of Fin-Jyv-A3 (91.8% = 67/73) was significantly higher than NE4049 (77.9% = 67/86; GLME LLR: $p = 0.021$). The nymphal spirochete load of strain Fin-Jyv-A3 (n = 67, mean = 7109, 95% CI = 4947–10216) was twice that of strain NE4049 (n = 67, mean = 3497, 95% CI = 2434–5026; LME LLR: $p = 0.038$). Thus, in single strain infections, strain Fin-Jyv-A3 outperformed strain NE4049 in both phenotypes.

For the co-infected mice, 91.5% of the nymphs (130/142) were infected with *B. afzelii*. Of the 130 infected nymphs, 17.7% (23/130) carried strain Fin-Jyv-A3 alone, 34.6% (45/130) carried strain NE4049 alone, and 47.7% (62/130) carried both strains. For these 142 nymphs, host-to-tick transmission was 59.9% (85/142) for strain Fin-Jyv-A3 and 75.4% (107/142) for strain NE4049. Thus in co-infected mice, strain NE4049 was the superior competitor because it had higher host-to-tick transmission than strain Fin-Jyv-A3.

Effect of competition on host-to-tick transmission: The effect of co-infection on host-to-tick transmission was analysed separately for each focal strain (Fig. 1) because the interaction between the focal strain and infection treatment was significant (GLME LLR: $p = 0.0134$). In experiment 1, host-to-tick transmission of strain Fin-Jyv-A3 was significantly lower for the co-infected mice (49.3% = 34/69), compared to the single strain mice (91.8% = 67/73; Fig. 1; GLME LLR: $p < 0.001$). In experiment 2, host-to-tick transmission of strain NE4049 was lower but not significantly so for the co-infected mice (69.9% = 51/73) compared to the single strain mice (77.9% = 67/86; Fig. 1; GLME LLR: $p = 0.487$). There was a significant negative effect of competition on the host-to-tick transmission of strain NE4049 when all 10 mice in the co-infection treatment were included in the analysis (see supplementary material).

Comparison of the total nymphal spirochete load between single strain and co-infection treatments: In experiments 1 and 2, there was no significant difference in the total spirochete load (as estimated by the *flagellin* qPCR) between the nymphs that had fed as larvae on the co-infected mice and the nymphs that had fed as larvae on the mice infected with a single strain (see the supplementary materials for details).

Effect of competition on the nymphal spirochete load: The nymphal spirochete load of the focal strain is the number of spirochetes of that strain in the nymph. With respect to the log10-transformed spirochete load, focal strain (LME LLR: p = 0.009) and infection treatment (LME LLR: p = 0.003) were significant, but their interaction was not (LME LLR: p = 0.715; Fig. 2). For focal strain Fin-Jyv-A3, the nymphal spirochete load in the co-infection group (n = 69, mean = 3257, 95% CI = 1918–5531) was reduced by more than half compared to the single strain infection group (Fig. 2; n = 73, mean = 7109, 95% CI = 4879–10368). For focal strain NE4049, the nymphal spirochete load in the co-infection group (n = 73, mean = 1929, 95% CI = 1252–2972) was reduced by almost half compared to the single strain infection group (Fig. 2; n = 86, mean = 3497, 95% CI = 2396–5094). The parameter estimates of the LME model also show a 50% reduction in the nymphal spirochete load of the focal strain when the mouse was co-infected with another strain.

For the 62 nymphs that were co-infected with both strains, the mean spirochete load of Fin-Jyv-A3 (mean = 3286, 95% CI = 2217–4872) was significantly higher than that of NE4049 (mean = 1543, 95% CI = 1041–2287; LME LLR: p = 0.0002). Thus in co-infected ticks, strain Fin-Jyv-A3 was the superior competitor because it had a higher spirochete load than strain NE4049.

DISCUSSION

Our study found that the competitive ability of each strain differed depending on the host type in the life cycle of this important tick-borne pathogen. In co-infected mice, strain NE4049 was the superior competitor because it had higher host-to-tick transmission than strain Fin-Jyv-A3. In contrast, in co-infected ticks, strain Fin-Jyv-A3 was the superior competitor because its spirochete load was higher than strain NE4049. To our knowledge, this study is the first demonstration that in multi-strain pathogens with a vector-borne life cycle, the winner of inter-strain competition in the vertebrate host can be the loser of inter-strain competition in the arthropod vector.

One of the central questions in ecology is to understand the factors that allow a community of species (or strains) to persist over time [5, 49-52]. Others and we have previously shown that a dozen strains of *B. afzelii* can co-exist at the spatial scale of a soccer field [41, 42, 44, 53]. Two independent long-term studies on *B. afzelii* in ticks and reservoir hosts have shown that the community of strains is stable over a time period of at least a decade [53, 54]. In our system, the central question is how a dozen strains of *B. afzelii* can persist in the same local Lyme disease system [42, 44, 53]? A general explanation for co-existence is the presence of trade-offs where the competitive hierarchy between genotypes is reversed between different states [14]. Below, we give three examples of how competitive hierarchy could be reversed between two or more strains. First, trade-off between performance when alone and in co-infection; for example, in single infections, strain Fin-Jyv-A3 had higher host-to-tick transmission than strain NE4049, but in mixed infections the relationship was reversed (Fig. 1). Second, trade-off between different host types in the lifecycle; for example, strain NE4049 outcompetes strain Fin-Jyv-A3 in the rodent host but the relationship is reversed in the tick vector. Third, intransitive competition relationships between strains; for example, strain A beats B, B beats C, and C beats A. In summary, there are different trade-offs that could stabilize a community of a dozen *B. afzelii* strains co-existing in the same local Lyme disease system.

To our knowledge, this study is also one of the first experimental demonstrations that strains of a vector-borne pathogen experience competitive interactions inside the arthropod vector. Our study adds to a growing literature on interactions between strains of vector-borne pathogens within their arthropod vectors [33, 35]. A study on mixed infections of rodent malaria parasites in mosquitoes found that malaria strains had a greater chance to establish infection and reach a high density if the mosquito was already infected by another strain, which is an example of cooperation or facilitation [33]. A study on the tick-borne bacterium *Francisella novicida* found that a wild-type strain excluded other strains from establishing infection in the tick vector [35]. Our results are in agreement with our previous study, which found indirect evidence for competition between strains of *B. afzelii* in wild *I. ricinus* nymphal ticks [44]. That study found that the number of spirochetes per strain decreased as the strain richness increased inside the nymphs [44]. Negative associations between the spirochete abundances of some strains inside the nymphal tick were also shown [44]. The present study found a large effect size of inter-strain competition in the tick vector; co-infection in the tick reduced the spirochete abundance of each strain by 50%. A critical question is whether competition between strains in the nymph influences the strain-specific nymph-to-host transmission success.

Competition between strains in the arthropod vector could have important consequences if the observed reductions in spirochete load influence strain-specific vector-to-host transmission. During the nymphal blood meal, spirochetes migrate from the midgut to the salivary glands [55, 56], and the nymph inoculates about 100 spirochetes into the vertebrate host [57]. This small inoculum size suggests that competition between strains in the nymphal tick could influence the strain-specific nymph-to-host transmission success. We have recently shown that *B. afzelii* strains that establish high spirochete loads in wild *I. ricinus* nymphs are more common in the field [44]. This observation suggests that strains with high nymphal spirochete loads are more competitive and have higher nymph-to-host transmission success [44]. Previous studies on other vector-borne pathogens have shown that the arthropod vector can limit the genetic diversity of strains that is transmitted to the vertebrate host [35, 58]. For example, Rego and colleagues (2014) used a set of genetically tagged clones of *B. burgdorferi* ss to show that co-infected ticks transmit a subset of clones to the rodent host [58]. This result shows that the arthropod vector can act as a genetic bottleneck when they transmit mixed infections to the vertebrate host. The vector bottleneck would be even more important, if some strains are better than others at achieving vector-to-host transmission from co-infected vectors.

Competition between microbial species or strains can occur by three different mechanisms: interference, exploitation, and apparent competition [4, 59-62]. In interference competition, microbes produce toxic substances that weaken the performance (transmission, growth, reproduction) of other species or strains [63-65]. This mechanism is unlikely because *B. burgdorferi* sl does not produce toxic substances [66]. Exploitation competition happens when pathogen strains compete over limited host resources such as nutrients or space [8, 67]. For example, many species of free-living and pathogenic bacteria compete over iron [68, 69]. A recent theoretical study of the microbiome found that rapid population expansion of bacteria followed by extrinsic resource limitation by the host exacerbates competitive interactions and enhances community stability [52]. This condition is likely to be met in ixodid ticks where a single resource pulse (the blood meal) is followed by a rapid expansion of the microbial community [70-72]. Others and we have shown that following the larval blood meal, the population of *B. burgdorferi* sl expands rapidly from an initial inoculum of about 100 bacteria to a spirochete population size inside the nymph that ranges from 2000 to 32000 cells [73-76]. We have also shown that the spirochete loads in the nymphs decrease dramatically over time, further suggesting that the resources inside the nymphal midgut are limiting [74]. Thus, exploitation competition over limited resources probably underlies the competitive interactions between the two strains of *B. afzelii* observed in this study. Finally, in apparent competition, the host immune response triggered by one strain affects the fitness of another strain [31, 77]. Ticks contain immune defences such as antimicrobial peptides and phagocytic cells [78-81]. The observation that the total spirochete load inside the nymph is low, suggests that the tick immune system restricts the spirochete population inside the nymphal midgut to certain limits.

Our study found direct evidence for competitive interactions between strains of *B. afzelii* in the rodent reservoir host. This result is in agreement with other studies on the North American LB system of *B. burgdorferi* ss, *I. scapularis* ticks, and *P. leucopus* mice [9, 45] that have shown that co-infection reduces host-to-tick transmission success. In addition, we found evidence for asymmetric competition: co-infection reduced host-to-tick transmission of strain Fin-Jyv-A3 by ~50%, but had no effect on the host-to-tick transmission of strain NE4049. This result is not novel, as one of the studies on *B. burgdorferi* ss also found evidence of asymmetric competition [9]. If we include the 4 mice in the co-infected group that became infected with strain Fin-Jyv-A3 alone, competition significantly reduced host-to-tick transmission by ~30% in both strains (see supplementary material for details). In summary, there is strong evidence that co-infection of strains of *B. burgdorferi* sl in the rodent host reduces host-to-tick transmission success.

Competitive exclusion occurs when one pathogen strain prevents another strain from establishing infection in the host [11-13]. In the present study, 4 of the 20 mice in the co-infection treatment became infected with strain Fin-Jyv-A3 but not strain NE4049. This result suggests competitive exclusion: strain Fin-Jyv-A3 prevented strain NE4049 from establishing infection in the host. There is some evidence that competitive exclusion is important in the field [29]. A field study on mixed strain infections of *B. afzelii* in bank voles found that strains carrying genetically similar *ospC* alleles were less likely to co-occur in the same host, a pattern that is consistent with competitive exclusion [29]. We have shown that the majority of *I. ricinus* nymphs carry mixed strain infections [42, 44], which means that simultaneous exposure to multiple strains is common in populations of wild reservoir hosts. Thus our observation that competitive exclusion occurred in 20.0% of mice simultaneously exposed to two strains, suggests that this phenomenon could be important in the field.

In nature, *Ixodes* nymphs are frequently infected with multiple strains of a given species of *B. burgdorferi* sl [40-44]. Our work on a wild population of *I. ricinus* in Neuchâtel, Switzerland found that ~80% of nymphs infected with *B. afzelii* carried more than one strain [42, 44]. In the present study, the rodents were infected with a maximum of two strains whereas in the field, rodents are often infected with more than two strains [10, 29, 54]. These differences in strain richness in the rodent host explain why the percentage of co-infected nymphs (43.7%) in our experiment was lower than what has been observed in nature. Our results are in agreement with a recent experimental study using the North American Lyme disease system, which found that 24.5% of nymphs that had fed as larvae on co-infected mice acquired both strains [9]. A field study in North America also showed that larval *I. scapularis* ticks that feed on wild reservoir hosts often acquire multiple strain infections [40]. A remaining question is why some nymphs acquire a subset of strains present in the vertebrate host whereas other nymphs acquire the complete set of strains?

In the present study, competition between strains of *B. afzelii* was shown in both the rodent host and the tick vector. Competition in the rodent host reduced host-to-tick transmission of strain Fin-Jyv-A3 but not strain NE4049. Competition in the tick vector reduced the bacterial abundance of both strains by 50%, but strain Fin-Jyv-A3 had a higher abundance than strain NE4049. Thus, strain NE4049 was the superior competitor in the rodent host, whereas strain Fin-Jyv-A3 was the superior competitor in the tick vector. Future studies should investigate whether inter-strain competition in the tick has important consequences for the strain-specific nymph-to-host transmission success.

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Author contributions: D.G. and M.J.V. conceived and designed the study. D.G. and A.S. conducted the experiment and performed the molecular work. A.S., A.G.-C. and J.D. helped develop the *ospC*-specific qPCR. J.D. did 454-sequencing on isolates Fin-Jyv-A3 and NE4049 to confirm their purity. C.C. isolated Fin-Jyv-A3 and created the nymphs infected with this isolate. O.R. helped with the experimental infections. D.G. conducted the statistical analyses. D.G. and M.J.V. wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval: The commission that is part of the "Service de la Consommation et des Affaires Vétérinaires (SCAV)" of Canton Vaud, Switzerland evaluated and approved the ethics of this study. The Veterinary Service of the Canton of Neuchâtel, Switzerland issued the animal experimentation permit used in this study (NE04/2016).

TABLES AND FIGURES

Table 1. The proportion of *B. afzelii*-infected nymphs is shown for each of the 33 mice in the study. For each mouse, the geometric mean spirochete load for the subset of infected nymphs and the 95% confidence interval (95% CI) are also shown. Here A3 and A10 refer to strains Fin-Jyv-A3 and NE4049, respectively.

Exp	Strains	Mouse ID	Infected nymphs /total nymphs (%)	Spiro load Mean	Spiro load 95% CI
1	A3	S01	8/8 (100.0%)	18,408	6,613-51,235
1	A3	S02	9/9 (100.0%)	4,885	1,861-12,824
1	A3	S03	9/9 (100.0%)	9,309	3,546-24,436
1	A3	S04	6/8 (75.0%)	9,513	2,917-31,023
1	A3	S06	6/7 (85.7%)	1,672	513-5,454
1	A3	S07	8/8 (100.0%)	6,722	2,415-18,710
1	A3	S08	8/9 (88.9%)	9,523	3,421-26,504
1	A3	S09	6/7 (85.7%)	8,643	2,650-28,185
1	A3	S10	7/8 (87.5%)	4,771	1,597-14,251
1	A3 + A10	S11	8/10 (80.0%)	2,188	786-6089
1	A3 + A10	S13	10/10 (100.0%)	8,730	3,494-21,809
1	A3 + A10	S14	7/9 (77.8%)	1,963	657-5,863
1	A3 + A10	S15	9/10 (90.0%)	4,786	1,823-12,565
1	A3 + A10	S16	10/10 (100.0%)	4,898	1,961-12,236
1	A3 + A10	S18	10/10 (100.0%)	5,117	2,048-12,783
1	A3 + A10	S19	8/10 (80.0%)	6,839	2,457-19,036
2	A10	S31	6/8 (75.0%)	5,888	1,806-19,202
2	A10	S32	8/10 (80.0%)	9,094	3,267-25,312
2	A10	S33	10/10 (100.0%)	9,162	3,667-22,890
2	A10	S34	8/10 (80.0%)	2,563	921-7,134
2	A10	S35	6/10 (60.0%)	2,247	689-7,329
2	A10	S36	9/9 (100.0%)	4,986	1,900-13,090
2	A10	S38	6/10 (60.0%)	614	188-2,003
2	A10	S39	7/9 (77.8%)	1,820	609-5,436
2	A10	S40	7/10 (70.0%)	2,138	716-6,387
2	A10 + A3	S21	7/9 (77.8%)	4,169	1,396-12,453
2	A10 + A3	S22	10/10 (100.0%)	5,808	2,325-14,509
2	A10 + A3	S23	8/9 (88.9%)	6,978	2,507-19,423
2	A10 + A3	S24	10/10 (100.0%)	6,223	2,491-15,547
2	A10 + A3	S26	10/10 (100.0%)	7,907	3,165-19,753
2	A10 + A3	S27	7/7 (100.0%)	7,268	2,433-21,712
2	A10 + A3	S28	8/9 (88.9%)	6,550	2,353-18,232
2	A10 + A3	S30	8/9 (88.9%)	2,447	879-6,813

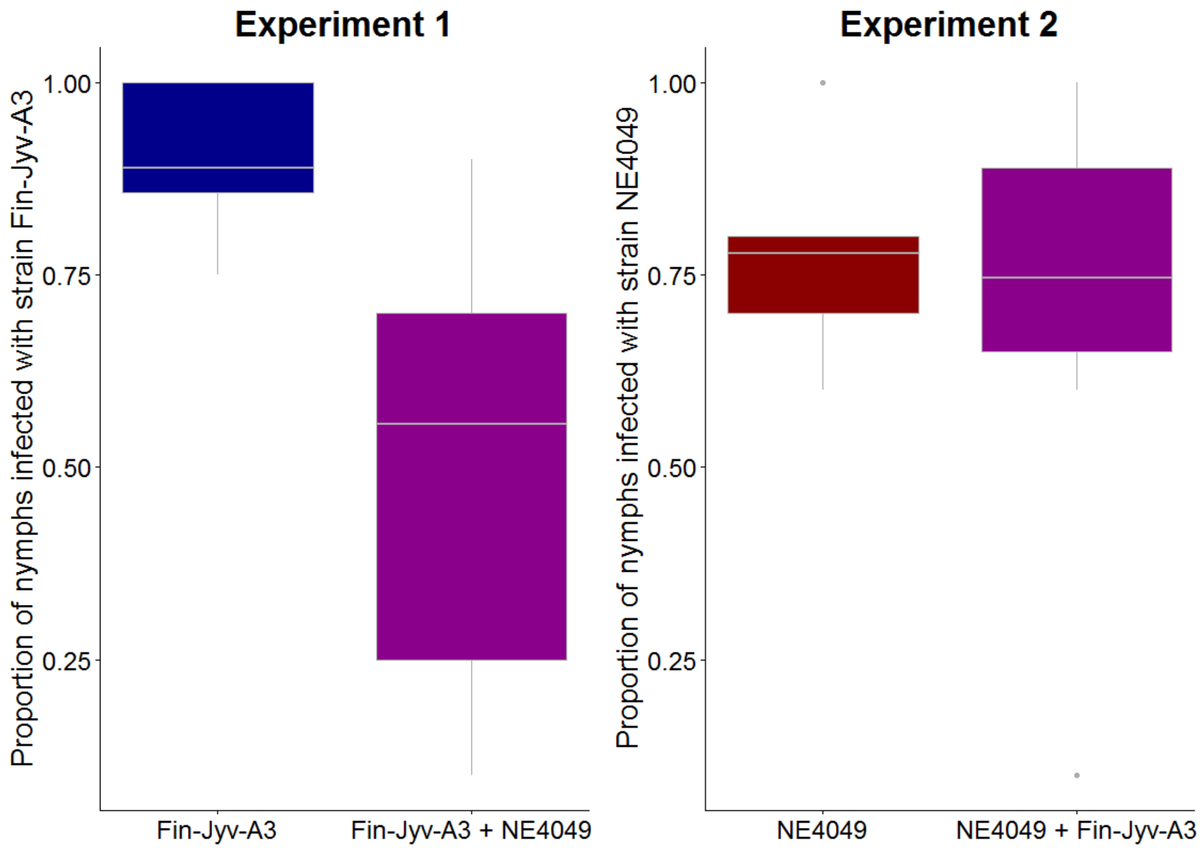

Figure 1. Co-infection reduces host-to-tick transmission for two strains of *B. afzelii*. In experiments 1 and 2, the focal *B. afzelii* strains are Fin-Jyv-A3 and NE4049, respectively. Experiment 1 shows that host-to-tick transmission of strain Fin-Jyv-A3 is reduced in the presence of strain NE4049. Experiment 2 shows that host-to-tick transmission of strain NE4049 is not affected in the presence of strain Fin-Jyv-A3. Each data point represents the mean for a single mouse (n = 33 mice). Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles).

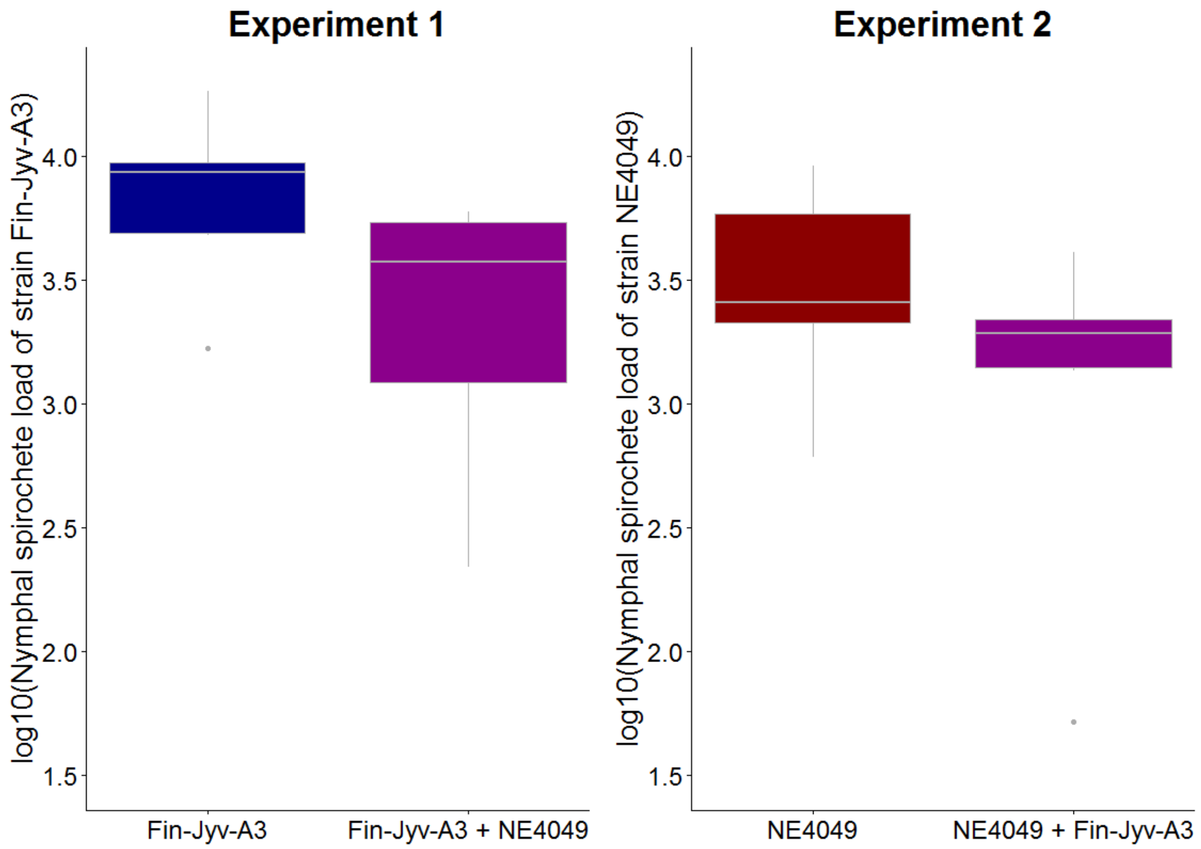

Figure 2. Co-infection reduces the spirochete load in *I. ricinus* nymphs for two strains of *B. afzelii*. In experiments 1 and 2, the focal *B. afzelii* strains are Fin-Jyv-A3 and NE4049, respectively. Experiment 1 shows that the nymphal spirochete load of strain Fin-Jyv-A3 is reduced by 50% in the presence of strain NE4049. Experiment 2 shows that the nymphal spirochete load of strain NE4049 is reduced by 50% in the presence of strain Fin-Jyv-A3. Each data point represents the mean for a single mouse (n = 33 mice). Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles).

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4. Chapter 2

Competition between strains of *Borrelia afzelii* in immatures *Ixodes ricinus* ticks is not affected by season

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The supplementary material of this chapter can be found in the appendix section.

ABSTRACT

Vector-borne pathogens often consist of genetically distinct strains that can establish co-infections in the vertebrate host and the arthropod vector. Co-infections (or mixed infections) can result in competitive interactions between strains with important consequences for strain abundance and transmission. Here we used the spirochete bacterium, *Borrelia afzelii*, as a model system to investigate the interactions between strains inside its tick vector, *Ixodes ricinus*. Larvae were fed on mice infected with either one or two strains of *B. afzelii*. Engorged larvae were allowed to moult into nymphs that were subsequently exposed to three seasonal treatments (artificial summer, artificial winter, and natural winter), which differed in temperature and light conditions. We used strain-specific qPCRs to quantify the presence and abundance of each strain in the immature ticks. Co-infection in the mice reduced host-to-tick transmission to larval ticks and this effect was maintained in the resultant nymphs at 1 and 4 months after the larva-to-nymph moult. Competition between strains in co-infected ticks reduced the abundance of both strains. This inter-strain competition occurred in the three life stages that we investigated: engorged larvae, recently moulted nymphs, and overwintered nymphs. The abundance of *B. afzelii* in the nymphs declined by 40.5% over a period of 3 months, but this phenomenon was not influenced by the seasonal treatment. Future studies should investigate whether inter-strain competition in the tick influences the subsequent strain-specific transmission success from the tick to the vertebrate host.

KEYWORDS: *Borrelia afzelii*, co-infection, inter-strain competition, *Ixodes ricinus*, transmission, Vector-borne pathogen, Lyme disease

INTRODUCTION

Many infections consist of multiple strains or genotypes of the same pathogen, which are called co-infections [1-3]. Co-infections (also referred to as mixed infections or multiple-strain infections) will result in positive or negative interactions between co-infecting strains that result in facilitation [4-6] or competition [7-14], respectively. In facilitation (positive interaction), the performance (e.g. transmission, fitness, abundance) of a particular strain in a mixed infection is enhanced compared to when this strain infects the host by itself. In competition (negative interaction), the performance of a particular strain in a mixed infection is reduced compared to the single-strain infection. Multiple-strain infections are important because interactions between strains in their host shape the optimal life history strategies of pathogen transmission and virulence, which is the level of harm that the pathogen causes in its host [1, 12].

In the case of vector-borne pathogens, mixed infections can occur in both the vertebrate host and the arthropod vector. Numerous studies have investigated interactions between pathogen strains in the vertebrate host [7-11, 15]. In contrast, studies on inter-strain interactions in the arthropod vector are rare [15-17], but these studies have shown that these interactions exist and that they influence the performance of the pathogen strains inside the arthropod vector. The population dynamics of vector-borne pathogens inside their arthropod vectors are also highly dependent on abiotic factors, such as temperature [18]. For example, warmer temperatures reduce the vectorial capacity of malaria mosquitoes [19] and high temperatures can clear the Lyme disease pathogen from ticks so that they are no longer infectious to mice [20]. Taken together, these observations suggest that temperature (and other abiotic variables) could influence the outcome of the interactions between strains in mixed infections in the arthropod vector, but to date there are no studies on this subject.

In the present study, we used the tick-borne spirochete, *Borrelia afzelii*, to test whether season (temperature and light) influences the competition between strains in mixed infections inside its long-lived tick vector. *B. afzelii* is the most common cause of Lyme borreliosis in Europe and is transmitted among small mammal reservoir hosts by the hard tick *Ixodes ricinus* [21]. Larvae acquire *B. afzelii* when they feed on an infected host (there is no transovarial transmission [22, 23]) and the engorged larvae moult into flat (unfed) nymphs by early fall. These flat nymphs overwinter in the soil [24, 25] and enter a diapause phase [26-28]. The nymphs become active the following spring and search for new hosts [28, 29]. From a life history perspective, the nymphs are the most important stage for tick-to-host transmission because they feed on competent reservoir hosts and because their density is an order of magnitude greater than adult ticks [30, 31].

In areas where Lyme borreliosis is endemic, mixed infections in the nymph are common [32-35]. A recent experimental infection study found that strains of *B. afzelii* experience competition inside *I. ricinus* nymphs [16]. The population size of *B. afzelii* inside the unfed nymph decreases over time suggesting that competition between strains could intensify with increasing nymphal age [36, 37]. There is indirect evidence that spirochete load in the nymph is an important phenotype for nymph-to-host transmission: *B. afzelii* strains with higher population sizes in nymphs are more common in the field [32]. In summary, Lyme disease is an interesting system for studying whether inter-strain competition changes over the life cycle of a long-lived arthropod vector and whether this competition is influenced by abiotic factors like temperature.

We recently used an experimental infection approach to show that strains of *B. afzelii* experience competition inside *I. ricinus* nymphs that were 1 month old [16]. As part of that study, we also collected engorged larvae, and we allowed a subset of nymphs to age under different seasonal treatments (i.e. different temperature and light conditions that represented summer versus winter), but these ticks were not analysed until now. The aim of the present study was to investigate whether competition between strains of *B. afzelii* occurs at different stages of the life cycle of immature *I. ricinus* ticks, and whether this competition is influenced by seasonal treatment. We made three predictions with respect to the interactions between interstrain competition, life cycle stage, and seasonal treatment. First, competition between strains would occur at each of the three life cycle stages: engorged larvae, 1-month-old nymphs, and 4-month-old nymphs. Second, the nymphal spirochete load would decrease with nymphal age and that competition between strains would be more intense in the older nymphs. Third, the nymphal spirochete load would decrease quickly under summer conditions but remain static under winter conditions.

MATERIAL AND METHODS

General: In a previous study, we investigated competition between two strains of *B. afzelii* (Fin-Jyv-A3 and NE4049) in the rodent host *Mus musculus* and in the tick vector *I. ricinus* [16]. We tested the effects of interstrain competition on two phenotypes of *B. afzelii*: strain-specific host-to-tick transmission and strain-specific spirochete load. Both of these phenotypes were measured in 1-month-old nymphs (n = 301) that had fed as larvae on experimentally infected mice, and that had been killed 1 month after the larva-to-nymph moult. What is new in the present study is that we investigated competition in the engorged larvae immediately following drop-off (n = 142), and in 4-month-old nymphs that were allowed to age under different seasonal treatments (n = 357). These two additional stages allowed us to investigate competition between strains of *B. afzelii* at three different time points of the tick life cycle.

Strains of *B. afzelii* and mice: *B. afzelii* strains NE4049 and Fin-Jyv-A3 were chosen for this study because both strains are highly infectious to rodent hosts [16]. Strains NE4049 and Fin-Jyv-A3 were obtained from an *I. ricinus* nymph in Neuchatel, Switzerland and from a bank vole (*Myodes glareolus*) in Jyväskylä, Finland, respectively. Both strains were passaged fewer than 5 times to avoid the loss of the plasmids that carry the virulence genes that are critical for infection [38, 39]. These strains have strain ID numbers 1961 and 1887 in the *Borrelia* multi-locus sequence type (MLST) database. Strain NE4049 has MLST 679 and *ospC* allele A10 and strain Fin-Jyv-A3 has MSLT 676 and *ospC* allele A3. The two *ospC* alleles used in this study (the A3 and A10 alleles) have a genetic distance of 23.19% and an amino acid distance of 62.57%. The concatenated sequences of these two MLSTs differ at 9 base pairs over 4785 bp (i.e. they are 99.81% similar).

The details of this experimental infection study were described in Genné et al (2018) and are shown in Figure 1. Briefly, we performed two independent experiments to test whether the presence of a competitor strain influenced the performance of the focal strain. In experiments 1 and 2, the focal strain was Fin-Jyv-A3 and NE4049, respectively. Each experiment contained two infection treatments: single infection with the focal strain and co-infection with both strains. Thus, 40 female *Mus musculus* BALB/c mice aged 5 weeks were randomly assigned to four different experimental infection groups (n = 10 per group): Fin-Jyv-A3 (Single infection), Fin-Jyv-A3 + NE4049 (Co-infection), NE4049 (Single infection), and NE4049 + Fin-Jyv-A3 (Co-infection). Mice were infected with the appropriate strains of *B. afzelii* via the bite of experimentally infected *I. ricinus* nymphs.

Mice in the co-infection treatment (Figure 1(A)), were infested with 5 nymphs putatively infected with strain Fin-Jyv-A3 nymphs and 5 nymphs putatively infected with strain NE4049 nymphs (total of 10 nymphs). Mice in the single strain infection treatments were infested with 5 nymphs putatively infected with the focal strain and 5 uninfected nymphs (total of 10 nymphs). Prior to the infestation, we determined the prevalence of infection by testing a random sample of Fin-Jyv-A3 nymphs (n = 10) and NE4049 nymphs (n = 14) using the *flagellin* qPCR. The prevalence of infection was 70.0% (7/10) for the Fin-Jyv-A3 nymphs and 71.4% (10/14) for the NE4049 nymphs. Thus, the expected number of infected nymphs to which the mice were exposed was 3.50 for strain Fin-Jyv-A3 and 3.57 for strain NE4049. The mean spirochete load in the subset of infected nymphs was ~380 for the Fin-Jyv-A3 nymphs and ~198 for the NE4049 nymphs. Assuming that the number of spirochetes inoculated by a nymph into a mouse is linearly related to the product of the number of infected nymphs and the mean spirochete load per infected nymph, we calculate that the infectious dose for strain Fin-Jyv-A3 (3.57 infected nymphs*380 spirochetes/nymph = 1356) was almost two times larger than the infectious dose for strain NE4049 (3.5 infected nymphs*198 spirochetes/nymph = 694 spirochetes).

Age classes of the ticks: At 34 days post-infection, each infected mouse was infested with ~100 pathogen-free *I. ricinus* larvae (Figure 1(B)). Following drop-off, ~50 engorged larvae were collected for each mouse and stored in individual Eppendorf tubes to facilitate random sampling. Each Eppendorf tube contained a piece of moistened paper towel to maintain high humidity. The engorged larvae were randomly assigned to be sacrificed at one of three ages: engorged larvae, 1-month-old nymphs, 4-month-old nymphs. Immediately following drop-off, up to 6 engorged larvae per mouse were frozen at -20°C for the engorged larvae age class. The remaining engorged larvae were allowed to moult into nymphs under standard laboratory conditions. At 1 month after the larva-to-nymph moult (22 January 2016), up to 10 nymphs per mouse were frozen at -20°C for the 1-month-old nymph age class. The remaining nymphs were randomly assigned to three different seasonal treatments (see below). At 4 months after the larva-to-nymph moult (16 April 2016), up to 15 nymphs per mouse were frozen at -20°C for the 4-month-old nymph age class. The tick age classes, mouse sample sizes, and tick sample sizes are shown in Table 1.

Seasonal treatment of the 4-month-old nymphs: When the 4-month-old nymphs were one month old, they were randomly assigned to one of three seasonal treatments: artificial summer, artificial winter, and natural winter (Figure 1(C)), hereafter referred to as phytotron, fridge, and underground, respectively. These three treatments were chosen to simulate summer conditions (warm temperature and long photoperiod) and winter conditions (cold temperature and no light) that are experienced by *I. ricinus* ticks in the natural environment. We chose to have no light in both winter treatments to simulate the natural situation. *Ixodes* ticks encounter very little light during the winter because they hide in the soil and under the leaf litter to protect themselves from cold temperatures [24, 25, 28]. The nymphs spent a total of 3 months in these three seasonal treatments (22 January 2016 to 15 April 2016).

In the artificial summer treatment, the nymphs were kept in a phytotron (4h–5h: 1 lumen (lm), 21.5°C; 5h–19h: 2 lm, 25°C; 19h–20h: 1 lm, 21.5°C; 20h–4h; 0 lm, 18°C) with a relative humidity of 85%. In the artificial winter treatment, the nymphs were kept in a fridge at a temperature of 4°C and with no light. For the natural winter treatment, the nymphs were kept in a plastic box (30 cm x 23 cm x 10 cm) that was buried in the soil at a depth of 10 cm in a forest in the botanical garden of Neuchâtel. The box contained three button logs that measured the temperature every thirty minutes. The mean daily average temperature in the natural winter treatment was 6.44°C (range = 3.72°C – 10.50°C; for details see section 1 in the supplementary material). In each of the three seasonal treatments, the nymphs were kept in their Eppendorf tubes.

Four months after the larva-to-nymph moult (15 April 2016), the 4-month-old nymphs were checked to determine whether they were alive or dead. The survival was very high, only 4 of the 444 nymphs had died (3, 0, and 1 in the artificial summer, artificial winter and natural winter, respectively). The next day (16 April 2016), all live 4-month-old nymphs were frozen at –20°C. The seasonal treatments, mouse sample sizes, and tick sample sizes are shown in Table 1.

DNA extraction: The engorged larvae, 1-month-old nymphs, and four-month-old nymphs were crushed in a TissueLyser II (Figure 1 (D)) using a previously described protocol [16, 40]. The crushed ticks were digested with proteinase K at 56°C overnight. Tick DNA was extracted using QIAGEN DNeasy 96 Blood and Tissue kit well plates and following the QIAGEN protocol. Each plate contained two *Anopheles gambiae* mosquitoes as negative DNA extraction controls. For each sample, the DNA was eluted into 65 µl of water.

General and strain-specific qPCR assays: The *B. afzelii* infection status of the ticks was assessed with a qPCR assay that targets a 132-bp fragment of the *flagellin* gene (Figure 1 (D)), as described previously [16]. The identities of the strains in the nymphs were determined using two strain-specific qPCRs (Figure 1 (D)). These qPCR assays amplify the same 143-bp fragment of the *ospC* gene but use different probes that are specific for *ospC* alleles A3 and A10, as described previously [16]. For each qPCR reaction, 3 µl of DNA template was used. The qPCRs were done using a LightCycler® 96 Multiwell Plate white (Roche). All the plates contained 80 tick DNA samples, 2 negative DNA extraction controls (mosquitoes), 2 negative controls for the qPCR (PCR-grade water), and 12 positive controls. For each of the three different qPCR assays (*flagellin*, *ospC* A3, and *ospC* A10), a sample of 81 ticks was tested twice to determine the repeatability of the assay [16]. For the *flagellin, ospC* A3, and *ospC* A10 qPCR, the repeatability of the log10-transformed spirochete loads was 98.3%, 97.8%, and 97.0%, respectively [16].

