University of South Bohemia in České Budějovice

Faculty of Science

# Identification of physical and chemical factors regulating gene expression and infectivity of the Lyme disease spirochetes

Master thesis

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**Master thesis**

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**Annotation**

*Borrelia afzelii* is one of the main Lyme disease causative agents. It is known that differential gene expression during the *Borrelia* life cycle can relate to infectivity for mammals, including humans. However, just a little is known about what affects these changes in expression. Here, we investigate the effect of temperature and common blood nutrients on *Borrelia afzelii* differential gene expression and transmission efficiency while stimulating the ticks with chosen substances.

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I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 10. 4. 2022 …….

Bc. Veronika Pavlasová

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**List of abbreviations**

| | |
|---|---|
| AA | amino acids |
| ABC | ATP-binding cassette |
| ATP | adenosine triphosphate |
| BBK32 | fibronectin-binding protein |
| BMD | *Borrelia miyamotoi* disease |
| bp | base pair |
| CDC | Center for Disease Control and Prevention |
| cDNA | complementary deoxyribonucleic acid |
| CNS | central nervous system |
| CTRL | control |
| DNA | deoxyribonucleic acid |
| G+/G- | Gram-positive/Gram-negative |
| Glc | glucose |
| GlcNAc | *N*-acetylglucosamine |
| Gly | glycine |
| kb | kilobase |
| LB/LD | Lyme borreliosis/Lyme disease |
| Mb | megabase |
| mRNA | messenger RNA |

| | |
|---|---|
| OspA | outer surface protein A |
| OspC | outer surface protein C |
| O/N | overnight |
| PBS | phosphate buffer saline |
| PCR | polymerase chain reaction |
| pmol | picomole |
| Pro | proline |
| PTS | phosphotransferase system |
| qPCR | quantitative polymerase chain reaction |
| qRT-PCR | quantitative reverse transcription polymerase chain reaction |
| RF | relapsing fever |
| RNA | ribonucleic acid |
| RT | room temperature |
| Salp15 | salivary protein 15 |
| SEM | standard error of mean |
| *s.l./s.s.* | sensu lato/sensu stricto |
| STARI | Southern Tick-Associated Rash Illness |
| TBEV | Tick-borne encephalitis virus |
| TBRF | Tick-borne relapsing fever |
| TROSPA | tick receptor for OspA |

# Table of contents

# 1 Introduction

## 1.1 Ticks

Ticks are obligate blood-feeding ectoparasites that can feed on a wide range of vertebrates, including mammals, birds, and reptiles. Systematically, these arachnids belong among the Parasitiformes, the order Ixodida. Recently, three families of ticks were established, Argasidae, Ixodidae, and Nuttalliellidae (Sonenshine & Roe, 2013). Argasidae and Ixodidae are the most represented ticks in the world, while Nuttalliellidae include only one known species, *Nutalliella namaqua* (Beati & Klompen, 2019).

### 1.1.1 Argasidae

Argasidae, or soft ticks, include the genera Antricola, Argas, Nothoaspis, Ornithodoros and Otobius (Guglielmone et al., 2010). Unlike hard ticks, they do not have a chitinized scutum on the dorsal side, and their round-shaped body is covered with a leathery cuticle. These ticks have multiple nymphal instars (multi-host life cycle) and do not mate on the host. Females can repeatedly feed on different hosts for short periods (minutes) and lay hundreds of eggs after each meal (Sonenshine & Roe, 2013).

### 1.1.2 Ixodidae

The genera Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Ixodes, and Rhipicephalus (Guglielmone et al., 2010) belong to the family Ixodidae (hard ticks). Members of the Ixodidae family are characterized by marked sexual dimorphism and only one nymphal stage. Mating occurs on the host, with the exception of the genus Ixodes, whose representatives commonly mate off the host. In contrast with soft ticks, hard ticks feed for several days on one host and then lay thousands of eggs (Sonenshine & Roe, 2013).

### 1.1.3 *Ixodes ricinus* lifecycle

The life cycle of a tick can be one-host, two-host, and three-host. The life cycle of the most common European tick *Ixodes ricinus* includes an egg stage and three subsequent active developmental stages: larva, nymph, and adult (**Fig. 1**). In nature, the lifecycle lasts about two to three years. Each active stage feeds only once and on a different host each time (three-host life cycle). Larval and nymphal feeding takes approximately 3 days, adult females feed for longer (7-10 days). After the blood meal, the tick molts into the next stage. While larvae and nymphs feed mainly on small animals (rodents, birds, reptiles), the adult females feed on large

mammals (deer, roe deer, wild boars). The female can imbibe up to 1 ml of blood and increase in size up to 100 times. After fertilization, the female lays thousands of eggs and dies. Adult males do not suck blood, their only mission is to fertilize the female (Sonenshine & Roe, 2013).

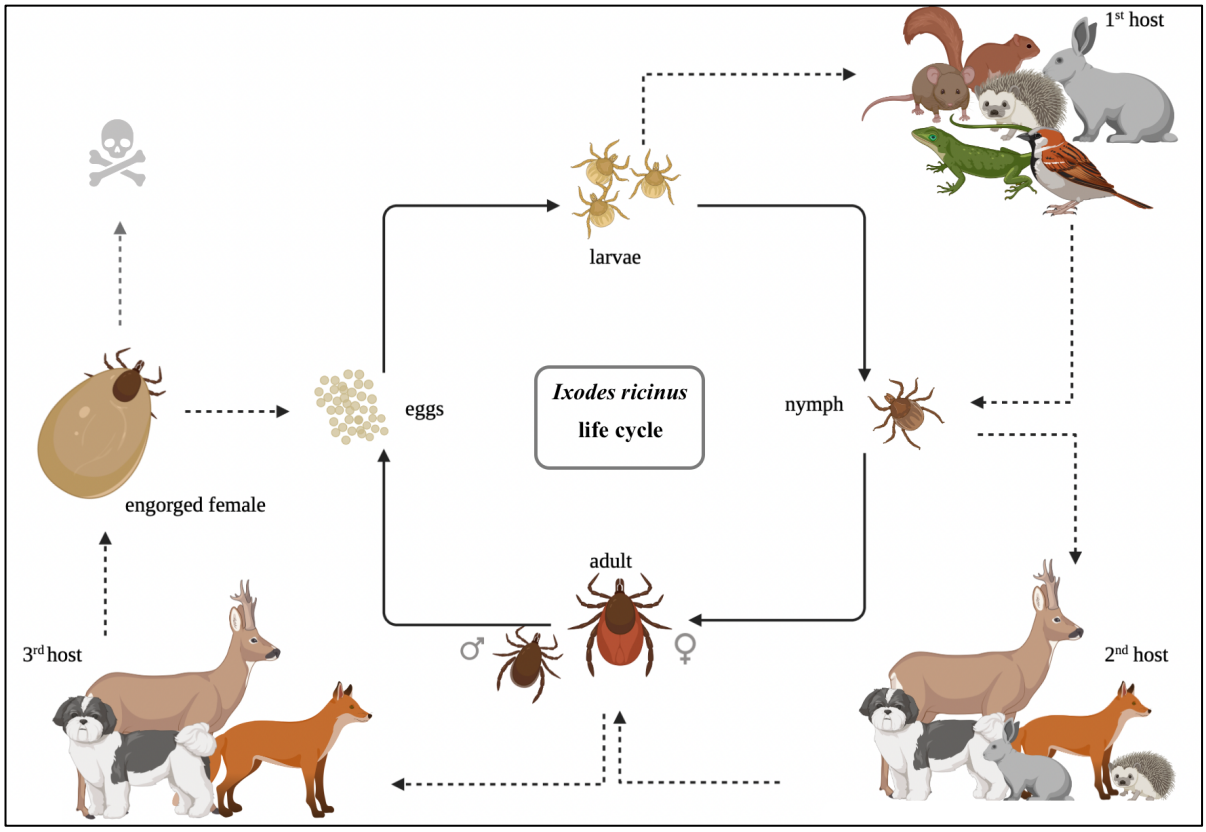


**Fig. 1: *Ixodes ricinus* three-host lifecycle.** Larval ticks usually feed on small vertebrates. After repletion, larvae detach and molt into nymphs that feed on another host, usually again a smaller vertebrate. Both, larval and nymphal feeding last about three days. Nymphs then molt into adults. The adult stage has a marked sexual dimorphism, females are larger and feed on blood, males are smaller and do not feed but actively search for females. Fertilization can occur both, on and off the host and is essential for full repletion of females. The females then lay about a thousand eggs and die (Sonenshine & Roe, 2013). Created with BioRender.com.

### 1.1.4 Tick transmitted pathogens

Ticks are well-known vectors of zoonotic pathogens. Tick-borne pathogens include viruses (e.g. tick-borne encephalitis virus and Powassan virus); bacteria causing Lyme disease, spotted fever rickettsiosis, and human anaplasmosis; or protozoan parasites causing babesiosis. Tick-borne diseases are emerging due to the geographical expansion of their tick vectors, particularly in the Northern Hemisphere (Rochlin & Toledo, 2020). Personal

protection has been recommended as the best defense against tick-borne diseases. Targeting and reducing the deer or rodent populations are the only available means of area-wide control to reduce tick populations, and therefore the risk of tick bite infection. Treatment with topical acaricides has not resulted in sustained reductions in tick populations and the incidence of tick-borne diseases in humans (Eisen et al., 2012; Grear et al., 2014).

A body of evidence shows that ticks have established a special bond with some of the pathogens during their coevolution, and this connection might be beneficial for both partners. It has previously been described that *Borrelia* infected ticks survive unpleasant conditions better, accumulate more fat reserves, have better survival rates and live longer (Herrmann et al., 2013; Herrmann & Gern, 2010, 2012). But not only *Borrelia*, also other pathogens share some positive reciprocity with ticks. Namely *Anaplasma phagocytophillum* has been shown to induce antifreeze glycoprotein and heat-shock proteins that increase tick survival (Neelakanta et al., 2010). Moreover, TBEV infected ticks have been shown to seek out humid environments to survive in dry conditions (Alekseev, 1996).

It is known that pathogens can target gene expression in ticks and subsequently use the products of these genes for survival and transmission. An example is Salp15, which is essential for the survival of *Borrelia burgdorferi* in the tick *Ixodes scapularis* (Ramamoorthi et al., 2005). But modulation of tick gene expression occurs in almost every tick-pathogen system, reviewed by Hajdušek et al., 2013. Successful targeting of these linkages could solve the growing problem of tick-borne diseases in the future.

## 1.2 Lyme disease

Lyme disease (borreliosis) is the most common vector-borne anthropozoonosis caused by different genospecies of *Borrelia burgdorferi sensu lato* complex. According to the Center for Disease Control and Prevention (CDC), the incidence of this disease is increasing in many countries.

In the United States, the main causative agent of Lyme disease is *Borrelia burgdorferi sensu stricto* (*s.s.*) transmitted by *Ixodes scapularis*, or *Ixodes pacificus* ticks, while in Europe, *Borrelia afzelii* and *Borrelia garinii*, transmitted by *Ixodes ricinus* ticks, are the main causative agents of the disease (Marques et al., 2021). In connection, this heterogeneity mirrors different clinical manifestations of Lyme disease due to host specialization and tissue tropism. In detail, *B. burgdorferi s.s.* is mainly associated with arthritis and neuroborreliosis, *B. afzelii* is characterized by chronic skin manifestations, and *B. garinii* causes neuroborreliosis

(Trevisan et al., 2020). The mechanism by which infection with different *Borrelia* species can lead to different clinical symptoms is unknown, but for example, differences in complement susceptibility may play a role. While *B. afzelii* is a resistant genospecies when it comes to contact with human complement, *B. garinii* is cleared within minutes, and *Borrelia burgdorferi s.s.* is considered a moderately resistant species that can survive in the blood for several days (Kraiczy et al., 2001). These differences in infectivity are probably related to the host spectrum of various *Borrelia* strains, for example, *B. afzelii* is preferentially found in mammals, whereas *B. garinii* is an avian specialist. *B. burgdorferi s.s.* can infect both, mammals, and birds.

In summary, the symptoms of Lyme disease include early localized infection of the skin (erythema migrans), progressive disseminated infection of the nervous system (neuroborreliosis), skin (acrodermatitis chronica atropicans), heart (Lyme carditis), and joints (Lyme arthritis). Other manifestations of Lyme borreliosis, such as borrelial lymphocytoma on the ear lobe and meningoradiculoneuritis also support the diagnosis (Stanek & Strle, 2018; Trevisan et al., 2020).

### 1.2.1 Vaccination against Lyme disease

Currently, there is no human vaccine against Lyme disease. First attempts to find one universal vaccination target failed due to the high heterogeneity of molecules on the surface of *Borrelia*. In their life cycle, *Borrelia* alternate between two completely different environments, the tick gut, and vertebrate tissues. Therefore, it is difficult to find a single molecule that is constantly expressed throughout the whole life cycle. The situation is further complicated by the existence of different species of *Borrelia*, especially in Europe.

