University of South Bohemia in České Budějovice
Faculty of Science

Influence of dietary components and redox enzymes on intestinal microbiota proliferation in the tick *Ixodes ricinus*

Master thesis

Bc. Matěj Kučera

Supervisor: RNDr. Petr Kopáček, CSc.

Consultant: Mgr. Jan Perner

České Budějovice 2015

Kučera M., (2015): Influence of dietary components and redox enzymes on intestinal microbiota proliferation of the tick *Ixodes ricinus*. Mgr. Thesis, in English 57 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

In this work, we have analysed the temporal dynamics of gut-dwelling bacteria and *Borrelia* in the gut of the deer tick *Ixodes ricinus*. Using quantitative PCR, we have shown that levels of the tick intestinal microflora are profoundly decreased at later stages of feeding on whole blood but not on serum. Even though we noted that host complement system manages to interfere with *Borrelia* viability *in vitro*, we did not see any effect of host complement on *Borrelia* acquisition in adult ticks *in vivo*. However, we revealed that host hemoglobin is essential for *Borrelia* proliferation in the tick gut. All together, these data imply that, during feeding, levels of gut-dwelling bacteria and *Borrelia* are determined by the host. While hemoglobin seems to be detrimental for gut-dwelling bacteria, *Borrelia* require it in order to proliferate. During off-host stage, we showed that levels of gut-dwelling microflora are regulated by an intestinal transmembrane enzyme Dual oxidase. In conclusion, we aimed, and mostly succeeded, to perform pilot experiments describing the biology of a complex process of regulating gut microflora in the vector *Ixodes ricinus* and extend it by its impact on *Borrelia* acquisition

Financial support

This work was funded by the Grant Agency of the Czech Republic, Project No. 13-11043S to Petr Kopáček

I hereby declare that I have worked on my master thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my master thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 22.4. 2015

Bc. Matěj Kučera

Acknowledgements

In this part, I wish to thank my supervisor Dr. Petr Kopáček for the opportunity to become a member of his team, for his scientific guidance and valuable advice. I thank Jan Perner for help in designing most of my experiments and for his assistance during data evaluation. I wish to thank Radek Šíma for his help and support in methodical issues. Big thank belongs to Jitka for her willingness to help me with dissecting and *in vitro* feeding experiments. I thank to all members of our laboratory for open discussions and overall great atmosphere in the lab.

Special thanks (in Czech) - Největší dík patří mým rodičům za umožnění studia a za jejich finanční i psychickou podporu během celého studia. Děkuji bratrovi a sestře za jejich optimismus a za jejich četnou kritikiku. Děkuji přítelkyni Ivetce za podporu, trpělivost a pomoc během psaní a za navracení na správnou cestu. Dík patří i všem mým přátelům, kvůli kterým jsem si celá studia báječně užil.

Contents

1 Introduction

1.1 Ticks

Ticks are blood-sucking obligate ectoparasites cabable of transmitting a broad variety of pathogens of animals and humans. These small arachnids from the order Parasitiformes constitute together with Acariformes and Opilioacariformes the subclass Acarina. There are two main tick families called Ixodidae and Argasidae differing not only in appearance but in the way of life in general. Ixodidae, also referred to as hard ticks, consists of 14 generas (Horak et al., 2002). Most important vectors from Ixodidae family are generas *Amblyomma*, *Dermacentor*, *Haemaphysallis*, *Hyalomma*, *Rhipicephalus* and the most numerous Ixodida (Horak et al., 2002). These ticks possess three active stages of a life cycle. Six-legged larvae hatched from the eggs, molt into eight-legged nymphs and the cycle is ended by the fertile adult females capable of laying thousands of eggs after previous blood ingestion and mating. Inter-stage periods are characterized by blood intakes from different vertebrate hosts that might differ also for developmental stages. Accordingly, Ixodidae are divided into 3-hosted, 2-hosted and single hosted parasites (Sonenshine, 1991). Ticks seeking the host using Haller`s organ, complex sensory organ for detection of scents, humidity, temperature and carbon dioxide (Sonenshine, 1991). The attachment of the ticks to their host is mediated by specialized harpoon-like structure called hypostome placed on the top of mouth area. Hypostome is anchored in the host body by products of salivary glands that provide also broad scale of bioactive compounds for successful host immunity modulation (Fingerle et Wilske, 2006). Protozoan, bacterial and viral pathogens are ingested together with blood (Jongejan et Uilenberg, 2004). Interestingly, both families of ticks (Ixodidae and Argasidae) are capable of transmitting closely related pathogens, like *Borrelia,* despite strikingly different life styles of the two families. After mosquitoes, ticks are second most important vectors of arthropod-borne pathogens (Sonenshine, 1991).

1.2 Blood meal digestion

It is supposed that blood feeding in ticks evolved 400 - 100 million years ago (Mans, 2011). Unlike other hematophagous arthropods where the blood is rapidly digested extracellularly in the midgut lumen (Billingsley, 1990), the blood digestion in tick constitutes a slow process that occurs solely in the midgut cells in the acidic environment of digestive vesicles (Sonenshine, 1991). Tick gut lumen is believed to be the store organ of the ingested blood

(Coons et al., 1986). Particularly precise hemoglobin degradation is required due to the potential toxicity of hemoglobin-released heme (Graca-Souza et al., 2006). Considering the tick model *I. ricinus*, the hemoglobinolysis is mediated by a spectrum of endopeptidases cleaving gradually the molecule of hemoglobin into smaller fragments (Horn et al., 2009). Importantly, primary hemoglobin cleavage is accompanied by forming of antimicrobial peptides with strong microbicidial activity (Fogaça et al., 1999). The role of cathepsin D and cathepsin L supported by legumain peptidase was demonstrated in this primal hemoglobin processing (Horn et al., 2009; Franta et al., 2010).

1.3 Microbiota

Microorganism ingested by arthropods co-evolved as symbionts or commensals and adapted for environment of specific tissues as gut, ovaries, Malpighian tubules or hemocoel (Eleftherianos et al., 2013). Classical symbiotic relationship of arthropods and microorganisms is demonstrated on termites and their cellulose-digesting microbiota (Ohkuma, 2003). Despite having constant species-specific core microbiota, the majority of microbiome is created by opportunistic microorganisms (Osei-Poku et al., 2012). Factors like geographical origin, ecological niche, nutrition sources and even sex can significantly influence the quantity and species-composition in populations or even individuals (Boissière et al., 2012; Osei-Poku et al., 2012; Zouache et al., 2011;Terenius et al., 2012; Valiente Moro et al., 2013). Using state of the art technologies, such as metagenonomics, the quantification and distribution of microorganisms within the tissues provide us with detailed insight into microbiota importance (Osei-Poku et al., 2012; Valiente Moro et al., 2013). Some tick microbiomes are currently known due to implementing of these technologies.

The metagenomic approach was applied for investigation of tick microbiome and microbiotic composition was described for tick species as *Ixodes ricinus, Rhipicephalus microplus* or *Amblyomma americanum* (Andreotti et al., 2011; Carpi et al., 2011; Menchaca et al., 2013; Budachetri et al., 2014). Proteobacteria phylum was detected as the main phylum of *I. ricinus.* Other abundant phyla were Actinobacteria, Spirochetes, Bacteroidetes and Firmicutes. Overall 108 identified genera are described considering *I. ricinus* tick. Bacterial genera *Stenotrophomonas, Pseudomonas, Rhodococcus* and *Propriobacterium* were confirmed to create core microbiota that was found in all examined *I. ricinus* ticks (Carpi et al., 2011). *I. ricinus* is additionally unique by hosting endosymbiotic bacteria

Candidatus midichloria mitochondrii. This bacterium was reported as the first endosymbiont residing within animal mitochondria (Sassera et al., 2006).

Gut microbiota of disease-transmitting parasites play an important role in determination of vector competence for pathogens (Dennison et al., 2014; Narasimhan et al, 2014). Mosquitoes with decreased or removed gut microbiota showed augmented permissiveness for *Plasmodium falciparum* infection (Noden et al., 2011). The restoration of gut microflora by serving the bacteria-enriched meal renewed the resistence against pathogen (Bahia et al., 2014; Noden et al., 2011). Absence of microbiota in the tick *Ixodes scapularis* substantially reduced colonization rate of their guts by *Borrelia burgdorferi*. Subsequently, it was confirmed that gut microbiota play a role in JAK/STAT pathway regulation (Narasimhan et al., 2014). Despite the insufficient knowledge of regulation tick immune responses (Kopáček et al., 2010), JAK/STAT pathway was implicated to play an important role in immune processes and gut remodeling of tick gut (Liu et al., 2012). Absence of intestinal microflora resulted in decreased STAT transcription and thus worsened adherence of spirochetes to gut epithelium (Narasimhan et al., 2014). Additionally, JAK/STAT pathway mediates the proper peritrophic matrix (PM) formation, the protective barrier of inner gut epithelium against abrasive food particles and pathogens (Hegedus et al., 2009; Kuraishi et al., 2011). The thinned PM, given by the absent STAT, resulted in decrease *Borrelia* quantity suggesting the role of intestinal microbiota in pathogen acquisition via JAK/STAT pathway regulation (Narasimhan et al., 2014; Liu et al., 2012).

Arthropod gut microbiota is able to activate the anti-pathogen response by inducing the host immune gene expression. Using microarray method, it was shown that gene expression of immune genes in two mosquito species markedly differs between mosquitoes containing intestinal microbiota and antibiotic-treated mosquitoes (Dong et al., 2009). The expression of immune genes in the mosquito *Aedes aegypti* was reported to be mediated by intestinal microbiota through Toll pathway. The expression of several antimicrobial peptides, cecropin, attacin or gambicin via Toll pathway was reported (Xi et al., 2008). *Drosophila melanogaster* orthologous genes of Toll pathway were also detected in *I. scapularis* after genome comparison with (Liu et al., 2012). Nevertheless, the involvement of Toll pathway in expression of antimicrobial peptides in ticks has been not described as of yet.

Another mechanism affecting vector competence is a competition for nutritional resources between commensal microflora and transmitted pathogens. For instance, *Wolbachia*, the

vertically transmitted intracellular bacteria, are symbionts of 60 % of all insect species. These bacteria have negative effect on mosquito-transmitted pathogens like *flavirus dengue virus* (DENV), *Chikungunya* virus (CHIKV) or *Plasmodium* (Moreira et al., 2009). DENV requires free fatty for its successful replication (Lee et al., 2008). *Wolbachia* completely lacks the genes coding for fatty acid desaturation enzymes and exploits the fatty acid from the host cells. High quantity of *Wolbachia* is able to deplete the fatty acid reserves and thus interrupt the DENV virus proliferation (Sinkins et al., 2013). *Wolbachia* requires cholesterol for its membrane formation. Depletion of cholesterol originating from the meal ingested by insects restricts the lifespan of CHIKV that needs the cholesterol for its successful endosomal fusion (Vancini et al., 2015). *Wolbachia* was firstly announced as common symbiotic bacteria also in ticks (Noda et al., 1997) but later it was demonstrated that detection of *Wolbachia* within ticks was caused by their interaction with tick-specific parasitoid wasp *Ixodiphagus hookeri* (Plantard et al., 2012).

Beside indirect effects on pathogen transmission, the intestinal microbiota exerts direct mechanism based on production of secondary metabolites. Gram negative bacteria (G^-) were reported as the important agents in inhibition of *Plasmodium falciparum* ookinetes within the mosquito guts (Cirimotich et al., 2011). *Enterobacter* sp., as the representative of the G^- bacteria, is marked as the successful non-immunity inducing *Plasmodium* inhibitor with suppressing efficiency about 99 %. These bacteria produce reactive oxygen species in a quantity dependent manner and thus directly inhibit the *Plasmodium* infection (Cirimotich et al., 2011). Metagenomic profiling of the bacterial communities of *I. ricinus* revealed the presence of *Enterobacter* (Carpi et al., 2011) but its role in immunity against tick-borne pathogens remains to be examined.

1.4 Tick-borne pathogens

Among pathogens that can be transmitted by the European hard tick *Ixodes ricinus* belong bacteria from the genus *Borrelia*, the causative agent of Lyme disease (Rizzoli et al., 2011). The guts of *I.ricinus* can harbor also bacteria of the order Rickettsiales, bacteria from the genus *Anaplasma* that causes granulocytic anaplasmosis in both humans and animals (Stuen et al., 2013). Bacterium *Candidatus Neoehrlichia mikurensis*, the genus *Rickettsia*, the bacteria *Francisella tularensis* or *Coxiella burnetii* and protozoan organism of the genus *Babesia* belong among organisms that were detected in *I.ricinus* and their pathogenic effect

on human or animals is either known or their emerging disease potential is considered (Li et al., 2012; Parola et al., 2013; Gray et al., 2010; Socolovschi et al., 2009; Hai et al., 2014). Selected tick-transmitted diseases, pathogens and their tick vectors are listed in the Table 1.

Table 1. Tick-transmitted diseases, pathogens and their vectors*

Disease	Pathogen	Vector
Lyme Borreliosis	*Borrelia burgdorferi* sensu lato (b)	*I. pacificus, I. persulcatus, I. ricinus, I. scapularis*
Rocky mountain spotted fever	*Rickettsia rickettsii* (b)	*Dermacentor andersoni, D. variabilis, Amblyomma cajennense, A. aureolatum, Rhipicephalus sanguineus*
Ehrlichiosis	*Anaplasma phagocytophilum* (b)	*I. pacificus, I. hexagonus, I. ricinus, I. scapularis*
Relapsing fever	*Borrelia* sp. (b)	*I. pacificus, I. persulcatus, I. ricinus, I. scapularis*
Tularemia	*Francisella tularensis* (b)	*D. variabilis*
Meningoencephalitis	TBEV (v)	*I. ricinus*
Colorado tick fever	CTF (v)	*D. andersoni, D. occidentalis, D. albipictus*
Crimean-Congo hemorrhagic fever	CCHF (v)	*Hyalomma* sp.
Babesiosis	*Babesia* spp (p)	*I. scapularis, Haemaphysalis longicornis*

* adapted according to de la Fuente et al., (2008). (b) - bacteria, (v) - viruses, (p) - protozoa

1.4.1 *Borrelia* sp.

Borrelia sp. are bacteria belonging into the phylum of Spirochetes that are not classified as either $Gram^+$ or $Gram^-$.They are causative agents of borreliosis, multisystemic disease that impacts heart, joints or central nervous system (Cook, 2014). Thirty six from overall 37 of known *Borrelia* genera can be transmitted by ticks, out of which 18 species are known to be transmitted by Ixodid ticks (Rudenko et al., 2011). In nature, *Borrelia* spirochetes are maintained by repeated interaction of mice and ticks (Barbour et Fish, 1993) although the transovarial transmission of *Borrelia miyamotoi* species was currently implied (Rollend et al., 2013). One of the factors affecting *Borrelia* acquisition from vertebrate host to tick vector is tick salivary protein Salp25D. This glutathione peroxidase homologue protects the spirochetes by detoxifying detrimental $^{\cdot}OH$ radicals acting on vector-pathogen-host interface (Narasimhan et al., 2007). Following the ingestion, spirochetes adhere to the tick gut via *Borrelia*-specific OspA and OspB proteins and colonize it (Neelakanta et al., 2007). The enhanced efficiency of gut colonization is facilitated by interaction of *Borrelia*

surface protein with tick gut receptors named TROSPA as demonstrated by Pal et al. (2004). After colonization and reproduction, *Borrelia* spirochetes decrease OspA and OspB expression to loosen the *Borrelia*-gut attachment and begin to penetrate the gut wall. In this process, OspC protein expression and BBE31 outer surface protein are required (Schwan et Piesman, 2002; Zhang et al., 2011). *Borrelia* enter the hemolymph and migrate to salivary glands from which they are transmitted into the host during the next feeding process (Benach et al., 1987; Ribeiro et al., 1987; Schwan et Piesman, 2002). For successful colonization and transmission, *Borrelia* must deal with immune systems of animal hosts (Berndtson, 2013). Capability of spirochetes to avoid animal immunity systems (vectors and hosts) involving mechanisms like: (**i**) exploiting tick salivary proteins to delay the immune response (Ramamoorthi et al., 2005); (**ii**) host complement inhibition (Schuijt et al., 2008), (**iii**) host plasminogen capturing (Önder et al., 2012), (**iv**) continuous surface antigen exchange (Zhang et al., 1997), (**v**) enhanced motility (Norris, 2012), (**vi**) advanced chemotaxis for immunity agents evasion (Sze et al., 2012), (**vii**) quorum sensing (Stevenson et Babb, 2002); (**viii**) horizontal gene transfer (Alitalo et al., 2002) or (**ix**) atypical morphology (Miklossy et al., 2008).