STATISTICAL ANALYSES

***B. afzelii* infection status of immature *I. ricinus* ticks:** The *flagellin* qPCR and the *ospC* qPCRs were used to determine the *B. afzelii* infection status of the ticks. Samples were excluded from the analysis whenever the *ospC* qPCR and the *flagellin* qPCR differed with respect to the infection status of the tick. Generalized linear mixed effects models (GLMMs) with binomial errors were used to analyse the infection status of the ticks (0 = uninfected, 1 = infected). The fixed factor that combined tick age class and seasonal treatment had 5 levels: engorged larvae, 1-month-old nymphs, phytotron 4-month-old nymphs, fridge 4-month-old nymphs, and underground 4-month-old nymphs. These levels were combined to create new factors with fewer levels: tick age (3 levels: larvae, 1-month-old nymphs, and 4-month-old nymphs), tick stage (2 levels: larvae and nymphs). Mouse ID was modelled as a random factor. To determine the statistical significance of the fixed factors, models that differed with respect to the fixed factor of interest were compared using log-likelihood ratio (LLR) tests. Similarly, comparing a model that includes the 5 levels of tick age class and seasonal treatment with a model that contains 3 levels of tick age, tests whether the seasonal treatment had a significant effect on the phenotype of the 4-month-old nymphs.

***B. afzelii* spirochete in immature *I. ricinus* ticks:** The total spirochete abundance inside the ticks (hereafter referred to as total spirochete load) was analysed for the subset of ticks infected with *B. afzelii*. The total estimate of the spirochete load inside each tick was estimated by correcting the spirochete load in 3 µl of DNA template to the total DNA extraction elution volume (i.e. multiplied by a factor of 21.67 = 65 µl/3 µl). The *flagellin* qPCR was used to estimate the total spirochete load in the ticks. In co-infected ticks, the strain-specific spirochete loads estimated by the *ospC* qPCRs were constrained to sum to the total spirochete load estimated by the *flagellin* qPCR [16]. Linear mixed effects models (LMMs) were used to analyse the spirochete loads, which were log10-transformed to normalize the residuals. The fixed factors were the same as for the GLMM of infection status. Mouse ID was modelled as a random factor.

Competition between strains over the life cycle of the immature tick: GLMMs with binomial errors and LMMs with normal errors were used to analyse the strain-specific prevalence and the strain-specific spirochete load, respectively. The fixed factor that combined tick age class and seasonal treatment (5 levels: engorged larvae, 1-month-old nymphs, phytotron 4-month-old nymphs, fridge 4-month-old nymphs, and underground 4-month-old nymphs), the strain (2 levels: Fin-Jyv-A3, NE4049), competition (2 levels: no, yes) and their interactions were fixed factors. Mouse identity was included as a random factor. As before, we tested whether the 5-level factor of tick age class and seasonal treatment could be reduced to tick age (3 levels: larvae, 1-month-old nymphs, and 4-month-old nymphs), or tick stage (2 levels: larvae and nymphs). To determine the statistical significance of the fixed factors, models that differed with respect to the fixed factor of interest were compared using LLR tests. The results from this stepwise regression approach were compared with a model selection approach based on the Akaike information criterion (AIC).

Statistical software: The statistical analyses were done in R v. 1.1.463 using the following R packages: base, lme4, emmeans, and MuMIn. The 'lmer' and 'glmer' functions (lme4 package) were used to create the GLMMs and LMMs. The 'anova' function (base package) was used to perform the log-likelihood ratio tests. The 'model.sel' function (MuMIn package) was used to perform the AIC-based model selection. The 'emmeans', 'contrast' and 'pairs' functions (emmeans package) were used for the post-hoc analyses of the GLMMs and LMMs (details of the post-hoc tests are shown in section 8 of the supplementary material).

RESULTS

Mice: Of the 40 mice used in this study, 2 mice died (S5 and S12), 1 mouse did not become infected (S37), and 4 mice in the co-infected treatments acquired only one strain (S17, S20, S25, S29). Ticks produced by these 7 mice were excluded from the study. For the engorged larvae and the 1-month-old nymphs, the final sample size was 33 infected mice that were distributed across the four infection treatments as follows: Fin-Jyv-A3 (n = 9), Fin-Jyv-A3 + NE4049 (n = 7), NE4049 (n = 9), and NE4049 + Fin-Jyv-A3 (n = 8). For the 4-month-old nymphs, the final sample size was 26 infected mice because some mice did not produce enough ticks to be included in this tick age group (Table 1).

Ticks: Of the 849 ticks in this study, 49 were excluded because the *flagellin* qPCR and the *ospC* qPCR disagreed with respect to tick infection status. Specifically, this criterion excluded 13.9% (23/165) of the larvae, 5.3% (17/318) of the 1-month-old nymphs, and 2.5% (9/366) of the 4-month-old nymphs. The final sample sizes for each of the five groups were as follows: engorged larvae (n = 142), 1-month-old-nymphs (n = 301), 4-month-old phytotron nymphs (n = 119), 4-month-old fridge nymphs (n = 120), and 4-month-old underground nymphs (n = 118).

Prevalence of *B. afzelii* in immature ticks: The infection prevalence is defined as the percentage of ticks that tested positive for *B. afzelii* (ignoring strain identity). The infection prevalence for the different tick ages (Table 2) was as follows: larvae (37.3% = 53/142), 1-month-old-nymphs (87.7% = 264/301), and 4-month-old nymphs (82.6% = 295/357). After combining all the nymphs, the prevalence of *B. afzelii* infection in the nymphs (85.0% = 559/658) was 2.3 times higher compared to the larvae (37.3% = 53/142). The statistical analysis of these infection prevalences is presented in section 3.4.

Effect of tick age and tick seasonal treatment on the prevalence of *B. afzelii* infection: We analysed the prevalence of *B. afzelii* infection in the immature *I. ricinus* ticks (ignoring strain identity) as a function of tick age and seasonal treatment. The seasonal treatment had no effect on the infection prevalence and we therefore combined all the 4-month-old nymphs into a single group (see section 2 in the supplementary material).

There was a significant effect of age on infection prevalence (LLR test of age vs null: Δ df = 2, Δ dev = 146.16 $p < 0.0001$). The infection prevalence in the larvae was significantly lower compared to the 1-month-old nymphs (emmeans: $p < 0.0001$) and compared to the 4-month-old nymphs (emmeans: $p < 0.0001$). The infection prevalence was not significantly different between the 1-month-old nymphs and the 4-month-old nymphs (emmeans: $p = 0.249$).

Spirochete load of *B. afzelii* in immature ticks: The spirochete load is defined as the total number of spirochetes in an infected tick (ignoring strain identity). The geometric mean spirochete loads were calculated for the subset of infected ticks (Table 2) and were as follows: larvae (n = 53; mean = 429; range = 28–269,154), 1-month-old nymphs (n = 264; mean = 5,055; range = 34–218,776), and 4-month-old nymphs (n = 295; mean = 3009; range = 36–275,423). The spirochete load in the 1-month-old nymphs was 11.8 times higher compared to the larvae and 1.7 times higher compared to the 4-month-old nymphs. Over the 3-month overwintering period, the nymphs lost spirochetes at a rate of 22.7 spirochetes per day. The statistical analysis of these spirochete loads is presented in section 3.6.

Effect of tick age and tick seasonal treatment on the *B. afzelii* spirochete load: We analysed the total *B. afzelii* spirochete load in the immature *I. ricinus* ticks (ignoring strain identity) as a function of tick age and seasonal treatment. The seasonal treatment had no effect on the nymphal spirochete load and we therefore combined all the 4-month-old nymphs into a single group (see section 3 in the supplementary material).

There was a significant effect of tick age on the tick spirochete load (LLR test of age versus null: Δ df = 2, Δ dev = 124.65, p < 0.0001; Table 2, Figure 2). The spirochete loads in the engorged larvae were significantly lower compared to the 1-month-old nymphs (emmeans: p < 0.0001) and compared to the 4-month-old nymphs (emmeans: p < 0.0001). The 1-month-old nymphs had a significantly higher spirochete load than the 4-month-old nymphs (emmeans: p = 0.0002). In summary, the spirochete load was lowest in the larvae, highest in the 1-month-old nymphs and intermediate in the 4-month-old nymphs (Table 2, Figure 2).

Effect of competition between strains on the strain-specific transmission to immature *I. ricinus* ticks: Here the response variable is the strain-specific infection prevalence in the immature ticks, as estimated by the *ospC* qPCR. To be conservative, the competition factor (two levels: no, yes) was based on the co-infection status of the mice rather than the co-infection status of the ticks (see section 4 in the supplementary material). Model comparison found justification for combining all of the nymphs into a single group (see section 5 in the supplementary material). Thus, the strain-specific prevalence was analysed as a function of three fixed factors: strain, competition, tick stage, and their interactions.

A classic step-wise model simplification approach using LLR tests found that the best model included strain, competition, tick stage, the competition:tick stage interaction, and the strain:competition interaction (see section 5 in the supplementary material). The AIC-based model selection approach converged on the same model (see section 7 in the supplementary material). The presence of two significant two-way interactions complicates the interpretation of the main effects and requires splitting the statistical analyses. We ran separate analyses for larvae and nymphs; this approach allowed us to independently test the effects of competition and strain for each tick stage.

In the larvae, the interaction between strain and competition was significant (GLME LLR: Δ df = 1, Δ dev = 8.228, p = 0.004), the effect of competition was therefore tested separately for each strain. For strain Fin-Jyv-A3, its prevalence was higher in larvae that fed on singly infected mice (45.5% = 20/44) compared to larvae that fed on co-infected (14.8% = 4/27) mice, and this difference was significant (GLME LLR: Δ df = 1, Δ dev = 5.173, p = 0.023). For strain NE4049, its prevalence was lower in larvae that fed on singly infected mice (15.0% = 6/40) compared to larvae that fed on co-infected mice (35.5% = 11/31), but this difference was not significant (GLME LLR: Δ df = 1, Δ dev = 3.16, p = 0.075). In summary, competition between strains significantly reduced transmission to engorged larval ticks for strain Fin-Jyv-A3 but not strain NE4049 (Table 3, Figure 3, Figure S3 in the supplementary material).

In the nymphs, the interaction between strain and competition was not significant (GLME LLR: Δ df = 1, Δ dev = 2.724, p = 0.091) and was removed from the model. Competition was significant (GLME LLR: Δ df = 1, Δ dev = 8.683, p = 0.003), but strain was not (GLME LLR: Δ df = 1, Δ dev = 0.024, p = 0.877). The prevalence of both strains was higher in nymphs that had fed as larvae on singly infected mice (81.8% = 279/341) compared to nymphs that had fed as larvae on co-infected mice (59.3% = 188/317; Table 3, Figure 3, Figure S3 in the supplementary material).

Effect of competition between strains on the strain-specific spirochete load in immature *I. ricinus* ticks: Here the response variable is the strain-specific spirochete load in the immature ticks, as estimated by combining the *ospC* qPCR and the *flagellin* qPCR. Model comparison showed that we could combine all of the 4-month-old nymphs into a single group (see section 6 in the supplementary material). Thus, the strain-specific spirochete load was analysed as a function of three fixed factors: strain, competition, tick age and their interactions.

A classic stepwise model simplification approach using LLR tests found that none of the interactions were significant and that the best model contained the three fixed factors of strain, competition, and tick age (see section 6 in the supplementary material). The AIC-based model selection approach converged on the same model (see section 7 in the supplementary material).

The three fixed factors all had significant effects on the spirochete load: tick age (LLR test: Δ df = 2, Δ dev = 86.393, p = < 0.001), strain (LLR test: Δ df = 1, Δ dev = 3.948, p = 0.047), and competition (LLR test: Δ df = 1, Δ dev = 10.589, p = 0.011) (Figure S4 in the supplementary material). The spirochete load in the engorged larvae was significantly lower compared to the 1-month-old nymphs (emmeans: < 0.0001) and the 4-month-old nymphs (emmeans: p < 0.0001) (Table 3, Figure 4, Figure S4(A) in the supplementary material). The 1-month-old nymphs had a higher spirochete load than the 4-month-old nymphs (emmeans: p < 0.0046; Figure 4, Figure S4(A) in the supplementary material). Strain Fin-Jyv-A3 always had a higher spirochete load in the tick than strain NE4049 (parameter estimates in Table S3 in supplementary material; Figure 4, Figure S4(B) in the supplementary material). Competition reduced the spirochete load of each strain and this effect was the same for engorged larvae, 1-month-old nymphs, and 4-month-old nymphs (Figure 4, Figure S4(C) in the supplementary material). In all three of these tick ages, the spirochete load of each strain was reduced in ticks that had fed on co-infected mice compared to ticks that had fed on mice infected with single strains (Figure 4, raw data in Table 4, parameter estimates in Table S3 in supplementary material, Figure S4(A) in the supplementary material).

DISCUSSION

Competition in the rodent host reduces host-to-nymph transmission: Our study found that co-infection in the rodent host reduced host-to-tick transmission success of both strains of *B. afzelii* to *I. ricinus* nymphs. This result is in agreement with other studies that have investigated how mixed strain infections of *B. burgdorferi* sl in the rodent host influence strain-specific transmission to *Ixodes* ticks [16, 41, 42]. Studies on other vector-borne pathogens have also shown that competition between strains in the vertebrate host reduces strain-specific transmission to the arthropod vector [13, 15], suggesting that this result is a general phenomenon in these systems. To date, all *B. burgdorferi* sl studies on the relationship between co-infection and host-to-tick transmission have measured the latter phenotype in young nymphs (i.e. sacrificed shortly after the larva-to-nymph moult) that have not overwintered [16, 41, 42]. In nature, the majority of *Ixodes* nymphs overwinter and search for a host the following spring when they are much older [28, 43, 44]. Hence, a new contribution of our study was to show that the effects of inter-strain competition in the rodent host persist in *I. ricinus* nymphs that were allowed to overwinter and age. We found that co-infection reduced the host-to-nymph transmission of both strains, which is different from previous studies (including ours) that found asymmetric competition [16, 41].

Interestingly, our study shows that reduced host-to-tick transmission can be detected in engorged larval ticks immediately following drop-off. This observation reinforces the idea that competition between strains in the tissues of the rodent host [45] reduces the probability that a given strain will colonize a feeding larval tick. The much lower infection prevalence in the engorged larvae compared to the nymphs is due to their much lower spirochete load, which the qPCR sometimes fails to detect. However, an alternative explanation for the observation that the spirochete load in engorged larvae is lower compared to the nymphs is that blood interferes with the efficacy of the qPCR assays [46-48]. We consider this explanation unlikely because an early study that used immunofluorescence microscopy also showed that *Ixodes scapularis* larvae acquire a small inoculum of *B. burgdorferi* sensu stricto (ss) spirochetes, which subsequently expands during the period of larva-to-nymph development [49]. Similarly, a recent study on the population dynamics of *B. afzelii* in immature *I. ricinus* ticks showed that qPCR and immunofluorescence microscopy gave the same results [37]. In summary, there is a general consensus that *Ixodes* larvae acquire a small inoculum of *B. burgdorferi* sl spirochetes, which grows to a larger size in the tick midgut during the period of larva-to-nymph development [36, 37, 49, 50].

Competition in the arthropod vector: To date, very few studies have investigated interactions between pathogen strains in the arthropod vector [15-17]. Inter-strain facilitation has been shown for malaria parasites in Anopheline mosquitoes [17], whereas inter-strain competition has been shown for *Francisella novicida* in *Dermacentor andersoni* ticks [15], and for *Borrelia afzelii* in *Ixodes ricinus* ticks [16]. Our study found that competition resulted in decreased spirochete loads for both strains and in all three tick stages in this study: engorged larvae, 1-month-old nymphs, and 4-month-old nymphs. This result is in agreement with our previous study, which was restricted to the 1-month-old nymphs [16]. The present study is an improvement on our previous study because it is based on a larger sample size (800 ticks versus 301 ticks) and because the results are more general; i.e. we show here that inter-strain competition occurs at three different time points over the first 5 months of the life cycle of immature *I. ricinus* ticks compared to only one time point [16]. An important remaining question is whether competition between strains of *B. burgdorferi* sl in the nymph influences subsequent nymph-to-host transmission.

Dynamics of *B. burgdorferi* sl spirochete populations in immature *Ixodes* ticks: The population size of *B. burgdorferi* sl spirochetes is highly dynamic over the life cycle of immature *Ixodes* ticks [36, 37, 49-52]. A recent study on the spirochete population dynamics of *B. afzelii* in *I. ricinus* found that engorged larvae acquired a small inoculum (~600 spirochetes), which subsequently expanded 34-fold to reach a peak spirochete population size (21,005 spirochetes) in 2-week old nymphs [37]. The results of our study were very similar; the engorged larvae acquired a small inoculum of *B. afzelii* (~400 spirochetes), which expanded and was 12 times higher in the 1-month-old nymphs (5,005 spirochetes). Numerous studies on *B. burgdorferi* ss in *I. scapularis* have shown that the spirochete population size increases dramatically after a blood meal in both larvae and nymphs [49-52].

The ability of *B. burgdorferi* sl to persist inside the nymph over long periods of time (8 to 12 months) is critical for the maintenance of Lyme disease in nature [53]. A recent study on the spirochete population dynamics of *B. afzelii* in *I. ricinus* nymphs found that the spirochete population size decreased by 71.3% between 2 and 6 weeks post-moult but reached a stable plateau after this period [37]. We have observed a similar decrease in spirochete population size between 1-month-old versus 4-month-old nymphs in the present study (decrease of 40.5%), and in a previous study (85.8% decrease) [36]. As we only measured the nymphal spirochete population size at two time points, we do not know whether it plateaued, as was shown in the other study [37]. A recent study on the genetic mechanisms underlying persistence of *B. burgdorferi* ss in *I. scapularis* found no evidence that the spirochete population size declined over time [53].

One explanation for the observed decrease in the spirochete population over time is bloodmeal digestion, which is a slow process in ixodid ticks that occurs in the gut epithelium rather than the gut lumen [54-56]. Proteomics studies have shown that digestion of the larval blood meal causes host proteins to decrease slowly over time, and some can be identified 10 months after the larva-to-nymph moult [57, 58]. The slow digestion of the blood meal could explain the gradual decrease of the spirochetes over time in the tick midgut [59]. We expected that the intensity of inter-strain competition would increase with this age-related decline in the spirochete population, but we found no evidence that competition was stronger in the 4-month-old nymphs compared to the 1-month-old nymphs. One limitation of our qPCR-based approach is that it allows us to estimate spirochete abundance but not spirochete viability, which may decline with advanced nymphal age.

Mechanisms of competition inside the tick vector: The mechanisms underlying the competition between *B. afzelii* strains in the tick remain unknown. Competition between parasite strains is classified into three different types: interference, exploitation, and apparent competition [12]. Interference competition is unlikely in *B. burgdorferi* sl, because spirochetes are not known to produce toxic substances [60]. In exploitation competition, strains compete over limited resources like space or nutrients. This type of competition is expected because nutrients in the tick midgut will disappear over time as the tick digests its bloodmeal [58]. Furthermore, studies have shown that ticks contain a relatively small number of spirochetes, suggesting that strains compete over space as well [16, 32]. In apparent competition, interactions between strains are mediated by the host immune system. The innate immune system of ticks uses antimicrobial peptides (e.g. lysozymes and defensins) as defence against microbial pathogens [61, 62]. Spirochetes in the tick hemolymph are cleared by hemocytes using phagocytosis [63-65]. However, we are not aware of any experimental evidence demonstrating that the tick immune system mediates competition between strains of *B. burgdorferi* sl.

Inter-strain competition and nymph-to-host transmission: Our study found that competition between strains reduced the strain-specific spirochete loads in *I. ricinus* nymphs. An important question is whether this inter-strain competition influences the subsequent nymph-to-host transmission success of the strains. Most *B. afzelii* spirochetes spend 8 months or more in the nymph midgut before achieving nymph-to-host transmission [28]. We expect that the temporal decline in quantity or viability of the nymphal spirochete population could eventually result in the loss of the less abundant strains from the co-infected nymph (i.e.

competitive exclusion). Studies on *B. burgdorferi* ss and *F. novicida* have found that ticks can limit the number of strains that are transmitted to the vertebrate host [15, 66]. The biology of spirochetes in blood-feeding nymphs further suggests that inter-strain competition could reduce strain-specific nymph-to-host transmission. During the nymphal blood meal, spirochetes migrate from the midgut to the salivary glands [67, 68], where their abundance is surprisingly low [52]. One study suggested that nymphs inoculate only ~100 spirochetes into the vertebrate host [69]. These observations suggest that *B. burgdorferi* sl strains that are the most abundant in co-infected nymphs are more likely to be transmitted to the reservoir host during the nymphal blood meal [32]. Future experimental infection studies should test whether co-infection in the nymph reduces strain-specific nymph-to-host transmission.

Relevance of our study to the situation in nature: The results of our laboratory experiment are relevant to understanding what happens in nature. Studies on wild *I. ricinus* nymphs found that 77-79% of the nymphs are co-infected with multiple strains and that nymphs carry an average of 2.4-2.9 strains of *B. afzelii* [32, 33]. Numerous studies in North America have found that wild *I. scapularis* ticks are commonly infected with multiple strains of *B. burgdorferi* ss [35, 70-73]. Thus, co-infected nymphs are the norm rather than the exception in areas where Lyme disease is endemic. A field study on *B. afzelii* in wild *I. ricinus* nymphs found indirect evidence that competition between strains in nymphs reduces the strain-specific abundance and strain-specific nymph-to-host transmission [32]. First, this study found the same pattern that we found in the present study: co-infection in the nymph reduced the abundance of the constituent strains [32]. Second, this study found a positive correlation between the strain-specific spirochete load and the strain-specific prevalence suggesting that *B. afzelii* strains with higher nymphal abundance have higher nymph-to-host transmission [32]. In summary, our controlled experiments found patterns of co-infection and competition that we have also found in surveys of wild *I. ricinus* populations.

No effect of seasonal treatment on spirochete load: We found no effect of seasonal treatment on the *B. afzelii* spirochete load in the 4-month-old nymphs. The seasonal treatments were chosen to simulate summer conditions (mean temperature of 22.4°C and 16 hours light: 8 hours darkness) and winter conditions (mean temperature of 4°C and 6.44°C and 24 hours darkness) that are experienced by *I. ricinus* ticks in the natural environment [24, 25, 28]. We expected that the nymphs exposed to summer conditions would have higher metabolism, faster digestion, and therefore lower spirochete population sizes in their midguts than nymphs exposed to winter conditions. We were surprised that the seasonal treatment had no effect on the spirochete population size in the nymphal midgut. This result suggests that the large differences in temperature and light conditions between the summer and winter conditions over a period of 3 months was not enough to influence tick metabolism, tick digestion of spirochetes, and the dynamics of the *B. afzelii* spirochete population inside the nymphs. Future studies should investigate whether more extreme conditions (e.g. hotter and colder temperatures) would influence the spirochete population dynamics and inter-strain competition inside the nymph vector. We had previously suggested that the temporal decline in nymphal spirochete load following the larva-to-nymph moult could be an artefact of housing our ticks under unnatural ‘summer-like’ conditions in the lab [36]. Thus, the present study demonstrates that the nymphal spirochete load also decreases over time when nymphs are housed under more natural ‘winter-like’ conditions. The survival of the nymphs over the 3-month winter treatments was very high

(99.1%) due to the mild temperatures (6.44° C in natural winter and 4° C in artificial winter). Storing ticks in separate Eppendorf tubes and burying them underground is a promising approach for measuring individual tick survival under natural winter conditions.

The infectious dose and the importance of using ticks instead of needles: In studies that investigate competition between pathogen strains, it is critical to control the infectious dose (ID) with which the vertebrate host is infected [13]. If the ID was consistently larger for one strain, we would expect this strain to have higher competitive success (all else being equal). There is evidence that when mice are infected with *B. burgdorferi* ss using artificial needle inoculation, a 10-fold difference in the ID can influence the spirochete load in the mouse tissues [74], and presumably the efficiency of host-to-tick transmission. In section 2.2 of the Methods, we assumed that the ID delivered by the nymphs is proportional to the product of the number of infected nymphs and the mean spirochete load per infected nymph, and we estimated that the ID for strain Fin-Jyv-A3 was two times higher than strain NE4049. Despite having a (theoretical) two-fold disadvantage in abundance during the nymphal tick bite (Figure 1(A)), strain NE4049 was still able to reduce the performance of strain Fin-Jyv-A3. This observation suggests that competitive interactions would remain even if both strains had the exact same ID.

Alternatively, one could infect mice via needle inoculation of spirochete cultures, which can be more easily quantified, but this artificial approach creates other problems. Tick saliva enhances the ability of *B. burgdorferi* sl pathogens to infect their vertebrate host [75]. For example, cultured spirochetes are an order of magnitude less infectious than the spirochetes in tick salivary glands [76, 77]. Tick saliva also increases the spirochete load of *B. burgdorferi* sl in rodent tissues [78]. A recent study found that the mode of inoculation (needle versus tick) influenced tissue tropism of *B. burgdorferi* sl [79]. In summary, there is lots of evidence that the mode of inoculation influences the infection phenotype. *B. burgdorferi* sl spirochetes have co-evolved with ticks and not needles. Using ticks will generally give a better reflection of what occurs in nature.

Conclusion: This study found that co-infection in the mice reduced the strain-specific host-to-tick transmission success to larval ticks and that this effect was maintained for both strains in the resultant nymphs at 1 and 4 months after the larva-to-nymph moult. This study also demonstrates that competition between strains of *B. afzelii* occurs in co-infected immature *I. ricinus* ticks. Inter-strain competition resulted in decreased spirochete loads for both strains in all three ages of immature ticks investigated in our study. The 3-month-long seasonal treatments did not affect the strain-specific spirochete load nor the intensity of inter-strain competition in the 4-month-old nymphs. Future studies should investigate whether inter-strain competition in the nymph influences the strain-specific nymph-to-host transmission success.

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Author contributions: DG and MV conceived and designed the study. DG and AS conducted the experiment and performed the molecular work. OR helped with the experimental infections. DG conducted the statistical analyses. DG and MV wrote the manuscript. All authors read and approved the final version of the manuscript

Ethics approval: The commission that is part of the "Service de la Consommation et des Affaires Vétérinaires (SCAV)" of Canton Vaud, Switzerland evaluated and approved the ethics of this study. The Veterinary Service of the Canton of Neuchâtel, Switzerland issued the animal experimentation permit used in this study (NE04/2016).

TABLES AND FIGURES

Table 1. Sample sizes are shown for immature *I. ricinus* ticks that were collected in our previous study (1-month-old nymphs) and the present study (larvae and 4-month-old nymphs). Ticks that had fed as larvae on mice infected with one or two strains of *B. afzelii* were sacrificed at three different ages: 2 days after drop-off, 1 month after larva-to-nymph moult, and 4 months after larva-to-nymph moult, which corresponds to two different stages: larva and nymph. The 4-month-old nymphs had been exposed to three different seasonal treatments. For each tick stage, tick age, and seasonal treatment, the number of infected mice, the number of ticks per mouse, and the total number of ticks are shown.

Stage	Age	Seasonal Treatment	Infected mice	Ticks per mouse	Total ticks
Larva	2 days	NA	33	5-6	142
Nymph	1 month	NA	33	10	301
Nymph	4 months	Combined	26	15	357
Nymph	4 months	Phytotron	26	5	119
Nymph	4 months	Fridge	26	5	120
Nymph	4 months	Underground	26	5	118

Table 2. The prevalence of *B. afzelii* infection in *I. ricinus* ticks and the *B. afzelii* spirochete loads in the subset of infected *I. ricinus* ticks are shown separately for the seasonal treatment, tick age, and tick stage. The spirochete load is the total number of spirochetes in the tick and is based on the *flagellin* qPCR. Shown are the geometric mean of the spirochete loads and the 95% confidence intervals (95% CI).

Factor	Level	Inf/Total	Prev (%)	Mean	95% CI
Treatment	Larva	53/142	37.3%	429	286–644
Treatment	1-month-old nymph	264/301	82.6%	5,055	4,215–6,063
Treatment	4-month-old phytotron nymph	95/119	79.8%	2,651	1,959–3,589
Treatment	4-month-old fridge nymph	98/120	81.7%	2,928	2,173–3,945
Treatment	4-month-old underground nymph	102/118	86.4%	3,477	2,596–4,657
Tick age	Larva	53/142	37.3%	429	286–644
Tick age	1-month-old nymph	264/301	87.7%	5,055	4,216–6,062
Tick age	4-month-old nymph	295/357	82.6%	3,009	2,534–3,574
Tick stage	Larva	53/142	37.3%	429	285–647
Tick stage	Nymph	559/658	85.0%	3,845	3,388–4,363

Table 3. The percentages of infected immature *I. ricinus* ticks are shown for each of the 8 unique combinations of tick stage, *B. afzelii* strain, and competition.

Stage	Strain	Competition	% Infected
Larvae	Fin-Jyv-A3	No	20/44 (45.5%)
Larvae	Fin-Jyv-A3	Yes	4/27 (15.8%)
Larvae	NE4049	No	6/40 (15.0%)
Larvae	NE4049	Yes	11/31 (35.5%)
Nymphs	Fin-Jyv-A3	No	127/147 (86.4%)
Nymphs	Fin-Jyv-A3	Yes	79/157 (50.3%)
Nymphs	NE4049	No	152/194 (78.4%)
Nymphs	NE4049	Yes	109/160 (68.1%)

Table 4. The spirochete loads in the subset of infected *I. ricinus* ticks are shown for each of the 12 unique combinations of tick age, *B. afzelii* strain, and competition. Mean is the geometric mean of the spirochete loads on the original scale. The 95% confidence interval (95% CI) for the geometric mean is also shown.

Tick Age	Strain	Competition	Mean	95% CI
Larva	Fin-Jyv-A3	No	513	241–1,090
Larva	Fin-Jyv-A3	Yes	146	27–786
Larva	NE4049	No	209	53–829
Larva	NE4049	Yes	338	122–934
1-month-old nymph	Fin-Jyv-A3	No	7,111	4,873–10,376
1-month-old nymph	Fin-Jyv-A3	Yes	3,352	1,972–5,697
1-month-old nymph	NE4049	No	3,493	2,394–5,098
1-month-old nymph	NE4049	Yes	1,875	1,216–2,892
4-month-old nymph	Fin-Jyv-A3	No	3,951	2,738–5,703
4-month-old nymph	Fin-Jyv-A3	Yes	1,410	923–2,154
4-month-old nymph	NE4049	No	2,520	1,851–3,429
4-month-old nymph	NE4049	Yes	1,960	1,350–2,847

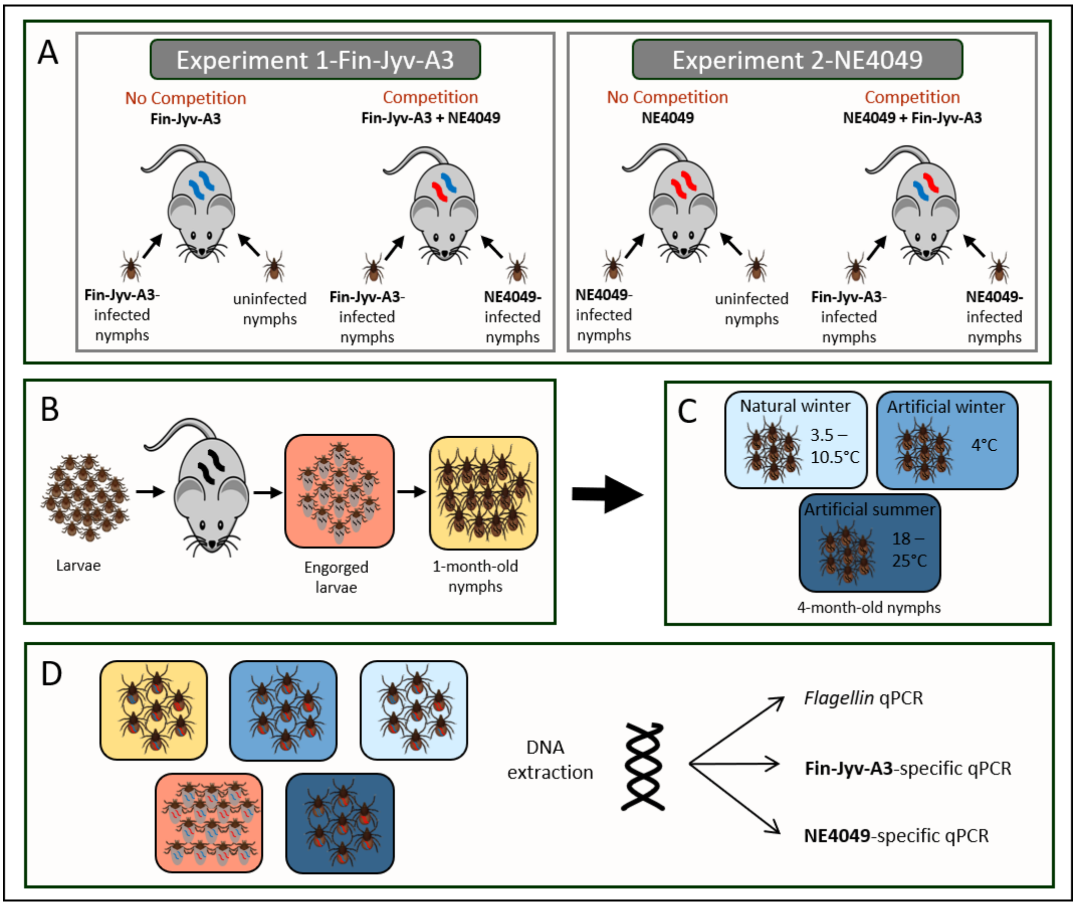

Figure 1. (A) The study was divided into two competition experiments that differed in the focal strain. In experiments 1 and 2, the focal strain was Fin-Jyv-A3 and NE4049, respectively. Each experiment was divided into two groups: no competition (infection with the focal strain only) and competition (infection with both strains). The sample size of each group was 10 mice. In the no competition treatment, each mouse was infected with 5 nymphs infected with the focal strain and 5 uninfected nymphs. In the competition treatment, each mouse was infected with 5 nymphs infected with strain Fin-Jyv-A3 and 5 nymphs infected with strain NE4049. (B) At 34 days post-infection, the mice were infested with ~100 larvae. Engorged larvae were allowed to moult into nymphs under laboratory conditions. (C) One month after the larva-to-nymph moult, the 1-month-old nymphs were exposed for 3 months to one of the three seasonal treatments: natural winter, artificial winter, artificial summer. (D) Ticks were sacrificed at three different ages: engorged larvae, 1-month-old nymphs, and 4-month old nymphs. Only the 4-month old nymphs had been exposed to three different seasonal treatments (natural winter, artificial winter, artificial summer). DNA was extracted from all ticks and the total number of spirochetes in each tick was estimated using *flagellin* qPCR. For the co-infected ticks, the abundance of strain Fin-Jyv-A3 and strain NE4049 was estimated using a strain-specific qPCR that targeted the *ospC* A3 allele and the *ospC* A10 allele, respectively.

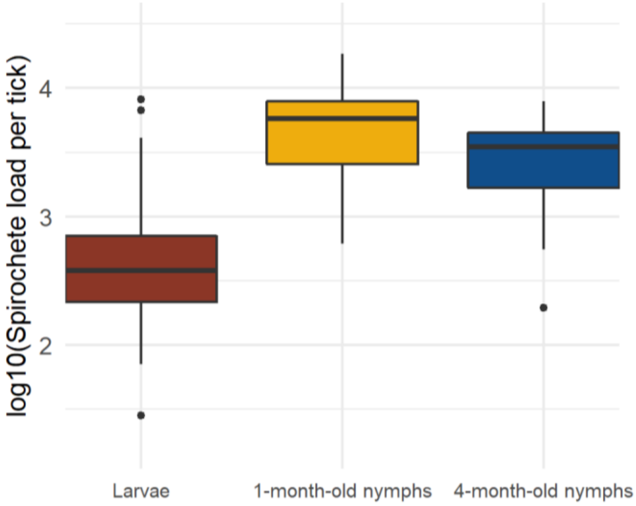

Figure 2. The *B. afzelii* spirochete loads in immature *I. ricinus* ticks change with the age of the tick. The spirochete load is lowest in the engorged larvae, highest in 1-month-old nymphs, and intermediate in 4-month-old nymphs. Each data point represents the mean for ticks sampled from the same mouse. The total numbers of ticks for each of the three tick ages are as follows: engorged larvae (n = 142), 1-month-old nymphs (n = 301), and 4-month-old nymphs (n = 357). Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles).

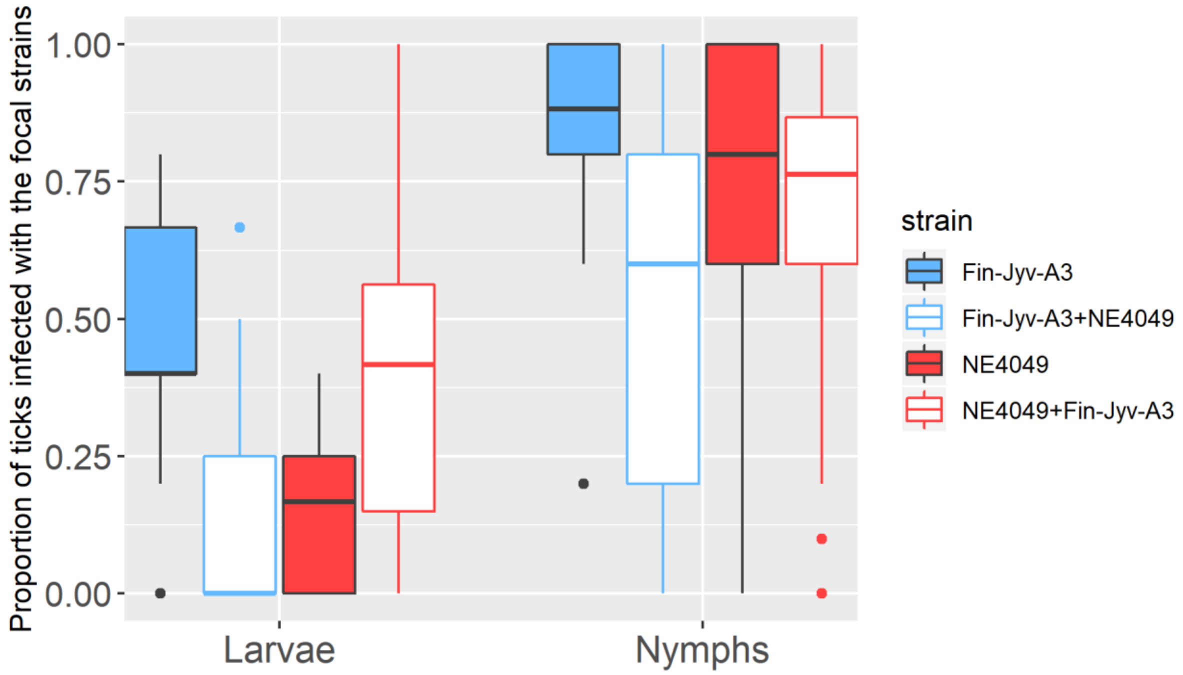

Figure 3. The proportion of immature *I. ricinus* ticks infected with *B. afzelii* is shown as a function of three factors: (1) tick age (engorged larvae, 1-month-old nymphs, and 4-month-old nymphs), (2) strain (Fin-Jyv-A3 in blue and NE4049 in red), and (3) competition (competition absent in solid colours versus competition present in white). The proportion of infected ticks is an estimate of host-to-tick transmission. The graph shows the raw data and not all observable differences are statistically significant. According to the parameter estimates of our statistical analysis, competition between strains in co-infected mice reduced host-to-tick transmission of strain Fin-Jyv-A3 to engorged larvae and nymphs and reduced the host-to-tick transmission of strain NE4049 to nymphs. In contrast, competition between strains in co-infected mice did not influence the host-to-tick transmission of strain NE4049 to engorged larvae. Each data points represents the mean for a single mouse. Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles).

