Aus dem Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Artificial feeding of the hard tick *Ixodes ricinus*

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Nina Militzer
Tierärztin aus Berlin

Berlin 2024
Journal-Nr.: 4457

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To

Thorsten,

Carsten,

Anna,

Mila,

Kimberly,

Ruby,

Blue,

Marvel

and all the other experimental animals

without this work would not have been possible.

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LIST OF ABBREVIATIONS

°C	Celsius
µg	Microgram
µL	Microliters
µm	Micrometers
µM	Micromol
χ2	Chi-square
16S rRNA	16S subunit of the ribosomal ribonucleic acid
3R	Replacement, Reduction and Refinement (of animal experiments)
75-W	75-Watt
A	Adults
ACE	Abundance-based coverage estimator
ATFS	Artificial tick feeding system*s
ATP	Adenosine triphosphate
bp	base pairs
C	Control group (**Chapter 3**)
CI	Confidence interval
CO_2	Carbon dioxide
CoA	Co-enzyme A
CLE	*Coxiella*-like endosymbiont
CRISPR/cas	Clustered regularly interspaced short palindromic repeats + CAS
CV	Coefficient of variation
df	Degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
e.g.	exempli gratia
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay

F	Females
F_0	Zero filial generation (parental generation)
F_1	First filial generation
FAD	Flavin adenine dinucleotide
FISH	Fluorescence *in situ* hybridization
FLE	*Francisella*-like endosymbiont
FU	Feeding unit
gDNA	genomic Deoxyribonucleic acid
GLM	Generalized linear
GSH	Glutathione
h	Hours
HGT	Horizontal gene transfer
IgE	Immunglobulin E
incl.	including
IQR	Interquartile range
IVG^+	*in vitro* with gentamicin treatment (**Chapter 3**)
IVG^-	*in vitro* without gentamicin treatment (**Chapter 3**)
L	Larvae
LC-MS	Liquid chromatography–mass spectrometry
LD	Light-dark
LMM	Linear mixed-effect model
M	Males
mm	Millimeter
mM	Millimole
mg	Milligram
min	Minutes
mol/L	Molar/ mol per milliliter
MWU	Mann-Whitney U (test)
n	Sample size
N	Nymphs
NA	Not applicable/ not mentioned

NADP	Nicotinamide adenine dinucleotide phosphate
NaCl	Sodium chloride
NGS	Next-generation sequencing
NMDS	Non-Metric Multidimensional Scaling
OTU	Operational taxonomic unit
p	Probability
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
QC	Quality control
qPCR	Qualitative polymerase chain reaction
RH	Relative humidity
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room temperature
s	Seconds
SAS	Semi-automated system
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SOP	Standard Operating Procedure
spp.	species pluralis
TBEV	Tick-borne encephalitis virus
TBP*s	Tick-borne pathogens
V1-9	Hypervariable region 1-9
$VitB^+$	with vitamin B supplementation (**Chapter 4**)
$VitB^-$	without vitamin B supplementation (**Chapter 4**)
vs.	versus

PREFACE

Introduction and study objectives

Artificial Tick Feeding Systems (ATFS) provide an alternative to laboratory tick rearing and live animal experimentation, thus contributing to the replacement, reduction, refinement and responsible use of animal experiments. Since the first reports on membrane feeding techniques for ticks in the early-mid 20th century (Hindle and Merriman 1912; Totze 1933), ATFS have been used worldwide for various applications including 1) tick rearing, and studies on 2) tick biology and physiology, 3) tick control and 4) the tick-microbiome-pathogen interface.

Ticks are obligatory hematophagous ectoparasites that can cause both direct and indirect damage, for instance by the transmission of a wide variety of pathogens. This is of relevance from a medical and veterinary point of view and particularly impedes the livelihoods of farmers in low- and middle-income countries where ticks and tick-borne diseases are widespread (Minjauw and McLeod 2003; de la Fuente et al. 2023). These circumstances emphasize the need for further insights to effectively control ticks and tick-borne diseases. Reports on acaricide resistances further highlight the current demand for trials based around novel compounds or other alternatives to existing tick control methods (Dzemo et al. 2022).

The most common hard tick species in Central and Western Europe is *Ixodes ricinus*. This three-host tick species is the primary vector for a range of tick-borne pathogens (TBPs) of human and veterinary medical concern, including *Borrelia burgdorferi* sensu lato, the causative agent of Lyme borreliosis, and Tick-Borne Encephalitis Virus (TBEV).

The **main objective** of this research project was to investigate tick feeding and fitness parameters in different life stages of *I. ricinus* consecutively fed using the ATFS. We additionally examined the effect of supplementing the blood meal offered in the ATFS with antibiotics on the tick's microbiome and fitness.

Chapter 1 of this cumulative thesis provides comprehensive background information on the systematics, morphology and biology of *I. ricinus,* as well as TBPs, insights into the tick microbiome and the current advances of ATFS. **Chapter 2** describes the consecutive feeding of all life stages of *I. ricinus* using an ATFS and **Chapter 3** compares the influence of gentamicin-supplementation to non-treated blood meals in the ATFS and ticks fed on cattle. Finally, **Chapter 4** provides a summarizing discussion on the two research studies, their results and gives an outlook for future research questions.

CHAPTER 1

Literature review

1.1. Systematics and biology of *Ixodes ricinus*

1.1.1. Taxonomy

Ticks (Ixodida) are obligate blood-feeding ectoparasites from the class of Arachnida, taxonomic phylum Arthropoda. The suborders Ixodida (ticks), Mesostigmata (mites), Opilioacarida and Holothyrida together form the order Parasitiformes in the subclass Acari (Nava et al. 2009).

The suborder Ixodida (ticks) is further divided into three families: the soft ticks (Agarsidae) with ~190 species, the hard ticks (Ixodidae) with ~714 species, and the monotypic Nuttalliellidae (Beati and Klompen 2019). The presence of a dorsal sclerotized shield (scutum) is characteristic for hard ticks. They can be further divided based on other morphological characteristics such as the presence of eyes, the number of festoons and the shape of the capitulum. Furthermore, the anal groove is positioned posterior to the anal pore in Metastriata and anterior to the anal pore in Prostriata. While there are eleven genera of Metastriata, there is only one genus within the Prostriata, namely *Ixodes.*

The genus *Ixodes* contains over 270 species (Guglielmone et al. 2014; Guglielmone et al. 2015) with at least 14 subgenera (Clifford et al. 1973; Keirans et al. 1999; Xu et al. 2003). The "*Ixodes ricinus* complex" includes different *Ixodes* species, which are relevant from a public health perspective due to their role as key vectors for tick-borne diseases in humans (Gray 1998; Estrada-Peña and Jongejan 1999; Keirans et al. 1999). Currently, for the northern hemisphere, there are four important species included in the *I. ricinus* complex: *I. ricinus, I. persulcatus, I. scapularis,* and *I. pacificus* (Gray et al. 2016). These four tick species are among the most abundant ticks in the northern hemisphere and act as main vectors for TBPs relevant to both humans and animals (Stanek et al. 2012).

1.1.2. Morphology

The body of the Acari is divided into two segments: the gnathosoma (mouthparts) and the idiosoma (abdomen). The capitulum includes the mouthparts; two palps and two chelicerae clasped around the hypostome.

As is the case in all *Ixodes* ticks, *I. ricinus* has an anal groove positioned anterior to the anal pore and does not have eyes (Estrada-Peña et al. 2018c). *Ixodes ricinus* adults show sex dimorphism; unfed females are larger than unfed males (♀♀: 3.0- 3.6 mm and ♂♂: 2.4- 2.8

mm) (Deplazes et al. 2012) and the chitin scutum common to all adults covers the entire dorsal abdomen in males but only approximately one third in females. The scutum is dark brown or black, while the body part that is not covered by the scutum, the so-called alloscutum, appears reddish or red brownish without any patterning. The alloscutum in females is longer than it is wide (Estrada-Peña et al. 2018c). The female hypostome is long and has a mean length of 0.5 mm, while the male hypostome has a mean length of 0.28 mm (Krober and Guerin 2007b). Ticks use their hypostome to traverse the dermis of the host to obtain the blood meal. When feeding to repletion, females can increase to a size of 11 x 7 mm (Arthur 1962). All adult ticks have four leg pairs, the most distal segment of which is called the tarsus. A sensory organ referred to as the Haller's organ is located on the tarsus of the first leg.

Ixodes spp. nymphs do not have festoons. The alloscutum of juveniles is dark brown or black and has no patterning. An unfed nymph is 1.3- 1.5 mm in diameter (Deplazes et al. 2012), which increases to up to 3 mm in the engorged state (Wheler 1906). Nymphs have four leg pairs. *Ixodes ricinus* larvae on the other hand have only three leg pairs and two pairs of hypostomal setae. Larvae are 0.5- 0.8 mm in size in the unfed state (Deplazes et al. 2012) and up to 1.43 mm in size in the engorged state (Wheler 1906).

1.1.3. Life cycle

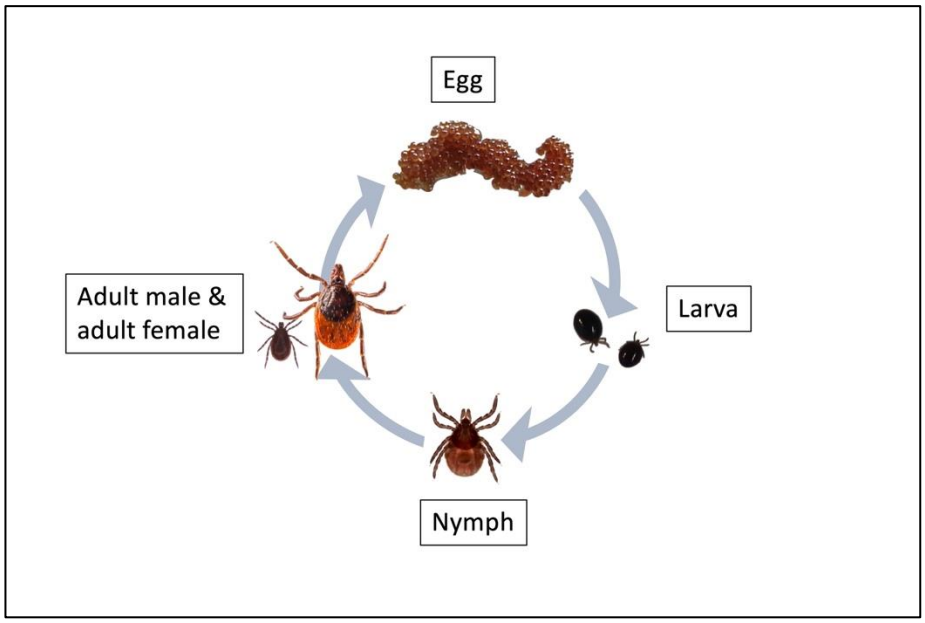

Figure 1: The life stages of *Ixodes ricinus* ticks: from egg to larva to nymph to adult female or adult male.

Figure created using Microsoft PowerPoint. © Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, and N. Militzer.

All hard ticks have four life stages: egg, larva, nymph and adult (Figure 1). The last three are the active life stages, each requiring a blood meal to either develop into the next life stage or produce offspring. *Ixodes ricinus* is a three-host tick, which means that the tick obtains each blood meal from a different individual host (Oliver Jr 1989). One can therefore distinguish between non-parasitic off-host phases and short parasitic on-host phases (Lees 1948; Needham and Teel 1991; Kahl 2018).

The feeding duration depends on the life stage of the tick and on the host species. While *I. ricinus* larvae generally feed for 2- 5 days, nymphs feed slightly longer for 3- 7 days and females require between 5 and 14 days to feed to repletion (Lees 1948; Lamontellerie 1965; Balashov 1972; Matuschka et al. 1990; Gray 1991; Needham and Teel 1991). The feeding duration may be longer depending on the animal host species (Balashov 1972; Matuschka et al. 1990).

After feeding on a host, the engorged tick detaches and a longer off-host phase ensues. At this stage, ticks are exposed to environmental conditions such as relative humidity and temperature and live in the vegetation (under leaves, hummus or soil). Within this period, the tick's survival is influenced by the its water balance, behavior (including diapause) and cold-hardiness (Needham and Teel 1991). Temperature and relative humidity are the primary external factors that influence tick survival (MacLeod 1935; Balashov 1972; Oliver Jr 1989; Needham and Teel 1991; Randolph et al. 2002; Balashov 2012). To regulate its water household, the tick can actively absorb water from high humidity in the air of its microclimate in the litter zone (Rudolph and Knülle 1974; Kahl and Knulle 1988; Perret et al. 2004).

The off-host phase in *I. ricinus* females consists of pre-oviposition, oviposition and the larval hatching period. Under quasi-natural conditions and in laboratory tick rearing experiments, the duration of pre-oviposition is commonly reported to be around 7- 28 days but can take up to 126 days. It is followed by the oviposition period which ranges from 3- 32 weeks (Nuttall 1913; Falke 1931; MacLeod 1936; Campbell 1948; Metianu 1951; Chmela 1969; Gray 1982). After oviposition is completed, it takes between 2.4- 8.6 weeks until the first larva hatches (Nuttall 1913; Falke 1931; Metianu 1951; Lamontellerie 1965). Some authors refer to the oviposition starting date as the beginning of the larvae hatching period, resulting in a range of around 8.5- 49 weeks (Campbell 1948; Chmela 1969; Gray 1982).

For engorged juveniles, the pre-molt period is influenced by temperature, seasonality and the tick's ability to go into behavioral and developmental diapause. A pre-molt period of 8- 22 weeks has been reported for spring-summer fed larvae, while 44- 57 week-long pre-molt periods are common for autumn-fed larvae. A pre-molt period of 8- 22 weeks was reported for

spring-summer fed nymphs, while the pre-molt period lasts 44- 57 weeks for autumn-fed nymphs (Chmela 1969; Gray 1982).

The life cycle of *I. ricinus* ticks is commonly 2- 3 years long: each active life stage consumes one blood meal, each of which typically occurs in a different calendar year (Campbell 1948; Gray 1991; Dantas-Torres and Otranto 2013; Grigoryeva and Shatrov 2022). However, all life stages can survive for up to 12- 15 months before the next blood meal intake, which means that the total duration of the whole life cycle can increase to 5 or even up to 7 years (Grigoryeva and Shatrov 2022). It is also possible to shorten the duration of the life cycle to as little as 1- 2 years if climate and temperature conditions permit (MacLeod 1935; Gray 1991; Balashov 2012; Dantas-Torres and Otranto 2013; Estrada-Peña and Fernández-Ruiz 2020). A life cycle duration of only approximately 6- 7.5 months has been reported for *I. ricinus* under natural conditions (Anastos 1957) and for the sister species *I. scapularis* under laboratory conditions (Kocan et al. 2015).

After molting or hatching, specific environmental conditions induce questing behavior in the tick. In contrast to endophilic ticks, *I. ricinus* is an exophilic tick and practices the ambush technique to find a host: the tick crawls up blades of grass or stalks of other plants and waits until a potential host passes by (Lees and Milne 1951; Uexküll and Kriszat 1956; Gray et al. 2016). The questing behavior is complemented by the tick pointing and sometimes waving its first pair of legs and is influenced by many factors such as the desiccation level of the tick, the tick‘s life stage, the biological age and its body size, the temperature and humidity on the ground vs. in the air, the number of sunlight hours in the day, gravity and geotropism (Lees 1948; Gigon 1985; Mejlon and Jaenson 1997; Randolph and Storey 1999; Perret et al. 2000; Randolph 2004; Balashov 2010). *Ixodes ricinus* larvae typically quest at 7- 19 cm from the ground, nymphs at 50- 59 cm, and adult females at 60- 79 cm (Gigon 1985; Mejlon and Jaenson 1997). It is assumed that an association exists between the questing height for each life stage and the “preferred” host species (Jaenson et al. 1994; Mejlon and Jaenson 1997; Randolph 2004; Randolph 2014). Vegetation type as well as the tick’s infection status also affect the vertical movement (Mejlon and Jaenson 1997; Herrmann et al. 2013; Herrmann and Gern 2015).

In Prostriata, spermiogenesis and spermatogenesis occurs immediately after the ecdysis of the nymph into the adult stage (Balashov 1956; Oliver Jr et al. 1972). Thus, *I. ricinus* regularly mate off-host (Gray 1987; Oliver Jr 1989). In rare instances, *I. ricinus* males can also be found attached to the host (Schulze 1942; Oliver Jr 1989), but they seemingly do not ingest blood (Balashov 1972).

1.1.4. Host range

Ixodes ricinus is considered a generalist tick and has been found to feed on more than 300 vertebrate species such as small mammals, livestock, birds, reptiles and humans (Milne 1949; Arthur 1962; Sonenshine et al. 2002). However, the choice of the host depends on the habitat, regional host abundance and distribution as well as the life stage of *I. ricinus.* As stated above, larvae quest closely to the ground and therefore mostly infest small mammals such as rodents (e.g. *Apodemus flavicollis, Myodes glareolus* or *Sorex* spp.) (Matuschka et al. 1991; Jaenson et al. 1994). However, larvae can also sometimes be found on larger animals such as deer or sheep (Matuschka et al. 1993; Talleklint and Jaenson 1994; Mejlon and Jaenson 1997; Carpi et al. 2008; Randolph 2014) and, under laboratory conditions, the presence of bovine olfactory stimuli has been reported to induce questing behavior in larvae (Osterkamp et al. 1999).

Ixodes ricinus nymphs have a broader host spectrum and commonly feed on livestock, deer, hedgehogs and hares (Matuschka et al. 1991; Mejlon and Jaenson 1997). Both juvenile stages are found on birds much more frequently than adults (Arthur 1962; Jaenson et al. 1994; Hasle et al. 2009; Hasle 2013).

Human beings are another important host for *I. ricinus* (Estrada-Peña and Jongejan 1999). It is the most common tick species found on humans in Europe; the nymphal life stage are the most common life stage found on humans and of greatest epidemiological relevance for the transmission of TBPs to humans (Gray 2002; Jameson and Medlock 2011; Wilhelmsson et al. 2013; Jahfari et al. 2016; Waindok et al. 2017; Guglielmone and Robbins 2018).

1.1.5. Seasonal activity

The seasonal activity of *I. ricinus* depends on the geographical location and the natural habitat (Gray 1991). In continental climate regions*, I. ricinus* shows a bimodal activity pattern throughout the year with one activity peak in spring and a second one from mid-summer until early fall (Hendrick et al. 1938; Lees and Milne 1951; Gray 1991; Cadenas et al. 2007; Daniel et al. 2015; Gray et al. 2016; Cayol et al. 2017). In some regions, a unimodal pattern is more common, where either the first or second peak of activity is smoothed out (Gray 1991; Perez et al. 2012; Schulz et al. 2014; Gethmann et al. 2020; Grigoryeva and Shatrov 2022). This tends to be the case in sheltered habitats, colder temperatures (in freezing climate zones or at higher altitudes) or warmer climates such as in Algeria (Jensen and Kaufmann 2003; Wongnak et al. 2022). *Ixodes ricinus* activity begins at approximately +5 to 7°C. The optimum temperature for *I. ricinus* activity is between 19 and 23°C in the air and 13 to 15°C close to the ground (Gray 1984; Kahl and Knülle 1988; Perret et al. 2000; Gethmann et al. 2020). Therefore, the activity of adults and nymphs in Central Europe typically starts in spring, while larval activity usually begins later and peaks in summer (Gray 1984; Estrada-Peña et al. 2004;

Gray 2008; Dantas-Torres and Otranto 2013). However, for larvae, a great variation in activity has been reported (Gray 1991). For adults and nymphs, both uni- and bimodal activity patterns can be observed. In continental climate zones, the nymphs dominantly occur in spring and adults in fall (Gray 1984; Estrada-Peña et al. 2004). Mild winter conditions can even permit questing behavior throughout the winter season (Lindgren et al. 2000; Estrada-Peña et al. 2004; Dautel et al. 2008; Daniel et al. 2015).

Seasonal patterns differ between life stages and are influenced by the ticks' ability to undergo dormancy. There are two forms of dormancy in ticks. Quiescence is an immediate, reversible response to external stressors such as very low or very high temperature (Belozerov 2009; Gray et al. 2016). Diapause is a reversible status, which occurs as a reaction to negative environmental conditions. During diapause, the tick is in a state of minimal activity which ensures its survival during winter (Belozerov 1982; Belozerov 2009; Gray et al. 2016; Tougeron 2019). One can further differentiate between behavioral diapause, where host-seeking behavior in unfed ticks is suppressed and developmental diapause where the development of engorged ticks or eggs is suppressed (Belozerov 1982). The main stimulus for entering diapause are photoperiod and temperature (Belozerov 1982; Gray et al. 2016).

Depending on the microclimate, diurnal activity patterns have been also reported (Lees and Milne 1951; Randolph and Storey 1999; Perret et al. 2003).

1.2. Relevance of *Ixodes ricinus* and tick-borne diseases

In general, tick bites from both hard and soft ticks have a negative impact on human and animal health. For instance, penetration of the host dermis can result in inflammatory or allergic responses. The transmission of neurotoxins can additionally cause tick toxicosis or paralysis. This is most commonly described after tick bites from ticks such as *Dermacentor andersoni, D. variabilis* and *I. holocyclus* (Mans et al. 2004; Estrada-Peña and Mans 2014). While there are some rare case reports of tick toxicosis after *I. ricinus* infested animals or humans, a definitive link could not be made because hosts were either simultaneously infested with other tick species or the tick in question was later identified as *I. gibbosus* (Gothe 1999). In addition, Gothe reported that laboratory tick rearing trials have not been able to produce tick toxicosis after *I. ricinus* infestation.

Another medical concern associated with tick bites is a red meat allergy, an immediate hypersensitivity type I reaction caused by an increased IgE-antibody level in response to alpha-Gal saccharine found in red meat (Commins et al. 2009). Previous studies have detected alpha-Gal saccharine in ticks including in *I. ricinus*, which supports the assumption that the

presence of tick-derived alpha-Gal leads to a sensitization by inducing an increased IgE response in humans after tick bites (Van Nunen et al. 2009; Hamsten et al. 2013; Apostolovic et al. 2020; Young et al. 2021).

Infestations with a large number of ticks can result in significant blood losses (Talleklint and Jaenson 1997; Deplazes et al. 2012). This is of particular concern in the field of veterinary medicine where massive tick infestations can occur, specifically in livestock or wildlife. The subsequent deterioration of animal health as well as decrease in quality of animal products such as leather can have a substantial economic impact (Jongejan and Uilenberg 2004; Gashaw and Mersha 2013). Secondary wound infection due to bacteria or other parasites is an additional concern associated with tick bites (Reck et al. 2014).

Tick infestation is additionally risky for both animals and humans because of the ticks' ability to act as transmitting agents for tick-borne diseases such as viruses, bacteria or parasites. This is classified as indirect harm to the host.

Ixodes ricinus is a vector for a variety of viruses such as the TBEV complex or the Louping-ill virus. While its exact role as a vector remains to be determined, Eyach virus, Grotenhout virus, Tribec virus, Lipovnik virus, Erve virus, Uukuniemi(-like) virus and the Kemerovo virus have also been isolated from *I. ricinus* (Rizzoli et al. 2014; Gondard et al. 2018; Migné et al. 2022b).

Ixodes ricinus is also one of the main vectors for the causative bacterial agents of Lyme-borreliosis *Borrelia burgdorferi* sensu lato. This includes human pathogenic agents such as *B. afzelii*, *B. bavariensis, B. burgdorferi* sensu stricto, *B. garinii, B. spielmanii;* potentially human-pathogenic agents such as *B. bisseti, B. finlandensis, B. lusitaniae* and *B. turdi* and the non-human pathogenic agent *B. valaisiana* (Casjens et al. 2011; Stanek et al. 2012; Margos et al. 2017; Steinbrink et al. 2022). In Europe, the most frequently detected agents in *I. ricinus* are *B. afzelii* and *B. garinii* (Steere et al. 2016; Estrada-Peña et al. 2018b; Steinbrink et al. 2022). Other *I. ricinus*-borne bacteria associated with human and/ or veterinary diseases are *Anaplasma* spp., *Bartonella* spp., *Borrelia miyamotoi, Francisella tularensis, Coxiella burnetti, Neoehrlichia mikurensis, Rickettsia* spp. and *Spiroplasma ixodetis* (Sprong et al. 2018; Azagi et al. 2020).

Ixodes ricinus is also a relevant vector for the transmission of parasites to animals and humans, in particular the *Babesia* species (e.g. *B. divergens, B. capreoli, B. venatorum* and *B. microti)* (Hildebrandt et al. 2021). In addition, *I. ricinus* has been reported to serve as an intermediate host for the helminth *Cercopithifilaria rugosicauda* (Winkhardt 1980; Ramos et al. 2013). The occurrence of parasites such as *Theileria* spp., *Hepatozoon* spp., *Trypanosoma* spp. or microsporidia in *I. ricinus* has been sporadically reported (Hamšíková et al. 2016; Luu et al. 2020; Fernández et al. 2022; Trzebny et al. 2022).

The abundance and wide geographical distribution of *I. ricinus* as well as its broad range of hosts renders this tick species particularly relevant in Europe (Figure 2). It is the most common hard tick species in Germany (Petney et al. 2012; Ribeiro et al. 2019). Tick distribution, population density and the subsequent impact on human and veterinary health are expected to be affected by climate change. Increases in temperature and humidity might prolong the duration of questing activity and shorten the duration of the tick life cycle. Changes in land use and recreational behavior of humans may alter the host population dynamics and hence, tick-host (animal and human) encounters. Changes in vegetation as well as increased occurrences of severe weather are likely to affect both host and tick populations (Medlock et al. 2013; Ogden and Lindsay 2016; Bouchard et al. 2019; Hvidsten et al. 2020; Diuk-Wasser et al. 2021; Gray et al. 2021; Zając et al. 2021; Jenkins et al. 2022). In general, an increase in average temperatures has already led to a more northward distribution of *I. ricinus* in Europe as well as an increased occurrence at higher altitudes (Talleklint and Jaenson 1998; Lindgren et al. 2000; Materna et al. 2008).

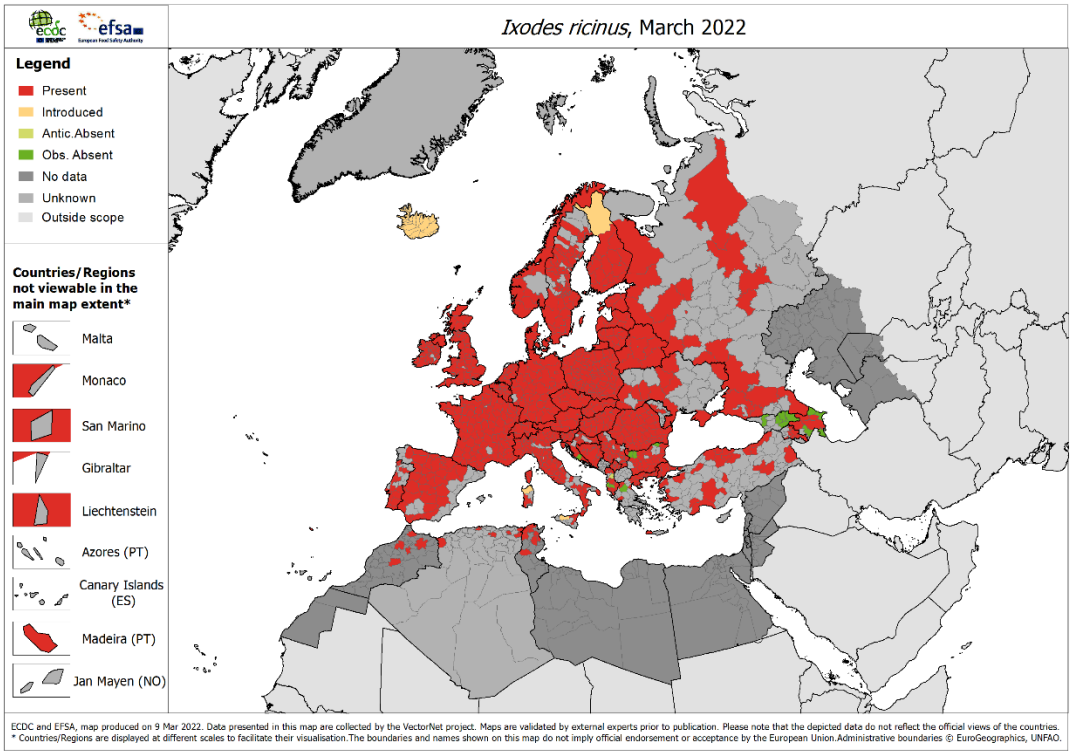

Figure 2: Distribution of *Ixodes ricinus* in Europe.
(Accessed on the 05.12.22 via
https://www.ecdc.europa.eu/sites/default/files/images/Ixodes_ricinus_2022_03.png).

Undiagnosed and untreated *I. ricinus*-borne diseases can have lifelong impacts on human and animal health, resulting in a high economic burden (Lohr et al. 2015; Van Den Wijngaard et al. 2017; Mac et al. 2019). In Europe, informative campaigns, citizen science projects and regular

expert reports have led to a high general awareness of ticks and tick-borne diseases among the population, but despite increased research attention, standardized transboundary tick control protocols and programs are still lacking (de la Fuente et al. 2023).

1.3. The microbiome of ticks

1.3.1. Definitions and general thoughts on the microbiome

Hard ticks may not only harbor pathogenic microorganisms, but also carry non-pathogenic ones and the tick microbiome has received increased attention in recent years. With regard to the development of novel tick control strategies, the composition of the microbiome, its diversity and interaction with TBPs is particularly of interest.

While the definition of microbiome varies slightly (Bass et al. 2019; Berg et al. 2020), it most commonly refers to microbiota such as bacteria, archaea, or eukaryotes such as fungi or parasites (Lederberg and McCray 2001; Marchesi and Ravel 2015) as well as non-living organisms such as viruses, phages, free DNA and structural elements all of which interact and function within a specific environment or habitat (Whipps et al. 1988; Berg et al. 2020).

Components of the microbiome can have varying effects on the host; beneficial (symbionts), neutral (commensals) or negative (pathogens, from opportunistic to obligate pathogenic) (Lederberg and McCray 2001; Bonnet et al. 2017). Whether a symbiont is classified as obligate or facultative is dependent on its origin, localization, function, transmission or the dependency of the host on the symbiont, some of which can be difficult to identify (Baumann 2005; Moran et al. 2008; Gupta and Nair 2020). Obligate symbionts have a co-dependent mutualistic relationship with their hosts and are essential for the survival of the arthropod (Bonnet et al. 2017; Stewart and Bloom 2020). They usually have reduced genome sizes, are vertically transmitted and intracellular (Song et al. 2022). Facultative symbionts on the other hand, are generally adapted to altered environmental influences (including temporal changes) and therefore may support adaptation of the arthropod to maximize reproduction, fitness or defense mechanisms (Bonnet et al. 2017). The effects of facultative symbionts have been studied for some arthropods, but are not well understood in ticks (Carpi et al., 2011; Bonnet et al., 2017; Duron et al., 2017; Duron and Gottlieb, 2020).

While pathogens are by definition harmful to the immediate host (here: the tick), they are often seen from a more anthropogenic perspective as causative agents for human or animal diseases. In this manuscript, “pathogenic” is used to refer to agents that are harmful to vertebrates.

The term "holobiont" takes on a more holistic perspective and includes the tick, all components of the microbiome (including pathogens and non-pathogenic microorganisms), the environment and interactions between the individual players (Bass et al. 2019; Berg et al. 2020). When the holobiont diverges from the healthy state, it is referred to as “pathobiont” or “disease state”, in which pathogenic microorganisms are able to thrive (Vayssier-Taussat et al. 2014; Bass et al. 2019). This dysbiosis can be caused by a low-diversity, low-density metabolic status (Bass et al. 2019; Berg et al. 2020). The concept of the pathobiome highlights the fact that the “disease state” of the tick is always a multifactorial condition, caused by more than just one pathogen (Bass et al. 2019).

It can be assumed that certain organisms have a “core microbiome”; microbiota which are consistently present among individuals who share similar habitats (Berg et al. 2020). Whether or not *I. ricinus* or other hard ticks have a core microbiome remains to be determined (Narasimhan and Fikrig 2015; Estrada-Peña et al. 2018b; Ross et al. 2018; Guizzo et al. 2020; Guizzo et al. 2022).

