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Functional role of keystone bacteria in host-
microbiota-pathogen interactions: implications
for the control of vector-borne diseases

*Rôle fonctionnel des bactéries clés dans les interactions hôte-microbiote-
pathogène : implications pour le contrôle des maladies à transmission
vectorielle*

Thèse de doctorat de l'université Paris-Saclay

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Title: Functional role of keystone bacteria in host-microbiota-pathogen interactions: implications for the control of vector-borne diseases

Keywords: Vector-borne diseases, vectors, microbiota, anti-microbiota vaccines, microbial networks, keystone taxa

Abstract: Animal and human pathogens, principally those transmitted by blood feeding arthropods such as tick and mosquitoes are a global public health concern. Although several strategies were developed and used to control vector-borne diseases (VBDs), half of the world's population still lives today at risk of VBDs. Therefore, the search of new strategies to block VBDs with better outcomes is needed. Several studies emphasize the pivotal role that vector microbiome has on vector fitness and vector competence. Vector microbiome perturbation has been proposed as a mean for the control of VBDs. In the present thesis, we used a network approach to study the relevance of keystone bacteria in vector microbiota and propose anti-microbiota vaccines as a tool for the precise manipulation of vector microbiome, with implications for the study of vector-microbiota interactions and the control of VBDs. The results obtained showed that keystone taxa are important and influential bacterial members of the vector microbiota. Firstly, we demonstrated that keystone taxa have an important role maintaining the functional stability of tick microbiome under heat stress. Secondly, targeting keystone taxa of vector microbiota with host antibodies we showed that anti-microbiota vaccines decrease pathogen colonization in ticks and mosquitoes.

Specifically, we showed that targeting keystone taxa using anti-microbiota vaccine decrease the connectedness of keystone taxa in the microbial network which has a cascading ecological impact resulting in an extensive modulation of the taxonomic and functional profile of tick microbiota. Notably, the perturbation of tick microbiome reduced *Borrelia afzelii*, the causative agent of Lyme borreliosis in Europe, colonization within *Ixodes ricinus* ticks. This outcome was not limited to the tick vector since we also demonstrated that perturbation of mosquitoes also decreased *Plasmodium relictum*, the causative agent of avian malaria, development in its natural vector *Culex quinquefasciatus*. Altogether, these results show (i) the important role of keystone taxa on vector bacterial community assembly (ii) the suitability of anti-microbiota vaccines as a precision microbiology tool for the study of vector-microbiota interactions and the perturbation of vector microbiome as a means to control VBDs and (iii) the major impact that vector microbiota has on pathogen colonization within the vector. The results of the thesis will inform novel approaches to develop transmission-blocking vaccines and control VBDs.

Titre : Rôle fonctionnel des bactéries clés dans les interactions hôte-microbiote-pathogène : implications pour le contrôle des maladies à transmission vectorielle

Mots clés : maladies vectorielles, vecteurs, microbiote, vaccin anti-microbiote, réseaux microbiens, taxons clés

Résumé : Les agents pathogènes animaux et humains, principalement ceux transmis par les arthropodes qui se nourrissent de sang tels que les tiques et les moustiques, constituent un problème de santé publique mondial. Bien que plusieurs stratégies aient été développées et utilisées pour lutter contre les maladies à transmission vectorielle (MTV), la moitié de la population mondiale vit encore aujourd'hui à risque de MTV. Par conséquent, la recherche de nouvelles stratégies pour bloquer les MTV avec de meilleurs résultats est nécessaire. Plusieurs études soulignent le rôle central que joue le microbiome vectoriel sur la physiologie des vecteurs et la compétence vectorielle. La perturbation du microbiome du vecteur a été proposée comme moyen de contrôle des MTV. Dans la présente thèse, nous avons utilisé des réseaux microbiens pour étudier l'importance des bactéries clés dans le microbiote du vecteur et nous proposons des vaccins anti-microbiote comme outil de manipulation précise du microbiome du vecteur, avec des implications pour l'étude des interactions vecteur-microbiote et le contrôle des MTV. Les résultats obtenus ont montré que les taxons clés sont des membres bactériens importants et influents du microbiote du vecteur. Premièrement, nous avons démontré que les taxons clés ont un rôle important dans le maintien de la stabilité fonctionnelle du microbiome des tiques sous stress thermique. Deuxièmement, en ciblant les taxons clés du microbiote vecteur avec des anticorps de l'hôte, nous avons montré que les vaccins anti-microbiote diminuent la colonisation des agents pathogènes chez les tiques et les moustiques.

Plus précisément, nous avons montré que le ciblage des taxons clés à l'aide d'un vaccin anti-microbiote diminue la connectivité des taxons clés dans le réseau microbien, ce qui a un impact écologique en cascade entraînant une modulation importante du profil taxonomique et fonctionnel du microbiote des tiques. Notamment, la perturbation du microbiome des tiques a réduit la colonisation de *Borrelia afzelii*, l'agent causal de la borréliose de Lyme en Europe, chez les tiques *Ixodes ricinus*. Ce résultat ne se limitait pas au vecteur tique puisque nous avons également démontré que la perturbation de microbiote des moustiques diminuait également le développement de *Plasmodium relictum*, l'agent causal du paludisme aviaire, dans son vecteur naturel *Culex quinquefasciatus*. Dans l'ensemble, ces résultats montrent (i) le rôle important des taxons clés sur l'assemblage de la communauté bactérienne vectorielle (ii) la pertinence des vaccins anti-microbiote comme outil de microbiologie de précision pour l'étude des interactions vecteur-microbiote et la perturbation du microbiome du vecteur en tant que moyens de contrôler les MTV et (iii) l'impact majeur que le microbiote vecteur a sur la colonisation des agents pathogènes au sein du vecteur. Les résultats de la thèse éclaireront de nouvelles approches pour développer des vaccins bloquant la transmission et ainsi, contrôler les MTV.

Résumé de thèse

Introduction

Vecteurs et maladies à transmission vectorielle

Un vecteur est un organisme vivant qui sert d'hôte intermédiaire pour la transmission d'agents pathogènes d'un hôte infecté à un autre (Hill et *al.*, 2005 ; Vector-Borne Diseases | EFSA). Les vecteurs sont souvent des arthropodes et comprennent les tiques, les moustiques, les poux, les puces, les moucheron, les acariens et les mouches. Les arthropodes vecteurs peuvent transmettre des virus, des bactéries ou des parasites qui sont responsables de diverses maladies humaines et animales, notamment le paludisme, la borréliose de Lyme, la trypanosomiase, l'encéphalite et la dengue, entre autres (Baxter et *al.*, 2017 ; Hill et *al.*, 2005). Ces maladies transmises par un arthropode à l'homme ou à l'animal sont appelées maladies à transmission vectorielle (MTV).

Charge mondiale des maladies à transmission vectorielle : Impact sur la santé

Les maladies infectieuses et parasitaires transmises par des vecteurs peuvent provoquer une morbidité et une mortalité graves dans le monde. Selon les estimations de l'Organisation mondiale de la santé (OMS), en 2020, 17 % de la charge mondiale de l'ensemble des maladies infectieuses sera due aux maladies à transmission vectorielle, causant plus de 700 000 décès par an (Vector-Borne Diseases). Cependant, le véritable impact des MTV est leur indice de morbidité élevé. Par exemple, le paludisme, une infection parasitaire transmise par les moustiques Anopheline, a touché environ 219 millions de personnes dans le monde ; la dengue, une infection virale transmise par les moustiques *Aedes*, a été estimée à 96 millions de cas dans le monde (Vector-Borne Diseases). Cette morbidité élevée est due au nombre important de personnes susceptibles de contracter des MTV. En effet, plus de 80 % de la population mondiale vit dans des zones à risque pour au moins une MTV. De plus, de nombreuses MTV sont co-endémiques et on estime que plus de la moitié de la population mondiale vit dans des zones à risque pour deux MTV ou plus (Vector-Borne Diseases). Ainsi, les MTV constituent une menace sérieuse pour la santé publique dans plusieurs pays (Hill et *al.*, 2005 ; Vector-Borne Diseases; Wilson et *al.*, 2020).

Principaux arthropodes vecteurs

Près de 90% des vecteurs impliqués dans les MTV sont constitués d'Acari (acariens et tiques) et de Diptères (moustiques). Au sein de ces groupes, la grande majorité des zoonoses vectorielles émergentes sont transmises par des tiques et des moustiques, notamment de la famille des Ixodidae et Culicidae, respectivement (Baxter et *al.*, 2017 ; Swei et *al.*, 2020). Ainsi, dans la présente thèse, nous nous intéresserons à ces deux familles de vecteurs.

Les tiques Ixodidae

Les tiques de la famille des Ixodidae sont connues sous le nom de "tiques dures". Elles sont des parasites hématophages obligatoires et la période d'alimentation peut varier de quelques jours à plusieurs semaines selon le stade de vie et l'espèce. Le volume de sang ingéré peut être supérieur à 100-200 fois leur masse corporelle pour les femelles adultes (Estrada-Peña, 2015 ; Kahl, 2018). Les tiques Ixodidés sont également caractérisées par un cycle de développement complexe qui comprend quatre stades de développement, à savoir l'œuf, la larve, la nymphe et l'adulte (mâle et femelle). Chaque stade de vie postembryonnaire doit prendre un repas de sang (Kahl, 2018) provenant du même hôte ou de différents hôtes pour assurer la suite du développement. En effet, les membres de la famille des Ixodidae subissent un cycle de vie à un hôte, deux hôtes ou trois hôtes (CDC - DPDx - Ticks, n.d.). Le cycle de vie à trois hôtes est le style de cycle de vie de la plupart des tiques d'importance pour la santé publique en Europe, notamment les tiques du genre *Ixodes*. La principale maladie transmise par les tiques *Ixodes* est la maladie de Lyme.

La maladie de Lyme : principale maladie transmise par les tiques

La maladie de Lyme, également appelée "borréliose de Lyme", est la maladie vectorielle la plus répandue en Europe et en Amérique du Nord (Bush & Vazquez-Pertejo, 2018). Les mois les plus propices à l'apparition de la maladie de Lyme, dans le nord-est des États-Unis et dans la majeure partie de l'Europe, sont les mois de juin et juillet, qui correspondent à la période active d'alimentation des tiques nymphales (Lyme Disease Charts and Figures : Historical Data | Lyme Disease | CDC). Les agents responsables de la maladie de Lyme sont des membres bactériens du complexe *Borrelia burgdorferi* sensu lato, qui comprend plus de 20 espèces de bactéries différentes. Ce sont des parasites obligatoires de forme spiralée qui dépendent fortement de leur hôte pour leur soutien nutritionnel (Bush & Vazquez-Pertejo, 2018). Les trois principaux responsables de la borréliose de Lyme humaine sont *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), *Borrelia afzelii* et *Borrelia garinii* (Baranton et al., 1992). Quatre espèces de tiques, à savoir *Ixodes ricinus*, *Ixodes persulcatus*, *Ixodes scapularis* et *Ixodes pacificus* sont les principaux vecteurs de la transmission de *B. burgdorferi* s.l. à l'homme et chacune d'entre elles a une localisation géographique naturelle différente. *I. ricinus* et *I. persulcatus* sont les principaux vecteurs en Europe et en Asie, tandis que *I. scapularis* et *I. pacificus* sont les principaux vecteurs dans l'est et l'ouest des États-Unis, respectivement (Bush & Vazquez-Pertejo, 2018 ; Ogden et al., 2009 ; Steere et al., 2016).

Cycle d'infection de *Borrelia burgdorferi* s.l.

Borrelia doivent être acquises à chaque génération puisqu'il n'existe pas de transmission transovarienne de cette bactérie (Radolf et *al.*, 2012). Le cycle d'infection de *Borrelia* consiste en une phase tique et une phase mammifère (Steere et *al.*, 2016). Les espèces *Borrelia* sont acquises par les larves d'*Ixodes* à partir d'un hôte animal infecté (également appelé hôte réservoir) et pénètrent dans l'intestin moyen des tiques (Bush & Vazquez-Pertejo, 2018 ; Steere et *al.*, 2016). Tout au long du cycle de vie annuel, *Borrelia* reste dans un état dormant dans l'intestin de la tique nymphale et se caractérise par l'expression de la protéine de surface externe (Osp) A, qui est impliquée dans la survie et la colonisation de la bactérie dans l'intestin moyen de la tique (Bush & Vazquez-Pertejo, 2018). Néanmoins, lorsque les nymphes d'*Ixodes* commencent à se nourrir de sang, les signaux associés à l'engorgement des tiques induisent la transformation des espèces de *Borrelia* d'un état adapté à la colonisation des tiques à un état prêt à infecter le mammifère (Steere et *al.*, 2016). Par la suite, grâce à la morsure de la tique et aux protéines salivaires de la tique qui modulent la réponse immunitaire de l'hôte, les spirochètes de *Borrelia* sont introduits profondément dans le derme de l'hôte et établissent une infection initiale chez l'hôte (Steere et *al.*, 2016). Enfin, les spirochètes se propagent dans les tissus de l'hôte en pénétrant dans le sang ou le système lymphatique. Le mammifère infecté devient alors un nouvel hôte réservoir qui peut transmettre les spirochètes à une autre génération de tiques. Les humains ou les chiens sont considérés comme des hôtes accidentels car la transmission de *Borrelia* de l'homme à la tique n'a pas été observée (Radolf et *al.*, 2012).

Moustiques Culicidae

Les moustiques sont des insectes volants, suceurs de sang, présents dans le monde entier et adaptés à un large éventail de conditions environnementales (Chandrasegaran et *al.*, 2020 ; Lee et *al.*, 2018 ; What Is a Mosquito ? | Mosquitoes | CDC). Ils ont un cycle de vie complexe qui comprend les stades d'œuf, de larve, de nymphe et d'adulte. La durée de vie des moustiques adultes est d'environ 2 à 4 semaines en fonction de certains facteurs tels que l'espèce, l'humidité, la température, entre autres. Seules les femelles prennent un repas de sang d'un hôte vertébré afin d'obtenir des ressources métaboliques et des protéines pour la production d'œufs et, par conséquent, pour augmenter leur forme physique (Takken et *al.*, 1998 ; Yan et *al.*, 2021). L'une des maladies infectieuses les plus graves transmises par les moustiques est le paludisme et le système expérimental le plus ancien pour l'étude de la biologie et de la transmission des parasites *Plasmodium* est le paludisme aviaire.

Le paludisme aviaire

Le paludisme aviaire a joué un rôle clé dans la compréhension des parasites du paludisme humain et dans le développement des premiers vaccins et médicaments antipaludiques (Rivero & Gandon, 2018). Les parasites responsables du paludisme aviaire appartiennent aux genres *Plasmodium* et à l'ordre des hémospodidés (phylum Apicomplexa). Plus de 50 espèces de paludisme aviaire ont été identifiées sur la base des caractéristiques morphologiques de leurs stades sanguins et de nouvelles espèces sont découvertes chaque année (Valkiunas & lezhova, 2018). En outre, sur la base de l'analyse du génome mitochondrial des *Plasmodium* spp., il pourrait y avoir beaucoup plus d'espèces que ce que l'on pensait auparavant (Bensch et al., 2009). Parmi le grand nombre d'espèces de *Plasmodium* aviaires, l'espèce la plus répandue est *Plasmodium relictum* (Hellgren et al., 2015).

P. relictum est classé comme l'une des espèces les plus invasives par l'Union internationale pour la conservation de la nature (UICN) (Lowe et al., 2000), il a été trouvé dans toutes les grandes régions géographiques à l'exception de l'Antarctique (Rivero & Gandon, 2018) et est responsable du déclin et de l'extinction d'un grand nombre de grimpeaux endémiques d'Hawaï (Hellgren et al., 2015). *P. relictum* est considéré comme un parasite généraliste car il a été trouvé dans plus de 300 espèces d'oiseaux différentes qui appartiennent à 11 ordres différents. (Bensch et al., 2009 ; Valkiunas & lezhova, 2018). Il est également considéré comme un généraliste dans l'utilisation des vecteurs étant transmis par différents genres de moustiques. Plus précisément, 3 espèces du genre *Culex*, à savoir *Culex quinquefasciatus*, *Culex Stigmatosoma* et *Culex tarsalis* ont été répertoriées comme vecteurs naturels, tandis que 20 autres espèces différentes des genres *Culex*, *Culiseta*, *Aedes* et *Anopheles* ont été prouvées expérimentalement comme vecteurs potentiels de *P. relictum* (Santiago-Alarcon et al., 2012).

Cycle de vie du paludisme aviaire

Le cycle de vie du paludisme aviaire est similaire à celui des espèces de *Plasmodium* humaines et mammifères dans leurs caractéristiques de base (Valkiunas & lezhova, 2018). Le parasite *Plasmodium* a une phase de reproduction sexuelle chez le moustique Culicidae et une phase asexuée chez les oiseaux. Les gamétocytes mâles et femelles de *Plasmodium* sont acquis lors de la prise de sang par les moustiques femelles d'un hôte aviaire infecté. Dans l'intestin moyen du moustique, les gamétocytes se différencient en gamètes femelles et mâles (Bruce et al., 1990 ; Silvestrini et al., 2000), qui fusionnent pour former un zygote diploïde, qui à son tour, subit une méiose et se différencie en oocinète mobile (Sinden et al., 1985). Par la suite, les ookinètes migrent vers la lame basale de l'intestin moyen du moustique où ils forment les oocystes (Sinden, 2009).

Au cours de plusieurs jours, chaque oocyste subit plusieurs cycles de mitose pour produire des milliers de sporozoïtes haploïdes, qui sont libérés lorsque l'oocyste se rompt (Sinden, 2009). Les sporozoïtes migrent ensuite à travers l'hémocèle vers la glande salivaire du moustique (Gerald et al., 2011), prêts à être transmis à un nouvel hôte aviaire non infecté lors de la prochaine prise de sang du moustique. Ce processus sexuel et la sporogonie des parasites *Plasmodium* chez les moustiques sont achevés en sept jours environ (Huijben et al., 2007). Le cycle chez les oiseaux commence lorsque le moustique injecte les sporozoïtes avec sa salive qui contient plusieurs enzymes pour améliorer l'absorption du sang et empêcher la coagulation (Huijben et al., 2007). Les sporozoïtes atteignent la circulation sanguine de l'oiseau et envahissent ensuite les cellules réticulo-endothéliales de plusieurs organes, dont la rate, et entament plusieurs cycles de réplication pour former les mérozoïtes (cycle exoérythrocytaire). Les mérozoïtes sont ensuite libérés dans la circulation sanguine. Contrairement aux mérozoïtes du paludisme humain, ces mérozoïtes sont incapables d'infecter immédiatement les globules rouges ; ils subissent plutôt un deuxième cycle exoérythrocytaire au cours duquel ils envahissent les macrophages de nombreux organes (Huff & Coulston, 1946). Les mérozoïtes résultants entrent dans le cycle érythrocytaire, où ils envahissent les globules rouges et se développent en trophozoïtes. Les parasites se divisent et finissent par libérer d'autres mérozoïtes dans la circulation sanguine. Une partie de ces mérozoïtes va envahir les globules rouges et réinitialiser le cycle de réplication asexuée. Les mérozoïtes restants entrent dans le cycle exoérythrocytaire secondaire ou envahissent de nouveaux globules rouges et se transforment en gamétocytes sexuels (mâles et femelles), qui est le stade de *Plasmodium* qui est infectieux pour les moustiques (Huijben et al., 2007 ; Rivero & Gandon, 2018).

Microbiote du vecteur : Pourquoi est-il important ?

L'intestin moyen est le premier organe dans lequel les microbes pathogènes ingérés avec le sang de l'hôte peuvent survivre et, dans la plupart des cas, envahir d'autres tissus de tiques (Sonenshine & Simo, 2021) ou de moustiques (Gabrieli et al., 2021). L'intestin moyen est également le microenvironnement optimal pour la survie et le maintien du microbiote du vecteur (Maitre et al., 2022).

Le microbiote désigne l'ensemble des micro-organismes qui vivent dans le tissu du vecteur et se compose de bactéries, d'archées, de champignons et de virus (Narasimhan & Fikrig, 2015 ; Wu-Chuang et al., 2021). Dans le présent document, le terme "microbiome" désigne les micro-organismes et leurs gènes, tandis que le terme "microbiote" désigne uniquement les microbes eux-mêmes. L'intérêt pour le microbiome s'est accru en raison de son rôle important sur la physiologie des vecteurs et, plus intéressant encore, sur leur compétence vectorielle (Caragata &

Short, 2022 ; Narasimhan & Fikrig, 2015 ; Wu-Chuang et *al.*, 2021). En effet, la diversité des microorganismes hébergés par le vecteur se trouve souvent à proximité des pathogènes que le vecteur transmet (Cirimotich, Ramirez, et *al.*, 2011). On peut donc imaginer que les microorganismes individuels et les communautés microbiennes du microbiote, qui interagissent avec les agents pathogènes transmis par le vecteur (Hajkazemian et *al.*, 2021 ; Wu-Chuang et *al.*, 2021), peuvent faciliter (M. Wang et *al.*, 2021) ou concurrencer (Bando et *al.*, 2013) la colonisation et le développement des agents pathogènes au sein du vecteur. Ces résultats ouvrent la voie à l'élaboration de nouvelles stratégies visant à interrompre la transmission des agents pathogènes par la modulation du microbiote des vecteurs. Une compréhension approfondie du microbiote est donc nécessaire. L'écologie microbienne a été appliquée à l'étude du microbiote et sera commentée dans la section suivante.

Écologie microbienne

L'écologie microbienne est apparue comme un domaine d'étude des interactions des microorganismes entre eux et avec leur environnement. Le microbiote des vecteurs est formé d'un vaste groupe de bactéries et comme ces bactéries ne sont pas isolées, elles peuvent interagir ou s'associer entre elles, directement ou indirectement. L'analyse standard du microbiote implique la recherche de bactéries qui diffèrent en abondance entre différentes conditions. Cependant, cette approche ne permet pas d'étudier les associations existantes entre les membres bactériens du microbiote (Röttgers & Faust, 2018). Cet inconvénient a incité la recherche de nouveaux outils pour étudier les communautés microbiennes tels que les réseaux de cooccurrence microbienne.

Un réseau est défini comme des instantanés temporaires ou spatiaux de toute collection d'unités interagissant potentiellement comme un système et est représenté par un ensemble de nœuds connectés par des arêtes. (Proulx et *al.*, 2005). Dans les réseaux microbiens, les nœuds représentent les taxons bactériens tandis que les arêtes indiquent les corrélations positives ou négatives entre deux taxons (Berry & Widder, 2014 ; Wu-Chuang, Obregon, et *al.*, 2022). Les interactions microbe-microbe peuvent être analysées à l'aide de l'analyse des réseaux microbiens, qui permet d'explorer les modèles de cooccurrence entre les membres d'une communauté microbienne très complexe (Faust & Raes, 2012 ; Fuhrman, 2009). Ces modèles de cooccurrence montrent comment des organismes particuliers dans une communauté peuvent apparaître parfois ou jamais ensemble dans une certaine condition et peuvent révéler des règles intéressantes d'assemblage de la communauté (Fuhrman, 2009). Les réseaux de cooccurrence sont déduits statistiquement et un large éventail de méthodes (par exemple, des techniques basées sur la distance et la corrélation) sont disponibles pour la construction de réseaux microbiens, avec des niveaux variables d'efficacité et de précision (Beiko et *al.*, 2018 ; Wu-Chuang, Obregon, et *al.*,

2022). Outre les associations, l'une des forces des réseaux biologiques est leur capacité à représenter les propriétés émergentes (Röttjers & Faust, 2018). Les propriétés émergentes sont celles qui sont exposées par le système dans son ensemble et ne peuvent pas être prédites si seulement une partie du réseau est analysée seule (Aderem, 2005). Ces propriétés émergentes peuvent expliquer des comportements d'un système complexe, comme leur robustesse ou leur modularité. La robustesse est une propriété *in silico* qui fait référence à la vulnérabilité d'un réseau à la suppression aléatoire ou ciblée de nœuds ; les réseaux vulnérables se décomposent en parties plus petites suite à la suppression de nœuds (Röttjers & Faust, 2018). La robustesse des réseaux peut être un outil de diagnostic pour la prédiction de la robustesse écologique, c'est-à-dire la résilience d'un écosystème face aux fluctuations du comportement de ses espèces membres ou de son environnement (Mumby et al., 2014 ; Röttjers & Faust, 2018). La modularité quantifie dans quelle mesure les réseaux peuvent être décomposés en éléments plus petits appelés modules (Röttjers & Faust, 2018). Ainsi, les modules d'un réseau microbien représentent un ensemble de nœuds qui ont de fortes interactions entre eux mais des connexions éparses avec les nœuds d'autres modules (Aderem, 2005).

En plus des associations bactériennes, l'analyse des réseaux de cooccurrence microbienne offre la possibilité d'identifier statistiquement des taxons clés (Berry & Widder, 2014). Bien qu'il n'y ait pas de consensus sur les meilleures métriques pour leur identification, il est clair que les taxons clés sont des bactéries très influentes sur la structure et le fonctionnement de la communauté microbienne (Agler et al., 2016 ; Banerjee et al., 2018 ; Layeghifard et al., 2017) et que leur suppression peut provoquer un changement drastique dans la composition ou le fonctionnement d'un microbiome (Banerjee et al., 2016). Chez les tiques, par exemple, l'appauvrissement d'un taxon clé identifié a eu un impact sur la performance des tiques pendant l'alimentation (Mateos-Hernández et al., 2020). L'identification de ces taxons peut aider à mieux comprendre le fonctionnement d'un système complexe et peut être une cible appropriée pour la modulation du microbiome vers un état souhaité.

Méthodes de lutte contre les vecteurs et les maladies à transmission vectorielle

Plusieurs méthodes, à l'efficacité variable, ont été développées et utilisées pour réduire les populations de vecteurs et contrôler les maladies vectorielles. La majorité des tactiques actuelles de lutte contre les vecteurs reposent en grande partie sur l'utilisation d'acaricides chimiques synthétiques (Benelli, 2022 ; Richardson et al., 2022). L'utilisation populaire des acaricides repose sur leur grande efficacité générale dans la lutte contre les arthropodes, mais divers effets néfastes leur ont été associés, notamment le développement d'une résistance aux acaricides chez de nombreux parasites médicaux et vétérinaires importants (Benelli et al., 2021 ; Hemingway et al.,

2016 ; McNair & Carol McNair, 2015), leur forte persistance dans les environnements et la présence de traces restantes de ces composés dans la chaîne alimentaire (Beugnet & Franc, 2012). En outre, ils présentent des effets létaux et potentiellement sublétaux sur les espèces non ciblées (par exemple, les abeilles domestiques) (Desneux et *al.*, 2006). Tous ces effets négatifs préoccupants ont incité à rechercher de nouvelles méthodes de contrôle plus sûres et respectueuses de l'environnement.

Les vaccins sont une méthode alternative utilisée pour le contrôle des vecteurs et des MTV. Les vaccins pour le contrôle des arthropodes vecteurs, tels que les tiques ou les moustiques, sont basés sur l'utilisation d'anticorps de l'hôte pour cibler les protéines des vecteurs essentielles à leur développement ou à leur reproduction. D'autre part, les vaccins bloquant la transmission sont conçus pour bloquer la transmission d'agents pathogènes entre le vecteur et l'hôte en ciblant les protéines du vecteur qui sont essentielles à la transmission de l'agent pathogène ou les protéines de l'agent pathogène exprimées dans les vecteurs (Maitre et *al.*, 2022 ; Neelakanta & Sultana, 2015). En outre, la modulation du microbiote a été proposée comme une stratégie de contrôle des MTV. Dans ce contexte, le vaccin anti-microbiote est apparu comme un nouvel outil pour la manipulation précise du microbiote des vecteurs.

Le vaccin anti-microbiote est basé sur la vaccination de l'hôte pour induire des anticorps spécifiques contre une bactérie. Plus tard, au cours du repas sanguin, ces anticorps seront absorbés par les vecteurs arthropodes hématophages et cibleront une bactérie spécifique du microbiote du vecteur (Mateos-Hernández et *al.*, 2020). Les résultats obtenus en ciblant le taxon clé à l'aide d'un vaccin anti-microbiote dans une condition expérimentale ont montré que cette technique peut avoir un impact sur la performance d'*I. ricinus* pendant l'alimentation (Mateos-Hernández et *al.*, 2020). Comme le vaccin anti-microbiote peut cibler des taxons bactériens spécifiques dans le microbiote des tiques (Mateos-Hernández et *al.*, 2020), il pourrait être utilisé pour épuiser les bactéries sélectives du microbiote des vecteurs qui facilitent le développement des agents pathogènes et/ou augmentent la compétence des vecteurs et être utilisé comme un moyen de bloquer la transmission des agents pathogènes.

Hypothèses et objectifs

Nous émettons l'hypothèse suivante :

Le ciblage de taxons clés du microbiote des vecteurs avec des anticorps de l'hôte aura un impact écologique en cascade réduisant le fitness des vecteurs et/ou leur compétence.

Pour tester cette hypothèse, la présente thèse a quatre objectifs :

1. Identifier les taxons clés du microbiote des tiques et évaluer leur rôle fonctionnel dans les communautés bactériennes en cas de stress thermique.
2. Caractériser les effets des vaccins anti-microbiote sur l'assemblage du microbiote des tiques.
3. Évaluer l'impact du vaccin anti-microbiote sur la colonisation de *B. afzelii* chez les tiques *I. ricinus*.
4. Évaluer l'impact du vaccin anti-microbiote sur la colonisation de *P. relictum* chez les moustiques *C. quinquefasciatus*.

Résultats

1. Rôle fonctionnel des taxons clés chez les tiques *Ixodes scapularis* sous stress environnemental

Dans la première partie du résultat de la thèse, nous avons voulu étudier le rôle fonctionnel des taxons dans le microbiote des tiques sous stress thermique. Les températures élevées peuvent induire des variations dans la composition et la diversité du microbiome des tiques et ainsi, influencer la hiérarchie des membres de la communauté en réponse à un changement environnemental. On suppose que ces modifications de la structure de la communauté entraînent des altérations de la présence et/ou de l'abondance des voies fonctionnelles dans le métagénome bactérien. Cette hypothèse a été testée en utilisant des ensembles de données publiées sur l'ARNr 16S de mâles *Ixodes scapularis* incubés à différentes températures (4, 20, 30 et 37 °C) en laboratoire. Les changements dans la structure de la communauté et les profils fonctionnels en réponse aux changements de température ont été mesurés en utilisant des réseaux de cooccurrence et l'inférence du métagénome. Les résultats des tiques élevées en laboratoire ont ensuite été comparés à ceux des tiques collectées sur le terrain. Les résultats obtenus avec des tiques élevées en laboratoire ont montré qu'une température élevée réduisait la diversité bactérienne, modifiait la structure de la communauté microbienne et diminuait le nombre de taxons clés. Notamment, quatre taxons (*Pseudomonas*, *Acinetobacter*, *Bradyrhizobium* et *Ralstonia*) ont été identifiés comme des bactéries clés à toutes les températures. En plus, nous avons trouvé que la diversité fonctionnelle du microbiome des tiques était contenue dans les quatre bactéries clés thermostables et les taxons bactériens auxquels ils sont reliés dans les réseaux de cooccurrence microbienne. Trois des taxons clés thermostables (*Acinetobacter*, *Bradyrhizobium* et *Ralstonia*) ont également été trouvés dans les tiques vivantes collectées à Massachusetts. En outre, la comparaison des profils fonctionnels des tiques élevées en laboratoire

et collectées sur le terrain a révélé l'existence d'un ensemble important de voies métaboliques qui sont présents dans les microbiotes des tiques de différente origine. Comme pour les tiques élevées en laboratoire, les taxons clés identifiés dans les tiques collectées sur le terrain et leurs consortiums (taxons co-occurents) étaient suffisants pour conserver la majorité des voies métaboliques dans le profil fonctionnel. Ces résultats suggèrent que les taxons clés sont essentiels à la stabilité et à la résilience fonctionnelle du microbiome des tiques en cas de stress thermique.

2. Modulation du microbiome des tiques par un vaccin anti-microbiote

Dans la deuxième partie du résultat de la thèse, nous avons voulu étudier l'impact du ciblage des bactéries clés par un vaccin anti-microbiote sur les profils taxonomiques et fonctionnels du microbiome des tiques. Pour cela, nous avons immunisé un modèle d'hôte vertébré (*Mus musculus*) avec des vaccins bactériens vivants ciblant des taxons clés (i.e., *Escherichia-Shigella*) ou non clés (i.e., *Leuconostoc*) du microbiote des tiques et nous avons testé l'impact des anticorps spécifiques des bactéries (Abs) sur la structure et la fonction du microbiote des tiques. Nous avons également étudié l'effet de ces vaccins anti-microbiote sur la composition du microbiote intestinal des souris. Nos résultats ont montré que le microbiote des tiques nourries sur des souris immunisées contre *Escherichia coli* présentait une abondance réduite d'*Escherichia-Shigella* et une plus faible diversité d'espèces par rapport aux tiques nourries sur des souris témoins immunisées avec un vaccin factice. L'immunisation contre les bactéries clés a restructuré la hiérarchie des nœuds dans les réseaux de cooccurrence et réduit la résistance du réseau bactérien à l'élimination des taxons. Les niveaux élevés d'IgM et d'IgG spécifiques d'*E. coli* étaient négativement corrélés avec l'abondance d'*Escherichia-Shigella* dans le microbiote des tiques. Ces effets n'ont pas été observés lorsque *Leuconostoc* était ciblé par la vaccination contre *Leuconostoc mesenteroides*. La prédiction des voies fonctionnelles dans le microbiome des tiques à l'aide de PICRUSt2 a révélé que la vaccination contre *E. coli* réduisait l'abondance de la voie de dégradation de la lysine dans le microbiome des tiques, un résultat validé par qPCR. En revanche, le microbiome intestinal des souris vaccinées n'a montré aucune altération significative de la diversité, de la composition et de l'abondance des taxons bactériens. Nos résultats ont démontré que les vaccins contre le microbiote des vecteurs sont un outil sûr, spécifique et facile à utiliser pour la manipulation du microbiome des vecteurs. Ces résultats guident les interventions pour le contrôle des infestations de tiques et de l'infection/transmission de pathogènes.

3. Effet de la modulation du microbiote des tiques par un vaccin anti-microbiote sur la colonisation de *Borrelia afzelii* chez la tique *Ixodes ricinus*

Comme a été démontré, les vaccins anti-microbiote ciblant les taxons clés du microbiote des tiques peuvent modifier l'alimentation des tiques et moduler les profils taxonomiques et fonctionnels des communautés bactériennes dans le vecteur. Cependant, l'impact du vaccin anti-microbiote sur le développement des pathogènes transmis par les tiques au sein du vecteur n'a pas été testé. Dans la troisième partie du résultat de la thèse, nous avons caractérisé la modulation du microbiote d'*Ixodes ricinus* en réponse à l'infection par *B. afzelii*, et nous avons constaté que le pathogène induit des changements dans la composition du microbiote, sa diversité bêta et la structure de l'assemblage des communautés bactériennes. La perturbation du microbiote des tiques par des anticorps anti-microbiote ou l'ajout d'une nouvelle bactérie commensale dans l'intestin des tiques entraîne encore des modifications différentes (sur le microbiote) par rapport à la modulation du microbiote induite par *B. afzelii*, ce qui se traduit par une charge plus faible de l'agent pathogène chez *I. ricinus*. Les réseaux de cooccurrence ont permis d'identifier les propriétés émergentes des communautés bactériennes qui définissent mieux les états réfractaires à l'infection par *Borrelia* du microbiote des tiques. Plus précisément, il a été constaté que des changements significatifs de taxons bactériens uniques, des interactions microbiennes-microbiennes élevées avec un ensemble unique de nœuds ainsi qu'une plus grande robustesse des réseaux microbiens peuvent définir les états réfractaires à l'infection par *Borrelia* dans le microbiote d'*I. ricinus*. Ces résultats suggèrent que *Borrelia* est très sensible aux perturbations du microbiote des tiques et que les écarts par rapport à la modulation induite par l'agent pathogène dans le microbiote du vecteur représentent un coût élevé pour le spirochète. L'analyse des réseaux apparaît comme un outil approprié pour identifier les propriétés émergentes du microbiote du vecteur associées aux états réfractaires à l'infection.

4. Impact du vaccin anti-microbiote sur la colonisation de *Plasmodium relictum* chez les moustiques *Culex quinquefasciatus*

Dans la quatrième partie des résultats de la thèse, nous avons testé si la vaccination anti-microbiote des oiseaux ciblant les Enterobacteriaceae dans les intestins des moustiques module le microbiote des moustiques et perturbe le développement de *P. relictum* chez son vecteur naturel *C. quinquefasciatus*. Des canaris domestiques (*Serinus canaria domestica*) ont été expérimentalement infectés par *P. relictum* et/ou immunisés avec des vaccins vivants contenant différentes souches d'*Escherichia coli*. L'immunisation des oiseaux a induit des anticorps spécifiques d'*E. coli*. Les communautés microbiennes de l'intestin moyen des moustiques nourris par des oiseaux infectés par *Plasmodium* et/ou immunisés par *E. coli* étaient différentes de celles

des moustiques nourris par des oiseaux témoins. Le vaccin anti-microbiote et/ou l'infection par *Plasmodium* ont également induit des changements dans l'abondance de plusieurs taxons. Les taxons bactériens affectés par l'infection ou la vaccination seule étaient différents de ceux affectés simultanément par la vaccination et l'infection, ce qui suggère que les vaccins anti-microbiote interfèrent avec la modulation du microbiote des moustiques induite par *Plasmodium*. En outre, la vaccination anti-microbiote restructure les communautés microbiennes dans l'intestin moyen des moustiques infectés par le *Plasmodium*. Notamment, la modulation du microbiote de l'intestin moyen des moustiques a été associée à une diminution significative de l'occurrence des oocystes et des sporozoïtes de *P. relictum* dans l'intestin moyen et les glandes salivaires de *C. quinquefasciatus*, respectivement. Une réduction significative du nombre d'oocystes a également été observée. Ces résultats suggèrent que les vaccins anti-microbiote peuvent être utilisés comme un nouvel outil pour contrôler la transmission du paludisme et potentiellement d'autres pathogènes vectoriels.

Conclusions

Les principales conclusions de la présente thèse sont les suivantes :

1. Les taxons clés jouent un rôle important dans le maintien de la stabilité fonctionnelle du microbiome des tiques en cas de stress thermique.
2. Le ciblage des taxons clés dans le vecteur tique à l'aide d'un vaccin anti-microbiote peut moduler le microbiome des tiques en termes de diversité, de taxonomie, de structure microbienne et d'interactions.
3. D'après les résultats présentés dans cette thèse, les vaccins anti-microbiote n'augmentent pas la mortalité des vecteurs.
4. La modulation du microbiote à l'aide d'un vaccin anti-microbiote a réduit la colonisation de *B. afzelii* chez les tiques *I. ricinus*.
5. La modulation du microbiote à l'aide d'un vaccin anti-microbiote réduit le développement de *P. relictum* chez les moustiques *C. quinquefasciatus*.
6. Le vaccin anti-microbiote est un outil approprié pour la manipulation précise du microbiote des vecteurs et pourrait être utilisé comme un vaccin bloquant la transmission pour contrôler les pathogènes transmis par les vecteurs.

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List of abbreviations

AgTRIO	<i>Anopheles gambiae</i> TRIO
AMPs	Antimicrobial peptides
BosR	<i>Borrelia</i> oxidative stress regulator
CI	Cytoplasmic incompatibility
<i>cyt-b</i>	Cytochrome-b
DENV-2	Dengue virus 2
EIP	Extrinsic incubation period
IAFGP	<i>Ixodes scapularis</i> antifreeze glycoprotein
ICS	Integrated control strategy
IgG	Immunoglobulin G
IMD	Immune deficiency
IRS	Indoor residual spraying
IUCN	International Union for Conservation of Nature
JAK	Janus kinase
JEV	Japanese encephalitis virus
LLINs	Long-lasting insecticidal nets
MPM	Meconial peritrophic matrix
NGS	Next-generation sequencing
Osp	Outer-surface proteins
PGRP	Peptidoglycan recognition proteins
PIXR	Protein of <i>Ixodes scapularis</i> with a Reeler domain
PM	Peritrophic matrix
ROS	Reactive oxygen species
RpoS	RNA polymerase alternative σ -factor
STAT	Signal transducer and activator of transcription
TBE	Tick-borne encephalitis
TBVs	Transmission-blocking vaccines
VBDs	Vector-borne diseases
WHO	World Health Organization
YFV	Yellow fever virus

CHAPTER I

Introduction

Vector and vector-borne diseases: an overview

Vectors and vector-borne diseases

A vector is a living organism that act as an intermediate host for the transmission of disease-causing pathogens from one infected host to another (Hill et al., 2005; *Vector-Borne Diseases* | EFSA). Vectors are frequently arthropods and include ticks, mosquitoes, lice, fleas, biting midges, mites and flies. Arthropod vectors can transmit viruses, bacteria, fungi or parasites that are responsible of a variety of human and animal diseases including malaria, Lyme borreliosis, trypanosomiasis, encephalitis and dengue, among others (Baxter et al., 2017; Hill et al., 2005). These diseases that are transmitted by an arthropod to humans or animals are called vector-borne diseases (VBDs). A list of vector-borne diseases and their associated vectors are available in Table 1.

Table 1. Vector-borne diseases, associated aetiological agents and arthropod vectors (Table adapted from Baxter et al., 2017; Hill et al., 2005; Schorderet-Weber et al., 2017; Shaw & Catteruccia, 2019; *Vector-Borne Diseases*)

Disease	Pathogen/parasite	Arthropod disease vector
Ticks		
Lyme disease	<i>Borrelia</i>	<i>Ixodes</i> sp.
Rocky Mountain spotted fever	<i>Rickettsia</i>	<i>Dermacentor</i> and <i>Rhipicephalus</i>
Tick-borne encephalitis (TBE)	Tick-borne encephalitis virus	<i>Ixodes</i> sp.
Human granulocytic anaplasmosis	<i>Anaplasma phagocytophylum</i>	<i>Ixodes</i> sp.
Human granulocytic ehrlichiosis	<i>Ehrlichia ewingii</i>	<i>Amblyomma americanum</i>
Human monocytic ehrlichiosis	<i>Ehrlichia chaffeensis</i>	<i>Amblyomma americanum</i>
Human babesiosis	<i>Babesia divergens</i>	<i>Ixodes ricinus</i>
	<i>Babesia microti</i>	<i>Ixodes</i> sp.
Relapsing fever (borreliosis)	<i>Borrelia hermsii</i>	<i>Ornithodoros hermsi</i>
	<i>Borrelia parkeri</i>	<i>Ornithodoros parkeri</i>
	<i>Borrelia turicatae</i>	<i>Ornithodoros turicata</i>
	<i>Borrelia miyamotoi</i>	<i>Ixodes scapularis</i>
Tularemia	<i>Francisella tularensis</i>	<i>Dermacentor reticulatus</i> , <i>Haemaphysalis concinna</i> and <i>Ixodes ricinus</i>

Q fever	<i>Coxiella burnetti</i>	Many tick genera
Crimean-Congo haemorrhagic fever	Crimean-Congo haemorrhagic fever virus	<i>Hyalomma</i> ticks
Mosquitoes		
Malaria	<i>Plasmodium falciparum</i> , <i>Plasmodium vivax</i> , <i>Plasmodium ovale</i> , <i>Plasmodium malariae</i>	<i>Anopheles</i> spp.
Avian malaria	<i>Plasmodium relictum</i>	<i>Culex</i> mosquitoes
Dengue haemorrhagic fever	DEN-1, DEN-2, DEN-3, DEN-4 flaviviruses	<i>Aedes aegypti</i>
Zika virus disease	Zika virus	<i>Aedes aegypti</i> and <i>Aedes albopictus</i>
O'nyong'nyong fever	O'nyong'nyong virus	<i>Anopheles funestus</i> and <i>Anopheles gambiae</i>
Chikungunya	Chikungunya virus	<i>Aedes albopictus</i> and <i>Aedes aegypti</i>
Yellow fever	Yellow fever flavivirus	<i>Aedes aegypti</i>
Encephalitis	Flavi-, alpha- and bunyaviruses	Various mosquito species
Rift Valley fever	Rift Valley fever virus	<i>Aedes</i> and <i>Culex</i> mosquitoes
Lymphatic filariasis	<i>Brugia malayi</i> , <i>Brugia timori</i> , <i>Wuchereria bancrofti</i>	<i>Anopheles</i> , <i>Culex</i> , <i>Aedes</i> and <i>Ochlerotatus</i> mosquitoes
Tularemia	<i>Francisella tularensis</i>	<i>Aedes cinereus</i> species
Flies		
Trypanosomiasis	<i>Trypanosoma brucei gambiense</i> , <i>Trypanosoma brucei rhodesiense</i>	<i>Glossina</i> spp. (tsetse fly)
Leishmaniasis	<i>Leishmania</i> spp.	<i>Lutzomyia</i> and <i>Phlebotomus</i> spp. sandflies
Sandfly fever (phlebotomus fever)	Naples virus, Sicilian virus and Toscana virus	Phlebotomine sandflies
Onchocerciasis	<i>Onchocerca volvulus</i>	<i>Simulium</i> spp. blackflies
Loiasis	<i>Loa loa</i>	<i>Chrysops</i> deer flies
Others		
Chagas disease	<i>Trypanosoma cruzi</i>	<i>Rhodnius</i> , <i>Triatomine</i> spp.
Plague	<i>Yersinia pestis</i>	<i>Xenopsylla</i> fleas
Typhus	<i>Rickettsia prowazekii</i>	<i>Pediculus humanus</i> lice
Louse-borne relapsing fever	<i>Borrelia recurrentis</i>	<i>Pediculus humanus humanus</i> body louse
Mansonellosis	<i>Mansonella perstans</i>	Biting midges (<i>Ceratopogonidae</i>) <i>Culicoides</i> sp.

Global burden of vector-borne diseases

Health impact

Infectious and parasitic diseases that are transmitted by vectors can cause severe morbidity and mortality in the world. According to estimations of the World Health Organization (WHO) in 2020, 17% of the global burden of all infectious diseases is due to vector-borne diseases causing more than 700.000 deaths annually (*Vector-Borne Diseases*). However, the true impact of VBDs is their high morbidity index. For example, malaria, a parasitic infection transmitted by Anopheline mosquitoes, affected approximately 219 million people globally; dengue, a viral infection transmitted by *Aedes* mosquitoes, have 96 million of estimated cases in the world (*Vector-Borne Diseases*). The high morbidity is due to the high number of people at risk of contracting VBDs. Indeed, more than 80% of the world's population live in areas at risk from at least one VBD. Furthermore, many of the VBDs are co-endemic and it is estimated that more than half of the global population live in area at risk for two or more VBDs (*Vector-Borne Diseases*, n.d). As shown in Figure 1, inhabitants of south Asia, the Americas and some parts of sub-Saharan Africa are at risk of five or more VBDs (Golding et al., 2015; Wilson et al., 2020). Thus, VBDs is a serious threat for public health in several countries (Hill et al., 2005; *Vector-Borne Diseases*, n.d.-a; Wilson et al., 2020).

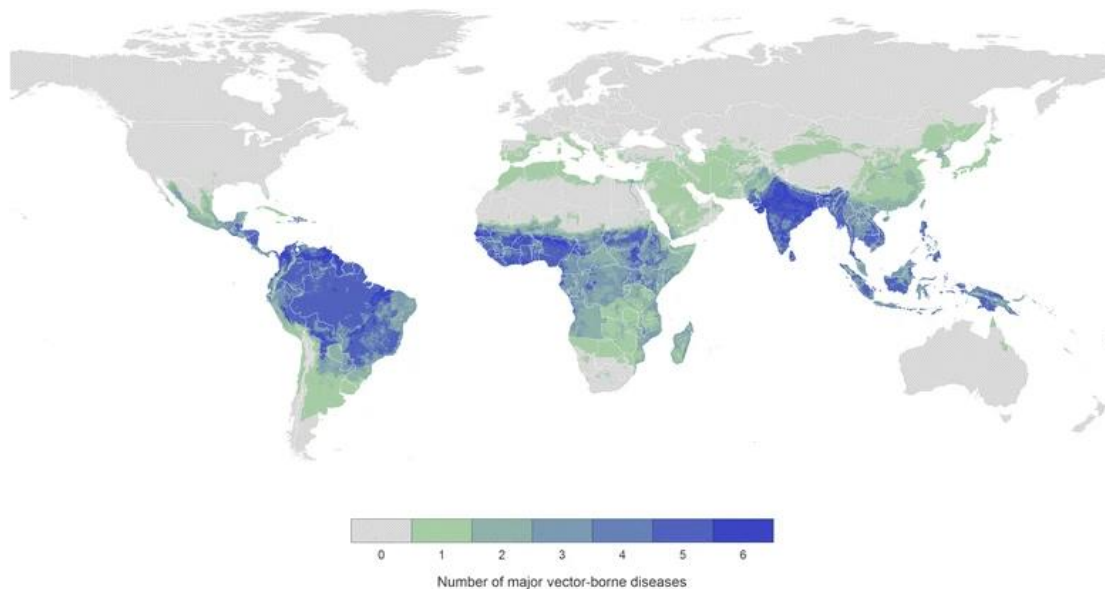


Figure 1. Global distribution of major vector-borne diseases (malaria, lymphatic filariasis, leishmaniasis, dengue, Japanese encephalitis, yellow fever, and Chagas disease). Figure from Golding et al., 2015.

Economic and social impact

VBDs have a significant social and economic burden for a vast number of countries and it is disproportionately greatest in low-income countries of tropics and subtropics area (*Vector-Borne Diseases*). A study that performed tracking spending on 106 endemic countries for malaria reported a €3.8 thousand million spending on 2016 and a 4.0% annually increase since 2000 (Haakenstad et al., 2019). In reference to Dengue, a systematic review showed that the cost of this VBD in just 18 endemic countries was €2.9 thousand million in 2015 (da Silva Oliveira et al., 2019). The direct economic impact of VBDs is also experienced in Europe, where Lyme disease is the leading cause. A study estimated the treatment cost for acute and chronic Lyme disease for 2018 for Europe within €10.1-20.1 billion (Davidsson, 2018). Furthermore, the imposed economic costs of VBDs can result in broad social costs by causing changes in household behaviors such as decisions related to reproduction, education, and economic matters, which, in turn, have a long-term effect on economic growth and development (Sachs & Malaney, 2002).

Factors influencing the spread and establishment of vector-borne diseases

Adaptation of the pathogens to the host and equally the adaptation of both, pathogens and host, to the environment are key points for the establishment of VBDs (Chala & Hamde, 2021; Lenz & Andrews-Polymeris, 2008). A number of ecological, environmental and demographic factors may have an important influence on this interplay among the pathogen, the host and the environment determining the emergence or re-emergence of VBDs (Chala & Hamde, 2021; Savić et al., 2014). Identification of these ecological drivers of VBDs is central for the understanding of disease risk (Kamiya et al., 2019). Changes in these factors may create suitable conditions for the contact of the pathogen with their natural host or the spreading of VBDs to new areas (Chala & Hamde, 2021). Determinants of the emergence or re-emergence of VBDs can be classified as climate and non-climate factors (Savić et al., 2014).

Climate change (i.e., long-term shifts in weather conditions) has already impacted the transmission of a wide range of VBDs in Europe (Semenza & Suk, 2018). Indeed, climate and weather-related factors were reported to account for 10% as a driver of VBDs (Swei et al., 2020). Climate change can affect the pathogens directly, impacting their survival, reproduction or their life cycle inside the vectors or human host or indirectly through the environment or habitat suitability alteration or contact patterns changes of the host with the pathogen or vector (Chala & Hamde, 2021). On the other hand, climate change can impact the vectors equally. Due to its ectothermic trait, arthropods vectors are sensitive to climatic factors (*Vector-Borne Diseases*|ECDC). For instance, climate change was implicated on the shift of ticks to elevated

altitudes and latitudes, notably of *Ixodes ricinus* ticks which is the vector of Lyme borreliosis and tick-borne encephalitis. Furthermore, climate change was also a factor for the expansion of mosquitoes' vector including *Aedes albopictus* which transmits VBDs as dengue, chikungunya and Zika (Semenza & Suk, 2018).

Temperature can impact the transmission of disease since vital parameters that determine the fitness of pathogens and vectors are highly temperature-sensitive (Kamiya et al., 2019). One important feature to be considered when studying VBDs risk is the extrinsic incubation period (EIP), which is defined as the time-course of pathogen incubation and maturation within arthropod vectors (Kamiya et al., 2019; Paaijmans et al., 2012). EIP has been reported to fluctuate with temperature variations (Kamiya et al., 2019; Paaijmans et al., 2012). For instance, Liu et al., 2017 demonstrated that the EIP for dengue virus-2 (DENV-2) in *A. albopictus* was shorter with high temperatures (32°C compared to 28, 23 and 18°C) and consequently, the vector competence of *A. albopictus* was increased.

Non-climatic factors have been also implicated in the emergence and re-emergence of VBDs. These factors include global human populations, international trade and commerce, international travel, intensification and modernization of agricultural practices, antimicrobial drug and pesticide use (Chala & Hamde, 2021; Jones et al., 2013; Perry et al., 2013; Tong et al., 2015). For instance, it has been reported that changes of land-use were the most commonly cited driver and account for 26% of all VBDs (Swei et al., 2020). Globalization and urbanization, for example, induce territorial expansion of vectors and pathogens (Gubler, 2011) causing some VBDs becoming threat worldwide (Chala & Hamde, 2021). Increased migration of rural to urban zones and unplanned urbanization has been associated to the creation of conducive environment for vectors development. This is because urbanization is characterized by agriculture intensification, socioeconomic changes and ecological fragmentation having an impact on the epidemiology of VBDs (Hassell et al., 2017). Furthermore, unplanned urbanization is often associated to a lack of appropriate system for basic services as water and waste management, which creates ideal habitats for the propagation of vector population (Hassell et al., 2017). Moreover, encroachment of human settlements and agriculture on wild ecosystems create a new environment where species from different habitats meets each other. This provides new chances for pathogen adaptation to the host and genetic diversification. (Jones et al., 2013). Finally, the construction of dams and irrigations, that may impact vector population densities, have been associated to outbreaks of some infectious diseases as Rift Valley fever (Chala & Hamde, 2021; Pepin et al., 2010). Hence, the direct impact of climate change alongside with

ecological drivers as temperature and other non-climatic factors need to be taken into account when assessing the risk and dissemination of VBDs in order to create effective control methods.

Major arthropod vectors

As shown in Figure 2, nearly 90% of the vectors involved in VBDs are comprised by Acari (mites and ticks) and Diptera (mosquitoes). Within these groups, the vast majority of emerging vector-borne zoonotic diseases are transmitted by ticks and mosquitoes, specially from the Ixodidae and Culicidae families, respectively (Baxter et al., 2017; Swei et al., 2020). Thus, in the next sections, we will focus on vectors within these two families.

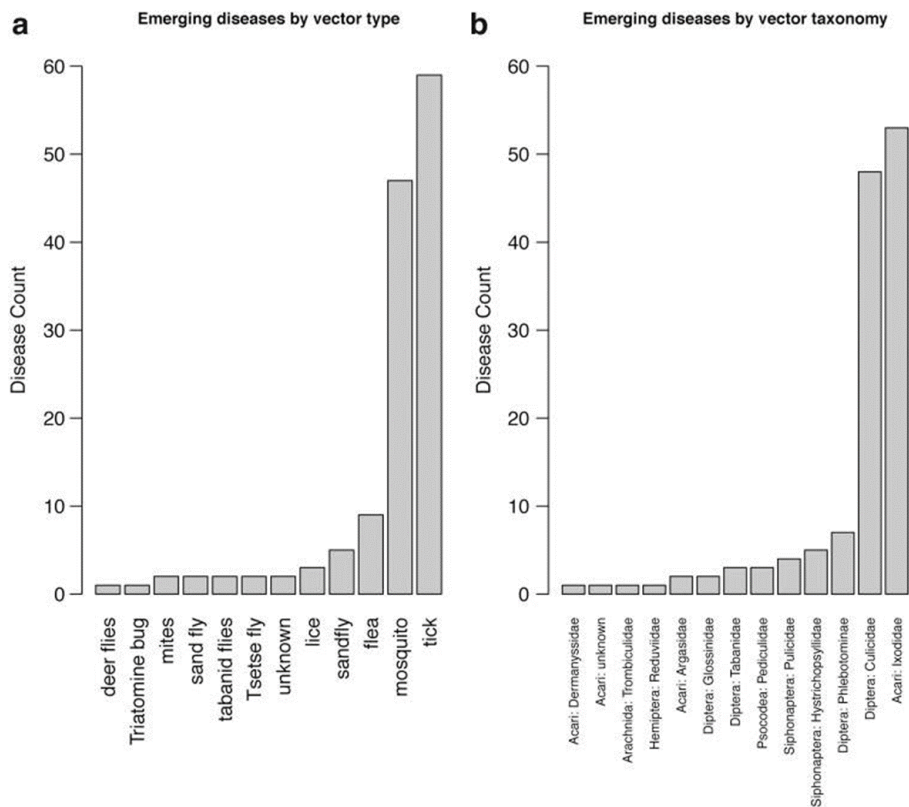


Figure 2. Primary vector types (a) and vector families (b) of emerging vector-borne diseases. Figure from Swei et al., 2020.

Ixodidae ticks

Ticks from the Ixodidae family are known as 'Hard ticks'. It is noteworthy that there are also 'soft' ticks that belong to the family Argasidae but it will not be cover by the present thesis.

Taxonomic classification

The formal taxonomic classification of hard ticks is as follows (*ITIS - Report: Ixodidae*) :

Kingdom	Animalia
Subkingdom:	Bilateria
Infrakingdom:	Protostomia
Superphylum:	Ecdysozoa
Phylum:	Arthropoda
Subphylum:	Chelicerata
Class:	Euchelicerata
Subclass:	Arachnida
Superorder:	Parasitiformes
Order:	Ixodida
Superfamily:	Ixodoidea
Family:	Ixodidae

The family Ixodidae regroups several genera (e.g., *Ixodes*, *Amblyomma*, *Anomalohimalaya*, *Archaeocroton*, *Bothriocroton*, *Cosmiomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Margaropus*, *Nosomma*, *Rhipicentor*, *Rhipicephalus* and *Robertsicus*) and more than 700 species (Estrada-Peña, 2015; *ITIS - Report: Ixodidae*),

Generalities of hard ticks

Hard ticks are obligate hematophagous parasites. They possess a sclerotized scutum and apically located gnathosoma as shown in Figure 3. (Estrada-Peña, 2015). The period of feeding can variate between several days to weeks depending on the life stage and species and the volume of blood ingested could be superior to 100-200 times their body mass for adult females (Estrada-Peña, 2015; Kahl, 2018). Ixodid ticks are also characterized by a complex developmental cycle that includes four developmental stages, namely egg, larva, nymph and adult (male and female). Each postembryonic life stage must take a blood meal (Kahl, 2018) from the same or different hosts to ensure further development. Members of the Ixodidae family undergo either one-host,

two-host or three-host life cycle (CDC - DPDx - Ticks). In the present thesis, we will focus in the three-host life cycle, which is the life cycle style of most ticks of public health importance in Europe, notably ticks of the genera *Ixodes*.