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5. Chapter 3

Competition between strains of *Borrelia afzelii* in the host tissues and consequences for transmission to ticks

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The supplementary material of this chapter can be found in the appendix section.

ABSTRACT

Pathogen species often consist of genetically distinct strains, which can establish mixed infections or co-infections in the host. In co-infections, interactions between strains can have important consequences for their transmission success. We used the tick-borne bacterium *Borrelia afzelii*, which is the most common cause of Lyme disease in Europe, as a model multi-strain pathogen to investigate the relationship between co-infection, competition between strains, and strain-specific transmission success. *Mus musculus* mice were infected with one or two strains of *B. afzelii*, strain transmission success was measured by feeding ticks on mice, and the distribution of each strain in the mouse tissues of different internal and external organs was measured using qPCR. Co-infection and competition reduced the tissue infection prevalence of both strains and changed the bacterial abundance of one of the two strains in some tissues. The ability of the *B. afzelii* strains to establish infection in the host tissues was strongly correlated with their transmission success to the tick vector. Our study demonstrates that co-infection and competition between pathogen strains inside the host tissues can have major consequences for their transmission success.

KEYWORDS: *Borrelia afzelii*, co-infection, competition, Lyme borreliosis, transmission, vector-borne pathogen

INTRODUCTION

Most pathogen species are composed of different strains [1], which can vary in traits, such as infection intensity, virulence, and transmission [2-4]. This pathogen strain diversity can result in mixed infections or co-infections where hosts are infected with multiple strains [1, 2, 5]. Mixed infections can alter the fitness of the co-infecting strains compared to when they are alone. However, the outcome of interactions between strains is highly variable and can range from facilitation to competition [5-7]. Furthermore, the performances of strains when they are alone in their host, are often not predictive of their performances when they are in a mixed infection [5, 8]. In summary, in systems were co-infections are common, interactions between strains may have important consequences for their transmission and hence the strain composition of the pathogen population.

Vector-borne pathogens are interesting systems for the study of mixed infections because pathogen strains can interact in both the vertebrate host [9-13] and the arthropod vector [14-17]. However, to our knowledge, only one study has shown a direct relationship between strain-specific abundance in the tissues of co-infected hosts and strain-specific host-to-vector transmission success [3]. In a rodent malaria system, the strain of *Plasmodium chabaudi* that established the highest abundance in the mouse blood was also the strain that had the highest mouse-to-mosquito transmission success [3]. Other vector-borne pathogens are found in tissues such as the skin, where the interactions between strains may be very different compared to a well-mixed liquid tissue such as the blood. Thus, studies of other systems will enhance our understanding of how inter-strain interactions in the vertebrate host shape strain-specific transmission success to the arthropod vector.

Our research group uses the tick-borne pathogen, *Borrelia afzelii,* as a model system to study interactions between strains in the rodent host and the tick vector. This spirochete bacterium belongs to the *Borrelia burgdorferi* sensu lato species complex and is one of the most common causes of Lyme disease in Europe. *B. afzelii* is transmitted by the hard tick *Ixodes ricinus* and uses small mammals (e.g. rodents and shrews) as reservoir hosts [18]. *I. ricinus* consists of three different stages, larva, nymph, and adult, and each stage requires a blood meal to moult to the next stage, or to produce eggs in the case of adult females. Vertical transmission of spirochetes is rare [19, 20], and larval *I. ricinus* ticks acquire *B. afzelii* during their first blood meal from an infected host. The skin, rather than the blood, is believed to be a critical organ for transmission of *B. afzelii* to feeding *I. ricinus* ticks [21-23].

B. afzelii contains dozens of genetically distinct strains [24, 25] and strain diversity is high at small spatial scales [9, 13, 26-30]. Both small mammal hosts [9, 13, 31] and *I. ricinus* ticks [26, 27] can be co-infected with multiple strains of *B. afzelii*. Field studies on wild small mammals have found associations that are consistent with inter-strain competition [13]. Co-infection in the rodent host reduces strain-specific host-to-tick transmission, but none of these studies quantified the strain-specific abundances in the host tissues [14, 15, 32]. Surprisingly, there are no controlled studies for any *B. burgdorferi* sl genospecies on whether interactions between strains in the tissues of the vertebrate host influence strain-specific host-to-tick transmission success.

In this study, we used two strains of *B. afzelii* to investigate their interactions in a suitable rodent host, the laboratory mouse *Mus musculus*. For mice infected with single strains and for co-infected mice, we quantified the strain-specific presence and abundance in different organs and the strain-specific host-to-tick transmission success. We predict that competition between strains will reduce the strain-specific presence and abundance in tissues of co-infected mice compared to mice with single strain infections. We predict that strains will exhibit preferences for different organs (strain-specific tissue tropism) and that the strength of inter-strain competition will differ among organs. We predict that the skin is a key organ for host-to-tick transmission and that in mixed infections, the strain that is most competitive in the skin will have higher strain-specific host-to-tick transmission success.

MATERIALS AND METHODS

Mice, ticks and *Borrelia afzelii* strains: 40 female 5-week-old pathogen-free *Mus musculus* BALB/c mice were used in this study. The *I. ricinus* ticks came from the laboratory colony that has been maintained at the University of Neuchâtel since 1978. The two strains of *B. afzelii* used in this study were NE4049 and Fin-Jyv-A3. Strain NE4049 was isolated from an *I. ricinus* tick in Switzerland, has multi-locus sequence type (MLST) 679, strain ID number 1887 in the *Borrelia* MLST database, and *ospC* major group (oMG) A10. Strain Fin-Jyv-A3 was isolated from a Finnish bank vole, has MSLT 676, strain ID number 1961 in the *Borrelia* MLST database, and oMG A3.

Ethics approval: The commission that is part of the "Service de la Consommation et des Affaires Vétérinaires (SCAV)" of Canton Vaud, Switzerland evaluated and approved the ethics of this study. The Veterinary Service of the Canton of Neuchâtel, Switzerland issued the animal experimentation permit used in this study (NE04/2016).

Experimental infection of mice via tick bite with one or two strains of *B. afzelii*: The study consisted of two independent experiments that differed with respect to the focal strain. In experiment 1, the focal strain was Fin-Jyv-A3; 10 mice were infected with strain Fin-Jyv-A3 (single infection) and 10 mice were simultaneously co-infected with strains Fin-Jyv-A3 and NE4049 (co-infection). In experiment 2, the focal strain was NE4049; 10 mice were infected with strain NE4049 (single infection) and 10 mice were simultaneously co-infected with strains NE4049 and Fin-Jyv-A3 (co-infection). All mice were infected via tick bite by using *I. ricinus* nymphs that had been experimentally infected with one of the two *B. afzelii* strains of interest (see section 1 in the supplementary material). Mice in the co-infection treatment were simultaneously infested with 5 nymphs infected with strain Fin-Jyv-A3 and 5 nymphs infected with strain NE4049. Mice in the single infection treatment were infested with 5 nymphs infected with the focal strain and 5 uninfected nymphs, so that each mouse was infested with a total of 10 nymphs. To confirm *B. afzelii* infection, we took a blood sample at 4 weeks PI and used the SERION® ELISA classic *Borrelia burgdorferi* IgG/IgM immunoassay to detect *Borrelia*-specific IgG antibodies.

Host-to-tick transmission of *B. afzelii*: To measure host-to-tick transmission, mice were infested with *I. ricinus* larvae at 5 weeks PI as previously described [14, 15]. The engorged larvae were allowed to moult into nymphs, which were sacrificed by freezing. For each mouse, a mean of 20 nymphs (range = 7 – 25) were tested for the two strains of *B. afzelii* as previously described [14, 15]. For each mouse, the percentage of nymphs infected with the focal strain (Fin-Jyv-A3 or NE4049) is an estimate of the strain-specific transmission success.

Mice dissection and DNA extraction: At 6 weeks PI, mice were euthanized with CO_2. Mice were dissected, and 6 organs were collected: bladder, left ear, right ear, heart, right ankle joint, and the section of dorsal skin where the nymphs had attached. For the left ear, right ear, and dorsal skin, ~1 mg of tissue was sampled using a type II forceps (2 mm diameter). For the bladder, heart, and ankle joint, we used a scalpel to cut ~20 mg of tissue. DNA from the mouse tissue samples was extracted with Qiagen DNeasy Blood and Tissue kit 96-well plates and following the Qiagen protocol. Extracted DNA was eluted in 150 µl of autoclaved milliQ water.

***Flagellin* qPCR:** The total spirochete load in the mouse tissues was estimated using a qPCR that targeted a 132-bp fragment of the *flagellin* gene [33] using a previously described protocol [14] (see section 1 in the supplementary material). All samples were processed three times, each of the three replicates was performed in a different plate on a different day. All the plates contained 3 negative DNA extraction controls (tissues from uninfected mice), 2 negative controls for the qPCR (PCR-grade water), and 4 standards containing 10^2, 10^3, 10^4, and 10^5 copies of the *flagellin* gene. For the three independent qPCR assays, the overall repeatability of the Cq values was 86.85%. The mean of the Cq values of the three independent qPCRs were used to calculate the mean total spirochete load in 3 µl of DNA template for each of the mouse tissue samples.

Nested strain-specific qPCRs: The two strains used in this study can be differentiated using the *ospC* gene; strains Fin-Jyv-A3 and NE4049 carry *ospC* major group alleles A3 and A10, respectively. The strain-specific spirochete load was estimated using two independent qPCR assays that targeted a 142-bp fragment of the *ospC* gene. However, because the spirochete load was very low in the mouse tissues, we first used a conventional PCR to enrich the number of *ospC* gene copies relative to the amount of mouse DNA. This PCR amplified a 657-bp fragment of the *ospC* gene using a previously described protocol [24] (see section 1 in the supplementary material). The amplicons from the conventional PCR were the template for the strain-specific *ospC* qPCR.

The amplicons from the conventional PCR were run in triplicate in the *ospC* A3 qPCR and in the *ospC* A10 qPCR to determine which strains were present. The primers are the same for the two strains and amplify a 142-bp fragment of the *ospC* gene. Strain specificity is determined by the probes, which are different for each *ospC* allele. This strain-specific qPCR protocol has been described elsewhere [14] (see section 1 in the supplementary material).

Repeatability of the strain-specific spirochete load: The repeatability of the strain-specific spirochete load was estimated by performing all three qPCR reactions in triplicate on the same set of 222 organ tissue samples. The overall repeatability (across all 222 organ tissue samples) of the spirochete load of strain Fin-Jyv-A3 and strain NE4049 was 78.87% and 87.04%, respectively (see section 1 in the supplementary material for details).

STATISTICAL ANALYSES

All the statistical analyses were done using R version 1.2.5019 using the following packages: base, car, lme4, and emmeans. The *lmer()* and *glmer()* functions (lme4 package) were used to create the GLMMs and LMMs. The *Anova()* function (car package) and the *anova()* function (base package) were used to perform the log-likelihood ratio tests. The *emmeans()* and the *pairs()* functions (emmeans package) were used for the post-hoc analyses of the GLMMs and LMMs.

Analysis of the *B. afzelii* tissue infection prevalence: The *flagellin* qPCR (which cannot distinguish between strains) was used to determine whether tissues were infected with *B. afzelii* or not. Tissue samples were considered infected with *B. afzelii* if at least two of the three replicate *flagellin* qPCR assays tested positive. The binomial response variable was whether *B. afzelii* was absent (0) or present (1) in a tissue sample, and it was analysed as a generalized linear mixed effects model (GLMM) with binomial errors.

Analysis of the total tissue spirochete load: The *flagellin* qPCR (which cannot distinguish between strains) was used to estimate the total *B. afzelii* spirochete load per mg of tissue in each organ. The spirochete loads estimated by the *flagellin* qPCR in 3 µl of DNA template were corrected to the total DNA extraction elution volume (150 µl), standardized by mg of tissue extracted, and log-10 transformed to improve normality. The standardized log10-transformed tissue spirochete load was analysed as a linear mixed effects model (LMM).

Effect of strain, co-infection, and organ on the strain-specific tissue infection prevalence: The focal strains in experiments 1 and 2 are Fin-Jyv-A3 and NE4049, respectively. In experiments 1 and 2, the tissue sample was considered to be infected with the focal strain (Fin-Jyv-A3 or NE4049) if at least two of the three replicate strain-specific qPCR assays (*ospC* A3 or *ospC* A10) tested positive. The binomial response variable was whether the focal strain was absent (0) or present (1) in a tissue sample, and it was analysed as a GLMM with binomial errors. The fixed factors were focal strain (two levels: Fin-Jyv-A3, NE4049), co-infection (two levels: no, yes), organ (six levels: bladder, left ear, right ear, heart, joint, dorsal skin), and their interactions. Mouse identity was modelled as a random factor. Models that differed with respect to the fixed factor of interest were compared using log-likelihood ratio (LLR) tests to determine statistical significance.

Effect of strain, co-infection, and organ on the strain-specific tissue spirochete load: The focal strains in experiments 1 and 2 are Fin-Jyv-A3 and NE4049, respectively. For the co-infected mice, the abundance of each strain in a given tissue was estimated by combining the total spirochete abundance of the *flagellin* qPCR with the relative abundances of the *ospC* qPCRs (see section 1 in the supplementary material for details). These strain-specific spirochete loads were log-10 transformed to improve normality and this variable was analysed using an LMM. The fixed factors, random factors, model simplification, and model analyses were the same as the analyses for the strain-specific tissue infection prevalence.

RESULTS

Eight mice were excluded from the study: 2 mice died during the experiment, 1 mouse was not infected, and 5 mice in the co-infected group only became infected with strain Fin-Jyv-A3 (i.e. strain NE4049 was excluded). The final sample size therefore consisted of 32 mice: strain Fin-Jyv-A3 alone (n = 9), strain Fin-Jyv-A3 in co-infection (n = 7), strain NE4049 alone (n = 9), and strain NE4049 in co-infection (n = 7).

The *B. afzelii* infection prevalence differs among organs: We estimated the prevalence of *B. afzelii* infection in the 6 mouse tissue samples (n = 192 tissue samples for 32 mice) using the *flagellin* qPCR. The rank order of tissue infection prevalence among organs (from highest to lowest) was bladder (32/32 = 100.0%), left ear (29/32 = 90.6%), right ear (29/32 = 90.6%), heart (27/32 = 84.4%), ankle joint (28/32 = 87.5%), and dorsal skin (20/32 = 62.5%). These significant differences in tissue infection prevalence among organs (GLMM LLR: Δ df = 5, Δ χ^2 = 22.368, $p < 0.001$) indicate that *B. afzelii* exhibits tissue tropism (see section 3 in the supplementary material for a comprehensive analysis).

The *B. afzelii* infection prevalence differs among strains: We tested whether the two strains differed in tissue tropism for the subset of mice infected with one strain (n = 18). We used a GLMM with binomial errors to analyse the tissue infection prevalence as a function of organ and strain. The interaction between focal strain and organ was not significant (GLMM LLR: Δ df = 5, Δ χ^2 = 6.184, p = 0.286). The tissue infection prevalence for strain NE4049 (90.7% = 49/54) was significantly higher (1.2x) than that of strain Fin-Jyv-A3 (74.1% = 40/54; GLMM LLR: Δ df = 1, Δ χ^2 = 5.924, p = 0.015). Thus, strain NE4049 was more successful at establishing infection in rodent tissues than strain Fin-Jyv-A3. As shown previously, the tissue infection prevalence differed significantly among organs (GLMM LLR: Δ df = 5, Δ χ^2 = 12.923, p = 0.024).

The total tissue spirochete load of *B. afzelii* differs among organs: We estimated the spirochete load of *B. afzelii* in the subset of infected mouse tissue samples (n = 165 for 32 mice) using the *flagellin* qPCR. If the heart, which had the lowest spirochete load, is set as the reference, then the spirochete load is higher in the bladder (5.3x), ankle joint (5.5x), dorsal skin (26.7x), right ear (121.7x), and left ear (151.8x) (Figure 1). These significant differences in tissue spirochete load among organs (LMM LLR: Δ df = 5, Δ χ^2 = 977.1, $p < 2.2e-16$) show that *B. afzelii* has tissue tropism (see section 4 in the supplementary material for a comprehensive analysis).

The total tissue spirochete load differs among strains: We tested whether the two strains differed in tissue bacterial abundance for the subset of mice infected with one strain (n = 18). We used an LMM to analyse the standardized log10-transformed strain-specific tissue spirochete loads as a function of organ and focal strain. The interaction between focal strain and organ was significant (LMM LLR: Δ df = 5, Δ χ^2 = 11.85, p = 0.037), and we therefore compared the tissue spirochete load between the two strains separately for each organ. The mean spirochete load in the ankle joints for strain Fin-Jyv-A3 (mean = 347; 95% CI = 184–655; units = spirochetes per mg of tissue) was significantly higher (3.8x) than that of strain NE4049 (mean = 98; 95% CI = 61–158; df = 101.5, t = 3.245, p = 0.0016). There were no significant differences in spirochete load between the two strains for the other five organs (see section 5 in the supplementary material). Thus, strain Fin-Jyv-A3 established higher spirochete loads in the joint tissues than strain NE4049.

Effect of strain, co-infection, and organ on the strain-specific tissue infection prevalence: The strain-specific tissue infection prevalence refers to whether a tissue was infected with the focal strain. We used a GLMM with binomial errors to analyse the strain-specific infection prevalence in the mouse tissues as a function of focal strain, co-infection, and organ. Non-significant terms were removed using stepwise model simplification; the best model included focal strain, co-infection and organ (see section 6 in the supplementary material). The tissue infection prevalence in the single strain group (82.4% = 89/108; n = 18 mice) was significantly higher (1.4x) compared to the co-infection group (59.5% = 50/84; n = 14 mice; Figure 2; GLMM LLR: Δ df = 1, Δ χ^2 = 14.504, p = 0.0001). The tissue infection prevalence of strain NE4049 (88.5% = 85/96; n = 16 mice) was significantly higher (1.6x) compared to strain Fin-Jyv-A3 (56.3% = 54/96; n = 16 mice; Figure 2; GLMM LLR: Δ df = 1, Δ χ^2 = 25.704, p < 0.000001). As shown previously, the strain-specific tissue infection prevalence differed significantly among organs (GLMM LLR: Δ df = 5, Δ χ^2 = 17.830 p = 0.003). Thus, co-infection resulted in competition between strains, which reduced their presence in the host tissues (see section 7 in the supplementary material for a comprehensive analysis).

Effect of strain, co-infection, and organ on the strain-specific tissue spirochete load: For the infected mouse tissue samples (n = 165), we used an LMM to analyse the strain-specific spirochete load in the mouse tissue samples as a function of focal strain, co-infection, and organ. A classic stepwise model simplification approach found that the three-way interaction was significant (LME LLR: Δ df = 4, Δ χ^2 = 10.633, p = 0.031), and the analysis was therefore divided by focal strain. For strain Fin-Jyv-A3, the interaction between co-infection and organ was significant (LME LLR: Δ df = 4, Δ χ^2 = 22.324, p = 0.0002); we therefore tested the effect of co-infection separately for each organ. In the bladder, co-infection significantly decreased (4.0x) the mean spirochete load of strain Fin-Jyv-A3 (Table 1; df = 58.9, t = 3.109, p = 0.003). In the ankle joint, co-infection significantly increased (5.9x) the mean spirochete load of strain Fin-Jyv-A3 (df = 67.4, t = -2.353, p = 0.022). The contrast between single infection and co-infection was not significant for the other 3 organs (see section 8 in the supplementary material). For strain NE4049, the interaction between co-infection and organ was not significant (LME LLR: Δ df = 5, Δ χ^2 = 3.152, p = 0.677). Co-infection was not significant (LME LLR: Δ df = 1, Δ χ^2 = 3.500, p = 0.061), but as before, organ had a significant effect on the strain-specific organ spirochete load (Table 1; LME LLR: Δ df = 5, Δ χ^2 = 600.226, p < 0.000001).

Relationship between host-to-tick transmission of the focal strain and the presence and abundance of the focal strain in the mouse organs: We used a GLMER with binomial errors to test whether variation in strain-specific host-to-tick transmission success depended on the co-infection-induced changes in the presence and abundance of the focal strain in the rodent tissues. We tested a number of host-related variables including the number of organs infected with the focal strain, the mean spirochete load of the focal strain across all organs, and the infection status and spirochete load of the organs either separately or combined in groups of internal and external organs.

The number of organs infected with the focal strain (GLMM LLR: Δ df = 1, Δ χ^2 = 8.401, p = 0.004) and the mean organ spirochete load of the focal strain (GLMM LLR: Δ df = 1, Δ χ^2 = 8.028, p = 0.005) both significantly increased the host-to-tick transmission of the focal strain (Figure 3; see sections 9 and 10 in the supplementary material). The strain-specific infection status of the right ear (GLMM LLR: Δ df = 1, Δ χ^2 = 8.485, p = 0.004) and the spirochete load of the focal strain in the right ear (GLMM LLR: Δ df = 1, Δ χ^2 = 9.192, p = 0.002) both significantly increased the host-to-tick transmission of the focal strain. None of the other organs had a significant effect (see sections 11 and 12 in the supplementary material). An AIC-based model selection analysis suggested that the strain-specific host-to-tick transmission depended on the infection status of the external organs (left ear, right ear, dorsal skin) and not the internal organs (bladder, heart, ankle joints) (see section 13 in the supplementary material).

DISCUSSION

Prevalence of *B. afzelii* in the tissues of the rodent host: Overall, the prevalence of *B. afzelii* infection in the organs was high; 5 of the 6 organs had an infection prevalence >80%. These results were similar to a recent experimental infection study with strain NE4049 in the same host (27 female BALB/c mice) where 92.6% of the tissue samples (100/108) tested positive for *B. afzelii* [34]. In the present study, we found that NE4049 established infection in more tissues than Fin-Jyv-A3, which suggests that NE4049 is more invasive or more infectious than Fin-Jyv-A3.

Spirochete load of *B. afzelii* in the tissues of the rodent host: The spirochete load of *B. afzelii* per mg of tissue differed greatly between the organs. For the subset of infected tissues, we found that the spirochete load was highest in the ears and the dorsal skin and lower in internal organs like the heart, bladder, and ankle joints. Other studies on *B. afzelii* and *B. burgdorferi* sensu stricto (ss) in rodent hosts, have also found that the spirochete load is higher in the skin compared to internal organs like the bladder and heart [34-38]. One explanation for this tissue tropism is that the skin is important for the host-to-tick transmission of *B. burgdorferi* sl [21-23], whereas the internal organs (e.g. bladder, heart, and joints) are a dead-end for spirochete transmission to ticks [39].

We found strain-specific tissue tropism in the mice that were infected with single strains; strain Fin-Jyv-A3 established a higher spirochete load in the ankle joints compared to strain NE4049. Previous studies on *B. afzelii* and *B. burgdorferi* ss in lab mice have shown differences in tissue spirochete load between strains [21, 38, 40, 41]. This strain-specific variation is important from a medical perspective because it has been associated with host pathology including carditis, arthritis, and ankle joint swelling [40, 41]. The tropism of Fin-Jyv-A3 for the joints suggests that this strain would cause more arthritis and joint swelling in the host compared to strain NE4049, as observed for strains of *B. burgdorferi* ss in lab mice [40, 41].

Competition between strains of *B. afzelii* in the tissues of the rodent host: Our study demonstrated that co-infection of mice with two strains of *B. afzelii* resulted in competitive interactions between strains in the host tissues. We measured the outcome of co-infection in two ways: (1) strain-specific tissue infection prevalence and (2) strain-specific tissue spirochete load. For strain Fin-Jyv-A3, co-infection reduced the tissue infection prevalence by 55%, indicating a strong effect of competition (Figure 2). The results of our study are in agreement with other studies on vector-borne pathogens that have shown that co-infection leads to competitive interactions in the vertebrate host [3, 10, 42]. Our study is the first experimental demonstration that strains belonging to the same *B. burgdorferi* sl genospecies compete inside the tissues of the vertebrate host. An uncontrolled field study in Sweden on small mammals naturally infected with *B. afzelii* previously found results consistent with inter-strain competition in the host [13]. Other studies on *B. afzelii* and *B. burgdorferi* ss have shown that co-infection in the rodent host reduced strain-specific transmission to ticks [14, 15, 32, 43], however, this result could be caused by interactions in the rodent host, the tick vector, or both. In contrast, our controlled study clearly shows that co-infection and competitive interactions reduce the ability of some *B. afzelii* strains to establish infection in the tissues of the rodent host.

With respect to organ spirochete load, we found that the outcome of the interactions between strains depended on the organ. In co-infected mice, strain Fin-Jyv-A3 experienced competition in the bladder, but facilitation in the ankle joints. We had expected that inter-strain competition would be most intense in the ears and dorsal skin because these organs are critical for host-to-tick transmission, but this was not the case. In contrast, we do not expect spirochetes in the internal organs to contribute to host-to-tick transmission and so the reason for significant inter-strain competition in the bladder is not clear. Strain Fin-Jyv-A3 has a tropism for joint tissues, and we note that while this strain was generally the loser in competition, it actually increased its abundance in the ankle joints of co-infected mice compared to when alone. Thus, when faced with a superior competitor, Fin-Jyv-A3 increased its abundance in the only tissue for which it had a tissue tropism compared to NE4049.

Strength of the infection in the rodent host and host-to-tick transmission: Host-to-vector transmission is one of the critical life history traits for any vector-borne pathogen and determines the frequency of a strain in nature and whether it is common or rare [28, 44, 45]. We demonstrated that inter-strain competition influenced the presence and abundance of the two strains in the rodent tissues, which subsequently influenced their strain-specific host-to-tick transmission. Both the tissue infection prevalence and the tissue spirochete load were positively related to host-to-tick transmission of the focal strain (Figure 3). Our study is in agreement with studies on the rodent malaria parasite (*P. chabaudi*) in lab mice, where strains with higher abundance in the blood have higher mouse-to-mosquito transmission [3, 46, 47]. Previous studies on *B. afzelii* found a positive relationship between the spirochete load in rodent ear tissues and host-to-tick transmission [21, 22], but these studies did not investigate inter-strain competition. Our study is the first experimental demonstration that competitive interactions between strains of *B. burgdorferi* sl in the host tissues determine strain-specific host-to-tick transmission success.

Inter-strain competition and strain diversity of *B. burgdorferi* sl pathogens in nature: In nature, there is substantial strain diversity of *B. burgdorferi* sl pathogens at small spatial scales [27]. Studies have shown that some strains are much more common than others, but that these differences in strain frequency are constant over time and space [26, 30] [48, 49]. A major question is to understand the ecological factors that maintain the strain composition of the pathogen population. The multiple niche polymorphism (MNP) hypothesis proposes that the different strains are adapted to different vertebrate reservoir hosts [50, 51]. The present study suggests that competitive interactions between strains in the host tissues can also have important consequences for their host-to-tick transmission. The MNP hypothesis and the inter-strain competition hypothesis are not mutually exclusive; for example, the competitive ability of strains could differ between vertebrate hosts. Future studies should investigate the relative importance of MNP versus inter-strain competition in maintaining the strain diversity of *B. burgdorferi* sl pathogens in nature.

Conclusions: For multi-strain vector-borne pathogens, we show that competition between strains in the host tissues can reduce host-to-vector transmission. Simultaneous co-infection of the rodent host can inhibit the ability of strains to invade and establish infection in the host tissues. Depending on the organ, co-infection and interactions between strains either increased or decreased the spirochete abundance of a given strain in the mouse tissues. The ability to establish infection in many tissues and the mean bacterial abundance were both positively related with strain-specific host-to-tick transmission success. Our results also suggest that the skin is the critical organ for the host-to-tick transmission of *B. afzelii*.

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Author contributions: DG and MJV conceived and designed the study. MR, AS, FB, and DG conducted the experiment and performed the molecular work. MR and AS developed the nested strain-specific qPCRs. OR helped with the experimental infections of the mice. ROMR performed the plasmid profile analysis of the *B. afzelii* strains. DG performed the statistical analyses. DG and MJV wrote the manuscript. All authors read and approved the final version of the manuscript.

TABLES AND FIGURES

Table 1. The strain-specific organ spirochete load is shown for each of the 24 unique combinations of focal strain, organ, and co-infection. There are two strains (Fin-Jyv-A3, NE4049), six organs (bladder, left ear, right ear, heart, right ankle joint, and the section of dorsal skin where the nymphs had attached), and two co-infection states (no, yes). The data are shown for the 32 mice that survived to the end of the study. The units of the strain-specific organ spirochete load are the number of spirochetes of the focal strain per mg of tissue. The estimates are based on combining the estimates of total spirochete abundance from the *flagellin* qPCR with the estimates of the strain-specific relative abundance from the strain-specific *ospC* qPCRs. Shown are the means and the 95% confidence intervals (95% CI).

Focal strain	Organ	Co-infection	Mean	95% CI
Fin-Jyv-A3	Bladder	no	157	93–265
Fin-Jyv-A3	Bladder	yes	39	18–85
Fin-Jyv-A3	Left ear	no	5 926	3 278–10 715
Fin-Jyv-A3	Left ear	yes	4 987	1 647–15 101
Fin-Jyv-A3	Right ear	no	2 533	1 336–4 803
Fin-Jyv-A3	Right ear	yes	1 151	380–3 484
Fin-Jyv-A3	Heart	no	33	19–57
Fin-Jyv-A3	Heart	yes	27	12–59
Fin-Jyv-A3	Joint	no	347	172–700
Fin-Jyv-A3	Joint	yes	2 051	677–6 212
Fin-Jyv-A3	Dorsal skin	no	1 074	533–2 164
Fin-Jyv-A3	Dorsal skin	yes	NA	NA
NE4049	Bladder	no	178	106–300
NE4049	Bladder	yes	170	94–307
NE4049	Left ear	no	5 782	3 323–10 062
NE4049	Left ear	yes	6 741	3 556–12 781
NE4049	Right ear	no	3 463	2 054–5 838
NE4049	Right ear	yes	6 506	3 598–11 763
NE4049	Heart	no	26	15–45
NE4049	Heart	yes	25	12–56
NE4049	Joint	no	98	58–166
NE4049	Joint	yes	174	96–314
NE4049	Dorsal skin	no	759	400–1 439
NE4049	Dorsal skin	yes	1 324	657–2 668

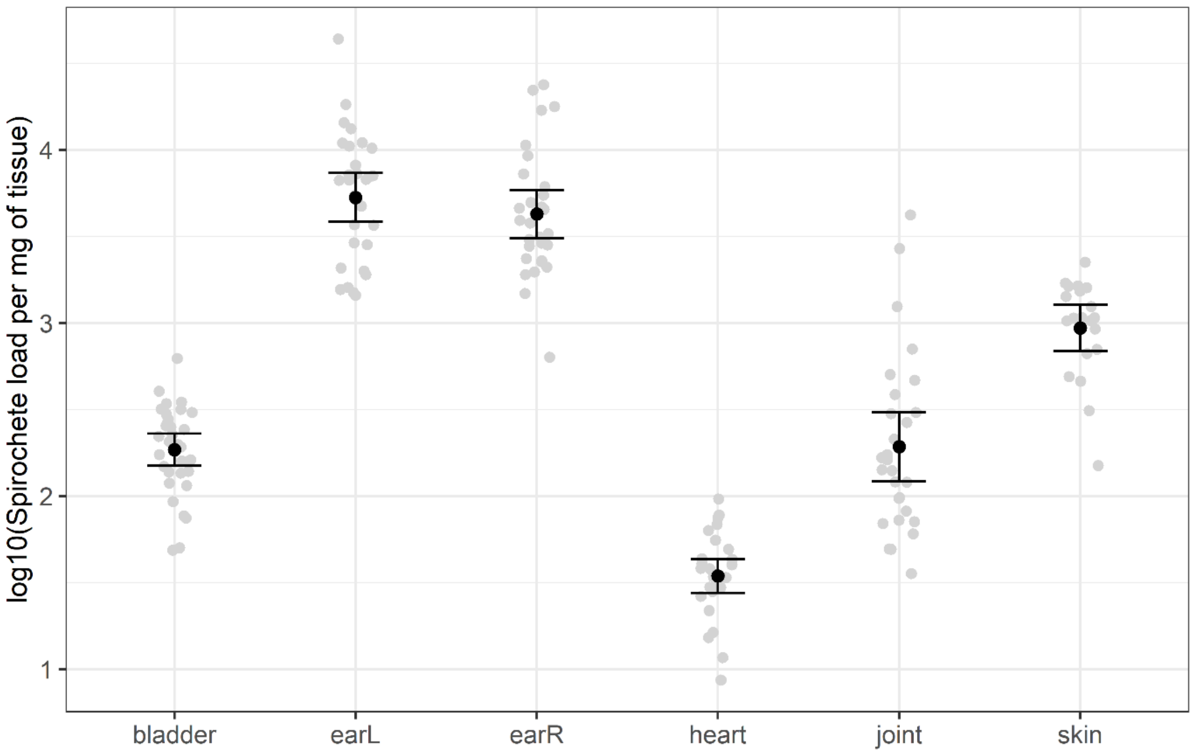

Figure 1. *B. afzelii* exhibits tissue tropism with respect to the spirochete load in the mouse tissues. The tissue spirochete load was estimated using a *flagellin* qPCR and was standardized per mg of tissue. The log10-transformed tissue spirochete load per mg of tissue is significantly different among the six mouse organs: bladder, left ear (earL), right ear (earR), heart, right ankle joint, and the section of dorsal skin where the nymphs had attached. Shown are the means and the 95% confidence intervals.

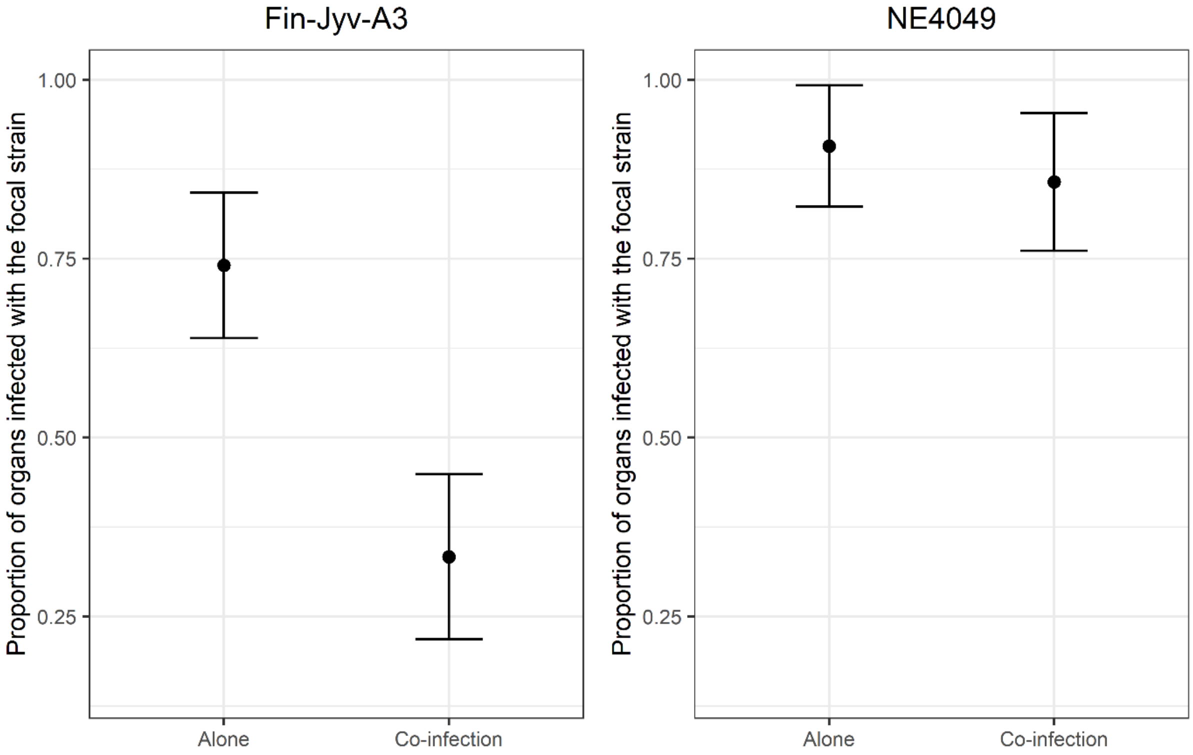

Figure 2. Competition between strains influences the number of tissue samples infected by the focal strain. The left and right panels show the results from two independent experiments where the focal strains are Fin-Jyv-A3 and NE4049, respectively. For both strains co-infection in the mouse reduced the infection prevalence in the tissue samples of the six organs indicating that the strains experienced competition. Shown are the means and the 95% confidence intervals.

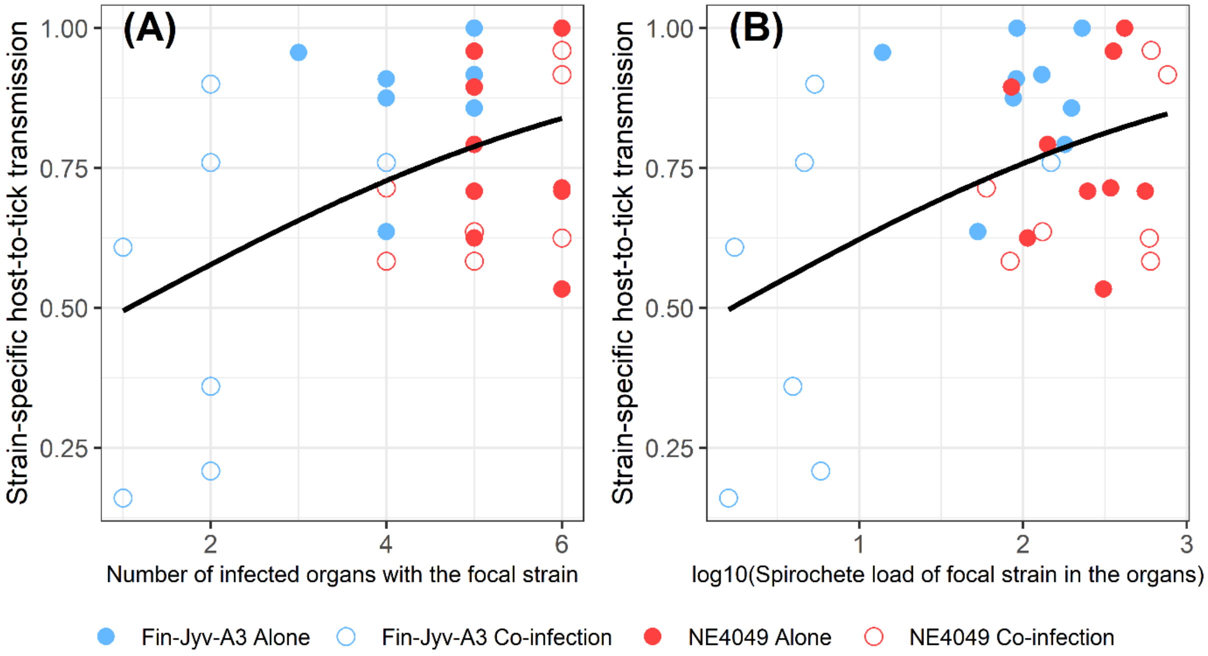

Figure 3. Host-to-tick transmission of the focal strain depends on (A) the number of organs that are infected with the focal strain or (B) the mean spirochete load of the focal strain in the mouse tissues. In (A) the six organs included the bladder, left ear, right ear, heart, right ankle joint, and the section of dorsal skin where the nymphs had attached. In (B) the spirochete load of the focal strain was averaged over the six organs. Strain Fin-Jyv-A3 and strain NE4049 are shown in blue and red symbols, respectively. Mice in the single strain groups and the co-infected groups are shown with solid and empty symbols, respectively. The black line shows the line of best fit from a GLM with binomial errors.

