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

Evaluation of tick protein extracts as anti-tick vaccines targeting *Ixodes ricinus* in cattle

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
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vorgelegt von
Sarah Knorr (geb. Pröhl)
Tierärztin aus Schmölln

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Dedicated to all experimental animals;
without them, this work would not have been possible

Table of Contents

List of Abbreviations

3 R	Replacement, Reduction and Refinement in relation to animal welfare
ABC	ATP-binding cassette
ANTIDotE	Anti-tick vaccine to prevent tick-borne diseases in Europe
AQPs	Aquaporins
ATP	Adenosine triphosphate
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
DDA	Dodecanoic acid
DEET	Diethyltoluamide
dsRNA	double-stranded RNA
ELI	Expression Library Immunization
Fer1/ Fer2	Ferritin1/ Ferritin2
GST	Glutathione S-transferase
LB	Lyme Borreliosis
LC-MS	Liquid chromatography–mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MG	Midgut
mRNA	messenger RNA
Osp	Outer Surface Protein
rFer	recombinant Ferritin
RNAi	RNA Interference
Salp15	15-kDa tick salivary gland protein
SG	Salivary gland
siRNA	Small interfering RNA
SUB	Subolesin
TBDs	Tick-borne Diseases
TBEV	Tick-borne encephalitis virus
WHO	World Health Organization
YSD	Yeast Surface Display

PREFACE

Introduction and Study Objectives

Ticks are found worldwide and can act as vectors for a wide variety of bacteria, protozoa and viruses that may cause tick-borne diseases (TBDs) and threaten the health of humans and animals. Around 900 tick species have been described and they can be divided into two large families: the hard and soft ticks. A third family, the Nuttaliellidae is monotypic. The hard ticks are the largest family, consisting of 14 genera with approximately 702 species. The systematics of the soft ticks are not fully elucidated, but it is thought to consist of at least four genera with >193 species (Guglielmone et al., 2010; Mans et al., 2019).

Hard ticks in particular may cause high economic losses in livestock in tropical and subtropical regions. Massive infestation with ticks leads to impaired production performance and poor leather quality. The health, welfare and economic impacts from tick-borne disease are more significant than the direct effects from the ticks themselves (Jongejan and Uilenberg, 2004).

Although economic losses in livestock due to tick bites are rare in Europe, humans and animals can also be affected by ticks and TBDs. Here, Lyme Borreliosis (LB) caused by *Borrelia burgdorferi* sensu lato and Tick-Borne Encephalitis (TBE) virus are transmitted by *Ixodes ricinus* ticks can severely affect human health. About 85,000 cases of LB are reported annually in Europe. Since surveillance methods in Europe vary and public awareness of LB differs between European countries, the occurrence of LB cannot easily be compared between countries. Nevertheless, increases in LB have been observed in several European countries during the past decade, and the geographic distribution of cases has also expanded (Lindgren and Jaenson, 2006; Vandekerckhove et al., 2021). In 2019, 25 European countries reported 3,411 cases of TBE to the European Surveillance System. The majority (n=3,246) could be confirmed as TBE positive, giving an incidence of 0.7 per 100,000 inhabitants (European Centre for Disease Prevention and Control, 2021). Other zoonotic pathogens such as *Babesia divergens, Babesia venatorum, Anaplasma phagocytophilum* and several *Rickettsia* sp. are also transmitted by *I. ricinus* and may cause illness in humans as well. In Europe, approximately 50 cases of Babesiosis have been described since 1950s, mostly caused by *B. divergens* in splenectomised or immunocompromised patients (Krause, 2019; Vannier et al., 2008). The first clinical case of human granulocytic anaplasmosis in Europe was reported in the early 1990s, after which around 300 additional cases have been reported (Matei et al., 2019). The spotted fever rickettsiaes transmitted by *I. ricinus* are less well defined and only a few case reports from Italy and Spain are available (Jado et al., 2007; Madeddu et al., 2012).

Control of tick infestations in animals mainly relies on antiparasitic drugs, which have several drawbacks including possible residues in food products, pollution of the environment or inducing resistance in ticks. An alternative and promising tick control strategy is the use of anti-tick vaccines. Vaccines hold promise as being sustainable, cost-effective, not affecting non-

target species and development of resistance against vaccines by ticks is considered less likely to occur.

The phenomenon of tick immunity, is known since the 1930s (Trager, 1939). Ticks fed on animals that were repeatedly infested with ticks showed increased mortality and reduced feeding and reproduction. This phenomenon could be mimicked by immunization with tick homogenates, which eventually led to the discovery by Australian scientists that immunization with native or recombinant Bm86, a tick midgut membrane-associated protein, protected cattle against infestations with the one-host cattle tick *Rhipicephalus microplus* (Willadsen et al., 1989). Bm86-based vaccines were subsequently commercialized and released on the market, in Australia as TickGARD® and in Latin American countries as Gavac®. Field use of Gavac® in Cuba between 1995 and 2003 resulted in an 87% reduction in acaricide use (Valle et al., 2004). In Australia, Jonsson et al. reported that vaccination of dairy herds with TickGARD® resulted in a reduction in tick numbers by 56% and had a positive effect on animal performance (Jonsson et al., 2000). Although immunization with Bm86 was shown to be effective against infestations with several tick species other than *R. microplus,* such as *Rhipicephalus annulatus, Rhipicephalus decoloratus* and *Hyalomma dromedarii* (Canales et al., 2009; de Vos et al., 2001; Fragoso et al., 1998; Pipano et al., 2003), it was not effective against several other tick species such as *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Amblyomma cajennense* (de Vos et al., 2001; Rodríguez-Valle et al., 2012). Studies showed that the immunization of rabbits with Bm86 homologues from *I. ricinus* (Ir86-1 and Ir86-2), did not confer protection against subsequent challenge with this species (Coumou et al., 2015; de Vos et al., 2001). To promote the development of a vaccine against *I. ricinus*, a consortium consisting of seven institutions under the acronym of ANTIDotE (**an**ti-tick vaccine to prevent **ti**ck-borne **d**iseases in **E**urope) was founded in 2013. This consortium aimed to identify and characterize *I. ricinus* proteins involved in feeding and pathogen transmission. The knowledge gained would be used to develop and evaluate anti-tick vaccines that could prevent multiple human tick-borne diseases. Strategies encompassing anti-tick vaccines to prevent transmission of pathogens to humans, animals or wildlife would be developed with relevant stakeholders with the ultimate aim of reducing the incidence of tick-borne diseases in humans (Sprong et al., 2014a). This thesis which was carried out within the framework of the ANTIDotE project, focuses on the tick-protective potential of *I. ricinus* tissue extracts.

The following chapter (**Chapter 1**) gives a general introduction to the vector biology of *I. ricinus* ticks, highlighting the importance of preventing tick-borne diseases associated with *I. ricinus* and presenting main strategies for tick control. In **Chapter 2**, the occurrence, abundance as well as clinical symptoms and treatment schemes of common TBDs in Europe are summarized

and discussed. This review also discusses the development of an effective anti-tick vaccine to prevent the transmission of TBDs.

The major bottleneck in tick vaccine development is the identification of tick protective antigens. **Chapter 3** describes immunization trials in which cattle were immunized with *I. ricinus* tissue extracts or recombinant ferritin 2, a protein that previously showed promise as a tick-protective antigen, followed by *I. ricinus* tick infestations. Since immunization with native protein extracts conferred a strong immune response and significantly reduced the feeding success of both *I. ricinus* nymphs and adults, **Chapter 4** describes a follow-up study that was conducted to identify and characterize immunodominant proteins present in these protein extracts. A summarizing discussion is presented in **Chapter 5**.

CHAPTER 1

Literature Review

1.1 Biological classification and morphology

Ticks are haematophagous arthropods that belong to the subclass Acari of the class Arachnida. The suborder Metastigmata consists of three families: Ixodidae (hard ticks), Argasidae (soft ticks) and the monotypic Nutallielidae. *I. ricinus* is an ixodid or hard tick species that is widespread in Europe. This species has major medical and veterinary relevance as it can transmit various TBDs, including Lyme Borreliosis (LB) and Tick-borne Encephalitis virus (TBEV).

As for all hard ticks, the body of *I. ricinus* is separated into an anterior region, the gnathosoma, which includes the basis capituli with paired palps, a tube shaped hypostome and paired chelicerae. The remaining part of the body is the idiosoma, which is oval shaped, bears the scutum, three or four pair of legs, the anal and, in adults, genital aperture and the spiracular plates. The body size of *I. ricinus* ticks depends on the life stage (larvae, nymph or adult), sex and whether the tick is in an unfed, feeding or engorged state. Some average size properties are summarized in Table 1. *Ixodes ricinus* can easily be confused with *Ixodes inopinatus*, a tick species with a similar morphology that also occurs in Europe. These species can be discriminated on minor morphological differences, e.g., by the appearance of the punctuations on the idiosoma or the lack of a marginal groove in *I. inopinatus* females (Estrada-Peña et al., 2014).

A distinct sex dimorphism exists for adult ticks. Females have a small black scutum that covers approximately one third of the dorsoanterior idiosoma. The part that is not covered by the scutum is reddish to brownish in colour in unfed females and is referred to as the alloscutum. Males are smaller than females and have a black scutum that covers the whole dorsal idiosoma (Deplazes et al., 2013; Sonenshine, 2014). Figure 1 shows an adult female and male *I. ricinus*.

Figure 1. Adult female (A) and male (B) of *I. ricinus*, © Institute for Parasitology and Tropical veterinary medicine, FU Berlin

Table 1. Morphological characteristics of *I. ricinus* life stages

Life stage	**Adults**			**Nymphs**		**Larvae**	
	female	female (engorged)	male	fasting	engorged	fasting	engorged
Size (mm)	3.0-3.6	10	2.4-2.8	1.3-1.5	2.0-3.0	0.5	1
Pair of legs	4	4	4	4	4	3	3
Color	reddish - brownish	greyish	black	transparent	greyish	transparent	greyish

Reference: Deplazes et al., 2013

1.2 Hosts and Life cycle

Ixodes ricinus pursues an ambush strategy when questing for hosts. Typically, they climb up on vegetation under suitable ambient conditions and wait for a passing host to attach to. Changes in light level, concentrations of carbon dioxide, lactic acid and other chemicals, humidity or temperature as well as vibrations can be detected by sensory organs, such as photo-sensitive cells located dorsolaterally and the Haller´s organ, which is located on the tarsal segment of the front legs. These organs allow *I. ricinus* to find suitable hosts and feeding sites (Perret et al., 2003; Šimo et al., 2014). A wide range of warm- and even cold-blooded vertebrates may serve as hosts (Lees, 1948). As all other ixodid tick species, *I. ricinus* ticks have three haematophagous life stages: the larvae, nymph and adult. Fully engorged females lay ~2,500 eggs and subsequently die (Honzáková et al., 1975). After several weeks larvae hatch from the eggs and will try to find a suitable host, typically a small rodent or bird. Once attached, the larvae engorge within two to three days, drop from their host and moult to nymphs. Nymphs may also feed on small mammals and birds, but may also attach to larger vertebrates including humans, where they feed for up to five days (Lees, 1948). Nymphs are considered of highest risk to public health because they are more likely to be infected than larvae and are present in larger numbers than adults. Moreover, due to their small size nymphs on humans may go unnoticed and can transmit TBDs affecting humans. Within the *Ixodes* genus, mating takes place on the host while feeding, but can also occur off-host in the vegetation (Gray, 1987; Sonenshine, 2014). Females will feed for approximately 7 to 10 days, detach upon engorgement and hide in a suitable microhabitat for oviposition. The complete life cycle takes approximately 3 years, but may be as long as 6 years (Chmela, 1969; Gray, 1991).

1.3 Distribution

As mentioned above, *I. ricinus* is widespread in Europe and occurs from Ireland to the Ural and from Sweden to North Africa (Estrada-Pena, 2001; Estrada-Pena et al., 2013). Both abundance and the geographical distribution appear to be expanding, especially northwards and to higher altitudes (Gray et al., 2009; Materna et al., 2005). This might be a result of climate change, as increased temperatures and rainfall, as well as shortened snow coverage periods have effects on tick and host populations. With increasing temperatures, the questing season may extend and development times between life stages shorten (Daniel et al., 2008). More frequent rainfall and shorter periods of frost lead to extended vegetation periods. This will offer mammalian hosts new habitats and increase the survival rate of both ticks and hosts. Ultimately, the distribution of ticks will follow the movement and extension of the distribution areas of their hosts (Jaenson and Lindgren, 2011). The spread of *I. ricinus* ticks depends mainly on the presence of suitable hosts; a higher host density will increase the abundance of ticks (Hofmeester et al., 2017). Reforestation or placing areas under nature protection may also increase the density and distribution of hosts and thereby have a positive effect on tick populations (Hobbs, 2009; Paul et al., 2016). Tick survival and abundance is determined by microclimate conditions, with moderate temperatures and high relative humidity being key requirements for successful development (Coipan et al., 2013; Estrada-Pena et al., 2013; Gray et al., 2009; Lindgren et al., 2000; Matei et al., 2017).

1.4 Medical importance

Lyme Borreliosis (LB) and Tick-Borne Encephalitis (TBE) are the main tick-borne diseases (TBDs) transmitted by *I. ricinus*. In Europe, Lyme borreliosis is commonly caused by five different genospecies that are part of the *Borrelia burgdorferi* sensu lato complex (Stanek et al., 2012). These spirochetes are transmitted during the blood intake of the tick. The majority of infected humans develop a red, sometimes itchy spot at the tick bite site, also known as erythema migrans. Other symptoms might be flu-like, including fever, listlessness or headaches. If the disease is not treated, severe headache, joint pain or heart palpitations with shortness of breath or tightness in the chest may occur (Wormser et al., 2006). In 2006, the World Health Organization (WHO) estimated the incidence of LB from available national data to about 85,000 cases per annum in Europe; recently, the annual case number was estimated to be 230,000 in Western Europe (Lindgren and Jaenson, 2006; Sykes and Makiello, 2017). The number of LB cases are increasing in most Western European countries, particularly in the northern and central part. This may in part be explained by an increased detection and awareness, but may also present a real increase in spread of the disease (Vandekerckhove et al., 2021).

The causative agent of TBE belongs to the Flaviviridae family. Different subtypes such as the European or Western, Siberian and Russian subtypes infect either the brain, the meninges or both. Typical symptoms in decreasing order of frequency are meningitis, meningoencephalitis and meningoencephalomyelitis. Since the 1990s, increasing incidences of TBE have been reported in several Central and Western European countries. In 2012, TBE became an obligatory notifiable disease in all countries of the European Union (2012/506/EU: Commission Implementing Decision of 8 August 2012). In 2019, 25 European countries reported 3,411 cases of TBE to the European Surveillance System of which 3,246 were confirmed as TBE positive, indicating an incidence of 0.7 per 100,000 inhabitants (European Centre for Disease Prevention and Control, 2021) Active immunization against TBE is an effective prophylaxis, therefore, two different European Medicines Agency (EMA)-authorized vaccines are in use in Europe, FSME-IMMUN® and ENCEPUR® (Kollaritsch H, 2011).
A detailed description for both TBDs, including their epidemiology, diagnosis, treatment and prevention as well as the importance of the other less widely distributed TBDs such as anaplasmosis, ehrlichiosis or babesiosis is presented in Chapter 2.

1.5 Veterinary importance

In general, the feeding of *I. ricinus* ticks causes little harm to their hosts. Only a massive infestation with ticks may have a negative impact on the performance of the host (Deplazes et al., 2013). As for humans, the main concern associated with *I. ricinus* infestations are the tick-borne pathogens that can be transmitted by the tick to the host. Tick-borne diseases (TBDs) associated with *I. ricinus* are caused by tick-borne pathogens such as *Borrelia* sp., *Babesia* sp., *Anaplasma* sp. and viruses (Deplazes et al., 2013). In Chapter 2, Table I provides an overview of the pathogens, which have been detected in or can be transmitted by *I. ricinus.* Moreover, this Chapter describes the epidemiology, clinical signs and recommended treatment for dogs, horses and cattle.

1.6 Tick control strategies

1.6.1 Chemical acaricides

Chemicals used to kill ticks and mites are Acaricides. Acaricides can be divided in different drug classes depending on their chemical active ingredients: arsenical preparations, chlorinated hydrocarbons (e.g., DDT and lindane), organophosphates (e.g., coumaphos), carbamates (e.g., carbaryl), formamidines (e.g., amitraz), pyrethroids (e.g., permethrin, flumethrin), macrocyclic lactones (e.g., ivermectin), phenylpyrazoles (e.g., fipronil), insect growth regulators (e.g., fluazuron), and isoxazolines (e.g., afoxalaner, fluralaner, sarolaner) (Nicholson et al., 2019).

Currently, synthetic pyrethroids are most frequently used for tick control worldwide, with spot-on and pour-on products being the main application methods for tick control in livestock. Isoxazolines were released on the veterinary market in 2014 and are increasingly being used for ectoparasite control on pets as they can be administered orally and offer long-lasting broad-spectrum protection against ticks and other ectoparasites (Nicholson et al., 2019). Table 2 provides an overview about the main acaricides used in Europe.

However, chemical tick control is associated with problems such as residues in animal products and the environment, concerns about the development of resistance against these products in ticks and increasing costs for regular treatment. Alternative tick control strategies, such as tick-resistant breeds, immunological or biological control, are therefore finding increased interest (Almazan et al., 2018; George et al., 2004; Shyma et al., 2015).

1.6.2 Synthetic and natural repellents

The application of repellents on skin or clothes can also prevent tick infestations. The American Centers for Disease Control and Prevention (CDC) suggests the use of synthetic repellents such as Diethyltolulamide (DEET) or Icaridin to discourage ticks and insects to feed and hence prevent vector-borne diseases (Centers for Disease Control and Prevention, 2020). Natural repellents, e.g. lemon eucalyptus oil or citronella can also be effective, but need to be reapplied in 30 to 60 min intervals to achieve a similar repellency as synthetic repellents (Yoon et al., 2015). The mode of action of most repellents is still not fully understood. It is supposed that these substances block olfactory receptors for volatile agents of human and animal odour or sweat, such as carbon dioxide or lactic acid. However, the assumption that DEET affects the ability to perceive carbon dioxide was refuted for insects by Ditzen et al. (Ditzen et al., 2008). Questing ticks are mainly guided by the Haller's organ, chemosensory sensilla on the tarsus of the foreleg. Not only the olfactory perception, but also infrared detection is ascribed to Haller's organ (Mitchell et al., 2017). Studies on *Amblyomma americanum* and *Dermacentor variabilis* showed that DEET exposure did not affect the olfactory attraction to carbon dioxide, but disrupted thermotaxis of ticks (Carr and Salgado, 2019). Koloski et al. hypothesized that DEET inhibits cytochrome p450s and cholinesterases. They found immediate and substantial reduced frequency of transcripts of cytochrome P450 genes and cholinesterases in *in vitro* assays with *Dermacentor variabilis* ticks, which was reversible over time (Koloski et al., 2019). According to these studies, the mode of action of DEET has not yet been conclusively clarified and requires further study.

Animal owners and consumers are increasingly looking for eco-friendly and sustainable tick control agents such as plant and root extracts. This includes garlic and coconut oil. Garlic (*Allium sativum*) has been used for thousands of years, not only in the kitchen, but also for its

medicinal properties. During the last decades, scientific studies were conducted to investigate the biologically active ingredients. Extraction of garlic oil by hydrodestillation determined sulphur compounds as the main components (Calvo-Gomez et al., 2004; Tunon, 2001). Several studies were performed to validate sulphur as tick control agent, but its efficacy could not be proven (Wharton, 1970; Wilkinson, 1970).

The repellent compound of coconut oil is lauric acid, a dodecanoic acid (DDA). The fur of companion animals can be rubbed with coconut oil or the solution can be given orally. Schwantes *et al.* performed laboratory screening tests (Bioassays and tests in humans) with different DDA-formulations against *I. ricinus*. All substances repelled nymphs and adults for at least 6 hours. DDA-based formulations gave 63-83% protection against nymphs and 75-88% protection against adults in humans, when the solution was applied on the skin (Schwantes et al., 2008). Comparable studies were not conducted for animals so far and could be difficult to generalize, since there are many breeds with different skin and fur conditions.
Essential oils extracted from different plants were also tested for their tick repellent efficacy. Several authors confirmed the efficacy in *in vitro* assays, an overview is presented in Table 3. It appears that biological tick control achieves results that are comparable to chemical tick control, but it must be considered that these studies were mainly performed in *in vitro* assays. Adenubi et al. compared several *in vitro* assays and found that various methods under different external conditions were performed. Non-standardized assays complicate comparison of the results. In addition, studies are frequently missing negative (ideally extractant or solvent) and/or positive controls (e.g., synthetic repellent), further complicating their interpretation (Adenubi et al., 2018). *In vivo* studies in which essential oils are evaluated as tick repellents in animals are rare. In a recent example of such a study, the efficacy of turmeric oil to prevent tick attachment to dogs was evaluated. In a period of four weeks, the owners of the animals were asked to spray the dogs' legs and belly before going for a walk. It was found that the animals in the treated group had close to significant fewer ticks than the negative control and untreated group (Goode et al., 2018). However, it should be mentioned that acaricide pre-treatment was not an exclusion criterion for participation in the study.

Table 2. List of approved acaricides for the control of mites or ticks on animals in Germany

	Chemical agent	Method of Application	Target Species
Ectoparasiticides for topical use	Amitraz	Strip	Bee
	Deltamethrin	Pour-on, Collar	Cattle, Sheep, Cat, Dog
	Doramectin	Pour-on, Injection	Cattle, Sheep
	Eprinomectin	Spot-on, Pour-on, Injection	Cat, Cattle, Sheep, Goat
	Fipronil	Spot-on, Spray	Cat, Dog
	Flumethrin	Pour-on, Collar	Cattle, Cat, Dog
	Formic acid 60%	Solution, Strip	Bee
	Permethrin	Spot-on,	Dog
	Phoxim	Shampoo, Pour-on	Sheep, Pig
	Propoxur	Spray, Shampoo, Collar	Cat, Dog
Ectoparasiticides for systemic use	Afoxolaner	Chewing tablet, Spot-on	Cat, Dog
	Fluralaner	Chewing tablet, Spot-on, Use in drinking water	Cat, Dog, Poultry
	Ivermectin	Injection, Paste, Pour-on	Cattle, Horse, Sheep, Pig
	Lotilaner	Chewing tablet	Cat, Dog
	Moxidectin	Chewing tablet, Spot-on, Injection, Pour-on, Suspension, Paste	Cat, Dog, Ferret, Cattle, Horse, Sheep
	Sarolaner	Chewing tablet, Spot-on	Dog
Repellent	Permethrin	Ear tag, Emulsion	Cattle, Horses

Reference: PharmNet.Bund-Arzneimittel-Informationssystem

Table 3. Repellency (%) by natural repellents (essential oils) in *in vitro* assays against *I. ricinus* nymphs

Essential oil	Repellency in %	Reference
Rosmarinus officinalis	40.0	(Elmhalli et al., 2019)
Salvadora persica	95.0	
Ammi majus	68.3	(El-Seedi et al., 2017)
Ammi visnaga	62.4	
Foeniculum vulgare	70.6	
Nerium oleander	60.0	
Artemisia herba-alba	84.2	
Calendula officinalis	82.0	
Conyza dioscoridis	94.0	
Matricaria recutita	40.0	
Ricinus communis	61.2	
Lawsonia inermis	58.3	
Lantana camara	63.3	
Artemisia abrotanum	56.8	(Tunon et al., 2006)
Dianthus caryophyllum	91.7	
Corymbia citriodora oil	85.0	(Jaenson et al., 2005)
Geranium oil	100.0	
Lavender oil	100.0	

1.6.3 Other strategies to prevent tick bites

To prevent tick infestations, it is recommended to prevent walking in tick habitats, e.g., by walking in the centre of trails and avoiding high grass or leaf litter. Light clothes should be worn on which ticks are easily detected, and tucking pants into socks acts as barrier for ticks to get to the skin. After staying in tick infested areas, clothes, the body as well as the coat of pets should be investigated for ticks. Attached ticks should be removed as soon as possible using forceps or dedicated tick-removal tools. Clothes and backpacks can be washed at ≥ 54 degrees or tumble-dried at high temperatures for at least 6 minutes to destroy ticks (Nelson et al., 2016).

1.6.4 Vaccination

During feeding, ticks introduce salivary proteins into the host that interfere with the hosts' defence mechanisms. In cattle and laboratory animals, such as mice, rabbits and guinea pigs repeated tick infestations lead to impaired tick feeding, resulting in reduced tick numbers, engorgement weight of females and fecundity – a phenomenon known as 'tick immunity' (Allen, 1989; Brossard, 1982; Newson and Chiera, 1989; Trager, 1939).

Tick immunity to *I. ricinus* was observed as natural and experimentally acquired resistance, too. In 1995, Kurtenbach et al. examined tick infestation rates and *B. burgdorferi* s.l. seroprevalence in small rodents in German woodlands, there *Apodemus ssp.* mice were more often infested by larvae than *Clethrionomys sp.* mice. Moreover, investigations regarding the specific infectivity potential of *B. burgdorferi* s.l. revealed that *Clethrionomys* plays a subordinate role compared to *Apodemus* in this area. These results suggest that *Clethrionomys* has developed protective immune responses induced by tick bites or *B. burgdorferi* infection (Kurtenbach et al., 1995). Corresponding to these results, experimental infestation with *I. ricinus* larvae in *Clethrionomys* and *Apodemus* mice led to acquired immunity in *Clethrionomys,* but did not in *Apodemus* (Dizij and Kurtenbach, 1995). A comprehensive recent review provides more insights into acquired tick immunity (Narasimhan et al., 2021).

It has been known for decades that tick immunity can also be artificially induced by immunization with whole tick extracts, single organs, tick cement cone material, recombinant proteins or by passive immunization through the transfer of immunoglobulins from tick-infested hosts (Agbede and Kemp, 1986; Allen, 1979; Banerjee et al., 2003; Kemp et al., 1989; Trager, 1939; Willadsen et al., 1992; Willadsen et al., 1989).

Active immunization with native tick proteins or homogenates was shown to lead to reduced tick numbers, lower engorgement weights and impaired fertility (oviposition and hatching of larvae). Over the last three decades, anti-tick vaccine development mainly focused on the cattle tick *R. microplus*, a one-host tick species that causes high economic losses in the (sub)tropics. Initial studies in which calves were vaccinated with tick protein extracts prepared from partially engorged females, showed reduced engorgement weight and fertility of adult female ticks, as well as significantly reduced tick numbers on vaccinated calves. Kemp et al. suggested that host antibodies bind on tick midgut cells, which are subsequently damaged and eventually result in impaired oviposition and hatching of larvae (Agbede and Kemp, 1986; Johnston et al., 1986; Kemp et al., 1986). Subsequent protein fractionations by electrophoresis, high speed centrifugation and gel permeation chromatography, followed by vaccination trials with increasingly simpler protein mixtures, finally led to the identification of the Bm86 protein (Willadsen et al., 1989). The Bm86 protein, a tick gut membrane-bound glycoprotein, was shown to be effective in both its native and recombinant form (Willadsen et

al., 1992; Willadsen et al., 1989). The Bm86 vaccine was subsequently in form of Gavac® and TickGARD® commercialized (de la Fuente et al., 2007a). Both vaccines were used for years in Latin America and Australia in integrated tick control strategies and were shown to reduce tick numbers and the number of required acaricide treatments. Use of the vaccines also improved the performance of dairy or beef cattle (Jonsson et al., 2000; Valle et al., 2004). Cross-protection of Bm86-based vaccines was observed for other tick species such as *Rhipicephalus annulatus, Hyalomma dromedarii* or *Rhipicephalus decoloratus* (Canales et al., 2009; de Vos et al., 2001; Fragoso et al., 1998), but the vaccine was not effective against *Amblyomma variegatum* and *Rhipicephalus appendiculatus* ticks (de Vos et al., 2001). Recombinant Bm86 orthologues were also evaluated in immunization trials, e.g., Ir86-1 and Ir86-2 for *I. ricinus*, Ba86 for *R. annulatus* or Haa86 for *Hyalomma anatolicum* (Azhahianambi et al., 2009; Canales et al., 2009; Coumou et al., 2015). Whereas immunization with Ba86 and Haa86 did reduce tick feeding success, immunization with Ir86-1 or Ir86-2 did not confer protection against *I. ricinus* infestations in rabbits.

