The role of GtxA in the pathogenesis of
Gallibacterium anatis in vivo and in vitro

Supervisor: Anders Miki Bojesen

This thesis has been submitted to the Graduate school of Health and Medical Sciences
University of Copenhagen, 15 December 2020
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Bo Tang  
PhD thesis 2020

Department of Veterinary and Animal Sciences  
Faculty of Health and Medical Sciences  
University of Copenhagen

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Submitted on: December 15th, 2020  
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Preface

The work described in this thesis was carried out from March of 2016 to December of 2020 at Department of Veterinary and Animal Sciences of University of Copenhagen under supervision of Professor Anders Miki Bojesen and Associate Professor Rikke Heidemann Olsen. The thesis was submitted to the Faculty of Health and Medical Sciences, University of Copenhagen. This work was supported by the Health and Medical Faculty of University of Copenhagen and The Chinese Scholarship Council.

Bo Tang
December 2020
Copenhagen, Denmark
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First, I would like to express my heartfelt gratitude to my professor, Anders Miki Bojesen, for his warm-heart encouragement and most valuable advice related to courses, experiments and other things, particularly for his professional comments on my English writing, with his help, encouragement and guidance, I have enhanced my skills within experimental design and overcome my biggest obstacle of English writing.

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Last but not least, I would like to extend my gratitude to my parents and my professors for their valuable encouragement and spiritual understanding during my overseas study. Here, in memory of my grandmother, who support me forever and give me love all the time, even though you did leave me, your spirit encourage me all my life, and I will try to be a good women like you for the rest of my life.
Summary - English

*G. anatis* biovar haemolytica could cause avian veterinary diseases in egg-laying hens resulting in respiratory and reproductive disorders with severe egg-drop and increased mortality. The virulence determinant, GtxA, which is in charge of *G. anatis’* haemolytic and leukotoxic activity has previously been recommended as a promising vaccine candidate. Currently, however, very little is known about the mechanisms that determine *G. anatis* host or cell specificity, although the recombinant protein GtxA-N has immunogenic and protective capacity in a *G. anatis* challenge model. However, recent research suggested that GtxA play important role in the pathogenesis of *G. anatis* in the natural host. Alternatively, the specific role of GtxA in the pathogenesis *in vivo* is sparsely understood. It is widely reported that bacterial interaction with immune cells e.g. avian macrophages is an effective way to assess specific protein-host interactions during a systemic infection in the natural host, to understand the host-pathogen interactions between *G. anatis* and different chicken tissue types, and evaluate the impact of GtxA on cultured macrophages, we used an avian pathogenic strain *G. anatis* 12656-12 wild-type and its isogenic gtxA deletion mutant (*AgtxA*) for inoculations in egg-laying hens and for co-cultivation with the chicken macrophage-like cell line HD11, respectively.

The ultimate goal of this Ph.D. thesis was to investigate the effect of GtxA during the infection of *G. anatis* in the natural host and macrophage HD11 cells, respectively. In this study, we hypothesized that *G. anatis* strains produce pore-forming toxins GtxA, which participate and trigger different immune system and apoptosis response in the host against *G. anatis* infection. Alternatively, they could describe adhesion and invasion characteristics of *G. anatis* strains to macrophage cells *in vitro*. And the results of this thesis are presented in two published papers investigating on the role of GtxA during the infection *in vivo* and *in vitro*.

In published paper I, to assess the importance of GtxA-host interactions during avian *G. anatis* infection, a total of 24 egg-laying hens infected with a *G. anatis* 12656-12 wild type and its isogenic *G. anatis* *AgtxA* was used as an *in vivo* model to evaluate and compare the gross pathology and the re-isolation rates of *G. anatis*. The results shown the virulence in the *G. anatis* *AgtxA* mutant was attenuated during infection in laying hens, indicating that GtxA had an important contribution to the severity of host-specific infections. In the attempt to identify the
immune response in the natural host, it was shown that \( \Delta gtxA \) mutant could regulate the acute phase reaction by promoting of TNF-\( \alpha \) production and interfere humoral immunity by suppression of secretion of IL-4 in poultry. It was also agreement with that \( \Delta gtxA \) mutant induce less cellular infiltration and inflammatory reaction in pathological results, which suggest that \( \Delta gtxA \) mutant could take part in a weaker host response possibly through partial destruction of the Th2-like pathway in the ovary tissue. In addition, it could be speculated that GtxA may participate in apoptosis suppression at an early stage in the ovary to promote \( G. anatis \) multiplication, which was a preferred locus of this bacterium. In a further study, we hypothesized that pore-forming toxins of \( G. anatis \) strains involved in mediating apoptosis through a similar calcium-dependent mechanism as \( E. coli \) or involved in mediating pro-inflammatory death pathway. These investigations may allow us to understand better the role of GtxA in the activation of certain apoptotic pathways and offer novel insights into the mechanisms of \( G. anatis \)-induced apoptosis in immune cells in the future.

In published paper II, in order to understand the host-pathogen interactions between \( G. anatis \) and macrophage-like HD11, it is pivotal to describe adhesion and invasion characteristics of \( G. anatis \) strains to macrophage cells and investigate the specific impact of GtxA for the cellular changes in the pathogenesis of \( G. anatis \) in vitro. Briefly, it was shown that the highest adhesion level of \( G. anatis \) wild-type was achieved at the peak of 60 min in the macrophage and intracellular amount of the \( \Delta gtxA \) mutant strain was lower than that of \( G. anatis \) WT and remained relatively lower level after 6 h PI, which not only imply that \( G. anatis \) wild-type has a higher capacity of adherence and invasion in initial processes of exposure in vitro, but also suggesting that GtxA contributes significantly to the severity of infections in collapsed cells.

We also demonstrated that GtxA seems to be able to attenuate the host inflammatory response at an initial infection through overexpression of IL-10 and low-level expression of TNF-\( \alpha \), while the \( \Delta gtxA \) mutant promoted a pro-inflammatory response through induced expression of IL-1\( \beta \), IL-6 and TNF-\( \alpha \). These results provided further understanding of the role of GtxA in the interaction with the avian immune system by inhibiting initial host inflammatory response and showed macrophages HD11 can be employed as biological agents for subsequent experiments related to commercial vaccine applications. Although we found GtxA has an alteration effect on the apoptotic response of splenic tissue infected by \( G. anatis \) in the spleen tissue, but the effect of
the GtxA toxin on the type of cell death was less clear. Considering the characteristics of Bcl-2 preventing cell death as an original defense mechanism against bacterial infection, our data showed that the production of the anti-apoptotic bcl-2 gene in the ΔgtxA mutant group was mainly down-regulated at 2 h PI rather than after 6 hours compared with the WT group, which indicating that in the early stages of *G. anatis* infection, GtxA may proactively prevent or delay cell damage through promoting anti-apoptotic responses in the host. Furthermore, flow cytometry analysis shown that the ΔgtxA group had fewer dead cells and more surviving cells than the WT group respectively indicating that GtxA has the ability to induce cell death caused by *G. anatis* in HD11 cells and the ΔgtxA mutant can attenuate dead cells caused by *G. anatis*. Although GtxA obviously induces cell death, it is not clear whether it is mainly due to necrosis or apoptosis. We hypothesize that GtxA may prevent cell apoptosis and thus become a chance of survival because it enables bacteria to replicate in the host cell. That is why the inhibitor efficacy assay would be used to examine the function of GtxA at activating the caspase-9-dependent apoptotic pathway that blocks apoptosis and promotes *G. anatis* replication in *G. anatis*-treated macrophage cells.

In conclude, this thesis has contributed to increase the knowledge about the effect of GtxA in the pathogenesis of *G. anatis* in vivo and vitro, especially during the early phases of attachment and invasion of avian host immune cells. This study demonstrated that GtxA plays an important role in stimulating both the innate and parts of the adaptive cellular immune system, obviously in mediating a Th2-like immune response against *G. anatis* infection in the ovary tissue. This provide further understanding of the role of GtxA in the interaction with the avian immune system. In addition, this thesis investigated that ΔgtxA mutant had significantly fewer lesions and microscopic changes in the ovary and had attenuated dead cells caused by *G. anatis* than those infected with *G. anatis* WT in the macrophage HD11. Our findings have implications for ΔgtxA mutant might be involved in modulating the apoptosis host defense to prolonged bacteria survival in the host. These observations play a particularly important role for aiming at optimizing a multivalent vaccine against *Gallibacterium anatis* in the future.
Sammendrag - Dansk


I den første publicerede artikel (artikel 1) er vigtigheden af GtxA-vært interaktion under infektion med G. anatis adresseret. I dette studie blev i alt 24 æglæggere inficeret eksperimentelt


I den anden publicerede artikel (artikel II) undersøgtes adhæsion og invasion af *G. anatis* i den makrofag-lignende celleline HD11 for at opnå større forståelse af vært (modelleret ved HD11 celleline)-patogen interaktioner. Samtidig blev det undersøgt hvilket betydning GtxA har for de cellulære ændringer og *in vitro* patogenesen. Opsummeret blev det vist at det højeste adhæsionsniveau af *G. anatis* WT stammen til makrofagerne blev opnået efter 60 min og det cellulære niveau af Δ*gtxA* mutanten på det tidspunkt var betydeligt lavere end WT stamme niveauet og forblev relativt lavere seks timer efter infektion. Disse resultater viser at GtxA er af betydning for *G. anatis*’ kapacitet til adhérer og invadere makrofager ved den initiale
eksponering, og desuden at GtxA bidrager signifikant til sværhedsgraden af infektion i kollapsede celler.