1.5 Host complement system

Host complement system constitutes the first antibacterial defense. Antibacterial mechanism is mediated by membrane attack complex formation causing the lysis of bacteria and by opsonization of bacteria with C3b and C4b complements and thus labeling them for phagocytosis by immune cells. Activation of mammalian complement system can be performed through three pathways - classical, alternative and lectin pathway. All pathways are activated upon recognition of specific molecules present on the microbial surface (pathogen-associated molecular patterns, PAMP). After PAMPs recognition the complement is formed that finally lead to the elimination of the microbe (Dunkelberger et Song, 2010)

On the other hand, the microbes developed their own active mechanisms to protect themselves against complement system. Mechanisms of binding C4b protein and thus diminishing bacteria lysis, production of proteases that degrade crucial complement components or mediate C3 component inactivation were described (Blom et al., 2009). *Borrelia* utilize the mechanism of complement system degradation within the tick vector and thus are uninfluenced by its detrimental effect (Rathinavelu et al., 2003).

1.6 Hemoglobin fragments

The pivotal role in microbiota control is performed by antimicrobial peptides derived from ingested hemoglobin. Antibacterial and antifungal peptide was described in the cattle tick *Boophilus microplus* and its origin in bovine α-hemoglobin was subsequently confirmed showing utilization of ingested hemoglobin in defense against bacterial overpopulation in tick guts. This peptide showed considerable anti-Gram$^+$ bacteria and anti-fungal activity (Fogaça et al., 1999). Similar hemoglobin-derived peptides were subsequently described in the gut lumen of *Ornithodoros moubata* and *D. variabilis* (Nakajima et al., 2003; Sonenshine et al., 2005). Antibacterial peptides bind to specific molecules on target membranes, cause lipid displacement and alteration of membrane structure leading to membrane collapse, its fragmentation and physical disruption (Shai, 1999; Zasloff, 2002).

1.7 Tick gut immunity

The absence of digestive proteases, neutral pH and high concentration of nutrients originating from blood would make the tick gut the ideal environment for bacteria proliferation. Therefore ticks evolved mechanisms for regulation of intestinal microbiome at tolerable level (Hajdušek et al., 2013).

1.7.1 Defensins

Defensins are the most spread group of antimicrobial peptides of many organisms including ticks (Johns et al., 2001). These 4 kDa long cationic peptides and their structure is fixed by three disulfide bonds formed between 6 conserved cystein residues (Kopáček et al., 2010). Defensins attack Gram$^+$ bacteria through perforation of phospholipid membranes (Gillespie et al., 1997). Johns et al. (2001) described synergistic effect of defensin and lysosyme in anti-*Borrelia* response. Induction of tick defensins expression in the tick gut was reported to be mediated by pathogen invasion (Nakajima et al., 2002).

1.7.2 Lysozyme

Lysozymes are antibacterial enzymes catalyzing hydrolysis of 1,4-β-D bond between N-acetyl muramic acid and N-acetyl-D-glukosamin residues in the bacterial membrane. This mechanism effectively eliminates G$^+$ bacteria (Bilej et al., 2010). Lysozyme activity against *M. luteus* was confirmed in tick *O. moubata*, where its expression was strongly up-regulated by blood intake suggesting its role in the digestion of ingested bacteria (Kopáček et al., 1999; Grunclová et al., 2003)

1.7.3 Redox balance

Reactive oxygen species (ROS) are chemically reactive oxygen-containing molecules formed as natural by-products of oxygen metabolism during mitochondria respiration and other metabolic processes. They can be divided into oxygen radicals (superoxide, hydroxyl) and nonradicals (hypochlorus acid, hydrogen peroxide). ROS can be generated enzymatically through transportation of electrons across the plasma membrane and thus generate superoxide radicals or other ROS (Bedard et Krause, 2007). Enzymes of such nature belong to nicotinamide adenine dinucleotide phosphate oxidases family (NADPH). ROS produced by NADPH enzymes play a role in antimicrobial defense, biosynthesis, cell signaling or apoptosis triggering (Ha et al., 2005; Oliveira et al., 2012; Apel et Hirt, 2004; Matés et Sánchez-Jiménez, 2000). The temporary ROS production and their proper aiming is required for beneficial functioning (Bae et al., 2010). On the other hand, the oxidizing properties of ROS can cause serious damage in case of excessive concentration. Damage of DNA, lipid peroxidation, amino acids oxidation or enzyme deactivation caused by oxidation, are the most often ROS-inflicted detriments (Covarrubias et al., 2008). Redox balance is the process of maintaining the production of ROS and degradation of ROS in equilibrium. This regulation is essential for normal functioning of redox processes within the cell (Balaban et al., 2005). ROS scavenging is provided by a whole spectrum of peroxidases for reducing hydrogen peroxide to water and superoxide dismutases for regulation of superoxide radicals. These enzymes together with other mechanisms provide the antioxidant defense system of cells (Pigeolet et al., 1990).

1.8 NADPH oxidase (NOX)

Members of NADPH family transport electrons through the membrane into the cytoplasm or across the membrane of inner-cell organels. Electron is transferred from NADPH to oxygen while releasing superoxide radical ($O_2^{\cdot}$) that is used for other ROS formation (Cross et Segal, 2004). All identified members of NADPH family possess conserved structure consisting of NADPH binding site, FAD-binding region, 6 conserved transmembrane domains and 4 conserved histidines that bind heme molecule (Bedard et Krause, 2007). Oliveira et al. (2012) presented the utilizing of superoxide radical in mosquito immunity defense against *Plasmodium*. Heme peroxidase 2 (HPX2) together with *A. gambiae* NADPH oxidase (NOX5) mediate nitration of *Plasmodium* that acts as

opsonization system inducing the mosquito complement system that eliminates the pathogen. Firstly, increased heme peroxidase 2 (HPX2) expression was observed after *Plasmodium* infection and its role in *Plasmodium* nitration was confirmed by HPX2 RNA interference. To perform the antiplasmodial function, hydrogen peroxide is required by HPX2. The augmented expression of NOX5 following the *Plasmodium* infection was detected and thus NOX5 was suggested as the main ROS source. NOX5 gene silencing showed the same effect as HPX2 silencing and the HPX2/NOX5 system was definitely confirmed as the main determinant of *Plasmodium* survival (Oliveira et al., 2012). One ortholog of NOX gene was identified within the *I. scapularis* genome (Pagel Van Zee et al., 2007). Therefore the presence of NOX in *I. ricinus* genome can be assumed. The role of tick NOX remains to be revealed.

1.9 Dual oxidase (DUOX)

Dual oxidase is another transmembrane enzyme of NADPH family described in many organisms ranging from amoeba to human. DUOX enzyme is named dual because of additional domain that is homologous to heme-containing peroxidases as myeloperoxidase or lactoperoxidase (Lambeth et al., 2000; De Deken et al., 2000). DUOX peroxidase domain of insects and *Caenorhabditis elegans* contains histidine at the same position as myeloperoxidase that allows the heme binding and thus typical peroxidase activity (Bae et al., 2010).

DUOX was presented as the enzyme playing a role in variety of physiological events such as stabilization of *D. melanogaster* wing (Anh et al., 2011), thyroid hormone formation (Ohye et Sugawara, 2010), water resistance providing in insect eggs (Dias et al., 2013) and others. DUOX play a very important role in the host defense against microorganisms. DUOX-dependent antimicrobial effect was discovered in mammalian airway epithelial cells. DUOX-generated H_2O_2 is converted by lactoperoxidase and in the presence of pseudohalide thiocyanate to hypothiocyanate, stronger oxidative reagent and antimicrobial agent compared to H_2O_2 (Geiszt et Leto, 2004; Wijkstrom-Frei, 2003; Fisher, 2009). Human bronchial epithelial cells were shown to efficiently kill bacteria including *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Staphylococcus aureus* through ROS production (Rada et al., 2008).

Antimicrobial effect mediated by DUOX was described in *D. melanogaster*. Flies lacking immune-regulated catalase (IRC), enzyme for H_2O_2 decomposition, were unable to cope with infection triggered by non-lethal bacteria and showed high mortality (Ha et al., 2005). This mortality was explained by ROS excess caused by IRC absence. ROS as the main agent of microbe elimination were confirmed and DUOX was marked as their main source after DUOX RNA interference showing inability of flies to control the intestinal bacteria proliferation (Ha et al., 2005). The determining factors for induction of DUOX-mediated pathogen elimination are membrane surface molecules of microbes. Recognizing of these molecules triggers the ROS production in a dose-dependent manner (Bae et al., 2010; Ha et al., 2005). Modulation of DUOX enzymatic activity is mediated through the pathway including inositol (1,4,5)-triphosphate mobilizing intracellular Ca^{2+} that activate ROS production via interaction with DUOX EF-hand (Ameziane-El-Hassani, 2005). DUOX enzyme was shown to be pivotal in pathogen elimination but simultaneously tolerates symbiotic microflora.

Oxidative killing of bacteria was reported even for mosquito vector *Aedes aegypti*. Robust ROS production was detected in sugar-fed mosquitoes but it dramatically decreased after blood ingestion idicating the role of heme. Adding heme into the sugar diet showed ROS decrease in a dose-dependent manner implying how mosquitoes protects themselves against highly cytotoxic radicals formed in presence of heme (Oliveira et al., 2011; Balla et al., 2007). The ROS production was attributed to DUOX as RNA interference-mediated DUOX silencing resulted in considerable ROS decrease that resulted in augmented bacteria proliferation (Oliveira et al., 2011). Inverse correlation was observed between the ROS level and bacteria proliferation. Heme presence caused considerable ROS decrease that reciprocally created favorable environment for microbe proliferation (Oliveira et al., 2011).

Role of DUOX in determination of vector competence for pathogen was elucidated in the mosquito *Anopheles gambiae*. In response to blood feeding, the formation of peritrophic matrix (PM) was observed (Kumar et al., 2010). This semi-permeable layer surrounds the blood meal and prevents the direct contact of blood with gut epithelium (Devenport et al., 2004). The PM formation is mediated by immunomodulatory peroxidase (IMPer) and DUOX that creates covalent bonds between tyrosine residues of proteins involved in PM production (Edens et al., 2001). The created PM barrier limits the permeability of immune elicitors into the PM-surrounded blood bolus and thus creates appropriate environment for *Plasmodium* survival. This mechanism makes mosquitoes more susceptible to *Plasmodium*

but simultaneously protect the mosquitoes and their commensal bacteria against deleterious effect of alternatively overproduced ROS (Kumar et al., 2010). Presence of PM was even described in the tick *I. scapularis* (Yang et al., 2014). The role of DUOX in tick PM formation was assessed by DUOX knockdown. RNA interference of DUOX caused inability of ticks to form PM and additionally activated specific tick innate immunity pathway genes. Absence of protective PM and up-regulation of immune response significantly impacted *B. burgdorferi* persistence within the tick vector (Yang et al., 2014).

2 Aims of work

1. To describe the impact of hemoglobin and the host complement components on intestinal microbiota proliferation using artificially-fed adult *I. ricinus* females.

2. Quantitative determination of spirochete load and numbers of intestinal bacteria in *B. afzelii* (CB43) infected adult ticks *I. ricinus* depending on diet composition.

3. Functional characterization of DUOX in adult female ticks *I. ricinus* using RNA interference.

4. Optimization of *in vitro* feeding for nymphs *I. ricinus*.

3 Material and methods

Table 2. Material used for work

DNA/RNA electrophoresis, PCR	
1× TAE buffer	40mM Tris-acetate, 1mM EDTA, pH 8.0
DEPC H_2O	0.1% (v/v) diethylpyrocarbonate diluted in distilled water
EtBr	Ethidium bromide 0.5 µg/ml
6× Loading Dye (Fermentas)	10mM Tris/HCl (pH 7.6), 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylencyanol, 60% (v/v) glycerol, 60mM EDTA
DNA Ladder	GeneRuler™ 100bp DNA Ladder (MBI Fermentas)
RNA Ladder	High Range RNA Ladder (MBI Fermentas)
PCR Master mix	FastStart PCR Master (Roche)
Media and chemicals for bacteria cultivation	
SOC medium	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5mM KCl, 10mM MgSO4, 20mM glucose, pH 7.0, sterile
LB (Luria-Bertani) medium	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0, sterile
Barbour-Stoenner-Kelly H medium	BSK-H complete medium (Sigma)
fungicide	Amphotericin (Sigma) 0.25% (w/v)
Artificial feeding	
Silicone	Wacker silicone E4
Silicone oil	FLUKA DC 200 silicone oil
Colour paste	Wacker FL colour paste
Hexane	n-Hexane (VWR chemicals)
Dichloromethane	Emparta® ACS Dichloromethane for analysis (MERCK)
Diet supplements	
gentamicin	0.5% (w/v) gentamicin (Sigma)
glucose	0.2% (w/v) glucose (Sigma)
ATP	10mM ATP (FLUKA), sterile, filtered (0.2 µm filter)
NaCl	0.9% (w/v) NaCl
Dissection	
Sterile PBS	8% (w/v) NaCl, 0,2% (w/v) KCl, 1.8% (w/v) Na_2HPO_4, 0.14% (w/v) KH_2PO_4 in 1000 ml distilled H_2O + 0.1% (v/v) diethyl pyrocarbonate in distilled H_2O, pH = 7, autoclaved
qPCR, chemistry	
qPCR Master mix	LightCycler® 480 Probes Master
RT-qPCR Master mix	FastStart SYBR Green Master

Table 3. Oligonucleotides used in experiments

TaqMan probes	
16S rRNA gene probe	CGTATTACCGCGGCTGCTGGCAC
IR actin gene probe	CCATCCAGGCTGTGCTCTC
flagellin gene probe	TGCTACAACCTCATCTGTCATTGTAGCATCTTTTATTTG
Used primers	
duox F/R (RNAi)	*duox* F: ATGGGCCCACTACGACGTCAAG *duox* R: ATTCTAGATCTCGAAGGACCGCATCGTG
ir-duox F1/R1 (RT-qPCR)	*ir-duox* F1: CCAACAACTTGCACGAAGG *ir-duox* R1: GGCGATCTCGAACTTGTACC
16S rRNA F/R	16S rRNA F: TCCTACGGGAGGCAGCAGT 16S rRNA R: GGACTACCAGGGTATCTAATCCTGTT
ir actin F/R	*ir actin* F: GATCATGTTCGAGACCTTCA *ir actin* R: CGATACCCGTGGTACGA
Elongation factor (EF) F/R	EF F: ACGAGGCTCTGACGGAAG EF R: CACGACGCAACTCCTTCAC
FlaF1/R1	FlaF1: AAGCAAATTTAGGTGCTTTCCAA FlaR1: GCAATCATTGCCATTGCAGA

3.1 Ticks rearing and treatment

Ticks used for all performed experiments were either collected by flagging from nature around České Budějovice or originated from tick colonies of the Institute of Parasitology (Czech Republic), where they were kept at 25 °C and 96% humidity. The tick bodies and hypostomes were treated with 3% H_2O_2 before every experiment.

3.2 Preparation of *Borrelia*-infected adult females ticks

Six weeks old female C3H/HeN mice (Jackson Laboratory, Sulzfeld, Germany) were infected by subcutaneous injections of 10^5 *Borrelia afzelii* (CB43) in 100 µl of BSK-H medium per mouse. Presence of spirochetes in ear biopsies was determined at weekly intervals by PCR. Total DNA was extracted using a NucleoSpin Tissue Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Detection of spirochetes was performed by qPCR (see qPCR). Probe and primers sequence are listed in the Table 3. Three weeks after the infection the presence of spirochetes was confirmed in all ear biopsy samples. At the 4th week after inoculation, non-infected nymphs were fed on infected mice until repletion (40 nymphs per mouse) and left to molt into adults. The

infection rate in adults was determined in 10 individual adult females using PCR with specific primers (Table 3). Infection rate of adult ticks was set to 40 %.

3.3 *In vitro* feeding

3.3.1 Animal hairs extract preparation

250 ml of dichloromethane (DCM) was added to 50 g of cut animal hair and lipid compounds were extracted by constant shaking at room temperature for 20 min. The half volume was collected, replaced with 100 ml of fresh DCM and shook for additional 20 min. The process was repeated one more time. All collected solutions were combined and solid parts were disposed by filtration. The concentration of animal odour extract was assessed by weighing the dry matter in 1 ml of the extract.

3.3.2 Feeding unit preparation

Feeding unit (FU) preparation was based on the published *in vitro* feeding assay protocol (Kröber and Guerin, 2007); except the exchange of glass FU to plastic FU (Miroslav Kubík - PLEXI, Lidická tř. 1121/222, České Budějovice 7). To prepare 10 membranes 15 g of the silicone, 0.15 g of the colour paste, 5 g of the silicone oil and 2.9 g of hexane were mixed in a plastic bowl to create a homogenous mixture. The upper edges of lens cleaning papers (Tiffen, Kodak) were glued to the smooth glass board. The silicone mixture was spread over the cleaning papers with the silicone spatula to the thin film and prepared membranes were let to dry overnight. The membrane thickness was measured with a micrometer on 4 different places and only those with 60 – 100 µm thickness were used for FU completion. The thin silicone layer was applied onto the FU bottom rim; FU were subsequently glued to the membranes. Membranes were scissors-shaped to adjust the FU form. For tick attachment support the circular grids were stuck to the FU bottom and the plastic tile spacers were glued to the grid center (Fig. 1). Before tick application, the FU membranes were perfumed with 640 ng cow scent per FU.

