Universidade Nova de Lisboa

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Regulation of the apoptosis pathway in *Rhipicephalus annulatus* ticks by the protozoan *Babesia bigemina*

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DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM PARASITOLOGIA MÉDICA

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Resumo

Regulação da via de apoptose em carraças *Rhipicephalus annulatus* pelo protozoário *Babesia bigemina*

Palavras-chave: Apoptose, *Babesia*, Carraça, Vacina.

As carraças são os vetores de agentes patogénicos com maior importância na área veterinária enquanto que em saúde humana aparecem em segundo, atrás dos mosquitos. Estes ectoparasitas hematófagos obrigatórios de vertebrados terrestres são capazes de transmitir um grande número de agentes patogénicos. Diferentes ixodídeos são responsáveis pela transmissão do protozoário *Babesia*, agente causal de babesiose numa ampla variedade de animais incluindo humanos. A carraça *Rhipicephalus annulatus*, considerado um dos mais importantes ectoparasitas do gado com grande impacto económico na produção animal, é o principal vetor de *Babesia bigemina*. O controlo de carraças e agentes patogénicos transmitidos por carraças baseia-se sobretudo no uso de acaricidas. Contudo, a acumulação de resíduos químicos nos animais e produtos derivados de animais, bem como o aparecimento de carraças resistentes aos acaricidas e contaminação ambiental evidenciam a necessidade de desenvolver alternativas económica e ambientalmente seguras como as vacinas. A identificação e caracterização de antigénios com uma função essencial no desenvolvimento da carraça e/ou no "fitness" do parasita ainda estão a limitar o desenvolvimento de vacinas contra carraças e agentes patogénicos transmitidos por carraças. Focar as interações vetor-agente patogénico é uma abordagem que permite a identificação de antigénios de carraça com um possível efeito tanto na carraça como no agente patogénico. Estas interações têm sido ajustadas ao longo de uma coevolução duradoura: os agentes patogénicos invadem o hospedeiro e, as células hospedeiras respondem, forçando os agentes patogénicos a desenvolver novas estratégias moleculares para ultrapassar os seus mecanismos de defesa. Alguns agentes patogénicos transmitidos por carraças parecem ter desenvolvido estratégias para manipular diferentes processos metabólicos, como a apoptose, mecanismo de defesa celular baseado no sacrifício individual em beneficio do tecido ou organismo. Com estas premissas, o principal objetivo deste estudo foi a caracterização da via de apoptose durante a infeção por *B. bigemina* nas glândulas salivares das carraças, uma barreira que os agentes patogénicos têm de ultrapassar e explorar para serem transmitidos com sucesso ao hospedeiro vertebrado. A informação sobre a regulação da apoptose por agentes patogénicos em carraças é escassa e nula no caso de *Babesia* spp. Assim, o sistema *R. annulatus – B. bigemina* foi usado para investigar se o parasita *Babesia* é capaz de afetar o processo celular de apoptose. Com base num catálogo de sialotranscriptómica previamente obtido, seis genes apoptóticos de carraças foram selecionados para estudos quantitativos de expressão genética. Três genes pró-apoptóticos, *DAP3, DAPK1* e *VDAC* demonstraram estar significativamente diferenciadamente expressos na infeção por *B. bigemina*. Os três genes anti-apoptóticos *AATF*, *BI-1* e *API5* não mostraram expressão diferencial significativa. No geral, os resultados sugerem que a *Babesia* pode ser capaz de reprimir parte da resposta celular apoptótica promovendo a sua sobrevivência e multiplicação nas células. Contudo, esta ação parece estar limitada à via intrínseca de apoptose. Estudos futuros podem esclarecer esta questão considerando mais genes de carraças para entender melhor as limitações desta regulação.

Abstract

Regulation of the apoptosis pathway in *Rhipicephalus annulatus* ticks by the protozoan *Babesia bigemina*

Keywords: Apoptosis, *Babesia*, Tick, Vaccine.

Ticks are the vectors of pathogens of major importance in the veterinary area whereas in human health they appear in second, behind the mosquitoes. These obligate hematophagous ectoparasites of terrestrial vertebrates are capable of transmitting a large number of pathogens. Different ixodid ticks are responsible for the transmission of the protozoan *Babesia*, causal agent of babesiosis in a wide variety of animals including humans. The tick *Rhipicephalus annulatus*, considered one of the most important ectoparasites of cattle with great economic impact on animal production, is the main vector of *Babesia bigemina*. The control of ticks and tick-borne pathogens (TTBP) is mainly based on the use of acaricides. However, the accumulation of chemical residues in animals and animal products, as well as the appearance of acaricide resistant ticks and environmental contamination evidence the need for the development of cost-effective and environmentally safe alternatives such as vaccines. Identification and characterization of antigens with a key role in the development of tick and/or parasite "fitness" are still limiting the development of anti-TTBP vaccines. Focusing the intricate vector-pathogen interactions is one approach that enables the identification of tick antigens with a potential effect on both tick and pathogen. These interactions have been adjusted through long-lasting coevolution: pathogens invade the host and host cells respond by forcing pathogens to develop new molecular strategies to bypass their defense mechanisms. Some tick-borne pathogens (TBP) appear to have developed strategies for manipulating different metabolic processes, such as apoptosis, a cellular defense mechanism based on individual sacrifice for the benefit of the tissue or organism. With these premises, the main objective of this study was the characterization of the apoptosis pathway during *B. bigemina* infection in the salivary glands (SG) of ticks, a barrier that pathogens have to overcome and exploit to be successfully transmitted to the vertebrate host. Information regarding apoptosis regulation in ticks by pathogens is scarce and nothing is known in the case of *Babesia* spp. Thus, herein the system *R. annulatus – B. bigemina* was used to investigate if *Babesia* parasite is able to affect the cellular process of apoptosis. Based on a previously obtained sialotranscriptomic catalogue, six tick apoptotic genes were selected for quantitative gene expression studies. Three pro-apoptotic genes *DAP3*, *DAPK1* and *VDAC* demonstrated to be significantly differentially expressed upon *B. bigemina* infection. The three anti-apoptotic genes *AATF*, *BI-1* and *API5* did not-show a significant differential expression. Overall, the results suggest that *Babesia* may be able to repress part of the cellular apoptotic response promoting survival and multiplication within cells. However, this action appears to be limited to the intrinsic apoptotic pathway. Future studies may clarify this issue considering more tick genes to better understand the limitations of such regulation.

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Abbreviations

µl	Microliter
µm	Micrometre
µM	Micromolar
AATF	Apoptosis antagonizing transcription factor
AGE	Agarose gel electrophoresis
AMPs	Antimicrobial peptides
API5	Apoptosis inhibitor 5
BI-1	Bax inhibitor 1-related
BID	BH3-interacting domain death agonist
bp	Base pair
cDNA	Complementary DNA
DAP3	Putative mitochondrial ribosome small subunit component mediator of apoptosis dap3
DAPK1	Death-associated protein kinase 1
DELE	Death ligand signal enhancer
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DR	Death receptors
dsDNA	Double-stranded DNA
EDTA	Ethylene diamine tetra acetic acid
ELF	Elongation factor
ER	Endoplasmic reticulum
g	Gram
g	G-force
i.v.	Intravenous
IFA	Indirect fluorescent antibodies
IFN	Interferon
kDa	Kilo Dalton

LIV	Louping ill virus
MD	midgut
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
NGS	Next-generation sequencing
nm	Nanometer
ºC	Celsius
PBS	Phosphate-buffered saline
PCD	Programed cell death
PCR	Polymerase chain reaction
qPCR	Real time PCR
RFU	Relative fluorescence units
RNA	Ribonucleic acid
RNAseq	RNA sequencing
rRNA	Ribosomal RNA
RT	Reverse transcription
s	Second
s.s.	*sensu stricto*
SG	Salivary glands
spp.	Species (plural)
TBD	Tick-borne diseases
TBE	Tris/Borate/EDTA
TBEV	Tick-borne encephalitis virus
TBP	Tick-borne pathogens
TNF	Tumor necrosis factor
UV	Ultraviolet
VDAC	Voltage-dependent anion channel
w/v	Weight per volume

1. Introduction

1.1. Ticks

Ticks (Acari: Ixodida) are arthropod ectoparasites of a huge variety of terrestrial vertebrates comprising reptiles, birds, amphibians and mammals (including humans) (Anderson and Magnarelli, 2008; Hajdušek *et al.*, 2013). Fossil records suggest that ticks originated 65–146 million years ago in the Cretaceous period from the Mesozoic Era (Figure 1) (Klompen and Grimaldi, 2001; Nava *et al.*, 2009). Currently, there are approximately 900 tick species divided into three families – Argasidae, Ixodidae and Nutalliellidae, this last one with only one species (Estrada-Peña, 2015). These arthropods exert a direct physical injury as a result of their biting and blood feeding activities and may cause allergic reactions and paralysis. Besides that, ticks act as vectors being capable to transmit the widest spectrum of pathogens such as bacteria, viruses and protozoa. This versatility is related with the fact of the ticks are strict obligate hematophagous arachnids, which allows them to acquire, and therefore, multiply, maintain and transmit disease-causing pathogens to their hosts (Anderson and Magnarelli, 2008; Hajdušek *et al.*, 2013). Ticks are the most important arthropod vectors of animal diseases (Arthur, 1962) and second to mosquitos as vector of human diseases (de la Fuente *et al.*, 2008; de la Fuente *et al.*, 2016c), whereby 10% of the known tick species represent high medical and veterinary concern all around the world (de la Fuente *et al.*, 2017), although their diversity is greatest in tropical and subtropical regions (Anderson and Magnarelli, 2008; Hajdušek *et al.*, 2013).

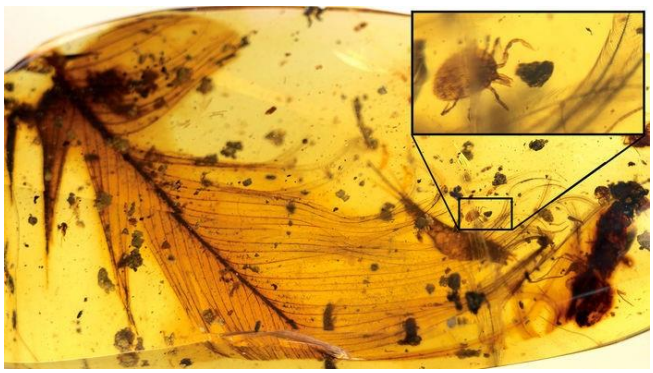

Figure 1 *Cornupalpatum burmanicum* hard tick in a feather, conserved in an amber (*adapted from Peñalver et al., 2017*).

Tick-borne diseases (TBD) have been noticeable by the last few decades regarding their emergence, resurgence and expansion in Europe, Asia and North America (Mansfield *et al.*, 2017), possibly due to the climate change, drug resistance, exploitation of land resources, global movement of people and animals, among others (Colwell *et al.*,

2011). Tick-borne encephalitis, Lyme disease, human granulocytic anaplasmosis, for example, are transmittable to humans making TBD a global threat not only to livestock but also to human health. Babesiosis, anaplasmosis and theileriosis undermine cattle health, welfare and fitness thereby causing significant economic losses to the livestock production (Hajdušek *et al.*, 2013). In Portugal, five endemic TBD are described, as many as in the other southern Mediterranean countries. These are the Lyme borreliosis, Q fever, Mediterranean spotted fever, tick-borne encephalitis and tick-borne relapsing fever (CEVDI Annual Report 2017).