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6. Chapter 4

Mixed strain infections reduce the transmission of a tick-borne pathogen

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The supplementary material of this chapter can be found in the appendix section.

ABSTRACT

For vector-borne pathogens, co-infections with multiple strains can be found in both the vertebrate host and the arthropod vector. Competitive interactions between strains can negatively influence the subsequent transmission of the strains. In this study, we used two strains of the bacterium *Borrelia afzelii* to test whether co-infection in the tick vector can affect the tick-to-host transmission of the strains. 100 test mice were individually infected with a single challenge nymph, which had fed as larvae on donor mice that were either infected with a single strain or co-infected. To determine the infection status of the test mice, we used both xenodiagnostic ticks and a tissue sample. We used strain-specific qPCR assays to detect the presence of each strain in the engorged challenge nymphs, the tissue sample of the test mice, and the xenodiagnostic ticks. However, our method was not able to reliably determine the infection status of the engorged challenge nymphs. To explain tick-to-host transmission of the strains, we used instead the strain-specific probability of infection in the challenge nymphs and the known infection status of the donor mice. We found that co-infection in the donor mice decreased the tick-to-host transmission of both strains. Furthermore, strains differed in their ability to establish an infection in the test mice. Our results therefore suggest that the presence of multiple strains in the vertebrate host negatively affects the entire transmission cycle of the bacteria (from one vertebrate host to the next). The second finding of this study suggests that strains can differ in their ability to exploit different parties of their life cycle, which can ultimately prevent them from competitive exclusion.

KEYWORDS: *Borrelia afzelii*, co-infection, competition, Lyme borreliosis, transmission, vector-borne pathogen

INTRODUCTION

The lifecycles of pathogens and parasites consists of different steps such as finding a host, establishing infection, reproduction/multiplication, and transmission to a new host [1]. Pathogens that maintain high abundance in the host tissues are expected to achieve better transmission. Different factors can influence pathogen abundance in the host tissues, and one of them is the presence of a co-infecting parasite. These co-infections or mixed infections are common, and occur when the host is infected with multiple pathogen species or with multiple genotypes of the same pathogen species [2-4]. Mixed infections in the host can lead to interactions between pathogen species or between pathogen strains. These interactions can enhance pathogen fitness in the case of facilitation [5] or reduce pathogen fitness in the case of competition [6, 7]. For vector-borne pathogens, the outcome of interactions between strains can depend on whether they occur in the vertebrate host or the arthropod vector.

Vector-borne pathogens experience two independent transmission events, from the host to the vector and from the vector to the host. Mixed infections of vector-borne pathogens occur in both the vertebrate host and the arthropod vector [1, 3, 4, 8-10]. Most studies on mixed infections in vector-borne systems have been done in the vertebrate host [11-15]. Competitive interactions between strains in the host have been shown to reduce their host-to-vector transmission success [14, 16-18]. Conversely, studies on mixed infections in arthropod vectors have found both facilitation [9], and competition [16, 19], with respect to the strain-specific abundance. However, whether competition between strains in the arthropod vector influences their vector-to-host transmission success remains unknown.

We use the tick-borne pathogen *Borrelia afzelii* as a model system to study how interactions between strains influence their transmission success. This spirochete bacterium belongs to the *B. burgdorferi* sensu lato genospecies complex and is one of the most common causes of Lyme borreliosis in Europe [20]. *B. afzelii* is transmitted by *Ixodes ricinus* ticks and uses small mammals as reservoir hosts [20]. The life cycle of *I. ricinus* consists of three stages: larva, nymph, and adult. Transovarial transmission of *B. burgdorferi* sl is rare [21, 22]; *I. ricinus* larvae acquire the bacterium after feeding on infected hosts. Newly infected larvae moult into infected nymphs, which overwinter and transmit the bacterium back to the vertebrate host population in the following year. Adult female ticks require a blood meal to produce eggs, and they usually feed on larger mammals (e.g. deer), which are incompetent hosts for *B. afzelii* [23, 24]. The nymph is therefore the most important stage for the transmission of *B. afzelii*, and the density of infected nymphs determines the risk of Lyme borreliosis [25].

Populations of *B. afzelii* consist of multiple genetically distinct strains [26, 27]. In areas where *B. afzelii* is endemic, the majority of the *I. ricinus* nymphs are co-infected with multiple strains [26, 28]. A field survey found an association between the frequency of a strain in the *B. afzelii* population and its bacterial abundance in nymphs [28]. This observation suggests that variation among strains in bacterial abundance in nymphs is associated with variation in nymph-to-host transmission success. Experimental studies on *B. afzelii* have shown that co-infection and competition reduce the bacterial abundance of strains in the nymph [16, 19]. This observation suggests that co-infection in the nymph will reduce the transmission success of the strains from the co-infected nymph to the host (hereafter referred to as the nymph-to-host transmission success). In the present study, we used an experimental approach to test whether co-infection and competition between two strains of *B. afzelii* in *I. ricinus* nymphs influence the strain-specific nymph-to-host transmission success. We expected that strain-specific nymph-to-host transmission success would be reduced in co-infected nymphs compared to nymphs infected with a single strain. We also expected that the two strains of *B. afzelii* would differ in their nymph-to-host transmission success and that this transmission phenotype would depend on the bacterial abundance in the nymph.

MATERIALS AND METHODS

Mice, ticks and *Borrelia afzelii* strains: 100 female 5-week-old pathogen-free *Mus musculus* BALB/c mice were used in this study. *I. ricinus* ticks were obtained from a laboratory colony that has been maintained since 1978 at the University of Neuchâtel. Strain Fin-Jyv-A3 was obtained from a bank vole in Finland, has *ospC* major group (oMG) A3, multi-locus sequence type (MLST) 676, and strain ID number 1961 in the *Borrelia* MLST database. Strain NE4049 was obtained from a Swiss *I. ricinus* tick, has oMG A10, MLST 679, and strain ID number 1887 in the *Borrelia* MLST database.

Ethics approval: The commission that is part of the “Service de la Consommation et des Affaires Vétérinaires (SCAV)” of Canton Vaud, Switzerland evaluated and approved the ethics of this study. The Veterinary Service of the Canton of Neuchâtel, Switzerland issued the animal experimentation permit used in this study (NE03/2018).

Overview of the study: The purpose of this study was to test whether co-infection in the nymphal tick with strains Fin-Jyv-A3 and NE4049 influences their nymph-to-host transmission success. We therefore compared the nymph-to-host transmission success of strains in co-infected nymphs versus nymphs infected with single strains. In a previous infection experiment [16, 19], we created three types of infected nymphs: (1) infected with strain Fin-Jyv-A3, (2) infected with strain NE4049, and (3) co-infected with both strains by feeding them as larvae on (1) mice infected with strain Fin-Jyv-A3, (2) mice infected with strain NE4049, and (3) mice co-infected with both strains (Figure 1). These nymphs were subsequently used to challenge the mice; each mouse was infested with one nymph belonging to one of the three infection types. Following the nymphal infestation, we collected the engorged nymphs and tested their strain-specific infection status. After allowing the infection to develop in the mice, we tested their strain-specific infection status using a tissue sample and xenodiagnosis.

Creation of the challenge nymphs: *I. ricinus* nymphs that carried three types of infection, strain Fin-Jyv-A3 alone, strain NE4049 alone, or both strains, were created as follows. In a previous experiment [16], larval ticks from our laboratory colony of *I. ricinus* were fed on mice carrying three types of infection: (1) strain Fin-Jyv-A3 alone (n = 10 mice), (2) strain NE4049 alone (n = 10 mice), (3) and both strains (n = 20 mice). The engorged larval ticks were allowed to molt into flat nymphs. For each of the 40 mice, we tested a random sample of these flat nymphs (range = 7 to 26 per mouse) to determine the probabilities of the three types of infection [16, 19]. For the second part of the study (see below), a subsample of 30 donor mice was selected because their flat nymphs had a high probability of carrying the three types of infection of interest (see Table S1 in the supplementary material). For the 9 donor mice in group 1, the mean probability that a flat nymph was infected with strain Fin-Jyv-A3 was 88.2% (range = 63.6% to 100.0%). For the 9 donor mice in group 2, the mean probability that a flat nymph was infected with strain NE4049 was 77.0% (range = 53.3% to 100.0%). For the 12 donor mice in group 3, the probability that a flat nymph was co-infected with both strains, infected with strain Fin-Jyv-A3 alone, or infected with strain NE4049 alone was 42.4% (range = 12.0% to 80.0%), 14.3% (range = 0.0% to 33.0%), and 30.3% (range = 8.0% to 72.0%), respectively.

Tick-to-host transmission of *B. afzelii*: 100 female, pathogen-free *M. musculus* BALB/c mice aged 5 weeks were used to test the efficacy of nymph-to-host transmission. The study consisted of two independent experiments where the focal strains were Fin-Jyv-A3 and NE4049. In experiment 1, test mice were exposed to Fin-Jyv-A3-infected nymphs (n = 12 mice) or to co-infected nymphs (n = 38 mice). In experiment 2, test mice were exposed to NE4049-infected nymphs (n = 12) or to co-infected nymphs (n = 38). All test mice were infested with a single challenge nymph aged 10 to 11 months that was randomly selected from one of the donor mice. Importantly, the probability that each challenge nymph carried the infection of interest was known because we had tested a random sample of flat nymphs for each donor mouse (see above). Each challenge nymph was placed in a plastic capsule that was attached to the shaved back of the test mouse, and that was checked daily to ensure that the nymph had attached to the test mouse. The capsules also allowed us to collect the engorged nymphs once they had detached from the test mice. The engorged nymphs were frozen at -20°C to determine their strain-specific infection status. To manage the workload, the study was conducted in two temporal blocks (A and B) that were separated by 1 month.

Ear tissue biopsies, IgG antibodies, and xenodiagnosis to determine *B. afzelii* infection status in the test mice: To confirm *B. afzelii* infection, blood and ear tissue samples were taken from the test mice at 4 weeks after the nymphal challenge. We used qPCR to detect *B. afzelii* in the ear tissue samples of the test mice and SERION® ELISA classic *Borrelia burgdorferi* IgG/IgM immunoassay to detect *Borrelia*-specific IgG antibodies in the blood samples of the test mice.

For vector-borne pathogens like *B. burgdorferi* sl, xenodiagnoses, which is the testing of vectors that have fed on a host, is a sensitive method for determining host infection status [29]. Five weeks after the nymphal challenge, each test mouse was infested with ~100 pathogen-free xenodiagnostic larval ticks from our laboratory colony of *I. ricinus*. Blood-engorged larvae were kept in individual Eppendorf tubes, and were allowed to moult into xenodiagnostic nymphs under standard laboratory conditions (20–25°C, 12h light: 12h dark). To maintain high humidity, each tube contained a piece of moistened paper towel. Four weeks after the larva-to-nymph moult, 10 xenodiagnostic nymphs were randomly selected for each test mouse and frozen at –20°C for DNA extraction. To test whether the spirochetes were viable, we placed up to 5 xenodiagnostic nymphs in BSK medium for each test mouse that had a sufficient number of ticks. The cultures were checked for the presence of live spirochetes on a weekly basis using a dark field microscope.

DNA extraction of ticks and mouse tissue samples: The engorged challenge nymphs and the four-week-old xenodiagnostic flat nymphs were crushed using a previously described protocol [30]. The crushed nymphs were digested with proteinase K at 56°C overnight. The DNA of the nymphs was extracted using QIAGEN DNeasy 96 Blood and Tissue kit well plates and following the QIAGEN protocol. Each plate contained 4 negative DNA extraction controls (*Anopheles gambiae* mosquitoes) and 1 water control. DNA from the ear samples of the test mice was extracted using QIAGEN DNeasy Blood & Tissue mini spin columns and following the QIAGEN protocol. Mouse ear tissue DNA and nymph DNA were eluted into 65 µl of water.

qPCR to determine infection and co-infection status: To determine the infection status and the co-infection status of the samples, three different qPCR assays were performed. A qPCR assay that targets a 132 bp fragment of the *flagellin* gene was used to determine the *B. afzelii* infection status of the samples (= *flagellin* qPCR). Two strain-specific qPCRs that amplify the same 143 bp fragment of the *ospC* gene, but that use different strain-specific probes, were used to identify the strains in the samples (= *ospC* qPCR). Finally, a nested strain-specific qPCR was used to identify the strains in samples that had low abundance of spirochetes (= nested qPCR). First, a PCR that targets a 657 bp fragment of the *ospC* gene [31] was used to amplify the number of *ospC* gene copies. The amplicons were then used as template in the two strain-specific qPCRs to identify the co-infection status of the samples. Flat nymphs were tested using the *flagellin* qPCR and the two strain-specific qPCRs that target the *ospC* gene. Ear tissue samples from the test mice were tested using the *flagellin* qPCR and the nested qPCR for strain identity. The engorged nymphs were tested with the *flagellin* qPCR, the *ospC* qPCR, and in triplicate with the nested qPCR.

The qPCR assays were done using a LightCycler® 96 Multiwell Plate white (Roche). All the plates contained 4 negative controls for the DNA extraction (mosquito DNA), 3 negative controls for the qPCR (water), and 12 positive controls at 4 different dilutions. For each qPCR assay, the 12 positive controls were used to determine the repeatability of the assay. For the *flagellin* qPCR, *ospC* A3 qPCR, and *ospC* A10 qPCR, the repeatability of the log10-transformed spirochete loads was 96.5%, 98.4%, and 96.2% respectively. The conventional *ospC* PCR (first step of the nested qPCR) was done using a Mastercycler® nexus (Eppendorf). All the plates contained 3 negative controls for the PCR (water).

RESULTS

***B. afzelii* infection status of the test mice:** The *B. afzelii* infection status of the test mice (yes, no) was based on three independent infection phenotypes: presence of *B. afzelii* in the ear punch biopsy, presence of IgG antibodies against *B. afzelii*, and presence of *B. afzelii* in the xenodiagnostic nymphs. We are highly confident in the *B. afzelii* infection status of the test mice because the correspondence between the ear punch biopsies and the xenodiagnostic nymphs was 100% and their correspondence with the IgG antibody response was 99%. After the nymphal challenge, 60.0% (60/100) of the test mice became infected with *B. afzelii*.

Strain-specific infection status of the test mice: The strain-specific infection status of the test mice (Fin-Jyv-A3, NE4049, co-infected, uninfected) was based on the combined results of the ear punch biopsy and the xenodiagnostic nymphs. The correspondence in the strain-specific infection status between the ear punch biopsy and the xenodiagnostic nymphs was 91% (91/100). The remaining 9 test mice were all classified as co-infected because they contained both strains after combining the results for the ear punch biopsy and the xenodiagnostic nymphs. The strain-specific infection statuses of these 9 test mice are hereafter based on those corrections.

***B. afzelii* infection status of the engorged challenge nymphs:** We were able to collect 81 engorged challenge nymphs; the 19 other engorged challenge nymphs could not be found.

We found that 71.6% (58/81) of the engorged challenge nymphs tested positive for *B. afzelii*. There were 9 engorged challenge nymphs that tested negative for *B. afzelii*, but that transmitted *B. afzelii* to the test mice, indicating that our qPCR-based method did not detect all of the infections in the engorged challenge nymphs.

Strain-specific infection of the engorged challenge nymphs: We compared the strain-specific infection status between the engorged challenge nymphs and the test mice. In 15 of 81 pairs, there was a mismatch in the strain-specific infection status between the engorged challenge nymph and the test mouse (see Tables S2, S3, and S4 in the supplementary information). We decided to not analyse the engorged challenge nymphs because the strain-specific infection status is unknown for the 19 missing nymphs.

Effects of the co-infection status of the donor mice and strain identity on the infection prevalence of the focal strain in the unfed challenge nymphs: In our previous work, we showed that co-infection in the 37 donor mice influenced the infection prevalence of the focal strain in the unfed challenge nymphs [16, 19]. In the present study, we wanted to test whether the effects of co-infection held for the subset of 30 donor mice from which the challenge nymphs (used to expose the test mice) were selected. We used a GLMM with binomial errors to model the infection prevalence of the focal strain in the unfed challenge nymphs. The fixed factors included donor mouse infection status (single strain or co-infection), strain identity (Fin-Jyv-A3 or NE4049), and their interaction; the identity of the donor mice was modelled as a random factor. The interaction between co-infection and strain was not significant (GLMM LLR: Δ df = 1, $\Delta\ \chi^2 = 3.526$, p = 0.065) and was removed from the model. Co-infection status of the donor mouse was significant (GLMM LLR: Δ df = 1, $\Delta\ \chi^2 = 6.239$, p = 0.012; Figure 2, Table S5 in the supplementary material) but strain was not (GLMM LLR: Δ df = 1, $\Delta\ \chi^2 = 0.004$, p = 0.947). Unfed challenge nymphs that had fed as larvae on co-infected donor mice had a lower infection prevalence of the focal strain compared to unfed challenge nymphs that had fed as larvae on donor mice infected with a single strain (Figure 2 and Table 2). Thus, the risk of exposure to the focal strain is lower in unfed challenge nymphs from the co-infected treatment compared to unfed challenge nymphs from the single strain treatment.

Effects of co-infection treatment origin of the challenge nymphs on the infection prevalence of the focal strain in the test mice: Based on the previous results, we expected higher infection prevalence in test mice exposed to the nymphs from the single infection treatment compared to the nymphs from the co-infection treatment. When experiments 1 and 2 were combined, the percentage of test mice infected with the focal strain in the single infection treatment (58.3% = 14/24) was significantly higher (1.8x) compared to the co-infection treatment (31.6% = 24/76; $\chi^2 = 4.464$, df = 1, p-value = 0.034). For the two independent experiments the results were as follows. In experiment 1, the percentage of test mice infected with the focal strain (Fin-Jyv-A3) was 66.7% (8/12) for the single infection treatment and 42.1% (16/38) for the co-infection treatment. In experiment 2, the percentage of test mice infected with the focal strain (NE4049) was 50.0% (6/12) for the single infection treatment and 21.1% (8/38) for the co-infection treatment (Table 2).

Effects of the co-infection treatment origin of the challenge nymphs and strain identity on the infection prevalence of the focal strain in the test mice: We used a GLMM with binomial errors to model the infection status of the focal strain in the test mice as a function of the following fixed factors: strain identity, co-infection treatment origin of the challenge nymph, their interaction, and block (A or B); the identity of the donor mice was modelled as random factor. The co-infection treatment origin of the challenge nymph refers to the co-infection status of the donor mouse from which that challenge nymph was issued. The interaction was not significant (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 0.099$ p = 0.753) and was removed from the model. Block was not significant (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 1.678$, p = 0.195), which means that the one-month age difference between the challenge nymphs of block A versus block B did not affect the probability of *B. afzelii* transmission from the challenge nymphs to the test mice. Strain identity (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 4.455$, p = 0.035) and co-infection status (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 5.593$, p = 0.018) were both significant (Figure 3, Table 2, and Table S6 in the supplementary information). Thus, challenge nymphs that originated from co-infected donor mice transmitted the focal strain to a lower percentage of test mice (31.6% = 24/76) compared to challenge nymphs that originated from singly infected donor mice (58.3% = 14/24). This important result shows that reduced transmission of the focal strain from co-infected donor mice to the larval ticks (Figure 2) had real and negative consequences for strain fitness when the resultant challenge nymphs fed on the next generation of test mice (Figure 3). Strain Fin-Jyv-A3 had a higher infection prevalence in the test mice (48% = 24/50) compared to strain NE4049 (28% = 14/50), suggesting that Fin-Jyv-A3 has higher nymph-to-host transmission than NE4049.

Effects of the probability of infection in the challenge nymphs and strain identity on the prevalence of the focal strain in the test mice: We repeated the previous analysis, but in the fixed effects structure we replaced the co-infection treatment origin of the challenge nymph with the probability that the challenge nymph was infected with the focal strain (hereafter referred to as the probability of exposure), which was based on the random sample of unfed challenge nymphs that was tested for each donor mouse. The interaction between the probability of exposure and strain identity was not significant and was removed from the model (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 0.307$, p = 0.580). Block was not significant (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 1.632$, p = 0.201), but strain identity (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 5.723$, p = 0.017) and the probability of exposure (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 6.406$, p = 0.011) were both significant (Figure 4, Table S7 in the supplementary material). We found the expected positive relationship between the probability of exposure to the focal strain and the probability that the test mouse became infected with the focal strain (Figure 4). This important result shows the mechanistic links between co-infection in the donor mice, reduced transmission of the focal strain from the co-infected donor mice to feeding larval ticks, and the subsequent reduced transmission of the focal strain from the challenge nymphs to the test mice. After correcting for the probability of exposure to the focal strain, strain Fin-Jyv-A3 still had a higher infection prevalence in the test mice compared to strain NE4049 (Figure 4), demonstrating that Fin-Jyv-A3 has higher nymph-to-host transmission than NE4049.

We used the parameter estimates to calculate the probability of nymph-to-host transmission for each strain given that the challenge nymph is actually infected (i.e. probability of exposure to the focal stain is 1.00). Following exposure to an infected challenge nymph that fed to repletion, the probability of nymph-to-mouse transmission was 1.6 times higher for strain Fin-Jyv-A3 (0.680) compared to strain NE4049 (0.425; Figure 4).

DISCUSSION

Effects of co-infection in the rodent host on the fitness of *B. afzelii* strains: We and others have previously shown that co-infection with strains of *B. burgdorferi* sl in the rodent host reduced the host-to-tick transmission success of the strains [16, 17, 19, 32]. Whether the effect of co-infection in the rodent host affected the fitness of the strains in the subsequent tick-to-host transmission step remained to be tested. For the sample of 30 donor mice from which the challenge nymphs were selected (to expose the test mice), we confirmed that co-infection in the donor mice reduced the transmission of the focal strain to the challenge nymphs (Figure 2). We then showed that challenge nymphs originating from co-infected donor mice transmitted the focal strain to a lower percentage of test mice compared to challenge nymphs originating from donor mice infected with a single stain, and this was true for both strains Fin-Jyv-A3 and NE4049 (Figure 3). Co-infection in the donor mice reduced the subsequent nymph-to-host transmission success of both strains by 46%. Thus, our study shows that co-infection in the donor mice (and the associated reduction in host-to-tick transmission) has a significant negative effect on the fitness of these strains in the next step of their life cycle (i.e. nymph-to-host transmission). In the third analysis, we replaced the co-infection treatment of origin of the challenge nymphs with the probability that the challenge nymph was infected with the focal strain (i.e. the probability of exposure). We found the expected result that the probability that a test mouse becomes infected with the focal strain depends on the probability of exposure to the focal strain. In summary, co-infection in the donor mice reduced host-to-tick transmission of the focal strain to the challenge nymphs, which in turn reduced the probability that the test mice were exposed to the focal strain. Whether co-infection in the nymph reduces nymph-to-host transmission of the focal strain (after correcting for the probability of exposure) remains to be tested.

***B. afzelii* strains differ in their probability of nymph-to-host transmission:** Following exposure to the challenge nymphs, a higher percentage of the test mice became infected with strain Fin-Jyv-A3 versus strain NE4049. After correcting for the probability of exposure, strain Fin-Jyv-A3 still infected a higher percentage of test mice compared to strain NE4049 (Figure 4). The probability of nymph-to-host transmission refers to the probability that the host becomes infected with the pathogen after having been exposed to an infected challenge nymph that fed to repletion. For strains Fin-Jyv-A3 and NE4049, we estimated the probability of nymph-to-host transmission to be 68.0% and 42.5%, respectively (Figure 4). Thus, the probability of nymphs-to-host transmission is 1.6 times higher for strain Fin-Jyv-A3 compared to strain NE4049.

To our knowledge, our study provides the first estimated of the probability of nymph-to-host transmission for any *B. burgdorferi* sl pathogen. Theoretical studies that model the epidemiology of Lyme disease typically assume a nymph-to-host transmission efficiency of 80.0%, but this estimate is not based on any published data [33]. Most infection studies challenge hosts with multiple nymphs, which makes it difficult to determine the probability of nymph-to-host transmission per infected tick bite [34-36]. For example, an infection study with *B. afzelii* strain NE4049 infested each rodent host with 3 challenge nymphs and found that the probability of nymph-to-host transmission was >95% [35]. In the present study we showed that a single infected *I. ricinus* nymph can transmit *B. afzelii* to its rodent host. However, exposure to an infected nymph does not guarantee transmission of the pathogen.

The mechanism underlying the higher nymph-to-host transmission success may be related to bacterial abundance in the nymphal tick. This idea is supported by a field study on wild *I. ricinus* nymphs, which found that *B. afzelii* strains that established a high spirochete load *I. ricinus* nymphs had higher frequency in the pathogen population [28]. In previous studies, we have shown that strain Fin-Jyv-A3 has a higher spirochete load in nymphs than strain NE4049, and this is true for singly infected nymphs, co-infected nymphs, 1-month-old nymphs and 4-month-old nymphs [16, 19]. Thus, the higher nymphal spirochete loads of strain Fin-Jyv-A3 compared to strain NE4049 may explain its higher nymph-to-host transmission success. An alternative explanation is that strain Fin-Jyv-A3 is better than strain NE4049 at invading the tick salivary glands [41, 42], or has a higher replication rate. When *Ixodes* nymphs begin their blood meal, spirochetes replicate in the midgut of the nymphs and migrate to the salivary glands for transmission [37-39]. Only a small number of spirochetes (~100) are transmitted to the host [40], therefore, differences in growth rates between the strains could impact their transmission success. Whether the strain-specific spirochete load in challenge nymphs explain the nymph-to-host transmission of the strains remains to be tested.

Maintenance of *B. afzelii* strain diversity in nature: Vector-borne pathogens can experience trade-offs in fitness or performance between the vertebrate host and the arthropod vector. The evidence that strains Fin-Jyv-A3 and NE4049 have superior performance in the tick vector versus the rodent host is as follows. Strain Fin-Jyv-A3 establishes higher spirochete loads in nymphal ticks compared to strain NE4049 [16, 19]. Strain Fin-Jyv-A3 also has significantly higher nymph-to-host transmission success compared to strain NE4049. In contrast, in co-infected mice, strain NE4049 infected more organs in the rodent host compared to strain Fin-Jyv-A3, which resulted in higher host-to-tick transmission success for strain NE4049 compared to strain Fin-Jyv-A3 [43]. Our work suggests that strain NE4049 is better adapted to the rodent host than strain Fin-Jyv-A3, whereas strain Fin-Jyv-A3 is better adapted to the tick vector than strain NE4049. Field studies on *I. ricinus* ticks and small mammal hosts have found that up to a dozen different strains of *B. afzelii* can coexist locally [26-28, 44]. We expect competition between strains that share a common niche to result in the loss of strain diversity over time [7]. However, long-term studies of strain diversity in populations of *B. afzelii* have shown that the strain composition is relatively constant over time [28, 44]. This long-term stability raises the obvious question of what ecological factors facilitate the maintenance of strain diversity in pathogen populations. Our study suggests that strain-specific adaptations to different steps of the pathogen life cycle could maintain the strain diversity of pathogen populations in nature.

Engorged nymphs: In 18.5% of the cases (15/81), there was a mismatch in the strain-specific infection status between the engorged challenge nymphs and the test mice. The high correspondence between the infection status of the flat nymphs and the mouse ear punch biopsies suggests that our strain-specific qPCR-based detection method is reliable for these two different types of materials. The probability of detecting *B. burgdorferi* sl spirochetes decreases when their number are very low. For this reason, we used a nested PCR to increase the number of *B. afzelii ospC* gene copies relative to the DNA of the tick and the host, before running the strain-specific qPCR. One explanation for the reduced sensitivity in detecting the strains in the engorged nymphs is the presence of mouse blood in the DNA extractions, which is known to interfere with the efficacy of qPCR [45-47].

Conclusion: The next step for this study will be to test whether the strain-specific spirochete load in the challenge nymphs explain the strain-specific infection prevalence in the test mice. We will also be looking for alternative analyses that could include the data we have on the engorged challenge nymphs.

TABLES AND FIGURES

Table 1. Comparison of the prevalence in the engorged challenge nymphs, the test mice, and the probability of infection the unfed challenge nymphs. The three types of infection include: infection with strain Fin-Jyv-A3 alone, infection with strain NE4049 alone, and co-infection with both strains. The acronyms Ch. Nymphs, En. Nymphs, Test Mice refer to the unfed challenge nymphs, the engorged challenge nymphs, and the test mice, respectively.

Groups	Ch. Nymphs	En. Nymphs	Test Mice
Group 1 (Fin-Jyv-A3)	N=12	N=12	N=12
Unrecovered	NA	25.0% (3/12)	NA
Fin-Jyv-A3	0.89 (0.64 – 1.00)	88.9% (8/9)	66.7% (8/12)
Uninfected	0.10 (0.00 – 0.36)	11.1% (1/9)	33.3% (4/12)
Group 2 (NE4049)	N=12	N=12	N=12
Unrecovered	NA	25.0% (3/12)	NA
NE4049	0.80 (0.53 – 1.00)	88.9% (8/9)	50.0% (6/12)
Uninfected	0.20 (0.00 – 0.47)	11.1% (1/9)	50.0% (6/12)
Group 3 (Fin-Jyv-A3+NE4049)	N=76	N=76	N=76
Unrecovered	NA	17.1% (13/76)	NA
Fin-Jyv-A3	0.15 (0.00 – 0.33)	30.2% (19/63)	34.2% (26/76)
NE4049	0.29 (0.08 – 0.72)	22.2% (14/63)	11.8% (9/76)
Coinfected	0.44 (0.12 – 0.80)	14.3% (9/63)	14.5% (11/76)
Uninfected	0.13 (0.00 – 0.29)	33.3% (21/63)	39.5% (30/76)

Table 2. The probability of infection with the focal strain is shown for the unfed challenge nymphs, the recovered engorged challenge nymphs and the test mice, according to the factors focal strain and infection. For experiments 1 and 2, the focal strain of *B. afzelii* was Fin-Jyv-A3 and NE4049, respectively. Co-infection (No, Yes) refers to whether the challenge nymph had fed on a donor mouse that was infected with a single strain or co-infected with both strains. For the engorged challenge nymphs and the test mice, infections are given in percentage. For the unfed challenge nymphs, it is the mean probability of infection that is given.

Focal Strain	Co-infection	Unfed Challenge Nymphs	Engorged Challenge Nymphs	Test Mice
Fin-Jyv-A3	No	0.897 (0.64 – 1.00)	88.9% (8/9)	66.7% (8/12)
Fin-Jyv-A3	Yes	0.593 (0.16 – 0.90)	47.1% (16/34)	42.1% (16/38)
NE4049	No	0.802 (0.53 – 1.00)	88.9% (8/9)	50.0% (6/12)
NE4049	Yes	0.719 (0.58 – 0.96)	31.0% (9/29)	21.1% (8/38)
Total			50.6% (41/81)	38.0% (38/100)

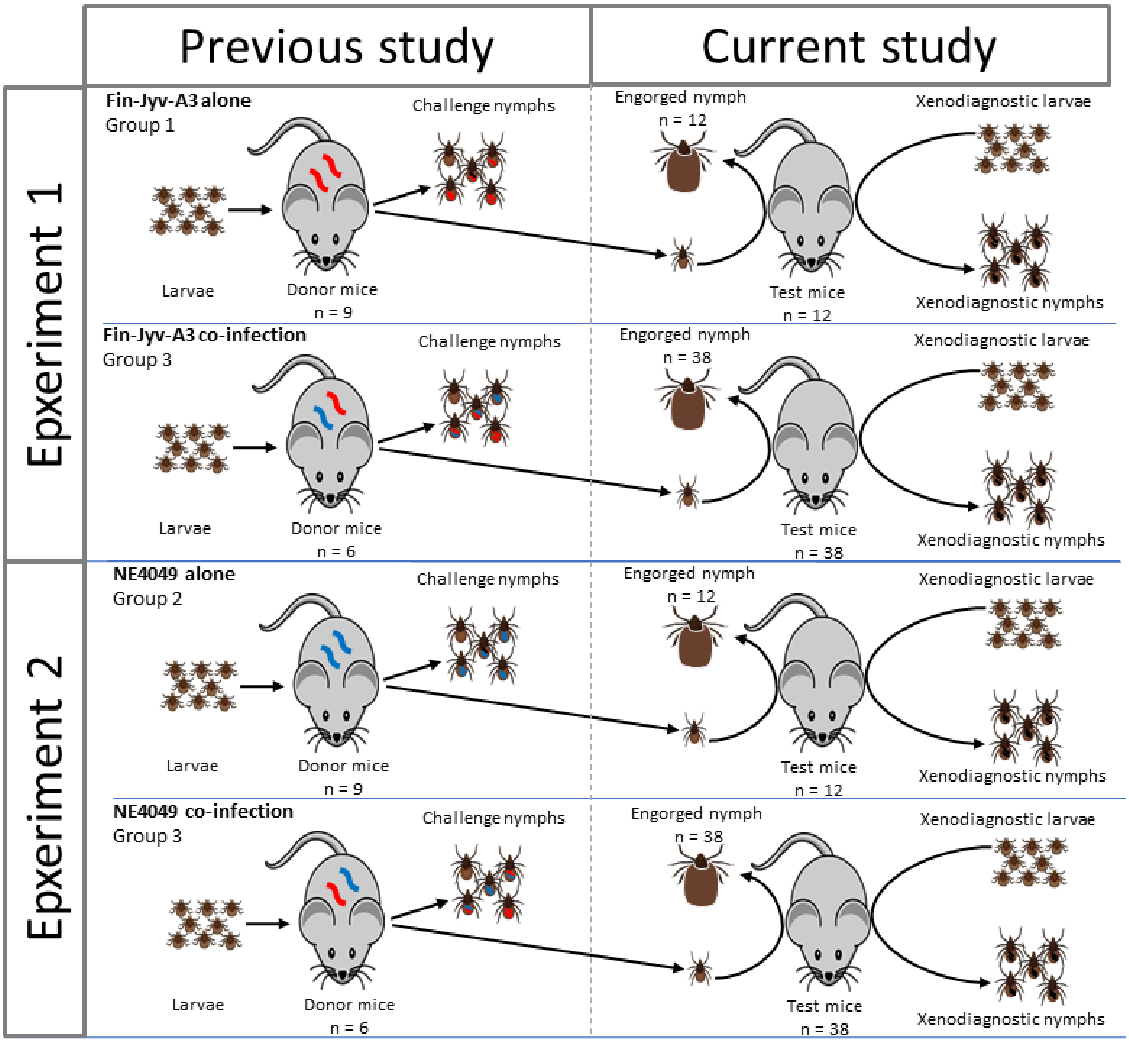

Figure 1. This study was divided into two independent experiments. In experiments 1 and 2, the focal strain was strain Fin-Jyv-A3 and strain NE4049, respectively. Each experiment included a single strain infection treatment and a co-infection treatment. In a previous study, we created the challenge nymphs by feeding larvae on the experimentally infected donor mice. For each donor mouse, we tested a random sample of unfed challenge nymphs to determine the probability of infection with the focal strain in the challenge nymphs. In the current study, we infested each of the 100 test mice with a single challenge nymph. The resultant engorged challenge nymphs were tested to determine their strain-specific infection status. The strain-specific infection status of the test mice was based on an ear tissue biopsy and xenodiagnostic ticks. Challenge nymphs from the single strain infection treatment were either infected with the focal strain or uninfected. Challenge nymphs from the co-infection treatment were either infected with strain Fin-Jyv-A3, strain NE4049, co-infected with both strains, or uninfected.

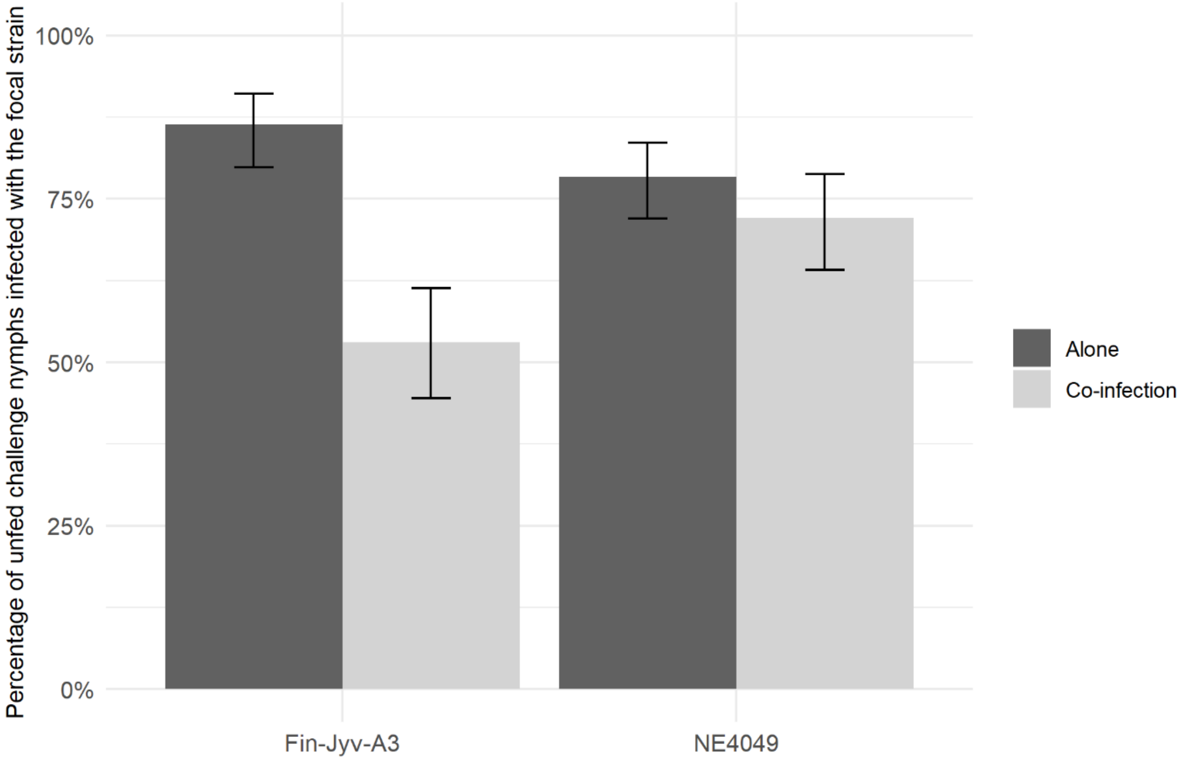

Figure 2. Co-infection in the donor mice reduced the efficacy of host-to-nymph transmission for both strains of *B. afzelii*. The percentage of unfed challenge nymphs infected with the focal strain is shown as a function of strain identity (Fin-Jyv-A3 versus NE4049) and co-infection status of the donor mice (alone versus co-infection). According to the parameter estimates of our statistical analysis, co-infection reduced the efficacy of host-to-nymph transmission for both strains, but there was no effect of strain identity. The graph shows the means and the 95% confidence intervals.