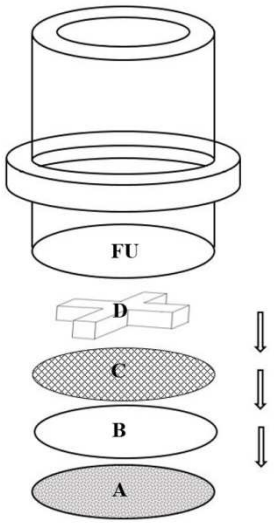

Figure 1. Feeding unit composition. FU - feeding unit, A - paper matrix, B - silicone layer, C - grid, D - plastic tile spacer

3.3.3 Diet preparation

Bovine blood (BL) was gained from the local slaughterhouse. It was manually defibrinated, filtered through a UV-steriled insect plastic mesh and supplemented with glucose to final concentration 0.2 % (w/v).

Serum (S) was prepared by blood centrifugation at 2 500 × g for 10 min in 4 °C with slow deceleration, serum phase collection and additional centrifugation at 10 000 × g for 10 min in 4 °C. Serum was also glucose-supplemented to the final concentration 0.2 % (w/v). Preparation of heat inactivated serum (HIS) was prepared the same way as the active serum, but before glucose supplementation, it was heated on 56 °C for 30 min on a water bath to inactivate the complement components. Prepared diets were stored at 4 °C.

Diets were applied in a six-well plate and, before the FU immersing, they were additionally supplemented with ATP and gentamicin if necessary. The diet (BL, S, HIS) volume of 3.1 ml was pipetted into each well and subsequently enriched with ATP to 1 mM final concentration. Diets prepared this way were used to feed ticks for subsequent quantification of the bacteria loads. Diets determined for other experiments were additionally supplemented with gentamicin to a final concentration 5 µg/ml.

3.3.4 Tick application

Fifteen female adult ticks were placed per one FU, diet was applied (see Diet preparation) and after 24 hours long adaptation in water bath, in temperature ranging from 30 to 35 °C, the non-attached females were taken out and the equal number of male tick was put inside each FU to ensure mating and full engorgement of the females. Dead male ticks were taking out within the duration of the experiment. Ticks for analysis have been forcibly removed from membrane in specified time points during the feeding and further processed.

3.4 *Borrelia* cultivation

Serum and HIS were prepared as mentioned above (see Diet preparation). For control of *Borrelia* proliferation, the Barbour-Stoenner-Kelly H medium (BSK-H) supplemented with fungicide (Table 1) was used. *B. afzelii* (strain CB43) (10^7 genomes/ml) was inoculated into 10 ml of BSK-H medium and subsequently incubated at 34 °C for a week. 100 µl of *Borrelia* (10^7 genomes/ml) cultivated in BSK-H medium was added into 100 µl of bovine active serum and into 100 µl of HIS and incubated at 34 °C for 22 hours. *Borrelia* development and distribution were assessed by light microscopy (400× magnification) in the dark field.

3.5 Genomic DNA (gDNA) extraction

Ticks were dissected under the stereo microscope with LED illumination (Stemi DV4, Zeiss), their whole guts were extracted into the Buffer T1 from NucleoSpin® Tissue kit (Macherey-Nagel) and gDNA was extracted according to the supplier`s instruction from the mentioned kit. Samples of tick feces were collected from blood-fed and serum-fed ticks and gDNA was isolated following the NucleoSpin®Blood kit protocol (Macherey-Nagel). Additional feces samples with 10^4 *E.coli* spike was prepared to verify the reliability of the isolation. Samples of diets (200 µl) were collected in six hours long intervals from each diet. gDNA from these samples was extracted by following the NucleoSpin®Blood kit (Macherey-Nagel) protocol. gDNA samples served as templates for qPCR analyses (see qPCR).

3.6 Total RNA isolation, complementary DNA (cDNA) synthesis

Whole tick midguts, salivary glands, ovaries and tracheas were dissected using binocular microscope (Stemi DV4, Zeiss) into sterile PBS (Table 2). Tissues from five ticks were pooled and homogenized in RA1 buffer (NucleoSpin® RNA kit, Macherey-Nagel) using an insulin syringe (29G). Total RNA was extracted using a NucleoSpin® RNA kit (Macherey-Nagel) according to the manufacturer's protocol and its concentration was measured by spectrophotometer (NanoDrop™ 1000). The quality of obtained RNA was verified on 1% agarose gel, where 100 ng of each RNA sample was loaded into the well. Transcriptor High Fidelity cDNA Synthesis Kit (Roche) was used for cDNA synthesis from 100 ng of prepared total RNA. cDNA samples served as templates for RT-qPCR analyses (see RT-qPCR).

3.7 Quantitative polymerase chain reaction (qPCR)

3.7.1 *I. ricinus actin* (*ir actin*) standard curve

Ten nymph guts were dissected, pooled and the gDNA was extracted following the NucleoSpin® Tissue (Macherey-Nagel) protocol. Prepared gDNA sample was diluted in a decimal row and the set of standard samples was prepared. Each sample was qPCR analyzed in technical triplicate using specific *IR actin* probe and primers (Table 3). Standard samples were stored at -20 °C and served as calibrator samples in future qPCR analyses.

3.7.2 *E. coli* standard curve

E. coli (One Shot® T10 Chemically Competent *E.coli*, Invitrogen) bacterial suspension (25 µl) was spread over the LB media plates supplemented with ampicilin (100 µg/ml) and incubated for 12 h at 37 °C. A single colony was picked up and incubated in 4 ml liquid LB medium with ampicilin in 37 °C to the optical density $OD_{600} = 1$, corresponding to ~10^9 genomes. One milliliter of the suspension was used for gDNA isolation following the NucleoSpin® Tissue kit (Macherey-Nagel) manufacturer`s protocol. The standard curve was prepared as described above using 16S rRNA gene specific probe and primers (Table 3).

3.7.3 Bacteria and *Borrelia* load quantification

For bacteria and *Borrelia* quantification, 5 gut samples from each time point of the feeding process were used for independent gDNA isolations and subsequent qPCR analyses. The detection of spirochetes was performed by amplification of 154bp long fragment of *flagellin* gene using *flagellin*-specific primers and probe (Table 3). Detection of bacteria load was performed by amplification of 466bp fragment of 16S rRNA gene using gene specific probe and primers (Table 3). Bacteria and *Borrelia* numbers were normalized to the *ir actin* gene concentration.

3.8 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

3.8.1 Quantification of gene expression

Gene transcript levels were quantified from 20-fold diluted cDNA templates in ratio of 2 µl per 25 µl PCR mixture using LightCycler® 480 SYBR Green I Master in a LightCycler® 480 System qPCR machine (Roche). Each sample was analyzed in technical triplicate. Used primers for gene amplification are listed in Table 2. Relative values were normalized to the PCR amplification of the cDNA for *I. ricinus* elongation factor 1α (EF) and the data were expressed as a relative expression to the sample with the highest expression levels (100 %).

3.9 dsRNA synthesis

The pair of *I. ricinus* dual oxidase (*ir-duox*) gene specific primers for PCR was designed according to the *duox* sequence of *I. scapularis* (VectorBase: ISCW007865). Designed primers primers had *ApaI* and *XbaI* restriction sites attached to them, listed in Table 3. A 339 bp-long fragment of *I. ricinus* dual *ir-duox* was amplified using ovarian cDNA of partially fed ticks as a template and prepared *ir-duox* primers, verified on 1% agarose gel and purified with Agarose Gel Extraction kit (Roche) according to supplier`s protocol. The reaction for the subsequent PCR restriction was mixed (Table 4.) and incubated at 37 °C for 2 hours.

Table 4. Purified PCR product restriction

	µl
Buffer TANGO 10×	3
ApaI, *XbaI* enzyme	1 each
PCR product	20
H_2O	6

Restricted PCR product was purified following the GenElute™ PCR Clean-Up kit protocol and subsequently cloned into the pll10 plasmid. Ligation reaction is indicated in Table 5. The reaction was incubated at 16 °C overnight.

Table 5. Ligation of restricted PCR product

	µl
Buffer 2×	5
Restricted pll10 plasmid	2
Restricted PCR product	2
T4 ligase	1

The whole reaction of plasmid with insert was transformed into 25 µl of One Shot® T10 Chemically Competent *E.coli* (Invitrogen), held 30 min on ice and after 60sec heat shock at 42 °C, 300 µl of SOC medium was added and the mixture was shaken for 90 min at 37 °C. After that, the mixture was spread on ampicilin supplemented (100 µg/ml) LB (LB-amp) plates and incubated at 37 °C overnight. 20 colonies of *E.coli* were transferred onto fresh LB-amp plates and the presence of insert was verified by colony PCR using M13 forward and reverse primers from TOPO cloning kit (Invitrogen). Positive *E.coli* colony was inoculated into 100 ml of liquid LB-amp and shaken at 37 °C for 15 hours. Plasmids were subsequently extracted according to NucleoBond® Xtra Midi / Maxi kit (Macherey-Nagel) protocol and isolated plasmids were restricted as displayed in Table 6. Two separate reactions were prepared for *ApaI* and *XbaI* restricted plasmids and were processed separately to create sense and antisense single strand RNA (ssRNA).

Table 6. Restriction of purified plasmid

	µl
10 µg of plasmid	3
Buffer TANGO 10×	5
ApaI / Xba I	6
H_2O	36

Subsequent purification of linearized plasmid was performed by adding 150 µl 10 mM Tris-HCl (pH=8) supplemented with 25 µl of proteinase K (25 µg/µl), 2mM $CaCl_2$ and 3.75 µl 10% sodium dodecyl sulfate. The mixture was incubated at 50 °C for 30 min. After adding of 80 µl of phenol-chloroform (Sigma), vortexing and centrifugation (15 000 × g for 5 min), the aqueous phase was collected into the fresh tube and 80 µl of chloroform was added into it, the mixture was vortexed and centrifuged (15 000 × g for 5 min) again. The aqueous phase was one more time collected, supplemented with 56 µl of isopropanol and vigorously vortexed. The reaction was placed into -20°C for 15 min and then centrifuged for 30 min at 15 000 × g. Supernatant was discarded, pellet was washed with 80% ethanol, mixture was centrifuged for 10 min/ 15 000 × g and the rest of the ethanol was left to evaporate. The pellet was diluted in 20 µl of DEPC H2O and the concentration was measured with spectrophotometer (NanoDrop™ 1000). ssRNAs were isolated using MEGAscript® RNAi Kit (TURBO DNA-free™ Kit, Ambion) according to the manufacturer`s protocol. ssRNAs were purified by adding 1 µl of DNAse (Ambion) and incubated at 37 °C for 15 min. Additionally 115 µl of RNAse free H_2O, 15 µl of ammonium acetate and 150 µl of phenol-chloroform (Sigma) was added. The mixture was vortexed, centrifuged 5 min / 15 000 × g and the aqueous phase was collected into the fresh tube. 150 µl of chloroform was added into it, the mixture was vortexed and centrifuged (15 000 × g for 5 min) again. The aqueous phase was one more time collected, supplemented with 110 µl of isopropanol and vigorously vortexed. The reaction was placed into -20 °C for 15 min and then centrifuged for 30 min/ 15 000 × g. The pellet was diluted in 20 µl of DEPC H_2O and the concentration was measured by spectrophotometer (NanoDrop™ 1000). Sense and antisense ssRNAs were mixed in the ratio 1:1 to final concentration 3 µg/µl. The mixture was placed into a cylinder filled with hot water and allowed to cool slowly at room temperature for 12 hours. The quality of double stranded RNA (dsRNA) was tested on 1% agarose gel.

3.10 Silencing gene expression by RNA interference (RNAi)

Unfed female ticks were stuck to double-sticky tape. dsRNA (0.5 µl, 3 µg/µl) was injected into hemocoel using a micromanipulator (Narishige). Control ticks were injected with the same volume of *gfp* dsRNA synthesized under the same conditions from linearized plasmid pll6 (Hajdušek et al., 2009). Both group (30 ticks per group) rested 96 h in a humid chamber (96 % of humidity) at room temperature. Group of ticks was dissected for individual tissues, total RNA was extracted from the collected samples and cDNA was prepared. Prepared cDNA samples were qRT-PCR analyzed using SYBR Green dye and specific *ir-duox* primers (Table 3). Second group of ticks was dissected for guts that were used for gDNA isolation and subsequent bacteria/*Borrelia* quantification.

Ir-duox dsRNA treated ticks and the control gfp dsRNA treated tick were put to feed on guinea pigs (15 females + 15 males per animal). Ticks were allowed to fully engorge until repletion, weighed, photographed and placed into separate vials to assess ensuing oviposition. Clutches were taken out and weighed.

3.11 Statistics

Data were analysed using an unpaired t-test with the significance level $P < 0.05$. For bar graphs, means ± SEM are used. All statistics were performed using GraphPad Prism 6 for Windows, version 6.04.

4 Results

4.1 Impact of dietary hemoglobin on bacteria levels in the tick gut

To assess the dynamics of tick gut microbiota in dependence on diet composition during the feeding process, an *in vitro* membrane feeding experiments were performed. To verify the antimicrobial effect of hemoglobin, two different diets, namely whole bovine blood and bovine serum, were prepared and served to ticks. DNA extracts obtained from whole guts of ticks fed for various time periods were used for quantitative PCR analyses using 16S specific probe. Additionally, the bacterial levels in the served diets and the numbers of bacteria excreted from the body were assessed. Substantially increasing intestinal microflora levels were observed in ticks fed on serum during the whole feeding period whereas profound decrease in bacterial counts at the late stage of feeding process was demonstrated in ticks fed on whole blood (Fig. 2 A, B). Considerable amount of bacteria was detected in the diet samples despite the antibiotic presence. On the contrary, only few bacteria were detected in tick feces (Fig. 2 C, D). Sample of feces with known concentration of *E.coli* added was used as a control of isolation (not shown). These observations may imply that host hemoglobin plays a role in regulating levels of intestinal microflora in the guts of *I. ricinus.*

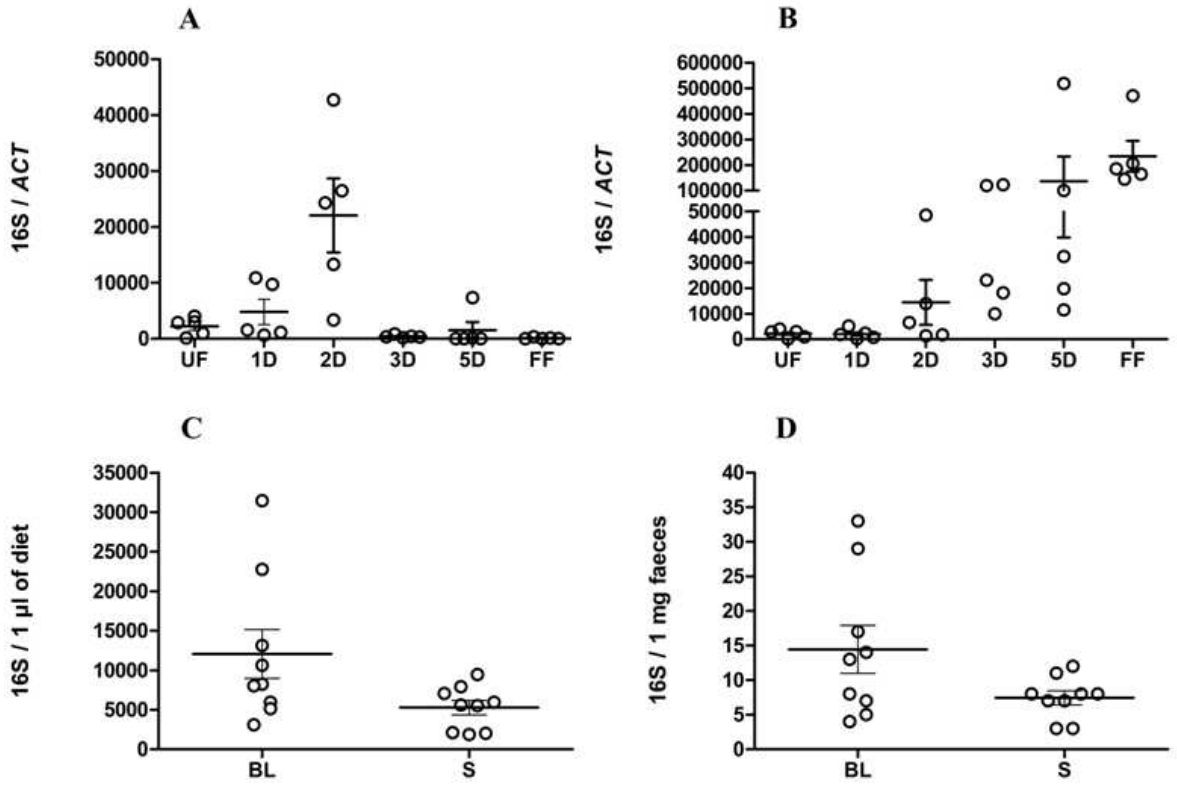

Figure 2. Influence of hemoglobin on bacterial load in tick guts, feces and served diets. Bacterial quantity was evaluated with qPCR and 16S specific probe. Each spot represents value of one biological sample obtained as the mean from technical triplicate. Standard errors of mean are shown. *UF* - unfed ticks, *1D*, *2D*, *3D*, *5D* - days of feeding, *FF* - fully fed ticks, *BL* - blood, *S* - serum. (**A**) qPCR quantification of bacteria load in tick guts when fed on whole blood. (**B**) qPCR quantification of bacteria load in tick guts when fed on serum. (**C**) qPCR quantification of bacterial load in served diets. (**D**) qPCR quantification of bacterial load in tick feces fed on different diets.