Tick and TBD control in animals is mainly based on the use of chemical acaricides (Valle and Guerrero, 2018). Several disadvantages as the toxicity to animals and humans, resistance of ticks to acaricides and environmental contamination led to the development of vaccines, cost-effective and environmentally safe alternatives (Domingos *et al.*, 2013). The only commercially available anti-tick vaccine is based on the recombinant antigen Bm86. Developed in the 1990s, this vaccine results in the reduction of the number, weight and reproductive capacity of engorging female *Rhipicephalus (Boophilus) microplus* (Canestrini, 1888) and *Rhipicephalus (Boophilus) annulatus* (Say, 1821), and consequently in the reduction of cattle tick infestations and tick-borne pathogens (TBP) (de la Fuente *et al.*, 2016b; Domingos *et al.*, 2013). However, more efficient vaccines need to be developed for the control of ticks and TBP since this vaccine has limited efficacy against all tick stages, different tick species and geographical *R. microplus* ticks. Next-generation sequencing (NGS) represents a fundamental tool to the in-depth study of tick molecular dynamics and TBP interface. Transcriptomics, proteomics and functional genomics (García-García *et al.*, 1999; Rodriguez-Valle *et al.*, 2012; Valle and Guerrero, 2018) have brought new insight regarding this subject characterizing tick antigens that could become therapeutic targets. The study of the molecular interactions between ticks and pathogens may evidence key molecules that could be tested as potential vaccines that would target not only ticks but also the pathogens they harbour.

Babesia sp.-tick relationship is one of the most important in the veterinary field. These intraerythrocytic apicomplexan organisms can be vectored by different ixodid tick and typically, different species are associated whit specific vertebrate and arthropod hosts. *B. bigemina* and *B. bovis* are mainly vectored by *R. microplus* and *R.annulatus* ticks, specially affecting cattle (Antunes *et al.*, 2012).

1.2. *Rhipicephalus annulatus* Life Cycle

Ixodid ticks life cycle is a complex process of morphological and physiological modifications in the same individual encompassing four development stages - egg, larvae, nymph and adult (male and female). After egg hatching, the six-legged larvae emerge and a blood meal is required in order to moult to the next immature stage, the nymph. In the same way as larvae, eight-legged nymph need a blood meal to develop into adults. Ixodid ticks feed once in each active stage, being that the immature stages feed during 2.5 to 8 days while the adults feed 5 to 12 days. Mating also occurs in the host, followed by female drop-off. Fertilized females may ingest 100 to 120 times their body mass in blood, essential for the proper maturation of the eggs (Anderson and Magnarelli, 2008; Estrada-Peña, 2015).

Rhipicephalus annulatus are one-host ticks (Figure 2), *i.e.*, all the stages develop on only one host, except the eggs that are deposited in suitable microenvironments (Figure 2). These ticks are part of the *Boophilus* complex, a *Rhipicephalus* subgenus, known as cattle ticks (Jongejan and Uilenberg, 2004).

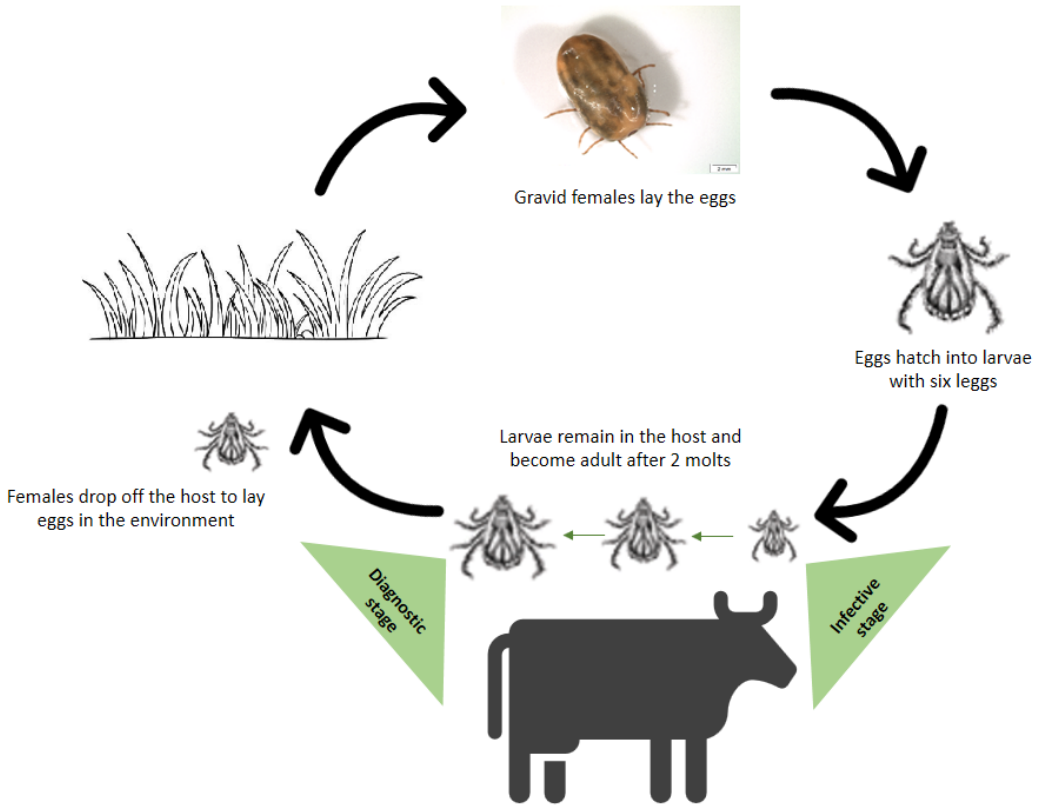

Figure 2 One-host ixodid life cycle (*original from Catarina Monteiro*). Dorsal view of an engorged *Rhipicehalus annulatus* female (*original and authorized by Sandra Antunes*).

These ixodid ticks, so-called pasture or field-dwelling ticks, can be found in the forest, brush and grassland habitats (Anderson and Magnarelli, 2008; Sonenshine and Roe, 2014). They take about three weeks to complete their life cycle (Jongejan and Uilenberg, 2004), depending on the conditions to which they are subject. Between the last are the abiotic environmental conditions, such as temperature, relative humidity and photoperiod (Anderson and Magnarelli, 2008) that "have a direct effect on tick development, questing activity and longevity" (de la Fuente *et al.*, 2017). Cattle are the preferred hosts for *R. annulatus*, occasionally occurring on other large animals, such as horses and deer. However, this tick species was already found in humans and dogs (CFSPH, 2007). This species is associated with the transmission of pathogens that cause babesiosis (*Babesia bovis* and *Babesia bigemina*) and anaplasmosis (*Anaplasma marginale*) (Peter *et al.*, 2009) which are regarded as very important diseases in cattle leading to large economic losses (Suarez and Noh, 2011; Bock *et al.*, 2004; Shkap *et al.*, 2007).

1.3. Babesiosis

Babesiosis is a worldwide TBD caused by apicomplexan hemoparasites of the protozoan genus *Babesia* (Piroplasmida: Babesiidae) (Chauvin *et al.*, 2009; Vannier *et al.*, 2015). It is the most common blood-borne disease of parasitic origin, following the trypanosomiasis, which affects many mammalian and some avian species (Gohil *et al.*, 2013; Hunfeld *et al.*, 2008). More than 100 *Babesia* spp. are reported and biologically distinct by their exclusive invasion of erythrocytes and transovarial transmission (*Babesia* spp. *s.s.*) in ixodid ticks, their primary vector (Chauvin *et al.*, 2009; Hunfeld *et al.*, 2008; Antunes *et al.*, 2017). *Babesia* spp. can be grouped according to their size. Example of the small babesiae (1.0– 2.5µm), in which the merozoites are smaller than the erythrocyte radius, are *B. ovis*, *B. microti*, *B. divergens* and *B. gibsoni*. Large babesiae (2.5–5.0µm), in which the merozoites are longer than the erythrocyte radius, are *B. bigemina*, *B. canis*, *B. major* and *B. caballi*, for example (Chauvin *et al.*, 2009; Hunfeld *et al.*, 2008).

It was Babes (1888) that first identified the commonly called *Babesia* as the intraerythrocytic microorganism responsible for the bovine hemoglobinuria or red water fever. Five years later, Smith and Kilbourne (1893) recognized the first arthropod-borne pathogen when established the tick as the vector of *B. bigemina*, the causative agent of

Texas Cattle Fever. Human babesiosis was only confirmed in 1956 (Skrabalo and Deanovic, 1957) and, ever since, babesiosis is considered as a potential life-threatening emerging zoonosis of humans (Hunfeld *et al.*, 2008; Vannier *et al.*, 2015). Human babesiosis is sporadically reported in Europe, Africa, Asia, Australia and South America (Vannier *et al.*, 2015) contrarily to the high endemicity area of Eastern United States, where *B. microti* has the major number of human cases attributed (Hunfeld *et al.*, 2008). The other known species also liable as causing disease in humans are "*Babesia microti* and *B. microti*-like, *Babesia duncani* and *B. duncani*-type organisms, *Babesia divergens* and *B. divergens*–like organisms, *Babesia venatorum*, and KO1" (Vannier *et al.*, 2015). The increasing outdoor activities are a proposed explanation for a higher exposure to TBP. However, more than 170 blood transfusion-transmitted cases have been reported, a fifth of which led to death. One only case of transplacental transmission is known (Vannier *et al.*, 2015).

The cattle industry is particularly impaired by *Babesia* parasites leading to enormous economic impact due to death, abortion, sterility, reduced meat, milk and leather production and the cost of treatments and prevention. *B. bigemina* and *B. bovis* are the foremost responsible species and are transmitted by the *R. annulatus* and *R. microplus* cattle ticks in tropical and subtropical regions (Antunes *et al.*, 2012; Gohil *et al.*, 2013) against to the northern Europe where *B. divergens* can be transmitted by the *Ixodes ricinus* tick (Gohil *et al.*, 2013). *B. ovata* and *B. major* are also known to cause disease in cattle (Suarez and Noh, 2011).

1.3.1. *Babesia* spp. Life Cycle

During a blood meal, *Babesia*-infected tick introduces sporozoites into the vertebrate host, which directly penetrate the erythrocytes, the only cells infected by *Babesia* species (Chauvin *et al.*, 2009; Uilenberg, 2006). Sporozoites differentiate into trophozoites that undergo asexual replication, resulting in two or four merozoites (Uilenberg, 2006; Vannier *et al.*, 2015), depending on whether it is a large or small *Babesia* spp., respectively (Bennett *et al.*, 2015). Upon rupture of the infected red blood cells, merozoites invade new host cells to repeat the replicative cycle (merogony) (Chauvin *et al.*, 2009; Gohil *et al.*, 2013; Vannier *et al.*, 2015), until death of the host or immune system riposte (Hunfeld *et al.*, 2008; Uilenberg, 2006). Gamogony takes place

when some trophozoites develop into gametocytes, ingested by ticks during feeding. Gamete differentiation and zygote formation occur in the tick gut. Zygote then transforms into kinete, which replicates by asexual division (Chauvin *et al.*, 2009; Hunfeld *et al.*, 2008; Uilenberg, 2006) and further invade tick ovaries and salivary glands through the hemolymph (Liu and Bonnet, 2014). Infection acquired during one life stage can be passed on to the next (transstadial transmission) although *Babesia* spp. *s.s.*, commonly called large *Babesia* spp., can also be transovarially transmitted via the eggs, whereby all the tick stages are potentially infective, persisting over several tick generations, even without new infections (Chauvin *et al.*, 2009; Hunfeld *et al.*, 2008; Uilenberg, 2006). Transovarial transmission is an absolute necessity for TBP infecting one-host tick species (Liu and Bonnet, 2014; Šimo *et al.*, 2017), such as the *R. annulatus* - transmitted *B. bigemina*. Once in the larvae, kinetes become sporozoites in the salivary glands (SG), which are only form transmitted to the vertebrate host during tick nymph stages for *B. bigemina* (Suarez and Noh, 2011). Figure 4 illustrates the life cycle of *B. bigemina*.