The traditional approach is to target antigens on the surface of *Borrelia*, such as OspA. But other alternative strategies have been also tested, such as targeting tick proteins that *Borrelia* need for transmission. Most of these alternative studies targeted tick salivary or midgut proteins. These vaccines do not target *Borrelia* itself but prevent transmission by reducing the attachment time of the infected ticks or reducing the efficiency of digestion (reviewed by de la Fuente et al., 2016). It has previously been shown, that *Borrelia* spirochetes require at least 24 hours to migrate from the tick gut to the tick bite site. Thus, reducing the tick attachment time might be a promising strategy in combating Lyme borreliosis (Cook, 2014; Pospisilova et al., 2019).

In 2017, a multivalent VLA15 vaccine targeting borrelial OspA was announced. This vaccine is designed to protect people against both American as well as European strains of Lyme disease spirochetes and combines six different OspA serotypes linked together (Nayak et al., 2020). It is currently the only candidate vaccine in clinical trials.

In 2020, another potential vaccine candidate, combining fused OspA with ferritin from *Helicobacter pylori*, was proposed. In contrast with VLA15 vaccine, which uses synthetic OspA fusion protein, this vaccine uses OspA that matches the native antigens of naturally occurring strains (Kamp et al., 2020).

Recent study has shown a different approach for vaccine development. The first formulation of an mRNA vaccine against the salivary proteins of the tick *Ixodes scapularis*. This vaccine is not directed against *Borrelia* spirochetes, but against the tick itself, and prevents transmission by promoting detachment of the tick. In this vaccination study, a combination of mRNAs encoding 19 salivary proteins encapsulated in lipid nanoparticles was tested in guinea pigs (Sajid et al., 2021).

### 1.2.2 Other tick-borne *Borrelia* related diseases

In addition to Lyme disease, *Borrelia* spirochetes also cause tick-borne relapsing fever (TBRF), and *Borrelia miyamotoi* disease (BMD, sometimes called hard tick relapsing fever). A third disease, Southern Tick-Associated Rash Illness (STARI), is often assigned to these illnesses, but the causative agent still waits to be described (Fong, 2017).

TBRF is a zoonotic disease caused by various species of *Borrelia*, *Borrelia hermsii*, *Borrelia parkeri*, and *Borrelia turicatae,* which are transmitted by ticks of the genus *Ornithodoros* worldwide. Relapsing fever is characterized by recurrent episodes of fever, and other, nonspecific symptoms, such as headache, arthralgia, shaking chills and abdominal distress. *Borrelia* cause massive, microscopically visible bacteremia that stimulates the immune system and causes febrile responses in patients (usually 3 days of fever followed by 7 days without fever) (Dworkin et al., 2008).

*Borrelia miyamotoi* was first described in 1995 when isolated from *Ixodes persulcatus* ticks. It was later detected in other tick species, such as *I. scapularis*, *I. pacificus*, and *I. ricinus*. It was originally classified as relapsing fever spirochete, but it was later shown, that *B. miyamotoi* has similar symptoms to Lyme disease. In 2011, first human case with symptoms resembling Lyme borreliosis, including fever, and flu-like symptoms, was described. After evaluation of some *B. miyamotoi*-suspected samples, additional cases have been reported in

the Netherlands, United States, France, Belgium, England, and Japan, counting hundreds of known cases (Kubiak et al., 2021).

In the same decade as BMD, another Lyme-like illness transmitted by *Amblyomma americanum* occurred. As the symptoms are very similar to Lyme disease, it was assumed that spirochetes of the *Borrelia* genus, specifically *Borrelia lonestari*, could possibly be the cause, but this has not yet been confirmed (Feder et al., 2011). The most obvious symptoms of Southern Tick-Associated Rash Illness (STARI) are erythema migrans, fatigue, fever, muscle and joint pain, but unlike Lyme disease, there are no chronic symptoms, such as arthritis, or neuroborreliosis (K. L. Clark et al., 2013).

## 1.3 *Borrelia*

The genus *Borrelia* belongs to the eubacterial phylum *Spirochaetes* and contains tick- or louse-borne spirochetes belonging to the Lyme borreliosis (LB) group, the relapsing fever (RF) group, or to the group of spirochetes that form intermittent clades (Margos et al., 2018; Tilly et al., 2008). LB and RF spirochetes share a common set of genetic and biological characteristics, namely they all share an obligate parasitic lifestyle, are transmitted between vertebrate hosts by arthropod vectors and can be transstadially transmitted within their arthropod vectors (**Fig. 2**). The genus *Ixodes* primarily transmits LB species, whereas Argasid ticks often vector the RF group, with a few exceptions, e. g. *Borrelia miyamotoi* is associated with *Ixodes* ticks. The third group is associated with reptile and echidna hosts and do not cluster with LB or RF group (Fukunaga & Koreki, 1995; Loh et al., 2017; Margos et al., 2018).

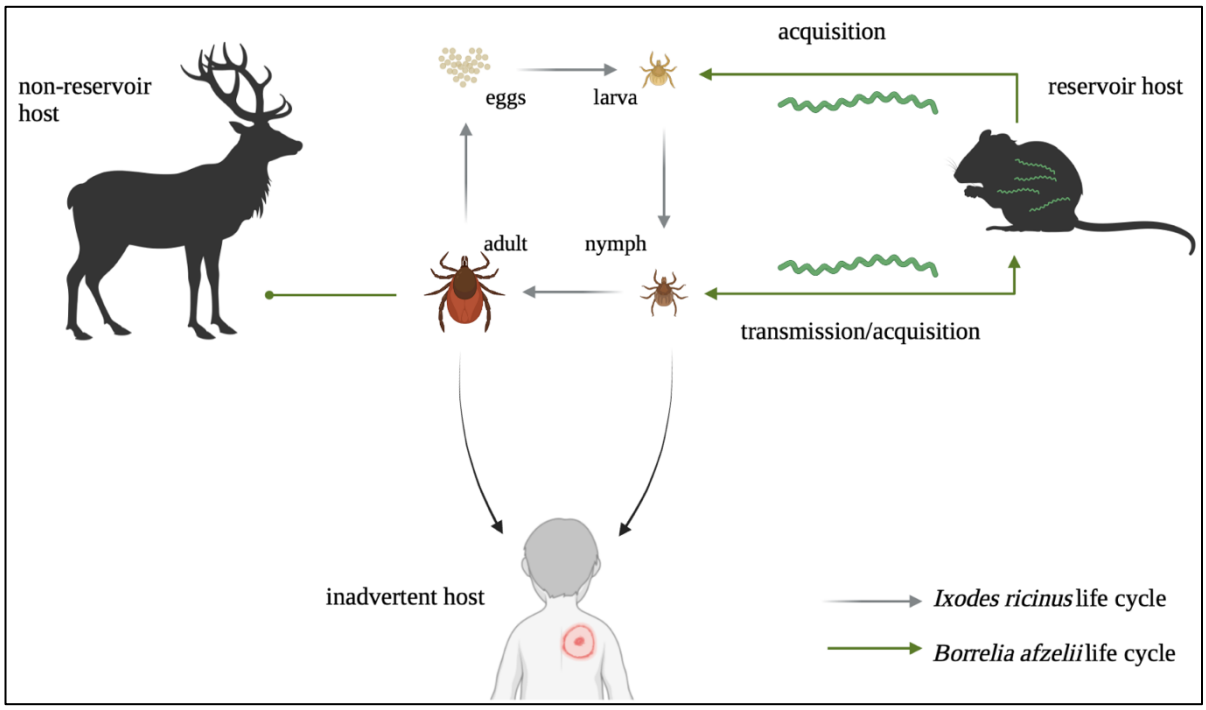


**Fig. 2: Lyme disease transmission.** Transovarial transmission of Lyme disease spirochetes is not likely (Rollend et al., 2013). Larval or nymphal ticks acquire *Borrelia* during feeding on infected reservoir host. After molting, *Borrelia* spirochetes are transmitted by infected nymphs or adult ticks to other reservoir hosts and to humans, who are unwilling hosts. Deer are important hosts for adult ticks but are not effective reservoirs. Created with BioRender.com.

### 1.3.1 *Borrelia* acquisition by feeding ticks

Larval or nymphal ticks acquire *B. burgdorferi* during blood feeding within the first 36 hours of attachment to an infected host (Couret et al., 2017; Hodzic et al., 2001). Spirochetes migrate from the murine dermis and enter the tick. Here, the spirochetes can be detected by qRT-PCR within the first day of tick attachment (Promnares et al., 2009).

After colonization of the tick gut, *Borrelia* persist in the gut lumen till the next blood meal. It is still not yet fully understood how *Borrelia* survive in the tick during molting and in the period until the next feeding. The lumen of unfed tick gut contains just a few nutrients. Most nutrients are stored in cellular endosomes of the gut epithelium, where they are inaccessible to extracellular pathogens (Sonenshine & Roe, 2013).

### 1.3.2 *Borrelia* transmission from tick to host

Soon after the infected tick starts feeding on the host, the *Borrelia* in the gut become activated and prepare to invade the host. The complete mechanism of activation has not been fully elucidated; however, it is known that triggering factors include temperature (Stevenson et al., 1995), pH (Ramamoorthy & Scholl-Meeker, 2001), osmolarity (Bontemps-Gallo et al., 2016), and possibly components of human blood. However, the overall knowledge of the nutritional factors triggering activation of *Borrelia* is surprisingly sparse.

At present, two models of *Borrelia* transmission from infected tick to the host have been suggested. *B. burgdorferi* is probably transmitted through the gut-salivary gland route and utilizes some of the tick salivary proteins during the transmission (Dunham-Ems et al., 2009). An alternative model of transmission has recently been proposed for *B. afzelii*. Unlike *B. burgdorferi*, *B. afzelii* avoids salivary glands and is transmitted directly via the gut-to-mouthpart route (Pospisilova et al., 2019). Interestingly, *B. afzelii* spirochetes are able to invade their host without the support of tick saliva, suggesting that the tick thus may serve as a mere vehicle that mediates bridging between tick and host tissues, where *Borrelia* eventually disseminate and cause disease (Perner et al., 2022).

### 1.3.3 Cell envelope

*Borrelia* is a coil-shaped bacteria with an average length of 20 μm and a diameter of 0.2 μm. However, individual spirochetes may vary in length, diameter, tightness, and regularity of the coils (Anguita et al., 2003). The flagella are localized in the periplasmic space between the outer and inner membrane (Meriläinen et al., 2015), while the filaments are connected at each cell pole to linearly arranged flagellar motors (7-11 at each end), converting chemiosmotic potential into motive force (Liu et al., 2009). This allows the spirochete to move relatively quickly through the viscous environment and invade host tissues being unseen by the vector or host immune system. Soon after entering the host body, spirochetes are seen interacting with capillaries and veins. They rely on dynamic genetic regulation and antigenic variability to invade multiple tissue types and evade the immune system, while the cell-forming peptidoglycan protects *Borrelia* from osmotic stress during migration from the tick midgut to the vertebrate host (DeHart et al., 2021). The roles of motility and chemotaxis are likely important in both, the adherence and penetration through blood vessels, and the interaction with immune cells in the liver (Charon et al., 2012; Moriarty et al., 2008).

*Borrelia* lack many of the classical virulence factors typically associated with pathogens, such as lipopolysaccharide, toxins, and a specialized secretion system (Tilly et al., 2008). Even though spirochetes might be weakly stained as Gram-negative bacteria, they are neither G+ nor G- due to the absence of lipopolysaccharides and their unusual cell wall. Their major lipid constituents are phosphatidylcholine, phosphatidylglycerol, and highly immunogenic but non-inflammatory glycolipids (Radolf et al., 2012).

### 1.3.4 Genome

The *Borrelia* genome consists of a linear chromosome (~1 Mb), and many plasmids, some of which are linear and others circular (approximately 600 kb in total), which is unusual in the bacterial world (Baril et al., 1989; Hyde & Johnson, 1988). Despite the atypical form of DNA, most of the genes encoded on the chromosome are common to the genomes of other bacteria. In contrast, the genes encoded on plasmids appear to be unique to the genus *Borrelia* (Fraser et al., 1997). The plasmids, encoding most of the differentially expressed outer-surface lipoproteins, show great variability, and are not equally represented in all strains. The high genetic diversity of *B. burgdorferi s.l.* is partly responsible for the multiple clinical manifestations in humans (Coipan et al., 2016; Crowder et al., 2010). Other experiments have also shown that some of the plasmids can be lost during multiplication of the bacteria in vitro (Barbour, 1988; Schwan et al., 1988).

### 1.3.5 Regulation of gene expression

*Borrelia* spirochetes can persist in the tick-host life cycle due to their ability to adapt to the environment in which they reside. The ability to respond to variable conditions, changes in pH (Carroll et al., 2000; Ramamoorthy & Scholl-Meeker, 2001), temperature (Schwan et al., 1995; Stevenson et al., 1995), cell density (Indest et al., 1997; Ramamoorthy & Philipp, 1998), or host factors (Liang et al., 2002; Revel et al., 2002), relates to the regulation of gene expression. In vitro experiments mimicking temperature-induced changes during tick attachment and reduced pH revealed altered expression of several genes encoding outer surface proteins of *Borrelia* spirochetes (Ramamoorthy & Scholl- Meeker, 2001). However, the regulation seems to be complex and multifactorial. Also, little is known about the key mechanisms that govern spirochete gene regulation in vivo.