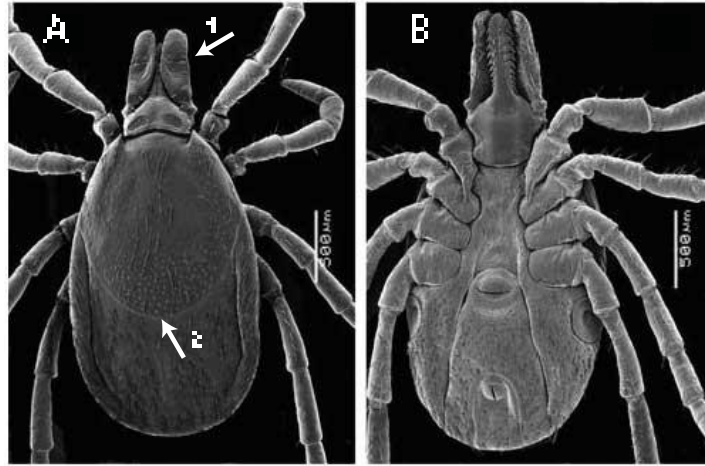


Figure 3. Main morphological features of the dorsal (A) and ventral (B) part of an unfed female *I. ricinus*, a representative of the family Ixodidae. The mouthpart, composed of hypostome, chelicera and palpes are called gnathosoma (arrow 1) and the main body part are called idiosoma, which has a hard plate on the dorsal part of the tick that is called scutum (arrow 2). Figure modified from Estrada-Peña, 2015.

Three-Host Ixodid tick life cycle

In this type of life cycle, the larval, nymphal and adult stage feed once in a different host (CDC - DPDx - Ticks; Estrada-Peña, 2015). The immature stages, namely larvae and nymphs, feed usually upon small hosts, such as rodents and birds, while the adult ticks feed upon large mammals, including carnivores and ungulates (Estrada-Peña, 2015). After repletion, the engorged larvae and nymph detach and drop off the host to molt in the environment, while engorged female lay the eggs (Estrada-Peña, 2015; Kahl, 2018; Leal et al., 2020). A diagram of the three-host life cycle is represented in Figure 4.

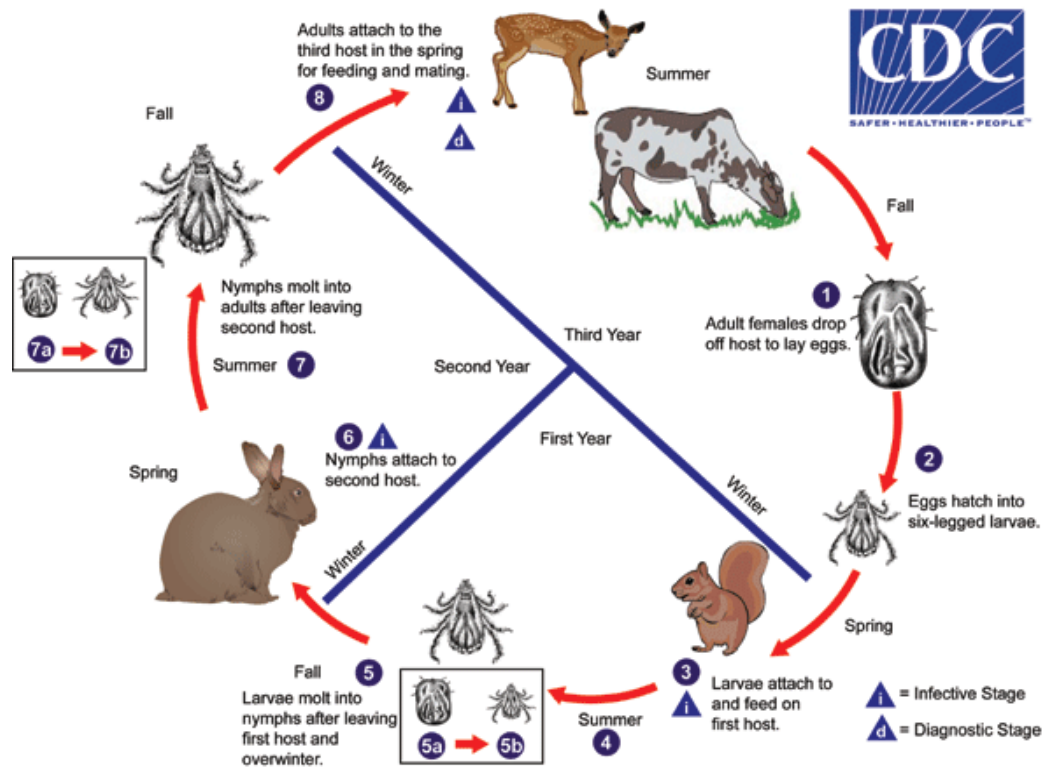


Figure 4. Schematic representation of three-host life cycle in Ixodid ticks. Figures from (CDC - DPDx - Ticks)

Biological attributes contributing to the efficiency of Ixodid ticks as vectors

The on-host (parasitic) period of Ixodid ticks is a window for the acquisition or transmission of pathogens. In this period, the host blood is uptaken by the ticks but there is also injection of a complex salivary cocktail (composed of several hundreds of pharmacologically active compounds) into the host to sustain the flow of blood in the feeding site and equally to counteract the host immune system (Estrada-Peña, 2015; Kahl, 2018; Nuttall & Labuda, 2004; Šimo et al., 2017). The exchange of materials that take place between the vector and the host make a perfect pathway for the transmission of pathogens (Kahl, 2018). Therefore, biological factors surrounding the bloodmeal period can play an important role in the vectorial capacity of the ticks. **The long feeding duration** in ixodid ticks, which is of 2-4 days in larvae, 3-5 days in nymphs and 7-10 days in adult females, gives pathogens a better chance to either leave the feeding tick and enter in the vertebrate host or passively acquired by the tick from the host (Kahl, 2018). Once the pathogen enters in the tick, midgut is the first organ they get a contact with. One particularity of the ticks is the **intracellular digestion of the blood meal** (Arthur, 1965; Nuttall, 2009). This fact may enhance the pathogen survival since the midgut environment becomes less hostile for the pathogens due to the reduced proteolytic enzymes levels (Kahl,

2018). Another factor favoring the entry of pathogens to the tick is the **large volume of blood uptaken** by the Ixodid ticks. For instance, during the blood meal, *I. ricinus* larvae are able to expand its body mass up to 20 times while nymphs are able to expand up to 40 times and adult females up to 200 times (Cupp, 1991; Kahl, 2018). The **three-host life cycle** configuration is also a factor that can enhance the acquisition or transmission of pathogens by the ticks. As they blood meal in different host in each stage, it increases the opportunity of acquiring pathogens from the reservoir host, particularly in the larval, nymphal and adult stage or transmitting the pathogens to uninfected host at the nymphal and adult stages (Dantas-Torres et al., 2012; Kahl, 2018). Furthermore, the **incomplete metamorphosis** that ticks undergo, where only the organs of the ectodermal origin (i.e., the cuticle, the tracheae, the salivary glands and the salivary ducts, except the midgut) degenerate and new tissues and cuticle are formed during the development to the next life stage, facilitates the survival and the transstadial transmission of the pathogens compared to other insects with complete metamorphosis (Kahl, 2018).

Lyme disease: principal tick-borne disease

Lyme disease, also called 'Lyme borreliosis' is the most prevalent vector-borne disease in both Europe and North America (Bush & Vazquez-Pertejo, 2018). It has been estimated that only in the United States, approximately 476.000 people are diagnosed and treated annually for Lyme disease for the period between 2010-2018 (*How Many People Get Lyme Disease? | Lyme Disease | CDC*) whereas more than 200.000 cases per year are reported in western Europe (Marques et al., 2021). The 90% of cases of Lyme disease in North America is concentrated in two regions of United States as observed in Figure 5: the northeast and mid-Atlantic region and the north-central region (Kugeler et al., 2021; Schwartz et al., 2017) while in Europe, the highest incidence of Lyme disease are found in Austria, the Czech Republic, Germany, and Slovenia in central Europe and in the Scandinavian and Baltic states in northern Europe (Marques et al., 2021).

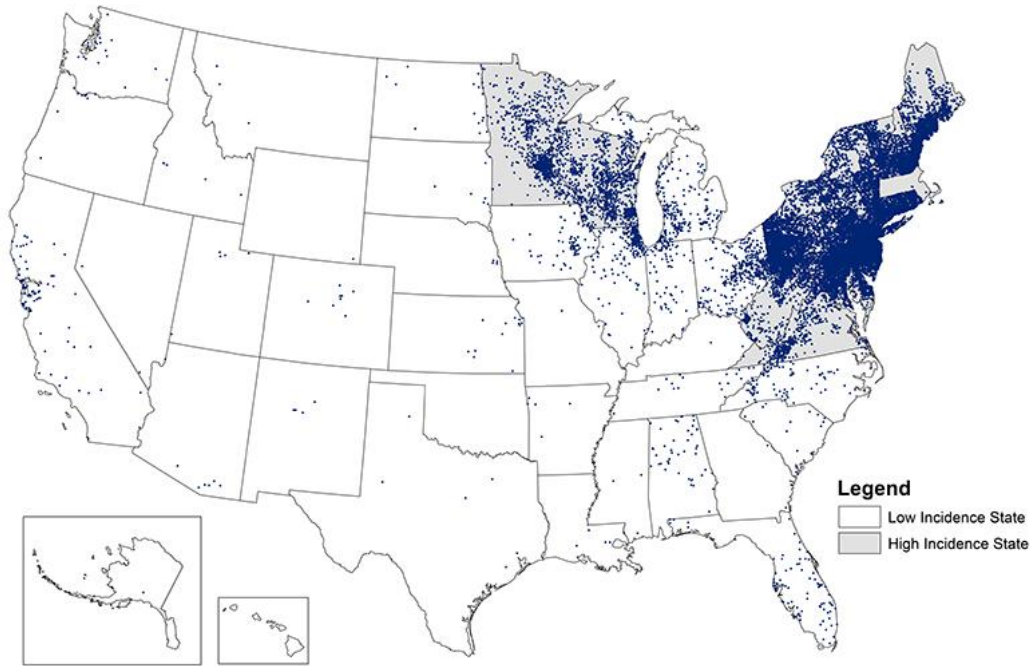


Figure 5. Map of Lyme disease incidence in United States in 2019. Each dot represents one case of Lyme disease and is placed randomly in the patient’s county of residence. Figure from *Lyme Disease Maps: Most Recent Year | Lyme Disease | CDC*

The peak months of Lyme disease onset, in northeastern United States and in most of Europe, are June and July, which correspond to the active period of feeding of nymphal ticks (see Figure 4) (*Lyme Disease Charts and Figures: Historical Data | Lyme Disease | CDC*). Nymphs are responsible for 90% of the human disease transmission due to their abundance and the increase in human activity during those months (late spring and early summer) (Carriveau et al., 2019). Nevertheless, it has been reported that the timing of the peak occurrence can vary geographically and from year to year. These variations are correlated with meteorological conditions such as temperature, humidity and rainfall that influence tick feeding and human behaviour (Moore et al., 2014).

Clinical manifestations of Lyme disease in humans are often characterized by the apparition of an expanding skin lesion, known as erythema migrans, which, if untreated, can progress to an early disseminated infection with neurological abnormalities and to a late infection accompanied of arthritis in North America or acrodermatitis chronica atrophicans in Europe (Steere et al., 2016).

The causal agents of Lyme disease are bacterial members of the *Borrelia burgdorferi* sensu lato complex, which includes 20 different genospecies. They are obligate parasites with spiral shape

that depend heavily on their host for nutritional support (Bush & Vazquez-Pertejo, 2018). The three primarily responsible for human Lyme borreliosis are *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), *Borrelia afzelii* and *Borrelia garinii* (Baranton et al., 1992). The three genospecies are transmitted by different species of ticks and are responsible for Lyme borreliosis in different geographical regions. *B. burgdorferi* sensu stricto are found mainly in United States while *B. afzelii* and *B. garinii* are found mostly in Europe (Steere et al., 2016). The three different strains are also responsible of differences observed in the clinical manifestations. Specifically, *B. burgdorferi* s.s. is particularly arthritogenic, *B. afzelii* primarily causes skin infections and *B. garinii* is especially neurotropic (Steere, 2001).

Four tick species, namely *I. ricinus*, *Ixodes persulcatus*, *Ixodes scapularis* and *Ixodes pacificus* are principally the major vectors for the transmission of *B. burgdorferi* s.l. to humans and each of them have different natural geographic locations as shown in Figure 6. In Europe, the principal vector is *I. ricinus*, which is able to transmit the three major genospecies of *B. burgdorferi* s.l. (Steere et al., 2016). In western Europe, beside *I. ricinus*, *I. persulcatus* is also a vector of Lyme borreliosis and it transmits *B. afzelii* and *B. garinii*, but is not known to transmit *B. burgdorferi* s.s. (Masuzawa, 2004). Furthermore, *I. persulcatus* is also present in Asia and can be found in western Russia, the Baltic countries, parts of Finland, central regions of eastern Russia, northern Mongolia, China and Japan (Steere et al., 2016). On the other hand, *I. scapularis* are distributed in the eastern and mid-western United States and some areas in middle southern and southeastern Canada (Bush & Vazquez-Pertejo, 2018; Ogden et al., 2009; Steere et al., 2016) while *I. pacificus* are found in the western United States (Bacon et al., 2008). Both of them transmits *B. burgdorferi* s.s. (Steere et al., 2016).

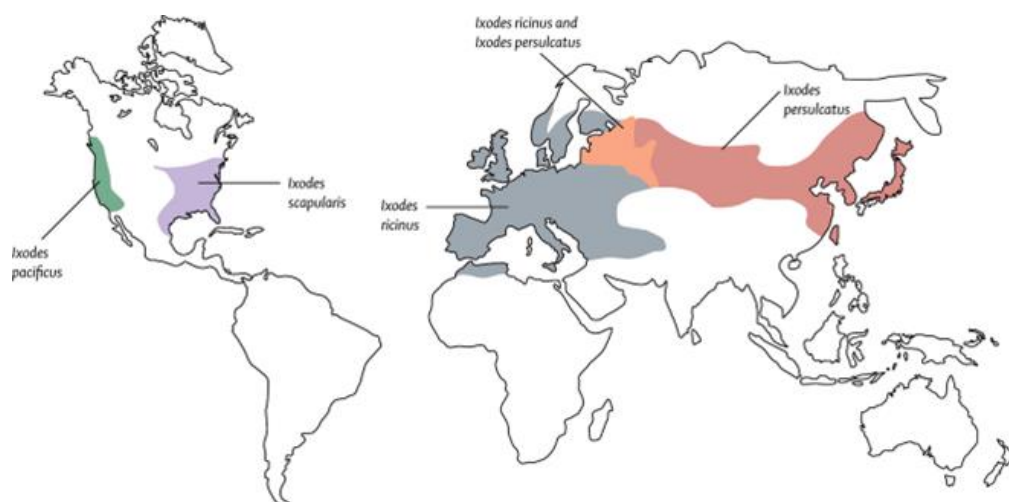


Figure 6. Distribution of the four major Ixodes ticks that transmits Lyme Borreliosis to humans. Figure from Stanek et al., 2012

Infection cycle of *B. burgdorferi* s.l. and the molecular mechanism implicated

B. burgdorferi spirochetes have to be acquired in each generation since there is no transovarial transmission of this bacteria (Radolf et al., 2012). The infection cycle of *B. burgdorferi* species consists of a tick and mammalian phase and the spirochete undergoes differential gene expression for survival in the different environments (Steere et al., 2016). When *Borrelia* species are acquired by *Ixodes* larvae from infected animal host (also called reservoir host) and enter to the midgut of the ticks, the bacteria express tick-phase lipoproteins (Bush & Vazquez-Pertejo, 2018; Steere et al., 2016). Throughout the year life cycle, *Borrelia* remains in a dormant state within the gut of nymphal tick and is characterized by the expression of the outer-surface protein (Osp) A, which is implicated in the survival and colonization of the bacteria in the tick midgut (Bush & Vazquez-Pertejo, 2018). Nevertheless, when *Ixodes* nymphs start blood feeding, signals associated to tick engorgement, such as the increase of temperature, the availability of nutrients, changes of oxygen tension and the decrease of pH, induce the transformation of *Borrelia* species from a state that is adapted for tick colonization to one that is primed for infection of the mammal (Steere et al., 2016). The transformation includes the expression of the RNA polymerase alternative σ -factor (RpoS) and *Borrelia* oxidative stress regulator (BosR) (Radolf et al., 2012). Specifically, RpoS induces the transcriptional activation of mammalian-phase-specific genes (among them, *ospC* implicated in the establishment of early infection in the mammal (Grimm et al., 2004)) while BosR induces the transcriptional repression of the tick-phase-specific genes (for example, *ospA*). Subsequently, through the tick bite and tick salivary proteins that modulate host immune response, *Borrelia* spirochetes are delivered deep into the host dermis and establish and initial infection in the host (Steere et al., 2016). In this process, it is implicated OspC which recruits tick salivary protein Salp15, a molecule with immunosuppressive properties, to the bacterial surface (Ramamoorthi et al., 2005). Finally, the spirochetes spread through host tissues by entering into the blood or lymphatic system (Steere et al., 2016). In this mammalian phase, *Borrelia* survives by evading host bactericidal antibodies. For this, the bacteria replace OspC with VlsE, another outer surface lipoprotein, which in turn, undergoes extensive antigenic variation to evade the host immune response (Zhang et al., 1997). The infected mammalian, then, becomes a new reservoir host that can transmit the spirochetes to another generation of ticks. It is noteworthy that the reservoir host vary according to the *Borrelia* genospecies implicated. In the northeastern United States, *B. burgdorferi* s.s. is acquired by *I. scapularis* larvae or nymphs from small rodents, particularly white-footed mice and chipmunks whereas rodents and migratory birds are the principal reservoirs in Europe for *B. afzelii* and *B. garinii*, respectively (LoGiudice et al., 2003; Radolf et al., 2012). Meanwhile, the infected nymphs (that infected the

new reservoir host) molt into infected adult ticks, nevertheless, they are not considered important for the maintenance of *Borrelia* species in the wild since they feed on large animals, such as deer, which are incompetent hosts for the bacteria (Radolf et al., 2012). Humans or dogs are considered incidental host or dead-end host since it has not been observed the transmission of *Borrelia* from humans to ticks (Radolf et al., 2012). Figure 7 depicts the enzootic cycle of *B. burgdorferi* s.l. species.

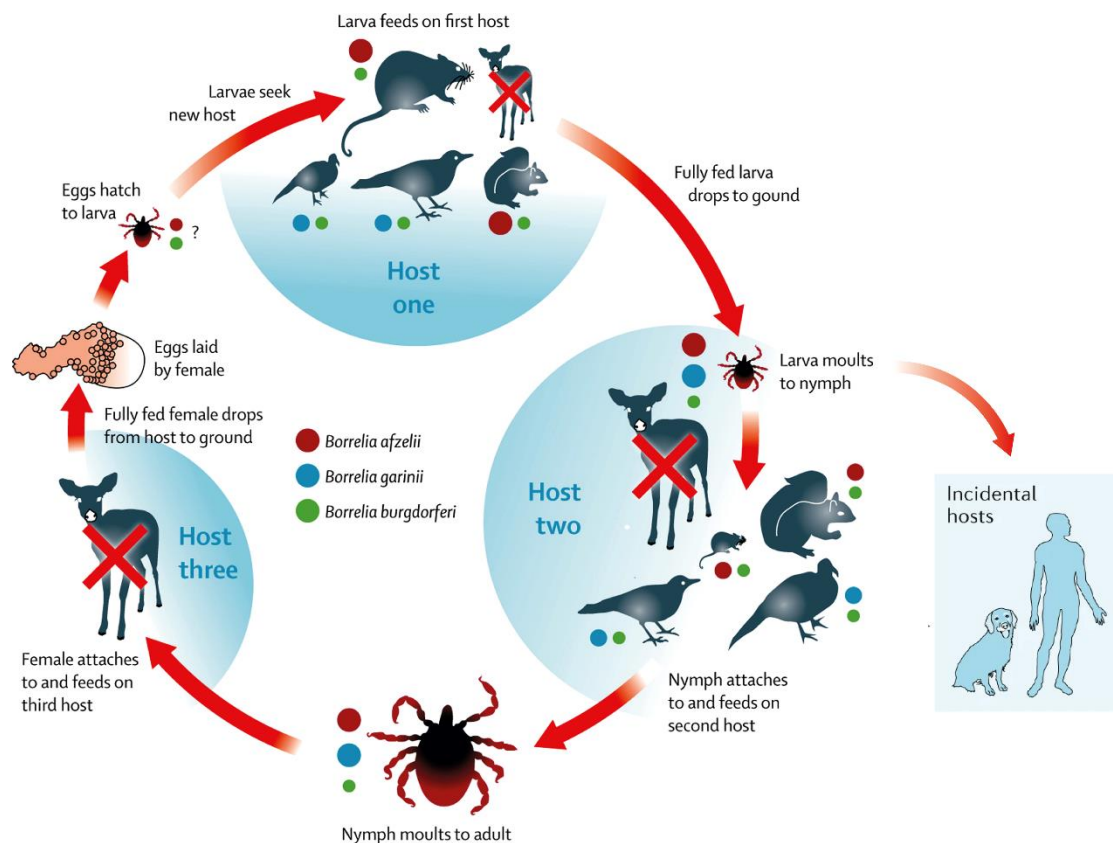


Figure 7. The enzootic cycle of European *B. burgdorferi* s.l. genospecies. The size of the colored circles indicates the relative involvement of the different vertebrate reservoirs for the different genospecies. Figure adapted from Kurokawa et al., 2020 and Stanek et al., 2012.

Culicidae mosquitoes

Taxonomic classification

The formal taxonomic classification of mosquitoes from the Culicidae family is as follows (*ITIS - Report: Culicidae*):

Kingdom:	Animalia
Subkingdom:	Bilateria
Infrakingdom:	Protostomia
Superphylum:	Ecdysozoa
Phylum:	Arthropoda
Subphylum:	Hexapoda
Class:	Insecta
Subclass:	Pterygota
Infraclass:	Neoptera
Superorder:	Holometabola
Order:	Diptera
Suborder:	Nematocera
Infraorder:	Culicomorpha
Family:	Culicidae

The family Culicidae regroups two subfamilies: Anophelinae and Culicinae, 41 genus and more than 3500 species.

Generalities of Culicidae mosquitoes

Mosquitoes are flying, blood-sucking insects that can be found worldwide and are adapted to a broad range of environmental conditions (Chandrasegaran et al., 2020; Lee et al., 2018; *What Is a Mosquito? | Mosquitoes | CDC*). Mosquitoes are characterized by a segmented body, a pair of wings, a pair of halteres, three pairs of long hair-like legs and elongated mouthparts. A more detailed parts of the mosquitoes is shown in Figure 8. They have a complex life cycle which consists of egg, larva, pupa and adult stages. The life span of adult mosquitoes is about 2 to 4 weeks depending on some factors such as species, humidity, temperature, among others. Only female mosquitoes take a blood meal from vertebrate host to obtain metabolic resources and proteins for egg production and hence, to increase their fitness (Takken et al., 1998; Yan et al., 2021)

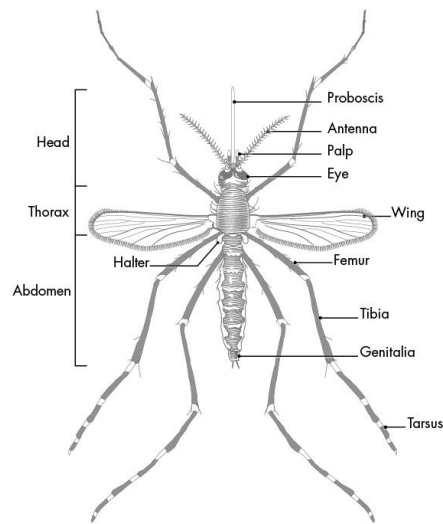


Figure 8. Schematic representation of parts of an adult mosquito. Figure from *What Is a Mosquito?* | *Mosquitoes* | CDC.

Life cycle of *Culex* species

The life cycle of all types of mosquitoes is similar, with small variations in the time of molting between stages and the site preference for egg oviposition (*Life Cycle of Culex Species Mosquitoes* | *Mosquitoes* | CDC). The development of their life cycle occurs in contrasting habitats. Indeed, immatures and adults are confined to aquatic and terrestrial habitats, respectively (Chandrasegaran et al., 2020). In Figure 9, life cycle of *Culex* species is represented. After blood meal, adult female *Culex* mosquito laid egg raft masses of approximately 200 coeval individuals on the surface of fresh or stagnant water (McCann et al., 2009). Larvae hatched from the eggs, live in the water and feed on microbial communities associated with decaying organic matter (Walker et al., 1991). The amount of nutrients stored at the larval stage will influence later the way that adult females seek nutrition and blood meals (Vantaux et al., 2016). Aquatic larvae molt several times until development to the pupa stage. These Pupae lacks of external mouthparts and do not feed during this stage. Finally, from the pupae emerges an adult mosquito that feed on plant sugars (Foster, 1995). In addition, adult females mosquitoes feed on blood to initiate the endocrine cascade that is implicated in egg maturation and oviposition (Muturi et al., 2019).

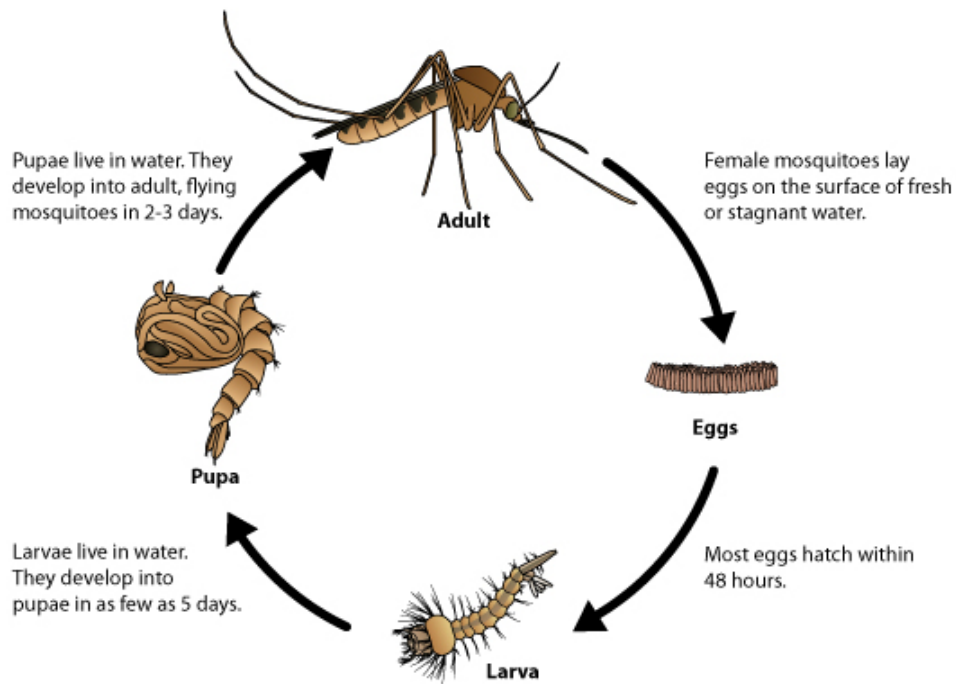


Figure 9. Schematic representation of the life cycle of *Culex* species. Figure from *Life Cycle of Culex Species Mosquitoes | Mosquitoes | CDC*

Factors affecting the vectorial capacity of mosquitoes

Mosquito-borne pathogens are acquired from infected host to mosquitoes and transmitted from infected mosquitoes, through their saliva, to a new host during the blood feeding time of adult mosquitoes. It has been reported that pathogen circulation presents particular requirements such as: (i) a mosquito vector competent to harbor infective forms in their salivary glands and (ii) a competent host into which the pathogen can multiply (Yan et al., 2021). Thus, the contact between competent vertebrate host-arthropod vector is vital for the complete transmission cycle of vector-borne pathogens (Takken & Verhulst, 2013). Nevertheless, these requirements did not impede the worldwide spreading of mosquito-borne diseases. Several factors can play a role in the vector competence of mosquitoes and therefore, the expansion of diseases transmitted by them. **Blood feeding patterns** of mosquitoes vary by species (Gibson & Torr, 1999). Indeed, some mosquito species feed opportunistically on a wide range of host (i.e., *Culex quinquefasciatus*) and others feed predominantly on a narrow range of host species (i.e., *Culex pipiens* feeds mainly on birds, *Culex molestus* feeds mainly on humans and birds) (Reisen, 2012). These heterogeneities in blood feeding patterns may influence the rates of host-vector contact and therefore, the risk of pathogen exposure to different individual hosts (Yan et al., 2021). Larvae **nutrition status** may influence also mosquitoes vectorial capacity since larvae with nutrition deprivation results in adults that are more susceptible of pathogens infection

(Carvajal-Lago et al., 2021). This is likely due to the fact that those larvae invest the available energy in their survival and development instead of their defense and immunity (Moller-Jacobs et al., 2014; Muturi et al., 2011). **Vector longevity** may also have an impact on pathogen transmission since a longer mosquito lifespan provides, on the one hand, more time for pathogens to complete its extrinsic incubation period within the vector and on the other hand, more potential for infective bites to hosts by infected mosquitoes; thus, favoring pathogen spreading (Vézilier et al., 2012). Interestingly, it has been demonstrated that *C. pipiens* mosquitoes infected experimentally with avian *Plasmodium* spp. increased mosquitoes longevity (Vézilier et al., 2012) meaning that pathogen induces changes in order to increase mosquitoes survival for their own benefit. Other factors such temperatures or the level of competition for resources during the larval stages can also impact the vectorial capacity of mosquitoes (Chandrasegaran et al., 2020). Indeed, **higher temperatures** is a principal driver in the variation of development rates of mosquitoes larvae (Couret & Benedict, 2014). For instance, an increase of temperature from 16 to 32°C is related to a 2.9-fold increase in the development rate of *Culex* mosquitoes (Ciota et al., 2014). **Elevated competition for resources** during the larval stages enhance the vectorial capacity of mosquitoes and this is because adult females that emerge from a competitive environment for resources seek hosts relatively early upon emergence and take multiple blood meals (Noden et al., 2016).

Avian Malaria: oldest experimental system for the study of *Plasmodium* parasites biology and transmission

Avian malaria has played a key role in the understanding of human malaria parasites and in the development of the first vaccines and antimalarial drugs (Rivero & Gandon, 2018). Parasites causing avian malaria are from the genera *Plasmodium* and belongs to the order Haemosporida (Phylum Apicomplexa). More than 50 avian malaria species have been identified based on morphological features of their blood stages and new species are discovered every year (Valkiunas & Iezhova, 2018). Moreover, based on mitochondrial genome analysis of avian *Plasmodium* spp., there might be many more species than previously thought (Bensch et al., 2009). Among the vast number of avian *Plasmodium* species, the most widespread species is *Plasmodium relictum* (Hellgren et al., 2015).

P. relictum is classified as one of the most invasive species by the International Union for Conservation of Nature (IUCN) (Lowe et al., 2000), it has been found in all major geographic regions except Antarctica (Rivero & Gandon, 2018) and is responsible of the decline and extinction of many of Hawaii's endemic honeycreepers (Hellgren et al., 2015). *P. relictum* is a morphologically defined species and has several lineages based on the sequence of the

cytochrome-b (*cyt-b*) gene, nevertheless, three of its *cyt b* lineages are particularly widespread: SGS1, GRW11, and GRW4. The worldwide distribution of each of these *P. relictum* lineages is shown in Figure 10. Using molecular tools, the lineage SGS1 have been identified as the lineage that infects more species of birds than any other *Plasmodium* lineage (Martínez-de la Puente et al., 2021).

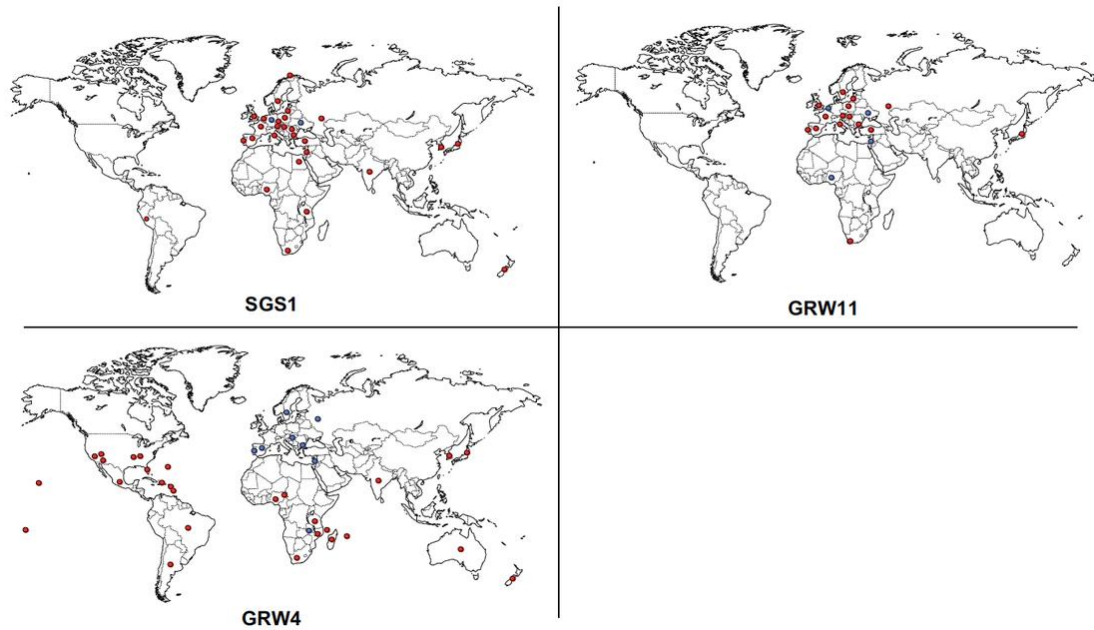


Figure 10. Global distribution of *P. relictum* *cyt b* lineages SGS1, GRW1 and GRW4. Red dots represent confirmed transmission events found in a resident bird species or juvenile bird before migration while the blue dots represent positive cases detected in adult migratory birds with tropical wintering areas. Figure from Hellgren et al., 2015.

P. relictum is considered a generalist parasite since it has been found in over 300 different bird species that belongs to 11 different orders. (Bensch et al., 2009; Valkiunas & Iezhova, 2018). It is also considered a generalist in vector usage being transmitted by different genera of mosquitoes. Specifically, 3 species of the genera *Culex*, namely *C. quinquefasciatus*, *Culex stigmatosoma* and *Culex tarsalis* have been listed as natural vectors while 20 other different species of the genera *Culex*, *Culiseta*, *Aedes* and *Anopheles* have been proven experimentally as potential vectors of *P. relictum* (Santiago-Alarcon et al., 2012). The infection virulence caused by *P. relictum* can vary between bird species and transmission areas. For instance, some bird species infected with *P. relictum* can develop light transient parasitemia, especially in endemic regions, while in other species it may lead to more critical symptoms such as acute anemia and organ pathology (Martínez-de la Puente et al., 2021).

Avian malaria life cycle

The life cycle of avian malaria is similar to those of human and mammal *Plasmodium* species in their basic features (Valkiunas & Iezhova, 2018). The parasite *Plasmodium* has a sexual reproduction phase in Culicidae mosquito and an asexual phase in birds.

Plasmodium male and female gametocytes is acquired during blood-feeding by female mosquitoes from an infected avian host. In the mosquito midgut, the gametocytes differentiate into female and male gametes (Bruce et al., 1990; Silvestrini et al., 2000), which fuse to form a diploid zygote, which in turn, undergo meiosis and differentiates into the motile ookinete (Sinden et al., 1985). Subsequently, the ookinetes migrate to the basal lamina of the mosquito midgut where they form the oocysts (Sinden, 2009). Over the course of several days, each oocyst undergoes several rounds of mitosis to produce thousands of haploid sporozoites, which are released when the oocyst ruptures (Sinden, 2009). The sporozoites, then, migrate through the haemocoel towards the salivary gland of the mosquito (Gerald et al., 2011) ready to be transmitted to a new uninfected avian host in the next blood feed of the mosquito. This sexual process and sporogony of *Plasmodium* parasites in the mosquitoes are completed in approximately seven days (Huijben et al., 2007). The cycle in the birds starts when the mosquito injects the sporozoites along with its saliva which contains several enzymes to enhance blood uptake and prevent clotting (Huijben et al., 2007). The sporozoites reach the bird's blood stream and subsequently invades the reticuloendothelial cells of several organs, including the spleen, and initiate several rounds of replication to form the merozoites (exoerythrocytic cycle). The merozoites are, subsequently, released into the bloodstream. Unlike the merozoites in human malaria, these merozoites are unable to infect the red blood cells immediately; instead, they undergo a second exoerythrocytic cycle during which they invade the macrophages in many organs (Huff & Coulston, 1946). The resulting merozoites enter to the erythrocytic cycle, where they invade the red blood cells and develop into trophozoites. The parasites divide and ends up releasing further merozoites into the bloodstream. A proportion of these merozoites will invade red blood cells and will reinitiate the asexual replication cycle. The remaining merozoites either enter the secondary exoerythrocytic cycle or invade new red blood cells and transform into sexual (male and female) gametocytes, which is the stage of *Plasmodium* that is infective to mosquitoes (Huijben et al., 2007; Rivero & Gandon, 2018). Figure 11 shows a simplified scheme of avian malaria life cycle.

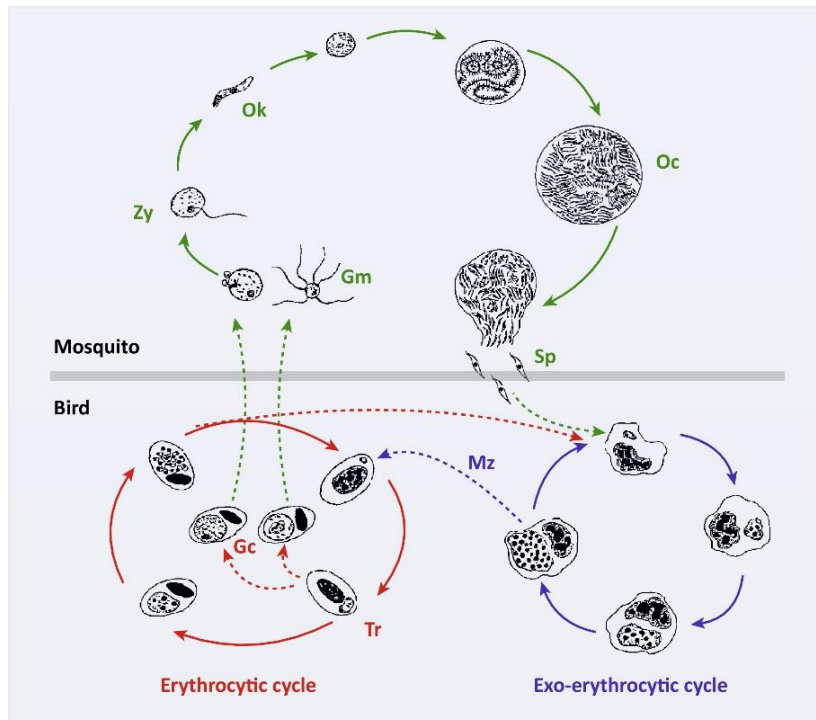


Figure 11. Simplified Avian Malaria Life Cycle. The upper panel shows the cycle of *Plasmodium* in the mosquito. Gm: gametes, Zy: zygote, Ok: ookinete, Oc: oocysts, Sp: sporozoites. The lower panel shows the cycle of *Plasmodium* in birds. Mz: merozoites, Tr: Trophozoites, Gc: gametocytes. Figures from Rivero & Gandon, 2018.

Vector microbiota

Vector microbiota: Why it is important?

Pathogens acquired by arthropod vectors, through the blood feeding, enter to the body of the vector and replicate or undergo developmental changes essentials for the transmission to a new host (Caragata & Short, 2022). The midgut is the first organ in which pathogenic microbes ingested with the host blood can survive and, in most cases, invade other tick (Sonenshine & Simo, 2021) or mosquito (Gabrieli et al., 2021) tissues. The midgut is also the optimal microenvironment for the survival and maintenance of the vector microbiota (Maitre et al., 2022).

Microbiota is referred to all microorganisms that dwell within the vector tissue and is composed of bacteria, archaea, fungi and viruses (Narasimhan & Fikrig, 2015; Wu-Chuang et al., 2021). In here, "microbiome" refers to the microorganisms and their genes whereas "microbiota" only refers to the microbes themselves. Interest on microbiome has been risen due to its important role on vector physiology and more interestingly, on vector competence (Caragata & Short, 2022; Narasimhan & Fikrig, 2015; Wu-Chuang et al., 2021). Indeed, the diversity of microorganisms harbored by the vector are often found in close proximity to the pathogens that the vector transmits (Cirimotich, Ramirez, et al., 2011). Thus, it is imaginable that individual microorganisms and microbial communities in the microbiota, which interact with vector-borne pathogens (Hajkazemian et al., 2021; Wu-Chuang et al., 2021), can facilitate (Wang et al., 2021) or compete (Bando et al., 2013) with pathogen colonization and development within the vector. These findings open the gate for the development of new strategies to interrupt pathogen transmission via modulation of the vector microbiota. However, to reach this goal, comprehension of the factors that influence vector microbiome and the biological interactions between the vector, its microbiome and vector-borne pathogens is first needed (Wu-Chuang et al., 2021). Although the next section is mainly focused on ticks, some examples from mosquitoes are also documented.

Factors influencing vector microbiota composition and diversity

Mounting evidences showed that diverse factors can shape the pattern of vector-associated microbiota. Different experiments in the field and under controlled conditions showed that vector microbiota can vary by intrinsic factors such as genetic traits, sex, species, age, life stage, and extrinsic factors like vector environment, diet and immunity (Muturi et al., 2019; Wu-Chuang et al., 2021). For instance, comparison of the taxonomic variability observed across microbiome of different tick **species** suggested that tick microbiome assemblages are not stochastic (Cabezas-Cruz et al., 2018). Rather, the phylogenetic structure of ixodid tick microbial communities supports the existence of a species-specific tick holobiont (Díaz-Sánchez et al.,

2019). We defined 'holobiont' as the single ecological unit integrated by the host and their microbiome.

The impact of **genetic traits** on tick microbiota composition is still not well described but it has been hypothesized that genetic traits may determine the permissiveness of ticks to polymicrobial colonization (Wu-Chuang et al., 2021). Indeed, characterization of the microbiota of *I. ricinus* ticks collected within the same site revealed an unequal distribution of the bacterial diversity suggesting that some *I. ricinus* strains are highly permissive to polymicrobial challenges and harbor diverse microbial communities, while others are not (Estrada-Peña et al., 2018). Indeed, it has been reported that approximately 80% of bacterial phylogenetic diversity was found in approximately 20% of ticks, regardless of the sampling sites (Estrada-Peña et al., 2018). Another evidence of unequal permissiveness to polymicrobial challenge was given by Ross et al., 2018, who showed that the majority of field-collected adult *I. scapularis* harbor limited internal microbial communities, while a minority of ticks harbors abundant midgut bacteria. Impact of genetic traits was also observed in mosquitoes, specifically in the Asian tiger mosquito *A. albopictus*, where a significant correlation between mosquitoes' genetic diversity and midgut microbiota diversity was found (Minard et al., 2015).

Microbiota diversity can be also impacted by the **sex** of the vector (Thapa et al., 2019a; van Treuren et al., 2015; Wu-Chuang et al., 2021). Analysis of *I. scapularis* and *Ixodes affinis* microbiomes by next generation sequencing (NGS) technologies showed that microbiomes of adult female ticks were significantly less diverse than those of male ticks (van Treuren et al., 2015). Interestingly, it has been observed that the microbiota of female ticks is dominated by a single taxon with a high relative abundance. For instance, a high relative abundance of *Rickettsia* has been found in *I. affinis* (van Treuren et al., 2015), *A. americanum* (Ponnusamy et al., 2014) and *I. scapularis* microbiota (Brinkerhoff et al., 2020; Hawlena et al., 2013). This high prevalence of *Rickettsia* in female ticks could be explained by the high rate of transovarial transmission of this bacteria, which have been reported in several tick species (Hauck et al., 2020; Macaluso et al., 2001; T. C. Moore et al., 2018). Similarly, in mosquitoes was also reported differences in the microbiota by sex. For instance, in *A. albopictus* a greater diversity was observed in females, contrary to ticks (Zouache et al., 2011). In field populations of malaria vector *Anopheles stephensi*, diversity of microbiota was also related to the sex. In this vector, female mosquito microbiota presented exclusive bacteria genera, namely *Cryseobacterium*, *Pseudomonas* and *Serratia*. In general, it has been found that the midgut of females is mostly colonized by members of the Gammaproteobacteria whereas the midgut of males is dominated by bacteria from the phylum Firmicutes (Minard et al., 2013).

Vector microbiome also changes with the progression of the life-cycle and **developmental stages** (Wu-Chuang et al., 2021). For example, it has been observed a progressive decrease in the species richness and diversity of microbiota through the different developmental stages (with the highest diversity at the larval stage) in ticks of different species such as *I. pacificus* (Chicana et al., 2019; Kwan et al., 2017; Swei & Kwan, 2017), *Dermacentor albipictus* (Chicana et al., 2019), *D. silvarum* (R. Zhang et al., 2020) and *A. americanum* (Menchaca et al., 2013). While the mechanisms underlying the decrease of microbiota diversity through the tick ontogeny are not well understood, it has been hypothesized that the existence of competitive interactions between tick microbiome bacteria or the gradual loss of unstable microbes through the tick development are associated to the loss of diversity (Chicana et al., 2019). Another hypothesis suggests that differences in the microbiota diversity are associated to particular changes in the functional profile. In line with this idea, it has been reported that sequences associated with the biosynthesis of amino acids and purine metabolism pathways were overrepresented in *D. silvarum* nymphs compared to other stages (R. Zhang et al., 2020).

Differences in the microbiota was also observed in mosquitoes. The four gradual stages of metamorphosis of mosquitoes – egg, larvae, nymph and adult – are intimately associated to their respective biotopes (terrestrial for adult mosquitoes and aquatic environment for the other stages). Thus, it is likely that the microbiota composition and diversity differs between stages, at least for the fraction of mosquito-associated microflora that is acquired from the surrounding environment (Minard et al., 2013). For instance, the midgut of mosquito larvae contains photosynthetic cyanobacteria acquired from breeding sites, which are not found in adult mosquitoes (Thiery et al., 1991). Moreover, it has been observed that microbial richness of late-instar larva of *Anopheles atroparvus* was significantly higher than wild-caught females (Birnberg et al., 2021). One possible explanation for the decrease in microbiota richness is the radical modification of the mosquito anatomy during its metamorphosis (Minard et al., 2013). In particular, a first meconial peritrophic matrix (MPM1) is formed early in the pupal stadium whereas a second MPM2 emerged during the adult stage (Moncayo et al., 2005). It has been suggested that MPMs can contribute to the sterilization of the adult midgut by sequestration of remaining larval gut bacteria, which, along with remaining meconial material are cleared off during adult emergence (Minard et al., 2013; Moll et al., 2001).

Microbiota composition can also be determined by the **transgenerational inheritance** (Wu-Chuang et al., 2021). While not the entire microbiota is transferred from one generation to another, it has been found that specific bacteria can be inherited from the mother to the offspring (vertical transmission). For instance, the microbiota richness and diversity in *I.*

scapularis ticks were similar between adult females and their eggs, with the endosymbiont *Rickettsia* being the dominant genus, suggesting the vertical transmission of the endosymbiont (Brinkerhoff et al., 2020). Similarly, the microbiota of *Dermacentor silvarum* females and eggs exhibited high similarity but in contrast to the former study, the dominant genus here was *Coxiella* (R. Zhang et al., 2020). Interestingly, *Coxiella* and *Rickettsia* were identified as nutritional endosymbionts (Hunter et al., 2015; Smith et al., 2015). Therefore, we can hypothesize that transgenerational microbiome inheritance includes bacteria that are indispensable for early tick development (Wu-Chuang et al., 2021).

Diet was also described as a strong factor that shapes vector microbiota (Wu-Chuang et al., 2021). In ticks, the impact of the blood meal was described in *I. pacificus*. Comparison of the microbiota of *I. pacificus*, feeding on different hosts, showed that the microbiota of *I. pacificus* nymphs fed on western fence lizards (*Sceloporus occidentalis*) presented significantly lower species richness compared to the microbiota of nymphs fed on mice (Swei & Kwan, 2017). Furthermore, it has been reported that *Haemaphysalis leporispalustris* and *D. albipictus* ticks, that feed predominantly on a single or limited range of hosts, have lower microbiome species richness and diversity compared to *I. pacificus* or *D. variabilis* ticks that feed on several host species (Chicana et al., 2019). In mosquitoes, the adult individuals exhibit different ecological behaviors in terms of nutritional capabilities according to sex. Both, female and male mosquitoes, feed on nectar and plant saps but females are also hematophagous (Minard et al., 2013). It has been reported that different diet regime (i.e., sugar meal and blood meal) in adult mosquitoes affects significantly the bacterial population structure. Indeed, it was found that blood meals reduced significantly microbiota community diversity and favored enteric bacteria (Wang et al., 2011).

Environmental factors are also a strong factor driving vector microbiome variation (Wu-Chuang et al., 2021). Given the observed spreading of vector-borne diseases related to global climate change, understanding the impact of changing environmental factors on vector microbiota is of high importance. Indeed, ecological setting where the vector come from can impact microbiota composition. For example, it was found that laboratory-reared or field-collected larvae and nymphs possess different microbiota composition (Kwan et al., 2017; Zolnik et al., 2016) suggesting that environmental factors have an impact on tick microbiota. Furthermore, temperature can also have an impact on the microbiota. An experimental trial studied the effect of temperature on *I. scapularis* ticks and found that the bacterial community composition and diversity changed at high temperatures (i.e., 30 °C and 37 °C) compared to ticks incubated at lower temperature (i.e., 4 °C and 20 °C) (Thapa et al., 2019a).

Comparison of the microbiota of ticks collected in different geographical sites showed that bacterial community or structure can change according to collection site (Carpi et al., 2011; Chandra & Šlapeta, 2020; Gall et al., 2017; Trout Fryxell & DeBruyn, 2016; van Treuren et al., 2015). It has been hypothesized that tick microbiota variation across different sampling sites could be the result of acquisition of microbes present in the soil. Supporting this idea, it has been found the existence of soil-associated bacteria in *I. scapularis* microbiota (Rynkiewicz et al., 2015; Zolnik et al., 2016). Nevertheless, some studies did not find an impact of the collection site on tick microbiota composition (Brinkerhoff et al., 2020; Hawlena et al., 2013). Rather, it was some arthropod traits such as life stages or tick species, and not environmental factors, that determined the bacterial community (Hawlena et al., 2013). One hypothesis about the differences observed among different studies is the existence of dominant species-specific endosymbionts that exclude other bacteria masking possible environmental effects (Hawlena et al., 2013).

Mosquitoes' microbiota is also susceptible to environmental factors. As previously described, Culicidae mosquitoes live in contrasting environments and some abiotic (e.g., temperature and humidity) factors can strongly influence their microbiota (Minard et al., 2013). While the exact proportion of bacterial species acquired from the environment is unknown, comparison of the microbiota between mosquitoes reared in a controlled, laboratory setting to those exposed to their natural environment can give a hint of the contribution of the ecology to the bacterial composition of mosquitoes' microbiota. Indeed, studies on the microbiota of *A. stephensi* (Rani et al., 2009) and *Anopheles gambiae* (Boissière et al., 2012; Y. Wang et al., 2011) mosquitoes showed a decreased bacterial diversity in lab-reared mosquitoes compared to their field-caught counterparts. The highest bacterial taxa richness observed in field-caught mosquitoes shows the extent to which bacteria are acquired and integrated in the vector microbiota from the habitat (Minard et al., 2013). The ecological origin of each species of mosquito can dictate its bacterial content. For instance, a fraction of the composition of adult mosquitoes' microbiota comes from the water during mosquito emergence (Lindh et al., 2005). Moreover, the bacterium *Acinetobacter*, which was frequently found in different species including *Aedes aegypti*, *C. pipiens*, *C. quinquefasciatus* was also found in mosquito larval breeding sites (Minard et al., 2013).

Finally, **vector immune system** can also have a role modulating vector microbiota. In ticks, several signaling pathways such as the immune deficiency (IMD), the Janus kinase (JAK), signal transducer and activator of transcription (STAT) and Toll receptor signaling pathway have been described as important components of the tick immune system (Gulia-Nuss et al., 2016; Smith & Pal, 2014). In the model organism *Drosophila melanogaster*, both beneficial and pathogenic

microbes can induce immune pathways which have a role in maintaining the location, density and diversity of the microbiome (Lesperance & Broderick, 2020). Indeed, recognition of cell wall components in Gram-positive and Gram-negative bacteria leads to the activation of Toll and IMD pathways, respectively (Hanson & Lemaitre, 2020) which in turn activate the transcription factors NF- κ B, from the Toll pathway, and Relish, from the IMD pathway, and results in the expression of different AMPs (Hanson & Lemaitre, 2020). These AMPs can then modulate host microbiota by keeping a balance in the abundance of the bacterial members (Mergaert, 2018; Zong et al., 2020). Additionally, AMPs can also control infection by invading bacteria, viruses or fungi (Hoffmann & Reichhart, 2002). In ticks, several canonical components of the immune pathways, notably in the IMD pathway, are missing (Fogaça et al., 2021); still, the production of AMPs by the ticks has been reported (Wu et al., 2022), although their relationship with the microbiota is not characterized in the same extent as in *Drosophila* (Wu-Chuang et al., 2021).

In mosquitoes, the IMD pathway is conserved (Christophides et al., 2002) and is activated in midgut epithelial cells in response to the proliferation of midgut microbiota that is triggered by the blood meal (Ferreira Barletta et al., 2017). Indeed, peptidoglycans of the bacterial cell walls is recognized by peptidoglycan recognition proteins (PGRP), which leads the activation of two isoform of Relish homolog, namely REL-2S and REL-2F, that are involved in the response against Gram-negative and Gram-positives bacteria, respectively (Meister et al., 2005). As in *Drosophila*, Relish induces the expression of AMPs. The modulation of AMPs on mosquito microbiota was demonstrated by Song et al., 2018, who silenced the PGRP-LD and observed in consequence an over-activation of the immune response which led to an over-expression of multiple AMPs in *A. stephensi* prior blood feeding. These AMPs, then, causes a reduction of the bacterial load in the mosquito gut. These results demonstrate the role that the immune system has on keeping host gut homeostasis.

Altogether, these results show that several intrinsic and extrinsic factors can contribute substantially to variation in vector microbiota composition as outlined in Figure 12.

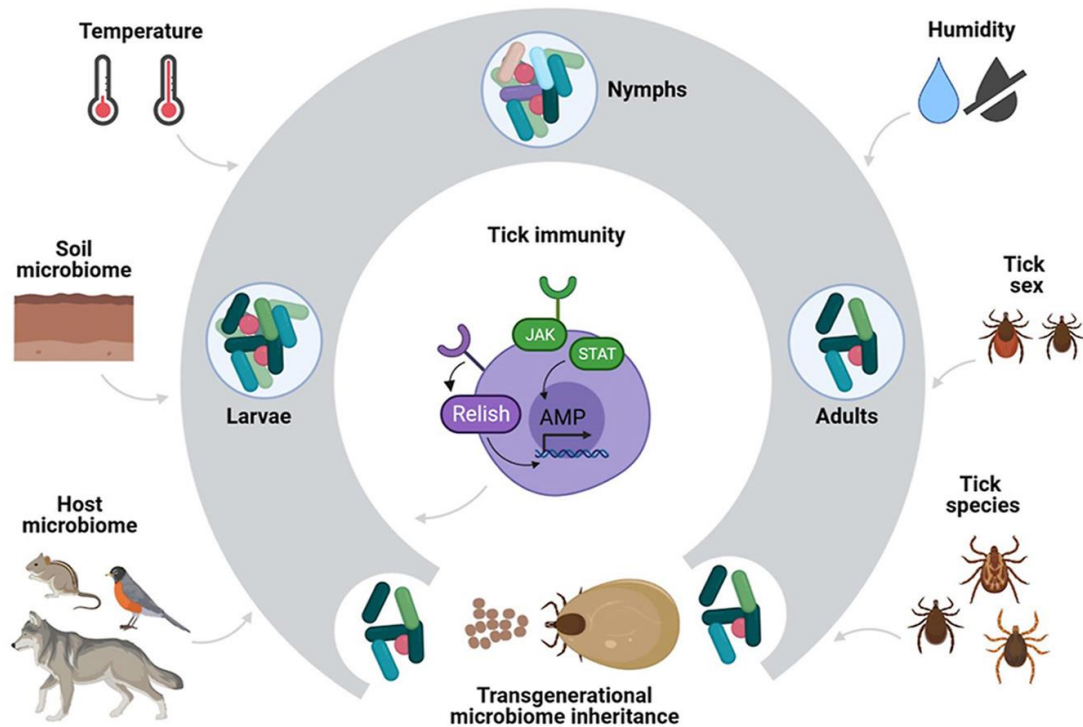


Figure 12. Factors influencing tick microbiota composition and diversity. Figure from Wu-Chuang et al., 2021

Vector-microbiota interactions

There is an increasing interest in determining the role of the microbiome of hematophagous arthropods vectors in their biology. One of the best-characterized contributions of arthropod microbiome is the nutritional complementation observed in obligate blood feeders. Indeed, because of their restrictive, blood-based diet, ticks lack important nutrients like B vitamins and other cofactors but these deficiencies are encountered by ticks via their association with symbiotic bacteria (Duron et al., 2018; Wu-Chuang et al., 2021). These microbes include members of the genera *Coxiella*, *Rickettsia* and *Francisella*. For example, *Rickettsia* endosymbionts (*Rickettsia buchneri*) encode the full set of gene orthologues (*folA*, *folC*, *folE*, *folKP* and *ptpS*) for *de novo* folate biosynthesis in *I. pacificus* and *I. scapularis* ticks (Hunter et al., 2015; Kurtti et al., 2015). Moreover, *R. buchneri* encodes 2 functional biotin operons indicating its potential to provide biotin in addition to folate to the tick host (Gillespie et al., 2012). Interestingly, it has been observed an increase in the relative abundance of endosymbionts through developmental stages in *Ixodes* spp (Chauhan et al., 2020; Thapa et al., 2019b; Zolnik et al., 2016) and is hypothesized that this accumulation of *Rickettsia* in female adults would facilitate the transovarial transmission of the endosymbiont to their eggs. Thus, transgenerational microbial inheritance in ticks includes bacteria that are indispensable for tick survival and development (Hunter et al., 2015; Narasimhan et al., 2021). Similarly, *Coxiella*- and

Francisella-like endosymbionts encodes for cofactor and vitamins, but they are not associated to *Ixodes* tick but rather to other tick species such as *A. americanum* and *Ornithodoros moubata*, respectively (Duron et al., 2018; Smith et al., 2015). The reason and the biological significance of the preferential association of endosymbionts to some tick genera remains to be elucidated. Several other experiments have demonstrated an association between the reduction of endosymbionts abundance, specifically *Coxiella*-like endosymbiont, and a decreased reproductive fitness (Ben-Yosef et al., 2020; Zhang, & Zhu, 2018; Zhang, Zhu, et al., 2018; Zhang et al., 2017; Zhong et al., 2007) or impairment in development to adult stage (Guizzo et al., 2017).

The role of some facultative symbionts in tick physiology was also studied. For instance, a study suggested that *Arsenophonus* sp. decreased the motility of *A. americanum*, *I. scapularis*, and *D. variabilis* and thus, impacted host-seeking success (Kagemann & Clay, 2013). Using another approach, where the functional profile of tick microbiome was depicted, Obregón et al. (2019) demonstrated that the tick microbiome contain genes involved in different metabolic pathways including carbohydrate, aminoacid, lipid and B vitamin metabolism. Interestingly, these genes were not identified in one but in different bacteria of the tick microbiome, giving the idea of the existence of functional redundancy in tick microbiome. Functional redundancy refers to the presence of the same genes and/or functional categories in different microbes. Indeed, it has been found that a single pathway can be contributed by up to 198 bacterial genera in *I. scapularis* microbiome (Estrada-peña et al., 2020). Such functional redundancy suggest that ticks select bacteria, that will conform their microbiome, using a functional filter, in other words, ticks modulate their microbiome selecting multiple bacteria that contribute to a functional profile.