4.2 *In vitro* effect of the host complement system on *B. afzelii* development

The effect of serum with active complement and HIS on bacterial development was previously described (Melching and Vas, 1971). To verify the borreliacidal effect of complement system on *B. afzelii* development and distribution, bovine serum and HIS were tested in their *in vitro* cultivation. The effect of different media was examined by light microscopy. It was repeatedly observed, that serum with active complement system caused profound *B. afzelii* clustering, whereas spirochetes cultivated in HIS displayed no or only limited clump formations (Fig. 3). This shows that host complement system may also determine *Borrelia* viability in the tick gut *in vivo*.

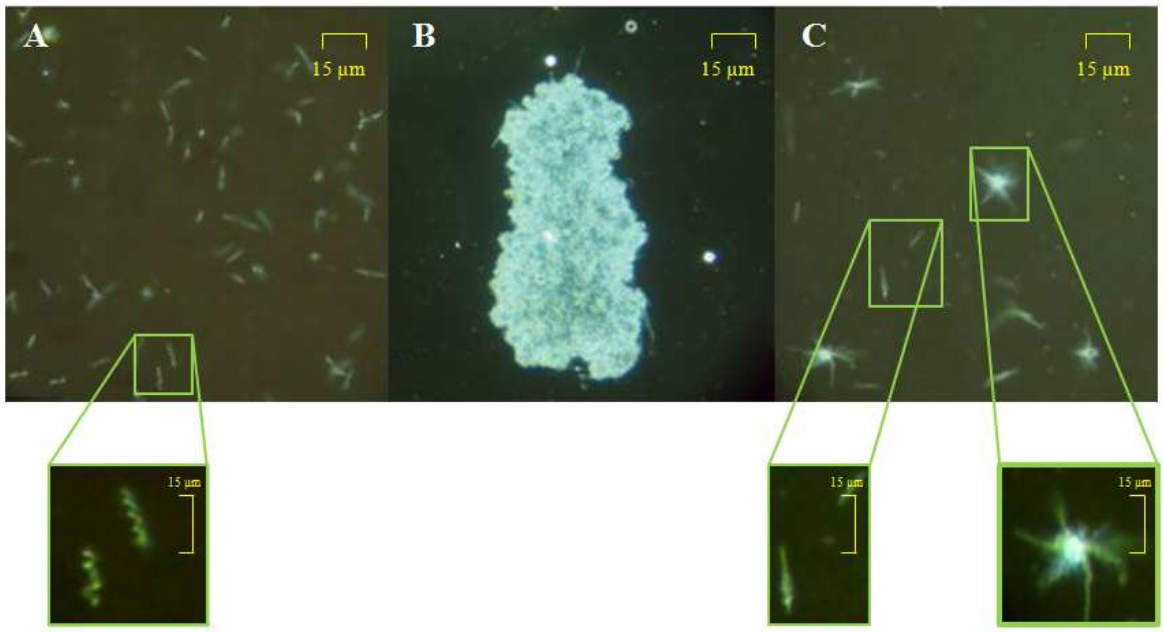

Figure 3. The effect of different media on *B. afzelii* cultivation. Light microscopy imaging of *B. afzelii* cultivation in (**A**) cultivation medium, (**B**) active serum, (**C**) HIS serum.

4.3 Impact of dietary components on intestinal microflora levels of *B. afzelii* infected ticks *in vivo*

For more profound assessment of influence of dietary components on tick gut microbiota development, and its effect on *B. afzelii* levels in the female ticks were tested. First, uninfected *I. ricinus* nymphs were fed *in vivo* on infected mice. Fully fed nymphs were allowed to molt into the adults, where the 40% infection rate was detected. To extend the understanding of the effect of hemoglobin and host complement system on the gut microbiota of *Borrelia*-infected ticks, the *in vitro* membrane feeding experiment was performed with bovine blood, serum and HIS. The influence of various diets on gut microbiota proliferation, *B. afzelii* proliferation and their mutual relationship during the feeding process was assessed by qPCR quantification. DNA extracts from whole guts were tested for bacteria and *Borrelia* presence using qPCR quantification with 16S and *Borrelia* specific probes, respectively. Intestinal bacterial dynamics analysed in *B. afzelii* infected ticks showed a similar course for blood and serum fed ticks as in the previously performed experiment (Fig. 4 B, D; Fig. 2 A, B). No apparent quantitative difference in the symbiotic bacteria was observed in ticks fed on serum and HIS (Fig. 4 D, F). Under set experimental conditions, the increasing *Borrelia* numbers were observed for all groups of ticks fed on different diets but the dynamics differed. Considering the blood fed ticks, the low *Borrelia*

numbers were detected in the middle of the feeding process. By contrast, in active serum-fed and HIS-fed ticks, the *Borrelia* numbers were peaking the 3rd day of feeding (Fig. 4 A, C, E). At the late phase of feeding process, numbers of *Borrelia* considerably increased in blood-fed ticks, whereas the opposite was observed in serum-fed and HIS-fed ticks. Based on the obtained data we suggested that bacteria are sensitive to hemoglobin-derived peptides whereas *Borrelia* may seem to require host hemoglobin to successfully proliferate. Next, we implied that host complement most likely does not contribute to tick midgut immunity. Additionally, we observed a correlation between symbiotic microflora numbers and pathogen counts. *Borrelia* quantity in blood-fed ticks increased at the moment, when the symbiotic intestinal microflora dropped and vice versa, pathogen numbers lowered when the symbiotic microbiota increased. The reason for the reciprocal relationship between pathogen and tick symbiotic microbiota remains unclear.

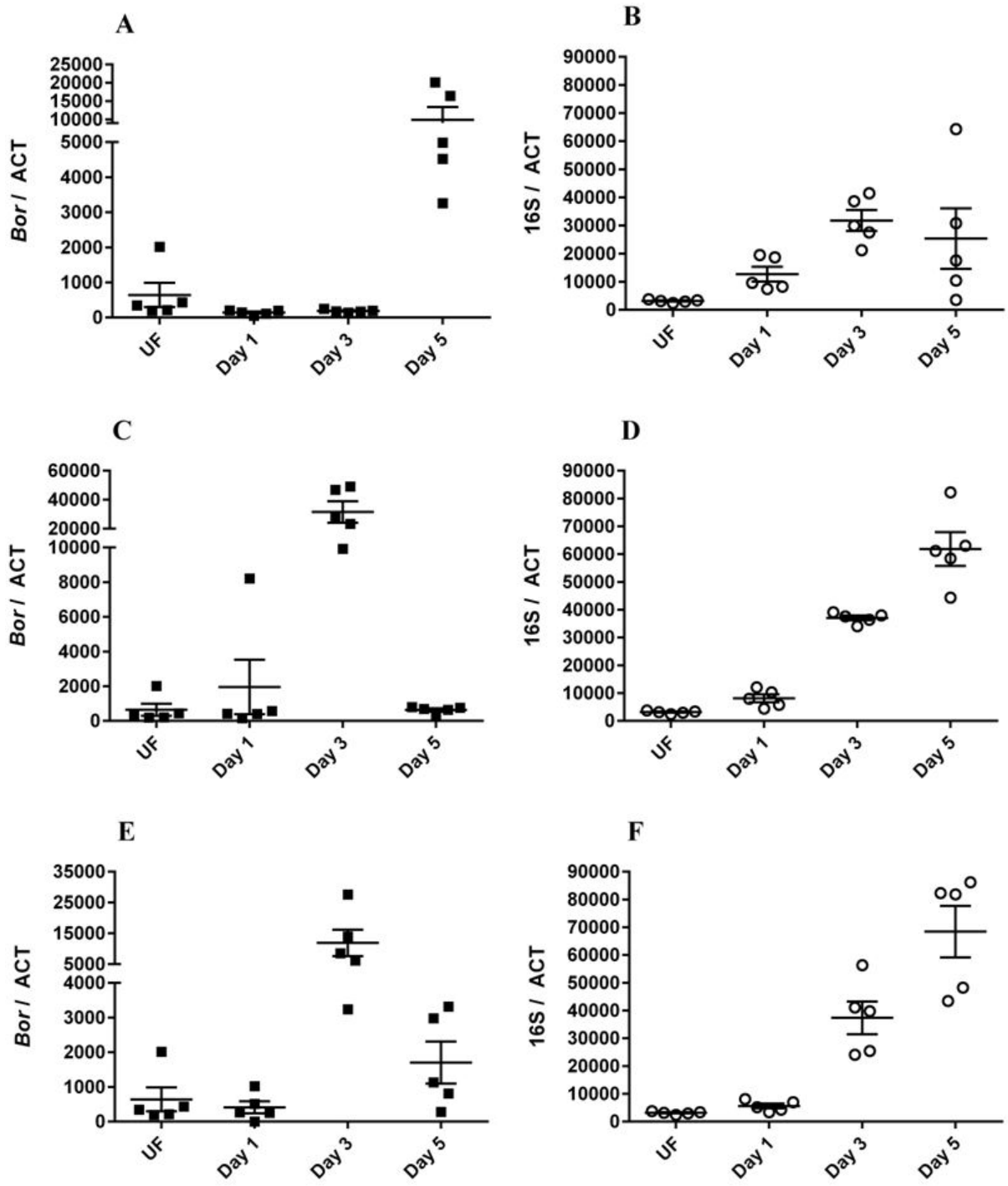

Figure 4. Influence of the different diets on bacteria/*Borrelia* load in guts of infected ticks. Bacteria (**B**,**D**,**F**) and *Borrelia* (**A**,**C**,**E**) quantification in guts of ticks fed on whole blood (**A**,**B**), serum (**C**,**D**), HIS (**E**,**F**) was performed using qPCR with 16S and *Borrelia* specific probes. The values shown indicate biological replicates. *UF* - unfed ticks, *1D*, *3D*, *5D* - days of feeding, (**A**) qPCR quantification of *Borrelia* load in tick guts when fed on whole blood. (**B**) qPCR quantification of intestinal bacteria load in tick guts when fed on whole blood. (**C**) qPCR quantification of *Borrelia* load in tick guts when fed on active serum. (**D**) qPCR quantification of intestinal bacteria load in tick guts when fed on active serum. (**E**) qPCR quantification of *Borrelia* load in tick guts when fed on HIS. (**F**) qPCR quantification of intestinal bacteria load in tick guts when fed on HIS.

4.4 Assessment of *ir-duox* in unfed ticks by RNAi

Dual oxidase (*duox*), one member of NADPH oxidases, was described as the main factor of epithelial immunity in guts of model organism *Drosophila melanogaster* (Ha et al., 2005). The gene coding for *duox* was identified in *I. scapularis* genome (IscW_ISCW007865) and primers designed according to *is-duox* sequence were used to identify the *duox* ortholog in *I. ricinus* (*ir-duox*). The full sequence of *ir-duox* was determined and transcription expression profiles within *I. ricinus* tissues were described in my bachelor thesis (Kučera M, 2012).

Function of *ir-duox* in tick gut microbiota regulation was assessed using *ir-duox* silencing by RNAi. dsRNA was synthesized and its quality verified by agarose gel electrophoresis (Fig. 5). After RNA quality assessment (Fig. 6 A, B), cDNA sets were prepared and reference transcript levels verified (Fig. 6 C). Substantial *ir-duox* silencing was observed in all examined tissues. RNAi efficiency ranged from 80 to 99.9 % for particular tissues in comparison to *gfp* dsRNA-injected control group (Fig. 7 A, B, C, D).

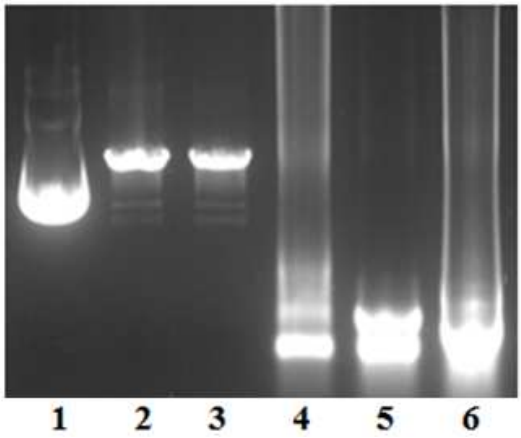

Figure 5. Control of *ir-duox* dsRNA quality. 1 - nonlinearized PLL plasmid with cloned *ir-duox* insert, 2 - *APAI* linearized PLL 10 plasmid, 3 - *XBAI* linearized plasmid, 4 - ssRNA sense 5 - ssRNA antisense, 6 - dsRNA of *ir-duox*.

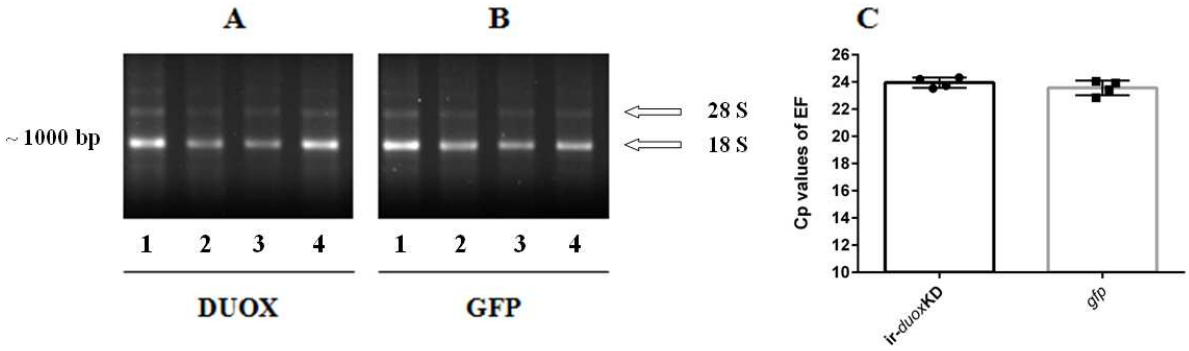

Figure 6. The control of the quality of isolated total RNA. All prepared total RNA samples were controlled on 1% agarose gel. 100 ng of each RNA was put on the gel. Each sample represents the pool of five tissues. Total RNA from *1* - gut, *2* - salivary glands, *3* - ovaries, *4* - trachea of (**A**) *ir-duox* dsRNA-injected ticks, (**B**) *gfp* dsRNA-injected ticks, (**C**) Verification of stable expressional level of the reference gene, *elongation factor* 1α (EF) in dsRNA-injected and *gfp* dsRNA-injected ticks.