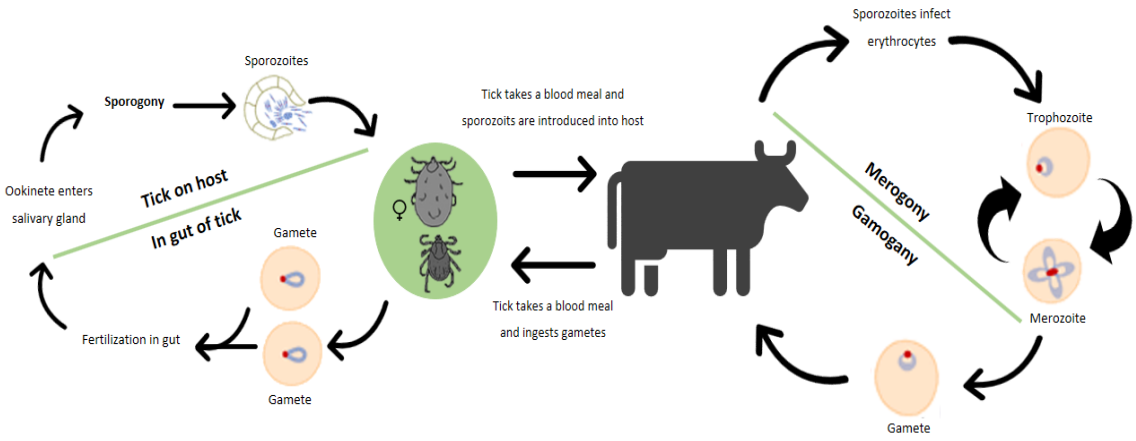

Figure 3 *Babesia* life cycle *(original from Catarina Monteiro)*. During a blood meal *Babesia*-infected tick introduces sporozoites into the vertebrate host, which penetrate the erythrocytes and differentiate into trophozoites that undergo asexual replication, resulting in merozoites. Upon rupture of the infected red blood cells, merozoites invade new cells to repeat the replicative cycle. Gamogony occurs when some trophozoites develop into gametocytes, ingested by ticks during feeding. Gamete differentiation and zygote formation occurs in the tick gut. Zygote then transforms into kinete, which replicate by asexual division and invade tick organs through the hemolymph.

1.3.2. *Babesia* spp. - Vector Interactions: The Importance of Tick Salivary Glands in Infection

Midgut (MD) and tick salivary glands (SG) are very important tissues during infection showing different roles during pathogen infection, multiplication, and transmission (Antunes *et al.*, 2017). In ticks, as hemoparasites, the MD is the first barrier

that pathogens encounter where initial uptake and replication occurs before migration into hemolymph and other tissues. SG are the vehicle for pathogen transmission during feeding and are the final place for replication, constituting another barrier that pathogens need to surpass (Alberdi *et al.*, 2016a,b) (Figure 4). Infection pressures cells to respond with the activation of several metabolic pathways including apoptosis (Bedner *et al.*, 1998; Mansfield *et al.*, 2017). It was described that during infection with Louping ill virus (LIV) and tick-borne encephalitis virus (TBEV), and the bacterium *Anaplasma phagocytophilum*, tick cell lines present a comparable transcriptional response, whereas an upregulation of key regulators of apoptosis was observed (Alberdi *et al.*, 2016b; Mansfield *et al.*, 2017). The protozoan parasites *Plasmodium* spp., *Giardia lamblia* and *Trichomonas vaginalis* also showed to inhibit apoptosis in host cells as a parasite survival strategy (Bruchhaus *et al.*, 2007; Shemarova, 2010). Yet, little is known about the influence of *Babesia* infection in the host tick. Apoptosis reflects the organism survival strategy, sacrificing some for the benefit of the whole. Thus, the ability to restrain this mechanism will be for sure an important advantage for pathogen survival.

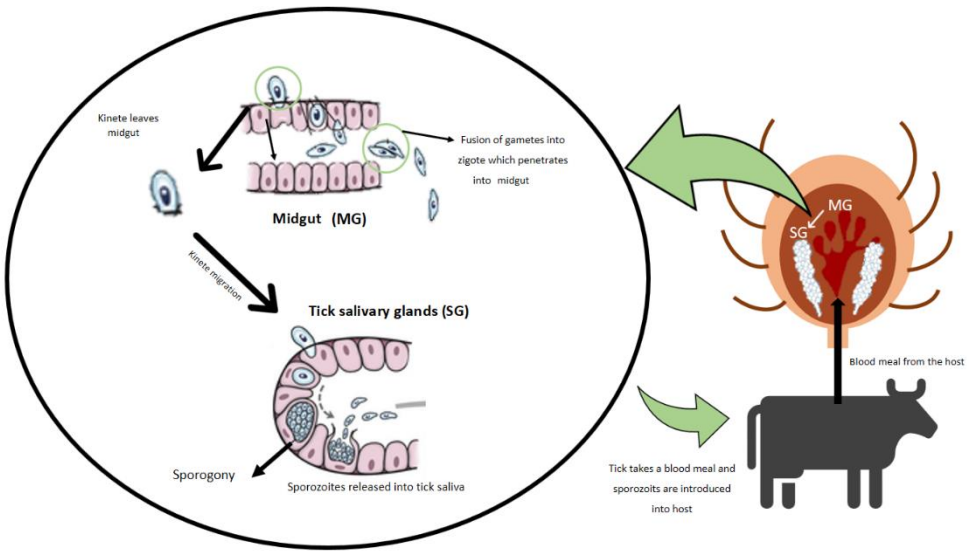

Figure 4 Midgut and tick salivary glands importance during pathogen infection (*adapted from https://veteriankey.com/babesiosis/*).

1.4. Apoptosis

Apoptosis, also called type I programmed cell death (PCD), is an evolutionarily conserved process (Ichim and Tait, 2016) consisting in a homeostatic mechanism to maintain cell populations in tissues (Elmore, 2007), with essential roles that range from proper development during ontogenesis to protection of viral, bacterial and parasitic

infections (Bruchhaus *et al.*, 2007). Apoptosis is associated with characteristic morphologic and biochemical signs, such as cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, exposure of phosphatidylserine and protein cleavage (Bruchhaus *et al.*, 2007; Elmore, 2007; Shemarova, 2010; Zandbergen *et al.*, 2010).

There are two major apoptotic signalling pathways - the intrinsic, so-called mitochondrial, and extrinsic, so-called death receptor, pathways (Figure 5), that are linked whereby molecules of one pathway can influence the other (Elmore, 2007). Various protein families promote or inhibit caspase activation, which is "essential for the morphological and biochemical hallmarks of apoptosis" (Ichim and Tait, 2016), upon binding to protein complexes such as the apoptosome or the death-inducing signaling complex (DISC) (Apoptosis Handbook, Novus Biologicals). Intrinsic apoptosis pathway is triggered by various stimulus, such like DNA damage and endoplasmic reticulum (ER) stress, which activate BH3-only protein family, resulting in the loss of mitochondrial integrity, release of cytochrome c, and the subsequent activation of caspase-9. In turn, caspase-9 activates caspase-3 and caspase-7, leading to apoptosis (Ichim and Tait, 2016; Portt *et al.*, 2011). In contrast, extrinsic apoptosis pathway is triggered by external stimulation and mediated by cell surface death receptors, such as Fas, tumor necrosis factor (TNF), or TRAIL receptors able to activate the initiator caspases-8 and -10 that cleave and activate the effector caspase-3 and caspase-7, leading to apoptosis (Ichim and Tait, 2016). Crosstalk between the intrinsic and extrinsic pathways can occur through caspase-8 cleavage and activation of the BH3-only protein BH3-interacting domain death agonist (BID), whose product is required in some cell types for death receptor-induced apoptosis (Ichim and Tait, 2016; Taylor *et al.*, 2008). A more detailed apoptosis signalling pathway is shown in Appendix 1.

The impact of the bacterial infection by *A. phagocytophilum* in the apoptotic pathway have been studied in the both tick and mammalian cells showing that this pathogen can manipulate cell machinery to counteract apoptosis (Carlyon and Fikrig, 2003; Lee and Goodman, 2006; Galindo *et al.*, 2012; Ayllon *et al.*, 2013, 2015; Alberdi *et al.*, 2016a,b). However, nothing is known about apoptosis related to the *Babesia* genus. It is important to study the death mechanism manipulation in host cells by *Babesia* in

order to provide information on new strategies for the prevention and control of babesiosis, such as the identification of new targets for drug and vaccine development.

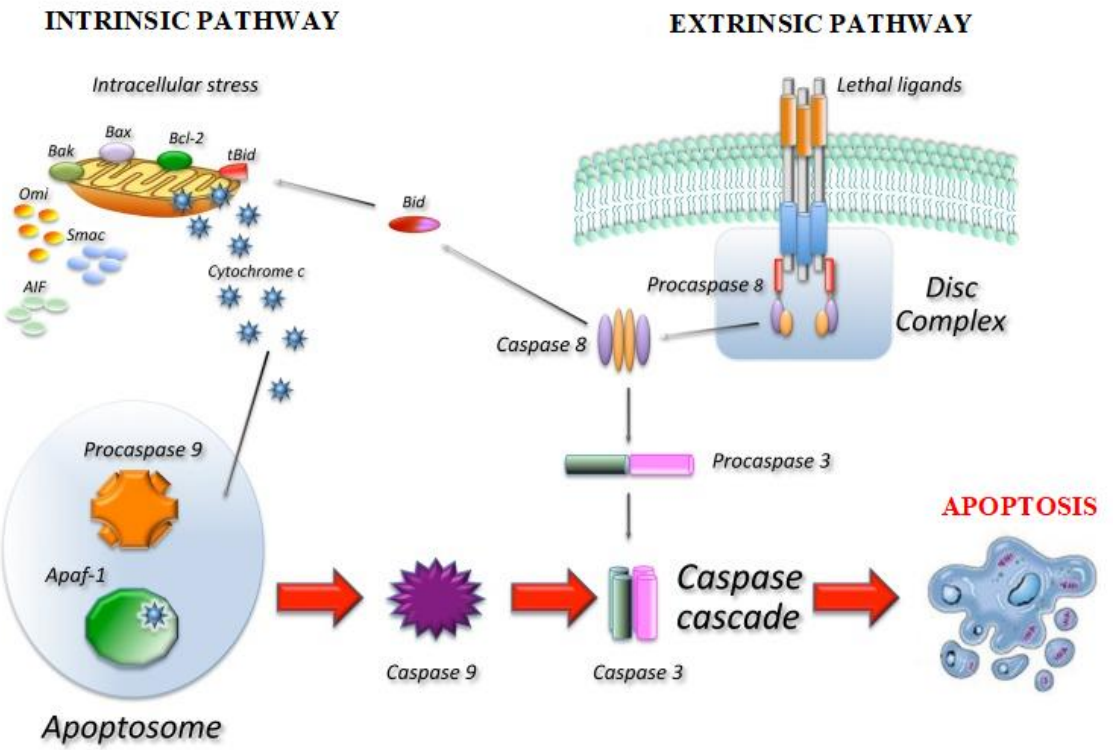

Figure 5 Intrinsic and extrinsic cell death pathways (*adapted from Favaloro et al., 2012*). The intrinsic pathway is initiated by stimulus such as DNA damage, ER stress and growth factor withdrawal leading with changes in the integrity of the mitochondrial membrane, regulated by Bcl-2 family proteins, that results in the release of pro-apoptotic proteins. In the cytoplasm, Cyt c interacts with Apaf-1, which recruits procaspase-9 that recruits the effector caspase-3, leading to apoptosis. The extrinsic pathway is initiated by ligand binding to cell surface death receptors such as TNF, Fas and TRAIL leading to FADD and/or TRADD recruitment. FADD recruits procaspase-8 that once in the cytosol leads to the DISC formation and its subsequent cleavage and activate the effector caspase-3, leading to apoptosis.

1.5. Aims

Infection facilitation by regulation of specific defense mechanism such as cell death confers some pathogens an important parasitic adaptive trait. Some studies have reported this pathogen ability focusing on the vertebrate host but information regarding the influence in ticks is scarce. The only tick-transmitted pathogen studied so far is *A. phagocytophylum* showing that as in vertebrate host cells also in the vector the bacteria is able to avoid destruction of the cells.