The influence of the microbiota on mosquito physiology was also studied. Unlike the ticks, mosquitoes are facultative blood feeders and do not appear to depend nutritionally on specific bacteria (Narasimhan et al., 2021). Nevertheless, it has been reported that microbiota can have a role on mosquito nutrition, specifically, in blood digestion. For instance, it has been demonstrated that *Enterobacter* sp. and *Serratia* sp., two bacteria that reside in the *A. aegypti* mosquito midgut, have hemolytic activities (Gaio et al., 2011). In *A. albopictus*, *Acinetobacter baumannii* and *Acinetobacter johnsonii* have been associated with improvement in blood protein digestion and nectar assimilation, respectively (Minard et al., 2013). Moreover, elimination of the majority of the microbiota in the midgut of female *A. aegypti* using antibiotics affected the lysis of red blood cells and slowed the digestion of blood proteins depriving mosquito from essential nutrients. Interestingly, the production of eggs was equally reduced in those antibiotic-treated mosquitoes (Gaio et al., 2011) demonstrating that microbiota has an important role on blood meal digestion and reproductive fitness on its mosquito host. Another example of the role of

mosquito microbiota was given by Coon et al. (2014), who showed that axenic (i.e., without microorganisms) *A. aegypti*, *A. gambiae* and *Aedes atropalpus* larvae failed to develop beyond the first instar. However, inoculation of *Escherichia coli* in these axenic larvae rescued development showing the critical role of the microbiota on Culicidae mosquitoes. Similarly, *A. albopictus* and *C. quinquefasciatus* requires the colonization of living bacteria in the gut for their development (Coon et al., 2016). Interestingly, it has been found that re-colonization of axenic larvae lower gut oxygen levels in the midgut (Coon et al., 2017). Thus, one explanation of how microbiota helps in mosquitoes' growth is that microbiota induces a gut hypoxia-response which could function as a growth signal via the activation of the insulin/insulin growth factor pathway and other processes with essential growth functions (Coon et al., 2017). It is noteworthy that one study showed that live bacteria were not required for the development of axenic larvae to adult mosquitoes. Indeed, these axenic larvae, maintained on sterilized agar plugs containing a high concentration of liver and yeast extract, could complete their development. However, these mosquitoes presented delayed development time and stunted growth compared to bacteria-colonized counterparts suggesting that the main role of the microbiota is to supply nutrients that are essential for mosquito development (Correa et al., 2018).

Mosquito microbiota can also modulate mosquito mating, reproduction behavior and preoviposition (Gao et al., 2020). For example, it has been demonstrated that two bacteria isolated from *C. pipiens*, *Klebsiella* sp. and *Aeromonas* sp., enhance oviposition (Díaz-Nieto et al., 2016). Genetic manipulation of the anti-*Plasmodium* immune genes in *A. stephensi* male mosquitoes decreased the bacterial loads in the midgut and reproductive organs and more interestingly, made them more attractive mates to wild-type females (Pike et al., 2017). Altogether, these results show the functional role of arthropod microbiota and its impact on vector physiology. Thus, arthropod microbiota can be used as a gate to control, for example, vector population via the modulation of the living bacteria that the vector hold.

Tripartite interactions between the vector, microbiome and transmitted pathogens

Mounting evidence suggests that the native microbes harbored by the ticks can play a critical role on the ability of colonization of pathogens within the vector or the efficiency of transmission to the mammalian host (Cirimotich, Ramirez, et al., 2011; Weiss & Aksoy, 2011; Wu-Chuang et al., 2021). In ticks, for example, the generation of a dysbiosed larvae impairs the colonization of the pathogen *B. burgdorferi* in *I. scapularis* midgut (Narasimhan et al., 2014). Specifically, these dysbiosed larvae presented an increase of the relative abundance of *Rickettsia*, *Thioclava* and *Delftia* and a decrease of relative abundance of *Aquabacterium*, *Brevibacterium* and *Novosphingobium* which resulted also in tick engorgement weights (Narasimhan et al., 2014).

Mechanistically, it was shown that the dysbiosed larvae had a decreased expression of STAT and peritrophin, a key glycoprotein scaffold of the glycan-rich mucus-like peritrophic matrix (PM) that separates the gut lumen from the epithelium (Narasimhan et al., 2014). Change in the expression of these proteins altered the tick gut PM integrity, which is essential for *B. burgdorferi* colonization in the gut epithelium and consequently, the number of epithelium-bound *B. burgdorferi* spirochetes was reduced (Narasimhan et al., 2014). This result shows how modulation in tick microbiota can impact pathogen colonization. On the other hand, it has also been reported the impact of the presence of *Borrelia* on its vector microbiota. Indeed, *B. burgdorferi* colonization increases the expression of a tick gut protein that has a Reeler domain, PIXR. Interestingly, RNA interference-mediated silencing of the gene encoding PIXR and anti-PIXR immunity in mice significantly decreased *B. burgdorferi* colonization in the tick gut (Narasimhan et al., 2017). Mechanistically, the abrogation of PIXR resulted in alterations in the gut microbiome, metabolome and immune responses (Narasimhan et al., 2017). Specifically, the taxonomic and pathways diversity was increased and the polysaccharide biosynthesis pathways, involved in biofilm formation, was overrepresented (Estrada-Peña et al., 2020). These changes in tick-associated microbiota as a consequence of PIXR abrogation, thus, increased biofilm formation and induced alteration in the PM barrier, which resulted in detrimental effects on *B. burgdorferi* colonization. These results, thus, suggest that *B. burgdorferi* induces PIXR, which inhibits the overexpression of bacterial biofilm and maintains the integrity of the PM barrier to enhance its colonization in the tick (Narasimhan et al., 2017). Moreover, it has been reported that the presence of *Borrelia* in ticks is associated with greater alpha diversity (Sperling et al., 2020) and presented a significance difference in the beta-diversity of their microbiota (Landesman et al., 2019) compared to *Borrelia*-negative ticks. However, two recent epidemiological studies (Brinkerhoff et al., 2020; Chauhan et al., 2020) did not find significant changes in the overall diversity or richness of the microbiome of *Borrelia*-infected *I. scapularis* but they revealed significant associations between the persistence of *B. burgdorferi* and the occurrence of specific microbial taxa in *I. scapularis* microbiota. These results suggest that *B. burgdorferi* requires a specific gut microbial composition for successful pathogen colonization in the vector (Brinkerhoff et al., 2020; Chauhan et al., 2020).

Another example of the influence of a pathogen to manipulate vector microbiota for their own benefit is given by the obligate intracellular bacterium *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis. Contrary to *Borrelia* who needs an intact PM integrity, *A. phagocytophilum* requires a thin and permeable PM for successful colonization (Abraham et al., 2017). Indeed, it was demonstrated that *A. phagocytophilum* induces the tick to express *I. scapularis* antifreeze glycoprotein (IAFGP), which in turn, alters bacterial biofilm formation.

Moreover, IAFGP perturbed tick gut microbiota, which influenced integrity of the peritrophic matrix and gut barrier facilitating gut colonization by this bacterium (Abraham et al., 2017). These results showed the existence of a functional link between the pathogen, the tick and its microbiota.

In mosquitoes, interactions of the vector, its microbiota and the pathogen were also documented. Mosquito gut microbiota has been regarded as an important player in defense mechanism and is considered as a major 'immunity organ' against pathogen infections (Maitre et al., 2022; Saraiva et al., 2016). For instance, a higher bacterial load in *Anopheles* mosquitoes was associated to a lower infection of *Plasmodium falciparum* (Cirimotich, et al., 2011; Dong et al., 2009; Pumpuni et al., 1993). Furthermore, specific bacterial taxa were also associated to different outcome of pathogen infection in mosquitoes. For example, it has been shown that gram-negative bacteria have the most associations with the *Plasmodium* parasite while gram-positive bacteria had no prominent effect on the development of malaria infection (Cirimotich, Ramirez, et al., 2011; Tchioffo et al., 2013). Indeed, some species of *Enterobacter*, *Escherichia*, *Serratia* and *Pseudomonas*, that can be found in *Anopheles* mosquitoes, can significantly reduce the intensity and prevalence of human and rodent malaria infection (Cirimotich et al., 2011). The presence of the commensal Enterobacteriaceae correlates positively with *Plasmodium* infection indicating that Enterobacteriaceae favors *P. falciparum* infection (Boissière et al., 2012). A specific strain of *Serratia marcescens*, isolated from a laboratory *A. aegypti* strain, facilitates arboviral infection (Wu et al., 2019).

The mechanisms used by members of the microbiota to modulate pathogen infection in mosquitoes are diverse. For example, an *Enterobacter* bacterium, isolated from wild mosquito population, can produce a short-lived anti-*Plasmodium* molecule, like reactive oxygen species (ROS), which in high concentrations can significantly reduce *Plasmodium berghei* (the agent of rodent malaria) intensities *in vitro* (Cirimotich et al., 2011). The bacterium *Asaia* sp. can act as an immune-modulator activating antimicrobial peptide expression in *A. stephensi* in presence of *P. berghei* (Capone et al., 2013). Furthermore, it has been showed that *S. marcescens* strain Y1, isolated from the gut of field-caught *Anopheles sinensis*, can modulate the immunity-related *Plasmodium* effector genes such as *TEP1* and *FBN9* which resulted in *Plasmodium* development inhibition (Bai et al., 2019). Moreover, several strains of *Serratia* spp. secrete serralyisin proteins and prodigiosin, which have a pathogen-killing effect *in vitro* (Castro, 1967; Welch, 1991). On the other hand, the bacterium *Asaia bogorensis* are able to remodel *A. stephensi* glucose metabolism in a way that increases midgut pH, resulting in the promotion of *P. berghei* gametogenesis (Wang et al., 2021).

Altogether, these results show the critical role that the native microbes of the vector have on pathogen infection. Therefore, vector microbiome manipulation can be used to disrupt and/or block the pathogen life cycle within the vector. Nevertheless, as specific bacteria can have positive or negative effects on pathogen colonization, the choice of which bacteria to tackle during microbiome manipulation should be considered with precaution to avoid enhancing effects on pathogen development.

Microbial ecology

Vector microbiota are formed by a vast group of bacteria and as these bacteria are not isolated, they can interact or associate with each other, directly or indirectly. The standard analysis of microbiota involves the search of bacteria that differ in abundance between different conditions. However, this approach does not allow the study of the existing associations between bacterial members of the microbiota (Röttjers & Faust, 2018). This inconvenient prompted the search of new tools for studying the microbial communities.

In this context, microbial ecology has emerged as a field for the study of the interactions of the microorganisms among each other and with their environment. The increasing use of high-throughput sequencing in the past couple of decades has allowed researchers to make progress in this field (Fuhrman, 2009). Disentangling microbe-microbe interactions is an essential step toward the understanding of the functional role of the microbiome or the impact of the microbiota on vector physiology and vector competence (Wu-Chuang, Obregon, et al., 2022).

Microbial co-occurrence networks

A network is defined as temporary or spatial snapshots of any collection of units potentially interacting as a system and is represented by a set of nodes connected by edges (Proulx et al., 2005). In microbial networks, nodes represent bacterial taxa while edges indicate positive or negative correlations between two taxa (Berry & Widder, 2014; Wu-Chuang, Obregon, et al., 2022). Microbe-microbe interactions can be analyzed using microbial network analysis, which allows the exploration of co-occurrence patterns among members in a highly complex microbial community (Faust & Raes, 2012; Fuhrman, 2009). These co-occurrence patterns shows how particular organisms in a community sometimes or never occur together under a certain condition and can reveal interesting community assembly rules (Fuhrman, 2009). The co-occurrence networks are inferred statistically and a wide range of methods (e.g., distance- and

correlation-based techniques) are available for the construction of microbial networks, with varying levels of efficiency and accuracy (Beiko et al., 2018; Wu-Chuang, Obregon, et al., 2022).

Depending on the type of data available, two types of networks can be constructed: directed and undirected networks (Figure 13). Undirected networks, the type of networks used in the present thesis, are inferred from cross-sectional datasets that do not contain temporal information; thus, the order of samples cannot be known (Röttjers & Faust, 2018). Therefore, undirected networks possess edges that do not have a directionality. In microbial ecology, directionality refers to whether one species affects another species, is affected by other species or both (Röttjers & Faust, 2018). Thus, positive correlations in undirected networks represent cooperation (e.g., transfer of complementary metabolites or quorum sensing) whereas negative correlations represent antagonism (competition for a limiting resource or direct interference) between two taxa (Berry & Widder, 2014; Wu-Chuang, Obregon, et al., 2022). On the other hand, directed networks are mostly inferred from time series datasets where the order of the samples is used to compute the associations (Röttjers & Faust, 2018). Directed networks are those where the edges have a directionality. Thus, depending on the direction of the association, it is possible to differentiate, for example, mutualism from commensalism in positive correlations or amensalism from competition in negative correlations (Röttjers & Faust, 2018).

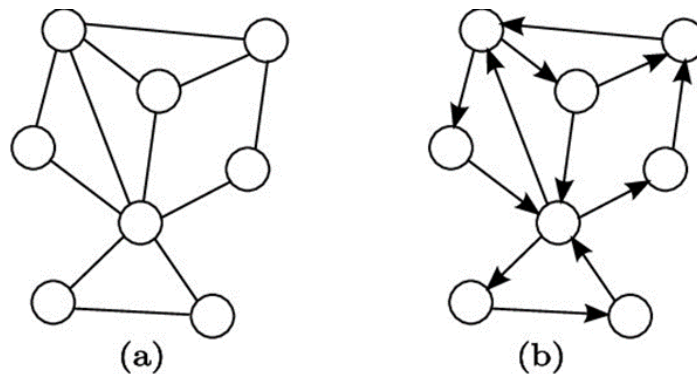


Figure 13. Visualization of an undirected (a) and directed (b) networks. Figure from Fionda & Palopoli, 2011.

Properties of microbial co-occurrence networks

Besides the associations, one of the strength of biological networks is their ability to represent emergent properties (Röttjers & Faust, 2018). Emergent properties are those that are exhibited by the system as a whole and cannot be predicted if only part of the network is analyzed on their own (Aderem, 2005). These emergent properties may explain behaviors of a complex system, such as their robustness or modularity. The robustness is an *in silico* property that refers

to a network's vulnerability to random or targeted node removal; vulnerable networks breaks up in smaller parts as a consequence of node removal (Röttjers & Faust, 2018). Network robustness may be a diagnostic tool for prediction of ecological robustness, i.e., the resilience of an ecosystem facing fluctuations in the behavior of its member species or its environment (Mumby et al., 2014; Röttjers & Faust, 2018). Modularity quantifies to what extent networks can be broken up into smaller components called modules (Röttjers & Faust, 2018). Thus, modules in a microbial network represent a set of nodes that have strong interactions between each other but sparse connections to nodes from other modules (Aderem, 2005). While the source of modularity in a microbial network is not entirely clear, it has been suggested that the modules may represent different niches that share a common functional role (Guidi et al., 2016) or niches that share a common habitat (Cram et al., 2015). Another property of microbial networks is the global clustering coefficient which quantifies the extent to which nodes cluster together (Röttjers & Faust, 2018). It has been suggested that this property may display cross-feeding and high global clustering coefficient might be indicative of degradation pathways or niche filtering, however, to really make biological interpretation of this property, more information about bacterial interactions is needed (Röttjers & Faust, 2018). A representative image of these properties is represented in Figure 14A.

Identification of keystone taxa

In addition to bacterial associations, microbial co-occurrence network analysis offers the possibility to statistically identify keystone taxa (Berry & Widder, 2014). Although there is not a consensus on which metrics are best for their identification, it is clear that keystone taxa are highly influential bacteria on the microbial community structure and functioning (Agler et al., 2016; Banerjee et al., 2018; Layeghifard et al., 2017) and their removal can cause a drastic shift in the composition or functioning of a microbiome (Banerjee et al., 2016). Several studies identified these keystone taxa in microbial communities and they have been linked to important functions. For example, in humans, *Ruminococcus bromii* was identified as a keystone taxa in the gut microbiome and it has an important role for the degradation of resistant starch in the human colon (Ze et al., 2012). In soil microbiome, *Acidobacteria*, *Frateuria* and *Gemmatimonas* in bacteria and *Chaetomium*, *Cephalotheca* and *Fusarium* in fungi were identified as keystone taxa, which have been linked to organic matter decomposition (Banerjee et al., 2016). In plant communities, nitrogen-fixing rhizobia have been proposed as keystone taxa since their abundance can improve plant productivity and community evenness (Van Der Heijden et al., 2006). In ticks, the depletion of a identified keystone taxa impacted tick performance during feeding (Mateos-Hernández et al., 2020). All these studies demonstrate the impactful role of

keystone taxa on their host; thus, identification of these taxa can help in a better understanding of the functioning of a complex system and can be suitable target for the modulation of the microbiome towards a desired state.

To identify these keystone taxa, network-based centrality measures (i.e., degree centrality, closeness centrality, betweenness centrality, eigenvector centrality) are often used since these measures may allow ranking the nodes in a complex network and quantifying their relative importance (Mason & Verwoerd, 2007). These measures indicate different characteristics of a node, for instance, degree refers to the number of edges that a node has in a network; closeness centrality indicates how close a node is to all other nodes in the network; betweenness centrality indicates how much a given node is in-between others and eigenvector centrality measures a node's importance while giving consideration to the importance of its neighbors (Figure 14B) (Golbeck, 2013). Different studies that identified keystone taxa have used different metrics. For instance, taxa with high degree, which are termed 'hubs' have been proposed as keystone taxa. The rationality behind this idea is that 'An important node is involved in a large number of interactions due to its high number of connections' (Banerjee et al., 2018; Mason & Verwoerd, 2007). It is noteworthy that not all network hubs are keystone taxa in real life microbial communities (Agler et al., 2016; Wu-Chuang, Obregon, et al., 2022) and some authors have questioned the suitability of this measure for the identification of keystone taxa (Banerjee, Schlaeppli, et al., 2019; Banerjee, Walder, et al., 2019; Röttjers & Faust, 2018). On the other hand, some studies (Banerjee et al., 2016; Jiang et al., 2017) highlighted as keystone taxa those with high betweenness centrality using the rationality that 'An important node will lie on a high proportion of paths between other nodes in the network' (Banerjee et al., 2016; Mason & Verwoerd, 2007). Others, however, suggest that keystone species tends to have a combination of low betweenness centrality values, high mean degree and high closeness centrality (Banerjee, Schlaeppli, et al., 2019; Banerjee, Walder, et al., 2019; Berry & Widder, 2014). In summary, there is not a consensus nor a guideline for the better measures to be taken into account for the identification of keystone taxa. The choice of the method or metrics used for the identification of keystone taxa should be done based on the aims of each study and predicted network keystone species should be accompanied by experimental validation to uncover their biological importance (Banerjee, Schlaeppli, et al., 2019; Banerjee, Walder, et al., 2019; H. Zheng et al., 2021). Experimental validation can be achieved by comparing the impact of the removal or addition of keystone candidate on the functioning and dynamics of microbial community (Röttjers & Faust, 2019; Ze et al., 2012).

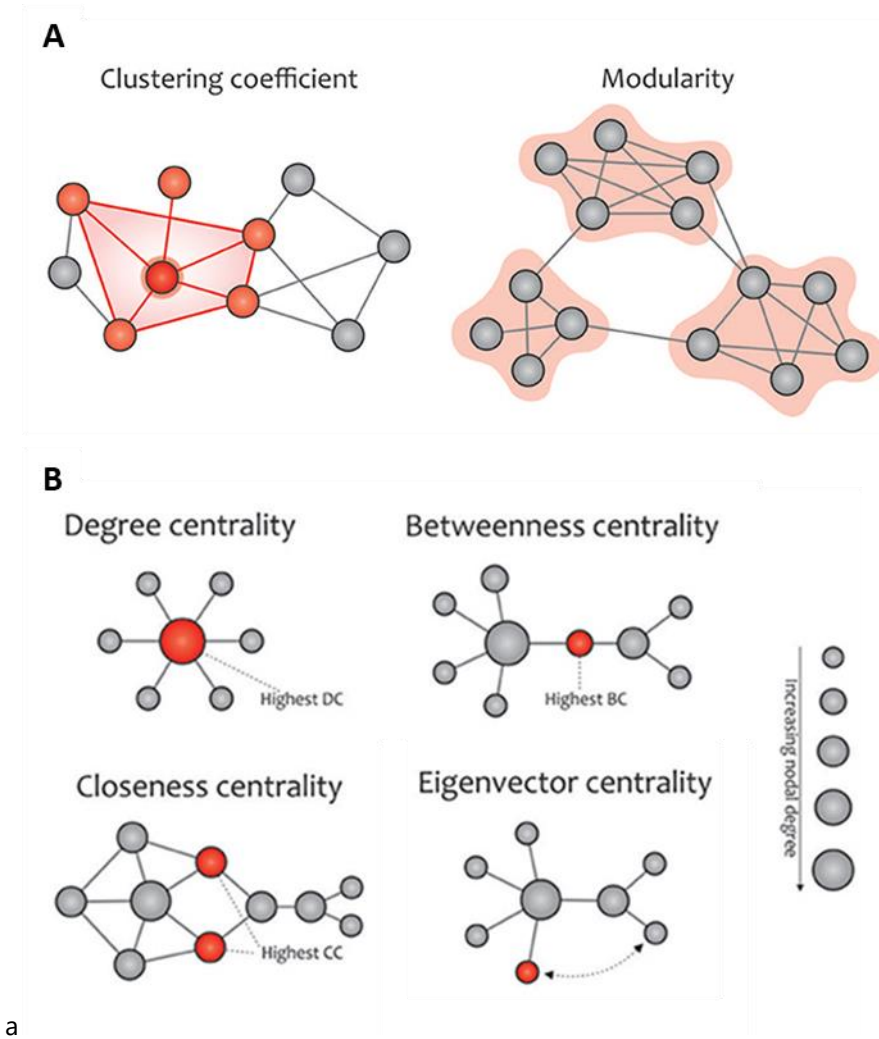


Figure 14. Graphical representation of global properties (A) and centrality measures (B) of a network. Figure modified from Farahani et al., 2019.

Control of vector-borne diseases

Methods to control vector and vector-borne diseases

The control of vector-borne diseases is one of the major challenges faced by the humanity. Indeed, the rapid and uncontrolled urbanization has intensified the concern in resolving these problems. Several methods, with variable effectiveness, have been developed and used for reducing vector populations and controlling VBDs. These methods are described below:

Acaricides

The majority of current vector control tactics rely heavily on the use of synthetic chemical acaricides (Benelli, 2022; Richardson et al., 2022). The major classes of conventional acaricides include organochlorines, synthetic pyrethroids, organophosphates, amidines, phenylpyrazoles, insect growth regulators and macrocyclic lactones (Rodriguez-Vivas et al., 2018). Each of these compounds have different mechanism of action but virtually, all acaricides are neurotoxins (Taylor, 2001). For more detailed mechanism of action of each group of acaricides, the reader is referred to Taylor, (2001). The popular use of acaricide rely on its general high effectiveness for control of arthropods, although each of them present different efficacies. For example, it was reported that within the macrocyclic lactones, milbemycin (94.84–100%) was the most effective, followed by spinosad (93.21–100%) and the avermectins (81.34–100%) for the control of the tick *Rhipicephalus microplus* (vector of pathogens such as *Babesia bovis*, *B. bigemina* and *Anaplasma marginale*). The phenylpyrazole group presented similar efficacy (99.90%) to the macrocyclic lactones while the amidine presented a lower efficacy (51.35–100%) followed by synthetic pyrethroids + organophosphate associations (68.91–81.47%). Finally, the synthetic pyrethroids (SPs) acaricides were the least effective (48.35–76.84%) for the control of *R. microplus* (Brito et al., 2011). The efficacy of acaricides was tested also in *I. scapularis* ticks and it was found that a single application of acaricide on residential properties could result in the reduction by 68-100% of host-seeking *I. scapularis* nymphs. (Hinckley et al., 2016).

A number of control routes has also been used to manage mosquitoes' populations, including classic use of chemical pesticides, long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (Benelli, 2022). LLINs are bed-nets that were pre-treated with a safe, residual insecticide for the purpose of killing and repelling vector mosquitoes and their effectiveness remains for multiple years without retreatment. (Lengeler, 2004; Ng'ang'a et al., 2021). IRS consist of spraying residual insecticides on the walls and other surfaces of a house with the aim of killing vector mosquitoes that rest on the sprayed surface after their blood-meal. Thus, IRS does not prevent people from being bitten by mosquitoes, but rather prevent the transmission of vector-borne pathogens from an infected mosquitoes to a naïve host (Prevention, 2019). The

importance of the use of these methods are reflected in the observed global decline in malaria cases since 2000 (Bhatt et al., 2015). Notably, it was reported that, in sub-Saharan Africa (region which accounts for more than 90% of malaria global cases), the use of LLINs reduced malaria incidence by 50% (Lengeler, 2004) and malaria mortality rates by 55% in children aged under 5 years (Eisele et al., 2010).

Although acaricides show effectiveness for control vector population, various detrimental effects have been associated to them. The repeated applications over time have led unavoidably to the overexposure to their active ingredients, which resulted in the development of acaricide resistance in many important medical and veterinary parasites (Benelli et al., 2021; Hemingway et al., 2016; McNair & Carol McNair, 2015). In fact, resistance to the acaricide has been observed in more than 600 species of arthropods (Bass & Field, 2011). Furthermore, these chemical products, mostly applied through environmental spraying/nebulization, are highly persistent in the environments and remaining traces of these compounds can be found in the food chain (Beugnet & Franc, 2012). Additionally, they present lethal and potential sub-lethal effects on non-target species (e.g., honey bees) (Desneux et al., 2006). All these concerning negative effects have prompted the search of new, safer and environmental-friendly control methods.

Integrated control strategy

The integrated control strategy (ICS) consists in the systematic combination of at least two compatible control methods (e.g., combined use between acaricides, entomopathogenic fungus, vaccines among others). Existing vaccines against vectors will be discussed in the next section. This ICS strategy was created in order to reduce the selection pressure that favor acaricide-resistant individuals (Rodríguez-Vivas et al., 2014), to decrease the intensity of tick infestation and the use of chemicals and acaricides and to lengthen the period between acaricide applications (Obregón-Alvarez et al., 2020). The ICS has been widely used, notably in the control of the cattle tick *R. microplus*. For instance, it was reported that the combined use of deltamethrin, a synthetic pyrethroid acaricide, and *Metarhizium anisopliae*, an entomopathogenic fungus induce the mortality of 30.92% of *R. microplus* ticks in cattle infested experimentally (Bahense et al., 2008). Another study reported that an integrated system employing vaccination against the antigen Bm86 (a protective tick antigen) and amidine treatments controlled near 100% of *R. microplus* populations resistant to pyrethroids and organophosphates (Redondo et al., 1999). Furthermore, the joint application of acaricides and the vaccine anti-Bm86 has reduced the times/year of use of acaricides and reduced significantly the level of tick infestation in Mexico (de la Fuente et al., 2007). The same combination (vaccine anti-Bm86+acaricide) reduced 87% the use of acaricides in Cuba and interestingly, it reduced

significantly the number of clinical cases of babesiosis (a VBD transmitted by *R. microplus*) (Rodriguez Valle et al., 2004).

In mosquitoes, the ICS is based on the environmental management, which consist of a broad range of measures designed to reduce mosquitoes populations and subsequently, the risk of disease transmission (Becker et al., 2010). The ICS in mosquitoes can include also the use of acaricides to get a successful result (*Success in Mosquito Control: An Integrated Approach | US EPA*). Several tactics are included in the integrated mosquito control strategy: (1) environmental manipulation of vector habitats in order to produce conditions unfavourable to vector breeding (e.g., changing aquatic habitats where the vectors develop, drain temporary pools of water); (2) changes to human habitation using structural barriers at the house (e.g., windows and door screens, nets) in order to reduce the reduce the contact between human/vector/pathogen and (3) control of mosquitoes at different developmental stage using larvicides to eliminate the immature stages or adulticides to eliminate emergent adult mosquitoes. (Becker et al., 2010; *Success in Mosquito Control: An Integrated Approach | US EPA*). It was reported that application of these measures for 3-5 years has declined by 70-95% malaria-related mortality and morbidity (Utzing et al., 2001). Altogether, these results show the advantage of using an integrated control strategy. Nevertheless, it still involves the repetitive use of acaricides meaning that vector resistance development and ecological damages are still an outcome. Furthermore, the use of ICS is threatened by poor management practices and relaxing of control measures. Therefore, the search for alternatives methods with long-lasting effect and safe methods for human and animal is still a need. One of the alternative methods that was developed is the vaccines.

Vaccines

The development of vaccines has had a great impact on improving human or animal health since they can induce long-lasting protective immune responses (de la Fuente et al., 2017). Vaccines targeting vector proteins, or non-protein antigens, expressed by vector-borne pathogens during infection in the host have been used as an alternative for vector and/or pathogen control (de la Fuente et al., 2017; Manning & Cantaert, 2019).

Vaccines for the control of vector populations

Vaccines for the control of vector arthropods, such as ticks or mosquitoes are based in the use of host antibodies to target vectors proteins essentials for their development or reproduction. For example, vaccination of bovines with the glycoprotein Bm86, the first protective tick antigen located in the membrane of tick gut cells (Gough & Kemp, 1993), induced the expression of antibodies anti-Bm86 in the cattle (Willadsen, 1997). Once these host antibodies are ingested

by the ticks during feeding, they bind to the surface of epithelial cells in the tick intestine causing cell lysis via antibody-mediated mechanisms of host defense, and provoking a reduction in *R. (Boophilus) microplus* tick number, weight, and reproductive capacity (Kemp et al., 1989). Another example is the vaccine against the dog tick *Rhipicephalus sanguineus* that use a peptide of 20 amino acids from the ribosomal protein P0 of *Rhipicephalus* ticks (pP0) as antigen (Rodríguez-Mallon et al., 2015). The Po protein is essential for the assembly of the 60S ribosomal subunit and is localized in the cell cytoplasm. Targeting Po in ticks by host antibodies leads to inactive ribosomes for protein synthesis and to the cell death (Jishu et al., 2002) causing a significant diminution in the number and weight of engorged females and eggs as well as a decreased viability of newly molted nymphs from larvae fed (Rodríguez-Mallon et al., 2012, 2015). A non-exhaustive list of vaccines targeting vector population are available in Table 2. In mosquitoes, the development of vaccines was mostly focused on targeting the transmission of mosquito-borne pathogens, rather than targeting mosquitoes' population and will be discussed in the next section.

Table 2. Example of vaccines that target vector population (Table adapted from de la Fuente et al., 2017; Zhang et al., 2011; Anisuzzaman et al., 2012)

Vaccine antigens	Vaccinated hosts	Targeted vector species
<i>Rhipicephalus</i> spp. BM86/BM95	cattle, camel, dog, deer	<i>Rhipicephalus microplus</i> , <i>Rhipicephalus annulatus</i> , <i>Rhipicephalus decoloratus</i> , <i>Rhipicephalus sanguineus</i> , <i>Hyalomma dromedarii</i>
<i>R. microplus</i> Metalloprotease	cattle	<i>R. microplus</i>
<i>R. microplus</i> Ribosomal protein P0	cattle	<i>R. microplus</i>
<i>I. ricinus</i> , <i>R. microplus</i> Ferritin 2	cattle, rabbit	<i>R. microplus</i> , <i>R. annulatus</i> , <i>I. ricinus</i>
<i>I. ricinus</i> , <i>R. microplus</i> Aquaporin	cattle, rabbit	<i>R. microplus</i> , <i>I. ricinus</i>
<i>Haemaphysalis longicornis</i> acid phosphatase (HL-3)	rabbit	<i>Haemaphysalis longicornis</i>
<i>H. longicornis</i> Longistatin	mice	<i>Haemaphysalis longicornis</i>

Transmission-blocking vaccines (TBVs)

Transmission-blocking vaccines are designed to block the transmission of pathogens between the vector and the host by targeting vector proteins that are essential for pathogen transmission or pathogen proteins expressed within the vectors (Maitre et al., 2022; Neelakanta & Sultana, 2015). For example, active immunization of mice with Salp15, an immunosuppressive tick protein used by *B. burgdorferi* to escape from host immunity during transmission from tick to host, showed significant protection (60%) of mice from acquisition of the pathogen (Dai et al., 2009). Similarly, immunization of mice with Salp25D, a tick-protein with antioxidant properties that protect *B. burgdorferi* from reactive oxygen species during transmission from host to tick, reduced the acquisition of spirochetes by ticks to threefold in comparison to the non-immunized controls (Narasimhan et al., 2007). An additional study showed that immunization of mice with tick histamine release factor, a protein expressed during the feeding phase of the tick, reduced the efficiency of tick feeding and 20-30% of immunized mice were protected from *B. burgdorferi* transmission (Dai et al., 2010). Others vaccines have targeted antigens expressed by the pathogen. For example, BBA52, an outer membrane surface-exposed protein that is preferentially expressed by *B. burgdorferi* in the feeding tick and thus, during transmission (Kumar et al., 2010), was selected as a possible target for blocking transmission of *B. burgdorferi*. Immunization of mice with BBA52 significantly decreased *B. burgdorferi* transmission from ticks to naïve hosts (Kumar et al., 2011). Moreover, it was shown that BBA52 antibodies blocks spirochete transmission by binds to pathogen surface rather than triggering a bactericidal mechanism (Kumar et al., 2011). Another vaccine targeting OspA, an outer surface protein of *B. burgdorferi*, was able to protect immunized mice from challenge with several strains of *B. burgdorferi* (Fikrig et al., 1990). A list of antigens considered for the formulation of vaccines to block tick-borne pathogens is available in Table 3.

Table 3. Transmission-blocking vaccine for tick-borne pathogens (Table adapted from de la Fuente et al., 2017).

Vaccine antigens	Vaccinated hosts	Targeted vector species	Targeted vector-borne pathogen
<i>R. microplus</i> Silk	cattle	<i>R. microplus</i>	<i>Anaplasma marginale</i>
<i>R. microplus</i> TROSPA	cattle	<i>R. microplus</i>	<i>Babesia bigemina</i>

Tick spp. Subolesin and Subolesin/Akirin chimeras	cattle, sheep, deer, rabbit, mouse	<i>R. microplus</i> , <i>R. annulatus</i> , <i>Ixodes scapularis</i> , <i>Ixodes ricinus</i> , <i>Hyalomma</i> spp., <i>Haemaphysalis</i> spp., <i>Amblyomma americanum</i> , <i>Dermacentor variabilis</i> , <i>Dermacentor reticulatus</i>	<i>Anaplasma phagocytophilum</i> , <i>Anaplasma marginale</i> , <i>Borrelia burgdorferi</i> s.l., <i>Babesia bigemina</i>
<i>I. scapularis</i> salivary protein (Salp) Salp15	mouse	<i>I. scapularis</i>	<i>Borrelia burgdorferi</i> s.l.
<i>I. scapularis</i> Salp25D	mouse	<i>I. scapularis</i>	<i>Borrelia burgdorferi</i> s.l.
<i>I. scapularis</i> TROSPA	mouse	<i>I. scapularis</i>	<i>Borrelia burgdorferi</i> s.l.
<i>I. scapularis</i> tick Histamine release factor (tHRF)	mouse	<i>I. scapularis</i>	<i>Borrelia burgdorferi</i> s.l.
<i>I. scapularis</i> tick Salivary lectin pathway inhibitor (TSPI)	rabbit	<i>I. scapularis</i>	<i>Borrelia burgdorferi</i> s.l.
<i>R. appendiculatus</i> Serpins (RAS)	cattle	<i>R. appendiculatus</i>	<i>Theileria parva</i>
<i>R. appendiculatus</i> cement protein 64P	guinea pig, hamster, rabbit, mouse	<i>R. appendiculatus</i> , <i>I. ricinus</i>	Tick-borne encephalitis virus
<i>B. burgdorferi</i> s.l. outer surface proteins (OspA/OspC)	mouse, hamster, dog, monkey, wild white-footed mouse, human		<i>Borrelia burgdorferi</i> s.l.
<i>B. burgdorferi</i> s.l. BBI36/BBI39	rodent		<i>Borrelia burgdorferi</i> s.l.

In mosquitoes, the majority of licensed vaccines for the control of mosquito-borne diseases target the pathogen as is typically done with vaccines for pathogens transmissible via respiratory secretions, fecal-oral exchange, or other bodily secretions (Manning & Cantaert, 2019). A list of these vaccines and their status can be found in Manning & Cantaert, 2019. Nevertheless, the development of transmission-blocking vaccines for mosquito-borne diseases was also considered. For example, TBVs for malaria are using as antigens proteins that are expressed specifically in the sexual or sporogonic stages of *Plasmodium* with the aim of blocking their development inside the mosquito, thereby reducing mosquito infectivity and prohibiting the spread of the disease (Carter et al., 2000). Pfs48/45, Pfs230 and Pfs25 are currently lead candidates for the development of TBVs (Chowdhury et al., 2009; Kapulu et al., 2015). Pfs48/45 and Pfs230 are proteins expressed on the surface of gametocytes and gametes of malaria parasites (Sauerwein & Eling, 2002) whereas Pfs25 is an antigen expressed on the surface of zygote and ookinete (Saxena et al., 2007). Interestingly, vaccination against these three proteins suppress effectively both oocyst burden and percentage of mosquitoes infected by *P. falciparum* gametocytes in *Anopheles* mosquitoes (Chowdhury et al., 2009; Kapulu et al., 2015). On the other hand, candidates for TBVs against arboviruses are mostly proteins from mosquitoes' machinery used by the virus for their life cycle within the host (Bakhshi et al., 2018). Other candidates considered for TBVs are mosquito saliva proteins (Bakhshi et al., 2018). Mosquito saliva contains complex mix of proteins that can facilitate transmission of mosquito-borne pathogens (Bakhshi et al., 2018; Schneider & Higgs, 2008). For example, the role of *A. gambiae* TRIO (AgTRIO), a mosquito salivary gland antigen, on the protection against *Plasmodium* infection was tested (Dragovic et al., 2018). It was shown that administration of AgTRIO antiserum to mice reduced *Plasmodium* liver burden and decreased parasitemia when exposed to infected mosquitoes. Active immunization with AgTRIO also shows a protective effect against *P. berghei* infection, however, this protection response was only partial (Dragovic et al., 2018).

In summary, vaccines represent a suitable alternative method for the control of VBDs. However, these vaccines have been controversial due among other limitations to the lack of complete protection against pathogen infection. Indeed, with the exception of yellow fever virus (YFV) and Japanese encephalitis virus (JEV), vaccine development for vector-borne diseases has been challenging (Shaw & Catteruccia, 2019). In fact, YFV and JEV are the only vaccines on the WHO-approved list of vector-borne vaccines for use without caveat (Manning & Cantaert, 2019). With a single immunization, the vaccine for YFV confers lifelong protective immunity in over 90% of vaccinated individuals (Gotuzzo et al., 2013). On the other hand, malaria vaccine that has now been made available, requires 3 to 4 doses and yields only 36% efficacy that wanes over time (Olotu et al., 2016). Similarly, a vaccine was also recently approved for dengue virus but with the

restriction that it can be only used in dengue-experienced persons over the age of 9 years old in hyperendemic areas. Therefore, much work remains to be done to improve vaccine candidates for blocking VBDs and the search for new strategies with better outcome is still required.

Microbiota manipulation as a mean for the control of vector-borne diseases

Vector microbiota may hold the key to vector-borne pathogen control, as mounting evidence show the impactful role that vector microbiota can have on vector physiology and pathogen life cycle (Wu-Chuang et al., 2021). Several strategies for microbiome manipulation were attempted and the current methods are based on the introduction of microorganisms with engineered or defined roles to block pathogen transmission. Most of these methods was described for mosquitoes and will be commented in the next section. Additionally, a new emerging tool called “anti-microbiota vaccine” will be presented at the end.

Paratransgenesis

Paratransgenesis is the genetic engineering of bacteria that can produce antipathogen effector molecules (Gao et al., 2020). For example, *Escherichia coli* was designed to express either a single-chain immunotoxin, or compounds such as salivary gland and midgut peptide 1 (SM1) or phospholipase-A2 to block *Plasmodium* development in the mosquito midgut (Riehle et al., 2007; Yoshida et al., 2001). Another example is *Pantoea agglomerans* strain, which was used to express five different antimalarial proteins at the same time, resulting in a significant suppression of the human malaria parasite *P. falciparum* and rodent malaria parasite *P. berghei* by up to 98%. Interestingly, *P. agglomerans*, unlike *E. coli*, is a common mosquito symbiotic bacterium symbiont and therefore, can persist longer in the mosquito gut (Wang et al., 2012). Another mosquito symbiont, *Asaia*, was used to conditionally express the antiplasmodial protein scorpion only when the mosquito is blood feeding which resulted in a significant inhibition of pathogen infection. The advantage of this method is the improvement of the bacterium fitness compared to strains that constitutively express the antiplasmodial effector, which may allow a longer survival and transmission through mosquito populations (Shane et al., 2018). Paratransgenesis are particularly suitable for vectors that have an outdoor biting behavior (which make strategies such as long-lasting insecticide-treated nets and indoor residual spraying less effective on them). However, a major inconvenient of paratransgenesis is their delivery method to natural mosquito populations (Shaw & Catteruccia, 2019). In fact, one central question in using paratransgenesis is how to ensure transgenic bacteria to disseminate and perpetuate in mosquito field populations. One strategy is using bacteria that can be transmitted vertically or horizontally. For

example, Wang et al., (2017) engineered a *Serratia* bacterial strain AS1, found in *Anopheles* ovary, to express antimalarial effectors and showed that it can successfully reduce *P. falciparum* transmission by mosquitoes. Interestingly, this particular strain is able to colonize the mosquito midgut and their reproductive organs, which facilitate its vertical and horizontal transmission within mosquito populations.

Wolbachia

The Intracellular bacterium *Wolbachia* has been used for the control of vector populations and for the control of vector-borne pathogens (Gao et al., 2020; Shaw & Catteruccia, 2019). *Wolbachia* infects 66% of the insect species in the world; it can be found naturally in some mosquitoes species such as *C. pipiens*, *A. albopictus*, and *A. gambiae* (Wilke & Marrelli, 2015) and it can be transmitted transovarially as they populate the female germline (Bian et al., 2013). In China, the release of *Wolbachia*-infected mosquitoes reduced drastically the *A. albopictus* field population (Zheng et al., 2019). The mechanism by which *Wolbachia* regulates vector population is via cytoplasmic incompatibility (CI). In CI, the mate between *Wolbachia*-infected males and uninfected females produce embryonic lethality of progeny, whereas the mating of infected males to infected females yields viable progeny (Gao et al., 2020). Thus, the release of a large group of *Wolbachia*-infected males sterilize the wild females causing population reduction (Shaw & Catteruccia, 2019). Besides restricting mosquito populations, *Wolbachia* can impact also pathogen transmission. For example, experimental introduction of the strain wMelPop of *Wolbachia* in *A. aegypti* directly inhibits the ability of chikungunya, dengue and West Nile viruses as well as *Plasmodium gallinaceum* (causal agent of avian malaria) to infect the mosquito (Hussain et al., 2013; Moreira et al., 2009). These effects were possibly mediated by the activation of mosquito innate immune system and by the competition for limiting resources that are required by the pathogen for its replication (Moreira et al., 2009). An additional study showed that the presence of *Wolbachia* within *A. aegypti* was associated with the resistance of Zika virus infection in the mosquitoes (Dutra et al., 2016). Interestingly, *Wolbachia* is not found in natural conditions in *A. aegypti* (Moreira et al., 2009). This suggests that introduction of uncommon members of the microbiome can disrupt potential co-evolution between pathogens and the microbiota (Maitre et al., 2022). It is noteworthy that it has been reported that certain strains of *Wolbachia* can facilitate the transmission of certain pathogens. For instance, the introduction of *Wolbachia* wMel to *A. aegypti* in Brazil and Vietnam increased the mosquito susceptibility to dengue infection (King et al., 2018). Another example was reported for *Wolbachia* wAlbB strain, who enhanced West Nile virus infection in *C. tarsalis* mosquitoes via downregulating the Toll immune pathway (Dodson et al., 2014). In summary, the use of *Wolbachia* seems a useful tool

for the control of vector-borne pathogens. However, the exact mechanism underlying the caused effects are not fully understood. Considering that some strains of *Wolbachia* can increase the transmission of some pathogen, it is strongly necessary a deeper understanding of the molecular mechanisms mediating the *Wolbachia*–pathogen interactions for an effective and safe use of this bacterium.

Fungi

Another method proposed for the control of pathogen transmission through microbiota manipulation is the use of fungi. For instance, two fungi, *Beauveria bassiana* and *Metarhizium anisopliae* are able to infect anopheline mosquitoes which causes progressive mosquito death (Bukhari et al., 2011; Scholte et al., 2005). Mechanistically, it was shown that *B. bassiana* generated a cross-kingdom microRNA-like RNA (bba-milR1) that can attenuate mosquito immunity and accelerates mosquito death (Cui et al., 2019). Interestingly, it has been also reported that *B. bassiana* modulate mosquito microbiota, which induced a down-regulation of antimicrobial peptides and dual oxidase in the midgut. These changes increased gut bacterial loads and caused the overgrowth of the opportunistic pathogen *S. marcescens* that translocated from the gut to the hemocoel, thus promoting mosquito death (G. Wei et al., 2017). Moreover, fungi can also be engineered to produce anti-pathogen effectors. For example, *M. anisopliae* was designed to produce SM1 and scorpine to block *Plasmodium* transmission and interestingly, it was shown that this method could reduce significantly the sporozoite. On the other hand, it has been reported that some fungus can have the contrary effects on pathogen transmission. For example, the fungus *Penicillium chrysogenum*, isolated from field-caught *A. gambiae* gut, suppressed the mosquito's innate immune system which rendered the mosquito more susceptible to *Plasmodium* infection (Muturi et al., 2016). Moreover, *Talaromyces*, another fungus isolated from mosquito midgut, was shown to enhance the susceptibility of *A. aegypti* to dengue virus by modulating gut trypsin activity (Angleró-Rodríguez et al., 2017). These results show that mosquito fungi can be used as a tool for the control of VBDs, but similarly to *Wolbachia*, a deeper understanding of the mechanism underlying the tripartite microbiota-vector-pathogen interactions is needed for the selection of the adequate fungus to combat VBDs while avoiding enhancement of other pathogen transmission (Gao et al., 2020).

Anti-microbiota vaccine: a new tool for microbiome manipulation

Depleting all or selective vector microbiota from bacteria that facilitates pathogen development and/or increase vector competence could be exploited as a means for blocking transmission. Nevertheless, the lack of tools for the **precise** manipulation of the vector microbiota is currently

a major limitation to develop novel transmission-blocking strategies. Recently, anti-microbiota vaccine was developed to target specific bacterial taxa in tick microbiota (Mateos-Hernández et al., 2020). Anti-microbiota vaccine is based on the vaccination of the host to induce specific antibodies against a bacterium. Later, during the blood meal, these antibodies will be uptaken by hematophagous arthropod vectors and would target a specific bacterium in the vector microbiota (Mateos-Hernández et al., 2020). Results obtained by targeting keystone taxon using anti-microbiota vaccine in an experimental condition showed that this technique can impact *I. ricinus* performance during feeding (Mateos-Hernández et al., 2020). Specifically, Mateos-Hernández et al., (2020), using a combination of next-generation sequencing and co-occurrence network analysis, identified bacteria of the family Enterobacteriaceae as a keystone taxon. Subsequently, they immunized mice with a live vaccine containing *E. coli* strain BL21 and they observed that the weight of *I. ricinus* nymphs that fed on *E. coli*-immunized mice increased significantly compared to ticks fed on mock-immunized mice (Mateos-Hernández et al., 2020). Additionally, they found high mortality of ticks fed on mice with high levels of antibodies against the carbohydrate α -Gal, broadly present in the tick microbiota. These results showed that anti-microbiota vaccine is a promising tool for microbiota manipulation and potentially for the control of vector and VBDs (Mateos-Hernández et al., 2020).

Hypothesis and objectives

Hypothesis and objectives

Vector-borne diseases are a global concern, particularly those transmitted by ticks and mosquitoes. Despite all the methods developed to control vector populations or blocking pathogen infection, half of the world's population lives today at risk of vector-borne diseases (Shaw & Catteruccia, 2019). Therefore, the search of new approaches for controlling VBDs is still needed. Vector microbiota has an important role not only on vector fitness, but also on vector competence. Moreover, vector microbiota may harbor keystone taxa that contribute significantly in vector physiology and in the dynamics of transmission/acquisition of vector-borne pathogens. Thus, we hypothesize that:

Targeting keystone taxa of vector microbiota with host antibodies will have a cascading ecological impact reducing vector fitness and/or vector competence

To test this hypothesis, the present thesis has four objectives:

1. To identify keystone taxa in tick microbiota and evaluate their functional role in the bacterial communities under environmental stress
2. To characterize the effects of anti-microbiota vaccines on tick microbiota assembly
3. To evaluate the impact of anti-microbiota vaccine on the colonization of *B. afzelii* in *I. ricinus* ticks.
4. To evaluate the impact of anti-microbiota vaccine on the colonization of *P. relictum* in *C. quinquefasciatus* mosquitoes

Results

Chapter II

Functional role of keystone taxa in *Ixodes scapularis* ticks under environmental stress



Thermostable Keystone Bacteria Maintain the Functional Diversity of the *Ixodes scapularis* Microbiome Under Heat Stress

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Abstract

Variations in the composition and diversity of tick microbiome due to high temperatures may influence the hierarchy of community members as a response to environmental change. Modifications in the community structure are hypothesized to drive alterations in the presence and/or abundance of functional pathways in the bacterial metagenome. In this study, this hypothesis was tested by using published 16S rRNA datasets of *Ixodes scapularis* males incubated at different temperatures (i.e., 4, 20, 30, and 37 °C) in a laboratory setting. Changes in community structure and functional profiles in response to temperature shifts were measured using co-occurrence networks and metagenome inference. Results from laboratory-reared ticks were then compared with those of field-collected ticks. The results from laboratory-reared ticks showed that high temperature altered the structure of the microbial community and decreased the number of keystone taxa. Notably, four taxa were identified as keystone in all the temperatures, and the functional diversity of the tick microbiome was contained in the four thermostable keystone their associated bacterial taxa. Three of the thermostable keystone taxa were also found in free-living ticks collected in Massachusetts. Moreover, the comparison of functional profiles of laboratory-reared and field-collected ticks revealed the existence of an important set of metabolic pathways that were common among the different datasets. Similar to the laboratory-reared ticks, the keystone taxa identified in field-collected ticks alongside their consortia (co-occurring taxa) were sufficient to retain the majority of the metabolic pathways in the functional profile. These results suggest that keystone taxa are essential in the stability and the functional resiliency of the tick microbiome under heat stress.

Keywords *Ixodes scapularis* · Microbiome · Heat stress · Microbial networks · Keystone bacteria · Functional redundancy

Introduction

The tick microbiome is an important player in tick physiology and vector competence [39, 52]. Efforts have been concentrated on the characterization of the microbial diversity as well as the understanding of the factors that modulate the tick microbiota. Diverse factors such as tick species, gender, developmental stage, blood meal, and host species among others modify the composition of the tick microbiome [12,

37, 45, 49, 51, 52]. Besides these biotic factors, the tick microbiome is also sensible to abiotic factors such as soil type, humidity, season and temperature variation [19, 28, 35, 45, 48, 50, 55]. Among these, the temperature was shown to be an important environmental variable that shapes microbial composition and diversity in *Ixodes scapularis* in a laboratory setting [48]. Specifically, the study of Thapa et al. [48] showed that the incubation of *I. scapularis* in a gradient of temperature, including 4 °C, 20 °C, 30 °C, and 37 °C at a constant humidity of 80–95%, have an impact on tick survival, where only 15% of ticks survived at 30 and 37 °C after 10 days of incubation. Moreover, they reported that incubation of ticks at high temperatures (30 and 37 °C) induces large changes in taxonomic composition and decreases bacterial diversity compared to ticks incubated at lower temperatures (4 and 20 °C).

Despite advances in the characterization of the taxonomic composition of the tick microbiome under abiotic stressors such as high temperature, little is known about the impact

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of abiotic disturbance on the functional profiles of the tick microbiome. Previous studies showed that the functional profiles of tick microbiomes are highly redundant [15, 16, 40]. For instance, Estrada-Peña et al. [15] showed that up to 198 bacterial genera could contribute to a single pathway in *I. scapularis* microbiome. Furthermore, analysis of the resistance of *I. scapularis* microbiome to disturbing factors such as *Anaplasma phagocytophilum* infection and exposure to an antimicrobial peptide showed that while these factors affected the taxonomic composition, they had limited impact on the functional traits of tick microbiome [16]. These results suggest that a moderate reduction in the abundance of any taxa or random loss of few taxa would have only a marginal impact on the overall function of the tick microbiome since other bacteria can also perform an identical function. Thus, redundancy can contribute to the functional resistance of the microbiome in stressful ecological conditions.

How the functional stability of the tick microbiome is maintained despite taxonomic disturbance is currently unknown. Microbial communities found in the soil, plants, and animals harbor keystone taxa, which play important roles in stabilizing species diversity and ecosystem function [2, 24, 54]. Keystone bacteria were recently identified in tick microbiota using co-occurrence networks [33, 34, 53]. Keystone taxa are important for the tick microbiome as their depletion using anti-microbiota vaccines was associated to major disturbance in the tick microbial community [33, 34, 53].

We hypothesized that the impact of heat stress on tick microbiota may drive changes in the structure and hierarchical organization of the community as well as its functionality, as central microbial taxa may lose their keystone-ness (i.e., the quality of being keystone [9, 36] at different temperatures and thus, impact the structure and functional traits of tick microbiome. In this study, 16S rRNA sequence data reported by Thapa et al. [48] was used to test whether keystone bacteria contribute to the functional stability of the microbiome of ticks incubated at different temperatures. Then, the 16S rRNA sequence data reported by Thapa et al. [49] was used to test whether keystone bacteria play a similar role in from wild-caught ticks.

Methods

Data Source

16S rRNA gene sequencing datasets published by Thapa et al. [48] and Thapa et al. [49] were used for the analysis. The original studies described the changes in taxonomic composition and bacterial diversity of the microbiome of *Ixodes scapularis* reared in the laboratory or collected from vegetation (detailed below). Datasets were generated

by amplification of hypervariable region four (V4) of the bacterial 16S rRNA gene using barcoded universal primers (515F/806R) and sequenced in an Illumina MiSeq system.

- (i) The study of Thapa et al. [48] investigated the impact of environmental temperature on bacterial microbiome by incubation of unfed, female and male *I. scapularis* ticks under different temperatures (4, 20, 30, and 37 °C) at a stable humidity of 80–95% for 10 days or until tick death.
- (ii) The study of Thapa et al. [49] examined the influence of sex and location on the bacterial microbiome of free-living *I. scapularis* ticks collected from vegetation in Texas (during autumn) and Massachusetts (during late spring), USA.

As female ticks from the mentioned studies [48, 49] were reported to have a low taxonomic richness, we concentrated our analysis on the microbiome of male ticks. The raw sequences data are publicly available at the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI) under the project accession numbers PRJNA471905 [48] and PRJNA464062 [49].

Original Raw Sequences Processing

The 16S data sets were downloaded from SRA repository [29], and deinterlaced using the SRA-Toolkit (<http://ncbi.github.io/sra-tools/>). The raw sequences (paired-end) were processed and analyzed with the microbiome analysis package QIIME2 (v. 2021.4) [7]. Briefly, fastq files were denoised and merged using DADA2 software package [10]. The resulting amplicon sequence variants (ASVs) were aligned with MAFFT [26]. Taxonomy annotation of ASVs was done using the q2-feature-classifier [6] classify-sklearn naive Bayes taxonomy classifier against the 16S SILVA database (release 132). The resulting taxonomic data tables were collapsed at genus level, then filtered excluding rare taxa (i.e., with less than 10 total reads and presents in less than 30% of samples of each dataset).

Microbial Diversity and Differential Taxonomic Composition Analysis

Alpha diversity metric (i.e., ASV richness) was estimated using the q2-diversity plugin in Qiime2 environment, and analyzed using Kruskal-Wallis statistical test. For assessment of the differential abundant taxa, the tables with taxonomic profiles were collapsed at genus level, and normalized using centered log ratio (clr) transformation. The comparisons were performed using Kruskal-Wallis statistical test through the ALDEx2 R package [17]. Furthermore, we identified the differential abundant taxa that directly responded to the continuous variable temperature, for which correlational

analyses were performed using `aldex.corr` function from the ALDEx2 R package. This tool returned the average Pearson coefficient and its significance for each feature.

Microbial Co-occurrence Networks

Co-occurrence networks were built for each temperature condition using the taxonomic profiles. Co-occurrence networks are graphical visualization of microbial community assemblies where nodes represent bacterial taxa and the connecting edges represent significant correlations between taxa. The Sparse Correlations for Compositional data (SparCC) method [18] implemented in R studio [43] was used to detect significant ($p < 0.05$) correlations (SparCC = 0.5) among bacterial taxa. The visualization and assessment of the networks was performed using the software Gephi 0.9.2 [4]. Network topological parameters were calculated: number of nodes and edges, modularity (the strength of division of a network into modules), network diameter (the shortest path between the two most separated nodes), average degree (the average number of links per node), weighted degree (the sum of the weight of all the edges connected to a node), and clustering coefficient (the degree to which nodes in a network tend to form clusters). For the module (i.e., cluster of taxa that co-occur more often among them than with other bacterial taxa in the network) detection, we used the Louvain method [5] available in Gephi 0.9.2. This algorithm assigns a modularity class value for each node; thus, nodes with the same value of modularity class belong to the same module.

Identification of Keystone Taxa in Microbial Networks

For the identification of keystone taxa, we used three different criteria: (i) eigenvector centrality, which measures the importance of a node in a co-occurrence network while considering the relevance of their neighbors [44], (ii) ubiquitousness (i.e., bacterial taxa present across all the samples at one condition); and (iii) abundance. Then, cut-off values of 5 and 0.75 were selected for the mean of the abundance and the eigenvector centrality, respectively. Scatter plot was prepared using the software GraphPad 8 Prism (GraphPad Software Inc., San Diego, CA, USA).

Prediction of Functional Traits in Tick Microbiome

For the metabolic profiling of each sample, PICRUSt2 software (v. 2.4.1) [14] was used for the prediction of functional gene abundances based on 16S rRNA amplicon sequences. Briefly, the ASVs were aligned and placed into a reference tree (NSTI cut-off value of 2) that contains 20000 full 16S rRNA sequences from prokaryotic genomes, which is used to infer gene family copy numbers of each ASVs and finally determine

gene family abundance per sample. Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO) [25], Enzyme Classification numbers (EC), and Cluster of Orthologous Genes (COGs) [47] were used as gene family catalogues for the predictions. Pathway profiles were inferred from structured pathway mapping based on MetaCyc database [11].

Functional Diversity and Contribution of Taxa to the Functional Profile of Tick Microbiome

Functional diversity among the different temperatures was assessed using the open-source software PAST (v. 4.07) [22]. Non-metric multidimensional scaling (NMDs), based on a distance matrix computed with Bray-Curtis similarity index and a PERMANOVA test, was used to compare the functional diversity among the four temperature conditions. A pairwise PERMANOVA test was applied later to determine differences between specific groups. A permutation test was also applied to test differences in the homogeneity of dispersions of the group using the function `betadisper` from the Vegan R-package.

Linkages between ASVs (collapsed at genus level) and predicted functions (pathways) were assessed using the function “Taxa contribution” from PICRUSt2 metagenome predictions.

The number of shared functions between the whole bacterial community and the bacterial taxa of interest as well as the number of shared keystone taxa amongst the tick microbiome at different temperature conditions was calculated using Venn diagrams through the online tool <http://bioinformatics.psb.ugent.be/webtools/Venn/>. The number of shared functions between laboratory and field datasets was done in R studio [43] using the package “Venn”.

Definition of Environmental Conditions of Field-Collected Ticks

The environmental variables included average maximum and minimum temperatures and precipitation of the months in which ticks were collected in Texas and Massachusetts, USA. The data were obtained from the TerraClimate repository (<http://www.climatologylab.org/terraclimate.html>), accessed on July 2021.