Assumed, *Borrelia* spirochetes are adapted to exploit minor changes in expression of regulatory genes that subsequently affect expression of downstream genes. Thus, a relatively small alteration in gene expression controls the production of a large number of target lipoproteins (Revel et al., 2002).

### 1.3.6 Changes in outer surface proteins during tick feeding

The outer surface of *Borrelia* is covered with many proteins, that help spirochetes survive in their environment. *Borrelia* spirochete changes two distinct environments during its life cycle, lumen of the tick gut, and tissues of the vertebrate host. By differential expression of surface proteins, the spirochete can flexibly adapt to the specific conditions of these two environments.

It has been documented, that during colonization of the tick gut, *Borrelia* spirochetes preferentially express OspA. It is synthesized when spirochetes are acquired by larval ticks from an infected vertebrate and coats the spirochetes from the time they enter the tick until they are transmitted to the next host. OspA is essential for successful colonization of the tick gut and protects the spirochetes from mammalian antibodies (Battisti et al., 2008). The tick receptor for OspA (TROSPA), firstly identified in *Ixodes scapularis*, ensures the adherence of *Borrelia* to the midgut surface (de Silva et al., 1996). The receptor is more abundantly synthesized in *Borrelia*-infected ticks, silencing of the *trospa* gene showed that the receptor is crucial for successful colonization and transmission of *Borrelia* (Pal, Li, et al., 2004). Moreover, *ospA* deletion mutants can be transmitted from infected mice to ticks but are not able to survive within the tick gut, demonstrating the key role of OspA in colonization of the tick gut (Yang et al., 2004).

When the infected tick starts to feed on the next host, *Borrelia* begin to synthesize OspC. Pioneering studies suggested that incoming blood meal, changes in pH and temperature lead to downregulation of *ospA* and upregulation of *ospC*, which is necessary for successful transmission (Pal, Yang, et al., 2004; Ramamoorthy & Scholl-Meeker, 2001; Schwan et al., 1995). Simultaneously, the ticks downregulate the production of TROSPA, which helps spirochetes to detach from the midgut. Spirochetes then travel through the hemolymph and salivary glands to infect the next host. However, later studies have shown, that there is no reciprocity in *ospA*/*ospC* expression, and even during tick feeding, *ospA* expression is maintained at detectable level (Dunham-Ems et al., 2012; Ohnishi et al., 2001). However, there is one thing that previous studies agree on. When *Borrelia* spirochete enters

the host, *ospC* expression is gradually downregulated as it is highly immunogenic. Moreover, when *Borrelia* establish stable infection in the host, OspA and OspC are hardly detectable. Instead, these molecules are replaced by others that help spirochetes to survive in a vertebrate host.

One of the proteins that help *Borrelia* to survive in the host is BBK32, which binds host fibronectin; it therefore plays an important role in the attachment of spirochetes to the extracellular matrix. BBK32 is associated with host infection but is not detectable in tick or *Borrelia* cultures (Probert & Johnson, 1998). It has been suggested that this protein has no role in acquisition or transmission of spirochetes but might have a partial role in host colonization. Silencing of *bbk32* had no effect on survival in the host (Li et al., 2006). In contrast, antibodies against BBK32 displayed protective immunity in mice (Probert & Johnson, 1998). Moreover, BBK32 inhibits the classical pathway of complement by blocking the activation of the C1 complex, which leads to higher resistance to host innate immunity and dampens the response of adaptive immune systems, because complement also promotes B-cell differentiation and T- cell immunity (Garcia et al., 2016).

### 1.3.7 Rrp2-RpoN-RpoS and Hk1-Rrp1 pathways

The regulation of gene expression in *Borrelia* is very complex. More than 10 % of protein-encoding genes are transcribed by an alternative RNA polymerase σ-factor, RpoS, which is transcribed by another alternative σ-factor, RpoN. This pathway modulates the differential expression of genes required during the vector-host phase, including *ospA*, and *ospC* (Caimano et al., 2016; Xu et al., 2010). However, the precise mechanism by which the upstream activator and response regulator Rrp2 is activated remains unclear.

Another gene regulation pathway during the *Borrelia* enzoonotic cycle, Hk1-Rrp1, has putative function in carbon metabolism, spirochete cell envelope maintenance, and adaptation to the vector or host environment. Creation of mutants lacking Hk1 resulted in normal survival in the tick gut or establishment of infection in inoculated mice. On the other hand, *Borrelia* Hk1 mutants inoculated into mice were not able to establish infection in feeding larvae (Caimano et al., 2011).

Therefore, spirochetes in unfed nymphs have both Rrp2-RpoN-RpoS and Hk1-Rrp1 pathways inactive, host-phase genes (*ospC, bbk32*) are repressed, but tick-phase genes (*ospA*) are maximally expressed (**Fig. 3**). Feeding serves as an activator of both the Rrp2-RpoN-RpoS and Hk1-Rrp1 pathways. The genes required for transmission are expressed, while tick-stage

genes become repressed. In addition, genes regulated independently on RpoS system are also induced, e.g., genes dependent on RpoD. RpoD is a housekeeping sigma factor regulating motility and, for example, synthesis of the flagellin gene, *flaB* (Ge et al., 1997). At this stage, Hk1-Rrp1 must be activated to protect *Borrelia* from elimination at the beginning of feeding. During infection of the mammalian host, Rrp2-RpoN-RpoS pathway is activated to promote the expression of genes required in the mammalian host, while repressing genes required in the tick, for example *bbk32*. Hk1-Rrp1 is inactivated during host infection (Caimano et al., 2011; He et al., 2011).

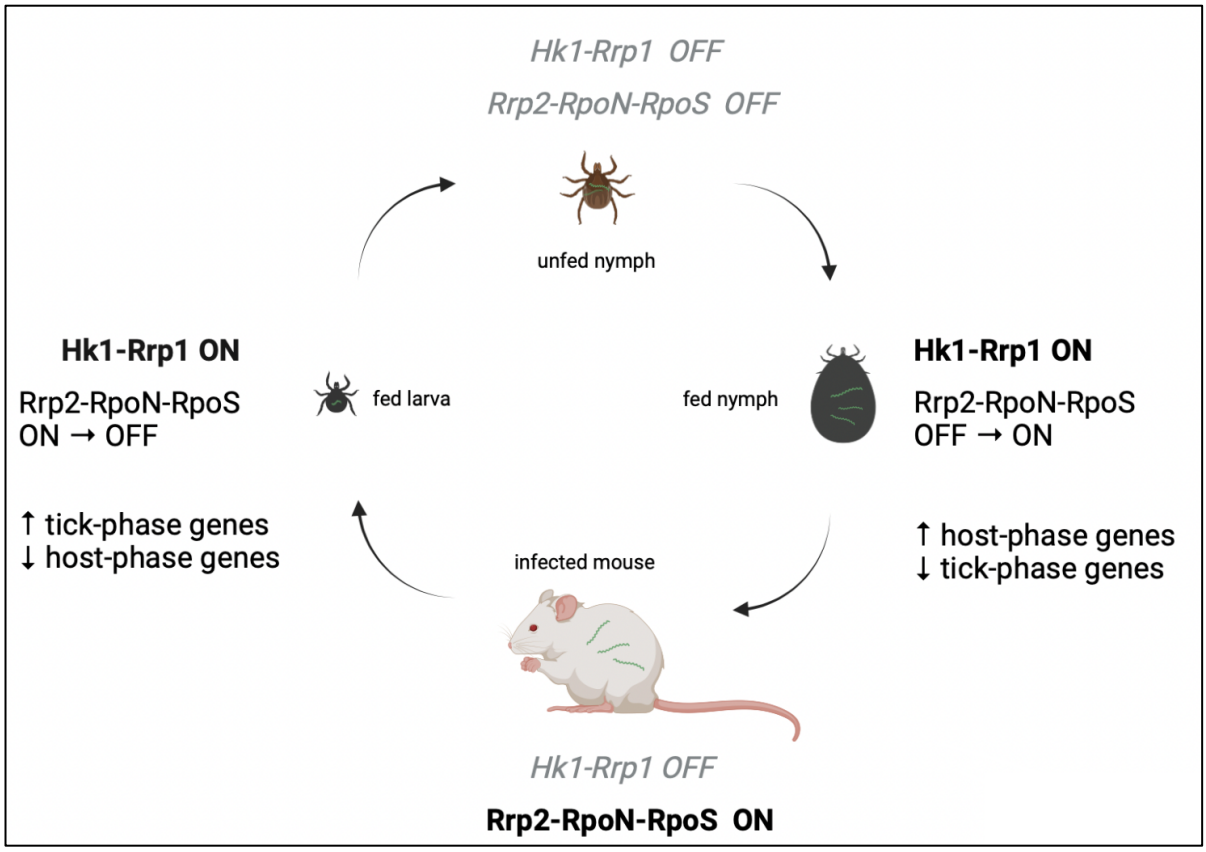


**Fig. 3: Changes in Rrp2-RpoN-RpoS/HK1-Rrp1 system during borrelial life cycle.** In unfed nymphs, both pathways are "OFF", leading to expression of tick-phase genes (*ospA*). Once the nymph starts feeding on infected mouse, Hk1-Rrp1 pathway is "ON", and Rrp2-RpoN-RpoS is slowly getting activated to start the expression of host-phase genes (*ospC*, *bbk32*). Once the spirochete reaches the mouse, Hk1-Rrp1 pathway is downregulated, but Rpr2-RpoN-RpoS system is "ON". During acquisition, Rrp2-RpoN-RpoS system is silenced, while Hk1-Rrp1 system is upregulated and stimulates the expression of tick-phase genes and simultaneously silences the host-phase genes. Created with BioRender.com.

# 2 Objectives

1. To test the effect of temperature on the expression of major surface molecules of *B. afzelii* spirochetes.
2. To test the effect of selected nutrients on the expression of major surface molecules of *B. afzelii* spirochetes.
3. To verify the infectivity of *B. afzelii* after stimulation with selected physical and chemical factors.

# 3 Materials and methods

## 3.1 Ticks

Ticks were obtained from the tick breeding facility of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences. Ticks were maintained in wet chambers at a constant temperature of 24 °C, humidity of 95 %, and a photoperiod corresponding to 49 ° north latitude (15 hours day / 9 hours night).

## 3.2 Mice

Both noninfected and CB43 infected mice were used in this work. Inbred, pathogen-free BALB/c mice (Charles River, Germany) were used for the inoculation experiments. CB43-infected BALB/c mice were used to generate infected nymphs. All experimental animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethic approval No. 25/2020.

## 3.3 Preparation of infected mice and ticks

*Borrelia afzelii* CB43 spirochetes (Štěpánová-Tresová et al., 2000) were grown in BSK-H (Sigma-Aldrich) medium at 33 °C for 5-7 days. Six weeks old female BALB/c mice were infected by subcutaneous injection of $10^5$ spirochetes in 100 µl of BSK-H medium per mouse. The presence of spirochetes in ear biopsies was verified 3 weeks after injection by standard PCR. When mice tested positive, uninfected *I. ricinus* larvae (approximately 100 larvae per mouse) were allowed to feed on. Fed larvae were enabled to molt, and nymphs were considered infected if at least 90 % were tested positive for *Borrelia*. Infected nymphs were ready for experiments 2-3 months after molting.

## 3.4 Nucleic acid isolation

### 3.4.1 DNA isolation

NucleoSpin Tissue Kit (Macherey-Nagel) was used for total DNA isolation from ticks as well as murine tissues according to the manufacturer‘s protocol.

### 3.4.2 RNA isolation

Total RNA was isolated using NucleoSpin RNA Kit (Macherey-Nagel) according to the manufacturer's protocol. The quality and concentration of RNA were measured on a NanoDrop™ 1000 (NanoDrop) spectrometer.

### 3.4.3 cDNA preparation

cDNA was prepared using Transcriptor First Strand cDNA Synthesis Kit (Roche). One μg of isolated RNA was used as a template for reverse transcription. cDNA synthesis was performed following the manufacturer's protocol. Transcribed cDNAs were diluted 10x and were used as templates for subsequent qRT-PCR analyses.

## 3.5 PCR

*Borrelia afzelii* spirochetes in ticks or murine tissues were detected by PCR. A 154 bp fragment of *flagellin* gene was amplified in 25 μl reaction volume containing 12.5 μl of FastStart PCR MasterMix (Roche), 4 μl of purified DNA, 10 pmol of each primer FlaF1A and FlaR1 and PCR water (Top-Bio) up to 25 μl. Primer sequences are reported in **Tab. 1**. The PCR program used for *Borrelia* detection consisted of 96 °C denaturation for 10 minutes followed by 40 cycles of 96 °C denaturation for 30 s, 60 °C annealing for 30 s, and 72 °C elongation for 1 min. The program finished with a 10 minute final extension at 72 °C.