The development of genomic tools such as cDNA library expression immunization (ELI) and RNA interference (RNAi) led to new approaches to identify proteins essential for tick survival and/or function that may therefore be effective tick-protective antigens. These include targets such as Ferritin 1 and 2, iron regulatory protein 1, saliva proteolytic enzyme inhibitors (serpin) or cement proteins (64TRP) that showed at least partial protection (reduced tick numbers or fecundity of females or nymphs) against *I. ricinus* infestations (Hajdusek et al., 2010; Hajdusek et al., 2009; Prevot et al., 2007; Trimnell et al., 2005). An overview of published immunization trials with potential tick-protective antigens targeting *I. ricinus* infestations is presented in Table 4. Although some of the evaluated antigens show promise as vaccine candidates, there remains a need for the identification of more effective antigens.

Table 4. Overview of conducted immunization trials with potential tick-protective antigens against *I. ricinus* infestation.

	Antigen	Method of Identification	Immunization			Tick Challenge		Results Tick Feeding (%)				Effect on Reproduction (%)		Reference
			Animal	Dosage (µg)	Interval	No. of ticks	Life stage	No. of ticks fed	Engorgement weight	Mortality	Moulting	Oviposition	Hatching	
Synthetic peptides targeting the neuropeptides innervating *Ixodes ricinus* salivary glands and hindgut	SIFamide (SIFa)	Study results of related species suggest similar mechanisms, which were identified by MALDI-TOF and immune-histochemical studies	Sheep	50	d0	1000	Larvae	+23	n/a	+2	+37	n/a	n/a	(Almazán et al., 2020b)
					d15									
					d30	48	Nymphs	-92	n/a	+2	+25	n/a	n/a	
			Mouse	10	d0									
					d14	20	Nymphs	+66	-8	-67	+33	n/a	n/a	
					d28									
	Myoinhibitory peptide (MIP)		Sheep	50	d0	1000	Larvae	+25	n/a	-5	+16	n/a	n/a	
					d15									
					d30	48	Nymphs	-37	n/a	+1	-53	n/a	n/a	
			Mouse	10	d0									
					d14	20	Nymphs	+30	-11	-12	+6	n/a	n/a	
					d28									
	SIFa+MIP		Sheep	50+50	d0	1000	Larvae	+22	n/a	-5	+37	n/a	n/a	
					d15									
					d30	48	Nymphs	-26	n/a	-1	-65	n/a	n/a	

	Antigen	Method of Identification	Immunization: Animal	Immunization: Dosage (µg)	Immunization: Interval	Tick Challenge: No. of ticks	Tick Challenge: Life stage	Results Tick Feeding (%): No. of ticks fed	Results Tick Feeding (%): Engorgement weight	Results Tick Feeding (%): Mortality	Moulting	Effect on Reproduction (%): Oviposition	Effect on Reproduction (%): Hatching	Reference
Ixodes ricinus serin protease inhibitors	*I. ricinus* serin protease inhibitor (IrSPI)	Proteins involved in the transmission of pathogens and induced by tick-borne pathogens	Sheep	50	d0 d15 d30	1000 48	Larvae Nymphs	+9 -83	n/a n/a	-29 +3	+19 +59	n/a n/a	n/a n/a	(Almazán et al., 2020a)
			Mouse	10	d0 d14 d28	20	Nymphs	+2	0	+27	+10	n/a	n/a	
	I. ricinus Lipocalin (IrLIP)		Sheep	50	d0 d15 d30	1000 48	Larvae Nymphs	+34 -93	n/a n/a	-57 +3	+75 +59	n/a n/a	n/a n/a	
			Mouse	10	d0 d14 d28	20	Nymphs	+13	+12	-9	+10	n/a	n/a	
	IrSPI+IrLIP		Sheep	50	d0 d15 d30	1000 48	Larvae Nymphs	+19 -50	n/a n/a	-65 +3	+88 -48	n/a n/a	n/a n/a	
	I. ricinus Immuno-suppressor (Iris)	Differential analysis using cDNA Expression Library of unfed and fed females	Mouse	20		15	Nymphs	n/a	-2	—	-2	n/a	n/a	(Prevot et al., 2007)
			Rabbit	50	d0 d21	100	Nymphs	n/a	-14	+367	-3	n/a	n/a	
			Rabbit	50		60	Adults	n/a	-15	+674	n/a	+4	n/a	
	I. ricinus aquaporin (IrAQP)	Essential tick protein in metabolism	Rabbit	50	d0 d14	200	Larvae	n/a	0	-22	-13	n/a	n/a	(Contreras and de la Fuente, 2017)

	Antigen	Method of Identification	Immunization: Animal	Immunization: Dosage (µg)	Immunization: Interval	Tick Challenge: No. of ticks	Tick Challenge: Life stage	Results Tick Feeding (%): No. of ticks fed	Results Tick Feeding (%): Engorgement weight	Results Tick Feeding (%): Mortality	Moulting	Effect on Reproduction (%): Oviposition	Effect on Reproduction (%): Hatching	Reference
	Subolesin/Akirin chimera Q38	cDNA Expression Library Immunization	Rabbit	50	d0 d14	200	Larvae	n/a	+15	+325	-34	n/a	n/a	(Contreras and de la Fuente, 2016)
Iron and heme metabolism proteins	Ferritin 1	Molecular characterization in *in silico*, positive evaluation with RNAi	Rabbit	25	d0 d21 d42	25	Adults	+4	+15	n/a	n/a	+19	-19	(Hajdusek et al., 2016)
	Iron regulatory protein1							+9	+22	n/a	n/a	+19	+6	
	Transferrin2							-11	+18	n/a	n/a	-1	-2	
	Ferrochelatase							-7	+14	n/a	n/a	-19	-1	
	Heme-binding lipoprotein							-9	+10	n/a	n/a	+8	+9	
	Ferritin 2	Essential protein in heme metabolism, positive evaluation with RNAi	Rabbit	100	d0 d14 d21	50	Adults	-43	-31	n/a	n/a	-71	-85	(Hajdusek et al., 2010)
Bm86 Homologues	Ir86-1	Tick protein extract fractionation	Rabbit	100	d0 d21 d42	50	Adults	No effect (no values available)			n/a	No effect (no values available)		(Coumou et al., 2015)
	Ir86-2			100										
	Ir86-1+Ir86-2			50+50										

Antigen	Method of Identification	Immunization			Tick Challenge		Results Tick Feeding (%)			Moulting	Effect on Reproduction (%)		Reference
		Animal	Dosage (µg)	Interval	No. of ticks	Life stage	No. of ticks fed	Engorgement weight	Mortality		Oviposition	Hatching	
Metalloproteinase Metis 1	Immuno-Screening of host defense, positive evaluation with RNAi	Rabbit	50	d0 d14 d21	50	Nymphs	n/a	-2	+25	-2	n/a	n/a	(Decrem et al., 2008)
					30	Adults	n/a	-53	n/a	n/a	-45	n/a	
Secreted cement protein 64P	64TRP2	Hamster	25	d0 d21	50-150	Nymphs	0	n/a	+94	n/a	n/a	n/a	(Trimnell et al., 2005)
		Rabbit	75		15	Adults	+10	-54	-1100	n/a	47	n/a	
	64TRP5	Hamster	25	d0 d21	50-150	Nymphs	0	n/a	+61	n/a	n/a	n/a	
		Rabbit	75		15	Adults	+15	-13	-980	n/a	7	n/a	
	64TRP6	Hamster	25	d0 d21	50-150	Nymphs	0	n/a	+44	n/a	n/a	n/a	
		Rabbit	75		15	Adults	-11	-17	-800	n/a	6	n/a	

Immuno-Screening of cDNA Library with tick-immune sera

Vaccinated groups were compared to trial-specific control groups using the following calculation: Difference (%) = 100[(Mean vaccinated group / Mean control group)-1]. Not available data is indicated by n/a; — indicates that no mortality was found in the control group.

CHAPTER 2

Control of Lyme borreliosis and other *Ixodes ricinus*-borne diseases

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Control of Lyme borreliosis and other *Ixodes ricinus*-borne diseases

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Abstract

Lyme borreliosis (LB) and other *Ixodes ricinus*-borne diseases (TBDs) are diseases that emerge from interactions of humans and domestic animals with infected ticks in nature. Nature, environmental and health policies at (inter) national and local levels affect the risk, disease burden and costs of TBDs. Knowledge on ticks, their pathogens and the diseases they cause have been increasing, and resulted in the discovery of a diversity of control options, which often are not highly effective on their own. Control strategies involving concerted actions from human and animal health sectors as well as from nature managers have not been formulated, let alone implemented. Control of TBDs asks for a "health in all policies" approach, both at the (inter)national level, but also at local levels. For example, wildlife protection and creating urban green spaces are important for animal and human well-being, but may increase the risk of TBDs. In contrast, culling or fencing out deer decreases the risk for TBDs under specific conditions, but may have adverse effects on biodiversity or may be societally unacceptable. Therefore, in the end, nature and health workers together must carry out tailor-made control options for the control of TBDs for humans and animals, with minimal effects on the environment. In that regard, multidisciplinary approaches in environmental, but also medical settings are needed. To facilitate this, communication and collaboration between experts from different fields, which may include patient representatives, should be promoted.

Keywords: Lyme borreliosis, Tick-borne encephalitis, Anaplasmosis, *Ixodes ricinus*, Transmission cycles, Vaccines, Prevention

Background

Ixodes ricinus is a hard tick species that transmits pathogens of medical and veterinary importance. It has recently become clear that the bite of *I. ricinus* by itself can also cause meat allergy [1, 2]. *Ixodes ricinus*-borne infectious diseases are a considerable health concern in many European countries for several reasons.

First of all, the European Center for Disease Prevention and Control has predicted that the incidence of tick-borne diseases (TBDs) will rise in the near future [3]. Several studies describe a long-lasting increase in the incidences of the two most commonly reported TBDs, namely Lyme borreliosis (LB) and tick-borne encephalitis (TBE) in several European countries [4–9]. Another trend is that human infections and diseases involving other tick-borne pathogens (TBPs), such as *Anaplasma phagocytophilum*, *Borrelia miyamotoi*, *Neoehrlichia mikurensis*, spotted fever rickettsiae and *Babesia* species, are emerging or being (re)discovered. Indeed, the number of studies describing infections and disease cases involving these agents is accumulating in the literature [10–17]. The severity and incidence of TBDs, other than LB and TBE, is unknown, awareness is low and adequate diagnostic modalities are often lacking in routine settings. Many of these pathogens are also of veterinary relevance, not only for livestock, but also for pet animals [18–21].

Secondly, the reliability of the diagnosis of LB and the efficacy of antibiotic treatments are publicly being questioned, including by some self-proclaimed experts and medical doctors [22, 23]. The (inter)national guidelines on the clinical diagnosis with recommendations for supporting laboratory diagnosis and treatment appear to

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be a matter of continuous debate [24]. This has led to considerable societal unrest. Furthermore, as *I. ricinus* is often infected with multiple zoonotic agents, it is still unclear to what extent co-infections are able to affect the course of LB [25]. Doubts and uncertainties about the severity, symptoms, diagnosis and treatment of TBDs are widespread in the media, and give rise to uncertainties and controversies between patients and health providers. This rising concern for TBDs has contributed to the formation of LB interest groups in many European countries, who actively seek public and political awareness, particularly for LB. In the Netherlands, for example, an association for LB patients presented a petition with more than 70,000 signatures of concerned citizens to the parliament for more awareness and research on LB and political attention [26].

Together, these concerns require actions of public and medical health professionals and require solid, evidence-based solutions, to minimize the concerns and disease burden of TBD. This review aims to link the knowledge on *I. ricinus* and TBDs from different disciplines, in order to formulate possible solutions and knowledge gaps to control ticks and TBDs. An excellent review on the public health concerns and the challenges to control of *I. scapularis*-borne diseases in the USA has been published [27]. Despite the differences in ecology, epidemiology, environmental and health care systems, there are overlapping research questions which can be tackled together.

Abundance and spread of *I. ricinus*

Understanding which factors drive population densities of disease vectors is an important step in assessing disease risk and formulating possible intervention strategies. *Ixodes ricinus* has a four stage life-cycle, i.e. egg, larva, nymph and adult, requiring only one blood meal during every active stage. The time for *I. ricinus* to complete its life-cycle varies between three and six years, mostly depending on climate and host availability. *Ixodes ricinus* employs an ambush strategy for host finding [28], which implies climbing the vegetation, clinging to the tips of stems, and waiting for a vertebrate host. Questing ticks cling to a host animal as the animal passes through vegetation. After feeding for a few days, ticks detach from the host and fall in the litter layer. It takes several months to molt into their next developmental stage, or, in the case of adult females, to lay several thousand eggs and subsequently die. Only a small fraction of the ticks complete the life-cycle: about 10% of the questing larvae will develop into a questing nymph, and then again between only 1 and 10% of the nymphs manage to develop into a questing adult.

Although *I. ricinus* can utilize a multitude of host species, these host species differ considerably in the numbers of ticks they feed, which further differs between the different tick life stages. In forest areas, larvae predominantly feed on rodents, nymphs feed on the highest variety of host, but mostly forest birds and rodents, whereas the key reproduction hosts for ticks are deer [29]. Although annual fluctuations in rodent densities affect the densities of nymphs the following year to some extent, the (local) presence of propagation hosts, mostly deer, is often the key factor for the presence of moderate tick densities in forested areas [30]. *Ixodes ricinus* spends almost its entire life in the vegetation. Temperature and relative humidity are key requirements for the development, survival and activity of *I. ricinus*. They are considered to be the principal factors limiting the geographic range of *I. ricinus* [31–33]. More locally, the survival time of ticks also strongly depends on (micro)climatic conditions. The large spatio-temporal fluctuations in the densities of questing ticks within a location is mostly determined by daily and seasonal weather conditions [34]. More generally, the climatic changes over the last decades have probably resulted in an increased length of the annual tick questing season [35], whether that has affected the population sizes of ticks is unknown.

These key requirements imply that *I. ricinus* is mainly found in deciduous woodland containing small mammals and deer, but in some areas with sufficient rainfall, large populations may occur in open habitats such as meadows, dune areas and moorland, where the majority probably feed on livestock [36]. Although very focal and often in low densities, *I. ricinus* has also been found in green areas in cities, such as parks and gardens [37, 38]. There, hedgehogs, rather than deer, might act as propagation hosts [39, 40].

Policy driven changes in abundance and spread

Although direct evidence is lacking, the increase in LB and TBE incidence is very likely caused in part by the increase and spread of *I. ricinus* populations [41]. Tick-suitable areas in Europe are expanding, particularly due to reforestation and other actions to restore and protect nature [35, 42–47]. For example, the protective status of wildlife has resulted in increases in their abundance and spread, particularly of deer populations. Expanding and creating ecological networks across Europe is not only beneficial for wildlife, but also for ectoparasites and their associated pathogens, allowing easier maintenance and spread to new areas. The current policy of some European countries is to create more green spaces in (sub)urban areas to improve human health and well-being, and to mitigate the effects health risks such as heat wave, air pollution and flooding (Committee on Climate Change 2014). It is important to realize, however, that these spaces may also enhance opportunities for contact between humans and *I. ricinus*, posing risks for acquiring TBDs [38, 48–50].

Transmission dynamics of TBDs

Pathogens can be acquired by ticks while feeding on infected hosts. In suitable tick vectors, TBPs have the ability to persist throughout the molting process to the next instar, a phenomenon called transstadial transmission. The efficiency of vertical transmission, from female tick to her offspring, varies from non-detectable for *B. burgdorferi* (*s.l.*) and *B. microti* [51, 52], to ~40% for *B. venatorum* [53] to close to 100% for *R. helvetica* [54]. *Ixodes ricinus* is capable to transmit more than twenty different (potentially) pathogenic parasites, bacteria and viruses via their blood meal to vertebrate hosts (Table 1). Pathogen transmission by ticks requires many often unexplored tick-pathogen interactions, from the migration of these pathogens from the gut to their secretion in tick saliva [55].

Vertebrate hosts can be regarded best as amplifying hosts for TBDs, their prominent role is to produce a sufficient number of newly infected ticks to close the enzootic cycles of pathogens. The infection dynamics of pathogens in vertebrate hosts varies in host range, tissue tropism and infection time. The host range of some pathogens, for example *B. lusitaniae* and *A. phagocytophilum* ecotype II, is relatively small with only a few vertebrate species being able to act as amplifying hosts, whereas the host range of others, such as *B. afzelii* and *A. phagocytophilum* ecotype I, is much broader. Tissue tropism varies from skin (*B. afzelii*), to blood (*Babesia* species), immune cells (*A. phagocytophilum*, *N. mikurensis*), endothelium (*R. helvetica*) and even to the central nervous system (TBEV, *B. garinii*). Sometimes, adequate immune responses are developed, for example against *B. miyamotoi* and TBEV, giving rise to short-term, limited infections. Other pathogens, such as *B. burgdorferi* (*s.l.*), *A. phagocytophilum* and probably also several *Babesia* species too can evade the immune system and cause chronic, long-lasting infections. Infections with *B. garinii* appear to be latent in thrushes (*Turdus iliacus*) for several months, but can then be reactivated by physiological cues [56]. Recurrent bacteremia also occurs in sheep, which remain infected persistently with *A. phagocytophilum* [57].

Transmission dynamics can also be affected at the (vertebrate) community levels *via* many, often poorly understood, mechanisms. For example, most vertebrates often are simultaneously or sequentially infected with multiple pathogens. Patterns of (co-)infection arise because infection by one microorganism affects susceptibility to others or due to inherent differences between hosts [58]. Another example is the dilution effect hypothesis, where diluting the abundance of transmission-competent hosts with non-competent hosts will reduce the probability of ticks feeding on transmission-competent hosts and consequently decreases the infection prevalence of pathogens in ticks [59]. This mechanism probably applies only in certain circumstances for a few TBPs, and even less often if considering abundance rather than prevalence of infected ticks [60]. Recently, we showed that mesocarnivores can lower the number of ticks feeding on reservoir-competent hosts, which implies that changes in predator abundance may have cascading effects on tick-borne disease risk [61].

Transmission dynamics can also be affected by weather and climatic conditions. The seasonal synchrony of larval and nymphal stages is an important driver of non-systemic transmission of TBEV *via* co-feeding of infected nymphs with uninfected larvae. This synchrony in tick activity and feeding, in turn, is affected by temperature patterns, in particular autumn cooling and spring warming [62, 63]. Climate change might therefore not only affect the distribution of ticks themselves, but also the distribution and nymphal infection rate of TBEV, and maybe also of other TBPs [41, 64].

These infection dynamics are important drivers for the abundance and spread of infected ticks, and therefore have major clinical implications, implications on the incidence, but also on the risk management and control of the associated diseases. For example, the geographical distribution of TBEV is multifocal [63] with relatively low infection rates, whereas Lyme spirochetes are more widespread with relatively high infection rates in *I. ricinus* [65]. Five genospecies of *B. burgdorferi* (*s.l.*) are commonly associated with LB in Europe: *B. afzelii*, *B. garinii*, *B. burgdorferi* (*sensu stricto*), *B. spielmanii* and *B. bavariensis* [23]. *Borrelia afzelii* is predominantly involved in cutaneous manifestations, such as erythema migrans (EM) and acrodermatitis chronica atrophicans (ACA), *B. garinii* and *B. bavariensis* in neuroborreliosis (LNB), and *B. burgdorferi* in Lyme arthritis (LA) [23, 66]. The incidence of the different manifestations of LB can be partially explained by their pathogenicity and by the relative occurrences of different genospecies in questing ticks [34]. Specific associations have also been found between vertebrates and *Borrelia* genospecies. For example, rodents and voles appear to contribute most to the transmission cycle of *B. afzelii*, whereas thrushes contribute most to the *B. garinii* and *B. valaisiana* cycles [29]. It is to be expected that local abundances of these animals in tick suitable recreational areas determine the risk of acquiring the specific disease manifestations [38, 45].

Epidemiology of TBDs

Measuring incidences and cost of illness (humans) or production loss (livestock) can guide decision-makers to prioritize health policies and initiate cost-effective actions to control diseases with the highest economic or societal impact [67]. As TBE is notifiable in many European countries, incidences and sometimes also cost of illness have been estimated [68]. This information has enabled the

Table 1 Pathogens detected in, or transmitted by, *I. ricinus*. Pathogens are defined here as microorganisms which have been implicated in disease, because of evidence of infection in patients. Some pathogens have only caused disease in immune compromised cases. For most pathogens the Koch's postulates have not been fulfilled and solid epidemiological evidence is lacking too [217]. Some pathogens, particularly *Bartonella, Francisella* and *Coxiella*, have other main modes of transmission. Transmission of *Hepatozoon* spp. by *I. ricinus* is not proven, but the infection of animals with *Hepatozoon* spp. usually involves the digestion of infected ticks. Finally, human infections of TBEV have also occurred through ingestion of contaminated, unpasteurized milk products [218], and other tick-borne pathogens have been transmitted *via* blood transfusion

Microorganism	Variants	Disease	Reference
Borrelia burgdorferi (*s.l.*)	*B. afzelii*	Human	[23, 91, 219]
	B. garinii	Human	
	B. burgdorferi (*s.s.*)	Human/ animal	
	B. spielmanii	Human	
	B. valaisiana	Human	
	B. bavariensis	Human	
	B. bissetti	Human	
	B. finlandensis	–	
	B. lusitaniae	Human	
	B. turdi	–	
Babesia species	*B. venatorum*	Human/animal	[220–222]
	B. divergens	Human/ animal	
	B. microti	Human	
	B. capreoli	Animal	
	B. odocoilei(-like)	–	
Spotted fever rickettsia	*R. helvetica*	Human	[223]
	R. monacensis	Human	
Anaplasma phagocytophilum	Ecotype I	Human /animal	[224]
	Ecotype II	–	
Borrelia miyamotoi	Russian	Human	[17]
	European	Human	
Neoehrlichia mikurensis		Human/animal	[225, 226]
Spiroplasma ixodetes		Human/animal	[227, 228]
Orbivirus	Kemerovo virus	Human	[229]
	Lipovnik virus	Human	
	Tribeč virus	Human	
Flaviviruses	Tick-borne encephalitis virus	Human	[18, 230, 231]
	Louping ill virus	Animal/ human	
Nairovirus	Grotenhout virus	–	[232]
Coltivirus	Eyach virus	Human	[230, 233]
Phlebovirus viruses	Uukuniemi(-like) virus	–	[234]
Midichloria midichondria		–	[235]
Hepatozoon species		–	[236]
Coxiella burnetti		Human/animal	[237]
Francisella tularensis	*F. tularensis holarctica*	Human/animal	[238, 239]
Bartonella species		Human	[240]

calculation of the cost-effectiveness of vaccination strategies against TBE in several countries [69], which further aided the formulation of various strategies to control TBE, from creating awareness alone to incidence-, travel- or profession-based vaccination advises to mass-vaccination campaigns [9, 70].

The epidemiology of LB is more complex. In most European countries LB is not notifiable and incidence estimates are often based on passive reporting laboratory surveillance or on incidental, systematic investigations [71, 72]. Early stages of LB are underreported in laboratory surveillances, because most cases are serologically negative at presentation. Furthermore, laboratory testing at that stage is often not required, hence not recommended by guidelines, for the diagnosis of EM. Most importantly, the clinical manifestations of LB differ enormously in incidence and disease burden. In the Netherlands, 95% of the LB cases are EM, 2% LNB, 2% LA, 0.9% ACA, 0.4% borrelial lymphocytoma, 0.1% Lyme carditis and 0.1% had ocular manifestations [71]. In Germany, comparable proportions were observed [73]. A recent study estimated the total disease burden of LB for the Netherlands. Although ~91% of the LB cases had EM, it only constitutes ~6% of the disease burden, whereas the ~5% cases which displayed persisting symptoms attributed to LB accounted for almost 90% of the disease burden [74]. Thus, controlling the incidence of patients with persisting symptoms attributed to LB, will have the highest impact on reducing disease burden, but hardly on the disease incidence.

Only a few studies have investigated the incidence of other TBDs transmitted by *I. ricinus*, such as anaplasmosis and babesiosis, in Europe [75]. For example, a seroepidemiological study estimated between 10 and 40 human anaplasmosis cases in Belgium per year [11, 76]. Human cases of other TBDs are being reported in the literature, mostly as case studies or series. In contrast, the exposure through bites of infected ticks in the general population and in risk groups such as forest workers is high. Based on molecular evidence alone, the probability of infection with a TBP other than Lyme spirochetes after a tick bite is roughly 2.4% [12]. Similarly, among patients with EM, the probability of a co-infection with another TBP is approximately 3% [12]. How often these infections cause disease or to what extent co-infections affect the course of LB needs further investigation. Infections with TBDs in humans is supported by many serological studies where antibody titers against for example *A. phagocytophilum* have been found in a few percent of human populations [77–79]. Nonetheless, the incidence and severity of the medical problems caused by these TBPs in many, if not all, European countries are unknown. One of the reasons for that is that current diagnostic tools for many of the TBDs are non-existing, of questionable quality, or poorly validated in the European setting. As a consequence, the awareness of other TBDs among physicians and the public is generally low. Therefore, to gain more knowledge on the incidence and nature of TBDs it is imperative to improve laboratory diagnostic tests and awareness.

Domestic animals are more prone to exposure to ticks than humans, as they generally spend more time outdoors, are in closer proximity to the ground and vegetation, and have coats that facilitate tick attachment. Since none of the TBDs associated with *I. ricinus* are notifiable in Europe, official information on TBD incidence in animals is not available. One exception is Q-fever caused by *Coxiella burnetii*, but the role of ticks in the epidemiology of the disease is considered to be negligible [80]. Most reports concern case descriptions, seroprevalence studies or molecular surveys looking at the occurrence of pathogens in ticks collected from the vegetation or animals, which says little about the actual incidence of clinical disease in animals.

Clinical aspects of TBDs: clinical presentation, diagnostics and treatment

Clinical presentation of LB and TBE

LB is divided in three partially overlapping stages, reflecting the duration of the infection and the severity of the disease [23, 81]. The first stage is characterized by the hallmark EM, an erythematous expanding skin lesion at the site of the tick-bite (Fig. 1), usually occurring 1–2 weeks after the tick-bite. When left untreated or unnoticed the infection can disseminate and cause early disseminated and eventually late disseminated LB, the second and third stage respectively [81]. The characteristic manifestations of early disseminated LB include other skin manifestations, such as lymphocytoma and multiple EM, carditis, oligoarthritis and neurological symptoms, such as meningo-(poly)radiculitis (Bannwarth syndrome) with or without cranial nerve involvement [82], amongst other rare manifestations. The central nervous system or the joints can also be affected in late disseminated disease, but the hallmark clinical manifestation of late LB is ACA [83].