I vores studier blev det også vist at GtxA synes at være i stand til at svække værtens inflammatoriske respons gennem en overekspresion af IL-10 og nedsat ekspression af TNF-α, da det blev belyst at ΔgtxA mutanten stimulerede et pro-inflammatorisk respons gennem induceret ekspression af IL-1β, IL-6 and TNF-α. Med disse resultater har vi opnået en større forståelse af betydningen af GtxA for G. anatis interaktion med det aviære immunsystem, vist ved at det initiale inflammatorisk respons er inhiberet af GtxA. Desuden har vi vist at HD11 makrofager kan anvendes som metode for at undersøge comerciëlle vacciner i fremtiden. Selvom vi har konstateret at GtxA har en modificerende effekt på det apoptotiske respons i miltvæv inficeret med G. anatis, var effekten af GtxA toksinet på typen af den inducerede celledød mindre klar. Det anti-apoptotiske gen Bcl-2 er forbyggende for celledød som en del immunsystemets forbyggende mekanismer ved bakteriel infektion. Vores data viser at Bcl-2 niveauet i ΔgtxA mutantgruppen var nedreguleret to timer efter infektion, men først efter seks timer i WT infektionsgruppen, og disse data tyder på at GtxA sandsynligvis forbygger eller udsætter celleskade i værten under de tidlige infektionsstadier, ved at fremme det anti-apoptotisk respons i værten.


Samlet set har denne afhandling bidraget til et øget kendskab til betydningen af GtxA for G. anatis- patogenesen både in vivo og in vitro, særligt under de tidlige faser af adhæsion og invasion af de aviære immune celler. Resultaterne i afhandlingen har påvist at GtxA spiller en
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered, regularly spaced arrays of short palindromic repeats</td>
</tr>
<tr>
<td>SCVs</td>
<td>Small colony variants</td>
</tr>
<tr>
<td>ICEs</td>
<td>Integrative and conjugative elements</td>
</tr>
<tr>
<td>AFLP</td>
<td>Amplified length polymorphism analysis</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in-situ hybridization</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>PFTs</td>
<td>Pore-Forming Toxins</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>CPAF</td>
<td>Chlamydia proteasome-like active factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of apoptotic protein</td>
</tr>
<tr>
<td>Annexin V</td>
<td>Annexin V-FITC</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP-biotin nick-end labeling</td>
</tr>
<tr>
<td>CIC</td>
<td>Cellometer Imaging Cytometer</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>BL-3 cells</td>
<td>Bovine lymphoblastoid cells</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ΔgtxA</td>
<td>Isogenic gtxA deletion mutant</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>PCOECs</td>
<td>Primary chicken oviduct epithelial cells</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
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<td><em>G. anatis</em></td>
<td><em>Gallibacterium anatis</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>VFDB</td>
<td>Platform called virulence factor database</td>
</tr>
<tr>
<td>T1SS</td>
<td>T1Stype I secretion system</td>
</tr>
<tr>
<td>OMVs</td>
<td>Outer membrane vesicles</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>gyrB</td>
<td>Gyrase subunit B gene</td>
</tr>
<tr>
<td>IgY</td>
<td>Special IgG</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>C1</td>
<td>Complement protein</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>NTHi</td>
<td>Non-typeable Haemophilus influenza</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
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<td><em>E. coli</em></td>
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1.1 Gallibacterium anatis

1.1.1 Description

*Gallibacterium anatis* (*G. anatis*) is an opportunistic pathogen in poultry. *G. anatis* was initially identified as *Pasteurella* -like “cloacal bacteria” when the organism were first isolated from the oviduct in hens suffering from “egg-peritonitis” (Kjos-Hanssen, 1950). Since then organisms named as *Pasteurella haemolytica*, *Actinobacillus salpingitis* or *Pasteurella anatis* were published during the following decades (Biberstein and Gills, 1962; Helfer and Helmboldt, 1977; Mushin et al., 1980; Neubauer et al., 2009). Subsequently, by using 16S rRNA sequencing analysis and ribosomal RNA hybridization techniques, researchers confirmed that *P. haemolytica* and *A. salpingitidis* belonged to the family *Pasteurellaceae* (De Ley et al., 1990; Dewhirst et al., 1992). Yet it was not until 2003 that *Gallibacterium* was established as a new genus of the *Pasteurellaceae* and proposed to include five different species of *Gallibacterium* namely *G. anatis*, *Gallibacterium melopsittaci*, *Gallibacterium salpingitis*, *Gallibacterium trehalosifermentans*, an unnamed species-like group termed “group V ” and three genomospecies species 1, 2 and 3 (Christensen et al., 2003; Neubauer et al., 2009). Later additional species were proposed meaning that genus currently comprise species (Bisgaard et al., 2009).

*G. anatis* appears to be the only species commonly causing disease. Pathogenic *G. anatis* has been reported from a range of different avian species, such as turkeys, ducks, pheasants, guinea fowl, geese and partridges (Mushin et al., 1980; Bojesen et al., 2003a; Bojesen et al., 2003b; Christensen et al., 2003; Bisgaard et al., 2009; Neubauer et al., 2009). Two phenotypically distinct biovars have been reported to date for *G. anatis*: a haemolytic biovar (*G. anatis* biovar haemolytica), which can be distinguished by its strong haemolytic phenotype and a non-haemolytic biovar *G. anatis*, which can by distinguished by its lack of hemolysis (Christensen et al., 2003; Persson and Bojesen, 2015). Particularly, *G. anatis* biovar haemolytica is a potential pathogenic bacterium commonly found in the upper respiratory tract and lower genital tract in poultry where it may cause salpingitis and peritonitis in egg-laying hens (Bojesen et al., 2004; Persson and Bojesen, 2015). The *G. anatis* core genome is relatively small when overlapping features were identified from comparing the strains UMN179, F149\(^T\), and 12656 -12, respectively (Figure 1) (Bojesen et al., 2003b; Bojesen et al., 2004; Johnson et al., 2013).
Growth of *G. anatis* is fast and most strains incubated in fresh brain heart infusion (BHI) broth at 37 °C will reach the late log-phase within approximately 3-4 hours (Christensen *et al.*, 2003). The characteristics of the haemolytic biovar *G. anatis* incubated on blood agar are circular, greyish, semi-transparent, smooth with an entire margin (1-2 mm) surrounded by a wide β-hemolytic zone (Christensen *et al.*, 2003)(Figure 2).

**Figure 1.** Venn diagram depicting shared and unique proteins among *G. anatis* strains UMN179, F149T, and 12656-12. Percentages are relative to UMN179 total predicted proteins (Johnson *et al.*, 2013).

**Figure 2.** The colony morphology of haemolytic biovar *G. anatis*. The haemolytic biovar *G. anatis* 12656-12 incubated on blood agar showed circular 1-2 mm shiny colonies surrounded by a wide β-hemolytic zone (Christensen *et al.*, 2003).

### 1.1.2 Epidemiology

After Kjos-Hansen initially discovered a cloacal bacteria isolated from egg-laying chickens suffering from salpingitis and peritonitis in 1950 (Kjos-Hanssen, 1950), researchers gradually reported its widespread distribution throughout the world. *G. anatis* has been reported from several European countries including Germany, Poland, Denmark, and Asian countries, such as Japan and China (Mráz *et al.*, 1976; Mirle, 1991; Suzuki *et al.*, 1996; Rzewuska *et al.*, 2007; Singh *et al.*, 2016). In addition to the reports from Demark information about the epidemiology of *G. anitas* has also been reported from other places including Africa, Australia, Mexico and North America (Gilchrist, 1963; Bisgaard, 1977; Addo and Mohan, 1985; Shaw *et al.*, 1990; Vazquez *et al.*, 2006; Johnson *et al.*, 2011; Jones *et al.*, 2013; Lawal *et al.*, 2017). In 1981, Bisgaard & Dam conducted a prevalence study on salpingitis in poultry in which *G. anatis* was isolated from 9.3% of egg-laying chickens with salpingitis and peritonitis lesions (Bisgaard and...
Dam, 1981). In addition, a histopathological study of 12-week-old chickens infected with *G. anatis* showed severe local damage in the respiratory tract, but that the tissue distribution of *G. anatis* in systemic organs was limited (Zepeda et al., 2010).