## 3.6 Gel electrophoresis

Amplified fragments were visualized by gel electrophoresis. Ethidium bromide (Sigma-Aldrich) stained 1% agarose gel in TAE buffer (**Appendix 1**) was used for the separation of PCR products. PCR product (25 μl) was mixed with 6 μl of DNA loading dye (Top-Bio). As size determinator, GeneRuler 100bp DNA Ladder (Thermo Fisher Scientific) was used. The electrophoresis run at 100 V for 25 min.

## 3.7 Quantitative PCR

### 3.7.1 Relative quantification (qRT-PCR)

cDNA prepared from isolated RNA served as a template for gene expression analysis using QuantStudio™ 6 Flex real time PCR system (Thermo Fisher Scientific). Reaction mixture contained 12.5 μl of FastStart Universal SYBR Green Master (Rox) (Roche), 10 pmol

of reverse and forward primer (RT- and BaospC primers, **Tab. 1**), and 5 µl of cDNA. Reactions were adjusted to 25 µl with PCR water. The amplification program started with initial denaturation at 95 °C for 10 minutes, followed by 50 cycles of 20 s denaturation at 95 °C, 30 s annealing at 60 °C, and 30 s elongation at 72 °C. Melting curve analysis was carried out for every pair of primers. Relative expressions of *ospA*, *ospC*, and *bbk32* were standardized to *flaB* using the ΔΔCt method (Pfaffl, 2001).

### 3.7.2 Absolute quantification (qPCR)

The total number of spirochetes in murine and tick tissues was determined by quantitative real-time PCR (qPCR) using the QuantStudio™ 6 Flex real-time PCR system (Thermo Fisher Scientific). Reaction mixture contained 12.5 µl of FastStart Universal Probe Master (Rox) (Roche), 10 pmol of each primer, 5 pmol of TaqMan probe, and 5 µl of DNA. The reaction was adjusted with PCR water up to 25 µl. The amplification program consisted of 95 °C initial denaturation for 10 minutes, followed by 50 cycles of 95 °C denaturation for 15 s, and annealing combined with elongation at 60 °C for 1 min in total. All samples were analyzed in triplicates to minimize random deviation. Primers FlaF1A, FlaR1, and TaqMan probe FlaProbe1 amplifying a fragment of *flagellin* gene were used for quantification of *Borrelia* spirochetes in murine tissues. Quantification of murine *ß-actin* was performed using MmAct-F and MmAct-R primers and a MmAct-P TaqMan probe.

Obtained Ct values were compared to the external standard curves for mouse *actin* and *Borrelia flagellin.* The spirochete load in murine tissues was expressed as the number of spirochetes per $10^5$ of murine *ß-actin* copies. The spirochete burden in ticks was calculated as the total number of spirochetes in the whole tick body.

**Tab. 1:** PCR primers and probes used for *Borrelia afzelii* detection and quantification in tick and murine tissues, and quantification of *B. afzelii* gene expression.

| Target | Name | Sequence (5‘-> 3‘) | Product size | Source |
|---|---|---|---|---|
| *Borrelia flagellin* | FlaF1A | AAGCAAATTTAGGTGCTTTCCAA | 154 bp | Schwaiger et al., 2001 |
| | FlaR1 | GCAATCATTGCCATTGCAGA | | |
| | TaqMan FlaProbe1 | TGCTACAACCTCATCTGTCATTG TAGCATCTTTTATTTG | | |
| *Mus musculus ß-actin* | MmAct-F | AGAGGGAAATCGTGCGTGAC | 137 bp | Dai et al., 2009 |
| | MmAct-R | CAATAGTGATGACCTGGCCGT | | |
| | MmAct-P | CACTGCCGCATCCTCTTCCTCCC | | |
| *ospC* lipoprotein | BaospC-F | GCAGGAGCCTATGCAATATCA | 150 bp | Pospisilova et al., 2019 |
| | BaospC-R | TTTGCCAAGATCTGCATGAC | | |
| *ospA* lipoprotein | RTospA-F | GGTTCTGGAGTGCTTGAAGG | 112 bp | Koči et al., 2006 |
| | RTospA-R | TGTTTTGCCATCTTCTTTG | | |
| *bbk32* lipoprotein | RTbbk32-F | CACGTCTTGACAACCTTGCT | 117 bp | |
| | RTbbk32-R | CCTTGCACTCACTTGAATATAG | | |
| *Borrelia flagellin* | RTflaB-F | GTTCATGTGGGAGCAAATCA | 120 bp | |
| | RTflaB-R | ACCCTCTTGAACAGGTGCAG | | |

## 3.8 Solution preparation

Solutions were prepared by dissolving chemicals in dd$H_2O$ and filtered through a 0.22 µM filter (Merck Millipore). Aliquots were stored at -20 °C and thawed at RT before use. For membrane feeding, modified Tyrode’s solution was prepared. The solution was sterile filtered and freshly made before every experiment. All solutions and buffers used are listed in **Appendix 1**.

## 3.9 Tick dissections and gut incubation

Infected *I. ricinus* nymphs were sticked to a glass slide and dissected using micro scissors (F.S.T) and autopsy tweezers (Dumont). Guts were then carefully washed in 1x PBS and placed into Eppendorf tubes for incubation either at RT or 37 °C with chosen solution. As a control, 1x PBS was used. The guts were incubated for 24 hours or 48 hours at RT or 37 °C according to the protocol.

## 3.10 Injection of ticks

### 3.10.1 Injection into hemolymph

Infected *I. ricinus* nymphs were sticked dorsally to double-sided adhesive tape attached to a slide and 32 nl of the solution was injected into hemolymph with sharpened microcapillary. As a control, sterile 1x PBS was used. Nymphs were then collected and placed into a wet chamber at RT O/N.

### 3.10.2 Injection into rectal sac

Infected *I. ricinus* nymphs were placed on double-sided tape and injected into the rectal sac using sharpened microcapillary. Care being taken not to puncture the rectum, 32 nl of solution was injected into every nymph. Treated nymphs were collected and placed into wet chamber O/N at RT.

## 3.11 Artificial feeding

### 3.11.1 Membrane feeding

In vitro feeding of ticks was performed in membrane feeding units according to the procedure developed by Kröber and Guerin (Kröber & Guerin, 2007), but the glass feeding unit was exchanged for plastic feeding unit. Bovine blood was collected at local slaughterhouse. Human blood was collected from consenting volunteers. Co-feeding method was used to increase feeding success of attached nymphs. Feeding units with adult females were placed over bovine blood for two days. After two days, feeding units with attached ticks were washed carefully and placed over examined solutions. Blood and serum were diluted in Tyrode's solution (one part of blood or serum and two parts of Tyrode's solution). Nymphs were then added to attach and let to feed for 24 or 48 hours.

### 3.11.2 Capillary feeding

Capillary feeding of nymphs was carried out according to the modified protocol of Broadwater et al. (Broadwater et al., 2002). Ticks were sticked by their dorsal surface to adhesive foam tape (Herlitz) and a sharpened microcapillary (Drummond) was placed over the hypostome. Nymphs were then allowed to feed for 2.5 h in 37 °C incubator with air humidity of 95 %. After removal of the capillaries, nymphs were allowed to rest at RT or 37 °C.

## 3.12 Mice infections

Tick guts were dissected 24 hours after stimulation. Five dissected tick guts in 100 µl of BSK-H were subcutaneously injected into the mouse. Infectious rates in mouse ear biopsies were checked 3 weeks post-infection. After 5 weeks, mice were euthanized and heart, bladder, and ear biopsies were dissected and tested for *Borrelia* presence using qPCR.

## 3.13 Statistical analysis

Data were analyzed by GraphPad Prism 9.3.1 for macOS. The error bars shown in graphs indicate the standard error of mean. Normality of all datasets was inspected using Shapiro-Wilk test followed by Q-Q plot. For comparison of multiple datasets, ordinary one-way ANOVA or Kruskal-Wallis tests were executed. As post hoc test, according to the previous analysis, Dunnett's or Dunn's multiple comparisons test were executed. Samples with a p-value $< 0.05$ were considered statistically significant.

# 4 Results

Previous research, especially on the American *Borrelia burgdorferi s.s.*, or less on *Borrelia afzelii*, has shown that the expression of the bacterial outer surface proteins changes during tick feeding (Pospisilova et al., 2019; Fikrig et al., 2000; Schwan & Piesman, 2000; Suk et al., 1995). These differential changes in gene expression then affect both, host-vector acquisition, and vector-host transmission of *Borrelia* (de Silva et al., 1996; Ohnishi et al., 2001; Schwan et al., 1995). Because the environment of the tick gut is very different from one in the vertebrate host (Sonenshine & Roe, 2013), *Borrelia* overcome large transcriptional changes to escape attention. The *ospA*, *ospC*, and *bbk32* genes are particularly well described in this connection.

Little is known about the factors regulating these transcriptional changes. This thesis presents a pilot study to test possible triggers of gene expression in the main European causative agent of Lyme borreliosis, *Borrelia afzelii*. The effect of temperature and two groups of common blood nutrients was examined using different approaches of tick stimulation. The two most abundant groups of nutrients – sugars and amino acids were considered the most interesting. Differential expression of *ospA*, *ospC,* and *bbk32* genes was tested by qRT-PCR, and the ability of stimulated spirochetes to develop infection was tested in the laboratory mouse model.

From the wide range of nutrients that enter the tick gut with the blood, those for which *Borrelia* has receptors were selected. Due to their parasitic lifestyle, spirochetes rely on glycolysis to produce ATP, but lack genes involved in the processing of essential nutrients, Krebs cycle enzymes, and oxidative phosphorylation, as well as being unable to synthesize amino acids or fatty acids which must be taken in externally (Corona & Schwartz, 2015; Radolf et al., 2012; von Lackum & Stevenson, 2005). In this regard, the cell envelope expresses a variety of receptors that facilitate the uptake of required nutrients and their transfer to the cytoplasm. Among them, the shared PTS (phosphotransferase system) for glucose and *N*-acetylglucosamine, the ABC (ATP-binding cassette) transporter for ribose, or glycine/proline receptors, play a pivotal role and have been in the center of our attention, mainly due to the demonstrable presence of ligands for these receptors in vertebrate blood, both free or bound to the cell wall of blood cells (Brandt et al., 2021; Chen et al., 2010; P. M. Clark et al., 2014; Dietzen, 2018; Fraser et al., 1997; Gross & Zellner, 1991; Krebs, 1950).

## 4.1 *Borrelia afzelii* gene expression in temperature stimulated ticks

In this experiment, we tested the effect of temperature on *Borrelia afzelii* gene expression in ticks maintained at ambient temperature (resembling an off-host stage) and at 37 °C (mimicking a situation where a tick feeds on its vertebrate host).

Unfed, *B. afzelii* infected *I. ricinus* nymphs were kept at RT, or at 37 °C for 24 hours and 48 hours, and the levels of *ospA*, *ospC*, and *bbk32* were analyzed by qRT-PCR.

The effect of temperature on the expression of borrelial *ospA*, *ospC*, and *bbk32* genes in unfed nymphs is shown in **Fig. 4**. There was no difference in *ospA* expression. On the other hand, significant upregulation of *ospC* [P < 0.0001, F(2,9) = 43.59], and *bbk32* [P < 0.0001, H(2) = 6.527] was observed in nymphs incubated at 37 °C for 24 hours. However, over the next 24 hours, the expression of both genes decreased to the levels comparable with nymphs treated at RT.

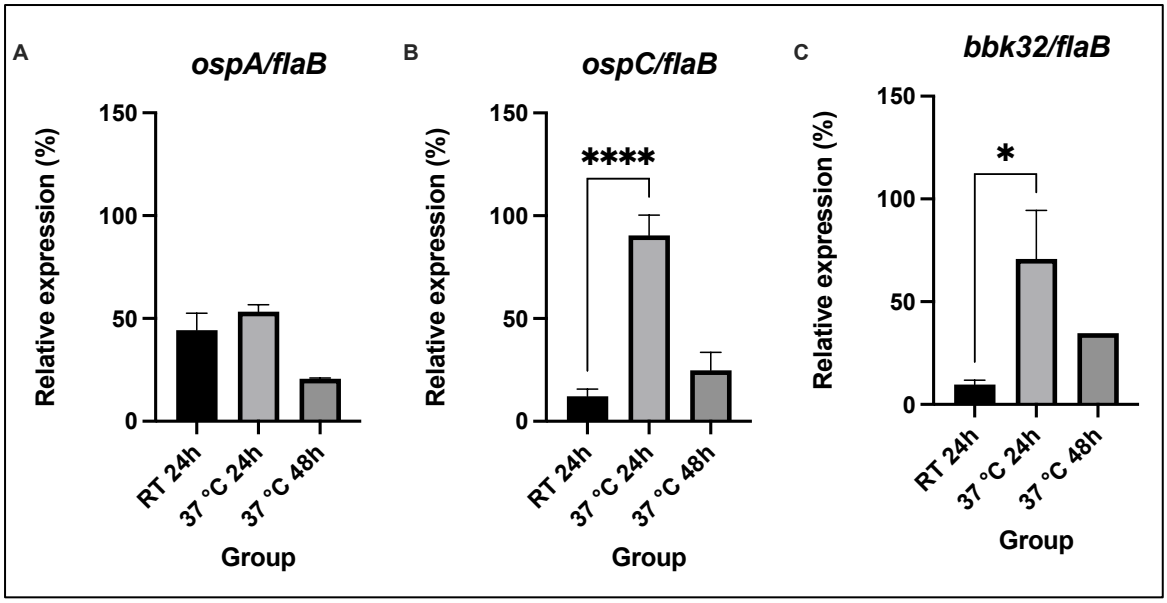


**Fig. 4: Expression of *B. afzelii* genes in nymphs incubated at different temperatures.** No significant difference in expression of *ospA* in nymphs incubated at RT and 37 °C was observed (**A**), but the expression of *ospC* (**B**) was highly upregulated in nymphs incubated at 37 °C during the first 24 hours [P < 0.0001, F(2,9) = 43.59]. Expression of *bbk32* (**C**) showed the same pattern [P = 0.007, H(2) = 6.527]. Each data point represents the mean of 3 individually analyzed samples. Error bars indicate standard error of mean. RT = room temperature, 37 °C 24h = kept at 37 °C for 24 hours, 37 °C 48h = kept at 37 °C for 48 hours.