Apart from these clear-cut manifestations, there are patients with non-characteristic complaints such as myalgia, arthralgia, fatigue, which are sometimes attributed to LB. These complaints are often long-lasting and can even be debilitating. The constellation of these symptoms is sometimes referred to as chronic Lyme, however this term seems to be an umbrella name for a variety of diseases and syndromes [22, 84]. Examples thereof include late disseminated LB, post-treatment LB syndrome (a post-infectious syndrome), a persisting *B. burgdorferi* (*s.l.*) infection after antibiotic treatment, or one of many other diagnoses misattributed to LB [85]. How to define “chronic Lyme” is more than a semantic discussion as a proper diagnosis [84], i.e. the identification of the nature and cause of an illness, greatly determines the care and cure of patients [85], and could lower the disease burden and medical costs.

Exposure to Lyme spirochetes in animals in Europe is common, with reported seroprevalences in Europe in

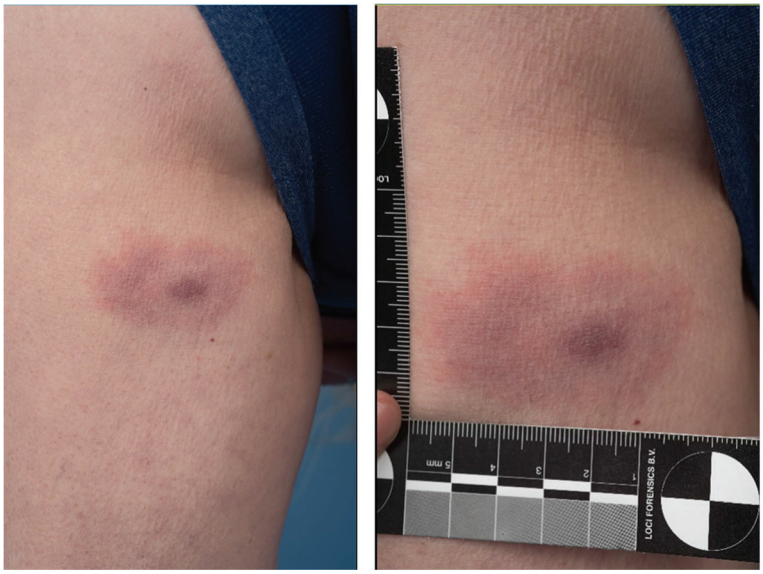

Fig. 1 Clinical representation of an EM, the most common manifestation of LB. A culture-proven EM (diameter ~6 cm) on the leg of a 62-year-old female. This patient presented with this slowly expanding macula with very faint central clearing as the only symptom. There was no known tick bite prior to the development of the lesion.

healthy dog populations ranging from 0.3% in southern Italy [86] to 26% in Serbia [87] and 7% in horses from in Italy [88] to 30% in France [89], but clinical disease with a conclusive LB diagnosis is rare. Clinical LB caused by *B. burgdorferi* (*s.s.*) has nonetheless been reported in dogs, horses and cats [90–95]. A broad spectrum of clinical signs has been associated to *Borrelia* infections in animals, including fever, lethargy, weight loss, (shifting) lameness, ataxia, uveitis, polyarthritis, glomerulopathy and neuritis [96–98]. This variation might to some extent be the result of unapparent co-infections with other pathogens, such as *A. phagocytophilum* [99, 100].

Ixodes ricinus ticks transmit the European variant of TBEV [101]. Although the majority of human infections are asymptomatic, the first symptoms are displayed between 2–28 days (median = 8) after a tick bite [102, 103]. Infections with the European TBEV usually display a typical biphasic course with a viremic phase of 2–10 days and a neurological phase of 1–21 days, separated by a period (median = 7 days) without symptoms [104]. In the first phase the most common symptoms are fever, fatigue and headaches [103]. After a temporary recovery, neurological symptoms appear in the second phase, ranging from mild meningitis to severe meningoencephalomyelitis [103]. Although TBEV-EU mortality is relatively low (1–2%), neurological sequelae, which can greatly affect the quality of life, often occur [105]. TBEV may also affect dogs and result in fever, change in behavior and various neurological symptoms (reviewed in [106]). A variant of the TBEV, Louping Ill virus causes acute encephalomyelitis, resulting in severe illness and death in livestock, especially sheep and red grouse [18].

Clinical presentation of other TBDs

The clinical spectrum of human granulocytic anaplasmosis ranges from subclinical and self-limiting to subacute, chronic or severe in the immunocompromised [107]. The incubation period is 1–2 weeks, after which non-characteristic symptoms (e.g. fever, flu-like symptoms) arise, accompanied by nausea, vomitus, abdominal pain and arthralgia in approximately one-third of the patients. A skin rash or neurological symptoms are less frequently observed, and the estimated fatality rate is less than 1% [108–112]. Animal species that may be affected by *A. phagocytophilum* include dogs, cattle, horses and sheep and clinical signs vary in severity but are usually non-specific such as fever, lethargy and anorexia [113]. To date, there are dozens of case reports of human neoehrlichiosis, the disease caused by *N. mikurensis,* describing acute and chronic infections characterized by fever, headache, nausea, arthralgia, haemorrhages and weight loss [16]. *Borrelia miyamotoi* causes hard tick-borne relapsing fever (HTBRF). The onset of symptoms starts approximately two weeks after a tick-bite with a sudden onset of high fever with signs of septicaemia accompanied by headache, myalgia, arthralgia, and coughing or even gastrointestinal symptoms. The relapsing fever episodes typically last three days, divided by seven relatively healthy days, although for HTBRF the typical relapsing fever pattern is not that often observed [114]. The general trend of the course of the disease is subsiding and self-limiting, but in rare cases, i.e. in highly immunocompromised individuals, the disease appears to be neuro-invasive [114–118]. The two spotted

fever rickettsias transmitted by *I. ricinus* are *R. helvetica* and *R. monacensis.* Their infections may cause vasculitis with fever, headache, myalgia and local lymphadenopathy. An inoculation eschar and generalized maculopapular rash, which are pathognomic for other spotted fever rickettsiosis, are rarely described for these genospecies [115, 119, 120]. The pathogenicity of *R. helvetica* is only partly established by several case series and reports in Europe [121–126]. *Rickettsia monacensis* infection is even less defined, although culture, molecular and serological evidence of human exposure has been reported [127–129].

In Europe, human babesiosis is caused by *B. microti, B. divergens* and *B. venatorum* [12, 130–132]. All *Babesia* species infect erythrocytes and cause haemolysis, leading to the clinical manifestations of fever, anaemia, jaundice, haemoglobinuria and potentially also renal insufficiency. Over 40 human babesiosis cases have been reported in Europe, mostly in asplenic patients. Other risk factors are immunosuppression, depletion of mature B-cells and old age [21]. Judging from the discrepancy between case reports and seroprevalence, an asymptomatic and/or self-limiting course is common [133]. Although serious infection appears to be uncommon, when acquired, the disease has a mortality rate of 42% in *B. divergens* and 5% in *B. microti* [134]. *Babesia divergens* is the causal agent of bovine babesiosis in Europe. The clinical picture is similar to that seen in humans, with a bimodal seasonal occurrence of the disease that is associated with *I. ricinus* activity. In cattle, an inverse age resistance phenomenon is present in which calves up to the age of 9–12 months are susceptible for infection, but resistant to disease [21].

Human infections with multiple TBDs and even with non-tick borne pathogens have been described [135, 136]. Co-infections have been shown to affect the course of LB causing a longer and more disabling course of disease [137–142]. Although co-infections in ticks are the rule rather than the exception [25, 34], the opposite is probably true in humans: co-infections seem to occur only occasionally [129, 143–146]. Our recent findings indicate that among patients with EM the probability of a co-infection with second TBP is merely 3% [12]. To date, there is no convincing evidence that infection with any other TBP or any other infectious agent, is associated with chronic Lyme [147, 148].

Diagnosis of LB

In diagnosing LB, the foremost tool for a physician is a thorough history and physical examination. An EM is considered a clinical diagnosis and additional laboratory testing for EM is discouraged [82]. For many other disseminated forms of LB, laboratory work-up, including a search for alternative explanations, may serve to aid the physician. Serology is the current standard as it has good diagnostic parameters with a sensitivity and specificity of more than 90–95% in LB patients with late (disseminated) manifestations [149]. Serology has some disadvantages. First, the sensitivity is low in early stages of LB, approximately 50% [149], which may lead to a wrong or delayed diagnosis. Secondly, approximately 5% of the general population - and even higher depending on the age, geographical region and the population examined - have antibodies against Lyme spirochetes, while not having active LB [150]. Thus, serology cannot always differentiate well between a past and a current *Borrelia* infection [151–153]. Additional tests include PCR or culture, which are only recommended for specific manifestations and specific tissues or fluids: on synovial fluid/tissue in the case of LA, on a skin biopsy in the case of an ACA or in some specific cases of LNB on cerebrospinal fluid (CSF) [154, 155]. For LNB, other laboratory tests are available, such as leukocyte count, intrathecal antibody production, or intrathecal CXCL-13 concentration, to support the presence of an infection in the central nervous system or other inflammatory conditions [156].

The diagnosis of any form of chronic Lyme is far more complicated. Some of these patients may benefit from (additional) antibiotic treatment, while others may be better helped with other forms of treatment or rehabilitation. A laboratory test that is able to adequately differentiate between a past and active *Borrelia* infection is desired for these patients. It has been hypothesized that cellular tests have this ability. Several of these tests are already commercially available, but their accuracy has not been adequately determined [157–160] and, therefore, warrants more research before they can be used in clinical practice [159]. In addition, there are many alternative methods, which are said to test for LB, but sound evidence for these methods (e.g. as dark field microscopy directly on blood, VEGA-test or bio-resonance) is lacking [161].

When deciding to test for a given condition, whether it be LB or any other disease, it is important not to only take into consideration what the technical performance of a test is, but to also consider the pre-test probability that the patient has the disease [155]. When the pre-test probability of LB is low, then - taking into account the current diagnostic parameters of serological tests and the incidence of IgG-seropositivity in the general population - the added value of testing is limited. Furthermore, the various LB manifestations, the pre-test probabilities as well as the population under study and their expectations vary greatly between primary, secondary, and tertiary care. This might also affect recommendations for the use of *Borrelia* serology in current guidelines and requires further investigation. In that regard, although not recommended in most guidelines,

it could be argued that testing for LB in patients with longer-lasting symptoms with a low pre-test probability in the primary care setting, could actually be helpful. In this situation, a negative test result would make an LB manifestation extremely unlikely, whereas a positive test result would require further investigation, e.g. referral to secondary or tertiary care center. Nevertheless, in some situations testing for LB is discouraged altogether, specifically when the patient is clinically diagnosed with an EM.

The non-specific clinical picture, together with a high seroprevalence, also complicate the diagnosis of LB in animals. The combination of a history to tick exposure within an endemic region, clinical signs consistent with LB, a positive test result, exclusion of differential diagnoses and response to treatment are required for a presumptive LB diagnosis in animals [162].

Diagnosis of TBE and other TBDs

TBEV infection is associated with general non-specific infectious biochemical and blood count results. CSF analysis usually shows pleocytosis with polymorphonuclear cells early, and mononuclear cells late, in the disease development [163]. Serology can be performed on both liquor and serum by IgM/IgG ELISA, which is the most common diagnostic method for TBEV-infection in dogs as well [106]. A four-fold rise in TBEV-specific antibodies in liquor or serum confirm the diagnosis. A neutralization assay is recommended in flavivirus endemic regions to avoid a false positive result [104, 164]. Imaging of the brain and/or myelum may result in focal abnormalities; however it does not contribute greatly to the diagnosis [163].

Diagnosis of other TBDs is based on the assembly of specific clinical characteristics, laboratory findings together with diagnostic tools in a setting of relevant epidemiological exposure. The main non-specific laboratory findings associated with other TBDs are general parameters found in infection, such as elevated inflammation parameters (C-reactive protein, erythrocyte sedimentation rate), leukopenia or leucocytosis, thrombocytopenia and anaemia, with or without elevated liver enzymes or kidney dysfunction. Especially in *A. phagocytophilum* and *N. mikurensis* infection, leukopenia is observed due to leukocyte infection [108, 165]. *Babesia* spp. can cause a distinct haemolytic anaemia due to erythrocyte infection with accompanying elevated bilirubin, reticulocytosis and decreased haptoglobin [166]. Thrombocytopenia appears to be most pronounced in anaplasmosis, babesiosis and HTBRF. In the rare severe cases of anaplasmosis and HTBRF with involvement of the central nervous system, the CSF can reveal pleocytosis [167, 168]. For some TBDs, there are additional, more specific, tests available, such as a buffy coat examination for *A. phagocytophilum* or peripheral blood smear with Giemsa staining in *A. phagocytophilum* and *Babesia* spp. to look for respectively morulae or merozoites by microscopy [169, 170].

Most additional targeted diagnostic tests in TBDs are either in the experimental phase or not widely validated (molecular tests), based on cross-reactivity between other species (serology), time-consuming, or difficult to perform and requiring a high level of expertise (cultures) [171–173]. In general, the sensitivity of available molecular tests for all these TBDs is high in the first week of disease and rapidly decreases over time, and after proper treatment. Therefore, a positive PCR result is helpful, but a negative result does not rule out the diagnosis. As an exception to the rule, *Babesia* spp. can be detected up to months to years after (un)treated infection [174, 175].

For most TBPs, there are no standardized antigens, or well-defined consensus as to what thresholds constitute a significant antibody titer. As a rule of thumb, serological tests are usually required to show a four-fold rise in antibody titer in convalescent sera. It should be noted that the onset of symptoms sometimes precedes the rise in antibody titer. In addition, because antibodies may persist beyond the clearance of infection, it can be difficult to distinguish between a past, recent or current infection [21, 169, 176]. For *A. phagocytophilum*, *Babesia* and *Rickettsia* spp. indirect fluorescent antibody tests are available, yet they make use of other strains or even genospecies than the ones found in Europe, with the exception of *B. microti* [166, 171, 177, 178]. Serological tests for *B. miyamotoi* are in the experimental phase and based on specific antigens (glycerophosphodiester phosphodiesterase (GlpQ) and more recently also variable major proteins (Vmps) identified in the available different isolates from Asia and the USA [179, 180]. These assays do not discriminate between the different relapsing fever *Borrelia* genospecies. There is no widely available and established serological test for the diagnosis of *N. mikurensis* infection.

Treatment of TBDs

LB is treated with antibiotics. The prognosis, especially when treated early in the course of the disease is good, although rarely antibiotic failure can occur. In contrast, persisting symptoms can be observed in approximately 5–20% of LB patients despite recommended antibiotic treatment [181, 182]. Therefore, this condition has been referred to as post-treatment LB [169]. It has been shown in multiple placebo controlled randomized trials that prolonged antibiotic treatment is not effective in treating these non-specific yet disabling and long-lasting symptoms [183–187]. LB in animals is also treated with antibiotics, usually with doxycycline given *per os* at 10 mg/kg every 12 or 24 h for a period of one month [162, 188]. In horses, the intravenous administration of oxytetracycline

(5 mg/kg/day) was more effective in clearing experimentally induced *Borrelia* infections than doxycycline treatment [189].

There is no causal treatment for TBEV. Treatment consists of supportive care and there is no evidence that steroids or immunoglobulins are beneficial [104]. Asymptomatic or subclinical infection frequently occurs for all of the other TBDs and thus infection does not necessarily require treatment. However, when symptomatic, treatment is, or may be, warranted. The large group of intracellular other TBDs, such as *A. phagocytophilum*, *N. mikurensis* and spotted fever rickettsia, as well as *B. miyamotoi* are all susceptible to doxycycline, which is the drug of choice [116, 169, 173, 190] for adults. For younger children, pregnant women and when the central nervous system is affected, specific alternatives exist. Therefore, in countries where doxycycline is recommended as the first line treatment for LB, these pathogens would be concomitantly treated. In countries where beta-lactams are the drug of first choice for LB, clinicians should have a higher level of suspicion for other TBDs, since these are likely not co-treated as such. Moreover, babesiosis requires a different treatment, consisting of azitromycine and atovaquone or clindamycine and quinine depending on the severity of the disease [169]. Cattle suffering from babesiosis are treated with imidocarb diproprionate. In Africa, diminazene aceturate is frequently used to treat bovine babesiosis caused by *B. bovis* or *B. bigemina*, but this product is not available in Europe [21].

Control of LB and other *I. ricinus*-borne diseases

Knowledge on *I. ricinus*, its associated pathogens and the diseases they cause have been increasing in many fields and many approaches to control or prevent TBDs have been investigated and proposed (Table 2). Excellent reviews and even (hand)books on this topic are available [191–195].

Personal preventive actions

Control of *I. ricinus*-borne diseases primarily consist of the promotion of personal preventive actions for the public and for risk groups, such as forest workers, by providing information and education. Such actions include avoiding high-risk habitats, wearing protective clothing, application of repellents, prompt removal of attached ticks, and seeking medical advice when developing symptoms (e.g. fever, skin rash) or another illness in weeks to months after a tick bite. Personal protective measures have poor rates of compliance and their effectiveness has been difficult to demonstrate in terms of reducing disease cases [196, 197]. For example, providing information and education has not resulted in a decline

Table 2 Present and potential measures[a] to control TBDs. This table is modified from Eisen & Gray [241]. There is not a single method that effectively controls all TBDs. National and local strategies, which combine several methods probably work best [191, 192]. Anti-tick vaccines blocking pathogen transmission in humans and domestic animals might encompass the silver bullet to control TBDs. Hygiene measures[b] involve checking for tick bites, prompt removal, and most importantly, seek medical advice when developing symptoms (e.g. fever, skin rash) or illness in weeks to months after a tick bite

Personal	Domestic animal	Residential	Vegetation	Fauna	Medical
Avoid tick habitats	Avoid tick habitats	Xeriscaping/ Hardscaping	Awareness for visitors		Increase awareness and knowledge of medical doctors
Protective clothing	Treatments with topical or systematic acaricides	Keep grass short, remove weeds, remove leaf litter and brush	Reduce tick abundance on sites with high recreational activities	Deer fencing	Technical improvement of laboratory tests
Repellents	Hygiene measures[b]	Remove harborages/ food for rodents and insectivores	Avoidance tick habitats/ directing visitor flows	Deer removal	Improvement of diagnostic/ clinical pathways
Acaricide-impregnated clothing		Fencing to exclude wildlife	Mowing/extensive grazing of paths and recreational sites	Topical acaricide for propagation hosts (deer)	Improve cure and care of patients with late LB and persisting complaints
Hygiene measures[b]		Move play/rest structures to low risk areas	Create open habitats rather than woodlands	Sheep mopping	Prophylactic antibiotic treatment after a tick bite
Control ticks on dogs/ cats and in gardens		Chemical/fungal acaricides		Topical acaricide/antibiotics for rodents	
sTBE vaccine	TBE vaccine			Oral LB vaccine for rodents[a]	
LB vaccine[a]	LB vaccine			Oral tick growth regulator/acaricide[a]	
Tick vaccine[a]	Tick vaccine[a]			Tick vaccine[a]	

in the incidence of LB in the Netherlands, not even after intensified efforts since 2003 [7].

Environmental-based approaches

Environmental-based approaches mostly rely on reduction of tick suitable habitats, the disruption of the tick life-cycle or interference with pathogen transmission. A major advantage of environmental-based control options is that most of them can readily be applied in various practical situations, as they involve existing nature management options, such as mowing, grazing or fencing [195]. Furthermore, controlling tick abundance or tick exposure reduces the risk of acquiring any TBD for both humans and domestic animals. So far, there has been little interest in Europe in environmentally-based preventive measures. Large-scale and long-term spraying with acaricides was carried out in Russia during the 1970s and 1980s in an attempt to control *I. persulcatus*, the main vector of the TBEV [198]. The widespread application of acaricides has been publicly criticized and has become socially undesirable, because of their detrimental effects on the ecosystem and biodiversity [191, 199]. Unlike in the USA, only a limited number of studies exploring environmental-based methods to control ticks have been conducted in Europe [30, 195, 200–202]. A wide range of acaricidal products in various formulations, which are effective against *I. ricinus,* is being used for tick control on domestic animals [203].

Health in All Policies

Most, if not all, of the available environmentally-based preventive and control measures suffer from the fact that they are not highly effective on their own [192]. Probably, long-term implementation of control strategies, i.e. the integrated use of two or more control measures, are necessary to effectively reduce disease risk. Only a few studies on the effectiveness of control strategies have been carried out in the USA, but not in Europe [192]. The successful implementation of environmentally-based preventive and control measures requires involvement of stakeholders from both nature management and human (and animal) health ('One Health'). Of key importance is that the environmental control options for TBDs are put into the context of other aims and ambitions, such as nature conservation, ecosystem services or heat mitigation in urban areas. Indeed, sectors involved in nature management and environmental planning are often more familiar with a so-called 'Health in All Policies' approach. The 'Health in All Policies' approach integrates and articulates many health considerations, far broader than infectious diseases alone, into policymaking across sectors. A future challenge is to integrate the risk of TBDs, but also of wildlife- and other vector-borne diseases, into the 'Health in All Policies' in local nature organizations, such as Municipal Health Services and nature owners, but also governmental institutions and (inter)national organizations responsible for nature and health.

Healthcare actions

More and better awareness of the epidemiology, clinical presentation and course of the various TBDs amongst physicians could raise a suspicion on these diseases in endemic regions. For example, the communication with health professionals on the presence of *B. miyamotoi* and TBEV in questing ticks in the Netherlands has resulted in the identification of the first cases of HTBRF and TBE [70, 168]. Clearly, there is room for the improvement of laboratory tests for the diagnosis of LB and especially other TBDs. Both direct (antigen tests, cultivation or molecular tests) and indirect tests (serology or cellular tests) could greatly aid in establishing the diagnosis. Rather than making one guideline for each tick-borne disease separately, it might be more advantageous to have one guideline for all TBDs for primary care centers with clear consensus on diagnostic testing and referral to secondary and tertiary care centers. Specialized guidelines for secondary and tertiary care centers can aid the diagnosis and treatment for more severe manifestations of LB, but also and for all variants of chronic Lyme. Finally, better knowledge on the course of the various diseases after treatment could prevent overdiagnosis and retreatment.

Vaccination

Where the risk of infection is high or the resulting disease severe, vaccines may be the most efficient and cost-effective means of prevention and control [204]. TBE is well under control in Austria because of mass vaccination programs. The available TBE vaccines have an effectiveness of ~98%. With a vaccination rate in the population of 85%, it is estimated that more than 4000 severe cases of TBE were prevented in Austria between 2000 and 2011 [205]. Remarkably, the vaccination coverage in many central and eastern European countries is low [206], despite predictions that TBE vaccination programs in central and eastern Europe can be cost-effective [207]. A vaccine protecting against LB is currently unavailable in Europe, but a potential vaccine has recently been tested in a Phase I/II trial [208, 209], and another LB vaccine is being developed for the European market as well [210]. Based on the experiences with a previous Lyme vaccine that was on the American market, with an effectivity between 62% and 85%, it remains to be seen whether a Lyme vaccine will be widely accepted and used, or only cost-effective for high risk groups [211, 212]. Ideally, one would like to have a single vaccine for humans, protecting against multiple TBDs [213]. Anti-tick vaccines targeting other tick species already exist and are being used in the veterinary field.

The strategy behind these vaccines is to locally control *Rhipicephalus* (*Boophilus*) tick species, and act as a safe and environmentally friendly alternative to acaricides [214, 215]. Application of anti-tick vaccines was shown to dramatically decrease the incidence of bovine babesiosis [216]. Whether anti-tick vaccines can also be used to (locally) eradicate *I. ricinus* populations and prevent human TBDs is difficult to predict due to its very large host range, yet is a topic of investigation [213].

Conclusion

Unfortunately, there is no silver bullet to control TBDs yet. In order to effectively control TBDs, "health" should be considered in a broader context, involving ecosystems, the environment, wildlife, animals and also curative and public health and policymaking. This implies a multidisciplinary approach and asks for international collaborations throughout Europe, but also multidisciplinary collaborations and approaches at local levels. Patient representatives or patient advocacy groups are part of such a multidisciplinary approach. In our experience, patients and researchers often have shared goals and convictions, yet comprehensive collaboration in the field of LB research seems rare. Patient advocates can have a valuable role in anything from designing the study and securing funding, to effectively communicating study results to patients and the general public.

Since the ecology and epidemiology of TBDs are diverse, yet greatly influence the burden of the different TBDs, these should also be considered. In addition, more awareness amongst physicians, prompt recognition of the various clinical symptoms and improved diagnostic tools could aid in combating TBDs in the future. A variety of personal and environment-based preventive and control measures exist, but suffer from the fact that they are not highly effective on their own. Combining them, and investing in fundamental as well as translational research, to be able to formulate (evidence-based) strategies on the control of TBDs might prove to be the way forward. Last, but most certainly not least, for most of the TBDs no vaccine exists and therefore research should most definitely focus on vaccine discovery and development. In that regard, vaccines targeting the tick vector, which could potentially prevent multiple TBPs, have the potential to become the next silver bullet, and require further investigation.

Abbreviations

ACA: Acrodermatitis chronica atrophicans; CSF: Cerebrospinal fluid; EM: Erythema migrans; GlpQ: Glycerophosphodiester phosphodiesterase; HTBRF: Hard tick-borne relapsing fever; LA: Lyme arthritis; LB: Lyme borreliosis; LNB: Lyme neuroborreliosis; TBD: Tick-borne disease; TBE(V): Tick-borne encephalitis (virus); TBP: Tick-borne pathogen; Vmps: Variable major proteins

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HS and JWH initiated the review and drafted the outline. All authors have written one or more sections of the review. All authors read and approved the final manuscript.

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CHAPTER 3

Preliminary Evaluation of Tick Protein Extracts and Recombinant Ferritin 2 as Anti-tick Vaccines Targeting *Ixodes ricinus* in Cattle

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Anti-tick vaccines have the potential to be an environmentally friendly and cost-effective option for tick control. In vaccine development, the identification of efficacious antigens forms the major bottleneck. In this study, the efficacy of immunization with recombinant ferritin 2 and native tick protein extracts (TPEs) against *Ixodes ricinus* infestations in calves was assessed in two immunization experiments. In the first experiment, each calf (n = 3) was immunized twice with recombinant ferritin 2 from *I. ricinus* (IrFER2), TPE consisting of soluble proteins from the internal organs of partially fed *I. ricinus* females, or adjuvant, respectively. In the second experiment, each calf (n = 4) was immunized with protein extracts from the midgut (ME) of partially fed females, the salivary glands (SGE) of partially fed females, a combination of ME and SGE, or adjuvant, respectively. Two weeks after the booster immunization, calves were challenged with 100 females and 200 nymphs. Blood was collected from the calves before the first and after the second immunization and fed to *I. ricinus* females and nymphs using an *in vitro* artificial tick feeding system. The two calves vaccinated with whole TPE and midgut extract (ME) showed hyperemia on tick bite sites 2 days post tick infestation and exudative blisters were observed in the ME-vaccinated animal, signs that were suggestive of a delayed type hypersensitivity (DTH) reaction. Significantly fewer ticks successfully fed on the three animals vaccinated with TPE, SGE, or ME. Adults fed on the TPE and ME vaccinated animals weighed significantly less. Tick feeding on the IrFER2 vaccinated calf was not impaired. The *in vitro* feeding of serum or fresh whole blood collected from the vaccinated animals did not significantly affect tick feeding success. Immunization with native *I. ricinus* TPEs thus conferred a strong immune response in calves and significantly reduced the feeding success of both nymphs and adults. *In vitro* feeding of serum or blood collected from vaccinated animals to ticks did not affect tick feeding, indicating that antibodies alone were not responsible for the observed vaccine immunity.