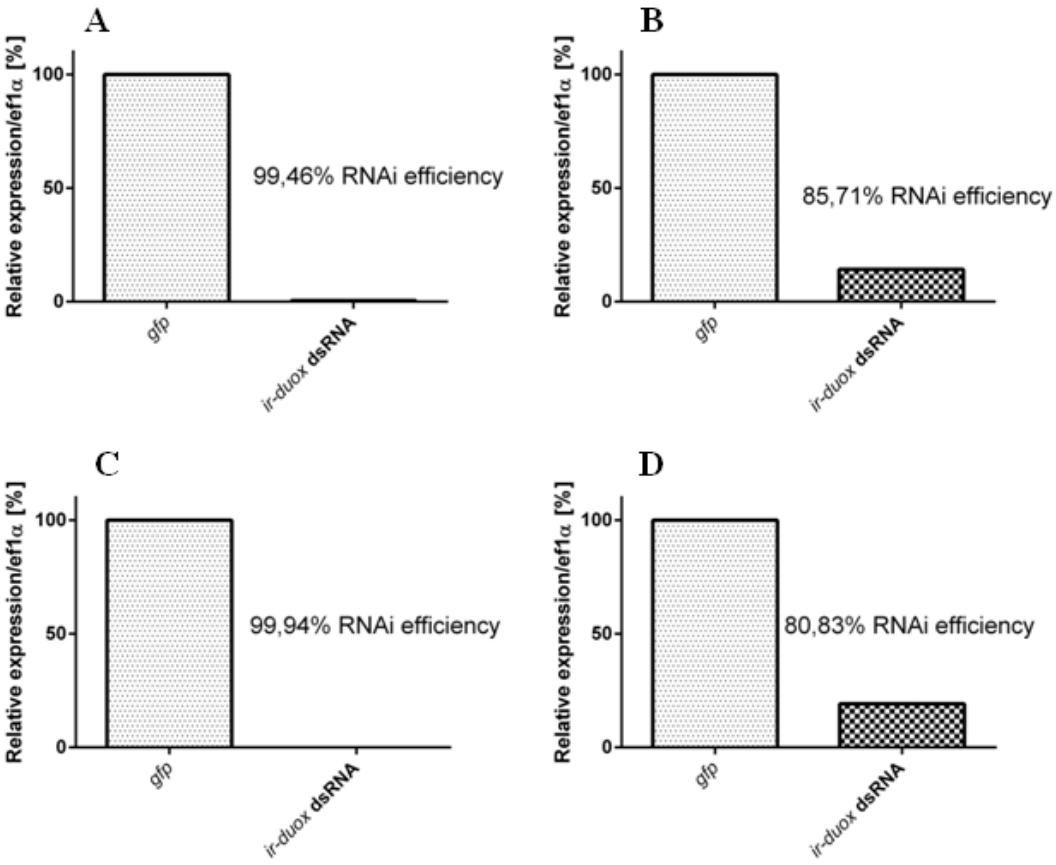

Figure 7. RT-qPCR verification of *ir-duox* RNAi effect in different tissues of unfed ticks. (**A**) in the guts, (**B**) in the salivary glands, (**C**) in the ovaries, (**D**) in the trachea. RT-qPCR was performed on tissue samples prepared by pooling of one tissue from five ticks together.

4.5 The effect of *Ir*DUOX RNAi on intestinal microbiota dynamics in unfed ticks

To evaluate the effect of *Ir*DUOX RNAi on the intestinal microbiota development, 9 unfed *ir-duox* dsRNA injected ticks, 9 *gfp* dsRNA injected ticks and 9 untreated ticks were dissected, their guts were extracted and total RNA was isolated from three pools of three dissected guts. Subsequently the cDNAs from these samples were prepared and tested for bacterial presence using qPCR and 16S specific probe (Fig. 8). The bacterial load in *ir-duox* KD ticks was significantly higher compared to the *gfp* control group and untreated group. This result confirms that *Ir*DUOX may play a role in the microbiota levels regulation within the tick gut. The necessity of *Ir*DUOX expression and the gut bacteria overpopulation impact on tick development was further examined.

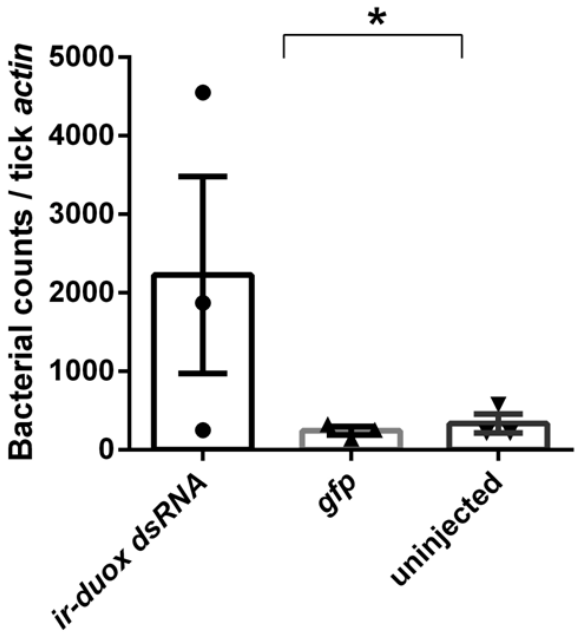

Figure 8. *Ir*DUOX RNAi effect on intestinal microbiota development in unfed *I. ricinus* females. Each dot presents the sample prepared by pooling of three guts. The standard errors of mean are shown. Asterisk indicate statistically significant difference (* - P<0,05).

4.6 *Ir-duox* RNAi impact on tick development

The impact of *Ir*DUOX RNAi-mediated silencing on tick engorgement and clutch sizes was assessed. *Ir-duox* dsRNA-injected ticks were allowed to feed naturally on guinea pigs until repletion. Afterwards, the engorged females were weighed and allowed to lay eggs. The egg clutches were weighed subsequently. *Gfp* ticks reach 83mg weight whereas *duox*-silenced ticks reached 15mg weight. Additionally, *ir-duox* dsRNA-injected ticks had darker, strongly

shriveled bodies (Fig. 9 A, B). These ticks were susceptible to mould and their viability was extensively decreased. It should be further assessed, whether the differences in phenotype were related to the bacterial overpopulation given the *Ir*DUOX silencing or that the lack of *Ir*DUOX affects another unknown mechanism of tick development. Fifteen *ir-duox* dsRNA injected ticks were let to lay the eggs out of which only 3 females survived three weeks after detachment the guinea pig. Only two *Ir*DUOX silenced females were able to lay eggs and clutch size were considerably smaller (in average, 15 mg) compared the egg clutches laid by *gfp* dsRNA injected ticks (in average, 83 mg) (Fig. 10). We suggest that *Ir*DUOX may play critical and possibly multiple functions in tick development and reproduction.

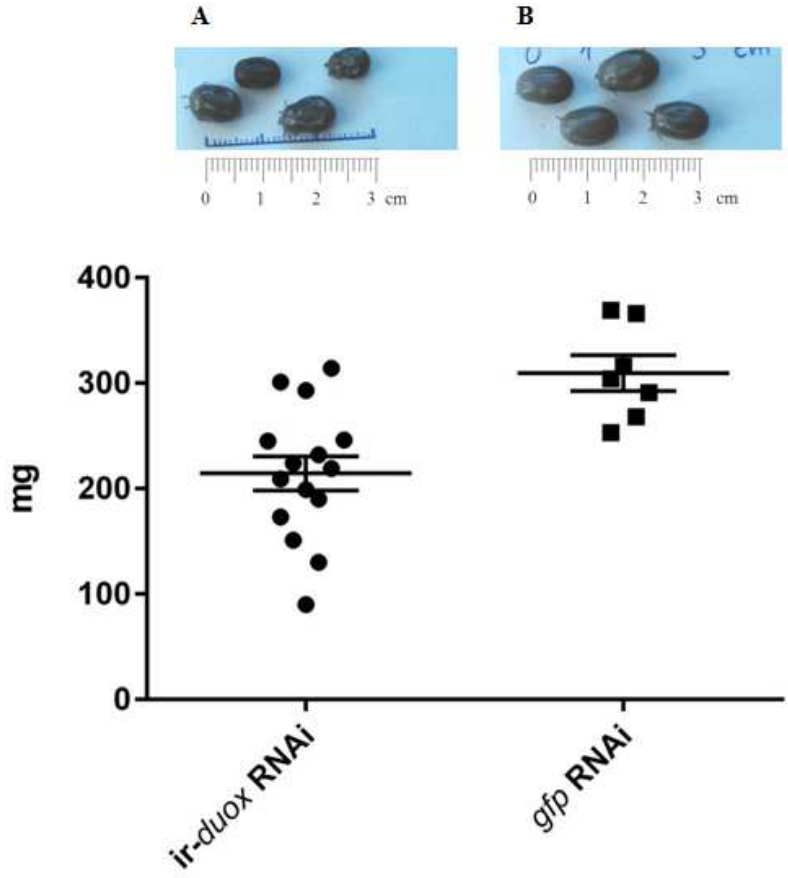

Figure 9. Impact of *Ir*DUOX absence on tick weight and phenotype. Weight differences of *ir-duox* RNAi impacted ticks with the control *gfp* dsRNA-injected ticks (**A**) Phenotype of *ir-duox* dsRNA injected ticks. (**B**) Phenotype of control *gfp* dsRNA-injected ticks.

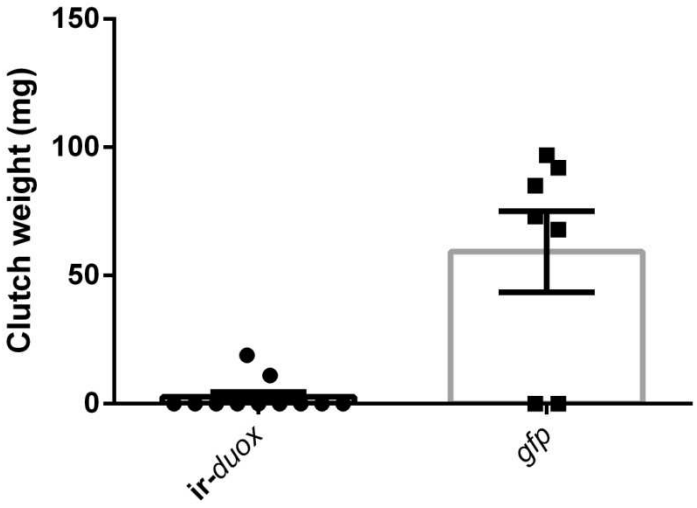

Figure 10. Impact of IrDUOX absence on tick oviposition. Weight differences of clutches of *ir-duox* RNAi impacted ticks with the control *gfp* dsRNA-injected ticks.

5 Discussion

5.1 Impact of dietary hemoglobin on bacteria levels in the tick gut

In this study the dynamics of intestinal microflora depending on different diets was assessed. The effect of whole blood, active serum or heat inactivated serum (HIS) on tick intestinal microflora proliferation during the feeding was assessed. Quantification of microbes was also performed in samples of diet and feces to evaluate the entering bacterial load and the excreted amount of bacteria.

Hemoglobin-rich diet, represented by the whole blood and hemoglobin-depleted diet represented by serum, were used for *in vitro* feeding experiments to describe the hemoglobin impact on tick intestinal microflora proliferation. Two groups of ticks were artificially fed on blood or serum until repletion. We detected similar bacterial increase in both groups up to the 2nd day of feeding after which substantial differences appeared. A significant decrease in bacterial numbers was observed in the later stage of feeding in blood-fed ticks compared to serum-fed ticks, the effect presumably caused by the presence of hemoglobin derived antimicrobial peptides. Our explanation is supported by the experiment performed by Hodson and Hirsh (1958) who firstly described the antimicrobial effect of hemoglobin. Determining the hemoglobin structure made it possible to describe the regions of hemoglobin that directly corresponded to elimination of different microbes (Parish et al., 2001). Finally, the substantial anti G^+ bacteria, anti G^- bacteria and anti-yeast activity of hemoglobin was presented (Parish et al., 2001). *In vivo* confirmation of hemoglobin microbicidal activity was described in the tick *Boophilus microplus*. Peptide with considerable bactericidal and fungicidal activity was detected in the tick gut and its origin in bovine α-hemoglobin was subsequently revealed (Fogaça et al., 1999) showing for the first time the utilization of host hemoglobin in vector antimicrobial defense. Horn et al. (2009) described the hemoglobin cleavage process of *I. ricinus*. It was shown that the large hemoglobin molecule cleavage to smaller fragments is induced by cathepsin L, cathepsin D and legumain peptidase. Dynamics profiles of expression of these peptidases were assessed and it was revealed that the cleavage of hemoglobin begins second day after tick attachment to the host (Horn et al., 2009; Franta et al., 2010). During the primary hemoglobin cleavage the antibacterial hemoglobin-derived peptides are formed (Horn et al., 2009, Cruz et al., 2010). We observed a significant decrease of intestinal microbiota in blood-fed ticks already after the 2nd day of feeding (Fig. 2A). Whether this phenomenon is associated with

accumulation of hemoglobin–derived antimicrobial peptides in the tick gut remains to be proved.

Interestingly, we detected constantly increasing bacterial quantity in ticks fed on serum (Fig. 2B). Because of presence of host immune agents, mainly the complement system, we presumed an adequate negative effect on bacteria proliferation as described by D`Amelio and Biselli (1994). Variety of mechanisms suppressing the action of the mammalian complement was described in vectors as well as in symbiotic or pathogenic microbes (Schroeder et al., 2009; Swe et al., 2014; Blom et al., 2009). Hematophagous parasite, the itch mite *Sarcoptes scabiei* utilizes serine proteases, called SMIPPs, capable of inactivation of all three known complement pathways through binding proteins responsible for complement system activation (Swe et al., 2014; Reynolds et al., 2014). Broad scale of anti-complement compounds was also described in salivary glands of different tick species. Interestingly, the most tick salivary anticomplement proteins were identified in *I. ricinus* (Schroeder et al., 2007). Tick anti-complement proteins are able to block alternative and classical pathway of complement system activation at different stages of the cascade activation (Schuijt et al., 2011; Valenzuela et al., 2000; Daix et al., 2007; Schroeder et al., 2007). Even the intestinal microbiota was reported to be active in complement system deactivation through its own mechanisms. Binding of C4b protein and thus suppression of bacteria lysis, production of proteases that mediate crucial complement components degradation, or C3 component inactivation were described (Blom et al., 2009). Collectively with that, we propose that tick is able to efficiently inactivate the host complement components at multiple levels. We suggest the model in which the ingested meal is enriched by anticomplement components originating in tick saliva. Complement inactivation might be further supported by compounds produced by unknown gut proteins and the anti-complement mechanism could be additionally strengthened by bacterial metabolites produced by commensal bacteria.

To verify our hypothesis that host complement system likely does not play an important role in tick intestinal microbiota regulation, we performed *in vitro* experiment where HIS was served to ticks. Heating of serum to 56 °C for 30 min was proven to inactivate all complement system components (Lesniak et al., 2010). Additionally, by heating the serum up to 56 °C the property of serum lipopolysaccharide-binding protein (LBP) to bind the $Gram^-$ bacteria-exposed surface lipopolysaccharides (LPS) is highly reduced (Mészáros et al., 1995). As expected, we detected the same bacteria proliferation dynamics

and even very similar bacteria numbers in ticks fed on both active serum and HIS. Therefore we conclude that ticks indeed possess mechanisms for efficient host complement inactivation that are likely beneficial for protection of their symbiotic microbiota.

Knowing the effect of hemoglobin and host complement components of serum on microbial proliferation within the tick guts, we decided to retrospectively quantify the bacterial load in the served diets. Despite their supplementation with gentamicin, unexpectedly high numbers of bacteria were detected in served diets after six hours of tick feeding. We detected twice higher bacteria quantity in the samples of blood diets than in the serum samples. These results show that gentamicin alone is not sufficient to ensure the aseptic diet for membrane feeding experiments. It may be because its antimicrobial activity is targeted mainly against $Gram^-$ bacteria, or by its limited stability at higher temperatures 30–35 °C used for the membrane feeding.

Presence of considerable bacterial quantity in blood samples is according to Olczak et al. (2005) explained an essential role of hemoglobin in microbiota proliferation as the source of iron. Nonetheless, most of the current works do not support this opinion and consider hemoglobin and hemoglobin-derived peptides, named hemocidins, as the agents of antimicrobial defense in both invertebrates and vertebrates (Fogaça et al., 1999; Mak et al., 2000; Liepke et al., 2003; Parish et al., 2001; Daoud et al., 2005). We conclude that for a strong hemoglobin-mediated microbicidial effect, the proteolytic cleavage of hemoglobin and formation of hemoglobin-derived peptides are needed.

Based on the determined bacteria numbers in different diets and the theoretical meal volume imbibed by *Ixodes* sp. female, we could perform a following extrapolation: In blood-fed ticks, 225× lower bacterial load was detected than expected whereas in serum fed-ticks, 14× higher bacterial load than theoretically estimated. These data strongly support that hemoglobin present in the diet is an important negative regulator of tick gut microbiome.

To extend our understanding of intestinal microbiota fate, we performed bacteria quantification even in tick feces. Obtained data surprisingly showed minimal bacteria load in tick feces regardless the diet served (approximately 15×10^3 bacteria per gram of tick feces). For comparison, the bacterial load of the human excrements constitutes 75×10^9 bacteria genomes per 1 gram of feces (5 million times more) (Rendtorff et Kasgarian, 1967; Stephen et Cummings, 1980). Our finding that tick feces are almost aseptic compared to the blood contents is quite interesting and requires further investigation. We hypothesize that

intestinal microbiota is an important element in tick physiology and thus tick must ensure its sustaining at the required level as the naturally fed ticks receive only limited amount of bacteria from the host blood which is supposed to be almost sterile The importance of arthropod microbiota presence has been currently reported for various organisms like mosquitoes (Damiani et al., 2010), termites and cockroaches (Dillon et Dillon, 2004), flies (Erkosar et Leulier, 2014) or ticks (Narasimhan et al., 2014). The sustaining of the certain microflora level was shown to participate in host metabolism or immune gene priming (Dennison et al., 2014). Important role of intestinal microflora quantity in determination of vector competence for pathogens was described in tick *I. scapularis* (Narasimhan et al., 2014). Taken together, more profound understanding of host-microbiota interaction could provide us implementation of new strategies against ticks.