## 4.2 *Borrelia afzelii* gene expression in nutrient stimulated ticks

Just a little is known about what triggers differential gene expression in *Borrelia* during tick feeding. Previous experiments have been conducted under in vitro conditions, particularly in *Borrelia* cultures or in ticks fed on the whole blood. As described earlier, physical factors such as pH, temperature, and osmolarity may have the desired effect (Ramamoorthy & Scholl-Meeker, 2001, Bontemps-Gallo et al., 2016). However, the overall knowledge of the chemical factors that contribute to the extensive transcriptional changes that *Borrelia* undergoes during its enzootic cycle is surprisingly sparse. Following experiments were performed to test the effect of selected nutrients on *B. afzelii* gene expression.

### 4.2.1 Incubation of homogenates from *B. afzelii* infected tick guts with selected nutrients

To investigate whether gut-associated *B. afzelii* could be stimulated by incubation in selected nutrients, guts from infected nymphs were dissected and placed into sterile solutions of sugars and amino acids for 24 hours. As described in the previous section (4.1), temperature contributes to differential gene expression, so the experiment was conducted at both, RT and 37 °C. Incubation longer than 24 hours was not possible without the use of antibiotics. After incubation, *B. afzelii* gene expression was analyzed by qRT-PCR.

Spirochetes extracted from stimulated guts showed differences in gene expression. Decreased levels of *ospA* and increased levels of *bbk32* genes were observed in spirochetes incubated at 37 °C, regardless of the type of chemical stimulation. However, the differences were not statistically significant. Expression of *ospC* was not affected by any type of stimulation (**Fig. 5**).

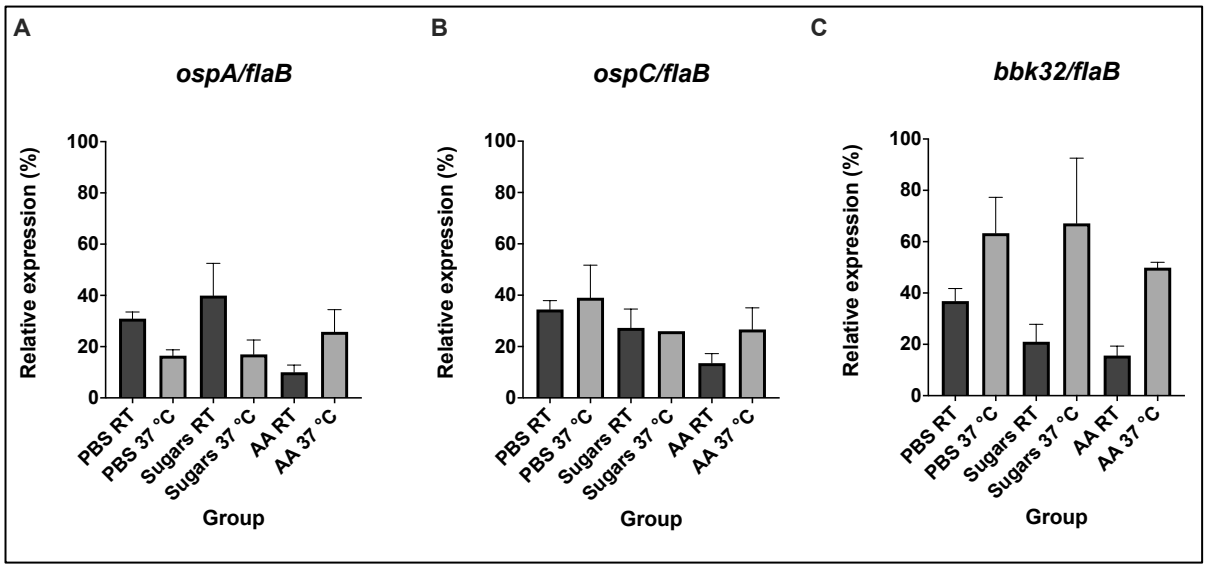


**Fig. 5: *Borrelia afzelii* gene expression in stimulated tick gut homogenates.** Expression of *ospA* (**A**), *ospC* (**B**), and *bbk32* (**C**) in *B. afzelii* spirochetes during stimulation of dissected tick guts at RT, or 37 °C for 24 hours with two groups of nutrients (Sugars and AA). PBS treatment was used as a control. Decreased levels of *ospA* and increased levels of *bbk32* regardless the type of stimulation are shown. Each datapoint represents mean of 2 individually analyzed samples. Error bars indicate standard error of mean. RT = room temperature, AA = amino acids.

### 4.2.2 Injection of nutrients into hemolymph of *B. afzelii* infected nymphs

The hemolymph is the circulatory fluid of ticks, comparable to the blood and lymph of vertebrates (Sonenshine & Roe, 2013). It supplies the tick gut, and by injecting nutrients into the hemocoel, these can get close to the gut and thus to the *Borrelia*.

In this experiment, selected nutrients were injected into the hemolymph of *B. afzelii* infected *I. ricinus* nymphs. After 24 hours of rest in a humid chamber at room temperature, total RNA was extracted from whole ticks and gene expression was quantified by qRT-PCR.

Compared to non-injected nymphs, upregulation of *ospA*, and downregulation of *ospC* and *bbk32* were observed in injected nymphs, regardless of the type of the nutrient (**Fig. 6**), however, the differences were not statistically significant.

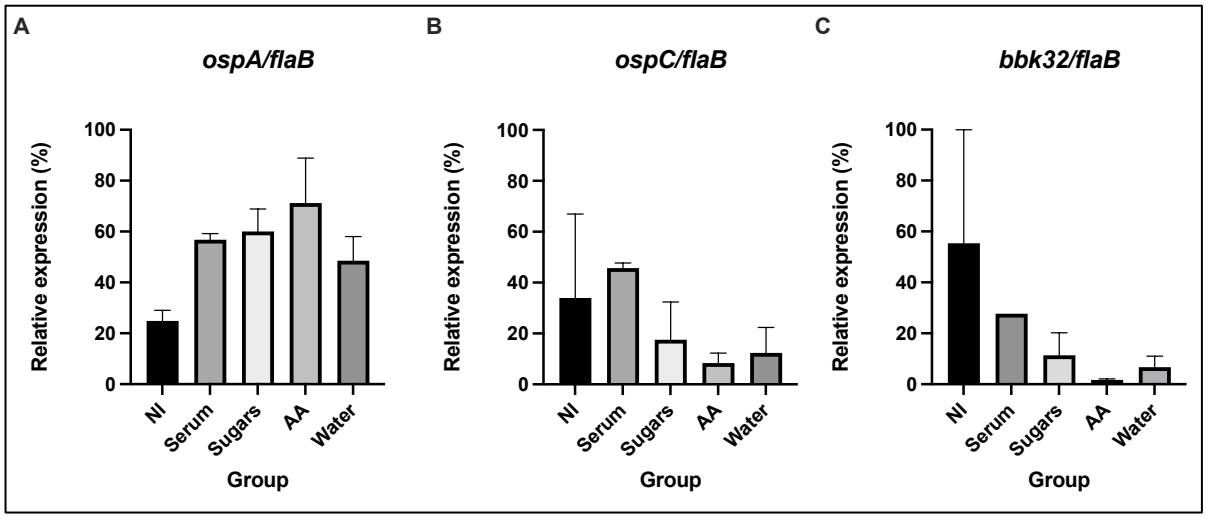


**Fig. 6: *Borrelia afzelii* gene expression after injection of nutrients into the hemocoel.** qRT-PCR analysis of *ospA*, *ospC*, and *bbk32* in *B. afzelii* spirochetes stimulated with microinjections of nutrients into tick hemocoel. An upregulation of *ospA* (**A**) and downregulation of *ospC* (**B**) and *bbk32* (**C**) were observed regardless of the type of stimulus. Each datapoint represents mean of 3 individually analyzed samples. Error bars indicate standard error of mean. NI = non-injected, AA = amino acids.

### 4.2.3 Injection of nutrients into rectal sac of *B. afzelii* infected nymphs

In this experiment, selected nutrients were injected into the rectal sac of *B. afzelii* infected nymphs. Compared to the previous experiment, nutrients were non-invasively delivered directly to the *Borrelia* resting in the tick gut. After 24 hours of rest in a humid chamber at room temperature, total RNA was isolated from whole nymphs and gene expression was analyzed by qRT-PCR.

*ospC* was significantly upregulated in nymphs injected with sugar mixture and insignificantly in all other groups. Interestingly, high upregulation of *ospC* was observed in water-injected nymphs, suggesting that mere dilution of gut contents may mobilize resting spirochetes (**Fig. 7B**). The expression of *ospA* and *bbk32* was not significantly altered in any of the groups tested (**Fig. 7A, C**).

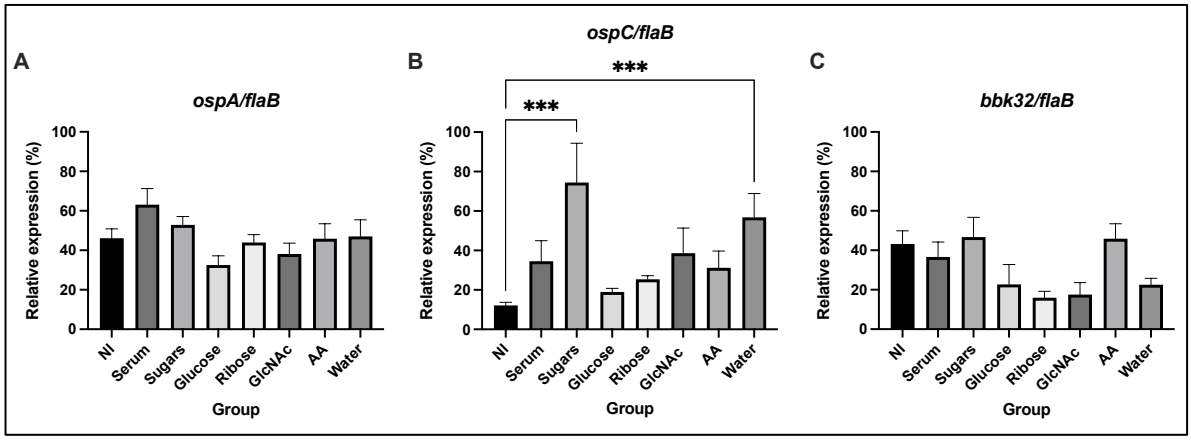


**Fig. 7: *Borrelia afzelii* gene expression after injection of nutrients into the rectal sac.** qRT-PCR analysis of *ospA*, *ospC*, and *bbk32* expression in *B. afzelii* stimulated by microinjection of nutrients into the tick rectal sac. There were not significant differences in expression of *ospA* (**A**), and *bbk32* (**C**). *ospC* expression was increased by all stimuli, and significantly upregulated after treatment with sugars [P = 0.0003, H(7) = 23.45] and water [P = 0.0007, H(7) = 23.45]. Each datapoint represents mean of 6 individually analyzed samples. Error bars indicate standard error of mean. NI = non-injected, GlcNAc = *N*-acetylglucosamine.

### 4.2.4 Infectivity of *B. afzelii* spirochetes stimulated by rectal sac injection

As published previously, OspC is considered a major virulence factor of *Borrelia.* Its expression starts with tick feeding and helps spirochetes to establish infection (Grimm et al., 2004). On the other hand, OspA levels are assumed to be downregulated for successful survival in the host (Yang et al., 2004).

In this experiment, *B. afzelii* infected ticks were stimulated by rectal sac injections of glucose, GlcNAc, and water. The ticks were kept in a humid chamber at RT for 24 hours. Then the tick guts were dissected and subcutaneously injected into mice. The spirochete levels in mice were measured five weeks after inoculation.