The recent advances in molecular techniques such as Next Generation Sequencing (NGS) have given rise to more insights into the tick microbiome. The first microbiome profile of an *I. ricinus* tick was established using amplicon-based sequencing, targeting the hypervariable region V6 of the prokaryotic 16S ribosomal RNA gene (Carpi et al. 2011). Overall, one or more hypervariable regions (V1-V9) can be assessed, of which the V3-V4 regions have been the most commonly used targets for *I. ricinus* (Papa et al. 2017; Estrada-Peña et al. 2018a; Aivelo et al. 2019; Hoffmann et al. 2020; Hamilton et al. 2021; Lejal et al. 2021; Aivelo et al. 2022; Guizzo et al. 2022; Krawczyk et al. 2022a; Maitre et al. 2022b). Besides metagenomic approaches, metatranscriptomic, metaproteomic, metabolomic as well as a combination of these approaches have been used to better understand microbiota communities and their activities in *I. ricinus* (Nakao et al. 2013; Vayssier-Taussat et al. 2013; Bonnet et al. 2014; Di Venere et al. 2015; Hernández-Jarguín et al. 2018; Kmeť and Čaplová 2021). While a broad array of information concerning taxonomy of microbiota has become available through sequencing (Turnbaugh et al. 2007; Brinkerhoff et al. 2020; Guizzo et al. 2020), recent literature highlights the scarcity of functional profiling (Lemanceau et al. 2017; Chicana et al. 2019; Couper et al. 2019; Estrada-Peña et al. 2020; Estrada-Peña and Fernández-Ruiz 2020; Maitre et al. 2022b). Even though cell cultures have been used to study tick-microbiome interactions (Grabowski and Hill 2017; Grabowski and Kissinger 2020; Bonnet et al. 2021; Rousseau et al. 2021), symbiont cultures from *I. ricinus* midguts have been reported to be unsuccessful (Guizzo et al. 2023).

1.3.2. Influences on the tick microbiome

The tick microbiome is extremely variable between tick species, genera, life stages, sexes and ages (Moreno et al. 2006; Lalzar et al. 2012; Rynkiewicz et al. 2015; Zolnik et al. 2016; Swei and Kwan 2017). The host, including the host blood and feeding status also influence the tick microbiome (Heise et al. 2010; Zhang et al. 2014; Rynkiewicz et al. 2015; Swei and Kwan 2017). Although the extent of interaction between the tick microbiome and TBPs or other symbionts is not extensively understood, the fact that interactions take place is highly likely (Narasimhan et al. 2014; Gall et al. 2016; Gil et al. 2020; Hamilton et al. 2021; Narasimhan et al. 2022).

Microbial communities within the tick are affected by environmental conditions such as temperature, seasonal or temporal changes (Lalzar et al. 2012; Thapa et al. 2019; Lejal et al. 2021; Aivelo et al. 2022) as well as geographic locations including differences in habitats or altitudes (Van Overbeek et al. 2008; Carpi et al. 2011; Williams-Newkirk et al. 2014; Van Treuren et al. 2015; Gall et al. 2017; Kueneman et al. 2021; Aivelo et al. 2022; Krawczyk et al. 2022a). Also, Lejal *et al.* reported on timely changes of the microbial composition of *I. ricinus*, mainly due to alterations in the immediate environment (Lejal et al. 2021).

The microbial composition is influenced by the direct acquisition of environment/ habitat bacteria, for instance from soil, plants or the host skin (Narasimhan and Fikrig 2015; Zolnik et al. 2016; Estrada-Peña et al. 2018a; Kmeť and Čaplová 2021; Narasimhan et al. 2021; Rousseau et al. 2021; Guizzo et al. 2022). Laboratory-reared ticks show differences in microbial compositions or tick symbionts when compared to field-collected ticks (Lo et al. 2006; Heise et al. 2010; Zolnik et al. 2016; Gall et al. 2017; Kwan et al. 2017; Wu-Chuang et al. 2021) and it is important to consider secondary contamination during tick sample preparation as a possible cause (Binetruy et al. 2019; Fernández-Ruiz et al. 2023).

1.3.3. Symbionts of Ixodes ricinus

The most common and dominant bacterial microbiota found in *I. ricinus* are *Candidatus* Midichloria mitochondrii (formerly known as “IricES1”, hereafter referred to as *M. mitochondrii*) (Lo et al. 2006; Bonnet et al. 2017; Krawczyk et al. 2022a).

Midichloria mitochondrii is a gram-negative alpha-proteobacterium (order: *Rickettsiales,* family: *Midichloriaceae*), which has a specific tropism for the female ovarian tick tissues and eggs (Lewis 1979; Beninati et al. 2004; Sacchi et al. 2004; Sassera et al. 2006; Epis et al. 2013). It has also been found in salivary glands, Malpighian tubes, tracheae and midguts and is rarely detected in males (Beninati et al. 2004; Sassera et al. 2006; Mariconti et al. 2012; Epis et al. 2013; Olivieri et al. 2019; Guizzo et al. 2020). The prevalence of *M. mitochondrii* is

particularly high in eggs, which suggests a vertical, transovarial transmission. The bacterial load then drops in larvae and shows a high variation in nymphs, most likely due to the lack of sex differentiation in this life stage (Sassera et al. 2008; Daveu et al. 2021). Further, it has been shown that *M. mitochondrii* loads are lower (or become lower over time) in laboratory-reared ticks in comparison to field-collected *I. ricinus* (Lo et al. 2006; Cafiso et al. 2019). Recent insights suggest that horizontal of *M. mitochondrii* between arthropods (possibly through co-feeding) is also possible (Epis et al. 2008; Cafiso et al. 2016; Buysse and Duron 2018).

Due to the presence of *M. mitochondrii* in tick salivary glands, their transmission to mammalian hosts during a blood meal may be possible. Reports of *M. mitochondrii* DNA and antibody responses in mammals (including humans) previously infested by ticks support this notion (Mariconti et al. 2012; Bazzocchi et al. 2013; Cafiso et al. 2016; Sgroi et al. 2022). While *M. mitochondrii* does not appear to have pathogenic effects on mammal hosts, the interaction with other (potentially) pathogenic TBPs warrants further research (Mariconti et al. 2012; Cafiso et al. 2016; Cafiso et al. 2019; Sgroi et al. 2022).

The primary localization of *M. mitochondrii* in female reproductive tissues and eggs suggests that it has the potential to affect reproductive fitness and development of *I. ricinus* (Lo et al. 2006; Sassera et al. 2011; Olivieri et al. 2019; Guizzo et al. 2020). It has been suggested that the bacterium acts as an additional energy/ adenosine triphosphate (ATP) source or supplies the tick with essential nutrients such as B vitamins that are missing from the blood meal (Sassera et al. 2011; Rio et al. 2016; Duron and Gottlieb 2020; Buysse et al. 2021; Floriano et al. 2023).

Given the high bacterial loads in female ticks during the first days of feeding, it seems likely that the high demand for energy and nutrients prior to oogenesis is supplemented by specific endosymbionts (Sassera et al. 2008; Sassera et al. 2011; Olivieri et al. 2019; Guizzo et al. 2020). Moreover, a recent study supported the hypothesis of the essential role of *M. mitochondrii* in larvae when *M. mitochondrii*-free tick lines proved to be sub-standard in comparison. However, further research is warranted to fully exclude possible other factors or microbes involved in this observation (Garcia Guizzo et al. 2023).

Other common bacterial symbionts in *I. ricinus* are *Coxiella/ Coxiella*-like endosymbiont (CLE), *Francisella/ Francisella*-like endosymbiont (FLE), *Spiroplasma, Rickettsia* and *Rickettsiella* and *Lariskella* (Duron et al. 2017; Papa et al. 2017; Špitalská et al. 2018; Aivelo et al. 2019; Maitre et al. 2022b). There have also been reports of *Wolbachia* and *Arsenophonus* in *I. ricinus* but this is most likely associated with a concurrent infection of the parasitic wasp *Ixodiphagus hookeri* which is known to harbor these bacteria (Plantard et al. 2012; Bohacsova et al. 2016; Lejal et al. 2021).

1.3.4. Roles and functions of symbionts

Symbionts can affect biological parameters such as feeding, fecundity, development success or fitness of the tick.

One example of tick-microbiome symbiosis is the production of B vitamins in obligate hematophagous parasites. The vertebrate blood meal is lacking nutrients such as B vitamins and their co-factors that are essential to the tick and genome analyses have shown that ticks are unable to produce these nutrients themselves (Buchner 1965; Sterkel et al. 2017; Buysse et al. 2021). The restricted blood diet, inability of ticks for self-synthetization of B vitamins and the parallel specification of symbionts to do so is thought to have led to this mutualistic and interdependent relationship resulting in a congruent phylogenetic relation (Duron et al. 2017; Duron and Gottlieb 2020). Similar interactions have been reported for other obligate hematophagous parasites such as tsetse flies, lice, bedbugs, triatomine bugs, and mites (Rio et al. 2016; Husnik 2018; Duron and Gottlieb 2020; Sonenshine and Stewart 2021). The microbial genera which are most commonly associated with vitamin B pathways in ticks are *Coxiella*/ CLE (Gottlieb et al. 2015; Smith et al. 2015; Guizzo et al. 2017; Ben-Yosef et al. 2020), *Francisella/* FLE (Duron et al. 2018; Gerhart et al. 2018; Buysse et al. 2021), *Rickettsia* (Hunter et al. 2015; Duron et al. 2017; Al-Khafaji et al. 2020), *M. mitochondrii* (Sassera et al. 2011), potentially *S. ixodetis, Wolbachia, Anaplasma phagocytophilum* (Hodosi et al. 2022), *Lariskella* (Buysse and Duron 2021) and *Rickettsiella*.

Furthermore, tick endosymbionts may affect the tick biology such as the tick feeding behavior (Zhang et al. 2017; Ben-Yosef et al. 2020; Zhong et al. 2021), the fecundity in females, development of juveniles, egg hatching or viability of larvae (Zhong et al. 2007; Zhang et al. 2017; Duron et al. 2018; Guizzo et al. 2023). Bacterial microbiota have additionally been reported to influence tick behaviors such as activity or movements (Kagemann and Clay 2013; Benelli 2020) and can influence the acquisition, maintenance and transmission of TBPs, either directly or by altering the tick's immune system (Narasimhan et al. 2014; Abraham et al. 2017; Narasimhan et al. 2017; Hamilton et al. 2021; Narasimhan et al. 2021).

In order to better understand the relationship between the microbial community and tick phenotypes, different dysbiosis techniques have been developed. The general aim of these experiments is to use antibiotic substances to reduce or eliminate specific bacteria in the microbiome and subsequently deduce their role. These antibiotics, most commonly from the tetracycline class, can be administered in a number of ways and an overview of publications in which this has been done is shown in Table 1. Ticks can be fed on live animals that have previously been treated with antibiotics or in an ATFS using a blood meal treated with

antibiotics. Alternatively, microinjection, washing procedures and the use of entomopathogenic fungi have been used.

Tick species	Life stage	Feeding status	Antibiotic substance	Administration method	Phenotype parameters	Samples for molecular analysis	Molecular/further analysis	Specific bacterial focus*	Reference
A. americanum	N, F	engorged	Rifampicin, tetracycline	Microinjection	A: sex proportion, weight; L: hatching proportion incl. alive and dead larvae counts	Whole tick (gDNA): eggs, unfed L+N+F	qPCR	*Coxiella, Rickettsia*	(Zhong et al. 2007)
I. pacificus	F	engorged	Ampicillin, ciprofloxacin, tetracycline	Animal-treated	F: oviposition duration, hatch duration, hatching proportion (incl. alive and dead larvae)	Whole tick (gDNA): eggs, unfed L	qPCR	*Rickettsia*	(Kurlovs et al. 2014)
I. scapularis	F, L	engorged, unfed	Gentamicin	Wash& maintenance, animal-treated	L: engorgement proportion, engorgement weight, molting proportion; N: engorgement weight	Whole tick: unfed, partly engorged& engorged N+L; midguts: N	Pyrosequencing, qPCR, RNAi, histology (EMSA, FISH)	*B. burgdorferi*	(Narasimhan et al. 2014)
D. andersoni	M	unfed	Oxytetracycline	Animal-treated	L: survival proportion, engorgement weight, molting proportion; N: engorgement proportion, engorgement weight, molting proportion	Salivary glands, midguts (gDNA): engorged M	454 pyrosequencing, PCR	*Rickettsia, Francisella*	(Clayton et al. 2015)
I. ricinus	F	engorged, unfed	Tetracycline	Microinjection, animal-treated	NA	Whole tick (gDNA): unfed, engorged L+N	qPCR	*M. mitochondrii*	(Ninio et al. 2015)
D. andersoni	A	unfed	Oxytetracycline	Animal-treated	NA	Whole tick, midgut, salivary glands (gDNA): unfed& engorged M, engorged N	16S rRNA, qPCR, ddPCR	*A. marginale, R. bellii, Francisella*	(Gall et al. 2016)
R. microplus	F, L	engorged, unfed	Tetracycline	Microinjection, animal-treated	Mean egg conversion factor, larvae hatch proportion	Salivary glands, midgut, Malpighian tubule, ovary (RNA): F; whole tick (RNA): eggs, M; whole ticks (gDNA): eggs, L; ovaries (gDNA), saliva (gDNA)	PCR, Illumina MiSeq (V4 16S rRNA), qPCR	*Coxiella*	(Guizzo et al. 2017)
H. longicornis	F	partly engorged	Tetracycline	Microinjection, animal-treated	F: engorgement weight, feeding duration, egg numbers, pre-oviposition duration, oviposition duration, hatching duration	Ovaries, Malpighian tubules (gDNA): partly engorged F	semi-quantitative PCR	*Coxiella*	(Zhang et al. 2017)
O. moubata	1st N (3-5), A	unfed	Rifampicin	ATFS	N: molting proportion into further nymph stages and into adults (incl. alive& dead), sex ratio, A: weight	Whole tick (gDNA): for comparison of antibiotic-treated/ vitamin B-treated groups; Salivary glands, midgut, Malpighian tubule, ovaries/testes (gDNA)	Multiplex PCR, Illumina MiSeq (V4 16S rRNA), PCR, qPCR, histology (FISH), Francisella sequencing	*Francisella, Rickettsia*	(Duron et al. 2018)
R. haemaphysaloides	F	engorged	Ciprofloxacin, kanamycin, ofloxacine, tetracycline	Microinjection	F: engorgement weight (prior antibiotic-treatment), oviposition duration, egg weights, egg conversion factor, hatching proportion& duration	Whole tick (gDNA): unfed A, eggs	Illumina MiSeq (V3-V4 16S rRNA), qPCR	*Coxiella, Rickettsia*	(Li et al. 2018a)
R. haemaphysaloides	F	engorged	Ciprofloxacin, kanamycin	Microinjection	L: engorgement weight	Whole tick (gDNA): unfed L+N	qPCR, nested PCR	*B. microtii*	(Li et al. 2018b)

R. sanguineus	N, A, e, L	various	Ofloxacine, rifampicin, tetracycline	Microinjection	N: molting proportion& duration; F: feeding duration, engorgement weight; egg weights; L: feeding proportion	Whole tick (gDNA): engorged N, unfed A, F+L+eggs in pools	qPCR, Illumina MiSeq (V4 16S rRNA)	*Coxiella*	(Ben-Yosef et al. 2020)
I. scapularis	F	various	Tetracycline (different doses)	ATFS, microinjection (or combination)	NA	Internal tissues (gDNA): eggs, unfed& engorged L+N, unfed A	qPCR	*R. buchneri*	(Oliver et al. 2021)
H. longicornis	N, A	various	Tetracycline (different doses), antibiotic mix: penicillin + streptomycin + gentamicin	Microinjection	N: engorgement weights	Whole tick, midgut, ovaries, Malpighian tubules (gDNA, RNA): A; whole tick, midgut, salivary gland, carcass (RNA): unfed, partly engorged& engorged N	qPCR (gDNA, RNA), Illumina HiSeq (RNA), N: gene knockdown, ELISA, A: LC-MS analysis, immunohistochemistry	*Coxiella*	(Zhong et al. 2021)
I. ricinus	e	NA	NA	Wash& maintenance	L: engorgement weight, molting proportion, molting duration; N: unfed molting weight, immature survival	Whole tick (gDNA): unfed L+N	qPCR, Illumina MiSeq (V3-V4 16S rRNA)	*B. afzelii*	(Hamilton et al. 2021)
I. ricinus	e	NA	NA	Wash& maintenance	L: engorgement weight, molting proportion, molting duration; N: unfed molting weight, immature survival	Whole tick (gDNA): unfed L+N	qPCR, ELISA	*B. afzelii*	(Hurry et al. 2021)
H. longocornis	N	unfed	Ampicillin, metronidazole, neomycin, vancomycin	Animal-treated	NA	Midgut (gDNA, RNA): unfed& partly engorged A	Illumina MiSeq (V3-V4 16S rRNA), qPCR (RNA, DNA), histology, Illumina novaseq	*B. microti*	(Wei et al. 2021)
I. ricinus	F	unfed, engorged	Tetracycline (different doses)	ATFS, microinjection (or combination)	F: engorgement weight, egg weights; L, N, A: engorgement proportions; N, A: molting proportions, oviposition proportion, hatching proportion	Whole tick or ovaries (gDNA): engorged fed ticks or eggs; ovaries (RNA): engorged F	qPCR, cell culture experiments, SDS-PAGE, western blotting, RNAi experiments	*M. mitochondrii*	(Guizzo et al. 2023)
O. moubata	F, N	unfed, partly engorged, engorged	Rifampicin, gentamicin	ATFS	F: engorgement weight, oviposition proportion& duration, egg weights, egg conversion factor, eggs: hatching& molting duration& proportion	Whole tick (gDNA): unfed N+A	qPCR (DNA)	*Francisella, Rickettsia*	(Taraveau et al. 2023)
I. scapularis	F	unfed	Gentamicin	ATFS	F: attachment proportion, engorgement proportion& weight, oviposition proportion, hatching proportion	Whole tick (gDNA): unfed& engorged L; Ovary, midgut, salivary glands, Malpighian tubes (gDNA): engorged F	qPCR (DNA)	*R. buchneri*	(Garcia Guizzo et al. 2023)
R. microplus	F	unfed	Tetracyclin and/ or entomopathogenic fungi	ATFS, topical treatment	F: survival over time after ATFS and fungi treatment	Engorged F (gDNA): midgut	Illumina MiSeq (V3-V4 16S rRNA)	*Coxiella*	(Mesquita et al. 2023)

Table 1: Overview on experiments with dysbiosis methods for microbiome or infection studies.

Life stage and feeding status at the time point of dysbiosis. L= larvae, N= nymphs, A= adults, F= females, M= males, NA= not applicable or not mentioned, ATFS= artificial tick feeding system. *Information is on bacteria species may include species name and/or its species -like endosymbionts.

1.3.5. Implications of tick microbiome studies

Besides offering further insights into the tick biology, physiology and evolutionary adaptations, the use of novel molecular technologies in tick microbiome studies has the potential to advance the domains of epidemiology, discovery and diagnostics of TBPs (Vayssier-Taussat et al. 2015). It has for instance been suggested that symbionts which are highly abundant in ticks, such as *M. mitochondrii* in *I. ricinus,* could be used as a tick-bite markers (Mariconti et al. 2012; Serra et al. 2019).

Altering specific bacterial microbiota in the tick microbiome or causing dysbiosis of the whole composition can disrupt the acquisition, maintenance or transmission of pathogens (Macaluso et al. 2002; Narasimhan et al. 2014; Gall et al. 2016; Abraham et al. 2017; Wei et al. 2021). Specifically targeting microbial components linked to tick survival or the TBPs-interface could therefore be used in the development of tick control strategies.

Such a dysbiosis could for instance be induced by antibiotic treatments or anti-microbiota vaccines. In addition, a recent report highlighted the effect of entomopathogenic fungi on the tick microbiota as a future tick control option (Mesquita et al. 2023). However, given the fact that an antibiotic treatment does not target specific bacteria and is controversial in light of increasing risks of antimicrobial resistance, anti-microbiota vaccines pose another option. In this case, ticks are fed on vaccinated host animals and thus acquire antibodies against specific bacteria or microbial components linked to nutrient synthesis, TBP-interface, the tick immune system or other survival factors (Mateos-Hernández et al. 2020; Mateos-Hernández et al. 2021; Bonnet et al. 2022; Maitre et al. 2022a; Wu-Chuang et al. 2022). Nevertheless, the development of anti-microbiota vaccines requires a stable and reliable tick microbiome and recent assumptions of a non-existing tick gut core microbiome challenge this approach (Ross et al. 2018; Guizzo et al. 2020; Guizzo et al. 2022). Moreover, the longevity of ticks and the vast number of influencing factors on the microbiome complicate the further development and application of such techniques and highlight the need for further research.

1.4. Artificial tick feeding systems

For more than a century, ATFS have been used to examine tick biology and sensory perceptions (Hindle and Merriman 1912; Totze 1933). Applications of ATFS have since expanded to include tick rearing, acaricide screening and studies of TBPs or microbiota. In general, we can differentiate between systems using membranes or skins and the capillary tube feeding technique.

1.4.1. The artificial tick feeding system components

Membrane feeding-based ATFS have four basic components: (a) a confined space for ticks, (b) the blood meal, (c) a membrane which separates the two and (d) a temperature control system to keep the blood meal heated. Nevertheless, environmental conditions and attachment stimuli also play an essential role in membrane feeding systems.

1.4.1.1. Feeding units

Tick containment is ensured by a confined space for ticks and referred to as a feeding unit (FU). It is situated either on top of the membrane, so the blood meal container is below the ticks, or underneath the membrane, in what is sometimes referred to as “upside down” feeding systems (Kuhnert 1996). A combination of both methods also has also been described (Kemp et al. 1986). In general, the FU is closed on one side by a membrane, separating the ticks from the blood meal, and by an air-permeable plug made of gauze (Kemp et al. 1975a), cotton wool (Kuhnert et al. 1995), muslin fabric (Musyoki et al. 2004) or a glass cover for short feeding experiments on the other side (Osborne and Mellor 1985).

The most common shape of the FU is a vertical cylinder or tube. The tubes can be made of acrylic glass or polystyrene (Krober and Guerin 2007b), plastic (Howarth and Hokama 1983; Young et al. 1996) or (borosilicate) glass (Waladde et al. 1991; Krull et al. 2017). A container with a slightly larger diameter than the FU contains the blood meal. A 50mL beaker glass (Barré et al. 1998; Krull et al. 2017), a 135mL plastic container (Howarth and Hokama 1983), a standard 6- well plate (Howarth and Hokama 1983; Krober and Guerin 2007b) or even honey jars have been used as blood meal containers (Kuhnert et al. 1995). In fact, the screw caps of the honey jars permit airtight sealing and could therefore be advantageous in infection trials that require high biosafety levels (Liebig et al. 2020).

The “upside down” feeding system was developed under the assumption that blood cells that sediment on the membrane due to gravity would be more accessible to the ticks in the FU. First attempts involved inverting the FU after tick attachment to support infection by intracellular pathogens (Kemp et al. 1986; Bonnet and Liu 2012; Vimonish et al. 2020). Although a good degree of membrane stability is required to avoid leakage of the blood meal into the tick FU, ticks exhibiting negative geotaxis are likely to show good attachment proportions.

A plethora of variations of the “upside down” feeding system has been developed: besides the typical cylindrical form made of glass or plastic (Kemp et al. 1986; Waladde et al. 1995; Musyoki et al. 2004; Hatta et al. 2012), rectangular cell-culture flasks (Voigt et al. 1993), modified Rutledge-feeders (Burkot et al. 2001; Bonnet et al. 2007; Migné et al. 2022b) or, more recently, triple-layered Plexiglas with the dimensions of a standard 24-well plate (Trentelman

et al. 2017; Trentelman et al. 2019) have been described. In the latter, the middle layer acts as the FU containing the ticks and is separated from the upper layer, which contains the blood meal, by a membrane. This ATFS has been successfully used for the feeding *Rhipicephalus* larvae for up to 72 h. However, it may be prone to contamination because the individual units are difficult to clean during experiments.

Soft tick feeding experiments are typically conducted in a similar cylindrical FU and are associated with positive results due to the relatively short feeding periods (several minutes to hours) (Mango and Galun 1977; Schwan et al. 1991).

1.4.1.2. The membrane

Important requirements for the membranes include stability to avoid leakages and flexibility to allow for the ticks to attach, detach and re-attach several times. Membrane perforations are detrimental to tick feeding experiments because they can cause an influx of the blood meal into the FU. Contamination, both bacterial and fungal, plays a particularly important role in hard tick feeding experiments due to the longer feeding duration of several days to weeks. To prevent contamination, the membrane should therefore be resistant to treatments such as washing, disinfection and autoclaving.

The membrane can either be of animal origin or artificial. Whole animal skins of rats (Hindle and Merriman 1912), sheep (Lees 1948), mice (Doube and Kemp 1979), gerbils (Bonnet et al. 2007), rabbits (Howarth and Hokama 1983; Voigt et al. 1993) or cattle (Kemp et al. 1975b; Voigt et al. 1993) were used in the first ATFS. The skins were prepared by shaving or shortening the fur (Howarth and Hokama 1983; Voigt et al. 1993), sometimes followed by washing processes. Other animal-origin membranes include the diaphragm of rats (Lees 1948) or guinea pigs (Totze 1933), thin Baudruche membranes (also called Goldbeater's membrane) made of cattle mucosa (Kemp et al. 1975a) or a sausage skin which could even be used as a FU itself (Lees et al. 1988; Lewis et al. 2019). Air cell membranes from chicken eggs have also been examined but were only successful for feeding of soft tick nymphs and adults (Burgdorfer and Pickens 1954) and for hard tick larvae (Pierce and Pierce 1956). It is plausible that the biggest advantage of using membranes from an animal origin are the olfactory and mechanic stimuli, which lead to a faster tick attachment. However, animal-derived membranes have a higher risk of contamination and putrefaction (Totze 1933). Therefore, intensive washing procedures using ethanol or antimicrobials are required prior to their use and each membrane can only be used a limited number of times (Musyoki et al. 2004; Bonnet et al. 2007; Hatta et al. 2012; Migné et al. 2022b).

In order to increase the flexibility and integrity of membranes, which is particularly useful during tick attachment and detachment, several authors reported the use of a silicone mixture to coat

the membranes, specifically when using Baudruche membranes (Waladde et al. 1979; Waladde et al. 1991; Trentelman et al. 2017), or membranes made of non-animal origin such as those made of cellulose, rayon or polyester (Stone et al. 1983; Kuhnert 1995; Krober and Guerin 2007b; Andrade et al. 2014). The use of a membrane made of pure silicone has also been tested on several occasions (Habedank and Hiepe 1993; Voigt et al. 1993; Habedank et al. 1994; de Moura et al. 1997; Barré et al. 1998). Synthetic membranes such as cellophane or Parafilm M have been used without a silicone coating with varying degrees of success; cracks and leakages as well as poor attachment of hard ticks was reported (Totze 1933; Habedank and Hiepe 1993; Habedank et al. 1994).

The hypostome length varies between individual tick species and life stages, which means that the membrane thickness must be adjusted accordingly (Voigt et al. 1993; Kuhnert et al. 1995; Krober and Guerin 2007a; Krull et al. 2017). For experiments involving *Ixodes* spp., silicone-coated membranes with a thickness of 60- 90 µm for nymphs and 50- 225 µm for adults have been used (Krober and Guerin 2007b; Oliver et al. 2016; Hart et al. 2018; Knorr et al. 2018; Koci et al. 2018; Fourie et al. 2019; Korner et al. 2020; Król et al. 2021; Tahir et al. 2021). In order to reduce fungal or bacterial contamination, antibiotic or antifungal components can be added to the silicone mixture (Barré et al. 1998; Tajeri and Razmi 2011). Prior to the use of silicone-covered membranes, FUs with attached membranes were commonly disinfected using ethanol or autoclaving procedures (Krober and Guerin 2007b; Krull et al. 2017).

Combinations of pre-feeding ticks on live animals followed by feeding in ATFS have also been reported. Feeding on live animals typically results in high attachment proportions. Partially fed ticks can subsequently be detached manually and transferred to an ATFS where they can feed to repletion (Stone et al. 1983). However, given the relatively low re-attachment success in the ATFS (Howarth and Hokama 1983), there have also been attempts to transfer animal skin with attached ticks to an ATFS after euthanasia of the animal and subsequently feeding the ticks to full engorgement there (Burkot et al. 2001; Hatta et al. 2012; Maeda et al. 2016).

1.4.1.3. The blood meal

Ticks are well known for their enormous increase in body weight during blood meal intake. Therefore, the blood meal is the most crucial part of feeding and rearing ticks.

While the use of artificial blood meal diets has been reported, animal-derived blood meals are most commonly used in ATFS. Blood from cattle (Stone et al. 1983; Krober and Guerin 2007b; Knorr et al. 2018), but also guinea pigs (Totze 1933), quails (Hart et al. 2018), rats (Schwan et al. 1991), swine (Mango and Galun 1977; de Carvalho Ferreira et al. 2014), sheep or goat (Barré et al. 1998; Gonzalez et al. 2017; Migné et al. 2022b), chickens (Liu et al. 2014) or even humans (Lees 1948; Tarshis 1958; Hart et al. 2018) has been used. The most common source

for blood is cattle, probably because it is relatively easily available in larger amounts from abattoirs. However, freedom of TBPs and absence of recent ectoparasiticide treatments of the donor animal are important to not endanger successful tick feeding or exert other detrimental effects on ticks fed by ATFS. In some experiments, sterile blood is preferred (Waladde et al. 1995; Knorr et al. 2018; Korner et al. 2020) and the use of serum or plasma (Waladde et al. 1979; Perner et al. 2016a; Trentelman et al. 2017), packed red blood cells (Howarth and Hokama 1983) or combinations of the two (Perner et al. 2016a; Knorr et al. 2018) can provide alternatives to the use of whole blood.

Another alternative to whole blood meals is the use of artificial diets such as sodium chloride, liquid gelatin or vegetable bouillon. Ticks may successfully attach to membranes when fed with these substances, but will not feed to repletion (Hindle and Merriman 1912; Totze 1933; Galun and Kindler 1965; Kemp et al. 1982). An artificial diet consisting of tissue cell culture medium supplemented with glucose or phosphate-buffered saline proved less effective than culture medium supplemented with animal serum or plasma components (Doube and Kemp 1979; Stone et al. 1983), which, in turn, proved inferior to whole blood meals (Voigt et al. 1993).

While anticoagulants are necessary to avoid coagulation of blood during collection and storage, they can also have an impact on tick feeding success (Lin et al. 2020). The best results have previously been reported for the use of heparinized blood with heparin concentrations of 2 IE/ mL (Habedank and Hiepe 1993), 20 IE/ mL (Barré et al. 1998; Krull et al. 2017), or 50 IE/ mL (Waladde et al. 1993; Waladde et al. 1995). Manually defibrinated blood also showed good results (Waladde et al. 1979; Voigt et al. 1993; Waladde et al. 1993; Krober and Guerin 2007b). The use of ethylenediaminetetraacetic acid (EDTA) or citrate as anticoagulants has been shown to inhibit feeding behavior in ticks and are thus not recommended for blood meal treatments (Voigt et al. 1993; Waladde et al. 1993; Asri et al. 2023).

To date, there is a scarcity of data on optimal blood meal storage. Blood meals are typically stored at +4- 6°C for a duration of one day (Waladde et al. 1993), up to one week (Kuhnert et al. 1995; Krull et al. 2017; Król et al. 2021) or up to 15 days (Asri et al. 2023). Freezing the blood meal has shown to have little to no negative effects on feeding success (Habedank and Hiepe 1993; Barré et al. 1998; Krull et al. 2017) but has also been associated with increased contamination levels after defrosting (Krull et al. 2017).

During each artificial feeding experiment, the blood meal must be changed at regular intervals. These intervals range from six times (Waladde et al. 1993) to once a day (Burkot et al. 2001; Bohme et al. 2018; Olivieri et al. 2018; Krawczyk et al. 2020). The most common approach is a bi-daily change with a time interval of approx. 10-14 h (Kuhnert et al. 1995; Waladde et al.

1995; Krober and Guerin 2007b; Korner et al. 2020; Koči et al. 2021). After changing the blood meal, the membrane can be cleaned using 0.9 % sodium chloride (Krober and Guerin 2007b; Korner et al. 2020) with or without a disinfectant agent (Guizzo et al. 2023). Many changes a day are laborious and costly over time. Nevertheless, they may have a positive impact on the contamination risk and may be deemed necessary based on the study objective.

The blood meal is often supplemented with phagostimulants such as 1- 2 $x10^{-3}$ mol/L of ATP or glutathione (GSH) (Galun and Kindler 1965; Waladde et al. 1979; Kemp et al. 1982; Hokama et al. 1987). Another common additive is glucose, which does not have a phagostimulant effect by itself, but does so in combination with ATP or GSH (Hosoi 1959; Galun and Kindler 1968; Kemp et al. 1982). For example, in *D. reticulatus*, higher glucose levels were associated with a positive effect on adult feeding (Krull et al. 2017) most likely due to its stabilizing effect on erythrocytes (Lemos et al. 2011).

