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Gene expression and infectivity of *Borrelia afzelii* in the course of tick feeding

Master thesis

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Annotation

Borrelia afzelii differential gene expression in the course of tick blood-feeding, and during chronic infection in mice was studied. Temperature effect on *B. afzelii* gene expression, and infectivity was investigated. Infection rates of mice immunized with *B. afzelii* tick gut antigen at various stages of tick blood-intake were analyzed.

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Tereza Pospíšilová

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1. Introduction

1.1. Ticks

Ticks, together with mites, are arachnids belonging to the superorder Parasitiformes, subclass Acari. To date, three families of ticks (Argasidae, Ixodidae, and Nuttalliellidae) characterized by different morphology and life strategy have been established. Argasidae (around 200 species) and Ixodidae (around 680 species) comprise most of the tick species, whereas south African *Nuttalliella namaqua* is a unique representative of Nuttalliellidae family (Horak et al., 2002).

Argasids, also known as soft ticks for its round-shaped body with leathery cuticle, include genera *Antricola*, *Argas*, *Nothoaspis*, *Ornithodoros*, and *Otobius.* Argasid ticks feed many times over their lifetime on a number of different hosts, and lay hundreds of eggs repeatedly. Tick engorgement is rapid, only lasting about few minutes. When feeding, surplus fluids are excreted via coxal glands (Nava et al., 2009).

Ixodid ticks possess a hard dorsal plate called scutum, so called hard ticks, and contain for instance genera *Amblyomma*, *Dermacentor, Hyalomma*, *Ixodes*, and *Rhipicephalus.* Most ixodid ticks have three hosts, one for each stage of the life cycle (larva, nymph, and adult). In contrast to soft ticks, hard ticks feeding lasts several days. Once the female is engorged, she drops off the host to lay a few thousands of eggs. Ixodid ticks use salivary glands to eliminate excess water during the blood intake (Sonenshine and Roe, 2013).

Ticks are the most important vectors of pathogens worldwide after mosquitoes (Sonenshine, 1991). A wide range of tick-borne pathogens involves protozoan infections (babesiosis, theileriosis), viruses (encephalitis), as well as bacterial diseases (borreliosis, rickettsiosis, anaplasmosis, and tularemia). Beside pathogenesis, massive tick infestation causes huge economic loses to the world livestock industry (Graf et al., 2004).

1.2. *Borrelia burgdorferi*

Borrelia burgdorferi is a spiral shaped bacteria of the spirochete class of the genus *Borrelia* (Tilly et al., 2008).

Unique architecture of borrelial cell plays a significant role in its pathogenesis. Its fluid and fragile double membrane envelope contains highly immunogenic but non-inflammatory glycolipids, instead of lipopolysaccharides, hence neither considered G^+ nor G^- (Ben-

Menachem et al., 2003). Several (7-14) periplasmic flagella anchored at both ends of spirochete to the cytoplasmic membrane twist around the protoplasmic cell cylinder forming the typical spiral cell shape. This extraordinary cell shape and structure enable spirochetes rapid chemotactic movement through viscous fluids, and successful evasion of the host tissues (Barbour and Hayes, 1986; Charon et al., 2012).

Borrelia possess one of the most complex bacterial genomes with a linear chromosome of ~1 Mb, and multiple (around 20) linear or circular plasmids varying among *Borrelia* strains (Casjens et al., 2012; Fraser et al., 1997). Besides conserved chromosomal housekeeping genes, many plasmid-encoded genes are also essential for *Borrelia* infectivity and survival. Modulation of surface lipoproteins (mostly plasmid-encoded) generate antigenic diversity that plays a key part in *Borrelia* pathogenesis. It is a mighty tool for successful evasion of the host defense mechanisms, and for the adaptation to specific conditions that *Borrelia* experience during its enzootic cycle (Purser and Norris, 2000). Similar mechanism of antigenic variation is also used by *Trypanosoma brucei*, and *Plasmodium falciparum* (Dzikowski et al., 2006; Liang et al., 2002; Taylor and Rudenko, 2006).

However, the overall knowledge of the machinery regulating differential gene expression and extensive transcriptional changes *Borrelia* undergoes throughout its enzootic cycle is surprisingly sparse (Iyer et al., 2015). So far, alteration of Borrelia gene expression controlled by Hk1–Rrp1 and Rrp2-RpoN-RpoS global regulatory systems have been described along with the chemotaxis CheA-CheY system (Caimano et al., 2011; He et al., 2011; Charon et al., 2012; Kostick et al., 2011). Nevertheless, it is more than likely that many of the spirochete's differentially expressed genes are controlled by regulatory pathways outside the Hk1-Rrp1 and RpoS networks (Iyer et al., 2015).

Apparently, RNA polymerase, sigma S (RpoS) serves as a master regulator in controlling expression of many genes involved in *B. burgdorferi* transmission and virulence (Caimano et al., 2007). *rpoS* transcription is initiated by formation of the upstream rpoS promoter complex containing another alternative σ-factor (RpoN), phosphorylated regulatory protein 2 (Rrp2), and *Borrelia* oxidative stress regulator (BosR) (Caimano et al., 2007; Ouyang et al., 2011). Hk1-Rrp1 system activates production of the second messenger cyclic-dimeric-GMP (c-di-GMP). C-di-GMP has a role in carbon metabolism, chemotaxis, and virulence as it governs the expression of genes essential for *Borrelia* survival within the tick (He et al., 2011). In contrast to the RpoN-RpoS pathway, which activates genes required for the vertebrate host colonization, the Hk1-Rrp1 functions exclusively during the tick phase of the enzootic cycle (Radolf et al., 2012; Samuels, 2011).

Furthermore, *Borrelia* metabolic pathways are extremely reduced and limited due to its parasitic lifestyle. *Borrelia* is an auxotroph for all amino acids, fatty acids, and nucleotides, with no requirement for iron. Furthermore, lacking enzymes for tricarboxylic acid cycle and oxidative phosphorylation, *Borrelia* derives energy instead from the fermentation of sugars via the Embden–Meyerhof pathway (Lackum and Stevenson, 2005; Radolf et al., 2012).

To conclude, *Borrelia* unique cellular architecture, differential gene expression, and extremely reduced metabolism, make the pathogen difficult to fight.

1.3. Lyme borreliosis

Lyme borreliosis is the most common vector-borne disease in the northern hemisphere (Jongejan and Uilenberg, 2004). It is caused by *B. burgdorferi* sensu stricto in the United States and *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* in Europe. *Borrelia* spirochetes are maintained in nature through an enzootic cycle involving small vertebrates and *Ixodes* ticks. Different *Borrelia* strains prefer different hosts based on the host complement system. *B. burgdorferi* s. s. is transmitted mostly by rodents and birds, however, *B. afzelii* spirochetes persist in rodents but not in birds, and *B. garinii* is an avian specialist (Kurtenbach et al., 2002). As the transovarial transmission of *Borrelia* in ticks is not likely, humans can contract Lyme disease only by feeding of infected tick nymphs or adults (Rollend et al., 2013). Nymphs therefore play a key role in the epidemiology of the Lyme disease.

Early stages of the disease involve flu-like symptoms and tiredness, and can be treated with antibiotics. As the pathogen migrates through the skin, characteristic rash called Erythema migrans may develop as well. In chronic stages, spirochetes develop systemic infection in tissues difficult to reach by the host immunity, and cause huge spectrum of problems ranging from arthritis (*B. burgdorferi* sensu stricto) to neuromuscular disorders (*B. garinii*). Reviewed by (Cook, 2014). To date, no vaccine is available preventing Lyme disease transmission.

1.4. Lyme disease transmission models

Understanding the complex interactions within the tick-*Borrelia*-host triangle is crucial for the development of efficient Lyme disease treatment and vaccine.