*B. afzelii* spirochetes from non-stimulated guts were not able to colonize the mice. In contrast, spirochetes from guts injected with GlcNAc and water developed infection in 80 % (4/5) of mice (**Tab. 2**, **Fig. 8**), further supporting the idea that dilution of gut contents activates dormant spirochetes. The mouse was considered infected if at least one tissue was positive for *Borrelia flagellin* five weeks after inoculation. The average number of spirochetes per $10^5$ mouse genomes along with SEM are shown in **Appendix 2**.

**Tab. 2: Development of infection in mice injected with stimulated guts from *B. afzelii* infected nymphs.** Mice were considered infected if at least one tissue was tested positive five weeks after inoculation. All mice in the control (NI) group tested negative, while 1/5 mice in the glucose-injected group and 4/5 mice in GlcNAc- and water-injected groups tested positive. NI = non-injected, GlcNAc = *N*-acetylglucosamine.

| Group | Infected/Total |
|---|---|
| NI | 0/5 |
| Glucose | 1/5 |
| GlcNAc | 4/5 |
| Water | 4/5 |

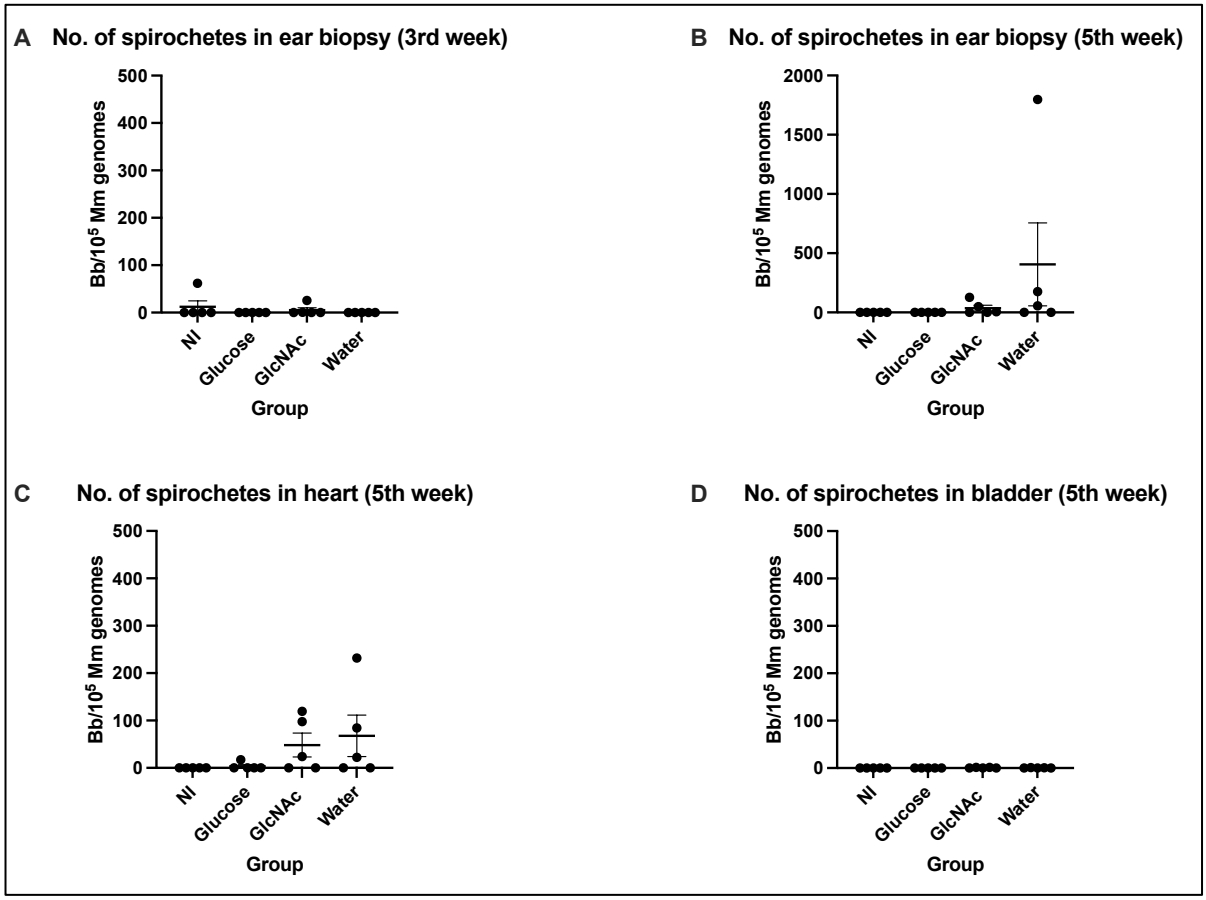


**Fig. 8: Spirochete levels in mice inoculated with stimulated guts from *B. afzelii* infected nymphs.** Number of spirochetes in ear (**A, B**), heart (**C**), and bladder (**D**) biopsies three and five weeks after inoculation with stimulated tick guts. Each column represents a mean number of spirochetes per $10^5$ mouse genomes. Error bars indicate standard error of mean. GlcNAc = *N*-acetylglucosamine, NI = non-injected.

### 4.2.5 Stimulation of *B. afzelii* by membrane feeding of nymphs

Membrane feeding is the most natural way of controlled feeding of ticks. It allows controlled modification of tick diet (addition or depletion of selected nutrients) and is therefore the method of choice in studies investigating nutrients essential for ticks and tick-transmitted pathogens.

In this experiment, we used an amended membrane feeding protocol described by Kröber & Guerin, 2007. We first conducted a pilot experiment to test the suitability of this approach for testing nutrients and their effect on *B. afzelii* gene expression. For the first two days, adult female ticks were fed on artificial feeding units supplemented with bovine blood. The adults here served as a support for nymphal ticks. Then, bovine blood was replaced with human blood, human serum, or Tyrode's solution (**Appendix 1**). Nymphs were then allowed to feed and were collected after 24 hours and 48 hours. Expression of *ospA*, *ospC,* and *bbk32* was analyzed by qRT-PCR. The expression of *ospA* was not significantly altered in any of tested groups (**Fig. 9A**). In contrast, the expression of *ospC* was upregulated in all groups compared to both controls, unfed nymphs, and nymphs fed with Tyrode's. However, only feeding on serum resulted in a statistically significant upregulation of *ospC* [$P = 0.0037$, $H(6) = 19.3$] (**Fig. 9B**). An increase of *bbk32* expression during the tick feeding was evident regardless of the type of treatment, however, the changes were not statistically significant (**Fig. 9C**).

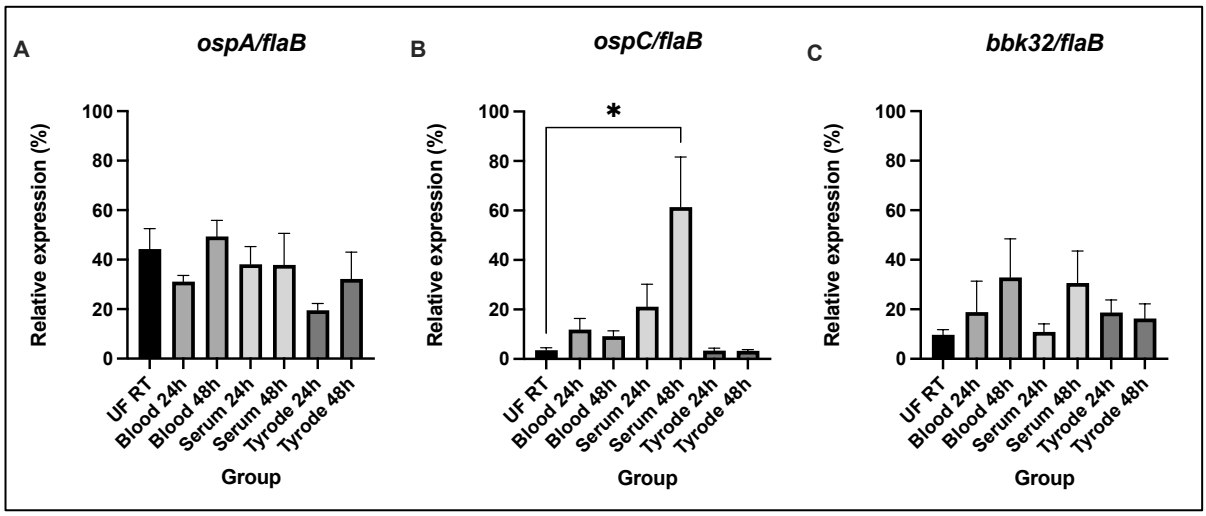


**Fig. 9: Gene expression in *Borrelia afzelii* during membrane feeding on human blood and serum.** Levels of *ospA* were comparable in all tested groups (**A**), while the expression of *ospC* (**B**) and *bbk32* (**C**) was altered in spirochetes from serum and blood-fed nymphs. However, *ospC* was significantly upregulated only in spirochetes from nymphs fed on serum for 48 hours [P = 0.0037, H(6) = 19.3]. Each datapoint represents mean of 3 individually analyzed samples. Error bars indicate standard error of mean.

### 4.2.6 Stimulation of *B. afzelii* by feeding nymphs on a defined diet

Membrane feeding of infected nymphs with blood and serum affected the expression of *ospC* and *bbk32* in *Borrelia* spirochetes. In the next experiment, we tested the effect of specific nutrients on *B. afzelii* expression. Selected nutrients (glucose, ribose, GlcNAc, amino acids) were diluted in Tyrode's solution and served as defined meal for *B. afzelii* infected nymphs.

Compared to spirochetes from unfed nymphs, *ospA* expression was markedly downregulated in spirochetes from nymphs fed for 24 hours, regardless of diet type (**Fig. 10A**). Of these, downregulation of *ospA* was statistically significant in spirochetes from GlcNAc-fed nymphs [P = 0.0495, H(10) = 24.73]. Interestingly, *ospA* expression was restored to its original level in ticks fed on glucose, ribose and GlcNAc for 48 hours. *ospC* was significantly upregulated in ticks fed on sugars [P = 0.0353, H(10) = 23.91] and GlcNAc [P = 0.014, H(10) = 23.91] for 48 hours (**Fig. 10B**). Similarly, *bbk32* expression was significantly upregulated in ticks fed on sugars for 48 hours [P = 0.0482, H(10) = 21.58] (**Fig. 10C**).

Surprisingly, no nymphs attached to the feeding units with Tyrode's solution supplemented with amino acid. Even the co-feeding females detached from the units when the bovine blood was changed to amino acid enriched Tyrode's solution. Thus, no data were obtained with this type of nutrient.