The blood is often supplemented with antimycotic and/ or antibiotic substances such as penicillin, streptomycin, gentamicin, tetracycline, ciprofloxacin, rifampicin, and phosphomycin to prevent bacterial or fungal growth over the course of the experiments (Kemp et al. 1975a; Kuhnert 1995; Krober and Guerin 2007b; Hart et al. 2018; Oliver et al. 2021; Guizzo et al. 2023). The antimycotic substances amphotericin b and nystatin have also been used as an addition to the blood meal (Kemp et al. 1975a; Kuhnert 1995; Bonnet et al. 2007; Migné et al. 2022b) or for the treatment of membranes during feeding experiments (Kuhnert 1995; Krober and Guerin 2007b).

Irradiation of the blood meal presents another sterilization method but was associated with lower feeding success (Krull et al. 2017). A range of short-term experiments (maximum six days in adults, shorter for juveniles (Waladde et al. 1993; Koci et al. 2018; Liebig et al. 2020; Ebert et al. 2023)) and some longer experiments (Korner et al. 2020; Garcia Guizzo et al. 2023) abstained from antimicrobial treatments altogether. This may be beneficial given the assumption that alterations in the tick microbiome caused by anti-infectious agents may have a negative impact on tick fitness, feeding, fecundity and/ or the ability to develop successfully (Zhong et al. 2007; Guizzo et al. 2017; Zhang et al. 2017; Duron et al. 2018; Li et al. 2018a; Ben-Yosef et al. 2020).

To reduce the negative impact of antibiotics in blood meals on the tick microbiome, a supplementation with a vitamin B cocktail has been suggested (Smith et al. 2015; Duron et al. 2018). Obligate hematophagous ectoparasites such as ticks rely on endosymbionts to execute vitamin B pathways, the numbers of which may change upon antibiotic treatment. This has been described for many other hematophagous ectoparasites, such as lice (Kirkness et al. 2010), kissing bugs (Lake and Friend 1968) or bed bugs (Hosokawa et al. 2010; Nikoh et al.

2014), but reports of diets supplemented with B vitamin are scarce (Duron et al. 2018; Artigas-Jerónimo et al. 2021; Taraveau et al. 2023).

1.4.1.4. The temperature control system

The temperature of the blood meal plays an essential role in attracting ticks to attach to the membrane, initiate and complete feeding (Totze 1933; Lees 1948; Voigt et al. 1993). Under natural conditions, ticks detach from the host when the host dies and the body temperature subsequently decreases (Nakao and Sato 1996; Tahir et al. 2020). It is therefore essential to keep the blood meal at a constant temperature of 35- 38 °C, for instance using a water bath (Hindle and Merriman 1912; Waladde et al. 1991; Kuhnert et al. 1995; Krober and Guerin 2007b; Krull et al. 2017). Water circulation systems have been specifically adapted to the "upside-down"- feeding system (Burkot et al. 2001; Bonnet et al. 2007; Migné et al. 2022b). Alternatively, incubators (Kemp et al. 1975a; Voigt et al. 1993; Korner et al. 2020), hot plates (Lees 1948; Waladde et al. 1979; Knorr et al. 2018), digital heating power sources (Vimonish et al. 2020) or even a desk lamp with a 75-W light bulb (Howarth and Hokama 1983) can be used to keep the temperature constant.

1.4.1.5. Environmental conditions

Environmental conditions also play an important role in tick activity, attachment and feeding. Arguably the most important role is played by the temperature. Previous experiments have used environmental temperatures equivalent to (Voigt et al. 1993; Waladde et al. 1995; Andrade et al. 2014; Trentelman et al. 2017; Korner et al. 2020) or lower to than the temperature of the blood meal (Waladde et al. 1991; Krull et al. 2017; Vimonish et al. 2020). At high environmental temperatures of ~37 °C, there is a risk that the ticks desiccate before attachment to the membrane.

To decrease the risk of tick desiccation, an optimal relative humidity (RH) is required. Feeding experiments are typically performed at a RH of 70- 80 % (Waladde et al. 1991; Knorr et al. 2018; Korner et al. 2020), with some researchers using higher levels of up to ~100 % (Voigt et al. 1993; Krober and Guerin 2007b; Koci et al. 2018; Tahir et al. 2021). The RH is regulated by either using an incubator, a water bath, a desiccator or a wet tissue/ fabric (Barré et al. 1998). If the RH is too high, droplet formation in the closed container may occur, causing the ticks to get entrapped with negative effects on feeding.

The light-dark (LD) rhythm is another factor of relevance. Data suggests that long-day LD rhythms that mimic summer conditions increase tick activity. The LD rhythms of 15:9 h or 16:8 h (Krober and Guerin 2007b; Krull et al. 2017) or even 12:12 h (Waladde et al. 1991; Kuhnert et al. 1995) have been used. There are also some reports of successful tick feeding in complete

darkness (Mango and Galun 1977; Waladde et al. 1995; Andrade et al. 2014; Trentelman et al. 2017).

Finally, the CO_2 level has been used to activate ticks in order to increase tick attachment. In previous experiments, CO_2 levels ranged between ~0.05- 10 % were used (Habedank and Hiepe 1993; Voigt et al. 1993; Waladde et al. 1995; Krull et al. 2017; Trentelman et al. 2017; Bohme et al. 2018). Higher CO_2 levels have been shown to stimulate tick activity and lead to increased engorgement weights (Voigt et al. 1993; Krull et al. 2017). Depending on the life stage of the tick, the effect is reversed once a certain threshold is surpassed (Voigt et al. 1993). The rather activating than attracting effect was also observed in an olfactometer experiment (Van Duijvendijk et al. 2017).

1.4.1.6. Additional tick stimuli

Olfactory and/ or mechanical stimuli are frequently added to membranes of artificial origin to attract ticks to the membrane. For example, in experiments involving *Ixodes* spp., extracts from cattle hair (Kuhnert 1996; Krober and Guerin 2007b; Knorr et al. 2018; Fourie et al. 2019; Răileanu et al. 2020), deer hair (Hart et al. 2018; Koci et al. 2018), sheep wool (Korner et al. 2020) or dog hair (Liebig et al. 2020; Tahir et al. 2021) were shown to act as olfactory stimuli. Other examples are the use of animal skin washes (Waladde et al. 1979; Waladde et al. 1991; Habedank and Hiepe 1993; Kuhnert 1996; Musyoki et al. 2004), sweat, saline, blood vestiges, exfoliated skin (Kuhnert 1996; de Moura et al. 1997; Fourie et al. 2013) or plain animal hair (Kemp et al. 1975a; Krober and Guerin 2007b).

In addition, the use of plain or pulverized tick feces (Waladde et al. 1991; Kuhnert et al. 1995; Waladde et al. 1995; Musyoki et al. 2004) or extracts thereof (Kuhnert 1996; Oliver et al. 2016) or exuviae of previous life stages (de la Vega et al. 2004) have been used as attachment stimuli.

Given the finding that ticks prefer to attach in corners, mechanical stimuli such as a mosquito net, cotton wool or plastic crosses can be included in the FU (Waladde et al. 1991; Habedank and Hiepe 1993; Krober and Guerin 2007b). Shaking or stirring devices have additionally been used in soft and hard tick feeding experiments (Kuhnert 1995; Ruheta et al. 2005; Garcia Guizzo et al. 2023). Besides providing a mechanical stimulus, they may help to reduce sedimentation of blood cells or pathogens, which could be particularly beneficial in infection studies to allow for an even distribution of pathogens in the blood meal (Tajeri et al. 2016).

1.4.2. The capillary feeding technique

While the membrane feeding technique has been widely used for soft and hard ticks, capillary feeding has mainly been applied in infection studies on hard ticks. The capillary feeding

technique was developed by Chabaud to study ticks' nutrient uptake (Chabaud 1950). In this method, a thin glass capillary is placed over the cleaned tick hypostome to "force-feed" it with a blood meal. Capillary tube feeding represents a fast and easy laboratory method of introducing substances such as pathogens, molecular markers, water or possible anti-tick-vaccine candidates into the tick with a blood meal (Burgdorfer 1957; Kocan et al. 2005; Soares et al. 2005; Antunes et al. 2014; Maldonado-Ruiz et al. 2020). However, this technique does not correspond with the natural feeding behavior of ticks so possible biases must be taken into consideration. For successful engorgement, the ticks must be fed on live animals before or after capillary tube feeding (Bonnet and Liu 2012). Blood coagulation in the tubes renders the replacements of tubes laborious (Lew-Tabor et al. 2014).

1.4.3. Automation of artificial tick feeding systems

To reduce the extensive workload associated with ATFS, scientists have attempted to automate parts of the process. Automated systems are advantageous because the blood meal may not need to be replaced frequently. First attempts of (semi-) automation were conducted in the 1970s, using filter paper to allow the blood meals to infiltrate at a slow rate (Kemp et al. 1975a) and were improved later by using a peristaltic pump either in a circular or non-circular system (Stone et al. 1983). Non-circular systems require a large amount of blood and are faced with a higher risk of contamination (Kuhnert 1995; Kuhnert 1996; Kuhnert et al. 1998; de la Vega et al. 2004). Circular system have also been developed recently and may require less blood (Vimonish et al. 2020).

Until today, only limited attempts were performed with (semi-) automated systems and thus only limited data is available on its effect on the tick feeding (Bohme et al. 2018).

1.4.4. Applications of artificial tick feeding systems

The ATFS can be used to gain insight into tick biology and physiology, the tick-microbiome-pathogen interface and tick control methods as well as providing opportunities to rear ticks in the laboratory.

A range of recent reviews has highlighted the increasing use of ATFS for ticks (González et al. 2021), in arthropods (Nijhof and Tyson 2018), for its application in vaccine development (Ndawula and Tabor 2020) and in viral (Talactac et al. 2018) or general infection studies (Bonnet and Liu 2012).

1.4.4.1. Tick biology and physiology

In vitro feeding studies by ATFS have primarily been used to study tick biology and physiology, particularly the complex feeding behavior and blood meal uptake. Early ATFS experiments

focused on the effects of different chemical and physical parameters on feeding activity and sensory physiology (Hindle and Merriman 1912; Totze 1933; Lees 1948). The ATFS enabled precise alterations of the blood meal, which gave rise to new insights into the role of albumin, heme and iron by observing tick fecundity and developmental fitness parameters (Perner et al. 2016b) or by molecular analyses of different tissues (Perner et al. 2016a; Sojka et al. 2016; Perner et al. 2018a; Perner et al. 2018b). A range of studies was performed on tick secretions such as tick paralyzing toxin found in blood meals of *I. holocyclus* adults (Stone et al. 1983) or cement components (Bullard et al. 2016). In other recent experiments, the ATFS was used to study the source of alpha-Gal in tick tissues (Crispell et al. 2019; Maldonado-Ruiz et al. 2022) and composition of water or ion channels for potential development of new tick control methods (Campbell et al. 2010; Li et al. 2019). In order to conduct studies on molecular compounds or their visualization in tick tissues, the ATFS can additionally be used to produce ticks at different stages of the engorgement cycle for dissection (Kim et al. 2014; Kim et al. 2016; Park et al. 2020).

1.4.4.2. Tick-microbiome-pathogen interface

Membrane feeding experiments may offer an alternative to infection experiments using live animals. Rather than injecting ticks with pathogens, the ATFS provides a system where pathogen acquisition and transmission occur under semi-natural conditions. Another advantage of ATFS in infection studies is a subsequent analysis of the blood meal for specific pathogens (González et al. 2021). This enables a reconstruction of the pathogen transmission dynamics, but only a few studies have described the acquisition and consecutive transmission experiments solely by *in vitro* feeding (Cotte et al. 2008; Bonnet et al. 2009; Răileanu et al. 2020; Vimonish et al. 2020; Król et al. 2021).

Currently, most infection experiments are conducted using a combination of membrane-based ATFS and live animal feedings (Howarth and Hokama 1983; Voigt et al. 1993; Waladde et al. 1993; Waladde et al. 1995; Young et al. 1996). For instance, in some studies, pathogen acquisition occurs in the ATFS and is followed by the feeding of *in vitro* infected ticks on animals to confirm pathogen transmission (Bonnet et al. 2007; Tajeri et al. 2016; Migné et al. 2022b). Instead, naturally infected ticks from infected animals can be fed on naïve blood in the ATFS (Fourie et al. 2013; Hart et al. 2018; Fourie et al. 2019). Alternatively, acquisition and transmission experiments are performed in an ATFS and live animals are subsequently injected with recently infected blood meal or the tick salivary glands in order to draw conclusions about the infectivity of the pathogens (Cotte et al. 2008; Vimonish et al. 2020). This also applies for *Ixodes* spp., as most studies only performed either acquisition or transmission experiments by ATFS without consecutive second feeding by ATFS (Liu et al. 2014; Oliver et al. 2016; Koci et al. 2018; Korner et al. 2020; Migné et al. 2022a).

For the infection, one could use blood meals from infected donor animals or the blood meal can be artificially spiked with (cultivated) pathogens (Bonnet et al. 2007; Cotte et al. 2008; Bonnet et al. 2009; Bouwknegt et al. 2010; de Carvalho Ferreira et al. 2014; Liu et al. 2014; Umemiya-Shirafuji et al. 2017; Koci et al. 2018; Korner et al. 2020; Liebig et al. 2020; Răileanu et al. 2020; Koči et al. 2021; Król et al. 2021). Naturally infected ticks collected by flagging (Krawczyk et al. 2020) or laboratory colonies have been used.

The ATFS permit alteration of the tick microbiome by treating the blood meal with antibiotics. Subsequently, the tick-microbiome interface and factors influencing tick fitness and the composition of tick symbionts can be studied in ATFS (Duron et al. 2018; Oliver et al. 2021; Guizzo et al. 2023).

1.4.4.3. Tick control

The ATFS are frequently used for drug discovery and screenings. A standardized system, the same blood meal source, a controlled laboratory method, a high reproducibility and the exact doses of substance are advantages of the ATFS for drug screening (Nijhof and Tyson 2018; González et al. 2021). Even though there are ATFS-based acaricide tests for hard ticks (Kuhnert et al. 1995; Krober and Guerin 2007b), they have primarily been used for soft ticks (Gassel et al. 2014; Williams et al. 2015; McTier et al. 2016; Perez-Sanchez and Oleaga 2017). A recent ATFS study evaluated the effect of acaricide-treated animal hair in the FU to the ticks' ability to acquire *Borrelia* spp. (Tahir et al. 2021).

The ATFS have also been used to study novel candidates for anti-tick vaccines. For example, functional analyses of specific proteins or genes can help to identify new target areas for potential tick control compounds. This can be combined with molecular methods such as RNA interference (RNAi) for specific knock-downs (Artigas-Jerónimo et al. 2021; Koči et al. 2021) or other specific inhibitors (Gobl et al. 2020). Both capillary feeding (Gonsioroski et al. 2012; Antunes et al. 2014; Lew-Tabor et al. 2014) and membrane feeding can be used to test the effect of antisera from vaccinated animals on tick feeding (Kemp et al. 1986; Trentelman et al. 2017; Knorr et al. 2018; Trentelman et al. 2019).

1.4.4.4. Tick rearing

Studying the biology of ticks under controlled conditions and developing new tick control strategies requires large numbers of ticks. For this purpose, ticks have been reared under laboratory conditions, particularly in animal experiments, for decades. The ATFS offers an *in vitro* alternative, which potentially tackles ethical and economic challenges of animal rearing, maintenance and experiments.

Mice and rabbits are frequently used as laboratory hosts for the establishment and maintenance of long-term *I. ricinus* tick colonies (Bouchard and Wikel 2005; Allan 2013). When using living animals to rear hard ticks, the number of ticks that can be fed on one animal is limited. Therefore, *I. ricinus* juvenile stages are commonly fed on smaller mammals such as mice (*Mus musculus*), guinea pigs (*Cavia porcellus*) or gerbils (*Gerbillus* spp.), while adult ticks are fed on larger mammals such as rabbits (*Oryctolagus cuniculus*), sheep (*Ovis gmelini*) or cattle (*Bos taurus*) (Allan 2013). A prerequisite for such experiments is secure animal housing and biosecurity measures to ensure that ticks will not escape. Ticks feeding on animals can be confined using sleeves, cotton ear bags or capsules attached to the animal's skin using adhesive tape (Enigk and Grittner 1953; Gregson 1966). Neck collars can be used to prevent the animal's grooming behavior. Daily tick collections or check-ups are potential stress situations for the host animal. Short-term sedation or anesthesia have been used to reduce stress levels. However, this requires the necessary material and know-how (Levin and Schumacher 2016; Nuss et al. 2017). In hard tick experiments, not only the tick rearing is time consuming, rearing host animals may arguably be even more of a timely factor. Furthermore, rabbits can develop tick-induced resistance (Rechav et al. 1989) and can therefore only be infested once or twice with ticks (Enigk and Grittner 1953; Bouchard and Wikel 2005; Troughton and Levin 2007). The number of animals required for tick rearing may result in high facilitation costs. While tick rearing on larger mammals appears more efficient because a larger number of ticks can be fed on a single animal, the requirements in terms of housing conditions, certification, staff training and veterinary care can be complex and expensive. Further, the choice of host animal must correspond with the host preference of the tick species. Consequently, the process of animal experiments has many costly, time-consuming and ethical factors. Since the 3R principle to reduce, refine and replace animal experiments was first published in 1959, the development and optimization of artificial feeding techniques for hematophagous ectoparasites has steadily increased (Russell et al. 1992; Costa-da-Silva et al. 2014).

Even though ATFS have many advantages, it is still facing major limitations, which will be highlighted in the summarizing discussion (**Chapter 4**).

CHAPTER 2

Artificial feeding of all consecutive life stages of *Ixodes ricinus*

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Artificial Feeding of All Consecutive Life Stages of *Ixodes ricinus*

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Abstract: The hard tick *Ixodes ricinus* is an obligate hematophagous arthropod and the main vector for several zoonotic diseases. The life cycle of this three-host tick species was completed for the first time in vitro by feeding all consecutive life stages using an artificial tick feeding system (ATFS) on heparinized bovine blood supplemented with glucose, adenosine triphosphate, and gentamicin. Relevant physiological parameters were compared to ticks fed on cattle (in vivo). All in vitro feedings lasted significantly longer and the mean engorgement weight of F_0 adults and F_1 larvae and nymphs was significantly lower compared to ticks fed in vivo. The proportions of engorged ticks were significantly lower for in vitro fed adults and nymphs as well, but higher for in vitro fed larvae. F_1-females fed on blood supplemented with vitamin B had a higher detachment proportion and engorgement weight compared to F_1-females fed on blood without vitamin B, suggesting that vitamin B supplementation is essential in the artificial feeding of *I. ricinus* ticks previously exposed to gentamicin.

Keywords: *Ixodes ricinus*; artificial tick feeding; in vitro tick feeding; vitamin B; life cycle

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1. Introduction

Ticks are obligate hematophagous arthropods and divided in three families: hard ticks (Ixodidae), soft ticks (Argasidae), and the monotypic Nuttalliellidae [1–3]. About ten percent of the approximately 900 known tick species are of medical or veterinary relevance and may cause direct damage due to their blood feeding habit, as well as indirect damage by acting as vectors for pathogens, including viruses, bacteria, and protozoan parasites [4,5]. In the northern hemisphere, four tick species belonging to the *Ixodes ricinus* species complex: *I. ricinus*, *I. scapularis*, *I. pacificus*, and *I. persulcatus* are of particular relevance as they may act as vectors for a number of zoonotic pathogens, including *Borrelia burgdorferi* sensu lato. In Europe, *I. ricinus* is widely distributed and can serve as a vector for other human pathogens, such as tick-borne encephalitis virus, *Babesia divergens*, and *Anaplasma phagocytophilum* as well [6,7]. *Ixodes ricinus* is a three-host tick species; all life stages (larvae, nymphs and adult females) require a blood meal from different hosts for their development. This tick species also has an extraordinary broad host range on which it can feed, ranging from small mammals to livestock, birds, reptiles and humans [8,9].

To facilitate research on hematophagous arthropods, such as mosquitoes, flies, and ticks, in vitro feeding techniques have found wide application [10–14]. In addition, they also contribute to the 3R principle to reduce, replace, and refine the use of animals in research. Artificial tick feeding systems (ATFS) have also found increased use in recent years to study tick biology, tick-pathogen interactions, drug development, and development of anti-tick vaccines under defined laboratory conditions [15–20]. ATFS also found a wide application for identifying different tick control targets or the development of anti-tick vaccines under defined laboratory conditions [17–20].

Feeding systems for hematophagous arthropods typically consists of four parts: (1) a unit containing the arthropods, (2) the blood meal, (3) a membrane that mimics skin and separates the arthropods from the blood meal, and 4) a temperature control system to heat the blood meal to a temperature corresponding to the body temperature of their homeothermic hosts [21–23].

In contrast to soft ticks and other hematophagous arthropods, which generally feed for short periods only, hard ticks feed for prolonged periods of up to several days or weeks; *I. ricinus* juvenile ticks typically feed for 3–5 days and adults for 7–12 days [24,25]. This long duration forms a major challenge in the artificial feeding process [26], as it, in combination with temperatures of approximately 37 °C, results in a higher risk of decay of the blood meal. This results in the need for regular blood changes, making the artificial feeding a laborious process, and the addition of antibiotics in the blood meal, which may affect the tick microbiome including nutritive symbionts [12,27–30]. In addition, hard ticks also have an intricate pre-feeding behavior [24,26], the mimicking of which in vitro can be complicated.

Hard ticks including *I. ricinus* are commonly reared on experimental animals [31], although reports on the in vitro feeding of nymph and adult *I. ricinus* have been published [15,32–34]. The only hard tick species for which successful feeding of all life stages has previously been reported is the tropical bont tick *Amblyomma hebraeum* [27]. Here, we report on the completion of the life cycle of *I. ricinus* in vitro by the feeding of all consecutive life stages using an ATFS and a comparison between relevant biological parameters of in vitro fed ticks and ticks fed on cattle, further referred to as in vivo fed ticks.

2. Materials and Methods

2.1. Tick Feeding

All ticks used for this study originated from a laboratory colony at the Institute of Parasitology and Tropical Veterinary Medicine of the Freie Universität Berlin. For the maintenance of this colony, larvae are routinely fed on laboratory gerbils (*Meriones unguiculatus*), nymphs on rabbits (*Oryctolagus cuniculus*), and adults on rabbits or calves (*Bos taurus*). All replete larvae and nymphs are kept at room temperature and >90% Relative Humidity (RH). Shortly after molting into the adult stage, ticks are separated by sex and stored at 12 °C and >90% RH in the dark. Replete adult ticks are kept in the dark at 20 °C, >90% RH.

For this study, 8 to 32-week-old and 8 to 15-week-old larvae were used for the in vivo and in vitro feeding, respectively. In vivo fed nymphs were fed at an age of approximately 2–4 months post molt, in vitro nymphs at 2–3 months. In vitro F_0-adult ticks were fed at an age of 7–9 months post molt, while in vitro F_1-adult ticks were fed at 5–7 months. Adults fed in vivo had an age range of 2–10 months post molt.

For our study, all life stages reported as in vivo fed ticks were fed inside linen ear bags on 14 to 18-week-old tick-naïve Holstein-Friesian calves. The ears were checked twice daily for engorgement starting at three or five days post-infestation for juvenile and adult ticks, respectively. Thirty female and 30 male *I. ricinus* adults, nine months after being fed on rabbits, were used to initiate the in vitro life cycle (F_0 adults). They were brought together in a desiccator kept at room temperature and >90% RH seven days before the start of the artificial feeding.

Blood used for in vitro feeding was drawn aseptically from cattle that grazed on pastures considered to be free of ticks; natural tick infestations were not observed during this period. All animal experiments were approved by the regional authorities for animal experiments (Landesamt für Gesundheit und Soziales, Berlin, Germany, 0387/17).

2.2. Artificial Tick Feeding System (ATFS)

The ATFS developed by Kröber and Guerin [1] was adapted as previously reported [35]. For containment of the blood, autoclaved 50 mL beaker glasses (SIMAXX, Bohemia Cristal, Selb, Germany) or sterile standard 6-well cell culture plates were used. Ten females and

10 males were placed in each feeding unit. Juvenile ticks were fed using a smaller feeding system, which fitted in a standard 12-well cell culture plate (Sarstedt, Nürnbrecht, Germany). Here, the feeding units were made up of a borosilicate glass tube (length 40 mm, inner diameter 15–16 mm; Neubert Glass Geschwenda, Germany and Glastechnik Rahm Mutterz GmbH, Switzerland), and a smaller rubber ring with an inner diameter of 18 mm (Emil Lux GmbH, Wermelskirchen, Germany). A moistened air-permeable foam plug (K-TK e.K., Retzstadt, Germany) was used instead of a plastic stopper for the smaller feeding units.

2.3. Artificial Membranes

The silicone mixture for the artificial membranes was produced as previously described [32,35]. A metal scraper (Emil Lux) was used to spread the silicone paste onto a matrix made of lens cleaning paper for adult ticks (Tiffen, Happauge, NY, USA) or goldbeater's membrane for juvenile ticks (20 µm thickness, Altenburger Pergament and Trommelfell GmbH, Altenburg, Germany). After overnight drying, the membranes were glued to glass tubes using silicone glue (Elastosil E41, Wacker, München, Germany). The membrane thickness varied from 40–50 µm, 50–70 µm, and 80–120 µm for larvae, nymphs, and adults, respectively. In the adult ATFS, a piece of 15 × 20 mm of glass fiber netting (Drahtwaren Driller GmbH, Freiburg, Germany) was glued on top of the membrane to provide a mechanical attachment stimulus. The feeding units were tested for leakage and disinfected for >10 min using ethanol (70%), followed by autoclaving of adult feeding units before use.

2.4. Animal Hair Extract

Membranes were treated with 0.35 mg (juvenile ATFS) or 0.525 mg (adult ATFS) of a low volatile mass (LVM) animal hair extract to increase their attractivity for ticks. This extract was prepared by immersing 50 g of freshly collected animal hair in a 2:1 chloroform-methanol mixture for 2 h, followed by immersion in a 1:1 chloroform-methanol mixture for a 2 h and a third immersion in a 1:2 chloroform-methanol mixture at 45 °C. The supernatant was collected after each immersion, vacuum filtered and subsequently concentrated by roto-evaporation. The extract was finally dissolved in a 1:2 chloroform-methanol mixture and stored at −80 °C. Extracts were diluted to appropriate working concentrations and stored at −20 °C. After application on the membrane, the extract was allowed to evaporate for at least 2 h before ticks were placed in the feeding unit.

2.5. Blood Meal and the Artificial Tick Feeding Procedure

Collected bovine blood was immediately treated with 20 IU/mL sodium-heparin (B. Braun, Melsung, Germany) and 2 g/L sterile glucose and stored at 4 °C for a maximum of one week. Prior to each blood change, 0.1M adenosine triphosphate (ATP, Carl Roth, Karlsruhe, Germany) dissolved in 0.9% autoclaved NaCl (VWR, Darmstadt, Germany), and 5 µg/mL gentamicin (Cellupur, Roth) were added to the blood, which was subsequently warmed to 37 °C in a water bath. The culture plates were warmed to ~37 °C using a heating plate (Hot Plate 062, Labotect, Göttingen, Germany). During the blood change, which took place every 10–14 h, the underside of the membrane was cleaned using sterile 0.9% NaCl and the feeding unit was transferred to a new cell culture plate containing freshly prepared blood meals. In general, in vitro tick feeding was performed in an incubator (ICH110C, Memmert, Schwabach, Germany) set at 20 °C, 80% RH and 4% CO_2. Adults were fed under a 16:8 light-dark-cycle, whereas juvenile stages were fed in the dark. For the in vitro F_1-adult feeding, ticks were split in different groups, which were either fed in autoclaved sterile 50-mL beaker glasses in a water bath (WNE 7, Memmert) heated to 37 °C or in an incubator as described above. Furthermore, for one group of F_1-adults the blood meal was supplemented with an aqueous sterile filtered solution of ten vitamin B components [29]. For each in vitro experiment, the temperature and RH inside the feeding

units were monitored by an iButton data logger (Maxim Integrated, San Jose, CA, USA) placed in a control feeding unit without ticks.

2.6. Data Collection of In Vitro and In Vivo Feedings

All in vitro feeding experiments were initiated in the evening. The tick feeding units were inspected with each blood change and the number of attached ticks was documented twice daily. Observations on the larvae started three days after the larvae were placed in the feeding units. For in vivo feeding experiments, the approximate number of larvae placed on an animal was calculated by dividing the weight of the larval batch with the calculated mean weight of a single unfed larva, as previously measured in batches on an analytic scale (Ohaus Discovery, Nänikon, Switzerland). After detachment, the juveniles were washed in water and air-dried. The detachment weight was measured using a balance scale (LC220S, Sartorius GmbH, Göttingen, Germany) for adults and an analytic scale for larvae and nymphs, whereby larvae were weighed in batches. All detachment weights were measured within 24 h. Only females with a weight higher than the female with the lowest detachment weight that still produced viable larvae, were taken into consideration for further analyses.

For individual females, the pre-oviposition period as well as the time between engorgement and hatching of the first larvae (pre-hatch period) were recorded. The egg batch mass was measured by an analytic scale after the first larvae had hatched or 80 days after female detachment. The hatching was scored qualitatively by estimation under stereo microscope (1: ≈0%, 2: ≈5%, 3: ≈50%, 4: ≈75%, 5: ≈100% hatching). The egg conversion ratio was calculated as egg mass divided by female engorgement weight. Molted adults were separated by sex and weighed using an analytic scale.

2.7. Video

Exemplary video recording of nymphs inside feeding units were done using a Wi-Fi otoscope camera (SB-10, Bysameyee, Shenzhen Shengyi Electronic Commerce Co, Ltd., Guangdongsheng, China) and edited in iMovie (10.1.6, Apple Inc., Cupertino, CA, USA).

2.8. Statistics

Statistical analyses and graphs were made in R version 3.6.0. For graphs, the ggplot2-package (3.3.1) [36] and the 95% confidence intervals of proportions were computed by binom.wilson from the "epitools"-package (version 0.5–1.0). Depending on normal distribution, statistical differences were calculated by either *t*-test with Welch correction or Mann–Whitney U test. For proportions of attachment, detachment, engorgement, molting, oviposition, female molting, and of deployed ticks reaching the next life stage, the Z-test was performed. Results are reported with 95% confidence intervals (CI), standard deviation or coefficient of variation (CV). For larvae hatch steps, median and interquartile range (IQR) were computed. A significance threshold of 0.05 was used. For adult ticks, individuals were taken into considerations, while for juveniles, depending on the feeding parameters either individual ticks, feeding unit batches or whole experimental replicates were taking into consideration.

To further quantify the effect of in vitro feedings on detachment weight for adults, egg batch mass and proportion of viable larvae-producing females (number of initially fed females/number of viable larvae batches), different mixed models by the glmer.nb function for count data due to overdispersion or lmer function for continuous data from the "lme4"-package (version 1.1–26) with a nested design were constructed. As fixed effect, the in vivo or in vitro feeding was assessed. To study the F_1-in vitro feeding, further fixed effects were considered: generation (F_0/F_1), vitamin B-supplementation and incubator/water bath feeding method. To account for repeated measures nested random effects for experiment treatment (in vitro/in vivo), experimental replicates (in vivo: 5, in vitro F_0: 1, in vitro F_1: 2), experimental units (in vivo: 1–2, in vitro F_0: 3, in vitro F_1: 1–2) were included.

3. Results

3.1. Feeding of F_0 Adult Ticks

The batch of F_0 adult ticks used to initiate the in vitro life cycle were fed in vitro in the winter season and had a maximum attachment of 20%. F_0-females only attached and engorged in two out of three feeding units, attachment was not observed in the third feeding unit. For comparison purposes, 450 female and 450 male ticks were fed in vivo on calves, in batches of 50 females and 50 males per ear between August 2018 and August 2020. An overview of the adult in vitro and in vivo feeding is shown in Table 1.

Table 1. Artificial feeding of F_0 and F_1 females in comparison to in vivo. Artificial feeding of 30 F_0-female *I. ricinus* ticks from in vivo origin compared to artificial feeding of F_1-in vitro generation female ticks in water bath with and without vitamin B components and to in vivo tick feeding on calves.