Keywords: *Ixodes ricinus*, anti-tick vaccine, salivary glands extract, midgut extract, ferritin, artificial tick feeding

INTRODUCTION

Ixodes ricinus is a tick species which is widespread in Europe and can transmit various bacterial, protozoal and viral pathogens of medical and veterinary importance, including the causal agents of Lyme borreliosis, tick-borne encephalitis (TBE) virus and babesiosis. Multiple studies have shown that the incidence of both Lyme borreliosis and TBE in several European countries have increased over the last decades (Smith and Takkinen, 2006; Fulop and Poggensee, 2008; Sykes and Makiello, 2017; Radzisauskiene et al., 2018). Lyme borreliosis is also the most common zoonotic vector-borne pathogen in the United States where *I. scapularis*, a sister species of *I. ricinus*, is the main arthropod vector (Schwartz et al., 2017).

The abundance and activity of *I. ricinus* ticks depends on abiotic factors, including relative humidity and temperature, as well as biotic factors such as adequate vegetation cover and vertebrate host availability (Randolph et al., 2002). *I. ricinus* is a three-host tick species with a broad host range; larvae and nymphs feed predominantly on rodents and birds, whereas the key reproduction hosts for adults are larger mammals (Gray et al., 1998). Control of *I. ricinus* and associated tick-borne diseases include personal preventive measures, such as the avoidance of tick habitats and a prompt removal of attached ticks, as well as environmental-based approaches, including habitat modification, a reduction of host densities or treatment of wildlife hosts with acaricides (Pound et al., 2012; Sprong et al., 2018). Another alternative targeted at controlling the tick vector is the use of anti-tick vaccines that would interfere with tick feeding and survival or pathogen transmission (Parizi et al., 2012; Sprong et al., 2014).

The first observations that animals repeatedly infested with ticks can develop an immune response that results in the rejection of ticks and that injection of tick extracts may also result in a partial immunity were made by William Trager in the 1930s (Trager, 1939a,b). This and similar studies formed the foundation for work by Australian scientists which led to the identification of the Bm86 antigen. This antigen is the principal component of the only commercialized anti-tick vaccine targeting an ectoparasite, the one-host tick *Rhipicephalus microplus*, to date (reviewed in Willadsen, 2004). The Bm86 protein was identified following multiple cycles of biochemical fractionation of immunogenic tick midgut extracts (MEs) followed by immunization trials with parasite challenges, with increasingly simpler protein mixtures being used for immunization in each successive cycle (Willadsen et al., 1989). Immunization with recombinant Bm86 was subsequently shown to be effective against *R. microplus* and a number of other tick species and homologs of Bm86 were identified in all main ixodid tick genera (de Vos et al., 2001; Nijhof et al., 2007). Immunization with two Bm86 orthologs isolated from *I. ricinus* was, however, not effective against conspecific tick infestations in rabbits (Coumou et al., 2015). More promising results for *I. ricinus* were obtained by immunization of rabbits with recombinant ferritin 2 (FER2). This protein is secreted by the tick midgut into the hemocoel and acts as an iron transporter, thus playing a pivotal role in the iron metabolism of ticks (Hajdusek et al., 2009). Immunization with recombinant FER2 in rabbits resulted in a reduction in tick numbers, engorgement weight and fertility rate of *I. ricinus* females feeding on immunized animals. Similar effects were observed for *R. annulatus* and *R. microplus* ticks feeding on cattle immunized with recombinant *R. microplus* FER2 (RmFER2) (Hajdusek et al., 2010). Other recombinant proteins that were evaluated for their efficacy in controlling *Ixodes* infestations in rabbits, mice and guinea pigs include subolesin (Almazan et al., 2005), tick cement protein 64TRP (Trimnell et al., 2005), the elastase inhibitor Iris (Prevot et al., 2007), sialostatin L2 (Kotsyfakis et al., 2008), a putative metalloprotease (Metis 1) (Decrem et al., 2008), anti-complement proteins IRAC I and IRAC II (Gillet et al., 2009), a cyclin-dependent kinase (Gomes et al., 2015), and aquaporin (Contreras and de la Fuente, 2017).

In this pilot study, we aimed to investigate if immunization of cattle to reduce *I. ricinus* tick feeding and reproduction is possible. To this purpose, we evaluated the use of recombinant FER2 as well as native tissue extracts. The cow was chosen as an animal model since it is a suitable host for infestations with high numbers of *I. ricinus* and also because large blood volumes can be safely drawn without affecting the animal's health (Wolfensohn and Lloyd, 2013). This facilitated the subsequent evaluation of collected antisera within an artificial tick feeding system (ATFS) to study individual components of the immunological response on tick feeding (Contreras et al., 2017).

MATERIALS AND METHODS

Calves and Ticks

Six-month-old Holstein-Friesian calves were purchased from a local dairy farm. Calves were housed on pastures of the Institute for Parasitology and Tropical Veterinary Medicine in Berlin during the first 7 weeks of the study (d0–d49). The pastures were considered to be free of ticks; natural tick infestations were not observed during this period. One week prior to tick challenge, they were moved to an enclosed cattle pen for the duration of the tick infestation. *I. ricinus* ticks for the *in vivo* challenge originated from the tick breeding unit of the Institute for Parasitology and Tropical Veterinary Medicine of the Freie Universität Berlin. For the *in vitro* feeding experiments, half of the adult *I. ricinus* ticks originated from the tick breeding unit. Prior to use, these were mixed with an equal number of adult *I. ricinus* ticks collected from the vegetation in and around Berlin, as previous (unpublished) work suggested that this might improve the attachment rate of ticks in the ATFS. All animal experiments were conducted with approval of the commission for animal experiments (LAGeSo, Berlin, registration number G0210/15).

Vaccine Preparation

Native Tick Protein Extracts

For the preparation of native tick protein extracts (TPEs), female *I. ricinus* ticks were prefer for 3–5 days on rabbits. Partially fed females weighing ∼30 to 70 mg were manually detached and washed for 30 s in 70% ethanol. They were subsequently dissected on a glass slide under ice-cold phosphate buffered saline (PBS, pH

7.2). The internal organs (midguts, salivary glands, Malpighian tubules, trachea, synganglia, and ovaries), were dissected and stored in PBS on ice. For preparation of the TPE used in the first immunization study, all internal organs except the midguts were pooled together. For the second immunization study, only the salivary glands and midguts were used for preparation of the salivary gland extract (SGE) and ME, respectively. Tissues were homogenized in an ultrasound homogenizer (Hielscher, UP100H) followed by centrifugation at 15,000 *g* for 30 min at 4°C. The supernatant of each extract was sterile filtered (0.2 μm non-pyrogenic filters, Sarstedt Germany) and stored at −20°C until use. Protein concentrations were measured by the CB-X^{TM} Protein Assay (G Biosciences, United States) according to the manufacturer's instructions.

Ferritin 2 (FER 2)

Recombinant protein ferritin 2 from *I. ricinus* (IrFER2) was expressed in *E. coli* strain BL21 as previously described (Hajdusek et al., 2010).

Prior to immunization, TPE, SGE, and ME or recombinant IrFER2 were emulsified in a homogenizer (IKA Turrax T25 Mixer) with 1.5 mg saponin in 1 mL of Montanide ISA V50 adjuvant (SEPPIC, France) as specified by the adjuvant's manufacturer.

Study Outline

All calves were vaccinated intramuscularly (i.m.) twice at an interval of 6 weeks. Two weeks after the second immunization, each calf was challenged with ticks. The first study was performed with three calves: one calf received 100 μg IrFER2, the second calf ∼12 mg TPE (4 ME: 1 other internal organs), and the third calf was vaccinated with Montanide ISA 50 adjuvant and saponin only. The second study was performed with four calves: one calf was vaccinated with ∼6 mg SGE, one with ∼9 mg ME, one with a ∼8 mg combination of ME and SGE (4 ME : 1 SGE) and a control calf with Montanide ISA 50 adjuvant and saponin only.

Tick Challenge

Two weeks after the second immunization, each animal was infested with 200 nymphs, 100 adult males, and 100 *I. ricinus* females. The ticks were equally divided over two linen bags, which were attached to the basis of the unshaved ears using adhesive tape (Leukoplast, BSN medical, Hamburg, Germany).

Following a resting period of 2 days to allow ticks to attach undisturbed, bags were checked twice per day and engorged nymphs or females were removed and weighed. Adult females were subsequently stored individually and nymphs were stored in small batches in glass tubes. The tubes containing the ticks were stored in a desiccator with 80% relative humidity (RH), which was placed in a climate chamber at 20°C and a light-dark-cycle of 14–10 h.

In vitro Tick Feeding

The ATFS used for the feeding of plasma or whole blood from the vaccinated calves to *I. ricinus* ticks *in vitro* was based on a previously published protocol (Krober and Guerin, 2007) which was further optimized in house (Krull et al., 2017). In short, tick feeding units were made of borosilicate glass tubes with a 28 mm inner diameter, 2 mm wall thickness, and height of 65 mm. The feeding units were closed on one end with a silicone membrane with a thickness of 80–120 μm for adults and 70–90 μm for nymphs. The silicone membranes were attached to the tick feeding unit using silicone glue. A square piece of glass fiber mosquito netting, approximately 20 mm × 20 mm in size, was glued to the silicone membrane inside the feeding units used for adult ticks to provide tactile stimuli. Silicone glue was applied to two sides of the square mosquito netting only, leaving sufficient space for ticks to crawl underneath the netting. Bovine hair extract (Krull et al., 2017) and bovine hair were dispersed over the silicone membrane to stimulate tick attachment. Ten females with 5–7 males, or 50 nymphs were placed in each feeding unit, which was subsequently placed in a climate chamber set at 20°C, 80% RH, 5% CO_2 and 14 h light–10 h dark cycle.

Before the first (d0) and 2 weeks after the second immunization (d56), 1.5 L blood was collected from each calf. Blood was centrifuged at 3,500 *g* for 30 min at 4°C to separate blood cells and plasma. Plasma was collected and stored at −20°C until use.

For the first study and nymphal tick feeding of the second study, collected plasma was mixed with the erythrocyte and buffy coat layer of bovine blood collected at a local slaughterhouse. Blood cells were washed twice and subsequently stored at 4°C in modified Vega y Martinez phosphate-buffered saline solution containing 20% glucose (Zweygarth et al., 1995). Blood cells were overlaid with two-thirds of buffer. Blood cells and stored plasma were mixed in a 2:1 ratio, resulting in whole blood with a packed cell volume (PCV) of 33%. Ticks were subsequently fed using the ATFS. Before each feeding, gentamycin (10 mg/mL) and 0.1 M adenosine triphosphate (ATP) were added to the blood meal.

During the second study, blood for the *in vitro* feeding of *I. ricinus* adults was collected from each calf on day 55, day 62, and day 68 in the presence of heparin (20 I.U./mL) and glucose (2 g/L). Prior to *in vitro* feeding, it was supplemented with gentamycin (10 mg/mL) and 0.1 M ATP and preheated to 37°C on a hot plate (Hot Plate 062, Labotect, Göttingen, Germany). Blood was changed twice per day. Detached and replete adults and nymphs were weighed and stored individually in a desiccator with 80% RH at RT.

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood was collected weekly from each calf in a serum tube (Microvette®, Sarstedt, Germany). After coagulation, blood samples were centrifuged at 2,800 *g* for 5 min. The serum was subsequently withdrawn and stored until use at −20°C. A 96-well plate (F-bottom, Medium binding; Greiner Bio-one) was coated with 2.5 μg/mL TPE, ME or SGE or 0.5 μg/mL IrFER2 in coating buffer (0.05 M carbonate-bicarbonate; pH 9.6) at 20°C for 1 h and at 4°C overnight. Serum samples diluted 1:200 in 0.05% PBS-Tween were added to each well and the plate was incubated at 37°C for 30 min. Mouse anti-bovine IgG antibody, horseradish peroxidase-conjugated (Acris Antibodies GmbH) was diluted

1:5000 in 0.05% PBS-T and incubated at 37°C for 30 min. O-phenylenediamine (OPD-P9187, Sigma Aldrich) was used as substrate and resulting color was measured with Epoch Microplate Spectrophotometer (BioTek) at 492 nm. The plate was washed three times with 0.05% PBS-Tween after each incubation step.

IgM, IgG1, and IgG2 antibody titration experiments were performed using eight serial dilutions, from 1:100 to 1:12,800, of sera from the first and second immunization experiment. Here, the amount of total protein used for coating was 0.5 μg/mL in carbonate buffer. The plates were then washed, blocked with 1% fetal bovine serum in PBS, incubated with the sera dilutions followed by specific secondary antibodies against bovine IgG1, IgG2, and IgM conjugated with horseradish peroxidase (Bio-Rad). The ELISAs were developed using HRP substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, United States) and read at 450 nm.

Western Blot

Five micrograms of purified Ferritin 2 or tissue extracts were resolved in 4–20% precast polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked for 2 h in PBS + 5% skim milk, followed by incubation with calf sera in PBS containing 0.1% Tween 20 (PBST) at 1:1000 dilution for 2 h. The membranes were washed three times with PBST, and incubated with a 1:10,000 dilution of anti-bovine IgG (Life Technologies) conjugated with horseradish peroxidase for a period of 1 h. After extensive washing the membranes were briefly incubated with HRP substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Life Technologies) and developed in a ChemiDoc Gel Imaging System (Bio-Rad).

Skin Sample Collection and Histological Processing

Biopsies were taken from euthanized animals of the second study at d68 from areas where ticks had fed and processed using routine histological techniques.

Statistical Analyses

Engorgement weight of adult ticks and nymphs were analyzed using GraphPad Prism version 5.03 for Windows, GraphPad Software, La Jolla, CA, United States[1]. For normally distributed data one-way ANOVA followed by Bonferroni's multiple comparison test was performed. Kruskal-Wallis test and Dunn's post-test was conducted for data with non-Gaussian distribution. $P \leq 0.05$ was considered significant for all tests. *P*-values for number of ticks and molting rate of nymphs were calculated with mid-p-exact test using OpenEpi version 3.01[2]. *P*-values were adjusted according to Holm correction in RStudio version 3.4.3.

[1]http://www.graphpad.com

[2]http://openepi.com

RESULTS

Immune Response

Indirect ELISA-results demonstrated that the IrFER2 and TPE vaccinated calf each developed antibody titers against recombinant IrFER2 and native TPE, respectively. Serum from the TPE vaccinated animal did not clearly recognize IrFER2 (**Figure 1**). These results were confirmed by Western blot, which also showed that immune sera from the TPE-immunized animal recognized ME, SGE and ovary proteins. Recombinant IrFER2 and native tick proteins were not recognized by pre-immune sera of the IrFER2 and TPE-immunized animals, respectively (**Figure 2**).

Calves from the second study, immunized with ME, SGE or a combination thereof developed higher antibody titers compared to the TPE-vaccinated calf from the first study. The calf immunized with ME showed a strong immune response against both ME and SGE and developed the highest antibody titer of all vaccinated calves (**Figure 3**). Antibody titration experiments predominantly showed a IgG1 and, to a lesser extent, IgG2 response in sera from the ME and SGE-immunized calves and the calf immunized with a combination of ME and SGE. A clear IgM response was not detected (**Figure 4**).

Tick Challenge

Two days post tick infestation, hyperemia and oedema on the tick bite sites on the ears of TPE vaccinated calf were observed (**Figure 5**). These reactions were not observed on the ears of the IrFER2 vaccinated or control calf. On the negative control, 70 female ticks engorged, with a mean weight of 245.8 ± 85.3 mg. This was not statistically different from the IrFER2-vaccinated animal, on which 87 ticks engorged with a mean weight of with 268.6 ± 82.0 mg. Only 22 ticks fed to repletion on the TPE-immunized calf, with a significantly reduced engorgement weight of 126.6 ± 86.9 mg ($p \leq 0.001$). The results for the nymphs fed on the TPE-vaccinated calf showed a similar trend: 14 from 200 nymphs were able to engorge compared to 128/200 and 145/200 nymphs from the control and IrFER2-vaccinated animal, respectively, but there was no significant difference between the engorgement weights (**Table 1**).

Control and SGE vaccinated calves of the second study did not show a clear skin response at 2 days post tick infestation. The calf immunized with a combination of SGE and ME showed similar inflammatory signs on the tick bite sites with hyperemia as observed in the first study. The ME vaccinated animal showed a severe cutaneous reaction with a papular reaction at the tick attachment site with serous exudation 2 days post infestation (**Figure 6**). Histological examination of the ears could only be performed at d68 post immunization after the tick infestation in the second immunization experiment. It showed an extensive infiltration of the dermis with eosinophils and macrophages in the ears of animals immunized with SGE, ME and a combination of both extracts that was absent in the skin of the control animal (**Figure 7**).

Adults fed on SGE (187.8 ± 102.4 mg, $n = 63$) or SGE and ME (140.6 ± 89.5 mg, $n = 25$) immunized calves had a significantly

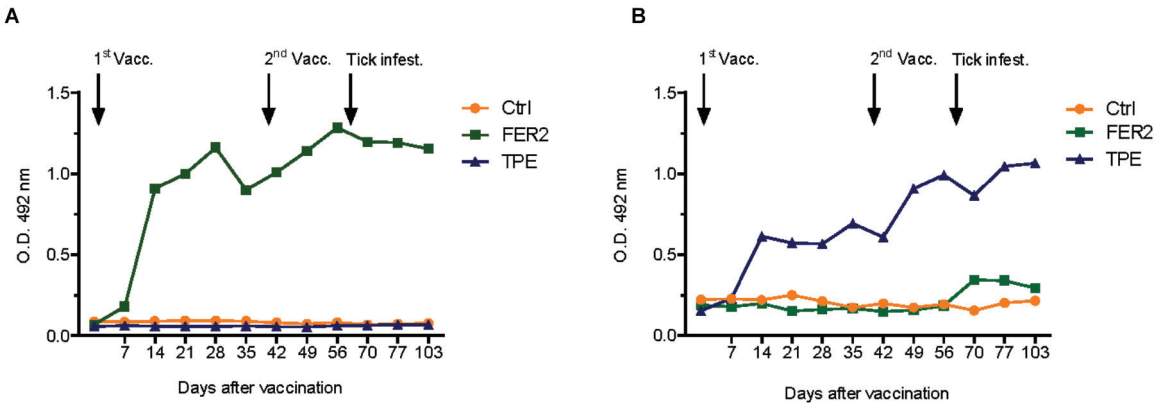

FIGURE 1 | Antibody response in vaccinated animals of the first cattle immunization study. Bovine serum (diluted 1:200) antibody titers to **(A)** recombinant ferritin 2 (FER2) and **(B)** tick protein extract (TPE) were determined by indirect ELISA in cattle vaccinated with FER2, TPE, and adjuvant control.

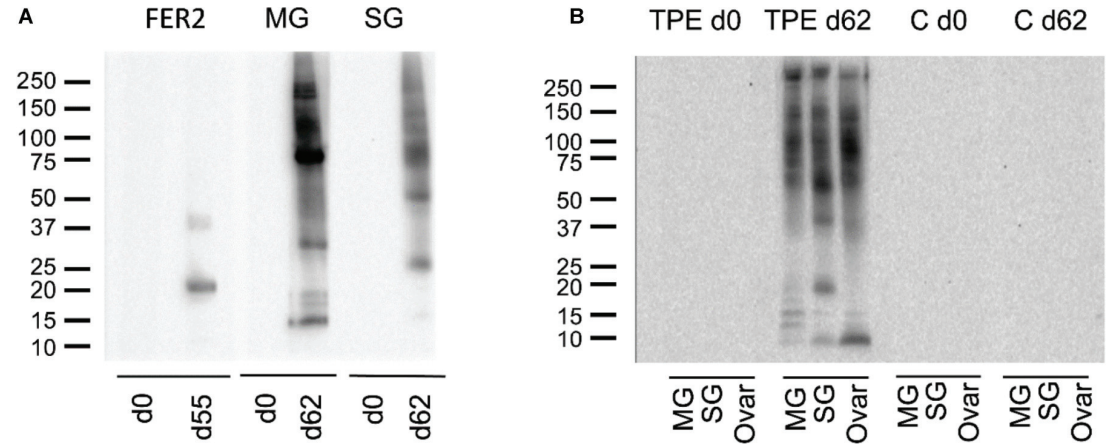

FIGURE 2 | (A) Western blot of recombinant ferritin 2 (FER2), native midgut (MG), and salivary gland (SG) extracts, probed with pre-immune and immune sera of calves vaccinated with recombinant ferritin 2 (lanes 1 and 2) and tick protein extract (lanes 3 to 6); **(B)** western blot of native MG, SG, and Ovaries (Ovar) extracts, probed with pre-immune and immune sera of the calf vaccinated with tick protein extracts (TPE) and the control calf (C).

($p \leq 0.001$) lower engorgement weight compared to adults fed on control (262.2 ± 70.8 mg, $n = 69$). Only two adult ticks were able to engorge on the ME vaccinated calf, with a significantly lower engorgement weight (79.0 ± 19.7 mg) in comparison to the control ($p \leq 0.05$). For all vaccinated calves, a reduced number (SGE $n = 23$; ME $n = 11$; SGE and ME $n = 20$) of nymphs could engorge compared to the Ctrl ($n = 92$), but no significant differences between engorgement weights were observed. Mean engorgement weights and statistical results for adult ticks and nymphs of the second study are shown in **Table 2**.

In vitro Tick Feeding

For the first study, the percentage of ticks that attached and successfully fed in the ATFS ranged from 33 to 44% per group for females and from 35 to 42% for nymphs. No significant differences in number of ticks that completed feeding and their engorgement weights were found. The attachment and engorgement rates of the second study were comparable to that of the first and ranged from 24 to 44% for both females and nymphs. Slightly higher female engorgement weights compared to that of the first *in vitro* study were recorded. Again, an effect of *in vitro* feeding of either stored plasma or fresh whole blood on tick engorgement was not observed. Mean engorgement weights and statistical results for adult ticks and nymphs of 1st and 2nd immunization study are shown in **Tables 1**, **2**, respectively.

DISCUSSION

Immunization with recombinant IrFER2 or native tick proteins elicited a clear antibody response in the immunized animals. Interestingly, sera of the animal vaccinated with ME also recognized salivary gland antigens in the indirect ELISA and vice versa (**Figure 3**), suggesting the presence of common epitopes in both ME and SGE. Similar findings were previously reported in cattle vaccinated with *R. microplus* gut membrane antigens (Opdebeeck and Daly, 1990) and recent data for *I. ricinus* also

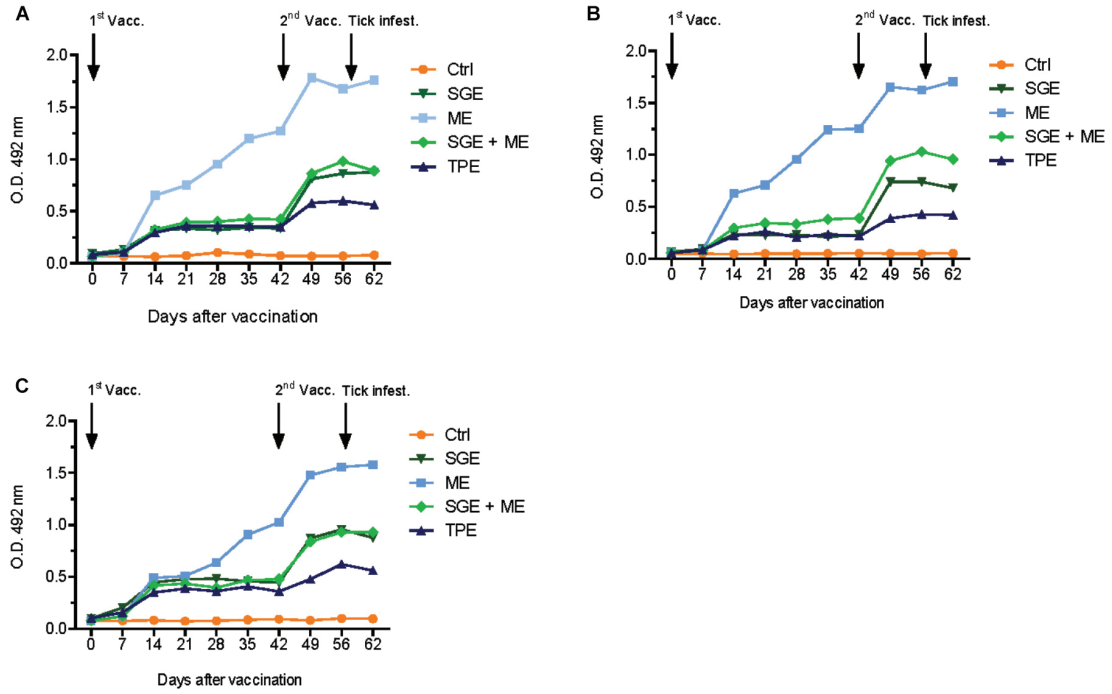

FIGURE 3 | Antibody response in vaccinated animals of the second cattle immunization study. Bovine serum (diluted 1:200) antibody titers to **(A)** midgut extract (ME), **(B)** salivary gland extract (SGE), and **(C)** SGE and ME were determined by indirect ELISA in cattle vaccinated with ME, SGE and a combination of ME and SGE and the adjuvant control. For comparison purposes, serum from the TPE-vaccinated animal from the first immunization study was also included in the ELISA.

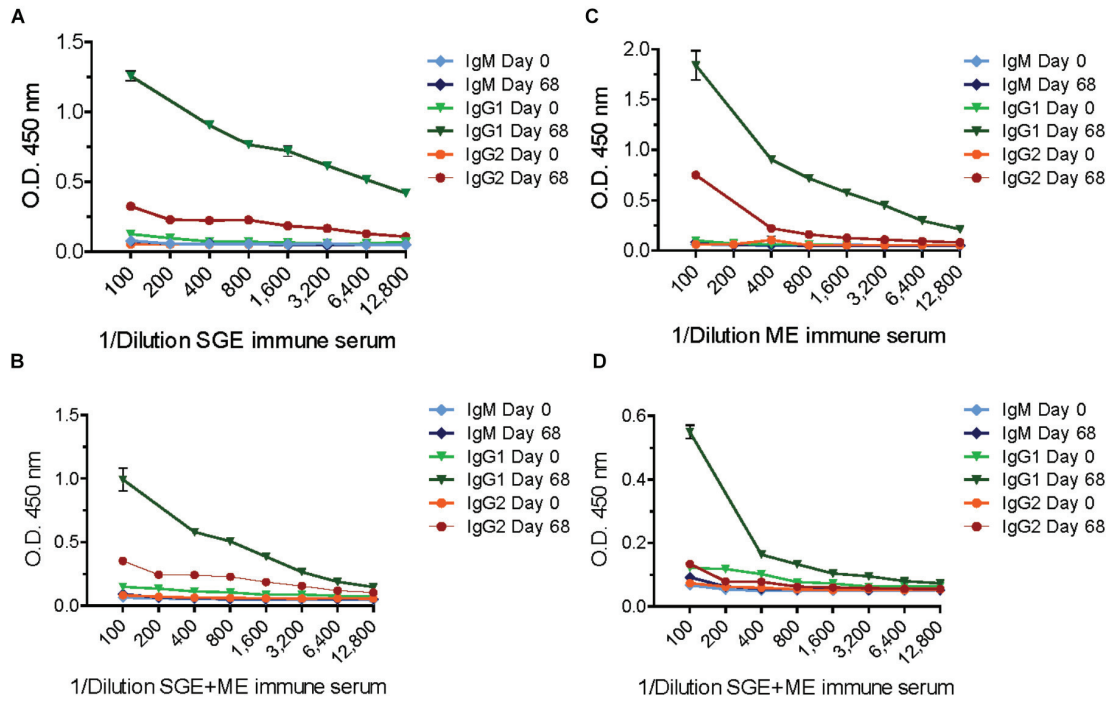

FIGURE 4 | Titrated IgM, IgG1, and IgG2 antibody response in animals vaccinated with salivary gland extract (SGE), midgut extract (ME) or a combination of both SGE and ME (SGE+ME). Plates were coated with 0.5 μg/mL SGE **(A,B)** or ME **(C,D)**, respectively.