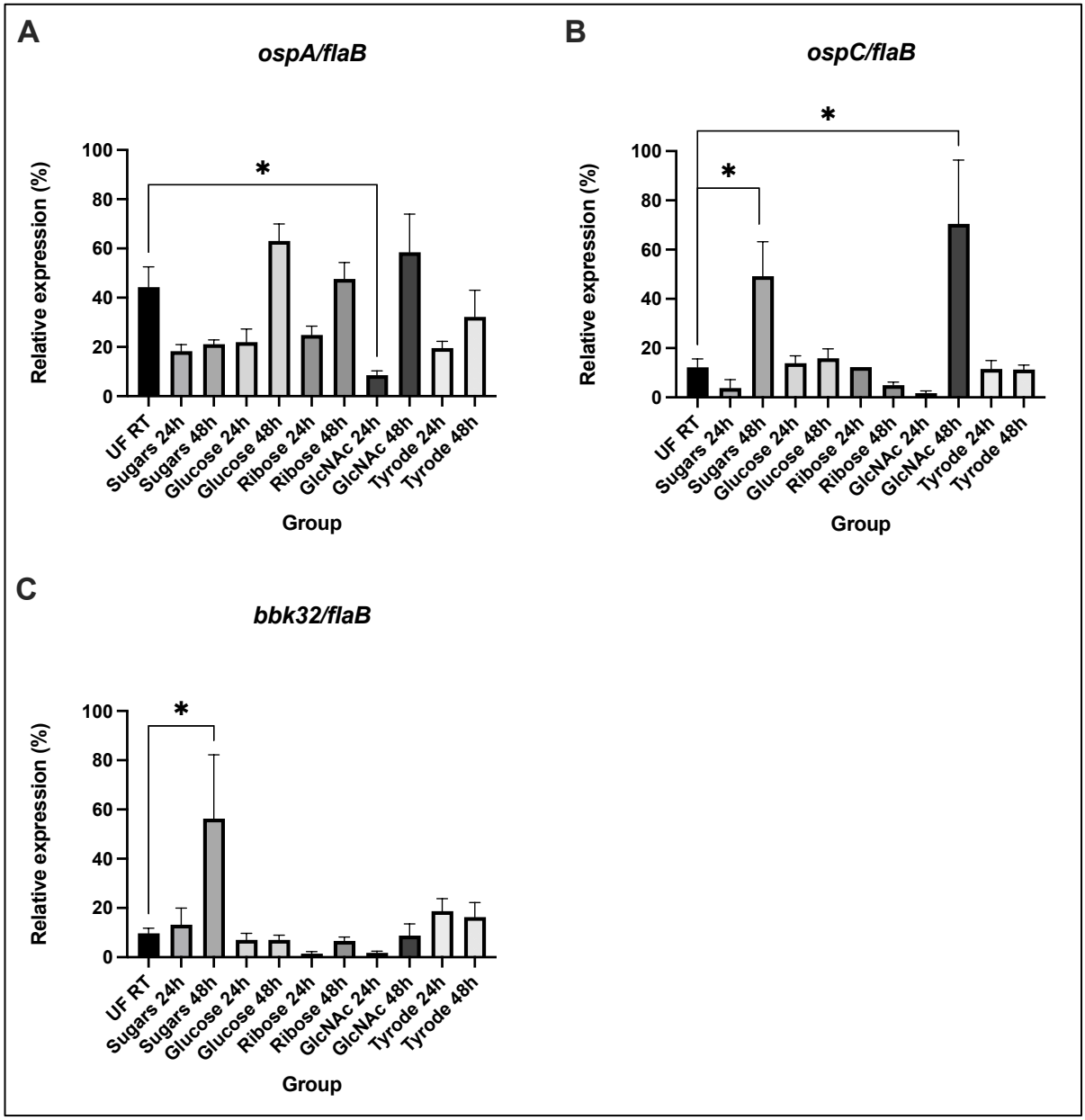


**Fig. 10: Gene expression in *Borrelia afzelii* during membrane feeding on specific nutrients.** Expression of *ospA* was downregulated in spirochetes from all ticks fed for 24 hours (a statistically significant downregulation in GlcNAc group [P = 0.0495, H(10) = 24.73]). The *ospA* expression was then restored in ticks fed on glucose, ribose and GlcNAc for 48 hours (**A**). *ospC* was significantly upregulated in spirochetes from nymphs fed on sugars [P = 0.0353, H(10) = 23.91] and GlcNAc for 48 hours [P = 0.014, H(10) = 23.91] (**B**). *bbk32* expression was significantly upregulated in spirochetes from nymphs fed on sugars for 48 hours [P = 0.0482, H(10) = 21.58] (**C**). Each data point represents mean of 3 individually analyzed samples. Error bars indicate standard error of mean. UF = unfed, GlcNAc = *N*-acetylglucosamine, RT = room temperature, 24h = fed for 24 hours in 37 °C, 48h = fed for 48 hours in 37 °C.

### 4.2.7 Stimulation of *B. afzelii* by capillary feeding of infected nymphs

Feeding ticks on artificial membranes is challenging, requiring high numbers of infected nymphs and adults. Despite advances in the formulation of minimal tick diets, it is not possible to feed ticks with a single nutrient(s). In addition, some nutrients (e.g. pure amino acids) are not accepted by ticks (see above). Therefore, we tested capillary feeding as a simple alternative to membrane feeding (Broadwater et al., 2002; Korshus et al., 2004).

Hypostomes of *B. afzelii* infected nymphs were immersed in capillaries prefilled with solutions of glucose, ribose, GlcNAc, mixed sugars, amino acids, and water. The nymphs were placed in a humid chamber and allowed to feed for 1.5 h at 37 °C and then allowed to rest for 24 hours at room temperature or 37 °C. Then, the expression of *ospA*, *ospC*, and *bbk32* was tested by qRT-PCR. A significant change in *ospA* expression was observed in the group fed on water [$H(11) = 33.99$, $P = 0.0352$]. No significant changes in gene expression were observed in any of the other tested groups. The only subtle changes were upregulation of *ospC* in the ribose- and water-stimulated group, and upregulation of *bbk32* in the glucose-stimulated group (**Fig. 11**). The observed changes were due to temperature rather than nutrient stimulation, as gene expression was affected in spirochetes from nymphs incubated at 37 °C.

Interestingly, the group of amino acid-fed ticks died within half an hour of capillary application. Therefore, this group was excluded from the analysis.

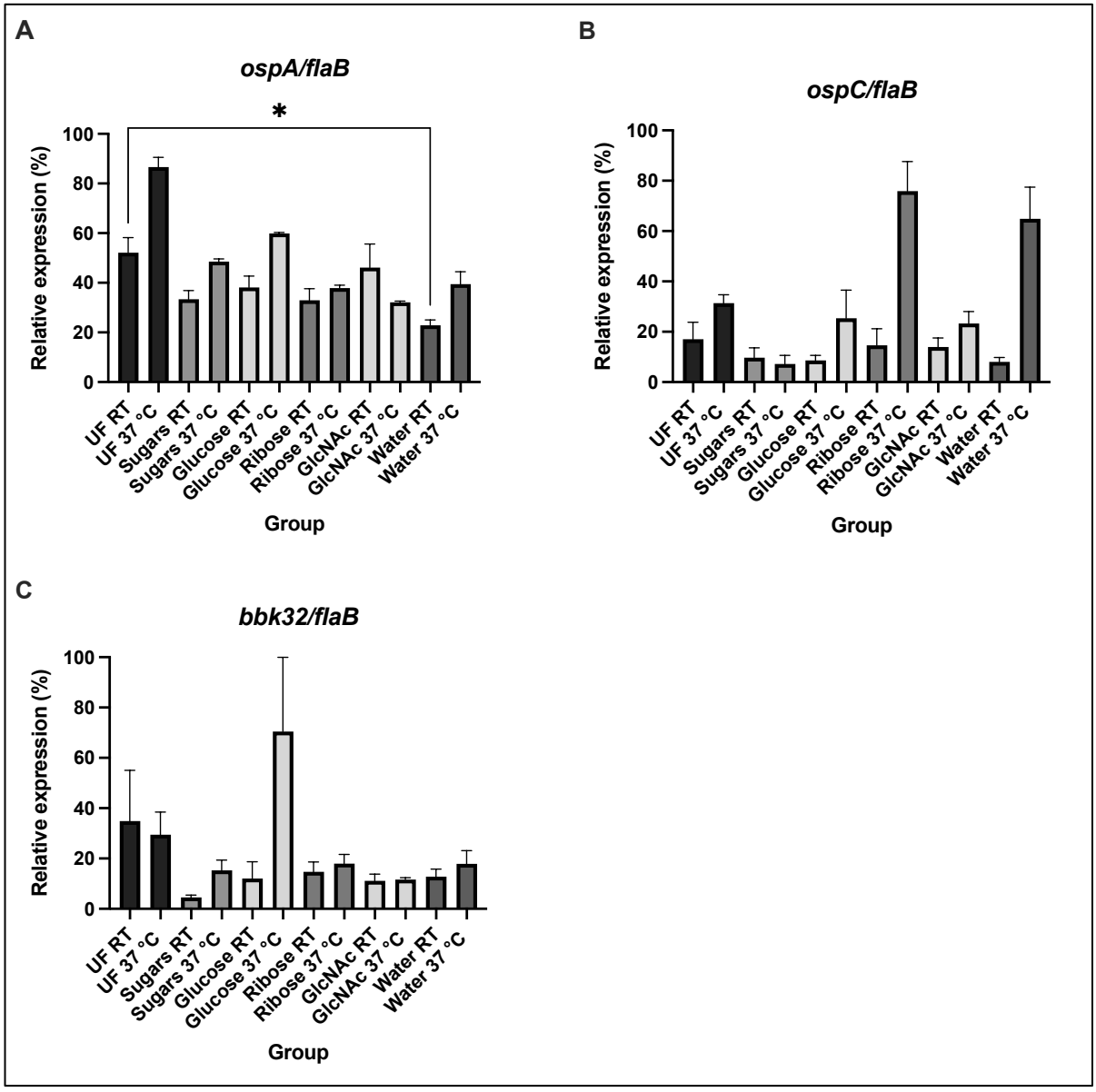


**Fig. 11: Stimulation of infected nymphs by capillary feeding.** Levels of *ospA* were at the same level in all groups except in group fed on water which was significantly downregulated during the first 24 hours (**A**). *ospC* was upregulated in spirochetes from nymphs fed on ribose and water (**B**). *bbk32* was upregulated in spirochetes from glucose-fed nymphs (**C**). No significant changes in *ospC* and *bbk32* expression were found in any of the groups tested. Each data point represents mean of 3 individually analyzed samples. Error bars indicate standard error of mean. UF = unfed, RT = room temperature, GlcNAc = *N*-acetylglucosamine. * RT = kept at RT after feeding, * 37 °C = kept at 37 °C after feeding.

# 5 Discussion

Uncovering the mechanisms behind the successful transmission of *Borrelia* spirochetes is an important first step toward improving strategies to combat Lyme borreliosis. To date, three main scientific approaches have been used to unravel the mechanisms of transmission and pathogenesis of *Borrelia*. Most of the previous research has focused on 1) testing *Borrelia* environmental responses in vitro, 2) feeding ticks with whole blood, or 3) creating *Borrelia* mutants. Moreover, this research has been conducted using the American model (*Ixodes scapularis* and *Borrelia burgdorferi s.s.*). Although it has led to significant discoveries concerning the life cycle of *Borrelia* and can to some extent be applied to our European model of *Ixodes ricinus*/*Borrelia afzelii*, the information is still incomplete and answers to important questions are missing. To fill the knowledge gap, we have recently established creative in vivo methods (artificial membrane feeding, capillary feeding, microinjections) to challenge *Borrelia* with defined chemical and physical stimuli. These methods allow us to test a broad range of compounds on wild-type *Borrelia* in its natural habitat, the tick gut.

*Borrelia* spirochetes use sophisticated strategies to survive during their enzootic cycle when they alternate between invertebrate vector and vertebrate host. It has been shown in many studies, that differential expression of several outer surface proteins plays a pivotal role in the survival and pathogenesis of *Borrelia* (Grimm et al., 2004; Yang et al., 2004). Modification of the expression of outer surface lipoproteins facilitates transmission and establishment of infection while evading the immune system. All of this is remotely controlled by regulatory pathways activated during tick feeding (Bontemps-Gallo et al., 2016; Ouyang et al., 2012).

In recent studies, the effect of temperature has been proposed and generally accepted as one of the main causes of differential gene expression in *Borrelia*. Exposure to elevated temperature mimicking mammalian host conditions has been shown to decrease the expression of *ospA* but increase the expression of *ospC* and *bbk32*. Whereas maintaining *Borrelia* at 23 °C simulating the tick environment resulted in the opposite effect (Alverson et al., 2003; He et al., 2007; Stevenson et al., 1995). In our experiments, there was no effect of elevated temperature on *ospA* expression during the first 24 hours, but it decreased slightly after 48 hours of incubation at 37 °C. This observation is consistent with other similar studies using *B. burgdorferi s.s.* and indicates that the regulation of OspA responds with a delay compared with the other lipoproteins tested, OspC and BBK32, which showed a huge increase during

the first 24 hours of stimulation (Ojaimi et al., 2003; Schwan & Piesman, 2000). Also, the decrease in OspA expression must probably be related to other parameters, such as contact with tick cells or osmolarity (Obonyo et al., 1999). Surprisingly, the expression of *ospC* and *bbk32* showed a significant decrease after 48 hours of stimulation, expression of both genes dropped to pre-stimulation levels. This could mean, that temperature is the very first factor affecting gene expression, but it is not sufficient to maintain this switch in gene expression in unfed ticks without the contribution of other factors.

Recently, several innovative approaches to study tick physiology and tick-pathogen interactions have been implemented. Membrane feeding has multiple uses and partially replaces the use of live animals in experiments (González et al., 2021). Capillary feeding and rectal sac injections have been introduced for artificial infections of ticks and to study tick-pathogen interactions (Korshus et al., 2004; Taank et al., 2020). Hemocoel injections have been used for RNAi silencing in ticks (Galay et al., 2016; Karim et al., 2008). Incubation of tick gut or whole tick homogenates is one of the primary methods for preparing *Borrelia* cultures from infected ticks (Wittenbrink et al., 1996).

In this work, we employed these methods to study the effect of selected nutrients on the expression of genes encoding surface antigens of gut-associated *Borrelia*. It was not possible to test the effect of nutrients on *Borrelia* in culture medium. With its adoption of a parasitic lifestyle, the bacterium is an auxotroph for all amino acids, nucleotides, and fatty acids. It also lacks genes encoding enzymes for the tricarboxylic acid cycle and oxidative phosphorylation. Therefore, *Borrelia* spirochetes grow only in a specific, complex medium containing all essential nutrients which precludes its use in similar types of experiments.

Therefore, stimulating *Borrelia* in its natural habitat - the tick gut appeared to be the best option. However, this meant determining the most appropriate method for this type of experiment that would be both tick-friendly and suitable for *Borrelia* stimulation.

Hemolymph injections have significantly advanced protein expression research in ticks and appear to be the most appropriate silencing method to replace the original injection of dsRNA into the tick gut. However, it proved to be unsuitable for the purpose of *Borrelia* stimulation. Compared to the other tested methods, we obtained opposite results when injecting nutrients directly into the hemolymph. This was probably because the nutrients only came into contact with the outer surface of the gut instead of the inner. Thus, the nutrients probably did not reach the *Borrelia* in the gut directly and they could not be stimulated. The observed differences in expression, elevated *ospA* and decreased *ospC* and *bbk32*, were more likely to be random variation in gene expression than a true effect of the injected nutrients.