The rationale of the present study is based on the assumption that tick-transmitted pathogens may use similar strategies to successfully infect and multiply within tick cells. The overall objective of the present Master thesis is to advance the understanding on the key

molecular interactions between the tick *Rhipicephalus annulatus* and *Babesia bigemina* in order to suggest a novel approach to antigen selection for vaccine development against ticks and tick-transmitted pathogens. Focusing on the apoptosis pathway, the transcriptional response of *R. annulatus* salivary glands to *B. bigemina* infection was investigated by qPCR. Different genes involved in the intrinsic and extrinsic apoptotic pathway were selected and their expression profiles analysed in order to characterize the metabolic pathway of apoptosis during *Babesia* sp. infection.

2. Material and Methods

2.1. *Rhipicephalus annulatus* Ticks

The non-infected and *Babesia bigemina*-infected *Rhipicephalus annulatus* female ticks used in this study were provided by the Kimron Veterinary Institute, Israel. Ticks were maintained at the tick rearing facilities of the Kimron Veterinary Institute, in accordance with standards detailed in the Guide for Care and Use of Laboratory Animals. Two 3-4 months old male Friesian calves free of babesiosis were used to obtain the ticks after being tested for antibodies to *Babesia* spp. infection do with an indirect fluorescent antibody (IFA) assay (Shkap *et al.*, 2005) and kept under strict tick-free conditions. One calf was inoculated i.v. with cryopreserved 2X 10^8 *B. bigemina* (Moledet strain), to obtain the infected ticks. Engorged adult female ticks were collected from both the infected and non-infected calves after feeding and maintained at 28ºC and 80% humidity. Ticks were promptly stored in 500µl of RNA later (Ambion, CA, USA) at –20ºC, partially opened to exposed internal organs to the solution. Ten of each control and test groups were randomly selected to proceed with tissue dissection.

2.2. Salivary Glands Extraction

Each tick was submerged in a drop of 1X phosphate-buffered saline (PBS) (VWR, Pennsylvania, USA), to prevent dehydration. With fine-tipped forceps, ticks were stabilized by holding the basis capitulum and the scutum removed to expose the organs (Fig. 6 – A). Salivary glands (SG) (Fig. 6 – B), located bilaterally along the legs of the tick (Fig. 6 – C), were removed with fine-tipped forceps and place in a new drop of PBS. This wash step was gently repeated until the SG were cleaned of blood. SG were dissected under a stereoscopic microscope at 3.5X magnification (Motic SMZ-171B, China).

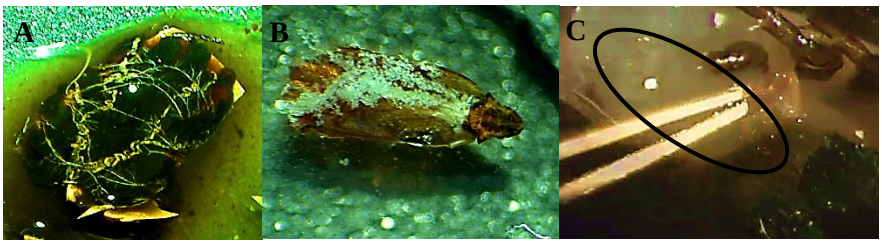

Figure 6 Representation of tick salivary glands dissection steps performed in *Rhipicephalus bursa* (*original from Catarina Monteiro*).

2.3. RNA, DNA Extraction and cDNA Synthesis

SG total RNA was promptly extracted and purified with the GRS FullSample Purification Kit GK26.0050 (GRISP Research Solutions, Porto, Portugal), according to the manufacturer´s instruction. Briefly, SG were disrupted in 2ml centrifuge tubes with 400µl of DRP lysis buffer (GRS kit) and 4µl of β-mercaptoethanol (Bio-Rad, CA, USA), using a VWR^{TM} pellet mixer (VWR). Five minutes later, sample lysates were centrifuged at 15.000g (Micro Star 17, VWR) for 90s and 0.8 volumes of 100% ethanol added to supernatants. Mixtures were transferred to RNA mini spin columns and centrifuged at 15.000g for 60s. One hundred µl of DNAse I mixed in reaction buffer was added to the spin columns placed in new collection tubes and incubate at room temperature for 10min. Afterwards, 400µl of wash buffer RNA 1 were added and columns centrifuged at 15.000g for 60s followed by the addition of 600µl of wash buffer RNA 2 and centrifugation at 15.000g for 60s. Flow-through was discarded in each step. To dry the matrix, RNA columns were centrifuged at 15.000g for 3min and transferred into 1.5ml microcentrifuge tubes. After, 25µl of nuclease-free water was pipetted to the center of the spin columns, incubated at room temperature for 3min and then centrifuged at 15.000g for 60s. The eluted RNA was promptly used or stored at -80°C. RNA concentration and purity were determined by measuring the ratio of the UV absorbance at 260nm and 280nm, using the Nanodrop-1000 V3.7.1 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and with QIAxcel (Qiagen, Hilden, Germany). The QIAxcel system uses capillary gel electrophoresis to enable fast separation of nucleic acids based on size, enabling higher detection sensitivity, less sample wastage and automated loading and analysis. Briefly, 1µl of each sample was pipetted into a 12-tube strip, one of them with 1µl of the QX RNA size marker. An equal volume of QX RNA denaturation buffer was added and the solution heated at 70ºC for 2min, then placed on ice for 1 min. After centrifuged, the 10µl total volume was completed with QX RNA dilution buffer and immediately analysed.

The cDNA was synthesized from 190ng/µl of RNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's directions, using a 100^{TM} Thermal Cycler (Bio-Rad). In summary, 4µl of 5X iScript reaction mix, 1µl of iScript reverse transcriptase and 9µl of nuclease-free water were used to a reaction mix of 20µl. The thermal protocol included a 5min priming step at 25ºC, 20min of reverse transcription

(RT) at 46ºC, 1min of RT inactivation and a hold optional step at 4ºC. cDNA was then stored at -20°C for downstream application.

2.4. Detection of *Babesia bigemina* in Ticks by Taq-man based qPCR

A TaqMan-based qPCR was performed to detect the presence of *B. bigemina* in the sampled ticks using the pair of forward and reverse primers BiF-BiR (BiF: 5´-AATAACAATACAGGGCTTTCGTCT-3´; BiR: 5´-AACGCGAGGCTGAAATACAACT-3´) and TaqMan fluorescence-labelled probe BiP (5´-TTGGAATGATGGTGATGTACAACCTCA-3´) that specifically amplify a fragment of the *18S* rRNA gene, previously described by Kim *et al.*, (2007). PCR amplifications were carried out on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) with the Xpert Fast Probe kit (Grisp). Triplicate 20µl reactions were performed with 10µl of Xpert Fast Probe 2X Mastermix, 400 nM of reverse and forward primers, 100nM of probe, 1µl of cDNA template and nuclease-free water up to the final volume. The conditions of the PCR were: initial denaturation 10min at 95ºC followed by 45 cycles: 20s at 95ºC and 1min at 55ºC. Negative controls were prepared with no template. PCR efficiency was determined generating sequence-specific standard curves with fivefold serial dilutions of DNA from the positive controls. The data was analysed using the Bio-Rad CFX Manager Software version 3.1. Samples with quantification cycle (Cq) values above 39 were considered negative for the presence of the pathogen.

2.5. Selection of Apoptosis Pathway Target Genes

Genes coding for putative mitochondrial ribosome small subunit component mediator of apoptosis dap3 (*DAP3*), death-associated protein kinase 1 (*DAPK1*), apoptosis antagonizing transcription factor (*AATF*), apoptosis inhibitor 5 (*API5*), bax inhibitor 1-related (*BI-1*) and voltage-dependent anion channel (*VDAC*) were chosen from a *R. annulatus* RNAseq based sialotranscriptomic catalogue previously obtained by the team. Sequence-specific primers used for qPCR (Table 1) were designed using Primer3 (v.4.0.0) program (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and synthetized at STABVIDA (Caparica, Portugal).

2.6. Gene Expression Analysis

Gene expression analysis were assessed in the SG by qPCR using the minimum information for publication of qPCR experiments (Bustin *et al.*, 2010).

2.6.1. qPCR Assay Optimization

In order to identify an annealing temperature that provides efficient and specific amplification of the targets, annealing temperatures for the primer sets were optimized by running a temperature gradient on an CFX Connect™ Real-Time PCR Detection System (Bio-Rad) with a thermal cycling comprising 95°C for 3min, 40 cycles of 95°C for 10s and 48ºC – 62ºC for 30s, and a melting curve step of 55°C - 95°C, 0.5°C/s. Primers concentrations were then optimized between 0.3µM and 1µM. Final annealing temperatures and primers concentrations are in Table 1.

2.6.2. qPCR Assay Design

qPCR assay using SYBR® Green-based detection was designed and carried out on an CFX Connect™ Real-Time PCR Detection System (Bio-Rad) with a thermal cycling protocol comprising a 3min denaturation and polymerase activation step at 95°C, 45 cycles of 95°C for 10s and 30s of specific annealing temperature for each primer sets in study. A melting curve step at the end of every amplification reaction, to ensure the production of single specific products and no primer-dimers as well as nonspecific products, is formed by easing the temperature in small increments (55°C - 95°C; 0.5°C/s) and monitoring the fluorescence signal at each step. When the double-stranded DNA (dsDNA) is denatured, SYBR® Green I Die is released and the fluorescence significantly reduced. Melting temperature (Tm) corresponds to the temperature at which 50% DNA denaturation occurs.

To ensure accuracy, triplicate 10µL reactions were composed by 5µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), forward and reverse primers (according to table 1), 1µl of cDNA and nuclease-free water (AMRESCO, OH, USA) to complete the final 10µL. Negative controls were prepared with water. In order to evaluate the amplification efficiency (E), standard curves using serial dilutions of a representative pool of blended infected and non-infected SG RNA samples was performed. Differential gene expression analysis was performed using the Bio-Rad CFX Manager Software version

3.1. Efficiency is established to be between 90% and 115%. The differential gene expression was considered significant when p-value < 0.05.

2.6.3. Gene Expression Normalization

For accurate and reliable gene expression analysis, normalization of cDNA concentration relative differences between samples of the genes of interest against reference genes is essential, in order to infer steady-state mRNA levels. Data were normalised using the genes elongation factor (*ELF*), Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), TATA box binding protein (*TATA*), *β-actin*, *β-tubulin* and *16S* as internal controls, once these genes are described as constitutively expressed (Nijhof *et al.*, 2009). Reference gene validation was based on the geNorm algorithm (Vandesompele *et al.*, 2002) and their expression stability measured with the coefficient variation (CV<0.5) and M-value (M<1) (Hellemans *et al.*, 2007), all included in the CFX Manager Software version 3.1 (Bio-Rad).

2.7. Amplicon Identity Confirmation

Amplicon identity was confirmed by the melt curve from qPCR and an agarose gel electrophoresis (AGE) as described above. The positive amplicons were sequenced at STABVIDA by using the Sanger sequencing technology and the obtained sequences compared with those that were deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed) and the transcripts previously obtained by the team.

Table 1 Primer sequences used for qPCR and respective optimal conditions.

Gene	Accession Number	Forward/reverse primer sequences (5´-3´)	Fragment Length (bp)	Temp anneal (ºC)	Primer (µM)
β-actin	AY255624	GACATCAAGGAGAAGCTYTGC CGTTGCCGATGGTGAT	127	58	0.6
GAPDH	CK180824	AGTCCACCGGCGTCTTCCTCA GTGTGGTTCACACCCATCACAA	123	53.5	0.7
TATA	CV453818	CTTGTCCTCACACACAGCCAGTT GTGAGCACGACTTTTCCAGATAC	122	59.8	
ELF	EW679365	CGTCTACAAGATTGGTGGCATT CTCAGTGGTCAGGTTGGCAG	109		0.5
β-tubulin	CK179480	AACATGGTGCCCTTCCCACG GCAGCCATCATGTTCTTTGC	140	56	
16S	MF946466	TTAACTGGGGCGGTTAAAAA AACATCGAGGTCGCAAACTT	147		
DAPK1	GACK01000273	AGGGTCACACGGACGTTATC GTCTCCATGCTCGTCAGTCA	173	61.6	
API5	GACK01008908	AACGTAACCCCCAGTTCCTC GCCACCTTCAGCTTGTTCTC	175	50	
BI-1	JO843858	GATGCCCAACACTGACACAG CTCCTGCCAGAACACCTTTC	159	54.5	
VDAC	GU994210	CGCGACCTGTTCAACAAGAA CGTGTTGTCGGTGTTCCATT	198	59.6	
DAP3	GACK01006243	CAACCTGACCACTACGCAGA GCAGGAGAGCTCCCATACAG	134	60	0.7
AATF	GACK01004895	AGGCAGTGCAGGTCTCCTTA GCTTCTGCAACTGGATCCTC	179	54	0.9

3. Results

3.1. Salivary Glands Dissection and RNA Extraction

In this study, 20 *R. annulatus* female ticks were used to extract total RNA. Ten ticks were fed in a *B. bigemina* infected calf and ten ticks were fed in a healthy calf, free of babesiosis. Before cDNA synthesis, RNA concentration was determined as described and the results are present in the following table; RNA quality was evaluated with QIAxcel and the result presented in Appendix 2.