Overall, three possible routes of *Borrelia* transmission from infected ticks to the host were proposed (via regurgitation, saliva, or feces). In the early studies, regurgitation of spirochetes from the tick gut directly to the host via hypostome was suggested (Benach et al., 1987; Burgdorfer, 1984). Evidence of regurgitation was indeed confirmed in *Amblyomma*

americanum and *Ornithodoros moubata* (Brown, 1988; Connat, 1991). Possible infection via contaminated feces was also considered, but swiftly rejected (Benach et al., 1987; Burgdorfer, 1984; Cook, 2014). In parallel, the salivary route of transmission was proposed, based on the study performed on *B. burgdorferi s.s.* / *I. scapularis* (Ribeiro et al., 1987). Since then, a number of studies supported the salivary route of *B. burgdorferi s.s.* transmission by *I. scapularis* (De Silva and Fikrig, 1995; Dunham-Ems et al., 2009; Fikrig and Narasimhan, 2006).

Indisputably, the risk of acquiring the Lyme disease increases with the length of tick attachment. However, as the spirochetal transmission times and virulence depend on the tick and *Borrelia* species, the minimum transmission time has never been established (Cook, 2014). Comparing widely studied American *B. burgdorferi/ I. scapularis* with European *I. ricinus* and *B. afzelii*, many studies show surprisingly divergent trends in transmission dynamics of American and European *Borrelia* species (Crippa et al., 2002; Hojgaard et al., 2008; Ohnishi et al., 2001).

1.4.1. *Borrelia burgdorferi/ Ixodes scapularis* transmission

1.4.1.1. The *Borrelia*–tick interface

Salivary route of *B. burgdorferi* transmission via *I. scapularis* present the nowadays generally accepted mechanism of Lyme disease transmission, and can be described as follows.

Naïve *I. scapularis* larvae acquire *Borrelia* spirochetes from infected vertebrate hosts within the first 24 hours of feeding. Spirochetes then multiply rapidly in feeding larvae and during the first days post-repletion. However, number of spirochetes decrease dramatically during subsequent molting (Piesman et al., 1990).

How exactly *Borrelia* recognize and migrate towards the feeding tick remains unknown. It has been found, that spirochetes act in response to the host neuroendocrine stress hormones, which are released to the tick bite site (Scheckelhoff et al., 2007). By the time the larvae are fully engorged, the spirochetal Hk1–Rrp1 pathway required for successful bacterial colonization of the larvae is active, whereas the Rrp2–RpoN–RpoS pathway (fully activated in the mammalian host) becomes inactive (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). This results in upregulation of tick-phase genes, such as ospA, which are repressed by RpoS (Caimano et al., 2005; Schwan et al., 1995). For instance, expression of spirochetal outer surface protein A (OspA) is induced in response to adrenaline and noradrenaline production in the host skin (Scheckelhoff et al., 2007). Moreover, OspA binding to the TROSPA receptor on

the surface of the midgut allows spirochetes to colonize the tick gut and also protects them from the host digestive and defense pathways (Battisti et al., 2008; Hajdusek et al., 2013; Pal et al., 2004a).

Tick digestion is intracellular, and the pH within the tick gut is almost neutral. To maintain the intestinal microflora at tolerable level, ticks possess defense mechanisms including antimicrobial defensins, lysozymes, and large antimicrobial peptides hemocidins. Nevertheless, the engorged tick gut full of blood derived nutrients provides *Borrelia* and other microorganisms suitable environment for its colonization (Hajdusek et al., 2013; Kopácek et al., 1999; Nakajima et al., 2002; Narasimhan and Fikrig, 2015; Sonenshine et al., 2005).

When the nutrients supply within the tick gut dwindle, both the Hk1–Rrp1 and the Rrp2–RpoN–RpoS systems are inactive, and only tick-phase genes are expressed (Radolf et al., 2012). Spirochetes within flat nymph are not completely dormant, but exist in poorly understood metabolic state that enables them to survive long period without blood meal (Radolf et al., 2012). Dramatic decrease of *B. burgdorferi* numbers during *I. scapularis* molting might be due to depleted amounts of N-acetylglucosamine – an important building block of integumentary chitin but also a key component for spirochetal development (Barbour and Hayes, 1986). Also limited availability of other nutrients might be the reason for halted proliferation of spirochetes in molted nymphs (Piesman et al., 1990).

1.4.1.2. Mammalian host colonization

As molted *I. scapularis* nymphs start feeding on the second host, spirochetes sense appropriate physiochemical stimuli that trigger their replication, and their numbers increase exponentially (De Silva and Fikrig, 1995). Spirochetes within feeding nymphs activate both the Hk1-Rrp1 and Rrp2–RpoN–RpoS pathway, and simultaneously downregulate OspA, and upregulate OspC production (Caimano et al., 2011, 2016; Schwan et al., 1995).The expression of the bacterial protein OspC is essential in the initial phase of spirochetal transmission, and for the establishment of early infection(Pal et al., 2004b; Schwan et al., 1995; Tilly et al., 2006). OspC perhaps facilitate penetration of tick salivary glands, and also enables binding of spirochetes to salivary gland proteins (Salp15), and host derived molecules, such as plasminogen (described later) (Önder et al., 2012; Pal et al., 2004b). In parallel, ticks downregulate production of TROSPA (Pal et al., 2004a). All together, these changes help spirochetes to detach from the midgut, penetrate into the hemolymph, migrate to the salivary glands, and infect vertebrate host.

As mentioned above, *Borrelia* exploits tick salivary proteins in favor of its transmission to the host (Hajdusek et al., 2013; Nuttall and Labuda, 2008; Ramamoorthi et al., 2005). Compounds present in the tick saliva facilitate painless bite, and enable tick feeding. These factors include anticoagulants, cytokine inhibitors, histamine binding factors, and complement inhibitors (Nuttall and Labuda, 2008; Šimo et al., 2017). For instance, spirochetal binding to tick salivary proteins Salp15, Salp20, and TSLPI (tick salivary lectin pathway inhibitor) protects the pathogen from antibody mediated killing, phagocytosis or bacterial lysis in the host. These molecules are significantly upregulated in *Borrelia* infected ticks, and help spirochetes to invade the host. (Dai et al., 2009; Hajdusek et al., 2013; Hovius et al., 2008; Ribeiro et al., 2006; Tyson et al., 2007).

In addition to tick saliva, spirochetes make use of host derived molecules, such as plasminogen, to successfully colonize host tissues (Coleman et al., 1997). *B. burgdorferi* is found primarily in connective tissue, which colonization require degradation of the insoluble extracellular matrix. To this end, spirochetes bind vertebrate protease plasminogen that degrades many blood plasma proteins including fibrin clots. OspC, a dominant surface protein during the tick-to-host transition, is a potent plasminogen receptor (Önder et al., 2012). Plasmin-coated spirochetes are able to migrate through mammalian extracellular matrix (Coleman et al., 1999). To conclude, beside tick saliva, binding of plasminogen to the cell surface is critical during the infectious cycle of *B. burgdorferi*.

Above all, antigenic variation and changes in gene expression of surface-exposed lipoproteins are fundamental for *B. burgdorferi* pathogenesis. The most dramatic modifications occur as *B. burgdorferi* migrates from the tick to the mammalian host. After down-regulation of OspA and up-regulation of OspC in the initial phase of transmission (He et al., 2008; Schwan et al., 1995) *Borrelia* sheds OspC, which is no longer required. To establish permanent infection in the host, *Borrelia* subsequently upregulates expression of highly variable antigens including VlsE , DbpA, and Bbk32 (Crother et al., 2004; Liang et al., 2002). Successful evasion of the host antibodies is enabled via expression of highly variable surface antigen VlsE. VlsE generates antigenic diversity through the recombination of sequences from silent cassettes into the expressed *vlsE* locus (Bankhead and Chaconas, 2007; Zhang and Norris, 1998). Decorin binding proteins DbpA and DbpB mediate adhesion to the host tissues (Hübner et al., 2001). With regard to diverse forms of adhesins, different *Borrelia* strains prefer colonization of different tissues, and consequently cause distinct symptoms like arthritis or carditis (Lin et al., 2014). Another surface exposed lipoprotein Bbk32 has been shown to bind fibronectin, heparin,

and other compounds of extracellular matrix (Fischer et al., 2006). Moreover, Bbk32 protects *Borrelia* from being destroyed by the host complement system (Garcia et al., 2016). Interestingly, environmental and physiochemical stimuli such as temperature, pH, and nutrients influence spirochetal gene expression even in vitro (Carroll et al., 2000; Ramamoorthy and Scholl-Meeker, 2001; Stevenson et al., 1995; Yang et al., 2001). All-together, without these adaptations the pathogen would be cleared off promptly by the host immune system.