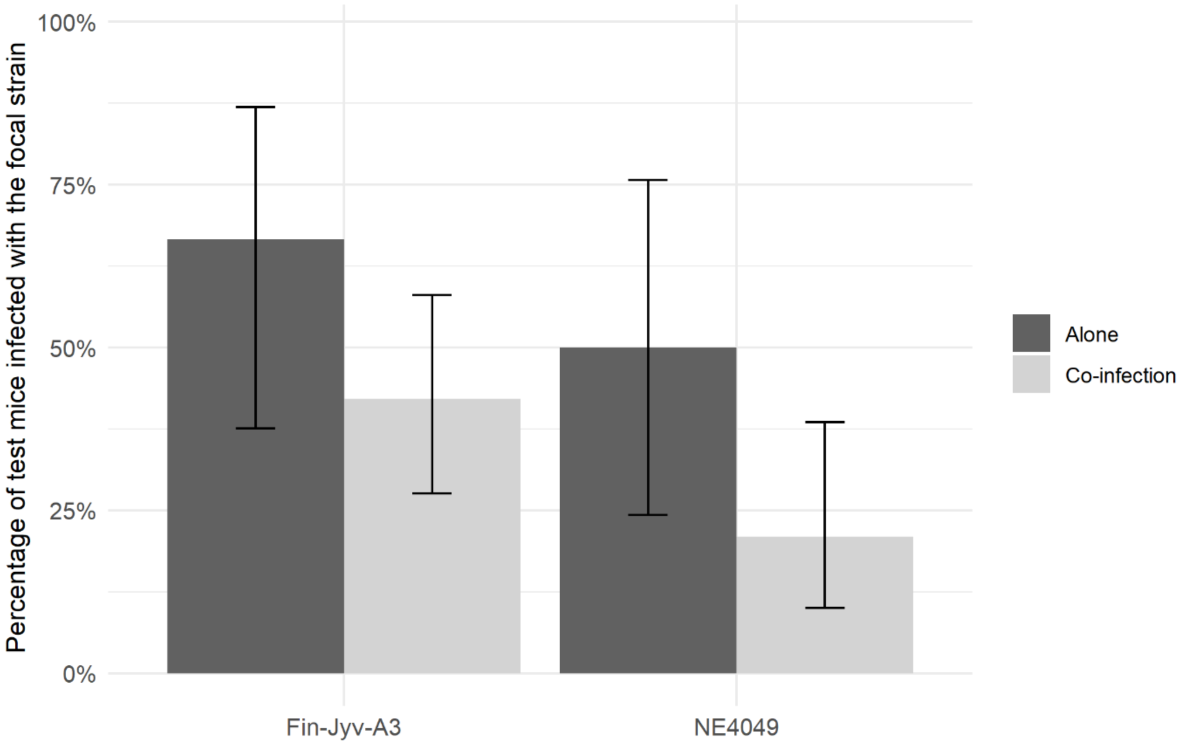

Figure 3. Co-infection reduced transmission of the focal strain of *B. afzelii* from the challenge nymphs to the test mice. Challenge nymphs that originated from the co-infected donor mice transmitted the focal strain of *B. afzelii* to a lower percentage of test mice compared to challenge nymphs that originated from the donor mice infected with single strains. The percentage of test mice infected with the focal strain is shown as a function of strain identity (Fin-Jyv-A3 versus NE4049) and co-infection treatment origin of the challenge nymphs (alone versus co-infection). The co-infection treatment origin of the challenge nymph refers to the infection status of the donor mice. According to the parameter estimates of our statistical analysis, co-infection reduced the probability of nymph-to-host transmission for both strains. The probability of nymph-to-host transmission for strain Fin-Jyv-A3 was higher than strain NE4049. The graph shows the means and the 95% confidence intervals.

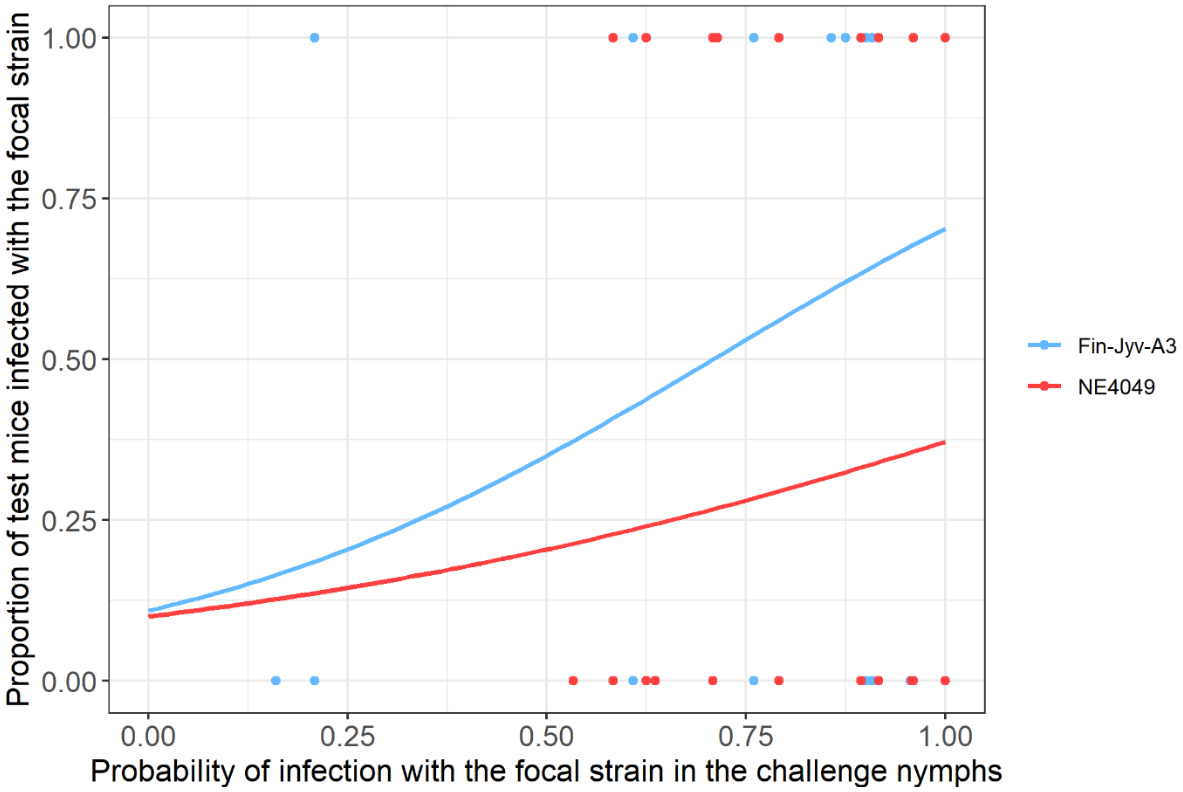

Figure 4. The probability that the test mouse becomes infected with the focal strain is shown as a function of strain identity (Fin-Jyv-A3 versus NE4049) and the probability that the challenge nymph is infected with the focal strain (i.e. the probability of exposure). According to the parameter estimates of our statistical analysis, there is a positive relationship between the probability of exposure to the focal strain and the probability that the test mouse becomes infected with the focal strain. When the probability of exposure is set to 1.00 (i.e. the test mouse is infested with a challenge nymph that is infected with the focal strain), the Y-intercepts indicate the probability of nymph-to-host transmission for the two strains. The probability of nymph-to-host transmission for strain Fin-Jyv-A3 (0.680) was 1.6 times higher than strain NE4049 (0.425). The lines of best fit were taken from a GLM with binomial errors where the fixed effects structure contained the probability of exposure and strain identity.

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7. General discussion

7.1. Co-infection and competition

This thesis used a multi-strain tick-borne pathogen, *B. afzelii*, as a model system to explore the effects of co-infection on transmission success of strains in different steps of the tick-borne life cycle. Specifically, we investigated how co-infection in the rodent host influenced the bacterial abundance and prevalence in the host tissues and subsequent host-to-tick and tick-to-host transmissions of strains. We also investigated how co-infection in the *I. ricinus* tick vector influenced the bacterial abundance and prevalence of strains in the tick. Co-infection can result in interactions between strains; the outcome of which can be classified as facilitation (or cooperation) or competition. The type of interaction (i.e. facilitation or competition) was determined by comparing the performances of the strains in single infections versus co-infections. In chapter 3, we showed that co-infection in the rodent host (*M. musculus*) led to competition between strains Fin-Jyv-A3 and NE4049. For both strains, co-infection in the mouse host reduced the infection prevalence in the different mouse organs. Co-infection in the mouse also affected the bacterial abundance of strain Fin-Jyv-A3 in the mouse tissues; its spirochete load decreased in the bladder and increased in the ankle joint. In chapters 1 and 2, we found that co-infection in the rodent host reduced the host-to-tick transmission success of both strains. We also found that co-infection and competition in the immature *I. ricinus* tick vector reduced the spirochete loads of both strains in the engorged larvae, 1-month-old nymphs and 4-month old nymphs. Finally, in chapter 4, we showed that co-infection in the donor mice reduced the subsequent nymph-to-host transmission success of the strains. In summary, we found that competitive interactions between *B. afzelii* strains occurred in both the vertebrate host and the tick vector and at most steps of the tick-borne life cycle for this particular pair of strains (Fin-Jyv-A3 and NE4049).

Co-infection and competition reduced the strain-specific abundance in the immature stages of the tick vector. This result is in agreement with a study on *B. afzelii* strains in field-collected *I. ricinus* nymphs; this study found patterns of spirochete abundance suggesting that co-infection and competition between strains of *B. afzelii* occur in the tick vector. Specifically, this study found that the number of spirochetes per strain in the nymph decreased with the number of strains (i.e. strain richness) in that nymph [32]. A field study on small mammals in Sweden also found that the mean spirochete load per strain decreased as the strain richness increased inside different species of small mammals [46]. In our study, co-infection in the rodent host did not reduce the strain-specific abundance in the organs of the rodent host, with the exception of strain Fin-Jyv-A3 in the bladder. Instead, we found that co-infection in the rodent host reduced the prevalence of both strains in the organs of the rodent host.

The mechanisms responsible for competition between *B. afzelii* strains are unknown in both the rodent host and the tick vector. Competition between pathogen strains can be mediated by three different mechanisms: (1) interference competition; for example, by the production of toxic compounds, (2) exploitation competition over limited resources such as space in tissues and host nutrients, and (3) apparent competition, which is mediated via the host immune response [10]. *B. burgdorferi* sl does not produce toxic compounds [132], direct interference competition is therefore unlikely in this system. Thus, exploitation competition over space and nutrients and apparent competition are both likely mechanisms underlying the competition between *B. afzelii* strains in both the rodent host and the tick vector.

We found that co-infection in the rodent host reduced the infection prevalence of both strains in the organs of the rodent host. This result suggests that there was competition over space or resources in those organs. Despite the fact that *B. burgdorferi* sl spirochetes are able to evade the rodent immune system and establish chronic infections [127], mice can control the spirochete abundance via components of both their innate and adaptive immune system [133-135] that are not strain-specific. A study of SCID mice (SCID = severe combined immunodeficiency), which lack an adaptive immune system (no B and T lymphocytes) has shown that these mice have much higher spirochete loads than immunocompetent mice [133]. Another study has shown that spirochetes are recognized and phagocytosed by host leukocytes [134]. Therefore, apparent competition could also explain the competition between strains that we observed in the rodent host.

The immune system of *Ixodes* ticks produces a range of molecules that could regulate the abundance of spirochetes in the tick vector [136]. For example, tick hemocytes (analogous to the white blood cells of vertebrate hosts) can eliminate spirochetes in the hemolymph (the fluid that travels through the open circulatory system of invertebrates) [137]. However, as hemocytes do not enter the tick midgut, which is where spirochetes spend most of their time, we do not expect hemocytes to regulate the spirochete population in the tick midgut. Exploitation competition over space and resources between *B. afzelii* strains is expected in ticks, because they have a carrying capacity and usually feed only once per year. The midgut of nymphs is a limiting environment, it contains a modest population size of ~10,000 spirochetes of *B. afzelii* [80]. This carrying capacity of the nymphal midgut reduced the strain-specific spirochete loads when the nymphs were co-infected with multiple strains [32, 138]. Ticks and spirochetes compete over nutrients, such as glucose derived from the blood meal [139]. Because ticks feed generally once a year, nutrients in the ticks are not restored before the following blood meal. Therefore, in ticks co-infected with multiple strains, one can expect fiercer competition over nutrients.

7.2. Host-to-tick transmission and nymph-to-host transmission

An important contribution of this PhD thesis is our demonstration that the effects of co-infection and inter-strain competition on bacterial presence and/or bacterial abundance in the rodent host, influenced the fitness of the strains in the subsequent transmission steps of the life cycle of this vector-borne pathogen. For *B. burgdorferi* sl parasites, few studies have shown that spirochete load in the tissues of the vertebrate host influences host-to-tick transmission success [82, 83], and to our knowledge, no studies have shown this phenomenon for multiple strain infections. The strain-specific ability to establish infection in the host tissues and the mean bacterial abundance of the strains in those tissues, which were altered by competition and co-infection, were both strong predictors of the transmission success of the strains from the infected host to the tick vector. We are aware of only one other study on co-infection and inter-strain competition that demonstrated a link between strain-specific abundance in the tissues of the vertebrate host and host-to-vector transmission success of the strains [22]. Ultimately, we found that co-infection and competition in the rodent host resulted in reduced host-to-tick transmission success for both strains. Our results are consistent with studies on *B. burgdorferi* ss in white-footed mice (*Peromyscus leucopus*), which found that co-infection in the host reduced the host-to-tick transmission success of the strains [44, 45]. We found that co-infection in the donor mice negatively influenced the subsequent nymph-to-host transmission of the strains. Thus, co-infection in the rodent host and the observed reduction in host-to-tick transmission of the focal strain had measurable consequences during the next step of the life cycle. For the nymph-to-host transmission step, the probability that the challenge nymph was infected with the focal strain and strain identity were predictors of whether the test mice would acquire the infection. In summary, co-infection and competition in the rodent host is a key inter-strain interaction that predicts the fitness of the strains, because of its influence on strain transmission to the ticks and on subsequent transmission to the vertebrate host.

7.3. Relationship between co-infection, inter-strain competition and virulence

We found that *B. afzelii* established different infection prevalence and different spirochete loads in different organs of *M. musculus,* which is evidence for tissue tropism. Our results are in agreement with a study on strains of *B. burgdorferi* ss in white-footed mice [140]. Organs differed with respect to their impact on the transmission of *B. afzelii* from the infected host to feeding ticks. We found that variation in the infection status or bacterial abundance in the ear, but not the internal organs (heart, bladder, joints), was positively related to host-to-tick transmission of *B. afzelii*. Identifying the tissues that drive transmission is important for understanding how selection on transmission shapes pathogen virulence. In rodent malaria for example, the blood is the critical tissue for host-to-mosquito transmission. In this system, high abundance of parasites in the blood increases transmission, and causes high virulence because the malaria parasites lyse the red blood cells [22]. In our system by contrast, the tissues where virulence is observed (carditis in the heart and arthritis in the joints) are not relevant for transmission. Thus, strains that have a tropism for joints but not for skin cause more virulence (e.g. arthritis) but have low transmission. Conversely, strains that have a tissue tropism for skin but not for joints have high transmission but low virulence. In summary, for pathogens like *B. burgdorferi* sl, which can invade many types of tissues, the link between pathogen abundance in host tissues, virulence, and transmission can be decoupled if virulence and transmission occur in different organs.

7.4. Inter-strain interaction in the vector

To date, most studies on mixed infections of vector-borne pathogens have focussed on the vertebrate host [12, 21, 43, 45]. Our study demonstrated that strains of *B. afzelii* compete inside their tick vector (*I. ricinus*), which reduces the bacterial abundance of the strains inside the tick, and this was true for three different ages of immature ticks (engorged larvae, 1-month-old nymphs and 4-month-old nymphs). Co-infection and competition reduced the spirochete load of both strains in the tick vector and strain Fin-Jyv-A3 always had higher spirochete loads than strain NE4049, and this pattern was true for three different ages of immature. Our work adds to a growing number of studies on mixed strain infections in the invertebrate vector [13, 39, 48, 50]. These studies have shown that interactions between vector-borne pathogen strains in the arthropod vector have important consequences for the transmission and fitness of strains. Interactions between strains in the vector can sometimes benefit the strains, and increase their density, as shown in mosquitoes co-infected with different strains of a malaria parasite [48]. However, we found that co-infection in the tick vector results in inter-strain competition as suggested by the observation that the strain-specific abundance is reduced in co-infected ticks [32]. Studies on mixed infections of other tick-borne pathogens have shown that ticks act as bottlenecks for strains by restricting the number of strains that are acquired from the host [49, 50] and the number of strains that are transmitted to the host [49]. Future studies on mixed infections of vector-borne pathogens should investigate the effects of inter-strain interactions in the arthropod vector, because the consequences of co-infection in the vertebrate host is not necessarily predictive of the consequences of co-infection in the arthropod vector.

7.5. Trade-off

An interesting result of this PhD thesis is that it provides evidence for the concept of trade-offs in the ability of vector-borne pathogens to exploit the different parties (vertebrate host and arthropod vector) in the vector-borne life cycle. Our work suggests that strains NE4049 and Fin-Jyv-A3 have specialized on the rodent host and the arthropod vector respectively (Table 1). This specialization allowed these two strains to mitigate the negative effects of co-infection. In co-infected mice, the negative effect of competition on the prevalence of the focal strain in the organs of the rodent host was less for strain NE4049 compared to strain Fin-Jyv-A3. For co-infected mice, the host-to-tick transmission success was reduced for both strains, but it was higher for strain NE4049 compared to strain Fin-Jyv-A3. Conversely, the spirochete load in the immature ticks was lower for strain NE4049 compared to strain Fin-Jyv-A3. After correcting for the probability that the challenge nymph was infected with the focal strain, the nymph-to-host transmission success was lower for strain NE4049 compared to strain Fin-Jyv-A3 Table 1). Thus, the single strain performance and/or the competitive ability of strains NE4049 and Fin-Jyv-A3 differed between the vertebrate host and the arthropod vector in this vector-borne life cycle. The strategy of strain NE4049 is that it infects a greater number of immature ticks, but its transmission to new vertebrate hosts will be less efficient. The strategy of strain Fin-Jyv-A3 is that it will infect fewer immature ticks, but its transmission to new vertebrate hosts will be more efficient. This PhD thesis provides another example of a trade-off, such as the competition-colonization trade-off [36], partitioning of host resources [10], or colonization of different organs in the host [39], that allows strains to overcome the negative effects of competition and avoid competitive exclusion.

Table 1. Comparison of the performance between strains NE4049 and Fin-Jyv-A3 under conditions of single strain infection versus co-infection in either the vertebrate host or the tick vector.

Phenotype	Host	Co-infection	Outcome	Winner
Prevalence in mouse organs	Mouse	Combined	NE4049 > Fin-Jyv-A3	NE4049
Host-to-tick transmission	Mouse	No	Fin-Jyv-A3 > NE4049	Fin-Jyv-A3
Host-to-tick transmission	Mouse	Yes	NE4049 > Fin-Jyv-A3	NE4049
Nymphal spirochete load	Tick	Combined	Fin-Jyv-A3 > NE4049	Fin-Jyv-A3
Nymph-to-host transmission	Tick	Combined	Fin-Jyv-A3 > NE4049	Fin-Jyv-A3

7.6. Diversity of *OspC* strains

A fundamental question in ecology and evolution is to understand how species diversity and strain diversity are maintained over time [24, 141, 142]. For *B. burgdorferi* sl, strain diversity can be measured using the *ospC* typing system. Based on this typing system, studies have found that substantial number of *B. burgdorferi* sl strains can coexist in local areas [32, 97, 98]. Longer term studies have shown that the frequencies of these *ospC* strains are relatively constant over space [31, 99] and time [32, 33]. The *ospC* strains have diversified through immune escape, to avoid the cross-reactive immune response of the host [101, 143], which is shown by the fact that the OspC antigen induces a highly strain-specific IgG antibody response [82, 116, 128]. The diversification of the *ospC* gene allows different *ospC* strains to infect the same host (either via simultaneous co-infection or via sequential infections) resulting in hosts with mixed strain infections. Two theories have been proposed to explain the balancing selection on *ospC* strain diversity in nature, negative frequency-dependent selection (NFDS) mediated by the adaptive immune system of the vertebrate host [99, 101] and multiple niche polymorphism (MNP) [41].

Under NFDS, common strains in nature have a fitness disadvantage. Wang et al. (1999) suggested a mechanism whereby vertebrate hosts are repeatedly bitten by nymphs carrying common strains resulting in a stronger antibody response against those strains, which could subsequently reduce host-to-tick transmission success of those common strains [101]. However, this mechanism seems implausible as there is no evidence that hosts that are chronically infected with a common strain would develop a stronger antibody response against that strain if they are repeatedly exposed to that particular strain. More recently, a study from our research group has suggested that maternal antibodies could mediate NFDS in nature [128]. We showed that female bank voles transmit strain-specific maternal antibodies to their offspring [128]. Most nymph-to-host transmission of *B. afzelii* occurs during the summer when rodent populations are reproducing. A common *ospC* strain is expected to have higher prevalence in the mothers, but the subsequent offspring generation (in the same transmission season) will have protective antibodies against this common strain. Thus, a rare strain compared to a common strain will have an advantage in infecting the offspring generation because the offspring generation is more likely to carry maternal antibodies against the common strain than the rare strain. So maternal antibodies could maintain the diversity of *ospC* strains in a local ecosystem and prevent any particular strain from becoming too dominant.

Under the MNP hypothesis, different *ospC* strains are adapted to different host species, therefore, the frequency of the strains depends on the abundance of their host species. The MNP hypothesis was originally proposed by Brisson and Dykhuizen (2004) who demonstrated that different small mammal species, such as white-footed mice, chipmunks, grey squirrels, and shrews differed in their host-to-tick transmission of the different *ospC* strains. However, a major limitation of the study was that the hosts had not been experimentally infected; instead these field-collected hosts had been exposed naturally. Thus, spatio-temporal variation in exposure to the different *ospC* strains is an alternative explanation for the observed host-specificity of the *ospC* strains in the study of Brisson and Dykhuizen (2004). Other studies that have investigated the host-specificity of the *ospC* strains found no support for the MNP hypothesis [33, 65].

We suggest that trade-offs in the competitive abilities of the strains can play a part in the maintenance of *ospC* strain diversity. Strain NE4049 was the superior competitor in the rodent host, whereas it was the inferior competitor in the tick vector. This trade-off can facilitate strain co-existence, because neither strain was the inferior competitor throughout the pathogen life cycle. When more than two strains are involved, a phenomenon such as intransitive competition [141], where species A outcompetes species B, species B outcompetes species C, and species C outcompetes species A, could explain the maintenance of strain diversity. Thus, in mixed infections, the outcome for each strain will depend on the competitive ability of the co-infecting strains, whether co-infection occurs in the vertebrate host or the tick vector, and most certainly on the number of strains involved in the co-infection. We point out that NFDS, MNP, and trade-offs in competitive abilities are not mutually exclusive hypotheses. For example, the competitive ability of strains could change depending on the species identity of the vertebrate host. In fact, the MNP hypothesis can be expressed as a mechanism with non-transitive competitive abilities, if strain A is superior to strains B and C in host 1, strain B is superior to strains A and C in host 2, and strain C is superior to strains A and B in host 3. It remains to be seen whether differences among host species or differences in competitive ability within host species are most important for maintaining strain diversity.

7.7. Bbsl infection

This PhD thesis is a first step in our understanding of competitive interactions between strains of *B. burgdorferi* sl. In the two experiments that were performed, interactions were investigated for only two strains using simultaneous co-infection. Thus, when we exposed a rodent host to co-infection, both strains had the same chance to establish in the rodent host. However, in nature, hosts can acquire strains either simultaneously (by exposure to a co-infected nymph) or sequentially (by being bitten repeatedly by nymphs carrying different strains). Mixed infections via sequential strain infection is not an important phenomenon in the tick vector. Nymphal ticks could acquire additional strains during the nymphal blood meal, but the resultant adult tick will either not feed (male adult ticks) or feed on larger mammals (female adult ticks), which are incompetent hosts for *B. burgdorferi* sl [79]. In nature, small mammal hosts are repeatedly bitten by nymphs that are infected with different strains of *B. burgdorferi* sl. Thus, strains often encounter vertebrate hosts that already harbour other strains, a phenomenon known as superinfection. An experimental study on superinfection in a rodent malaria system showed that competitive suppression was stronger with increased duration of the time interval between the inoculation of the primary and secondary strains [21]. This study also showed that strains of the rodent malaria parasite differed in their ability to achieve superinfection [21]. A study on *B. burgdorferi* ss in white-footed mice used sequential exposure with two different strains to create co-infections in the rodent host [45]. While both strains where able to establish infection in mice previously infected with the resident strain, the host-to-tick transmission success of the secondary strain was reduced in the presence of a resident infection [45]. When the inferior strain had to establish infection in host carrying a superior resident strain, the host-to-tick transmission success of the inferior strain remained low [45]. We expect that co-infection in the vertebrate host driven by sequential exposure to nymphs carrying different strains will likewise result in competition between strains that will affect their host-to-tick transmission success and subsequent fitness.

In summary, this PhD thesis examined the interactions between two strains of *B. afzelii* over the different steps of their life cycle. We found that strains compete in both the rodent host (*M. musculus*), and their vector (*I. ricinus*). Co-infection and competition in the vertebrate host reduced pathogen transmission to the tick and subsequent transmission back to the next vertebrate host. Thus, this PhD thesis provides new insight in the importance of co-infection for the fitness of pathogen strains.

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10. Appendix

10.1. Supplementary material of chapter 1

Title: Competition between strains of *Borrelia afzelii* inside the rodent host and the tick vector

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Supplementary material

SUPPLEMENTARY MATERIAL AND METHODS

***Flagellin* gene qPCR:** The *flagellin* qPCR was used to detect *B. afzelii* infection in the nymphal ticks and to quantify the total spirochete load in the nymphal ticks. This qPCR assay can detect and quantify the *flagellin* gene of *B. burgdorferi* sensu lato (sl) including *B. afzelii* strains Fin-Jyv-A3 and NE4049 used in the present study. The *flagellin* qPCR amplified a 132 bp fragment of the *flagellin* gene [1]. The qPCR protocol was as follows. The wells were filled with a mixture of 5.8 µl of water, 10 µl of 2x Master Mix (FastStart Essential DNA probes Master, Roche), 0.4 µl of 20 µM forward primer FlaF1A, 0.4 µl of 20 µM reverse primer FlaR1, 0.4 µl of 10 µM Flaprobe1, and 3 µl of DNA template. The thermocycling conditions consisted of 10 min at 95°C for denaturation, followed by 50 cycles of 30 sec at 60°C and 10 sec at 95°C.

Description of the strain-specific *ospC* qPCR: The Finnish strain Fin-Jyv-A3 and the Swiss strain NE4049 carry *ospC* major groups (oMGs) A3 and A10, respectively. A strain-specific qPCR assay that was able to quantify the gene copy number of each oMG allele was developed. The qPCR used a common set of primers and two different probes that were specific for each oMG allele. The qPCR primers amplified a 142 bp fragment that is located between bp 482 and 623 of U01894 in the *ospC* gene. The forward primer is 5'-GCT GTT AAA GAA GTT GAG ACT TTG G-3' and the reverse primer is 5'-GAT ATT GCA TAG GCT CCT GCT A-3'. The probe for oMG allele A3 hybridized with a 36 bp sequence that is located between bp 558 and 593 of U01894 in the *ospC* gene. The probe for oMG allele A10 hybridized with a 27 bp sequence that is located between bp 562 and 588 of U01894 in the *ospC* gene. The sequence for the A3 probe was FAM 5'-TGA TGG CAC TTT AGA TAA CGA AGC AAA TCA CAA YGG-3' and the sequence for the A10 probe was YYE 5'-GGT TTA GCT GCT GAT GCG GCT GAT CAC-3'.

Protocol for the *ospC*-strain specific qPCR: A 142 bp fragment of the *ospC* gene was amplified to detect and quantify the copy number of oMG alleles A3 and A10. Different probes were used to distinguish the oMG alleles in the qPCR assays. The 20 µl qPCR mixture consisted of 10 µl of 2× Master Mix (FastStart Essential DNA Probes Master, Roche Applied Science), 5.8 µl of water, 0.4 µl of 20 µM forward primer OspC_qPCR_For, 0.4 µl of 20 µM reverse primer OspC_qPCR_Rev, 0.4 µl of 10 µM of either A3_Probe or A10_Probe, and 3 µl of DNA template. The thermocycling conditions included a denaturation step at 95°C for 10 min followed by 50 cycles of 52°C for 20 s, 72°C for 20 s and 95°C for 10 s using a LightCycler® 96 (Roche Applied Science, Switzerland). All the plates contained negative controls for DNA extraction (mosquito DNA), negative controls for the qPCR (water), and 4 standards. The standards contained 10^2, 10^3, 10^4 and 10^5 copies of the *ospC* gene in 3 µl, respectively. The LightCycler® 96 software (Roche Applied Science, Switzerland) calculated the standard curves and the absolute number of spirochetes present in each positive sample. The total spirochete load for each tick was calculated by multiplying the spirochete load in 3 µl of tick DNA template by the appropriate correction factor.

Production of standards for the *flagellin* qPCR and *ospC* qPCR: The plasmid containing the *flagellin* gene was kindly provided to us by Reinhard Wallich [2]. The production of the plasmids containing *ospC* genes A3 and A10 was previously described in Jacquet et al. [3]. Briefly, the *ospC* gene, corresponding to the full OspC protein without its leader peptide, was amplified using primers modified from Earnhart et al. [4]. The forward primer contained a BamH1 restriction site (underlined) in the 5′ end (5′-GT ATA GGA TCC AAT AAT TCA GGG AAA GGT GG-3′) and the reverse primer contained a HincII restriction site (underlined) in the 5′ end (5′-C ATG GTC GAC TTA AGG TTT TTT TGG ACT TTC TGC-3′). DNA was ligated by T/A cloning to a pGEM-T Easy plasmid (PROMEGA) and then digested with BamH1 and HincII restriction enzymes. Plasmids carrying the *ospC* gene were transformed into *Escherichia coli* bacteria.

The recombinant *E. coli* bacteria were cultured in Luria-Bertani medium with ampicillin to a density of ~1.0*10^9 cells/ml. The cultures were centrifuged, re-suspended in 1.0 ml of 1xPBS, and plasmid DNA was extracted using the Wizard® *Plus* SV Minipreps DNA Purification System of Promega and following the manufacturer's instructions. The DNA concentrations of the resulting minipreps were measured using a Thermo Scientific™ NanoDrop 2000. The size of the pGEM-T Easy plasmid containing the *ospC* gene is 3565 bp. To calculate the number of plasmids in the miniprep, the DNA concentration of the miniprep was divided by the size of the plasmid. For each *ospC* allele, the minipreps were appropriately diluted with 1xWater PCR grade (ROCHE) to give 4 standards containing 10^2, 10^3, 10^4, and 10^5 *ospC* gene copies. The same approach was used to create the standards for the *flagellin* gene.

Validation of the strain-specific ospC qPCR: An experiment was conducted to determine the ability of the strain-specific *ospC* qPCR assays to detect the target oMG allele in the presence of the other oMG allele. The minipreps of the pGEM-T Easy plasmid containing oMG alleles A3 and A10 were used to create 7 mixtures, where the frequencies of the focal oMG allele (e.g. A3) were 5%, 10%, 30%, 50%, 70%, 90%, and 95% and where the frequencies of the other oMG allele were 95%, 90%, 70%, 50%, 10%, and 5%, respectively. For each of the 7 mixtures, the final density was diluted to 350 *ospC* gene copies/µl so that the 3 µl DNA template in each qPCR reaction contained a total of 1050 *ospC* gene copies (i.e., for the two oMG alleles combined). Each of the 7 mixtures was tested with the *ospC* allele A3 qPCR and the *ospC* allele A10 qPCR and each qPCR assay contained 5 standards with 10^1, 10^2, 10^3, 10^4, and 10^5 gene copies of the relevant oMG allele. This approach allowed us to estimate the number of copies of each oMG allele in each of the 7 mixtures. For each oMG allele, the number of gene copies estimated by the strain-specific *ospC* qPCR was modelled as a linear regression of the theoretical number of gene copies in the DNA template. For oMG allele A3, the theoretical abundance explained 98.4% of the variation in the estimated abundance and this relationship was highly significant ($F_{1,\ 10}$ = 632.3, p = 2.2e-10). For oMG allele A10, the theoretical abundance explained 98.9% of the variation in the estimated abundance and this relationship was highly significant ($F_{1, 12}$ = 1115, p = 3.3e-13). We note that the A3-specific qPCR was not able to detect oMG allele A3 in the mixture where its frequency was the lowest (5%) and where the theoretical abundance of oMG allele A3 was 52.5 gene copies (0.05*1050 gene copies = 52.5 gene copies). In contrast, the A10-specific qPCR was able to detect oMG allele A10 in all mixtures. This experiment shows that our strain-specific *ospC* qPCR is able to reliably estimate the abundance of oMG alleles A3 and A10 in mixtures where the frequency of these two alleles ranges from 5% to 95%.

SUPPLEMENTARY STATISTICAL METHODS

Comparison of the total nymphal spirochete load between single strain and co-infection treatments: In experiment 1, the mean total spirochete load (as estimated by the *flagellin* qPCR) of the nymphs in the Fin-Jyv-A3 group (n = 67, mean = 7109, 95% CI = 4947–10216, units = spirochetes per nymph) was higher than in the co-infection group (n = 62, mean = 4579, 95% CI = 3141–6676, units = spirochetes per nymph) but this difference was not significant (Figure S1; LME: $\Delta \chi^2$ = 2.529, Δ df = 1, p = 0.112). In experiment 2, the mean total spirochete load (as estimated by the *flagellin* qPCR) of the nymphs in the NE4049 group (n = 67, mean = 3497, 95% CI = 2434–5026, units = spirochetes per nymph) was lower than in the co-infection group (n = 68, mean = 5685, 95% CI = 3966–8148, units = spirochetes per nymph) but this difference was not significant (Figure S1; LME: $\Delta \chi^2$ = 2.730, Δ df = 1, p = 0.0985).

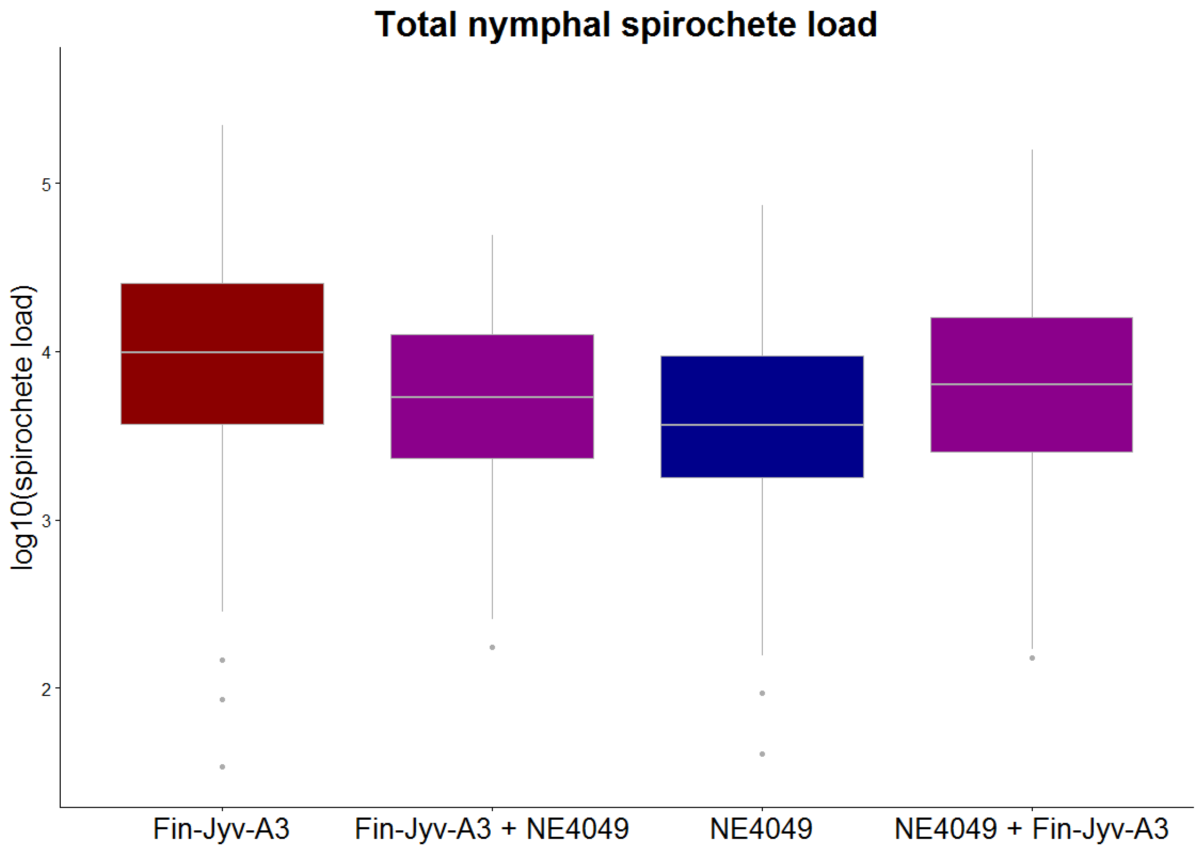

Figure S1. The total spirochete load of the subset of *Borrelia afzelii*-infected *I. ricinus* nymphs is similar among the four infection treatments: Fin-Jyv-A3, Fin-Jyv-A3 + NE4049, NE4049, and NE4049 + Fin-Jyv-A3). Each data point represents the mean for a single mouse (n = 33 mice). Shown are the medians (white line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles).