The transmission of *G. anatis* was investigated by Bisgaard in 1977 showing that haemolytic *G. anatis* has a wide distribution in the hatching and feeding process of Danish poultry with different biosafety levels, However, a haemolytic *G. anatis in vivo* infection model could not confirm the association between biosecurity and *G. anatis* prevalence (Bisgaard, 1977). In contrast, Bojesen et al. (2003) shown that the prevalence proportions were significantly related to the level of biosafety observed in the crowd in across-sectional study based on different layers of biological systems (Bojesen et al., 2003a). In the same study, no evidence of vertical transmission of *G. anatis* was observed.

In general, the transmission of *G. anatis* is believed to happen through horizontal transfer yet a number of reports have suggested that transmission possibly can take place through vertical transmission (Janetschke and Risk, 1970; Matthes et al., 1969; Mushin et al., 1980; Shapiro, 2013). Before Paudel et al., reported that cockerels could get infected in their reproductive organs fulfilling Koch's postulates in 2014, no information was given about the precise bacteriological genotypes in egg-laying chickens with salpingitis (Bisgaard and Dam, 1981; Paudel et al., 2015; Paudel et al., 2014).

Apart from the information on the prevalence and transmission of haemolytic *G. anatis* in animal hosts, including human immunosuppressed individuals seems to be at the highest risk for acquiring a *G. anatis* infection. *E.g.* there has been a report on an immunosuppressed 26-year-old woman suffering from bacteremia and diarrhea, most likely due to ingestion of food contaminated with *G. anatis* (Aubin et al., 2013). *G. anatis* can be isolated from different kinds of organisms including parakeets, geese, Budgerigars wild birds and the most prevalent host of *Gallibacterium* was chicken (Christensen et al., 2003; Bisgaard et al., 2009). In addition to indigenously available poultry categories such as guinea fowls, ducks, turkeys, and pigeons, some species have also been examined from pheasants, partridges, psittacine birds and web-footed birds (Bisgaard, 1993; Oguntunji, 2014).
1.1.3 Pathogenesis

As described in section 1.1.3, *G. anatis* is a genus in the Gram-negative *Pasteurellaceae* family and has a wide range of avian hosts (Christensen et al., 2003; Bisgaard et al., 2009). Interestingly, the haemolytic biovar of *G. anatis* has been comprehended as part of the normal symbiotic flora in the upper respiratory tract and the lower genital tract in healthy chickens (Beug et al., 1979; McWhinney et al., 1992; Frey et al., 1993; Bojesen et al., 2003a; Paudel et al., 2014). However, it is now evident that, *G. anatis* is to considered a potentially pathogenic bacterium, causing infection primarily related to the reproductive and respiratory systems in poultry, in which salpingitis and peritonitis are the most common clinical manifestations (Mirle, 1991; Jordan et al., 2005; Neubauer et al., 2009). Today, *G. anatis* is recognized as constituting a important cause of bacterial disease in hens resulting in respiratory and reproductive disorders with severe eggdrop, increased mortality and lowered animal welfare worldwide (Mirle, 1991; Jordan et al., 2005; Neubauer et al., 2009). Overall, *G. anatis* have been isolated and reported from several clinical cases, primarily those associated with salpingitis, peritonitis, decreased egg production and clinically respiratory syndrome, oophoritis, salpingitis and peritonitis lesions (Hacking and Pettit, 1974; Mushin et al., 1980; Shaw et al., 1990; Suzuki et al., 1996; Jordan et al., 2005; Neubauer et al., 2009), haemorrhagic and follicle degeneration, ruptured follicles and choana lesions (Addo and Mohan, 1985; Paudel et al., 2014). In conclusion, these reports showed that *G. anatis* time may be considered as an opportunistic (or secondary) pathogen associated with infected chicken with lesions (Bisgaard, 1977; Mushin et al., 1980; Shaw et al., 1990).

However, other findings have indicated that *G. anatis* play an important role in the process of veterinary infection by single-pathogen-induced or mixed-pathogen-induced lesions in chicken models (Bisgaard and Dam, 1981; Shaw et al., 1990). *G. anatis* has also be observed in mixed-pathogen infections associated with *E. coli* arousing reproductive tract disorders (Bisgaard and Dam, 1981; Trampel et al., 2007). Stokholm et al.(2010), inspected laying hens from 11 commercial farms and results demonstrated that *E. coli* and *G. anatis* biovar haemolytica, together with *P. multocida* have caused local co-infection (Stokholm et al., 2010).

Because of these early findings, *G. anatis* was presumed to have an important impact on the infection process with other bacterial pathogens (Bisgaard and Dam, 1981; Mirle, 1991).
However, it was difficult to distinguish whether the lesions were caused by *G. anatis* or caused by *E. coli*, and role of *G. anatis* in the lesions when co-segregating with *E. coli* has not be elucidated completely (Jordan *et al.*, 2005; Dziva and Stevens, 2008). *E. coli* mainly has been involved in the infection of the reproductive tract in chickens for many years as a single infection, until the indistinguishable phenomenon caused by an overgrowth of *Pasteurella* -like pathogens was investigated by the occurrence of *G. anatis* biovar haemolytica in hens with clinical symptoms of egg peritonitis (Jordan *et al.*, 2005; Dziva and Stevens, 2008; Neubauer *et al.*, 2009). Therefore, *G. anatis* was considered the main pathogen causing salpingitis and peritonitis in chicken. It was also further supported by other recent examples that *G. anatis* is associated with the pathological damage of the reproductive organs with, giving rise to egg peritonitis and an occasionally increased mortality (Mushin *et al.*, 1980; Bojesen *et al.*, 2004). Paudel *et al.*, described the observation of gross pathological lesions of SPF layers intranasally incubated with wild-type *G. anatis* in 2014, while confirmed the pathogenesis of *G. anatis* by infecting the birds experimentally has proved a good direct correlation between experimental infection with *G. anatis* and the pathological changes and clinical diagnosis findings observed under natural infection (Figure 3) (Paudel *et al.*, 2014). Based on these early findings, *G. anatis* was recently considered to be a pivotal major causative pathogen for causing reproductive tract lesions.
Figure 3. The overall pathological damage of the SPF layer experimentally infected with \textit{G. anatis}.
(a): Pericarditis, 7 d.p.i. (b): Follicular inflammation with large amounts of hemorrhage and lymphatic swelling and rupture in the lymphatic tissue (black arrow), 30 d.p.i. (c): There are a large number of necrotic cells in the liver (black arrow), 28 d.p.i. (d): Eggs and cheese-like tissues are separated from the abdomen of the chicken (black arrow), 28 d.p.i. (e): Haemorrhagic follicles (black arrow), 38 d.p.i. (f): Atherosclerotic ruptured and injured follicle (black arrow), 38 d.p.i. (Paudel et al., 2014).

Recent scientific discoveries have shown that \textit{G. anatis} infection involves not only colonization of important parts of the reproductive tract but also an infection of the gastrointestinal flora (Torok et al., 2011; Kaakoush et al., 2014). For example, Torok et al., explained in 2011 that different feeding methods can be used to identify and characterize the intestinal flora-related functions in broilers and then Kaakoush analyzed the gastrointestinal microbiota of 31 age-appropriate broilers to further improve our understanding of the interaction between bacteria and avian hosts, while the consumption of these taxa competing with these bacteria may be a sensible strategy to eliminate them in the chicken's gastrointestinal tract (Torok et al., 2011; Kaakoush et al., 2014). Additionally, \textit{G. anatis} was also involved in the infection of immunosuppressed chickens or mimicking natural infections in natural chicken (Bojesen et al., 2004; Paudel et al., 2013), and as previously mentioned there are few reports of \textit{G. anatis-} associated infections in humans that may only found in severely immunocompromised patients (Gautier et al., 2005; Aubin et al., 2013).