Table 2 Concentration of RNA extracted from *Rhipicephalus annulatus* salivary glands measure in Nanodrop. A260/280: ratio of the sample absorvance at 260 and 280 nm; A260/230: ratio of the sample absorvance at 260 and 230 nm.

RNA	ng/µl	A260/280	A260/230
NOT_INF_RNA1	845,36	2.14	2.22
NOT_INF_RNA2	1058,92	2.15	2.24
NOT_INF_RNA3	1782,05	2.01	2.08
NOT_INF_RNA4	425,55	2.07	2.27
NOT_INF_RNA5	631,13	2.11	2.15
NOT_INF_RNA6	190,68	2.17	2.29
NOT_INF_RNA7	85,97	2.15	2.08
NOT_INF_RNA8	737,3	2.11	2.15
NOT_INF_RNA9	833,3	2.14	2.20
NOT_INF_RNA10	1113,3	2.13	2.23
INF_RNA1	1664.75	2.06	2.18
INF_RNA2	1111.58	2.10	2.19
INF_RNA3	1655.68	2.04	2.12
INF_RNA4	1344.20	2.10	2.23
INF_RNA5	952.42	2.16	2.21
INF_RNA6	1270.32	2.14	2.26
INF_RNA7	1046.64	2.15	2.20
INF_RNA8	221.0	2.16	2.09
INF_RNA9	871.6	2.14	2.18
INF_RNA10	219.0	2.15	2.08

3.2. *Babesia bigemina* Infection Confirmation in Tick Salivary Glands

To confirm the infection by *B. bigemina* in the ticks, it was used a qPCR assay. The TaqMan fluorescence-labelled probe used increases the specificity of the reaction. This assay was developed to amplify a fragment of the *18S* rRNA gene. The absence of amplification in the negative controls and the use of positive samples validates the results.

The ten SG from ticks fed on a *B. bigemina* infected calve were found to be positive while the ten SG from ticks that fed on the naïve calve were found to be negative (Figure 7).

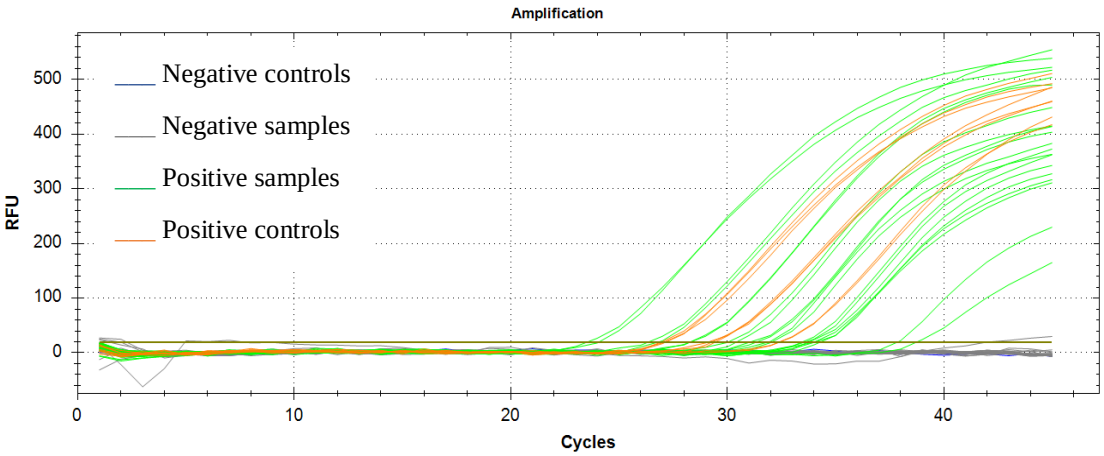

Figure 7 Detection of *Babesia bigemina* in *Rhipicephalus annulatus* salivary glands by TaqMan assay. The curves represent the relative fluorescence units (RFU) per cycle of amplification. The negative samples are in grey, positive samples in green, positive controls in orange and negative controls in blue.

3.3. qPCR Optimization

Gene expression of the six-targeted apoptosis-related genes (*DAP3*, *DAPK1*, *AATF*, *API5*, *BI-1*, *VDAC*), as well as those of the reference genes (*β-actin*, *GAPDH*, *TATA*, *ELF*, *β-tubulin*, *16S*) was accessed through a qPCR assay using SYBR® Green-based detection, as described above. After testing different annealing temperatures and the best primer concentration (the final conditions of each gene are described in table 1), the assays were validated by the absence of amplification in the negative controls and by the efficiency of each reaction plate (Table 2). Reaction efficiency, which describes how much the target is being produced in each cycle, was automatically calculated by the CFX manager software that displays it under the standard curve using serial dilutions of a representative SGs pool, whose slope of the derived line are created with, at least, two points but as many points as possible were used.

In order to normalize the expression of the genes in study, their relative quantity was normalized to the relative quantities of the reference genes. Reference genes stability between the different conditions (infected/uninfected) was calculated with CFX manager software. It is established that a correct and accurate normalization is achieved by using two to five reference genes (Vandesompele *et al.*, 2002). Herein, only the combinations

of reference genes showing a CV below 0.5 and M below 1 were used assuring the most stable reference genes.

Table 3 Final amplification efficiencies of the references and target genes.

Gene	Amplification efficiency (%)
β-actin	91.4
GAPDH	100.1
TATA	105.7
ELF	99.3
β-tubulin	103.1
16S	88.3
DAPK1	87.0
API5	109.1
BI-1	101.5
VDAC	98.6
DAP3	88.0
AATF	100.3

3.4. Apoptotic Pathway Genes Differential Expression

For the evaluation of differential expression of the selected genes, the relative expression of the genes was measure in both the conditions studied, infected and uninfected and after compared. If the relative expression in the control condition is higher than in the test condition then the gene is assumed to be down-regulated while if the relative expression in the control is lower than in the test condition, the gene is up-regulated. This regulation is represented by the fold change that can be either positive or negative. The statistical significance of such result is given by the p-value. Overall our results show that from the six studied genes *DAP3* and *API5* are more expressed in the infected samples while *DAPK1*, *VDAC*, *BI-1* and *AATF* are more expressed in the control samples (uninfected). Significant differential expression was demonstrated in *DAP3*, *DAPK1* and *VDAC* (p-value < 0.05). Detailed information regarding each gene is below.

DAP3 gene expression showed to be significantly up-regulated in the *R. annulatus* ticks SG when infected with the *B. bigemina* parasite (fold-change = 4.204; p = 0.005) (Figure 8). Normalization was made using the *16S* and *β-tubulin* reference genes (CV = 0.074; M-value = 0.212).

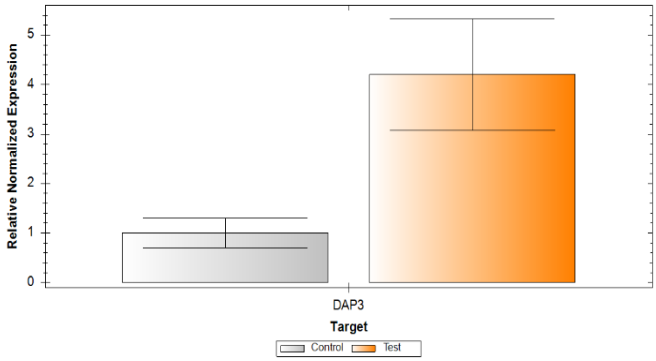

Figure 8 Relative normalized expression of the apoptotic *DAP3* gene in *Rhipicephalus annulatus* salivary glands. The grey column corresponds to the uninfected SG and the orange to the *B. bigemina* infected SG. Infection results in an upregulation (fold-change = 4.204; p = 0.005).

DAPK1 gene expression showed to be significantly down-regulated in the *R. annulatus* ticks SG when infected with the *B. bigemina* (fold-change = -3.546; p = 0.011) (Figure 9). Normalized expression was calculated using the *β-tubulin*, *β-actin*, *ELF* and *TATA* as reference genes (CV= 0.359; M-value= 0.766).

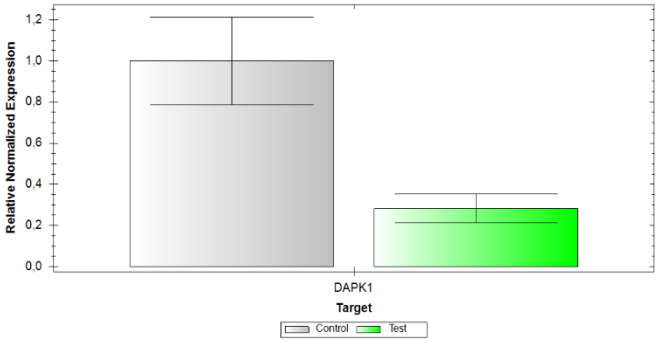

Figure 9 Relative normalized expression of the apoptotic *DAPk1* gene in *Rhipicephalus annulatus* salivary glands. The grey column corresponds to the uninfected SG and the green to the *B. bigemina* infected SG. Infection results in a downregulation (fold-change = -3.546; p = 0.011).

Similarly, the expression of the gene ***VDAC*** showed to be significantly down-regulated in the *R. annulatus* ticks SG when infected with *B. bigemina* (fold-change = -1.512; p = 0.034) (Figure 10). The expression normalization was made using the *β-tubulin*, *β-actin*, *ELF* and *TATA* reference genes (CV= 0.310; M-value= 0.664).

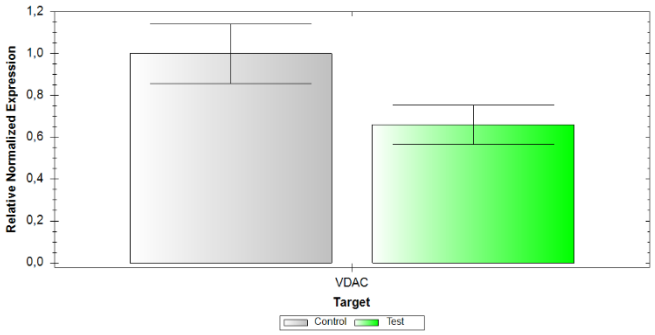

Figure 10 Relative normalized expression of the apoptotic *VDAC* gene in *Rhipicephalus annulatus* salivary glands. The grey column corresponds to the uninfected SG and the green to the *B. bigemina* infected SG. Infection results in a downregulation (fold-change = -1.512; p = 0.034).

In the case of the ***AATF*** gene expression, under the conditions undertaken in the present study, it was demonstrated that in infected SG there is a down-regulation of the gene without statistical significance (fold-change = -1.016; p = 0.086) (Figure 11). Normalization was assured against the *β-tubulin*, *β-actin* and *ELF* reference genes (CV = 0.245; M-value = 0.617).