5.2 Impact of dietary components on intestinal microflora levels of *B. afzelii* infected ticks *in vivo*

We evaluated the dynamics of the pathogen *B. afzelii* proliferation in dependence on different diets served. Considerable differences in *Borrelia* dynamics were observed in ticks fed on full blood in contrast to ticks fed on active serum or HIS. In blood-fed ticks, we detected the substantial increase in *Borrelia* numbers the 5^{th} day after attachment. Piesman et al. (2001) studied *Borrelia* dynamics in nymphs of *I. scapularis*. *Borrelia* increase was detected the second day post-attachment and it was followed by marked decrease the 4^{th} day post-attachment towards the end of nymphal feeding. The dynamics of *Borrelia* load in artificially-fed adult *I. ricinus* females might be quite differ from that reported for *I. scapularis* nymphal stage fed on infected mice.

Narasimhan et al. (2014) described the relationship of *Borrelia* and intestinal microflora in larvae of *I. scapularis*. Perturbed gut microbiota markedly reduced *Borrelia* colonization. Increasing microbiota was shown to up-regulate the expression of peritrophin via JAK/STAT pathway. PM-surrounded area provides *Borrelia* spirochetes a shelter against the hostile environment within the gut lumen. Absence of intestinal microbiota resulted in decreased STAT expression and thinning of PM. Decrease in *Borrelia* numbers was suggested to be caused by compromised permeability of the barrier and exposure of *Borrelia* to deleterious blood meal components and immunity elicitors (Narasimhan et al., 2014). Our observation do not support the model described by Narasimhan. We observed substantial

increase of *Borrelia* numbers at the same moment when symbiotic intestinal microbiota dropped. Opposite to that, we detected significant decrease of *Borrelia* immediately after intestinal microflora balanced the *Borrelia* quantity. It is known that *Borrelia* do not possess the pathway for heme synthesis (Posey et Gherardini, 2000). Based on our observations, we speculate that hemoglobin-derived peptides possess detrimental effect on commensal microbiota, whereas *Borrelia* may seem to require heme for its own proliferation. Introduction of efficient *in vitro* feeding for nymphs would bring the opportunity to describe the interplay of pathogen and symbiotic microflora more profoundly and make more relevant comparisons with the results published on the interplay between *I. scapularis* nymphs and *B. burgdorferi* spirochetes.

5.3 The effect of *Ir*DUOX RNAi on intestinal microbiota dynamics in unfed ticks

Previously we determined, that *I. ricinus* code for a good ortholog of predicted *I. scapularis* genome (98% nucleotide identity) (Kučera, 2012). We have verified its spatial and temporal expression. In this work, using RNAi, we managed to profoundly silence its expression in all examined tissues. As DUOX is a transmembrane protein, we reasoned that it might have a slower turn-over and therefore we addressed our questions as late as 5 days after dsRNA injection. Even though we tried to verify the RNAi effect also at protein level, using previously prepared antibodies, we failed to do that. To put it alternatively, we failed to verify the antibody specificity (data not shown). However, we managed to observe a striking phenotype correlating with silenced *ir-duox* levels. We noted that *ir-duox* silenced ticks have strikingly increased levels of intestinal microflora. We reasoned that this effects is linked to the absence of *Ir*DUOX in the gut epithelium and suggested that oxidative disbalanced environment in the gut may, directly or indirectly, caused the bacteria up-regulation.

The role of DUOX in intestinal microbiota regulation of invertebrates was firstly described by Ha et al. (2005) in the fly *Drosophila melanogaster*. Flies with silenced expression of DUOX showed significantly increased mortality because of their inability to regulate intestinal microbiota proliferation leading to infection. It was further demonstrated that overpopulation of microbes was caused by DUOX-produced ROS disbalance (Ha et al., 2005). Oliveira et al. (2011) described the role of DUOX in mosquito model, *Aedes aegypti*. Sugar-fed mosquitoes were able to successfully regulate its intestinal microflora implementing DUOX-produced ROS. Considerable lowered ROS production was recorded

after the blood ingestion implying the role of heme in ROS production. Decrease of ROS in the presence of hemoglobin was suggested as a compensation of the pro-oxidative effect of heme ingestion and as a protection against hydroxyl radicals that could be potentially formed in Fenton reaction (Oliveira et al., 2011). In this work we demonstrated the role of *Ir*DUOX in tick gut. However, based on previously performed transcriptional profiling (Kučera, 2012) , the site of highest expression was found in ovaries. Therefore, we may anticipate the importance of *Ir*DUOX in ovaries and other tissues. The essential role of DUOX in oviposition was reported for the hematophagous triatomine bug *Rhodnius prolixus* (Dias et al., 2013). *R. prolixus* DUOX silenced bugs showed lower size of the clutch and the eggs exerted the higher susceptibility to desiccate. It was shown that loss of ROS upon DUOX silencing resulted in the deteriorated ability to cross-link proteins of egg chorion and that these eggs were unable to sustain the water (Dias et al., 2013). Accordingly, we suggested that the phenotype changes observed in ticks could be also related to water management. Whether the changes in fecundity and oviposition are anyhow related to the changes in tick gut microbiota remains to be investigated.

In this study, we presented the importance of ROS producing dual oxidase enzyme on microbiota regulation and also implied its role in tick reproduction. Further description of DUOX and DUOX-mediated effect might result in development of new strategies against ticks and diseases they transmit.

6 Conclusion

Differences in *I. ricinus* intestinal microbiota proliferation depending on different diets were described. It was demonstrated that hemoglobin presence in the meal substantially limits the proliferation of gut microflora in *I. ricinus*. By contrast, host complement components did not seem to be involved in tick microbiota regulation as demonstrated by quantification of tick gut microflora in ticks fed on active and heat inactivated serum. Considerable intake of bacteria originating from the served meal was described. Sustaining of microbes within tick guts was delineated for two groups of differently fed ticks.

The dynamics of *B. afzelii* during the feeding process of *I. ricinus* was presented and the influence of served meal on changes in spirochete proliferation was described. We showed that *B. afzelii* proliferates in the tick gut through the feeding process only if host hemoglobin was available.

Ir-duox double stranded RNA was prepared and the RNA interference experiment was performed in adult *I. ricinus* females. The efficient DUOX silencing was shown in four tick tissues. Based on the RNAi experiment, the important role of DUOX on tick intestinal microbiota regulation was described. Additionally, the changed phenotype and negative effect of DUOX silencing on tick oviposition was shown.

A method of artificial feeding for *I. ricinus* nymphs was implemented and optimized. The essential factors for nymphal surviving and successful artificial feeding on membrane were described. Achievement of artificial feeding *I. ricinus* nymphs presents an important milestone towards future studies of tick-borne pathogen transmission.

7 Literature

Alitalo A, Meri T, Lankinen H, Seppälä I, Lahdenne P, Hefty PS, Akins D, Meri S. 2002, Complement inhibitor factor H binding to Lyme disease spirochetes is mediated by inducibleexpression ofmulti ple plasmid-encoded outer surface protein E paralogs., J Immunol., 169(7):3847-53.

Ameziane-El-Hassani R, Morand S, Boucher JL, Frapart YM, Apostolou D, Agnandji D, Gnidehou S, Ohayon R, Noël-Hudson MS, Francon J, Lalaoui K, Virion A, Dupuy C. 2005, Dual oxidase-2 has an intrinsic Ca2+-dependent H2O2-generating activity., J Biol Chem., 280(34):30046-54.

Andreotti R, Pérez de León AA, Dowd SE, Guerrero FD, Bendele KG, Scoles GA. 2011, Assessment of bacterial diversity in the cattle tick Rhipicephalus (Boophilus) microplus through tag-encoded pyrosequencing., BMC Microbiol., 11(1):6.

Anh NT, Nishitani M, Harada S, Yamaguchi M, Kamei K. 2011, Essential role of Duox in stabilization of Drosophila wing., J Biol Chem., 286(38):33244-51.

Apel K, Hirt H. 2004, Reactive oxygen species: metabolism, oxidative stress, and signal transduction., Annu Rev Plant Biol., 55:373-99.

Bae YS, Choi MK, Lee WJ (2010). Dual oxidase in mucosal immunity and host-microbe homeostasis., Trends Immunol., 31:278–87.

Bahia AC, Dong Y, Blumberg BJ, Mlambo G, Tripathi A, BenMarzouk-Hidalgo OJ, Chandra R, Dimopoulos G. 2014, Exploring Anopheles gut bacteria for Plasmodium blocking activity., Environ Microbiol., 16(9):2980-94.

Balaban RS, Nemoto S, Finkel T. 2005, Mitochondria, oxidants, and aging., Cell., 120(4):483-95.

Balla J, Vercellotti GM, Jeney V, Yachie A, Varga Z, Jacob HS, Eaton JW, Balla G. 2007, Heme, heme oxygenase, and ferritin: how the vascular endothelium survives (and dies) in an iron-richenvironment., Antioxid Redox Signal., 9(12):2119-37.

Barbour AG, Fish D. 1993, The biological and social phenomenon of Lyme disease., Science., 260(5114):1610-6.

Bedard K, Krause KH. 2007, The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology., Physiol Rev., 87(1):245-313.

Benach JL, Coleman JL, Skinner RA, Bosler EM. 1987, Adult Ixodes dammini on rabbits: a hypothesis for the development and transmission of Borrelia burgdorferi., J Infect Dis., 155(6):1300-6.

Berndtson K. 2013, Review of evidence for imine evasion and persistent infection in Lyme disease., Int J Gen Med., 6:291-306.

Bilej M, Procházková P, Silerová M, Josková R. 2010, Earthworm immunity., Adv Exp Med Biol., 708:66–79.

Billingsley PF. 1990, The Midgut Ultrastructure of Hematophagous Insects., Annu Rev Entomology., 35: 219-248.

Blom AM, Hallström T, Riesbeck K. 2009, Complement evasion strategies of pathogens-acquisition of inhibitors and beyond., Mol Immunol., 46(14):2808-17.

Boissière A, Tchioffo MT, Bachar D, Abate L, Marie A, Nsango SE, Shahbazkia HR, Awono-Ambene PH, Levashina EA, Christen R, Morlais I. 2012, Midgut microbiota of the malaria mosquito vector Anopheles gambiae and interactions with Plasmodium falciparum infection., PLoS Pathog., 8(5):e1002742.

Budachetri K, Browning RE, Adamson SW, Dowd SE, Chao CC, Ching WM, Karim S. 2014, An insight into the microbiome of the Amblyomma maculatum (Acari: Ixodidae)., J Med Entomol., 51(1):119-29.

Carpi G, Cagnacci F, Wittekindt NE, Zhao F, Qi J, Tomsho LP, Drautz DI, Rizzoli A, Schuster SC. 2011, Metagenomic profile of the bacterial communities associated with Ixodes ricinus ticks., PLoS One., 6(10):e25604.

Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, Dimopoulos G. 2011, Natural microbe-mediated refractoriness to Plasmodium infection in Anopheles gambiae., Science., 332(6031):855-8.

Cook MJ. 2014, Lyme borreliosis: a review of data on transmission time after tick attachment., Int J Gen Med., 8:1-8.

Coons LB, Rosell-Davis R, Tarnowski BI. 1986, Blood meal digestion in ticks, in: Sauer, J.R., Hair, J.A. (Eds.), Morphology, Physiology, and Behavioural Biology of Ticks. Ellis Horwood Ltd., John Wiley & Sons, New York, pp. 248-279.

Covarrubias L, Hernández-García D, Schnabel D, Salas-Vidal E, Castro-Obregón S. 2008, Function of reactive oxygen species during animal development: passive or active?, Dev Biol., 320:1–11.

Cross AR, Segal AW. 2004, The NADPH oxidase of professional phagocytes-prototype of the NOX electron transport chain systems., Biochim Biophys Acta., 1657:1–22.

Cruz CE, Fogaça AC, Nakayasu ES, Angeli CB, Belmonte R, Almeida IC, Miranda A, Miranda MT, Tanaka AS, Braz GR, Craik CS, Schneider E, Caffrey CR,Daffre S. 2010, Characterization of proteinases from the midgut of Rhipicephalus (Boophilus) microplus involved in the generation of antimicrobial peptides., Parasit Vectors., 3:63.

Daix V, Schroeder H, Praet N, Georgin JP, Chiappino I, Gillet L, de Fays K, Decrem Y, Leboulle G, Godfroid E, Bollen A, Pastoret PP, Gern L, Sharp PM, Vanderplasschen A. 2007, Ixodes ticks belonging to the Ixodes ricinus complex encode a family of anticomplement proteins., Insect Mol Biol., 16(2):155–166.

D'Amelio R, Biselli R.1994, The role of complement in anti-bacterial defence., Ann Ital Med Int., 9(3):173-7.

Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, Capone A, Ulissi U, Epis S, Genchi M, Sagnon N, Faye I, Kang A, Chouaia B, Whitehorn C, Moussa GW, Mandrioli M, Esposito F, Sacchi L, Bandi C, Daffonchio D, Favia G. 2010, Mosquito bacteria symbiosis: the case of Anopheles gambiae and Asaia., Microb Ecol., 60(3):644-54.

Daoud R, Dubois V, Bors-Dodita L, NedjarArroume N, Krier F, Chihib NE, Mary P, Kouach M, Briand G, Guillochon D. 2005, New antibacterial peptide derived from bovine hemoglobin., Peptides.**,** 26(5):713-9.

De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE, Miot F. 2000, Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family., J Biol Chem., 275(30):23227-33.

de la Fuente J, Estrada Pena A, Venzal JM, Kocan KM, Sonenshine DE. 2008, Overview: Ticks as vectors of pathogens that cause disease in humans and animals., Front Biosci., 13:6938-46.

Dennison NJ, Jupatanakul N, Dimopoulos G. 2014, The mosquito microbiota influences vector competence for human pathogens., Curr Opin Insect Sci., 3:6-13.

Devenport M, Fujioka H, Jacobs-Lorena M. 2004, Storage and secretion of the peritrophic matrix protein Ag-Aper1 and trypsin in the midgut of Anopheles gambiae., Insect Mol Biol., 13(4):349-58.

Dias FA, Gandara AC, Queiroz-Barros FG, Oliveira RL, Sorgine MH, Braz GR, Oliveira PL. 2013, Ovarian dual oxidase (Duox) activity is essential for insect eggshell hardening and waterproofing., J Biol Chem., 288(49):35058-67.

Dillon RJ, Dillon VM. 2004, The gut bacteria of insects: nonpathogenic interactions., Annu Rev Entomol., 49:71-92.

Dong Y, Manfredini F, Dimopoulos G. 2009, Implication of the mosquito midgut microbiota in the defense against malaria parasites., PLoS Pathog., 5(5):e1000423.

Dunkelberger JR, Song WC. 2010, Complement and its role in ikate and adaptive immune responses., Cell Res., 20(1):34-50.

Edens WA, Sharling L, Cheng G, Shapira R, Kinkade JM, Lee T, Edens HA, Tang X, Sullards C, Flaherty DB, Benian GM, Lambeth JD. 2001, Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox., J Cell Biol., 154(4):879-91.

Eleftherianos I, Atri J, Accetta J, Castillo JC. 2013, Endosymbiotic bacteria in insects: guardians of the immune system?, Front Physiol., 4:46.

Erkosar B, Leulier F. 2014, Transient adult microbiota, gut homeostasis and longevity: novel insights from the Drosophila model., FEBS Lett., 588(22):4250-7.

Fingerle V, Wilske B. 2006, Ticks, tick bites and how best to remove the tick., MMW Fortschr Med., 148:30–2.

Fisher H. 2009, Mechanisms and function of DUOX in epithelia of the lung., Antioxid Redox Signal., 11(10):2453-65.

Fogaça AC, da Silva PI Jr, Miranda MT, Bianchi AG, Miranda A, Ribolla PE, Daffre S. 1999, Antimicrobial activity of a bovine hemoglobin fragment in the tick Boophilus microplus., J BiolChem., 274(36):25330-4.

Franta Z, Frantová H, Konvičková J, Horn M, Sojka D, Mareš M, Kopáček P. 2010, Dynamics of digestive proteolytic systém during blood feeding of the hard tick Ixodes ricinus., Parasit Vectors., 10.1186/1756-3305-3-119.

Franta Z, Sojka D, Frantová H, Dvořák J, Horn M, Srba J, Talacko P, Mareš M, Schneider E, Craik CS, McKerrow JH, Caffrey CR, Kopáček P. 2011, IrCL1 - the haemoglobinolytic cathepsin L of the hard tick, Ixodes ricinus., Int J Parasitol., 41(12):1253-62.

Geiszt M, Leto TL. 2004, The Nox family of NAD(P)H oxidases: host defense and beyond., J Biol Chem., 279(50):51715-8.

Gillespie JP, Kanost MR and Trenczek R 1997, Biological mediators of insect immunity., Annu Rev of Entomol., 42:611–643.