Results

Decreased Bacterial Diversity at High Temperature is Associated with Shifts in Bacterial Community Assembly

Analysis of bacterial diversity using single nucleotide exact amplicon sequence variants (ASV) showed that the

microbiome of *I. scapularis* ticks incubated at 37 °C presented a significant decreased microbial richness compared to those incubated at 4 °C, 20 °C, and 30 °C (pairwise Kruskal-Wallis, $p < 0.05$, Supplementary Figure S1), which is consistent with the results of Thapa et al. [48]. Differential analysis showed that the abundance of 44 taxa changed significantly among the four temperatures (Kruskal-Wallis, $p < 0.05$, Supplementary Figure S2). Differences in taxa abundance among the different conditions came predominantly from microbiome of ticks incubated at 37°C, where most taxa presented decreased abundance compared to the microbiome of ticks incubated at 4, 20, and 30 °C. Moreover, correlation analysis showed that most taxa that changed significantly in abundance among the four conditions were strongly correlated to the temperature gradient (Spearman rank correlation, $p < 0.05$; Supplementary Figure S2). Specifically, all these taxa presented a negative correlation with the increase of the environmental temperature.

We then asked whether the changes in diversity and taxonomic composition induced by the temperature shift impacted the bacterial community assembly in *I. scapularis* microbiome. To answer this question, bacterial co-occurrence networks were inferred and used for the visualization and quantification of underlying interactions among bacteria of the tick microbiome. Network analysis showed distinct co-occurrence of bacterial genera across the different temperatures tested. Visual inspection of networks revealed that changes in environmental temperature reshape the bacterial interaction patterns with a decrease in the number of co-occurring taxa as temperature increases (Table 1; Fig. 1). For instance, the number of nodes decreased towards the increase of the temperature, while the number of edges decreased not only at 37 °C but also at 20 °C and 30 °C compared to the network at 4 °C. In addition, the modularity was decreased and the network diameter was increased at 37 °C. Moreover, the number of connections per node was significantly lower at 37 °C. These results suggest that

Table 1 Topological features of the microbial co-occurrence networks from microbiome of male *I. scapularis* incubated at different temperatures

Topological features	4 °C	20 °C	30 °C	37 °C
Nodes	143	129	127	59
Edges	1860	902	1009	108
Positive (%)	940 (50.5)	539 (59.7)	647 (64.1)	80 (74.1)
Negative (%)	920 (49.5)	363 (40.2)	362 (35.9)	28 (25.9)
Modularity	16.422	0.944	0.624	0.273
Network diameter	5	6	5	8
Average degree	26.014	13.984	15.89	3.661
Weighted degree	0.486	2.169	4.136	1.36
Clustering coefficient	0.611	0.52	0.516	0.609

the decrease in bacterial abundance and diversity induced at 37 °C is also associated with the reduction of microbe-microbe interactions.

A Fraction of Highly Connected Keystone Taxa is Maintained Along the Temperature Gradient

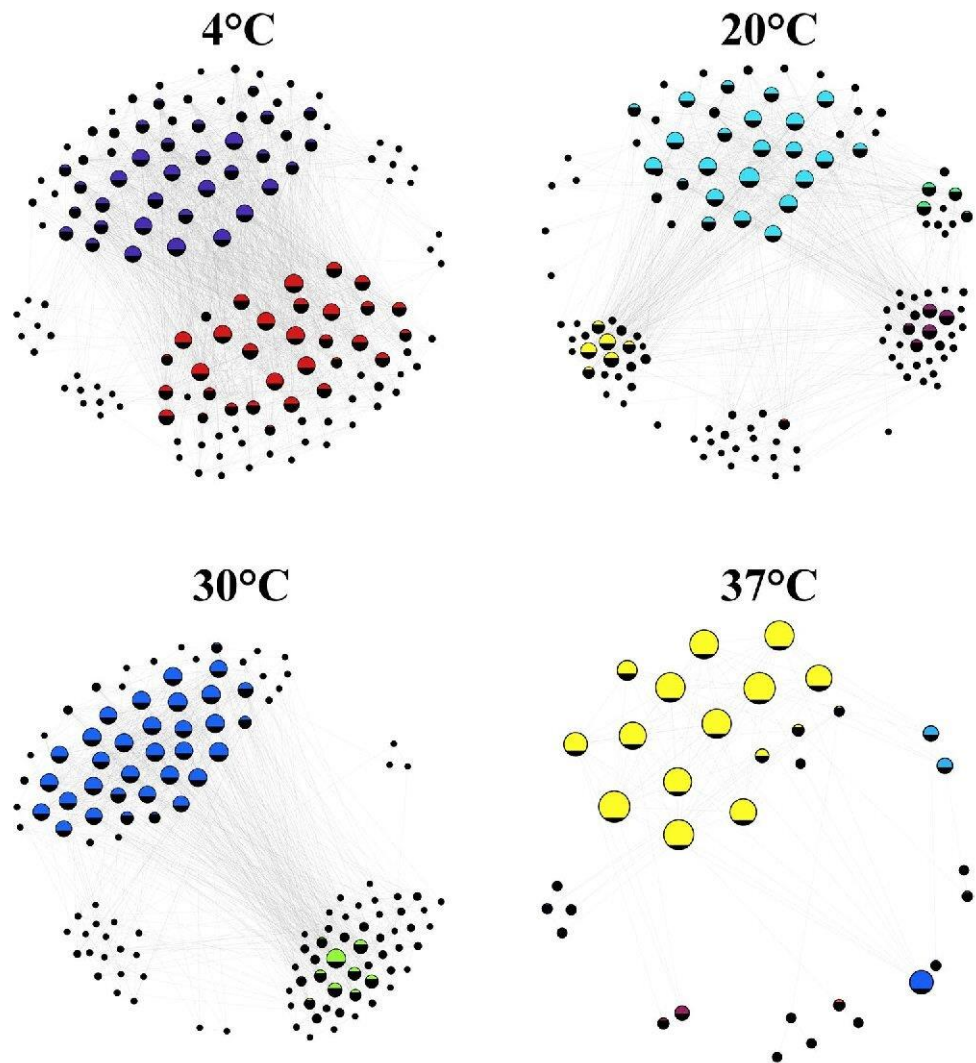
We next asked whether the microbial networks at different temperatures support keystone taxa. The keystone taxa of each network were identified based on ubiquitousness, abundance, and high eigenvector centrality in the networks. Various ubiquitous bacteria, with high eigenvector centrality (> 0.75) and abundance ($\text{clr} > 5$) were defined as keystone taxa (Fig. 2). However, the results show that the number of keystone taxa decreased with the increase in temperature, since 15, 14, 7, and 4 keystone taxa were identified in ticks incubated at 4, 20, 30, and 37 °C, respectively (Fig. 2). Notably, four taxa (i.e., *Pseudomonas*, *Ralstonia*, *Acinetobacter*, and *Bradyrhizobium*) were identified as keystone in the different temperatures. These taxa are referred hereafter as thermostable keystone taxa. The network analysis further showed that the thermostable keystone taxa were positively connected between them in all the temperatures (Fig. 3).

The 4 thermostable keystone taxa were directly connected to 66, 39, 38, and 15 nodes at 4, 20, 30, and 37 °C, respectively. From these, 41, 32, 31, and 8 taxa were connected to all the thermostable keystone taxa at 4, 20, 30, and 37 °C, respectively (Fig. 4). A detailed analysis of nodes connected to *Pseudomonas*, *Ralstonia*, *Acinetobacter*, and *Bradyrhizobium* in the four conditions (hereafter referred to as “subnetworks”) revealed the existence of a group of 8–10 bacteria that persist as neighbors of each thermostable keystone taxa across the gradient of temperature (Fig. 5). Moreover, the type of connections between the thermostable keystone taxa and their respective persistent neighbors were all positive at 4 °C, except the negative connection between *Pseudomonas* or *Acinetobacter* and *Sediminibacterium*. Interestingly, the positive connection was kept at 20, 30, and 37 °C (Supplementary Table 1). These results suggest that thermostable keystone taxa maintain stable ecological associations with selected members of the microbial communities. It is noteworthy that the keystone taxa and their positively associated neighbor represents only 27.3% (39, total = 143), 24.8% (32, total = 129), 24.4% (31, total = 127), and 27.1% (16, total = 59) of the total of bacteria presents in the co-occurrence networks at 4, 20, 30, and 37 °C, respectively.

Thermostable Keystone Bacteria Keep the Functional Stability of the Tick Microbiome

To study the impact of different temperatures on the metabolic potential of tick microbiome, we explored the functional diversity from predicted pathway profiles. The

Fig. 1 Co-occurrence networks in *I. scapularis* under heat stress. Co-occurrence bacterial networks were inferred from the microbiota of ticks incubated at 4, 20, 30, and 37 °C. Nodes represent bacterial taxa and connecting edges stand for a co-occurrence correlations (SparCC > 0.5). Node sizes are proportional to the eigenvector centrality value and node colors are based on modularity class metric. Only nodes with at least one connection are displayed. Modules in each network were colored



non-metric multidimensional scaling (NMDS) plot and a paired PERMANOVA test showed that the functional traits of ticks incubated at 37 °C were significantly different from ticks incubated at 4, 20, and 30 °C ($F = 5.716$, $p < 0.05$, Fig. 6). A permutation test for the evaluation of the homogeneity of dispersions in the four groups indicated that the functional traits of ticks incubated at 37 °C were significantly more disperse than ticks incubated at 20 and 30 °C ($F = 3.91$, $p < 0.05$).

We next tested the contribution of the thermostable keystone taxa and their direct neighbor (Fig. 3) to the functional profiles of tick microbiome. We found that the thermostable keystone taxa and their direct neighbors (i.e., including those with positive and negative interactions) contributed to more than 99% of the predicted pathways regardless the incubation temperature (Fig. 7, Supplementary Table 2). Interestingly, when only the taxa that co-occur positively with the thermostable keystone taxa in the subnetworks were considered for the analysis, the contribution to the functional

profile remains identical to the precedent result (Supplementary Figure S3, Supplementary Table 3). Hence, the thermostable keystone taxa along with the bacteria that co-occur positively with them were capable to provide most of the metabolic functions regardless of the temperature gradient.

Field-Collected Ticks Contain Identical Keystone Taxa Found in Laboratory-Reared Ticks

In order to determine if the keystone taxa found in a laboratory setting are the same in field-collected ticks, we used published 16S rRNA datasets from *I. scapularis* collected from vegetation in Texas and Massachusetts, USA [49]. The authors reported that ticks were collected in different seasons to follow the tick activity levels in different locations. Thus, collection of ticks in Massachusetts was done during late spring (May 2017) while ticks from Texas were collected during autumn (December 2016, 2017, and November 2017). The average for maximum temperature in Texas was

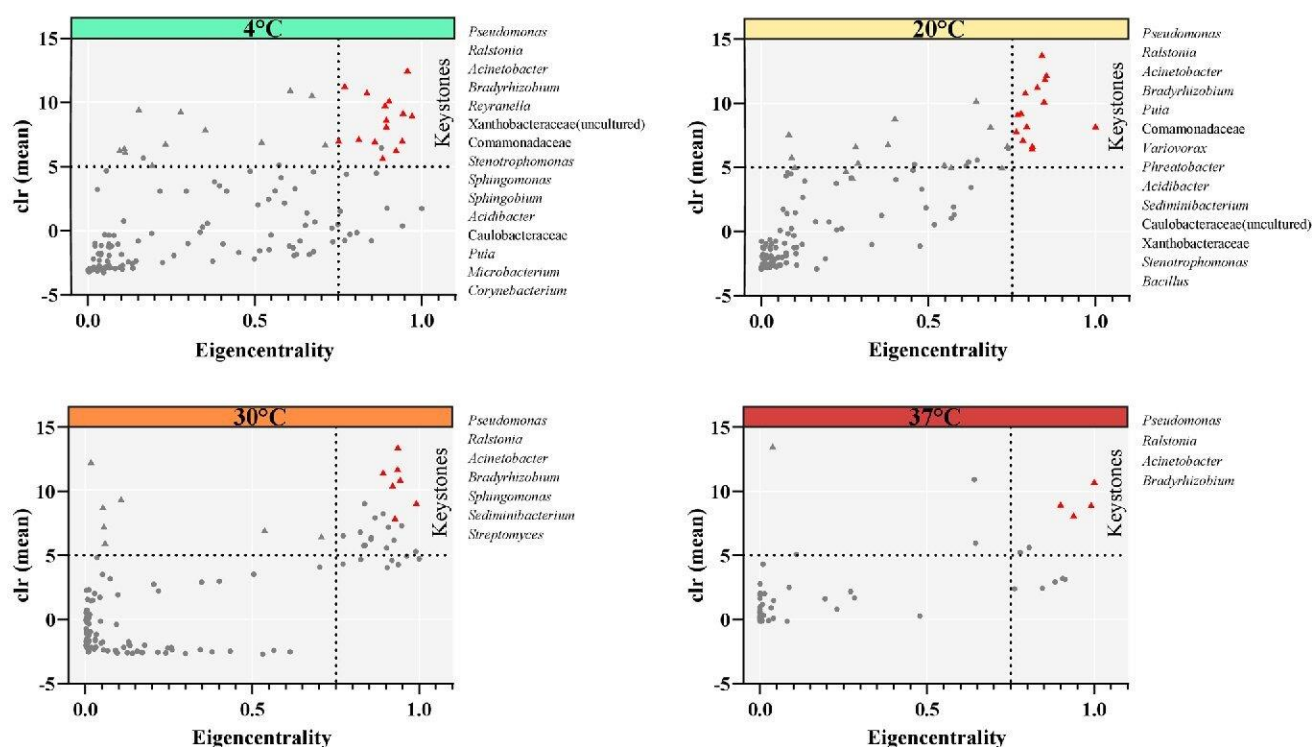


Fig. 2 Keystone taxa at different temperatures Scatter plot showing the mean relative abundance, expressed as center log ratio (clr) value, and the eigenvector centrality of each bacterial taxon (dots or triangle) of the co-occurrence networks at different temperatures. The triangles represent bacterial taxa that are present across all the samples of *I. scapularis* incubated at a certain temperature. The vertical dotted line represents the eigencentality cutoff value of 0.75 and the

horizontal dotted line represents the clr mean cutoff value of 5. Red triangles that are above and to the right of the horizontal and vertical dotted line, respectively, represent the identified keystone bacteria at each temperature. The list of keystone taxa is displayed next to the scatter plots. Note that the first four keystone taxa remain the same in all temperatures

of 15 °C, 15 °C, and 22 °C while the minimum temperature was of 7 °C, 6 °C, and 13 °C in December 2016, December 2017, and November 2017, respectively. The monthly rainfall during these months was between 18 and 31 mm. On the other hand, in May 2017 at Massachusetts, the averages for maximum and minimum temperatures were of 16.8 °C and 8.9 °C, respectively while the monthly rainfall during this period was 151 mm. Our analysis revealed that the microbial community of ticks collected in different locations harbour different keystone bacteria (Fig. 8). Surprisingly, these keystone taxa (i.e., *Acinetobacter*, *Ralstonia* and *Bradyrhizobium*) identified in *I. scapularis* of Massachusetts were the same as three of the thermostable keystone taxa found in ticks derived from a laboratory setting. These results suggest that the identification of keystone taxa from laboratory-reared ticks can accurately reflect the microbial community hierarchy of some populations of free-living ticks.

As in the laboratory-reared tick dataset, we analyzed the contribution of the keystone taxa and their direct neighbor to the functional profile of field-caught tick microbiome. We found that the keystone taxa and their subnetwork contribute to 99.3% and 98.1% of predicted pathways in the

microbiome of ticks collected in Massachusetts (Fig. 9A, Supplementary Table 4) and Texas (Fig. 9B, Supplementary Table 4), respectively. Furthermore, we found that the majority of the metabolic pathways (i.e., 373, total 430; 86.7%) were common among laboratory-reared and field-collected ticks (Fig. 9C, Supplementary Table 5).

Discussion

Several studies have demonstrated that the taxonomic composition and diversity of tick microbiome is highly influenced by diverse biotic [12, 37, 52] and abiotic factors [19, 45, 48, 50]. Yet, the functional traits of tick microbiome and their possible changes associated to environmental factors remains largely unknown. In this study, the impact of heat stress on the structure and functional profiles of the microbial communities of laboratory-reared and field-collected *I. scapularis* was assessed. To this aim, we used published and publicly accessible 16S rRNA datasets [48, 49]. As the study of Thapa et al. [48] reported that the microbiome of laboratory-reared female ticks was dominated by *Rickettsia* and

Fig. 3 Subnetworks of the local connectivity of the thermostable keystone taxa in the co-occurrence networks. The direct neighbors (light green nodes) of the thermostable keystone taxa *Pseudomonas* (blue nodes), *Acinetobacter* (red nodes), *Bradyrhizobium* (green nodes), and *Ralstonia* (yellow nodes) were identified from the co-occurrence network built at 4, 20, 30, and 37 °C. Positive and negative interactions between co-occurring bacteria are represented by the red and blue edges, respectively

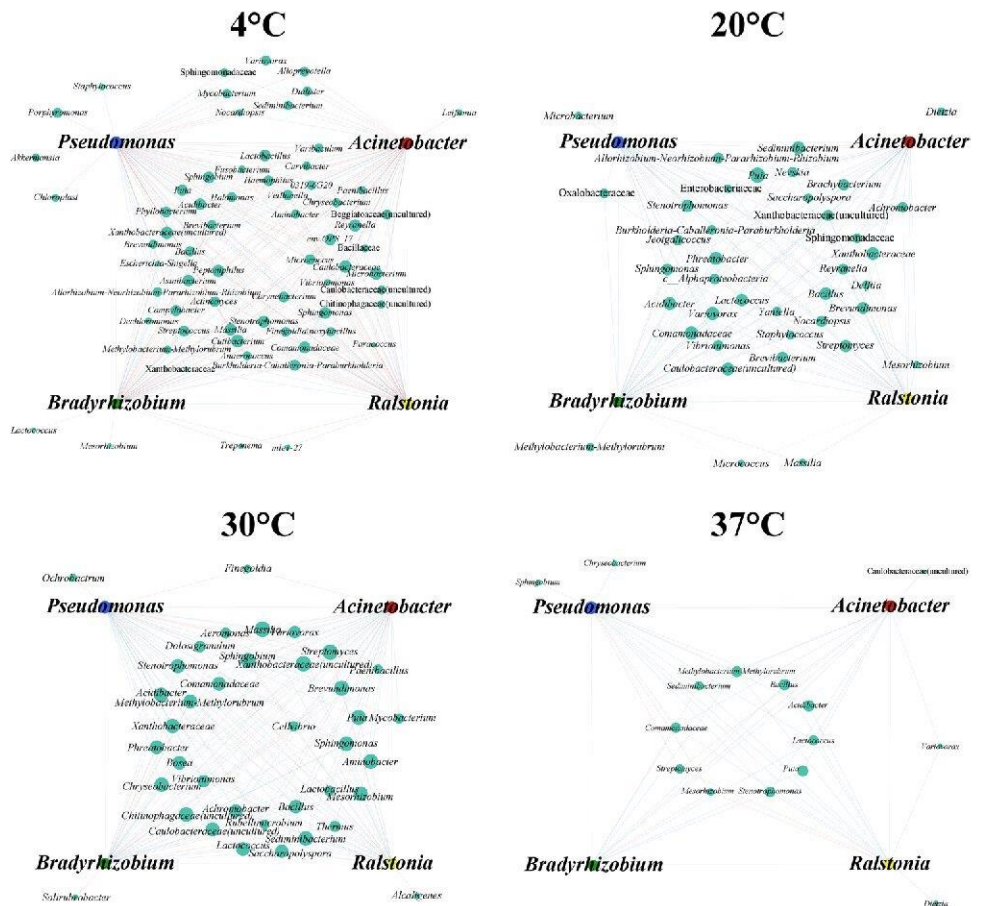
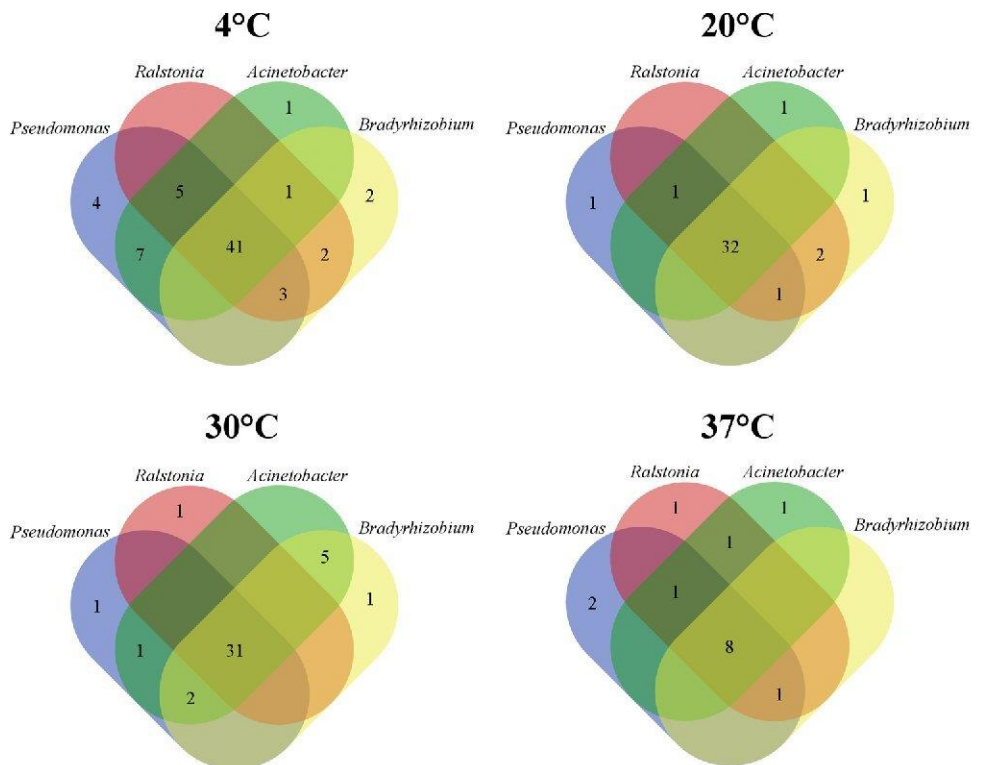


Fig. 4 Number of neighbor taxa shared by the thermostable keystone taxa in the different temperatures. Venn diagram showing the number of bacteria that are common or unique among the taxa connected to the four thermostable keystone taxa *Pseudomonas*, *Acinetobacter*, *Bradyrhizobium*, and *Ralstonia* in the different temperatures



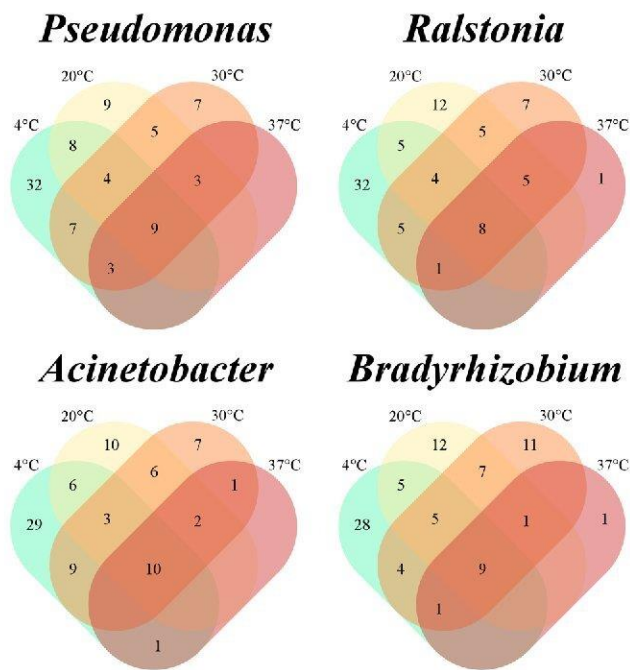


Fig. 5 Number of taxa that persist as neighbors for each thermostable keystone taxa across the gradient of temperature. The number of bacteria genera shared by each thermostable keystone taxa, *Pseudomonas*, *Ralstonia*, *Acinetobacter*, and *Bradyrhizobium*, in the four temperatures is displayed as a Venn diagram

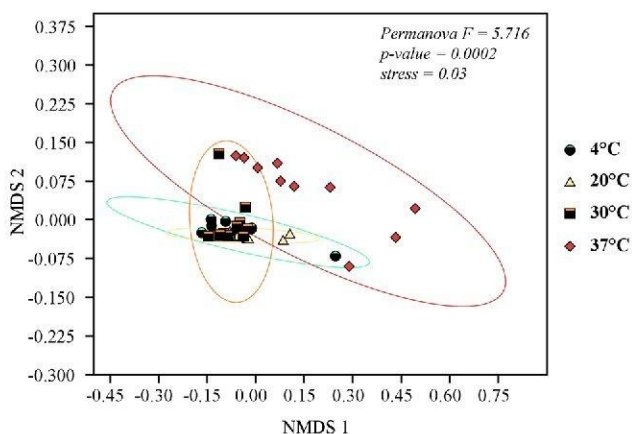


Fig. 6 Impact of temperature on the diversity of the functional profiles. Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis similarity matrix of predicted pathways for each sample at 4 (dots), 20 (triangle), 30 (square), and 37 °C (diamond). Groups are surrounded by ellipses (95% Confidence interval around centroid). Permutational analysis of variance (PERMANOVA) F and p values are displayed at the top right of NMDS plot

hence, it had a lower microbial diversity compared to male ticks, we decided to focus only on male dataset. The higher bacterial diversity of the *I. scapularis* male can spur more information of changes in microbial interactions and their contribution to the functional profile. Our results support

the stability of the functional microbiome despite changes in taxonomic composition due to heat stress. Notably, a fraction of microbes composed by four thermostable keystone taxa and their invariably associated microbial partners carry almost the totality of functions of the metagenome underlying the functional redundancy of tick microbiome.

Consistent with the results of Thapa et al. [48], we found a decrease in the diversity and relative abundance of bacterial taxa in the tick microbiome under heat stress. Community modifications were also reflected in the co-occurrence networks of ticks incubated at the different temperatures (i.e., 4, 20, 30, and 37 °C). The greater loss of bacterial taxa and decreased interconnection between them was observed in the networks inferred from microbial communities of ticks incubated at 37 °C. This microbial network presented the highest diameter and the lowest modularity, compared to microbial networks of ticks incubated at 4, 20, and 30 °C. Estrada-Peña et al. [16] showed that disturbing factors such as anti-tick immunity, *Anaplasma phagocytophilum* infection and antimicrobial peptide treatment increased the diameter of tick microbiome networks, which was associated with lower network robustness compared to the networks without disturbance. Furthermore, high network modularity has been proposed to be a proxy of microbiome stability [27]. Network parameters thus suggest that heat stress reduces the robustness and stability of tick microbial communities. Shifts on microbial interaction patterns as result of temperature variation has been previously described in soil bacterial communities [3, 8, 13, 20, 54].

Co-occurrence networks can also be used to identify keystone taxa which have high influence and importance in the microbial community [1, 2, 23]. We used three parameters (i.e., eigenvector centrality, ubiquitousness, and abundance) to infer the keystone taxa in the tick microbiome [33]. As discussed by Wu-Chuang et al. [53], these criteria differ from those used by other authors to infer bacterial keystone taxa in microbial communities. The impact of keystone taxa, predicted with the criteria used in this study, on tick physiology [33] and tick microbiome structure and function [33, 34] has been validated using anti-tick microbiota vaccines [33, 52, 53]. In this study, the number of predicted keystone taxa in tick microbiome decreased with high temperatures suggesting that environmental temperature variation can change the influence the importance of some bacteria in the community.

Despite a decrease in the number of keystone taxa, we found a core of four keystone bacterial genera that was systematically identified across the gradient of temperature. These thermostable keystone taxa were associated to a set of bacteria that together contributed to more than 99% of the predicted pathways regardless the incubation temperature. These findings suggest that thermostable keystone taxa may provide functional stability to the tick microbiome under heat stress. Similar result were found in soil bacterial

Fig. 7 Functional contribution of thermostable keystone taxa and their direct neighbor. Venn diagram showing the number of metabolic pathways contributed by the thermostable keystone taxa and their positively and negatively associated direct neighbors (subnetwork) and compared to the total of predicted functions for the whole bacterial community at 4, 20, 30, and 37 °C

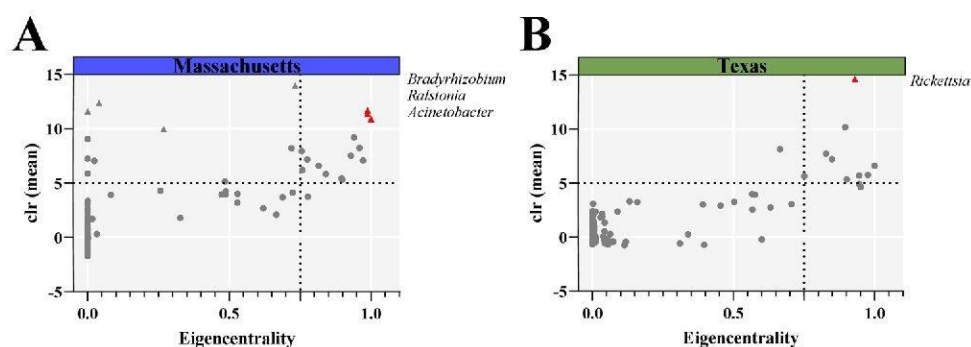
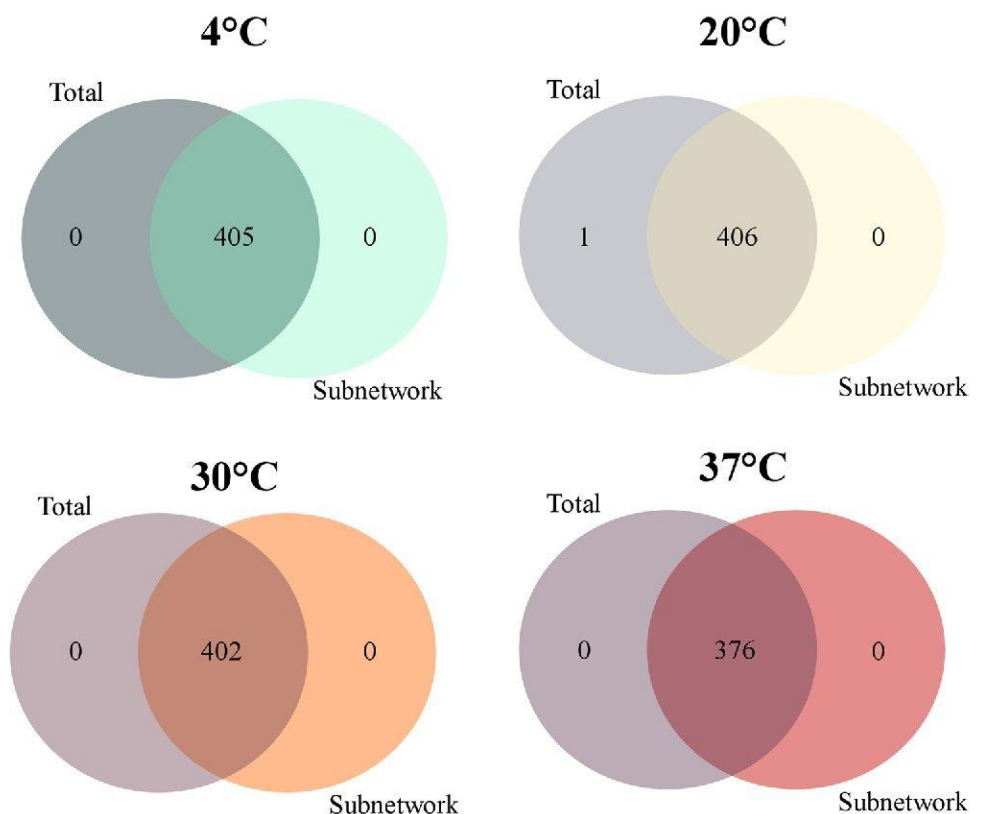


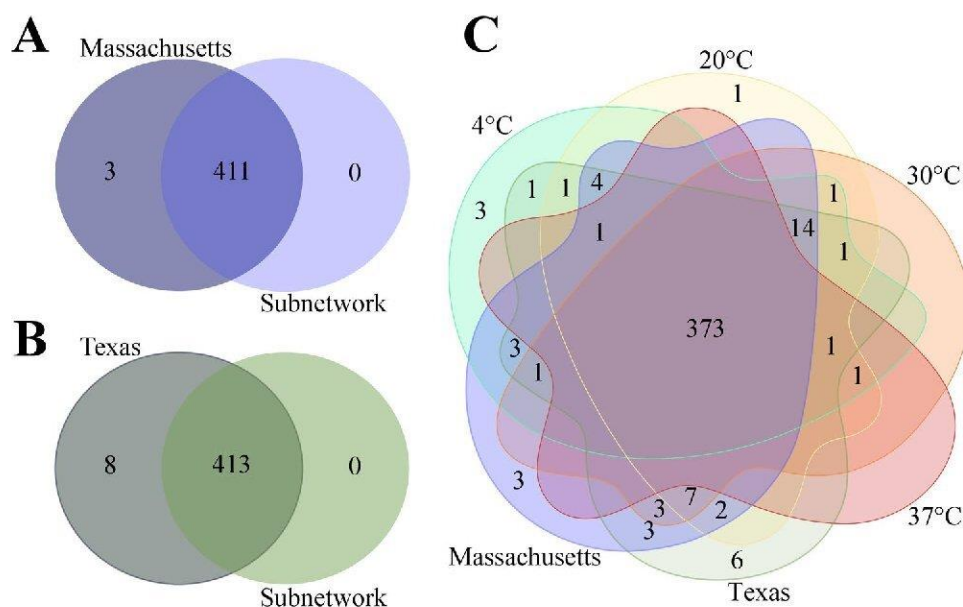
Fig. 8 Keystone taxa in field-collected ticks. Scatter plot showing the mean relative abundance (expressed as clr) and the eigencentality of each bacterial taxon (dots or triangle) of the microbial community from *I. scapularis* collected from Massachusetts (A) and Texas (B). The red dots or triangle represent bacterial taxa that are present across all the samples of *I. scapularis* collected from different loca-

tions. The vertical dotted line represents the eigencentality cutoff value of 0.75 and the horizontal dotted line represents the clr mean cutoff value of 5. Red triangles that are above and at the right of the horizontal and vertical dotted line, respectively, represent the identified keystone bacteria at each location. The list of keystone taxa is displayed next to the scatter plots

communities of two sites of the Atacama Desert, west of the Andes Mountains [32]. These authors showed that beside a persistent group of bacteria, the most abundant functional roles were conserved despite changes in environmental variables [32]. It is noteworthy that the keystone taxa with their positively correlated neighbors represent only a small fraction of the whole bacterial community suggesting that several bacteria contribute to the same set of functions in the tick microbiome, as previously described by Obregón et al. [40] and [15, 16]. A limitation here is that the accuracy

of functional inferences based in PICRUSt2 varies across sample types and functional categories [46]. Although the accuracy of PICRUSt2 predictions for tick microbiome has not been assessed, a recent study reported a sharp decrease in PICRUSt2 performance for inference in non-human (i.e., gorilla, mouse, and chicken) and environmental samples (i.e., soil), compared with human samples [46]. Having said that, it is worth mentioning that functional inference of tick microbiome using PICRUSt2 have been validated by PCR and qPCR in two independent studies [33, 34]. In both

Fig. 9 Field-collected and laboratory-reared ticks share a functional core. Venn diagram showing the number of metabolic pathways contributed by the keystone taxa and their direct neighbors compared to the total number of predicted pathways for the whole bacterial community of ticks collected from vegetation of Texas (A) and Massachusetts (B). Venn diagram showing the number of functional pathways shared among tick microbiomes of laboratory-reared and field collected ticks (C)



studies, validation by PCR/qPCR concurred with PICRUSt2 predictions of pathway presence and abundance [33, 34].

Notably, three of the thermostable keystone taxa identified in laboratory-reared ticks were also found in field-collected *I. scapularis* from Massachusetts. These results suggest that the microbiome of ticks studied in a controlled environment can reflect accurately the hierarchical organization of microbial communities in some populations of free-living ticks. Similarly, Narasimhan et al. [38] found that field-collected and lab-reared nymphs presented similar gut microbial composition at phylum and genus levels. Although not in the microbiome context, other studies have also shown that laboratory data can reflect physiological or behavioral features of field-collected ticks [41, 42]. In contrast, *I. scapularis* ticks collected from Texas were found to harbour *Rickettsia* as the only keystone taxon. Different abiotic or biotic factors influencing tick populations and their microbial communities in Texas (collected in late spring) and Massachusetts (collected in autumn) may modify microbiota composition and/or influence the hierarchical organization of microbial communities.

Conclusions

In this study, we found that heat stress induces changes in the tick microbial community including a reduction in the number of co-occurring bacteria and keystone taxa. Despite drastic alterations in the bacterial community structure, a core of keystone taxa persisted across the gradient of temperature. These thermostable keystone taxa and their shared set of positively correlated neighbors represented approximately 99%

of the functional diversity of the tick microbiome. The results obtained under laboratory conditions were partially validated in the field, as one of the tested populations of field-collected ticks harbored identical keystone taxa as the laboratory-reared ticks. The results suggest that keystone taxa keep the functional stability of the tick microbiome under environmental variations in temperature. Furthermore, we found the existence of a functional core shared by laboratory-reared and field-collected ticks, underlying that redundancy can contribute to the functional resistance of the microbiome in stressful ecological conditions. In summary, our study provides an insight into the role of keystone taxa in keeping the functional stability of the tick microbiome under abiotic stressors.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00248-021-01929-y>.

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Authors' Contributions A.C.C. and D.O. conceived the study. A.W.C., D.O., and A.E.P. performed the analyses. A.W.C. prepared the figures and drafted the manuscript. All authors revised and accepted the last version of the manuscript. All authors read and approved the final manuscript.

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Data Availability All material relevant to this publication are available in the manuscript.

Code Availability Not applicable

Declarations

Ethics Approval Not applicable

Consent to Participate Not applicable

Consent for Publication Not applicable

Conflicts of Interest The authors declare that they have no conflicts of interests.

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Chapter III

Impact of anti-microbiota vaccine on *Ixodes ricinus* microbiome and *Borrelia afzelii* colonization

Chapter IIIa

Modulation of tick microbiome by anti-microbiota vaccine



Anti-Microbiota Vaccines Modulate the Tick Microbiome in a Taxon-Specific Manner

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The lack of tools for the precise manipulation of the tick microbiome is currently a major limitation to achieve mechanistic insights into the tick microbiome. Anti-tick microbiota vaccines targeting keystone bacteria of the tick microbiota alter tick feeding, but their impact on the taxonomic and functional profiles of the tick microbiome has not been tested. In this study, we immunized a vertebrate host model (*Mus musculus*) with live bacteria vaccines targeting keystone (i.e., *Escherichia-Shigella*) or non-keystone (i.e., *Leuconostoc*) taxa of tick microbiota and tested the impact of bacterial-specific antibodies (Abs) on the structure and function of tick microbiota. We also investigated the effect of these anti-microbiota vaccines on mice gut microbiota composition. Our results showed that the tick microbiota of ticks fed on *Escherichia coli*-immunized mice had reduced *Escherichia-Shigella* abundance and lower species diversity compared to ticks fed on control mice immunized with a mock vaccine. Immunization against keystone bacteria restructured the hierarchy of nodes in co-occurrence networks and reduced the resistance of the bacterial network to taxa removal. High levels of *E. coli*-specific IgM and IgG were negatively correlated with the abundance of *Escherichia-Shigella* in tick microbiota. These effects were not observed when *Leuconostoc* was targeted with vaccination against *Leuconostoc mesenteroides*. Prediction of functional pathways in the tick microbiome using PICRUSt2 revealed that *E. coli* vaccination reduced the abundance of lysine degradation pathway in tick microbiome, a result validated by qPCR. In contrast, the gut microbiome of immunized mice showed no significant alterations in the diversity, composition and abundance of bacterial taxa. Our results demonstrated that anti-tick microbiota vaccines are a safe, specific and an easy-to-use tool for manipulation of vector microbiome. These results guide interventions for the control of tick infestations and pathogen infection/transmission.

Keywords: anti-microbiota vaccines, tick, microbiome modulation, keystone bacteria, networks analysis

INTRODUCTION

Ticks, like many multicellular eukaryotes, harbor a very diverse group of commensal, symbiotic, and pathogenic microorganisms that collectively comprise the microbiome (1, 2). This complex microbial system and the tick have evolved an intimate relationship relevant for tick development, nutritional adaptation, reproductive fitness, ecological plasticity, and immunity (3–6). Mounting evidence shows that non-pathogenic midgut bacteria may also affect tick vector competence and susceptibility to pathogens transmitted by ticks (7–10). The development of high-throughput sequencing technologies and bioinformatics tools in the last decade has significantly improved our knowledge of the phylogenetic and genetic diversity, dynamics, and ecology of the microbial communities in several tick species (2). However, the vast majority of studies are restricted to the analysis of the taxonomic composition of the tick microbiome and except for few of them (11–14), the functional significance of bacterial community structure and diversity remains largely unexplored.

Recent functional metagenomics studies have shown that exploring the taxonomic composition and variability of the tick microbiome underestimates the multidimensional nature of the tick hologenome and that analysis solely based on taxonomic profiles has limited biological significance (11–14). The native tick microbiome is likely composed of bacteria, archaea, fungi, protozoans and viruses with diverse functional capacities, which are engaged in a complex network of cooperative and competitive interactions (1, 2). Some of these microorganisms, known as keystone species, co-occur with many others and may have a large regulatory effect on the structure, organization, and function of the tick microbiome. The ubiquitousness of the keystone taxa is likely associated with important resources they provide to the overall microbial community and/or the tick host (11, 12, 14). This suggests that keystones are an essential component of the functional networks and therefore represent ideal targets for the rational manipulation of the microbial composition and function. The functional capacity of the tick microbiome is not equal to the overall number of its individual components, as microbial species strongly and frequently interact with one another and form a complex functional network (13, 14), which can thus be considered as a fundamental unit in microbial communities of ticks.

Understanding the microbe-microbe relationships is a critical step for predicting their holistic consequences on tick physiology and vector competence (13–15). Microbial co-occurrence networks represent a useful approach to measure the keystone of taxa and infer potential interactions between nodes of the functional networks (15, 16). In a recent study, Mateos-Hernández et al. (15, 17) introduced anti-tick microbiota vaccines as a tool to target tick microbiota bacteria. The bacterial family Enterobacteriaceae was identified as a keystone taxon in the microbiome of *Ixodes ricinus* and *Ixodes scapularis*. Immunization with the Enterobacteriaceae bacterium *Escherichia coli* elicited an anti-*E. coli* IgM and IgG antibody (Ab) response associated with increased engorgement weight of *I. ricinus* nymphs that fed on C57BL/6 mice and high mortality in ticks that fed on α -1,3-galactosyltransferase (α 1,3GT)-deficient C57BL/6 mice compared with the ticks that fed on the

control group. High mortality in *I. ricinus* concurred with a wide distribution of genes encoding α 1,3GT genes in the microbiota of *I. ricinus* and the host's immune response to α -Gal, a glycan synthesized by bacterial α 1,3GT enzymes. The results suggested that tick microbiome disturbance by immune targeting of keystone bacteria and/or bacterial surface molecules such as α -Gal altered the tick-microbiome homeostasis and impacted tick feeding and survival.

In this study, we investigated the impact of immunization with keystone bacteria on the taxonomic and functional profiles and the structure of the microbial communities associated with the tick microbiome. Our findings showed for the first time that host immunization with keystone bacteria is a promising tool for the manipulation of the tick microbiome with the potential to reveal functional mechanisms of the tick-microbiota interactions and spur new strategies to control ticks and tick-borne pathogens.

MATERIALS AND METHODS

Ethics Statement

All procedures were performed at the Animal Facility of the Laboratory for Animal Health of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Maisons-Alfort, France, according to French and International Guiding Principles for Biomedical Research Involving Animals (2012). The procedures were reviewed and approved by the Ethics Committee (ComEth, Anses/ENVA/UPEC), with permit number E 94 046 08.

Mice and Housing Conditions

Six-week-old C57BL/6 (Charles River strain code 027) mice were purchased from Charles River (Miserey, France) and maintained in Green line ventilated racks (Tecniplast, Hohenpeissenberg, Germany) at -20 Pa, with food (Kliba nafaj, Rinaustrasse, Switzerland) and water *ad libitum*. The mice were kept at controlled room temperature (RT, 20–23°C) and a 12-hour (h) light: 12-h dark photoperiod regimen. The animals were monitored twice a day (d) by experienced technicians and deviations from normal behaviors or signs of health deterioration were recorded, and reported.

Bacterial Cultures and Live Bacteria Immunization

Representative bacteria of the keystone genus *Escherichia-Shigella* (i.e., *Escherichia coli*) and the non-keystone genus *Leuconostoc* (i.e., *Leuconostoc mesenteroides*) were selected to be included in live bacteria vaccine formulations, aiming to test the impact of host immune response against “keystone” bacteria on tick microbiota composition, stability and functionality, and tick performance. The selection of these bacteria as live vaccines was based on our previous results (15) that showed that the genus *Escherichia-Shigella* of the family Enterobacteriaceae was among the top keystone taxa (i.e., high relative abundance, ubiquitousness, and eigencentality) identified in *Ixodes* microbiota. Based on the same study (15), we selected the genus

Leuconostoc of the family Leuconostocaceae as non-keystone bacteria with low keystone-ness (i.e., low relative abundance, ubiquitousness, and eigencentality) in the microbiome of *Ixodes*.

The Gram-negative bacterium *E. coli* BL21 (DE3, Invitrogen, Carlsbad, CA, USA) was prepared as previously described (15). Briefly, *E. coli* was grown on Luria Broth (LB, Sigma-Aldrich, St. Louis, MO, USA) at 37°C under vigorous agitation, washed with phosphate buffer saline (PBS) 10 mM NaH₂PO₄, 2.68 mM KCl, 140 mM NaCl, pH 7.2 (Thermo Scientific, Waltham, MA, USA), resuspended at 3.6 × 10⁴ colony-forming unit (CFU)/mL, and homogenized using a glass homogenizer. The gram-positive bacterium *L. mesenteroides* (strain LBH1148, INRAE collection) was grown on MRS broth (Difco, Bordeaux, France) at 37°C without agitation and resuspended and homogenized following the same procedures as for *E. coli*. Six-week-old, C57BL/6 mice were immunized subcutaneously with either *E. coli* (4, 1 × 10⁶ CFU per mouse) or *L. mesenteroides* (4, 1 × 10⁶ CFU per mouse) in a water-in-oil emulsion containing 70% MontanideTM ISA 71 VG adjuvant (Seppic, Paris, France), with a booster dose two weeks after the first dose. Control, C57BL/6 (n = 4) mice received a mock vaccine containing PBS and adjuvant.

Bacterial Protein Extraction

Escherichia coli and *L. mesenteroides* were washed twice with PBS, centrifuged at 1000× g for 5 min at 4°C, resuspended in 1% Trion-PBS lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) and homogenized with 20 strokes using a glass balls homogenizer. The homogenate was then centrifuged at 300× g for 5 min at 4°C and the supernatant was collected. Protein concentration was determined using the Bradford Protein Assay (Thermo Scientific, San Jose, CA, USA) with Bovine Serum Albumin (BSA) as standard.

Mouse Feces and Sera Sample Collection

Fecal and blood samples were collected on sterile tubes. Blood samples were collected on d0, d14, d30 and d46 in animals from all experimental groups and were incubated for 2h at RT, without anticoagulant, allowing for clotting, and then centrifuged at 5000× g for 5 min at RT, twice. Fresh feces were collected from each animal on d0, d30 and d46. Fecal samples were stored in sterile tubes at -20°C before genomic DNA extraction.

Indirect ELISA

The levels of Abs reactive against bacterial proteins were measured in mice sera as previously reported (15). The 96-well ELISA plates (Thermo Scientific, Waltham, MA, USA) were coated with 0.5 µg/mL (100 µL/well) of *E. coli* or *L. mesenteroides* protein extracts and incubated for 2 h with 100 rpm shaking at RT. Subsequently, plates were incubated overnight at 4°C. The antigens were diluted in carbonate/bicarbonate buffer (0.05 M, pH 9.6) and incubated overnight at 4°C. Wells were washed three times with 100 µL of PBS containing 0.05% (vol/vol) Tween 20 (PBST), and then blocked by adding 100 µL of 1% Human Serum Albumin (HSA)/PBS for 1 h at RT and 100 rpm shaking. After three washes, sera samples, diluted 1:50 in 0.5% HSA/PBS, were added to the wells and incubated for 1 h at 37°C with shaking. The plates were washed

three times and HRP-conjugated Abs (goat anti-mice IgG and IgM) (Sigma-Aldrich, St. Louis, MO, USA) were added at 1:1500 dilution in 0.5% HSA/PBST (100 µL/well) and incubated for 1 h at RT with shaking. The plates were washed three times and the reaction was developed with 100 µL ready-to-use TMB solution (Promega, Madison, WI, USA) at RT for 20 min in the dark, and then stopped with 50 µL of 0.5 M H₂SO₄. Optimal antigen concentration and dilutions of sera and conjugate were defined using a titration assay. The optical density (OD) was measured at 450 nm using an ELISA plate reader (Filter-Max F5, Molecular Devices, San Jose, CA, USA). All samples were tested in triplicate and the average value of three blanks (no Abs) was subtracted from the reads. The cut-off was determined as two times the mean OD value of the blank controls.

Immunofluorescence

Escherichia coli and *L. mesenteroides* were washed three times with PBS, centrifuged at 1000x g for 5 min, fixed with 4% paraformaldehyde for 30 min and blocked with 1% human serum albumin (HSA, w/v in PBS) for 1h at RT. Bacterial cells were then incubated for two days at 4°C with pooled sera (from all vaccinated mice, d30) of mice immunized either against *E. coli*, *L. mesenteroides* or the mock vaccine at a dilution of 1:20 (v/v in PBS). Thereafter, bacteria were washed three times with PBS followed by incubation with Alexa Fluor 488 conjugates anti-mouse antibody (Ab) against IgM (Life technologies, Eugene, OR, USA; A21042) and IgG (Life technologies, Eugene, OR, USA; A11029) at a dilution of 1:1000 (v/v in 1% HSA) for 3h at RT. After washing with PBS, bacteria were stained with 2µg/µL of 4',6-diamidino-2-phenylindole (DAPI) and mounted in ProLong Diamond Antifade (Life Technologies, Eugene, OR, USA; P36961). Image acquisition was performed using a Leica confocal microscope (Leica, Wetzlar, Germany) with 63X oil immersion objective. Representative pictures were assembled in Adobe Illustrator and fluorescence was slightly enhanced using Adobe Photoshop CS6 (Adobe System Incorporated, California, USA).

Tick Infestation

Unfed *I. ricinus* nymphs were obtained from the colonies of UMR-BIPAR, Maisons-Alfort, France. We used previously validated protocol for *I. ricinus* nymphal stages feeding on mouse (17). Briefly, the mice were anesthetized by isoflurane and the 2 cm outer diameter EVA-foam capsule (Cosplay Shop, Brugge, Belgium) was glued on their shaved backs using a non-irritating latex glue (Tear Mender, USA). Each mouse was infested with twenty *I. ricinus* nymphs on study d40. The individual ticks were deposited, one by one, to the capsule by forceps via a slit in the plastic lid that close the capsule. The ticks feeding were visually monitored twice a day.

DNA Extraction and 16S rRNA Sequencing

Genomic DNA was extracted from unfed nymphs and fully-engorged nymphs. Before DNA extraction, nymphs were washed two times in miliQ sterile water and one time in 70% ethanol. Ticks were pooled (5 ticks per pool) and crushed with glass beads using a Precellys24 Dual homogenizer (Bertin Technologies, Paris, France)

at 5500× g for 20 s. Genomic DNA was also extracted from mouse fecal samples in *E. coli*-immunized and mock-immunized mice. Tick and fecal genomic DNA was extracted using a Nucleospin tissue DNA extraction Kit (Macherey-Nagel, Hoerd, France). Each DNA sample was eluted in 100 µl of sterile water. Genomic DNA quality (OD260/280 between 1.8–2.0) was measured with NanoDrop™ One (Thermo Scientific, Waltham, MA, USA). More than 900ng of DNA at ≥ 20 ng/µL concentration were sent for amplicon sequencing of the bacterial 16S rRNA gene, which was commissioned to Novogene Bioinformatics Technology Co. (London, UK). Libraries were prepared with NEBNext® Ultra™ IIDNA Library Prep Kit (New England Biolabs, MA, USA). A single lane of Illumina MiSeq system was used to generate 251-base paired-end reads from the V4 variable region of the 16S rRNA gene using barcoded universal primers (515F/806R) in samples from unfed nymphs (n = 6 tick pools), nymphs engorged on *E. coli*-immunized (n = 9), *L. mesenteroides*-immunized (n = 5), or mock-immunized (n = 7) mice. Mouse fecal samples from *E. coli*-immunized (n = 12, 4 samples per group per time point, d0, d30 and d46) and mock-immunized (n = 12) mice were sequenced as described above. The raw 16S rRNA sequences obtained from tick samples, and mice fecal samples were deposited at the SRA repository (Bioproject No. PRJNA725498). Two extraction reagent controls were set in which the different DNA extraction steps were performed using the same conditions as for the samples but using water as template. DNA amplification was then performed on the extraction control in the same conditions as for any other sample.

16S rRNA Sequences Processing

The analysis of 16S rRNA sequences was performed using QIIME 2 pipeline (v. 2019.1) (18). The sequences in the fastq files were denoised and merged using the DADA2 software (19) as implemented in QIIME 2. The amplicon sequence variants (ASVs) were aligned with q2-alignment of MAFFT (20) and used to construct a phylogeny with q2-phylogeny of FastTree 2 (21). Taxonomy was assigned to ASVs using a classify-sklearn naïve Bayes taxonomic classifier (22) based on SILVA database (release 132) (23). Only the target sequence fragments were used in the classifier (i.e., classifier trained with the primers) (24, 25). Taxa that persisted across serial fractions of the samples using QIIME 2 plugin feature-table (core-features) were considered ubiquitous (18).

Relative Quantification of Bacterial Genes *atoB* and *eutD* by qPCR

To quantify the relative abundance of *E. coli* *atoB* and *eutD* genes in the tick microbiome, a qPCR was carried out using primers for *atoB* (*atoB*-F ‘AAGCACGCTCTGGTTATCGT’ and *atoB*-R ‘AATGTAGCGCCAGTTCATCC’) *eutD* (*eutD*-F ‘GGTGGATGGCGAGTTACAGT’ and *eutD*-R ‘CACGCTACAACCACGAGAGA’) designed using Primer-BLAST. Gene amplification was performed with SYBR Green LightCycler 480 Master mix (Roche, Meylan, France) and the conditions as follow: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The CT values were

recorded, and the relative levels of bacterial DNA were normalized against tick *rsp4* as housekeeping gene. Fold change in relative quantities were calculated using the $2^{-\Delta\Delta Ct}$ ratio method.

Bacterial Co-Occurrence Networks, Identification of Keystone Taxa and Attack Tolerance Test

Co-occurrence networks were inferred for each dataset, based on taxonomic profiles, collapsed at the genus level. Correlation matrices were calculated using the SparCC method (26), implemented in the R environment. The topological parameters, i.e., the number of nodes and edges, weighted degree, centrality metrics and the hub-score of each node, the diameter of the network, modularity, and clustering coefficient were calculated for each network. Network calculations and visualizations were prepared with the software Gephi 0.9.2 (27). Three criteria were used to identify keystone nodes within the networks as previously described (15): (i) high eigenvector centrality values, (ii) ubiquitousness, and (iii) the combination of high relative abundance and eigenvector centrality values. The resistance of these taxonomic networks to taxa removal (i.e., attack tolerance) was tested on these taxonomic networks. The purpose was to measure their resistance to the systematic removal of nodes, either by a random attack with 100 iterations, or by a directed attack, removing the nodes according to its value of betweenness centrality (the highest, the first). The analysis of the network resistance was done with the package NetSwan for R (28).

Prediction of Functional Traits in the Tick Microbiome

The 16S rRNA amplicon sequences from each data set were used to predict the metabolic profiling of each sample. PICRUSt2 (29) was used to predict the metagenomes from 16S rRNA amplicon sequences. Briefly, the AVSs were placed into a reference tree (NSTI cut-off value of 2) contained 20,000 full 16S rRNA sequences from prokaryotic genomes, which is then used to predict individual gene family copy numbers for each AVS. The predictions are based on Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO) (30). The output of these analyses included pathways and EC (Enzyme Commission number) profiling; the pathways were constructed based on the MetaCyc database (31).

Statistical Analysis

Differences in relative Ab levels (i.e., OD) between groups of immunized mice in the different time points were compared using two-way ANOVA with Bonferroni multiple comparison tests applied for individual comparisons. Microbial diversity analyses were carried out on rarefied ASV tables, calculated using the q2-diversity plugins. The alpha diversity (richness and evenness) was explored using Faith’s phylogenetic alpha diversity index (32) and Pielou’s evenness index (33). Differences in α -diversity metric between groups were assessed using Kruskal-Wallis test (alpha= 0.05). Bacterial β -diversity was

assessed using the Bray Curtis dissimilarity (34), and compared between groups using the PERMANOVA test. The differential abundant taxa and functional feature (KO genes and pathways) were explored between bacteria- and PBS-immunized mice, the differential features were detected by comparing the log₂ fold change (LFC) using the Wald test as implemented in the compositional data analysis method DESeq2 (35).