1.1.4 Virulence factors

In the past, we have identified and characterized a variety of virulence factors, and understood to a certain extent that they play a key role in the pathogenesis of bacterial diseases, especially identifying new drug targets and designing new vaccines (Persson et al., 2018; Van Driessche et al., 2020). The interaction between susceptible hosts and pathogenic factors are still updated on
the platform called virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm). According to the pathogenic mechanism of virulence and function (Figure 4)(Wu et al., 2008), recent studies have found decisive factors in different categories of virulence factors, such as Vibrio vulnificus RTX-like toxin, Fimbriae, Outer membrane vesicles, Polysaccharide capsules, Capsule, Biofilm formation, Iron acquisition, PhoP/PhoQ two-component system and other virulence determinants (Moxon and Kroll, 1990; Finlay and Falkow, 1997; Hall-Stoodley and Stoodley, 2005; Lloyd et al., 2007; Bojesen et al., 2011; Bager et al., 2013b; Persson and Bojesen, 2015; Pérez-Reytor et al., 2018). In general, the ability of pathogenic bacteria to cause disease in susceptible hosts is determined by a single factor or a combination of different virulence factors at different stages of infection. Recently, several putative virulence factors of G. anatis were discovered as described in the following sections.

Figure 4. The main virulence factors of pathogenic bacteria. (A) Gram-positive and (B) Gram-negative bacteria (Wu et al., 2008).

1.1.4.1 Gallibacterium toxin A (GtxA)

The importance of RTX-toxins for virulence and pathogenicity of bacteria belonging to the family of Pasteurellaceae is linked to their hemolytic and especially cytotoxic abilities (Frey et al., 1993; Tatum et al., 1998; Frey, 2011). In vivo infection models of pigs infected with mutant strains of Actinobacillus pleuropneumoniae that could not produce RTX-toxins in the host, have been used to determine the function of RTX-toxins (Reimer et al., 1995; Kamp et al., 1997; Liu et al., 2009). The results showed that the infected pigs in the mutant group had diminished
lesions resulting in lower mortality and reduced morbidity compared to the outcome of infection with the wildtype strain. From other in vivo studies investigating RTX deficient bacterial strains, a significant decline in virulence has been reported from A. pleuropneumoniae and Mannheimia haemolytica (Reimer et al., 1995; Kamp et al., 1997; Tatum et al., 1998). These previous findings support the observation of RTX-toxins and their construction of isogenic leukotoxin-deficient mutants of the Pasteurellaceae family, emphasizing the profound role of RTX toxins in the pathogenesis of these bacteria (Frey, 2011). In recent years, other toxins of different bacteria, such as the adenylate cyclase (CyaA) toxin of Bordetella pertussis, RTX toxin (rtxA) of Kingella negevensis, pore-forming RTX toxin (HlyA) of uropathogenic E. coli secreted by a type I secretion system (T1SS), have been reported (Welch, 2001; Opota et al., 2017; O’Brien et al., 2019; Benz, 2020; Oscarsson et al., 2020).

Similar to other members of Pasteurellaceae, Kristensen et al. identified and characterized the cytolytic RTX-toxin GtxA in Gallibacterium, which is responsible for hemolytic leukotoxic properties and the cytolysis (Figure 5)(Kristensen et al., 2010; Kristensen et al., 2011). GtxA is usually transcribed from a four-gene operon comprising the genes rtxD, rtxC, rtxA and rtxB (Persson and Bojesen, 2015). There are two domains in the structure of GtxA including 2026 amino acids in G. anatis, and its C-terminal regulates the haemolytic activity of GtxA, while the N-terminal is not necessary for haemolytic properties with unknown function (Figure 5) (Kristensen et al., 2010). The acylation of GtxA is necessary for the activation of RTX-toxin, which is also based on the expression of GtxC protein, responsible for the acylation of conserved Gly-Asp-rich part of GtxA (Figure 5)(Kristensen et al., 2010). Therefore, the C-terminal domain of GtxA of G. anatis has a structure with homology to other RTX toxins such as HlyA in E.coli and AvxA from Avibacterium paragallinarum (Küng and Frey, 2013) and the schematic diagram of GtxA and HlyA domain organization was further identified by Persson et al. in 2015(Figure 6) (Persson and Bojesen, 2015). Recently, the protective and immunogenic capacity of three recombinant proteins FlfA, GtxA-C and GtxA-N was recently evaluated in an in vivo challenge model by Persson et al. (2018), and a cross-protective efficacy of these recombinant proteins was not completely observed. while ΔtolR OMVs functioned as an adjuvant have a cross-protective potential with GtxA by inducing antibody production, it also supplementary the possibilities for researching new prophylactic methods to prevent infections with G. anatis (Pors et al., 2016; El-Adawy et al., 2018; Persson et al., 2018).
Figure 5. Two domains in the structure of GtxA GtxC of *G. anatis* 12656-12. (A) The genetic organization of *gtxC*, *gtxA* and their flanking genes in *G. anatis* 12656-12. The downstream of *gtxC* is confirmed as the transcriptional terminator (B) The genetic organization of *gtxA* and the special RTX-domain. The labelled part of the N-terminal is rich in glycine aspartic acid. Arrows indicate ORF. K indicates conserved lysine residues (and Lys1607 and Lys1484) (Kristensen *et al.*, 2010).

Figure 6. The comparison of domain organization between the GtxA from *G. anatis* biovar *haemolytica* strain 12656-12 and HlyA from *E. coli*. The homology area is shown with a dotted line, showing that the coverage of HlyA and GtxA reaches 34%, and the identity reaches 29%. The abnormal genetic domain organization of the GtxA toxin is a new type of RTX-like protein containing 2038 amino acids. The lysine residues in HlyA are located at Lys564 and Lys690, while the homologous residues of GtxA Lys1484 and Lys1607 are acetylated by GtxC to the active toxin. RTX C-terminal of GtxA (pfam08548) has a homologous domain with C-terminal of HlyA (pfam08548) included peptidase M10 serralysin and the Ca$^{2+}$-binding repeats (COG2931) (Persson and Bojesen, 2015).

Recently, significant progress has been made in the knowledge of *G. anatis* pathogenesis in mammalian hosts, most of which have become evident from epidemiological investigations focused on prevalence or from studies of *G. anatis* infection *in vivo* (Bojesen *et al.*, 2004). Despite these studies, only little has been reported on specific virulence factors governing the pathogenesis of *G. anatis* *in vivo* and *in vitro* infections (Persson and Bojesen, 2015). Furthermore, GtxA has been proven to be immunogenic and therefore suggested as a promising possible vaccine candidate (Pedersen *et al.*, 2015). It has been found that isogenic mutant strains
lacking parts of \textit{gtxA} not only lost their haemolytic, but also its cytolytic activity \textit{in vitro} (Kristensen \textit{et al.}, 2010; Kristensen \textit{et al.}, 2011; Persson and Bojesen, 2015), in addition to causing attenuation of virulence in an experimental infection in egg-laying hens (\textit{Paper1}). To investigate the effect of GtxA during the infection process in the natural host, studies to evaluate and compare lesions found in egg-laying hens after experimental infection with \textit{G. anatis} wild-type and its isogenic \textit{gtxA} deletion mutant of \textit{G. anatis} (\textit{ΔGtxA}) have been carried out. Compared with the findings after infection with GtxA-producing wild-type strain, GtxA has lost its virulence in the natural host infected with the \textit{ΔGtxA} strain by lower gross lesions and histopathology in pre-experiment. There was clear evidence that GtxA contributes markedly to the pathogenesis of \textit{G. anatis} upon experimental infection in its natural host (\textit{Paper1}).

In many instances, avian macrophages are a good cell line for research on defence against microbial infections and immune system regulation (Beeckman \textit{et al.}, 2007; He \textit{et al.}, 2012; de Freitas Neto \textit{et al.}, 2013; Xu \textit{et al.}, 2013). Particularly, GtxA has been proven to induce severe cytotoxicity on avian macrophage HD11 cells (Kristensen \textit{et al.}, 2010). However, the role of GtxA in colonization and infection in avian macrophage is still unclear. The present study aimed to describe adhesion and invasion characteristics of \textit{G. anatis} strains to macrophage cells \textit{in vitro}. The role of GtxA in \textit{G. anatis} modulation of antimicrobial activity, intracellular survival and cell invasion in avian macrophage HD11 cells is presented in paper 2 of this thesis.

\textbf{1.1.4.2 Fimbria}

A better understanding of the multiple molecular mechanisms of attachment between the adhesive molecules on the surfaces of bacteria and those receptors on host cell membranes is typically the first step in the prevention of serious bacterial infections (Beachey, 1981; Gyimah and Panigrahy, 1988). Bacterial adhesins and related structures exhibit precise selectivity for driving a high physiochemical affinity with host cells by targeting molecules, recognizing molecular motifs and binding to the specific receptors of microbial cell surface called adhesion/receptor interaction (Klemm and Schembri, 2000). These interactions can usually activate the complex molecular crosstalk at the host-pathogen interface, leading to multiple consequences, including activation of the inherent host defence system and subversion of cellular processes (Beachey, 1981; Soto \textit{et al.}, 2016). In many cases, adhesins expressed by several pathogens such as \textit{Hemophilus}, \textit{Actinobacillus} and \textit{Pasteurella} organisms, can be used to
prepare potential vaccine candidates in the process of preventing microbial infections (Jacques and Paradis, 1998; Mullen et al., 2007). In particular, one of the most important adhesins is fimbria, which is protruding a hair-like structure from the bacterial cell surface during bacterial infection (Krogfelt et al., 1991; Klemm and Schembri, 2000).