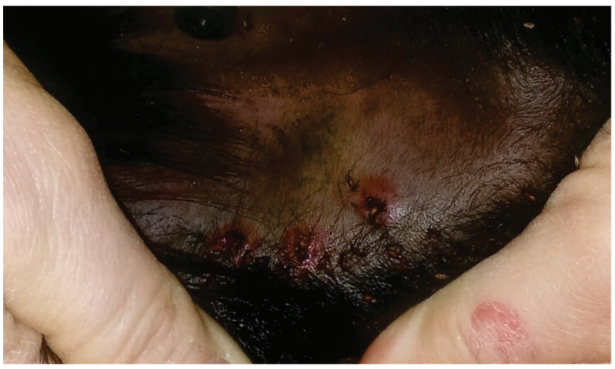

FIGURE 5 | Photograph of the ear from the TPE vaccinated cow, 2 days after tick infestation, showing hyperaemia and oedema around the tick feeding sites.

indicates the presence of shared transcripts and proteins between salivary glands and midguts (Schwarz et al., 2014; Perner et al., 2016). It is not known if these common epitopes are peptides or carbohydrates; efforts to deglycosylate the tissue extracts by enzymatic deglycosylation or sodium hydroxide to investigate this in more detail were not successful (data not shown). It is also striking that the strongest cutaneous response was observed in the ME-vaccinated animals, with a marked papular swelling at the tick attachment site and extensive serous exudate, a finding that supports the presence of shared epitopes between ME and saliva proteins. The regurgitation of midgut proteins during the feeding process might form an alternative explanation for the observed cutaneous response. There is some evidence that ticks may regurgitate gut contents during feeding (Brown, 1988) and it has also been suggested that pathogens might be transmitted by this route (Burgdorfer et al., 1989). However, the presence of a pharyngeal valve in ticks, which is considered to be an effective barrier preventing regurgitation, argues against this, making regurgitation of gut contents a little understood and controversial phenomenon (Sonenshine and Anderson, 2014). The skin reaction became apparent at 48 h after the ticks were placed on the ear, which together with the observed response is indicative of a type IV delayed hypersensitivity (DTH) reaction. Nymphs in particular might have become trapped in the exudate and died as a result, which may have contributed to the low number of ticks recovered from this animal. DTH reactions have also been observed in immunization studies targeting other hematophagous arthropods; immunization with a 15 kD salivary gland protein of the sandfly vector of leishmaniosis, *Phlebotomus papatasi* (Valenzuela et al., 2001) resulted in a humoral and strong DTH responses upon subsequent exposure to *P. papatasi* and immunization with the 64 TRP antigen derived from the cement cone of *R. appendiculatus* ticks was also shown to induce strong humoral and DTH responses (Trimnell et al., 2005).

Although the FER2 immunized calf developed a high antibody titer, tick feeding on this calf was not impaired. This is in contrast to previous findings of a immunization experiment in which the tick number, engorgement weight, oviposition and fertility of *I. ricinus* feeding on immunized rabbits ($n = 2$) were reduced after threefold immunization with 100 μg IrFER2 (Hajdusek et al., 2010). The small group size or use of individual animals in both studies, animal species-specific differences and differences in the immunization schedule might explain these contrasting findings. Recombinant IrFER2 produced in *E. coli* was not recognized by serum from the TPE vaccinated animal. This can be explained by the assumption that the amount of ferritin 2 in the TPE was minute, as it is mainly expressed in the midgut but secreted into the tick plasma, which was the only tissue where the protein could previously be detected by Western Blot (Hajdusek et al., 2009).

Immunization of calves with native TPEs from adult ticks was more successful in inhibiting tick feeding. Immunization with extracts containing midgut proteins (TPE, ME and a combination of both ME and SGE) in particular resulted in a significant reduction in the number of females and nymphs that fed successfully and also in a significant reduction in the engorgement weights of females. As the native proteins were extracted from adult females, the observed effect on nymphs could be explained by the presence of conserved proteins in both nymph and adult ticks. A recent proteomic study indeed showed a considerable overlap between proteins identified in nymphal and adult salivary glands and midguts (Schwarz et al., 2014).

The effect of immunization with soluble midgut proteins prepared from adult ticks on tick feeding has been previously evaluated for a number of metastriate tick species, including *Amblyomma variegatum*, *Dermacentor andersoni*, *Hyalomma anatolicum*, *H. dromedarii*, *R. annulatus, R. microplus*, *R. appendiculatus*, *R. sanguineus* and the soft tick *Ornithodoros erraticus* (Allen and Humphreys, 1979; Jongejan et al., 1989; Wong and Opdebeeck, 1989; Rechav et al., 1992; Kimaro and Opdebeeck, 1994; Szabo and Bechara, 1997; Banerjee et al., 2003; Manzano-Roman et al., 2006; Nikpay and Nabian, 2016). The reported efficacy of immunization of these trials was, however, rarely as high as reported in this study, with reductions in tick numbers ranging from 63% for ticks feeding on the animal immunized with a combination of SGE and ME, to 97% in the ME-immunized animal. This could again be due to the small number of animals used in this study and/or the mechanical disturbance of ticks caused by the serous exudate at the tick feeding site in the ME-vaccinated animal, but it does indicate that the immunization procedure followed was successful and that the development of tick immunity against *I. ricinus* through immunization in cattle is possible. The optimal amount of native TPEs that confers tick immunity in animals is not known; amounts used in previous studies with cattle ranged from three immunizations with 100 μg salivary gland proteins (Nikpay and Nabian, 2016) to one immunization with 200 mg midgut protein followed by three booster immunizations with 150 mg midgut protein (Essuman et al., 1991). Differences in the tick and host species used, as well as differences in the preparation of antigen extracts and the presentation of vaccine efficacy, make it difficult to draw any conclusions on this matter from previous studies. We therefore followed a pragmatic approach by using the maximum amount of proteins that could be extracted from the partially fed females, which turned out to be in the range of ∼6 to ∼12 mg protein.

TABLE 1 | Tick challenge and *in vitro* tick feeding results of 1st study.

Study	Life stage		Ctrl	TPE	IrFER2	TPE d0
Tick challenge	Adults	No	70[ab]	22[ac]	87[bc]	
		EW (mg)	245.8 ± 85.3[d]	126.6 ± 86.9[de]	268.6 ± 82.0[e]	
	Nymphs	No	128[f]	14[fg]	145[g]	
		EW (mg)	3.6 ± 0.9	3.1 ± 1.0	3.5 ± 0.9	
		Molting rate (%)	70[h]	36[hi]	71[i]	
		Sex of nymphs	54♀ 35♂	2♀ 3♂	59♀ 44♂	
In vitro	Adults	No	39	33	44	40
		EW (mg)	193.8 ± 74.8	177.8 ± 71.9	190.9 ± 79.3	201.4 ± 74.8
	Nymphs	No	84	79	70	
		EW (mg)	2.6 ± 0.9	2.7 ± 0.9	2.6 ± 0.8	
		Molting rate (%)	45[j]	49[k]	9[jk]	
		Sex of nymphs	16♀ 20♂	27♀ 17♂	2♀ 4♂	

No, number of engorged ticks; EW, engorgement weight. Mean values ± SD tested in a one-way analysis of variance (ANOVA) followed by Kruskal-Wallis test for multi-group comparisons. P-values for number of ticks and molting rate (in percent) were tested by mid-p-exact test. P-values were adjusted according to Holm correction. Significantly differences are indicated by letters and p-values. The sex of nymphs was determined after molting.
[a]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [b]Mid-P-exact test, p-value adjustment by Holm correction; $P = 0.0036$. [c]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [d]Kruskal-Wallis test, Dunn's post hoc-test; $p < 0.0001$. [e]Kruskal-Wallis test, Dunn's post hoc-test; $p < 0.0001$. [f]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [g]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [h]Mid-P-exact test, p-value adjustment by Holm correction; $P = 0.0355$. [i]Mid-P-exact test, p-value adjustment by Holm correction; $P = 0.0355$. [j]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [k]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$.

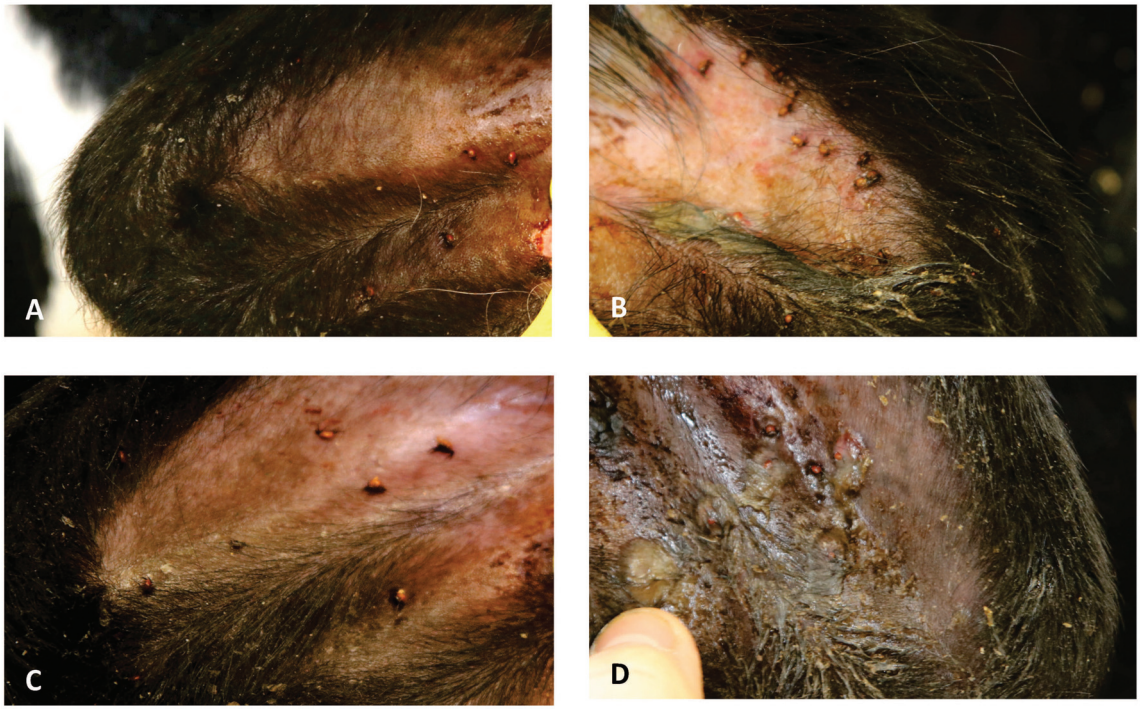

FIGURE 6 | Photographs of the ears from the vaccinated calves of the second immunization experiment, 2 days post-infestation. **(A)** Control animal; **(B)** SGE+ME-immunized calf; **(C)** SGE-immunized calf; **(D)** ME-immunized calf.

Feeding of blood from the vaccinated animals *in vitro* using the ATFS showed that the humoral response of the immunized animals alone did not affect tick feeding. It differs in this regard to the mode of action of the Bm86 vaccine, where the *in vitro* feeding of IgG1 antibodies in particular was shown to cause tick gut damage in *R. microplus* (Kemp et al., 1989). Further identification of the tissue extract antigens that were primarily responsible for the observed protective immune responses would be of great interest in the future development of vaccines targeting *I. ricinus* ticks.

Control of *Ixodes* tick infestations in important reproduction hosts such as deer by using the "4-Poster" acaricide dispensing

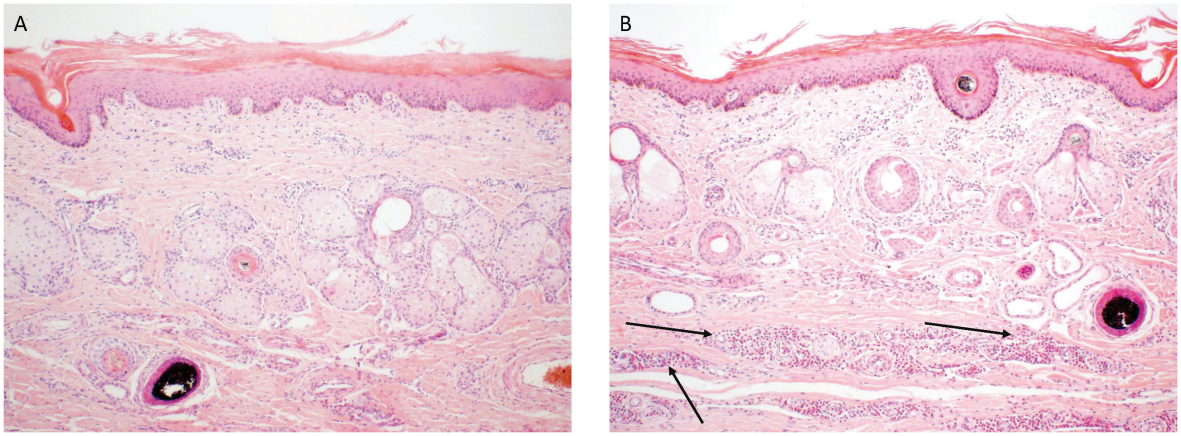

FIGURE 7 | Histopathology of the ear skin from the control **(A)** and ME-immunized animal **(B)**. The arrows indicate the mixed inflammatory infiltrate mainly composed of eosinophils and macrophages in the dermis of the ME-immunized animal. The biopsies were taken at d68 post immunization and stained with hematoxylin and eosin (100× magnification).

TABLE 2 | Tick challenge and *in vitro* tick feeding results of 2nd study.

Study	Life stage		Ctrl	SGE+ME	ME	SGE
Tick challenge	Adults	No	67^{ab}	25^{acd}	2^{bce}	63^{de}
		EW (mg)	262.1 ± 70.8^{fg}	140.6 ± 89.5^{f}	79.0 ± 19.7	187.8 ± 102.4^{g}
	Nymphs	No	92^{hij}	20^{h}	11^{i}	23^{j}
		EW (mg)	3.5 ± 1.0	3.2 ± 1.0	3.6 ± 0.7	3.8 ± 1.1
		Molting rate (%)	86^{k}	15^{kl}	55^{m}	100^{lm}
		Sex of nymphs	42♀ 37♂	0♀ 3♂	6♀ 0♂	15♀ 8♂
In vitro	Adults	No	23	23	18	32
		EW (mg)	256.4 ± 102.5	205.3 ± 68.9	195.7 ± 72.0	239.3 ± 92.4
	Nymphs	No	48^{no}	87^{n}	65	76^{o}
		EW (mg)	2.8 ± 0.9	2.8 ± 0.9	2.7 ± 0.9	2.7 ± 0.8
		Molting rate (%)	54^{pq}	34	26^{p}	29^{q}
		Sex of nymphs	10♀ 16♂	8♀ 22♂	5♀ 12♂	7♀ 15♂

No, number of engorged ticks; EW, engorgement weight. Mean values ± SD tested in a one-way analysis of variance (ANOVA) followed by Kruskal-Wallis test for multi-group comparisons. P-values for number of ticks and molting rate (in percent) were tested by mid-p-exact test. P-values were adjusted according to Holm correction. Significantly differences are indicated by letters and p-values. The sex of nymphs was determined after molting.
[a]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [b]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [c]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [d]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [e]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [f]Kruskal-Wallis test, Dunn's post hoc-test; $p < 0.0001$. [g]Kruskal-Wallis test, Dunn's post hoc-test; $p < 0.0001$. [h]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [i]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [j]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [k]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [l]Mid-P-exact test, p-value adjustment by Holm correction; $p < 0.0001$. [m]Mid-P-exact test, p-value adjustment by Holm correction; $p = 0.0133$. [n]Mid-P-exact test, p-value adjustment by Holm correction; $P = 0.0002$. [o]Mid-P-exact test, p-value adjustment by Holm correction; $P = 0.0127$. [p]Mid-P-exact test, p-value adjustment by Holm correction; $P = 0.0173$. [q]Mid-P-exact test, p-value adjustment by Holm correction; $P = 0.0292$.

device was shown to significantly reduce nymphal tick densities in the northeastern United States over time (Brei et al., 2009). Immunization of wildlife hosts against ticks could form an alternative means of reducing tick abundance and the risk for acquiring tick-borne diseases, but will depend on the identification of effective antigens and the availability of suitable vaccine delivery systems such as oral immunization or ballistic delivery of vaccines (Sharma and Hinds, 2012).

Although this work was limited by the small number of animals used due to cost constraints, the results nevertheless indicate that tick immunity against *I. ricinus* can be elicited in cattle upon immunization with native TPEs. Future work will focus on the analysis of immunogenic antigens to identify potential tick-protective vaccine candidates.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Landesamt for Gesundheit and Soziales, Berlin, Germany. The protocol was approved by the Landesamt für Gesundheit und Soziales, under registration number G0210/15.

AUTHOR CONTRIBUTIONS

SK, JA, JT, JH, and AN conceived and designed the study. OH and PK contributed materials. SK, JC, JA, and AN performed the experiments. OK performed the histopathological analyses. SK and AN analyzed the data and drafted the manuscript. All authors read and approved the final manuscript submitted for publication.

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Conflict of Interest Statement: 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.

CHAPTER 4

Identification and Characterization of Immunodominant Proteins from Tick Tissue Extracts Inducing a Protective Immune Response against *Ixodes ricinus* in Cattle

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Identification and Characterization of Immunodominant Proteins from Tick Tissue Extracts Inducing a Protective Immune Response against *Ixodes ricinus* in Cattle

Sarah Knorr [1,†], Sophia Reissert-Oppermann [1,†], Julen Tomás-Cortázar [2], Diego Barriales [3], Mikel Azkargorta [3], Ibon Iloro [3], Félix Elortza [3], Sophia Pinecki-Socias [1], Juan Anguita [3,4], Joppe W. Hovius [5] and Ard M. Nijhof [1,*]

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Abstract: *Ixodes ricinus* is the main vector of tick-borne diseases in Europe. An immunization trial of calves with soluble extracts of *I. ricinus* salivary glands (SGE) or midgut (ME) previously showed a strong response against subsequent tick challenge, resulting in diminished tick feeding success. Immune sera from these trials were used for the co-immunoprecipitation of tick tissue extracts, followed by LC-MS/MS analyses. This resulted in the identification of 46 immunodominant proteins that were differentially recognized by the serum of immunized calves. Some of these proteins had previously also drawn attention as potential anti-tick vaccine candidates using other approaches. Selected proteins were studied in more detail by measuring their relative expression in tick tissues and RNA interference (RNAi) studies. The strongest RNAi phenotypes were observed for MG6 (A0A147BXB7), a protein containing eight fibronectin type III domains predominantly expressed in tick midgut and ovaries of feeding females, and SG2 (A0A0K8RKT7), a glutathione-S-transferase that was found to be upregulated in all investigated tissues upon feeding. The results demonstrated that co-immunoprecipitation of tick proteins with host immune sera followed by protein identification using LC-MS/MS is a valid approach to identify antigen–antibody interactions, and could be integrated into anti-tick vaccine discovery pipelines.

Keywords: *Ixodes ricinus*; immunoprecipitation; anti-tick vaccines; midgut; salivary glands

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1. Introduction

Ixodes ricinus is a three-host ixodid tick species that is widely distributed in Western Europe. It is the predominant vector of several pathogens of medical and veterinary relevance, including tick-borne encephalitis virus (TBEV), *Borrelia burgdorferi* sensu lato (the causal agent of Lyme borreliosis), and *Babesia divergens* [1]. Besides their capacity to transmit a wide variety of pathogens, it was recently demonstrated that the saliva of *I. ricinus* contained galactose-α-1,3-galactose (α-Gal)-carrying proteins, which are associated with the induction of an anti-α-Gal immune response in humans and may result in red meat allergy [2,3]. The prevention and control of diseases associated with *I. ricinus* relies

on a combination of methods including the avoidance of tick habitats, the prompt removal of ticks, the use of repellents and acaricides, and landscaping measures [4].

Vaccines targeting ticks and the blocking of pathogen transmission are attractive alternative control options, and their development is drawing increasing interest [4–7]. Anti-tick vaccines targeting the common cattle tick *Rhipicephalus microplus* have been successfully developed and commercialized in the last century [8,9]. These vaccines are based on a single recombinant antigen, Bm86, a protein located in the microvilli of the tick midgut. This protein was identified following a laborious and long process involving multiple immunization trials, in which the effect of immunization with increasingly simpler fractions of tick midgut extracts on tick infestation was evaluated [10]. The artificial feeding of blood and plasma of animals immunized with tick midgut extracts showed that antibodies, in the presence of complement, were able to damage tick gut cells, thereby reproducing some of the detrimental effects observed in *R. microplus* ticks fed directly on immunized cows [11]. Antisera raised against recombinant Bm86 also partially inhibited larval engorgement of one-host *R. australis* (formerly *R. microplus*) ticks [12]. Our group recently immunized calves with different organ tissue homogenates of *I. ricinus* and found that the immunization with the soluble extracts of all tick organs, salivary glands, or midgut alone also conferred significant protection against subsequent challenge with *I. ricinus* nymphs and adults [13]. The identification of antibody–antigen complexes responsible for disrupting tick feeding could therefore be of relevance in the identification of tick-protective antigens.

In this study, we co-immunoprecipitated tick tissue extracts with pre- and post-immune sera from the *I. ricinus* immunization trial followed by label-free liquid chromatography–mass spectrometry (LC-MS/MS) to identify proteins that were differentially recognized by the serum of calves immunized with salivary gland extracts (SGE) or midgut extracts (ME). The results were partially validated by Western blotting, and the gene expression profile of selected antigens in tick organs was measured by quantitative reverse transcription PCR (RT-PCR). Finally, we analyzed the loss-of function phenotype for 10 of the identified proteins by RNA interference (RNAi).

2. Materials and Methods

2.1. Ticks and Animals

All *I. ricinus* ticks used originated from a laboratory colony maintained at the tick breeding unit of the Institute for Parasitology and Tropical Veterinary Medicine of the Freie Universität Berlin.

2.2. Protein Extracts and Antisera

Salivary glands and midguts were dissected from washed ticks and kept in sterile PBS on ice. The tissues were subsequently homogenized using an ultrasonic homogenizer (Hielscher, UP100H) and centrifuged at 15,000× *g* for 30 min at 4 °C. The supernatant was filtered through 0.4 µm and 0.2 µm non-pyrogenic filters (Sarstedt, Nümbrecht, Germany) and stored at −20 °C. The bovine antisera used originated from calves immunized with tick protein extracts, as recently described [13]. Antisera were collected at day 0 (pre-immune sera) and day 68 (post-immune sera).

2.3. Direct Antigen Co-Immunoprecipitation

Control and immune calf sera IgG were purified using the Melon Gel IgG Spin Purification Kit (Thermo Scientific, Rockford, IL, USA), following the manufacturer's protocol. Protein content was then measured in midgut and salivary gland lysates using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Direct immunoprecipitation was performed using the Pierce Direct IP Kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions. Enrichment was carried out by incubation of 100 µL of the AminoLink Plus Coupling Resin slurry with 20 µL purified serum that was immobilized onto the aldehyde-activated agarose resin at room temperature for 2 h. One

milligram of the tissue lysates was added to each antibody-coupled resin in a spin column. The column was incubated with gentle shaking at 4 °C overnight to form antibody–antigen complexes. After several washes, the antigens were eluted in 100 µL.

2.4. Tryptic Digestion

SDS-PAGE bands of the immunoprecipitated antigens were cut and washed in milli-Q water. Reduction and alkylation were performed using ditiothreitol (10 mM DTT in 50 mM ammonium bicarbonate) at 56 °C for 20 min, followed by iodoacetamide (50 mM iodoacetamide in 50 mM ammonium bicarbonate) at room temperature for another 20 min in the dark. Gel pieces were dried and incubated with trypsin (12.5 µg/mL in 50 mM ammonium bicarbonate) for 20 min on ice. After rehydration, the trypsin supernatant was discarded; gel pieces were hydrated with 50 mM ammonium bicarbonate, and incubated overnight at 37 °C. After digestion, tryptic peptides were recovered and dried in an RVC2 25 speedvac concentrator (Christ, Osterode, Germany). The peptides were resuspended in 10 µL 0.1% formic acid and sonicated for 5 min prior to analysis.

2.5. LC-MS/MS Analysis

LC was performed using an NanoAcquity nano-HPLC (Waters, Milford, MA, USA) apparatus equipped with a Waters BEH C18 nano-column (200 mm × 75 um ID, 1.8 µm). A chromatographic ramp of 120 min (5 to 60% ACN) was used with a flow rate of 300 nL/min. Mobile phase A was water containing 0.1% v/v formic acid, while mobile phase B was ACN containing 0.1% v/v formic acid. A lock mass compound, [Glu1]-Fibrinopeptide B (100 fmol/µL), was delivered by an auxiliary pump of the LC system at 500 nL/min to the reference sprayer of the NanoLockSpray (Waters) source of the mass spectrometer. For each run, 0.5 µg of each sample was loaded.

For mass spectrometry, we used a Synapt G2Si ESI Q-Mobility-TOF spectrometer (Waters) equipped with an ion mobility chamber (T-Wave-IMS) for high-definition data acquisition analyses. All analyses were performed in positive-mode ESI. Data were post-acquisition lock mass-corrected using the double-charged monoisotopic ion of [Glu1]-Fibrinopeptide B. Accurate mass LC-MS/MS data analysis was performed in HDDA mode, which is an enhanced form of data-dependent acquisition that enhances signal intensities using the ion mobility separation step.

Database searching was performed using MASCOT 2.2.07 (Matrixscience, London, UK) against a custom UNIPROT–Swissprot/Trembl database filled only with entries corresponding to *Ixodes* and *B. burgdorferi*. For protein identification, the following parameters were adopted: carbamidomethylation of cysteines (C) as a fixed modification and oxidation of methionines (M) as variable modifications, 15 ppm of peptide mass tolerance, 0.2 Da fragment mass tolerance, up to 3 missed cleavage points, and peptide charges of +2 and +3. Only peptides with a false discovery rate <1% were selected.