Correlations between tick microbiota bacteria abundance and mice Abs levels were calculated with the ANOVA-Like Differential Expression (ALDEx2, v. 1.22.0) correlation analysis (aldex.cor function) as implemented in R (v. 4.0.3). Unpaired non-parametric Mann-Whitney U test was used to compare the tick parameters (i.e., time to complete feeding, the weight of engorged ticks and tick mortality) between groups. Two-way ANOVA and Mann-Whitney U test analyses were performed in the GraphPad 5 Prism software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

RESULTS

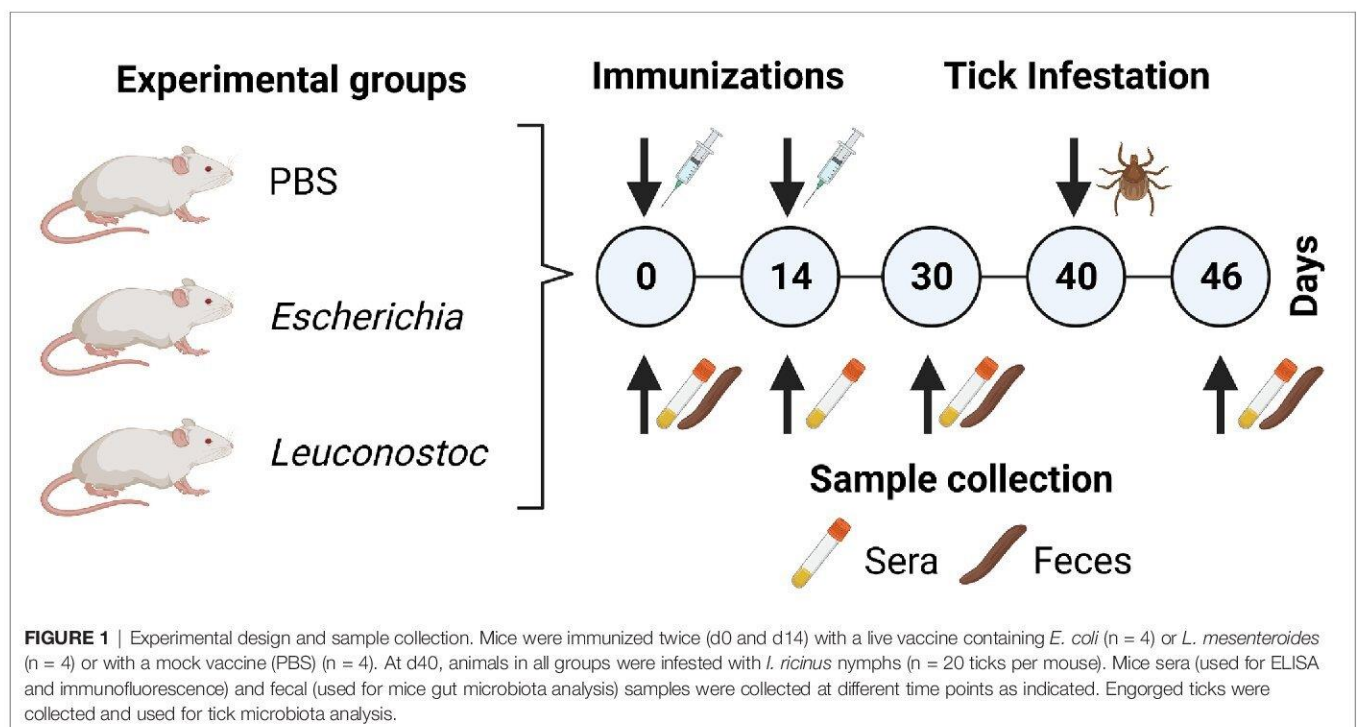
Vaccination Against Keystone Bacteria Increased Tick Engorgement Weight

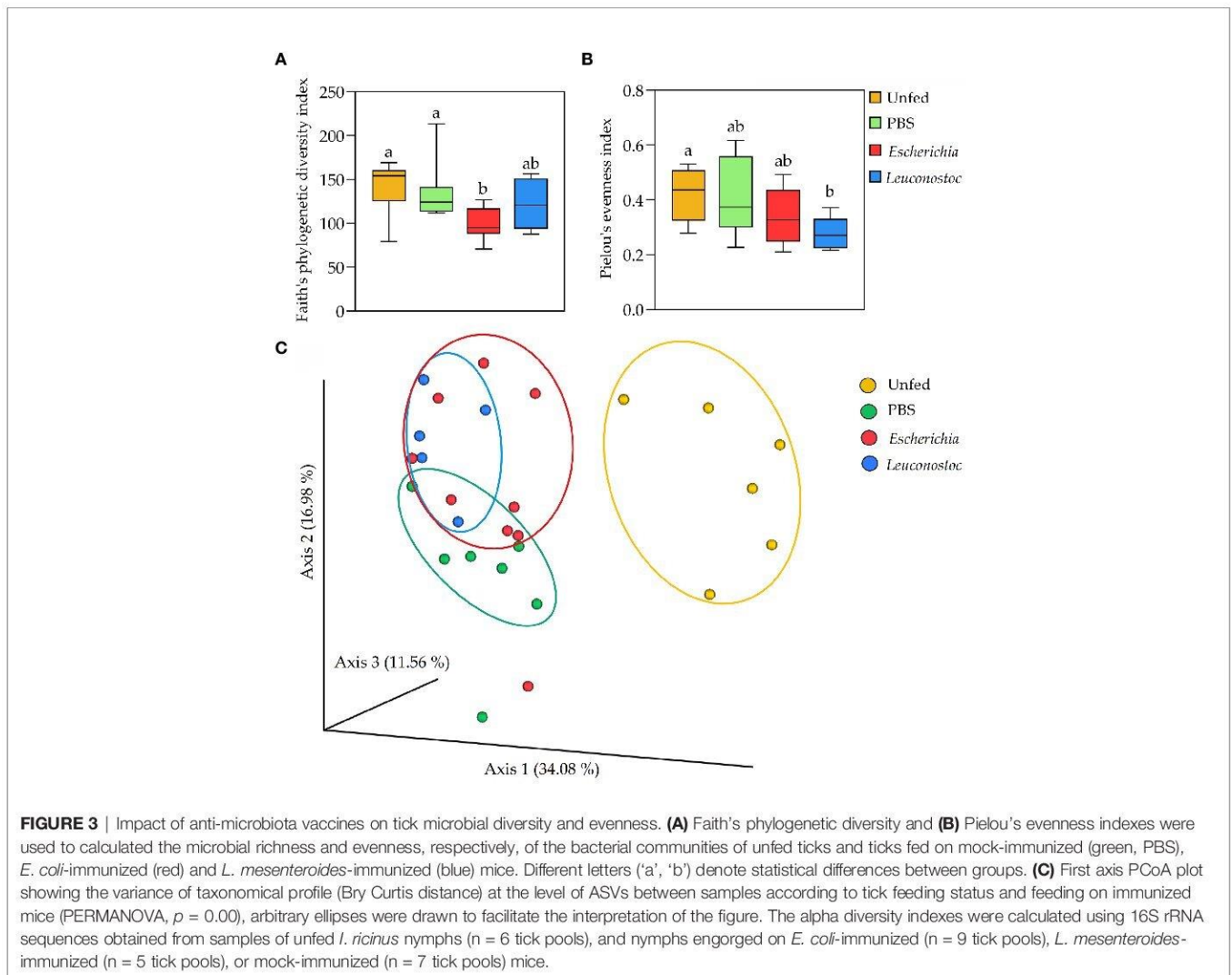
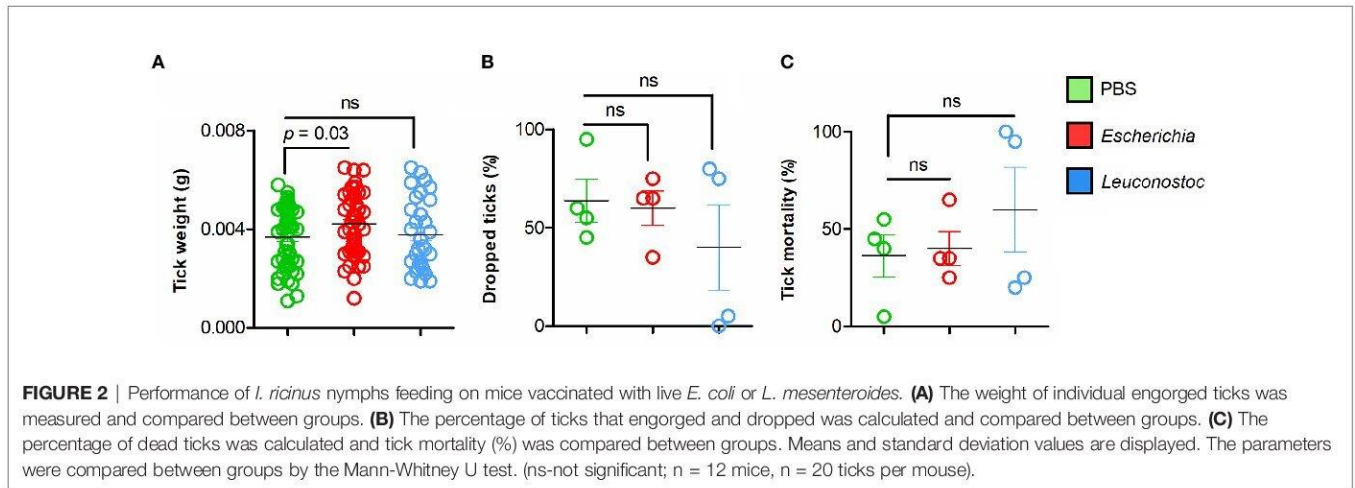
Following the immunization protocol, each mouse was infested with 20 *I. ricinus* nymphs (Figure 1). Time to complete feeding, weight of engorged ticks and tick mortality were recorded and compared between bacteria-immunized groups and control group (immunized with a mock vaccine). A significant increase (Mann-Whitney U test, $p = 0.03$) in weight was recorded in nymphs fed on *E. coli*-immunized mice compared with nymphs fed on mice of the control group (Figure 2A). This was not the case for ticks engorged

on *L. mesenteroides*-immunized mice (Mann-Whitney U test, $p > 0.05$). There were no significant differences (Mann-Whitney U test, $p > 0.05$) in the total number of ticks that dropped naturally (Figure 2B) or the mortality of ticks (Figure 2C) that fed on *E. coli*-immunized or *L. mesenteroides*-immunized mice compared with the control group. No mortality or adverse reactions were observed in mice immunized with *E. coli* or *L. mesenteroides*.

Vaccination Against Keystone Bacteria Reduced Bacterial Diversity in *I. ricinus* Microbiota, and Does Not Affect Mouse Gut Microbiota

The impact of anti-microbiota vaccines on the diversity, composition and abundance of tick microbiota bacteria was assessed after 16S rRNA amplicon sequencing of DNA extracted from unfed *I. ricinus* nymphs or from nymphs engorged on *E. coli*-immunized, *L. mesenteroides*-immunized, or mock-immunized mice. Vaccination with *E. coli* reduced the bacterial diversity associated with the tick microbiota ($H = 8.6$, $p = 0.03$, Figure 3A), but had no significant impact ($H = 5.8$; $p = 0.12$) on the species evenness, compared to unfed nymph (Figure 3B). Conversely, vaccination with the non-keystone bacterium *L. mesenteroides* had no significant impact on bacterial diversity (Figure 3A), but reduced significantly the species evenness (Figure 3B). Overall, the comparison of the diversity indexes of unfed and fed ticks revealed that anti-microbiota vaccination interferes with the normal dynamics of tick microbiota, regardless of the keystone status of the bacteria used in the vaccine formulation. Accordingly, a Principal Coordinates Analysis (PCoA) showed that the profiles of both groups of ticks that fed on bacteria-immunized mice were very





similar compared to mock-immunized or unfed ticks ($F = 5.30$; $p = 0.00$, **Figure 3C**).

To rule out a negative effect of anti-microbiota vaccine on host microbiome, we collected mouse feces at different time

points (**Figure 1**) and compared mouse gut microbiota before (d0) and after (d30 and d46) vaccination in the *E. coli*-immunized and mock-immunized mice. Results showed non-significant differences in the microbial richness ($p > 0.05$)

between the groups in the different time point, as measured by Faith's phylogenetic index (**Supplementary Figure S1A**). PERMANOVA (Adonis) test revealed that the factor time modified the Bray Curtis dissimilarity index ($F= 5.49$; $p = 0.001$) in both groups. However, no significant differences in the Bray Curtis dissimilarity index ($F = 0.67$; $p = 0.67$) or in the interaction vaccine/time ($F= 1.36$; $p = 0.17$) were observed between groups (*E. coli*-immunized vs. mock-immunized mice) (**Supplementary Figure S1B**). Furthermore, we observed no significant differences in the taxa abundance between groups at each time point (**Supplementary Figure S1C**). Notably, the relative abundance of *Escherichia-Shigella* in the mouse feces was not affected by the anti-microbiota vaccine containing *E. coli* (**Supplementary Figure S1D**).

The PCR amplification of extraction reagent negative controls (see methods) did not produce DNA products. In addition, rarefaction curves showed that there was sufficient number of

reads to draw a reliable list of bacterial genera within each tick and mouse sample (**Supplementary Figure S2**).

E. coli-specific Abs Are Associated With Reduced Abundance of the Keystone Taxon *Escherichia-Shigella*

Taxa composition and abundance analysis showed significant changes in the abundance of several bacterial genera in ticks fed on mock-immunized mice compared with unfed ticks (**Figure 4A**). The relative abundance of several taxa changed significantly in the ticks engorged on either *L. mesenteroides*-immunized (**Figure 4B**), or *E. coli*-immunized (**Figure 4C**) mice compared with the control group. The taxa with significant changes in abundance [measured as centered log ratio (clr)] are displayed in **Figures 4D, E** for *L. mesenteroides*-immunized and *E. coli*-immunized mice, respectively. Notably, the abundance of *Escherichia-Shigella*, but not *Leuconostoc*, was

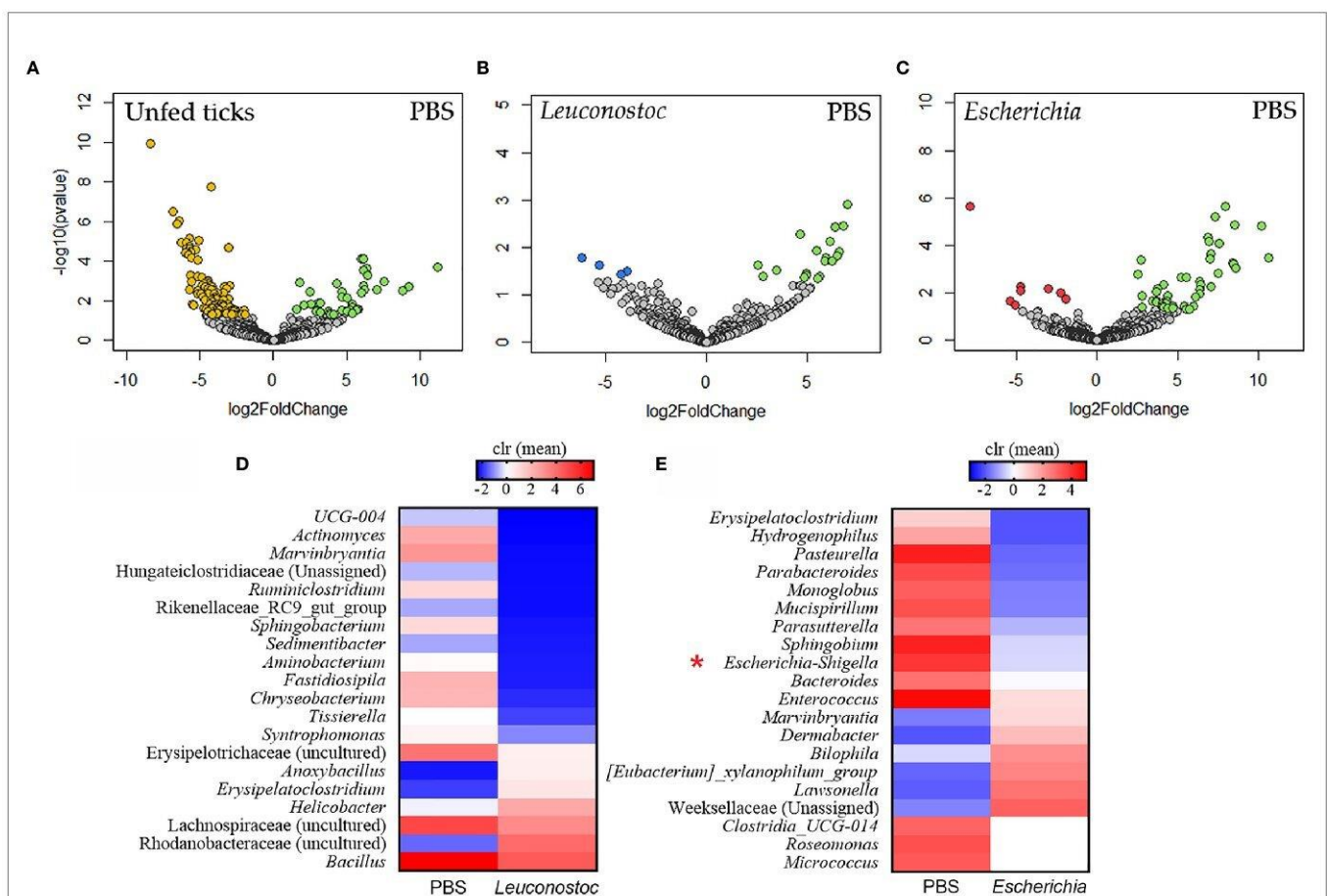


FIGURE 4 | Impact of anti-microbiota vaccines on the taxonomic profiles of tick microbiome. Volcano plot showing differential bacterial abundance in ticks of the different groups: **(A)** unfed ticks vs. ticks fed on mock-immunized mice (PBS), **(B)** ticks fed on mock-immunized vs. *L. mesenteroides*-immunized mice, and **(C)** ticks fed on mock-immunized vs. *E. coli*-immunized mice. The yellow (unfed ticks), blue (ticks fed on *L. mesenteroides*-immunized mice), and red (ticks fed on *E. coli*-immunized mice) dots indicate taxa that displayed both large magnitude fold-changes and high statistical significance favoring disturbed or control group (green dots), while the gray dots are considered as not significantly different between groups. Relative abundance (calculated as clr transformed values) of the 20 top bacterial taxa with the highest significant differences on ticks fed on mock-immunized vs. *L. mesenteroides*-immunized mice **(D)** and on ticks fed on mock-immunized vs. *E. coli*-immunized mice **(E)**, as detected by the DeSeq2 algorithm (Wald test, $p < 0.001$). *Escherichia-Shigella* was marked in the figure (red asterisk). The taxonomic profiles of the tick microbiota were characterized using 16S rRNA sequences obtained from samples of unfed *I. ricinus* nymphs ($n = 6$ tick pools), and nymphs engorged on *E. coli*-immunized ($n = 9$ tick pools), *L. mesenteroides*-immunized ($n = 5$ tick pools), or mock-immunized ($n = 7$ tick pools) mice.

significantly reduced in ticks that fed on *E. coli*-immunized mice compared with the control group (Figure 4E). In contrast, the abundance of *Escherichia-Shigella* or *Leuconostoc* was not significantly affected in ticks that fed on *L. mesenteroides*-immunized mice (Figure 4D).

Immunization with live *E. coli* elicited IgM and IgG responses specific to *E. coli* (Figure 5A). Strong and specific immune reaction of mouse IgM against *E. coli* was confirmed by immunofluorescence (Supplementary Figure S3A). The immunofluorescence reaction of anti-*E. coli* IgG against *E. coli* was comparatively less intense (Supplementary Figure S3A). Immunization with live *L. mesenteroides* elicited only marginal levels of IgM on d30, and the Ab levels had dropped by d46 (Figure 5B), suggesting that in contrast to *E. coli*, *L. mesenteroides* was poorly immunogenic as a live vaccine in the conditions used here. Consistent with the low IgM response raised by *L. mesenteroides* immunization, a poor recognition of *L. mesenteroides* by mouse anti-*L. mesenteroides* IgM was observed by immunofluorescence (Supplementary Figure S3B). A poor reaction was observed when anti-*L. mesenteroides* IgG detection was used in the immunofluorescence (Supplementary Figure S3B), which was consistent with a non-significant increase of anti-*L. mesenteroides* IgG after immunization with live *L. mesenteroides* (Figure 5B).

No significant cross-reaction to *E. coli* proteins was detected in the IgM and IgG fractions of the sera of mice immunized with the live vaccine containing *L. mesenteroides* (Supplementary Figure S4A). A weak increase in anti-*E. coli* IgM on d30 and d46, and no increase in anti-*E. coli* IgG were observed in *L. mesenteroides*-immunized mice. However, an increase in anti-*L. mesenteroides* IgM (d14, d30 and d46) and IgG (d30 and d46)

was detected in response to *E. coli* vaccination (Supplementary Figure S4B).

To test a possible association between the increase in *E. coli*-specific IgM and IgG after vaccination and the reduced abundance of *Escherichia-Shigella*, we performed an ALDEx2 correlation analysis between the abundance of all bacterial taxa at genus level and Abs levels. Significant negative correlations were found between the levels of anti-*E. coli* IgM ($r_s = -0.60, p = 0.01$) and IgG ($r_s = -0.57, p = 0.02$) in mice sera and the abundance of *Escherichia-Shigella* in the tick microbiota. Negative correlations between anti-*E. coli* IgM ($r_s = -0.57, p = 0.02$) and IgG ($r_s = -0.64, p = 0.01$) levels and an additional bacterial genus (0.18%, total 533 taxa), *Parabacteroides* (Family Porphyromonadaceae) was also found. A positive correlation was found between the genus *Lawsonella* (Order Corynebacteriales), and anti-*E. coli* IgM ($r_s = 0.65, p = 0.01$) and IgG ($r_s = 0.68, p = 0.008$) levels. The abundance of no taxa was found to correlate with the levels of both anti-*E. coli* IgM and IgG in the *L. mesenteroides*-immunized mice. In addition, no statistically significant correlations were found between the anti-*L. mesenteroides* IgM and IgG levels and the abundance of *Leuconostoc*, or any other taxa identified in the tick microbiome. Taken together, these results suggest that anti-*E. coli* immunization in mice reduces the *Escherichia-Shigella* abundance within the tick microbiome in a taxon-specific manner.

Escherichia coli Vaccination Reduced the Keystoneness of *Escherichia-Shigella* and the Tolerance of Co-Occurrence Networks to Taxa Removal

The impact of live bacteria immunization on the structure of the tick microbial communities was visualized and quantified using

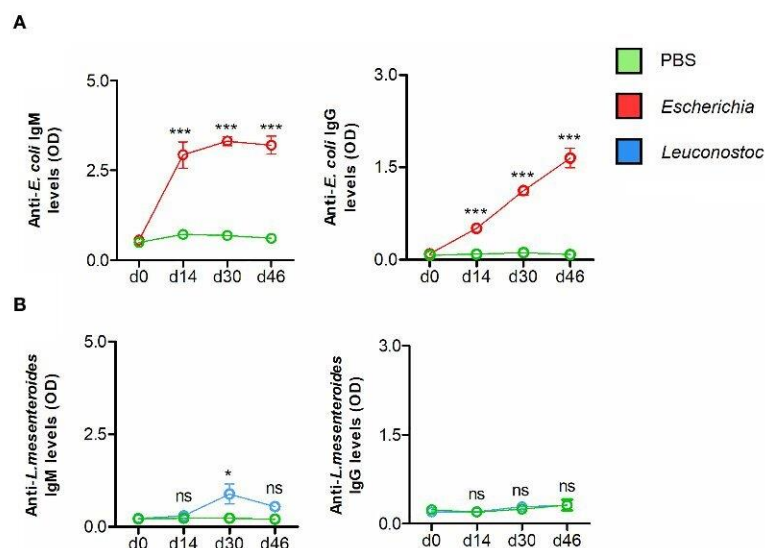


FIGURE 5 | Antibody response of mice vaccinated with live *E. coli* or *L. mesenteroides*. The levels of IgM and IgG specific to (A) *E. coli* and (B) *L. mesenteroides* proteins were measured by semi-quantitative ELISA in sera of mice immunized with *E. coli* (red) and *L. mesenteroides* (blue), respectively. Antibody levels of bacteria-immunized mice were compared with those of mock-immunized (green, PBS) mice. Means and standard error values are shown. Results were compared by two-way ANOVA with Bonferroni test applied for comparisons between control and immunized mice. (* $p < 0.05$, *** $p < 0.0001$; ns-not significant; 1 experiment, $n = 12$ mice and three technical replicates per sample).

co-occurrence networks. In accordance with their classification as non-keystone and keystone taxa, *Leuconostoc* (Figure 6A) and *Escherichia-Shigella* (Figure 6B) had low and high connectivity, respectively, in the microbial community of ticks fed on mock-immunized. Visual inspection of the local connectedness around *Leuconostoc* and *Escherichia-Shigella* revealed that the number of co-occurring taxa increased (Figure 6C) and decreased (Figure 6D) in the networks of tick microbiota exposed to anti-*L. mesenteroides* and anti-*E. coli* Abs, respectively. Notably, the eigenvector centrality value of *Leuconostoc* in the networks inferred from ticks fed on *L. mesenteroides*-immunized mice (eigenvector 0.11) was very similar to that of *Leuconostoc* in the control network (eigenvector 0.12). In contrast, the eigenvector centrality value of *Escherichia-Shigella* decreased 95 times in the network of ticks fed on *E. coli*-immunized (eigenvector 0.01) compared with those fed on mock-immunized mice (eigenvector 0.95). Visual (Supplementary Figure S5) and numerical (Table 1) comparison of the

networks showed that, in addition to the local connectedness effect, anti-microbiota vaccination had a large impact on the structure of the microbial community of ticks. For instance, the number of edges in the co-occurring networks of ticks that fed on *E. coli*-immunized and *L. mesenteroides*-immunized mice decreased and increased, respectively, compared with the control network (Table 1).

Co-occurrence networks were tested for attack tolerance. In this analysis, the resistance of the networks to random or directed removal of nodes was measured and the proportion of taxa removal needed to reach a loss in connectivity of 0.90 was recorded for each network. A proportion of 0.58 randomly removed nodes in the control (Figure 7A), 0.55 in the *E. coli* (Figure 7B) and 0.66 in the *L. mesenteroides* (Figure 7C) networks produced a 0.90 connectivity loss. The same loss in connectivity (i.e., 0.90) was observed when a smaller proportion of highly central nodes, 0.23, 0.14 and 0.53 was removed from the control, *E. coli* and *L. mesenteroides* networks, respectively

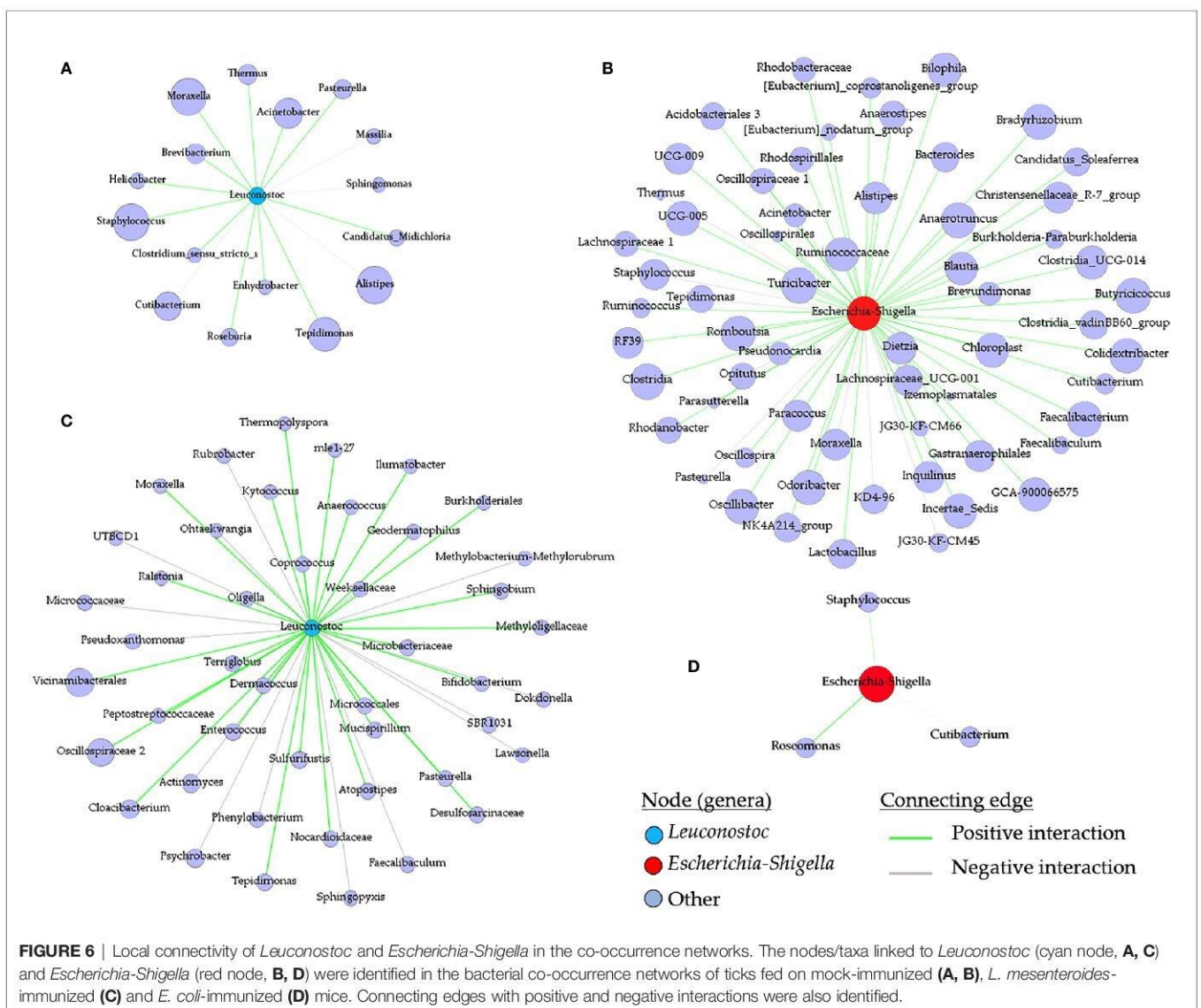


TABLE 1 | Topological parameters of co-occurrence networks.

Network features	Control (PBS)	Vaccinated with <i>L. mesenteroides</i>	Vaccinated with <i>E. coli</i>
Nodes	525	503	518
Edges	2930	7098	910
Positive	2159 (73.7%)	3862 (55.5%)	723 (79.4%)
Negative	771 (26.3%)	3228 (45.5%)	187 (20.6%)
Network Diameter	9	7	10
Average degree	11.2	28.3	8.4
Weighted degree	4.4	2.2	1.7
Average path length	3.4	2.8	3.9
Modularity	1.1	4.9	0.9
Number of modules	22	49	33
Average clustering coefficient	0.6	0.6	0.5

(Figure 7). Thus, immune targeting of the keystone taxon *Escherichia-Shigella* decreased the attack tolerance of the bacterial co-occurrence network. The random and directed removal curves within the network of ticks from *L. mesenteroides*-immunized mice revealed high similarity, which was not the case in the other two networks. This result suggests an unstructured hierarchy of nodes in the co-occurrence network of ticks from *L. mesenteroides*-immunized mice, and a reshaping in the hierarchy of nodes in the co-occurrence network of ticks from *E. coli*-immunized mice compared to the control network.

Escherichia coli Vaccination Reduced the Abundance of Lysine Degradation Genes in Tick Microbiome

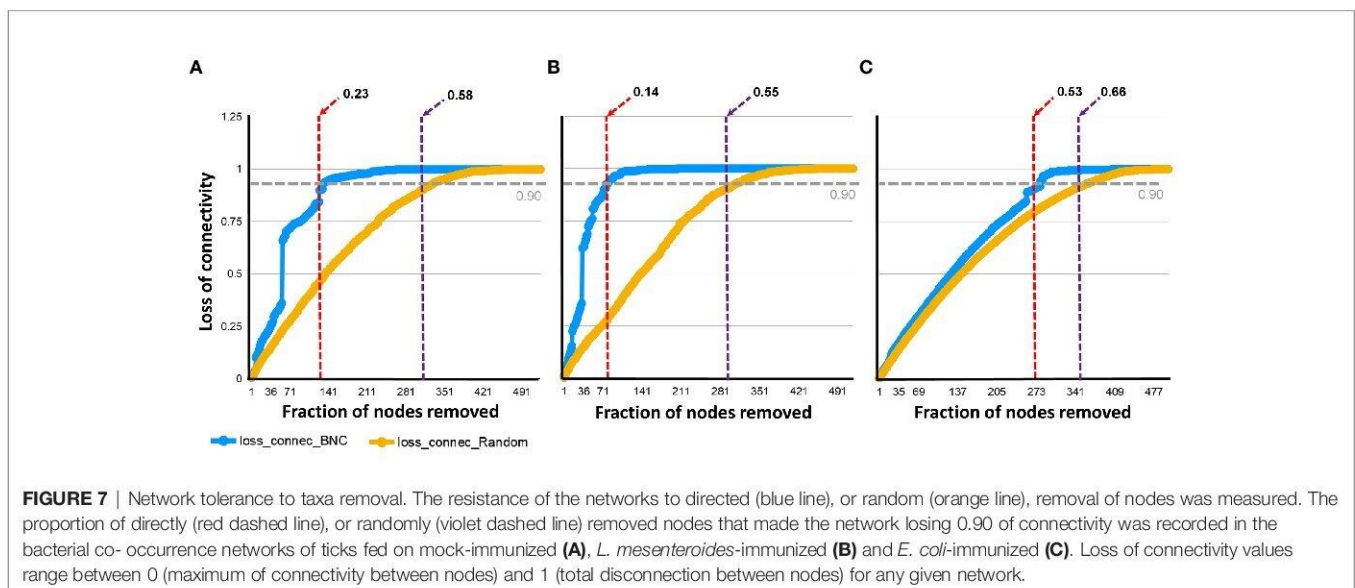
We analyzed microbial putative functions displayed by the microbiome of unfed ticks and those that fed on *L. mesenteroides*-immunized and *E. coli*-immunized mice by PICRUST2 and compared them with the predicted functions of bacterial communities present in the ticks fed on mock-immunized mice (Figure 8). Significant differences were found

in the relative abundance (fold changes) of several putative genes (KO) in the *I. ricinus* microbiome of nymphs fed on mock-immunized mice compared to unfed ticks (Figure 8A, Supplementary Table S1). Major changes in the putative gene profiles were also observed in the microbiome of tick from *L. mesenteroides*-immunized (Figure 8B, Supplementary Table S2) and *E. coli*-immunized (Figure 8C, Supplementary Table S3) mice.

Fold changes in the relative abundance of several pathways were found in unfed ticks (Figure 8D), *L. mesenteroides*-immunized (Figure 8E) and *E. coli*-immunized (Figure 8F) mice, compared to ticks fed on mock-immunized mice. The abundance of 115 pathways changed significantly (81 and 34 with decreased and increased abundance, respectively, Log2fold change > 1, $p < 0.05$) in ticks that fed on mock-immunized mice compared to unfed ticks (Supplementary Table S4). Among them, 96 were found exclusively in the microbiome of ticks fed on mock-immunized mice compared to unfed ticks and in none of the vaccinated groups (Figure 9A). Four pathways (i.e., methanogenesis from acetate, super pathway of glycerol degradation to 1,3-propanediol, superpathway of (Kdo)2-lipid A biosynthesis, and CMP-legionaminic acid biosynthesis I) changed significantly in the three groups of fed ticks (Figure 9) and they may represent functional changes induced by blood feeding in the bacterial communities independent of the treatment.

Thirteen pathways had a log2fold change lower than -1 in the functional prediction of the microbiome of ticks fed on *L. mesenteroides*-immunized mice, compared to the control group of ticks fed on mock-immunized mice (Supplementary Table S5). Only one of them, tetrahydromethanopterin biosynthesis (PWY-6148, Log2fold change = -5.5, Kruskal-Wallis test, $p = 0.02$), changed exclusively in the ticks of the *L. mesenteroides*-immunized group (Figure 9B).

Fourteen pathways had a log2fold change lower than -1 in the functional prediction of the microbiome of ticks fed on *E. coli*-immunized mice, compared to the control group (Supplementary



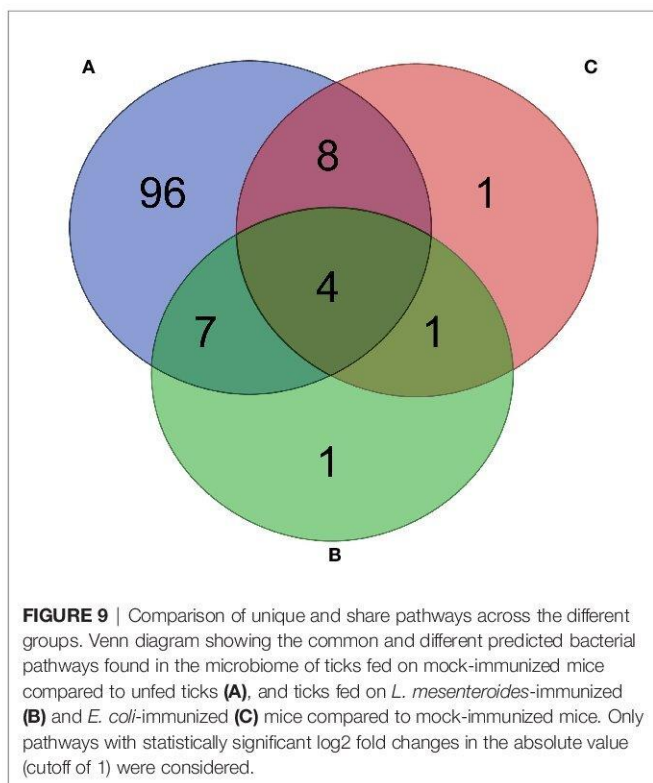
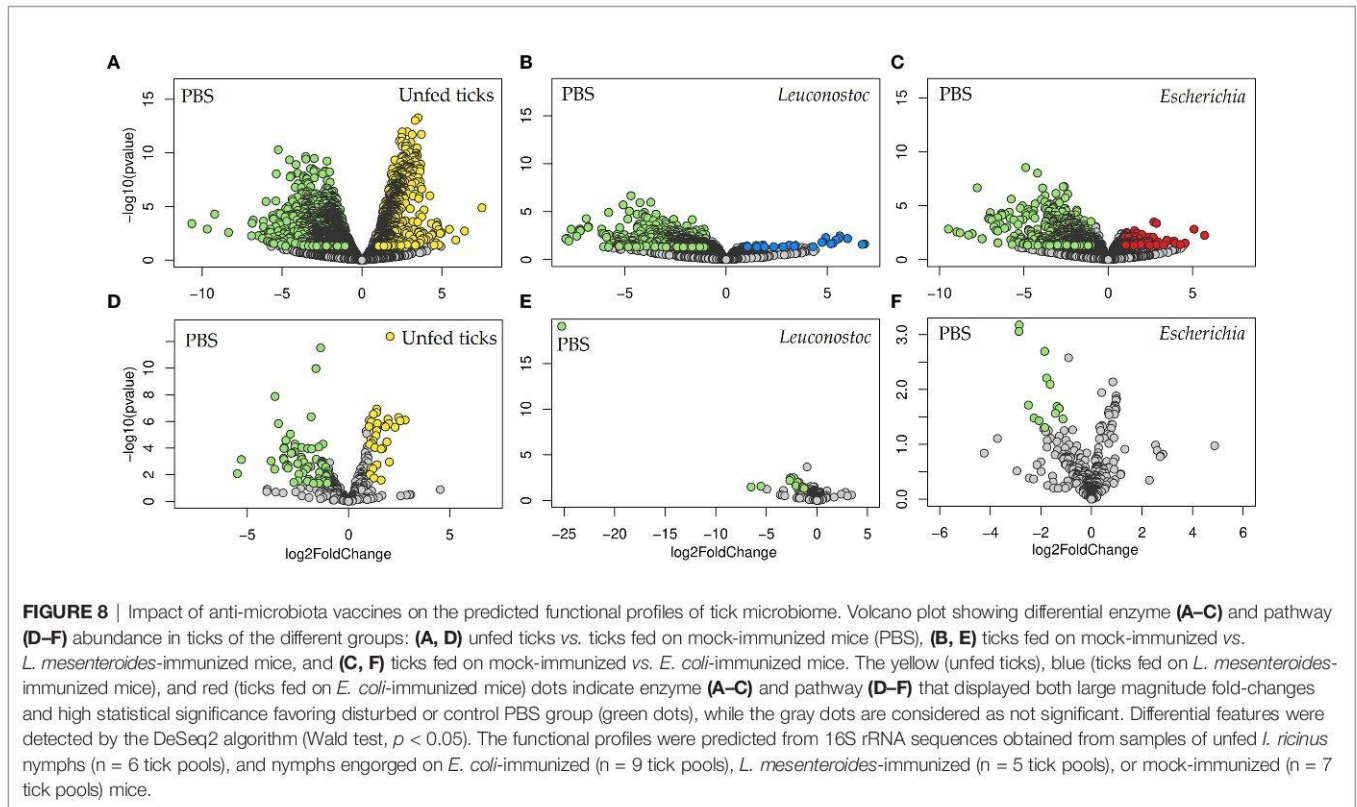


Table S6. A significant decrease in the relative abundance of the L-lysine fermentation to acetate and butanoate pathway (P163-PWY, Log2fold change = -1.6, Kruskal-Wallis test, $p = 0.008$) was

found exclusively in ticks fed on *E. coli*-immunized mice (Figure 9C). The pathway P163-PWY is composed of ten enzymatic steps that transform L-lysine into acetate and butanoate (Figure 10A). The bacterial genes *atoB* and *eutD* encoding for the enzymes acetyl-CoA acetyltransferase (EC.2.3.1.9) and phosphate acetyltransferase (EC.2.3.1.8) of the P163-PWY pathway in *E. coli* were selected for validation of the PICRUST2 prediction. The relative abundance of the genes *atoB* and *eutD* was significantly lower in ticks fed on *E. coli*-immunized mice compared to the control group (Figure 10B).

DISCUSSION

Several studies have shown that the tick microbiome is a gate to access tick physiology and vector competence (1, 36). Reduced bacterial loads have been associated with lower reproductive fitness after antibiotics treatment in ticks (3, 36–40). Considering that antibiotics can target several bacteria species simultaneously, studies using broad-spectrum antimicrobial compounds make impossible establishing causal links between specific taxa and alterations in tick physiology and/or vector competence. Recently, we showed for the first time that anti-tick microbiota vaccines impact tick performance during feeding (15). Immunization with the Enterobacteriaceae bacterium *Escherichia coli* elicited anti-*E. coli* IgM and IgG which were associated with increased engorgement weight of *I. ricinus* nymphs that fed on C57BL/6 mice and high mortality in ticks that fed on α -1,3-galactosyltransferase (α 1,3GT)-deficient

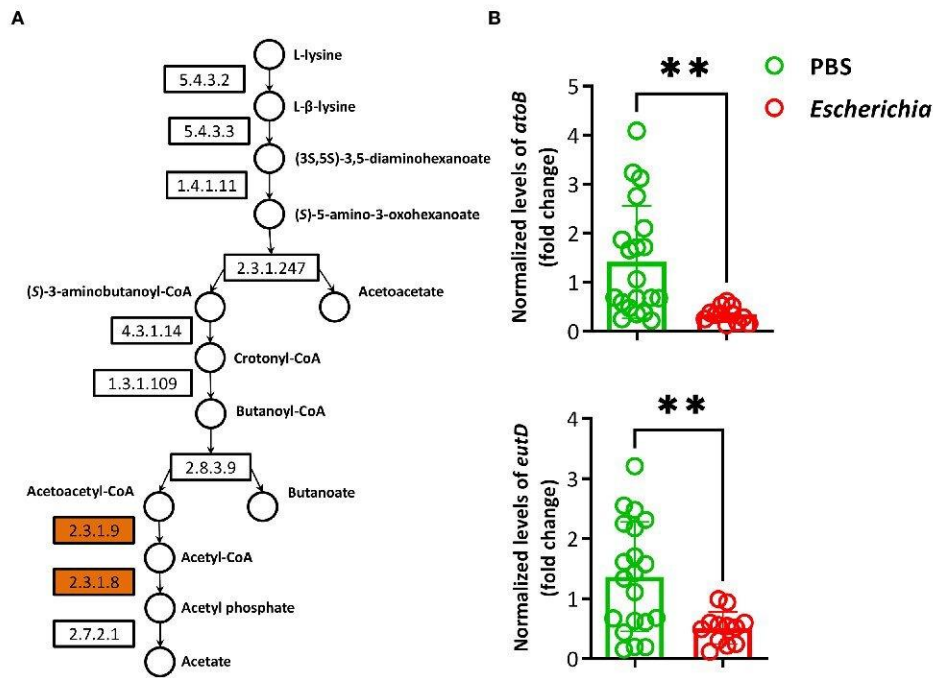


FIGURE 10 | Pathway P163-PWY and relative abundance of genes *atoB* and *eutD*. **(A)** The pathway P163-PWY was retrieved from MetaCyc database. The KEGG codes of the enzymes (boxes) and metabolites (close to the circles) involved in the pathway were added. Enzymes encoded by the genes *atoB* (EC.2.3.1.9) and *eutD* (EC.2.3.1.8) were highlighted (orange). **(B)** Normalized relative *atoB* (EC.2.3.1.9) and *eutD* (EC.2.3.1.8) levels were measured by qPCR in DNA samples of ticks fed on *E. coli*-immunized mice using gene-specific primers and normalizing against tick *rsp4* with the $2^{-\Delta\Delta Ct}$ ratio method. Results are relative to *atoB* and *eutD* in the control group (PBS). Individual technical replicates, means and standard deviation values are displayed. Results were compared by Mann–Whitney U test. (** $p < 0.001$; $n = 6$ DNA samples of ticks fed on *E. coli*-immunized mice, $n = 10$ DNA samples of the control group, two technical replicates per samples).

C57BL/6 mice compared with the ticks that fed on the control group, immunized with a mock formulation of PBS and adjuvant (15).

Considering that host Abs and complement acquired during tick feeding not only retain their immune functions, but also access several tick tissues (40–45), we hypothesized that anti-tick microbiota vaccines could be used as a precision microbiology tool to target selected taxa in the tick microbiome. Here, we showed that immunization with a live *E. coli* vaccine elicited bacterial-specific Abs of the isotypes IgM and IgG, which were associated with the reduction of the relative abundance of *Escherichia-Shigella* in engorged ticks. Therefore, it can be presumed that when ingested during blood feeding, the host Abs can reach the locations of tick microbiota bacteria. Abs induced against particular tick proteins can also reach and react with the corresponding tick protein within tick tissues (43, 46). For example, host Abs against the glycoprotein Bm86, predominantly located in the membrane of tick gut cells (46), modulate tick proteome (47) and bind to the surface of epithelial cells in the tick intestine causing cell lysis (43). The results suggest that host Abs bind and promote the killing of Gram-negative bacteria in the tick microbiota. Upon contact with tick microbiota bacteria, a step-wise activation process may result in the deposition of host complement proteins and insertion of Membrane Attack Complex (MAC) pores into Gram-negative bacterial membranes. This can lead to direct killing of Gram-

negative, but not Gram-positive bacteria (48–51). Even though the interaction of polyreactive antibodies with the bacteria surface stimulates the deposition of complement proteins on Gram-positive, this does not result in MAC-mediated lysis of this type of bacteria (48, 51). This suggests that anti-tick microbiota vaccines may be most effective against Gram-negative bacteria. In our study, the live vaccine using the Gram-positive bacterium *L. mesenteroides* had low immunogenicity. Therefore,

Targeting the keystone bacteria *Escherichia-Shigella* with host Abs also reduced the keystone-ness of this taxon in the networks, which was associated with a global modulation of the microbial community structure. The resulting community had a reduced alpha diversity and changes in the taxonomic and predicted functional profiles. In addition, the co-occurring network analysis showed that *Escherichia-Shigella*-depleted communities had fewer nodes and the connectivity between them was weak and more susceptible to taxa extinction when compared with the control group of ticks that fed on mock-immunized mice. Removal of keystone species has strong disturbing effects, resulting in loss of microbiota biodiversity in different ecological settings (52–55). Depletion of keystone species could also result in microbial dysbiosis that impairs the integrity of the gut ecosystem, as seen in vertebrates (56, 57). The impact of *E. coli* removal has been tested experimentally on a synthetic consortium of 14 human gut microbes (57). Removal of *E. coli* resulted in the highest impact on biomass and growth rates,

indicating major roles of this microorganism on a synthetic microbial consortium (57).

Here we used PICRUSt2 (29) to predict the metagenomes of tick microbiome using 16S rRNA amplicon sequences. The prediction showed that ticks fed on *E. coli*-immunized mice had a significant reduction in the relative abundance of the pathway P163-PWY. This result was validated by quantifying the abundance of two *E. coli* genes *atoB* and *eutD* encoding for enzymes involved in lysine fermentation via P163-PWY. The reduced abundance of these two *E. coli* genes concurs with the reduced abundance of *Escherichia-Shigella* in ticks fed on *E. coli*-immunized mice. This result suggests that bacterial community modulation by anti-microbiota vaccines could impact the functional profiles associated with the tick microbiome. Considering that lysine is an essential amino acid and that the tick genome does not encode for lysine synthesis enzymes (58), it is possible that a reduction of lysine degradation by tick microbiome might result in higher levels of free lysine available for tick metabolism. This could potentially explain the higher body weight of ticks fed on *E. coli*-immunized mice compared to mock-immunized and *L. mesenteroides*-immunized mice. The limitation here is that we used pathway prediction and the availability of free lysine in the gut of ticks fed on *E. coli*-immunized mice was not experimentally tested. Further investigation is needed to examine the metabolites dynamics *in vivo* in response to bacterial community modulation by anti-*E. coli* IgM and IgG.

CONCLUSIONS

Immunization with a live *E. coli* vaccine elicited an anti-*E. coli* IgM and IgG response that reduced the abundance of the keystone taxon *Escherichia-Shigella* in the tick microbiome. The results suggest that host Abs bind and kill *Escherichia-Shigella* bacteria in the tick microbiome, a conclusion supported by two evidences: (i) a negative correlation between the levels of both anti-*E. coli* IgM and IgG and the relative abundance of *Escherichia-Shigella* in the tick microbiome and (ii) the binding of anti-*E. coli* IgM and IgG to *E. coli ex vivo*. Thus, anti-tick microbiota vaccines can be used to target specific taxa within the tick microbiota through host antibodies (59). We also showed that tick engorgement, microbiome bacterial diversity and microbial community structure can be disturbed by vaccination with *E. coli* highlighting the important role that keystone microbiota bacteria have in tick performance and microbiome. Changes in the abundance of predicted enzymes and pathways suggest that the scope of anti-tick microbiota vaccine is not limited to the modulation of the tick microbiome at the taxonomic level, but it may also regulate the functions associated with the microbiome. As in our previous study (15), here we validated the use of PICRUSt2 as a suitable tool to detect functional changes in the tick microbiome. In summary, targeting keystone bacteria of the tick microbiota by host Abs seems to be a suitable tool for the modulation of tick microbiome to study the role of a specific taxon in tick physiology. Anti-tick microbiota vaccine can also be a powerful tool to evaluate the functional contribution of a specific taxon in tick microbiota on

pathogen colonization and transmission. These results guide precise interventions for the control of tick infestations and pathogen infection/transmission (59).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA725498.

ETHICS STATEMENT

The procedures were reviewed and approved by the Ethics Committee (ComEth, Anses/ENVA/UPEC), with permit number E 94 046 08.

AUTHOR CONTRIBUTIONS

AC-C, DO, JM, and LM-H conceived the study. LM-H, AW-C, JM, JB, and AH performed the experiments and acquired the data. DO, LM-H, SD-S, AE-P, AC-C, AH, and AW-C analyzed the data. DO, AC-C, AW-C and AE-P prepared figures and supplementary materials. LGB-H, ET-M, NV, and JF contributed reagents and other resources. AC-C, LS, LB-H, and JF supervised the work. AC-C, LM-H, AW-C, AH, and DO drafted the first version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.704621/full#supplementary-material>

Supplementary Figure 1 | Impact of anti-microbiota vaccines on fecal microbiota of mice. **(A)** Comparison of ASV richness between *E. coli*-immunized (red) and mock-immunized mice. **(B)** PCoA on Bray Curtis dissimilarity from *E. coli*-immunized (red) and mock-immunized mice (green) at d0 (T0), d30 (T2) and d46 (TF), compared by permutational (Adonis) test with 999 permutations. **(C)** Dendrogram heatmap on the taxonomic profile from all the samples in both vaccination groups at the three different time points. **(D)** The relative abundance of

the top 20 most abundant taxa, including *Escherichia-Shigella* (red asterisk) is displayed.

Supplementary Figure 2 | Rarefaction curves estimated from reads obtained for ticks and mice. (A) Rarefaction curves for unfed tick samples (light orange), and samples of ticks fed on mock-immunized (dark orange), *E. coli*-immunized (dark blue) and *L. mesenteroides*-immunized (light blue) mice are displayed. (B) Rarefaction curves for fecal samples in *E. coli*-immunized (dark blue) and mock-immunized mice are displayed.

Supplementary Figure 3 | Immunocytochemistry of *E. coli* and *L. mesenteroides* using sera of immunized mice. Fixed *E. coli* (A) and *L. mesenteroides* (B) were stained with pooled sera of mice immunized with a live *E. coli* vaccine (*Escherichia*), live *L. mesenteroides* vaccine (*Leuconostoc*) or mock vaccine (PBS). Examples of positive reaction are displayed (white arrows in inserts). Alexa fluor 488 conjugated anti-mouse antibody specific to the isotypes IgM and IgG were used as a secondary antibody. Negative control staining (Control) was performed using only the secondary antibody. Blue color indicates the nuclei visualized by 4',6-diamidino-2-phenylindole (DAPI). Images were obtained using 63X magnification and digital zoom. Scale bars are 2µm.

Supplementary Figure 4 | Antibody response of mice vaccinated with live *E. coli* or *L. mesenteroides*. The levels of IgM and IgG specific to (A) *E. coli* and (B) *L. mesenteroides* proteins were measured by semi-quantitative ELISA in sera of mice immunized with *L. mesenteroides* (blue) and *E. coli* (red), respectively, at different time points, d0, d14, d30 and d46. Antibody levels of bacteria-immunized mice were compared with those of mock-immunized (green, PBS) mice. Means and standard error values are shown. Results were compared by two-way ANOVA with Bonferroni test applied for comparisons between control and immunized mice. (* $p <$

0.05, ** $p <$ 0.001, *** $p <$ 0.0001; ns-not significant; 1 experiment, $n = 12$ mice and three technical replicates per sample.

Supplementary Figure 5 | A schematic representation of the co-occurring microbial taxa in the microbiome of ticks fed on mock-immunized (A), *L. mesenteroides*-immunized (B) and *E. coli*-immunized (C) mice. Circles (nodes) are bacterial genera and edges the co-occurrence between taxa. Equal colors mean clusters of taxa that co-occur more frequently among them than with other taxa. The size of the circles is proportional to the eigencentality of each taxon in the resulting network. The nodes *Escherichia-Shigella* (red) and *Leuconostoc* (cyan) were identified and labelled (lighting symbol).

Supplementary Table 1 | Predicted genes (KO) with differential abundance in the ticks that fed on mock-immunized mice compared to unfed ticks.

Supplementary Table 2 | Predicted genes (KO) with differential abundance in the ticks that fed on *L. mesenteroides*-immunized compared to mock-immunized mice.

Supplementary Table 3 | Predicted genes (KO) with differential abundance in the ticks that fed on *E. coli*-immunized compared to mock-immunized mice.

Supplementary Table 4 | Pathways with differential abundance in the ticks that fed on mock-immunized mice compared to unfed ticks.

Supplementary Table 5 | Pathways with differential abundance in the ticks that fed on *L. mesenteroides*-immunized compared to mock-immunized mice.

Supplementary Table 6 | Pathways with differential abundance in the ticks that fed on *E. coli*-immunized compared to mock-immunized mice.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter IIIb

Effect of the modulation of tick microbiota by anti-microbiota vaccine on *Borrelia afzelii* colonization in *Ixodes ricinus* tick

Microbiota perturbation by anti-microbiota vaccine reduces the colonization of *Borrelia afzelii* in *Ixodes ricinus*

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Abstract

Background

Ticks can transmit a broad variety of pathogens of medical importance, including *Borrelia afzelii*, the causative agent of Lyme borreliosis in Europe. Tick microbiota is an important factor modulating, not only the vector physiology, but also the vector competence. Anti-microbiota vaccines targeting keystone taxa of tick microbiota can alter tick feeding and modulate the taxonomic and functional profiles of bacterial communities in the vector. However, the impact of anti-microbiota vaccine on tick-borne pathogen development within the vector has not been tested.

Results

Here, we characterized the *Ixodes ricinus* microbiota modulation in response to *B. afzelii* infection, and found that the pathogen induces changes in the microbiota composition, its beta diversity and structure of bacterial community assembly. Tick microbiota perturbation by anti-microbiota antibodies or addition of a novel commensal bacteria into tick midguts causes departures from the *B. afzelii*-induced modulation of tick microbiota which resulted in a lower load of the pathogen in *I. ricinus*. Co-occurrence networks allowed the identification of emergent properties of the bacterial communities which better defined the *Borrelia* infection-refractory states of the tick microbiota.

Conclusions

These findings suggest that *Borrelia* is highly sensitive to tick microbiota perturbations and that departures from the modulation induced by the pathogen in the vector microbiota pose a high cost to the spirochete. Networks analysis emerges as a suitable tool to identify emergent properties of the vector microbiota associated with infection-refractory states. Anti-microbiota vaccines can be used as a tool for microbiota perturbation and control of important vector-borne pathogens.

Keywords: Anti-microbiota vaccine, vector microbiota, Lyme borreliosis, *Borrelia afzelii*, *Ixodes ricinus*

Background

Ticks are vectors of a multitude of pathogens that can cause infectious diseases of medical and veterinary importance. One major example of tick-borne disease is Lyme borreliosis [1], which is caused by a genospecies complex of the spirochete *Borrelia burgdorferi* sensu lato (s.l.) [2]. Among the genospecies, *Borrelia afzelii* is the causal agent of most cases of Lyme borreliosis in Europe [3]. *Borrelia* is maintained in nature owing to biological transmission mediated by ticks of the genus

Ixodes [4]. *Borrelia* spirochetes are generally acquired by the larval or nymphal stages of ticks that fed blood on an infected vertebrate host [5]. Once ingested by the ticks, the spirochetes enter and colonize the gut. After tick molting and in the next blood meal, *Borrelia* migrates from the gut to the salivary gland and are transmitted, by nymphs or adults, to a new host along with tick saliva [2, 3]. In this cycle of acquisition, colonization and transmission of *Borrelia* spirochetes by ticks, the group of endogenous bacteria that form the tick microbiota might have a pivotal role on it.

Several studies have demonstrated that microbiota can shape the vector competence for pathogens in different arthropods [6–10]. In ticks, for example, the antibiotic-based disruption of the microbiota of *Dermacentor andersoni*, the Rocky Mountain wood tick, reduced the acquisition of the pathogen *Francisella novicida* [11]. Furthermore, the level of *F. novicida* was positively correlated with a decrease of *Francisella* endosymbionts quantity in the microbiota of *D. andersoni* demonstrating a positive relationship pathogen-endosymbiont [11]. Perturbation of *Ixodes scapularis* microbiota to a dysbiotic state reduced *B. burgdorferi* colonization in larvae [9]. *Borrelia burgdorferi* abundance in *I. scapularis* ticks was negatively correlated with the abundance of some bacterial taxa such as *Pseudomonas* or *Staphylococcus* and positively correlated with *Sphingomonas* [12]. Associations between commensal bacteria and pathogen levels in ticks suggest intimate pathogen-microbiota interactions that could facilitate or limit pathogen colonization in the vector. Targeting specific bacteria of vector microbiota that facilitate pathogen colonization could be a possible method of control through transmission-blocking vaccines.

In general, experimental manipulation of the microbiota has been achieved by antibiotic exposure or sterile-rearing conditions of the vector. However, these methods induce global changes in the microbiota and make difficult the depletion of specific bacteria. Recently, anti-microbiota vaccine was proposed as a precise tool for microbiota manipulation [13, 14]. Notably, identification of the keystone taxon (i.e., highly connected taxa driving community composition and function), Enterobacteriaceae, and subsequent vaccination against it induced host antibodies that were ingested by the vector during the blood meal and correlated with a decreased abundance of Enterobacteriaceae in *Ixodes ricinus* microbiota [14]. Furthermore, anti-microbiota vaccine impacted tick physiology by increasing tick weight during feeding [13] and modulated tick microbiota composition and diversity in a taxon-specific manner [14]. The impact of anti-microbiota vaccines on pathogen development was shown in *Plasmodium relictum* and the mosquito vector *Culex quinquefasciatus* [15]. Immune targeting of vector-associated Enterobacteriaceae modulated *C. quinquefasciatus* microbiota composition and diversity and decreased the occurrence and abundance of *P. relictum* in the midguts and salivary glands of the mosquitoes [15].

In this study, we aim to test whether the manipulation of tick microbiota by anti-microbiota vaccination of host mice against the keystone taxon Enterobacteriaceae reduces *B. afzelii* colonization in the vector *I. ricinus*. Comparison of the normal tick microbiota with that exposed to *B. afzelii* infection, anti-microbiota antibodies, and a novel commensal bacterium allowed the identification of infection-permissive and infection-refractory states of the microbial communities. The results will inform novel interventions for the control of Lyme borreliosis and other vector-borne diseases.

Materials and methods

Ethics statement

In vivo experiments were performed at the Animal Facility of the Laboratory for Animal Health of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Maisons-Alfort, France, according to French and International Guiding Principles for Biomedical Research Involving Animals (2012). The procedures were reviewed and approved by the Ethics Committee (ComEth, Anses/ENVA/UPEC), with animal experimentation permit number E 94 046 08.