To sum up, *Borrelia* antigenic variation and changes in gene expression, tick salivary protein mediated immunosuppression, plasminogen binding and destruction of the extracellular matrix, adhesins allowing colonization of the host tissues, and complement inhibition via Bbk32 enhance the survival of *B. burgdorferi* in the mammal. Reviewed by (Tracy and Baumgarth, 2017).

1.4.2. *Borrelia afzelii/ Ixodes ricinus* transmission

Lyme disease research in Europe lags far behind USA. To date, only a few papers have been published regarding transmission of European *Borrelia* strains by *I. ricinus* ticks (Crippa et al., 2002; Koci et al., 2006; van Duijvendijk et al., 2015, 2016). Whereas studies performed on American *B. burgdorferi/ I. scapularis* support the salivary route of *Borrelia* transmission, latest data on European *I. ricinus/ B. afzelii* put “gut to mouth” route back into the game, and even doubts spirochetal dependence on the tick.

The alternative way of *B. afzelii* transmission avoiding *I. ricinus* hemocoel and salivary glands is supported by our recent research. In brief, results of our experiments carried out on *I. ricinus/B. afzelii* mouse transmission model can be summarized as follows: Firstly, *B. afzelii* is transmitted by *I. ricinus* to the host during the first day of tick attachment. *B. afzelii* spirochetes are present in murine skin immediately the first day of the tick attachment, however, its infectivity is delayed at least of one day. Number of *B. afzelii* spirochetes in the nymphal midgut decrease during feeding and no spirochetes could be detected in salivary glands. We further demonstrated that tick saliva is not essential for *B. afzelii* infectivity and the spirochetes become infective yet in the tick midgut during feeding (Pospisilova, 2016). Moreover, silencing of tick immune molecules or elimination of phagocytosis in tick hemocoel by injection of latex beads had no obvious impact on *B. afzelii* transmission (Honig Mondekova et al., 2017; Urbanová et al., 2017, 2018). As a whole, our data remarkably support the concept of direct gut to mouth transmission and testify against the salivary route of *B. afzelii* transmission by *I. ricinus*.

To conclude, *B. afzelii* has a unique transmission cycle that remarkably differs from the

generally accepted salivary route of *B. burgdorferi* transmission by *I. scapularis*. Regurgitation, or rather active reverse migration of spirochetes from midgut to the mouthpart should be considered as a potential way of transmission of *B. afzelii* from *I. ricinus* nymph to the host. *B. afzelii* also seems to be less dependent on its tick vector. *B. afzelii* starts its transmission yet the first day of the tick attachment, most likely directly from the midgut to the feeding cavity. Physio-chemical stimuli in feeding *I. ricinus* activate *B. afzelii* RpoN-RpoS pathway and trigger massive change in its outer surface protein expression, which seems to be the main requirement for successful host colonization. The 24-48 hours time window between the tick attachment and transmission of infectious spirochetes is a key period in the whole process (Pospisilova, 2016). These findings have important implications towards development of efficient vaccines or drugs against European Lyme borreliosis.

1.5. Vaccination

At the present time, no vaccine against Lyme disease is available. Until now, all vaccination attempts failed because of *Borrelia* great strain diversity and antigenic variability (Bunikis et al., 2004; Kenedy et al., 2012). As mentioned above, *Borrelia* plays a stealth pathogen difficult to target (Fikrig and Narasimhan, 2006). Therefore, current vaccination strategies incline rather to the development of anti-tick vaccines that impair the tick feeding, thus might prevent the pathogen transmission in general (Schuijt et al., 2011; Sprong et al., 2014). For instance, an anti-tick vaccine derived from a tick cement protein 64TRP impairs the tick feeding and protects against the tick borne encephalitis virus (TBEV) (Labuda et al., 2006). Furthermore, immunization with tick antigens including Salp15, TSLPI, and tick histamine release factor tHRF, or its blocking by RNAi impairs the tick feeding, and even decrease the number of transmitted spirochetes (Dai et al., 2010; Ramamoorthi et al., 2005; Schuijt et al., 2011). However, exploiting any of these molecules protect from *Borrelia* infection. Reviewed by Hajdusek and colleagues (Hajdusek et al., 2013).

1.6. Future directions

Recently, several global transcriptomic, and microarray studies have been performed in vivo, and even in vitro, in order to better characterize *Borrelia* gene expression patterns in different conditions as well as at different stages of *B. burgdorferi* enzootic cycle (Adams et al., 2017; Arnold et al., 2016; Iyer et al., 2015; Iyer and Schwartz, 2016; Popitsch et al., 2017a; Wu et al., 2015). The results clearly show, that spirochetes exhibit unique expression profiles during each tick stage and during mammalian infection. Importantly, none of these in vivo profiles resembles that exhibited by in vitro-grown spirochetes (Iyer et al., 2015). As a whole, environmental sensing by *B. burgdorferi* drives an extensive modulation of cell envelope, motility, and metabolism. Current mapping of the *B. burgdorferi* transcriptome is gradually uncovering the world of numerous non annotated m-RNAs, operon and promotor structures, as well as non-coding RNAs (antisense RNA, sRNA). As a whole, these findings should gradually improve our understanding of *B. burgdorferi* regulatory pathways, and define the contribution of individual genes to spirochete survival in nature and virulence. To conclude, better understanding of *Borrelia* gene regulation, and identification of the critical genes responsible for *Borrelia* infectivity should lead to the final development of efficient vaccines blocking Lyme disease transmission.

2. Objectives

1. *B. afzelii* gene expression analysis in the course of tick blood-feeding, and during chronic infection in mice.
2. Analysis of temperature effect on *B. afzelii* gene expression, and infectivity.
3. Mice immunization with *B. afzelii* tick gut antigen at various stages of tick blood-intake.

3. Materials and Methods

3.1. Laboratory animals

I. ricinus larvae and nymphs were obtained from the breeding facility of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences. Ticks were maintained in wet chambers at 2 °C, with 95% humidity, and day/night period set to 15/9 h. For both, infected and uninfected *I. ricinus* nymphs preparation, the larvae were fed on either infected, or uninfected mice, allowed to molt to nymphs, and after 4–6 weeks were used for further experiments. Inbred, pathogen free C3H/HeN mice (Jackson Laboratory, Germany), were used for the pathogen transmission experiments. All experimental animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 137/2008.

3.2. Infection of mice and ticks

Low passage *Borrelia afzelii* CB43 spirochetes (Stěpánová-Tresová et al., 2000), were grown in BSK-H medium (Sigma-Aldrich, USA) at 33 °C for 5–7 days. Six weeks old female C3H/HeN mice were infected by subcutaneous injections of 10^5 spirochetes in 100 µl of BSK-H medium per mouse. Presence of spirochetes in ear biopsies was determined 3 weeks post injection by standard PCR.

Four weeks after inoculation, uninfected *I. ricinus* larvae were fed on infected mice (~100 larvae per mouse) and allowed to molt. Nymphs were considered to be infected if >90% of them were PCR positive.