Three methods to estimate the strain-specific spirochete loads in the nymphs: We used three different methods to estimate the strain-specific spirochete loads in the nymphs. The first method used the gene copy number estimated by the strain-specific *ospC* qPCR without adjustment to the total spirochete load estimated by the *flagellin* qPCR. The second method, multiplied the relative abundance of the strain-specific *ospC* qPCR by the total spirochete load. For example, if the Fin-Jyv-A3-specific qPCR and the NE4049-specific qPCR detected 1000 and 3000 spirochetes, the relative abundances of strains Fin-Jyv-A3 and NE4049 were 0.25 and 0.75, respectively. If the total spirochete load estimated by the *flagellin* qPCR was 6000 spirochetes, then the corrected spirochete loads of strains Fin-Jyv-A3 and NE4049 were 1500 and 4500 spirochetes, respectively. In the third method, the linear regression relationship between the *flagellin* gene copy number and the *ospC* gene copy number was determined for each oMG (all data were log10-transformed) using the subset of infected nymphs from the single strain infection treatments. For ticks infected with both strains, these linear regression equations were used to convert the *ospC* gene copies to *flagellin* gene copies. Similar to method 2, these strain-specific spirochete loads were then adjusted to sum to the total spirochete load as estimated by the *flagellin* qPCR. For the nymphs that were infected with only one strain, methods 2 and 3 used the spirochete load estimated by the *flagellin* qPCR. In the main manuscript, we present the strain-specific spirochete load estimates from method 3.

SUPPLEMENTARY RESULTS

Repeatability of the nymphal spirochete load: For a random sample of 81 nymphal ticks, we obtained duplicate estimates of the *flagellin* gene copy number, the A3 oMG gene copy number, and the A10 oMG gene copy number. For the subset of infected nymphs (n = 71, 45, and 35 nymphs for the *flagellin, ospC* A3, and *ospC* A10 qPCR, respectively), we calculated the repeatability of the log10-transformed gene copy number. For the *flagellin, ospC* A3, and *ospC* A10 qPCR, the repeatability of the log10-transformed spirochete loads was 98.3%, 97.8%, and 97.0%, respectively.

Correlations in nymphal spirochete load between the qPCR assays: For the subset of nymphs in the Fin-Jyv-A3 group, the correlation in the log10-transformed nymphal spirochete load between the *flagellin* qPCR and the *ospC* A3-specific qPCR was positive and highly significant ($r = 0.914$, $df = 65$, $t = 18.164$, $p < 2.2e-16$). Similarly, for the subset of nymphs in the NE4049 group, the correlation in the log10-transformed nymphal spirochete load between the *flagellin* qPCR and the *ospC* A10-specific qPCR was positive and highly significant ($r = 0.825$, $df = 65$, $t = 11.785$, $p < 2.2e-16$). Finally, for the subset of ticks that had fed on the co-infected mice, the correlation in the log10-transformed nymphal spirochete load between the *flagellin* qPCR and the sum of the *ospC* A3-specific qPCR and the *ospC* A10-specific qPCR was positive and highly significant ($r = 0.898$, $df = 128$, $t = 23.047$, $p < 2.2e-16$).

Effect of competition on host-to-tick transmission including all the mice from the co-infection treatment: In the main manuscript, the analysis of competition on host-to-tick transmission excluded 4 mice in the co-infection treatment. These 4 mice were excluded from the analysis because they were only infected with strain Fin-Jyv-A3 and were therefore not co-infected. Of these 4 mice, 2 came from experiment 1 where the focal strain is Fin-Jyv-A3 and 2 mice came from experiment 2 where the focal strain is NE4049. Here, we re-analysed the effect of competition on host-to-tick transmission using the data set that included these 4 mice and using the same statistical approach described in the main manuscript. Briefly, we used generalized linear mixed effects (GLME) models with binomial error terms to model host-to-tick transmission as a function of two fixed factors: focal strain (2 levels: Fin-Jyv-A3, NE4049) and co-infection treatment (2 levels: single strain infection, double strain infection), and their interaction. Mouse identity was modelled as a random factor. Nested models were compared using log-likelihood ratio (LLR) tests to determine the significance of the two fixed factors and their interaction.

The interaction term was not significant and was therefore removed from the model (GLME LLR: $\Delta \chi^2 = 0.952$, Δ df = 1, $p = 0.329$). The focal strain was not significant (Figure S2; GLME LLR: $\Delta \chi^2 = 1.630$, Δ df = 1, $p = 0.202$), but the effect of the co-infection treatment was significant (Figure S2; GLME LLR: $\Delta \chi^2 = 8.829$, Δ df = 1, $p = 0.003$). Thus, after including all the mice in the study, the effect of co-infection on host-to-tick transmission was the same for both of the focal strains. For focal strain Fin-Jyv-A3, host-to-tick transmission in the co-infection group (59.1% = 52/88) was lower compared to the single infection group (91.8% =

67/73). For focal strain NE4049, host-to-tick transmission in the co-infection group (56.0% = 51/91) was lower compared to the single infection group (77.9% = 67/86).

In the main manuscript where the 4 mice were excluded, the effect of competition on host-to-tick transmission was asymmetric for the two strains. Host-to-tick transmission of strain Fin-Jyv-A3 was reduced by competition with strain NE4049, but the reverse was not true, host-to-tick transmission of strain NE4049 was not affected by competition with strain Fin-Jyv-A3. The 4 mice that were excluded had been assigned to the co-infection treatment, but only became infected with strain Fin-Jyv-A3 following the nymphal challenge. After including these four mice in the analysis, the interaction between strain and competition disappeared and the main effect of competition became significant for both strains.

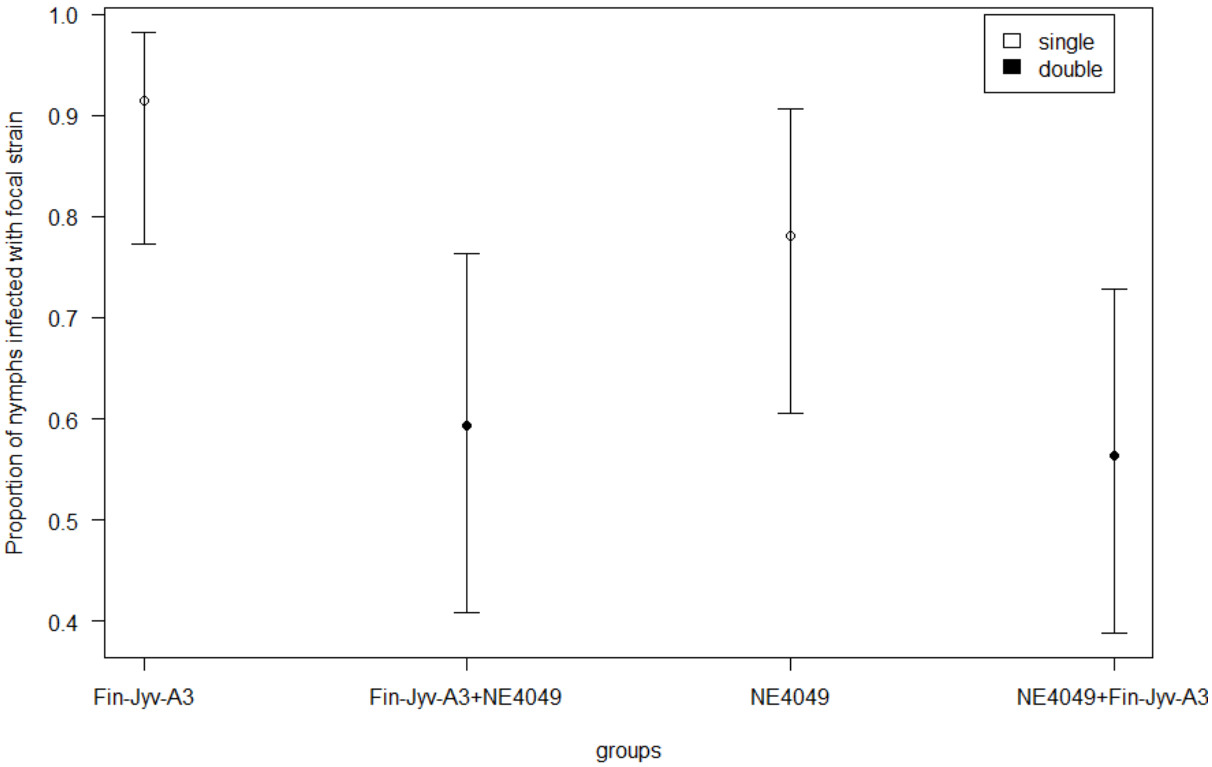

Figure S2. Competition between strains in *Ixodes ricinus* nymphs decreases the probability of host-to-tick transmission of the focal strain. In experiment 1, host-to-tick transmission of strain Fin-Jyv-A3 was reduced in the presence of strain NE4049. Similarly, in experiment 2, host-to-tick transmission of strain NE4049 was reduced in the presence of strain Fin-Jyv-A3. In Figure S2, all the mice in the study were included in the analysis (n = 37 mice). In the main manuscript, 4 mice in the co-infection group were excluded from the analysis (n = 33 mice) because they were only infected with strain Fin-Jyv-A3 (i.e., they were not co-infected). Shown are the means (central dot) and the 95% confidence intervals.

Three methods of calculating the strain-specific spirochete loads in the co-infected nymphs: In nymphs with mixed infections, the strain-specific spirochete load was calculated using three different methods: method 1, method 2, and method 3. Method 1 used the estimates of gene copy number of each oMG allele from the strain-specific *ospC* qPCR without correction to the gene copy number of the estimates of spirochete abundance from the *flagellin* qPCR.

Method 2 converted the estimates from the first method to frequencies and multiplied these frequencies by the estimates of spirochete abundance from the *flagellin* qPCR. In other words, method 2 created a constraint where the abundance estimates of the *ospC* gene from the strain-specific *ospC* qPCRs must sum to the estimate of spirochete abundance from the *flagellin* qPCR. Method 3 used the subset of nymphs infected with a single strain (i.e., that had fed as larvae on the mice infected with a single strain) to model the log10(abundance) estimate of the *flagellin* qPCR as a linear regression of the log10(abundance) estimate of the strain-specific *ospC* qPCR. This strain-specific linear regression was used to convert the estimates of *ospC* gene copy number to an estimate of the *flagellin* gene copy number for each strain. Similar to method 2, these regression-corrected estimates of abundance were then converted to frequencies and multiplied by the estimates of spirochete abundance from the *flagellin* qPCR. In other words, the third method corrects for potential differences in amplification efficiency between the two *ospC* strain-specific qPCRs and then creates the same constraint as the second method where the estimates of abundance must sum to the estimate of spirochete abundance from the *flagellin* qPCR.

For all three methods, the log10-transformed spirochete load of the focal strain was modelled as a linear mixed effects (LME) model with normal errors. The fixed factors included focal strain (2 levels: Fin-Jyv-A3, NE4049), the infection treatment (2 levels: single strain infection, double strain infection), and their interaction. Mouse identity was included as a random factor. All three methods of calculating the strain-specific spirochete load resulted in the same conclusions with respect to the significance of the fixed factors. The interaction term was not significant for methods 1, 2, and 3 (Table S1; $p_1 = 0.7911$, $p_2 = 0.0847$, and $p_3 = 0.6479$, respectively). The effect of the focal strain (equivalent to experiment) was always significant for methods 1, 2, and 3 (Table S1; $p_1 = 1.1e\text{-}10$, $p_2 = 0.0001$, and $p_3 = 0.0093$, respectively). For all three methods, strain Fin-Jyv-A3 had a higher spirochete load in the nymph than strain NE4049 (Table S2). The effect of the infection treatment was always significant for methods 1, 2, and 3 (Table S1; $p_1 = 0.0004$, $p_2 = 0.0004$, and $p_3 = 0.0053$, respectively). For all three methods, the spirochete load of the focal strain was lower in co-infected nymphs compared to nymphs infected with only the focal strain (Table S2).

Table S1. The three different methods of calculating the strain-specific spirochete abundances in the *Ixodes ricinus* nymphs all give the same results with respect to the statistical significance of the fixed factors strain, infection treatment, and their interaction. For each of the three methods, nested models were compared using log-likelihood ratio tests to determine the statistical significance of the fixed factors of interest. For each of the three methods, differences in degrees of freedom between nested models, differences in residual deviance between nested models, and the associated p-values are shown.

Method	Factor	$\Delta \chi^2$	Δ df	p
1	Strain:infection	0.070	1	0.791
1	Strain	41.64	1	1.1e-10
1	Infection	12.37	1	0.0004
2	Strain:infection	2.97	1	0.0847
2	Strain	14.849	1	0.0001
2	Infection	12.213	1	0.0004
3	Strain:infection	0.208	1	0.6479
3	Strain	6.765	1	0.0093
3	Infection	7.758	1	0.0053

Table S2. The three different methods of calculating the strain-specific spirochete abundances in the *Ixodes ricinus* nymphs all give same results with respect to the parameter estimates of the fixed factors strain, infection treatment, and their interaction. Shown are the parameter estimates from the models that include the main effect of strain (2 levels: Fin-Jyv-A3, NE4049) and the main effect of the infection treatment (2 levels: single strain infection, double strain infection). Spirochete loads in *I. ricinus* nymphs are lower for strain NE4049 compared to strain Fin-Jyv-A3 (the contrast NE4049 - Fin-Jyv-A3 is always negative). Spirochete loads of the focal strain in *I. ricinus* nymphs are lower in the double strain treatment compared to the single strain infection treatment (the contrast Double - single is always negative). For each of the three methods of calculating the strain-specific spirochete abundances, the parameter estimates (intercepts and contrasts), standard errors, degrees of freedom, t values, and the associated p-values are shown.

Method	Factor	Estimates	Std. Error	df	t value	p
1	Single Fin-Jyv-A3	4.106	0.070	25.1	58.58	<2e-16
1	NE4049 - Fin-Jyv-A3	-0.842	0.087	26.4	-9.71	3.31e-10
1	Double - single	-0.342	0.089	27.4	-3.85	0.0006
2	Single Fin-Jyv-A3	3.918	0.085	26.7	46.10	<2e-16
2	NE4049 - Fin-Jyv-A3	-0.452	0.105	28.2	-4.31	0.0002
2	Double - single	-0.407	0.107	29.2	-3.80	0.0007
3	Single Fin-Jyv-A3	3.832	0.081	24.9	47.16	<2e-16
3	NE4049 - Fin-Jyv-A3	-0.278	0.100	26.3	-2.78	0.01002
3	Double - single	-0.298	0.102	27.2	-2.91	0.00706

Effect of distinguishing between co-infection status in the mouse versus the tick: In the analysis of the log10-transformed spirochete load of the focal strains in the main manuscript, the infection status of the nymphs (single strain infection, double strain infection) was identical to the infection status of the mice on which they had fed as larval ticks. In other words, all nymphs that had fed as larvae on a co-infected mouse were considered to be co-infected regardless of whether they actually contained a single strain infection or a double strain infection. In the main manuscript, we showed that 91.5% of the nymphs (130/142) that had fed as larvae on the co-infected mice were infected with *B. afzelii*. Of these 130 nymphs, 17.7% (23/130) were infected with strain Fin-Jyv-A3 alone, 34.6% (45/130) were infected with strain NE4049 alone, and 47.7% (62/130) were co-infected with both strains. In other words, the co-infection status of the mouse predicts the co-infection status of the nymph about 50% of the time.

Here we re-analysed the log10-transformed spirochete load of the focal strains where the fixed factor 'infection treatment' reflected the infection status of the nymphal tick rather than the infection status of the mouse. This reclassification of the nymphal infection status did not change the results. The interaction between the focal strain and the nymphal infection status was not significant (Table S3; $\Delta \chi^2 = 1.919$, Δ df =1, p = 0.166). After removing the interaction from the model, the effects of the focal strain (Table S3; $\Delta \chi^2 = 7.776$, Δ df =1, p = 0.0053) and nymphal infection status (Table S3; $\Delta \chi^2 = 11.356$, Δ df =1, p = 0.0008) were highly significant. The mean spirochete load in *I. ricinus* nymphs was significantly higher for strain Fin-Jyv-A3 compared to strain NE4049 (Table S4). The mean spirochete load of the focal strain in *I. ricinus* nymphs was lower in the co-infection treatment compared to the single strain infection treatment (Table S4). In other words, co-infection in the nymphal tick reduced the spirochete load of the focal strain of *B. afzelii*.

Table S3. Competition between strains of *Borrelia afzelii* in *Ixodes ricinus* nymphs reduces the spirochete load of the focal strain regardless of whether the infection status refers to the mouse or the nymph. Mouse infection status refers to the situation where all nymphs that fed as larvae on a co-infected mouse were classified as ‘co-infected’ regardless of the actual infection status of the nymph. Tick infection status refers to the situation where all nymphs are classified according to their actual infection status. Thus, nymphs that fed as larvae on co-infected mice but that only acquired a single strain are classified as having a single strain infection. For each method of classifying nymph infection status, nested models were compared using log-likelihood ratio tests to determine the statistical significance of the fixed factors of interest. For each method of classifying nymph infection status, differences in degrees of freedom between nested models, differences in residual deviance between nested models, and the associated p-values are shown.

Infection status	Factor	$\Delta \chi^2$	Δ df	p
Mouse	Strain:infection	0.2085	1	0.6479
Mouse	Strain	6.7645	1	0.0093
Mouse	Infection	7.7577	1	0.0053
Tick	Strain:infection	1.919	1	0.166
Tick	Strain	7.7756	1	0.0053
Tick	Infection	11.356	1	0.0008

Table S4. Competition between strains of *Borrelia afzelii* reduces the spirochete load in *Ixodes ricinus* nymphs regardless of whether the infection status refers to the mouse or the nymph. Infection status refers to whether the single strain or double strain infection status was determined by the mouse (on which the nymph has fed as a larva) or the nymph. Shown are the parameter estimates from the models that include the main effect of strain (2 levels: Fin-Jyv-A3, NE4049) and the main effect of the infection treatment (2 levels: single strain infection, double strain infection). Spirochete loads in *I. ricinus* nymphs are lower for strain NE4049 compared to strain Fin-Jyv-A3 (the contrast NE4049 - Fin-Jyv-A3 is always negative). Spirochete loads of the focal strain in *I. ricinus* nymphs are lower in the double strain treatment compared to the single strain infection treatment (the contrast Double - single is always negative). For each of the two methods of determining the nymphal infection status, the parameter estimates (intercepts and contrasts), standard errors, degrees of freedom, t-values, and the associated p-values are shown.

Infection status	Factor	Estimates	Std. Error	df	t value	p
Mouse	Single Fin-Jyv-A3	3.832	0.081	24.9	47.16	<2e-16
Mouse	NE4049 - Fin-Jyv-A3	-0.278	0.100	26.3	-2.78	0.01002
Mouse	Double - single	-0.298	0.102	27.2	-2.91	0.00706
Tick	Single Fin-Jyv-A3	3.832	0.080	27.9	45.52	<2e-16
Tick	NE4049 - Fin-Jyv-A3	-0.303	0.106	25.7	-2.85	0.0085
Tick	Double - single	-0.371	0.110	27.9	-3.38	0.0012

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10.2. Supplementary material of chapter 2

Title: Competition between strains of *Borrelia afzelii* in immature *Ixodes ricinus* ticks is not affected by season

Authors: Dolores Genné, Anouk Sarr, Olivier Rais, and Maarten J. Voordouw

Supplementary material

SECTION 1 – Seasonal treatment of the 4-month-old nymphs:

For the natural winter treatment, the nymphs were kept in a plastic box (30 cm x 23 cm x10 cm) that was buried in the soil at a depth of 10 cm in a forest in the botanical garden of Neuchâtel (47°00’02.2”N, 6°56’15.1”E) for a period of three months (22 January 2016 to 15 April 2016). The box contained three button logs that measured the temperature every 30 minutes. Over the three-month period that the ticks were buried underground, the mean daily average, mean daily maximum, and mean daily minimum temperatures were respectively 6.44°C (range = 3.72°C – 10.50°C), 6.63°C (range = 3.79°C – 10.55°C), and 6.29°C (range = 3.58°C – 10.36°C; Fig. S1).

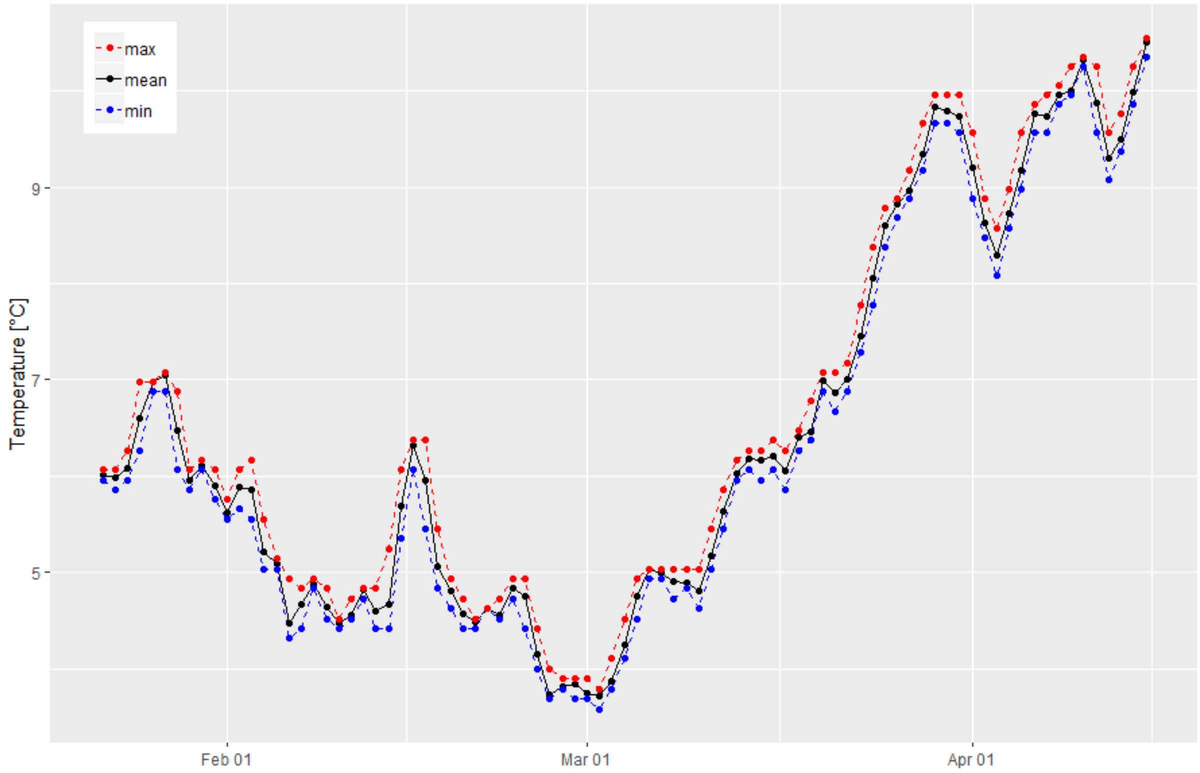

Figure S1. The daily average temperatures (black line), daily maximum temperatures (red line), and daily minimum (blue line) temperatures are shown for the soil at a depth of 10 cm at the Botanical Garden of Neuchâtel over a period of three months (from 22 January 2016 to 15 April 2016). Temperature was measured by temperature buttons that were placed in boxes that were buried underground. The *I. ricinus* nymphs in the natural winter treatment were exposed to this temperature treatment.

SECTION 2 – Effect of tick age and tick seasonal treatment on the prevalence of *B. afzelii* infection

We wanted to test whether tick age (larva, 1-month-old nymph, and 4-month old nymph) and seasonal treatment of the 4-month-old nymphs (phytotron, fridge, and underground) influenced tick infection status. Model comparison showed that the model with 5 treatments could be collapsed into a model with three tick ages with no loss of information (LLR test of treatment vs age: Δ df = 2, Δ dev = 2.236, p = 0.327). Similarly, when the analysis was restricted to the subset of 4-month-old nymphs, there was no effect of seasonal treatment on infection prevalence (LLR test of 4-month-old vs null: Δ df = 2, Δ dev = 2.254 p = 0.324). The rest of the statistical analysis is presented in the main manuscript in the section titled “Effect of tick age and tick seasonal treatment on the prevalence of *B. afzelii* infection”.

SECTION 3 – Effect of tick age and tick seasonal treatment on the *B. afzelii* spirochete load

We wanted to test whether tick age (larva, 1-month-old nymph, and 4-month old nymph) and seasonal treatment of the 4-month-old nymphs (phytotron, fridge, and underground) influenced the tick spirochete load. We therefore created a boxplot that shows the tick spirochete load as a function of these five groups of ticks (Figure S2). This boxplot shows that tick age has an important effect on tick spirochete load. In contrast, the seasonal treatment did not affect the spirochete loads of 4-month-old nymphs. Model comparison showed that the model with 5 treatments could be collapsed into a model with three tick ages with no loss of information (LLR test of treatment vs age: Δ df = 2, Δ dev = 2.369, p = 0.306). Similarly, when the analysis was restricted to the subset of 4-month-old nymphs, there was no significant effect of the seasonal treatment on the spirochete load (LLR test of 4-month-old vs null: Δ df = 2, Δ dev = 2.866 p = 0.239). The rest of the statistical analysis is presented in the main manuscript in the section titled “Effect of tick age and tick seasonal treatment on the *B. afzelii* spirochete load”.

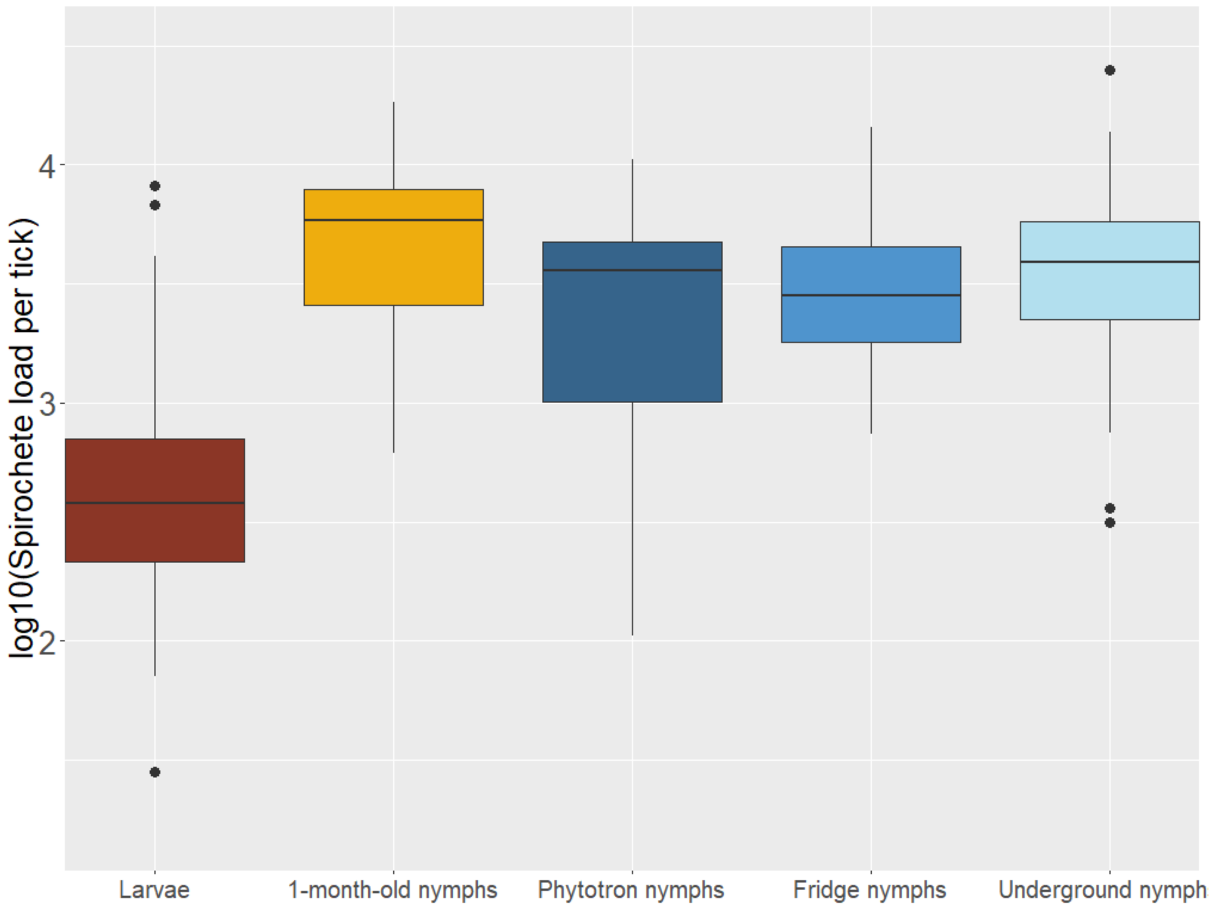

Figure S2. The *B. afzelii* spirochete loads inside the ticks are shown for each of the five treatments: engorged larvae, 1-month-old nymphs, 4-month-old phytotron nymphs, 4-month-old fridge nymphs, and 4-month-old underground nymphs. The spirochete loads were log10-transformed to normalize the residuals. The three seasonal treatments (phytotron, fridge, underground) had no effect on the spirochete load of the 4-month-old nymphs. Each data point represents the mean for the ticks sampled from the same mouse. The total number of ticks for each of the 5 treatments are as follows: larvae (n = 142), 1-month-old nymphs (n = 301), 4-month-old phytotron nymphs (n = 119), 4-month-old fridge nymphs (n = 120), and 4-month-old underground nymphs (n = 118). Shown are the medians (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum values (whiskers), and the outliers (solid circles).

SECTION 4 – Infection status of the mouse versus infection status of the tick

A co-infected mouse can produce ticks infected with strain Fin-Jyv-A3, ticks infected with strain NE4049, and ticks co-infected with both strains. In contrast, a mouse infected with a single strain can only produce ticks infected with that particular strain. Thus, for the statistical analyses of the strain-specific spirochete load (SSSL), the co-infection status of each tick can be decided using either the co-infection status of the mouse that produced the tick, or the co-infection status of the tick. The problem with analysing the SSSL in the tick (response variable) as a function of the co-infection status of the tick (explanatory variable) is that both of these variables were determined using the same qPCR protocol. Using the same data to generate both the response variable and the explanatory variable is likely to produce biased results and should be avoided. In contrast, the co-infection status of the mouse was determined using six mouse tissue samples (unpublished data) and all of the ticks that fed on that mouse. Thus, from a philosophy of science perspective, we should use mouse co-infection status rather than tick co-infection status to model the SSSL in the tick.

We can also use a model selection approach to determine which of these two explanatory variables, mouse co-infection status or tick co-infection status, is better for explaining variation in the SSSL in the tick. We therefore analysed the SSSL in the tick as a function of mouse co-infection status, tick co-infection status, strain, and tick age using linear mixed effects models (LMMs). The SSSL in the ticks were log10-transformed to normalize the residuals. All the models were compared using the Akaike information criterion (AIC); the models with the lowest AIC score are considered the best models. Interestingly, the best models used mouse co-infection status rather than tick co-infection status (Table S1). For this reason, the statistical analyses in the main manuscript used mouse co-infection status rather than tick co-infection status.

Table S1. Model selection results are shown for the linear mixed effects models of the log10-transformed strain-specific spirochete loads (SSSL) in the ticks. The models are ranked according to their AIC score. The explanatory variables were mouse co-infection status (M), tick co-infection status (T), strain (S), tick age (A), and their interactions. Shown for each model are the model rank (Rank), fixed effects structure of the model, model degrees of freedom (Df), log-likelihood (logLik), Akaike information criterion (AIC), difference in the AIC value from the top model (ΔAIC), model weight (Weight1; expressed as a %), and cumulative weight (Weight2; expressed as a %).

Rank	Fixed effects structure	Df	logLik	AIC	ΔAIC	Weight1	Weight2
1	SSSL ~ M:S+A+M+S	8	-502.7	1021.8	0.0	22.1	22.1
2	SSSL ~ A+M+S	7	-504.2	1022.5	0.8	14.9	37.0
3	SSSL ~ A:S+M:S+A+M+S	10	-501.1	1022.6	0.8	14.7	51.7
4	SSSL ~ A:S+A+M+S	9	-502.4	1023.2	1.4	10.7	62.4
5	SSSL ~ A+M	6	-506.1	1024.4	2.7	5.8	68.2
6	SSSL ~ A:T+A+T+S	9	-503.3	1025.0	3.3	4.3	72.4
7	SSSL ~ A:M+M:S+A+M+S	10	-502.3	1025.0	3.3	4.3	76.7
8	SSSL ~ A:T+A:S+A+T+S	11	-501.5	1025.6	3.8	3.2	79.9
9	SSSL ~ A+T+S	7	-505.7	1025.7	3.9	3.1	83.0
10	SSSL ~ A:M+A+M+S	9	-503.7	1025.9	4.1	2.9	85.9
11	SSSL ~ A:S+A+T+S	9	-503.9	1026.1	4.4	2.5	88.4
12	SSSL ~ A:M+A:S+M:S+A+M+S	12	-500.8	1026.3	4.5	2.3	90.7
13	SSSL ~ A:M+A:S+A+M+S	11	-502.2	1026.9	5.2	1.7	92.3
14	SSSL ~ A:T+T:S+A+T+S	10	-503.3	1027.1	5.3	1.5	93.9
15	SSSL ~ A:M:S+A:M+A:S+M:S+A+M+S	14	-499.4	1027.6	5.8	1.2	95.1
16	SSSL ~ A:T+A:S+T:S+A+T+S	12	-501.5	1027.7	5.9	1.1	96.2
17	SSSL ~ T:S+A+T+S	8	-505.7	1027.7	6.0	1.1	97.3
18	SSSL ~ A+T	6	-508.0	1028.1	6.3	0.9	98.2
19	SSSL ~ A:S+T:S+A+T+S	10	-503.9	1028.2	6.5	0.9	99.1
20	SSSL ~ A:T:S+A:T+A:S+T:S+A+T+S	14	-500.9	1030.7	8.9	0.3	99.4
21	SSSL ~ A+S	6	-509.5	1031.1	9.3	0.2	99.6
22	SSSL ~ A+S	6	-509.5	1031.1	9.3	0.2	99.8
23	SSSL ~ A	5	-511.2	1032.5	10.8	0.1	99.9
24	SSSL ~ A	5	-511.2	1032.5	10.8	0.1	100.0
25	SSSL ~ M+S	5	-547.4	1104.8	83.1	0.0	100.0
26	SSSL ~ M	4	-548.7	1105.4	83.7	0.0	100.0
27	SSSL ~ T+S	5	-551.3	1112.7	90.9	0.0	100.0
28	SSSL ~ T	4	-552.8	1113.6	91.9	0.0	100.0
29	SSSL ~ S	4	-552.9	1113.8	92.1	0.0	100.0
30	SSSL ~ S	4	-552.9	1113.8	92.1	0.0	100.0
31	SSSL ~ 1	3	-554.1	1114.2	92.5	0.0	100.0
32	SSSL ~ 1	3	-554.1	1114.2	92.5	0.0	100.0

The acronyms for the explanatory variables are as follows: SSSL = strain-specific spirochete load in the tick, M = mouse co-infection status, T = tick co-infection status, S = strain, A = tick age.

SECTION 5 – Effect of competition between strains on the strain-specific transmission to immature *I. ricinus* ticks

We wanted to test the effects of competition, strain, tick stage, and tick seasonal treatment on the strain-specific transmission from the rodent host to *I. ricinus* ticks. Model comparison showed that there was no effect of the seasonal treatment on the strain-specific prevalence in the 4-month-old nymphs and that we were justified in combining the 4-month-old nymphs into a single group (LLR test of treatment vs age: Δ df = 8, Δ dev = 5.271, p = 0.728). Further model comparison showed that the three tick ages could be collapsed into two tick stages with no loss of information (LLR test of stage vs age: Δ df = 4, Δ dev = 4.846, p = 0.304). Thus, the strain-specific prevalence was analysed as a function of three fixed factors: strain, competition, tick stage, and their interactions.

A classic step-wise model simplification approach using log-likelihood ratio (LLR) tests found that the three-way interaction (LLR: Δ df = 1, Δ dev = 3.355, p = 0.067) and the two-way interaction between strain and tick stage (LLR: Δ df = 1, Δ dev = 0.076, p = 0.783) were not significant. In contrast, the two-way interactions between competition and tick stage (LLR: Δ df = 1, Δ dev = 6.736, p = 0.0094), and between strain and competition (LLR: Δ df = 1, Δ dev = 4.760, p = 0.029) were significant. In summary, the best model included the following fixed effects: strain, competition, tick stage, competition:tick stage interaction, and strain:competition interaction. The parameter estimates for this model are shown in Table S2. The rest of the statistical analysis is presented in the main manuscript in the section titled "Effect of competition between strains on the strain-specific transmission to immature *I. ricinus* ticks". The AIC-based model selection approach converged on the same model (see model 1 in Table S4 in section 7).

Table S2. The parameter estimates are shown for the best GLMM model from the step-wise regression analysis of the strain-specific prevalence in the immature *I. ricinus* ticks. The best model included the main effect of strain (Fin-Jyv-A3, NE4049), the main effect of competition (no, yes), the main effect of tick stage (larvae, nymphs), the interaction between tick stage and competition, and the interaction between strain and competition. The parameters include an intercept (defined as strain Fin-Jyv-A3 in a larva with no competition), main effects (expressed as the difference between the levels of a factor), and interactions. Shown are the parameter estimates (Estimates), standard errors (SE), z-values, and the associated p-values (p).