It has been reported that F17-like fimbriae, a virulence factors strain produced by bovine pathogenic E. coli strains were involved in septicemia and diarrhea outbreaks in cattle and sheep. Bihannic et al., showed in a study infecting calves with E. coli mutants F17-A and F17-G that these fimbriae may be used as vaccine candidates (Bertin et al., 1996; Le Bouguénec and Bertin, 1999; Stordeur et al., 2002; Bihannic et al., 2014). By using three sequenced genomes of G. anatis, some putative adhesin genes were identified and speculated to encode putative hemagglutinin (Johnson et al., 2013; Kudirkienė et al., 2014). Additionally, Lucio et al. demonstrated that fimbriae of G. anatis F149T have fimbria-like structures similar to E.coli by immune-gold electron microscopy, and plays an important role in adhesion of G. anatis to chicken oropharyngeal epithelial cells and colonization of the upper respiratory tract (Lucio et al., 2012). Several reports confirmed that F17-like fimbriae of G. anatis is encoded by four genes called flfA, flfC, flfD and flfG and that the F17-like fimbria is associated with the recognition-binding process in the host (Bager et al., 2013a; Johnson et al., 2013; Persson and Bojesen, 2015). The latest update showed that G. anatis has five fimbriae clusters (Flf, Flf1, Flf2, Flf3 and Flf4) based on phylogeny-based approach (Kudirkienė et al., 2014; Montes-García et al., 2016; Elbestawy et al., 2018). Moreover, fimbriae subunit protein FlfA has been proposed as a vaccine candidate against G. anatis (Figure 7) (Bager et al., 2013a): Studies have shown that FlfA does not only partial protects the host individually, but also acts as a recombinant protein offering improved cross-protection against heterologous G. anatis in egg-laying hens vaccinated with outer membrane vesicles containing FlfA (Bager et al., 2013b; Pors et al., 2016; Persson et al., 2018).
Figure 7. Immunogold electron microscopy with the anti-FlfA immune serum of *G. anatis* 12656-12. The pictures A, B and C were taken from different population and cells were detected with a secondary antibody conjugated to 10-nm gold particles. (A) Observation of fimbrial in WT cell at a low-level population. (B) Observation of fimbrial structures in WT cell at a hyper fimbriated population. (C) Observation without fimbrial in FlfA mutant cells (Bager *et al.*, 2013a).

### 1.1.4.3 Other virulence factors

**Outer membrane vesicles, Capsule, Biofilm, Metalloproteases, CRISPRs**

Outer-membrane vesicles (OMVs) are composed of spherical buds from the outer membrane produced by Gram-negative bacteria. OMVs are beneficial for bacteria to interact with their environment (Schwechheimer and Kuehn, 2015). OMVs have been observed their functional versatility including promoting higher bacterial survival rates and regulating the interaction of the reproductive tract flora such as *E. coli*, *Salmonella* spp. and *Acinetobacter baumannii*, which is conducive to pathogenicity (Negrete-Abascal *et al.*, 2000; Coutte *et al.*, 2003; Schwechheimer and Kuehn, 2015). Moreover, they also play diversified metabolic roles in Gram-negative bacteria (Yonezawa *et al.*, 2009; Kulp and Kuehn, 2010; MacDonald and Kuehn, 2012). Ragnhild *et al.*, showed that OMVs are produced by *G. anatis* under *in vitro* growth conditions. In the same study transmission electron microscopy (TEM) were applied to detect changes in OMVs protein profile, which may provide the basis for future preparation of potential vaccines (Bager *et al.*, 2013b). It has recently been illustrated that OMVs produced by *G. anatis* develop with protein content varying under specific growth conditions, indicating they are spherical structures that could cope with diversity in a complex environment (Bager *et al.*, 2013b; Persson and Bojesen, 2015). The latest development is that OMVs can be used as an adjuvant to participate in cross-immune protection against *G.*
anatis at the laboratory level, and Antenucci et al., has found that hydrostatic filtration can expand the immune protection efficiency of OMV in practical applications (Persson et al., 2018; Antenucci et al., 2020; Narasinakuppe Krishnegowda et al., 2020).

**Capsule**

Among the many recently identified virulence genes, the presence of a thin capsule-like structure has been identified in ferritin-stained bacterial cells by TEM (Wiles and Mulvey, 2013). Extracellular polysaccharides constitute the main components of bacterial capsules and its functional importance in the interaction between the bacteria and the hosts have been documented in e.g. Mannheimia haemolytica and Pasteurella multocida (Singh et al., 2011; Wiles and Mulvey, 2013). They play an important role in the wide range of activities including pulmonary colonization, immune evasion and cell-cell interactions in the pathogenesis of transport fever of pigs (Singh et al., 2011; Harper et al., 2012). Recently, there has been much progress in the understanding the capabilities of the capsule of Gallibacterium, e.g. a capsule-knockout mutant ΔgexD of G. anatis 12656-12 has been confirmed to be less serious virulent than its wild-type strain (Bojesen et al., 2011). At the same time, the ΔgexD mutant capsule possibly causes the exposure of antigens resulting in a serious nonspecific immunity activated by pathogen-associated molecular patterns (PAMPS) which were hidden or suppressed under normal conditions (Bojesen et al., 2011; Persson and Bojesen, 2015).

**Biofilm**

Other known virulence factors include biofilm, which is produced by variety of bacterial species and composed of polysaccharide, protein, and DNA. Biofilm production increase the bacterial survival rate (Costerton et al., 1999; Donlan, 2002; Høiby et al., 2010). Particularly, the increased antibiotic resistance of bacteria in biofilms is linked to treatment failure and chronic bacterial infections in both humans and animals (Costerton et al., 1999; Høiby et al., 2010). The bacterial biofilm of G. anatis is generated by structural cells embedded in a polymer matrix, attached to the surface for biofilm formation (Vaca et al., 2011; Johnson et al., 2013). Interestingly, major variation among different strains has been observed, as strains may be defined as “weak”, “moderate”, “strong” biofilm producers (Johnson et al., 2013). It has been shown that biofilm formation might have a critical impact on certain clades of G. anatis even though there was no obvious correlation in pathogenesis (Johnson et al., 2013).

**Metalloproteases**

During normal development and tumor progression during organogenesis, Chang et al. found that metalloproteases are playing an essential role in a wide range of biological events, such as
remodeling of the extracellular matrix (ECM) to vascularization, cell migration and cell proliferation (Nelson et al., 2000; Chang and Werb, 2001). It has been proposed that HA-L haemagglutinin from *H. paragallinarum* strain 46-C3, observed agglutinating activity in other poultry pathogens such as *Avibacterium paragallinarum*, but the role of agglutinins during the *G. anatis* infection has not been further determined (Barnard et al., 2008). Metalloproteases secreted by *G. anatis* have been shown to participate in the process of interfering or promoting immune invasion by degrading chicken IgG (Garcia-Gomez et al., 2005; Zepeda et al., 2010).

Considering agglutinating avian erythrocytes as an important characterization of a *Gallibacterium* genomospecies 2, hemagglutination is associated with the expression of hemagglutinin or adhesin that binds to red blood cell surface receptors (Zepeda et al., 2010; Ramirez-Apolinar et al., 2012). Although we do not clearly understand the role of metalloproteinases in pathogenicity of *G. anatis*, three kinds of metalloproteases proteins have been identified by genetic analysis of *G. anatis* containing an extracellular protein with an important domain of metal-dependent endonuclease, especially a zinc metalloprotease (Zepeda et al., 2010; Johnson et al., 2013; Persson and Bojesen, 2015).

**CRISPRs**

Clustered, regularly spaced arrays of short palindromic repeats (CRISPR) are widely present in the genomes of several bacteria and they may confer resistance to phages, (Bolotin et al., 2005; Sorek et al., 2008). The presence of gene fragments in the CRISPR structure not only play a role in a DNA degradation step but also participate in the defense system by providing adaptive immunity against foreign invading DNA (Bolotin et al., 2005; Horvath and Barrangou, 2010). Several CRISPRs of *G. anatis* have been identified and investigated for their function in the adaptive bacterial immune system against foreign invasive DNA (Johnson et al., 2013).

Moreover, CRISPRs have the potential ability to antagonize and limit the natural transformation of *G. anatis* (Kristensen et al., 2012).