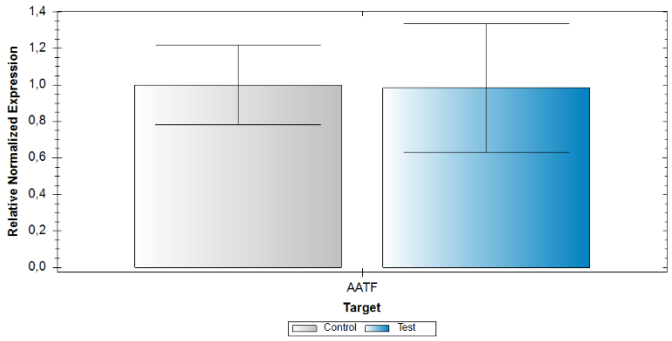

Figure 11 Relative normalized expression of the apoptotic *AATF* gene in *Rhipicephalus annulatus* salivary glands. The grey column corresponds to the uninfected SG and the blue to the *B. bigemina* infected SG. Infection results in a downregulation (fold-change = -1.016; p = 0.086).

After normalization with the *β-tubulin*, *β-actin* and *ELF* reference genes (CV = 0.224; M-value= 0.541), it was demonstrated that the ***BI-1*** gene is more expressed in the control samples (non-infected) in comparison to the infected samples (Figure 12). However, there is no a statistical significance (fold-change = -1.724; p = 0.441).

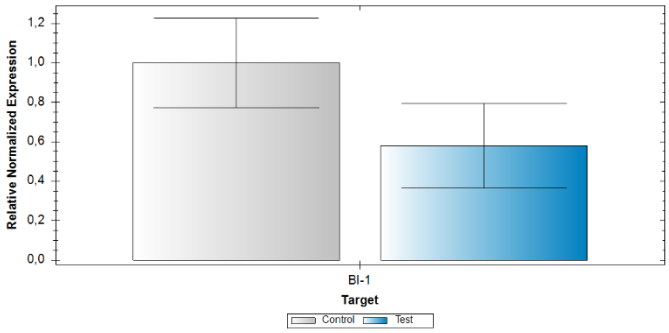

Figure 12 Relative normalized expression of the apoptotic *BI-1* gene in *R. annulatus* SG. The grey column corresponds to the uninfected SG and the blue to the *B. bigemina* infected SG. Infection results in a downregulation (fold-change = -1.724; p = 0.441).

Finally**, *API5*** gene relative expression was evaluated in tick SG in *B. bigemina* infected and non-infected SG. Results show that in infected SG there is an overexpression of this gene but without being statistically significant (fold-change = 2.514; p = 0.295) (Figure 13). For expression normalization the *16S* and *β-tubulin* reference genes were used (CV = 0.151; M-value = 0.438).

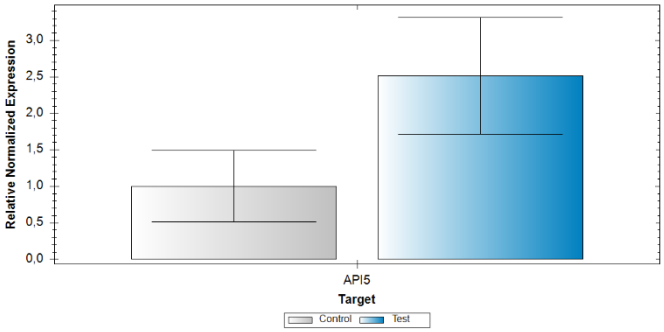

Figure 13 Relative normalized expression of the apoptotic *API5* gene in *Rhipicephalus annulatus* salivary glands. The grey column corresponds to the uninfected SG and the blue to the *B. bigemina* infected SG. Infection results in a downregulation (fold-change = 2.514; p = 0.295).

3.5. Amplicon Identity Confirmation

For confirmation of the identity of the amplified cDNA fragments three different strategies were used. The first approach was the analysis of the melt temperature (Table 4), followed by AGE (Appendix 3) and finally, Sanger sequencing was used to validate the identity of the genes studied. From the analysis of the melt curves, it was perceived that only one amplicon per pair of primers was being formed. All the samples and/or replicates that did not show the correct melt peak were discarded from the analysis.

Table 4 Study genes melting temperature.

Reference gene	Melting temperature (ºC)
DAP3	81.0-81.5
DAPK1	83.5-84.0
AATF	80.0-80.5
API5	82.5-83.5
BI-1	81.0
VDAC	85.0-85.5

The AGEs confirmed that the fragments amplified during the qPCRs presented the expected size (the fragment sizes are described in table 1 and AGE results are in appendix 3). Afterwards, randomly selected PCR products were purified using a commercial kit. Amplicons were sequenced and the obtained sequences compared with those that were deposited in the NCBI database and the transcripts previously obtained by the team. Results are shown in table 5 and appendix 4. With the exception of *API5*, sequencing confirmed the identity of all target genes. The quality of the *API5* obtained sequence did not allow a correct alignment with the available sequence. Nevertheless, the previous analysis suggest that the fragment being amplified is the correct one. The *BI-1* and *AATF* query cover values of 27 and 22%, respectively, are low because the obtained sequences were compared with the available ones from *Amblyomma maculatum* and *Rhipicephalus pulchellus*, respectively thus less homology between sequences can be expected.

Table 5 Alignment of obtained amplicons with target genes.

	Query cover	E value	Identity	Accession number
DAPK1	90 %	3e-68	100 %	GACK01000273
DAP3	84 %	7e-29	92 %	JAA58791
VDAC	97%	1e-81	99%	GU994210
API5	-	-	-	-
BI-1	27%	7e-08	100%	JO843858
AATF	22%	4e-07	100%	GACK01004895

4. Discussion

The protozoan parasites *B. bigemina* mainly vectored by the *R. annulatus* ticks are responsible for bovine babesiosis commonly named tick fever or red water fever. Due to the great impact on cattle health and economy is urgent the search for more effective strategies for the control of tick and TBP. The molecular interactions at the vector-pathogen interface may be targeted in order to impair pathogen infection and/or multiplication within the tick or block transmission to the vertebrate host. Research has shown that during the long-lasting tick-pathogen co-evolution, microorganisms have developed important strategies to manipulate or modulate tick response to infection, without impairing tick survival, enhancing their capacity of infection, replication and transmission guaranteeing the survival of both (de la Fuente *et al.*, 2016a; Šimo *et al.*, 2017). Moreover, it was demonstrated that tick gene expression is modified in response to pathogen infection in different tick stages and various tick organs, including SG (Antunes *et al.*, 2012; Heekin *et al.*, 2012, 2013; Sunyakumthorn *et al.*, 2012; Cotté *et al.*, 2014; Alberdi *et al.*, 2016b; Cabezas-Cruz *et al.*, 2016, 2017; Mansfield *et al.*, 2017; Martins *et al.*, 2017; Kalil *et al.*, 2017; Šimo *et al.*, 2017; Thangamani *et al.*, 2017). SG are an important barrier that pathogen need to overcome and exploit in order to successfully be transmitted to the vertebrate host (Alberdi *et al.*, 2016b; Šimo *et al.*, 2017), making this tissue a primary target in the discovery of potential protective tick antigens. Within this tissue, pathogens need to interact with SG proteins in order to invade cells and multiply inside them before being released to the vertebrate bloodstream taking advantage of the vasodilator, anticoagulant, anti-inflammatory, and immunosuppressive properties of saliva (Šimo *et al.*, 2017). During SG infection different metabolic processes are affected, including the important apoptosis pathway (Ayllón *et al.*, 2015). As previously mentioned, several pathogens, especially those capable of invading and multiplying within host cells (Ashida *et al.*, 2011), as *B. bigemina*, have developed mechanisms to inhibit the cellular process of apoptosis. Information regarding pathogen influence in tick apoptotic response is scarce and few systems, as the *A. phagocytophylum* – *Ixodes* sp., are partially studied so far. Infection of tick SG with *A. phagocytophilum* inhibit the intrinsic pathway of apoptosis by down-regulating Porin expression, favoring bacterial infection, and tick cells respond to infection, promoting the apoptosis through induction of the extrinsic apoptotic pathway (Ayllón *et al.*, 2015). However, nothing is known about apoptosis in the *Babesia* genus. It is important to study the PCD manipulation in host cells by *Babesia* in order to provide information on new strategies for the prevention and control of babesiosis, such as the

identification of new targets for drug and vaccine development. Base on this, six apoptotic genes of a *R. annulatus* RNAseq based sialotranscriptomic catalogue of genes up-regulated in an infected tick population were selected and evaluated in *B. bigemina* infected and uninfected *R. annulatus*. The results obtained are discussed below.

Mitochondrial Ribosome Small Subunit Component Mediator of Apoptosis dap3 (DAP3)

The commonly called death associated protein 3 (DAP3), highly conserved from yeast to human (Mukamel and Kimchi, 2004), is a 46-kDa protein containing a GTP binding domain and a P-loop, essentials for the induction of cell death (Mukamel and Kimchi, 2004). It is believed to be involved in both the intrinsic and extrinsic apoptotic pathways (Wazir *et al.*, 2015b). Kissil and collaborators (1995) firstly described DAP3 interaction with the extrinsic pathway through the antisense RNA-mediated inactivation of the *DAP3* in HeLa cells that shown to be protected from IFN-γ-induced cell death. Nowadays, it is known that DAP3 interfere with the extrinsic pathway by Fas, TNF-α or TRAIL death receptors (DR) stimulation (Wazir *et al.*, 2015a; Xiao *et al.*, 2015) although recent studies suggest that DAP3 association is strongest with Fas receptor-related DISC and its actions are enabled by binding with death ligand signal enhancer (DELE) (Wazir *et al.*, 2015a). Other studies have shown DAP3 interaction with the intrinsic pathway of apoptosis intimately related to the mitochondrial biogenesis (Berger *et al.*, 2000). It was demonstrated that a loss of *DAP3* expression leads to lethal mice embryonic mutations (Kim *et al.*, 2007) and overexpression of human, mouse and nematode *DAP3* is sufficient to cause cell death as a result of mitochondrial fragmentation (Mukamel and Kimchi, 2004; Xiao *et al.*, 2015). In the present study, *DAP3* gene was found to be overexpressed under *B. bigemina* infection of *R. annulatus* female ticks (fold-change = 4.204; $p = 0.005$). This result suggests that infection is leading to the natural cell response with the activation of apoptosis. Due to the role of DAP3 in both the extrinsic and intrinsic pathways, it is not clear which is via that is being induced.

Death-Associated Protein Kinase I (DAPK1)

Death-associated protein kinase 1 (DAPK1) belongs to a phylogenetically widespread family of five calcium-regulated serine/threonine (Ser/Thr) kinases that mediates cell death (Chuang and Chisholm, 2014; Singh *et al.*, 2016). It is a highly

conserved gene from invertebrates to chordates and mammals (Singh *et al.*, 2016) and in *Caenorhabditis elegans* and *Drosophila*, the only encoded DAPK family member (Chuang and Chisholm, 2014). In mammals, *DAPK1* gene is transcribed into a single mRNA of 6.3kb (Singh *et al.*, 2016) encoded for a 160 kDa protein with a conserved $Ca2^{+}$/CaM autoregulatory domain, 10 ankyrin repeats, 2 putative P-loop consensus sites and a ROC-COR domains, which overlap with a cytoskeletal-binding region, a death domain and a serine-rich C-terminal tail (Chuang and Chisholm, 2014; Shiloh *et al.*, 2014; Singh *et al.*, 2016). *DAPK1* is a critical component in the ER stress-induced cell death pathway (belonging to the intrinsic apoptotic pathway) that transmits upstream signaling events into two distinct directions, caspase activation and autophagy, leading to cell death. Stimuli include Fas, INF-γ, and TNF-α (Singh *et al.*, 2016). *DAPK1* can activate the tumor suppressor activity of p53 and "p53 activation molecularly links DAPK to the classical death pathway involving mitochondrial-based activation of the caspase cascade" (Bialik and Kimchi, 2006). This process requires the presence of p19ARF that promotes the ubiquitin-dependent degradation of p53 (Bialik and Kimchi, 2006). Currently identified as a putative tumor suppressor gene, nothing is known about their role in parasitic infections. In the present study, *DAPK1* gene shown to be significantly downregulated (fold-change = -3.546; p = 0.011). A deficiency in DAPK1 mRNA levels affects the intrinsic apoptotic pathway since it could no longer interact with p53. This potential *DAPK1* modulation by *B. bigemina* benefits the parasitic dissemination within cells.