Factor	Estimates	SE	z-value	p
Intercept: Fin-Jyv-A3/Larvae/No	-0.479	0.455	-1.053	0.292
Strain: NE4049 - Fin-Jyv-A3	-1.177	0.581	-2.026	0.043
Competition: Yes - No	-1.133	0.707	-1.601	0.109
Tick stage: Nymph - Larva	3.016	0.348	8.674	< 0.001
Interaction: Strain:Competition	1.911	0.837	2.282	0.023
Interaction: Competition:Tick stage	-1.301	0.500	-2.602	0.009

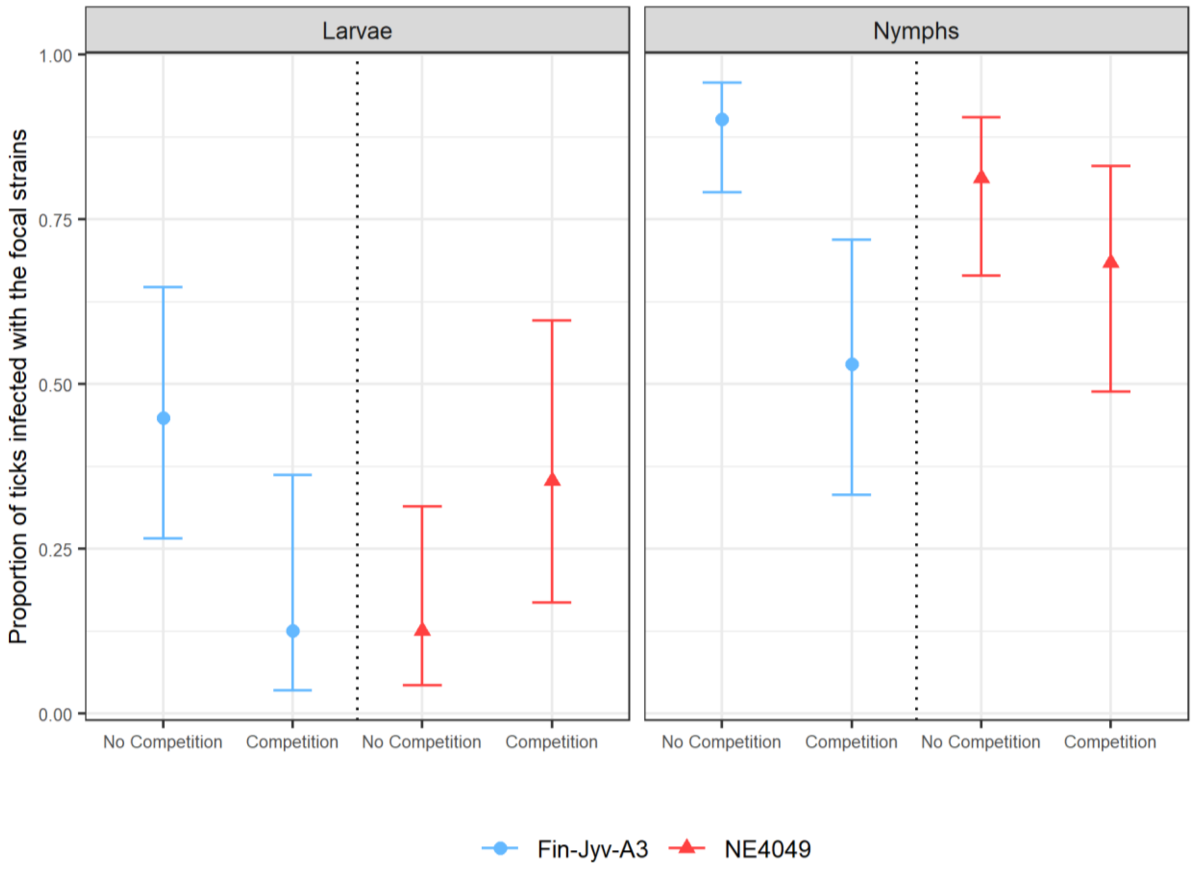

Figure S3. The proportion of immature *I. ricinus* ticks infected with *B. afzelii* is shown as a function of three factors: (1) tick age (engorged larvae, nymphs), (2) strain (Fin-Jyv-A3 in blue and NE4049 in red), and (3) competition (no competition versus competition). The proportion of infected ticks is an estimate of host-to-tick transmission. The 1-month-old nymphs and 4-month-old nymphs were combined because our statistical analysis found no difference between these two types of nymphs. According to the parameter estimates of our statistical analysis, competition between strains in co-infected mice reduced host-to-tick transmission of strain Fin-Jyv-A3 to engorged larvae and nymphs and reduced the host-to-tick transmission of strain NE4049 to nymphs. In contrast, competition between strains in co-infected mice did not influence the host-to-tick transmission of strain NE4049 to engorged larvae. The graph shows the means and the 95% confidence intervals.

SECTION 6 – Effect of competition between strains on the strain-specific spirochete load in immature *I. ricinus* ticks

We wanted to test the effects of competition, strain, tick stage, and tick seasonal treatment on the strain-specific spirochete load in the subset of *I. ricinus* ticks infected with *B. afzelii*. Model comparison showed that there was no effect of the seasonal treatment on the *B. afzelii* spirochete load in the 4-month-old nymphs and that we were justified in combining the 4-month-old nymphs into a single group (LLR test of treatment vs age: Δ df = 8, Δ dev = 6.016, p = 0.645). No further model simplification was justified because the 1-month-old nymphs and 4-month-old nymphs were significantly different (LLR test of age vs stage: Δ df = 4, Δ dev =

13.827, p = 0.008). Thus, the strain-specific spirochete load was analysed as a function of three fixed factors: strain, competition, tick age, and their interactions.

The log10-transformed strain-specific spirochete load was analysed as a function of three fixed factors: strain, competition, tick age, and their interactions. A classic step-wise model simplification approach using LLR tests found that the three-way interaction was not significant (LLR: Δ df = 2, Δ dev = 2.933, p = 0.231) and it was removed from the model. None of the two-way interactions were significant (LLR test of competition:age interaction: Δ df = 2, Δ dev = 0.468, p = 0.792, LLR test of strain:age interaction: Δ df = 2, Δ dev = 2.942, p = 0.230, LLR test of strain:competition interaction: Δ df = 1, Δ dev = 2.725, p = 0.099) and they were removed from the model. In summary, the best model included the main effects of strain, competition, and tick stage. The parameter estimates for this model are shown in Table S3. The rest of the statistical analysis is presented in the main manuscript in the section titled "Effect of competition between strains on the strain-specific spirochete load in immature *I. ricinus* ticks". The AIC-based model selection approach converged on the same model (see model 2 in Table S5 in section 7).

Table S3. The parameter estimates are shown for the best LMM model from the step-wise regression analysis of the strain-specific spirochete load in the immature *I. ricinus* ticks. The best model included the main effects of strain (Fin-Jyv-A3, NE4049), competition (no, yes), and tick age (larva, 1-month-old nymph, 4-month-old nymph). The parameters include an intercept (defined as strain Fin-Jyv-A3 in a larva with no competition) and main effects (expressed as the difference between the levels of a factor). Shown are the parameter estimates (Estimates), standard errors (SE), degrees of freedom (df), t-values, and the associated p-values (p).

Parameter	Estimates	SE	df	t-value	p
Intercept: Fin-Jyv-A3/Larva/No	2.681	0.117	164.619	23.000	< 0.001
Strain: NE4049 - Fin-Jyv-A3	-0.170	0.084	24.825	-2.038	0.052
Competition: Yes - No	-0.292	0.085	25.047	-3.454	0.002
Tick age: 1-month nymph - larva	1.080	0.110	495.073	9.797	< 0.001
Tick age: 4-month nymph - larva	0.887	0.111	507.922	7.969	< 0.001

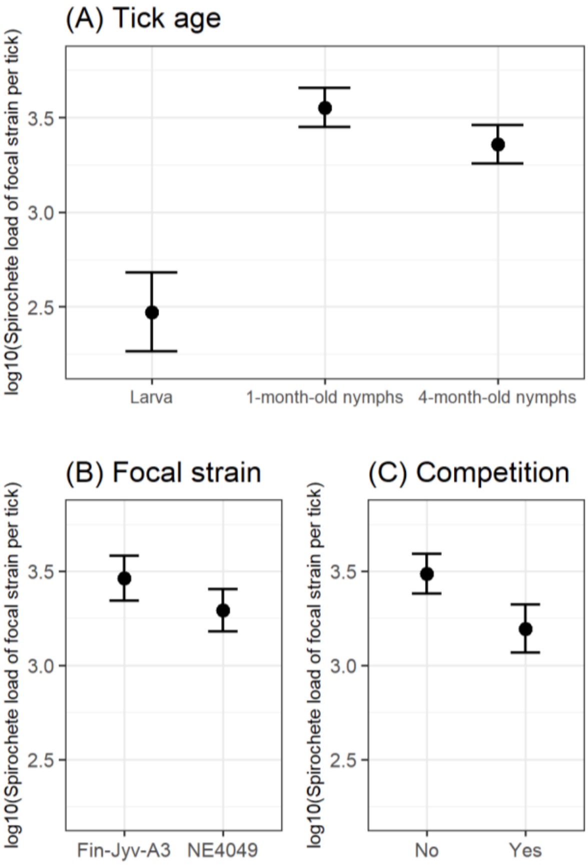

Figure S4. The *B. afzelii* spirochete loads in immature *I. ricinus* ticks are shown as a function of three factors: (A) tick age (engorged larvae, 1-month-old nymphs, and 4-month-old nymphs), (B) strain, and (C) competition. The spirochete loads were log10-transformed to normalize the residuals. According to the parameter estimates of our statistical analysis, competition between strains reduced the spirochete loads of both strains in all three types of ticks. The graph shows the means and the 95% confidence intervals.

SECTION 7 – Model selection using the Akaike information criterion (AIC)

For the analysis of the strain-specific prevalence and the strain-specific spirochete load in immature *I. ricinus* ticks, we used a classic step-wise model simplification approach to identify the best model (see section 5 and section 6, respectively). Specifically, we compared nested models using log-likelihood ratio (LLR) tests and removed non-significant interaction terms until we obtained a model where all of the remaining factors (or their interactions) were statistically significant. We also used AIC-based model selection to determine whether a different approach would identify the same model as being the best one.

In the analysis of the strain-specific prevalence in immature *I. ricinus* ticks, a classic stepwise model simplification approach using LRR tests found that the best model contained the three fixed factors: strain, competition, and tick stage and the interactions between strain and competition, and between competition and tick stage (see section 5). The AIC-based model selection approach also identified this model as the best (see model 1 in Table S4). In the analysis of the strain-specific spirochete load in immature *I. ricinus* ticks, a classic stepwise model simplification approach using LRR tests found that the best model contained the three fixed factors: strain, competition, and tick age (see section 6). The AIC-based model selection approach also identified this model as the best (model 2 in Table S5). In Table S5, the second-place model is actually the best model because it has fewer parameters than the first-place model and because ΔAIC < 1 (i.e. the simpler second-place model is within 1 AIC unit of the more complex first-place model).

Table S4. Model selection results are shown for the generalized linear mixed effects models of the strain-specific transmission (SST) to immature *I. ricinus* ticks. The models are ranked according to their AIC score. The explanatory variables were competition (C), strain (S), and tick stage (T). Shown for each model are the model rank (Rank), fixed effects structure of the model, model degrees of freedom (Df), log-likelihood (logLik), Akaike information criterion (AIC), difference in the AIC value from the top model (ΔAIC), model weight (Weight1; expressed as a %), and cumulative weight (Weight2; expressed as a %).

Rank	Fixed effects structure	Df	logLik	AIC	ΔAIC	Weight1	Weight2
1	SST ~ S+C+T+S:C+T:C	7	-414.2	842.6	0.0	37.2	37.2
2	SST ~ T+C+S+T:C+T:S+C:S+T:C:S	9	-412.5	843.3	0.7	26.8	64.0
3	SST ~ S+C+T+S:C+T:S+T:C	8	-414.2	844.6	2.0	13.9	77.9
4	SST ~ S+C+T+T:C	6	-416.7	845.5	2.9	8.9	86.8
5	SST ~ S+C+T+S:C	6	-417.6	847.2	4.6	3.7	90.5
6	SST ~ S+C+T+T:S+T:C	7	-416.6	847.3	4.7	3.6	94.1
7	SST ~ C+T	4	-419.9	847.9	5.3	2.7	96.7
8	SST ~ S+C+T+S:C+T:S	7	-417.6	849.3	6.7	1.3	98.1
9	SST ~ S+C+T	5	-419.8	849.7	7.1	1.1	99.1
10	SST ~ S+C+T+T:S	6	-419.8	851.7	9.0	0.4	99.5
11	SST ~ T	3	-423.0	852.1	9.5	0.3	99.9
12	SST ~ S+T	4	-422.9	853.9	11.3	0.1	100.0
13	SST ~ C	3	-478.2	962.3	119.7	0.0	100.0
14	SST ~ S+C	4	-478.2	964.4	121.8	0.0	100.0
15	SST ~ 1	2	-480.7	965.4	122.8	0.0	100.0
16	SST ~ S	3	-480.7	967.4	124.8	0.0	100.0

The acronyms for the response and explanatory variables are as follows: SST = strain-specific transmission, C = competition, S = strain, and T = tick stage.

Table S5. Model selection results are shown for the linear mixed effects models of the log10-transformed strain-specific spirochete loads (SSSL). The models are ranked according to their AIC score. The explanatory variables were competition (C), strain (S), and tick age (A). Shown for each model are the model rank (Rank), fixed effects structure of the model, model degrees of freedom (Df), log-likelihood (logLik), Akaike information criterion (AIC), difference in the AIC value from the top model (ΔAIC), model weight (Weight1; expressed as a %), and cumulative weight (Weight2; expressed as a %).

Rank	Fixed effects structure	Df	logLik	AIC	ΔAIC	Weight1	Weight2
1	SSSL ~ A+C+S+C:S	8	-502.7	1021.8	0.0	27.3	27.3
2	SSSL ~ A+C+S	7	-504.2	1022.5	0.7	18.5	45.8
3	SSSL ~ A+C+S+A:S+C:S	10	-501.1	1022.6	0.8	18.2	64.0
4	SSSL ~ A+C+S+A:S	9	-502.4	1023.2	1.4	13.3	77.3
5	SSSL ~ A+C	6	-506.1	1024.4	2.6	7.2	84.5
6	SSSL ~ A+C+S+A:C+C:S	10	-502.3	1025.1	3.3	5.3	89.8
7	SSSL ~ A+C+S+A:C	9	-503.8	1025.9	4.1	3.5	93.3
8	SSSL ~ A+C+S+A:C+A:S+C:S	12	-500.8	1026.3	4.5	2.8	96.1
9	SSSL ~ A+C+S+A:C+A:S	11	-502.2	1026.9	5.1	2.1	98.2
10	SSSL ~ A+C+S+A:C+A:S+C:S+A:C:S	14	-499.4	1027.6	5.8	1.5	99.7
11	SSSL ~ A+S	6	-509.5	1031.1	9.3	0.3	99.9
12	SSSL ~ A	5	-511.2	1032.5	10.7	0.1	100.0
13	SSSL ~ C+S	5	-547.4	1104.8	83.0	0.0	100.0
14	SSSL ~ C	4	-548.7	1105.5	83.7	0.0	100.0
15	SSSL ~ S	4	-552.9	1113.8	92.0	0.0	100.0
16	SSSL ~ 1	3	-554.1	1114.2	92.4	0.0	100.0

The acronyms for the response and explanatory variables are as follows: SSSL = log10-transformed strain-specific spirochete loads, C = competition, S = strain, and A = tick age.

SECTION 8 – Post-hoc tests

Pairwise comparisons for GLMMs and LMMs were done using the functions ‘emmeans’, ‘contrast’, and ‘pairs’ in the emmeans package. Tables S6, S7, and S8 show the results of the post-hoc tests.

Table S6. Pairwise comparisons of *B. afzelii* infection prevalence among tick ages: The *B. afzelii* infection prevalence was modelled as a function of tick age and seasonal treatment using GLMMs. Tick age was significant, and we therefore used post-hoc pairwise comparisons to determine which of the three tick ages (engorged larvae, 1-month-old nymphs, and 4-months-old nymphs) were significantly different from each other. For each pairwise comparison, the z-ratio and the associated p-values (p) are shown. These p-values are presented in the main manuscript in the section titled "Effect of tick age and tick seasonal treatment on the prevalence of *B. afzelii* infection".

qPCR	Pairwise comparison	z-ratio	p
flagellin and *ospC*	Larva – 1-month-old nymph	-10.146	<0.0001
flagellin and *ospC*	Larva – 4-month-old nymph	-9.432	<0.0001
flagellin and *ospC*	1-month-old nymph – 4-month old nymph	1.590	0.249

Table S7. Pairwise comparisons of total *B. afzelii* spirochete load among tick ages: The total *B. afzelii* spirochete load for the subset of infected ticks was modelled as a function of tick age and seasonal treatment using LMMs. Tick age was significant, and we therefore used post-hoc pairwise comparisons to determine which of the three tick ages (engorged larvae, 1-month-old nymphs, and 4-months-old nymphs) were significantly different from each other. For each pairwise comparison, the degrees of freedom (df), the z-ratio, and the associated p-values (p) are shown. These p-values are presented in the main manuscript in the section titled "Effect of tick age and tick seasonal treatment on the *B. afzelii* spirochete load".

qPCR	Pairwise comparison	df	t-ratio	p
flagellin	Larva – 1-month-old nymph	593.13	-11.824	<0.0001
flagellin	Larva – 4-month-old nymph	608.00	-9.346	<0.0001
flagellin	1-month-old nymph – 4-month old nymph	608.86	4.065	0.0002

Table S8. Pairwise comparisons of strain-specific spirochete load among tick ages: The strain-specific spirochete load for the subset of infected ticks was modelled as a function of strain, competition, and tick age using LMMs. Tick age was significant, and we therefore used post-hoc pairwise comparisons to determine which of the three tick ages (engorged larvae, 1-month-old nymphs, and 4-months-old nymphs) were significantly different from each other. For each pairwise comparison, the degrees of freedom (df), the z-ratio, and the associated p-values (p) are shown. These p-values are presented in the main manuscript in the section titled "Effect of competition between strains on the strain-specific spirochete load in immature *I. ricinus* ticks".

qPCR	Pairwise comparison	df	t-ratio	p
ospC	Larva – 1-month-old nymph	499.17	-9.754	<0.0001
ospC	Larva – 4-month-old nymph	513.33	-7.899	<0.0001
ospC	1-month-old nymph – 4-month old nymph	513.28	3.150	<0.0046

10.3. Supplementary material of chapter 3

Title: Competition between strains of *Borrelia afzelii* in the host tissues and consequences for transmission to ticks

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Supplementary material

SECTION 1 – Experimental methods and molecular methods

Creation of infected nymphs: Female BALB/c mice were infected with either strain Fin-Jyv-A3 or strain NE4049 via needle inoculation. *I. ricinus* larvae were fed on the infected mice at 4 weeks post-infection (PI) and the engorged larvae were collected and kept individually in Eppendorf tubes. The Eppendorf tubes contained a piece of moistened paper towel to increase the relative humidity and ensure high larva-to-nymph moulting success. After the larva-to-nymph moult, we randomly selected a sample of nymphs to determine the prevalence of *B. afzelii* infection. For strain Fin-Jyv-A3 and strain NE4049, the prevalence of infection in the nymphs was 70.0% (7/10) and 71.4% (10/14), respectively.

Host-to-tick transmission of *B. afzelii*: To measure host-to-tick transmission, mice were infested with larval ticks at 5 weeks PI as previously described (1, 2). The engorged larval ticks were allowed to moult into nymphs, which were sacrificed by freezing at either 1 month or 4 months after the larva-to-nymph moult. For each mouse, 10 one-month-old nymphs and up to 15 four-month-old nymphs (mean of tested nymphs per mouse = 20.3, range = 7 – 25) were analysed for *B. afzelii* infection as previously described (1, 2). Thus, for each mouse, we obtained two estimates of host-to-tick transmission; one estimate was based on 10 one-month-old nymphs, the other estimate was based on up to 15 four-month-old nymphs. We had previously shown that the age at which the nymph was frozen (1 month or 4 months) does not affect the infection prevalence of the two strains of *B. afzelii* (2). We are therefore justified in combining these two groups of nymphs to obtain a single and best estimate of host-to-tick transmission success.

Correlation in host-to-tick transmission between the 1-month-old nymphs and the 4-month-old nymphs: To measure host-to-tick transmission, each mouse was infested with larval ticks that were allowed to moult into nymphs that were frozen at either 1 month or 4 months of age. Host-to-tick transmission of the focal strain was measured as the proportion of 1-month-old nymphs and 4-month-old nymphs infected with the focal strain. We found a significant positive correlation in the host-to-tick transmission of the focal strain between the 1-month-old nymphs and the 4-month old nymphs ($r = 0.745$, $df = 28$, $t = 5.901$, $p < 0.00001$). The two groups of nymphs were therefore combined to give a single and best estimate of host-to-tick transmission success.

***Flagellin* qPCR:** The total spirochete load in the mouse tissues was estimated using a qPCR that targeted a 132-bp fragment of the *flagellin* gene (3). The qPCRs were performed using the LightCycler$^{®}$ 480 Multiwell Plate 96 white (Roche). The wells were filled with a mixture of 5.8 µl of water, 10.0 µl of Master Mix (FastStart Essential DNA probes Master, Roche), 0.4 µl of 20.0 µM forward primer FlaF1A, 0.4 µl of 20.0 µM reverse primer FlaR1, 0.4 µl of 10.0 µM Flaprobe1, and 3.0 µl of DNA template. The thermocycling conditions consisted of 10 min at 95°C for denaturation, followed by 50 cycles of 30 sec at 60°C and 10 sec at 95°C.

Conventional PCR to amplify the *ospC* gene: The spirochete load was very low in the mouse tissues, and we therefore used a conventional PCR to enrich the number of *ospC* gene copies relative to the amount of mouse DNA. This PCR amplified a 657-bp fragment of the *ospC* gene using a previously described protocol (4). The forward and reverse primers were 5'-ATGAAAAAGAATACATTAAGTGC-3' (positions 306–328 of U01894) and 5'-ATTAATCTTATAATATTGATTTTAATTAAGG-3' (positions 963–933 of U01894), respectively. The mixture contained 14.8 µl of water, 5.0 µl of Mastermix, 1.5 µl of 10.0 mM forward primer, 1.5 µl of 10.0 mM reverse primer, 0.2 µl of Taq, and 2.0 µl of DNA template. The thermocycling program was 94°C for 3 min, 35 cycles of [94°C for 45 s, 52°C for 45 s, and 72°C for 45 s], 72°C for 7 min, and cooling at 10°C. Each plate contained 2 wells filled with the reagents and water and 3 wells that had DNA from uninfected mice as template. The amplicons from the conventional PCR were the template for the strain-specific *ospC* qPCR.

Strain-specific qPCRs targetting the *ospC* gene: The amplicons from the conventional PCR were run in triplicate in the *ospC* A3 qPCR and in the *ospC* A10 qPCR to determine which strains were present. The primers are the same for the two strains and amplify a 142-bp fragment of the *ospC* gene. Strain specificity is determined by the probes, which are different for each *ospC* allele. The qPCRs were performed using the LightCycler® 480 Multiwell Plate 96 white (Roche). The wells were filled with 10.0 µl of 2× Master Mix (FastStart Essential DNA Probes Master, Roche Applied Science), 5.8 µl of water, 0.4 µl of 20.0 mM forward primer OspC_qPCR_For, 0.4 µl of 20.0 mM reverse primer OspC_qPCR_Rev, 0.4 µl of 10.0 mM A3_Probe or A10_Probe, and 3.0 µl of DNA template (1). Each plate contained 5 wells filled with the negative controls from the PCR and 2 wells filled with the reagents of the qPCR and water.

Repeatability of the strain-specific spirochete load: The repeatability of the strain-specific spirochete load was calculated as follows. The total spirochete abundance in the mouse tissues was estimated using the three independent *flagellin* qPCRs. Similarly, the relative abundance of strains Fin-Jyv-A3 and NE4049 was calculated using the three independent *ospC* A3 qPCRs and the three independent *ospC* A10 qPCRs. For each strain, the three estimates of total spirochete load were paired with the three estimates of relative abundance to calculate three estimates of the strain-specific spirochete load for each of the 222 organ tissue samples (37 mice* 6 organs per mouse = 222 organ tissue samples). The overall repeatability (across all 222 organ tissue samples) of the spirochete load of strain Fin-Jyv-A3 and strain NE4049 was 78.87% and 87.04%, respectively. All six *ospC* qPCRs were done on the same set of *ospC* amplicons, so our estimate of the repeatability does not include variation due to differences among amplicons coming from different conventional PCR reactions.

Estimation of the strain-specific spirochete load: The focal strains in experiments 1 and 2 are Fin-Jyv-A3 and NE4049, respectively. For the co-infected mice, the abundance of each strain in a given tissue was estimated by combining the total spirochete abundance of the *flagellin* qPCR with the relative abundances of the *ospC* qPCRs. For example, if the total spirochete load for a given tissue sample was 200 spirochetes per mg of tissue, and the relative abundances of strains Fin-Jyv-A3 and NE4049 were 75% and 25% respectively, then the strain-specific abundances of strains Fin-Jyv-A3 and NE4049 were 150 and 50 spirochetes per mg of tissue, respectively. For each of the 222 tissue samples, we had 3 estimates of total spirochete load and 3 estimates of the relative abundance, which allowed us to estimate 3 independent estimates of the strain-specific spirochete load. We calculated an average strain-specific spirochete load for each of the 222 tissue samples by taking the geometric mean of the 3 independent estimates. These averaged strain-specific spirochete loads were used in the statistical analyses of the strain-specific spirochete loads.

SECTION 2 – Analysis of the total spirochete load

The spirochete loads analysed in this study were standardized by mg of tissue extracted. When the spirochete loads were standardized by mg of DNA and compared to the spirochete loads standardized by mg of tissue, the correlation was positive and highly significant (r = 0.946, df = 220, t = 43.11, p < 2.2e-16).

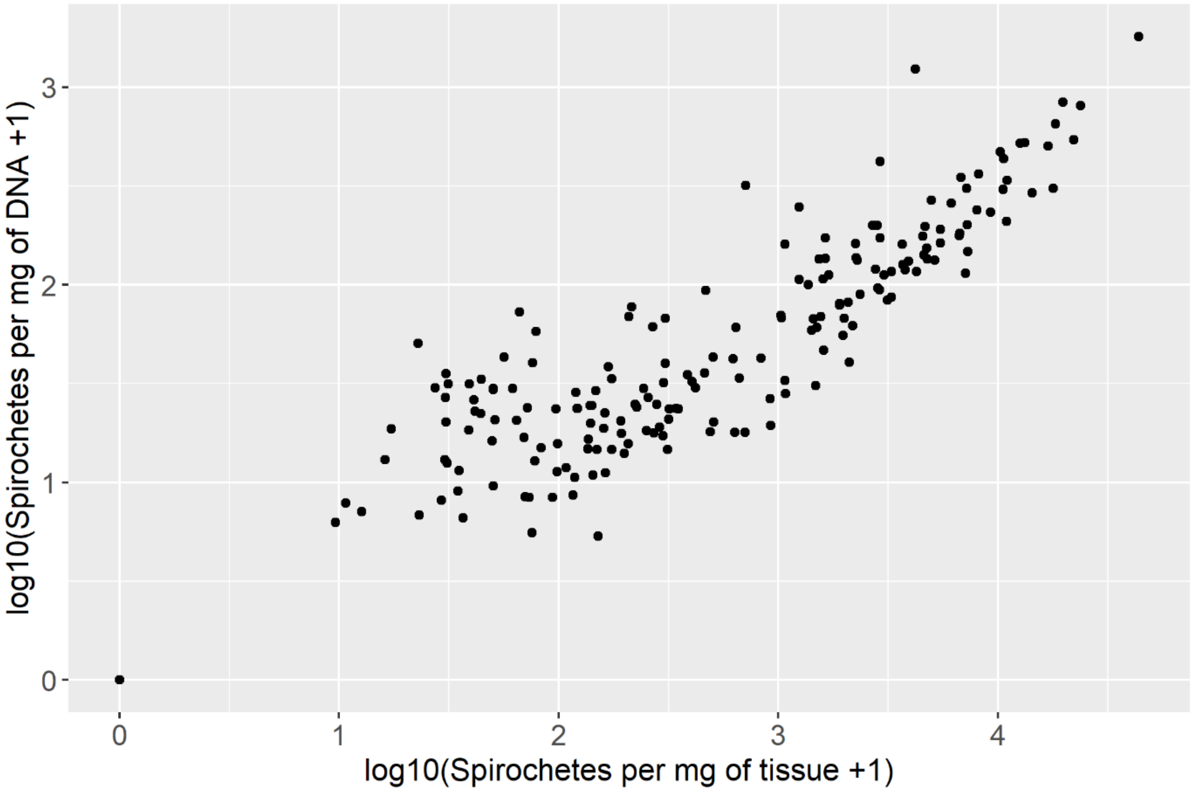

Figure S1. The two methods of standardizing the spirochete load in the mouse tissues give very similar results. The tissue spirochete loads were expressed as the spirochetes per mg of tissue (horizontal axis) or the spirochetes per mg of DNA (vertical axis).

SECTION 3 – The *B. afzelii* tissue infection prevalence differs among organs

The *flagellin* qPCR, which cannot distinguish between strains, was used to determine whether tissues were infected with *B. afzelii* or not. Tissue samples were considered infected with *B. afzelii* if at least two of the three replicate *flagellin* qPCR assays tested positive. The sample size was 32 mice with 6 tissues per mouse for a total of 192 tissue samples. The binomial response variable was whether *B. afzelii* was absent (0) or present (1) in a tissue sample, and it was analysed as a generalized linear mixed effects model (GLMM) with binomial errors. The fixed factors were focal strain (two levels: Fin-Jyv-A3, NE4049), co-infection (two levels: no, yes), organ (six levels: bladder, left ear, right ear, heart, joint, dorsal skin), and their interactions. Mouse identity was modelled as a random factor. Models that differed with respect to the fixed factor of interest were compared using log-likelihood ratio (LLR) tests to determine statistical significance.

We analysed the prevalence of *B. afzelii* infection in the mouse tissue samples as a function of focal strain, co-infection, and organ. The effect of the three-way interaction between focal strain, co-infection, and organ was not significant (LMM LLR: Δ df = 5, $\Delta \chi^2 = 0.168$, p = 0.999). The two-way interactions between co-infection and organ (LMM LLR: Δ df = 5, $\Delta \chi^2 = 0.518$, p = 0.991), between co-infection and focal strain (LMM LLR: Δ df = 1, $\Delta \chi^2 = 0.030$, p = 0.863), and between focal strain and organ (LMM LLR: Δ df = 5, $\Delta \chi^2 = 0.734$, p = 0.981) were not significant. The main effect of co-infection (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 3.042$, p = 0.081) and focal strain (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 3.549$, p = 0.060) were not significant, whereas organ (GLMM LLR: Δ df = 5, $\Delta \chi^2 = 22.368$, p < 0.001) was significant.

SECTION 4 – The total spirochete load of *B. afzelii* differs among organs

The spirochete loads estimated by the *flagellin* qPCR in 3 µl of DNA template were corrected to the total DNA extraction elution volume (150 µl), standardized by mg of tissue extracted, and log-10 transformed to improve normality. The standardized log10-transformed tissue spirochete load was analysed as a linear mixed effects model (LMM). The fixed factors were focal strain (two levels: Fin-Jyv-A3, NE4049), co-infection (two levels: no, yes), organ (six levels: bladder, left ear, right ear, heart, joint, skin), and their interactions. Mouse identity was modelled as a random factor. Statistical significance of fixed factors was determined by comparing nested models with LLR tests.

The effect of the three-way interaction between strain, co-infection, and organ on the log10-transformed total spirochete load per mg was not significant (LMM LLR: Δ df = 5, $\Delta \chi^2 = 4.629$, p = 0.463). The two-way interactions between co-infection and organ (LMM LLR: Δ df = 5, $\Delta \chi^2 = 5.945$, p = 0.312), between co-infection and focal strain (LMM LLR: Δ df = 1, $\Delta \chi^2 = 1.585$, p = 0.208) and between focal strain and organ LMM LLR: Δ df = 5, $\Delta \chi^2 = 10.047$, p = 0.074) were not significant. The main effects of focal strain (LMM LLR: Δ df = 1, $\Delta \chi^2 = 0.109$, p = 0.741) and co-infection (LMM LLR: Δ df = 1, $\Delta \chi^2 = 3.626$, p = 0.057) were not significant, whereas organ was significant (LMM LLR: Δ df = 5, $\Delta \chi^2 = 305.82$, p < 2.2e-16).

Table S1. The total organ spirochete load (based on *flagellin*-qPCR) is shown for each of the 24 unique combinations of focal strain, organ, and co-infection. The units of the total organ spirochete load are the number of spirochetes per mg of tissue. Shown are the means and the 95% confidence intervals (95% CI).

Focal strain	Organ	Co-infection	Mean total	95% CI
Fin-Jyv-A3	Bladder	no	157	93–266
Fin-Jyv-A3	Bladder	yes	221	122–401
Fin-Jyv-A3	Left ear	no	3,515	1,937–6,378
Fin-Jyv-A3	Left ear	yes	5,926	3,266–10,753
Fin-Jyv-A3	Right ear	no	2,533	1,331–4,821
Fin-Jyv-A3	Right ear	yes	4,957	2,732–8,994
Fin-Jyv-A3	Heart	no	33	19–57
Fin-Jyv-A3	Heart	yes	50	26–95
Fin-Jyv-A3	Joint	no	347	172–703
Fin-Jyv-A3	Joint	yes	271	149–491
Fin-Jyv-A3	Dorsal skin	no	1,074	531–2,173
Fin-Jyv-A3	Dorsal skin	yes	700	318–1,540
NE4049	Bladder	no	178	105–301
NE4049	Bladder	yes	204	112–369
NE4049	Left ear	no	5,782	3,312–10,096
NE4049	Left ear	yes	6,541	3,605–11,869
NE4049	Right ear	no	3,463	2,047–5,856
NE4049	Right ear	yes	7,450	4,106–13,518
NE4049	Heart	no	26	15–45
NE4049	Heart	yes	38	19–78
NE4049	Joint	no	98	58–166
NE4049	Joint	yes	214	118–389
NE4049	Dorsal skin	no	759	399–1,445
NE4049	Dorsal skin	yes	1324	654–2,679

SECTION 5 – The total tissue spirochete load differs among strains

To test whether bacterial abundance differed among the two strains, we analysed the standardized log10-transformed tissue spirochete loads as a function of organ and focal strain for the subset of mice infected with one strain (n = 18). The interaction between focal strain and organ was significant (LMM LLR: Δ df = 5, $\Delta \chi^2 = 11.85$, p = 0.037), which is evidence for strain-specific tissue tropism. We therefore used the emmeans function to compare the tissue spirochete load between the two strains separately for each organ. The mean spirochete load in the ankle joints for strain Fin-Jyv-A3 was higher than that of strain NE4049 and this difference was significant (df = 101.5, t = 3.245, p = 0.0016). Whereas the differences in spirochete load between strain Fin-Jyv-A3 and strain NE4049 in the bladder (df = 98.0, t = -0.363, p = 0.717), the left ear (df = 100.3, t = 0.074, p = 0.941), the right ear (df = 100.7, t = -0.653, p = 0.516), the heart (df = 99.7, t = 0.590, p = 0.557), and the skin (df = 102.3, t = 0.616, p = 0.540) were not significant.

Table S2. The *B. afzelii* spirochete loads in the mouse tissues are shown separately for the 12 unique combinations of strain and organ. There are two strains (Fin-Jyv-A3, NE4049) and six organs (bladder, left ear, right ear, heart, right ankle joint, and the section of dorsal skin where the nymphs had attached). The data were calculated for the subset of 18 mice that were singly infected with strain Fin-Jyv-A3 (n = 9) or strain NE4049 (n = 9). The units of the spirochete load are the total number of spirochetes per mg of tissue and the estimates are based on the *flagellin* qPCR. Shown are the geometric mean of the spirochete loads and the 95% confidence intervals (95% CI).

Focal strain	Organ	Mean	95% CI
Fin-Jyv-A3	Bladder	157	98–252
Fin-Jyv-A3	Left ear	5,926	3,467–10,129
Fin-Jyv-A3	Right ear	2,533	1,420–4,520
Fin-Jyv-A3	Heart	33	20–54
Fin-Jyv-A3	Joint	347	184–655
Fin-Jyv-A3	Dorsal skin	1,074	569–2,024
NE4049	Bladder	178	111–285
NE4049	Left ear	5,782	3,502–9,547
NE4049	Right ear	3,463	2,158–5,555
NE4049	Heart	26	16–43
NE4049	Joint	98	61–158
NE4049	Dorsal skin	759	426–1,355

SECTION 6 – Effect of strain, co-infection, and organ on the strain-specific tissue infection prevalence

The focal strains in experiments 1 and 2 are Fin-Jyv-A3 and NE4049, respectively. In experiment 1, the tissue sample was considered to be infected with Fin-Jyv-A3 if at least two of the three replicate *ospC* A3 qPCR assays tested positive. In experiment 2, the tissue sample was considered to be infected with NE4049 if at least two of the three replicate *ospC* A10 qPCR assays tested positive. The sample size for the analysis is 192 tissue samples. The binomial response variable was whether the focal strain was absent (0) or present (1) in a tissue sample, and it was analysed as a GLMM with binomial errors. The fixed factors were focal strain (two levels: Fin-Jyv-A3, NE4049), co-infection (two levels: no, yes), organ (six levels: bladder, left ear, right ear, heart, joint, dorsal skin), and their interactions. Mouse identity was modelled as a random factor. Models that differed with respect to the fixed factors of interest were compared using log-likelihood ratios (LLR) tests to determine the statistical significance of the fixed factors.

A classic step-wise model simplification approach using LLR tests found that the three-way interaction was not significant and was removed from the analysis (GLMM LLR: Δ df = 5, $\Delta \chi^2 = 0.502$, p = 0.992). The two-way interactions between co-infection and organ (GLMM LLR: Δ df = 5, $\Delta \chi^2 = 1.301$, p = 0.935), between focal strain and organ (GLMM LLR: Δ df = 5, $\Delta \chi^2 = 3.580$, p = 0.611), and between focal strain and co-infection (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 3.065$, p = 0.080) were not significant and were removed from the analysis. The main effects of focal strain (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 25.704$, p = 3.98e-07), co-infection

(GLMM LLR: Δ df = 1, Δ χ^2 = 14.504, p = 0.0001), and organ (GLMM LLR: Δ df = 5, Δ χ^2 = 17.83, p = 0.003) were significant.

Table S3. The strain-specific tissue infection prevalence is shown for each of the 12 unique combinations of focal strain, organ, and co-infection.