	Adult Feeding Experiment				**Statistical Analyses (Test, *p*-Value, df = Degrees of Freedom)**		
	In Vitro			**In Vivo** $n = 450$			
	F_0 $n = 30$	**F_1**			**In Vitro F_0 to In Vivo**	**In Vitro F_1 (Vitamin B) to In Vivo**	**In Vitro F_0 to In Vitro F_1 (Vitamin B)**
		No Vitamin B, $n = 10$	**Vitamin B,** $n = 21$				
Maximum attachment (%)	20 (CI: 9–37)	90 (CI: 59–98)	95 (CI: 77–99)	—	—	—	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 35{,}119$
Detachment (%)	20 (CI: 9–37)	50 (CI: 23–76)	90 (CI: 71–97)	71 (CI: 66–75)	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 33.312$	Z-test, $p = 0.0513$, df = 1, $\chi^2 = 3.8$	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 24.55$
Mean duration until detachment (days)	11.5 ± 0.8 (CV: 7.3)	11 ± 0.7 (CV: 6.4)	12.3 ± 2.5 (CV: 20.1)	6.9 ± 1.5 (CV: 21.3)	MWU, $p < 0.0001$, W = 1860	MWU, $p < 0.0001$, W = 5893.5	MWU, $p = 0.921$, W = 59
Mean detachment weight (mg)	136 ± 44.9 (CI: 89–183.3)	112 ± 37.5 (CI: 72.5–151)	180 ± 64.1 (CI: 149–211)	231 ± 72.3 (CI: 222.9–238.9)	*t*-test, $p = 0.003$, df = 5.5	*t*-test $p = 0.003$, df = 20.832	*t*-test, $p = 0.088$, df = 12.109
Oviposition of all detached ticks (%)	67 (CI: 29–90)	40 (CI: 11–77)	79 (CI: 56–91)	91 (CI: 87–94)	Z-test, $p = 0.0452$, df = 1, $\chi^2 = 4.011$	Z-test, $p = 0.088$, df = 1, $\chi^2 = 2.912$	Z-test, $p = 0.539$, df = 1, $\chi^2 = 0.377$
Mean duration of oviposition (days)	22.8 ± 11.4 (CV: 49.9)	19 ± 4.2 (CV: 22.3)	12.8 ± 5.4 (CV: 36.7)	35.8 ± 16.2 (CV: 45.1)	MWU, $p = 0.128$, W = 322.5	MWU, $p < 0.0001$, W = 407	MWU, $p = 0.071$, W = 51.5
Mean egg mass (mg)	46 ± 27.8 (CI: 1.7–90.2)	7.13 ± 3.37 (CI: 0–37.4)	56 ± 27.2 (CI: 40.5–71.9)	116 ± 39.5 (CI: 111–121)	*t*-test, $p = 0.0137$, df = −3.195	*t*-test, $p < 0.0001$, df = 16.215	*t*-test, $p = 0.544$, df = 4.781
Mean egg conversion factor	28.5 ± 10.5 (CI: 11.8–45.2)	6.5 ± 3.5 (CI: 0–38.3)	30.4 ± 12.3 (CI: 23.3–37.4)	47.5 ± 10.2 (CI: 46.2–48.7)	*t*-test, $p = 0.0351$, df = 3.0917	*t*-test $p = 0.0002$, df = 14.022	*t*-test, $p = 0.775$, df = 5.621
Larvae producing females per egg batches (%)	75 (CI: 30–95)	100 (CI: 34–100)	80 (CI: 54–93)	81 (CI: 76–85)	Z-test, $p = 0.745$, df = 1, $\chi^2 = 0.1056$	Z-test, $p = 0.894$, df = 1, $\chi^2 = 0.018$	Z-test, $p = 0.828$, df = 1, $\chi^2 = 0.048$
Mean larvae hatch duration (days)	59.7 ± 1.5 (CV: 2.6)	69.5 ± 0.7 (CV: 1)	64.4 ± 12.8 (CV: 19.9)	67.2 ± 6.4 (CV: 9.5)	MWU, $p = 0.071$, W = 115	MWU, $p = 0.011$, W = 661	MWU, $p = 0.995$, W = 16.5
Mean larvae hatching step (1–5)	3 (IQR: 0)	3 (IQR: 2)	5 (IQR: 2)	5 (IQR: 2)	MWU, $p = 0.072$, W = 165	MWU, $p = 0.625$, W = 960.5	MWU, $p = 0.282$, W = 7.5
Larvae prod. females per all fed females (%)	10 (CI: 3–26)	20 (CI: 5–51)	57 (CI: 36–76)	52 (CI: 47–57)	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 20.268$	Z-test, $p = 0.673$, df = 1, $\chi^2 = 0.177$	Z-test, $p = 0.0003$, df = 1, $\chi^2 = 13.224$

Data on individual ticks, mean values ± standard deviation and 95% confidence interval (CI) or coefficient of variation (CV). For larvae hatch steps, median and interquartile range (IQR) was computed. Statistical tests were performed by Z-test for proportions and by either *t*-test with Welch correction for normal distribution or by Mann–Whitney U (MWU) test for non-normal distribution, respectively. Additionally, degrees of freedom (df) and chi-square (χ^2) were computed.

In general, in vivo F_0 adult females reached a higher detachment weight (231 ± 72.3 vs. 136 ± 44.9 mg) and egg mass (116 ± 39.5 vs. 46 ± 27.8 mg) than in vitro F_0 females. The mean detachment weight in vivo was thereby 94 mg higher (linear mixed-effect model (LMM), CI: 21.33–166.66, $p = 0.011$) and the egg mass weight oviposited by the in vivo fed females was 69 mg higher (LMM, CI: 21.69–116.81, $p = 0.004$, number of observations (obs): 256). All in vitro feeding durations were longer compared to in vivo feeding (Figure 1). The duration of the in vitro feeding was significantly longer than the in vivo feeding

(Mann–Whitney U-test, $p < 0.0001$, Figure 1c). However, the pre-oviposition period was significantly shorter for in vitro fed ticks, as was the mean pre-hatch period (Table 1).

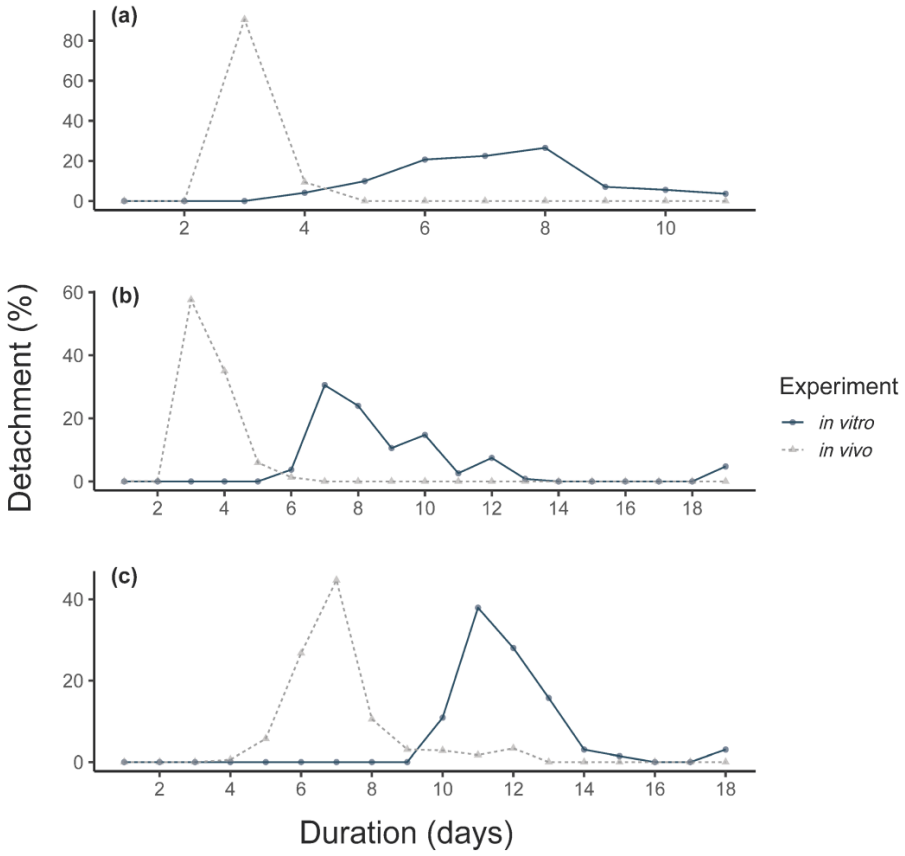

Figure 1. Mean feeding duration of (**a**) larvae, (**b**) nymphs and (**c**) adults ($F_0 + F_1$) in vitro compared to in vivo fed ticks fed on calves (means). Detachment is presented as a % of all detached ticks.

3.2. Feeding of Larvae

F_1 larvae in vitro feeding was performed in two experimental replicates with six feeding units each during the summer season. A total of 1003 larvae were fed in vitro with an average of 84 (CI: 64–103) larvae per feeding unit. To calculate the approximate number of in vivo fed larvae, the mean weight of unfed larvae was measured in batches of 83–103 larvae. Here, the mean weight of a single unfed larva was calculated to be approximately 0.0223 ± 0.0012 mg. Based on this calculation, approximately 11,737 larvae with a mean of 2347 (CI: 1044–3651) larvae were fed per calf's ear in three independent experiments in spring, summer, and autumn season.

For the in vitro ticks, an average attachment proportion of 60% was observed, with a range of 12–95% per feeding unit (Figure 2). In vitro fed larvae showed a significant higher engorgement and molting proportion compared to in vivo fed larvae (Table 2). However, the feeding duration of larvae fed in vitro was longer compared to in vivo fed larvae (Figure 1a). For larvae, contamination occurred for 3/12 feeding units after ~7 days of feeding. A total of 446 nymphs (44%) successfully molted from the in vitro fed larvae.

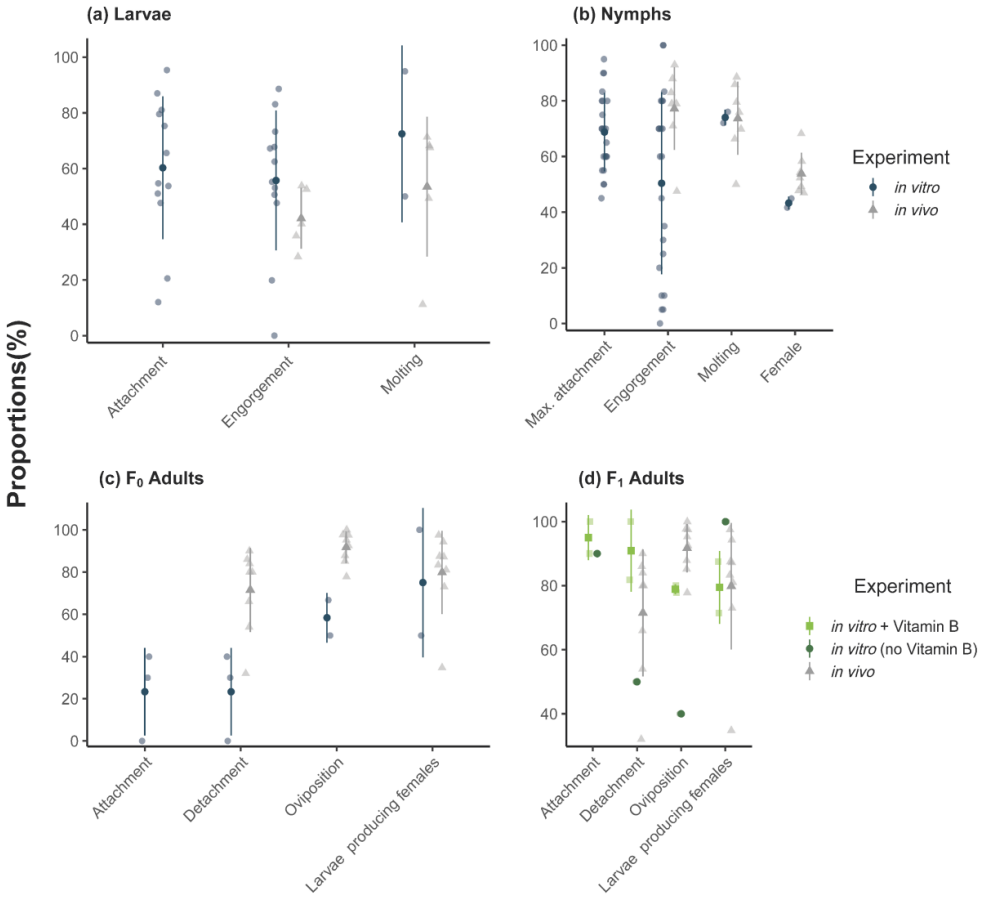

Figure 2. Mean proportions (%) per feeding unit for (**a**) larvae, (**b**) nymphs and (**c**) F_0 adults and (**d**) F_1 adults (with and without vitamin B) in vitro compared to in vivo (means ± SD). Molting proportion: molted ticks per engorged tick; oviposition proportion: egg batches per detached female; larvae production: viable larvae producing females per egg batch.

Table 2. Artificial feeding of *I. ricinus* larvae.

	Larvae Feeding Experiment		Statistical Analyses (Test, *p*-Value, df = Degrees of Freedom) In Vitro to In Vivo
	In Vitro ***n* = 1003**	**In Vivo** ***n* = 11,737**	
Attachment on day 3 (%)	60 (CI: 57–63)	—	—
Engorgement (%)	55 (CI: 52–58)	41 (CI: 40–42)	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 76.44$
Mean duration until first engorged tick (days) *	4.8 ± 0.6 (CV: 12.5)	3 (CV: 0)	MWU, $p = 0.001$, W = 55
Mean duration of feeding experiment (days) *	9.6 ± 1.3 (CV: 13.5)	3.8 ± 0.4 (CV: 11.8)	MWU, $p = 0.0018$, W = 55
Mean engorgement (mg) **	0.43 ± 0.02 (CV: 4.8)	0.53 ± 0.03 (CV: 6.4)	MWU, $p = 0.0003$, W = 1
Molting per engorged tick (%)	83 (CI: 76–84)	59 (CI: 57–60)	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 97$
Proportion of deployed larvae reaching the next life stage (%)	44 (CI: 41–48)	24 (CI: 23–25)	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 199.15$

n = number of used ticks, mean values ± standard deviation and a 95% confidence interval (CI) or coefficient of variation (CV). Statistical tests were performed by Z-test for proportions and by either *t*-test with Welch correction for normal distribution or by Mann–Whitney U (MWU) test for non-normal distribution, respectively. Additionally, degrees of freedom (df) and chi-square (χ^2) were computed. * = per feeding unit in vitro ($n = 12$) or per experiment in vivo ($n = 5$); ** = per weighted larvae batch (in vitro $n = 8$, in vivo $n = 12$).

3.3. Feeding of Nymphs

A total of 426 nymphs were fed in vitro at two occasions with a total of 21 feeding units containing 20 nymphs each and one unit containing six nymphs. The in vitro feeding of nymphs was performed in autumn. For the in vivo feeding of nymphs, a total of 800 nymphs were fed on four occasions with 100 nymphs per calf's ear in spring, summer, and autumn.

For the in vitro feeding, maximum nymphal attachment was observed after an average of four days. The first engorged nymphs per unit were collected after a mean of 6.9 days, significantly later than in vivo (2.9 days, Figure 1b). Leakage occurred in 3/21 feeding units and visible contamination was observed in 57% (12/21) of the feeding units.

The proportion of nymphs that engorged in vitro was lower than in vivo (Figure 2b, Table 3) as was their engorgement weight (in vitro: 2.82 mg, in vivo: 3.32 mg). The weights of both males (in vitro: 0.81 mg, in vivo: 0.98 mg) and females (in vitro: 1.32 mg, in vivo: 1.68 mg) that molted from in vitro engorged nymphs was significantly lower compared to the weights of adults fed as nymphs in vivo as well. A total of 157 (67 females and 90 males) F_1-adults successfully molted from the in vitro fed nymphs.

Table 3. Artificial feeding of *I. ricinus* nymphs.

	Nymph Feeding Experiment		**Statistical Analyses (Test, *p*-Value, df = Degrees of Freedom) In Vitro to In Vivo**
	In Vitro *n* = 426	**In Vivo *n* = 800**	
Maximum attachment (%)	68 (CI: 63–73)	—	—
Engorgement (%)	49 (CI: 44–54)	74 (CI: 70–86)	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 71.67$
Mean duration until first engorged tick (days) *	6.9 ± 1.4 (CV: 20.7)	2.9 ± 0.4 (CV: 2.5)	MWU, $p < 0.0001$, W = 119
Mean duration of feeding experiment (days) *	11.4 ± 2.9 (CV: 25)	5.3 ± 0.8 (CV: 14.3)	MWU, $p < 0.0001$, W = 171
Mean engorgement weight (mg)	2.82 ± 0.84 (CV: 29.7)	3.32 ± 0.96 (CV: 28.9)	MWU, $p < 0.0001$, W = 41,300
Molting rate per engorged tick (%)	75 (CI: 68–80)	73 (CI: 69–76)	Z-test, $p = 0.6117$, df = 1, $\chi^2 = 0.26$
Rate of females per molted adults (%)	43 (CI: 35–51) ♀= 67; ♂= 90	54 (CI: 49–59) ♀= 232; ♂= 197	Z-test, $p = 0.0145$, df = 1, $\chi^2 = 5.98$
Mean weight of female& male (mg)	♀1.32 ± 0.3 (CI: 1.25–1.39) ♂0.81 ± 0.16 (CI: 0.77–0.84)	♀1.68 ± 0.25 (CI: 1.65–1.72) ♂0.98 ± 0.18 (CI: 0.95–1)	♀: *t*-test, $p < 0.0001$, df = 97.74 ♂: *t*-test, $p < 0.0001$, df = 198.54
Proportion of deployed nymphs reaching the next life stage (%)	37 (CI: 32–42)	54 (CI: 50–57)	Z-test, $p < 0.0001$, df = 1, $\chi^2 = 31.33$

n = number of used ticks, mean values ± standard deviation and a 95% confidence interval (CI) or coefficient of variation (CV). Statistical tests were performed by Z-test for proportions and by either *t*-test with Welch correction for normal distribution or by Mann–Whitney U (MWU) test for non-normal distribution, respectively. Additionally, degrees of freedom (df) and chi-square (χ^2) were computed. * = per feeding unit in vitro (*n* = 22) or per experiment in vivo (*n* = 7).

3.4. Feeding of F_1-Adults In Vitro

The 51 F_1-females were split in groups. The first group of 20 females was fed under the same conditions as the F_0-adults. Although the proportion of attached females was 75% (CI: 41–100), only one female detached after 13 days with a detachment weight of 55 mg. She produced a viable egg batch of 14 mg that started hatching after 68 days. Almost all eggs hatched successfully.

The second and third F_1-adult group were given a blood meal supplemented with vitamin B (*n* = 21 females) or without vitamin B (*n* = 10 females). Both groups were fed in a water bath system (Table 1).

The mean detachment weight and mean egg mass were higher for in vivo fed females (Figure 3). The effect of vitamin B supplementation on female detachment weight and egg mass are presented in Table 4 (with F_1-adults with vitamin B in a water bath environment as intercept). There were significantly lower detachment weights for F_1 adults fed in vitro without vitamin B regardless of incubator or water bath feeding (Table 4). We also observed a negative effect of the absence of vitamin B supplementation on egg masses and on the proportion of females producing viable larvae batches. Only 9% of viable larvae batches were produced by F_1-females fed without vitamin B in the incubator (generalized linear mixed-effect model (GLM), 0.09, CI: 0.01–0.64 $p = 0.017$) and only 35% of viable larvae batches were produced by F_1-females fed without vitamin B in a water bath environment (GLM, 0.35, CI: 0.08–1.56, $p = 0.168$).

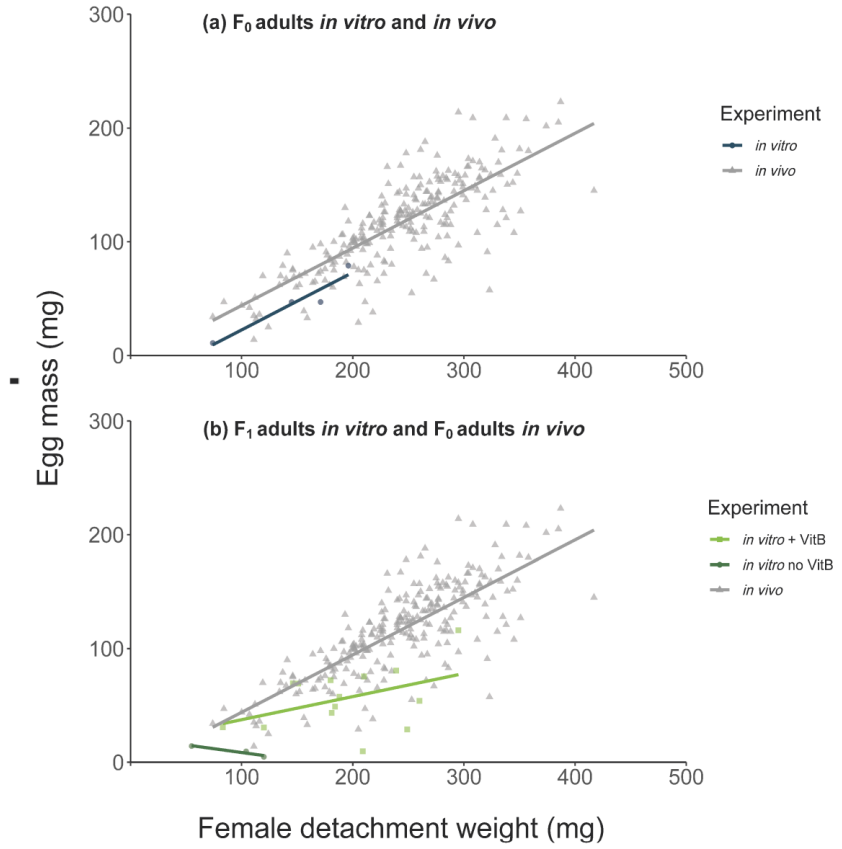

Figure 3. Egg mass (mg) and female detachment weight (mg) from (**a**) F_0 in vitro and in vivo ticks and (**b**) F_1 in vitro (water bath, with and without vitamin B) and F_0 in vivo-fed ticks.

Table 4. Detachment weights and egg masses of in vitro and in vivo fed adult ticks analyzed by four linear mixed-effect models (LMMs) using in vitro F_1-adults + vitamin B + water bath as reference group.

	Group	Estimates (mg)	95% CI	*n*	*p*
Detachment weight	F_1 + vitamin B + water bath	reference			
	F_0, no vitamin B + incubator	−43.52	(−95.64, 8.61)	31	0.102
	F_1, no vitamin B + incubator	−124.68	(−238.88, −0.48)	31	0.032
	F_1, no vitamin B + water bath	−56.48	(−112.43, −0.54)	31	0.048
	in vivo	+50.51	(4.55, 96.48)	338	0.031
Egg mass	F_0, no vitamin B + incubator	−10.22	(−39.65, 19.2)	21	0.496
	F_1, no vitamin B + incubator	−42	(−95.73, 11.72)	21	0.121
	F_1, no vitamin B + water bath	−49.09	(−88.33, −9.86)	21	0.014
	in vivo	+59.5	(30.04, 88.96)	266	<0.001

The addition of vitamin B to the blood meal in ticks fed in a water bath environment resulted in increased detachment and egg mass, although these were still lower than those observed for in vivo fed ticks (Table 4). The positive effect of vitamin B supplementation in a water bath environment to F_1 ticks was also observed for the proportion of females producing viable larvae batches, which were not statistically significant from the in vivo group (GLM, 0.89, CI: 0.41–1.91, $p = 0.763$).

It was observed on several occasions that attached females started to turn black after ~7 days of feeding and soon thereafter died. This occurred for 13/15 attached females fed on blood without vitamin B supplementation in an incubator and for 2/9 females fed on blood without vitamin B supplementation in a water bath, but was not observed for the 20 females that successfully engorged on a blood meal with vitamin B supplementation in a water bath environment.

4. Discussion

Over 25 years ago, the completion of the life cycle of *Amblyomma hebraeum* in vitro was reported [27], which until now remained the only ixodid tick species for which this was done. Although *Ixodes* nymphs and adults have successfully been fed in vitro using silicone-based membranes [19,28,32,33,37–40], only prepared animal skin membranes have previously been used for the artificial feeding of *I. ricinus* larvae [41]. We here report on the feeding of all consecutive *I. ricinus* life stages using a modified ATFS and include a comparison to *I. ricinus* ticks fed on calves.

4.1. Feeding Duration

Generally, hard ticks feed for longer periods than soft ticks [26]. In our study, in vivo fed ticks exhibited similar feeding durations as previously reported [24,25,42,43]. Significantly longer feeding durations were observed for all *I. ricinus* life stages fed in vitro. This finding was most apparent in F_0 adults and correspond to previous studies on *Ixodes* spp. [28,38,40]. Reports on feeding duration of in vitro fed ticks are quite heterogeneous, possibly due to differences in the ATFS, blood meal diets, season, tick fitness, as well as differences between tick species and their feeding behavior. It appears, for example, that adults of several metastriate tick species do not exhibit prolonged feeding durations when compared to in vivo feeding [27,44,45]. Prolonged feeding duration may be caused by longer pre-attachment times [12].

Several attempts have been made to reduce the pre-attachment duration by increasing the attractiveness of the artificial membrane, for instance by the addition of animal hair extract, tick feces or tick feces extract, animal hair or rubbing the membrane on live animals [27,46–48]. For our study, we decided to use only cow hair extract, as other additions might increase contamination risk [49]. In addition, the CO_2 level was set at 4% to stimulate tick activity [50]. Increased CO_2 levels were previously reported to increase engorgement proportions and/or detachment weights of in vitro fed *D. reticulatus* [35] and *Amblyomma* ticks [22,27].

While the attachment proportions of nymphs in vitro in individual feeding units is relatively stable and fluctuated between 45–90%, stronger fluctuations were found for adults (0–100%) and larvae (12–95%). In this study, we observed a higher maximum attachment proportion for F_1 adult ticks fed in a water bath (90 or 95%) compared to incubator-fed F_0 or F_1 adults, with attachment proportions of 20% and 75%, respectively. This might be explained by the presence of a natural light and circadian rhythm in the uncovered water bath. Another option could be an overstimulation by increased CO_2 levels, which was also observed in previous adult in vitro feeding, albeit at a higher CO_2 level of 10% [22]. However, as F_0 ticks showed a lower attachment proportion than F_1 ticks in both incubator and water bath, seasonality might also play a role, as F_0 ticks were fed during winter and F_1 ticks during summer. Another possible explanation might be an adaptation of ticks to in vitro feeding, as was for instance reported for mosquitos [51].

The complex feeding behavior and the long feeding duration of hard ticks make the evaluation of the effect of different artificial feeding conditions time-consuming, but it would be interesting to conduct further studies on the effect of factors, such as seasonality, tick age, and environmental conditions, have on the in vitro feeding success.

4.2. Tick Weights

In our study, significantly lower detachment weights were observed for all in vitro fed life stages compared to in vivo fed ticks, although the mean engorgement weights of both in vitro (0.43 mg) and in vivo (0.53 mg) fed larvae were still in the range of previous reports of larvae fed on animals (0.373–0.563 mg) [52–54].

The lower engorgement weights of in vitro fed *I. ricinus* nymphs were also in agreement with findings from previous studies (means 2.8–3 mg) [19,40]. The in vitro data showed a bimodal distribution, which is common for *Ixodes* nymphs and is related to the sex of the adult, with nymphs that molt into females having a higher engorgement weight than nymphs that molt into males [55–57]. Differences between the sex ratios of adults (67 females: 90 males in vitro, 232 females: 197 males in vivo), could at least partially explain the lower mean engorgement weights of nymphs.

The unfed F_1 adult weights were significantly lower in in vitro experiments than in in vivo experiments (unfed: ♀$p < 0.0001$, df = 97.74 ♂$p < 0.0001$, df = 198.54). The lower weight of unfed adults may be caused by the accumulative effect of the reduced engorgement weights observed for larvae and nymphs, which may eventually have resulted in smaller adult ticks. The mean weight of unfed larvae (0.0223 mg) used to calculate the approximate number of larvae used for the in vivo feedings was also lower compared to a previous report (0.034 mg) [55]. To avoid manipulation of larvae for in vivo feeding, we used this measuring method to calculate the number of deployed larvae. In contrast, in vitro deployed ticks were able to count since a smaller number of larvae were used per unit compared to a calf's ear.

Furthermore, feeding in a water bath vs. incubator environment appeared to have an impact on detachment weights of adult ticks. In the incubator, detachment weights of the F_1 adults were on average 55 mg lower than the F_0 adults, but in the water bath, F_1 ticks showed similar detachment weights as F_0 ticks (F_1: 112 ± 38 mg vs. F_0: 136 ± 45 mg). The availability of a natural light source and light-dark rhythm in the water bath might have been responsible for these differences. A larger sample size would be necessary to obtain a better understanding of the effects of different feeding environments on *I. ricinus* engorgement. For ticks fed on cattle, our findings correspond with previous findings concerning detachment weights [19,58].

4.3. Completion of Life Stages

In our study, a larger proportion of in vitro fed larvae successfully fed and molted into nymphs (44%) than in vivo fed larvae (24%). Since bovine blood was used in the ATFS, we opted for cattle for all in vivo feedings even though larvae are commonly lab-reared on mice or rabbits [31,59]. As cattle may not be the preferred hosts of *I. ricinus* larvae, it is plausible that the feeding of larvae on cattle ears reduced their in vivo feeding success.

In contrast, the proportion of nymphs that engorged in vitro (49%) was lower than in vivo (74%). This corroborates findings from previous experiments involving silicone membranes [19,40]. Experiments using animal skin membranes tend to produce higher engorgement proportions [41], presumably because ticks are more attracted to animal skin than to artificial silicone-based membranes. In our study, 75% of in vitro engorged nymphs molted successfully, which was similar to the molting success of in vivo engorged nymphs (72%) and higher than the in vitro *I. ricinus* molting success described in other studies [19,40]. However, the total outcome as proportion of molted adults from all fed nymphs was significantly lower in vitro (37%) compared to in vivo (54%).

Similar deficits were observed for in vitro F_0 adult feeding: the F_0 generation of in vitro fed adult ticks showed significantly lower detachment, oviposition and outcome

of viable larvae batches than the in vivo F_0 generation. It appeared that this could be improved by feeding F_1 adults in a water bath with a blood meal supplemented with vitamin B. Here no significant differences in detachment proportion and outcome of larvae producing females compared to in vivo fed females were observed.

4.4. Strength and Limitations

The miniature 12-well plate ATFS version presents a material-saving opportunity, as it results in a reduction of the amount of blood required. This can be of particular interest to studies in which valuable compounds such as novel drug compounds or antibodies raised against tick antigens within studies focusing on the development of anti-tick vaccines are added to the blood meal to examine their effect on tick feeding [18,60–62]. An additional advantage of this ATFS is that it also facilitates the feeding of juvenile life stages, which cannot easily be fed using capillary- or tube-based feeding systems [63].

A major limitation of the ATFS is the long feeding durations and associated risk of contamination. To limit this risk, calves were used as blood donors instead of blood collected during exsanguination at an abattoir [35,49]. Furthermore, only sterile or autoclaved materials and blood meal ingredients were used. In general, contamination started appearing after ~7 days of in vitro feeding and manifested itself by darkening and foul smell of the blood meal, and an increased white-yellow mucus on the blood side of the membrane. Fungal contamination inside the feeding unit was enhanced by small leakage of the membrane that introduced blood into the unit. When recognized quickly, ticks could be manually detached, washed and introduced in a new feeding unit or the dried blood can be discarded from the unit. Nymphs in particular tended to cluster and attach in corners of the feeding units, which may increase the risk of leakage (Supplementary Materials Figure S1, Video S1).