Mice and housing conditions

Wild-type female C3H/HeN (Charles River strain code 025) mice of six weeks old were purchased from Charles River (Miserey, France) and kept for adaptation for one week before experimental manipulation. During the study, mice were maintained in green line ventilated racks (Tecniplast, Hohenpeissenberg, Germany) at -20 Pa, with food (Kliba nafaj, Rinaustrasse, Switzerland) and water *ad libitum*. The mice were kept at controlled room temperature (RT, 20-23°C) and a 12-hour (h) light: 12-h dark photoperiod regimen. The number of mice per cage was limited to five. Animals were monitored twice a day (d) by experienced technicians and deviations from normal behaviors or signs of health deterioration were recorded and reported.

Bacterial cultures

Borrelia afzelii CB43 was donated by Dr. Ryan O. M. Rego from the Institute of Parasitology of Czech Academy of Sciences, České Budějovice, Czechia. Low passage isolates of *B. afzelii* CB43 were grown in Barbour-Stoenner-Kelly (BSK) -H (Sigma-Aldrich, St. Louis, MO, USA) media containing 6% rabbit serum and were kept at 33°C for seven days. *Escherichia coli* BL21 (DE3, Invitrogen, Carlsbad, CA, USA) was grown on Luria Broth (LB, Sigma-Aldrich, St. Louis, MO, USA) at 37°C under vigorous agitation overnight.

Experimental infection of mice with *B. afzelii*

For *Borrelia* infection, 1×10^6 *B. afzelii* CB43 contained in 250 μ L of BSK-H media was injected subcutaneously (100 μ L) and intraperitoneally (150 μ L) in the C3H/HeN mice. Normal mice were injected with BSK-H media alone, following the same protocol as described before. Blood samples were collected in animals from all experimental groups 3 weeks after inoculation to confirm the infection by western blot [16]. Additionally, the right ankle joint, the heart and the skin, were collected from each mouse of all experimental groups at the endpoint of the experiment to confirm the infection by qPCR (see below).

Live bacteria immunization

Live bacteria vaccine was prepared using *E. coli* BL21 (DE3, Invitrogen, Carlsbad, CA, USA) as previously described [13] Briefly, *E. coli* culture was washed with phosphate buffer saline (PBS) 10 mM NaH₂PO₄, 2.68 mM KCl, 140 mM NaCl, pH 7.2 (Thermo Scientific, Waltham, MA, USA), resuspended at 3.6×10^4 colony-forming unit (CFU)/mL, and homogenized using a glass homogenizer. C3H/HeN mice were immunized subcutaneously with 100 μ L of *E. coli* BL21 (1×10^6 CFU per mouse) in a water-in-oil emulsion containing 70% Montanide™ ISA 71 VG adjuvant (Seppic, Paris, France), with a booster dose two weeks after the first dose. Control mice received a mock vaccine containing PBS and adjuvant.

Tick Infestation

Unfed *I. ricinus* larvae were obtained from the colonies of UMR-BIPAR, Maisons-Alfort, France. Mice were anesthetized by isoflurane and the 2 cm outer diameter EVA-foam capsule (Cosplay Shop, Brugge, Belgium) was glued on their shaved backs using non-irritating latex glue (Tear Mender, USA). Each mouse in the different groups was infested with one hundred *I. ricinus* larvae at day 30 (Figure 1). The ticks, placed in a syringe, were deposited to the capsule by slowly pushing the plunger and then, a plastic lid was used to close the capsule. Tick feeding was visually monitored twice a day. Engorged larvae were collected in sterile tubes with holes and maintained with a light-dark (12 h/12 h) cycle in an incubator with >97% relative humidity at 22 °C.

Sera sample preparation

Blood samples were collected on sterile tubes on day 0 and day 30 in animals from all experimental groups. Additionally, blood samples were collected at days 14, 45 and 52 in mice from the PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups. Blood samples were incubated for 2h at RT, without anticoagulant, allowing for clotting, and then centrifuged at $5000 \times g$ for 5 min at RT, twice. Sera samples were then separated and stocked in new sterile tubes at -20°C until use.

Bacterial protein extraction

Lysates of *Borrelia afzelii* culture were prepared to perform western blots. 7 mL of culture of *B. afzelii* with a density of at least 1×10^7 /mL were centrifuged at 8000 rpm for 10 minutes at 20°C. The supernatant was then removed and the bacterial pellet was washed twice with 1mL cold HN-Buffer, centrifuged at 8000 rpm for 10 min at 20°C. The resulting pellet was re-suspended in 200 µL of bacterial protein extraction (B-PER) buffer (Thermo Scientific, Waltham, MA, USA) and incubated at RT for 10min. The lysate was stored at -20°C until use. *Escherichia coli* protein extraction was done to perform ELISA. *E. coli* culture were washed twice with PBS, centrifuged at 1000× g for 5 min at 4°C, resuspended in 1% Trion-PBS lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) and homogenized with 20 strokes using a glass balls homogenizer. The homogenate was then centrifuged at 300× g for 5 min at 4°C and the supernatant was collected. *B. afzelii* and *E. coli* protein concentration was determined using the Bradford Protein Assay (Thermo Scientific, San Jose, CA, USA) with Bovine Serum Albumin (BSA) as standard.

Western blot

Infectivity of *Borrelia afzelii* in infected mice was checked by western blot using the sera of mice as primary antibodies. Lysates of *B. afzelii* were mixed with an equal volume of 2X Laemmli buffer (Thermo Scientific, Waltham, Massachusetts, USA) for a final quantity of 20 µg of protein/lane and were denatured by heat at 100°C for 10 min. Prepared lysates were loaded in 4-15% Mini-PROTEAN TGX Stain-Free Protein gel (Bio-rad, Hercules, CA, USA) and SDS-page electrophoresis was run then at 120V for 1 h. Proteins were then transferred onto nitrocellulose membrane (Bio-rad, Hercules, CA, USA) using semi-dry transfer method. Blotting was performed for 30 min at 25V in a transfer cell (Trans-Blot SD, Bio-Rad, Hercules, CA, USA). The immunoblotting was done by blocking the membrane with 1% Bovine Serum Albumin (BSA)/PBS (Sigma-Aldrich, St. Louis, MO, USA) for 2h at RT, followed by incubation with mice sera in a dilution of 1:100 in PBS at 4°C overnight. The next day, membranes were washed in PBS three times for 10 min with gentle rocking. Then, membranes were incubated with HRP-conjugated antibodies (Abs, goat anti-mouse IgM) (Sigma-Aldrich, St. Louis, MO, USA) at 1:2000 dilution in PBS for 1 h at RT with gentle rocking. Membranes were washed three times and protein detection was performed by chemiluminescence using Pierce ECL western blotting substrate (Bio-rad, Hercules, CA, USA). Membranes were incubated with ECL reagent for 3 min and membrane pictures were taken using ChemiDoc™ Touch Imaging System (Bio-rad, Hercules, CA, USA).

Indirect ELISA

The levels of Abs reactive against bacterial proteins were measured in mice sera as previously reported [13]. The 96-well ELISA plates (Thermo Scientific, Waltham, MA, USA) were coated with 100 μ L per well of 0.5 μ g/mL of *E. coli* BL21 protein extracts and incubated for 2 h at RT with gentle continual shaking at 100 rpm. Subsequently, plates were incubated overnight at 4°C. The antigens were diluted in carbonate/bicarbonate buffer (0.05 M, pH 9.6). The next day, wells were washed three times with 100 μ L of PBS containing 0.05% (vol/vol) Tween 20 (PBST), and then blocked by adding 100 μ L of 1% Human Serum Albumin (HSA)/PBS for 1 h at RT and gentle continual shaking at 100 rpm. After three washes, sera samples, diluted 1:700 in 0.5% HSA/PBS, were added to the wells and incubated for 1 h at 37°C at 100 rpm. The plates were washed three times and 100 μ L per well of HRP-conjugated Abs (goat anti-mice IgG and IgM) (Sigma-Aldrich, St. Louis, MO, USA) were added at 1:1500 dilution in 0.5% HSA/PBST and incubated for 1 h at RT at 100 rpm. The plates were washed three times and the reaction was developed with 100 μ L ready-to-use TMB solution (Promega, Madison, WI, USA) at RT for 20 min in the dark, and then stopped with 50 μ L of 0.5 M H₂SO₄. Optimal antigen concentration and dilutions of sera and conjugate were defined using a titration assay. The optical density (OD) was measured at 450 nm using an ELISA plate reader (Filter-Max F5, Molecular Devices, San Jose, CA, USA). All samples were tested in triplicate and the average value of three blanks (no Abs) was subtracted from the reads.

Tick capillary feeding

Capillary feeding was carried out using unfed *I. ricinus* nymphs. Glass capillary tubes of 3.5" (Drummond Scientific, Broomall, PA, USA) were filled with a solution containing 1×10^6 spirochetes of *B. afzelii* in BSK-II alone or combined with 5×10^6 cells/mL of *E. coli* BL21. The proportion of *E. coli* BL21 to *B. afzelii* (5:1) was selected based on the abundance found for both bacteria in the normal group. Ticks were fixed on plastic Petri dishes with a double-sided adhesive tape and the filled capillary tubes were placed over ticks' mouthparts. Ticks were left to feed for 4 hours in a humidity chamber at 33°C. After feeding, ticks were detached from the double-sided tape, collected in a sterile tube with holes and maintained in an incubator with >97% relative humidity at room temperature for two- and six-hours prior DNA extraction.

Tick microinjection

Microinjection experiment was carried out in unfed nymphs. Microinjection needles were fabricated by heating and pulling 1mm glass capillary tubes in a glass micropipette puller device (Sutter Instrument, Novato, CA, USA). A solution of *B. afzelii* in BSK-II media (1×10^6 spirochetes/mL) alone or combined with *E. coli* BL21 (5×10^6 cells/mL) were used for microinjection. Ticks were temporarily

immobilized on a double-sided tape and microinjections were performed into the anal pore of unfed nymphs with a volume of 8 nL. Microinjected ticks were incubated at room temperature for six hours in an incubator at >97% relative humidity prior DNA extraction.

DNA extraction

Genomic DNA was extracted from fully-engorged larvae, nymphs and mice tissues. DNA from individual fully engorged larvae were extracted 15 days after the feeding. DNA from individual nymphs were extracted at the end of the period of incubation from the capillary feeding and microinjection experiments. DNA from mice tissues were extracted at the endpoint (day 52) of the experiment. Individual ticks were crushed with disposable probe while mice tissues were crushed with glass beads using a Precellys24 Dual homogenizer (Bertin Technologies, Paris, France) at 5500× g for 20 s two times. Tick and mice tissues genomic DNA was extracted using a Nucleospin tissue DNA extraction Kit (Macherey-Nagel, Hoerd, France). Each DNA sample from ticks and mouse tissue was eluted in 20 µl and 50 µL of sterile water, respectively. Genomic DNA quality (OD260/280 between 1.8 –2.0) was measured with NanoDrop™ One (Thermo Scientific, Waltham, MA, USA).

Quantification of *B. afzelii* load by qPCR

For the detection of *B. afzelii* in whole larvae or in mice tissues, a pre-amplification step was performed to improve pathogen DNA detection. For that, total DNA was pre-amplified using the PreAmp Master Mix (Fluidigm, CA, USA) according to the manufacturer's instructions. Primers targeting the gene 23S rRNA for *Borrelia* spp. (23S rRNA-F 'GAGTCTTAAAAGGGCGATTTAGT', 23S rRNA-R 'CTTCAGCCTGGCCATAAATAG') were pooled by combining an equal volume of each primer for a final concentration of 200 nM. The reaction was performed in a final volume of 5 µL containing 1 µL Perfecta Preamp 5X, 1.25 µL pooled primer mix, 1.5 µL distilled water and 1.25 µL DNA. The thermocycling program consisted of one cycle at 95°C for 2 min, 14 cycles at 95°C for 15 s and 4 min at 60°C. At the end of the cycling program, the reactions were diluted 1:10 in Milli-Q ultrapure water. Subsequently, a qPCR was carried out using the same aforementioned primers and an additional probe (23S rRNA-probe 'AGATGTGGTAGACCCGAAGCCGAGT') in a LightCycler 480 (Roche, Meylan, France). The reaction mixture contained 6 µL of FastStart universal probe master (Roche), 0.12 µL of 20 µM of primers 23S rRNA-F, 23S rRNA-R and TaqMan probe 23S rRNA-probe, 2 µL of pre-amplified DNA sample and Milli-Q ultrapure water up to 12 µL. The amplification program consisted of: 95°C for 5 min, 45 cycles of 95°C for 10 s and 60°C for 15 min. The spirochetes burden in ticks was obtained by interpolation of the CT value in a standard curve of 'number of spirochetes vs CT' and then was normalized by the quantity of DNA in each sample.

Detection of Enterobacteriaceae by PCR

DNA extracted from whole nymph was used to detect Enterobacteriaceae using the following pair of primers: F-Enterobacteriaceae 'ATGGCTGTCGTCAGCTCGT', R-Enterobacteriaceae 'CCTACTTCTTTTGCAACCCACTC' (from [17]) which target the 16S rRNA gene for Enterobacteriaceae. The reaction was performed in a final volume of 50 μ L containing 5 μ L 10X buffer, 4 μ L of dNTP, 1 μ L of each primer, 0.25 μ L of Taq polymerase (Takara, Shiga, Japon), 1 μ L DNA and 37.75 μ L of distilled water. The mixtures were amplified for 40 cycles at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for one minute, with a final extension at 72°C for 3 minutes in an automated thermal cycler (Perkin-Elmer Cetus, Gouda, The Netherlands). Aliquots containing 3 μ L of each amplified product, 1 μ L of gel loading buffer (Thermo Scientific, Waltham, MA, USA) and 2 μ L of distilled water were electrophoresed in 1.0% (wt/vol) agarose gel, with a molecular size marker (Thermo Scientific, Waltham, MA, USA) in parallel. DNA from a culture of *E. coli* BL21 was used as a positive control. Electrophoresis in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer (Lonza Biosciences, Basel, Switzerland) was performed at 90 V for 1.5 hours. The gel was stained with GelGreen (Biotium, Fremont, CA, USA) and photographed under ultraviolet light illumination.

Illumina library preparation and sequencing of the 16S rRNA gene

At least 400 ng of fully-engorged larvae DNA at ≥ 20 ng/ μ L concentration were sent for amplicon sequencing of the bacterial 16S rRNA gene, which was commissioned to Novogene Bioinformatics Technology Co. (London, UK). Libraries were prepared with NEBNext® Ultra™ IIDNA Library Prep Kit (New England Biolabs, MA, USA). A single lane of Illumina MiSeq system was used to generate 251-base paired-end reads from the V4 variable region of the 16S rRNA gene using barcoded universal primers (515F/806R) in samples from larvae engorged in normal mice ($n = 10$), larvae engorged on *B. afzelii*-infected ($n = 10$), *E. coli*-immunized and *B. afzelii*-infected ($n = 8$) or mock-immunized and *B. afzelii*-infected ($n = 10$) mice. The raw 16S rRNA gene sequences obtained from tick samples were deposited at the SRA repository (Bioproject No. PRJNA870490).

Controls and identification and removal of contaminants

Two extraction reagent controls were set in which the different DNA extraction steps were performed using the same conditions as for the samples but using water as template. DNA amplification was then performed on the extraction control in the same conditions as for any other sample. Possible contaminating DNA in samples for 16S rRNA gene sequencing was statistically identify with 'decontam' package [18] using the 'prevalence' method. The prevalence is defined as the presence or absence across sample and the method used compares the prevalence of each sequence feature in

true samples to the prevalence in negative controls to identify contaminants. Then, contaminants were removed from the dataset before downstream microbiome analysis [18].

Analysis of 16S rRNA gene amplicon sequences

The analysis of 16S rRNA gene sequences was performed using QIIME 2 pipeline (v. 2021.4) [19]. Using DADA2 software [20] implemented in QIIME2, 16S rRNA gene sequences were first demultiplexed and then quality trimmed based on the average quality per base of the forward and reverse reads. The total length was trimmed at 180 and 154 in forward and reverse reads, respectively. Consequently, reads were merged and chimeric variants were removed. The resulting representative sequences were taxonomically assigned using a pre-trained naïve Bayes taxonomic classifier [21] based on SILVA database version 132 [22] and the 515F/806R primer set. The resulting taxonomic data tables were collapsed at genus level and taxa with less than 10 total reads and presents in less than 30% of samples of each dataset were removed. The taxonomic data tables were used for network analysis and keystone taxa identification. For convenience, in this study we refer to the *Borrelia* genus as a single genus, in the sense presented by [23], and did not consider the division of the genus *Borrelia* into two genera: the emended genus *Borrelia* containing only the members of the relapsing fever *Borrelia*, and the genus *Borrelia* containing the members of the Lyme disease *Borrelia* (i.e., *B. burgdorferi* s.l. complex) [24]. This does not imply that we are taking any position on the current debate on this issue [25].

Construction of bacterial co-occurrence networks, identification of keystone taxa and attack tolerance test

Co-occurrence network analyses were performed using the Sparse Correlations for compositional data (SparCC) method [26] implemented in R studio [27]. Taxonomic data tables were used to calculate the correlation matrix. Correlation coefficients with magnitude > 0.75 or < -0.75 were selected. Network visualization and calculation of topological features and taxa connectedness (i.e., number of nodes and edges, modularity, network diameter, average degree, weighted degree, clustering coefficient and centrality metrics) was performed using the software Gephi 0.9.2 [28]. The robustness of co-occurrence networks was tested with an attack tolerance test using the package NetSwan for R [29]. For this, networks were subjected to systematic removal of nodes using a directed attack where nodes are removed in decreasing order of their betweenness centrality (BNC) value (i.e., number of times a node is found on the shortest path between other nodes).

Comparative network analysis

Comparison of the similarity of the most central nodes between two networks was done with the package "NetCoMi" [30] in R studio using the read count taxonomic tables. "Most central" nodes are

defined as those nodes with a centrality value above the empirical 75% quartile. The comparison returns Jaccard's indexes for each of four local measures (i.e., degree, betweenness centrality, closeness centrality, eigenvector centrality) of the sets of most central nodes as well as for the sets of hub taxa between the two networks compared. Thus, the Jaccard's index express the similarity of the sets of most central nodes as well as the sets of hub taxa between the two networks. Jaccard index of 0 indicates completely different sets while a value of 1 indicates equal sets of most central nodes or hub taxa between the compared networks [30].

Statistical analysis

Taxonomic, pathway and enzyme data tables, which consisted of sequencing-read counts, was used as input of the R package 'ALDEx2' [31], which performed centered log-ratio (clr) transformation for all features in all the samples. Taxa, pathway and enzyme abundances were compared using the R package 'DeSeq2' [32]. The number of shared direct neighbor of the reference taxon *Escherichia-Shigella* in the different experimental groups was visualized using Venn diagrams implemented in the online tool <http://bioinformatics.psb.ugent.be/webtools/Venn/>. Alpha and beta-diversity of bacterial taxa were carried out on rarified ASV tables. The alpha-diversity was explored using the Pielou's evenness and Faith's phylogenetic metrics. Differences in alpha-diversity metrics between groups were tested using a Kruskal-Wallis test. Beta-diversity was explored using the Jaccard similarity and the Weighted Unifrac measures and compared between the groups using a PERMANOVA test. Betadisper function in R was used to determine the dispersion of samples based on Bray-Curtis distance matrix and an analysis of variance (ANOVA) test was used for comparison of the dispersion of the samples between the groups. For testing similarity of most central nodes, two p-values $P(J \leq j)$ and $P(J \geq j)$ for each Jaccard's index, which represent the probability that the observed value of Jaccard's index is "less than or equal" or "higher than or equal", respectively, to the Jaccard value expected at random, were calculated. Differences in relative Ab levels (i.e., OD) between groups of immunized mice in the different time points were compared using two-way ANOVA with Bonferroni multiple comparison tests applied for individual comparisons. Cluster analysis of different samples was based on Jaccard distance matrix and was done using the package 'Vegan' [33] in R using the Ward method. Unpaired non-parametric Mann-Whitney U test was used to compare the tick parameters (i.e., percentage of ticks that dropped naturally, percentage of larvae that molt into nymphs and tick mortality) and the load of *B. afzelii* in ticks between groups. Kruskal-wallis test with Dunn's multiple comparisons test were used to compare the loss of connectivity when removing 5 to 7% of nodes among all the experimental conditions. Mann-Whitney U test and Kruskal wallis test followed by Dunn's multiple comparisons test were performed in the GraphPad 8 Prism software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

Results

Borrelia afzelii modulates the tick microbiota

To study the impact of *B. afzelii* infection on tick microbiota, *I. ricinus* larvae were fed on *Borrelia*-infected mice and uninfected normal mice (Fig. 1a).

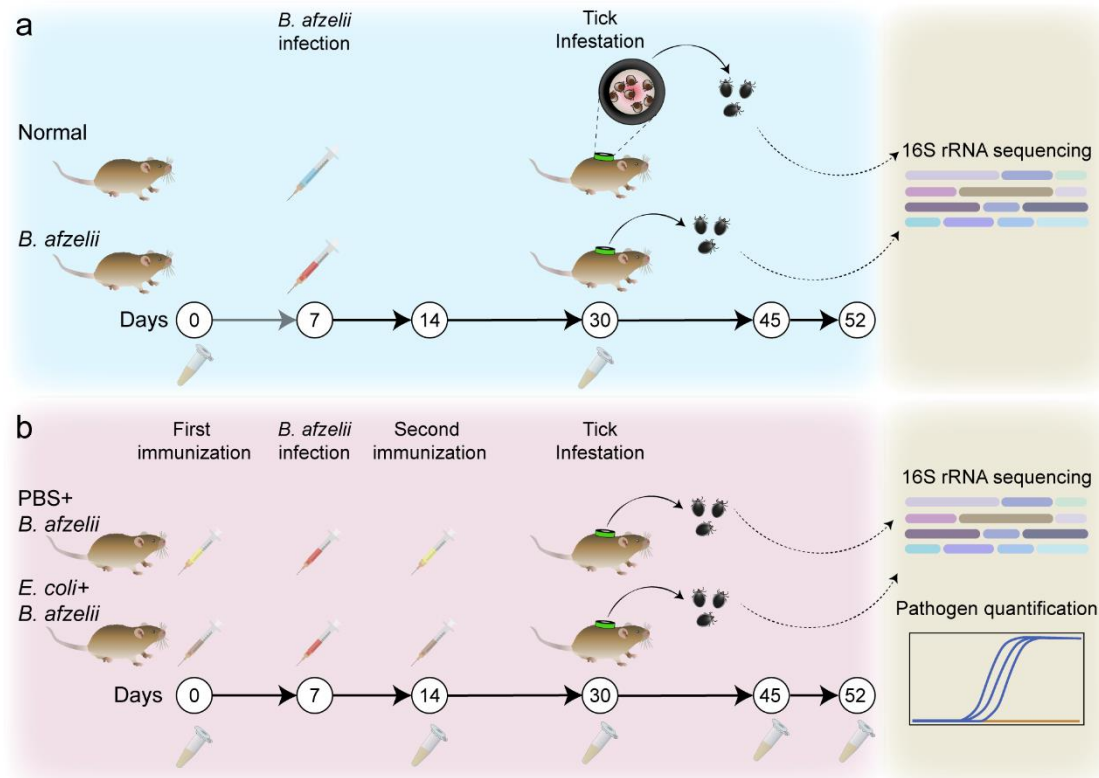


Fig. 1. Experimental design and sample collection. (a) Mice were experimentally infected with a volume containing spirochetes of *B. afzelii* and its culture media BSK-II ($n = 10$) while the normal group received an injection of the same volume containing only BSK-II. ($n = 10$). At day 30 (3 weeks post-infection), mice were infested with *I. ricinus* larvae ($n = 100$ per mouse). Sera of mice were recollected to check infection by western blot and engorged ticks were collected and used for tick microbiota analysis. (b) Mice were immunized with a live vaccine containing *E. coli* BL21 ($n = 5$) or with a mock vaccine (PBS) ($n = 5$) at day 0. Subsequently, mice were experimentally infected with *B. afzelii* at day 7 followed by a booster shot of the live or mock vaccine at day 14. Mice from both groups were then infested with *I. ricinus* larvae ($n = 100$ ticks per mouse) at day 30. Mice sera were collected at different timepoints as indicated for ELISA experiments and engorged ticks were collected and their DNA extracted for tick microbiota analysis and pathogen level quantification.

Subsequently, bacterial community composition and diversity of tick microbiota were analyzed using 16S rRNA gene profiling. Analysis of alpha diversity indexes showed that faith phylogenetic diversity

(Fig. 2a) as well as the evenness (Fig. 2b) did not differ between the ticks fed on *B. afzelii*-infected mice and the normal ticks (Kruskal-Wallis, $p > 0.05$). However, beta diversity analysis of tick microbiota revealed that *B. afzelii* infection led to a shift in the bacterial community composition and abundance, compared to the normal group, as measured using the Jaccard index (PERMANOVA, $F = 1.84$, $p = 0.001$, Fig. 2c) and Weighted unifrac distance (PERMANOVA, $F = 2.34$, $p = 0.005$, Fig. 2d), respectively. Furthermore, a permutation test for the evaluation of the homogeneity of dispersions based on Bray-Curtis distance matrix revealed no significant differences in the dispersion of the bacterial community between the two groups ($F = 2.43$, $p > 0.05$).

The impact of *B. afzelii* infection on the bacterial community assembly was assessed by construction of microbial co-occurrence networks. Visual inspection of the networks showed that infection with *B. afzelii* caused a shift in the bacterial community assembly patterns (Fig. 3a,b). Analysis of the topological features of the networks revealed an increased number of nodes and specially, of edges in the microbial co-occurrence networks inferred from microbiota of ticks fed on *B. afzelii*-infected mice compared to the normal network (Table 1). Similarly, the modularity and the average degree increased in the *B. afzelii* network compared to the normal one (Table 1). The observed Jaccard index for all the local centrality measures tested (i.e., degree, betweenness centrality, closeness centrality, eigenvector centrality and hub taxa), except for the betweenness centrality, was higher than expected by random for the comparisons between the normal and *B. afzelii* networks (Supplementary Table S1), suggesting high similarity in the hierarchical organization of nodes in the two networks.

Significant changes in the abundance of 65 taxa were found between ticks fed on *B. afzelii*-infected mice and the normal group (Kruskal-Wallis, $p < 0.05$, Supplementary Fig. S1). Among those, the abundances of 51 and 14 bacterial taxa increased and decreased respectively in ticks from *B. afzelii* group. The 20 taxa with the highest fold changes (Fig. 2e) in their abundance are presented in Fig. 2f. As expected, the taxon *Borrelia* changed significantly and its abundance was higher in the microbiota of ticks fed on *B. afzelii*-infected mice (Wald test, $p = 0.02$, Fig. 2f) compared to the normal group.

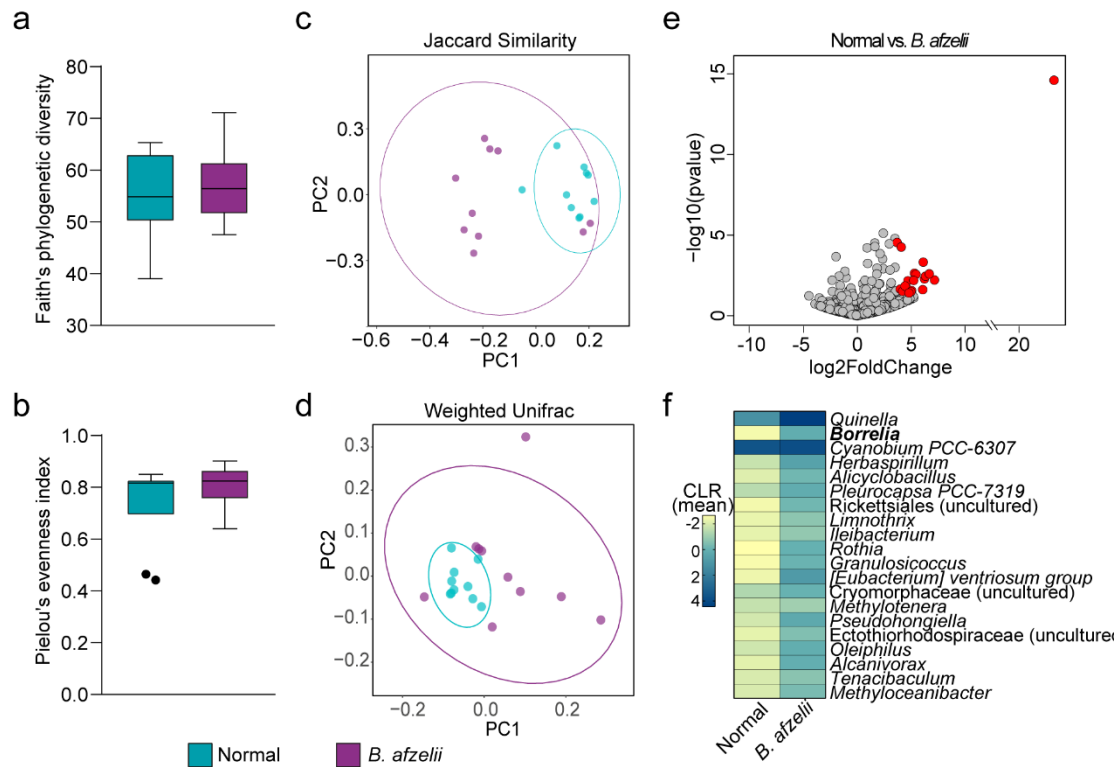


Fig. 2. Impact of *B. afzelii* infection on the diversity and taxonomic profile of tick microbiota.

(a) Faith's phylogenetic diversity and (b) Pielou's evenness indexes were used to measure the richness and evenness, respectively, of microbiota of ticks fed on *B. afzelii*-infected and normal mice (Kruskal-wallis, $p > 0.05$). Beta diversity of tick microbiota were analyzed with the (c) Jaccard and (d) Weighted Unifrac indexes to measure the similarity between the bacterial communities in the different experimental conditions (PERMANOVA, $p < 0.05$). (e) Volcano plot showing the differential microbial abundance in tick microbiota from the normal and *B. afzelii* groups. (f) Heatmap representing the abundance (expressed as CLR) of the top 20 taxa with the highest absolute value of log2foldchange. Rarified table of ASVs, used to measure the alpha and beta diversity, and taxonomic table were obtained from 16S rRNA gene sequences from ticks fed on normal mice ($n = 10$ individual larvae) and *B. afzelii*-infected mice ($n = 10$ individual larvae).

Table 1. Topological features of the microbial co-occurrence networks

Topological Features	Experimental groups	
	Normal	<i>B. afzelii</i>
Nodes ^a ;	626 (208) ⁱ	645 (230) ⁱ
Edges ^b	474	1008
-Positives	386	688
-Negatives	88	320
Modularity ^c	0.908	1.476
Modules ^d	56	45
Network diameter ^e	11	11
Average degree ^f	1.514	3.126
Weighted degree ^g	0.771	0.977
Clustering coefficient ^h	0.409	0.568

^aNodes represents bacterial taxa with co-occurrence correlation SparCC > or < -0.75; ^bEdges represent the number of connections/correlations; ^cmodularity is the strength of division of a network into modules; ^dModules are sub-communities of bacteria that co-occur more frequently among each other than with other taxa; ^enetwork diameter is the shortest path between the two most separated nodes; ^faverage degree is the average number of links per node; ^gweighted degree is the sum of the weight of all the edges connected to a node; ^hclustering coefficient is the degree to which nodes in a network tend to form clusters; ⁱtotal nodes and nodes with at least one edge are inside brackets.

Interestingly, the abundance of the keystone taxon *Escherichia-Shigella* decreased significantly in the *B. afzelii* group, compared with the normal group (Wald test, $p = 0.03$, Supplementary Fig. S1). The genus *Escherichia-Shigella* was described as a keystone taxon in the microbiome of *Ixodes* ticks [13, 14]. In addition, vaccine-induced antibodies specific to *Escherichia-Shigella* modulated the tick microbiota and induced changes on the tick physiology [13] and reduced vector competence of mosquitoes for *P. relictum* [15]. Visual inspection of the sub-networks of local interaction of *Escherichia-Shigella* showed that *B. afzelii* infection increased the number of direct neighbors co-occurring with *Escherichia-Shigella*, compared with the normal sub-network (Fig. 3c,d). Most of the

nodes connected to the taxon *Escherichia-Shigella* in the normal sub-network were also present in the *B. afzelii* sub-network (Fig. 3e, Supplementary Table S2), and the type of connection between them (i.e., positive or negative correlation) was conserved (Fig. 3f). We also found 23 unique nodes in the *B. afzelii* sub-network, where 12 and 11 nodes have negative and positive co-occurrence correlations, respectively, with *Escherichia-Shigella*. Further characterization of the importance of the genus *Escherichia-Shigella* in the co-occurrence networks revealed an increased closeness centrality, betweenness centrality and eigenvector centrality (Table 2). Notably, the betweenness centrality was the measure that changed the most where it increased six times in the network of ticks fed on *B. afzelii*-infected mice compared to those fed in normal mice. These results show that despite the abundance of *Escherichia-Shigella* is lower in *B. afzelii*-infected ticks, probably due to an increased in bacterial competition, the importance of this taxon increases in the networks. Altogether, the results showed that *B. afzelii* infection led to a shift in the tick microbiota characterized by changes in the beta diversity, bacterial abundance, some network properties and the relative importance of *Escherichia-Shigella*.

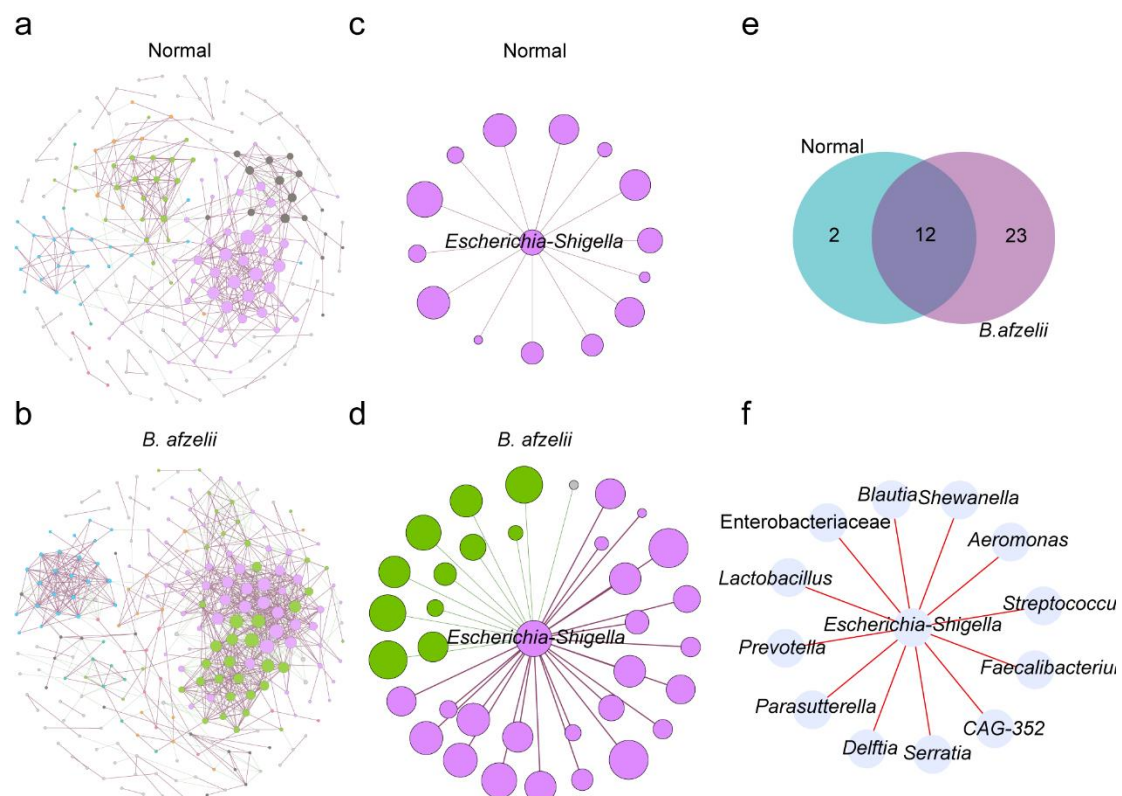


Fig. 3. Impact of *B. afzelii* infection on microbial community assembly. Bacterial co-occurrence networks were inferred from 16SrRNA gene sequences obtained from ticks fed on (a) control and (b) *B. afzelii*-infected mice. Sub-networks of the local connectivity of *Escherichia-Shigella* were extracted from the (c) normal and (d) *B. afzelii* co-occurrence networks. Nodes

represent bacterial taxa and edges stand for co-occurrence correlation (SparCC > 0.75 or < -0.75). Node size is proportional to the eigenvector centrality value and node color is based on the modularity class. Thus, nodes with the same color belong to the same cluster. Positive and negative interactions between co-occurring bacteria are represented by the dark red and green edges, respectively. Only nodes with at least one connecting edge are displayed. (e) Venn diagram showing the number of bacterial taxa that are common or unique among the neighbors directly connected to *Escherichia-Shigella* in the normal and *B. afzelii* groups. (f) Direction of associations of common direct neighbor to the taxon *Escherichia-Shigella* between the normal and *B. afzelii* groups. Red edges indicate positive co-occurrence associations in both groups.

Table 2. Centrality measures of the taxon *Escherichia-Shigella* in the normal and *B. afzelii* networks

Experimental groups	Closeness centrality ^a	Betweenness centrality ^b	Eigenvector centrality ^c
Normal	0.256	0.001	0.635
<i>B. afzelii</i>	0.350	0.006	0.903

^aCloseness centrality indicates how close a node is to all other nodes in the network; ^bbetweenness centrality indicates how much a given nodes is in-between others; ^ceigenvector centrality measures a node's importance while giving consideration to the importance of its neighbors

Anti-microbiota vaccine alters the tick microbiota shift induced by *B. afzelii* and decreases pathogen infection in ticks

The above results and previous evidence [9], led us to the hypothesis that departures from *Borrelia*-permissive states of the tick microbiota could alter pathogen colonization in the tick vector. To test this hypothesis, we altered the tick microbiota by targeting the keystone taxon *Escherichia-Shigella* [14] in *B. afzelii*-infected ticks and measured the impact on pathogen fitness. Immunization with an *E. coli*-based live vaccine was followed by experimental infection with *B. afzelii* and subsequent tick infestation on mice (Fig. 1b). *Borrelia afzelii* infection was confirmed by qPCR in different mice tissues (Supplementary Table S3) and by western blot using mice sera against *B. afzelii* protein extracts (Supplementary Fig. S2). Vaccination of mice with *E. coli* elicited an immune response where increased levels of antibodies IgM (Fig. 4a) and IgG (Fig. 4b) specific to *E. coli* was observed in mice sera

compared to the control group, which received a mock vaccine. This immune response was maintained over time, at least 52 days after the first immunization (Fig. 4).

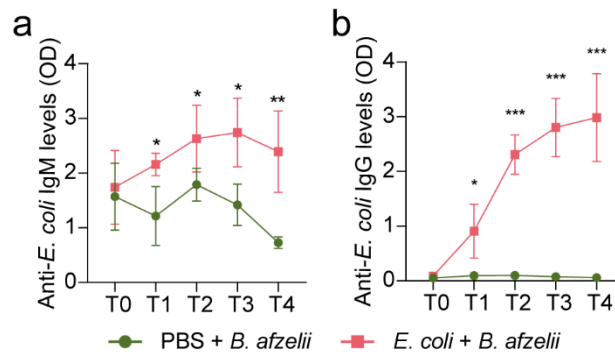


Fig. 4. Antibody response of mice infected with *B. afzelii* and vaccinated with live *E. coli* or mock vaccine. The levels of (a) IgM and (b) IgG specific to *E. coli* proteins were measured by semi-quantitative ELISA in sera of *B. afzelii*-infected mice immunized with *E. coli* (pink) and a mock vaccine (green, PBS). Means and standard error values are shown. Results were compared by two-way ANOVA with Bonferroni test applied for comparisons between control and immunized mice. (* $p < 0.05$, ** $p < 0.01$; 1 experiment, $n = 4$ mice per experimental group and three technical replicates per sample).

Differences in the microbiota of ticks fed on mice infected with *B. afzelii* and immunized with the anti-microbiota or the mock vaccine were assessed by comparison of the alpha and beta diversity of the bacterial communities. Vaccination with *E. coli* had no significant impact on the bacterial diversity (Kruskal-Wallis, $p > 0.05$, Fig. 5a) or in the species evenness (Kruskal-Wallis, $p > 0.05$, Fig. 5b). Similarly, the beta diversity did not reveal a separate clusterization of the experimental groups as measured with the Jaccard index (PERMANOVA, $p > 0.05$, Fig. 5c) and weighted unifracs (PERMANOVA, $p > 0.05$, Fig. 5d).

Visual inspection of the microbial co-occurrence networks constructed from the microbiota of ticks fed on PBS+*B. afzelii* and *E. coli*+*B. afzelii* mice showed that the anti-microbiota vaccine modulated the bacterial community assembly (Fig. 6a,b), which was further confirmed by the topological features of these networks (Table 3). Specifically, the number of positive and negative edges increased notably in the *E. coli*+*B. afzelii* networks compared to the PBS+*B. afzelii* network (Table 3). Similarly, other topological features as the modularity, number of modules, the average degree and network diameter increased in the *E. coli*+*B. afzelii* network compared to its control (Table 3). Testing the Jaccard index for the local network centrality measures revealed that the degree (Jacc = 0.389, $p = 0.03$) and the closeness centrality (Jacc = 0.404, $p = 0.009$) had Jacc values higher than expected by random for the comparisons between the two networks (Supplementary Table S4). Differential analysis of the abundance of each taxon in the microbiota of ticks showed that the abundance of 46

bacterial taxa changed significantly between ticks of the PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups (Kruskal-Wallis, $p < 0.05$, Supplementary Fig. S3). Specifically, the abundance of 23 bacterial taxa increased and also 23 taxa decreased in the microbiota of ticks fed on immunized and *B. afzelii*-infected mice compared to its control group. The top 20 taxa with the highest fold changes differences between the two groups is represented in Fig. 5e and are listed in Fig. 5f.

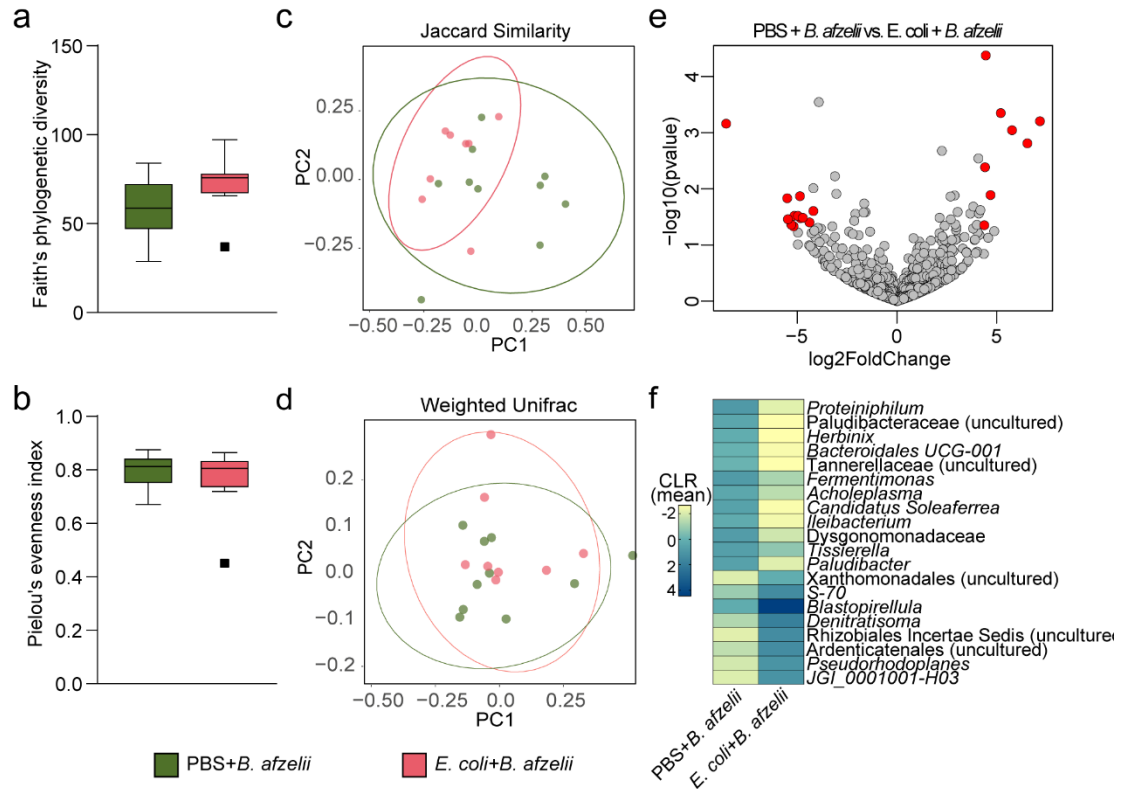


Fig. 5. Impact of anti-microbiota vaccine and *B. afzelii* infection on the diversity and taxonomic profile of tick microbiota. (a) Faith's phylogenetic diversity and (b) Pielou's evenness indexes were used to measure the richness and evenness, respectively, of microbiota of ticks fed on PBS+*B. afzelii*-infected and *E. coli*+*B. afzelii* mice (Kruskal-wallis, $p > 0.05$).

Beta diversity of tick microbiota were analyzed with the (c) Jaccard and (d) Weighted Unifrac indexes to measure the similarity between the bacterial communities in the different experimental conditions (PERMANOVA, $p > 0.05$). (e) Volcano plot showing the differential microbial abundance in tick microbiota from the PBS+*B. afzelii*-infected and *E. coli*+*B. afzelii* groups. (f) Heatmap representing the abundance (expressed as CLR) of the top 20 taxa with the highest absolute value of $\log_2(\text{fold change})$. Rarified table of ASVs, used to measure the alpha and beta diversity, and taxonomic table were obtained from 16S rRNA gene sequences from ticks fed on mock-immunized and *B. afzelii*-infected mice ($n = 10$ individual larvae) and *E. coli*-immunized and *B. afzelii*-infected mice ($n = 8$ individual larvae).

Table 3. Topological features of the microbial co-occurrence networks

Topological Features	Experimental groups	
	PBS+ <i>B. afzelii</i>	<i>E. coli</i> + <i>B. afzelii</i>
Nodes	739(204)	735(378)
Edges	1124	2002
-Positives	867	1421
-Negatives	257	581
Modularity	0.963	1.1
Module	28	73
Network diameter	11	12
Average degree	3.042	5.448
Weighted degree	1.384	1.951
Clustering coefficient	0.590	0.471

To investigate if live bacteria immunization had an impact on the importance of *E. coli* in tick microbiome, sub-networks composed by the taxon *Escherichia-Shigella* and the direct neighbor were constructed. Comparisons of the sub-networks revealed that the number of co-occurring taxa with the taxon *Escherichia-Shigella* decreased in the networks of microbiota of ticks fed on *E. coli*-immunized and *B. afzelii*-infected mice (Fig. 6c), compared to those fed on mock-immunized and *B. afzelii*-infected mice (Fig. 6d). Comparison of the taxonomic identity of the direct neighbors between the PBS+*B. afzelii* and *E. coli*+*B. afzelii* sub-networks showed that the direct co-occurring taxa of the genus *Escherichia-Shigella* were mostly unique for each experimental condition and only 12 were shared between the sub-networks (Fig. 6e, Supplementary Table S5). A detailed comparison of the type of co-occurrence correlation between the taxon *Escherichia-Shigella* and the common taxa between the two sub-networks revealed that the type of connection was kept in the PBS+*B. afzelii* and *E. coli*+*B. afzelii* sub-networks (Fig. 6f). In contrast to the increase in the centrality measures of the taxon *Escherichia-Shigella* in the *B. afzelii* network compared to the normal group, we observed a decrease in all the three centrality measures (i.e., closeness, betweenness and eigenvector

centralities) in the microbiota of ticks fed on on *E. coli*-immunized and *B. afzelii*-infected mice compared to those fed on mock-immunized and *B. afzelii*-infected mice (Table 4).

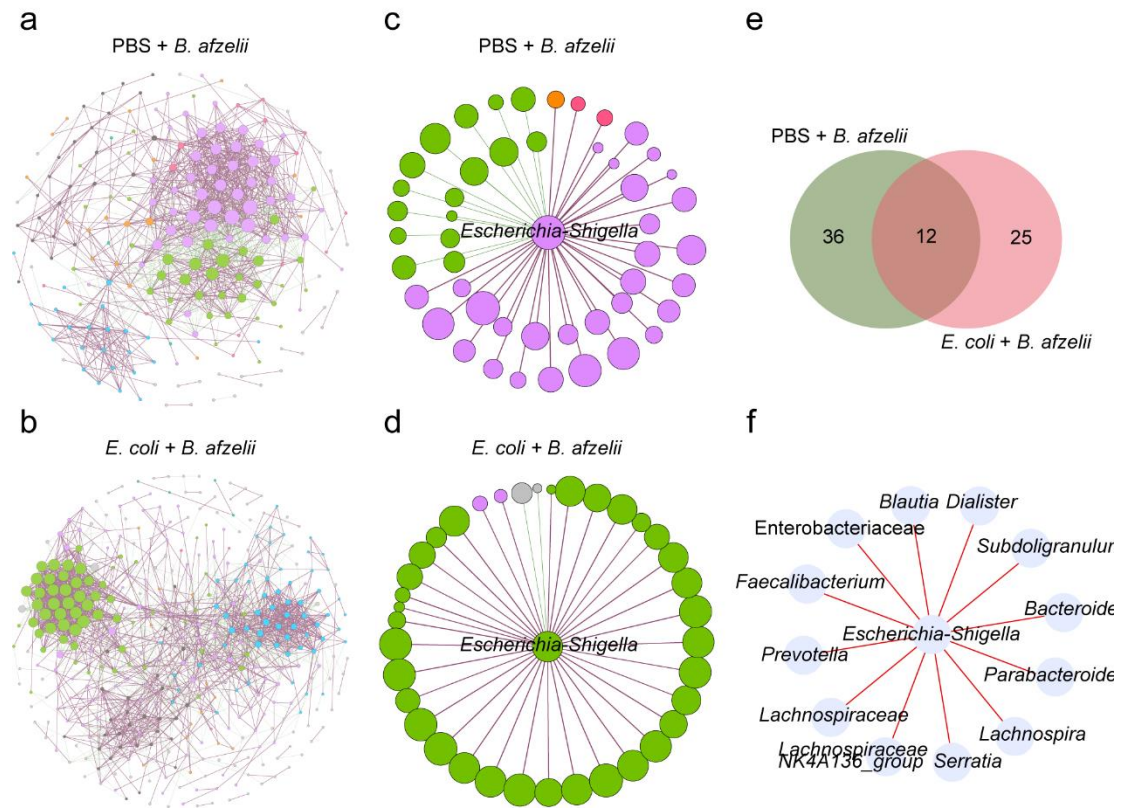


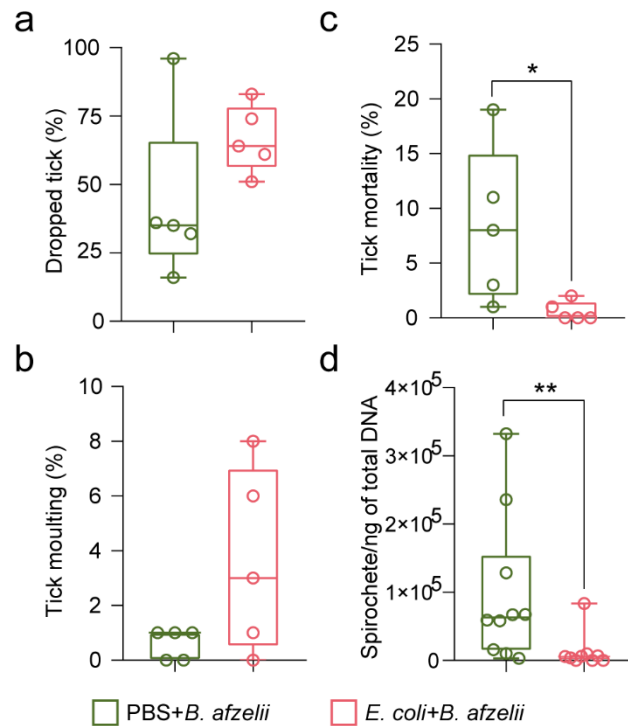
Fig. 6. Impact of anti-microbiota vaccine and *B. afzelii* infection on microbial community assembly.

Bacterial co-occurrence networks were inferred from 16SrRNA sequences obtained from ticks fed on (a) mock-immunized and *B. afzelii*-infected mice and (b) *E. coli*-immunized and *B. afzelii*-infected mice. Sub-networks of the local connectivity of *Escherichia-Shigella* were extracted from the (c) PBS+*B. afzelii* and (d) *E. coli*+*B. afzelii* co-occurrence networks. Nodes represent bacterial taxa and edges stand for co-occurrence correlation (SparCC > 0.75 or < -0.75). Node size is proportional to the eigenvector centrality value and node color is based on the modularity class. Thus, nodes with the same color belong to the same cluster. Positive and negative interactions between co-occurring bacteria are represented by the dark red and green edges, respectively. Only nodes with at least one connecting edge are displayed. (e) Venn diagram showing the number of bacterial taxa that are common or unique among the neighbors directly connected to *Escherichia-Shigella* in the PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups. (f) Direction of associations of common direct neighbor to the taxon *Escherichia-Shigella* between the PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups. Red edges indicate positive co-occurrence associations in both groups.

Table 4. Centrality measures of the taxon *Escherichia-Shigella* in the PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups.

Experimental groups	Closeness centrality	Betweenness centrality	Eigenvector centrality
PBS+ <i>B. afzelii</i>	0.431	0.004	1
<i>E. coli</i> + <i>B. afzelii</i>	0.295	0.003	0.898

To test the impact of the anti-microbiota vaccine on tick physiology and pathogen fitness several tick-performance parameters and *Borrelia* levels were compared between the PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups. Results showed no significant differences in the percentage of ticks that dropped naturally (Fig. 7a) nor in the percentage of larvae that molt to nymphs (Fig. 7b) between the two groups. However, a significant decrease in the mortality of ticks from *E. coli*+*B. afzelii* group was observed compared to ticks from PBS+*B. afzelii* group (Mann-Whitney test, $p = 0.02$, Fig. 7c). Interestingly, the *B. afzelii* levels in ticks fed on *E. coli*-immunized and *B. afzelii*-infected mice were also significantly lower than in ticks fed on mock-immunized and *B. afzelii*-infected mice (Mann-Whitney test, $p = 0.0056$, Fig. 7d). Here we showed that anti-microbiota vaccines can disturb the tick microbiota and reduce *Borrelia* colonization, while previous studies showed that tick microbiota perturbation by sterile-rearing of ticks or exposure to antibiotics also reduced *Borrelia* colonization [9].



Adding novel commensal bacteria reduces *B. afzelii* load in *I. ricinus* nymphs

To test whether other means of microbiota perturbation (i.e., addition of a novel commensal bacteria, [34–39]) also reduced *Borrelia* colonization, we used artificial capillary feeding and microinjection in the anal pore to introduce simultaneously *E. coli* BL21 and *B. afzelii* in *I. ricinus* nymph. By using *E. coli* BL21, a strain long kept in laboratory settings [40, 41], we ensured a novel interaction within the tick host, allowing us to rule out an evolutionary history between the added bacterium, the tick and the *Borrelia*, which may have reduced its impact in the microbiota.

After 2 (capillary feeding) and 6 (capillary feeding and microinjection) hours (h) incubation, we compared the pathogen loads in ticks exposed to *E. coli* and *B. afzelii* with those exposed only to *B. afzelii* spirochetes. The presence of added *E. coli* in nymphs after capillary feeding or microinjection was confirmed by PCR (Supplementary Fig. S4). After 2h and 6h incubation, *B. afzelii* levels were 55.3

(Mann-Whitney test, $p < 0.0001$, Fig. 8a) and 2.4 (Mann-Whitney test, $p > 0.05$, Fig. 8b) times lower in nymphs capillary fed with *E. coli*, respectively, compared to nymphs fed only with *B. afzelii*. For nymphs microinjected with *E. coli* and *B. afzelii* and incubated for 6h, *B. afzelii* load decreased 2.0 times compared to nymphs injected only with *B. afzelii* (Mann-Whitney test, $p = 0.01$, Fig. 8c). This suggests that *Borrelia* is highly sensitive to tick microbiota perturbations and that deviations from the modulation induced by the pathogen in the vector microbiota pose a high cost to the spirochete.

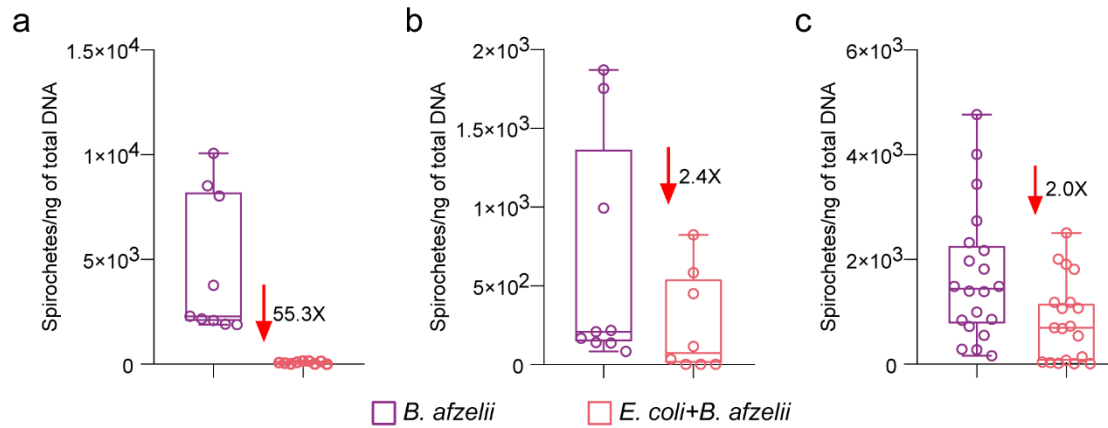


Fig. 8. Impact of the introduction of *E. coli* on *B. afzelii* infection. The load of spirochetes of *B. afzelii* were measured in *I. ricinus* nymphs fed artificially with *E. coli+B. afzelii* and incubated for (a) 2h or (b) 6h after the feeding and in (c) nymphs injected with *E. coli+B. afzelii* in the anal pore and incubated for 6h after microinjection. The results were compared to nymphs that received only *B. afzelii* spirochetes. Means and standard deviation values are displayed. *B. afzelii*, $n = 6-7$ nymphs/condition; *E. coli+B. afzelii*, $n = 6-7$ nymphs/condition. Mann-Whitney test, * $p < 0.05$, *** $p < 0.001$.

Defining *B. afzelii* infection refractory states in the *I. ricinus* microbiota

A global comparison between the four different experimental condition (i.e., normal, *B. afzelii*, PBS+*B. afzelii* and *E. coli+B. afzelii*) was performed aiming to define ecological properties of the infection refractory states. We first compared the taxonomic profile among all the experimental groups and we found that a high number of bacterial taxa (i.e., 535 taxa) were shared among the 4 groups. Only 2 to 9 taxa were exclusively found in each experimental condition (Fig. 9a). When comparing the list of taxa whose abundance changed significantly between the normal and *B. afzelii* groups (Supplementary Figure S1) and between the PBS+*B. afzelii* and *E. coli+B. afzelii* groups (Supplementary Figure S3), we observed that only 8 taxa changed significantly their abundance in the normal-*B. afzelii* comparison as well as in the PBS+*B. afzelii* and *E. coli+B. afzelii* comparison.