Graça-Souza AV, Maya-Monteiro C, Paiva-Silva GO, Braz GR, Paes MC, Sorgine MH, Oliveira MF, Oliveira PL. 2006, Adaptations against heme toxicity in blood feeding arthropods., Insect Biochem Mol Biol., 36(4):322-35.

Gray J, Zintl A, Hildebrandt A, Hunfeld KP, Weiss L. 2010, Zoonotic babesiosis: overview of the disease and novel aspects of pathogen identity., Ticks Tick Borne Dis., 1(1):3-10.

Grunclová L, Fouquier H, Hypša V, Kopáček P. 2003, Lysozyme from the gut of the soft tick Ornithodoros moubata: the sequence, phylogeny and post-feeding regulation., Dev Comp Immunol., 27:651-60.

Ha EM, Oh CT, Bae YS, Lee WJ. 2005, A direkt role for dual oxidase in Drosophila gut immunity., Science., 310(5749):847-50.

Hai VV, Almeras L, Socolovschi C, Raoult D, Parola P, Pagès F. 2014, Monitoring human tick-borne disease risk and tick bite exposure in Europe: available tools and promising future methods., Ticks Tick Borne Dis., 5(6):607-19.

Hajdušek O, Sojka D, Kopáček P, Burešová V, Franta Z, **Š**auman I, Winzerling J, Grubhoffer L. 2009, Knockdown of proteins involved in iron metabolism limits tick reproduction and development., Proc Natl Acad Sci U S A., 106(4):1033-8.

Hajdušek O, Šíma R, Ayllón N, Jalovecká M, Perner J, de la Fuente J, Kopáček P. 2013, Interaction of the tick immune system with transmitted pathogens., Front Cell Infect Microbiol., 3:26.

Hegedus D, Erlandson M, Gillott C, Toprak U. 2009, New insights into peritrophic matrix synthesis, architecture, and function., Annu Rev Entomol., 54:285-302.

Hodson D, Hirsch JG. 1958, The antibacterial activityof hemoglobin., J Exp Med., 107(2):167-83.

Horak IG, Camicas JL, Keirans JE. 2002, The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida): a Word list of valid tick names., Exp Appl Acarol., 28(1-4):27-54.

Horn M, Nussbaumerová M, Sanda M, Kovárová Z, Srba J, Franta Z, Sojka D, Bogyo M, Caffrey CR, Kopácek P, Mares M. 2009, Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics., Chem Biol., 16(10):1053-63.

Johns R, Sonenshine DE, Hynes WL. 2001, Identification of a defensin from the hemolymph of the American dog tick, Dermacentor variabilis., Insect Biochem Mol Biol., 31:857-65.

Jongejan F, Uilenberg G. 2004, The global importance of ticks., Parasitology., 129: S3–14.

Kopáček P, Vogt R, Jindrák L, Weise C, Safařík I. 1999, Purification and characterization of the lysozyme from the gut of the soft tick Ornithodoros moubata., Insect Biochem Mol Biol., 29:989-97.

Kopáček P, Hajdušek O, Burešová V, Daffre S. 2010, Tick innate immunity., Adv Exp Med Biol., 708:137-62.

Kröber T, Guerin PM. 2007, In vitro feeding assays for hard ticks., Trends Parasitol., 23(9):445-9.

Kučera M. 2012, Molekulární charakterizace NADPH oxidázy ve střevě klíštěte Ixodes ricinus (unpublished)

Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C. 2010, A peroxidase/dual oxidase system modulates midgut epithelial immunity in Anopheles gambiae., Science., 327(5973):1644-8.

Kuraishi T, Binggeli O, Opota O, Buchon N, Lemaitre B. 2011, Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in Drosophila melanogaster., Proc Natl Acad Sci U S A., 108(38):15966-71.

Lambeth JD, Cheng G, Arnold RS, Edens WA. 2000, Novel homologs of gp91phox., Trends Biochem Sci., 25(10):459-61.

Lee YR, Lei HY, Liu MT, Wang JR, Chen SH, Jiang-Shieh YF, Lin YS, Yeh TM, Liu CC, Liu HS. 2008, Autophagic machinery activated by dengue virus enhances virus replication., Virology., 374(2):240-8.

Lesniak A, Campbell A, Monopoli MP, Lynch I, Salvati A, Dawson KA. 2010, Serum heat inactivation affects protein corona composition and nanoparticle uptake., Biomaterials., 31(36):9511-8.

Li Y, Terkawi MA, Nishikawa Y, Aboge GO, Luo Y, Ooka H, Goo YK, Yu L, Cao S, Sun Y, Yamagishi J, Masatani T, Yokoyama N, Igarashi I, Xuan X. 2012, Macrophages are critical for cross-protective immunity conferred by Babesia microti against Babesia rodhaini infection in mice., Infect Immun., 80(1):311-20.

Liepke C, Baxmann S, Heine C, Breithaupt N, Ständker L, Forssmann WG. 2003, Human hemoglobin derived peptides exhibit antimicrobial activity: a class of host defense peptides., J Chromatogr B Analyt Technol Biomed Life Sci., 791(1-2):345-56.

Liu L, Dai J, Zhao YO, Narasimhan S, Yang Y, Zhang L, Fikrig E. 2012, Ixodes scapularis JAK-STAT pathway regulates tick antimicrobial peptides, thereby controlling the agent of human granulocytic anaplasmosis., J Infect Dis., 206(8):1233-41.

Mak P, Wójcik K, Silberring J, Dubin A**.** 2000, Antimicrobial peptides derived from heme-containing proteins: hemocidins., Antonie Van Leeuwenhoek., 77(3):197-207.

Mans BJ. 2011, Evolution of Vertebrate Hemostatic and Inflammatory Control Mechanisms in Blood-Feeding Arthropods., J Innate Immun., 3:41–51.

Matés JM, Sánchez-Jiménez FM. 2000, Role of reactive oxygen species in apoptosis: implications for cancer therapy., Int J Biochem Cell Biol., 32(2):157-70.

Melching L, Vas SI. 1971, Effects of serum components on gram-negative bacteria during bactericidal reactions., Infect Immun., 3(1):107-15.

Menchaca AC, Visi DK, Strey OF, Teel PD, Kalinowski K, Allen MS, Williamson PC. 2013, Preliminary assessment of microbiome changes following blood-feeding and survivorship in the Amblyomma americanum nymph-to-adult transition using semiconductor sequencing., PLoS One., 8(6):e67129.

Mészáros K, Aberle S, White M, Parent JB. 1995, Immunoreactivity and bioactivity of lipopolysaccharide-binding protein in normal and heat-inactivated sera., Infect Immun., 63(1):363-5.

Miklossy J, Kasas S, Zurn AD, McCall S, Yu S, McGeer PL. 2008, Persisting atypical and cystic forms of Borrelia burgdorferi and local inflammation in Lyme neuroborreliosis., J Neuroinflammation., 5:40.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL. 2009, A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium., Cell., 139(7):1268-78.

Nakajima Y, van der Goes van Naters-Yasui , Taylor D, Yamakawa M. 2002, Antibacterial peptide defensin is involved in midgut immunity of the soft tick, Ornithodoros moubata., Insect Mol Biol., 11:611–8.

Nakajima Y, Ogihara K, Taylor D, Yamakawa M. 2003, Antibacterial hemoglobin fragments from the midgut of the soft tick, Ornithodoros moubata (Acari: Argasidae)., J Med Entomol., 40(1):78-81.

Narasimhan S, Sukumaran B, Bozdogan U, Thomas V, Liang X, DePonte K, Marcantonio N, Koski RA, Anderson JF, Kantor F, Fikrig E. 2007, A tick antioxidant facilitates the Lyme disease agent's successful migration from the mammalian host to the arthropod vector., Cell Host Microbe., 2(1):7-18.

Narasimhan S, Rajeevan N, Liu L, Zhao YO, Heisig J, Pan J, Eppler-Epstein R, Deponte K, Fish D, Fikrig E. 2014, Gut microbiota of the tick vector Ixodes scapularis modulate colonization of the Lyme disease spirochete., Cell Host Microbe., 15(1):58-71.

Neelakanta G, Li X, Pal U, Liu X, Beck DS, DePonte K, Fish D, Kantor FS, Fikrig E. 2007, Outer surface protein B is critical for Borrelia burgdorferi adherence and survival within Ixodes ticks., PLoS Pathog., 3(3):e33.

Noda H, Munderloh UG, Kurtti TJ. 1997, Endosymbionts of ticks and their relationship to Wolbachia spp. and tick-borne pathogens of humans and animals., Appl Environ Microbiol., 63(10):3926-32.

Noden BH, Vaughan JA, Pumpuni CB, Beier JC. 2011, Mosquito ingestion of antibodies against mosquito midgut microbiota improves conversion of ookinetes to oocysts for Plasmodium falciparum, but not P. yoelii., Parasitol Int., 60(4):440-6.

Norris SJ. 2012, How do lyme borrelia organisms cause disease? The quest for virulence determinants., Open Neurol J., 6:119-23.

Ohkuma M. 2003, Termite symbiotic systems: efficient bio-recycling of lignocellulose., Appl Microbiol Biotechnol., 61(1):1-9.

Ohye H, Sugawara M. 2010, Dual oxidase, hydrogen peroxide and thyroid diseases., Exp Biol Med (Maywood)., 235(4):424-33.

Olczak T, Simpson W, Liu X, Genco CA. 2005, Iron and heme utilization in Porphyromonas gingivalis., FEMS Microbiol Rev., 29(1):119-44.

Oliveira JH, Gonçalves RL, Lara FA, Dias FA, Gandara AC, Menna-Barreto RF, Edwards MC, Laurindo FR, Silva-Neto MA, Sorgine MH, Oliveira PL. 2011, Blood meal-derived heme decreases ROS levels in the minut of Aedes aegypti and allows proliferation ofintestinal microbiota., PLoS Pathog., 7(3):e1001320.

Oliveira Gde A, Lieberman J, Barillas-Mury C. 2012, Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity., Science., 335(6070):856-9.

Önder Ö, Humphrey PT, McOmber B, Korobova F, Francella N, Greenbaum DC, Brisson D. 2012, OspC is potent plasminogen receptor on surface of Borrelia burgdorferi., J Biol Chem., 287(20):16860-8.

Osei-Poku J, Mbogo CM, Palmer WJ, Jiggins FM. 2012, Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya., Mol Ecol., 21(20):5138-50.

Pagel Van Zee J, Geraci NS, Guerrero FD, Wikel SK, Stuart JJ, Nene VM, Hill CA. 2007, Tick genomics: the Ixodes genome project and beyond., Int J Parasitol., 37(12):1297-305.

Pal U, Li X, Wang T, Montgomery RR, Ramamoorthi N, Desilva AM, Bao F, Yang X, Pypaert M, Pradhan D, Kantor FS, Telford S, Anderson JF, Fikrig E. 2004, TROSPA, an Ixodes scapularis receptor for Borrelia burgdorferi., Cell., 12;119(4):457-68.

Parish CA, Jiang H, Tokiwa Y, Berova N, Nakanishi K, McCabe D, Zuckerman W, Xia MM, Gabay JE. 2001, Broad-spectrum antimicrobial activity of hemoglobin., Bioorg Med Chem., 9(2):377-82.

Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, Abdad MY, Stenos J, Bitam I, Fournier PE, Raoult D. 2013, Update on tick-borne rickettsioses around the world: a geographic approach., Clin Microbiol Rev., 26(4):657-702.

Piesman J, Schneider BS, Zeidner NS. 2001, Use of quantitative PCR to measure density of Borrelia burgdorferi in the midgut and salivary glands of feedingtick vectors., J Clin Microbiol., 39(11):4145-8.

Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, Zachary MD, Remacle J. 1990, Glutathione peroxidase, superoxide dismutase, and katalase inactivation by peroxides and oxygen derived free radicals., Mech Ageing Dev., 51(3):283-97.

Plantard O, Bouju-Albert A, Malard MA, Hermouet A, Capron G, Verheyden H. 2012, Detection of Wolbachia in the tick Ixodes ricinus is due to the presence of the hymenoptera endoparasitoid Ixodiphagus hookeri., PLoS One., 7(1):e30692.

Posey JE, Gherardini FC. 2000, Lack of a role for iron in the Lyme disease pathogen., Science., 288(5471):1651-3.

Rada B, Lekstrom K, Damian S, Dupuy C, Leto TL. 2008, The Pseudomonas toxin pyocyanin inhibits the dual oxidase-based antimicrobial system as it imposes oxidativestress on airway epithelial cells., J Immunol., 181(7):4883-93.

Ramamoorthi N, Narasimhan S, Pal U, Bao F, Yang XF, Fish D, Anguita J, Norgard MV, Kantor FS, Anderson JF, Koski RA, Fikrig E. 2005, The Lyme dinase agent exploits a tick protein to infect the mammalian host., Nature., 436(7050):573-7.

Rathinavelu S, Broadwater A, de Silva AM. 2003, Does host komplement kill Borrelia burgdorferi within ticks?, Infect Immun., 71(2):822-9.

Rendtorff RC, Kashgarian M. 1967, Stool patterns of healthy adult males., Dis Colon Rectum., 10(3):222-8.

Reynolds SL, Pike RN, Mika A, Blom AM, Hofmann A, Wijeyewickrema LC, Kemp D, Fischer K. 2014, Scabies mite inactive serine proteases are potent inhibitors of the human complement lectin pathway., PLoS Negl Trop Dis., 8(5):e2872.

Ribeiro JM, Mather TN, Piesman J, Spielman A. 1987, Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae)., J Med Entomol., 24(2):201-5.

Rizzoli A, Hauffe H, Carpi G, Vourc H G, Neteler M, Rosa R. 2011, Lyme borreliosis in Europe., Euro Surveill., 16(27).

Rollend L, Fish D, Childs JE. 2013, Transovarial transmission of Borrelia spirochetes by Ixodes scapularis: a summary of the literature and recent observations., Ticks Tick Borne Dis., 4(1-2):46-51.

Rudenko N, Golovchenko M, Grubhoffer L, Oliver JH Jr. 2011, Updates on Borrelia burgdorferi sensu lato complex with respect to public health., Ticks Tick Borne Dis., 2(3):123-8.

Sassera D, Beninati T, Bandi C, Bouman EA, Sacchi L, Fabbi M, Lo N. 2006, 'Candidatus Midichloria mitochondrii', an endosymbiont of the tick Ixodes ricinus with a uniqueintramitochondrial lifestyle., Int J Syst Evol Microbiol., 56(Pt 11):2535-40.

Schroeder H, Daix V, Gillet L, Renauld JC, Vanderplasschen A 2007, The paralogous salivary anti-complement proteins IRAC I and IRAC II encoded by Ixodes ricinus ticks have broad and complementary inhibitory activities against the complement of different host species., Microbes Infect., 9(2):247–250.

Schroeder H, Skelly PJ, Zipfel PF, Losson B, Vanderplasschen A. 2009, Subversion of complement by hematophagous parasites., Dev Comp Immunol., 33(1):5-13.

Schuijt TJ, Hovius JW, van Burgel ND, Ramamoorthi N, Fikrig E, van Dam AP. 2008, The tick salivary protein Salp15 inhibits the killing of serum-sensitive Borrelia burgdorferi sensu lato isolates., Infect Immun., 76(7):2888-94.

Schuijt TJ, Coumou J, Narasimhan S, Dai J, Deponte K, Wouters D, Brouwer M, Oei A, Roelofs JJ, van Dam AP, van der Poll T, Van't Veer C, Hovius JW, Fikrig E. 2011, A tick mannose-binding lectin inhibitor interferes with the vertebrate complement cascade to enhance transmission of the lyme disease agent., Cell Host Microbe., 10(2):136-46.

Schwan TG, Piesman J. 2002, Vector interactions and molecular adaptations of lyme disease and relapsing fever spirochetes associated with transmission by ticks., Emerg Infect Dis., 8(2):115-21.

Shai Y. 1999, Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides., Biochim Biophys Acta., 1462(1-2):55-70.

Sinkins SP. 2013, Wolbachia and arbovirus inhibition in mosquitoes., Future Microbiol., 8(10):1249-56.

Socolovschi C, Mediannikov O, Raoult D, Parola P. 2009, The relationship between spotted fever group Rickettsiae and ixodid ticks., Vet Res., 40(2):34.

Sonenshine DE, Hynes WL, Ceraul SM, Mitchell R, Benzine T. 2005, Host blood proteins and peptides in the midgut of the tick Dermacentor variabilis contribute to bacterial control., Exp Appl Acarol., 36(3):207-23.

Sonenshine DE. 1991, Biology of Ticks, Volume 1, Oxford University Press, New York, Oxford

Stephen AM, Cummings JH. 1980, The microbial contribution to human faecal mass., J Med Microbiol., 13(1):45-56.

Stevenson B, Babb K. 2002, LuxS-mediated quorum sensing in Borrelia burgdorferi, the lyme disease spirochete., Infect Immun., 70(8):4099-105.