To further prevent bacterial growth, gentamicin was added to the blood meal, but this could not completely prevent contamination. Previous research suggests that antibiotics have a negative influence on the tick microbiome and consequently on tick fitness and fecundity [27,30,35]. Studies showed that after antibiotic treatment of ticks, hosts or blood meals, the endosymbiont density and/or composition changed, which resulted in a reduction of reproduction fitness [29,30,61,64]. or a decrease in development [65]. The use of antibiotics in the blood meal in this study could therefore explain the relatively low weights and proportions observed in the in vitro experiments. The negative impact of antibiotics on tick endosymbionts that may result in reduced tick fitness can be explained by the role that these endosymbionts appear to play in vitamin B pathways of obligate hematophagous parasites. It was previously shown that decreasing levels of tick endosymbionts, for instance as a result of antibiotic treatment, had an impact on vitamin B synthesis and tick survival [29,30,66–68]. Tick endosymbionts such as *Coxiella*-like, *Rickettsia*-like, but also *Wolbachia, Midichloria*, or *Francisella* are in focus of the vitamin B pathways [29,69,70]. To prevent a lack of vitamin B available to the tick caused by a disruption of the tick's endosymbionts, a vitamin B supplementation to the ticks' diet has been suggested [29]. In our study, one group of F_1 adults therefore received a vitamin B supplement as previously described [29,71]. The vitamin B supplement group continually showed higher detachment weights, egg masses, and higher detachment and oviposition proportions than the non-supplemented group, improving the in vitro F_0 adult feeding and rendering it nearly as successful as the in vivo group. Fewer attached females died during feeding in the vitamin B supplemented group (0/20) than in the non-supplemented group (9/20). Dead females were observed with black and sometimes spherical bodies similar to previous descriptions [67]. Furthermore, for F_1 adults, we observed a positive effect on weights and proportions. In contrast, this clear effect on weights was not seen for F_0 adults (data not shown). Nevertheless, we observed a less number of dead ticks at the end of F_0 adult feeding experiments with vitamin B supplementation and a positive effect on the proportion on females producing viable larvae batches (data not shown). It should be noted that the

addition of antibiotics to the blood meal may also impact the growth of pathogens, which should be considered in studies aiming to study tick-pathogen interactions.

While the positive effect of vitamin B supplementation in an ATFS without antibiotics may be negligible [29], further research regarding its effect on the feeding of larvae and nymphs would be justified.

5. Conclusions

All consecutive life stages of the hard tick *I. ricinus* were fed by artificial feeding and compared to data collected from experimental *I. ricinus* infestations on cattle. The data showed that the in vitro feeding of F_1 larvae was as effective as larvae feed in vivo on cattle, but the in vitro feeding of F_1 nymphs and adults was not as successful as the in vivo feeding on cattle. The complex feeding behavior of ixodid ticks such as *I. ricinus* and the prolonged duration of in vitro feeding resulted in an increased risk of contamination in the ATFS over time. The use of sterile blood, a sterile workflow and the supplementation of antibiotics to the blood meal may delay contamination, but the effect of antibiotics on tick fecundity requires further evaluation. The addition of vitamin B components to the diet of F_1 adults appeared to have a positive effect on tick feeding and fecundity. This suggests that vitamin B supplementation is essential for *I. ricinus* ticks previously exposed to antibiotic treatment, probably due to the detrimental effect of antibiotics on nutritive tick symbionts that would otherwise provide ticks with these vitamins.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/vaccines9040385/s1, Figure S1: Impressions on artificial feedings, Video S1: Artificial feeding of *I. ricinus* nymphs.

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Supplementary Materials

Artificial feeding of all consecutive life stages of *Ixodes ricinus*

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Video S1: https://www.mdpi.com/article/10.3390/vaccines9040385/s1 (accessed on 28.01.2024)

CHAPTER 3

Changes in the *Ixodes ricinus* microbiome associated with artificial tick feeding

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Changes in the *Ixodes ricinus* microbiome associated with artificial tick feeding

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Artificial tick feeding systems (ATFS) can be used to study tick biology and tick-pathogen interactions. Due to the long feeding duration of hard ticks, antibiotics are commonly added to the *in vitro* blood meal to prevent the blood from decaying. This may affect the ticks' microbiome, including mutualistic bacteria that play an important role in tick biology. This effect was examined by the consecutive feeding of *Ixodes ricinus* larvae, nymphs, and adults *in vitro* with and without the supplementation of gentamicin and in parallel on calves. DNA extracted from unfed females was analyzed by 16S rRNA sequencing. The abundance of *Candidatus Midichloria mitochondrii*, *Rickettsia helvetica* and *Spiroplasma* spp. was measured by qPCR in unfed larvae, nymphs, and adults. Larvae and nymphs fed on calves performed significantly better compared to both *in vitro* groups. Adults fed on blood supplemented with gentamicin and B vitamins had a higher detachment proportion and weight compared to the group fed with B vitamins but without gentamicin. The detachment proportion and weights of females did not differ significantly between ticks fed on calves and *in vitro* with gentamicin, but the fecundity was significantly higher in ticks fed on calves. 16S rRNA sequencing showed a higher microbiome species richness in ticks fed on calves compared to ticks fed *in vitro*. A shift in microbiome composition, with *Ca*. *Midichloria mitochondrii* as dominant species in females fed as juveniles on calves and *R*. *helvetica* as the most abundant species in females previously fed *in vitro* was observed. Females fed *in vitro* without gentamicin showed significant lower loads of *Ca*. M. mitochondrii compared to females fed *in vitro* with gentamicin and ticks fed on calves. *Spiroplasma* spp. were exclusively detected in female ticks fed on cattle by qPCR, but 16S rRNA sequencing results also showed a low abundance in *in vitro* females exposed to gentamicin. In conclusion, the employed feeding method and gentamicin supplementation affected the ticks' microbiome composition and fecundity. Since these changes may have an impact on tick biology and vector competence, they should be taken into account in studies employing ATFS.

KEYWORDS

***Ixodes ricinus*, *Midichloria*, *Rickettsia helvetica*, *Spiroplasma*, *in vitro* feeding, artificial feeding, vitamin B**

1. Introduction

Artificial tick feeding systems (ATFS) in which ticks are fed on artificial membranes or animal skin *in vitro* have been widely used, for instance in studies on tick biology, tick-pathogen interactions and the screening of anti-tick compounds under controlled laboratory conditions (Waladde et al., 1979; Kuhnert et al., 1995; Krober and Guerin, 2007; Antunes et al., 2014; Król et al., 2021; Militzer et al., 2021). In addition, ATFS contribute to the 3R principle to Reduce, Refine and Replace animal experimentation in science. Membrane-based ATFS typically consist of a tick containment unit, a membrane which mimics the skin, a blood meal and a heating device to warm the blood to 37–39°C (Nijhof and Tyson, 2018).

Hard ticks have a long feeding duration of several days to weeks and this period is usually extended when ticks are fed *in vitro* (Kuhnert et al., 1995; Militzer et al., 2021). This makes the use of ATFS challenging as the blood meal may decay due to microbial growth. The blood meal is therefore regularly changed and routinely treated with antimicrobials such as penicillin, streptomycin, rifampicin, phosphomicin, ciprofloxacin and gentamicin, or antimycotic substances such as amphotericin b or nystatin to prevent the blood from decaying (Kemp et al., 1975; Kuhnert et al., 1995; Krober and Guerin, 2007; Oliver et al., 2021). It is plausible that the addition of antimicrobial compounds to the blood meal will also affect the tick microbiome composition, but little is known about the extent of this effect.

It was previously shown that a dysbiosis of the ixodid tick microbiome after injection of ticks with antibiotics resulted in reduced fecundity, feeding, survival and/or development (Zhong et al., 2007; Kurlovs et al., 2014; Guizzo et al., 2017; Li et al., 2018; Ben-Yosef et al., 2020; Zhong et al., 2021). Alternative antibiotic treatment methods, such as feeding ticks on antibiotic-treated animals were also reported to have a negative effect on tick fecundity (Clayton et al., 2015; Zhang et al., 2017; Duron et al., 2018). Dysbiosis could also be induced by wash procedures or sterile maintenance (Narasimhan et al., 2014; Hamilton et al., 2021; Hurry et al., 2021). To our knowledge, only a single study has so far described the dysbiosis of hard ticks by feeding *Ixodes scapularis* female ticks on blood treated with antibiotics through an artificial membrane (Oliver et al., 2021). Changes to the tick microbiome were also shown to affect the vector competence of ticks: dysbiosed *I. scapularis* larvae were for instance less prone to *Borrelia* colonization (Narasimhan et al., 2014), whereas *A. phagocytophilum* colonization was increased in *I. scapularis* nymphs fed on gentamicin-treated mice infected with *A. phagocytophilum* (Abraham et al., 2017).

In Europe, *Ixodes ricinus* is the most widely distributed tick in Europe and the main vector for tick-borne pathogens causing Lyme Borreliosis and Tick-Borne Encephalitis in humans. Like all hard ticks, *I. ricinus* requires a blood meal in each parasitic life stage, i.e., as larvae, nymph and adult, in order to develop and reproduce. In *I. ricinus*, *Candidatus* M. mitochondrii (hereafter *M. mitochondrii*) is the most commonly reported maternally inherited symbiont (Gofton et al., 2015; Aivelo et al., 2019). Other bacteria associated with *I. ricinus* are *Rickettsiella* spp., *Borrelia* spp., *Spiroplasma*, *Rickettsia* spp., *A. phagocytophilum*, and *Candidatus* Neoehrlichia (van Overbeek et al., 2008; Gofton et al., 2015; Aivelo et al., 2019; Garcia-Vozmediano et al., 2021; Lejal et al., 2021). Maternally inherited bacterial endosymbionts play an important role in nutrition, defense and immune pathways. As the blood meal is lacking B vitamins and co-enzymes, it has been suggested that these and other nutrients could be provided to the tick by their endosymbionts (Gottlieb et al., 2015; Smith et al., 2015; Duron et al., 2018; Duron and Gottlieb, 2020). Biosynthesis pathways for certain B vitamins and cofactors were shown to be present in the genomes of some endosymbionts, including *M. mitochondrii* (Duron et al., 2017; Olivieri et al., 2019; Buysse et al., 2021).

The objective of this study was to compare the *in vitro* feeding of *I. ricinus* on bovine blood using an ATFS to a control group of *I. ricinus* ticks fed on cattle (C). To assess the influence of antibiotic treatment on tick feeding parameters in the ATFS, we compared ticks fed *in vitro* on gentamicin-treated blood (IVG^+) to ticks fed *in vitro* on blood without antibiotics (IVG^-). 16S rRNA sequencing was used to identify microbial communities in the ticks. The abundance of three of the most common species present in unfed females: *Rickettsia helvetica*, *M. mitochondrii* and *Spiroplasma* spp. was subsequently quantified by qPCR for samples from unfed larvae, nymphs, females and males.

2. Materials and methods

2.1. Ticks and the *in vivo* feeding

All *I. ricinus* ticks originated from a laboratory colony of the Institute for Parasitology and Tropical Veterinary Medicine of the Freie Universität Berlin. The feeding of each life stage was done in parallel for the IVG^-, IVG^+ and C groups. The study started with the feeding of *I. ricinus* F_0-larvae at 2–5 months post hatching in May–June 2019. The F_0-larvae were the offspring of four females from the laboratory colony. Nymphs that molted from these larvae were fed at 3–4 months post molting between October and November 2019, while the resulting adults were fed between 2 and 8 months after molting between July and September 2020. Ticks of the C group were fed on the ears of tick-naïve Holstein-Friesian calves that were 3.5–4.5 months of age. The estimated number of unfed larvae used was calculated by dividing the weight of the larvae batch by the calculated mean weight of a single unfed larva measured by an analytic scale. For the feeding, the base of each ear was covered with fabric-based tape (Leukoplast, BSN medical, Hamburg, Germany) after which linen ear bags containing equal amounts of ticks were placed over the ears. The ear bags were subsequently attached to the tape at the base of the ear by a second piece of Leukoplast. Detached ticks were collected twice daily. All animal experiments were approved by the regional authority for animal experimentation (LaGeSo, Berlin, 0387/17).

2.2. *In vitro* feeding (IVG$^+$ and IVG$^-$)

All feeding experiments were performed as previously reported (Militzer et al., 2021). Aseptically withdrawn heparinized bovine blood was supplemented with 2 g/L glucose and 0.1 M adenosine triphosphate (ATP, Carl Roth). Due to previous experiences with the artificial feeding of *I. ricinus* adults with blood supplemented with gentamicin, such as long feeding durations and the observation that some attached ticks turned black and died, we decided to supplement the blood meals for adults of both groups (IVG$^+$ and IVG$^-$) with B vitamin components (Militzer et al., 2021). For the ticks fed on blood supplemented with antibiotics (IVG$^+$), 5 µg gentamicin (Cellupur, Carl Roth, Karlsruhe, Germany) was added per mL of blood.

After feeding to repletion, larvae and nymphs of all groups (IVG$^+$, IVG$^-$, and C) were stored at room temperature (RT) and > 90% relative humidity (RH) under a natural light–dark regime. Adult ticks were stored at 20°C, >90% RH in darkness.

2.3. Sample preparation and DNA extraction

Immediately before each feeding experiment, unfed tick samples were collected and stored at −20°C. Prior to DNA extraction, all unfed ticks were surface-sterilized as previously described (Binetruy et al., 2019b). This was performed by washing the ticks in 1% commercial bleach for 30 s, followed by a rinsing for 1 min in three successive baths of DNA-free water. Individual females were quadrisected and nymphs and males were bisected to facilitate subsequent homogenization by crushing with a pestle. Only sterile tubes, scalpel blades and pestles were used. Genomic DNA extraction was performed using the Nucleospin Tissue XS kit (Macherey-Nagel) following the manufacturer's protocol, with an overnight lysis step and a final elution volume of 40 µL. All eluates were evaporated at RT for 10 min to remove residual ethanol. Extraction was performed for individual nymph and adult samples ($n = 5–8$ per experimental group) or in batches for larvae ($n = 13–20$). Negative controls were included for each batch of extracted DNA and consisted of tubes without tick material that were processed together with the tick samples.

2.4. Bacterial 16S rRNA sequencing

DNA from unfed ticks and negative controls were used for further NGS analysis. To amplify a 466 bp fragment spanning the V3-V4 region of bacterial 16S rRNA, primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNN GGGTATCTAAT-3′) were used. The whole amplification, library preparation and sequencing workflow including prior quality control was performed by Novogene Inc (Beijing, China). Briefly, quality control (QC) was performed on a 1% agarose gel electrophoresis. Tick DNA was subsequently diluted to 1 ng/µL using sterile water and subjected to PCR using Phusion High-Fidelity PCR Master Mix (New England Biolabs), followed by agarose gel electrophoresis. Only samples showing a bright band between 400 and 460 bp were used for library generation. The purification of PCR product mixtures was performed using the Qiagen Gel Extraction kit (Qiagen, Germany). Libraries were generated by NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) and quantified by Qubit and quantitative PCR (qPCR). Sequencing was performed on a Novaseq 6000 (Illumina) with a sequencing depth of 50 k raw reads per sample.

2.5. Bioinformatics and statistical analyses of NGS data

Processing of the sequence data including Operational Taxonomic Unit clustering was performed by Novogene Inc. Paired-end reads were merged by FLASH software (V1.2.7.) (Magoc and Salzberg, 2011) and further quality-filtered by QIIME (V1.7.0.) (Caporaso et al., 2010). Chimera sequences were identified and eliminated using the UCHIME algorithm (Edgar et al., 2011). Sequence analysis was performed by Uparse software (V7.0.1001). Mothur software was used against SSUrRNA database of the SILVA reference database (Wang et al., 2007; Quast et al., 2013) for species annotation, with a cut-off at ≥97% similarity. MUSCLE (3.8.31) (Edgar, 2004) software was used for further phylogenetic analyses. Alpha and beta diversity were analyzed by R (V 4.2.2). For data processing, the phyloseq package was used (McMurdie and Holmes, 2013). We further used the decontam package to identify possible contaminants by comparing the OTU abundance of negative controls to samples (Davis et al., 2018). Here, the prevalence method with a threshold of 0.5 was used. This was followed by trimming OTUs which were not present in any samples from our data subset. Further analysis was performed with this data subset. Alpha diversity for species richness included Chao1 and abundance-based coverage estimator (ACE), while for species diversity Shannon and Simpson index, the index of sequencing depth and observed species were included. Here, the Wilcoxon test was performed for statistical analysis and the graphs were computed by phyloseq package, ggplot2 and ggpubr packages (Wickham, 2011; McMurdie and Holmes, 2013; Kassambara, 2020). Beta diversity measures included weighted and unweighted UniFrac, focusing on relative abundances by using the Bray-Curtis distance measure computed by the phyloseq package. Further, the Non-Metric Multidimensional Scaling (NMDS) was computed by phyloseq package and a Principal Component Analysis (PCA) was computed and visualized by the MicrobiotaProcess package (McMurdie and Holmes, 2013; Xu and Yu, 2022). Statistical analyses for dissimilarity measures were performed by Permutational Multivariate Analysis of Variance (PERMANOVA) by the vegan package ($n = 999$ permutations; Oksanen et al., 2013). For beta diversity graphs, the phyloseq package was used (McMurdie and Holmes, 2013). For all statistical tests, a statistical significance level at $p < 0.05$ was set.

TABLE 1 Primers used in this study.

Organism	Target gene	Primer name	Sequence (5′–3′)	Annealing temperature for PCR (°C)	Product size (bp)	Reference
Ca. Midichloria mitochondrii	*gyrB*	gyrB-F	CTTGAGAGCAGAACCACCTA	61.5	125	Sassera et al. (2008)
		gyrB-R	CAAGCTCTGCCGAAATATCTT			
Ixodes ricinus	*Calreticulin*	calF	ATCTCCAATTTCGGTCCGGT	64.5	109	
		calR	TGAAAGTTCCCTGCTCGCTT			
Rickettsia spp.	*gltA*	Rickettsia_gltA_F1	GCTCTTCTCATCCTATGGCTATTA	59.1	499	This study
		Rickettsia_gltA_R2	TCCTTAGCTTTAGCTATATATTTAGG			
Rickettsia helvetica	*gltA*	Rhelvetica_qPCR_F2	GGAAGCAGACTACAAACTTACTGC	–	173	This study
		Rhelvetica_qPCR_R2	CTTTATATTTCGTACAAGGCGTTG			
Spiroplasma spp.	*rpoB*	Spiro_rpoB_qPCR_F1	CCAAAAGGTCAAACACAATCAAC	62.1	127	This study
		Spiro_rpoB_qPCR_R1	TACCTTGAACAATTCCAGCACC			
Spiroplasma	*gyrA*	Spixo_gyrA_F2	CCAGATGCAAGAGATGGATTG	56	561	Binetruy et al. (2019a)

2.6. Sequencing

The *Rickettsia* and *Spiroplasma* spp. detected by bacterial 16S rRNA sequencing were further identified by amplifying a ~ 499 bp region of the *Rickettsia gltA* gene and a ~ 561 bp fragment of the *Spiroplasma* DNA gyrase subunit A (*gyrA*) gene (Table 1), followed by amplicon sequencing (LGC Genomics, Berlin, Germany) and BLASTn analysis.

2.7. Plasmid DNA for standard curves

To generate plasmid DNA for standard curves that were used in the qPCR, PCR products were amplified using S7 Fusion polymerase (Mobidiag, Espoo, Finland). Each reaction mixture consisted of 5 µL of 5X HF buffer, 1 µL of each forward and reverse primer (10 µM), 0.5 µL of dNTP (2 mM), 0.25 µL polymerase, 1 µL DNA and nuclease-free water up to a reaction volume of 25 µL. Cycling conditions were 98°C for 8 s followed by 35 cycles of 94°C for 5 s, annealing for 20 s, and 72°C for 15 s, with a final extension step at 72°C for 1 min. Amplicons of the expected size were cleaned using the DNA Clean & Concentrator-5 kit (Zymo Research, Freiburg, Germany) and cloned in the pSC-B-amp/kan vector (Strataclone Blunt Cloning Kit, Agilent). Plasmids were isolated using the GenUp Plasmid Kit (Biotechrabbit, Berlin, Germany) and sequenced by LGC Genomics. Ten-fold serial dilution stocks with known copies of each plasmid DNA were prepared and stored at −20°C.

2.8. Quantification of endosymbionts

To measure the relative level of three of the most abundant bacteria identified by bacterial 16S rRNA sequencing in all unfed life stages (including the same female tick DNA samples as used for 16S sRNA sequencing), we performed qPCRs using the primers listed in Table 1. Novel primers were manually designed using NetPrimer software[1] based on nucleotide sequence alignments made in BioEdit 7.0.5.3.[2] qPCR reaction mixtures consisted of 10 µL Luna Universal mastermix (New England Biolabs, Frankfurt am Main, Germany), 1 µL of each primer (10 µM), 1 µL DNA and 7 µL nuclease-free water. Cycling conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and annealing/elongation at 60°C for 1 min in a CFX96 cycler (Bio-Rad Laboratories GmbH, Feldkirchen, Germany). All samples were run in technical duplicates. A no-template control and serial dilutions of plasmid DNA were included in each run. Results were normalized against the *I. ricinus calreticulin* (*cal*) gene as a reference gene in CFX Maestro software (Bio-Rad).

2.9. Statistical analyses for feeding and quantitative PCR data

For tick feeding experiments, analyses were computed in R (V 4.2.2.) (R Core Team, 2013) either by the Mann–Whitney U test or the *t*-test with Welch-Correction depending on normal distribution, the Z-test for proportions followed by degrees of freedom (df) and Chi-Square ($\chi 2$). Confidence intervals (CI) were computed at the 95% level and CIs for proportions were computed by the binom. wilson function from the epitools package (V 0.5–10.1). Graphs of feeding parameters and relative bacterial abundance were produced using the ggplot2 package (3.3.1) with a significance level of $p < 0.05$ (Wickham, 2011). The graphs and data analysis concerning the qPCR data on target gene/ housekeeping gene ratio were performed

1 http://www.premierbiosoft.com/netprimer/

2 http:///www.mbio.ncsu.edu/BioEdit/bioedit.html

in GraphPad Prism 9.3.1 (Graphpad Software Inc., La Jolla, United States), for which a Mann–Whitney test was performed and a value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Artificial tick feeding with and without antibiotics and tick feeding on cattle

All consecutive life stages (larvae, nymphs, adults) were successfully fed in the IVG$^+$ and the IVG$^-$ group. For the C group, feeding of consecutive larvae and nymphs on calves was also successful but feeding of the adult ticks on a calf failed for unknown reasons. Adults originating from a different larval batch to obtain comparative data for the *in vitro* feeding were used instead. Since only the microbiome of unfed larvae, unfed nymphs and unfed adult ticks originating from the same larval batches were analyzed, this did not affect the results from 16S rRNA sequencing or the qPCR analysis.

Overall, we fed an estimated number of 1,505 (IVG$^-$), 709 (IVG$^+$), and 1160 (C) F_0-larvae. For the consecutive feeding of nymphs that molted from these larvae, a total of 96 (IVG$^-$), 136 (IVG$^+$) and 200 (C) nymphs were used. These ticks were again fed after molting as adults. Here, 25 (IVG$^-$), 10 (IVG$^+$) and 100 (C) females were fed.

3.1.1. Larvae feeding

In general, we observed a statistically significant positive effect of gentamicin supplementation in the IVG$^+$ larvae group compared to IVG$^-$ for the proportion of engorged larvae and molting proportion per engorged larvae (Figure 1A; Supplementary Table S1). Although IVG$^+$ larvae showed a statistically significant higher engorgement proportion than control ticks, the molting proportion was significantly higher for larvae fed on calves.

3.1.2. Nymphal feeding

The positive effect of gentamicin observed for *in vitro* fed larvae was not seen for *in vitro* fed nymphs. Here, nymphs of the IVG$^-$ group had a higher engorgement- and molting proportion (Figure 1B; Supplementary Table S2). The weight of engorged nymphs and unfed females did not significantly differ between IVG$^+$ and IVG$^-$ (Figures 2A,B; Supplementary Table S2). No significant difference was observed for the engorgement proportion between IVG$^+$ nymphs compared to C group nymphs, but nymphs of the IVG$^+$ group did have lower engorgement weights, molting proportion and unfed female weights.

3.1.3. Adult feeding

IVG$^+$ females did not significantly differ in terms of detachment proportion and detachment weight compared to C group females. However, egg masses and the proportion of viable larvae-producing females were higher for the C group when compared to IVG$^+$. Parameters of females did not significantly differ between the IVG$^+$ and IVG$^-$ (Figures 2C,D; Supplementary Table S3).

3.2. Bacterial 16S rRNA sequencing

The DNA concentration from 14/19 unfed female tick samples (IVG$^+$: 6/6, IVG$^-$: 1/5, C: 7/8) was high enough to pass quality control analysis and was subjected to 16S rRNA amplification and sequencing. The DNA concentration of some extracts from individual ticks, in particular in the IVG$^-$ female group without gentamicin, was considered to be too low for 16S rRNA sequencing. DNA extracted from pools of larvae, individual nymphs and males did not pass the quality control of the service provider and were not sequenced. Due to the low sample size, IVG$^-$ was excluded for further analysis.

In total, 986,684 reads, resulting in 984,005 effective tags after quality filtering and comparison with reference databases were obtained (Supplementary material S1). The mean number of reads per sample in unfed females from the IVG$^+$ and C groups were 76,004 (± 11,109) and 74,064 (± 4,493), respectively. Removing contaminants by decontam package resulted in a mean library size of 75,968 (± 11,109) for IVG$^+$ and 73,820 (± 4,439) for C group. Overall, females fed as larvae and nymphs on calves had a more diverse microbiome composition compared to females of the IVG$^+$ group (Figure 3). All samples except the single IVG$^-$ female, contained *Rickettsia* and *M. mitochondrii* OTUs. *Streptomyces* ($n = 13/14$) and *Spiroplasma* ($n = 12/14$) were also detected in most samples.

The relative abundance of the bacteria differed between the two groups of females. As shown in Figure 3, the relative abundance of IVG$^+$ reared female ticks was dominated by *Rickettsia* OTUs, while females reared on calves had a higher relative abundance of *M. mitochondrii* and *Spiroplasma*.

The diversity indices were statistically compared between IVG$^+$ and C females (Supplementary Figure S1). In general, there was a tendency for higher diversity means within the C female group, which were significant for the Shannon (Wilcoxon, $p = 0.008$) and the Simpson index (Wilcoxon, $p = 0.004$). Furthermore, PCoA of unweighted and weighted UniFrac and NMDS distances of IVG$^+$ females showed a narrow spectrum in comparison to the much broader cluster of control females (Supplementary Figure S2). This bacterial community structure differed between IVG$^+$ and C females (weighted UniFrac: df = 1(11), $p = 0.002$, unweighted UniFrac: df = 1 (11), $p = 0.53$).

3.3. Sequencing of *Rickettsia* and *Spiroplasma* spp.

The *Rickettsia* species detected by 16S rRNA sequencing was identified as *R. helvetica* following sequencing of the *gltA* gene. The *gltA* sequence was 100% identical (499/499 nt) to that of the *R. helvetica* C9P9 reference strain (GenBank Accession Number

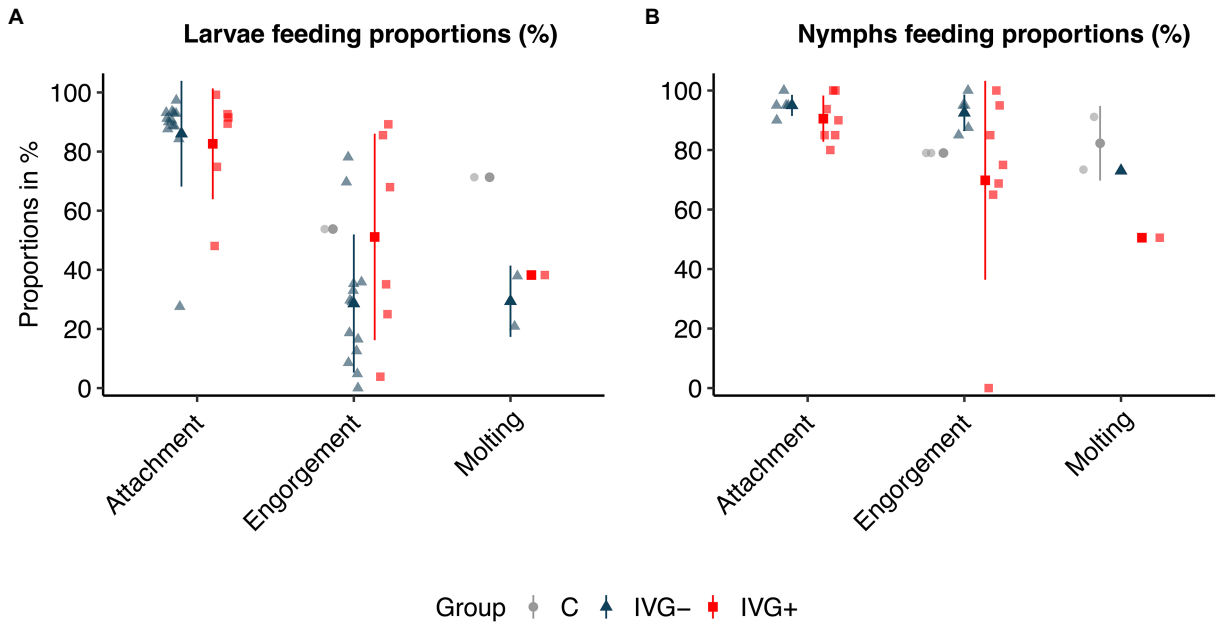

FIGURE 1
Attachment, engorgement, and molting proportions of *Ixodes ricinus* larvae **(A)** and nymphs **(B)**. Proportions are presented in % per feeding unit or per animal experiment. Bars present means ± standard deviation. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$, *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves.

CM001467). The partial *gyrA* sequence of the *Spiroplasma* species showed most identity (506/507 nt, 99.8%) to *Spiroplasma ixodetis* isolated from *I. ricinus* (MK267048) and *Ixodes uriae* (MK267049).

3.4. Quantitative analyses of bacterial loads

The qPCR data processing was limited to the three main bacterial species detected by 16S rRNA sequencing: *R. helvetica*, *M. mitochondrii*, and *Spiroplasma* species.

The qPCR data for the three F_0-larvae batches showed no significant differences for the main bacterial species: all three batches were positive for *M. mitochondrii* and *R. helvetica*, but negative for *Spiroplasma* (Supplementary Table S4).

The *M. mitochondrii* abundance was significantly higher in unfed IVG$^+$ and C females compared to the corresponding nymphal stages (Mann–Whitney test, $p = 0.0034$ and $p = 0.0129$, respectively), but this was not the case for unfed IVG$^-$ females and nymphs (Mann–Whitney test, $p = 1.0$). *Midichloria mitochondrii* was not detected in male ticks. The *M. mitochondrii* bacterial loads were significantly higher for the C females compared to IVG$^-$ (Mann–Whitney test, $p = 0.0132$; Figure 4).

Rickettsia helvetica was also present in all F_0-larvae groups (Figure 5). The relative number of *R. helvetica* bacteria increased in the nymphal IVG$^+$ and IVG$^-$ ticks, but not in the C group. The relative number of *R. helvetica* was significantly higher in IVG$^+$ nymphs compared to the C group (Mann–Whitney test, $p = 0.0043$), but did not statistically differ compared to IVG$^-$ (Mann–Whitney test, $p = 0.30$), although some IVG$^-$ nymphs tested negative for *R. helvetica* by qPCR. IVG$^+$ and IVG$^-$ ticks remained positive as adults at similar levels as nymphs, with significantly higher levels in the IVG$^+$ group compared to the C and IVG$^-$ group as both females (Mann–Whitney test, $p = 0.0298$ and $p = 0.0398$, respectively) and males (Mann–Whitney test, $p = 0.0225$ and $p = 0.0225$, respectively).

Although *Spiroplasma* was detected by 16S rRNA sequencing in most (5/6) IVG$^+$ females (albeit in low numbers) and in all C females in higher numbers, they were only detectable by qPCR in the C females (Figure 6).