Focal strain	Organ	Co-infection	Prevalence
Fin-Jyv-A3	Bladder	No	100% (9/9)
Fin-Jyv-A3	Bladder	Yes	57.1% (4/7)
Fin-Jyv-A3	Left ear	No	77.8% (7/9)
Fin-Jyv-A3	Left ear	Yes	28.6% (2/7)
Fin-Jyv-A3	Right ear	No	66.7% (6/9)
Fin-Jyv-A3	Right ear	Yes	28.6% (2/7)
Fin-Jyv-A3	Heart	No	88.9% (8/9)
Fin-Jyv-A3	Heart	Yes	57.1% (4/7)
Fin-Jyv-A3	Joint	No	55.6% (5/9)
Fin-Jyv-A3	Joint	Yes	28.6% (2/7)
Fin-Jyv-A3	Skin	No	55.6% (5/9)
Fin-Jyv-A3	Skin	Yes	0% (0/7)
NE4049	Bladder	No	100% (9/9)
NE4049	Bladder	Yes	100% (7/7)
NE4049	Left ear	No	88.9% (8/9)
NE4049	Left ear	Yes	85.7% (6/7)
NE4049	Right ear	No	100% (9/9)
NE4049	Right ear	Yes	100% (7/7)
NE4049	Heart	No	88.9% (8/9)
NE4049	Heart	Yes	57.1% (4/7)
NE4049	Joint	No	100% (9/9)
NE4049	Joint	Yes	100% (7/7)
NE4049	Skin	No	66.7% (6/9)
NE4049	Skin	Yes	71.4% (5/7)

SECTION 7 – Effect of co-infection on the strain-specific prevalence in the mice

In this analysis, we ignored the effect of organ and treated each of the 6 tissue samples as an estimate of whether the mouse was infected or not. We analysed the strain-specific prevalence in the mouse tissues as a function of focal strain, co-infection, and their interaction using a generalised linear model (GLM) with binomial errors. The ratio of the residual deviance (22.647) to the residual degrees of freedom (28) was 0.81, indicating that the residuals were not overdispersed. The interaction between co-infection and focal strain was not significant (GLM: Δ df = 1, Δ dev = 2.485, p = 0.115) and was removed from the analysis. Both co-infection (GLM: Δ df = 1, Δ dev = 14.405, p < 0.001) and focal strain (GLM: Δ df = 1, Δ dev = 28.331, p < 0.0000001) were significant.

SECTION 8 – Effect of strain, co-infection, and organ on the strain-specific tissue spirochete load

For the subset of infected mouse tissue samples (n = 190), we analysed the strain-specific tissue spirochete load of *B. afzelii* in mouse tissue samples as a function of focal strain, co-infection, organ, and their interactions. The organ "skin" for strain Fin-Jyv-A3 had to be removed from the analysis because all the tissue samples in the co-infected group tested negative for focal strain Fin-Jyv-A3 in experiment 1. A classic stepwise model simplification approach using LLR tests found that the three-way interaction was significant (LME LLR: Δ df = 4, $\Delta\ \chi^2 = 10.633$, p = 0.031), and the analysis was therefore divided by focal strain.

For strain Fin-Jyv-A3, the interaction between co-infection and organ was significant (LME LLR: Δ df = 4, $\Delta\ \chi^2 = 22.324$, p = 0.0002) and we therefore used the emmeans function to test the effect of co-infection in each organ. In the bladder, co-infection reduced the mean spirochete load of strain Fin-Jyv-A3 by a factor of 4.0 (emmeans: df = 58.9, t = 3.109, p = 0.003). In the ankle joint, co-infection increased the mean spirochete load of strain Fin-Jyv-A3 by a factor of 5.9 (emmeans: df = 67.4, t = -2.353, p = 0.022). The contrast between single infection and co-infection was not significant for the other 3 organs: left ear (emmeans: df = 65.8, t = 0.447, p = 0.656), right ear (emmeans: df = 66.2, t = 1.659, p = 0.102), and heart (emmeans: df = 60.3, t = 0.409, p = 0.684).

For strain NE4049, the interaction between co-infection and organ was not significant (LME LLR: Δ df = 5, $\Delta\ \chi^2 = 3.152$, p = 0.677). Co-infection was not significant (LME LLR: Δ df = 1, $\Delta\ \chi^2 = 3.500$, p = 0.061), but organ had a significant effect on the strain-specific organ spirochete load (LME LLR: Δ df = 5, $\Delta\ \chi^2 = 600.226$, p < 2e-16).

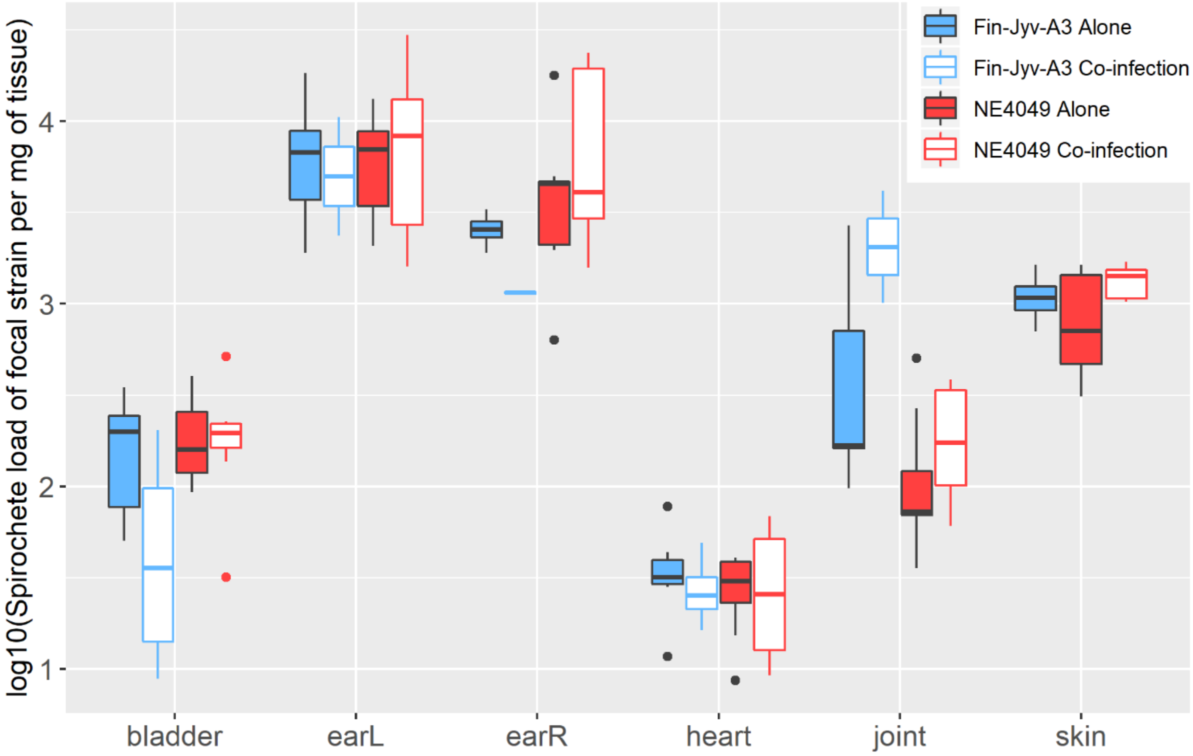

Figure S2. The spirochete load in the mouse tissues depends on organ, strain, and co-infection. The largest differences in tissue spirochete load occur between the six organs. Strains Fin-Jyv-A3 and NE4049 are shown with blue and red colours, respectively. Single infection and co-infection are shown by the empty and solid boxes, respectively. The boxplots show the median (black line), 25th and 75th percentiles (edges of the box), minimum and maximum values (whiskers), and outliers (solid circles).

SECTION 9 – Relationship between host-to-tick transmission of the focal strain and the number of organs infected by the focal strain

We wanted to test whether host-to-tick transmission of the focal strain was dependent on the infection status of the focal strain in any particular organ. For each mouse, we summed the infection status of the focal strain across all six organs. We used a GLMER with binomial errors to model host-to-tick transmission of the focal strain as a function of the number of organs infected with the focal strain. The number of organs infected with the focal strain had a significant and positive effect on the host-to-tick transmission of the focal strain (Figure S3; GLMM LLR: Δ df = 1, Δ χ^2 = 8.401, p = 0.004).

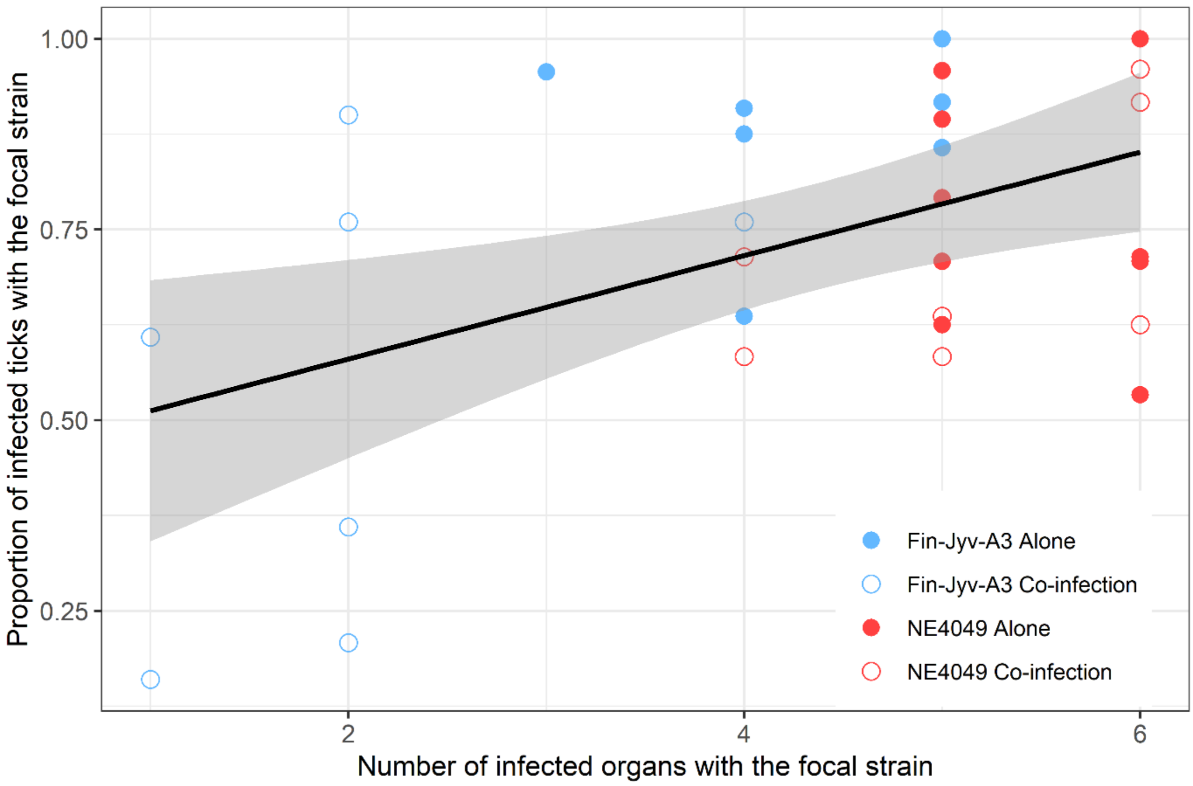

Figure S3. Host-to-tick transmission of the focal strain depends on the number of organs that are infected with the focal strain. Strain Fin-Jyv-A3 and strain NE4049 are shown in blue and red symbols, respectively. Mice in the single strain groups and the co-infected groups are shown with solid and empty symbols, respectively. The black line shows the line of best fit and the grey area shows the 95% confidence limits; both were estimated from a GLM.

SECTION 10 – Relationship between host-to-tick transmission of the focal strain and the mean organ spirochete load of the focal strain

We wanted to test whether host-to-tick transmission of the focal strain was dependent on the spirochete load of the focal strain in any particular organ. For each mouse, we averaged the log10-transformed spirochete load of the focal strain across all six organs. We used a GLMER with binomial errors to model the host-to-tick transmission of the focal strain as a function of the mean log10-transformed spirochete load of the focal strain in the organs (hereafter referred to as organ spirochete load for convenience). The mean organ spirochete load of the focal strain had a significant positive effect on the host-to-tick transmission of the focal strain (Figure S4; GLMM LLR: Δ df = 1, $\Delta\ \chi^2$ = 8.028, p = 0.005).

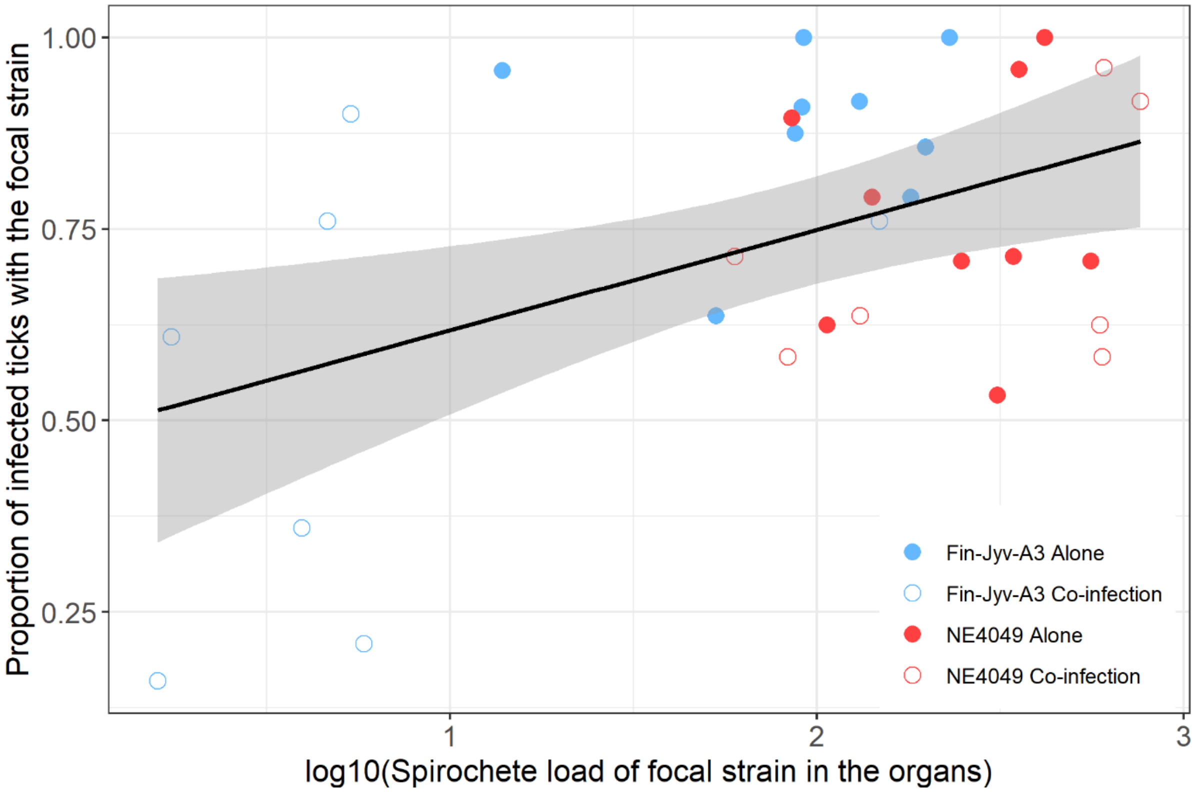

Figure S4. Host-to-tick transmission of the focal strain depends on the mean spirochete load of the focal strain in the mouse tissues. The spirochete load of the focal strain was averaged over six organs: bladder, left ear, right ear, heart, right ankle joint, and the section of dorsal skin where the nymphs had attached. Strain Fin-Jyv-A3 and strain NE4049 are shown in blue and red symbols, respectively. Mice in the single strain groups and the co-infected groups are shown with solid and empty symbols, respectively. The black line shows the line of best fit and the grey area shows the 95% confidence limits; both were estimated from a GLM.

SECTION 11 – Relationship between host-to-tick transmission of the focal strain and the infection status of the organs

We wanted to test whether host-to-tick transmission of the focal strain was dependent on the infection status of any particular organ. We used a GLMER with binomial errors to model host-to-tick transmission of the focal strain as a function of the infection status of each of the six organs: bladder, left ear, right ear, heart, ankle joint, and dorsal skin. We used step-wise removal of the least significant organ to simplify the model. The organs were removed in the following order: bladder (GLMM LLR: Δ df = 1, $\Delta\ \chi^2$ = 0.156, p = 0.693), ankle joint (GLMM LLR: Δ df = 1, $\Delta\ \chi^2$ = 0.368, p = 0.544), left ear (GLMM LLR: Δ df = 1, $\Delta\ \chi^2$ = 0.251, p = 0.616), heart (GLMM LLR: Δ df = 1, $\Delta\ \chi^2$ = 1.116, p = 0.291), and dorsal skin (GLMM LLR: Δ df = 1, $\Delta\ \chi^2$ = 2.594, p = 0.107). The infection status of the right ear had a significant effect on the host-to-tick transmission of the focal strain (GLMM LLR: Δ df = 1, $\Delta\ \chi^2$ = 8.485, p = 0.004).

Figure S5. Host-to-tick transmission of the focal strain depends on the prevalence of the right ear with the focal strain. The black line and grey area show the line of best fit and the 95% confidence limits estimated from the GLM.

SECTION 12 – Relationship between host-to-tick transmission of the focal strain and the spirochete load of the focal strain in each organ

We wanted to test whether host-to-tick transmission of the focal strain was dependent on the spirochete load of the focal strain in any particular organ. We used a GLMER with binomial errors to model host-to-tick transmission of the focal strain as a function of the spirochete load of each of the six organs: bladder, left ear, right ear, heart, ankle joint, dorsal skin. We used step-wise removal of the least significant organ to simplify the model. The organs were removed in the following order: bladder (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 0.0004$, p = 0.985), left ear (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 0.853$, p = 0.356), ankle joint (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 0.393$, p = 0.531), heart (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 1.579$, p = 0.209), and dorsal skin (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 2.307$, p = 0.129). The spirochete load of the focal strain in the right ear had a significant positive effect on the host-to-tick transmission of the focal strain (GLMM LLR: Δ df = 1, $\Delta \chi^2 = 9.192$, p = 0.002).

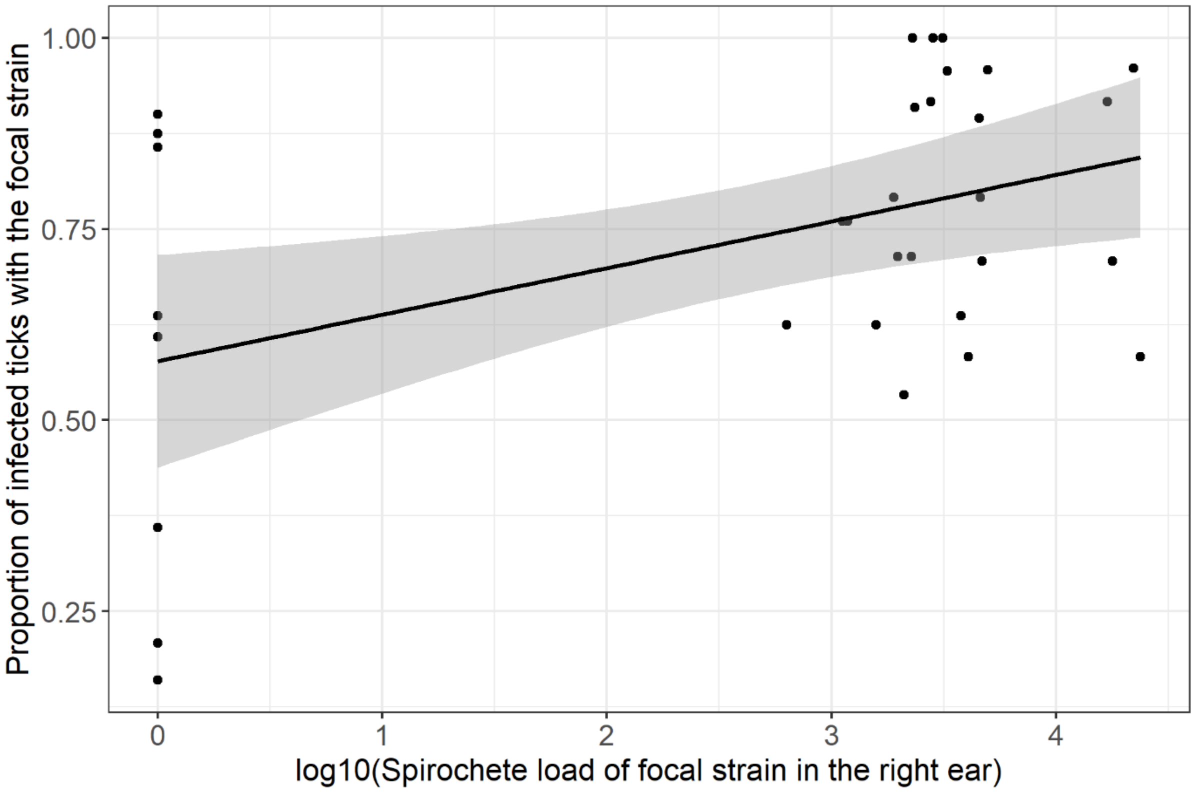

Figure S6. Host-to-tick transmission of the focal strain depends on the spirochete load of the focal strain in the right ear. The black line and grey area show the line of the best fit and the 95% confidence limits established from the GLM.

SECTION 13 – AIC-based model selection of the relationship between presence and abundance of the focal strain in the rodent tissues and the strain-specific host-to-tick transmission

We had previously shown that co-infection in the mouse decreases the efficiency of transmission of the focal strain from the infected mouse to immature *I. ricinus* ticks [1, 2]. We hypothesized that co-infection in the mouse changes the presence and abundance of the focal strain in the mouse tissues, which subsequently influences the efficacy of strain-specific host-to-tick transmission. We further hypothesized that exterior organs, such as the left ear, right ear, and dorsal skin should be more important for host-to-tick transmission than interior organs like the bladder, heart, and ankle joints.

We used generalized linear mixed effect models (GLMM) with binomial errors to model the strain-specific host-to-tick transmission success as a function of a variety of host-related factors. These host-related factors included mouse co-infection status and focal strain, which we had tested in earlier studies [1, 2]. New to the present study, we tested whether strain-specific host-to-tick transmission success depended on measures of strain-specific infection success, such as the mean number of tissues infected with the focal strain (summed across all six organs) and the mean tissue spirochete load of the focal strain (averaged across all six organs), as well as the strain-specific infection status and the strain-specific spirochete load of the individual organs, either all together or grouped into interior and exterior organs. Mouse identity always entered the model as a random effect. As many of these host-related variables are strongly associated or correlated, we used AIC-based model selection to compare the ability of different models to explain the variation in strain-specific host-to-tick transmission success.

The AIC-based model selection found that the top 16 models had 95.4% of the support (Table S4). The top 5 models were all within 1 AIC unit of the lowest AIC score. These models contained focal strain and co-infection (model 1, support = 13.9%), the spirochete load of the right ear (model 2, support = 13.2%), the number of organs infected with the focal strain (model 3, support = 11.5%), the infection status of the right ear (model 4, support = 10.1%), and the mean spirochete load of the focal strain in the infected organs (model 5, support = 9.9%). Models that included the external organs (right ear, left ear, and dorsal skin; support = 52.0%) had 8 times more support than models that included the internal organs (bladder, heart, ankle joint; support = 6.4%). These model selection results show that the presence and abundance of the strain in the external organs are most important for determining the strain-specific host-to-tick transmission success.

Inspection of the model-averaged parameter estimates of individual models found that many of the host-related factors were statistically significant. In Table S5, statistical significance of the model-averaged parameter estimates is shown by 95% confidence limits that do not overlap zero. The number of organs infected with the focal strain and the mean organ spirochete load of the focal strain were both significant predictors of the strain-specific host-to-tick transmission (Table S5). The infection status of the right ear and the spirochete of the right ear were both significant predictors of the strain-specific host-to-tick transmission (Table S5). In contrast, the infection status or spirochete load of internal organs like the bladder, heart, and ankle joint were not significant predictors of the strain-specific host-to-tick transmission (Table S5).

Table S4. Model selection results are shown for the GLMMs of the strain-specific host-to-tick transmission. Strain-specific host-to-tick transmission is a binomial variable that indicates whether the nymphs that fed as larvae on the infected mice were infected or not with the focal strain. The models are ranked according to their AIC score. The explanatory variables were co-infection (C), focal strain (F), number of organs infected with the focal strain (N), mean organ spirochete load of the focal strain (M), the strain-specific infection status of the bladder (S.B), the heart (S.H), the joint (S.J), the dorsal skin (S.S), the left ear (S.El), the right ear (S.Er), the strain-specific spirochete load in the bladder (L.B), the heart (L.H), the joint (L.J), the dorsal skin (L.S), the left ear (L.El), the right ear (L.Er). Shown for each model are the model rank (Rank), fixed effects structure of the model, model degrees of freedom (Df), log-likelihood (logLik), Akaike information criterion (AIC), difference in the AIC value from the top model (AIC), model weight (Weight1; expressed as a %), and cumulative weight (Weight2; expressed as a %).

Rank	Fixed effects structure	Df	logLik	AIC	ΔAIC	Weight1	Weight2
1	SSN ~ F*C	5	-337.1	684.2	0.0	13.9	13.9
2	SSN ~ L.Er	3	-339.1	684.3	0.1	13.2	27.1
3	SSN ~ N	3	-339.3	684.6	0.4	11.5	38.6
4	SSN ~ S.Er	3	-339.4	684.9	0.6	10.1	48.7
5	SSN ~ M	3	-339.4	684.9	0.7	9.9	58.6
6	SSN ~ L.Er+L.El+L.S	5	-337.8	685.6	1.4	6.8	65.4
7	SSN ~ S.Er+S.El+S.S	5	-338.0	686.1	1.9	5.4	70.8
8	SSN ~ L.Er+L.El	4	-339.1	686.2	2.0	5.1	75.9
9	SSN ~ F+C	4	-339.3	686.7	2.5	4.0	79.9
10	SSN ~ S.Er+S.El	4	-339.4	686.8	2.6	3.8	83.7
11	SSN ~ L.S	3	-340.4	686.9	2.7	3.6	87.3
12	SSN ~ S.S	3	-340.5	687.1	2.9	3.3	90.6
13	SSN ~ L.B	3	-341.1	688.3	4.1	1.8	92.4
14	SSN ~ S.J	3	-341.6	689.2	5.0	1.2	93.5
15	SSN ~ L.B+L.H+L.J+L.Er+L.El+L.S	8	-336.6	689.5	5.3	1.0	94.5
16	SSN ~ S.B	3	-341.8	689.6	5.4	0.9	95.4
17	SSN ~ 1	2	-343.0	689.9	5.7	0.8	96.2
18	SSN ~ L.J	3	-342.0	690.0	5.8	0.8	97.0
19	SSN ~ S.B+S.H+S.J+S.Er+S.El+S.S	8	-337.2	690.7	6.5	0.6	97.6
20	SSN ~ S.B+S.H+S.J	5	-340.3	690.8	6.6	0.5	98.1
21	SSN ~ L.B+L.H+L.J	5	-340.6	691.2	7.0	0.4	98.5
22	SSN ~ S.H	3	-342.6	691.3	7.1	0.4	98.9
23	SSN ~L.El	3	-342.7	691.4	7.2	0.4	99.3
24	SSN ~L.H	3	-342.7	691.5	7.3	0.4	99.7
25	SSN ~ S.El	3	-342.8	691.6	7.4	0.3	1.0

Table S5. Model-averaged parameter estimates are shown for the GLMMs of host-to-tick transmission in Table S4. Strain-specific host-to-tick transmission is a binomial variable that indicates whether the nymphs that fed as larvae on the infected mice were infected or not with the focal strain. Shown are the parameter types, the parameter names, the parameter estimates, and the 95% confidence limits (LL = lower limit and UL = upper limit). Estimate 1 is averaged over all the models in the set. Estimate 2 is averaged over the subset of models with a cumulative support of 95%. The 95% confidence limits are for estimate 2. Statistically significant parameter estimates are shown in bold-face type.

Type	Name	Estimate 1	Estimate 2	95% LL	95% UL
Intercept	Strain Fin-Jyv-A3, Single strain	0.489	0.489	-1.360	2.339
Contrast 1	Strain NE4049 – Strain Fin-Jyv-A3	-0.100	-0.559	-1.793	0.675
Contrast 2	**Co-infection – Single strain**	**-0.335**	**-1.872**	**-3.161**	**-0.583**
Contrast 3	**Co-infection: Strain NE4049**	**0.239**	**1.724**	**0.180**	**3.268**
Slope 1	**L.Er**	**0.097**	**0.372**	**0.111**	**0.633**
Slope 2	**N**	**0.045**	**0.388**	**0.125**	**0.650**
Slope 3	**S.Er**	**0.259**	**1.300**	**0.346**	**2.254**
Slope 4	**M**	**0.073**	**0.738**	**0.226**	**1.249**
Slope 5	L.El	-0.009	-0.066	-0.323	0.192
Slope 6	L.S	0.030	0.263	-0.033	0.559
Slope 7	S.El	-0.021	-0.204	-1.159	0.752
Slope 8	L.S	0.074	0.795	-0.087	1.678
Slope 9	L.B	0.012	0.389	-0.447	1.225
Slope 10	S.J	0.015	0.671	-0.390	1.731
Slope 11	L.H	0.005	0.292	-0.317	0.900
Slope 12	L.J	0.005	0.210	-0.192	0.613
Slope 13	S.B	0.019	0.927	-0.786	2.640
Slope 14	S.H	0.007	0.486	-0.479	1.451

SECTION 14 – The plasmid profile of *b. afzelii* strains Fin-Jyv-A3 and NE4049

We used PCR to determine whether the two strains used in this study, Fin-Jyv-A3 and NE4049, contained all of the plasmids necessary to complete the life cycle of *B. afzelii*. We tested the strains for the following 16 plasmids: lp 17, lp 28-2, lp 28-3, lp 25, lp 28, lp 28-4, lp 28-7, lp 28-8, lp 30, lp 34, lp 38, cp 26, cp 32-5, cp 32-7, cp 32-9, cp 32-10. Total genomic DNA was prepared from approximately 8 ml of *Borrelia* culture using the Wizard genomic DNA kit (Promega, Madison, WI) as per the manufacturer's instructions. The total plasmid contents of both strains of *B. afzelii* were compared using primers specific for plasmids based on the complete genome sequence of PKo and ACA-1 [5]. In addition, primers specific for the genes *pncA* and *adeC* were used to confirm their presence [5].

The plasmid profile of *B. afzelii* strains Fin-Jyv-A3 and NE4049 are compared to those of *B. afzelii* strains PKo and CB43 (Table S4). Of the 16 tested plasmids, strains Fin-Jyv-A3 and NE4049 contained 15 and 14 plasmids, respectively. Both strains were missing cp 32-7, which was present in both strains PKo and CB43. Strain NE4049 was also missing cp 32-5, which was also missing in strain CB43, but this plasmid was present in strains Fin-Jyv-A3 and PKo. In summary, the two strains that we used in this study, Fin-Jyv-A3 and NE4049, contained all of the plasmids that are necessary to complete the life cycle of *B. afzelii*.

Table S6. The plasmid profile is shown for four strains of *B. afzelii*: PKo, CB43, Fin-Jyv-A3 and NE4049. The 16 plasmids tested were as follows: lp 17, lp 28-2, lp 28-3, lp 25, lp 28, lp 28-4, lp 28-7, lp 28-8, lp 30, lp 34, lp 38, cp 26, cp 32-5, cp 32-7, cp 32-9, cp 32-10. The '+' and '-' symbols indicate whether a plasmid was present or absent for a given strain. Some of the plasmids were tested for with different primer sets.

Plasmids	PKo	CB43	Fin-Jyv-A3	NE4049
lp 17	+	+	-	+
ACA-1 lp 17	+	+	+	+
lp 28-2	+	+	-	+
ACA-1 lp 28-2	+	+	+	+
lp 28-3	+	+	-	+
ACA-1 lp 28-3	+	+	+	+
lp 25	+	+	+	+
lp 28	+	+	+	+
lp 28-4	+	+	+	+
lp 28-7	+	+	+	+
lp 28-8	+	+	+	+
lp 30	+	+	+	+
lp 34	+	+	+	+
lp 38	+	+	+	+
cp26	+	+	+	+
cp 32-5	+	-	+	-
cp 32-7	+	+	-	-
cp 32-9	+	+	+	+
cp 32-10	+	+	+	+

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10.4. Supplementary material of chapter 4

Title: Mixed strain infections reduce the transmission of a tick-borne pathogen

Authors: Dolores Genné, Anouk Sarr, Olivier Rais, and Maarten J. Voordouw

Supplementary material

SECTION 1 – Creation of the challenge nymphs

Table S1. For the second part of the study, a subsample of 30 donor mice was selected because their flat nymphs had a high probability of carrying the three types of infection of interest. For the 30 donor mice, this table show their identity (Mouse ID), their focal strain (Focal strain), their infection treatment (Infection), the number of challenge nymphs that tested positive with strain Fin-Jyv-A3 alone (Fin-Jyv-A3), the number of challenge nymphs that tested positive with strain NE4049 alone (NE4090), the number of challenge nymphs that were co-infected (Co-infection), the number of challenge nymphs that were uninfected (Uninfected), and the number of challenge nymphs that were tested (Total).

Mouse ID	Focal Strain	Infection	Fin-Jyv-A3	NE4049	Co-infection	Uninfected	Total
S01	Fin-Jyv-A3	Single	8	0	0	0	8
S02	Fin-Jyv-A3	Single	9	0	0	0	9
S03	Fin-Jyv-A3	Single	22	0	0	2	24
S04	Fin-Jyv-A3	Single	20	0	0	2	22
S06	Fin-Jyv-A3	Single	14	0	0	8	22
S07	Fin-Jyv-A3	Single	22	0	0	1	23
S08	Fin-Jyv-A3	Single	19	0	0	5	24
S09	Fin-Jyv-A3	Single	6	0	0	1	7
S10	Fin-Jyv-A3	Single	7	0	0	1	8
S11	Fin-Jyv-A3	Co-infection	1	18	3	3	25
S13	Fin-Jyv-A3	Co-infection	1	1	8	0	10
S14	Fin-Jyv-A3	Co-infection	5	5	9	4	23
S15	Fin-Jyv-A3	Co-infection	0	14	5	5	24
S16	Fin-Jyv-A3	Co-infection	7	5	12	1	25
S19	Fin-Jyv-A3	Co-infection	6	2	13	4	25
S21	NE4049	Co-infection	2	11	3	6	22
S22	NE4049	Co-infection	0	6	18	1	25
S23	NE4049	Co-infection	0	7	15	2	24
S24	NE4049	Co-infection	8	4	10	2	24
S28	NE4049	Co-infection	8	2	12	2	24
S30	NE4049	Single	2	11	4	7	24
S31	NE4049	Single	0	17	0	2	19
S32	NE4049	Single	0	19	0	5	24
S33	NE4049	Single	0	26	0	0	26
S34	NE4049	Single	0	10	0	4	14
S35	NE4049	Single	0	15	0	9	24
S36	NE4049	Single	0	23	0	1	24
S38	NE4049	Single	0	8	0	7	15
S39	NE4049	Single	0	17	0	7	24
S40	NE4049	Single	0	17	0	7	24

SECTION 2 – Strain-specific infection of the engorged nymphs

Table S2. This contingency tables show the correspondence between the infection status of the engorged challenge nymphs (rows) and the test mice (columns) from group 1 (infected with strain Fin-Jyv-A3 alone). EN refers to engorged challenge nymphs and MICE refers to test mice.

EN\MICE	Fin-Jyv-A3	Uninfected	Total
Fin-Jyv-A3	6	2	8
Uninfected	0	1	1
Unrecovered	2	1	3
Total	8	4	12

Table S3. This contingency tables show the correspondence between the infection status of the engorged challenge nymphs (rows) and the test mice (columns) from group 2 (infected with strain NE4049 alone). Highlighted in yellow is a test mouse that became infected while its engorged challenge nymph tested negative. EN refers to engorged challenge nymphs and MICE refers to test mice.

EN\MICE	NE4049	Uninfected	Total
NE4049	3	5	8
Uninfected	1	0	1
Unrecovered	2	1	3
Total	6	6	12

Table S4. This contingency tables show the correspondence between the infection status of the engorged challenge nymphs (rows) and the test mice (columns) from group 3 (co-infected with strains Fin-Jyv-A3 and NE4049). Highlighted in yellow are cases for which there is a mismatch in the strain-specific infection status between the engorged challenge nymphs and the test mice. EN refers to engorged challenge nymphs and MICE refers to test mice.

EN\MICE	Fin-Jyv-A3	NE4049	Coinfected	Uninfected	Total
Fin-Jyv-A3	14	2	0	3	19
NE4049	3	3	1	7	14
Coinfected	0	0	9	0	9
Uninfected	6	1	1	13	21
Unrecovered	3	3	0	7	13
Total	26	9	11	30	76

SECTION 3 – Effects of the co-infection status of the donor mice and strain identity on the infection prevalence of the focal strain in the unfed challenge nymphs

Table S5. The parameter estimates are shown for the GLMM model from the regression analysis of the strain-specific prevalence in the challenge nymphs. The model included the main effect of strain identity (Fin-Jyv-A3, NE4049) and the main effect of donor mouse infection status (single infection, co-infection). The parameters include an intercept (defined as strain Fin-Jyv-A3 in single infection) and main effects (expressed as the difference between the levels of a factor). Shown are the parameter estimates (Estimates), standard errors (SE), z-values, and the associated p-values (p).

Factor	Estimates	SE	z-value	p
Intercept: Fin-Jyv-A3/single infection	1.818	0.390	4.659	<0.00001
Strain: NE4049	-0.029	0.442	-0.067	0.947
Infection: co-infection	-1.112	0.445	-2.498	0.013

SECTION 4 – Effects of the co-infection treatment origin of the challenge nymphs and strain identity on the infection prevalence of the focal strain in the test mice

Table S6. The parameter estimates are shown for the GLMM model from the regression analysis of the strain-specific prevalence in the test mice. The model included the main effect of strain identity (Fin-Jyv-A3, NE4049) and the main effect of donor mouse infection status (single infection, co-infection). The parameters include an intercept (defined as strain Fin-Jyv-A3 in single infection) and main effects (expressed as the difference between the levels of a factor). Shown are the parameter estimates (Estimates), standard errors (SE), z-values, and the associated p-values (p).

Factor	Estimates	SE	z-value	p
Intercept: Fin-Jyv-A3/single infection	0.814	0.486	1.674	0.094
Strain: NE4049	-0.920	0.439	-2.095	0.036
Infection: co-infection	-1.170	0.497	-2.348	0.019

SECTION 5 – Effects of the probability of infection in the challenge nymphs and strain identity on the prevalence of the focal strain in the test mice

Table S7. The parameter estimates are shown for the GLMM model from the regression analysis of the strain-specific prevalence in the test mice. The model included the main effect of strain identity (Fin-Jyv-A3, NE4049) and the co-variate probability of infection in the challenge nymphs. The parameters include an intercept (defined as strain Fin-Jyv-A3) and main effects (expressed as the difference between the levels of a factor). Shown are the parameter estimates (Estimates), standard errors (SE), z-values, and the associated p-values (p).

Factor	Estimates	SE	z-value	p
Intercept: Fin-Jyv-A3/single infection	-1.843	0.779	-2.367	0.018
Strain: NE4049	-1.057	0.444	-2.381	0.017
Probability of infection	2.596	1.028	2.525	0.012