2.6. Cloning and Purification

The MG9 (A0A131YAQ2) and SG4 (A0A0K8RQF1) genes were cloned by overlapping PCR from midgut or salivary gland cDNA and cloned as EcoRI-XhoI or NcoI-SalI fragments, respectively, into the pHIS-parallel 2 expression vector. Sequence-confirmed clones were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 16 h at 20 °C in *E. coli* BL21 C41(DE3). The bacterial cells were then lysed and centrifuged. The expressed proteins were extracted from the inclusion bodies using the following protocol: the pellet was thoroughly homogenized in 50 mM Tris (pH 8), 300 mM NaCl, 1 mM DTT, and 2% Triton X-100, followed by an incubation at 37 °C for 30 min. The sample was ultracentrifuged at 96,000× g for 30 min and the pellet was homogenized again in 50 mM Tris (pH 8), 300 mM NaCl, and 1 mM DTT, and incubated at 37 °C for 30 min. After a second ultracentrifugation, the pellet was homogenized in 50 mM Tris pH 8, 300 mM NaCl, 1 mM DTT, and 7 M urea. The denatured proteins were refolded by dialysis in PBS overnight with an intermediate exchange of buffer to a final concentration of 2 M urea.

2.7. Western Blotting

For the validation or recognition of tick antigens by bovine immune sera, 5 µg of each extract or purified protein were boiled at 95 °C for 10 min, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane at 200 V for 1 h. The membranes were blocked with 5% non-fat milk in Tris-buffered saline solution containing 0.01% Tween-20 (TBS-T). The membranes were immunoblotted with diluted pre-immunization control sera (d0) and post-immunization sera (d68) (1:500) at 4 °C overnight.

2.8. Bioinformatics Analysis

The BLASTp tool was used to infer the potential function by homology. Signal peptides were predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/, accessed on 15 August 2019). Transmembrane helices were predicted using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/, accessed on 15 August 2019).

2.9. RNA Isolation

Tissues were dissected from unfed *I. ricinus* females and females pre-fed for 3–5 days on rabbits. Three biological replicates were made for each tissue type. Prior to dissection, ticks were washed for 30 s in 70% ethanol. Dissections were performed on a glass slide under ice-cold phosphate-buffered saline (PBS, pH 7.2). Internal organs were stored in TRI Reagent (Sigma-Aldrich, Taufkirchen, Germany) on ice and homogenized by passage through 24- and 27-gauge needles. Total RNA was subsequently isolated by chloroform phase separation and isopropanol precipitation, followed by DNase treatment (Thermo Fisher Scientific, Darmstadt, Germany). Sample concentrations and purity were measured using a Synergy HT Spectrophotometer (Bio-Tek Instruments, Bad Friedrichshall, Germany).

2.10. Quantitative RT-PCR

cDNA was synthesized from 100 ng of DNA-free RNA from the salivary glands, midguts, Malpighian tubules, ovaries, and fat bodies of unfed and partially fed *I. ricinus* females using the iScript cDNA synthesis kit (Bio-Rad laboratories, Feldkirchen, Germany) according to the manufacturer's instructions and stored at −20 °C. Quantitative RT-PCRs were performed for 15 targets identified in the LC-MS/MS analysis as being differentially recognized by immune sera and for the genes targeted by RNAi (see below). Two references genes, elongation factor 1-alpha (ELF1A) [14] and ATP synthase subunit g (ATP5L), were used for normalization purposes. ATP5L was used as a reference gene as it was recently found to be an abundant and stably expressed gene in *Borrelia afzelii*-infected as well as uninfected *I. ricinus* ticks in a quantitative transcriptomics study [15]. A list of primers used for quantitative RT-PCRs is presented in Table 1. All PCRs were conducted in a Bio-Rad CFX qPCR cycler. RT-PCR amplification mixtures (25 µL) contained 12.5 µL of Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories), 2.5 µL cDNA template, 400 nM of both forward and reverse primer, and 8 µL water. The cycling conditions were 30 s at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. A melt curve analysis was performed from 65 to 95 °C with a 0.5 °C increment with 2 s/step. All assays included a no-template control for each gene. Gene expression was analyzed using CFX Maestro software (Bio-Rad).

Table 1. Details of the quantitative RT-PCRs performed in this study.

Target	UNIPROT ID	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Amplicon Length (bp)
ELF1A		CAAGATTGGTGGTATCGGCA	GACCTCAGTGGTGATGTTGGC	106
ATP5L		CGAAAGCCATCACTACCCTG	TCTCCACCTTGGCATACTTGAC	85
MG1	A0A131XWI1	GGATGTGGTTTTACCCCGTC	CCCTCTTGAGCGTTGGATG	115
MG2	V5IFB6	CTCTGGAAACCTGGACAACG	GCAGCGTGAAAGATAGAGTCC	131
MG3	V5HWP4	AATCGCCAGTTGTCAGAAGC	TCAAGCCGACAGCAAATATG	76
MG4	V5I2L3	CACTGGTCATCTCCTGGCTC	CGTGCTCTTGTACATAAGGTCTG	137
MG5	A0A0K8R8I3	GTCTCTGCTTCGGTGTCTCC	GGCGACTTGAGGTTGTAGG	83
MG6	A0A147BXB7	GAGACTCCCAAGGACAAGAACC	TGTAGAGATATTTTTGCCACCAGG	78
MG7	V5IJN2	CCGAAGTCTCCAAGGGTCC	ACCGACTCCATCGTCAAAAAG	116
MG8	A0A147BMG4	GACAACACCACGGCACAGG	GGTGTAGGGCTTGAAGTTGTAGAA	92
MG9	A0A131YAQ2	GGGGATTTCCGAAGCCAC	CTGAAGATATTGTTGACGGGGTC	146
MG10	V5H492	AAACGGGCATCAGCAAAGC	TTGTTGAGATCGCCAGCAGAC	97
MG11	A0A131XS30	CATTCGTAGATCACACCCTGC	CGGCGATTCGTAGCGTG	107
SG1	V5HWD5	CCACTACGAAGGCTACCACAA	CCTATTCAGCCCTGTCCATC	56
SG2	A0A0K8RKT7	TTGCCTACGAGATGCTGTCC	TGAACTTGTCCGACTTGAGGT	135
SG3	A0A0K8RPW5	AGTTTACGAGCTTCTCTTGCC	TCCGTCGTGAACACTACCG	102
SG4	A0A0K8RQF1	CTTCCGAAGAGTGTCAGGGTGA	GTGCCGAATGCCGACTGC	108

2.11. RNA Interference

For the RNAi experiment, cDNA was synthesized from RNA isolated from tick tissues using the Superscript III first-strand cDNA synthesis kit (Thermo Fisher Scientific, Darmstadt, Germany) according to the manufacturer's instructions. Oligonucleotide primers (Sigma-Aldrich, Taufkirchen, Germany) containing a T7 promotor sequences at the 5′-end were used to amplify partial fragments of the genes coding for 10 target genes and green fluorescent protein (GFP) (Table 2). PCR products were purified using the DNA Clean and Concentrator kit (Zymo Research, Freiburg im Breisgau, Germany) following the manufacturer's recommendations and used as templates to produce dsRNA using the T7 Ribomax Express RNAi system (Promega, Walldorf, Germany) according to the manufacturer's instructions.

In the RNAi experiment, the effect of silencing the expression of 10 genes identified by the LC-MS/MS analysis was evaluated. Ticks were divided into 10 groups of 40 female ticks each that were subsequently injected with 0.5 µL of dsRNA (1×10^{12} molecules/µL dissolved in 10 mM Trish-HCl, pH 7 and 1 mM EDTA) coding for one of the selected targets. Female *I. ricinus* ticks were injected in the lower right quadrant using a 10 µL syringe with a 33-gauge needle (Hamilton) mounted on a micromanipulator. As a negative control, 80 ticks were equally divided over two groups and injected with dsRNA coding for GFP. Following injection with dsRNA, ticks were incubated at RT and 90% relative humidity for 24 h. The ticks were thereafter fed on six rabbits, with one group per ear. The two negative control groups injected with GFP dsRNA fed on different rabbits. For the confirmation of gene silencing by quantitative RT-PCR, the RNA from the salivary glands or midguts of six female ticks fed for five days from each group were collected and analyzed in three biological triplicates of two ticks each. Engorged females were weighed individually, and oviposition data were not recorded.

Table 2. List of primers used for dsRNA synthesis. All primers contained a T7 promoter sequence (5′-TAATACGACTCACTATAGG-3′) on their 5′ end. GFP: green fluorescent protein.

Target	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Amplicon Length (bp)
MG1	T7-CCCTTCCATCTTGCGGTAGC	T7- CGAACGAAGAGCGGAACG	393
MG2	T7-GAATCCCCAGTCCAAGATGATC	T7- CTTTCGTGACCGCTCGTTC	538
MG4	T7-CAGACATCGGCAAGGGTG	T7-GAGCCAGGAGATGACCAGTG	229
MG6	T7-GCGGACGAAGAGGAATACG	T7-GCTAAGAGTAACATTGGTGTATCC	493
MG7	T7-ACCACATCTGCCAACGGAG	T7-ATCCCAAGTAGGAAGCCGTT	257
MG8	T7-ACTTTGCTTTCTTGGCATCGG	T7-GTCGTATGTGTTGCCTTTGTCG	429
MG9	T7-GGTGGCATTGACAACGCTCTC	T7-GAACTTCTTCGTCGCTTCCTTG	400
MG10	T7-GGCTCCAGAAAACACAATCCTC	T7-CCTTTTCCGTGGTAGAATGGG	666
MG11	T7- CCAGGATGGGAAAGTGCGAC	T7- GAACGCCAGCGAACCAGG	243
SG2	T7-CCAAACCTGCCCTACTACCTG	T7-GGACAGCATCTCGTAGGCAAT	324
GFP	T7-GGCCACAAGTTCAGCGTGTC	T7-GCTTGATGCCGTTCTTCTGC	415

2.12. Statistical Analysis

Statistical analysis of data from the weights of ticks after feeding was performed using GraphPad Prism version 5.03 for Windows. The proportion of ticks that successfully engorged were analyzed by chi-squared test. Tick weights were compared between the experimental and combined GFP-injected groups using a *t*-test with Welch's correction. Quantitative RT-PCR data was analyzed using the Bio-Rad CFX Maestro software. *p*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Identification of Differentially Recognized Proteins from ME and SGE by Immune Sera of Calves Immunized with ME and SGE

In order to identify immunodominant proteins from SGE and ME, we immunoblotted midgut and salivary gland extracts using pre-immunization control sera (d0) and post immunization sera (d68) from calves. Both sera showed specific recognition of proteins in SGE and ME extracts that were not recognized by control sera (Figure 1).

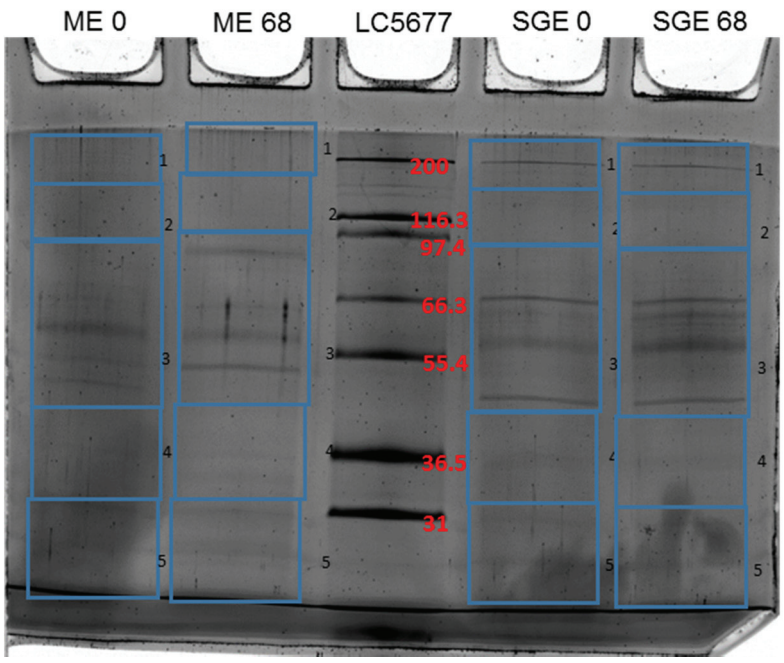

Figure 1. The SDS-PAGE gel of the immunoprecipitation complexes was cut as depicted, followed by in-gel tryptic digestion for the proteomic analysis. Abbreviations—ME: midgut extract; SGE: salivary gland extracts; LC5677: Mark12 protein marker (Invitrogen).

SGE antisera identified 167 unique peptides which could be linked to 55 proteins in the Uniprot database. Alignment of these proteins with their closest BLASTp hits revealed that four of the Uniprot entries (V5I085/A0A131Y1S2 and V5HWD5/A0A0K8RPW5) actually represented different fragments of two proteins, reducing the total number of uniquely identified proteins to 53. Of these proteins, five were only recognized by control sera and 24 exclusively by immune sera. A total of 24 proteins were identified in SGE-immunoprecipitates with both d0 and d68 sera (Table 3). In the ME, 78 unique peptides were found that could be linked to 46 proteins. Again, four UNIPROT entries (V5H0K4/A0A147BSS4 and A0A131Y7G4/A0A090X8W5) actually represented different fragments of two proteins, reducing the number of unique proteins to 44 proteins. A total of eight proteins were identified in ME-immunoprecipitates with both d0 and d68 sera, while 14 were identified by the control sera, and 22 by the immune sera (Table 4). Finally, 13 proteins were present in both SGE and ME immunoprecipitates, 10 of which in both d0 and d68 sera. A single protein, heat shock protein 60 (A0A131XPM3), was recognized by d68 sera of both SGE and ME immunized calves.

Table 3. List of proteins identified by LC-MS/MS from *Ixodes ricinus* salivary gland extracts (SGE) following co-immunoprecipitation with antisera raised against SGE. The Mascot score reflects the combined scores of all observed mass spectra that matched to amino acid sequences within the respective protein. MW: molecular weight.

Uniprot Accession Code	Protein Name	# of Unique Peptides	Mascot Score d0	Mascot Score d68	MW (kDa)	pI	Signal Peptide
V5H4T2	Trifunctional purine biosynthetic protein adenosine-3 *	1	41.20	71.91	7.3	8.19	No
V5HMC9	Small nuclear ribonucleoprotein G, putative	1		54.37	8.4	8.54	No
V5IF42	Myosin-2 essential light chain	1	40.41		9.7	6.51	No
V5HG94	60S ribosomal protein L22	2	76.25	88.50	9.9	10.01	No
A0A0K8RQM9	Small nuclear ribonucleoprotein Sm D3	1		58.85	13.2	10.13	No
V5IJC3	60s ribosomal protein L11	1	128.84	79.57	13.2	10.62	No
A0A0K8RIJ1	Histone H2A	1		50.33	13.4	10.73	No
A0A131Y512	40S ribosomal protein S16	1		32.98	14.9	10.04	No
V5HG43	Stromal cell-derived factor 2, putative	1	34.15		15.2	10.32	No
A0A090XEK9	Myosin, essential light chain	2	41.67	44.63	15.5	4.94	No
V5HWD5	Metalloproteinase **(=SG1)** [a]	1		44.26	16.9	9.92	No
A0A0K8RC23	40S ribosomal protein S13	1		33.57	17.2	10.68	No
A0A0K8RL33	Superoxide-dismutase	1		35.85	18.1	6.64	Yes
V5HD78	60S ribosomal protein L6	4	62.85	57.09	18.6	10.33	No
V5I150	60S ribosomal protein L5-A	3	94.73	50.00	19.0	7.84	No
V5I135	Alpha-crystallin A chain *	1	54.14		20.3	7.64	No
V5HXA8	60S ribosomal protein L18	2	60.03	131.89	21.5	11.62	No
V5H3S3	60S ribosomal L23	2		67.50	21.6	11.39	No
A0A0K8RQ35	40S ribosomal protein S8	1		40.95	21.9	10.30	No
A0A0K8RKT7	Glutathione S-transferase **(=SG2)**	1		39.95	25.5	7.88	No
A0A0K8RPW5	Metalloproteinase **(=SG3)** [a]	1		45.77	26.6	9.45	Yes
V5I164	Tropomyosin, isoform close to X4	3	145.00	57.96	26.6	5.34	No
A0A0K8RHG9	Tubulin alpha chain	1		62.27	27.1	5.57	No
A0A0K8RQF1	Toll-like receptor, putative **(=SG4)**	1		41.27	27.6	8.31	Yes
A0A131XW65	60S ribosomal protein L7	1	79.15	70.36	29.2	10.98	No
A0A0K8RG40	40S ribosomal protein S4	3	74.40	81.92	29.6	10.29	No
A0A0K8RG01	Glyceraldehyde-3-phosphate dehydrogenase 2, isoform X1 *	1		51.70	36.0	7.84	No
V5HG89	ATP synthase subunit beta	2		87.40	36.3	5.03	No

Table 3. *Cont.*

E3SS18	Translation elongation factor EF1-alpha *	4	63.01	184.99	36.7	8.27	No
A0A147BVX5	Venom metalloproteinase antarease-like TtrivMP_A	1	34.45		38.8	5.83	Yes
Q5D579	Actin *	9	401.51	335.42	41.5	5.85	No
A0A0K8RDN7	Protein N-myc downstream-regulated gene 3 (NDRG3) isoform X1	2	46.54	88.18	44.6	6.54	No
A0A0K8RCY6	Tubulin beta chain	3	40.59	127.53	45.1	5.97	No
A0A131XPA0	Eukaryotic translation initiation factor 3 subunit M	1		36.48	45.2	5.97	No
A0A0K8RMJ6	60S ribosomal protein L4	1		37.81	46.6	11.19	No
V5I085	Microsomal triglyceride transfer protein large subunit [b]	1	36.01	58.33	48.0	6.89	No
A0A0K8R4C2	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit	1		37.19	48.8	6.05	Yes
A0A0K8R4D7	Cytochrome b-c1 complex subunit 2	3	105.77	115.65	48.9	8.62	No
A0A131Y1S2	Microsomal triglyceride transfer protein large subunit [b]	2	36.65	51.53	49.7	8.97	Yes
V5I095	S-adenosylhomocysteine hydrolase-like protein	4		93.01	50.5	6.54	No
A0A131XNF3	Processing peptidase beta subunit, putative *	2	105.08	87.93	53.4	6.15	No
A0A0K8RCY2	Metis1	4	149.35	176.93	55.5	7.58	Yes
A0A131XPM3	Heat shock protein 60 *	5		151.93	59.3	5.62	No
A0A0K8RCE8	Heat shock 70 kDa protein cognate 4 *	2	60.97	115.44	59.8	7.43	No
A0A0K8RP16	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1	3		115.81	67.5	7.53	Yes
A0A090XC63	Moesin/ezrin/radixin homolog 1 isoform X1 *	7	81.17	206.20	70.1	5.66	No
A0A0K8RIU3	Heat shock protein, putative *	3	66.94	102.00	72.6	5.41	Yes
V5HP83	Coatomer subunit alpha	2		61.57	75.6	8.79	No
A0A0K8R8N9	Heat shock protein HSP 90-alpha	1		37.81	84.2	5.02	No
V5HRY6	Sodium/potassium-transporting ATPase subunit alpha-B	3		144.31	91.7	5.16	No
A0A131XXE4	F-box only protein 11	1	37.74		99.1	7.06	No
A0A131XWG4	Coatomer subunit beta	2		59.80	103.2	5.21	No
V5GY25	Clathrin heavy chain 1 *	3	35.11	119.33	190.7	5.81	No
V5I4B8	Myosin heavy chain, muscle isoform X3 *	40	1378.54	1750.99	222.0	6.09	No
V5I3C9	Myosin heavy chain, non-muscle isoform X1 *	15	354.64	650.15	227.5	5.55	No

* indicates a protein identified by LC-MS/MS in both ME and SGE following co-immunoprecipitation with antisera raised against ME and SGE, respectively. Entries with the same superscript letter in the protein description [a,b] represent different fragments of the same protein.

3.2. Expression of Recombinant Immunodominant Proteins and Validation by Western Blot

Validation of the LC-MS/MS analysis was performed by the recombinant expression of two identified proteins that were differentially recognized by the immune sera: a putative Toll-like receptor (SG4, UNIPROT ID A0A0K8RQF1) from the SGE and an uncharacterized protein (MG9, UNIPROT ID A0A131YAQ2) from the ME. Western blot analysis confirmed the differential recognition of these two proteins (Figure 2).

Table 4. List of proteins identified by LC-MS/MS from *Ixodes ricinus* midgut extracts (ME) following co-immunoprecipitation with antisera raised against ME. The Mascot score reflects the combined scores of all observed mass spectra that matched to amino acid sequences within the respective protein. MW: molecular weight.

Uniprot Accession Code	Protein Name	# of Unique Peptides	Mascot Score d0	Mascot Score d68	MW (kDa)	pI	Signal Peptide
V5H4T2	Trifunctional purine biosynthetic protein adenosine-3 *	1	52.26		7.3	8.19	No
V5HY31	Histone H4-like, putative	1		37.81	9.8	10.43	No
A0A0K8RK48	Uncharacterized protein	1	43.68	44.01	13.2	7.83	Yes
A0A0K8RQA6	Ubiquitin	1	47.08		14.5	9.82	No
V5H0K4	Pantetheinase, putative [a]	1		161.39	16.3	6.54	Yes
A0A131Y7G4	Uncharacterized protein [b]	1	58.35	84.84	18.2	8.29	Yes
V5I2L3	ADP/ATP translocase, putative **(=MG4)**	1		42.62	19.3	9.58	No
V5I135	Alpha-crystallin A chain *	1	110.22		20.3	7.64	No
A0A090X8W5	Uncharacterized protein [b]	1	73.12	61.59	21.7	8.94	Yes
A0A131YAP7	Tropomyosin isoform X15/X16	1	61.58		23.6	4.74	No
V5HHC0	Uncharacterized protein	1	58.35	95.16	23.8	9.85	Yes
A0A131XX88	60S ribosomal protein L19	1	44.69		24.2	11.43	No
A0A131XRL8	Cathepsin L	1		72.31	27.7	5.48	No
A0A131XWI1	Salivary secreted cytotoxin, putative **(=MG1)**	1		66.40	32.0	9.33	No
V5HBQ2	Lysosomal Pro-X carboxypeptidase	1		40.01	33.9	5.08	No
V5HWP4	Uncharacterized protein **(=MG3)**	1		48.01	35.0	7.01	No
A0A0K8RNA0	Malate dehydrogenase	2	73.48		35.8	9.09	No
A0A0K8RG01	Glyceraldehyde-3-phosphate dehydrogenase 2 isoform X1 *	1	39.91		36.0	7.84	No
E3SS18	Translation elongation factor EF1-alpha *	3	54.98	36.81	36.7	8.27	No
Q5D579	Actin *	7	248.34	38.91	41.5	5.85	No
A0A0K8RCB1	Enolase	1	38.63		47.1	6.01	No
A0A131XPI3	Aminopeptidase, putative W07G4.4	1		34.48	47.6	8.32	No
A0A147BSS4	Pantetheinase [a]	1		206.82	52.5	7.01	Yes
A0A131XNF3	Processing peptidase beta subunit, putative *	1	50.89		53.4	6.15	No
V5HEY6	Alpha-L-fucosidase	1		55.17	53.5	6.92	Yes
V5HB74	Retinal dehydrogenase 1	4	181.55	90.92	54.6	6.89	No
V5IJN2	Calcium-activated chloride channel regulator **(=MG7)**	1		39.96	55.1	4.96	No
A0A131Y0J3	Alpha-aminoadipic semialdehyde dehydrogenase	1	39.78		58.7	6.79	No
A0A131XPM3	Heat shock protein 60 *	1		89.26	59.3	5.62	No
A0A0K8RCE8	Heat shock 70 kDa protein cognate 4 *	3	171.54		59.8	7.43	No
A0A131XQI6	Moesin/ezrin/radixin homolog 1 *	1	97.17		62.5	5.52	No
V5HN24	Beta-hexosaminidase subunit beta	1		38.14	63.4	5.40	No
A0A0K8RIU3	Heat shock protein, putative *	3	179.61		72.6	5.41	Yes
A0A0K8R8I3	Uncharacterized protein (cubilin-like?) **(=MG5)**	2		80.81	75.8	6.67	Yes
V5IFB6	Integrin beta-PS (**=MG2**)	3		193.69	83.8	5.17	No
V5GPX7	Alpha-actinin isoform X2	1		38.52	89.4	6.13	No
A0A131YAQ2	Uncharacterized protein **(=MG9)**	1		71.18	94.3	6.62	Yes
A0A147BXB7	Cell adhesion molecule, putative **(=MG6)**	3		70.59	104.8	6.34	Yes
A0A147BMG4	Uncharacterized protein **(=MG8)**	1		34.22	109.1	5.95	Yes
A0A0K8RQE7	Lysosomal alpha-mannosidase-like	1		62.70	109.3	7.56	No
V5H492	Integrin alpha-PS1 **(=MG10)**	1		43.73	110.3	6.14	No
V5H7Z4	Alpha-2-macroglobulin-like protein	2		114.53	152.0	5.59	Yes
V5GY25	Clathrin heavy chain 1 *	1	47.55		190.7	5.81	No
V5I4B8	Myosin heavy chain, muscle isoform X3 *	6	178.22	52.04	222.0	6.09	No
V5I3C9	Myosin heavy chain, non-muscle isoform X1 *	6	68.25	71.36	227.5	5.55	No
A0A131XS30	MAM and LDL-receptor class A domain-containing protein 1 **(=MG11)**	1		40.27	420.4	5.49	No

* indicates a protein identified by LC-MS/MS in both ME and SGE following co-immunoprecipitation with antisera raised against ME and SGE, respectively. Entries with the same superscript letter in the protein descriptions [a,b] represent different fragments of the same protein.

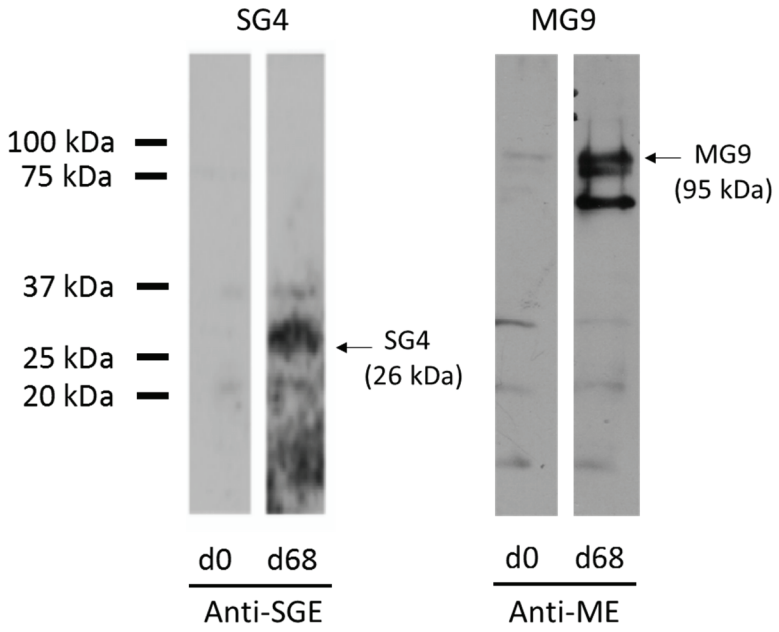

Figure 2. SG4 and MG9 Western blot as validation for the immunoprecipitation employed to identify new vaccine antigens (d0: control sera; d68: immune sera).