4. Discussion

4.1. Effects on tick feeding

In general, ticks from IVG$^+$ and IVG$^-$ showed lower engorgement and detachment weights and a longer feeding duration compared to ticks from the C group. This was in accordance to previous work, where artificially fed ticks were compared to ticks fed on calves (Militzer et al., 2021). The highest proportion of larvae that engorged was found in the IVG$^+$ group. It should be noted that although all life stages of *I. ricinus* can feed on large ruminants, they are not commonly used as experimental animals for the feeding of *I. ricinus* larvae, so there is little information available on the feeding efficacy of larvae on cattle (Jaenson et al., 1994; Hofmeester et al., 2016; Levin and Schumacher, 2016). This may explain the limited engorgement proportion observed for the control group larvae. We nonetheless

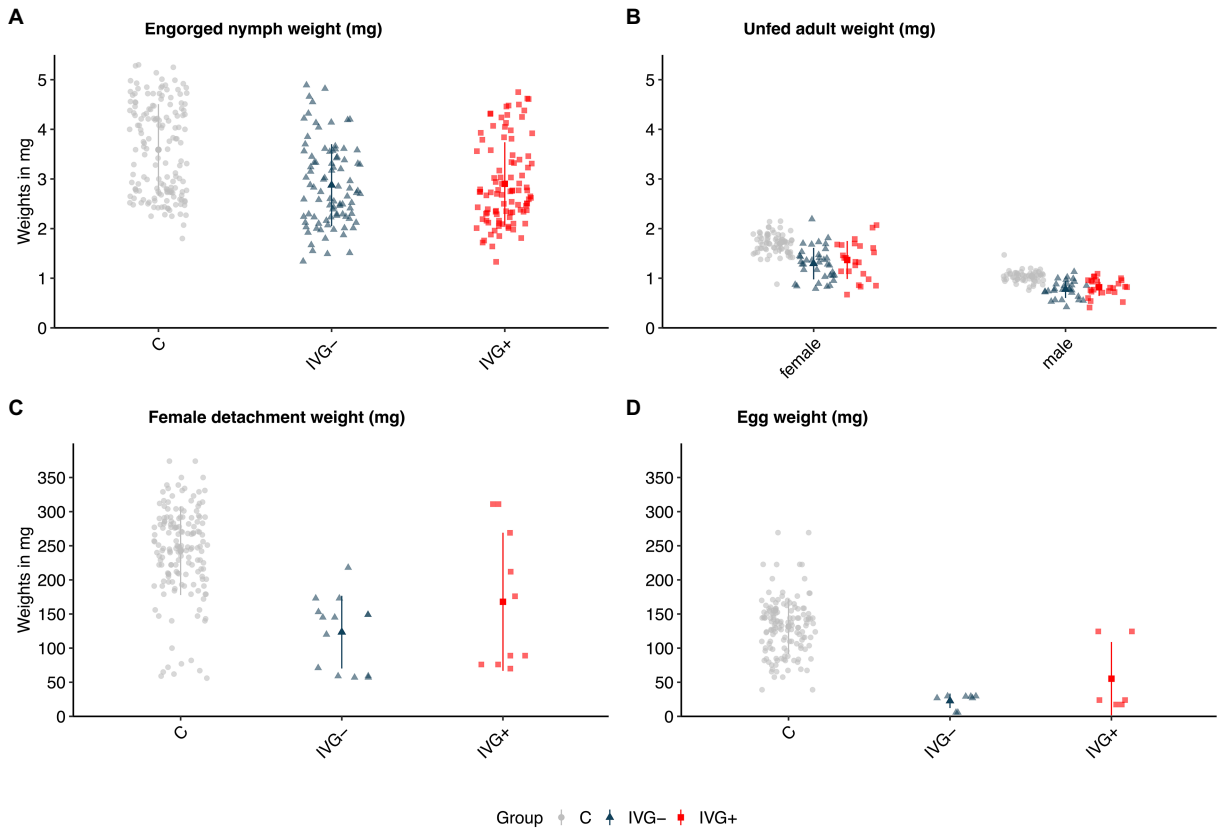

FIGURE 2
Weights (in mg) of **(A)** engorged *Ixodes ricinus* nymphs, **(B)** molted unfed adult *I. ricinus* females and males, **(C)** engorged and detached *I. ricinus* females, and **(D)** egg batches of *I. ricinus* females. Bars present means ± standard deviation. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$; *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves.

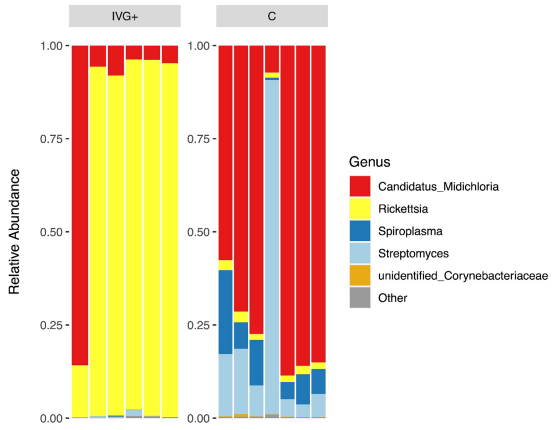

FIGURE 3
Relative abundances of the top five genera for *in vitro* reared females with gentamicin supplementation (IVG$^+$, *n*=6), and *in vivo* reared females (C, *n*=7).

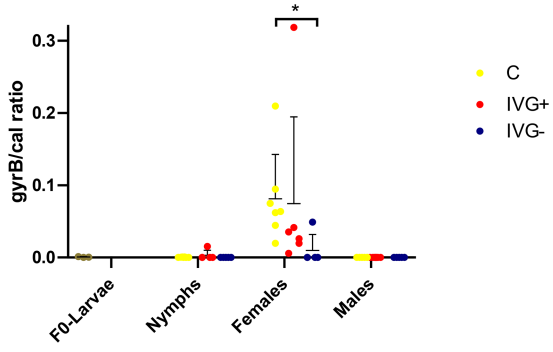

FIGURE 4
qPCR profiles (relative expression) of *Midichloria mitochondrii*. Each dot represents the relative *gyrB* expression in a single sample. The tick *calreticulin* gene was used as a housekeeping gene. Bars represent mean with the standard deviation shown in error bars. Statistical analysis by Mann–Whitney test performed in Graphpad. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$, *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves. *p<0.05.

preferred to use only cattle as hosts or blood source for all experiments to reduce variation between experiments, as the source of the blood meal was previously shown to affect tick feeding and molting (Koch and Hair, 1975; Brunner et al., 2011). The molting proportions were lower for IVG$^+$ and IVG$^-$ fed larvae compared to the C larvae, which might be explained by their

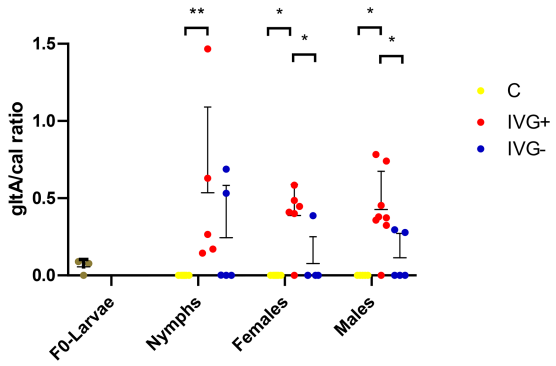

FIGURE 5
qPCR profiles (relative expression) of *Rickettsia helvetica*. Each dot represents the relative *gltA* expression in a single sample. The tick *calreticulin* gene was used as a housekeeping gene. Bars represent mean with the standard deviation shown in error bars. Statistical analysis by Mann–Whitney test performed in Graphpad. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$, *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves. *p<0.05, **p<0.01.

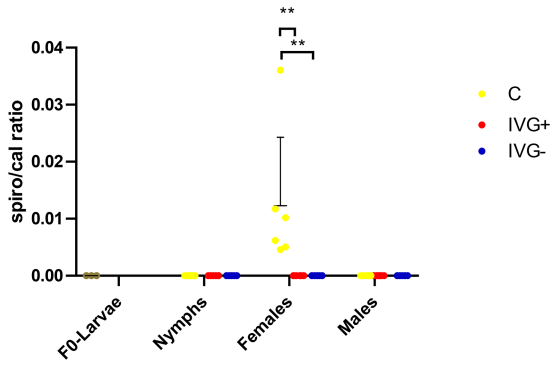

FIGURE 6
qPCR profiles (relative expression) of *Spiroplasma* spp. Each dot represents the relative *rpoB* expression in a single sample. The tick *calreticulin* gene was used as a housekeeping gene. Bars represent mean with the standard deviation shown in error bars. Statistical analysis by Mann–Whitney test performed in Graphpad. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$, *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves. **p<0.01.

significantly lower engorgement weight. Previous studies on *Amblyomma americanum* showed that the molting success of larvae and nymphs was associated to their engorgement weight; ticks that failed to reach a "critical" engorgement weight did not molt (Koch, 1986). Similar results were observed for *Rhipicephalus sanguineus* nymphs (Ben-Yosef et al., 2020).

The supplementation of the blood meal with gentamicin and B vitamins resulted in higher engorgement or detachment weights, in particular for females (IVG$^+$). In general, female ticks typically have a longer feeding duration than juvenile ticks, which increases the risk of bacterial contamination of the blood meal. This may have led to a reduction in the blood meal quality and prevented the ticks from fully engorging. Juvenile *I. ricinus* ticks did feed successfully without antibiotics, corroborating results of other studies where antibiotics were also omitted from the blood meal, for instance to prevent possible interference of antibiotics with pathogen acquisition or transmission (e.g., Waladde et al., 1993; Koci et al., 2018; Korner et al., 2020). It is important to note here that data on the artificial feeding of consecutive hard tick life stages is very limited (Kuhnert et al., 1995; Militzer et al., 2021). A possible cumulative effect of antibiotics on ticks and their endosymbionts cannot be ruled out and need to be studied further.

4.2. Effects on the microbiome diversity

In our study, the microbiome of female ticks fed as larvae and nymphs on the ears of calves was more diverse than that of the IVG$^+$ group and possibly from that of the IVG$^-$ group, for which only limited 16S rRNA sequencing data was available. This difference in variety could be explained by the feeding process: C ticks fed on calves were exposed to a wider variety of bacteria, for instance from the microbiome of the bovine skin and cerumen, compared to the IVG$^+$ and IVG$^-$ groups that were fed in a relatively sterile laboratory environment. They may therefore have acquired a more diverse set of bacteria from their environment by oral or cuticular routes compared to the IVG$^+$ ticks. In addition, the microbiome variety could have been reduced by the exposure of IVG$^+$ ticks to gentamicin, but the lack of sufficient data for the IVG$^-$ group prevents the drawing of definitive conclusions in this regard.

Interestingly, most of the IVG$^+$ and IVG$^-$ females (n = 6/7) had a high relative abundance of *R. helvetica* and low relative abundances of *M. mitochondrii* and *Spiroplasma* spp. This was in contrast to the C females, where most ticks (n = 6/7) showed a high relative abundance of *M. mitochondrii*, a finding supported by qPCR data. However, when C and IVG$^+$ groups were directly compared, the difference was not significant. This was mostly due to one sample of the IVG$^+$ group, which had an exceptionally high *M. mitochondrii/calreticulin* ratio of 0.318, corroborating its 16S rRNA sequencing result (Figures 3, 4).

Midichloria mitochondrii is abundant in various tick species collected from the field, with a reported prevalence ranging from 54.8 to 100% in *I. ricinus* females (Lo et al., 2006; Sassera et al., 2006; Duron et al., 2017; Garcia-Vozmediano et al., 2021). A prevalence below 100% may suggest that the symbiosis is not obligatory for *I. ricinus* to survive, or that other symbionts take over this role when *Midichloria* is absent (Krawczyk et al., 2022a). The number of *Midichloria* was shown to decrease after the molt of *I. ricinus* and increase during blood meal intake, suggesting that it may be relevant for tick development, for instance by providing the tick with essential nutrients that are missing in the blood meal (Sassera et al., 2008; Olivieri et al., 2019). In females, *Midichloria* have mainly been found in ovaries, which ensures its maternal transition (Epis et al., 2013; Olivieri et al., 2019). In our study, the analysis for *M. mitochondrii* was performed in unfed ticks, i.e., prior to feeding, which may explain the relatively low bacterial loads found compared to previous reports (Sassera et al., 2008).

Of note, the laboratory tick colony originated from ticks flagged in a study site that was previously shown to have a low *Midichloria* prevalence (36.4%) in nymphs (Garcia-Vozmediano et al., 2021). Furthermore, *M. mitochondrii* was not detected in male ticks, corroborating previous reports describing a low *Midichloria* abundance in males (Lo et al., 2006; Sassera et al., 2008; Lejal et al., 2020; Garcia-Vozmediano et al., 2021). Future male nymphs have also been reported to have lower *Midichloria* loads than future female-nymphs (Epis et al., 2013; Daveu et al., 2021). Since differentiation between future male and future female was not possible at the time of testing, this may also have influenced the detection of *M. mitochondrii* in nymphs in our study.

Ixodes spp. ticks have been shown to harbor several *Rickettsia* species (Kurtti et al., 2015; Hajduskova et al., 2016; Duron et al., 2017; Nováková and Šmajs, 2018). So far, *R. helvetica*, *Candidatus* R. mendelii, *R. monacensis*, *R. raoultii*, *R. slovaca*, and *Candidatus* R. thierseensis have been detected in *I. ricinus* ticks, with *R. helvetica* being the most common *Rickettsia* species found (Simser et al., 2002; Hajduskova et al., 2016; Schötta et al., 2017, 2020). The 16S rRNA sequencing results and additional *gltA* sequencing suggest that the *I. ricinus* ticks used for this study only contained *R. helvetica*. As the sequence of the 16S rRNA V3-V4 region of *R. helvetica* differs from that of the other *Rickettsia* species that have been associated with *I. ricinus*, the presence of these *Rickettsia* spp. in ticks used for this study is not plausible.

Rickettsia spp. are gram-negative intracellular alpha-proteobacteria that can be categorized in several groups (Salje, 2021). *Rickettsia helvetica* belongs to the spotted fever group (SFG), a group that contains several pathogenic species such as *R. rickettsi* and *R. conorii*, the causal agents of Rocky Mountain spotted fever and Mediterranean spotted fever, respectively, but also contains *Rickettsia* species of undetermined pathogenicity. Even though previous literature reported the detection of *R. helvetica* in a small number of diseased humans, disease causation has not been convincingly demonstrated and the pathogenicity of *R. helvetica* remains to be determined (Nilsson et al., 1999, 2010; Azagi et al., 2020).

qPCR results showed that *R. helvetica* was the predominant bacterial species in IVG$^-$ and IVG$^+$ groups, confirming the 16S rRNA sequencing data for IVG$^+$ (Figures 3, 5). This was a striking finding, as all three F_0-larvae batches with which the study started contained similar amounts of *R. helvetica* (Figure 5 and Supplementary Table S4). This suggests that artificial feeding led to a positive selection for *R. helvetica* in the majority of the analyzed samples. We hypothesize that this may have been caused by interactions between *R. helvetica* and the microbiome, which was less varied compared to the C group, leading to a dysbiosis that could have facilitated *R. helvetica* colonization of the ticks. Interestingly, a recent study described a significant reduction in the microbiota diversity in *I. ricinus* nymphs collected from humans that were infected with *R. helvetica* (Maitre et al., 2022). The authors hypothesized that *R. helvetica* may modulate the tick microbiome to facilitate colonization whereas our results raise the question if a high *R. helvetica* abundance could not actually be the result of a reduced tick microbiome diversity. The presence of *R. helvetica* in bovine blood used as a blood meal source could be an alternative explanation for the increased *R. helvetica* abundance. Although there are no reports on the detection of *R. helvetica* in bovine blood, it has been detected in the blood of other ruminants such as domestic goats (*Capra hircus*), roe deer (*Capreolus capreolus*) and sika deer (*Cervus nippon yeiensis*; Inokuma et al., 2008; Stefanidesova et al., 2008; Rymaszewska, 2018). However, the original blood samples used for the artificial feeding were not available anymore to test this hypothesis. Although blood collected from the same donor cattle several months after the use of their blood for artificial feeding of larvae tested negative for the presence of *R. helvetica* DNA by PCR (results not shown), this alternative hypothesis cannot be fully excluded. The observed high abundance of *R. helvetica* in IVG$^+$ and IVG$^-$ ticks could be useful for experimental studies in which a high pathogen abundance in ticks is advantageous. On the other hand, it also shows that the composition of the tick microbiome should be taken into account in ATFS acquisition and transmission studies, as ATFS itself may have a direct effect on the tick microbiome and tick-borne pathogen abundance. Successful colonization of *Ixodes* ticks with the causal agent of Lyme Borreliosis, *Borrelia burgdorferi sensu stricto*, has for instance been associated with a higher microbiome diversity (Narasimhan et al., 2017; Sperling et al., 2020). This should be considered in the experimental design of ATFS acquisition and transmission models for this pathogen.

A third species that was particularly abundant in the C females was *Spiroplasma* (Figure 6). *Spiroplasma ixodetis* is considered to be a facultative symbiont and has previously been detected in *I. ricinus* ticks, but its effect on ticks has not been clarified yet (Duron et al., 2017; Lejal et al., 2021). *Spiroplasma* spp. in *Ixodes* ticks are thought to maternally inherited (Beliavskaia et al., 2021) and although we did detect *Spiroplasma* OTUs in both the IVG$^+$ and C females, we could not detect *Spiroplasma* DNA by qPCR in the F_0-larvae to confirm transovarial transmission. However, this may also have been caused by limitations in the sensitivity of the used qPCR for the detection of *Spiroplasma*. A previous study on the microbiome of *I. ricinus* nymphs collected from the vegetation near Paris, France, showed a decreased abundance of *Spiroplasma* in *Rickettsia*-positive samples (Lejal et al., 2021), which corroborates with our findings where *Spiroplasma* was not detected by qPCR in ticks with a high *R. helvetica* abundance. This negative association is suggestive of competition or niche partitioning between *Spiroplasma* and *R. helvetica* (Krawczyk et al., 2022a,b).

Endosymbionts such as *Midichloria* are thought to play an important role in tick biology by providing essential B vitamins to ticks (Duron et al., 2018; Duron and Gottlieb, 2020). The most common bacteria associated with providing essential B vitamins other than *Midichloria* are *Coxiella*-like endosymbionts, *Francisella*, and some *Rickettsia* spp (Hunter et al., 2015; Duron et al., 2017). Although the production of a core set of B vitamins (biotin, riboflavin and folate) is usually associated with a single nutritional symbiont for each tick species (Duron et al., 2017), it was recently suggested that in some tick species a dual

endosymbiosis occurs whereby a second endosymbiont provides B vitamin components that the other endosymbiont cannot produce (Buysse et al., 2021). Previous analyses showed that the genome of *M. mitochondrii* contains genes for the synthesis of biotin and folate, but does not seem to have all genes required for the synthesis of riboflavin (Buysse et al., 2021). It leaves the question from which source *I. ricinus* obtains riboflavin, provided that the levels found in blood are insufficient. The genome of *R. helvetica* does not have a functional riboflavin pathway and it would be interesting to examine if the genomes of other bacteria associated with *I. ricinus*, such as *S. ixodetis*, *Rickettsiella* or perhaps *Streptomyces* species would have functional B vitamin synthetic pathways. If so, this might also explain how *I. ricinus* ticks in which *M. mitochondrii* is absent obtain essential B vitamin components. We also observed a negative association between *Midichloria* and *R. helvetica*. The same negative association was found in a previous study in which nearly 14,000 questing *I. ricinus* nymphs were screened by qPCR for tick-associated microorganisms (Krawczyk et al., 2022b). In contrast, other studies reported a positive association between *Midichloria* and *Rickettsia* spp., both in questing ticks and ticks collected from humans (Budachetri et al., 2018; Lejal et al., 2021; Maitre et al., 2022). These contrasting results may in part be explained by factors found to be of influence the microbiome composition of ticks that differed between the studies, such as environmental temperature and the identity of hosts on which the ticks fed (Swei and Kwan, 2017; Thapa et al., 2019).

A major limitation of this study is the low sample size for the 16S rRNA sequencing, due to low DNA yields. It has previously been reported that the extraction of DNA from single *I. scapularis* ticks and samples with a low biomass may result in low yields (Ammazzalorso et al., 2015). To overcome the lack of 16S rRNA sequencing data for the juvenile life stages, additional qPCRs were performed for larvae and nymphs, in which constant results for the tick *calreticulin* gene were obtained (Supplementary Table S4). It is known that low biomass samples are at a higher risk for contamination sequences than higher biomass samples (Salter et al., 2014; Eisenhofer et al., 2019; Lejal et al., 2020). Pooling of ticks would have been an alternative to increase DNA yields for sequencing and to have robust samples against biases and contamination challenges. Although pooling of ticks gives only limited insights in microbial communities and diversities, it could have been an alternative in combination with qPCR (Krawczyk et al., 2022a). Future studies should take these observations into account.

Another limitation of this study is the relatively low number of ticks that could consecutively be fed from the larval to the adult stage. The resulting sample size was too low to conduct further statistical analyses on eggs and F_1-larvae. The absence of B vitamin components in the blood meals offered to the *in vitro* fed larvae and nymphs could have negatively influenced tick fitness and development at these stages. The optimal dose of B vitamin supplementation and its effect on the larvae and nymphs should be examined in more detail in future studies aimed at optimizing the artificial feeding of *I. ricinus*.

In conclusion, we examined the microbiome of *I. ricinus* under different experimental conditions by feeding all consecutive life stages of *I. ricinus* by ATFS on blood meals with (IVG^+) or without gentamicin (IVG^-) and comparing the feeding parameters to those of ticks fed simultaneously on calves (C). The tick microbiome composition was studied by 16S rRNA sequencing and qPCRs for *M. mitochondrii*, *R. helvetica*, and *Spiroplasma* spp. The results showed a shift of the ticks' microbiome, with the symbiont *M. mitochondrii* being the dominant genus for females fed as larvae and nymphs on calves and *R. helvetica* being the most abundant bacteria in females that were fed as juveniles *in vitro*. IVG^- females showed significant lower loads of *M. mitochondrii* compared to the other groups. *Spiroplasma* spp. loads also differed: while exclusively detected in C female ticks by qPCR, 16S rRNA sequencing results also showed low relative abundances in IVG^+ females. Collectively, the results showed that the employed feeding techniques affect the fecundity and microbiome composition of ticks, with a decreased microbiome diversity in artificially fed ticks fed on blood supplemented with gentamicin. These effects should be taken into account in studies employing ATFS.

Data availability statement

The data presented in the study are deposited in the NCBI BioProject repository, accession number PRJNA905798.

Ethics statement

All animal experiments were approved by the regional authorities for animal experiments (LaGeSo, Berlin, 0387/17).

Author contributions

AN, NM, and SPS conceptualized this study. NM carried out methodology. AN and NM performed the formal analysis and wrote the original draft. All authors read and approved the final manuscript.

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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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1050063/full#supplementary-material

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Supplementary Materials

Changes in the *Ixodes ricinus* microbiome associated with artificial tick feeding

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Supplementary Figure S1: Impact on alpha diversity indices of *in vitro* reared females with gentamicin treatment (IVG^+, n=6) to *in vivo* reared female ticks (C, n=7).

Supplementary Figure S2: Beta-diversity means of *in vitro* with gentamicin reared female tick (IVG^+, n=6), *in vitro* without gentamicin treatment female ticks (IVG^-, n=1), and *in vivo* reared female ticks (C, n=7).

Supplementary Table S1: Data on *in vitro* (with gentamicin IVG^+ and without IVG^-) and *in vivo* (C) feeding of larvae ticks presented as mean ± standard deviation (SD) and the 95% confidence interval (CI) or coefficient of variation (CV).

Supplementary Table S2: Data on *in vitro* (with gentamicin IVG^+ and without IVG^-) and *in vivo* (C) feeding of nymphs presented as mean ± standard deviation (SD) and the 95% confidence interval (CI) or coefficient of variation (CV).

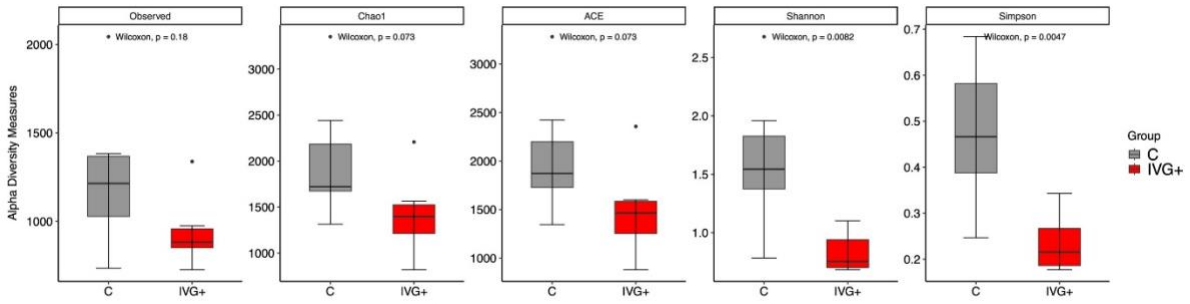
Supplementary Table S3: Data on *in vitro* (with gentamicin IVG^+ and without IVG^-) and *in vivo* (C) feeding of adult female ticks presented as mean ± standard deviation (SD) and the 95% confidence interval (CI) or coefficient of variation (CV).

Supplementary Table S4: Results of each developmental stage of mean DNA copy numbers (cal= calreticulin gene (*I. ricinus*), gyrB= *Ca*. M. mitochondrii, gltA= *R. helvetica*, rpoB= *Spiroplasma* spp.) by qPCR.

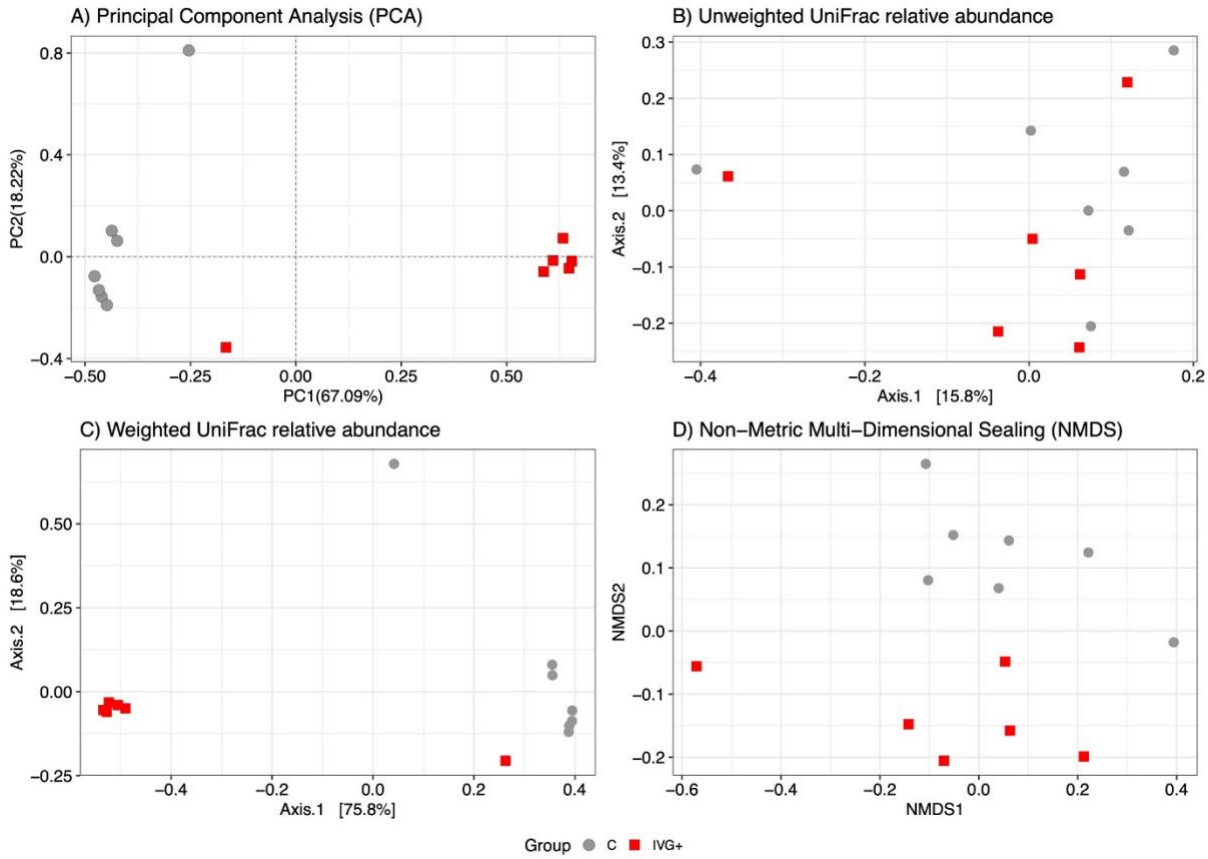
Supplementary material S1: Data Sheet 2. https://www.frontiersin.org/articles/file/downloadfile/1050063_supplementary-materials_datasheets_2_xlsx/octet-stream/Data%20Sheet%202.xlsx/1/1050063?isPublishedV2=False (accessed on 28.01.2024)

Supplementary Material

1 Supplementary Figures



Supplementary Figure 1: Impact on alpha diversity indices of *in vitro* reared females with gentamicin treatment (IVG^+, n=6) to *in vivo* reared female ticks (C, n=7): Observed species, Chao1, ACE, Shannon and Simpson index. For statistical significances (p-value) a Wilcoxon-test was performed.



Supplementary Figure 2. Beta-diversity means of *in vitro* with gentamicin reared female tick (IVG^+, n=6), *in vitro* without gentamicin treatment female ticks (IVG^-, n=1), and *in vivo* reared female ticks (C, n=7): A) Principal component analysis (PCA), B) principal coordinate analysis (PCoA) of B) unweighted and C) weighted UniFrac distances, and D) Non-metric Multi-Dimensional Sealing are presented.

Supplementary Table 1. Data on *in vitro* (with gentamicin IVG^+ and without IVG^-) and *in vivo* (C) feeding of larvae ticks presented as mean ± standard deviation (SD) and the 95% confidence interval (CI) or coefficient of variation (CV). Proportions analyzed by Z-test, analysis on engorgement weight and feeding duration analyzed by duration by Mann Whitney U test (MWU). p-value, df = degrees of freedom, $\chi 2$ = chi-square. *Larvae: per feeding unit IVG^+ (n= 6), IVG^- (n= 13), *in vivo* per feeding experiment (n= 1) **Larvae: per weighted larvae batch IVG^+ (n= 4), IVG^- (n= 3) or per experiment *in vivo* (n= 1).

Parameter	*in vitro*		*in vivo* (C)	Statistical Analyses (p-Value, df = Degrees of Freedom, χ^2 = Chi-square)		
	Without gentamicin (IVG^-)	With gentamicin (IVG^+)		IVG^- to IVG^+	IVG^- to C	IVG^+ to C
Estimated number of larvae used	1505	709	1160			
Engorgement (%)	25 (CI: 22 – 27)	61 (CI: 56 – 64)	54 (CI: 50 – 57)	df= 1, χ^2= 260.2, p< 0.0001	df= 1, χ^2= 229.5, p< 0.0001	df= 1, χ^2= 7.79, p= 0.0052
Mean duration of feeding experiment (days)*	5.8 ± 1.4 (CV: 3.7 – 4.4)	6.3 ± 0.5 (CV: 3.3 – 4.4)	4	MWU, W= 31, p= 0.652		
Mean engorgement weight (mg)**	0.358 ± 0.09 (CI: 0.135 – 0.582)	0.4 ± 0.01 (CI: 0.389 – 0.41)	0.529	MWU, W= 31, p= 0.652		
Molting per engorged tick (%)	29 (CI: 25 – 34)	38 (CI: 34 – 44)	71 (CI: 67 – 75)	df= 1, χ^2= 7.8, p= 0.0051	df= 1, χ^2= 165.2, p< 0.0001	df= 1, χ^2= 105.5, p< 0.0001

Supplementary Table 2: Data on *in vitro* (with gentamicin IVG^+ and without IVG^-) and *in vivo* (C) feeding of nymphs presented as mean ± standard deviation (SD) and the 95% confidence interval (CI) or coefficient of variation (CV). Proportions analyzed by Z-test, analysis on feeding duration and engorgement weight by Mann Whitney U test (MWU), and analysis on molted female weight by t-test. p-value, df = degrees of freedom, $\chi 2$ = chi-square. F: female ticks, M: male ticks. *Nymphs: per feeding unit IVG^+ (6 x n= 20, 1x n= 16), IVG^- (4 x n= 20, 1 x n= 16), *in vivo* per feeding experiment (2x n= 100).