3.3. Nucleic acid isolation and cDNA preparation

Total DNA was extracted from *I. ricinus* nymphs as well as murine tissues using a NucleoSpin Tissue Kit (Macherey-Nagel, Germany) following manufacturer's protocol. Quality of extracted DNA was assessed by standard PCR either amplifying a 600 bp part of murine chromosome 2 (primers Mm-600F, Mm-600R), or 600 bp part of tick genomic sequence (primers Ir-600F, Ir-600R); **(Tab. 1).**

Total RNA was isolated from *I. ricinus* nymphs, and murine tissues using NucleoSpin RNA (Macherey-Nagel) according to manufacturer's protocol. RNA quality was then checked on the 1% agarose gel, and RNA concentration measured on NanoDrop™ 1000

spectrophotometer (NanoDrop, USA). 1 µg of isolated RNA subsequently served as a template for reverse-transcription into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Switzerland). Prepared cDNAs were then 10x diluted, and served as templates for the subsequent quantitative expression qRT-PCR analyses.

3.4. PCR

Detection of spirochetes in ticks, and in the murine tissues was performed by nested PCR amplification of a 222 bp fragment of *Borrelia* 23S rRNA gene. 25 µl reaction volume contained 12.5 µl of FastStart PCR Master (Roche), 10 pmol of each primer (Bor-1, Bor-2 for the first round; Bor-3, Bor-4 for the second round; **Tab. 1**), template (4 µl of purified DNA for the first round, 1 µl aliquot of the first PCR product in the second round), and PCR water up to 25 µl. Amplification program for the first round consisted of denaturation at 95°C for 10 minutes, and then 40 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 30 seconds. The program was finished by 72°C incubation for 7 minutes. The amplification program for the second round was the same except the annealing temperature was 58 °C.

3.5. Quantitative PCR

Total loads of spirochetes in tick and murine DNA samples were determined by quantitative real-time PCR (qPCR) using QuantStudio™ 6 Flex real time PCR system (Thermo Fisher Scientific, USA). 25 µl reaction volume contained 12.5 µl of FastStart Universal Probe Master (Rox) (Roche), 10 pmol of primers FlaF1A and FlaR1, 5 pmol of TaqMan probe Fla Probe1(Schwaiger et al., 2001) (**Tab. 1**), and 5 µl of purified DNA. The remaining reaction volume was adjusted with PCR water. Amplification program consisted of denaturation at 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 15 seconds and annealing + elongation at 60°C for 1 min. All samples were analyzed in triplicates.

Similarly, quantification of murine ß-actin was performed using primers MmAct-F and MmAct-R and TaqMan probe MmAct-P (Dai et al., 2009) (**Tab. 1**). Reaction and amplification conditions were the same as described above.

Spirochete burden in murine tissues was determined as a number of spirochetes per 10^5 of murine ß-actin copies using external *B. burgdorferi flagellin* and *M. musculus actin* standard curves. Spirochete burden in ticks was calculated as a total number of spirochetes in whole tick body using external *B. burgdorferi flagellin* standard curve.

3.6. Relative quantification (qRT-PCR)

cDNA preparations from *B. afzelii* infected *I. ricinus* nymphs as well as murine tissues were made in independent triplicates and served as templates for the following quantitative expression analyses by relative qPCR. Samples were analyzed by QuantStudio™ 6 Flex real time PCR system (Thermo Fisher Scientific). 25 µl reaction volume contained 12.5 µl of FastStart Universal SYBR Green Master, Rox (Roche), 10 pmol of each **RT-** primer (**Tab. 1**) and 5 µl of cDNA template. The remaining reaction volume was adjusted with PCR water. Amplification program consisted of denaturation at 95°C for 5 minutes, followed by 50 cycles of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 30 seconds, and elongation at 72 °C for 30 seconds. Each primer pair was inspected for its specificity using melting curve analysis. Relative expressions of *ospA*, *ospC*, *dbpA*, and *bbk32* were normalized to *flaB* using the ΔΔCt method(Pfaffl, 2001).

Tab. 1: PCR primers and probes for detection and quantification of spirochetes in ticks and murine tissues; control primers for DNA presence in samples.

Target	Name	Sequence (5'→3')	Product size	Source
Borrelia flagellin	**FlaF1A** **FlaR1** **TaqMan FlaProbe1**	AAGCAAATTTAGGTGCTTTCCAA GCAATCATTGCCATTGCAGA TGCTACAACCTCATCTGTCATTGTA GCATCTTTTATTTG	154 bp	Schwaiger et al. 2001
Mus musculus B-actin	**Mm actin F** **Mm actin R** **Mm actin P**	AGAGGGAAATCGTGCGTGAC CAATAGTGATGACCTGGCCGT CACTGCCGCATCCTCTTCCTCCC	137 bp	Dai et al. 2009
Ixodes ricinus	**Ir-600R** **Ir-600F**	GACCTGCACGAAAATGATTG GAGGCATGAGGGTGTGTTTT	600 bp	Šíma R. unpublished new primers
Mus musculus	**Mm-600F** **Mm-600R**	GCTTCTGGAAGAACCACAGG AAGCACTTCGAACCACTGCT	600 bp	
Borrelia 23S rRNA	**Bor1** **Bor2**	AGAAGTGCTGGAGTCGA TAGTGCTCTACCTCTATTAA	260 bp	
	Bor3 **Bor4**	GCGAAAGCGAGTCTTAAAAGG ACTAAAATAAGGCTGAACTTAAAT	222 bp	
OspA lipoprotein	**RTospA-FN** **RTospA-RN**	CGCATGGGATTCAAAAACTT TGGTACCTGCGGAGTCGTAT	119 bp	
OspC lipoprotein	**RTospC-F** **RTospC-R**	GCAGGAGCCTATGCAATATCA TTTGCCAAGATCTGCATGAC	150 bp	
DbpA lipoprotein	**RTdbpA-F** **RTdbpA-R**	TACGCGTCGCTGACTTAACA CTTTTGCGGCGTTGAGTATTA	127 bp	
Bbk32 lipoprotein	**RTbbk32-F** **RTbbk32-R**	CACGTCTTGACAACCTTGCT CCTTGCACTCACTTGAATATAG	117 bp	Koci et al. 2006
Borrelia flagellin	**RTflaB-F** **RTflaB-R**	GTTCATGTGGGAGCAAATCA ACCCTCTTGAACAGGTGCAG	120 bp	

3.7. Gel electrophoresis

PCR products were visualized using gel electrophoresis. Ethidium bromide (Sigma-Aldrich, USA) stained 2% agarose gel in TAE buffer was used for PCR products separation and size determination. 10 μl of PCR sample subsequently mixed with DNA loading dye (Top-Bio, Czech Republic) was loaded on the gel. PCR product size was determined according to GeneRuler 100bp DNA Ladder (Thermo Fisher Scientific).

3.8. Statistical analysis

Data were analyzed by GraphPad Prism 6 for Windows, version 6.04. Unpaired Student's t-test was used for statistical significance evaluation, with Ap value of $P<0.05$ considered to be statistically significant. Error bars show standard error of the mean.

3.9. Preparation of tick gut antigen and vaccination experiment

Guts from unfed, 24h fed, 48h fed and 72h fed *B. afzelii* CB43 infected *I. ricinus* nymphs were dissected into PBS (50 guts dissected into 500 μl of sterile PBS). Dissected tick guts in PBS were then immediately disrupted using insulin syringe, and sonicated 3x30 sec with 60 sec break (on ice, cycle 0.5, amplitude 55%) using UP200S ultrasonic processor (Hielscher, Germany). Sonicated guts were then mixed with either complete (CFA) or incomplete (IFA) Freund's adjuvant (Sigma-Aldrich), and injected into mice.

Mice challenged 2 vaccination rounds: First boost (6 sonicated guts/mouse in 60 μl PBS + 60 μl CFA); Second boost in 14 days (6 sonicated guts/mouse in 60 μl PBS + 60 μl IFA).

21 days after second boost, *B. afzelii* CB43 infected ticks were fed on immunized mice until repletion (5 nymphs/mouse). Presence of spirochetes in ear biopsies was determined two, three and four weeks after tick infestation by qPCR.