Interestingly, most of the taxa were unique for each set of comparisons, where 38 taxa were found exclusively in the comparison between the normal and *B. afzelii* groups and 57 in the comparison between the PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups (Fig. 9b). Furthermore, cluster analysis of the microbiota based on the Jaccard index showed that the samples from different experimental conditions clustered in three different groups (Fig. 9c, Supplementary Fig, S5). Each cluster was formed mostly by tick microbiota samples from one experimental condition, namely *E. coli*+*B. afzelii*, normal and *B. afzelii* groups. Samples from PBS+*B. afzelii* clustered in two different groups, with samples from *B. afzelii* or *E. coli*+*B. afzelii* groups. It is noteworthy that all the samples of tick microbiota from the *E. coli*+*B. afzelii* group clustered in one separated group which is not the case for the other experimental conditions.

Subsequently, we compared networks emergent properties to determine their possible contribution to the *B. afzelii* infection refractory state in tick microbiota. Comparison of the identity of the nodes that are involved in microbe-microbe interactions showed that 85 bacterial taxa are common in the networks of all experimental groups (Fig. 9d). Interestingly, the network that presented the highest number of unique taxa (i.e., 157 nodes) was the one inferred from *E. coli*+*B. afzelii* group compared to the normal (i.e., 22 nodes), *B. afzelii* (i.e., 35 nodes), PBS+*B. afzelii* (i.e., 13 nodes). Moreover, to determine how much of the bacterial diversity is translated to microbe-microbe interactions, we compared the observed features versus the number of nodes (Fig. 9e) or edges (Fig. 9f) found in the microbial co-occurrence networks. We observed that the normal, *B. afzelii* and PBS+*B. afzelii* group with similar number of observed features presented similar number of interacting nodes in the microbial co-occurrence networks (Fig. 9e). In contrast, for a similar number of observed features, the number of edges increased two times in the *B. afzelii* and PBS+*B. afzelii* group compared to the normal one (Fig. 9f). Interestingly, the *E. coli*+*B. afzelii* group with the highest bacterial diversity had also the highest number of interacting nodes and bacterial associations (Fig. 9f). Finally, we tested the robustness of the networks by measuring their tolerance to directed taxa removal and compared it among all the experimental groups. Network inferred from the *E. coli*+*B. afzelii* group presented the lowest values of connectivity loss after directed removal of nodes (Fig. 9g), suggesting the highest tolerance to taxa removal in this network. Statistical comparison of the loss of connectivity for 5 to 7% of nodes removed showed a significant difference between the *E. coli*+*B. afzelii* and the normal, *B. afzelii* or PBS+*B. afzelii* groups (Fig. 9g). All these results showed that significant changes of unique bacterial taxa, high microbial-microbial interactions with unique set of nodes as well as a higher robustness of microbial networks can define the *Borrelia*-infection refractory states in the *I. ricinus* microbiota.

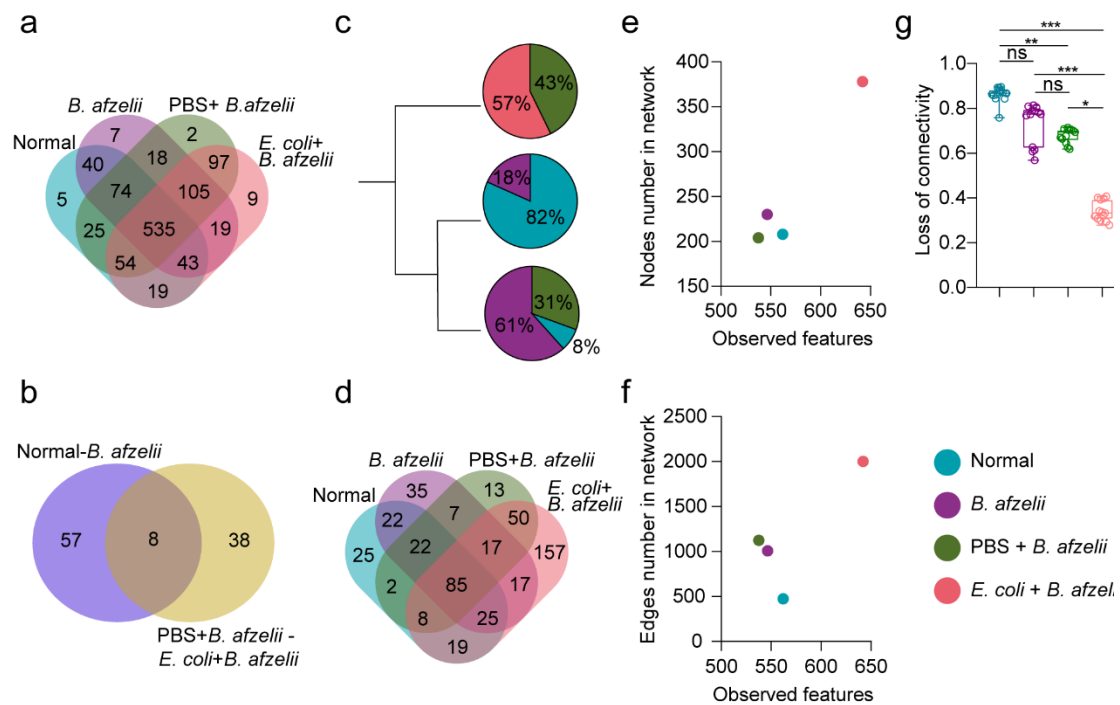


Fig. 9. *B. afzelii* infection-refractory state of *I. ricinus* microbiota. Venn diagram showing (a) the common and unique bacterial taxa in tick microbiota for the normal, *B. afzelii*, *PBS+B. afzelii* and *E. coli+B. afzelii* groups (b) the shared and unique taxa whose abundance changed significantly between the normal-*B. afzelii* and *PBS+B. afzelii*- *E. coli+B. afzelii* comparisons, (c) Dendrogram of clustering for samples of tick microbiota from different experimental conditions, (d) Venn diagram showing the common and unique nodes found in microbial co-occurrence networks from all conditions. Scatter plot showing the mean of observed features versus number of (e) nodes and (f) edges found in the microbial co-occurrence networks and (g) Scatter plot showing the loss of connectivity when 5 to 7% of nodes are removed from the microbial co-occurrence network.

Discussion

Microbiota perturbation is a promising avenue to develop measures to control vector-borne diseases, as vector microbiota influences vector competence [42–44]. Here, we first characterized the interaction of tick microbiota with the pathogen *B. afzelii* and subsequently, we showed how perturbation of pathogen-induced microbiota led to an infection-refractory state that reduced *Borrelia* colonization in tick vectors.

Other studies found that the presence of *Borrelia* within the vector changed significantly the tick microbiota. For example, *I. ricinus* nymphs molted from larvae that fed on *B. afzelii*-infected mice presented a less abundant but more diverse bacterial microbiome [45]. Differential abundances of

several taxa were also found between *B. afzelii*-infected and non-infected ticks [45]. In contrast, other study found no association between the microbiome diversity of a tick and its probability of carrying *B. burgdorferi* but specific microbial taxa were associated with pathogen presence in individual ticks [46]. These results suggest that *Borrelia* infection induced modifications on tick microbiota associated, particularly, to changes in the abundance of bacterial members. Our results also showed that *B. afzelii* produces major shifts in the bacterial community assembly and increases co-occurrence of bacteria, suggesting higher rates of microbe-microbe interactions in infected ticks. Modification of bacterial taxa associations induced by the presence of different pathogens such as *Borrelia*, *Anaplasma* and *Rickettsia* was also previously reported [47]. However, unlike our results, Lejal et al., (2021) found that bacterial correlations were lower in the network from *Borrelia*-positive ticks compared to the network from uninfected ticks. These differences could be due to the *Borrelia* species that was considered. In the study of Lejal et al., (2021), samples were considered positive for *Borrelia* if it was detected by microfluidic PCR and the bacterial taxa of the same genera was detected by 16S rRNA gene sequencing. Thus, in their study, the *Borrelia* species is unknown. Different species could lead to different results but further comparative studies on the impact of different pathogen species on tick microbiota is needed to affirm this hypothesis. Despite differences found between studies, the evidence shows consistently that the presence of pathogen in the vector modulates the vector microbiota towards a state compatible with pathogen colonization.

Considering that pathogens, such as *Borrelia*, have to overcome several barriers (e.g., evading tick immune defences or avoiding endocytic digestion in tick gut epithelial cells) to persist in the tick until the next blood meal, they have to activate mechanisms to modulate the gut environment in order to facilitate their colonization and persistence within the vector [3]. Mechanistically, it was shown that *B. burgdorferi* induces the expression of an *I. scapularis* gut gene encoding for PIXR (protein of *I. scapularis* with a reeler domain) [48]. Abrogation of PIXR function *in vivo* resulted in alterations in the gut microbiome, metabolome and immune responses and RNA interference-mediated knockdown of PIXR decreased *B. burgdorferi* colonization, which suggest that *B. burgdorferi* induces PIXR expression to enhance colonization in the tick [48]. Similarly, *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis, induces the expression of a gene encoding for *Ixodes scapularis* antifreeze glycoprotein (IAFGP), which perturbs the tick gut microbiota and the integrity of the peritrophic matrix and gut barrier in order to facilitate the infection [49]. The broad effects that the pathogen has on tick gut may create an environment that favors or reduces the fitness of some bacteria, which may explain changes in the diversity, composition or abundance of taxa in the microbiota. Furthermore, microbiota modulation could lead to the disappearance or emergence of microbial-microbial interactions. In agreement with this, here we found that the keystone taxon *Escherichia-Shigella* [13] was associated to more bacterial taxa in *B. afzelii* network compared to the

control network. However, the understanding of the exact role of these newly established associations on pathogen persistence within the vector needs further investigation.

Vector microbiota is considered a gate to access vector fitness and competence [50]. Inducing changes in normal tick microbiota can result in pathogen colonization impairment [9]. Here, we showed that tick fed on mice immunized with the Enterobacteriaceae bacteria *E. coli* BL21 presented lower level of *B. afzelii* load within the vector. These results are in concordance with a previous study where mosquitoes fed on immunized birds against two strains of *E. coli*, namely O86:B7 and BL21, had reduced number of *P. relictum* (causal agent of avian malaria) oocysts in the midguts [15]. Reduction of *Plasmodium* infectivity was owed to the alteration of *Plasmodium*-induced modulation of the mosquito microbiota [15]. Similarly, in the present study, we found that anti-microbiota vaccine produced changes in the microbial community assembly different from those induced by *B. afzelii*. Interestingly, we also found that anti-microbiota vaccine reduced the relative importance of the taxon *Escherichia-Shigella* compared to the same taxon in PBS+*B. afzelii* group. These results suggest that targeting Enterobacteriaceae with host antibodies induce the modulation of the vector microbiota triggering a cascading ecological impact on the whole tick microbiota that resulted in the impairment of pathogen colonization. Supporting this idea, Narasimhan et al. (2014) reported that an experimentally-induced dysbiosed microbiota in ticks reduced *B. burgdorferi* colonization in tick midgut. It was also reported that this outcome was associated with a lower expression of the transcription factor STAT and the glycoprotein peritrophin that affected the integrity of the peritrophic matrix, which is essential for *B. burgdorferi* successful colonization. Whether these mechanisms are implicated in the effects of anti-microbiota vaccine remains to be elucidated.

Enterobacteriaceae was suggested to have a role in *B. burgdorferi* colonization since its abundance was negatively correlated with the pathogen abundance [51]. Here, we found that antibodies-mediated targeting of Enterobacteriaceae modulate the tick microbiota and reduced *B. afzelii* load within the tick. These results suggest that the commensal bacteria Enterobacteriaceae may have a key role on pathogen acquisition. However, when we experimentally introduced *E. coli* BL21 with *B. afzelii* to the midgut of *I. ricinus* nymphs by capillary feeding or microinjection, we found surprisingly that *B. afzelii* level on these nymphs were significantly lower compared to nymphs that only received *B. afzelii*. These results suggest that the observed reduction on the pathogen load is not due to the shift in the abundance of Enterobacteriaceae. It is rather likely due to the modulation of the microbiota (by the depletion or emergence of some bacteria) towards a different state from the one induced by *B. afzelii*, specifically, a *Borrelia*-refractory state incompatible with pathogen development. It has been shown that alteration of the vertebrate gut microbiota composition either by antibiotic treatment [9] or by addition of probiotics [36] can inhibit the colonization of pathogenic bacteria in the gut. When

we defined this infection- refractory state by comparing taxonomic profile, abundance, and emerging properties of tick microbiota, we found that the main differences between the normal, the pathogen-permissive and the infection-refractory state relied mostly in the fluctuation of the abundance of unique taxa and the emergent properties of microbial networks. Emergent properties is one of the strength of biological networks and they can help explaining the behaviour of complex systems [52, 53]. We found that changes induced by *B. afzelii* in terms of interacting nodes, microbial-microbial interactions and robustness of the microbial network were not different as much as those induced by *E. coli*+*B. afzelii* compared to the normal network. Notably, a large-edge number, a higher number of new interconnecting taxa as well as a high robustness was found in the *E. coli*+*B. afzelii* network compared to the normal or *B. afzelii* networks. Contrasting results were found in tick microbiota after disturbance with an anti-tick vaccine, *A. phagocytophilum* infection and antimicrobial peptide, where a higher number of associations, but lower robustness were found [54]. These results suggest that tick microbiota is highly sensible to perturbations that led to changes in the emergent properties of microbial networks. Specifically, here, we found that perturbation of the microbiota with anti-microbiota vaccine induced drastic changes in the bacterial community assembly that led to an unsuitable stage for *B. afzelii* colonization within the vector.

Finally, we found that ticks fed on mice immunized with *E. coli* BL21 and infected with *B. afzelii* had significantly lower mortality rates compared with the PBS+ *B. afzelii* group. This result is in contrast with one study where they did not found evidence that *B. afzelii* infection and reduction of larval microbiota (by egg surface sterilization with bleach and ethanol) impacted tick survival [55]. Differences could be explained by the different developmental stage where the measure was done. In our study, tick mortality was measured in engorged larvae while in the study of Hurry et al. (2021), survival rates were measured in nymphs. We hypothesized that the diminution of the mortality of *E. coli*+*B. afzelii* larvae could be due to the lower *B. afzelii* load within the ticks, which could favor to the fitness of the vector.

Conclusions

We found that *B. afzelii* infection modulates the *I. ricinus* microbiota in terms of beta diversity, composition, abundance and microbial co-occurrence. The broad effects induced by the pathogen on tick microbiota are likely the result of the pathogen generating an environment conducive for its colonization within the vector. Disrupting this infection-permissive microbiome state may be an alternative to block pathogen colonization and its subsequent transmission to a new host. Effective chains of infection of vector-borne pathogens involve a competent vector, an infective pathogen and

an infection-permissive microbiome [56] and mismatch of one of these components can result in the inability of the pathogen to efficiently colonize the vector gut and/or the inability of the vector to transmit pathogens [56]. Here, we showed that anti-microbiota vaccine targeting *Escherichia-Shigella* can shape *I. ricinus* microbiome towards an infection-refractory state, by shifting the abundance of several bacterial members of the microbiota, and increasing microbe-microbe interactions and robustness, which impacted the whole tick microbiota and resulted in a lower *B. afzelii* load within the vector. Therefore, anti-microbiota vaccine is a suitable tool for the manipulation of the microbiome towards a desired state and can be used for the control of vector-borne diseases.

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Chapter IV

Impact of anti-microbiota vaccine on *Plasmodium relictum* colonization in *Culex quinquefasciatus* mosquitoes



Anti-Microbiota Vaccine Reduces Avian Malaria Infection Within Mosquito Vectors

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Animal and human pathogens that are transmitted by arthropods are a global concern, particularly those vectored by mosquitoes (e.g., *Plasmodium* spp. and dengue virus). Vector microbiota may hold the key to vector-borne pathogen control, as mounting evidence suggests that the contributions of the vector microbiota to vector physiology and pathogen life cycle are so relevant that vectorial capacity cannot be understood without considering microbial communities within the vectors. Anti-tick microbiota vaccines targeting commensal bacteria of the vector microbiota alter vector feeding and modulate the taxonomic and functional profiles of vector microbiome, but their impact on vector-borne pathogen development within the vector has not been tested. In this study, we tested whether anti-microbiota vaccination in birds targeting Enterobacteriaceae within mosquito midguts modulates the mosquito microbiota and disrupt *Plasmodium relictum* development in its natural vector *Culex quinquefasciatus*. Domestic canaries (*Serinus canaria domestica*) were experimentally infected with *P. relictum* and/or immunized with live vaccines containing different strains of *Escherichia coli*. Immunization of birds induced *E. coli*-specific antibodies. The midgut microbial communities of mosquitoes fed on *Plasmodium*-infected and/or *E. coli*-immunized birds were different from those of mosquitoes fed on control birds. Notably, mosquito midgut microbiota modulation was associated with a significant decrease in the occurrence of *P. relictum* oocysts and sporozoites in the midguts and salivary glands of *C. quinquefasciatus*, respectively. A significant reduction in the number of oocysts was also observed. These findings suggest that anti-microbiota vaccines can be used as a novel tool to control malaria transmission and potentially other vector-borne pathogens.

Keywords: anti-microbiota vaccines, avian malaria, mosquitoes, networks, microbiota

INTRODUCTION

Mosquitoes are vectors of major human diseases such as dengue (caused by dengue virus), and malaria (caused by *Plasmodium* spp.) (1). According to literature, there are more than 50 avian *Plasmodium* species and new species are discovered every year (2) and infection by these parasites is common in some bird species (3). Among the *Plasmodium* species affecting birds, *P. relictum* is listed among the most invasive organisms in the world, infecting more than 300 bird species and is prevalent all around the world (4). *Plasmodium relictum* is transmitted mostly by *Culex* mosquitoes, including *Culex pipiens* and *Culex quinquefasciatus* (5). The midgut is the first organ in which *P. relictum* ingested with the host blood can survive (3). From the midgut lumen, the parasite traverses the peritrophic membrane and epithelial layer of the midgut and develops to oocysts (3). The oocysts invade other vector tissues such as salivary glands to complete the sporogonic development (3). In general, the susceptibility of mosquitoes to *Plasmodium* parasites infection is under genetic control (6–8), but the large variability in oocyst number among closely related mosquitoes indicates that environmental factors also play a role. Of special interest are the interactions between the vector, its microbiota and transmitted pathogens, since commensal bacteria interact with mosquito-borne pathogens (9) and can facilitate (10) or compete (11) with pathogen colonization and development within the vector midguts, prompting research into microbiota manipulation and transmission-blocking strategies (12). Depleting vector microbiota from bacteria that facilitates pathogen development could be exploited as a mean for blocking transmission. For example, the bacterium *Asaia bogorensis* increases midgut pH promoting *Plasmodium berghei* gametogenesis within *Anopheles stephensi* (10), and high abundance of Enterobacteriaceae increases *Plasmodium falciparum* infection in *Anopheles gambiae* midgut (13), making the reduction of these bacterial species a sound strategy to reduce pathogen infection in the vector and potentially block transmission to the host.

Although targeting specific commensal bacteria could block pathogen transmission, the lack of tools for the precise manipulation of the vector microbiota is currently a major limitation to developing novel transmission-blocking strategies. Specific host antibodies (Abs) are easily induced by vaccination, and once taken with the blood meal, they remain functional within the vector tissues and can bind symbionts (14) and other bacterial microbiota (15) within hematophagous arthropods (16). Surprisingly, host Abs specific to bacterial microbiota had never been used for microbiota manipulation and transmission blocking strategies (16). It was recently shown that anti-microbiota vaccines modulate the tick microbiota in a taxon-specific manner (17). Firstly, combining next-generation sequencing (NGS) and network analysis, Enterobacteriaceae was identified as a keystone taxon (i.e., highly connected taxa driving community composition and function) in the microbiota of *Ixodes ricinus* ticks (17, 18). Secondly, the abundance of vector-associated Enterobacteriaceae decreased in ticks fed on mice immunized with a live bacteria vaccine containing *Escherichia coli* (17). Thirdly, vaccination against Enterobacteriaceae had cascading ecological impacts on the whole tick microbiome by reducing bacterial diversity and

modulating the functional profiles of the microbiome (17). Fourthly, decreased Enterobacteriaceae abundance was correlated with high levels of *E. coli*-specific Abs (17). Last but not least, no mortality or sign of pain were observed after the vaccination in the mice (17, 18) and the fecal microbiota of immunized mice showed no significant alterations (17). Here we tested whether modulation of mosquito microbiota by anti-microbiota vaccination of host birds against commensal Enterobacteriaceae disrupts *P. relictum* development within midguts and salivary glands of the vector *C. quinquefasciatus*.

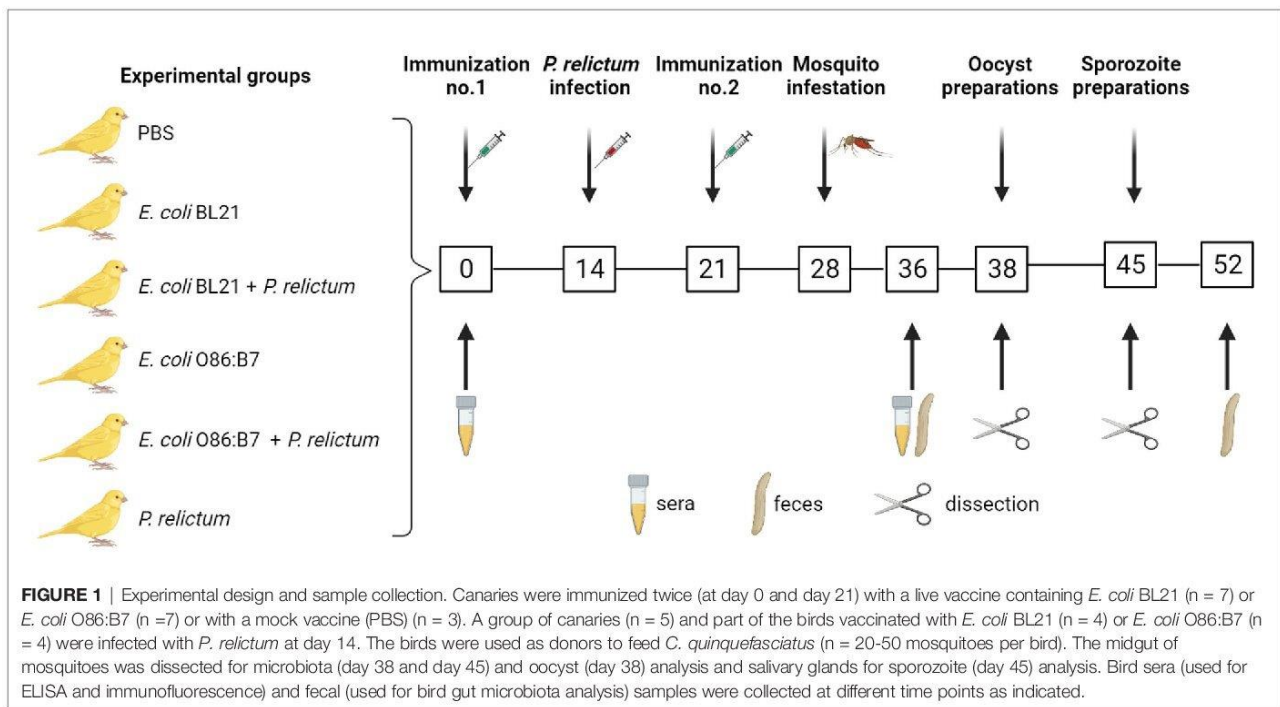
RESULTS

Anti-Microbiota Vaccination in Birds Interferes With Plasmodium-Induced Modulation of Mosquito Midgut Microbiota

Immunization was followed by experimental malaria infection and mosquito infestation (Figure 1). Anti-microbiota vaccination of birds with *E. coli* O86:B7 or *E. coli* BL21 increased the levels of IgY specific to *E. coli* (Figure 2A). Anti-*E. coli* IgY also increased in the sera of birds that received both anti-microbiota vaccines and infection with *P. relictum* (Figure 2A). No significant change was observed in the levels of anti-*E. coli* Abs in birds that received the mock vaccination (PBS) or those only infected with *Plasmodium* (Figure 2A). Following the bacterial immunization and *P. relictum* infection in birds, *C. quinquefasciatus* mosquitoes were allowed to feed on the birds to acquire the parasites and/or anti-*E. coli* Abs. The midguts of fed mosquitoes were dissected and used for microbiota analysis.

Bacterial community composition and diversity were compared between groups to assess the impact of anti-microbiota vaccines and *P. relictum* infection on the mosquito midgut microbiota. Analysis of alpha diversity indexes showed that overall, the phylogenetic richness (Figure 2B) and evenness (Figure 2C) did not differ between experimental groups (Kruskal-Wallis, $p > 0.05$). However, pairwise comparisons between groups revealed a reduced taxonomic richness in mosquitoes from *E. coli* O86:B7 + *P. relictum* group compared to *P. relictum*-infected mosquitoes (Kruskal-Wallis, $p = 0.036$, Figure 2B). Beta diversity analysis revealed a significant difference in Bray Curtis dissimilarity index between the groups (PERMANOVA, $F = 2.40$, $p = 0.001$). Specifically, *Plasmodium*-infected mosquito microbiota shows a tendency to separate from the other groups. Furthermore, dispersion analysis of Bray Curtis dissimilarity index did not show significant differences between groups (BetaDisper $F = 1.01$, $p = 0.426$, Figure 2D).

Further characterization of the impact of anti-*E. coli* O86:B7 immunization and *Plasmodium* infection on mosquito midgut microbiota was achieved using differential abundance analysis. Significant changes in the abundance of 20 and 7 bacterial genera were detected in the midgut microbiota of mosquitoes fed on *P. relictum*-infected (Figures 3A, B) or *E. coli* O86:B7-immunized canaries (Figures 3C, D), respectively, compared to those fed on mock-immunized canaries. This suggests that *P. relictum* infection and the anti-microbiota vaccine disturb the mosquito



microbiota. We next asked whether anti-microbiota vaccination could interfere with pathogen-induced modulation of the mosquito microbiota. The midgut microbiota of mosquito exposed to both *P. relictum* infection and anti-*E. coli* O86:B7 Abs had 23 and four taxa with significant changes in abundance compared to mosquitoes fed on *Plasmodium*-infected (Figures 3E, F), and *E. coli* O86:B7-immunized canaries (Figures 3G, H), respectively. Vaccination with *E. coli* BL21 was also associated with changes in the abundance of several bacterial taxa, compared with the control mock-vaccinated and with *Plasmodium* infection groups (Figure 4). Notably, compared with the mock-vaccinated group, the bacterial taxa affected by infection or vaccination alone were different between them and from those affected simultaneously by vaccination and infection (Figure 5). These results suggest that *P. relictum* infection and the anti-microbiota vaccines modulate the mosquito midgut microbiota in different ways and that the anti-microbiota vaccines interfere with the *Plasmodium*-induced modulation of the mosquito microbiota.

To rule out a negative effect of anti-microbiota vaccine on host microbiota, we collected bird feces after mosquitoes infestation (d36) and at the end of the experiment (d52). We compared fecal microbiota of mock-immunized, *E. coli* BL21-immunized and *E. coli* O86:B7-immunized birds. The results showed non-significant differences in the microbial richness (Kruskal-Wallis, $p > 0.05$), as measured by Faith's phylogenetic index (Supplementary Figure S1A). Similarly, species evenness did not differ between the different experimental groups (Kruskal-Wallis, $p > 0.05$, Supplementary Figure S1B). Regarding the beta diversity of the microbial communities in bird guts, Bray Curtis dissimilarity index did not show significant

differences among mock-immunized, *E. coli* BL21-immunized and *E. coli* O86:B7-immunized birds (PERMANOVA, $F = 1.35$, $p = 0.25$).

Anti-Microbiota Vaccination Re-Structures the Microbial Communities in Midgut of Plasmodium-Infected Mosquitoes

Bacteria co-occurrence networks were used to further characterize the impact of *Plasmodium* infection and anti-microbiota vaccines on the mosquito microbiota. Visual inspection of the taxonomic networks revealed that anti-microbiota vaccines and malaria infection, together and separately, changes network topology, compared with the control mock vaccine group (Figure 6 and Table 1). Jaccard index was used to test for similarities (Jacc = 0, lowest similarity and Jacc = 1, highest similarity) in selected local network centrality measures (i.e., hub taxa, degree, betweenness centrality, closeness centrality, and eigenvector centrality) of the different networks. The observed Jaccard index for the betweenness centrality was higher than expected by random for the comparison between *E. coli* BL21 and *E. coli* O86:B7 networks (Jacc = 0,418, $p = 0.01$) (Supplementary Table S1). In addition, except for the Jaccard index of betweenness centrality in the PBS - *P. relictum* (Jacc = 0,432, $p = 0.004$), PBS - *E. coli* BL21 (Jacc = 0,512, $p = 0.000002$) and PBS - *E. coli* O86:B7 (Jacc = 0,522, $p = 0.000001$) network comparisons, the observed Jaccard indexes of the other centrality measures were lower than expected by random in most networks (Supplementary Table S1). This suggests low similarity between compared networks. Topological differences, together with dissimilarity in local network centrality measures, indicate a major shift in the

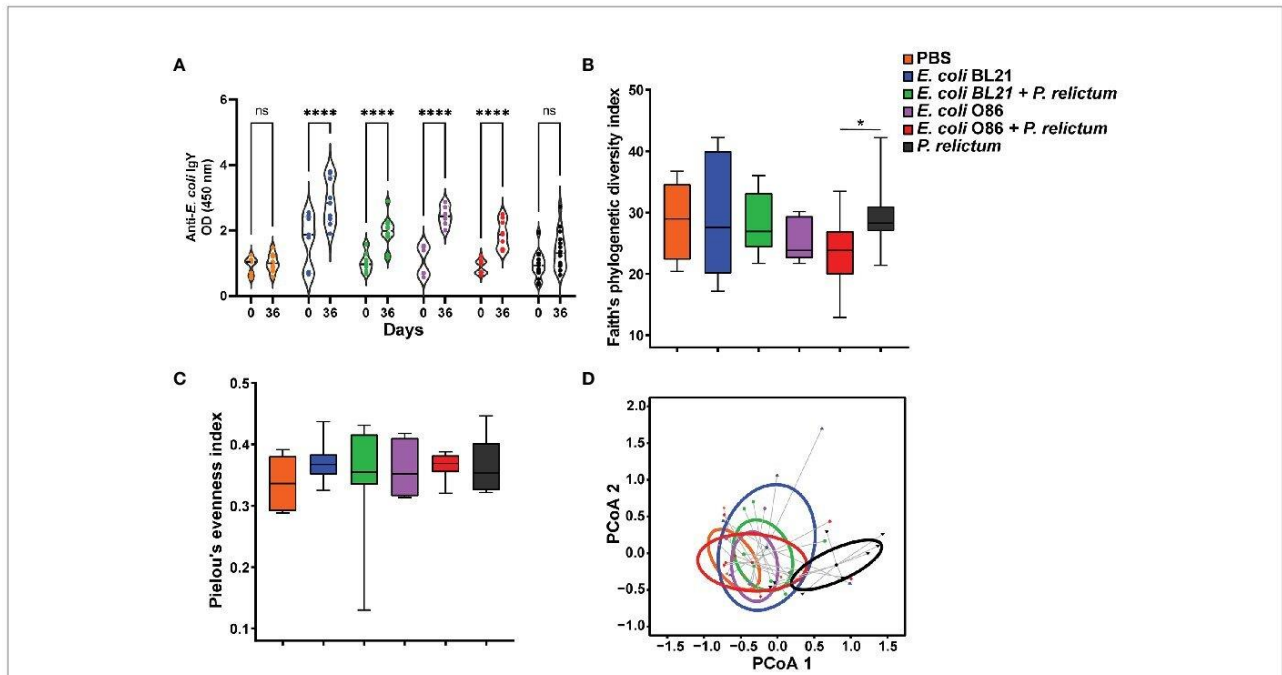


FIGURE 2 | Impact of anti-microbiota vaccine and *P. relictum* infection on bird antibody response and mosquitoes' microbial diversity. **(A)** Levels of IgY specific to *E. coli* proteins were measured by semiquantitative ELISA in sera of canaries before immunization (day 0) and in sera of canaries immunized with a mock vaccine, *E. coli* BL21 and *E. coli* O86:B7 alone or challenged with *P. relictum* (day 36 after first vaccination) (**** $p < 0.00001$; ns, not significant). **(B)** Faith's phylogenetic diversity and **(C)** Pielou's evenness indexes were used to measure the richness and evenness, respectively, of microbiota of mosquitoes fed on canaries immunized with a mock vaccine, *E. coli* BL21 and *E. coli* O86:B7 alone or challenged with *P. relictum*. The asterisk indicates significant differences between groups (pairwise Kruskal-wallis, $p = 0.03$). **(D)** Beta diversity of mosquito microbiota in the different experimental conditions represented in PCoA plot obtained by Betadisper function. There were no differences in the intragroup dispersions (variances) (ANOVA, $p = 0.426$), whereas differences were found in the community composition between groups (PERMANOVA, $p = 0.001$), specifically the group infected with *P. relictum* alone was different from the other groups. Taxonomic profiles at the level of ASVs, used to measure the alpha and beta diversity, were obtained from 16S rRNA sequences from midgut of mosquitoes fed on mock-immunized ($n = 5$ mosquitoes midgut pool), *E. coli* O86:B7-immunized ($n = 5$ mosquitoes midgut pool), *E. coli* O86:B7-immunized and challenged with *P. relictum* ($n = 8$ mosquitoes midgut pool) and challenged only with *P. relictum* ($n = 8$ mosquitoes midgut pool).

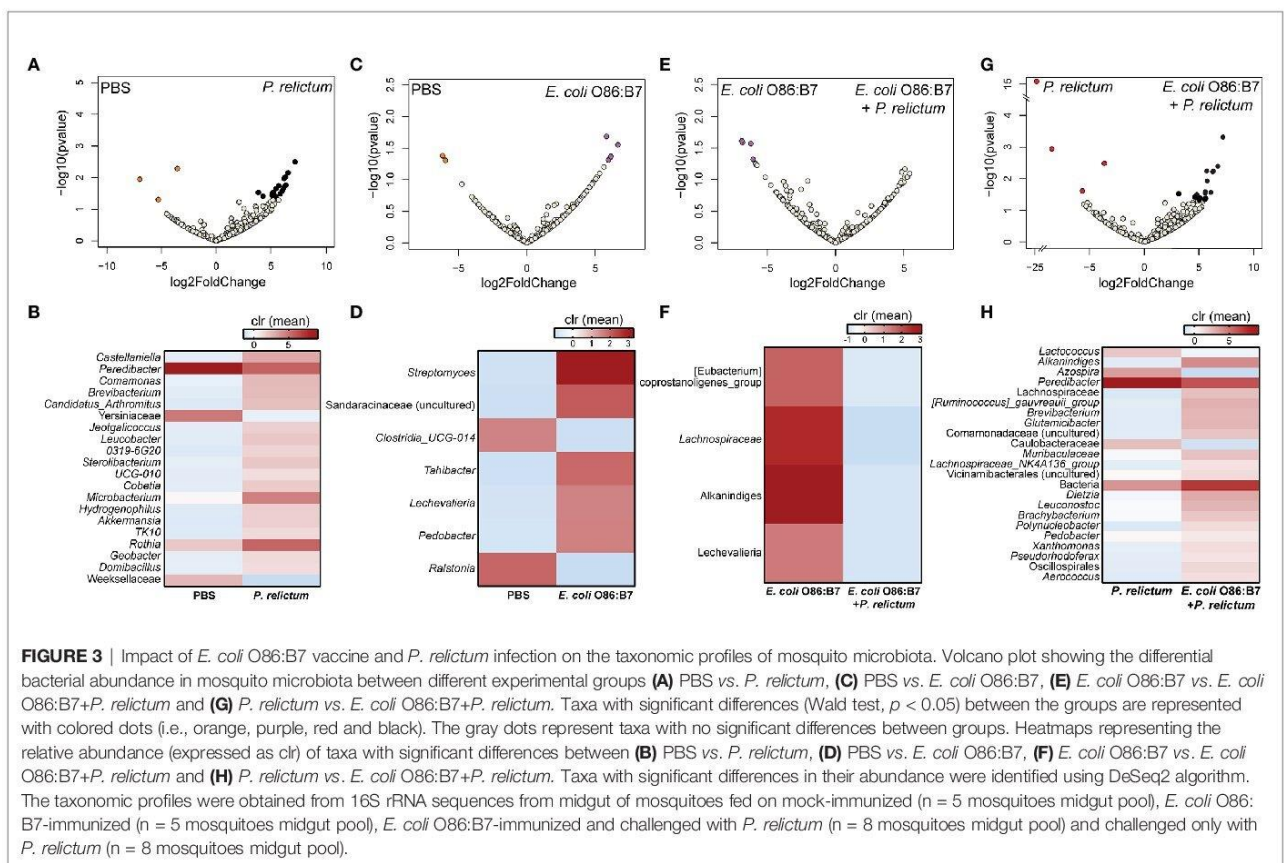
community structure induced by anti-microbiota vaccines and malaria infection.

High variation of local connectedness of *Escherichia-Shigella* was observed in co-occurrence sub-networks of experimental groups (Figure 7). Specifically, the number of direct neighbors co-occurring with *Escherichia-Shigella* in the microbiota of mosquitoes fed on birds of the different experimental groups decreased in comparison to the sub-network of the microbiota of mosquitoes fed on mock-vaccinated birds. Although the number of edges directly connected to *Escherichia-Shigella* was similar in the sub-networks of the microbiota of mosquitoes fed on mock-immunized (47 co-occurring taxa) and *E. coli* O86:B7-immunized birds (45 co-occurring taxa), an increase of negative co-occurrence correlation was observed in the latter compared to the former group. A detailed analysis of the nodes connected to *Escherichia-Shigella* revealed the inexistence of shared taxa among the different sub-networks (Supplementary Figure S2). In addition, we used the eigenvector centrality metric to evaluate the “keystoneness” (importance of a node within the network) of *Escherichia-Shigella* in the different microbial networks. Eigenvector centrality value of *Escherichia-Shigella* in the sub-network of mosquitoes fed on *E. coli*

BL21-immunized (eigenvector 0.03) and *E. coli* O86:B7-immunized birds (eigenvector 1) decreased and increased, respectively, compared to that in the control sub-network (i.e., mock vaccine group, eigenvector 0.29). Interestingly, eigenvector centrality values of *Escherichia-Shigella* decreased dramatically in the groups *E. coli* BL21+*P. relictum* (eigenvector 0.07), *E. coli* O86:B7+*P. relictum* (eigenvector 0), compared with the *P. relictum* infected mosquito group (eigenvector 0.16). Variable local interactions and “keystoneness” of *Escherichia-Shigella* between networks suggest that this taxon is affected by anti-microbiota vaccines and *Plasmodium* infection. More importantly, *Escherichia-Shigella* losses importance in the microbiota of mosquito exposed simultaneously to anti-*E. coli* Abs and *P. relictum*.

Anti-Microbiota Vaccination Reduces Plasmodium Infection Within Mosquito Tissues

Mosquito survival was recorded and compared between groups. Results showed a significant difference in survival rate between the groups (Fisher's exact test, $p = 0.013$). However, pairwise comparison of individual groups revealed significant differences



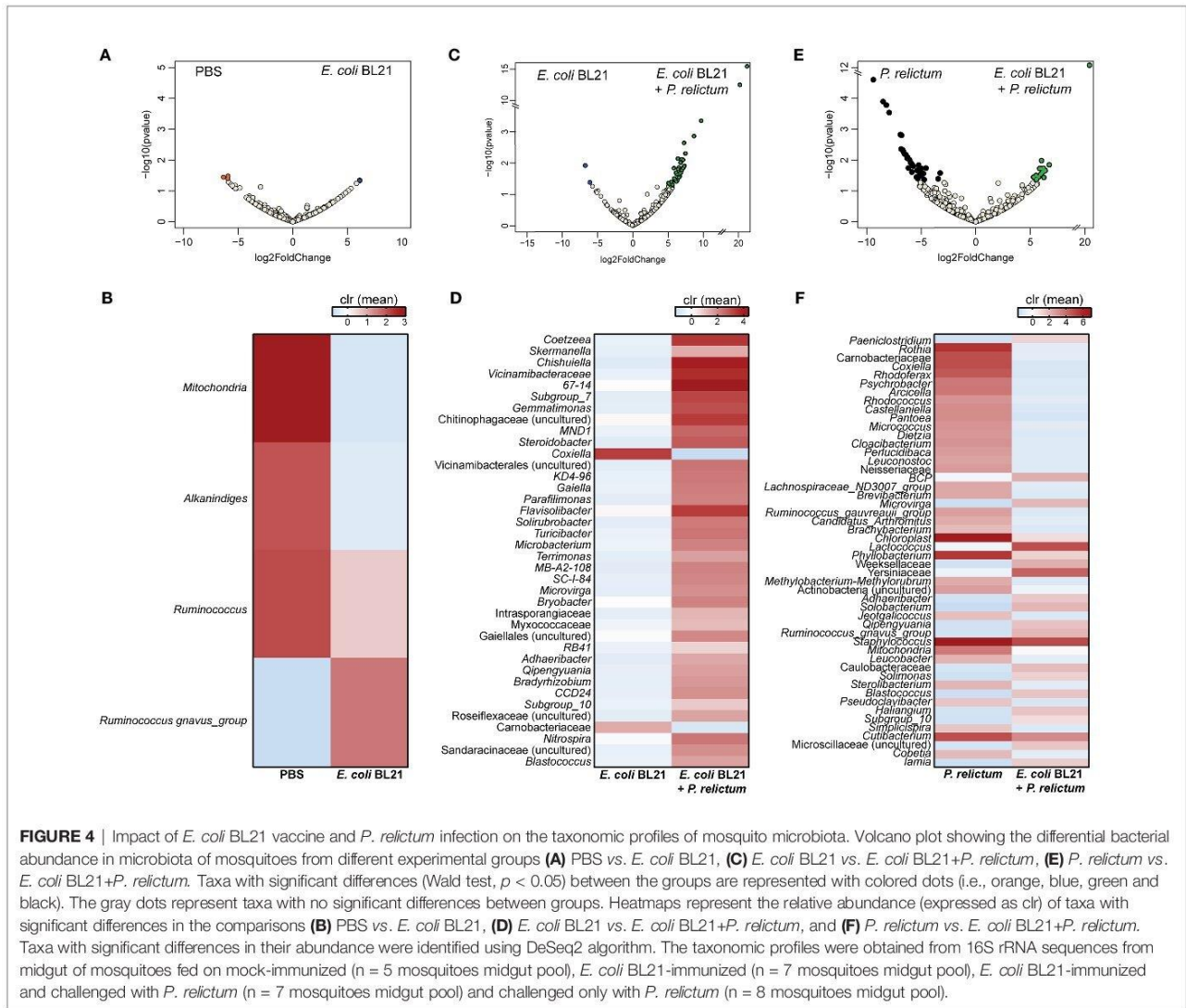
only in the survival of mosquitoes fed on birds of the *E. coli* BL21 and *E. coli* BL21-*P. relictum* groups between them ($p = 0.02$) and with the others (Figure 8). Mosquitoes in the *E. coli* BL21 and *E. coli* BL21+*P. relictum* groups had the highest and lowest survival rates.

The occurrence (i.e., number of mosquitoes in which at least one oocyst was found) and number of *P. relictum* oocysts in midguts and occurrence of sporozoites in salivary glands of mosquitoes were measured. The results showed a significant overall difference in the occurrence of oocysts between the groups (Fisher’s exact test, $p = 0.03$). Pairwise comparisons revealed that the occurrence of *Plasmodium* oocysts was significantly lower in the midguts of mosquitoes fed on birds vaccinated with *E. coli* O86:B7 ($p = 0.01$), but not with *E. coli* BL21 ($p > 0.05$), compared with mosquitoes infected with *P. relictum* and not exposed to anti-*E. coli* Abs (Figure 9A). The number of oocysts was significantly lower in midguts of mosquitoes fed on birds vaccinated with both *E. coli* O86:B7 ($p = 0.01$) and *E. coli* BL21 ($p = 0.001$), compared with control mosquitoes (Figure 9B). We then hypothesized that decreased oocysts load in midguts might be associated with lower sporozoite infection in salivary glands. Results showed a significant difference between sporozoite occurrence between the groups (Fisher’s exact test, $p < 0.001$). The occurrence of sporozoites in salivary glands of mosquitoes fed on *E. coli* O86:B7-immunized, but not *E. coli* BL21-immunized ($p > 0.05$), birds

was significantly lower ($p = 0.001$) than the control group (i.e., *P. relictum* infection alone) (Figure 9C).

DISCUSSION

In this study, we showed that anti-microbiota vaccination of birds with two strains of *E. coli* O86:B7 and BL21 modulate *C. quinquefasciatus* midgut microbiota. These results concur with a previous report in which live bacteria vaccine were used as a tool for the manipulation of tick microbiome (17, 18). During feeding, hematophagous ectoparasites ingest blood from the vertebrate host, along with host immune molecules. Host Abs and/or complement proteins have been detected in the guts of ticks (19–24), mosquitoes (25, 26), sandflies (27, 28), and tsetse flies (29). Once ingested, host immune components can remain active from a few hours to months depending on the species of blood-sucking arthropod, raising the possibility that vertebrate Abs could interact with pathogens and microbiota (16). Empirical work shows that host Abs can target vector-borne pathogens within ticks (30) and mosquitoes (31–33). Targeting pathogen proteins expressed within the arthropod vectors is the rationale behind transmission-blocking vaccines (32–34). Functional host Abs have also been shown to interact with symbionts in *Rhodnius prolixus* (14) and *Glossina morsitans* (35), as well as with bacterial microbiota in mosquitoes (15) and



ticks (17, 18). Vaccination of mice with *E. coli* BL21 induced anti-*E. coli* Abs that when ingested with the blood decreased Enterobacteriaceae abundance within the tick microbiota (17). Targeting commensal *Escherichia* in ticks with anti-*E. coli* Abs reduced the connectivity of this taxon in the co-occurring networks (17). Similarly, in this study we observed reduced connectivity of *Escherichia* in the co-occurring networks of mosquitoes fed on birds vaccinated with *E. coli* BL21, but not in those vaccinated against *E. coli* O86:B7. However, we did observe a switch in the bacterial connectivity pattern of mosquitoes fed on *E. coli* O86:B7 compared to the control group PBS. In the control group, the majority of *Escherichia* correlations with its direct neighbor nodes were positive, while in the *E. coli* O86:B7 group most correlations were negative. These results show that antibody-mediated disturbance of the microbiome had cascading ecological impacts on the whole tick microbiome with strong impact on the structure of the microbial community of the vectors.

Recent research on vector-pathogen-microbiota interactions shows that microbial communities within vectors strongly influence pathogen colonization and transmission (36). Despite recent advances in vector microbiota research, the lack of tools for the precise and selective manipulation of the vector microbiome is currently a major limitation to disrupt pathogen-microbiome interactions in a taxon-specific manner (12, 37). Here we showed that mosquitoes fed on birds immunized with the Enterobacteriaceae bacteria *E. coli* O86:B7 and *E. coli* BL21 had reduced numbers of *P. relictum* oocysts in the midguts. The results support the potential role of commensal Enterobacteriaceae as a key player in the early development of *Plasmodium* within the vector. In agreement with this, high abundance of Enterobacteriaceae was previously associated with increase *P. falciparum* infection in *A. gambiae* midgut (13), and several studies showed that the ookinete-oocyst transition of *Plasmodium* spp. is strongly influenced by resident midgut microbiota in different mosquito species (38–41). However, the directionality (facilitation vs. competition) of midgut

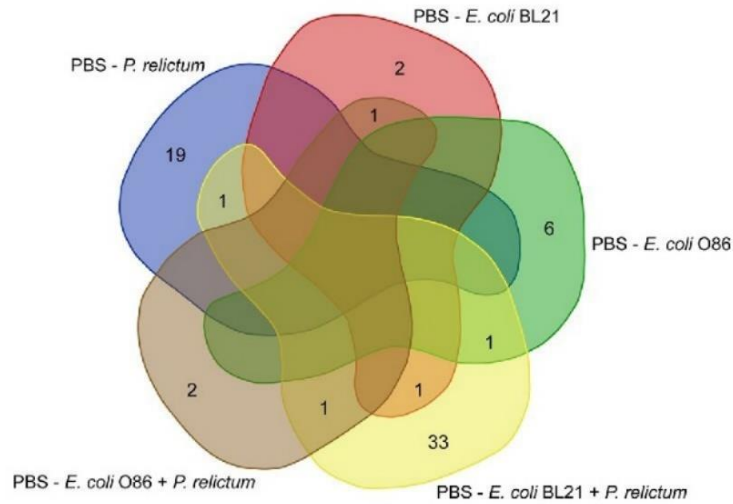


FIGURE 5 | Comparison of unique and shared taxa across different experimental groups. Venn diagram showing the common and different taxa with significant changes in their abundance between vaccinated and infected groups, compared to the mock-vaccinated group (PBS).

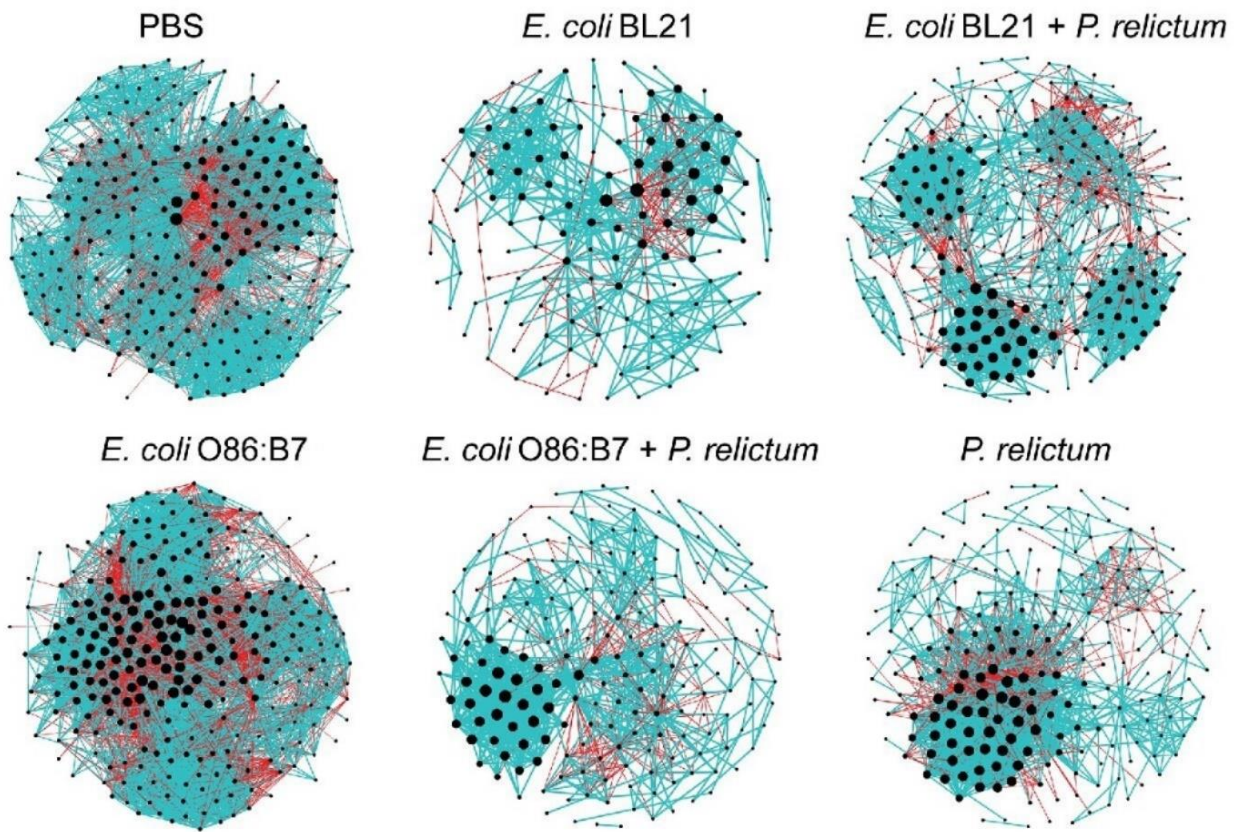


FIGURE 6 | Co-occurrence networks of *C. quinquefasciatus* microbiota in the different experimental groups. Bacterial co-occurrence networks were inferred from the microbiota of mosquitoes fed on control (PBS), vaccinated and/or infected birds. Nodes represent bacterial taxa and connecting edges stand for a co-occurrence correlation (SparCC > 0.75). Node sizes are proportional to the eigenvector centrality value. Edges representing positive or negative correlations were colored in blue and red, respectively. Only nodes with at least one connection are displayed.

TABLE 1 | Topological parameters of co-occurrence networks.

Network features	PBS	<i>E. coli</i> BL21	<i>E. coli</i> BL21 + <i>P. relictum</i>	<i>E. coli</i> O86:B7	<i>E. coli</i> O86:B7 + <i>P. relictum</i>	<i>P. relictum</i>
Nodes	231	137	267	261	199	221
Edges	4417	591	1787	6629	991	1709
Positive	3,041 (68.9%)	476 (80.5%)	1,386 (77.6%)	3,827 (57.7%)	822 (82.9%)	1,232 (72.1%)
Negative	1,376 (31.2%)	115 (19.5%)	401 (22.4%)	2,802 (42.3%)	169 (17.1%)	477 (27.9%)
Network diameter	3	10	13	4	10	12
Average degree	38.242	8.628	13.386	50.797	9.96	15.466
Weighted degree	12.07	4.265	6.113	7.018	5.287	5.83
Average path length	1.909	3.47	3.871	1.993	3.609	3.716
Modularity	1.459	0.974	1.105	2.996	0.785	0.872
Number of modules	6	18	27	13	20	32
Average clustering coefficient	0.719	0.556	0.621	0.658	0.538	0.623

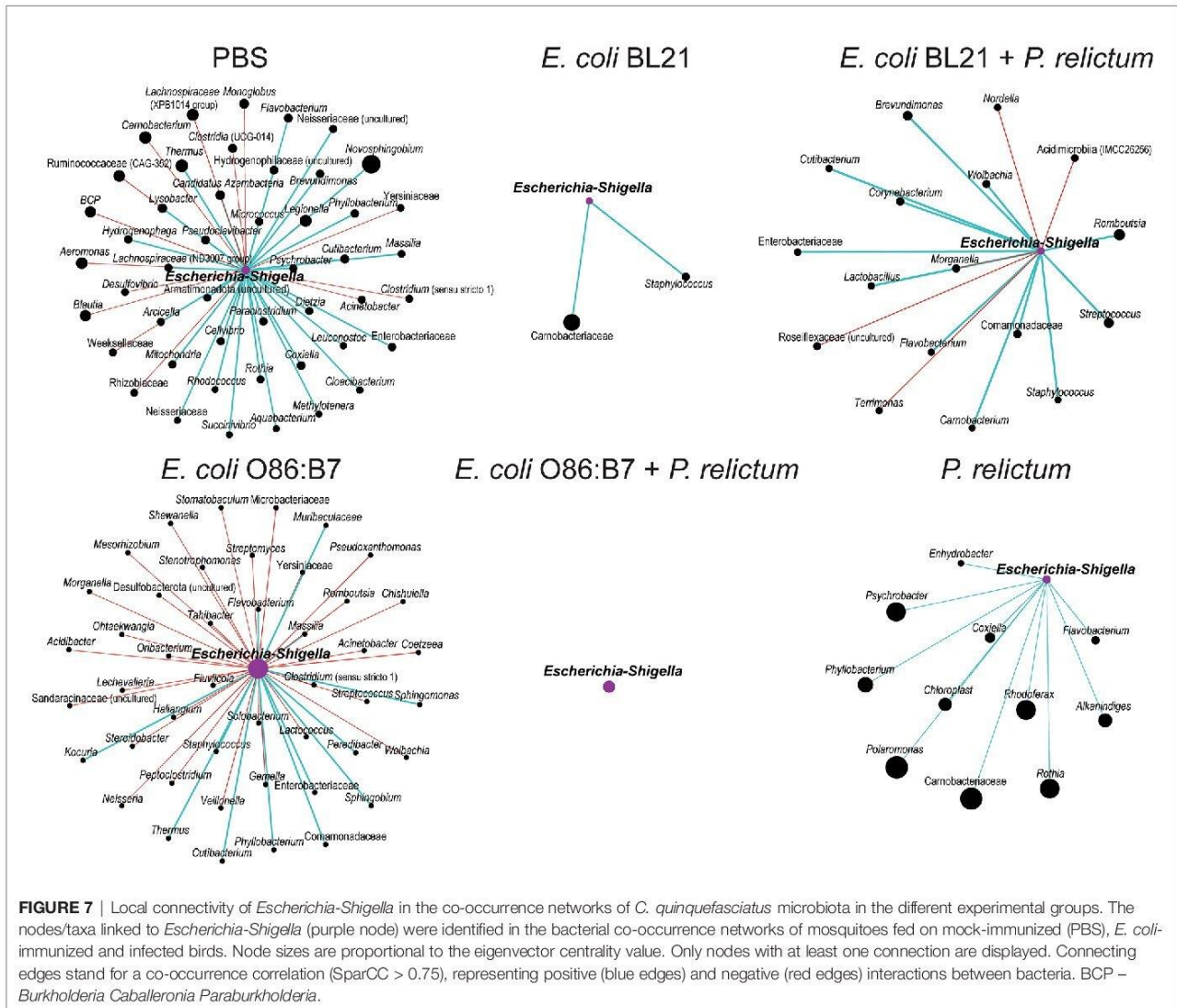


FIGURE 7 | Local connectivity of *Escherichia-Shigella* in the co-occurrence networks of *C. quinquefasciatus* microbiota in the different experimental groups. The nodes/taxa linked to *Escherichia-Shigella* (purple node) were identified in the bacterial co-occurrence networks of mosquitoes fed on mock-immunized (PBS), *E. coli*-immunized and infected birds. Node sizes are proportional to the eigenvector centrality value. Only nodes with at least one connection are displayed. Connecting edges stand for a co-occurrence correlation (SparCC > 0.75), representing positive (blue edges) and negative (red edges) interactions between bacteria. BCP – *Burkholderia Caballeronia Paraburkholderia*.

microbiota contribution to infection cannot be simplified to the abundance of a single taxon, while neglecting global changes of the microbial community as a whole. For example, experimental exposure of mosquitoes to a cocktail of penicillin and

streptomycin (PS) reduced the proportion of microbiota-resident Enterobacteriaceae 92-fold, while simultaneous exposure to PS and *Plasmodium* infection increased the prevalence and intensity of *P. berghei* oocyst in *A. gambiae* midguts (42). This is not expected if

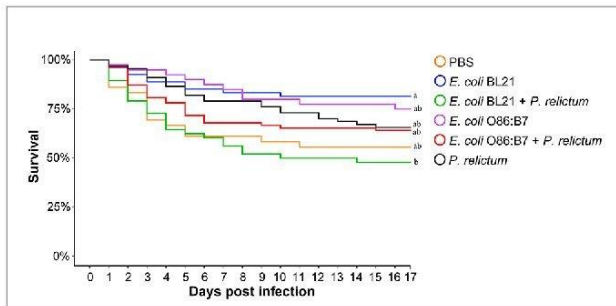


FIGURE 8 | Survival of blood-fed mosquitoes throughout the experiment. The highest mortality was observed in the mosquito group fed on donors vaccinated with *E. coli* BL21 and infected with *P. relictum*, which significantly differed from the group fed on the birds vaccinated with *E. coli* BL21 ($p = 0.02$). Survival of mosquitoes between other groups did not differ significantly. Survival of mosquitoes was estimated by Fisher's exact test with Bonferroni correction. Different letters at the end of the curves indicate statistically significant differences in survival rate ($p < 0.05$).