Stuen S, Granquist EG, Silaghi C. 2013, Anaplasma phagocytophilum--a widespread multi-host pathogen with highly adaptive strategies., Front Cell Infect Microbiol., 3:31.

Swe PM, Reynolds SL, Fischer K. 2014, Parasitic scabies mites and associated bacteria joining forces against host complement defence., Parasite Immunol., 36(11):585-93.

Sze CW, Zhang K, Kariu T, Pal U, Li C. 2012, Borrelia burgdorferi needs chemotaxis to establish infection in mammals and to accomplish its enzootic cycle., Infect Immun., 80(7):2485-92.

Terenius O, Lindh JM, Eriksson-Gonzales K, Bussière L, Laugen AT, Bergquist H, Titanji K, Faye I. 2012, Midgut bacterial dynamics in Aedes aegypti., FEMS Microbiol Ecol., 80(3):556-65.

Valenzuela JG, Charlab R, Mather TN, Ribeiro JM. 2000, Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, Ixodes scapularis., J Biol Chem., 275(25):18717-23.

Valiente Moro C, Tran FH, Raharimalala FN, Ravelonandro P, Mavingui P. 2013, Diversity of culturable bacteria including Pantoea in wild mosquito Aedes albopictus., BMC Microbiol., 13:70.

Vancini R, Hernandez R, Brown D. 2015, Alphavirus entry into host cells., Prog Mol Biol Transl Sci., 129:33-62.

Wijkstrom-Frei C, El-Chemaly S, Ali-Rachedi R, Gerson C, Cobas MA, Forteza R, Salathe M, Conner GE. 2003, Lactoperoxidase and human airway host defense., Am J Respir Cell Mol Biol., 29(2):206-12.

Xi Z, Ramirez JL, Dimopoulos G. 2008, The Aedes aegypti toll pathway controls dengue virus infection., PLoS Pathog., 4(7):e1000098.

Yang X, Smith AA, Williams MS, Pal U. 2014, A dityrosine network mediated by dual oxidase and peroxidase influences the persistence of Lyme disease pathogens within the vector., J Biol Chem., 289(18):12813-22.

Zasloff M. 2002, Antimicrobial peptides of multicellular organisms., Nature., 415(6870):389-95.

Zeidler MP, Bausek N. 2013, The Drosophila JAK-STAT pathway., JAKSTAT., 2(3):e25353.

Zhang JR, Hardham JM, Barbour AG, Norris SJ. 1997, Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes., Cell., 89(2):275-85.

Zhang L, Zhang Y, Adusumilli S, Liu L, Narasimhan S, Dai J, Zhao YO, Fikrig E. 2011, Molecular interactions that enable movement of the Lyme disease agent from the tick gut into the hemolymph., PLoS Pathog., 7(6):e1002079.

Zouache K, Raharimalala FN, Raquin V, Tran-Van V, Raveloson LH, Ravelonandro P, Mavingui P. 2011, Bacterial diversity of field-caught mosquitoes, Aedes albopictus and Aedes aegypti, from different geographic regions of Madagascar., FEMS Microbiol Ecol., 75(3):377-89.

8 Supplement

8.1 Optimization of feeding units for *I. ricinus* nymphs

Introduction of artificial *in vitro* feeding for *I. ricinus* nymphs would allow as to perform better experiments in shorter time and in higher number of ticks as nymphs feed for only 4 days and fit 50 in one FU as opposed to 8 days of feeding and fitting 10 adult ticks in one feeding unit. Introduction of artificial feeding of nymphs would further bring a substantial benefit for studying of pathogen-transmission by the ticks. Also, high-throughput tests like drug feeding and scoring ticks for engorgement rates and fitness or testing of candidate vaccines without extensive use of laboratory animals. Because of much higher quantity of available nymphs in comparison with adult ticks, statistically more significant data could be provided. Taken together, introduction of *in vitro* feeding for *I. ricinus* nymphs could accelerate the research of this ectoparasite. Therefore, we expanded our effort to introduce efficient and repeatable method of artificial feeding of nymphs by adjusting the method established for adult hard ticks (Kröber and Guerin, 2007). All performed attempts for method optimization are displayed in Fig.11. Feeding process of nymphs is displayed in Figure 12. Hereafter, we briefly describe the steps leading to the final optimization of the method changing: **A**) thickness and elasticity of the membrane, **B**) Chemical composition of the membrane, **C**) Influence of environmental conditions and different animal scent, **D**) collective influence of all factors that had positive impact on nymph feeding.

A. Set of feeding units (FUs) with commonly used membrane (FP) was prepared following the protocol (see 3.3.2. Feeding unit preparation). We hypothesized that this membrane might be insufficiently flexible and too hard for nymph attachment. Therefore, we introduced a new type of membrane (OS) that was prepared the same way except for omitting the paper matrices. We tested different thicknesses of FP and OS membranes. Additionally, we prepared new animal scent from doe hairs (3.3.1. Animal hairs extract preparation) and tested it in two different concentrations on both, FP and OS membranes. Fifty nymphs were used for each FU.

Results: Nymphs were unable to penetrate the membrane and we recorded high rate of mortality. No attachment was recorded in this experiment.

B. We chose only OS membranes for further optimization and changed the chemical composition of OS membranes by adding twice more silicone oil and twice more hexane. Additionally we applied second silicone layer on each prepared membrane and spread it over

with silicone spatula to create grid-like structure to facilitate tick attachment. Control adult ticks were added into FUs to verify if the membrane can be penetrated. Twenty nymphs were used for each FU.

Results: Together with adult ticks, few nymphs were able to penetrate the OS membrane with efficiency of penetration ranging from 7 – 17 % in different FUs but neither adult ticks nor nymphs survived more than couple of hours. Ticks were dried out and unable to finish the feeding. We proposed importance of humidity for nymphal feeding and aimed at optimization of FUs and the physical conditions of feeding.

C. In next experiment we utilized only OS membranes and introduced the new one. This new membrane (C) was created by spreading the silicone mixture over the fine-cloth, the curtain. Silicone mixture for this new membrane was prepared by adding 4-fold more silicone oil than used for FP membranes preparations and no hexane. Cow scent and doe scent were tested in different concentrations. By then used cotton wool plugs were replaced by more breathable caps to secure the humidity inside FUs. We further hypothesized that ticks are unable to recognize the spot for attachment because of equal temperature on the membrane and in the surrounding environment. Therefore, temperature gradient was provided by placing the water bath into the cooling box set on 24 °C. 20 nymphs were used for each FU

Results: No success was recorded with OS membranes perfumed with doe scent but some attached ticks were observed in the cow scent-perfumed OS membranes. Efficiency of attachment on this membrane was 35 – 45 %. Additionally we detected the same efficiency of attachment on newly implemented C membrane perfumed with cow scent. We also recorded relatively high efficiency of feeding finishing that reached ~ 38 % for SO membrane and 25 % for C membrane. Based on these observations we suggested the essentiality of humidity for surviving of nymphs. Additionally we demonstrated that thickness of membranes does not play such important role in the feeding process. Nymphs were able to penetrate even through 140 nm-thick membrane.

D. To confirm the functionality of optimized artificial feeding we decided to implement all positive factors in one experiment with larger amount of nymphs. Six FUs with SO membrane and six FUs with C membrane were prepared. Each FU was perfumed with 640 ng of cow scent. 20 nymphs were used for each FU. The water box was set to 35°C whereas the temperature inside the cooling box was 24°C. Additionally we added the source of light into the cooling box to provide the 10 h photoperiod.

Results: Implemented conditions seemed suitable for feeding of nymphs. Relatively high portion of nymphs in both types of FUs were able to successfully finish the feeding process. We recorded ~ 38 % efficiency of feeding process finishing for FUs with SO membrane and ~ 42 % efficiency in FUs with C membrane (Fig 10). Both membranes showed to be usable for nymphal artificial feeding but C membrane was determined for further testing due to its easier preparation and overall better manipulation given by included cloth matrix.

Conclusion: We were able to optimize the artificial feeding for nymphal stage of *I. ricinus* with efficiency of finishing the feeding process about 40 %. We described some chemical and physical factors that play a role in nymphal *in vitro* feeding, most notably: humidity and temperature gradient. New type of membrane that is rigid enough for manipulation, can be penetrated by ticks, it is easily prepared and always possess the same thickness was introduced. In the future we will test the artificial feeding in other physical conditions and we plan to implement chemical factors as pheromones to increase the efficiency of nymphal *in vitro* feeding.

		Metrix	Width	Nr.of layers	S : SO : H	Grid	Plastic cross	Scent	Conc. (ng)	Pr.of AF	Type of cap	Temp.gradient	Photoperiod	Eff. Of attach.	Eff. Of attach. (%)	End of suck.	End of suck. (%)
A	1.	FP	90	1	3 : 1 : 0,6	Y	Y	C	500	N	CW	N	N	100/2	2	0	0
	2.	FP	90	1	3 : 1 : 0,6	Y	Y	C	50	N	CW	N	N	50/1	2	0	0
	3.	FP	90	1	3 : 1 : 0,6	Y	Y	D	500	N	CW	N	N	100/0	0	0	0
	4.	FP	90	1	3 : 1 : 0,6	Y	Y	D	50	N	CW	N	N	50/0	0	0	0
	5.	OS	40	1	3 : 1 : 0,6	Y	Y	C	500	N	CW	N	N	100/0	0	0	0
	6.	OS	40	1	3 : 1 : 0,6	Y	Y	C	50	N	CW	N	N	50/1	2	0	0
	7.	OS	40	1	3 : 1 : 0,6	Y	Y	D	500	N	CW	N	N	100/1	1	0	0
	8.	OS	40	1	3 : 1 : 0,6	Y	Y	D	50	N	CW	N	N	50/0	0	0	0
	9.	OS	50	1	3 : 1 : 0,6	Y	Y	C	500	N	CW	N	N	100/0	0	0	0
	10.	OS	50	1	3 : 1 : 0,6	Y	Y	C	50	N	CW	N	N	50/0	0	0	0
	11.	OS	50	1	3 : 1 : 0,6	Y	Y	D	500	N	CW	N	N	100/4	4	0	0
	12.	OS	50	1	3 : 1 : 0,6	Y	Y	D	50	N	CW	N	N	50/0	0	0	0
	13.	OS	60	1	3 : 1 : 0,6	Y	Y	C	500	N	CW	N	N	100/6	6	0	0
	14.	OS	60	1	3 : 1 : 0,6	Y	Y	C	50	N	CW	N	N	50/2	4	0	0
	15.	OS	60	1	3 : 1 : 0,6	Y	Y	D	500	N	CW	N	N	100/1	1	0	0
	16.	OS	60	1	3 : 1 : 0,6	Y	Y	D	50	N	CW	N	N	50/2	4	0	0
	17.	OS	70	1	3 : 1 : 0,6	Y	Y	C	500	N	CW	N	N	100/0	0	0	0
	18.	OS	110	1	3 : 1 : 0,6	Y	Y	C	500	N	CW	N	N	100/0	0	0	0
B	1.	OS	40	2	3 : 2 : 1,2	Y	Y	C	500	Y	CW	N	N	17/3	17,6	0	0
	2.	OS	50	2	3 : 2 : 1,2	Y	Y	C	500	Y	CW	N	N	30/2	6,7	0	0
	3.	OS	60	2	3 : 2 : 1,2	Y	Y	C	500	Y	CW	N	N	27/2	7,4	0	0
C	1.	OS	50	2	3 : 2 : 1,2	Y	Y	C	640	N	BC	Y	N	20/7	35	7	35
	2.	OS	60	2	3 : 2 : 1,2	Y	Y	C	640	N	BC	Y	N	40/18	45	17	42,5
	3.	OS	50	2	3 : 2 : 1,2	Y	Y	D	800	N	BC	Y	N	20/0	0	0	0
	4.	OS	60	2	3 : 2 : 1,2	Y	Y	D	50	N	BC	Y	N	20/0	0	0	0
	5.	OS	60	2	3 : 2 : 1,2	Y	Y	D	500	N	BC	Y	N	20/0	0	0	0
	6.	OS	60	2	3 : 2 : 1,2	Y	Y	D	5000	N	BC	Y	N	20/0	0	0	0
	7.	C	140	2	3 : 4 : 0	N	N	C	500	N	BC	Y	N	20/7	35	5	25
D	1.	OS	50-90	2	3 : 2 : 1,2	Y	Y	C	640	N	BC	Y	Y	120/49	40,8	45	37,5
	2.	C	140	2	3 : 4 : 0	N	N	C	640	N	BC	Y	Y	120/52	43,3	50	41,6

Figure 11. Optimization of artificial feeding for nymphs *I. ricinus*. * (continue on the next page)

***Figure 11. Optimization of artificial feeding for nymphs *I. ricinus*.** (**Matrix**) – membrane included - FP = Filtrating paper, OS = Only silicone, C = Cloth; (**Width**) – membrane thickness given in nanometers; (**Nr.of layers**) - number of membrane layers, 1 = smooth membrane, 2 = membrane with wrinkles; (**S : SO : H**) - ration of the basic substances for membrane creation, S = silicone, SO = silicone oil, H = hexane; (**Grid**) – the presence of grid in FU; (**Scent**) – animal scent used for FU perfuming; (**Conc. ng**) = used concentration of animal scent in ng; (**Pr.of AF**) - presence of adult female tick in the FU; (**Type of cap**) – CW = cotton wool, BC – breathable cap; (**Temp. gradient**) - N = same temperature on membrane and in surrounding environment, Y = differing temperatures on membrane and in surrounding environment; (**Photoperiod**) - N =no light/dark cycle, Y = 10h light/14h dark cycle; (**Eff.of attach.**) - Efficiency of attachment, number of nymphs that successfully penetrated through membrane; (**Eff.of attach %**) - Efficiency of attachment given in percentage; (**End of suck.**) - Number of nymphs that successfully finished the feeding (**End of suck. %**) - Number of nymphs that successfully finished the feeding given in percentage.

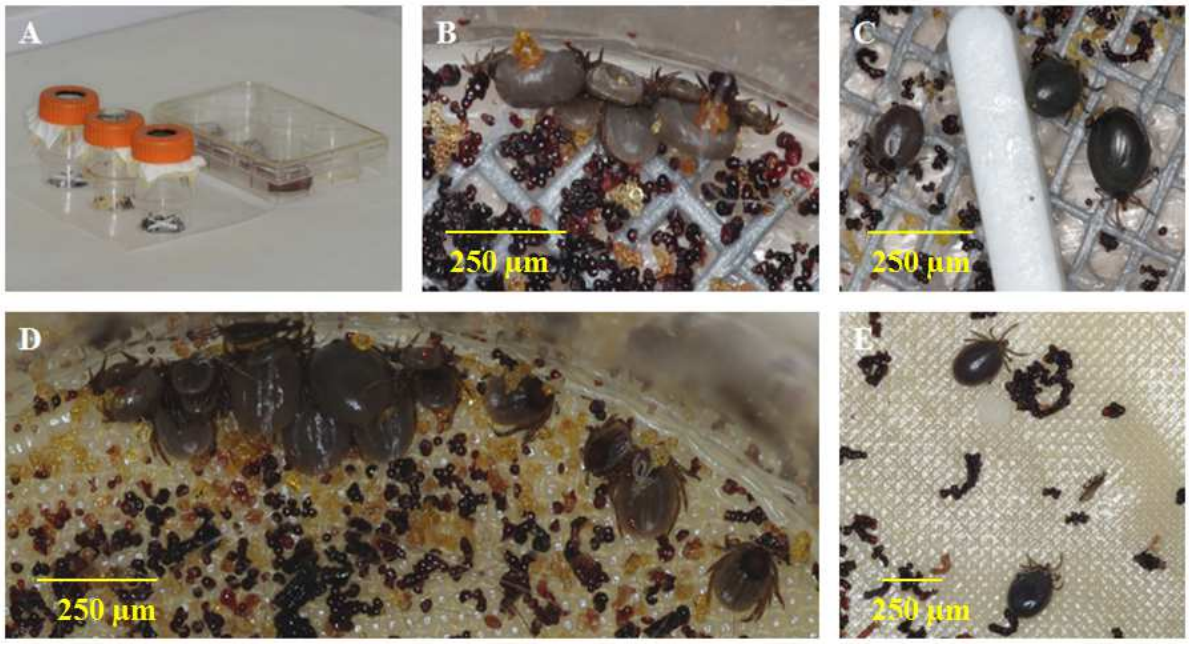

Fig 12. Demonstration of artificial feeding introduction for nymphs *I. ricinus*. Two types of membrane - silicone-only and cloth-including were tested. 20 nymphs were used per each FU. (**A**) - Prepared feeding units and six-well plate with diet; (**B**) - nymphs attached to silicone-only membrane; (**C**) - fully-fed nymphs fed on silicone-only membrane; (**D**) - nymphs attached to cloth-including membrane; (**E**) - fully-fed nymphs fed on cloth-including membrane