4. Results

4.1. *B. afzelii* gene expression during tick feeding and mouse infection

Former research performed mainly on American *B. burgdorferi* has shown connection between the bacteria altered protein expression, and its infectivity (Fikrig et al., 2000; Ohnishi et al., 2001; Schwan et al., 1995; Tilly et al., 2006). Previous gene expression studies performed on *B. burgdorferi* documented that during its enzootic cycle the pathogen undergoes huge transcriptional changes in genes responsible for its infectivity. For instance, genes encoding OspA, OspC, DbpA, and Bbk32 are among those well described.

To examine whether *B. afzelii* CB43 differential expression of orthologous genes may also contribute to the pathogen infectivity, qPCR analysis of *B. afzelii* CB43 spirochetes extracted from the guts of *I. ricinus* nymphs in different feeding stages as well as from mice 4 weeks post infection was performed.

Gene encoding OspA was abundantly expressed in unfed *I. ricinus* nymphs, downregulated during the tick feeding, and hardly detectable in mouse tissues with chronic infection. In stark contrast to *ospA*; *ospC* and *dbpA* mRNA levels were low in unfed ticks, whereas highly upregulated during the following tick feeding. Both *ospC* and *dbpA* genes were then steadily expressed in mice with permanent infection. Similarly, *bbk*32 transcription was highly induced in the course of the tick feeding with a subsequent gradual up-regulation during the chronic mammalian infection **(Fig. 1)**.

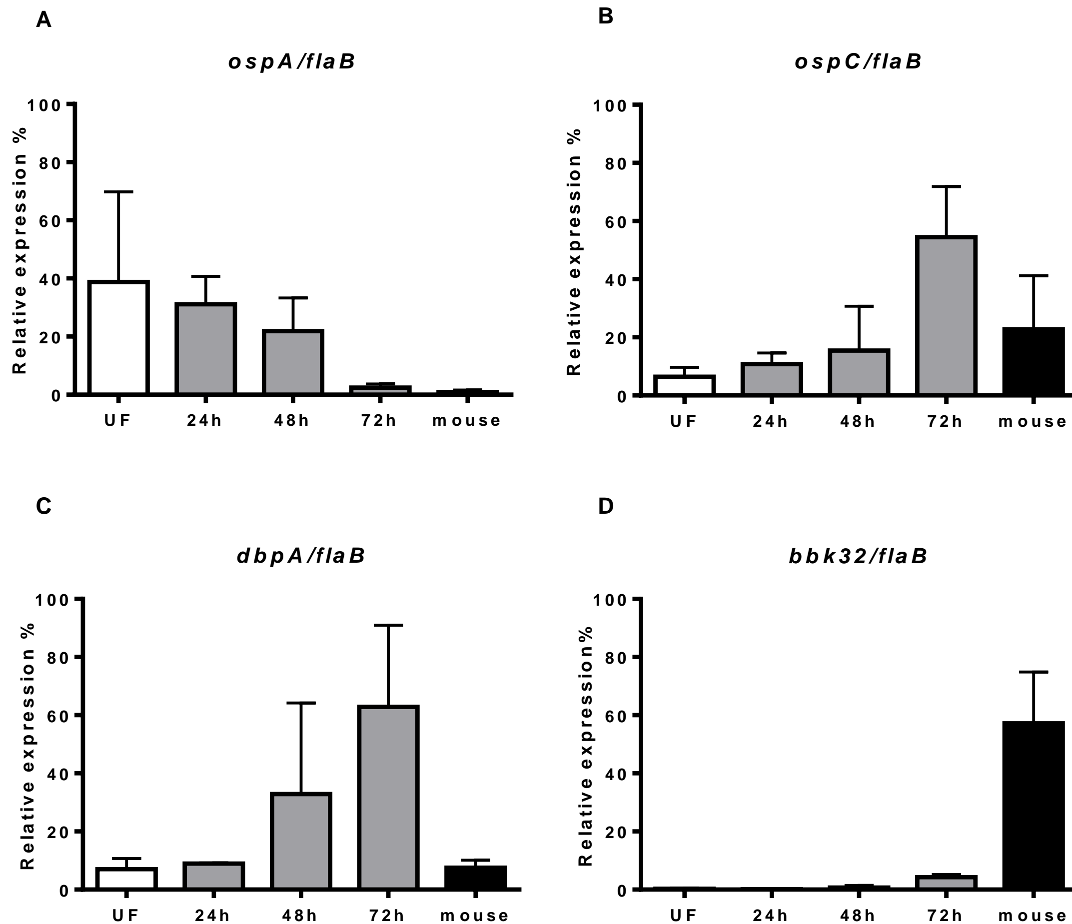

Fig. 1: *B. afzelii* gene expression during tick feeding and mouse infection. (A-D) qPCR analysis of *ospA*, *ospC*, *dbpA* and *bbk32* gene expression in *B. afzelii* spirochetes during tick feeding and mouse infection. **(UF)** unfed, *B. afzelii* CB43 infected nymphs, **(24h-72h)** *B. afzelii* CB43 infected nymphs detached from mouse at 1st-3rd day of the blood intake, **(mouse)** mouse with chronic *B. afzelii* CB43 infection, examined 4 weeks after detachment of infected ticks. Each datapoint represents mean of 3 individually analyzed samples. Error bars indicate standard error of mean.

4.2. Gene expression in *B. afzelii* spirochetes cultured at different temperatures

Prior research carried out on cultured *B. burgdorferi* spirochetes have documented massive modification of bacterial cell envelope in response to different environmental stimuli, including temperature.

To determine whether cultured *B. afzelii* spirochetes also change its gene expression in response to different temperature treatment, *B. afzelii* spirochetes were grown at 20 °C and 33 °C resembling the tick, and mammalian host environment.

Overall, all four analyzed *B. afzelii* genes including *ospA*, *ospC*, *dbpA* and *bbk32* showed differences in gene expression when treated at different temperatures. Whereas *ospA* was highly expressed in unfed ticks, and so at lower temperatures, increased temperature led to upregulation of *ospC, dbpA*, and *bbk*32, which are required for successful invasion of warm-blooded host **(Fig. 2).**