Parameter	*in vitro*		*in vivo* (C)	Statistical Analyses (p-value, df = Degrees of Freedom, χ^2 = Chi-square)		
	Without gentamicin (IVG^-)	With gentamicin (IVG^+)		IVG^- to IVG^+	IVG^- to C	IVG^+ to C
Number of fed ticks	96	136	200			
Engorgement (%)	93 (CI: 85 – 96)	70 (CI: 61 – 77)	79 (CI: 72 – 84)	df= 1, χ^2= 16.5, p< 0.0001	df= 1, χ^2= 7.9, p= 0.005	df= 1, χ^2= 3.2, p= 0.075
Mean duration until feeding experiment (days)*	10 ± 1.2 (CV: 12.2)	8 ± 1.1 (CV: 13.7)	6 ± 0	MWU, W= 27, p= 0.032		
Mean engorgement weight (mg)	2.88 ± 0.83 (CI: 2.7 – 3.05)	2.92 ± 0.83 (CI: 2.74 – 3.09)	3.59 ± 0.92 (CI: 3.45 – 3.73)	MWU, W= 4324, p= 0.793	MWU, W= 3980, p< 0.0001	MWU, W= 4331, p< 0.0001
Female proportion (%)	57 (CI: 44 – 68)	48 (CI: 34 – 62)	56 (CI: 47 – 64)	df= 1, χ^2= 0.57, p= 0.4487	df= 1, χ^2 <0.0001, p= 1	df= 1, χ^2= 0.7, p= 0.419
Molting per engorged tick (%)	73 (CI: 63 – 81)	51 (CI: 40 – 60)	82 (CI: 75 – 87)	df= 1, χ^2= 8.9, p= 0.003	df= 1, χ^2= 2.4, p= 0.125	df= 1, χ^2= 27.2, p< 0.0001
Weight of molted adults (mg)	F: 1.29 ± 0.32 (CI: 1.19 – 1.4) M: 0.78 ± 0.17 (CI: 0.71 – 0.84)	F: 1.37 ± 0.38 (CI: 1.2 – 1.53) M: 0.82 ± 0.17 (CI: 0.74 – 0.89)	F: 1.7 ± 0.2 (CI: 1.65 – 1.75) M: 1.0 ± 0.1 (CI: 1.0 – 1.1)	F: df= 40 p= 0.445	F: df= 52 p< 0.0001	F: df= 26, p< 0.0001

Supplementary Table 3: Data on *in vitro* (with gentamicin IVG$^+$ and without IVG$^-$) and *in vivo* (C) feeding of adult female ticks presented as mean ± standard deviation (SD) and the 95% confidence interval (CI) or coefficient of variation (CV). Due to the failure to feed adults *in vivo* in July 2020, adult *in vivo* data had to be conducted using ticks from a new tick batch (also fed as larvae and nymphs on calves) in parallel to *in vitro* feedings. Proportions analyzed by Z-test, analysis on durations by Mann Whitney U test (MWU), and analysis on weights and masses by t-test. p-value, df = degrees of freedom, $\chi 2$ = chi-square. F: female ticks, M: male ticks.

Parameter	*in vitro*		*in vivo* (C)	Statistical Analyses (p-value, df = Degrees of Freedom, χ^2 = Chi-square)		
	Without gentamicin (IVG$^-$)	With gentamicin (IVG$^+$)		IVG$^-$ to IVG$^+$	IVG$^-$ to C	IVG$^+$ to C
Number of fed ticks (females : males)	25 : 20	10 : 10	100 : 100			
Detachment >55mg (%)	40 (CI: 23 – 59)	70 (CI: 39 – 89)	87 (CI: 79 – 92)	df= 1, $\chi 2$= 1.4, p= 0.108	df= 1, χ^2= 25.4, p<0.0001	df= 1, χ^2= 2.1, p=0.146
Mean detachment weight (mg)	129 ± 53 (CI: 91 – 167)	172 ± 97 (CI: 81 – 262)	233 ± 74 (CI: 217 – 249)	df= 8, p= 0.322	df= 13, p< 0.0001	df= 6, p= 0.152
Mean duration until detachment (days)	17.3 ± 6.2 (CV: 36)	15.3 ± 6.8 (CV: 44.2)	6.9 ± 1.2 (CV: 17.2)	MWU, W= 29, p= 0.587	MWU, W= 856.5, p< 0.0001	MWU, W= 600, p< 0.0001
Oviposition (%)	50 (CI: 23–76)	43 (CI: 15 – 75)	86 (CI: 77 – 92)	df= 1, $\chi 2$= 0.08, p= 0.772	df= 1, $\chi 2$= 8.13, p= 0.004	df= 1, $\chi 2$= 8.62, p= 0.003
Mean pre-oviposition duration (days)	23.2 ± 14.9 (CV: 64.3)	21.3 ± 12.7 (CV: 53)	13.9 ± 1.8 (CV: 13.3)	MWU, W= 7, p= 1	MWU, W= 266, p= 0.094	MWU, W= 166, p= 0.236
Mean egg mass (mg)	23 ± 12 (CI: 4 – 41)	55 ± 60 (CI: 0 – 204)	132 ± 41 (CI: 122 – 141)	df= 2, p= 0.449	df= 8, p< 0.0001	df= 2, p= 0.156
Larvae producing females (%)	8 (CI: 2 – 25)	20 (CI: 5 – 51)	71 (CI: 61 – 79)	df= 1, χ^2= 1, p= 0.313	df=1, χ^2= 32.7, p< 0.0001	df= 1, χ^2= 10.6, p= 0.001

Supplementary Table 4. Results of each developmental stage of mean DNA copy numbers (cal= calreticulin gene (*I. ricinus*), gyrB= Ca. *M*. mitochondrii, gltA= *R. helvetica*, rpoB= *Spiroplasma* spp.) by qPCR; n= sample size per group, SD= standard deviation, IVG^+ = *in vitro* with gentamicin treatment, IVG^- = *in vitro* without gentamicin, C= *in vivo* ticks fed on calves.

copy numbers		**cal copy number**			**gyrB copy number**			**gltA copy number**			**rpoB copy number**		
Stage	**Group**	**Mean**	**n**	**SD**	**Mean**	**n**	**SD**	**Mean**	**n**	**SD**	**Mean**	**n**	**SD**
F_0-Larvae		13143	3	3701	8,0	3	7,69	841,00	3	730	0	3	
Nymphs	IVG-	5823	5	1188	0,2	5	0,42	1556,00	5	2275	0	5	
	IVG+	74454	5	94153	28,8	5	57,5	14429,00	5	12969	0	5	
	C	6959	8	1059	1,9	8	2,23	0,51	8	0,46	0	8	
Females	IVG-	251209	5	69653	2367,0	5	5293	18637,00	5	41673	0	5	
	IVG+	249803	6	155306	11434,0	6	9580	103945,00	6	74715	0	6	
	C	240673	7	85157	14425,0	8	9755	0,20	8	0,2	2421	7	2019
Males	IVG-	15670	5	4738	0,0	5		1644,00	5	2327	0	5	
	IVG+	13311	8	4038	0,0	8		5806,00	7	838	0	8	
	C	27876	5	5820	0,0	5		0,08	5	0,103	0	5	

CHAPTER 4

Summarizing discussion

Although *I. ricinus* is the most common hard tick species in Western and Central Europe and acts as a major vector for diseases of human and veterinary medical concern, our knowledge of its biology and its vector biology is still limited. Laboratory tick rearing and experiments are necessary in the development of novel control options, such as anti-tick vaccines and the discovery and testing of novel acaricidal drugs. For this purpose, ATFS may provide a useful alternative method to feeding experiments on live animals. Over the course of the last decade, different set-ups of ATFS have been used in tick feeding experiments. However, the exact consequences of using ATFS on the biology of different tick species and its application in addressing specific experimental research questions are often not clear. Thus, the overarching objective of the work performed as part of this thesis was to establish and further study ATFS as a tool for feeding all life stages of *I. ricinus*. **Chapter 1** provides a brief overview of the biology of *I. ricinus* and ATFS methodology. **Chapter 2** describes the first successful feeding of all consecutive life stages of *I. ricinus* in an ATFS and thus provides further insight into the use of *in vitro* tick feeding in comparison to feeding on experimental animals. In **Chapter 3**, we explore the use of gentamicin-treated blood meals in ATFS and its effect on the tick microbiome. A detailed discussion of the strengths and bottlenecks of ATFS as well as an outlook for future research questions are included in the summarizing discussion in **Chapter 4**.

4.1. Artificial tick feeding system as an alternative to animal experiments

A major strength of ATFS is the opportunity to contribute significantly to the **3R principle** of Replacing, Reducing and Refining animal experiments (Russell et al. 1992). This refers to all attempts to substitute or replace the use of live animals, reduce of the number of animals used in animal experiments and improve or refine the conditions under which animals are used in experiments. This can include attempts to reduce the number of interventions performed on an animal as well as to decrease the severity of interventions (Russell et al. 1992). In recent times, other “Rs” such as responsibility, reproducibility or reliability have been added to the list (Klein and Bayne 2007; Aske and Waugh 2017; Percie du Sert et al. 2020).

In theory, the use of artificial feeding systems for ticks as well as other arthropods supports the replacement of the animal component in the feeding process (Costa-da-Silva et al. 2014). Their increased employment in feeding experiments or even mass rearing of facultative blood-feeding species such as mosquitos (Foster 1995; Gonzales et al. 2018) as well as obligate

hematophagous species with short feeding periods such as bed bugs, tsetse flies or soft ticks (Mews et al. 1977; Endris et al. 1986; Montes et al. 2002; Aak and Rukke 2014) is therefore unsurprising. However, the longer feeding duration of hard ticks in general, but as also shown in **Chapter 2** and **3** for ATFS in particular, renders the use of ATFS for mass rearing for these species more challenging.

Nevertheless, at this point in time, often ATFS still rely on products of animal origin such as the blood meal, the broadly used Baudruche membrane or membranes derived from animal skin (Bonnet et al. 2007; Migné et al. 2022b). While artificial membranes have been developed using materials like silicone or cellulose, and both options are associated with advantages and disadvantages, direct comparisons have not yet been performed. Hence, a complete replacement of all animal components remains rare in tick *in vitro* feeding experiments and particular attention must also be paid to ethical methods of husbandry, material collection and euthanasia of the animals involved in regard to the 3Rs.

Spotlight on artificial diets

The blood meal used in the vast majority of ATFS (including the experiments described in **Chapter 2** and **3**) represents another important animal-origin component of many feeding experiments. In order to achieve a replacement at this level, further research into **artificial diets for ticks** is required.

Generally, the blood meal of vertebrates has low concentrations of carbohydrates, lipids and vitamins and mainly consists of protein, heme and iron (Sterkel et al. 2017; Whiten et al. 2018). An effective artificial blood meal must mimic these characteristics and additionally contain feeding stimulants and essential nutrients for the tick to develop successfully and produce viable offspring. Attention must also be paid to the absence of phagoinhibitory substances or contaminants as well as substances which interfere with arthropod behavior or immunity or negatively impact the tick microbiome (Gonzales and Hansen 2016). Further, physical-mechanical (viscosity, temperature, texture) and chemical (molarity, pH) characteristics may play a role and should be given some consideration (Lance and McInnis 2021). For mosquitos, several artificial blood meal diets have been studied, with iron, amino acids, sugars, cholesterol and phagostimulant substances proving to be of particularly high relevance (Gonzales and Hansen 2016).

For ticks, nutrients derived from blood meal digestion and the tick microbial community are hemoglobin, iron, carbohydrates, lipid but also cofactors and vitamins. Although vertebrate blood lacks some of these potential components, the role of the interaction between the blood

meal and the tick microbiome is the missing piece of the puzzle to provide essential nutrients to the tick.

Hemoglobin in the blood meal is essential for female hard ticks to produce viable offspring due their inability to synthetize heme (Perner et al. 2016b; Perner et al. 2022). Hemoglobin-depleted blood meals have also been associated with poorer feeding results in juvenile ticks, giving rise to the assumption that heme is similarly essential in juvenile feeding or development (Kemp et al. 1975a; Voigt et al. 1993; Trentelman et al. 2017). For soft ticks, successful feeding of several generations on fetal calf serum was reported (Ruheta et al. 2005), which may be explained by their ability to deposit heme and thus a reduced need for regularly dietary hemoglobin (Hatalová et al. 2023). Thus, it seems plausible that heme, derived from red blood cells, should be a mandatory addition to any artificial diet for hard ticks (Perner et al. 2016b).

The nutritional roles of iron and amino acids are not fully understood yet and seem to be interact with the microbial community of the tick. In terms of amino acids – serotonin, chorismate or tryptophan were able to restore the nutritional deficiency caused by the loss of the *Coxiella* symbiont in *Haemaphysalis longicornis* (Zhong et al. 2021). However, whether this is the case for other tick species such as *I. ricinus* needs to be further investigated.

Further, carbohydrates such as glucose or lipids such as cholesterol should be taken into consideration to supplement artificial blood meal diet (Perner et al. 2016a; Cabezas-Cruz et al. 2017; Hu et al. 2020). However, more research is required on their effects on tick parameters in ATFS.

Arthropods rely on micronutrients such as vitamins (particularly B vitamins) and co-factors (e.g. flavin adenine dinucleotide (FAD), coenzyme A (CoA), nicotinamide adenine dinucleotide phosphate (NADP+) (Sonenshine and Stewart 2021)) most likely for feeding, survival, development or reproduction (Hosokawa et al. 2010; Nikoh et al. 2014; Duron et al. 2018; Serrato-Salas and Gendrin 2023). However, limited information is available on the specific functions of each micronutrient for ticks. The vitamin B cocktail used in **Chapter 2** and **3** for adult ticks and in other studies (Duron et al. 2018; Artigas-Jerónimo et al. 2021) could offer a sufficient vitamin B source. It is likely that similar results could be achieved in artificial blood meals, but a series of research questions must be answered before definitive conclusions can be drawn. For instance, which life stages specifically rely on vitamin B supplementation and to what extent? What are the needs and effects over time and tick generations (F_0 vs. F_1 generations)? What vitamin B dosages are necessary to achieve sufficient supplementation throughout the tick life cycle?

Besides the benefits in regard to the 3R principle, artificial diets are likely to be less prone to contamination, easier to standardize and reproduce as well as being more cost effective as

commercial sterile blood meals and accessible. The use of transparent artificial diets in infection studies may allow better visualization of the transmission of substances or pathogens to and from the artificial diet. Improved survival or growth conditions for pathogens may also be achieved when using an artificial diet. In the studies described in **Chapter 2** and **3**, a bovine blood meal was used to allow for better comparison to *in vivo* feeding on calves. Overall the research question, study design, tick species, life stage, accessibility, financial aspects, material resources, and ethical guidelines must be considered in each individual case when choosing a nutrient source for ticks in ATFS.

When materials of animal origin are employed in tick feeding experiments, specific refinement steps must be taken wherever possible. As described in **Chapters 2** and **3,** the use of bovine blood usually requires live blood from donor animals. Factors such as breed, temperament, age and sex can have an effect on the stress threshold of animals and must be considered when choosing appropriate donor animals (Murphey et al. 1981; Haskell et al. 2014). The animals should then be handled regularly and trained young to accustom them to the sampling procedure. Positive reinforcement training is an effective tool and has been used for husbandry training of various animals (Schapiro et al. 2001; Laule 2003; Lomb et al. 2021). However, the research regarding cattle is relatively limited (Lomb et al. 2021; Nawroth 2022; Heinsius et al. 2023). Stress-free housing conditions and the use of calming odors have additionally proven to be beneficial (Nawroth 2022).

In the studies described here, we were able to achieve successful consecutive feeding of all three life stages of *I. ricinus* (**Chapter 2**). Furthermore, **Chapter 2** and **3** give data for membrane-based *I. ricinus* larvae feeding. This, as well as the comparison to *in vivo* feedings, provides valuable insight into suitable sample sizes in tick feeding experiments, which can act as a guideline for future research in order to reduce the number of ticks and animals used for tick feeding. While some validation remains necessary to establish gold standards, this is a first step towards a reduction of animal experiments in future tick feeding experiments. Mass rearing of ticks and feeding to full repletion in the ATFS remains challenging and a series of unanswered questions prevail. Data on consecutive feeding and its effect on tick fitness and fecundity is still particularly rare. Nevertheless, the data presented here illuminates the advantages of ATFS and highlights their potential to replace individual steps including acquiring partly fed ticks (Bullard et al. 2016; Kim et al. 2016; Kim et al. 2018; Liebig et al. 2020; Maldonado-Ruiz et al. 2022) and, eventually, the whole feeding process.

In conclusion, ATFS have the potential to contribute considerably to the 3R principle in research on ticks and tick-borne diseases but further experiments are necessary to overcome the challenges for consecutive feeding and the mass rearing of ticks.

4.2. Artificial tick feeding systems provide opportunities to mimic the natural feeding process of ticks within a controlled laboratory setting

Under controlled laboratory settings, it is possible to study the effect of individual factors on the tick and its microbiome. Examples include environmental conditions such as temperature or humidity as well as a reduction of potential environmental contaminants to the tick microbiome.

As described in **Chapter 3**, ticks fed on live animals show a higher microbial diversity than ticks fed artificially. Ticks fed on live animals come into direct contact with the host animal and its environment, whereas ticks in ATFS can be fed in disinfected or autoclaved FUs, with sterile blood meals with the possible addition of antibiotics in an incubator system or water bath. While the full extent to which the commensal microbiota on the host skin influences the tick microbiota is not known, it is plausible that a relationship between these factors exists. However, with relatively short on-host phases and long periods of time spent off-host, contact to microbial communities in the environment such as in soil or on plants may also play a significant role (Narasimhan et al. 2014; Brinkerhoff et al. 2020; Li et al. 2022).

Microbiota can be acquired and transmitted vertically (transovarial, transstadial) from the female tick via the ovaries/ eggs and to other life stages or can be acquired through direct contact via cuticular or oral routes. In addition, the acquired blood meal will also have an influence on the tick's microbial community. As emphasized in **Chapter 1,** the host species and its blood meal composition, the infection status and microbial composition of the host population as well as the tick's individual immune system all play a role in shaping the tick microbiome (Swei and Kwan 2017; Chicana et al. 2019; Krawczyk et al. 2022a; Narasimhan et al. 2022).

As stated above, in contrast to the relatively sterile surroundings in an ATFS, ticks fed on live animals are exposed to a plethora of bacteria from the environment and host skin (Narasimhan et al. 2014; Brinkerhoff et al. 2020; Guizzo et al. 2022). Some of these microbes are potentially transient and could influence the tick's innate immune system and thus the overall tick microbiome (Narasimhan et al. 2021). This may have been the case for the *in vivo* fed ticks in the **Chapter 3**, potentially explaining the different bacterial diversity seen compared to ATFS-fed ticks. Furthermore, the tick immune responses may have been influenced by the potentially

transient microbiota encountered as all F_0-larvae derived from females fed *in vivo* were later applied to the ATFS. This immune response may have led to changes of the microbial composition resulting potentially in a removal of bacteria or an inability to survive the altered environment in the ATFS. However, further research is needed to investigate these hypotheses.

In the experiments described in **Chapter 3**, ticks fed by ATFS showed higher loads of *R. helvetica* than ticks fed on live animals. This gives rise to the question of where individual symbionts and pathogens in ticks originate. Although the transmission of *R. helvetica* through bovine blood meals appears to be unlikely (as described in detail in **Chapter 3**), no comparative analyses of the blood meal were performed. This was a limitation of the study and an analysis of the microbial composition of the blood meal offered *in vitro* prior to its use should be considered in future experiments as to not inadvertently infect ticks with TBPs.

In certain studies, ATFS are particularly valuable because, as opposed to live animal experiments, it allows for to collect the tick saliva during the feeding process, to bypass the animal host immune response or to avoid a dilution effect (Bullard et al. 2016; Perner et al. 2018b; Filatov et al. 2023). Moreover, the blood meal can be customized and adapted to the specific question at hand. For instance, coloring compounds added to the blood meal can help to support the visualization of the feeding process in arthropods (Kocan et al. 2005; Ten Bosch et al. 2022), provide details on the feeding habits of *I. ricinus* males as found in our studies and elsewhere (Tahir et al. 2021), and potentially shed light on enigmatic processes such as tick regurgitation and its potential role in pathogen transmission (Brown 1988; Connat 1991; Pospisilova et al. 2019).

The blood meal can also be altered through supplementation (or lack) of exact, predefined concentrations of certain compounds such as heme, vitamin B, antibiotic or acaricidal substances and potential anti-tick vaccine candidates (Perner et al. 2016b; Duron et al. 2018; Knorr et al. 2018). Each compound can be tested individually for its effect on the tick and the potential effect on the tick microbiome can be explored. Not only the blood meal can be supplemented, but also the FU itself, for instance with acaricide-treated animal hair (Tahir et al. 2021). Thus, the controlled artificial feeding setting is ideal for testing candidates for anti-tick vaccines or acaricidal drug screenings.

Vitamin B supplementation to the blood meal was shown to restore negative effects caused by antibiotic treatments. For adult tick feedings in **Chapter 2 and 3**, the recipe for B vitamins that were added to the blood meal was based on previous publications (Lake and Friend 1968; Duron et al. 2018). However, the optimal dosage of B vitamins for *I. ricinus* remains unclear and this could be further investigated, specifically regarding the three “key” B vitamins

riboflavin (B2), biotin (B7) and folic acid (B9) (Buysse et al. 2021). While overdoses may not be likely as B vitamins are water-soluble and excreted quickly in animals, higher doses of certain B vitamins such as biotin have been shown to lead to a decrease in fecundity in insects (Benschoter and Paniagua G 1966; Pillai and Madhukar 1969) and possibly in blood-sucking bed bugs (Hickin et al. 2022). However, these findings are controversial as some studies reported a neutral or even positive effect of providing additional B vitamins to the artificial diet of for blood-sucking arthropods (Michalkova et al. 2014; Snyder and Rio 2015; Duron et al. 2018; Ju et al. 2020). Besides the complex B vitamin cocktails previously used (including the studies described here), a recent study showed that the addition of only the three key B vitamins to the blood meals for soft ticks restored negative effects caused by certain antibiotics (Taraveau et al. 2023).

Antibiotic treatment of the blood meal may reduce or eliminate symbionts involved in vitamin B pathways. In the studies described here**,** all adult ticks (**Chapter 2**: F_1 adults only) were fed on a blood meal supplemented with B vitamins, regardless of whether it had been treated with an antibiotic. Juvenile ticks were fed on blood meals that had not been supplemented with B vitamins. Preliminary observations suggest that F_1-larvae fed on gentamicin-treated blood meals for more than one generation benefit from vitamin B supplementation and showed higher engorgement and molting proportions (unpublished observations). This suggests that more research will be required to study the combined use of B vitamins and antibiotics in the artificial feeding of juvenile ticks over a longer time period. The potential interaction between antibiotic-treated blood meals and vitamin B supplementation over two consecutive feedings was recently described in a soft tick (Taraveau et al. 2023) and may differ to hard ticks such as *I. ricinus*. As previously described for insects, quantifying B vitamins in *I. ricinus* tissue, for example using biomarkers (Douglas 2017), could provide further insight into the base-line levels of key B vitamins (Michalkova et al. 2014; Snyder and Rio 2015; Douglas 2017; Ju et al. 2020).

To further study the potential interaction between antibiotic treatment and B vitamins, the experimental study outline should include positive and negative controls resulting in four groups ($VitB^+$ IVG^- / $VitB^+$ IVG^+ / $VitB^-$ IVG^- / $VitB^-$ IVG^+). Due to the limited feeding success for *I. ricinus* adults during the study described in **Chapter 2** and the low number of females reared in the study described in **Chapter 3**, we decided to supplement both study groups (with and without antibiotics, IVG^+ and IVG^-) for adult feeding with a vitamin B cocktail. This has been in line with our study question, as we intended only to study the effect of *in vitro* vs. *in vivo* feeding and the impact of antibiotic treated *in vitro* feedings, but does limit the study results described in **Chapter 3**.

Spotlight on artificial tick feeding system used for Ixodes ricinus *microbiome investigations*

In the study described in **Chapter 3**, qPCR data showed a rapid increase in *M. mitochondrii* in *I. ricinus* females in particular for the antibiotic-treated *in vitro* group (IVG^+) and the *in vivo* C group as opposed to juveniles. This was presented as *gyrB/ cal* ratio referring to *M. mitochondrii* genes per tick genes. However, *gyrB/ cal* ratios were low for all life stages. Previous studies have shown that *M. mitochondrii* loads tend to be low in unfed ticks and increase during feeding (Sassera et al. 2008; Olivieri et al. 2019). This is assumed to be related to an increase in metabolic activity in the tick during feeding and is also seen during oviposition and the molting process (Sassera et al. 2008; Taraveau et al. 2023). All ticks employed in **Chapter 3** study had molted (2- 8 months post molt) and were collected immediately prior to feeding as unfed tick samples, which could explain these numbers. In addition, the tick colonies used in this study were bred under laboratory conditions, which has been associated with decreased loads of *M. mitochondrii* for as long after 5- 10 generations (Lo et al. 2006; Cafiso et al. 2019).

In addition, albeit not statistically significant (most likely due to an outlier), results from 16S rRNA sequencing and qPCR showed that *I. ricinus* females that had been fed by ATFS showed lower abundances of *M. mitochondrii* compared to those fed *in vivo* (**Chapter 3**). Nonetheless, although low, the prevalence of *M. mitochondrii* was 100 % in IVG^+ and C females but *M. mitochondrii* was only detected in 1/5 IVG^- female samples.

To date, only limited data is available on the exact role and function of *M. mitochondrii*. As previously shown, the genome of *M. mitochondrii* includes B vitamin synthesis pathways (in particular biotin and folate), which suggests a nutritional role for *I. ricinus* as blood meals are lacking B vitamins (Sassera et al. 2011). For a long time, the role of *M. mitochondrii* was assumed to be of an obligate nature due to its potential involvement in vitamin B pathways, its primary location in reproductive tissues and high prevalence found in nature. Recently, there has been a shift in the assumption considering *M. mitochondrii* rather a facultative symbiont of *I. ricinus* ticks. As outlined in **Chapter 1**, obligate symbionts maintain a mutualistic co-dependent relationship with their hosts, making them indispensable to the survival of the tick. In contrast, facultative symbionts may be beneficial to the host but are not strictly essential for host survival. Facultative symbionts often have free-living phases and are generally acquired transiently by their host (Song et al. 2022). The prevalence of *M. mitochondrii* in *I. ricinus* is extremely variable, ranging from 54.8- 100 % in wild-caught females and even lower in laboratory tick colonies (Lo et al. 2006; Garcia-Vozmediano et al. 2021). In addition, it appears to be able to transfer to ticks and other arthropods horizontally and complete co-cladogenesis

has not been confirmed (Epis et al. 2008; Buysse and Duron 2018; Al-Khafaji et al. 2019; Floriano et al. 2023), which provides indications that it actually is a facultative symbiont.

However, a recent study did report a reduced feeding success in *M. mitochondrii*-free *I. ricinus* larvae compared to wild-type larvae (Guizzo et al. 2023). This could be indicative of a bigger dependency of *I. ricinus* on *M. mitochondrii* than previously thought. Exploring whether these negative effects that were achieved by antibiotic treatments can be rescued by vitamin B supplementation could provide valuable insight into the role of *M. mitochondrii* but was not performed in this experiment. Nevertheless, an interference of other symbionts affected through the antibiotic treatment could not be ruled out (Guizzo et al. 2023).

As *M. mitochondrii* seems to be able to synthetize biotin and folate, but not riboflavin, this raises the question on how *I. ricinus* obtains riboflavin (considered to be the third key B vitamin)? One could extend this question to **how does *I. ricinus* obtain essential B vitamins if *M. mitochondrii* is not present?** In our study, only 1/5 IVG^- females tested positive for *M. mitochondrii* so it may be possible that in the absence of *M. mitochondrii*, other symbionts take over the functions of B vitamin synthesis. According to Buysse *et al*., FLE and *M. mitochondrii* co-occur in *Hyalomma marginatum* ticks. They deduced that the demand for essential B vitamins is catered to in a complementary manner through dual symbiosis ticks (Buysse et al. 2021). Similar observations of complementary metabolic activity have been made in aphids (Manzano-Marín et al. 2020; Manzano-Marín et al. 2023).

Based on the 16S rRNA sequencing outcomes presented in **Chapter 3** and other previous studies, *R. helvetica, S. ixodetis, Rickettsiella* spp. or *Streptomyces* spp. were also abundantly present and may be candidates for complementary vitamin B synthesis in *I. ricinus*.

Previous studies have shown an involvement of *Rickettsia* spp. in biotin and folate pathways in *Ixodes* spp. ticks (Hunter et al. 2015; Al-Khafaji et al. 2020), but no reports exist on riboflavin pathways. While it is unclear whether this is transferrable to *I. ricinus* ticks, a negative correlation appears to exist between the presence of *R. helvetica* and the presence of *M. mitochondrii*, as well as between *S. ixodetis* and *M. mitochondrii* in *I. ricinus* (Krawczyk et al. 2022a; Krawczyk et al. 2022b). Results from our study were in line with this observation, which supports the notion that an absence of *M. mitochondrii* could potentially be compensated through *Rickettsia* activity. In our study, *R. helvetica* was predominantly present in both *in vitro* groups. As *R. helvetica* is not found in bovine blood (as discussed in **Chapter 3**), it is highly unlikely that an acquisition occurred via the blood meal. Instead, this result suggests that a positive selection for *R. helvetica* occurred in the ATFS.

In addition, it has been shown that the genetic make-up of *S. ixodetis* allows for a certain degree of involvement in the synthesis of folate and other B vitamins in insects (Yeoman et al.

2019). This can be augmented by a co-infection with *A. phagocytophilum* (Hodosi et al. 2022), which was however not detected in our samples. A recent study showed that *S. ixodetis* did not have an effect on reproductive parameters in experimentally infected *H. longicornis* ticks (Ogata et al. 2023). In our study in **Chapter 3**, in line with previous research, the presence of *S. ixodetis* was positively correlated with the presence of *M. mitochondrii* and negatively correlated with the presence of *R. helvetica* (Lejal et al. 2021; Krawczyk et al. 2022a; Krawczyk et al. 2022b), which suggests that the two compete for existence within the tick. In addition, *S. ixodetis* was only found in C group females by qPCR, which suggests that perhaps only *in vivo* fed females benefitted from the complementary activity.

While there is a scarcity of data on the role of *Rickettsiella* spp. in the tick, it has been reported to be involved in all B vitamin pathways except for biotin in the poultry red mite *Dermanyssus gallinae* (Price et al. 2021; Fukatsu et al. 2023). However, *Rickettsiella* was not detected in our samples using 16S rRNA sequencing. This is in line with findings from a previous study, where ticks collected from the same geographical location as the laboratory colony used here tested negative for *Rickettsiella* (Garcia-Vozmediano et al. 2021).

Streptomyces spp. may also be of interest in the vitamin B pathways. It is a transient facultative symbiont and has been shown to provide antimicrobial compounds to mites (Swe et al. 2019; Thapa et al. 2019). In our 16S rRNA sequencing samples, it was found in *in vivo* fed females (n= 8/8) and in ticks previously fed using the ATFS (n= 6/7).

Besides these, there may be a range of other bacteria, potentially facultative and transient, that could be involved in essential B vitamin supplementation in the tick host. However, it is clear that we still lack information on the extend of the roles and functions of transient microbiota. Recently, the importance of transient bacteria for a dual symbiosis theory in aphids based on horizontal gene transfer (HGT) was highlighted (Manzano-Marín et al. 2023).

In general, microbes are known to perform HGT with other microbes or potentially with eukaryotes. Horizontal gene transfer has been suggested by reports for *Rickettsia* spp. in insects, but also in *I. scapularis* including an operon covering even the complete biotin synthesis pathway (Gillespie et al. 2012; Davison et al. 2022). Further, HGT was described for the transfer of genetic elements involved in the biotin and folate pathways in ticks (Gillespie et al. 2012; Smith et al. 2015; Buysse et al. 2021) whereas thus far, HGT of genetic material involved in the riboflavin pathway has only been reported for insects (Manzano-Marín et al. 2020).

Another explanation to consider is that we may have failed to detect one (or more) symbionts in *I. ricinus* of relevance for supplying the tick with B vitamins. For instance, the analysis methodology included the use of the hypervariable regions V3-V4 only and a conservative

QIIME pipeline (Prodan et al. 2020). Further, the sampling and sample preparation techniques including tick washing procedures may have impacted the results. The timing of sampling and the use of individual ticks rather than pools increased the risk of contamination (Hoffmann et al. 2020; Fernández-Ruiz et al. 2023). The use of genomic DNA samples does for example not allow for differentiation between metabolic active/ inactive or dead cells and with the use of 16S rRNA and qPCR analysis to detect specific microbial species, we risked overlooking other microbial actors such as viruses, archaea and eukaryotes such as fungi or parasites. Very little is known about their roles in the microbiome of ticks (Stewart and Bloom 2020; Liu et al. 2022).

To date, the role of vitamin B in the tick is still shrouded in mystery. Extensive dietary studies including genomic analysis and quantification of B vitamin concentrations could shed light on the actual provision of vitamin B content in blood meals (Strong et al. 1941; Schweigert and Pearson 1947; Kahoun et al. 2022), the demand the tick has for each individual B vitamin and how these B vitamins are acquired. An additional focus should be on the impact of the different blood meal sources in three-host generalists like *I. ricinus* as well as the tick's ability to store B vitamins for the long duration between two blood meals, as has been described for certain species of insect that feed on vitamin-poor plant phloem sap (Blow et al. 2020). The hypothesis that selection pressure and cost-benefit balance have given rise to the existence of microbiome-free animals including arthropods could be explored as a result (Hammer et al. 2019; Krawczyk et al. 2022a). The data presented in **Chapter 3** could serve as a basis for future studies on the role of vitamin B and the dual-symbiosis hypothesis.