Therefore, we further focused only on methods in which nutrients are delivered directly into the tick gut, e.g., artificial membrane feeding, capillary feeding and injection into the rectal sac.

When the tick begins to feed, warm blood enters its gut, *Borrelia* awakens from its dormant state and prepares to move into the host body. This requires activation of Rrp2-RpoN-RpoS alternative sigma factor pathway and associated changes in the expression of outer surface proteins. The creation of mutants lacking expression of these selected genes, or their receptors, has been instrumental in the discovery of the functions of individual outer surface proteins (Li et al., 2006; Tilly et al., 2006; Yang et al., 2004). The major outer surface lipoprotein OspA plays a pivotal role in colonization of the tick gut. Spirochetes entering the tick gut upregulate expression of *ospA* and bind to the TROSPA receptor on the tick gut. (Figlerowicz et al., 2013; Pal, Li, et al., 2004). The use of antibodies against OspA prevented *Borrelia* from effectively colonizing the tick gut (Pal et al., 2001). It has been described that spirochetes downregulate *ospA* during tick feeding, leading to reciprocal activation of the Rrp2-RpoN-RpoS pathway and subsequently activating transcription of *ospC* and *bbk32*, along with other genes that play a role in the transmission of *Borrelia*. However, other studies suggest that *ospA* is expressed to some extent throughout the *Borrelia* life cycle (Schwan & Piesman, 2000). In this study, we used various methods to show that *ospA* expression decreased during the first 24 hours and surprisingly increased after 48 hours of feeding. Downregulation of *ospA* was also detected in in vitro experiments with dissected guts incubated at 37 °C. On the other hand, no changes in *ospA* expression were observed when ticks were injected into rectal sac. Overall, this suggests that the regulation of *ospA* expression is related to tick feeding and temperature, which is in agreement with previous studies.

However, we didn't observe any effect of different nutrients on *ospA* expression. Similarly, there were no significant changes in *ospA* expression when ticks were fed on whole blood or serum. Stimulation with water and GlcNAc significantly decreased *ospA* expression in the first 24 hours. However, this decrease disappeared after 48 hours and *ospA* expression increased to levels higher than the initial state, and the same pattern was observed in all other groups. One reason for this could be the lower viscosity of water and water solutions used in this experiment compared to blood and serum, which is three times (whole blood) and two times (serum) lower. This could cause faster uptake of liquid and thus a stronger response, which is probably only an initial stress response caused by the sudden dilution of the gut contents that mimics the blood intake. After finding out that it is not blood, probably due to the absence of another associated stimulus, the expression rises again.

Rapid *ospC* upregulation appears to be crucial in the early phase of *Borrelia* transmission and host colonization. Deletion of *ospC* led to inability to establish infection in mice (Grimm et al., 2004; Seemanapalli et al., 2010; Tilly et al., 2006). The role of *ospC* has not been fully elucidated, but it appears that it has an important antiphagocytic role (Carrasco et al., 2015) and helps *Borrelia* survive activity of human complement (Caine et al., 2017). Consistent with this, *ospC* is strongly upregulated during tick feeding. Interestingly, *Borrelia* spirochetes in culture express only minute amounts of OspC, likely indicating that spirochetes require a specific stimulus present in host blood (Ellis et al., 2014; Pospisilova, 2018). This theory is consistent with our in vitro observations that there was no differential expression in dissected tick guts incubated in various nutrient solutions and temperatures. However, membrane and capillary feeding, and rectal sac injection resulted in massive upregulation of *ospC* when serum and sugars were used as stimuli. The fact that rectal sac injection stimulated expression of *ospC* probably means that expression of this gene is not primarily triggered by feeding but by the composition of the "meal". Interestingly, the injection of water also led to the upregulation of *ospC*. This was probably due to its low osmolarity, which is one of the physical factors influencing the differential expression of outer surface molecules in *Borrelia*.

In summary, we have shown that *ospC* expression is influenced by sugars. GlcNAc has the strongest effect when injected into the rectal sac but also during membrane feeding. Capillary feeding, however, suggested the importance of ribose. In our experiments, glucose had no desired effect neither on *ospA* nor on *ospC*, although it is the most abundant sugar in the blood.

Previous studies have suggested that feeding is the only trigger that can cause successful activation of *Borrelia* and thus persistent infection in mice (Crippa et al., 2002; Piesman, 1993). Our results are partially consistent with these findings. Indeed, it has been shown that homogenates from unstimulated unfed infected nymphs are unable to induce persistent infection in mice. However, we have shown that the feeding process itself does not play a decisive role in establishing the infection. The infectivity of *Borrelia* could be triggered also by specific stimuli. Spirochetes stimulated with GlcNAc or water via injection into the rectal sac induced infection in 80 % of mice in contrast with glucose (20 %). Therefore, GlcNAc will be the subject of further investigation.

BBK32 belongs to the group of adhesins, which mimic host integrins, molecules that facilitate attachment and migration (Tracy & Baumgarth, 2017). It contributes to the pathogenesis of *Borrelia burgdorferi* during the host phase through binding of fibronectin, which is ubiquitously present on cell surfaces. BBK32 also inhibits host complement system,

and thus helps *Borrelia* escape attention of the host immune system (Garcia et al., 2016). Although a lot is known about BBK32 function, not much is known about its activation. Previous studies have suggested its upregulation during tick feeding, but most abundantly within the host (Pospisilova et al., 2019). Similar to *ospC*, *bbk32* has been shown to be modulated by increased temperature (He et al., 2007). Our results are consistent with an effect of temperature. Moreover, the presence of blood and serum in tick meal also appears to upregulate the expression of *bbk32*. We also observed the effect of mixed sugars and glucose along with incubation at 37 °C on *bbk32* expression. In vertebrate host, *Borrelia* use glucose as their main source of energy (Caimano et al., 2016; von Lackum & Stevenson, 2005). This increase in *bbk32* expression might be then explained by the synergy between temperature and glucose, which mimics the conditions in the host blood.

Our research at the tick – pathogen – host interface seeks to answer unanswered questions in the regulation of surface protein expression. Some questions have already been answered, but new questions have arisen. Amino acids are the building blocks of proteins necessary for the synthesis of a wide variety of compounds. Although concentrations of circulating amino acids are under homoeostatic control, they are also influenced by diet, metabolism, or genetic factors. Although they are a common component of vertebrate blood, our observations have shown, that they can have a strong effect on tick feeding. A strong repellent effect could not be overlooked in membrane feeding. No ticks attached to the membrane placed over the amino acid solution. Also, when feeding ticks through capillaries, the amino acid solution caused death in 100 % of the ticks. The reason for this interesting phenotype is currently unknown but will be the subject of further research. Perhaps, the effect could be due to glycine. Monoterpenoid esters of glycine were tested as spatial repellents against mosquitos. However, their mode of action has not yet been fully elucidated (Nesterkina et al., 2018). Besides being the protein precursor, glycine is also a neurotransmitter in the central nervous system (CNS). To date, not much is known about CNS signaling in ticks. However, glycine signaling pathway seems to be conserved as it is widespread among a variety of organisms, such as mammals (Schmidt & Thompson, 2016), fruit flies (Frenkel et al., 2017), and spiders (Meyer & Poehling, 1987). Disruption of glycine signaling, e.g., by hyperglycinemia, might lead to serious neurological problems, and death (Hennermann, 2006).

The mechanisms of regulation of *Borrelia afzelii* gene expression investigated in this work have outlined answers to some previously unanswered questions and will be complemented by following experiments.

First, infected nymphs will be stimulated with both, different temperatures and various sugars, and then used for transmission experiment to support our results. In planned experiment, we will focus mainly on the importance of GlcNAc and ribose, which showed an effect on *ospC*, the main virulence factor (Grimm et al., 2004).

Second, because these stimuli can directly affect not only *ospC* expression but also regulatory molecules, we will test the effect of nutrient stimulation on Rrp2-RpoN-RpoS pathway, with a special focus on the RpoS sigma factor which directly regulates the expression of *ospC*, but also other genes. OspC has been classified with other borrelial lipoproteins, such as DpbA, OspF, and the Mlp family, which seem to be regulated by similar environmental cues (Eggers et al., 2004; Yang et al., 2005).

Finally, gene expression in *Borrelia* is undoubtedly a complexly regulated process involving all three parties, spirochete, host, and vector. Therefore, we would like to test glycerol in connection with expression of tick-phase expressed genes as it serves as the main energy source of *Borrelia* in unfed ticks (Pappas et al., 2011).

# 6 Conclusion

- Analysis of *B. afzelii* gene expression showed that increased temperature induces initial upregulation of *ospC* and *bbk32*, however, this effect does not last longer than 24 hours. After 48 hours, the expression of both genes returned to their initial level. It indicates that temperature is one of the first stimuli leading to *Borrelia* activation, but without other factors, temperature is not sufficient to maintain permanent changes in gene expression.
- The expression of *ospA* is not affected by any type of stimulus used, at least during the first 48 hours. Experiments with nutrient stimulation confirmed that blood plays an important role in the differential expression of *bbk32* and *ospC*. More importantly, it suggests that blood-derived sugars play an important role and may serve as complementary factors to temperature stimulation.
- The transmission experiment demonstrated that spirochetes from stimulated but unfed ticks were able to induce infection in mice.
- The discovery of the repelling and killing effect of amino acids is interesting and tempting for further investigation.

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# 8 Appendices

**Appendix 1: List of buffers and media used in this work.**

| Buffer | Composition |
|---|---|
| **1x PBS** | 8 g NaCl (Lach:ner), 0.2 g $KH_2PO_4$ (Lach:ner), 2.9 g $Na_2HPO_4.12H_2O$ (Sigma), 0.2 g KCl (Penta) in 1 l $dH_2O$, pH 7.4 |
| **50x TAE buffer** | 242 g TRIS-HCl (Sigma), 100 ml 0.5M EDTA (pH=8, Sigma), 57.1 ml 100% acetic acid (Merck) in 1 l $dH_2O$ |
| **1x TAE buffer** | 50x diluted 50xTAE buffer (20 ml 50xTAE in 1 l $dH_2O$) |
| **1% agarose gel** | 0.3 g agarose (Sigma) + 30 ml 1x TAE buffer |
| **Tyrode's solution** | 8 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$ (Lach:ner), 0.1 g $MgCl_2$ (Lach:ner), 0.05 g $NaH_2PO_4$ (Sigma), 1 g $NaHCO_3$ (Lach:ner) in 1 l $dH_2O$, pH 7.4 |
| **Sugar mix solution (stock solution)** | 0.25 g Glc (Sigma), 0.25 g GlcNAc (Sigma), 0.25 g ribose (Sigma) in 50 ml $dH_2O$, pH 7.4<br><br>working solution = 150x diluted stock solution in Tyrode's solution |
| **Glucose solution (stock solution)** | 0.25 g Glc in 50 ml $dH_2O$, pH 7.4<br><br>working solution = 150x diluted stock solution in Tyrode's solution |
| **GlcNAc solution (stock solution)** | 0.25 g GlcNAc in 50 ml $dH_2O$, pH 7.4<br><br>working solution = 150x diluted stock solution in Tyrode's solution |
| **Ribose solution (stock solution)** | 0.25 g ribose in 50 ml $dH_2O$, pH 7.4<br><br>working solution = 150x diluted stock solution in Tyrode's solution |
| **AA solution (stock solution)** | 0.25 g Pro (Sigma), 0.25 g Gly (Merck) in 50 ml $dH_2O$, pH 7.4<br><br>working solution = 150x diluted stock solution in Tyrode's solution |

**Appendix 2: The average number of spirochetes in tissues of mice inoculated with microinjection-stimulated tick guts.**

| | Ear 3rd week | | | Ear 5th week | | |
|---|---|---|---|---|---|---|
| **Group** | **I/T** | **Mean** | **SEM** | **I/T** | **Mean** | **SEM** |
| NI | 1/5 | 12.34422 | 27.60252 | 0/5 | 0 | 0 |
| GlcNAc | 2/5 | 5.218971 | 11.34526 | 4/5 | 36.39933 | 54.70898 |
| Glucose | 0/5 | 0 | 0 | 1/5 | 0.031909 | 0.071351 |
| Water | 2/5 | 34.22726 | 52.6044 | 3/5 | 405.7464 | 781.2431 |
| | **Heart 5th week** | | | **Bladder 5th week** | | |
| **Group** | **I/T** | **Mean** | **SEM** | **I/T** | **Mean** | **SEM** |
| NI | 0/5 | 0 | 0 | 0/5 | 0 | 0 |
| GlcNAc | 3/5 | 48.26891 | 56.5089 | 2/5 | 0.612923 | 0.809798 |
| Glucose | 1/5 | 3.44413 | 7.701308 | 0/5 | 0 | 0 |
| Water | 3/5 | 67.72394 | 98.05839 | 3/5 | 0.324105 | 0.359576 |

I/T = number of infected mice/total number of mice in group, Mean = mean of spirochetes load per group per $10^5$ mouse genomes, SEM = standard error of mean.