Voltage-Dependent Anion Channel (VDAC)

The voltage-dependent anion channel (VDAC), commonly called mitochondrial porin, is a protein of the outer mitochondrial membrane that plays a central role in the intrinsic apoptotic pathway, leading to cell life or death, mediated by its association with various ligands and proteins (Shoshan-Barmatz *et al.*, 2010). With a molecular weight of 30–35kDa, VDAC "consists of a polypeptide having unfolded alternating hydrophobic and hydrophilic amino acids, forming from 13 to 19 transmembrane β-chains composed of a single α-helix at the amino terminus". This conformation separates the apolar environment from the polar one, forming a wide barrel-shaped channel through the membrane (Rodríguez-Hernández *et al.*, 2015). It is known that some pathogens have developed strategies to manipulate apoptosis in order to prevail within the host cells,

namely by interfering with the mitochondrial membrane permeability. VDAC allows the flux of small molecules into the mitochondrial intermembrane space and the release of pro-apoptotic molecules, such as the cytochrome c, into the cytosol (Rodríguez-Hernández *et al.*, 2015). VDAC upregulation is strongly correlated with apoptosis induction and several mechanisms have been proposed. Among them, mitochondrial outer membrane permeabilization (MOMP) increase, VDAC oligomerization, increase of VDAC-ANT complexes and ROS production (Shoshan-Barmatz *et al.*, 2010). A BmVDAC-like protein that participates during *B. bigemina* invasion of *R. microplus* midgut cells was recently identified by Rodríguez-Hernández and collaborators (2012) through proteomic analysis. Based on PCR results, BmVDAC gene expression shown to be significantly increased in infected ticks compared to uninfected ones at 24h post-repletion (Rodríguez-Hernández *et al.*, 2015). *VDAC* also showed to be critical in *T. gondii* infection with a modified expression in infected cells (Nelson *et al.*, 2008). "In mosquitoes, VDAC plays a role during *Plasmodium* sp. invasion of the midgut; likewise, the dissemination of *B. burgdorferi* through the tick midgut might be associated with the ability of VDAC to bind a tissue-type plasminogen activator" (Antunes *et al.*, 2017). Contrary to the results obtained by Rodríguez-Hernández *et al.*, (2015), in the present study, *VDAC* gene was significantly down-regulated under *B. bigemina* infection of *R. annulatus* ticks (fold-change = -1.512; p = 0.034). Once *VDAC* is required for apoptosis induction, the results obtained suggest that the parasite influences the levels of *VDAC* mRNA. The different tick species used in the studies may explain the difference or even different infection status or levels. Nevertheless, more studies focusing this molecule are necessary to clarify the *Babesia* spp. potential manipulation of *VDAC* expression.

Apoptosis Antagonizing Transcription Factor (AATF)

Apoptosis antagonizing transcription factor (AATF), also called Che-I, Ded (Passananti and Fanciulli, 2007) and Traube (the murine ortholog) (Desantis *et al.*, 2015), is a RNA polymerase II binding protein of 558 aminoacids, highly conserved among eukaryotic species (Lezzi and Fanciulli, 2015; Passananti and Fanciulli, 2007). This protein consists of an "N-terminal acidic domain, a canonical leucine zipper, and three LXXLL motifs for nuclear receptor binding. It also contains two nuclear and two putative nucleolar localization signals" (Lezzi and Fanciulli, 2015). With a nuclear and nucleolar localization (Lezzi and Fanciulli, 2015), *AATF* promotes de cellular transcription process

by binding specific transcription factors to the general transcription machinery (Passananti and Fanciulli, 2007). For example, after DNA damage AATF are released from the E2F target genes and recruits to p53 promoter (Floridi and Fanciulli 2007; Hopker *et al.*, 2012; Passananti and Fanciulli, 2007) where "AATF binds to the *PUMA*, *BAX* and *BAK* promoter regions to repress p53-driven expression of these pro-apoptotic genes" (Höpker *et al.*, 2012). So, AATF belongs to the intrinsic apoptotic pathway. AATF anti-apoptotic activity was originally identified by Page and collaborators (1999) in rats for its ability to antagonize the Dlk-induced apoptosis (Lezzi and Fanciulli, 2015). Up to now, information about the anti-apoptotic activity of this protein derives from studies performed in neural tissue but nothing is known about *AATF* possible involvement in the control of parasitic infection. In the present study, *AATF* expression under *B. bigemina* infection of *R. annulatus* ticks did not show significant regulation (fold-change = -1.016; p = 0.086). Interestingly, it was noticed that a mouse embryo mutant for *Traube* shows a decrease in the number of ribosomes and *Drosophila AATF* mutant restrain the development of the egg chamber at the same stage as the mutants affecting the synthesis of ribosomes (Lezzi and Fanciulli, 2015). In this context, more studies need to be performed to understand if *B. bigemina* is able to restrict *AATF* transcription, for example, through the reduction of ribosomal activity as a way of escape from *AATF* anti-apoptotic activity.

Bax Inhibitor-1 (BI-1)

Bax inhibitor-1 (BI-1) is a multi-spanning membrane protein of the endoplasmic reticulum (ER) with anti-apoptotic activity (Bultynck *et al.*, 2012; Urra *et al.*, 2013). It was originally identified by Xu and Reed (1998) in cDNA library screens for human proteins capable of suppressing BAX-induced apoptosis in yeast. BAX, a Bcl-2 family member, promotes the intrinsic apoptotic pathway when induced by ROS (Rolland *et al.*, 2014). During ER stress, calcium and protein disulfide isomerases (PDI) are released from the ER to the cytosol, inducing apoptosis by MOMP deregulation. In addition, "BiP at the cytosol translocates to the plasma membrane, where it serves as a cell surface receptor for the pro-apoptotic protein Par-4" (Urra *et al.*, 2013). BI-1 acts like an anti-apoptotic protein by increasing ER calcium content (Urra *et al.*, 2013), as well as reducing ROS production (Bultynck *et al.*, 2012; Rolland *et al.*, 2014). Furthermore, BI-1 was shown to protect certain cells from TRAIL, a tumor necrosis factor (TNF) family member

(Chae *et al.,* 2003). BI-1 is highly conserved across diverse species and the identified BI-1 plant homologs shown to be implied on an evolutionarily conserved cytoprotective pathway (Henke *et al.*, 2011; M'Angale and Staveley, 2017), namely against exposure to certain pathogens. *Arabidopsis BI*-1 showed to protect transgenic plants from apoptosis induced by ectopic expression of mammalian BAX. In barley, *BI*-1 was overexpressed in response to fungal inoculation. A downregulation of *BI*-1 in tobacco cells results in rapid cell death upon carbon starvation and the treatment of cultured rice cells with cytotoxic extracts from rice blast fungus down-regulates *BI*-1 expression (Chae *et al.*, 2003). In this study, tick *BI-1* gene expression was not demonstrated to be differentially regulated upon infection (fold-change = -1.724; p = 0.441) suggesting that *Babesia* is not able to interact with this molecule. More studies need to be performed on this gene in order to better understand if parasites can induce the expression of these anti-apoptotic genes.

Apoptosis Inhibitor 5 (API5)

Apoptosis inhibitor 5 (API5), also known as AAC-11 protein (anti-apoptosis clone 11), FIF (fibroblast growth factor 2-interacting factor) and MIG8 (cell migration-inducing gene 8) is a 55 kDa apoptosis-inhibiting nuclear protein (Koci *et al.*, 2012; Mayank *et al.*, 2015). It was originally identified in mouse BALB/c3T3 fibroblasts, human cervical carcinoma CUMC-6 cells and immortalized primary liver THLE-3 cells (Koci *et al.*, 2012) and found in protists, plants and animals (Mayank *et al.,* 2015). API5 regulates the intrinsic apoptotic pathway possibly through protein-protein interactions mediated by HEAT and ARM repeats with partners such as FGF-2, Acinus, ALC1 and AIP1/2. Once connected, API5 suppresses E2F1 transcription factor-induced apoptosis, binds to Acinus and protects it from cleavage by caspase-3, interacts with ALC1 that promotes cell proliferation and interacts with DEAD-box RNA helicases AIP1/2, regulating apoptosis (Han *et al.*, 2012). Influenza A virus (IAV) was shown to interact with API5, suppressing its expression, thus potentiating the E2F-dependent apoptotic pathway and ensuring viral replication (Mayank *et al.*, 2015). Similar results were obtained in a *Drosophila* genetic screening that identified API5 as a suppressor of apoptosis mediated by E2F in multiple cell types (Koci *et al.*, 2012). FGF-2 is able to protect cells from apoptosis, whereby it is involved in cell proliferation and tumorigenesis (Han *et al.*, 2012). In this study, *API5* showed to be more expressed under *B. bigemina* infection but no statistical significance was found (fold-change = 2.514; p = 0.295) suggesting that the parasite is able to

manipulate the expression of this gene that consequently will repress cell apoptosis benefiting *Babesia* maintenance within the cells. However, more studies need to be performed to clarify the role of parasites in the regulation of such gene.

4.1. General Conclusions

Once pathogens invade a host cell, their survival and multiplication rely on temporally and spatially regulated host cell-pathogen interactions. Therefore, the main objective of the present Master thesis was to evaluate the expression profile of the selected apoptotic genes in *B. bigemina* infected and uninfected *R. annulatus* ticks by qPCR, in order to evaluate the parasite ability to manipulate cellular apoptosis. The data collected herein, point to a strong interaction between *Babesia* parasites and tick apoptosis metabolic pathway. The downregulation of *DAPK1* and *VDAC* and the upregulation of *API5* suggest that the intrinsic apoptosis pathway is being repressed by the infection but cells are counteracting with the induction of *DAP3* expression perhaps via the extrinsic pathway, such as it was found in the *A. phagocytophilum* infected *Ixodes* ticks. Thus, pathogen proteins are interacting with host proteins, suppressing or hijacking the normal function of tick proteins. Also, this study shows that anti-apoptotic genes seem to be more difficult to manipulate. To benefit parasites, their expression must be induced inhibiting cell death and the mechanism to produce this effect is more complex. The results reported here clearly show that more studies need to be conducted focusing this particular tick-pathogen interaction and that *B. bigemina* is, to some extent, able to interact with host cells promoting its survival and transmission. Understanding this interplay is fundamental for the development of novel tick control measures.