3.3. Expression Profile of Selected Genes in Different Tissues of Ixodes ricinus Females

Eleven ME proteins and four SGE proteins were subsequently selected for further analysis based on their differential recognition by d68 sera, having putative extracellular exposure or being predicted to be secreted. The expression profile of these proteins was determined by quantitative RT-PCR. The results corroborated findings of the proteomic analysis since proteins identified by LC-MS/MS from SGE and ME immunoprecipitates were expressed in the salivary glands and midguts, respectively. Two salivary gland proteins (SG1 and SG3) that were predicted to be different fragments of the same metalloproteinase had similar expression profiles. A few proteins were expressed in salivary glands or midguts exclusively, such as the metalloproteinase mentioned above and two uncharacterized midgut proteins with unknown homology (MG8 and MG9). However, most of the proteins were expressed in multiple tick tissues (Figure 3).

3.4. Effect of Gene Silencing of Selected Candidates on I. ricinus Adult Feeding

Female ticks were injected with dsRNA complementary to GFP (control), SG2, MG1, MG2, MG4, MG6, MG7, MG8, MG9, MG10, or MG11, and were subsequently allowed to feed on rabbits. The proportion of ticks that were engorged was significantly lower for the MG2 ($p = 0.0003$), MG6 ($p < 0.0001$), MG8 ($p = 0.0026$), MG10 ($p < 0.0001$), and SG2 ($p = 0.0003$) groups compared to the GFP control groups. The engorgement weights of both GFP-injected control groups were not significantly different, and the engorgement weights obtained in the experimental groups were compared to the weights of the combined control groups. Significantly lower engorgement weights were found for MG4 ($p = 0.0025$), MG6 ($p= 0.0139$), MG9 ($p = 0.0213$), and SG2 ($p = 0.0356$) (Figure 4). Quantitative RT-PCR showed that the expression of the target genes was silenced in each respective group, with the exception of MG11, where a non-significant increase in MG11 gene expression levels was found in the ticks injected with MG11 dsRNA (Figure 5). Significant gene silencing was found for MG1, MG2, MG7, MG9, MG10, and SG2 (*t*-test, $p < 0.05$). The lower expression levels detected for MG4 and MG6 were not significant.

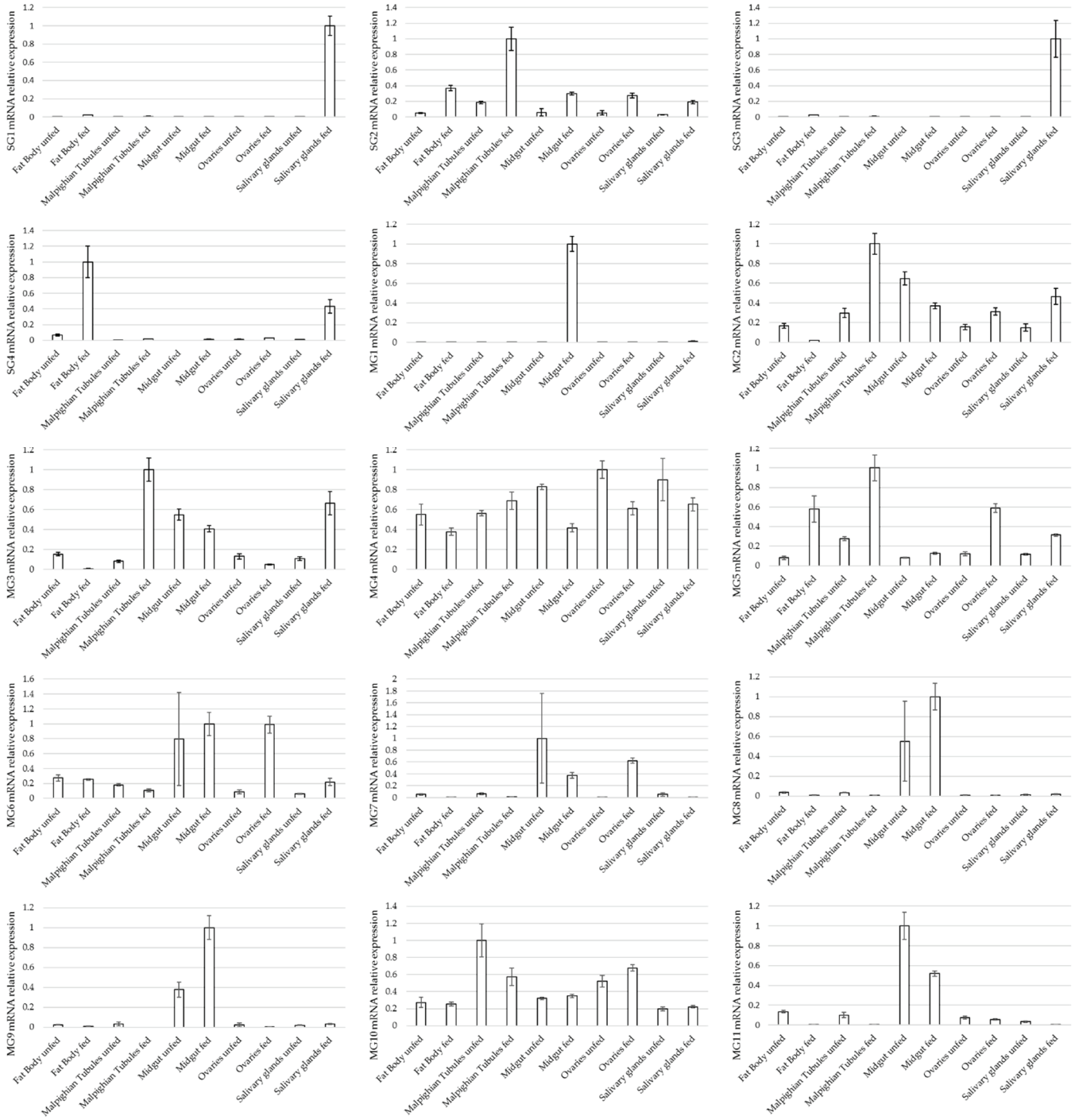

Figure 3. Relative gene expression of selected candidates identified by LC-MS/MS analysis of unfed and partially fed *Ixodes ricinus* females. Bars represent the standard deviation of the normalized gene expression.

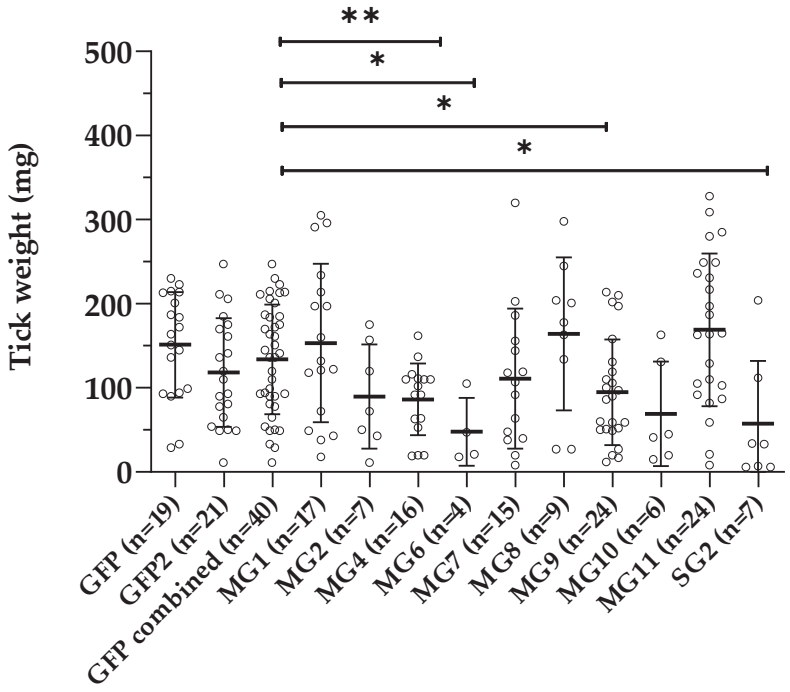

Figure 4. Weight of replete *Ixodes ricinus* females injected with dsRNA coding for GFP (control groups) and 10 gene targets identified by LC-MS/MS. Errors bars represent the standard deviation (SD). The asterixis indicate a significant difference in weight; * ($p < 0.05$), ** ($p < 0.005$).

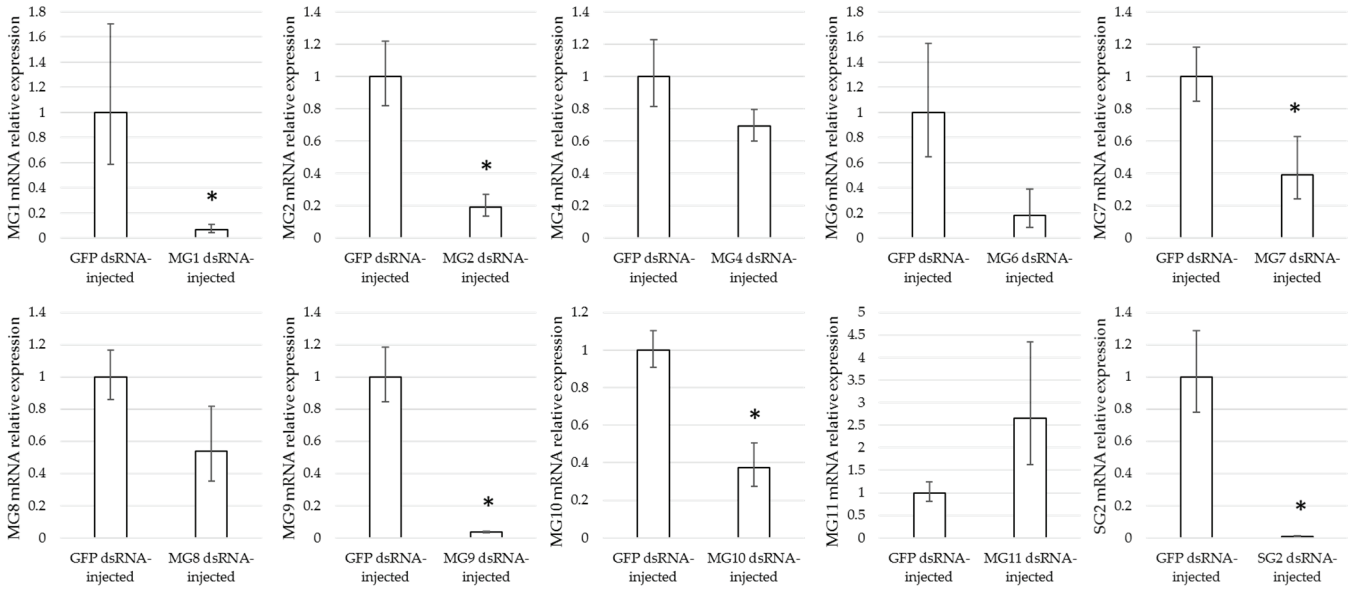

Figure 5. mRNA expression levels of target genes, relative to the expression in GFP-injected ticks. Compared to the control ticks, only the expression of target genes MG1, MG2, MG7, MG9, MG10, and SG2 was significantly knocked down in the respective experimental groups. * indicates a significant difference between the control and experimental group ($p < 0.05$).

4. Discussion

The main bottleneck in the development of successful anti-tick vaccines is the identification of tick-protective antigens that are effective in limiting tick infestations when applied as recombinant antigens. Several approaches have been followed to identify tick-protective antigens, including studies of the immune response in tick-immune hosts, the evaluation of the increasingly simpler native protein extracts in vaccination, and challenge

trials, as well as the identification of antigens that are crucial for the survival or function of ticks, for instance using functional genomic tools such as RNAi [7]. In this study, we used a combination of these approaches, starting with sera of calves immunized with tick extracts that showed a strong immune response upon *I. ricinus* tick challenge, which significantly hampered the feeding of both nymphs and adults [10]. The sera were used for the co-immunoprecipitation of tissue extracts, and differentially recognized proteins were subsequently identified by label-free LC-MS/MS, a step greatly facilitated by the increasing amounts of proteomic and genomic data that have become available for ticks over the last years [16]. Several differentially recognized antigens were subsequently characterized in more detail by determining their tissue expression profiles and loss-of-function phenotype by RNAi.

A considerable number of proteins identified by co-immunoprecipitation together with LC-MS/MS have previously also drawn attention as possible anti-tick vaccine candidates by other approaches. These include homologs of glutathione-S-transferase (SG2) [17–19], tropomyosin [20,21], ubiquitin and elongation factor EF1-alpha [22,23], myosin light chain [24], heat shock protein 70 [25], cathepsin L [26], enolase [27], antigen B from *R. microplus* (AAN15115) with 67.4% (663/984 amino acid (AA)) identity to MG8 [28], a glyceraldehyde-3-phosphate dehydrogenase from *Haemaphysalis flava* (AVK70348) with 88.2% (293/332 AA) identity to A0A0K8RG01 [29], and SG1/SG3 (A0A0K8RPW5/V5HWD5), a metalloproteinase with 60.2% (136/226 AA) identity to metis 5 from *I. ricinus* (CAO00629) [30,31].

Glutathione-S-transferases (GSTs) play a role in the excretion of toxic metabolites, and the partially characterized GST SG2 (A0A0K8RKT7) shares 88.3% (197/223) AA identity with DmGSTM1, a mu-class GST from *Dermacentor marginatus* ticks that was recently evaluated as an anti-tick vaccine [17,18]. SG2 also had a similar expression pattern in the tissues of adult females to DmGSTM1 [17]. Gene silencing of GSTs was previously shown to increase the susceptibility of *Rhipicephalus sanguineus* and *Haemaphysalis longicornis* ticks to the ectoparasiticides permethrin and flumethrin, respectively [32,33]. In our study, RNAi-mediated silencing of the expression of SG2 resulted in a smaller proportion of ticks that were engorged, with engorged ticks having significantly lower engorgement weights compared to the control group. Gene silencing of *gst* also significantly reduced the engorgement weights of *R. microplus* females [22], but not of *R. sanguineus* females [32]. These differences may have been caused by differences in the *gst* isoform targeted, experimental procedures, and/or RNAi efficiency.

The second protein that showed a clear loss-of-function phenotype in the RNAi study was MG6 (A0A147BXB7). This ~105 kDa protein is predicted to contain a signal peptide, eight fibronectin type III domains, and a single transmembrane protein. The fibronectin type III domain is one of three types of internal repeats found in fibronectin, a glycoprotein that connects cells to the extracellular matrix, plays a role in cell signaling, and may also act as a target for bacterial adhesion. Fibronectin type III domains also frequently occur as tandem repeats in cell surface proteins and in the extracellular regions of some cell surface receptors [34,35]. In ticks, Ixofin3D, an *I. scapularis* midgut protein containing a signal peptide, four putative fibronectin III domains and a transmembrane protein were shown to play a role in the aggregation of *Borrelia burgdorferi* on the gut epithelium [36]. Silencing of other fibronectin type III domain-containing proteins expressed in the gut of *Anopheles arabiensis* mosquitoes disrupted gut homeostasis following feeding and reduced mosquito longevity [37]. Taken together, it is tempting to speculate that MG6 could also act as a modulator for the bacterial population structure in the tick gut, whereby silencing of MG6 could lead to reduced feeding success and increased tick mortality due to disruption of the gut homeostasis. Additional studies will be required to examine the physiological function of MG6 and its potential as a tick-protective antigen within an anti-tick vaccine in more detail.

Although the injection of dsRNA in the haemocoel of ticks usually results in a systemic RNAi response, the RNAi efficiency may vary between target genes and experiments. In our study, silencing levels ranged from ~96% in the expression of MG9 (A0A131YAQ2) to

a complete absence of gene silencing in ticks injected with MG11 (A0A131XS30)-dsRNA, suggesting that MG11 is a refractory target gene for RNAi (Figure 5). In insects, other factors than the target gene, such as the targeted species, strain, tissue and life stage have also been reported to play a role in RNAi efficiency [38]. These and other factors, such as the optimal dsRNA amount for RNAi, have not yet been systematically investigated in ticks, although differences in RNAi efficiency between tick cell lines have been reported [39].

Significantly lower engorgement weights were found for the MG4-silenced females, despite a limited reduction (~31%) in MG4 transcript levels. MG4 is a putative ADP/ATP translocase also known as the adenine nucleotide translocator (ANT) protein, which exchanges ADP/ATP through the mitochondrial inner membrane and is essential for the cellular energy metabolism. In most eukaryotes, multiple ANT proteins are present, with some paralogs being exclusively expressed in testicular germ cells, where they are thought to be essential for spermatogenesis by supplying meiotic cells with ATP [40]. The ubiquitous expression of MG4 and its RNAi phenotype suggest that this protein has a critical role in organismal homeostasis. The high level of amino acid sequence homology of vertebrate and arthropod ANTs [40] may however limit the usability of this protein as an anti-tick vaccine antigen.

Significantly lower engorgement weights were also found for MG9-silenced females. Since the coding sequence for MG9 (A0A131YAQ2) does not contain a stop codon, it is likely to be a truncated version of *I. ricinus* protein V5ICT5, with which it shares 99% AA sequence identity. V5ICT5 is an uncharacterized protein of 1003 amino acids with a predicted mass of 113 kDa. It has a signal peptide, three apple domains, and a transmembrane domain. MG8 (A0A147BMG4), the silencing of which led to a significant reduction in females that fed successively but had no effect on the engorgement weight, has a similar structure with a signal peptide, four apple domains as well as a transmembrane protein. Apple domains are characterized by six cystine residues at highly conserved positions that through the formation of disulfide bonds form a structure which resembles an apple when drawn [41]. These domains are also present on plasma proteins such as factor XI and prekallikrein, where they are essential for binding of substrates [42,43]. The exclusive expression of MG8 and MG9 in the tick midgut, their upregulation upon feeding, and RNAi phenotypes warrant further studies into the function of these proteins.

The proportion of ticks that were successfully engorged was reduced in the MG2 (V5IFB6) and MG10 (V5H492)-silenced females, homologs of the integrin beta and integrin alpha subunits, respectively. Integrins function as cell surface receptors, providing a transmembrane link between the extracellular matrix and the cytoskeleton. Silencing of the expression of integrin beta subunits also had detrimental effects on the development of other arthropods such as the Oriental tobacco budworm, *Helicoverpa assulta*, and the beet armyworm, *Spodoptera exigua* [44,45].

5. Conclusions

Co-immunoprecipitation of tick tissue extracts with bovine immune serum raised against these extracts followed by LC-MS/MS analysis led to the identification of immunodominant proteins. This included several proteins that had previously raised interest as potential anti-tick vaccine antigens. Gene silencing of seven out of 11 selected immunodominant proteins targets resulted in a significantly decreased engorgement weight (MG4, MG6, MG9, and SG2) and/or a significant reduction in the number of ticks that successfully engorged (MG2, MG6, MG8, MG10, and SG2) compared to GFP-injected control groups. Although definite proof in the form of vaccination trials against these proteins remains outstanding, we tentatively conclude that the followed approach may be useful in anti-tick vaccine antigen discovery pipelines.

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review and editing, J.T.-C., J.A., F.E., J.W.H.; visualization, A.M.N.; supervision, A.M.N.; project administration, A.M.N.; funding acquisition, J.A., J.W.H., A.M.N. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All the data are included within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 5

Summarizing Discussion

5.1 Tick vaccine development

In Europe, *I. ricinus* is a widespread hard tick and transmits the causative agents of Lyme Borreliosis, Tick-borne Encephalitis, Babesiosis and other diseases. Beside the transmission of pathogens, it was recently observed that red meat allergy can also be associated with tick bites (Apostolovic et al., 2020; Hamsten et al., 2013). Thus, tick control is of medical and veterinary importance and can be carried out through several methods, whereby the use of synthetic acaricides have become the mainstay for tick control on animals over the last decades. The widespread and intensive use of these chemicals have led to the development of acaricide resistance against different chemical classes in several tick species, particularly in one-host *Rhipicephalus* ticks infesting cattle in the tropics and subtropics. The short generation times of these ticks, with up to five generations per year (Cruz et al., 2020), results in a need for frequent acaricide application and thereby an increased selection pressure for resistance. The first cases of resistance in *R. microplus* ticks are typically reported within a few years after introduction of new drug classes (Abbas et al., 2014). First indications of acaricide resistance were recently also reported for *I. scapularis*, a sister species of *I. ricinus* and vector for LB in North America, in a study in which the permethrin susceptibility of two *I. scapularis* field populations was compared to that of a laboratory colony. The field populations originated from an area where tick control was exercised by permethrin 4-poster devices, an area without tick control and a laboratory colony. The susceptibilities of the field populations were significantly lower than that of the laboratory ticks. Although the reported results were significant, they must be viewed critically as the comparison was not made with an established susceptible colony (Burtis et al., 2021). Acaricide resistance has not yet been reported for *I. ricinus*. However, in *in vitro* models using *I. ricinus* cell lines, the upregulation of ATP-binding cassette (ABC) genes after exposure to amitraz, fipronil and permethrin was observed (Mangia et al., 2018). ABC transporters are integral membrane proteins expressed in all organisms and are essential for several physiological processes. They play a crucial role in pumping toxic substances outside the cell (Holland and Blight, 1999) and may act as a multidrug detoxification system in *R. microplus* ticks (Pohl et al., 2012). The upregulation of ABC genes in an *I. ricinus* cell line is associated with drug resistance as it reduces the drug concentration inside the cell (Mangia et al., 2018), suggesting that this tick species may be able to develop resistance against acaricides through this mechanism.

In comparison to chemical substances, where a single point mutation in enzymes which metabolize the substance can suffice to reduce or even block their efficacy, vaccine antigens are typically targeting multiple epitopes. Hence, they might be less susceptible to resistance mechanisms (Willadsen, 2004). Moreover, vaccines could be applied specific to humans/animals and present no danger for non-target species or the environment. The main bottleneck in the development of anti-tick vaccines is the identification of effective antigens. To promote the development of a vaccine against *I. ricinus*, a consortium of seven institutions under the acronym ANTIDotE (**an**ti-tick vaccine to prevent **ti**ck-borne **d**iseases in **E**urope) was founded in 2013. This consortium aimed to identify and characterize *I. ricinus* proteins involved in feeding and pathogen transmission with the overarching aim of developing anti-tick vaccines that could block pathogen transmission (Sprong et al., 2014b).

Three different approaches have been used to identify tick antigens that can prevent tick infestations and/or inhibit pathogen transmission when administered as a vaccine: 1) investigation of immune response mechanisms of tick-immune hosts; 2) identification of tick proteins that are essential for survival of the parasite and 3) separation of effective crude extracts to elicit protective antigens (Willadsen, 2004). All three approaches were pursued in the ANTIDoTE project, but the studies reported in this thesis focused on the third approach: a crude tick protein extract of partially fed *I. ricinus* females was used for the immunization of calves. The immunized animals developed a strong immune response and tick feeding on these animals was shown to be impaired (**Chapter 3**). The immunodominant proteins were subsequently identified by immunoprecipitation followed by LC-MS/MS. Ten of the identified immunodominant proteins were examined in more detail by determining their expression profile and a loss-of-function study using RNAi (**Chapter 4**), providing further information on their potential as anti-tick vaccine antigens.

1.) Investigation of immune response mechanisms of immune hosts

Although the phenomenon of acquired tick immunity was first reported in the 1930s, the immune response mechanisms behind it have not yet been fully elucidated.

While in susceptible animals cellular inflammatory response is the main defence mechanism, acquired tick resistance is associated with cutaneous basophil hypersensitivity and is correlated with increased histamine concentration at the tick bite site. Resistant cattle show an earlier onset of proinflammatory reactions resulting in basophil recruitment and high histamine levels at tick bite site. Basophils belong to the group of granulocytes and occur only in a proportion less than 1% of peripheral blood leukocytes, they express the high-affinity Immunoglobulin E receptor on the cell surface and release mediators such as histamine (Stone et al., 2010). In tick-resistant cattle, guinea pigs and rabbits, extensive accumulation of basophils was observed in the second or subsequent infestation, indicating basophils play a

crucial role in acquired tick resistance (Allen et al., 1977; Brossard and Fivaz, 1982; Trager, 1939). Brown et al. provided indirect proof of this hypothesis, when resistant guinea pigs failed to reject ticks after treatment with anti-basophil-serum (Brown et al., 1982).

The role of the effector molecule histamine was investigated by several authors in different animal species, they observed in anti-histaminic treated tick-resistant animals abrogation of tick rejection (Wikel, 1982; Willadsen et al., 1979). Moreover, subcutaneous histamine injection led to detachment of larval ticks in cattle, guinea pigs and rabbits (Brossard, 1982; Kemp and Bourne, 1980). These results confirm that histamine significantly contributes to tick resistance.

Besides cellular mechanisms, the role of complement in acquired tick resistance was evaluated, too. It was shown that only complement activated by alternative pathways was responsible for successful tick rejection (Wikel, 1979; Wikel and Allen, 1978). Moreover, examination of skin structure of tick-resistant and non-resistant cattle breeds revealed that physical attributes of the skin, including the capacity for wound healing, also play a role in tick rejection (Jonsson et al., 2014).

In 2011, Schuijt et al. reported the identification and characterization of *I. scapularis* antigens associated with tick immunity using the Yeast Surface Display (YSD). With this method, tick salivary proteins, that reacted with sera from rabbits repeatedly infested with *I. scapularis* nymphs, were identified. Nymph feeding was impaired when ticks were fed on rabbits immunized with a cocktail vaccine consisting of recombinant versions of three of the identified antigens (Schuijt et al., 2011). A similar approach was used in the ANTIDoTE project with sera from tick-immune humans. Sera of forestry workers who reported >20 tick bites per year with or without inflammatory signs and of a control group with no previous exposure to ticks were collected and screened by the YSD method using *I. ricinus* salivary proteins as bait. Tick salivary gland proteins that were recognized by anti-tick immune responses were further analysed, resulting in the identification of 12 different tick salivary gland proteins. Three potential vaccine candidates were subsequently selected for vaccination studies based on their proportion, their reactivity with immune sera, their expression in the early tick feeding stage and their reactivity with *I. scapularis* tick immune rabbit sera (Trentelman et al., 2021).

2.) Identification of tick proteins, which are essential for survival of the parasite

Over the last decades, many tick proteins thought to play important roles in tick biology or pathogen transmission have been identified and probed as tick-protective vaccine candidates. Host animals vaccinated with tick antigens will develop an immune response, the effect of which can be measured by experimentally infesting vaccinated animals with ticks to examine tick feeding success. *Rhipicephalus microplus* infestations in livestock have a significant economic impact and therefore received particular attention. The main achievements in anti-

tick vaccine development for *R. microplus* were previously reviewed by several authors (Almazan et al., 2010; de la Fuente et al., 2007a; Parizi et al., 2009).
Findings for other tick species such as *Haemaphysalis*, *Dermacentor*, *Amblyomma* and *Ornithodoros* species are summarized in a comprehensive paper by Neelakanta and Sultana (Neelakanta and Sultana, 2015). The following sections focus on the achievements related to *Ixodes* sp.

2.1 Proteins essential to the biology of the tick

Several classes of tick proteins that are involved in the digestion, reproduction and development of ticks were examined as tick-protective antigens in the last years. Many of these tick proteins are highly conserved among different tick species and might therefore be useful in a vaccine targeting multiple tick species.