**Other virulence factors**

Apart from the virulence factors discussed above, there are also other factors of *G. anatis* which are considered potentially important in the pathogenesis. These exact role of these potential virulence factors of *G. anatis* is still not fully understood, but is including chaperone- usher fimbriae, integrative conjugative elements (ICEs), hemagglutination and small colony variants (SCVs) (Harbourne, 1962; Johnson et al., 2013; Bager et al., 2014; Kudirkienè et al., 2014)
Integrative and conjugative elements (ICEs) is self-propagating mobile genetic elements. ICEs are a significant component regulating the flow of horizontal gene in bacteria, and their evolution is usually transferring via conjugation, integrating into and replicating along with the host chromosome (Wozniak and Waldor, 2009; Zakharova et al., 2015). The study by Wozniak et al., has provided an in-depth understanding of the evolution of the SXT/R391 family of ICEs by comparative analyses of the DNA sequences of ICE genomics, shown that they have highly conserved core genes fractured by restricted insertions of specific variable genes related to antibiotic resistance (Wozniak and Waldor, 2009). However, the rudimentary understanding of ICEs will be addressed in future studies.

Hemagglutinin

After the putative hemagglutinin gene was identified from G. anatis UMN179 by the genomic analysis, Montes et al., observed that a 65 kDa hemagglutinin purified from G. anatis F149 is considered to be a virulence factor for G. anatis (Johnson et al., 2013; Montes-García et al., 2016). In the Ramirez’s study, the hemagglutinating activity of G. anatis strains was studied by using fresh erythrocytes of 19 different species including Harris’s hawk (Parabuteo unicinctus), chicken, house finch (Carpodacus mexicanus), human and ect., indicating that some G. anatis strains can agglutinate avian and mammalian erythrocytes (Zepeda et al., 2010; Ramirez-Apolinar et al., 2012).

Small colony variants

Apart from early findings, there are also other virulence factors such as small colony variants (SCVs) observed on poultry strain of Pasteurella haemolytica (Harbourne, 1962). It has been reported that SCVs is a part of pathogenic microorganism, recurrent infections and increased resistance to antimicrobials, which is especially helpful in the treatment of G. anatis (Proctor et al., 2006; Kudirkienė et al., 2014). Multiple virulence factors play a key role in the infectious process of G. anatis, but the deep pathogenesis and characterization of potential disease elements in vivo and in vitro remain to be determined.

1.1.5 Diagnosis

As mentioned in section 1.1.3., G. anatis is an opportunistic bacterium causing disease in the chicken, and it is arduous to identify the molecular diagnosis of disease caused by G. anatis from other bacterial infection by non-distinctive clinical features. Fortunately, biochemical testing and genotyping methods have gradually evolved to address this barrier for the correct diagnosis of G. anatis infections (Dousse et al., 2008; Huangfu et al., 2012). The diagnostic method originally
used was a bacteriological test on cultured blood agar plates to distinguish the properties of bacterial colony morphology (Bojesen et al., 2003b; Christensen et al., 2003; Narasinkuppe Krishnegowda et al., 2020). Dousse et al. have developed a routine phenotypic identification method that is a cost-efficient method for distinguishing *G. anatis* from other species of the *Pasteurellaceae* family isolated from different animals (Dousse et al., 2008). Moreover, it is a less sensitive method to diagnose and confirm the presence of *G. anatis* biovar 1, 2 and 4 in serum samples by serological detection (Wang et al., 2011). Because of the uncertainty and poorly sensitive methods for diagnosis, different genotypic methods should be used to correctly identify the various species of *Gallibacterium*.

In 2003, amplified length polymorphism analysis (AFLP) was used to characterize the genotypic diversity of different *G. anatis* isolates, which not only specifically fingerprinted these strains but also identified a specific pathogenic strain called *G. anatis* 12656-12 (Bojesen et al., 2003c). The isolates of strains were characterized by bio-typing, PFGE and detected by the fluorescent in-situ hybridization (FISH) method in chickens targeting the 16S rRNA of *Gallibacterium* (Bojesen et al., 2003c; Christensen et al., 2003). The FISH method is a rapidly specific method for identifying the *G. anatis* based on polymerase chain reaction (PCR). Primers are selected to target the 23S and 16S rRNA genes producing different sizes of amplicons including approx. 790 bp (all *Gallibacterium* isolates), 1030bp (*G.genomospecies* 1 and 2 ) and 1080bp (*G. anatis*) (Bojesen et al., 2007). In addition to traditional diagnosis methods, Alispahic et al. also developed a reliable method called matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which was a facilitated approach to identify and characterize *Gallibacterium* strains by proteomic phenotypic fingerprinting (Alispahic et al., 2011a, b). Interestingly, the indirect ELISA diagnostic method is a primary application for the detection of monoclonal antibodies (mAbs) against *G. anatis* in hybridoma cell lines, which has the potential advantage of detecting *G. anatis* from other pathogens (Li et al., 2012). With the development of the latest diagnostic methods, Wang et al., developed a specific real-time quantitative PCR for identification of *G. anatis* by amplification of the gyrase subunit B gene (*gyrB*) (Wang et al., 2016).
2.1 The general host response to bacterial infections

2.1.1 The basic immune system

Based on the review in the Janeway’s immunobiology and latest research progress on the OMV vaccines, this section will provide an overview of the immune system in the avian and how its response to bacterial infection. The main focus will be on the immunological effect of toxin GtxA. Such knowledge is required to understand how the complex network of the avian immune system responds during infection of G. anatis.

By breaking through the physical obstacles and increasing capillary permeability, bacteria rapidly trigger macrophages and dendritic cells response to initial pathology attack as a non-antigen specific gatekeeper of the innate system. In addition to the complement system, which assists in the elimination of germs near the surface of the host epithelium, the most important slower-acting defenses are adaptive immune response based on pathogen-molecular recognition, such as antibody neutralization humoral response and cellular regulatory response resulting in sets of antibacterial properties -inflammatory factors and cytokines (Stilgoe et al., 2013; Wigley, 2013). In addition to three main lines of defense including the humoral response and cellular mediated response, how bacterial pathogens replicate in the host and how the host participates in the process of eliminating or promoting bacterial infections is worthy of further exploration (Figure 8) (Kindt et al., 2007).

It has been shown that OMV as adjuvant combined with GtxA-N does not have the cross-protective ability against G. anatis, while the inoculation of OMV and FlfA induce cross-protective against the damage in salpinx after challenge infection (Pedersen et al., 2015; Persson et al., 2018; Zhang et al., 2019). These results are consistent with those studies of OMVs using a simple technique hydrostatic filtration with OMVs combined with GtxA-N or FlfA, showing that these OMVs can protect chickens against G. anatis infection (Antenucci et al., 2020). Some studies have shown that GtxA plays a key role in the pathogenesis of G. anatis in the natural host, and could provide a valuable model for investigating the role of specific RTX toxins on basic immunological mechanisms (Kristensen et al., 2011; Johnson et al., 2013; Pedersen et al., 2015; Zhang et al., 2017). Therefore, combined with my research and the basic immune system described above, we would explore the immune regulation mechanism during bacterial infection from the following aspects.
Figure 8. Bacteria respond to the basic immune system. The pathogen breaks through the physical defense of epithelial cells and penetrates sets of local infection: Bacteria trigger non-antigen specific gatekeeper of acquired immunity after the destruction of the first line of defense. Various chemical and enzymatic systems act as an immediate antibacterial barrier near these epithelial cells by migrating from blood vessels to tissues and releasing inflammatory mediators. With the increase of vasodilation and vascular permeability, innate lymphoid cells such as macrophage and dendritic cells can coordinate a rapid cell-mediated defense. The specific recognition of the pathogen-antibody reaction can slowly activate humoral response to eliminate and neutralize bacteria. Picture modified from Kuby Immunology (Kindt et al., 2007).