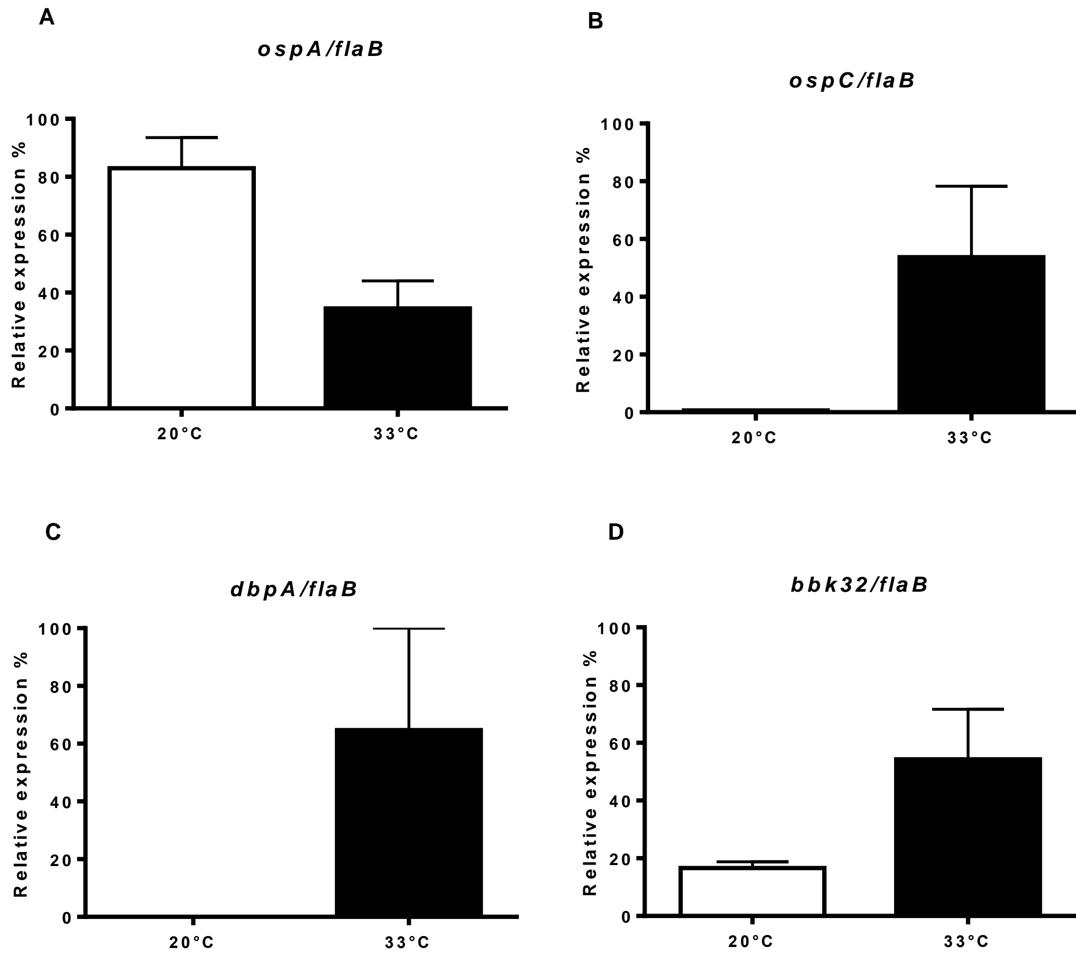

Fig. 2: Gene expression in *B. afzelii* spirochetes cultured at different temperatures. (A-D) qPCR analysis of *ospA*, *ospC*, *dbpA* and *bbk32* gene expression in *B. afzelii* spirochetes cultured at different temperatures **(20 °C and 33 °C)**. Each datapoint represents mean of 3 individually analyzed samples. Error bars indicate standard error of mean.

4.3. Gene expression in *B. afzelii* spirochetes extracted from *I. ricinus* nymphs incubated at different temperatures.

Former studies have reported that treating *B. burgdorferi* at increased temperatures led to increased transcription of genes responsible for *B. burgdorferi* infectivity (Popitsch et al., 2017b; Stevenson et al., 1995; Tokarz et al., 2004).

To investigate whether *B. afzelii* spirochetes also sense and act in response to increased temperature by altered gene expression even in vivo, spirochetes isolated from guts of unfed nymphs treated at 20 °C and 33 °C for 48 hours were analyzed by qPCR.

Similarly to cultured, temperature-treated spirochetes, spirochetes extracted from guts of unfed nymphs showed different phenotype. High levels of *ospA* and *dbpA* were expressed by spirochetes in ticks incubated at 20 °C, compared to lower *ospA* and *dbpA* levels in ticks kept at 33°C. On the contrary, increased levels of *ospC* and *bbk32* were detected in ticks maintained at 33°C, in comparison with ticks incubated at 20 °C **(Fig. 3).**

All together, these results imply that temperature shift related with blood meal uptake trigger the expression of particular genes governing infectivity of *B. afzelii* spirochetes.

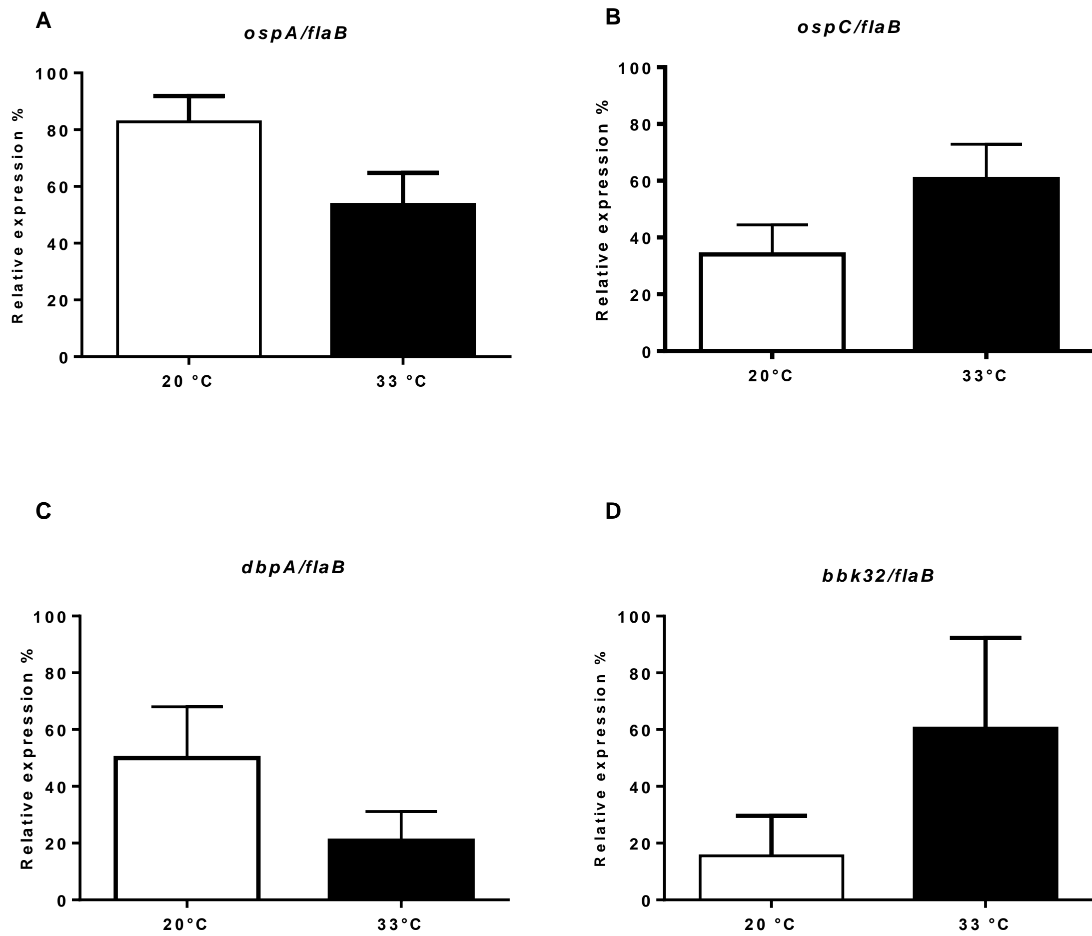

Fig. 3: Gene expression in *B. afzelii* spirochetes extracted from *I. ricinus* nymphs incubated at different temperatures. (A-D) qPCR analysis of *ospA*, *ospC*, *dbpA* and *bbk32* gene expression in *B. afzelii* spirochetes extracted from *I. ricinus* nymphs incubated for 2 days at different temperatures **(20 °C, 33 °C)**. Each datapoint represents mean of 3 individually analyzed samples. Error bars indicate standard error of mean.

4.4. *B. afzelii* infectivity in temperature stimulated ticks

Transmission efficiency and infectivity of *Borreli*a spirochetes increases with the length of the tick attachment. *Borrelia* infectivity is closely associated with massive transcriptional changes triggered by the tick feeding and intake of the warm blood (Ohnishi et al., 2001; Piesman, 1993). Results of our previous experiments have shown that increased temperature, resembling conditions of warm-blood-intake, leads to gene expression shift in *B. afzelii* spirochetes.

To test whether *B. afzelii* spirochetes in flat *I. ricinus* nymphs become infectious for mice solely by temperature shift, *B. afzelii* infected ticks were incubated at 33 °C for 48 hours. Unfed *B. afzelii* infected tick guts were then dissected and inoculated to mice (5 guts/ mouse). As a control, guts dissected from *B. afzelii* infected ticks incubated at 20 °C for 48 hours were injected into mice. 3 weeks after inoculation, infection was scored in murine tissues by PCR. Homogenates derived from temperature stimulated, *B. afzelii* infected *I. ricinus* nymphs caused infection in 2/5 mice. All 5 mice in the control group remained uninfected **(Tab 2).**

To sum up, our results show that treating *B. afzelii* infected ticks at elevated temperatures led to increased *B. afzelii* infectivity. Temperature stimulated *B. afzelii* spirochetes in unfed *I. ricinus* ticks were infectious for the vertebrate host, yet before the beginning of tick blood feeding. Moreover, our data support the hypothesis that increased temperature resembling the warm-blooded host environment is among the key stimuli sparking *Borrelia* infectivity.