Host hemostasis and inflammation responses pose powerful barriers for haematophagous arthropods, so mosquitoes, flies and ticks have evolved counteracting mechanisms to evade these responses. Saliva proteolytic enzyme inhibitors (serpins) are for instance essential for blood intake and might be good targets for tick-protective vaccines (Prevot et al., 2007). Immunization trials with recombinant serpins led to increased tick mortality and prolonged engorgement times (Prevot et al., 2007). Moreover, serpins in other tick species, such as *Ambylomma* (Tirloni et al., 2019), *Rhipicephalus* (Mulenga et al., 2003; Tirloni et al., 2016) or *Haemaphysalis* (Sugino et al., 2003) suggest that serpins are highly conserved sequences and could provide cross-protection for different tick species.
In 2008 Decrem et al. identified two Metalloproteases (metis 1 and metis 2) from *I. ricinus*. The gene expression of metis 1 and 2 were shown to be induced during blood ingestion and an *ex vivo* model showed that metalloproteases can interfere with the host's fibrinolysis. Immunization trials with recombinant Metis 1 and Metis 2 led neither to delayed tick feeding nor to increased mortality rates, although the engorgement weights and oviposition were impaired (Decrem et al., 2008). Once the blood meal, which can be several hundred times the weight of unfed ticks, is ingested, the iron level in the tick increases rapidly. For ticks, iron is both essential for reproduction but also potentially toxic. Thus, ticks have evolved strategies to manage iron and heme excess after the blood intake. Tick proteins, which are essential for iron and heme metabolism were identified and used as vaccine candidates as well. In **Chapter 3**, recombinant Ferritin 2 was used as vaccine but could not impair tick feeding in the reported immunization trial. In 2009 Hajdusek et al. evaluated the effect of Ferritin 1 (fer1), Ferritin 2 (fer2) and iron regulatory protein 1 (irp1) knockdown using RNA silencing in *I. ricinus.* They reported that silencing of fer1 reduced the engorgement weight by ~50%, decreased oviposition and prevented hatching of larvae. Knockdown of irp1 led to reduced hatching

(~66%), although repletion and oviposition were not altered. Without fer2 more than 50% of ticks were not able to engorge (Hajdusek et al., 2009). Fer2 was tested as a vaccine in rabbits, which resulted in a reduction of tick numbers, engorgement weight and fertility of more than 30% compared to the negative control (Hajdusek et al., 2010). However, this effect was not reproducible in cattle using fer2 as vaccine (**Chapter 3**). The small group size or use of individual animals in both studies, differences specific to the animal species and variations in the immunization schedule might explain these contrasting findings.

Proteins involved in iron and heme metabolism are promising vaccine targets. Characterization of fer1 and fer2 in several tick species indicates highly conserved sequences (Galay et al., 2014a; Githaka et al., 2020; Kopácek et al., 2003). In *H. longicornis* ticks gene expression profiles showed upregulation after blood feeding in all life stages (Galay et al., 2013). Nevertheless, most of the studies focused on adult females (Galay et al., 2014b; Hajdusek et al., 2010; Manjunathachar et al., 2019), presumably due to their enormous blood intake, they absolutely depend on successful iron binding or efflux.

Proteins of processes that are upregulated after the blood meal in every life stage, came into focus as anti-tick vaccine targets, too.

Aquaporins (AQPs) are crucial for transporting water and neutral solutes. In ticks, AQPs have been detected in the digestive tract, the Malpighian tubules and salivary glands (Holmes et al., 2008). These evolutionarily highly conserved proteins play an important role in water homeostasis and osmoregulatory stress following the blood intake (Campbell et al., 2010). Promising results were reported by Contreras et al., when vaccinating rabbits with AQP1, which led to increased *I. ricinus* larval mortality and reduced ability to moult to nymphs (Contreras and de la Fuente, 2017).

Subolesin (SUB) is an evolutionary conserved molecule that is involved in regulation of genes affecting immune responses, blood digestion, reproduction, and development. The protein was identified as a protective antigen by Almazán et al. in 2003 using cDNA expression library immunization. In this study, a cDNA expression library from *I. scapularis* tick embryo cells was constructed and cDNA clone pools were used for the immunization of mice, followed by a challenge with *I. scapularis* larvae. cDNA pool-induced immunity of mice were detected by reduced engorgement ability and impaired moulting to nymphs. Clones that induced immunity were sequenced, compared to sequence databases and characterized (Almazán et al., 2003). This approach led to the identification of SUB as a protective antigen. Several vaccination trials showed effective control of tick infestations by a reduction in tick numbers, weight, and fecundity. Moreover, transmission of different tick-borne pathogens was also reduced after SUB vaccination (de la Fuente et al., 2011; Merino et al., 2011).

In 2013 Moreno-Cid et al. reported that vaccination of mice with SUB and subsequent tick challenge with *I. ricinus* larvae led to reduced moulting to nymphs but larval mortality was not

affected significantly (Moreno-Cid et al., 2013). In contrast, Contreras and de la Fuente showed significantly increased *I. ricinus* larval mortality (4.25-fold) in vaccinated rabbits compared to controls, using recombinant SUB epitopes as a vaccine. Furthermore, larval moulting was reduced by 38%. They demonstrated efficient reduction of tick survival and moulting and suggest SUB as candidate for the control of multiple tick species (Contreras and de la Fuente, 2016).

However, it should be noted that many studies were focused on the first developmental stage of *Ixodes* sp. This might be due to the ease of handling and low costs of small animals that are suitable for infestations with larger numbers of larvae. The evaluation of the antigen's efficacy in other life stages would require a repetition of these vaccination studies, for instance in larger animals suitable for feeding *I. ricinus* adults on.

2.2 Proteins associated with pathogen transmission

The approach to use salivary gland proteins that are involved in pathogen transmission as vaccine candidates, could potentially kill two birds with one stone by reducing both tick infestation and inhibit pathogen transmission. Ticks stay attached to the host for multiple days to complete blood feeding and need to produce immunosuppressive proteins in order to do so. There is increasing evidence that pathogens also use these tick proteins to evade the host's immune system. The centre of attention has mainly focused on the relationship of *I. scapularis* and *Borrelia burgdorferi* sensu stricto. Different tick proteins, which were shown to facilitate pathogen transmission were identified and studied in more detail. Das et al. for instance identified Salp15, a 15-kDa salivary gland protein, as one of the most immunodominant antigen in engorged *I. scapularis* ticks (Das et al., 2001). The vaccine potential of Salp15 was evaluated in mice by Dai et al. in 2009. They reported that Salp15 antiserum significantly protected mice from *B. burgdorferi* infection and demonstrated that Salp15 antiserum also enhanced the protective capacity of antibodies against *B. burgdorferi* antigens. Mice that were actively vaccinated with Salp15 were also significantly protected (Dai et al., 2009).

The outer surface protein C (OspC) of *Borrelia burgdorferi* is produced by the pathogen when migrating from the tick gut to the salivary glands, where it was shown to bind to Salp15. Although, it was recently reported by Melo et al., that oral immunization with recombinant OspC did not prevent infection of mice with *B. burgdorferi* (Melo et al., 2016), a vaccine combination of Salp15 and OspC could inhibit pathogen transmission in two different ways: 1) Salp15-antibodies could neutralize the immunosuppressive effect of Salp15 and lead to impaired tick feeding and 2) antibodies could bind to Salp15 that is already bound to OspC on the surface of *B. burgdorferi* and induce phagocytic immune cells to eliminate the pathogen (Hovius et al., 2007b).

As mentioned above, research is mainly focused on *I. scapularis* proteins. However, its sister species *I. ricinus* is the main vector for Lyme disease in Europe so researchers have looked whether similar proteins are expressed in *I. ricinus* and examined if these could have the same potential as anti-tick vaccines. In 2007 Hovius et al. identified three Salp15 homologues in *I. ricinus*. The presence of different Salp15 homologues might be due to different preferential pathogen-tick interactions or a separate Salp15 homologue for each life stage of the tick (Hovius et al., 2007a). It is also an indication that there is redundancy in the tick's salivary proteins, and if this assumption is confirmed, vaccination efforts might be frustrated.

3.) Separation of effective crude extracts to elicit protective antigens

An easier, but laborious and time-consuming way to identify protective antigens is the use of native tick protein extracts. As mentioned in **Chapter 1**, this approach led to the identification of Bm86, a tick gut membrane-bound glycoprotein, which has been used in a recombinant form as a vaccine against *R. microplus* infestations. The use of parasite extracts for immunization is described for other parasite species too. The stomach or barber's pole worm *Haemonchus contortus* is one of the most common nematode parasite of small ruminants in tropical and subtropical areas. Similar to tick immunity, small ruminants can develop immunity against *H. contortus* by repeated parasite infestations (Barger et al., 1985). In 2015, the Barbervax® vaccine targeting *H. contortus* was commercialized. It is based on the vaccination of sheep with native integral gut membrane proteins of *H. contortus*, resulting in the generation of antibodies. The feeding worm imbibes these antibodies with its blood meal, which causes a destruction of the worm's intestine and leads to increased mortality and reduced fertility of the parasite. Like Bm86-based vaccines, it efficacy is correlated to antibody titres so regular booster vaccinations are required (Nisbet et al., 2016). A similar strategy was conducted to develop a vaccine against the red poultry mite, *Dermanyssus gallinae*. Here, immunization with native soluble mite extracts led to efficient reduction of mite infestations, initially in *in vitro* studies using vaccinated hen's blood fed to mites in an artificial feeding system, and later in field studies as well (Bartley et al., 2017; Bartley et al., 2015). However, recombinant expressed versions of immunogenic antigens (Vitellogenin, a Serpin and a protein of unknown function) did not induce reliable protection against mite infestation and would require further optimization (Bartley et al., 2017).

The work described in **Chapters 3 and 4** of this thesis followed a similar approach to find protective antigens against *I. ricinus*. Native tick extracts from partially fed females were prepared and used for the immunization of calves. Animals immunized with native tick protein extracts showed a strong cutaneous response at the tick bite site as well as impaired tick feeding. Markedly, a significant reduction of the number of females and nymphs that fed

successfully and a significant reduction of the engorgement weights of females was observed (Knorr et al., 2018).

In addition to traditional methods, the technical progress over the last years evolved new techniques to identify and assess potential vaccine candidates.
The assembly of the *I. scapularis* genome (Gulia-Nuss et al., 2016) facilitated the identification of tick proteins and biochemical pathways that could be targeted by anti-tick vaccines. With increasing genomic information for other tick species, such as draft genome assemblies for *I. ricinus* (Charrier et al., 2018; Cramaro et al., 2015), *R. microplus* (Barrero et al., 2017), and several other tick species (Jia et al., 2020) becoming available, the identification of highly conserved genes is facilitated and may be useful in the development of vaccines that can target multiple tick species.

Another valuable tool for functional gene analysis is RNA interference (RNAi), whereby the inoculation of double-stranded RNA (dsRNA) leads to the specific degradation of mRNA, resulting in post-transcriptional gene silencing (de la Fuente et al., 2007b). With the possibility to silence the expression of specific genes, RNAi became a popular method for the screening of tick-protective antigens and different approaches of dsRNA inoculation have been described. The most common method is the injection of dsRNA, whereby dsRNA is injected manually into each individual with special syringes. However, the injection success depends on experience and skills of personnel and is only suitable for larger specimens such as nymphs or adults. An alternative for the induction of RNAi in smaller life stages is the electroporation of dsRNA (Karim et al., 2010). Although this method appears to be less laborious and less traumatic for the ticks compared to injection, only one other research group reported successful electroporation of tick eggs after these were de-waxed (Ruiz et al., 2015). Another non-invasive dsRNA delivery method by the capillary feeding of dsRNA to pre-fed nymphs was also reported (Soares et al., 2005), but this method is rather laborious too.

The development of *in vitro* feeding assays for haematophagous arthropods such as mosquitos, flies and ticks provides effective tools to investigate their feeding behaviour and biology (Rutledge L.C., 1964). Moreover, the use of *in vitro* systems adheres to the 3R concept (**R**educe, **R**eplace, **R**efine) for humane animal experimentation. The number of experimental animals that are used for tick feeding or experimental infection with tick-borne pathogens, can be reduced by the use of *in vitro* feeding systems. Such feeding assays are available for different tick species including *I. ricinus* and are continuously being improved, as recently described by several authors (Böhme et al., 2018; González et al., 2017; Krull et al., 2017; Militzer et al., 2021; Trentelman et al., 2017). In the 1980s Kemp et al. succeeded in feeding

R. microplus ticks in an artificial feeding system with blood of vaccinated cattle and gained comparable results to ticks fed on animals. Different blood ingredients were examined to reveal the *in vivo* immune pathways that were responsible for tick damage. To this end, several assays were performed with or without complement, different antibody classes or lymphocytes (Kemp et al., 1986; Kemp et al., 1989). These studies demonstrate the potential applications of *in vitro* tick feeding systems in anti-tick vaccine development. In **Chapter 3**, results of *in vitro* feeding assays are presented in which blood from two vaccination trials performed with cattle was used. Although, *I. ricinus* is not a major problem in cattle except for the transmission of *Babesia divergens* and *Anaplasma phagocytophilum,* which can cause severe diseases (Springer et al., 2020; Woldehiwet, 2006), cattle can provide blood/serum for artificial feeding assays in sufficiently high volumes, making them potentially useful for the evaluation of antisera using artificial tick feeding systems.

In **Chapter 3** *in vitro* feeding assays were conducted with plasma or whole blood, but could not reproduce the results obtained in the *in vivo* trials. This may be caused by the absence of specific immunological components, such as leukocytes and complement in the *in vitro* system, but also by the reduced number of ticks that successfully attached and fed *in vitro* compared to ticks fed on animals. Although, Kröber and Guerin described an artificial feeding system for *Ixodes* sp. (Kröber and Guerin, 2007), which was subsequently adapted for the feeding of all consecutive life stages of *I. ricinus*, it remains challenging to achieve feeding results similar to those seen in ticks fed on animals. Reduced attachment, engorgement and fecundity, which lead to reduced egg masses and reduced larval hatching was even observed in control groups in *in vitro* feeding assays in **Chapter 3**. Only for larval stages comparable numbers and engorgement weights to *in vivo* feeding have been reported. Moreover, antibiotics added to the blood meal might reduce tick symbiont numbers and eventually tick fecundity (Militzer et al., 2021). While it is clear that there are still challenges that need to be overcome to obtain successful and robust *in vitro* feeding of all life stages, the system nonetheless holds promise for enabling reproducible and reliable studies under standardized conditions. Moreover, tick feeding and mortality can be monitored accurately and costs for laboratory animals could be reduced. Artificial feeding systems can therefore be useful for many studies, including acaricide testing or the evaluation of anti-tick vaccine candidates, at least in early research and screening stages (Artigas-Jerónimo et al., 2021; Contreras et al., 2017).

The identification of immunodominant proteins from extracts used for the successful vaccination of calves is described in **Chapter 4**, which builds on the use of cattle as the tick host. In these trials (**Chapter 4**), sera from vaccinated calves were immunoprecipitated with *I.*

ricinus salivary gland or midgut extracts. Antibody-antigen complexes were analyzed by liquid chromatography–mass spectrometry (LC-MS/MS), which resulted in the identification of 99 immunodominant proteins. Gene expression profiles were subsequently determined for 15 selected proteins and the expression of ten proteins was silenced by RNAi in adult females to observe its effect on tick feeding. Significantly decreased engorgement weights were found in ticks in which the expression of MG4, MG6, MG9 and SG2 (Salivary gland protein) was silenced and reduced tick numbers were observed for the MG2, MG6, MG8, MG10 and SG2-silenced groups. Based on these experiments, the genes that resulted in the most significant reduction in feeding success or engorgement weights when silenced were MG6 (=Phosphatidylinositol phosphatase PTPRQ, putative) and SG2 (=Glutathione S-transferase Mu 3). Further studies to evaluate whether these proteins would be effective as recombinant vaccines would be of interest. The identified proteins which have highly similar homologues in other tick species, such as Metalloproteinase, Glutathione S-transferase (GST), ADP/ATP translocase, Integrin alpha and beta, could also be considered as potential vaccine candidates and could be investigated further for their potential to induce cross-protection against different tick species.

The most promising antigen based on the RNAi studies reported in **Chapter 4**, is Glutathione S-transferase (SG2). This protein is thought to be involved in the excretion of toxic metabolites and GSTs have previously been evaluated as tick-protective antigens in other tick species by various research groups. Huercha et al. reported reduced female engorgement rate, lower egg masses and reduced larval hatching when adult ticks were fed on rabbits vaccinated with recombinant *Dermacentor marginatus* GST (Huercha et al., 2020). *In silico* analysis performed by Sabadin et al. revealed more than 80% protein sequence similarity for *Haemaphysalis longicornis*, *Rhipicephalus appendiculatus* and *R. microplus* GSTs, suggesting that GST sequences are highly conserved (Sabadin et al., 2017). Cattle and rabbits were immunized with rGST from *H. longicornis* in vaccination trials. Subsequent tick challenge with *R. microplus* on cattle and *R. appendiculatus* on rabbits led to decreased adult tick number, weight, and fertility, resulting in an overall vaccine efficacy of 57% and 67%, respectively (Parizi et al., 2011; Sabadin et al., 2017). However, nymphal tick feeding of *R. appendiculatus* as well as feeding of *R. sanguineus* on rabbits remained unimpaired (Sabadin et al., 2017). Similar results were reported by Ndawula et al. when evaluating rGSTs from *Amblyomma variegatum* and *Rhipicephalus decoloratus* as a vaccine against *R. sanguineus* infestations, which hampered female engorgement weight, oviposition and hatching but not to a level that was significantly different from the control group (Ndawula et al., 2019). The results of the conducted studies expose the difficulty of designing an effective and efficient recombinant vaccine, even when antigens are highly conserved amongst different tick species.

5. 2 Conclusion

Although the advantages of anti-tick vaccines are obvious, effective vaccines that reduce tick infestation or even inhibit / block pathogen transmission are not yet available for *I. ricinus*. The major bottleneck in anti-tick vaccine development is the identification of protective antigens. The main approaches for the identification of such antigens are 1.) investigation of immune responses of immune hosts, 2.) usage of essential tick proteins as vaccine candidates or 3.) separation of native tick extracts to elicit protective antigens. Recently developed methods such as RNAi, genomic and proteomic databases have facilitated a faster identification of potential antigens and were employed in the studies reported in this thesis.

Ferritin 2 has an essential function to ticks and was evaluated as a recombinant tick antigen in cattle, but could not impair nymphal or adult tick feeding. In contrast, immunization with native tick extracts led to significantly reduced tick numbers and engorgement weights. In subsequent studies, immunodominant tick proteins were identified and promising antigens were selected for further studies. Gene silencing of Phosphatidylinositol phosphatase (MG6) and Glutathione S-transferase (SG2) by RNAi showed significantly impaired feeding of adult female ticks. While these results look promising, immunization trials with recombinant versions of MG6 and SG2 followed by tick challenge will be required to evaluate the tick protective efficacy of these antigens. This should ideally include as challenge with nymphal ticks, as this life stage is of major epidemiological relevance in relation to the transmission of TBDs to humans.

Although it remains challenging to develop an effective anti-tick vaccine which provides cross-protection for different species and inhibit tick-borne pathogen transmission, researchers have more efficient tools available than ever. Our studies provided further insight into *I. ricinus* antigens that may be of interest for further examination as anti-tick vaccine antigens.

Summary

Ticks are obligate haematophagous ectoparasites, which can transmit different pathogens such as bacteria, protozoa or viruses. Tick-borne diseases occur all over the world and threaten the health of humans and animals. A novel and promising tick control strategy is the development of anti-tick vaccines. Therefore, seven European institutes joined forces to develop an anti-tick vaccine, which inhibit transmission of common pathogens in Europe, such as Borrelia, Babesia or the tick-borne encephalitis virus. Within the framework of the ANTIDotE (Anti-tick vaccine to prevent tick-borne diseases in Europe) project, this PhD thesis is focused on immunization experiments with calves and subsequent identification of immunodominant proteins.

Chapter 1 gives a general introduction to *Ixodes ricinus* ticks, highlights the importance of preventing tick-borne diseases and highlights different strategies of tick control. In Chapter 2, the occurrence, abundance as well as clinical symptoms and treatment schemes of common TBDs in Europe transmitted by *Ixodes ricinus* are summarized and discussed. Two small-scale immunization trials are described in Chapter 3. In these studies, crude protein extracts and recombinant Ferritin 2 were tested as anti-tick vaccine candidates. *Ixodes ricinus* ticks fed on tick protein extract-vaccinated calves were reduced in number and obtained lower repletion weights. Moreover, pronounced inflammatory signs were observed on the tick bite sites. Tick feeding was not impaired in the Ferritin 2 vaccinated calf. *In vitro* tick feeding assays using plasma or whole blood from vaccinated animals could not reproduce the *in vivo* feeding results, indicating that antibodies alone were not responsible for the observed vaccine immunity.

In Chapter 4, immune sera from salivary glands or midgut extract vaccinated calves were used for co-immunoprecipitation of tick tissue extracts followed by LC/MS-MS analyses. This resulted in the identification of 46 immunodominant proteins. The relative gene expression in tick tissues of unfed and partially fed females were measured for 15 proteins. Ten out of the 15 candidates were selected for further RNAi studies. The strongest RNAi phenotypes were observed for MG6 (A0A147BXB7), a protein containing eight Fibronectin type III domains predominantly expressed in tick midgut and ovaries of feeding females, and SG2 (A0A0K8RKT7), a glutathione-S-transferase that was found to be upregulated in all investigated tissues upon feeding. The results showed that co-immunoprecipitation of tick proteins with host immune sera, followed by protein identification using LC-MS/MS is a valid approach to identify antigen-antibody interactions and could be integrated in anti-tick vaccine discovery pipelines.

The studies of this thesis present possibilities for the evaluation and identification of potential anti-tick vaccine candidates. Furthermore, immunodominant proteins of the species *I. ricinus* were identified and characterized, which could contribute to the development of an anti-tick vaccine against the vector *I. ricinus.*

Zusammenfassung

Evaluierung von *Ixodes ricinus* Proteinextrakten als Impfstoff-Kandidaten in Rindern

Zecken sind blutsaugende Parasiten, die zur Gattung Acari zählen und weltweit verbreitet sind. Sie können verschiedene Krankheitserreger, wie z.B. Bakterien, Protozoen oder Viren übertragen. Durch Zecken übertragene Krankheiten kommen weltweit vor und bedrohen die Gesundheit von Menschen und Tieren. Eine neue und vielversprechende Bekämpfungsstrategie stellt ein Anti-Zecken-Impfstoff dar. Sieben europäische Institute haben sich mit dem Ziel zusammengeschlossen, einen Anti-Zecken-Impfstoff zu entwickeln, der die Übertragung verschiedener Pathogene, wie Borrelien, Babesien oder FSME Viren, die vor allem in Europa vorkommen, hemmt. Im Rahmen des Projekts ANTIDotE (Anti-Zecken-Impfstoff zur Verhinderung von durch Zecken übertragenen Krankheiten in Europa) wurde diese Doktorarbeit erstellt und verschiedene Impfstudien mit Rindern und die anschließende Identifizierung von immundominanten Proteinen durchgeführt.

Kapitel 1 gibt eine allgemeine Übersicht zu *Ixodes ricinus* Zecken, deren Vektorkompetenz für durch Zecken übertragene Krankheiten sowie Bekämpfungsstrategien. Im zweiten Kapitel werden Verbreitung, Häufigkeit, klinische Anzeichen und Behandlungsmethoden der häufigsten durch Zecken übertragenen Krankheiten besprochen.

In Kapitel 3 werden die Ergebnisse von zwei Pilot-Impfstudien vorgestellt, bei denen Zeckenproteinextrakte und rekombinantes Ferritin 2 als Anti-Zecken-Impfstoffe getestet worden*. I. ricinus* Zecken, die Blut von mit Proteinextrakt geimpften Rindern aufnahmen, wurden in ihrer Anzahl reduziert und wiesen ein geringeres Vollsauggewicht auf. Außerdem wurden ausgeprägte Entzündungsanzeichen an den Zeckeneinstichstellen beobachtet. Die Impfung mit rekombinanten Ferritin 2 beeinträchtige die Blutaufnahme der angesetzten Zecken nicht. In *in vitro* Assays, in denen Plasma oder Vollblut der geimpften Tiere verfüttert wurden, konnten die Ergebnisse der *in vivo* Studie nicht reproduziert werden, dass deutet darauf hin, dass Antikörper nicht allein für die beobachtete Impfimmunität verantwortlich waren.

In Kapitel 4 wurden Seren von mit Speicheldrüsen- oder Zeckendarmextrakt geimpften Kälbern für die Co-Immunpräzipitation mit Zeckengewebeextrakten verwendet. Mithilfe von Flüssigchromatographie mit Massenspektrometrie-Analysen (LC-MS) konnten 46 immundominante Proteine identifiziert werden. Für 15 Proteine wurde die relative Genexpression in Zeckengeweben von nicht gefütterten und teilweise gefütterten Weibchen gemessen. Zehn von diesen 15 Kandidaten wurden für eine RNAi-Studie ausgewählt. Die stärksten RNAi-Phänotypen wurden für MG6 (A0A147BXB7) beobachtet, ein Protein mit acht Fibronektin-Typ-III-Domänen, welches hauptsächlich im Darm und in den Eierstöcken von gefütterten Weibchen exprimiert wird, und SG2 (A0A0K8RKT7), einer Glutathion-S-

Transferase, die in allen untersuchten Geweben nach der Fütterung hochreguliert ist. Die Ergebnisse zeigen, dass die Co-Immunpräzipitation von Zeckenproteinen mit Immunseren und anschließender Proteinidentifizierung mittels LC-MS, ein valider Ansatz zur Identifizierung von Antigen-Antikörper-Wechselwirkungen ist und zur Entwicklung von Anti-Zecken-Impfstoffen genutzt werden kann.

In dieser Arbeit werden Methoden zur Identifizierung und Evaluierung von Anti-Zecken-Impfstoffen vorgestellt und eigene Ergebnisse diskutiert. Die identifizierten immundominanten Proteine der Spezies *I. ricinus* wurden charakterisiert und können einen Beitrag zur Entwicklung eines Impfstoffes gegen den Vektor *I. ricinus* leisten.

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Disclosure of the own share in the body of work

The share of the authors who were involved in the publications of this work is listed under the following criteria:

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2. Design of experiments
3. Test execution
4. Analysis of test data
5. Compilation of the manuscript

Review: **Control of Lyme borreliosis and other *Ixodes ricinus*-borne diseases**

1. Sprong, Hovius
5. Sprong, Azagi, Hoornstra, Nijhof, Knorr, Baarsma, Hovius

Publication I: **Preliminary Evaluation of Tick Protein Extracts and Recombinant Ferritin 2 as Anti-tick Vaccines Targeting *Ixodes ricinus* in Cattle**

1. Knorr, Nijhof
2. Knorr, Nijhof, Anguita, Hovius
3. Knorr, Anguita, Cortazar, Trentelman, Kershaw
4. Knorr, Nijhof
5. Knorr, Nijhof

Hajdusek and Kopáček contributed materials

Publication II: **Identification and Characterization of Immunodominant Proteins from Tick Tissue Extracts Inducing a Protective Immune Response against *Ixodes ricinus* in Cattle.**

1. Knorr, Anguita, Hovius, Nijhof
2. Knorr, Anguita, Nijhof
3. Knorr, Reissert-Oppermann, Tomás-Cortázar, Barriales, Azkargorta, Iloro, Elortza, Pinecki-Socias
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Conflict of Interest

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

Statement of Authorship

I hereby declare that I am the sole author of this PhD thesis and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution in order to obtain a degree.

Berlin, 14–January–2022 Sarah Knorr