2.1.2 Antibody - humoral response

According to the description in Kuby Immunology, several mechanisms of antibodies play a key role in host defense (Figure 9) (Kindt et al., 2007). It was generally believed that neutralizing antibodies was an effectively immuno-prophylactic therapy to prevent diseases caused by pathogenic species in Pasteurellaceae (Frey and Kuhnert, 2002). In particular, three antibodies including IgM, special IgG (IgY) are related to a chronic or late anti-infection. The antibody response is manifested in activating of the complement system. Furthermore, locally mucosal secreted IgA expressed in the chicken, together with the already mentioned that GtxA-N-specific IgY antibody could increase the protection against G. anatis in serum and egg yolk from specific chickens immunized with recombinant GtxA-N by increasing Ig Y antibody titers (Zhang et al., 2019). Although the recombinant protein GtxA-N has immunogenic and protective capacity in a G. anatis challenge model (Bager et al., 2014; Pors et al., 2016; Persson et al., 2018), the titers of GTXA specific antibodies IgA or IgM was much more limited.
Figure 9. Roles of antibodies in host defense. 1. The left panels: unbound toxins can recognize receptors on target cells where antibodies can inactivate and neutralize intact bacterial cells. The toxin-antibody complex does not react to the receptor that destroys bacteria, but it is recognized as foreign objects by phagocytes and then swallowed and destroyed according to opsonization. 2. The middle panels: opsonization and phagocytosis of bacterial cells in extracellular space. The variable regions of antibodies bind to antigens (red) on bacterial cells, and their Fc regions recognize Fc receptors (yellow) expressed by phagocytes resulting in ingestion and degradation of macrophages. 3. The right panels in plasma: activation of the complement system. The bound antibody deposits complement protein (blue) on the surface of the bacteria, which leads to the formation of pores that directly lyse the bacteria in some cases or ingested and destroy the bacteria by phagocytes at last. Figure modified from Kuby Immunology (Kindt et al., 2007).

Our current studies have shown that titers of GtxA-specific IgG in poultry serum from G. anatis inoculation group (G. anatis wild-type and its isogenic AgtxA mutant) was unchanged during the infection period (paper 1), which indicated that GtxA is not involved in stimulating the immune system to trigger an adaptive response as mentioned previously (Pedersen et al., 2015; Pors et al., 2016). In addition, our results indicated that G. anatis AgtxA group with minor pathological changes in the oviduct was identical to lower pro-inflammatory response corresponding to the re-isolation results in the HE staining. In general, GtxA was not the single bacterial factor which were fully responsible for reproduction lesions in the chicken oviduct in the in vivo model (Johnson et al., 2013; NarasinaKuppe Krishnegowda et al., 2020). It could be speculated if other bacterial factors, e.g. F17-like fimbria, would be involve in promoting a strong pro-inflammatory
response *in vivo*. Further research on avian macrophages may help clarify this aspect in the future (Bojesen *et al.*, 2004; Chen *et al.*, 2011).

## 2.1.3 Complement system

There are nearly 20 different protein molecules on the surface of the bacteria involved in the activating the complement system in two main ways, the classical and alternative pathway (*Figure 10*). Certain polysaccharides immediately activate the system to participate in innate immune response, or the Fc segment of the antibody binds to antigen involved in acting first complement protein (C1) to turn the complement system on a series of reactions (specific immune response), resulting in the peptide diffusing away and being cleaved into a final complex complement protein called membrane attack complex (MAC), which triggers a constellation of events to help destroy the pathogen and eliminate the infection: opsonisation, chemotaxis, inflammation, lysis, apoptosis (Janeway Jr *et al.*, 2001; Noris and Remuzzi, 2013).

![Complement systems](image)

*Figure 10. Complement systems.* There are classical pathway, bacterial endotoxin-alternative pathway and lectin pathway-mannose binding lectin following a sequence of events to destroy the pathogen and eliminate the infection. It can be activated by a number of different foreign molecules on the surface of pathogen and cleaved into a series of differentiated complement complexes when it binds to an antigen-antibody complex. C3 is cleaved into C3a which acts as a chemotactic factor and an inflammatory paracrine and C3b which bounds to C3 convertase at the surface of the microorganism and then splits sequentially and finally binds to a growing complex C6, C7, and C8. Next, complement C9 proteins that form a structure called a membrane attack complex (MAC), which create holes in the cell membrane of the cell producing a cascade of reactions:
opsonisation, chemotaxis, inflammation, lysis and apoptosis. Figure modified from Janeway (Janeway et al., 2001).

Protective vaccine against G. anatis is lacking, mainly due to the high genetic variability of these bacteria. Precious studies have shown SBA assay is an efficient way to detect the antibacterial effect of bacterial vaccines in Pasteurella-like family, for example, Haemophilus influenza, Salmonella enterica and Avian pathogenic E. coli (Ahmadi et al., 2013; Ercoli et al., 2015; Han et al., 2018). Compared with traditional SBA detection methods, this new one is faster and more cost-effective, and Ercoli et al., found that it can be used to discover the effectiveness of antibodies against recombinant antigens to Non-typeable Haemophilus influenza (NTHi) in vitro killing, as well as a detailed tool for predicting vaccine efficacy and searching for candidate protective vaccines (Ercoli et al., 2015). However, the specific immune mechanism of G. anatis has not been deeply understood. Using an in vivo challenge model is a good method to elaborate the antibacterial effectiveness of antibodies directed against G. anatis in vitro and evaluate complement-mediated functional activity of both infections- and vaccine-induced antibodies and optimizes the selection of protective antigens against G. anatis (McIntosh et al., 2015).

2.1.4 Cellular mediated response

In addition to the above-mentioned antibacterial adaptive immune system in the host, helper CD4⁺ T cells can differentiate into Th1 involved in cell-mediated responses and Th2 that activates B cells to participate in regulating humoral responses, which play a crucial role in the host defense through a series of downstream cytokines to eliminate the replication of germs (Nawab et al., 2019). The functions of avian cytokines involved in the chicken immune system can be obtained from Avian Cytokines-an overview (Giansanti et al., 2006; Karaffová et al., 2015), but the production of avian cytokines against G. anatis is rarely discussed. Our gross lesions and histopathology results have shown that the G. anatis ΔgtxA mutant in the laying hens result in less severe pathology compared with the WT group. Previous studies have shown that the primary chicken oviduct epithelial cells are stimulated to secrete cytokines such as IL-6, TNF-α and IFN-γ to participate in the response against G. anatis infection (Zhang et al., 2017). The importance of the production of avian cytokines against pathogenic G. anatis in the reproductive tract is not yet fully understood. In the spleen and ovary the production of IL-4 and TNF-α mRNA changed significantly in wild-type group, which suggest that GtxA play a key role in Th2-like immune response during the G. anatis infection (paper 1). IL-4 was expressed
at a 2-fold increase in target ovary and a relatively lower performance in the spleen at 2 days post infection (pi), which indicates that after originally entering the circulatory system, pathogens migrate into spleen as a critical tissue for clearance of remaining *G. anatis*, eventually causing a slightly delayed response. Moreover, these results indicated that the ΔgtxA mutant could regulate the acute phase reaction by promoting of TNF-α production and obstruct humoral immunity by suppression of secretion of IL-4 in poultry (Chtanova *et al.*, 2001) (paper 1).

Moreover, there is no significant difference in the amount of IL-6 and IL-10 between *G. anatis* ΔgtxA group and WT group in the above-mentioned tissues, which indicates that GtxA may preferentially modulate adaptive immune response rather than the innate immune response. This could like happened through partial destruction of the Th2-like pathway. This would be in accordance with a previous report that reported that a live attenuated mutant of *Salmonella Montevideo* and YopB mutant of *Y. enterocolitica* is beneficial to antibacterial host defense by inducing INF-α response (Burdack *et al.*, 1997; Lalsiamthara and Lee, 2017).

Zhang *et al.* observed that pro-inflammatory cytokines were expressed after *G. anatis* exposure in immune cells, on primary chicken oviduct epithelial cells (Zhang *et al.*, 2017). In our studies (paper 1), down-regulation of pro-inflammatory IL-10 and increased release of TNF-α and IL-1β was shown in *G. anatis* ΔgtxA mutant experimental group. These results indicated that GtxA may primarily have anti-inflammatory activity through moderating the IL-10 expression of macrophages. Combing previous results, GtxA not only stimulates the innate and partial adaptive cellular immune response of the ovary and spleen but also triggers host response disorders by inhibiting the expression of pro-inflammatory cytokines (paper 1 & paper 2). Our studies have contributed to a novel immunological understanding of GtxA and confirm that macrophages HD11 cells can be used for subsequent vaccine experiments.

### 2.2 Apoptosis

The virulence determinants encoded by multiple pathogens are involved in mediating a variety of responses to bacterial infection. One of the regulation responses is called programmed cell death. On one hand, the purpose of pathogen-induced host cell death pathways is to limit infection by eliminating critical immune cells or escaping host defenses. On the other hand, inhibiting the death pathway can promote the absorption of nutrients and the proliferation of pathogens in the infected cells. Both apoptosis and necrosis participate in the process of the highly regulated cell
death and mediate cell biological development and homeostasis. It is worth noticing that apoptosis abolishes individual cells without inducing an inflammatory response, in contrast to necrosis. Several ways have been evolved by bacterial pathogens to prevent apoptosis and facilitate replication within the cell, which provides a survival advantage by bacterial anti-apoptotic strategies. Therefore, activation or prevention of cell death reminds us that it may play a key role in the study of bacterial pathogenesis (Weinrauch and Zychlinsky, 1999; Faherty and Maurelli, 2008).