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6. Appendix

Appendix 1

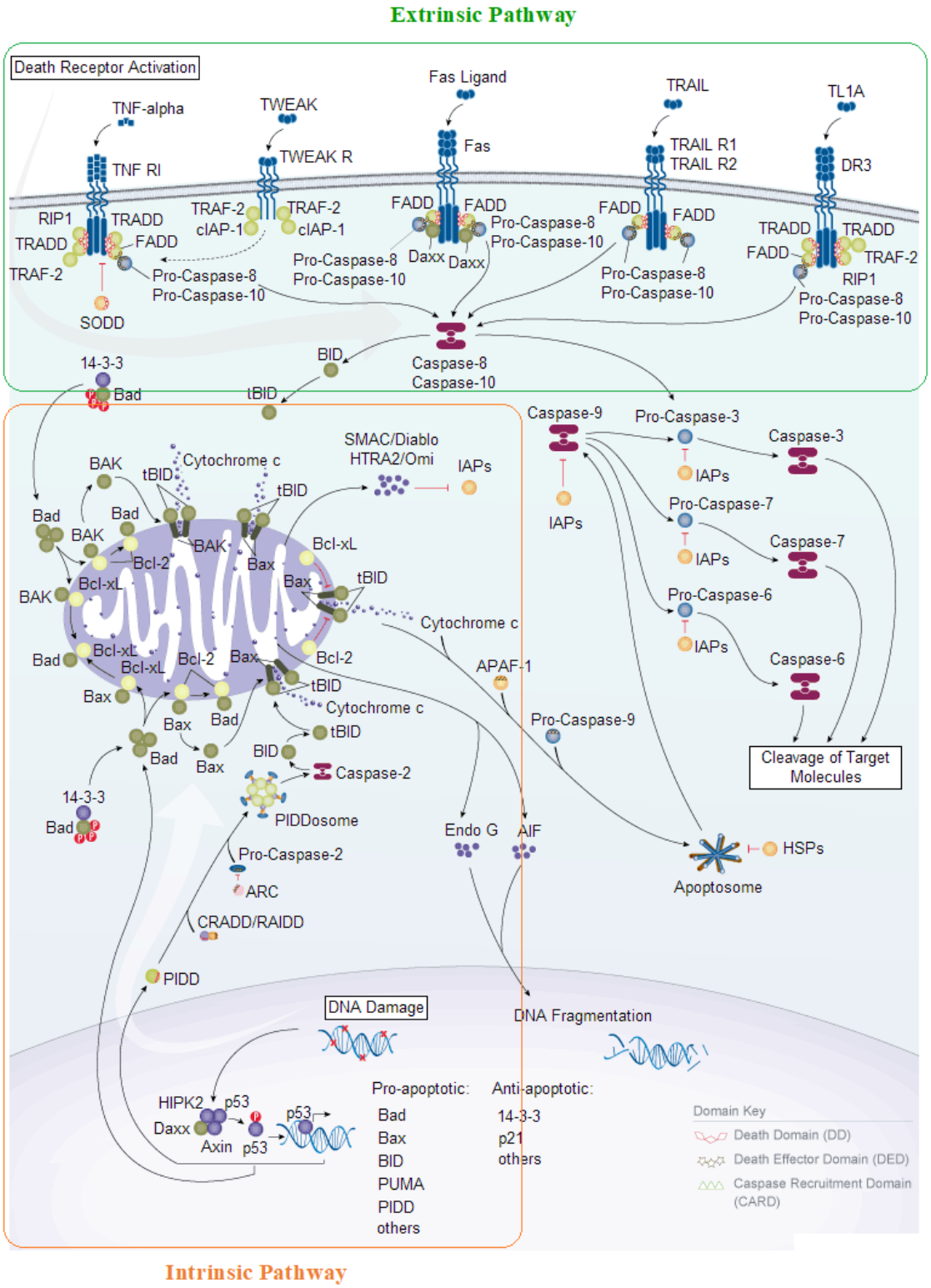

Figure 14 Detailed apoptosis signaling pathway *(adapted from https://www.rndsystems.com/pathways/apoptosis-signaling-pathway). .*

Appendix 2

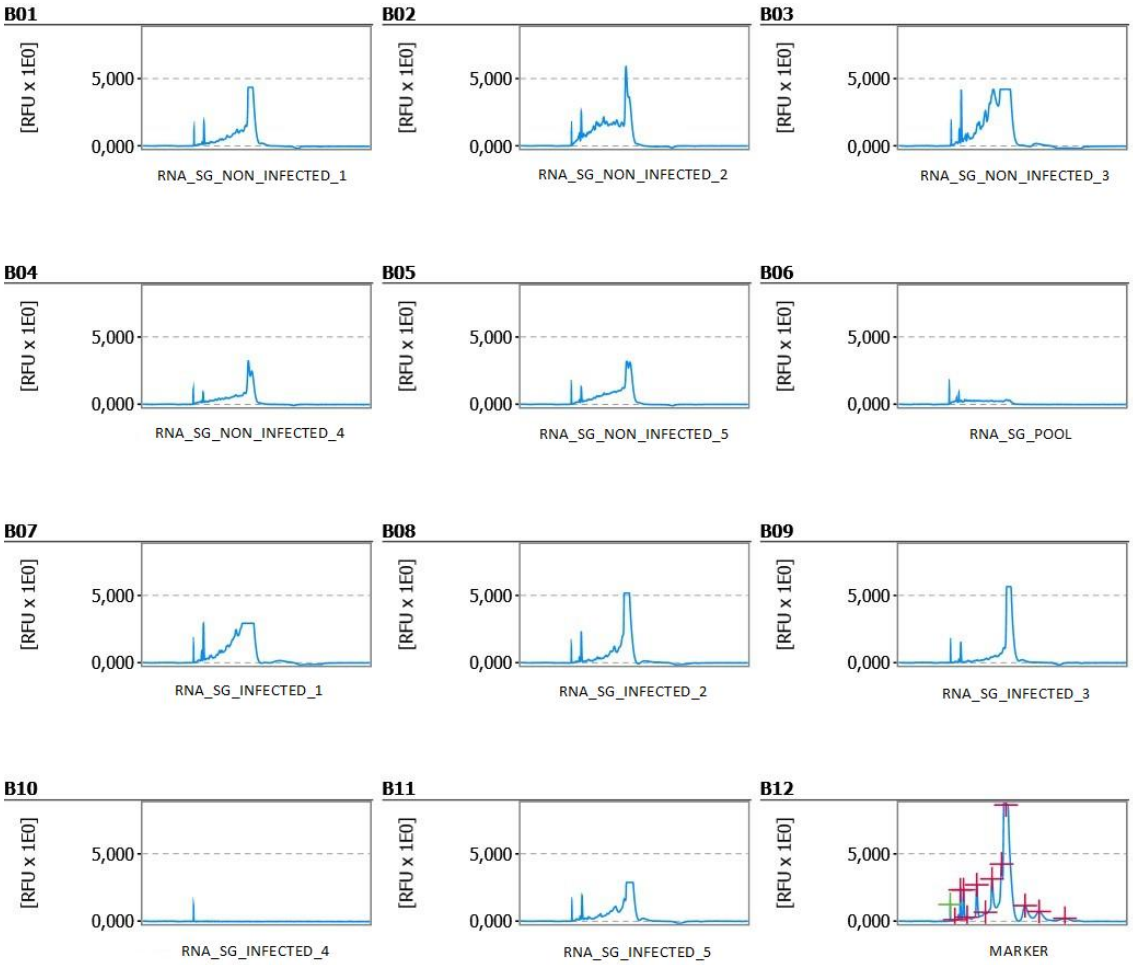

Figure 15 Qualitative analysis of the RNA samples from the SG of the non-infected and *Babesia bigemina*-infected *Rhipicephalus annulatus* ticks. Electropherogram view obtain from the QIAxel analysis. RFU – relative fluorescence units.

Appendix 3

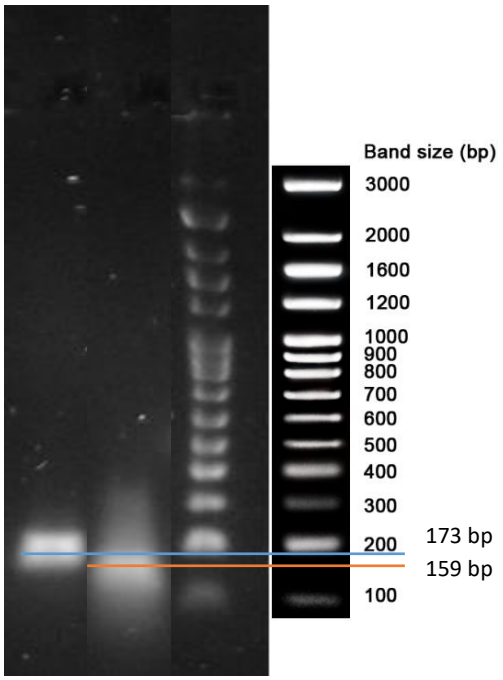

Figure 16 Confirmation of the *DAPK1* and *BI-1* amplicon size. Lane 1: *DAPK1* amplicon; Lane 2: *BI-1* amplicon; Lane 3: NZYDNA Ladder VII (nzytech). Samples were electrophoresed on a 1.5% Agarose/SYBRSafe gel, 0.5x TBE.

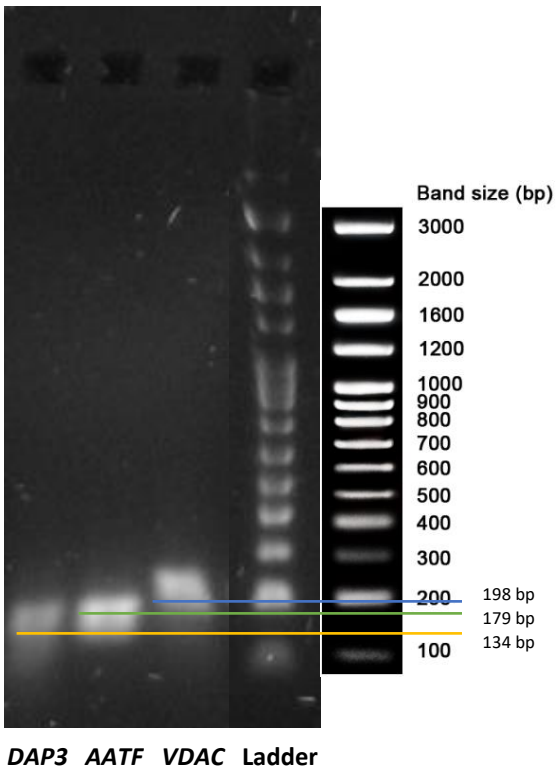

Figure 17 Confirmation of the *DAP3*, *AATF* and *VDAC* amplicon size. Lane 1: DAP3 amplicon; Lane 2: AATF amplicon; Lane 3: VDAC amplicon; Lane 4: NZYDNA Ladder VII (nzytech). Samples were electrophoresed on a 1.5% Agarose/SYBRSafe gel, 0.5x TBE.

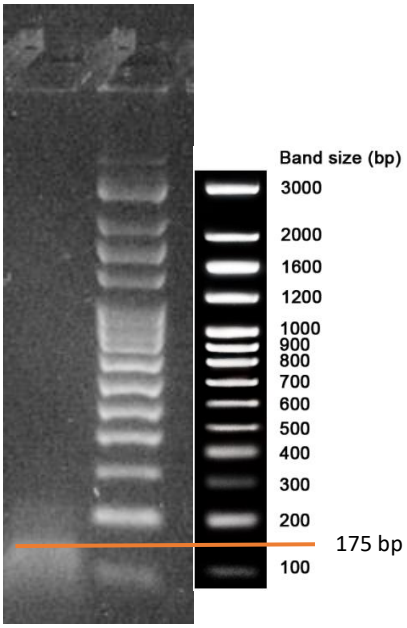

Figure 18 Confirmation of the *API5* amplicon size. Lane 1: API5 amplicon; Lane 2: NZYDNA Ladder VII (nzytech). Sample were electrophoresed on a 1.5% Agarose/SYBRSafe gel, 0.5x TBE.

Appendix 4

Table 6 Sequences of the amplicons obtained by Sanger sequencing.

Gene	Sequence
DAPK1	NNNNNNNNNNNGNNTCTCGGTCGACTGCCAGAACAAGTCGGGCGAGACTGC CCTGCATGTCGCTGCCCGCTACGGTCACCACCCAGCGGTCAAACTTCTTTGCT CCTTTGGTGCCAACATTAATGTGACTGACGAGCATGGAGAC
API5	NNNNNNNNNNNNNNCNNNNNNNNNNNGCNNCGACTCTTTTGTTNNGNANCC GTCNNNAGCCCTGNNNAANAACTGAAGGTGGNNNNGNCA
BI-1	NNNNNNNNNGTNNNANNCTCTNGCGGANTAATGCCCCTGACACACGGGCAC CCTGAAGTCCTTTAGACAAGGGGACATCTTTCGGAAAGGTGTTCTGGCAGGA GA
AATF	CNNTNANNNGGTGANCTGAGTNAGAAACTTTTGGCGAAGATTCCTGTGCCCC AATAAGAAACGGCAAAGTTACACACCTTTCCGAGGATCCAGTTGCAGAAGC
DAP3	NNNNGGNNAACCNCTGACAAACNNCNTGGATCATGGAGNTGAACGCATGAA GCACGCTTGCAGCTGTATGGGAGCTCTCCNNCCN
VDAC	GGGAGCTCGACTGCAGTCGACCACCCAGACGGGCGTCGAGTTCAACGTGAG CGGCACCAGCCTCAATGACACCGGCAAGGTGAACGCCTCGCTCGAGACCAA GTACAAGGTTCCCGAGTACGGGCTCACGCTCAAGGAAAAATGGAACACCGA CAACACGA