Tab 2: *B. afzelii* CB43 infectivity in temperature stimulated ticks.

Tick incubation temperature	20 °C	33 °C
no. of *B. afzelii* infected mice / no. of inoculated mice	**0/5 (0%)**	**2/5 (40%)**

4.5. Immunization with *B. afzelii* infected tick guts

Borrelia spirochetes undergo complex cell remodeling during its transmission cycle resulting in the production of a great variability of *Borrelia* surface antigens enabling secret evasion of the host immune system. The most essential changes in *Borrelia* protein expression occur at the early stages of the tick feeding. For that reason, development of efficient vaccine targeting *Borrelia* single proteins is so problematic.

Therefore, in the following experiment, we applied different approach. Rather than vaccination of mice with a single spirochetal protein, the main goal of our experiment was to identify the crucial timepoint at which immunization with gut derived spirochetes protects the mice from the *B. afzelii* infection. In other words, to identify the right "cocktail" of spirochetal antigens suitable for mice immunization.

To that end, mice were immunized with guts from *B. afzelii* CB43 infected nymphs at different stages of feeding. (Preparation of tick gut antigen is described in the Methods). Mice in the control group were immunized with PBS + Freund's adjuvants. All vaccinated mice were then challenged with *B. afzelii* infected ticks. Infection was subsequently scored in murine ear biopsies by qPCR. Surprisingly, mice immunized with antigen prepared from unfed ticks were significantly protected 2 weeks post infestation, compared to the control group of mice vaccinated solely with PBS + Freund's adjuvants. Steep decrease of the spirochetal burden was also seen in mice vaccinated with guts from 24 hours fed, *B. afzelii* infected nymphs. On the contrary, mice immunized with gut extracts from 48 hours fed, *B. afzelii* infected nymphs did not seem to be protected at all (**Fig. 4**).

Overall, our results indicate that antigen derived from *Borrelia* infected guts might serve as an efficient vaccination target.

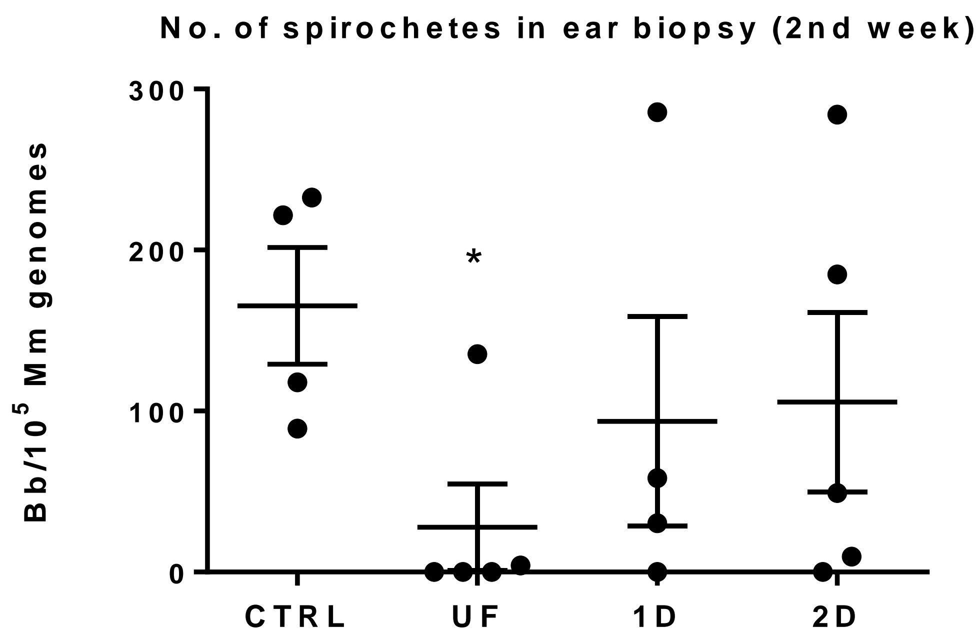

Fig. 4: Immunization with *B. afzelii* infected tick guts. Number of spirochetes in immunized mice 2 weeks after infestation of *B. afzelii* infected *I. ricinus* nymphs. **(CTRL)** mice immunized with PBS, **(UF)** mice immunized with guts from unfed, *B. afzelii* infected nymphs, **(1D, 2D)** mice immunized with guts from 24 and 48 hours fed, *B. afzelii* infected nymphs. Each column represents a mean number of spirochetes per 10^5 *M. musculus* genomes. *($P < 0.05$). Error bars indicate standard error of mean.

5. Discussion

Understanding the process of *Borrelia* transmission from the tick to the host is crucial for development of strategies preventing Lyme disease. Until now, most of the Lyme disease research has been performed in the USA using *I. scapularis* ticks, *B. burgdorferi* s.s. spirochetes and mice as vertebrate host. Research on the European *Borrelia* strains lags far behind the USA. To fill this information gap, we have recently implemented a reliable mouse transmission model for the European Lyme borreliosis involving *B. afzelii* spirochetes as the most common causative agent of borreliosis in Europe, and *I. ricinus* ticks. This allows us to investigate the interactions within the pathogen-vector-host triangle. Moreover, it enables us to study the pathogen acquisition, and to quantitatively track the growth kinetics as well as the infectivity of *B. afzelii* during *I. ricinus* life cycle. Our results can be then compared with data known for the *I. scapularis*/*B. burgdorferi* s.s. model. Finally, potential candidate vaccines can be tested using this *B. afzelii* transmission model.

B. burgdorferi requires coordinated mechanisms of gene regulation to survive throughout its enzootic cycle. Numerous studies provided evidence that differential expression of surface antigens plays a crucial role in *Borrelia* pathogenesis. Modulation of surface lipoproteins during the pathogen transmission from the tick to the mammal allows successful evasion of the host defense mechanisms and facilitates establishment of a permanent infection. The process of a massive cell remodeling is controlled by the Rrp2-RpoN-RpoS pathway which is activated by the tick feeding (Caimano et al., 2007). Being more specific, the most radical changes in *B. burgdorferi* s.s. gene expression and infectivity occur at the early stages of *I. scapularis* feeding (Iyer et al., 2015; Ohnishi et al., 2001). Similar pattern was observed also for *B. afzelii* transmitted by *I. ricinus*. Alike *B. burgdorferi* s.s., *B. afzelii* spirochetes in unfed *I. ricinus* nymphs produce high levels of OspA on its cell surface which is essential for its survival within the tick gut. Subsequently, as the tick feeding starts, *B. afzelii* spirochetes downregulate *ospA* production in response to altered physiological conditions in the tick gut, and activate expression of *ospC*, *dbpA*, *bbk32* and other key genes responsible for *Borrelia* infectivity.

Previous studies have also reported that *B. burgdorferi* s.s. spirochetes activate Rrp2-RpoN/RpoS pathway, and so change its gene expression and protein synthesis even in vitro in response to different environmental stimuli including temperature, and pH (Hyde et al., 2007; Popitsch et al., 2017a; Tokarz et al., 2004). Likewise, *B. afzelii* spirochetes grown at 33 °C (mimicking the mammalian host conditions) downregulate *ospA*, and upregulate *ospC*, *dbpA*

and *bbk32* expression. Whereas treating *B. afzelii* cultures at 20 °C (imitating the tick environment) causes *ospA* upregulation, and *ospC*, *dbpA* and *bbk32* downregulation. Moreover, analogous pattern in gene expression shift was observed also with *B. afzelii* spirochetes isolated from midguts of unfed *I. ricinus* nymphs incubated for 48 hours at 20 °C and 33 °C, respectively. *ospA* levels were high at 20 °C and low at 33 °C, whereas *ospC*, and *bbk32* levels increased with elevated temperature (33 °C). To sum up, spirochetes were activated in increased temperature. It indicates that temperature is one of the key signals responsible for alteration of transcriptional and polypeptide profiles of transmitted *B. afzelii*.