The ATFS offers a promising tool to perform standardized studies on the tick microbiome in future. In contrast to **artificial infection techniques** such as capillary tube feeding, tick injections or tick immersion, the membrane-based ATFS offers an opportunity to mimic the natural transmission routes of microbiota. Ticks are pool feeders and the tick's salivary glands and saliva are important components of the tick-host interface (Nuttall 2019) and play a big role in the transmission of pathogens. As a result, ATFS experiments have shown promising results in TBEV infection studies when compared to the immersion technique (Migné et al. 2022b). Further, ATFS can be used to study different pathogen transmission routes such as via feces, which can be easily collected in the FU and subsequently analyzed (Korner et al. 2020).

Different research questions may nonetheless call for different infection techniques and the pathogen pathways within the tick must be taken into consideration to maximize the benefits of employing ATFS in infection studies. The infection status of the tick and the blood meal must

for instance be known, as pathogens are a part of the microbiome and interact with the microbiome and the host. Limitations must also be taken into consideration. For instance, with a lack of immunologically active components from animal hosts, the relevance of ATFS experiment results and the transferability to vector competence in nature may become compromised.

Vector competence describes the ability of an arthropod (= the tick) to acquire, replicate and transmit pathogens to the susceptible, naïve host, evoking an infection/ disease (de la Fuente et al. 2017; Mota et al. 2023). For ticks, this also includes a transstadial/ transovarial transmission (or “transstadial passage”) to the next life stage (Kahl et al. 2002). To test the vector competence, at least two consecutive feedings (one using an infected blood meal and the second using a naïve blood meal) are required.

To establish a successful *in vitro* infection model, solely testing pathogen DNA in newly developed tick samples or in naïve blood meal most likely seems not to be sufficient as genomic components may persist in the tick and thus only gives limited results on whether the pathogens are still vivid or even infectious. Possible alternatives include DNA detection in salivary glands (depending on the pathogen (Estrada-Peña et al. 2013)), detection of pathogen RNA (Koci et al. 2018; Migné et al. 2022b) or recultivation, all of which have been described for ATFS- infected *Ixodes* spp. (Oliver et al. 2016; Korner et al. 2020; Król et al. 2021). However, proofing the ability to evoke an infection or disease, animal models are still inevitable. For instance, they have been included in some experiments to confirm transmission of viable and infectious pathogens from the ATFS- infected tick to the naïve blood meal either by injecting “infected” blood meals into the animals or by infesting the animal by infected ticks (Oliver et al. 2016; Vimonish et al. 2020; Migné et al. 2022b).

Standardized laboratory conditions are advantageous in ATFS-based infection experiments because the dose of pathogens can be standardized. However, with the enormous variety and combinations of pathogen species, strains, cultivation passage, tick species and life stages, information regarding optimal infection dose, confirmed uptake of pathogens including risk of sedimentation over time and potential instability or survival rate in the blood meal as well as differences between tick feeding success is often unavailable. Detailed laboratory protocols for each tick species, life stage and different pathogens are required to maximize the benefits of the ATFS.

Recently, the **tick-microbiome-pathogen** interface has received increased attention in research and there is no doubt that an interaction between them exists (Cabezas-Cruz et al. 2018; Bonnet and Pollet 2021; Mota et al. 2023). Nevertheless, information on the exact interplays, genetic data and information regarding function of individual players is still lacking.

With cultivation of symbionts still limited, the complex, bi- (or multi) directorial relationships and different interactions of environmental and obligate microbiota are difficult to understand. To date, different reports of interactions after dysbiosis of the tick microbiome have been observed: from dysbiosed ticks less prone to *Borrelia* spp. to dysbiosed ticks with increased acquisition of *Anaplasma* spp. to an assumed redundant role of environmentally acquired microbiota to *Borrelia* spp. transmissions (Narasimhan et al. 2014; Abraham et al. 2017; Narasimhan et al. 2022). The tick-microbiome-pathogen interaction has been described extensively with differences emerging between bacterial and tick species, as well as dysbiosis method (Gall et al. 2016; Swei and Kwan 2017; Budachetri et al. 2018; Hamilton et al. 2021) and yet, the role of the ticks' immune pathways remain to be further unraveled (Narasimhan et al. 2014; Narasimhan et al. 2022).

Antibiotic treatment of the blood meal in ATFS provokes dysbiosis. However, the ability to eliminate individual microbiota through antibiotic treatment is highly dependent on the antimicrobial agent, the mode of action (bactericidal vs. bacteriostatic), mechanisms of action, chemical structure, activity spectrum (narrow spectrum vs. broad spectrum) and dosage. For instance, gentamicin, a broad-spectrum antibiotic used in our studies, is likely to disturb the overall bacterial microbiome without a specific effect on individual classes of bacteria. Antimicrobial resistance or lower sensitivity to certain antimicrobial agents, as described for *Rickettsia* spp. and gentamicin, also plays a role (Rolain et al. 1998).

To date, the information regarding vector competence of *I. ricinus* with regard to the tick-microbiome interaction is limited. The results described in **Chapter 3** suggest that the ATFS causes a dysbiosis leading to a reduction in microbiome diversity in the tick. This may has supported the colonization by and transmission of *R. helvetica.* Maitre *et al*. (2022) proposed that instead, infection with *R. helvetica* has an effect on microbiome diversity (Maitre et al. 2022b). Since we were unable to confirm the microbial diversity in our F_0 larvae, at this point in time we are unable to conclude which hypothesis is correct and further studies are required to do so. The lower sensitivity of *Rickettsia* to gentamicin could also explain these results. If gentamicin, as a broad-spectrum antibiotic, causes reduced numbers of other microbiota or environmental bacteria, *R. helvetica* could benefit from the free-up space and experience a colonization advantage.

Further, the role of *M. mitochondrii* in pathogen acquisition and transmission is still uncertain. It was suggested that it may play a "bridging role" between symbionts and environmental acquired bacteria due to its positive correlation (Mota et al. 2023). As mentioned above, **Chapter 3** showed a negative association between *M. mitochondrii* and *Rickettsia* spp. However, in the literature, observations on various associations were made (Lejal et al. 2021;

Krawczyk et al. 2022a; Krawczyk et al. 2022b). Nevertheless, the pure detection of co-occurrence between symbionts and pathogens does not necessarily indicate a functional role in vector competence. Overall, interactions between symbionts and (potential) pathogens should be considered in the study design of infection experiments.

4.3. Potential for cost-reduction through use of the artificial tick feeding systems

A potential strength of the ATFS is its cost-efficiency. As stated in **Chapter 1**, the use of live animals in tick rearing requires adequate housing facilities, trained caretaker staff and veterinary care, all of which is extremely costly. In an ATFS with an artificial blood meal or with blood acquired from external sources, all of these expenses become irrelevant.

The recently developed 12-well plate format (**Chapter 2** and **3**) requires less blood meal per tick. Thus, the blood meal to feed 50 *I. ricinus* nymphs was reduced to 2.25- 2.5 mL, as opposed to the previously ranged 2.6- 5.17 mL required in a 6-well-plate design nymphs (**Chapter 2**, (Korner et al. 2020; Răileanu et al. 2020; Król et al. 2021)).

The 12-well plate design is additionally beneficial because it offers the possibility to have easily more replicates within one experimental group. Further, in infection studies and acaricide screenings, it is likely that the experiment can be performed with lower amounts of the active compound or the pathogens needed. The use of a 24-well-plate has been reported (Trentelman et al. 2017; Trentelman et al. 2019), but there are doubts about the practicality of this system.

Further, the use of an artificial diet has been shown to reduce costs in mosquito-feeding experiments (Gonzales and Hansen 2016; Gonzales et al. 2018). However, to date, no cost comparisons between artificial and animal-based tick feeding experiments have been published.

While currently uncommon in tick feeding, 3D-printing has shown promising results in producing artificial feeding systems for mosquitos with estimated manufacturing costs amounting to one third of the costs involved in standard glass feeders (Witmer et al. 2018; Graumans et al. 2020). When using 3D-printing to manufacture feeding systems, the materials must be chosen carefully and tested in regard to their permeability to the blood meal, longevity and material waste. In addition, the feeding system must ideally be easy to clean or autoclave, which Witmer *et al.* proposed to achieve through the use of a higher printing resolution or polishing techniques (Witmer et al. 2018). On the other hand, the feeding system must be stable and free of any residues that may interact with active agents used for drug screenings or infection studies. The use of 3D-printing could help to standardize the design of feeding

systems and improve accessibility across research facilities, which could indirectly contribute to cost reduction (Witmer et al. 2018). Previously 3D-printed hydrogel mosquito feeders (Li et al. 2020) could potentially be adapted to produce membranes for ATFS. These chitosan or gelatin-based membranes additionally contribute to the 3R principles by reducing the need for animal-based membranes. Also, 3D-printing could be used for developing (semi-) automated blood-circulation systems (Janson et al. 2023).

Besides of all the possible advantages of ATFS, they will only be cost-efficient if the feeding results are successful, reliable and reproducible.

4.4. Limitations and challenges of artificial tick feeding systems

One major challenge of ATFS is their **inferior feeding successes** for hard ticks compared to *in vivo* feedings. As shown in **Chapter 2** and **3**, we observed longer feeding durations for all life stages, general lower engorgement weights and lower proportions of hatching success of larvae producing females.

The inferior results of ATFS for *I. ricinus* have also been reported elsewhere (Liu et al. 2014; Knorr et al. 2018). In Table 2, previously published results for the feeding of *I. ricinus* nymphs and adults were compiled and compared to the results described in this thesis. As previous data for *in vitro* feeding of *I. ricinus* larvae on silicone-based membranes is not available, this life stage is not included in this Table.

		Literature	**Chapter 2 & 3***
Nymphs	Mean attachment proportion (%)	24-44	68
	Mean engorgement proportion (%)	46- 50	49- 93
	Mean engorgement weight (mg)	2.8- 3	2.82- 2.92
	Mean proportion of molted adults per engorged nymphs (%)	11.4- 92	51- 75
Adults	Mean attachment proportion (%)	75- 100	20- 95
	Mean engorgement proportion (%)	24- 90	20- 90
	Mean detachment weight (mg)	160- 256.4	112- 180

	Mean proportion of larvae producing females (number of successful larvae hatched batches/ number of fed females) (%)	35- 53.5	8- 57

Table 2: Compiled information from selected peer-reviewed published articles on membrane-based ATFS of *Ixodes ricinus* ticks from control groups in comparison to results from **Chapter 2** and **3** (* all experimental groups for *in vitro* feedings included).

Literature for nymph data: (Knorr et al. 2018; Korner et al. 2020; Król et al. 2021); Literature for adult data: (Krober and Guerin 2007b; Krober and Guerin 2007a; Knorr et al. 2018; Korner et al. 2020; Artigas-Jerónimo et al. 2021; Król et al. 2021).

Critical points to consider are the **attachment stimuli** in order to shorten the time to attachment and increase the proportion of successfully attached ticks to the membrane. The membrane thickness also plays a crucial role and should be adapted to the length of the tick's hypostome (Krober and Guerin 2007a). Recently, thinner membranes have been associated with better feeding results for adult feeding of *I. scapularis* (Garcia Guizzo et al. 2023).

As explained in **Chapter 1**, animal hair or animal hair extracts are also commonly applied to the membranes to enhance their attractiveness. In both of our studies, the attachment success for adult ticks was highly variable. Factors that may have played a role were the actual age of the ticks used (sum of time spent in each life stage), the season during which the feeding experiment was conducted (e.g., in **Chapter 2** F_0- adult tick feeding was performed in February, which is not the preferred feeding season for *I. ricinus* adults in nature) and the environmental conditions during feeding.

Substances such as glucose or ATP can be added to the blood meal to serve as additional phagostimuli (Galun and Kindler 1965; Galun and Kindler 1968; Krull et al. 2017). Recent studies were unable to find a positive effect of ATP additives on feeding success in the sister species *I. scapularis* (Garcia Guizzo et al. 2023). However, in this study, ATP was used without additional glucose even though it has been shown that glucose enhances the phagostimulant effect of ATP (Galun and Kindler 1968). In the same study, the authors reported a positive effect of the changed LD rhythm on tick feeding (Garcia Guizzo et al. 2023).

The attachment success of *I. ricinus* is generally expected to be higher in an ATFS with membranes made of animal skin. However, when data from animal skin-based membranes (Bonnet et al. 2007; Liu et al. 2014; Migné et al. 2022b) are compared with data from Table 2, similar engorgement weights and proportions can be noticed.

To date, there is a scarcity of information regarding the effectiveness of specific attachment stimuli for different tick species and life stages. This is unsurprising given the large number of

factors involved in tick feeding such as the age, mating status, the water balance of the tick as well as environmental conditions such as the season, LD rhythm, temperature, the temperature gradient close to the membrane, RH and CO_2 concentrations. The role of each of these parameters warrants further research and particular attention should be paid to the relationship between environmental temperature, RH and contamination of the ATFS.

In future, the mass rearing of ticks for research purposes will remain necessary and before ATFS could replace tick rearing on live animals, the consecutive feeding of multiple life stages and long-term propagation of tick colonies remains to be optimized. To date, **the completion of an entire life cycle** of hard ticks in an ATFS (e.g., from F_0 adults to viable F_1 larvae) has only been achieved for *Amblyomma hebraeum* (Kuhnert et al. 1995) and *I. ricinus* (**Chapter 2**).

Table 2 highlights the fact that parameters indicative of feeding success in the ATFS, such as engorgement proportions or engorgement weights, are **highly variable**. According to Migné *et al,* a relatively high level of "unpredictability" is to be expected in tick feeding (Migné et al. 2022b) and we can confirm that it is extremely challenging to produce reproducible data when using the ATFS for feeding of *I. ricinus*. However, even in feeding experiments using live animals, success is far from guaranteed. In **Chapter 3**, for instance, the *in vivo* feeding of adult ticks on live calves was unsuccessful and no specific explanation could be found.

Besides the attachment stimuli, a main area of concern in ATFS is the **risk of contamination**. The long feeding duration of hard ticks combined with the necessary temperatures and humidity levels create a favorable environment for contaminants. Questionable sterility of the individual components of the ATFS increases the risk of contamination and can result in an antibiotic or antimycotic treatment becoming necessary throughout the feeding process (Kuhnert et al. 1995; Krober and Guerin 2007b; Sebastian et al. 2023).

Hence, close attention must be paid to obtain adequately packaged samples of sterile blood and additives and adhere to sterile technique when handling and storing materials. This may also include handling of blood from larger container vs. using small aliquots or also the storage conditions of components (e.g. frozen or stored at 4°C). Other possible sources of contamination include the sand or water bath, particularly if high temperatures and/ or RH levels lead to the formation of water droplets in a in a closed incubator setting. Poorly disinfected Baudruche membranes or tick attachment stimuli (tick feces, animal hair) can also lead to contamination. Further, the tick itself poses another threat: microbiota present on the tick cuticle or external surface including the hypostome as well as contaminants present in excrements or saliva can be pose another source of contaminants within the ATFS.

To prevent and treat contamination, **antibiotic and/ or antimycotic substances** have been used since the early days of ATFS. The most frequently used antibiotics are broad-spectrum antibiotics (such as gentamicin as used in **Chapter 2** and **3)**, which target several bacteria at the same time regardless of potential beneficial effects. Of course, many bacteria present in and around the tick are beneficial to the tick but broad-spectrum antibiotics do not differentiate between useful bacteria and disruptive bacteria. Hence, substances like gentamicin have been described to perturb the tick microbiome (Narasimhan et al. 2014; Narasimhan et al. 2022).

Similarly, Kuhnert *et al*. have suggested that besides destroying harmful fungi in the ATFS, the antimycotic substance nystatin also eliminates yeast-like organisms previously described to be involved in the vitamin B supply chain in mosquitos. The use of antimicrobial substances has therefore been linked to a vitamin B deficiency (Kuhnert et al. 1995). Vitamin B supplementation has recently been shown to counteract these detrimental effects and thus highlights the link between the two (Duron et al. 2018).

The use of antibiotic/ antimycotic treatments has been associated with negative feeding results. For example, in adult tick feeding experiments with feeding duration of more than one week, some attached females were found black and deflated (Duron and Gottlieb 2020; Sebastian et al. 2023). This observation was described for soft tick nymphs (Duron et al. 2018).

In **Chapter 2**, we described a similar phenomenon, which occurred after approximately seven days of attachment. This observation was particularly made in adults fed in the incubator and without B vitamin supplementation. Switching to a water bath system and supplementing B vitamins appeared to have a restorative effect, reducing the number of black and dead attached females. Hence, in the study described in **Chapter 3**, adult tick feeding was performed in a water bath with vitamin B supplementation and resulted in 0/10 dead females in the IVG^+ group and 2/25 dead females in the IVG^- group. Given the small sample size of F_1 females used in the studies described here, more research is required to provide a statistically sound basis for these findings. Furthermore, investigations must be performed to distinguish between effects of the water bath and the effects of the vitamin B supplementation.

Two recent studies have reported on successful feeding of *Ixodes* spp. ticks to full repletion in an ATFS without the addition of antibiotics (Korner et al. 2020; Garcia Guizzo et al. 2023). In the study performed by Guizzo *et al*., *I. scapularis* ticks fed on a blood meal treated with gentamicin showed similar engorgement proportions and weights as ticks fed on an untreated blood meal. However, the proportion of oviposition was significantly reduced in the gentamicin group (Garcia Guizzo et al. 2023). On the contrary, in the study described in **Chapter 3**, females in the gentamicin group showed slightly higher engorgement proportions and weights than females in the untreated group with no statistically significant differences in oviposition

between the two. In an *in vitro* feeding experiment performed on *D. reticulatus* females, gentamicin treatment of the blood meal had a significantly positive effect on oviposition proportion and egg batch weights (Krull et al. 2017). Similarly, positive effects of gentamicin treated blood meals in female soft ticks were also reported previously (Taraveau et al. 2023).

One possible explanation for these contradictory results from our studies on *I. ricinus* and the previous study on its sister species *I. scapularis* could be the fact that *Rickettsia* spp. are not susceptible to gentamicin (Rolain et al. 1998). The population of *R. buchneri* was shown to increase slightly in *I. scapularis* after gentamicin treatment (Narasimhan et al. 2022). Similarly, the population of *R. helvetica* increased in IVG^+ adults in the study described in **Chapter 3**. As Guizzo *et al.* performed their experiments with *I. scapularis*, most likely harboring its obligate symbiont *R. buchneri*, these different results highlight the different roles of bacteria from the same genus in different tick species. However, the considerable differences in study design must also be acknowledged when comparing the findings. For instance, the sample sizes varied between 10 and 25 female ticks in **Chapter 3** study vs. 60 female ticks in the study performed by Guizzo *et al.* (Garcia Guizzo et al. 2023).

In the **Chapter 3** study, a water bath was used for the adult feeding experiment whereas a 37°C incubator was used in the study by Korner *et al.* for all life stages (Korner et al. 2020). Glucose supplementation and disinfection protocols differed between all three studies and both Korner *et al*. and Guizzo *et al*. considered *Ixodes* spp. adults or nymphs as individual entities (Korner et al. 2020; Garcia Guizzo et al. 2023). In our **Chapter 3** study on the other hand, the experiment began with F_0 larvae, which were consecutively fed until the engorged adult life stages. This is important to note because potential cumulative effects of consecutive antibiotic treatments cannot be ruled out at this point in time.

Another point to note is that the other *I. ricinus* laboratory population used in the study by Korner *et al*. was from another origin as the laboratory population used in our studies (Korner et al. 2020). As mentioned above, *M. mitochondrii* loads tend to differ between different laboratory populations, likely due to differences in the rhythm of cross-breeding with wild ticks. As a result, differences in *in vitro* feeding results may be expected.

In summary, tick feeding dynamics and feeding success are likely to vary between individual populations. Despite the fact that the ATFS provides an opportunity to standardize protocols, the dearth of data that is necessary to do so for individual tick species, life stages, tick generations and origins, as well as environmental conditions and other ATFS-dependent factors is not available at this point in time. This underlines the need for more consistent approaches, for instance through the establishment of Standard Operating Procedures (SOPs)

to enhance reproducibility. These SOPs should be openly available and could be in written or video format, as recently produced for *I. scapularis* (Khoo et al. 2022).

A third challenge associated with the use of ATFS in hard tick feeding is the **high workload.** This is mainly due to the requirement of two blood meal changes per day and as shown in **Chapter 2** and the significantly longer feeding durations in comparison to *in vivo*. This is resulting in a laborious and time-consuming task (Migné et al. 2022b). Some studies have been successful with only one blood meal change per day (Olivieri et al. 2018; Krawczyk et al. 2020) but this approach is likely unsuitable for microbiome and infection studies, specifically when working without antimicrobial substances.

While semi-automated and automated feeding systems have existed for quite some time, detailed reports are few and far between (overview given in **Chapter 1**). An automated system adapted from the “Hemotek” mosquito feeder, has recently shown promising results for ticks (Vimonish et al. 2020; Vimonish et al. 2021; Asri et al. 2023). Here, a silicone-covered Baudruche membrane, separates the FU from the blood container that is located above the FU. The blood container was connected to a peristaltic pump so that the blood meal could be changed and the FU cleaned without interrupting the feeding process. This is an advantage because interruptions to the feeding process may cause the ticks to detach, leading to poorer feeding success. However, the blood meal change by a conservative membrane-based ATFS usually requires only a couple of seconds and thus should not pose an issue.

However, in this system, just like in any “upside-down” ATFS, ticks tend to attach in clusters. This, as well as gravity make these systems more prone to leakages and highly dependent on particularly stable membranes (Asri et al. 2023).

In an upside-down feeding system, the air-permeable end of the FU is located at the bottom preventing droplets of condensed water falling into the FU. The blood meal was changed every 8- 12 h and a cleaning step was included each time (Vimonish et al. 2020; Asri et al. 2023). Asri *et al*. also performed a detailed assessment of the ticks every 12 h, which additionally increased the workload (Asri et al. 2023). Video or photographic recording could potentially replace the manual inspection in the future.

Asri *et al*. used 30- 60 adult *Rhipicephalus appendiculatus* ticks per FU (Asri et al. 2023), Vimonish *et al.* used 50- 120 adult *D. andersoni* ticks per FU (Vimonish et al. 2020) and up to 600 adult *R. appendiculatus* ticks (Vimonish et al. 2021). With such highly variable sample sizes, it is difficult to make comparable claims about the occurrence of leakages, tick feeding success and fecundity parameters. Novel techniques like 3D-printing could pave the way to refining and standardizing these feeding systems and generating comparable data.

SUMMARY

The artificial tick feeding systems (ATFS) offer a promising alternative to animal experiments. They can be used for studies of tick biology and physiology, for experiments investigating the tick microbiome, novel tick control strategies and the tick-(microbiome)-pathogen interface as well as to facilitate tick rearing. At a time where globalization and global warming is affecting the prevalence, distribution and host encounters of ticks, research regarding ticks and tick-borne diseases is particularly important.

The junior research group "Tick-borne Zoonoses" was founded to develop innovative and practical approaches to studying the vector biology of Europe's most common hard tick species, *Ixodes ricinus*. This PhD project specifically focused on the use of ATFS for feeding of *I. ricinus* ticks. **Chapter 1** provides an introduction to the biology and relevance of *I. ricinus* in Europe, as well as delving into an explanation of the different components and applications of ATFS.

In **Chapter 2**, results from our first study are used to compare and contrast tick feeding of multiple consecutive life stages of *I. ricinus* between the *in vitro* feeding by ATFS and *in vivo* on live cattle. Findings showed that artificially fed ticks were generally inferior to ticks fed on live cattle and all life stages showed significantly longer feeding durations. However, in larvae, higher engorgement and molting proportions indicated that the ATFS was more effective than *in vivo* feeding. Further, the feeding and fecundity parameters for F_1 adults improved after B vitamin supplementation and a water bath system.

The prolonged feeding durations commonly observed in the ATFS are associated with an increased risk of contamination, even if the blood meal is supplemented with the antibiotic gentamicin. However, both bacterial contamination and antibiotic treatment have been linked to negative effects on feeding success. To further investigate the effects of using gentamicin in the ATFS on the tick microbiome, ticks were consecutively fed on cattle blood with and without gentamicin in an ATFS. A multimethod approach involving amplicon sequencing of 16S rRNA and further quantification of common bacteria using qPCR was used. **Chapter 3** describes the results and compares the findings to ticks fed on live cattle. Despite facing challenges around the extraction and sequencing of DNA from individual ticks, we were able to show that in female ticks fed on live cattle the dominant symbiont was *Candidatus* Midichloria mitochondrii. In female ticks fed in the ATFS using a gentamicin-treated blood meal, the most abundant bacterium was *Rickettsia helvetica.* Hence, we were able to deduce that both fecundity and microbial composition are likely to be influenced by the ATFS. These findings should be taken into account for future studies using the ATFS.

ZUSAMMENFASSUNG

Die künstliche Schildzeckenfütterung von *Ixodes ricinus*

Künstliche Schildzeckenfütterungssysteme bieten eine vielversprechende Alternative für Tierversuche, um die Biologie und Physiologie von Zecken zu untersuchen, aber auch, um mehr Erkenntnisse über das Zeckenmikrobiom, bei der Entwicklung von Zeckenbekämpfungsstrategien und auch die Schnittstelle zwischen Zecke-Mikrobiome-Krankheitserreger zu gewinnen und schlussendlich auch, um die Zeckenaufzucht zu erleichtern. Die Erforschung von Zecken und von durch Zecken übertragenen Krankheiten ist in Zeiten der globalen Erwärmung und deren einhergehenden Ausweitung der Verbreitung und Auftreten von Zecken unerlässlich.

Dieses Dissertationsprojekt ist Teil der Nachwuchsgruppe "Tick-borne Zoonoses", die mit innovativen, praktischen und molekularen Ansätzen die Vektorbiologie der häufigsten europäischen Zeckenart, *Ixodes ricinus*, weiter versucht aufzuklären. Der Schwerpunkt dieser Dissertation liegt auf der künstlichen Schildfütterungssystemen von *I. ricinus*. Zum besseren Verständnis gibt Kapitel 1 eine Einführung in die Biologie und Bedeutung von *I. ricinus* in Europa und beleuchtet die verschiedenen Komponenten und Anwendungen von ATFS.

Es folgt Kapitel 2, in dem der erfolgreiche Abschluss des Lebenszyklus von *I. ricinus* mittels künstlicher Fütterung nachgewiesen wurde. Die künstlich gefütterten Zecken waren im Allgemeinen den *in vivo* an lebenden Rindern gefütterten Zecken unterlegen und haben eine deutlich längere Fütterungsdauer aufweisen. Allerdings wiesen bei den Larven die höheren Anteile an Saug- und Häutungsvorgängen darauf hin, dass die künstliche Fütterung effektiver war als die *in vivo* Fütterung. Darüber hinaus wurden die Fütterungs- und Fruchtbarkeitsparameter der adulten F_1 durch die B-Vitamin-Supplementierung und einer Wasserbadanwendung verbessert. Um die Auswirkungen der Verwendung von Gentamicin im künstlichen Fütterungssystem auf das Zeckenmikrobiom weiter zu untersuchen, wurden Zecken nacheinander mit Rinderblut mit und ohne Gentamicin gefüttert. Es wurde ein mehrheitlicher Ansatz mit Amplikon-Sequenzierung von 16S rRNA und anschließender Quantifizierung häufiger Bakterien mittels qPCR-Analyse angewendet. Kapitel 3 beschreibt die Ergebnisse und vergleicht sie mit *in vivo* gefütterten Zecken. Trotz den Herausforderungen einer erfolgreichen DNA-Extraktion aus einzelnen Zecken und deren Sequenzierungsanalyse wurde bei den *in vivo* gefütterten Weibchen eine andere mikrobielle Zusammensetzung beobachtet als mittels der künstlichen Fütterung: *in vivo* wurden in den Weibchen vor allem *Candidatus* Midichloria mitochondrii identifiziert, während bei Weibchen, die zuvor künstlich mit antibiotisch behandelten Blutmahlzeiten gefüttert wurden, *Rickettsia helvetica* die vorherrschende Bakterienspezies war. Diese Ergebnisse zeigen, dass die künstliche

Schildzeckenfütterung höchstwahrscheinlich die Fruchtbarkeit und die Zusammensetzung des Mikrobioms beeinflusst hat und dies für künftigen Studien mittels dieser Technik berücksichtigt werden sollte.

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Oral and Poster Presentations

Militzer N, Pinecki Socias S and Nijhof A M (2022): **Changes in the *Ixodes ricinus* microbiome associated with artificial tick feeding**, Zoonoses 2022 - International Symposium on Zoonoses Research, 2022, October 05-07, Berlin, Germany oral presentation and co-chair of the One Health plenary session.

Militzer N, Hoffmann-Köhler P and Nijhof A M (2021)**: The artificial feeding of all successive life stages of *Ixodes ricinus,*** 14th International Symposium on Ticks and Tick-borne Diseases 2021, March 24-26, Weimar, Germany.

Król N, **Militzer N,** Nijhof A M, Kempf V, Pfeffer M and Obiegala A (2021): **Experimental infection of *Ixodes ricinus* ticks with different *Bartonella* species via artificial feeding***,* 14th International Symposium on Ticks and Tick-borne Diseases 2021, March 24-26, Weimar, Germany.

Militzer N, Hoffmann-Köhler P and Nijhof A M (2021): **The artificial feeding of all successive life stages of *Ixodes ricinus***, 29th Annual Meeting of the German Society for Parasitology 2021, March 15-17, Berlin, Germany.

Militzer N (2020): **Life in the lab: artificial feeding and infection trials of the hard tick *Ixodes ricinus***, DFG Research Training Group 2046 Annual Retreat 2020, October 30, Berlin, Germany.

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Król N, **Militzer N**, Nijhof A M, Kempf V, Pfeffer M, Obiegala A (2019): **Experimental infection of *Ixodes ricinus* ticks with different *Bartonella* species via artificial feeding**, 9th International Conference on *Bartonella* as Emerging Pathogens 2019, September 18-20, Paris, France.

Militzer N, Pinecki Socias S and Nijhof A M (2019): **A comparison between *Ixodes ricinus* nymphs fed *in vitro* and on calves,** Zoonosis 2019 **-** International Symposium on Zoonoses Research, 2019, October 16-18, Berlin, Germany.

Król N, **Militzer N**, Nijhof A M, Kempf V, Pfeffer M and Obiegala A (2019): **Experimental infection of *Ixodes ricinus* ticks with different *Bartonella* species via artificial feeding,** Zoonosis 2019 **-** International Symposium on Zoonoses Research, 2019, October 16-18, Berlin, Germany.

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Militzer N, Pinecki Socias S and Nijhof A M (2019): **A comparison between *Ixodes ricinus* nymphs fed *in vitro* and on calves**, Junior Scientists Zoonoses Meeting 2019, June 20-22, Berlin, Germany.

Militzer N, Pinecki Socias S and Nijhof A M (2019): **A comparison between *Ixodes ricinus* nymphs fed *in vitro* and on calves**, 13th International Symposium on Ticks and Tick-borne Diseases 2019, March 28-30, Weimar, Germany.

Militzer N, Pinecki Socias S, Krull C and Nijhof A M (2018): **Artificial feeding of the hard tick *Ixodes ricinus;*** 11th Doktorandensymposium DRS “Biomedical Sciences”, 2018, September 21, Berlin, Germany, **1st** price for oral presentation.

Militzer N, Pinecki Socias S and Nijhof A M (2018): **The artificial feeding of *Ixodes ricinus.*** Junior Scientist Zoonoses Meeting 2018, June 07-09, Hamburg, Germany.

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DISCLOSURE

Author contributions to the published peer-reviewed articles of this dissertation according to the following criteria:

1. Idea and concept of the study
2. Design of experiments
3. Data analysis
4. Text execution
5. Compilation of the manuscript

Chapter 2: Artificial Feeding of All Consecutive Life Stages of *Ixodes ricinus*

1. Nijhof A M, Militzer N
2. Militzer N, Nijhof A M, Hoffmann-Köhler P
3. Militzer N, Bartel A
4. Militzer, N
5. Militzer N, Bartel A, Clausen P-H, Hoffmann-Köhler P, Nijhof A M

Chapter 3: Changes in the *Ixodes ricinus* microbiome associated with artificial tick feeding

1. Nijhof A M, Militzer N, Pinecki Socias S
2. Militzer N
3. Militzer N, Nijhof A M
4. Militzer N, Nijhof A M
5. Nijhof A M, Militzer N, Pinecki Socias S

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CONFLICT OF INTEREST

In the context of this work, there are no conflicts of interest due to contributions from third parties.

DECLARATION OF INDEPENDENCE

I hereby certify that I have prepared this thesis independently. I certify that I have used only the sources and aids indicated. I also declare that I have not submitted the dissertation in this or any other form to any other institution as a dissertation.

Berlin, 17.05.2024 Nina Militzer