Enterobacteriaceae facilitates *Plasmodium* infection (13). However, regardless that the impact of Enterobacteriaceae on parasite infectivity may differ between *Plasmodium* species (e.g., *P. berghei* vs. *P. falciparum*), the decreased of Enterobacteriaceae has been associated with a 19-fold increase of *Asaia* sp. in PS-exposed *A. gambiae* (42). Commensal *Asaia bogorensis* remodels glucose metabolism and increases midgut pH which in turn induces *P. berghei* gametogenesis and facilitates parasite infection of *A. stephensi* (10). Thus, the decrease of Enterobacteriaceae abundance may be balanced by the increase of another taxon (e.g., *Asaia* sp.) that also facilitates infection with a net effect of increased *Plasmodium* infectivity. Our results showed that

anti-microbiota vaccines altered the *Plasmodium*-induced modulation of the mosquito microbiota, resulting in a global microbial community transformation and the reduction of *Plasmodium* infectivity.

We also found a significant reduction of sporozoites occurrence in the salivary glands of mosquitoes fed on *E. coli* O86:B7-immunized birds, but no in those fed on *E. coli* BL21-immunized birds. This could be explained by differences in the levels of the glycan Gal α 1-3Gal (α -Gal) in these two *E. coli* strains. The strain *E. coli* O86:B7 expresses high levels of α -Gal (43), which is not the case for *E. coli* BL21 (44, 45). Even when *E. coli* BL21 produces low levels of α -Gal, immunization with this bacterium induces anti- α -Gal (18), as low-abundant antigens could also induce immune responses. However, immunization with *E. coli* O86:B7 may induce anti- α -Gal Abs at levels even higher than *E. coli* BL21. This is relevant because as previously shown in ticks (18), α -1,3-galactosyltransferase genes, and possibly α -Gal, may be widely distributed in mosquito bacterial microbiota, increasing the number of bacterial taxa targeted by Abs induced by *E. coli*-vaccination. Another point to consider is that α -Gal has been detected on the surface of *P. falciparum*, *P. berghei* and *Plasmodium yoelii* sporozoites (43). Whether *P. relictum* sporozoites express α -Gal within *C. quinquefasciatus* salivary glands and whether anti- α -Gal Abs could target this *Plasmodium* stage within mosquito salivary glands remain to be tested.

Interestingly, mosquitoes fed on birds immunized with *E. coli* BL21 or *E. coli* O86:B7 had the highest survival rates, although the differences were significant only for mosquitoes in the *E. coli* BL21 group, compared with the *P. relictum*+*E. coli* BL21 group. Similar impact on survival was observed on mosquitoes fed on blood supplemented with PS (42). Exposure of *A. gambiae* to PS

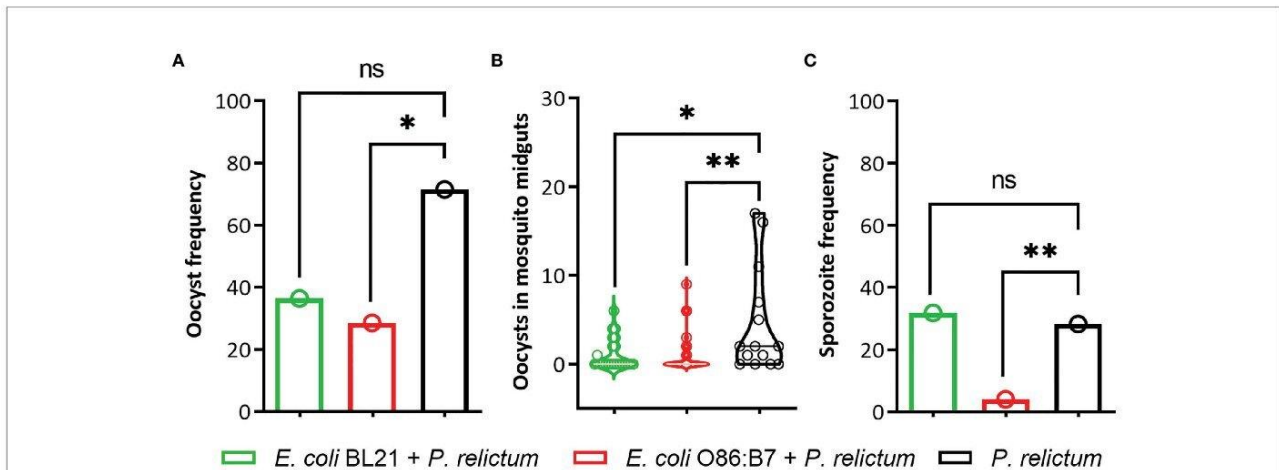


FIGURE 9 | Impact of anti-microbiota vaccines on *P. relictum* development in infected mosquitoes. Three groups of *P. relictum*-infected birds were used as infection donors for mosquitoes. One of the groups was vaccinated with *E. coli* BL21, the other with *E. coli* O86:B7, and a third group was a control (only infection). **(A)** The percentage of mosquitoes in which at least one oocyst was found in the midgut by microscopy and compared between the groups was measured. **(B)** The number of oocysts in the midgut of *P. relictum*-infected mosquitoes was calculated and compared between the groups. **(C)** The percentage of mosquitoes in which sporozoites were detected in salivary glands by microscopy or PCR was measured and compared between the groups. The parameters were compared between groups by Fisher's exact test with Bonferroni correction (oocyst and sporozoite frequency) and the Mann-Whitney U test (number of oocyst). (* $p < 0.05$; ** $p < 0.001$; ns, not significant; $n = 14$ -53 mosquitoes per group).

increased survival and fecundity, which are known to augment vectoral capacity (42). Our results can be interpreted as if microbiota modulation by *E. coli* BL21 vaccination: (i) increases *C. quinquefasciatus* fitness, and/or as if microbiota modulation by *E. coli* BL21 vaccination together with *Plasmodium* infection (ii) increases *P. relictum* virulence on mosquitoes and/or (iii) induces changes in the microbiome that impose a high cost on mosquito midguts homeostasis.

CONCLUSIONS

The vector microbiome can be assembled in different possible states, some of which may be incompatible with pathogen infection and/or transmission, while others increase vector competence or could increase or reduce vector fitness. Unraveling how to modulate these different states in a precise manner offers a powerful tool to develop novel transmission-blocking vaccines (16). Our results support the use of anti-microbiota vaccines to target vector commensal bacteria that facilitate pathogen infection (16, 46). In addition to taxon-specific effects, the community-level effects and cascading ecological impact of anti-microbiota vaccines on vector microbiota might induce infection-refractory states in the vector microbiome. Effective infection by vector-borne pathogens involves competent vectors, infective pathogens, and an infection-compatible microbiome (16). Mismatch of at least one of these components can result in an impaired ability of the vector to support the pathogen life cycle. For example, one strategy used to reduce the vector competence for pathogens is the genetic modification of insects that no longer transmit pathogens (47). Our results provide strong evidence that alterations in the vector midgut microbiomes, without the need to altering vector and/or pathogen genetics, affect pathogen infection in the vector. Therefore, deviations from infection-compatible microbiomes could block transmission and disease development. Anti-microbiota vaccines can be used as a microbiome manipulation tool for the induction of infection-refractory states in the vector microbiome for the control of major vector-borne pathogens such as malaria.

MATERIAL AND METHODS

Ethical Statement

All procedures were performed at the Nature Research Centre in Vilnius, Lithuania, according to Lithuanian and International Guiding Principles for Biomedical Research Involving Animals (2012). Infection experiments and other procedures were reviewed and approved by the Lithuanian State Food and Veterinary Service, Ref. No. 2020/07/24-G2-84. The assessment of the animal health and all described procedures were implemented by trained professionals (under licenses 2012/02/06-No-208, and 2016/01.29-No-344).

Birds and Housing Conditions

Seven-months-old domestic canaries were purchased commercially and kept for adaptation for one-month before

experimental procedures. Birds were kept at the Nature Research Centre vivarium (License No. LT-61-13-003) under standard living conditions for birds. Experimental animals were housed in cages, up to three birds per cage. The facilities were under a controlled temperature of 21°C, which was maintained throughout the time that the experimentation lasted. Animals were supplied with a standard food for canaries and water *ad libitum*.

Experimental Design

Domestic canaries (*Serinus canaria domestica*) were used as a model for avian malaria infection, anti-microbiota vaccination and as donors of *P. relictum* to mosquitoes. Before the experimental procedures began, birds were randomly separated into six groups as displayed in **Figure 1**. Birds in one group received a mock vaccination containing PBS and adjuvant. Another group of birds was only infected with *P. relictum*. All birds in the other four groups received anti-microbiota vaccines and in two of these groups, birds were additionally infected with *P. relictum*. After *Plasmodium* infection and antibody titers to anti-microbiota vaccines were confirmed, *C. quinquefasciatus* mosquito females were fed on the birds for pathogen acquisition experiment. In total, 22 experimental birds from all groups were exposed to mosquito bites as described in the sections below. Engorged mosquitoes (n = 663) were used for midgut and/or salivary glands preparations. Mosquito tissues were used for oocyst (midguts) and sporozoites (salivary glands) counting. Mosquito midguts were also used for DNA extraction and microbiota analysis using bacterial 16S rRNA amplicon sequencing. Bird feces were collected to test for an impact of anti-microbiota vaccines on bird gut microbiota. Experimental procedures are described below.

Bacterial Cultures and Live Bacteria Immunization

Representative bacteria of the genus *Escherichia-Shigella* were selected to be included in live bacteria vaccine formulations as previously reported (17, 18). Anti-microbiota vaccinations were used to test the impact of host immune response against targeted bacteria on mosquito microbiota composition and structure, mosquito survival and *Plasmodium* infection. The *Escherichia coli* strains BL21 (DE3, Invitrogen, Carlsbad, CA, USA), and O86:B7 (ATCC[®] 12701TM) were selected. The two bacterial strains were prepared as previously described (17, 18). Briefly, *E. coli* was grown on Luria Broth (LB, Sigma-Aldrich, St. Louis, MO, USA) at 37°C under vigorous agitation, washed with phosphate buffer saline (PBS) 10 mM NaH₂PO₄, 2.68 mM KCl, 140 mM NaCl, pH 7.2 (Thermo Scientific, Waltham, MA, USA), resuspended at 3.6×10^4 colony-forming unit (CFU)/mL, and homogenized using a glass homogenizer. Eight-month-old, canaries were immunized subcutaneously with *Escherichia* sp. in 50 μ L (4, 1×10^6 CFU per bird) of a water-in-oil emulsion containing 70% (w/w) Montanide[™] ISA 71 VG adjuvant (Seppic, Paris, France), with a booster dose two weeks after the first dose. Control birds received a mock vaccine containing PBS and adjuvant. All reagents used for bacterial preparation were apyrogenic.

Bacterial Protein Extraction

Escherichia coli strains were washed twice with PBS, centrifuged at 1,000× g for 5 min at 4°C, resuspended in 1% Trion-PBS lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) and homogenized with 20 strokes using a glass balls homogenizer. The homogenate was then centrifuged at 300× g for 5 min at 4°C and the supernatant was collected. Protein concentration was determined using the Bradford Protein Assay (Thermo Scientific, San Jose, CA, USA) with Bovine Serum Albumin (BSA) as standard. Bacterial protein extracts were used in the indirect ELISA to measure anti-*E. coli* Abs in bird sera.

Thawing the Cryopreserved Avian Malaria Sample

The cryopreserved *P. relictum* strain SGS1 was thawed and used to infect donor canaries. Tubes containing *Plasmodium*-infected avian blood, conserved frozen in liquid nitrogen, were thawed as described by Dimitrov et al. (48). Briefly, thawed samples were mixed with 12% NaCl (1/3 of thawed sample amount). After 5 min equilibration, one volume of 1.6% of NaCl was added followed by centrifugation at 10,000 rpm for 5 min. After centrifugation, the supernatant was removed and 1.6% NaCl (1/3 of original sample) was added and centrifuged again. The same procedure was repeated three times with 0.9% NaCl solution. The final mixture was diluted with 0.9% NaCl and sub-inoculated into two canaries.

Experimental Infection of Birds

All birds from the mock-immunized group and birds randomly selected ($n = 4$) from *E. coli* BL21-immunized and *E. coli* O86:B7-immunized groups were experimentally infected with *P. relictum* (SGS1) using the protocol described by Palinauskas et al. (49). Each experimental bird was sub-inoculated with a mixture (0.10 mL) of infected blood, 3.7% sodium citrate and 0.9% saline in proportion 4:1:5 into the pectoral muscles. Each bird received approximately 1×10^5 of mature *P. relictum* meronts. The duration of experimental time before exposure to the mosquitoes was 13 days post inoculation (dpi). This time was sufficient to develop higher parasitemia in the blood for malaria parasites. Birds with suitable infection levels were exposed to feed mosquitoes. Parasitemia was examined every 4 days by taking blood from the brachial vein as described in the section below.

Maintenance of Mosquitoes

For experimental infection of *P. relictum* in mosquitoes, we used the P. B. Šivickis parasitology laboratory-reared *C. quinquefasciatus* mosquitoes. The colony was maintained as described in Žiegytė et al. (50). Mosquitoes were kept in a nylon netted cage (65×65×65 cm) under controlled conditions (room temperature $23 \pm 1^\circ\text{C}$; humidity 75-80%; photoperiod 17:7 light:dark). Adult insects were provided with cotton wools saturated with 5% saccharose solution. Mosquito females were randomly separated from the main colony into smaller cages (about 300 mosquitoes) for each experimental group. For experimental infection we used insects of the same age, approximately one week after hatching.

Mosquito Exposure to Vaccinated Birds and Infection With Plasmodium

We evaluated gametocytemia in all donor birds immediately after mosquito exposure. The gametocytemia of *P. relictum*-infected donor birds varied between 0.005% – 1.5%, *E. coli* BL21-immunized birds – 0.02% – 2% and *E. coli* O86:B7-immunized birds – 0.01% – 3.5%. For experimental exposure to mosquito bites, the donor bird was carefully immobilized and fixed in a paper tube, leaving only the legs exposed for the mosquitoes (51). The tube was placed into a separate mosquito cage with separated female mosquitoes taken from the main colony. The bird was kept up to one hour, or when approximately 40 fully saturated mosquitoes were counted. Engorged insects were separated into small cages (17.4 × 17.5 × 17.5 cm) and kept there up to 17 days post exposure (dpe) under the same rearing conditions as described above. All cages were additionally provided with cups containing water for oviposition. Mosquitoes were exposed to donor birds immunized with *E. coli* BL21, *E. coli* O86:B7 and mock vaccine 28 days post first vaccination (dpv1). *Plasmodium*-infected donor birds were used for mosquito infestation 28 dpv1 (i.e., 14 dpi). Exposed mosquitoes were dissected gradually for preparations of different sporogonic stages and sampling for microbiota analysis.

Blood Sample and Bird Feces Collection

Blood samples were taken from birds by puncturing brachial vein using microcapillaries. A small drop of blood was used to make smears for microscopy to estimate the development of parasites in the blood. Smears were air-dried, fixed with absolute methanol and stained as described by Valkiūnas et al. (52). A fraction of blood (20–30 μL) was placed in SET-buffer for molecular analysis (PCR, see below) to confirm the lineage in recipient birds. The rest of the blood (100 μL) was used to obtain serum for immunological analysis. Before centrifugation the blood was incubated for 2 h at room temperature, allowing it to coagulate. Then samples were centrifuged at 5,000× g for 5 min and serum separated in microtube and kept in a freezer at -15°C until processing. The blood for smears and SET-buffer was collected on days 0, 4, 8, 12, after inoculation of parasites. The blood for serum was taken on days 0 and 36 after the first vaccine inoculation. Fresh feces were collected from each bird in sterile tubes on days 36 and 52 and were stored at -20°C before genomic DNA extraction.

Microscopic Examination

We used an Olympus BX61 light microscope (Olympus, Japan) to examine blood smears and preparations of mosquito tissues. Parasitemia was calculated as a percentage by actual counting of the number of parasites per 10,000 erythrocytes as described by Godfrey et al. (53). The infection intensity in the mosquito was evaluated by counting the oocysts in the midgut. Successful sporogony was determined by examining salivary glands preparations and confirming sporozoite development.

Indirect ELISA

The levels of Abs reactive to bacterial proteins were measured in bird sera as previously reported (17, 18), with small modifications. The 96-well ELISA plates (Thermo Scientific, Waltham, MA, USA)

were coated with 50 ng/mL (100 µL/well) of *E. coli* BL21 protein extracts in carbonate/bicarbonate buffer (0.05 M, pH 9.6) and incubated for 2 h with 100 rpm shaking at RT. Subsequently, plates were incubated overnight at 4°C. Wells were washed three times with 100 µL of PBS containing 0.05% (vol/vol) Tween 20 (PBST), and then blocked by adding 100 µL of 1% Human Serum Albumin (HSA)/PBS for 1 h at RT and 100 rpm shaking. After three washes, sera samples, diluted at 1:200 in 0.5% HSA/PBS, were added to the wells and incubated for 1 h at 37°C and 100 rpm shaking. The plates were washed three times and HRP-conjugated Abs (goat anti-turkey IgG) (MyBioSource, San Diego, CA, USA) were added at 1:1,000 dilution in 0.5% HSA/PBST (100 µL/well) and incubated for 1 h at RT with shaking. The plates were washed three times and the reaction was developed with 100 µL ready-to-use TMB solution (Promega, Madison, WI, USA) at RT for 20 min in the dark, and then stopped with 50 µL of 4% H₂SO₄. Optimal antigen concentration and dilutions of sera and conjugate were defined using titration assays. The optical density (OD) was measured at 450 nm using an ELISA plate reader (Filter-Max F5, Molecular Devices, San Jose, CA, USA). All samples were tested in triplicate and the average value of three blanks (no Abs) was subtracted from the reads. The cut-off was determined as two times the mean OD value of the blank controls.

Evaluation of Mosquito Survival and Collection of Midguts and Salivary Glands for Testing *P. relictum* Development and Midgut Microbiota Analyses

The survival of mosquitoes was estimated by daily checking the mosquito cages at 10 am and counting dead insects until 17 dpe. On 10 dpe, mosquito midguts were dissected for estimation of developed oocysts and microbiota analysis. Before dissection, mosquitoes were euthanized by shaking vigorously to stun them in insect aspirator. Wings and legs of the insects were removed before dissection, which was performed under the binocular stereoscopic microscope. Each mosquito was carefully separated in two segments, the thorax with head and abdomen. The abdomen was placed in the drop of saline and the midgut of the mosquito was extracted. The midgut was stained according to Kazlauskienė et al. (51) for counting oocysts of *Plasmodium* parasite. For microbiota analysis, unstained midguts were pooled up to 10 in sterile microtubes and frozen at -20°C. To eliminate contamination of samples, new dissecting needles were used for each dissected insect. On 17 dpe, each mosquito was carefully separated in two segments, the thorax with head and abdomen. Salivary glands were extracted from the thorax, placed in a separate sterile drop of saline and grinded to make a smear to record the presence of sporozoites (51). The remnants of salivary gland preparations and thorax were fixed in SET-buffer for PCR analysis. The abdomen, the same as on day 10, was placed in a drop of saline to prepare the samples for the midgut microbiota analysis.

DNA Extraction and PCR for *P. relictum* Identification

Total DNA for PCR analysis was extracted from the blood remnants of mosquitoes using an ammonium acetate extraction protocol by Sambrook & Russel (54). A nested PCR protocol

described by Hellgren et al. (55) was used to confirm *P. relictum* infection in the donor birds and test mosquitoes for positive parasite sporozoite development. For the first PCR we used the primers HaemNFI [5'-CATATATTAAGAGAAITATGGAG-3'] and HaemNR3 [5'-ATAGAAAGATAAGAAATACCATTC-3'] (55). In the second PCR a mitochondrial *cyt b* gene (478 bp) was amplified using the primers HaemF [5'-ATGGTGCTTTTCGATATATGCATG-3'] and HaemR2 [5'-GCATTATCTGGATGTGATAAATGGT-3'] (56). For PCR mix we used 12.5 µL of DreamTaq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 µL of nuclease-free water, 1 µL of each primer and 2 µL of template DNA (extracted DNA or products of first PCR). *P. relictum*-positive samples were determined by running 2 µL of second PCR product on 2% agarose gel. For parasite lineage confirmation, samples containing parasite DNA were sequenced from the 5' end using the HAEMF primer on an ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, USA) as described by Bensch et al. (56). The BLAST search tool (National Centre for Biotechnology Information website: <http://www.ncbi.nlm.nih.gov/BLAST>) was used to determine SGS1 lineage.

DNA Extraction and 16S rRNA Sequencing for Microbiota Analysis

Genomic DNA for microbiota analysis was extracted from frozen midguts of engorged mosquitoes and from fecal samples of birds using a Pure Link Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, CA, USA). Each DNA sample was eluted in 100 µL of elution buffer. Genomic DNA quality (OD_{260/280} between 1.8–2.0) was measured with NanoDrop™ One (Thermo Scientific, Waltham, MA, USA). More than 850 ng of DNA at ≥ 8.5 ng/µL concentration were sent for amplicon sequencing of the bacterial 16S rRNA gene, which was commissioned to Novogene Bioinformatics Technology Co. (London, UK). Libraries were prepared with NEBNext® Ultra™ II DNA Library Prep Kit (New England Biolabs, MA, USA). A single lane of Illumina MiSeq system was used to generate 251-base paired-end reads from the V4 variable region of the 16S rRNA gene using barcoded universal primers (515F/806R) in samples from mosquitoes engorged on *E. coli* BL21-immunized (n = 7 midgut pools), *E. coli* BL21-immunized and *Plasmodium*-infected (n = 7), *E. coli* O86:B7-immunized (n = 5), *E. coli* O86:B7-immunized and *Plasmodium*-infected (n = 8), *Plasmodium*-infected (n = 8) or mock-immunized (n = 5) birds. The raw 16S rRNA sequences obtained from mosquito samples were deposited in the SRA repository, Bioproject No. PRJNA778616. One extraction reagent control was set in which the different DNA extraction and amplification steps were performed using the same conditions as for the samples but using water as template.

16S rRNA Sequences Processing

The analysis of 16S rRNA sequences was performed using QIIME 2 pipeline (v. 2021.4) (57). The sequences in the fastq files were denoised and merged using the DADA2 software (58) as implemented in QIIME 2. The amplicon sequence variants (ASVs) were aligned with q2-alignment of MAFFT (59) and used

to construct a phylogeny (60). Taxonomy was assigned to ASVs using a classify-sklearn naïve Bayes taxonomic classifier (61) based on SILVA database (release 132) (62). The taxonomic data tables were collapsed at genus level and filtered excluding taxa with less than 10 total reads and present in less than 30% of samples of each dataset.

Bacterial Co-Occurrence Networks

Co-occurrence networks were inferred for each experimental condition based on taxonomic profiles. Correlation matrices were calculated using the Sparse Correlations for Compositional data (SparCC) method (63), implemented in the R Studio (64). Network visualization and calculation of topological features and taxa connectedness (i.e., the number of nodes and edges, network diameter, average degree, weighted degree, average path length, modularity, number of modules, average clustering coefficient) was performed using the software Gephi 0.9.2 (65).

Statistical Analysis

Statistical analysis was performed using R program (version 4.0.4) (66). Differences in oocyst and sporozoite frequency between the groups of infected mosquitoes were compared using Fisher's exact test with Bonferroni comparison tests. The numbers of oocysts formed in mosquito midguts were compared between infected groups by the Mann-Whitney U test. Differences in relative Ab levels (i.e., OD) among groups of immunized birds in the different time points were compared using two-way ANOVA with Bonferroni multiple comparison tests applied for individual comparisons. Microbial diversity analyses were carried out on rarefied ASV tables, calculated using the q2-diversity plugins. The alpha diversity (richness and evenness) was explored using Faith's phylogenetic alpha diversity index (67) and Pielou's evenness index (68). Differences in α -diversity metric between groups were assessed using Kruskal-Wallis test ($\alpha=0.05$). Bacterial β -diversity was assessed using the Bray Curtis dissimilarity (69) and compared between groups using the PERMANOVA test. Betadisper function was used for the construction of PCoA plot, and an ANOVA test was used to compare the dispersion of the samples by groups. The differential features were detected by comparing the log₂ fold change (LFC) using the Wald test as implemented in the compositional data analysis method DESeq2 (70). The number of shared co-occurring taxa among different experimental groups was done in R studio using the package "Venn".

Differential Network Analysis

Comparison of the similarity of the most central nodes between two networks was done with the package "NetCoMi" (71) in R studio using the read count taxonomic tables. "Most central" nodes are defined as those nodes with a centrality value above the empirical 75% quartile. The comparison returns Jaccard's indexes for each of four local measures (i.e., degree, betweenness centrality, closeness centrality, eigenvector centrality) of the sets of most central nodes as well as for the sets of hub taxa between the two networks compared. Thus, the Jaccard's index express the similarity of the sets of most central

nodes as well as the sets of hub taxa between the two networks. Jaccard index of 0 indicates completely different sets while a value of 1 indicates equal sets of most central nodes or hub taxa between the compared networks. The two p -values $P(J \leq j)$ and $P(J \geq j)$ for each Jaccard's index are the probability that the observed value of Jaccard's index is 'less than or equal' or 'higher than or equal', respectively, to the Jaccard value expected at random, which is calculated taking into account the present total number of taxa in both sets [based on Real and Vargas (72)].

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/sra>, PRJNA778616.

ETHICS STATEMENT

All procedures were performed at the Nature Research Centre in Vilnius, Lithuania, according to Lithuanian and International Guiding Principles for Biomedical Research Involving Animals (2012). Infection experiments and other procedures were reviewed and approved by the Lithuanian State Food and Veterinary Service, Ref. No. 2020/07/24-G2-84. The assessment of the animal health and all described procedures were implemented by trained professionals (under licenses 2012/02/06-No-208, and 2016/01.29-No-344).

AUTHOR CONTRIBUTIONS

AC-C and VP conceived the study. JA, RŽ, JM, EP, LM-H, and VP performed the experiments and acquired the data. JA, AW-C, and DO analyzed the data. JA, AW-C, and AC-C prepared the figures. AC-C, VP, and JM contributed reagents and other resources. AC-C, VP, and DO supervised the work. JA, AW-C, AC-C, and VP drafted the first version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.841835/full#supplementary-material>.

Supplementary Figure S1 | Impact of anti-microbiota vaccines on fecal microbiota of birds. Comparison of ASV richness and evenness, measured with **(A)** Faith's phylogenetic diversity index and **(B)** Pielou's evenness index, respectively, among the microbiota of mock-immunized, *E. coli* BL21-immunized and *E. coli* O86:B7-immunized birds.

Supplementary Figure S2 | Number of shared co-occurring taxa of *Escherichia-Shigella* among the sub-networks of different experimental group. Venn diagram showing the number of bacteria that are common or unique among the taxa that co-occur directly to *Escherichia-Shigella* in the different experimental group.

Supplementary Table S1 | Jaccard indexes of local centrality measures. Jaccard's indexes for each of local centrality measures (i.e., degree, betweenness centrality, closeness centrality, eigenvector centrality and hub taxa) of the sets of most central nodes for pairwise network comparisons. The two *p*-values, $P(J \leq j)$ and $P(J \geq j)$, for each Jaccard's index were added.

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Chapter V

General discussion

Vector-borne diseases pose a considerable public health problem worldwide, principally those vectored by ticks and mosquitoes. Studies emphasized that the interactions among pathogen, vector and microbiota play a key role for the establishment of vector-borne diseases (Wu-Chuang et al., 2021). Indeed, vector microbiota has become an exciting topic due to its pivotal role on vector biology processes, including nutrition, digestion, development, immune responses functions and more importantly, on vector competence (Huang et al., 2020; Kurokawa et al., 2020; Wu-Chuang et al., 2021).

With the advance and increasing accessibility of next-generation sequencing technologies, the understanding of the microbiota has been growing in the past two decades. Most of the studies characterize the microbiota composition and analyze differential abundances of bacterial members of the microbiota (when different conditions are studied). These analysis methods, although very informative, neglect completely the bacterial community as a whole and the possible interactions or associations between microbial taxa. Understanding microbial structure can give new insights that cannot be seen when studying individually the bacteria from the microbiota (Hirano & Takemoto, 2019). For example, it was reported that the colonization of the model organism *Caenorhabditis elegans* with a protective symbiont against a pathogen had minimal impact on the host microbiota diversity (Dahan et al., 2020). However, when a network approach was used to characterize the impact of the protective microbial symbiont, it was found that the symbiont become a keystone taxon in the host microbiota, induced significant changes in the host bacterial community structure and possibly altered the functional profile of the host microbiome (Wu-Chuang, Bates, et al., 2022). Therefore, microbial networks should be included in microbiome analysis for a deeper understanding of the functioning of the microbiome.

It is noteworthy that interpreting these networks is not straightforward and the biological interpretation from network properties is not clear (Röttjers & Faust, 2018). The use of microbial networks has limitations, some of them associated to the limitations in 16S rRNA marker gene data (Röttjers & Faust, 2018). For example, the low resolution found sometimes on sequencing data may prevent the differentiation between two different strains or species, hence, analysis of networks to study closely related taxa is not possible (Röttjers & Faust, 2018). Moreover, in the inferred networks, edges that represent indirect associations cannot be distinguished from direct associations, thus, when two taxa are associated in a network, it will be difficult to know if the association between them is due to a direct interaction or due to a third taxa or an environmental factor exerting influence in both taxa (Röttjers & Faust, 2018). Furthermore, in undirected edges, the type of symbiotic relationship from an association is not known. In other words, we cannot distinguish if a positive association between two taxa are referred to commensalism or mutualism and similarly, for negative

association, we cannot distinguish competition from amensalism (Röttgers & Faust, 2018). Finally, the inferred associations are also sensitive to the sampling since bacteria that share niches are more likely to co-occur. If the sampling was done in a heterogeneous environment, differing niche specialization can cause spurious associations (Röttgers & Faust, 2018). However, this last problem can be overcome easily in an experimental setting by splitting samples by different experimental conditions. Despite these limitations, microbial networks are a popular and useful tool since they can integrate multiple types of information and may represent systems-level behaviour. They can be used for the study of emergent properties such as the robustness of the bacterial community or the modularity where the modules can represent different niches (Aderem, 2005). Furthermore, as they are sensitive to external factors, the use of microbial networks under different experimental conditions can therefore give valuable information about the impact of the factor studied on the behaviour of the microbial community. Moreover, microbial networks allow the identification of keystone taxa and targeting these taxa may have an impactful effect on the microbiota.

In the present thesis, we used microbial networks to represent the complex world of vector microbiota under different conditions and we used this approach (i) to determine the effects of environmental factors, such as the temperature, on tick bacterial community structure (ii) to characterize the influence of anti-microbiota vaccine on tick microbiome (iii) to evaluate the impact of the modulation of bacterial community on pathogen development within the vector. We found that keystone taxa have a role where they maintain the functional stability of tick microbiome under environmental stress. The functional role of keystone taxa was visualized in other contexts. For example, keystone taxa in soil microbiome have been found to sustain its stability through specialized metabolic functions (Xun et al., 2021). Thus, this role of keystone taxa to maintain microbiome stability seems to be similar and conserved irrespective of the origin of the microbiome. Therefore, targeting these keystone taxa may be a suitable methodology to create extensive modulation of the microbiota. In this context, anti-microbiota vaccine was created as a tool for the precise manipulation of vector microbiota. We found that targeting keystone taxa of tick microbiota using anti-microbiota vaccine induced important modulation on the tick microbiome in terms of diversity and microbial structure. Interestingly, this modulation resulted in the impairment of the development of the pathogen *B. afzelii* within the tick *I. ricinus*. Similarly, modulation of the microbiota in mosquitoes vector using anti-microbiota vaccine also affected *P. relictum* development in *C. quinquefasciatus*. These results demonstrate the important role that vector microbiota has on pathogen development. Impairment of pathogen colonization within the vector via the modulation of the microbiota has already been demonstrated in several studies that used antibiotics treatment (Gall et al., 2016; Gendrin et al., 2015, 2016; Narasimhan et al., 2014; N. Wei et al., 2021). However, these methods are not suitable to use for blocking vector-borne pathogens due to the increasing antibiotic resistance in different bacterial

strains. Furthermore, the effects of antibiotics are wide and unspecific and several taxa can be depleted, even those that might have an inhibitory effect on the pathogen. We hypothesized that anti-microbiota vaccine can be used to control vector-borne pathogens. However, at this point, some questions arise: In what epidemiological context can anti-microbiota vaccine used? What are the limitations of anti-microbiota vaccines?

An effective anti-microbiota vaccine should induce levels of host antibodies sufficient to be acquired by the vector in quantities that, once in the vector tissues, they can bind, inactivate and/or kill commensal bacteria of the vector microbiota. The subsequent modulation of vector microbiota by host antibodies should induce infection-refractory states that limit pathogen colonization and/or transmission to a new host. As the activity of an anti-microbiota vaccine depends on the time necessary for host antibodies to modulate the vector microbiota and induce infection-refractory states, anti-microbiota vaccines will be useful to block pathogen transmission from an infected and immunized host to a naïve host. Anti-microbiota vaccines may not be useful to protect an immunized host against pathogen transmission by vector not previously exposed to host antibodies, specially, if the pathogen carried by the vector is transmitted to the host within minutes from the bite of the vector. For example, tick-borne encephalitis (TBE) is transmitted with the saliva of *I. ricinus* in the first minutes of feeding (Süss, 2003). Although we still do not know the time that anti-microbiota vaccine takes to induce the modulation of vector microbiota, we hypothesized that it is unlikely that, in the case of TBE, the host antibodies (acquired by the vector during the feeding) will induce the sufficient modulation of vector microbiota within the first minutes in order to generate an impact on the pathogen load and in its transmission. However, in the case of Lyme disease, where in most cases, the tick must be attached for 36 to 48 hours or more before the pathogen *Borrelia* can be transmitted to the host (Transmission | Lyme Disease | CDC), we hypothesized that this timeframe could be sufficient for host antibodies to induce a modulation of tick microbiota that will impact on pathogen colonization and its subsequent transmission to the host.

Initially, identification of keystone taxa for subsequent use as anti-microbiota vaccine was based on three criteria: ubiquitousness, high eigenvector centrality and high relative abundance. These criteria were selected using the rationality that 'A candidate for vaccination should be an important taxon that are connected with equally important neighbors and should be present in all the ticks and in high quantity for a good efficacy of the vaccine'. Subsequently, the attenuated live bacteria candidate is used for the formulation of anti-microbiota vaccine. However, this approach poses a major limitation since most of the bacteria in the vector microbiota are unable to grow in standard laboratory media, which make isolation of uncultured keystone bacteria a major challenge. One way to contour this problem is using antigenic proteins in the bacterial microbiota of the vectors. Indeed,

using shotgun metagenomics for the mapping of antigenic proteins in vector microbiota could help to identify antigenic proteins from bacterial candidates and used as an alternative of live bacteria vaccines.

Conclusions

Conclusions

The main conclusions found in the present thesis are:

1. Keystone taxa has an important role maintaining the functional stability of tick microbiome under heat stress.
2. Targeting keystone taxa in the tick vector using anti-microbiota vaccine can modulate the tick microbiome in terms of diversity, taxonomy, microbial structure and interactions.
3. Based on the results presented in this thesis, anti-microbiota vaccines do not increase vector mortality.
4. Modulation of the microbiota using anti-microbiota vaccine reduced the colonization of *B. afzelii* in *I. ricinus* ticks.
5. Modulation of the microbiota using anti-microbiota vaccine reduced the *P. relictum* development in *C. quinquefasciatus* mosquitoes.
6. Anti-microbiota vaccine is a suitable tool for the precise manipulation of vector microbiota and could be used as a transmission-blocking vaccine to control vector-borne pathogens.

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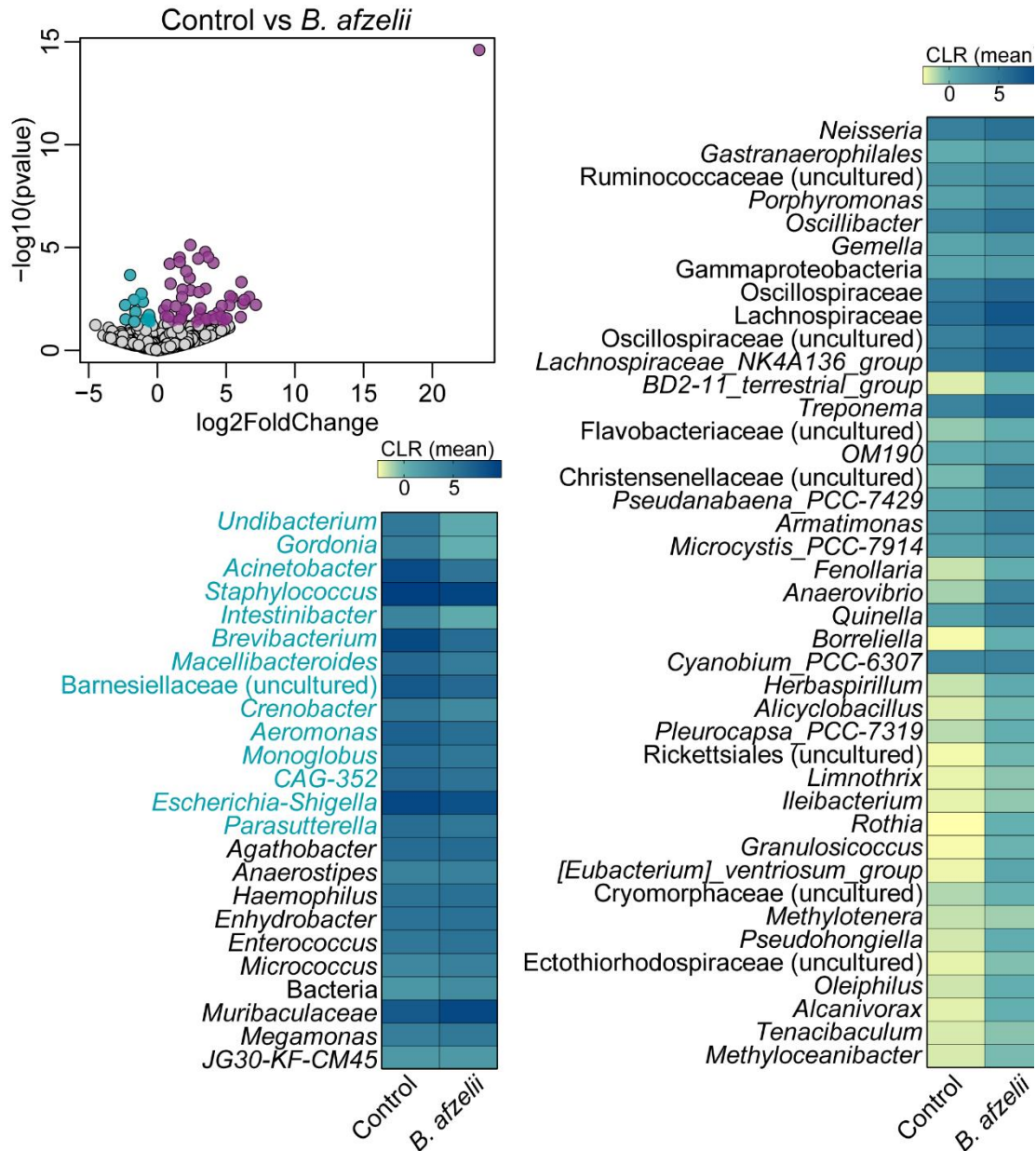
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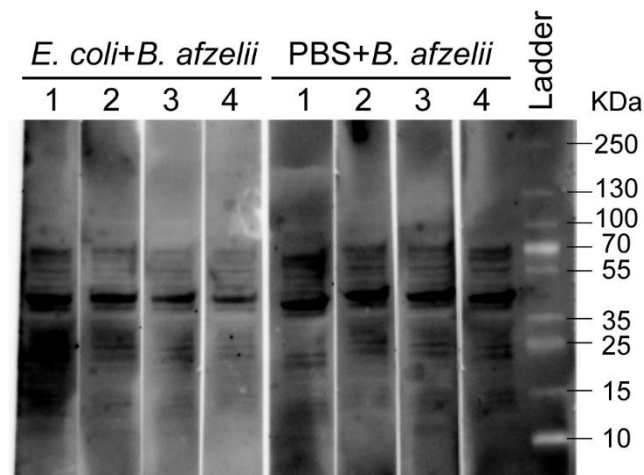
Annex

Supplementary materials of the results 'Microbiota perturbation by anti-microbiota vaccine reduces the colonization of *Borrelia afzelii* in *Ixodes ricinus*'

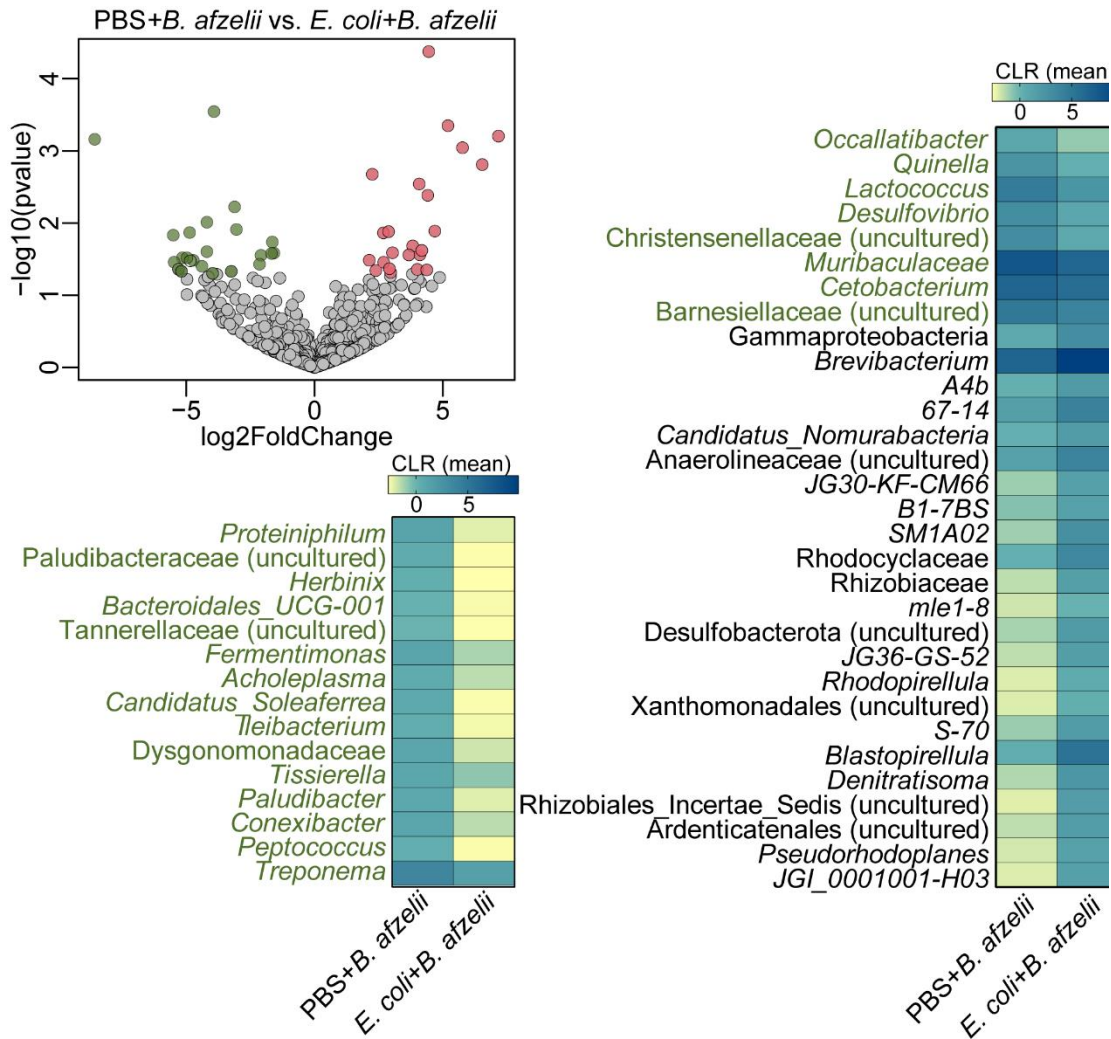


Supplementary Fig. S1. Changes in the taxonomic profile of tick microbiota after *B. afzelii* infection. Volcano plot showing the differential microbial abundance in tick microbiota from the normal and *B. afzelii* groups. Turquoise and purple dots represent bacterial taxa whose abundances significantly decreased and increased, respectively, in the microbiota of ticks from *B. afzelii* group compared to the control group. Heatmaps represent the abundance (expressed as CLR) of all the taxa with significant differences between the normal and *B. afzelii* groups. Taxa whose abundance decreased significantly in the *B. afzelii* group are annotated in turquoise.

Taxonomic table used for the differential abundance analysis were obtained from 16S rRNA gene sequences from ticks fed on normal mice ($n = 10$ individual larvae) and *B. afzelii*-infected mice ($n = 10$ individual larvae).



Supplementary Fig. S2. Detection of *Borrelia* proteins. Proteins of *Borrelia* were detected by western blot using sera of mice experimentally infected with *B. afzelii* and immunized with a live vaccine containing *E. coli* BL21 or a mock vaccine (PBS).

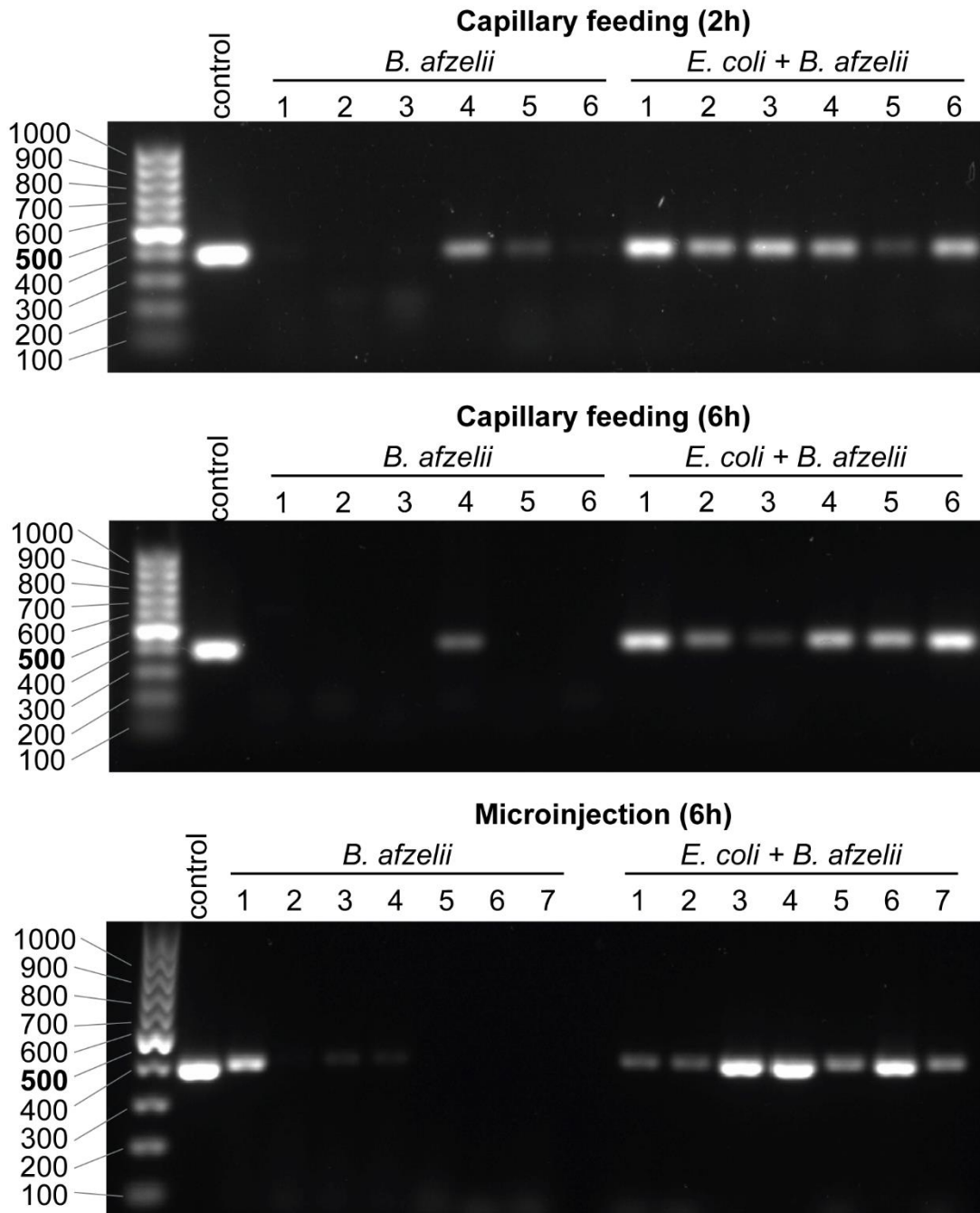


Supplementary Fig. S3. Changes in the taxonomic profile of tick microbiota after *B. afzelii* infection and anti-microbiota vaccine immunization.

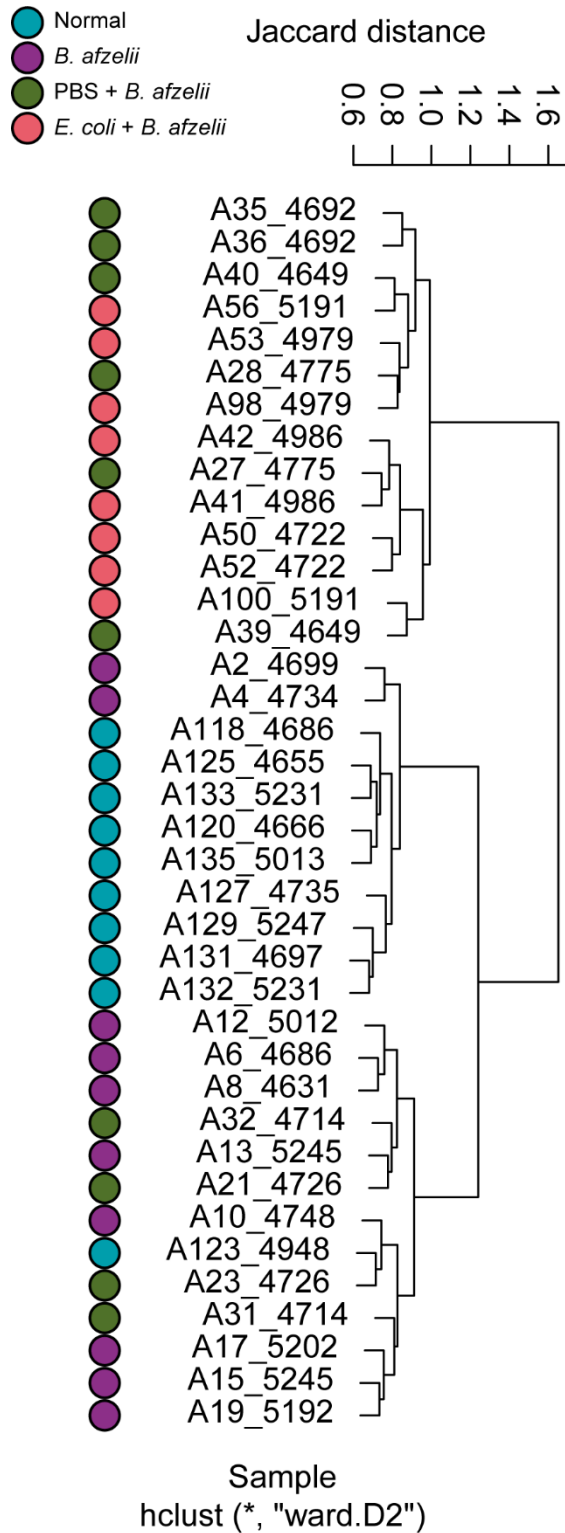
Volcano plot showing the differential microbial abundance in tick microbiota from the PBS+*B. afzelii* and *E. coli+B. afzelii* groups.

Green and pink dots represent bacterial taxa whose abundances significantly decreased and increased, respectively, in the microbiota of ticks from *E. coli+B. afzelii* group compared to the PBS+*B. afzelii* group. Heatmaps represent the abundance (expressed as CLR) of all the taxa with significant differences between the PBS+*B. afzelii* and *E. coli+B. afzelii* groups. Taxa whose abundance decreased significantly in the *E. coli+B. afzelii* group are annotated in green.

Taxonomic table used for the differential abundance analysis were obtained from 16S rRNA gene sequences from ticks fed on PBS+*B. afzelii* mice ($n = 10$ individual larva) and *E. coli+B. afzelii* mice ($n = 8$ individual larva).



Supplementary Fig. S4. Detection of Enterobacteriaceae by PCR. Representative images of the gel of electrophoresis showing bands corresponding to the 16S rRNA gene for Enterobacteriaceae. Different panels represent different experiments: Ticks were given *B. afzelii* or *E. coli+B. afzelii* by capillary feeding and incubated for 2h after the feeding (upper panel), capillary feeding and incubated for 6h after the feeding (middle panel) or by microinjection and incubated for 6h after the injection (lower panel). Each lane represents a different tick from groups. For the positive control was used DNA extracted from a culture of *E. coli* BL21.



Supplementary Fig. S5. Cluster analysis of different samples of tick microbiota. Dendrogram based on Ward's method of clustering for samples of tick microbiota from the normal, *B. afzelii*, PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups.

Supplementary Table S1. Jaccard indexes of local centrality measures for the comparison between normal and *B. afzelii* groups.

Parameters	Normal vs. <i>B. afzelii</i>			
	Jacc	P(<=Jacc)	P(>=Jacc)	
degree	0.444	0.999701	0.000495	***
betweenness centr.	0.36	0.789257	0.260987	
closeness centr.	0.45	0.999903	0.000164	***
eigenvec. centr.	0.413	0.995517	0.006582	**
hub taxa	0.413	0.995517	0.006582	**

Jaccard's indexes for each of local centrality measures (i.e., degree, betweenness centrality, closeness centrality, eigenvector centrality and hub taxa) of the sets of most central nodes for pairwise network comparisons. The two p-values, $P(J \leq j)$ and $P(J \geq j)$, for each Jaccard's index were added.

Supplementary Table S2. Common and unique neighbor nodes of the taxon *Escherichia-Shigella* between the normal and *B. afzelii* groups

Condition	Number of nodes in each condition	Nodes
<i>B. afzelii</i> and Normal	13	<i>Lactobacillus</i>
		<i>CAG-352</i>
		<i>Faecalibacterium</i>
		<i>Delftia</i>
		<i>Prevotella</i>
		<i>Serratia</i>
		Enterobacteriaceae
		<i>Parasutterella</i>
		<i>Streptococcus</i>
		<i>Aeromonas</i>
		<i>Shewanella</i>
		<i>Escherichia-Shigella</i>
<i>Blautia</i>		
Normal	2	<i>Subdoligranulum</i>
		<i>Akkermansia</i>

<i>B. afzelii</i>	23	Rikenellaceae,uncultured
		<i>Romboutsia</i>
		<i>Enhydrobacter</i>
		<i>Bacteroidales_UCG-001</i>
		Hungateiclostridiaceae,uncultured
		<i>Anaerovibrio</i>
		<i>PeH15</i>
		<i>Fusicatenibacter</i>
		<i>Caldicoprobacter</i>
		<i>Kapabacteriales</i>
		<i>[Ruminococcus]_torques_group</i>
		<i>Cetobacterium</i>
		Dysgonomonadaceae,uncultured
		<i>Roseburia</i>
		<i>Lachnospira</i>
		<i>Bacteroidales_RF16_group</i>
		<i>Leuconostoc</i>
		Christensenellaceae,uncultured
		Barnesiellaceae,uncultured
		<i>Bacteroides</i>
<i>Izemoplasmatales</i>		
<i>Treponema</i>		
<i>Macellibacteroides</i>		

Supplementary Table S3. Mice tissues positive for *B. afzelii* in PBS+*B. afzelii* and *E. coli*+*B. afzelii*

N° mouse	PBS+<i>B. afzelii</i>
1	-
2	Skin
3	Right ankle joint, Heart, Skin
4	Right ankle joint, Heart, Skin
5	-
N° mouse	<i>E. coli</i>+<i>B. afzelii</i>
1	-
2	Right ankle joint
3	Right ankle joint
4	Heart
5	Heart

Supplementary Table S4. Jaccard indexes of local centrality measures for the comparison between PBS+*B. afzelii* vs. *E. coli*+*B. afzelii* groups

Parameters	PBS+<i>B. afzelii</i> vs. <i>E. coli</i>+<i>B. afzelii</i>			
	Jacc	P(<=Jacc)	P(>=Jacc)	
degree	0.389	0.974878	0.033578	*
betweenness centr.	0.33	0.493642	0.563469	
closeness centr.	0.404	0.993517	0.009155	**
eigenvec. centr.	0.378	0.948538	0.066104	.
hub taxa	0.378	0.948538	0.066104	.

Supplementary Table S2. Common and unique neighbor nodes of the taxon *Escherichia-Shigella* between the PBS+*B. afzelii* and *E. coli*+*B. afzelii* groups

Condition	Number of nodes in each condition	Nodes
<i>E. coli</i> + <i>B. afzelii</i> and PBS+ <i>B. afzelii</i>	13	<i>Faecalibacterium</i>
		Lachnospiraceae
		<i>Serratia</i>
		Lachnospiraceae_NK4A136_group
		<i>Lachnospira</i>
		<i>Parabacteroides</i>
		<i>Subdoligranulum</i>
		<i>Prevotella</i>
		Enterobacteriaceae
		<i>Escherichia-Shigella</i>
		<i>Bacteroides</i>
		<i>Dialister</i>
		<i>Blautia</i>
PBS+ <i>B. afzelii</i>	36	<i>Bacillus</i>
		Oxyphotobacteria_Incertae_Sedis,Unknown_Family
		CAG-352
		<i>Bilophila</i>
		<i>Pseudanabaena_PCC-7429</i>
		<i>Fusicatenibacter</i>
		<i>Brevundimonas</i>
		<i>Candidatus_Nomurabacteria</i>
		<i>Candidatus_Zambryskibacteria</i>
		<i>Roseburia</i>
		<i>Alloprevotella</i>
		<i>Microcystis_PCC-7914</i>
		<i>Akkermansia</i>

		<i>Agathobacter</i>
		<i>Calothrix_KVSF5</i>
		<i>Aeromonas</i>
		OM190
		<i>Tychonema_CCAP_1459-11B</i>
		<i>Colidextribacter</i>
		<i>Brevibacterium</i>
		<i>Chloroplast</i>
		<i>Gracilibacteria</i>
		<i>Clostridium_sensu_stricto_1</i>
		<i>Cetobacterium</i>
		<i>[Eubacterium]_coprostanoligenes_group</i>
		<i>Succinispira</i>
		<i>Armatimonas</i>
		<i>Clostridia_UCG-014</i>
		<i>Streptococcus</i>
		<i>Parcubacteria</i>
		JG30-KF-CM45
		Christensenellaceae,uncultured
		<i>Muribaculaceae</i>
		Barnesiellaceae,uncultured
		<i>Cyanobium_PCC-6307</i>
		<i>Erysipelotrichaceae_UCG-003</i>
<i>E. coli+B. afzelii</i>	25	<i>[Eubacterium]_ruminantium_group</i>
		<i>Blastocatella</i>
		<i>Sutterella</i>
		<i>Ralstonia</i>
		<i>Cupriavidus</i>
		<i>Sphingomonas</i>
		<i>Actinomyces</i>
		<i>Micrococcus</i>
		<i>Porphyromonas</i>
		<i>Treponema</i>
		<i>Lautropia</i>

		<i>Paenarthrobacter</i>
		<i>Ruminococcus</i>
		<i>Lachnoclostridium</i>
		<i>Geobacillus</i>
		<i>Delftia</i>
		<i>Rikenellaceae_RC9_gut_group</i>
		<i>Coprococcus</i>
		<i>Dorea</i>
		<i>[Eubacterium]_ventriosum_group</i>
		<i>[Ruminococcus]_gnavus_group</i>
		<i>UCG-002</i>
		<i>Aurantimicrobium</i>
		<i>Tumebacillus</i>
		<i>Gemella</i>