Undoubtedly, transmission efficiency and infectivity of *Borreli*a spirochetes increases with the length of the tick attachment (Ohnishi et al., 2001; Piesman, 1993). Transmission times and virulence primarily depend on the tick and *Borrelia* species (Cook, 2014). For instance, *B. afzelii* spirochetes seem to be transmitted earlier than *B. burgdorferi* (Crippa et al., 2002). Results of prior studies imply that *B. burgdorferi* spirochetes in unfed ticks are noninfectious when inoculated to mammals, and that *Borrelia* spirochetes become infectious for the mammalian host within the tick midgut at the early period of the tick feeding (Ohnishi et al., 2001; Piesman, 1993) . However, our experiment demonstrated that *B. afzelii* CB43 infectivity might be triggered even without the tick feeding, solely by unfed tick maintenance at elevated temperature (33°C). Surprisingly, homogenates derived from temperature stimulated, *B. afzelii* infected *I. ricinus* nymphs caused infection in 2/5 mice. Thus, our results are in contrast with Piesman (1993) who reported that incubation of unfed ticks at 37°C does not produce infectious homogenates (Piesman, 1993). Host infection was detected only if the inoculated nymphs had previously been attached to a host for at least 24 hours (Crippa et al., 2002; Piesman, 1993).

To sum up, our results show that treating *B. afzelii* infected ticks at increased temperatures led to increased tick/*B. afzelii* infectivity. Temperature stimulated *B. afzelii* spirochetes in unfed *I. ricinus* ticks are infectious for the vertebrate host, yet before the beginning of tick blood feeding. Moreover, our data support the hypothesis that increased temperature resembling the warm-blooded host environment is among the key stimuli sparking *Borrelia* infectivity. Importantly, our results indicate that yet tick temperature stimulation alone might successfully activate the expression of the key genes standing behind *B. afzelii* infectivity.

Until now, all vaccination attempts thwarting *Borrelia* transmission were unsuccessful (Schuijt et al., 2011; Steere et al., 1998). *Borrelia* spirochetes pursue sophisticated strategy of complex bacterial cell remodeling that generate vast variability of *Borrelia* surface antigens enabling secret evasion of the host immune system. For that reason, development of efficient vaccine targeting borrelial surface antigens is so problematic. To circumvent these obstacles,

we applied different vaccination strategy. Rather than immunization of mice with a single *Borrelia* recombinant protein, we prepared a complex antigen derived from *B. afzelii* infected tick guts at different stages of feeding and tested its ability to protect mice against *Borrelia* transmission. Surprisingly, mice immunized with antigen prepared from unfed, *B. afzelii* infected ticks were significantly protected against *B. afzelii* transmission, compared to the control group of mice vaccinated solely with PBS. Steep decrease in spirochetal transmission was also seen in mice vaccinated with guts from 24 hours fed, *B. afzelii* infected nymphs. Overall, our results indicate that it might be feasible to exploit antigen derived from *Borrelia*-infected guts as an efficient vaccination target.

However, in the following experiment, it will be essential to distinguish whether the protective immune response was developed against *B. afzelii* molecules, or against the tick gut itself. To that end, antigen derived from unfed, *B. afzelii* infected tick guts will be used for mice immunization to confirm the significant protection against *Borrelia* infection. In parallel, mice in the 2nd experimental group will be immunized with antigen derived from uninfected tick guts to see any potential effect on *Borrelia* transmission. Nevertheless, it actually appears that the protective immunity is developed rather against *Borrelia* antigens. The possible explanation why the protection rate in mice vaccinated with antigen prepared from 24 hours and 48 hours fed ticks decreases is that the number of *B. afzelii* spirochetes within the tick drops rapidly during the tick feeding (Pospisilova, 2016).

For efficient Lyme disease vaccine development, it is crucial to understand the extensive alteration of gene expression that *Borrelia* undergoes during its lifecycle. Clearly, the mechanisms of *B. burgdorferi* adaptation for survival in the tick vector are critical for environmental persistence as well as pathogenesis of the spirochete. However, the molecular events that contribute to these processes, particularly during mammalian infection have remained largely unknown. So far, there is still lack of *Borrelia* global transcriptomic RNA-seq studies performed in vivo. However, after having a closer look, the reason behind is quite clear.

Conducting a global *Borrelia* transcriptomic study in vivo bares numerous obstacles that have to be overcome to obtain reliable data. Firstly, it is difficult to extract enough *Borrelia* RNA from ticks. The RNA sample is always a mixture of sequences from different species (tick, mouse, and tick gut microbiome including *Borrelia* RNA). Consequently, abundant ribosomal RNA has to be removed from the total RNA. Next, Poly-A selection of contaminating eukaryotic RNA follows. Thus, at the end of purification procedure, low yields of bacterial RNA are harvested. Yet worse, subsequent amplification of bacterial RNA is undesirable as in quantitative gene expression studies preferential amplification of particular *Borrelia* sequences

might lead to data misinterpretation. Moreover, number of spirochetes within the tick gut decrease during feeding which makes the *Borrelia* RNA purification from the tick even more difficult. Overall, obtaining sufficient amount of high quality *B. burgdorferi* RNA from in vivo sources necessary for robust transcriptome analysis present a formidable technical barrier. Furthermore, the data obtained from RNA-seq might be difficult to identify, align, and interpret as there is always an RNA mixture of different bacterial species.

Microarray and Chip hybridization analysis possess an alternative to global RNA-seq studies. The main advantage is low RNA input requirement. For instance, SurePrint G3 Human Gene Expression Microarray (Agilent Technologies) require down to 10 ng for 8x60K formats and 25 ng for all other formats. In comparison, a minimum of 100 ng rRNA-depleted RNA is required for transcriptome sequencing (https://www.seqme.eu/documents/ngs-ssg-en.pdf). Elucidation of *Borrelia* complete genome sequence (Fraser et al., 1997) enables production of the whole genome arrays. Only specific *Borrelia* sequences should hybridize on the designed microarray platform, and so only the signal of hybridized sequences is examined. Self-hybridization and cross-hybridization is a potential concern with this procedure.

In conclusion, performing a global in vivo *Borrelia* transcriptomic analysis would be highly beneficial for gaining further insights into *Borrelia* gene regulation machinery. Next, better understanding of a complex *Borrelia* gene regulation should help us to subsequently target the particular gene expression changes responsible for *Borrelia* infectivity occurring during the first 24-48 hours of the tick feeding (Pospisilova, 2016). Finally, identification and disruption of crucial points of *Borrelia* regulatory pathways should lead to an ultimate development of efficient vaccine thwarting *Borrelia* transmission.

6. Conclusion

Analysis of *B. afzelii* gene expression during tick feeding and during chronic infection of mice has shown that increased temperature resembling conditions of the tick blood-intake leads to gene expression shift in *B. afzelii* spirochetes. Importantly, similar gene expression pattern was induced even by *B. afzelii* culture incubation at 20 °C and 33 °C, respectively, and also by maintenance of whole ticks at corresponding temperatures. Importantly, our results indicate that yet temperature stimulation alone might successfully activate the expression of the key genes standing behind *B. afzelii* infectivity.

Subsequent transmission experiment, which tested the effect of temperature stimulation on *B. afzelii* infectivity, demonstrated that *B. afzelii* infectivity might be triggered even without the tick feeding, solely by unfed tick maintenance at increased temperature (33 °C). Thus, elevated temperature increases *B. afzelii* infectivity.

Furthermore, immunization with antigen prepared from unfed, *B. afzelii* infected tick guts significantly protected mice against *B. afzelii* infection.

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