In this section, we introduce the identification of differences in the two ways cell death using experimental techniques and increase the basic understanding of apoptosis. This chapter introduces the viewpoint of eukaryotic cell death and discusses the diversity of processes that mediate cell death. Moreover, the following general aspects of apoptosis will be discussed on the basis of several different topics, such as basic molecular mechanisms of bacteria-induced multiple apoptosis, Pore-Forming Toxins (PFTs) induce multiple cellular responses, caspase-mediated apoptosis in the ovary and the interaction between RTX toxins and target cells (Zychlinsky and Sansonetti, 1997; Weinrauch and Zychlinsky, 1999; Benz, 2020).

This will provide an overview of cell death in the chickens, in relation to G. anatis pathogenicity (Kroemer et al., 1998; Weinrauch and Zychlinsky, 1999; Fink and Cookson, 2005; Elmore, 2007; Faherty and Maurelli, 2008; Kulp and Kuehn, 2010; Davison, 2011; Wiles and Mulvey, 2013; Benz, 2016, 2020).

2.2.1. Apoptosis and necrosis - Bacterial pathogens and apoptosis

Apoptosis is often associated with pathogenic microorganisms (Weinrauch and Zychlinsky, 1999; Kobayashi et al., 2003). Apoptosis and necrotic cell death can be distinguished by identifying the morphological and biochemical changes caused by each of the two mechanisms. It has been reported that necrotic cells exhibit organelle destruction, swelling, chromatin flocculation, and release of intracellular substances (Moss et al., 1999; Weinrauch and Zychlinsky, 1999). Compared with the cell necrosis resulting in overwhelming injuries, apoptosis known as programmed cell death (PCD) cause less damage to the host’s tissues. In apoptosis, there is cleavage of nuclear DNA into oligonucleosomal fragments, activation of downstream apoptosis genes participated in the regulation of cell-intrinsic suicide program, and then the maintenance of cell membrane integrity with the formation of apoptotic bodies (Moss et
besides, apoptotic cells are characterized by the exposure of surface molecules to undamaged cell cadavers for phagocytosis. In particular, in the absence of phagocytosis, apoptotic bodies selectively tend to undergo lysis and with necrosis or secondary apoptotic as the outcome (Fink and Cookson, 2005). The mechanistic description of pathogen-induced cell death in eukaryotic cells was reviewed by Fink and Cookson and cell death have been respectively characterized as apoptosis, pyroptosis, and necrosis in the variety of molecular pathways (Figure 11) (Fink and Cookson, 2005).

**Figure 11. Multiple pathways of cell death.** Normal living cells respond to death stimulant by activating multiple molecular pathways that cause cell death (Fink and Cookson, 2005).

**Molecular mechanisms of bacteria-induced apoptosis**

Different pathway utilization can significantly affect the biological systems that cause cell death, which is characterized by first activating the initiator caspase and then activation of the effector caspase to cleave cellular substrates. Given examples of inhibition of apoptosis during bacterial infection (Figure 12), we reviewed multiple mechanisms of their bacterial pathogenesis. The molecular mechanisms of bacteria-induced apoptosis have been studied for many different species. E.g. it has been reported that *Chlamydia* produces Chlamydia proteasome-like active factor (CPAF) for inhibition and degradation of pro-apoptotic proteins, which companied with BH3 domain inhibiting the release of pro-survival Bcl-2 protein (Fischer *et al.*, 2004; Faherty and Maurelli, 2008).
In 2005, the effector SopB protein secreted by *Salmonella* through the type III secretion system, results in activation of the 3-kinase/Akt (PI3K / Akt) pathway, which prevents cytochrome c (CytoC) release (Knodler *et al.*, 2005). In addition to activation of nuclear factor kappa B (NF-kB) activated by *Bartonella, Rickettsia, and Ehrlichia, N. meningitidis* the *Anaplasma* also triggers the PI3K / Akt pathway, which blocks the release of CytoC and triggers the production of inhibitors of apoptotic protein (IAP) (Knodler *et al.*, 2005; Faherty and Maurelli, 2008).

![Figure 12. The mechanism by which bacterial pathogens inhibit apoptosis at different points in the apoptotic pathway.](image)

2.2.2. Molecular methods to identify and distinguish the apoptosis and necrosis

Apoptosis has four main characteristics at the molecular biology level: chromatin condensation, DNA fragmentation, cytoplasmic vacuolization and cell death, without lysed or completely destroyed neighboring cells (Faherty and Maurelli, 2008). Fortunately, detection techniques have matured in recent years and several molecular biology methods have been used to identify and distinguish apoptosis and necrosis death (Tsujimoto, 1997; Glasser *et al.*, 2001; Anilkumar *et al.*, 2017). Condensed and/or dissolved atomic nucleus in apoptotic cells were detected by using the Immunofluorescence staining, cleavage of nuclear DNA into oligonucleosomal fragments were visualized by the TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay (Johnson and Bridgham, 2002; Faherty and Maurelli, 2008), internucleosomal DNA fragmentation was detected on DNA- Electrophoresis gel by PCR (Moss *et al.*, 1999; Anilkumar *et al.*, 2017),
apoptosis and necrosis of cells were assessed using Flow Cytometry assay (Lizard et al., 1999; Ludovico et al., 2002). There are several molecular techniques to identify and distinguish apoptosis and necrosis from similar characteristics of cell death including Immunofluorescence staining and TdT-mediated dUTP-biotin nick-end labeling assay (TUNEL), cytometry assay, DNA- Electrophoresis gel, Intracellular ATP level as a rapidly important method for detecting cell death modes (Tsujimoto, 1997; Glasser et al., 2001; Kao et al., 2011).

**Immunofluorescence staining**
Fluorescence microscopy provides a rapid and convenient method to distinguish typical features of apoptosis. Staining with the Hoechst 33342 or Annexin V-FITC (Annexin V) / propidium iodide (PI) can be used for detecting condensed and/or dissolved atomic nucleus in apoptotic cells. For example, there was no potential DNA fragmentation and immunofluorescence binding observed in the isothiocyanate (FITC)-conjugated annexin V staining of J774-A1 cells infected with E. coli strain LF82 at 24 h.p.i., indicating that the adherent invasive E. coli strain LF82 was able to survive in the macrophage, and their replication does not induce any death in infected cells (Glasser et al., 2001).

**Flow Cytometer**
In addition to displaying a bright colorful nucleus with concentration or fragmentation, there is also another staining mode that can be used to distinguish normal cells, apoptotic cells and dead cell populations, namely flow cytometry (Johnson and Bridgham, 2002; Chan et al., 2011; Kao et al., 2011). Chan et al., investigated the dose-response effects of heat and drug-induced cell death by evaluating the amount of Annexin V-labeled (apoptotic) and/ or PI-labeled (necrotic) Jurkat cells ,and this is the first case of necrosis/apoptosis detection using the Cellometer Imaging Cytometer(CIC) compared to the conventional ones (Chan et al., 2011).

**DNA fragmentation - Electrophoresis gel**
Alpha toxin as a pore-forming agent was first confirmed by Hamed et al., showing that it induced extensive DNA fragmentation and cytolysis through Ca-permeable transmembrane channels in cell lines infected with E.coli (Hameed et al., 1989). Bhakdi et al., (1989) first demonstrated that alpha-toxin is involved in the cytotoxicity of E.coli hemolysin regulated by permeabilization, which caused permeabilization of target cell membranes by Ca$^{2+}$/ K$^+$ cationic fluxes and the release of host metabolites e.g ATP (Bhakdi et al., 1989). Jonas et al., (1994) showed that HlyA induced internucleosomal DNA degradation in stimulated T cells, whereas no fragmentation was observed in unactivated lymphocytes, and whether the fragmentation they observed were accidental events that may be associated with cellular ATP (Jonas et al., 1994).
ATP

Apoptosis and necrosis constitute two different ways of cell death, with distinct morphological features. It is difficult to distinguish them by using simple histopathology and molecular biology methods. Not only is there a common step shared in both apoptosis and necrosis, but also because anti-apoptotic Bcl-2 proteins and caspase inhibitors can be simultaneously involved in the inhibition of cell death patterns (Eguchi et al., 1997; Moss et al., 1999; Fink and Cookson, 2005). However, the level of intracellular ATP in cells is one of the most significant physiological differences between apoptotic and necrotic cells both in vitro and in vivo (Eguchi et al., 1997; Tsujimoto, 1997). Since the determinant of cell death fate has been directly addressed the question is whether cell apoptosis is entirely ATP-dependent since intracellular ATP depletion itself induced necrosis (Eguchi et al., 1997). In 2004, Izyumov proposed that apoptosis in vitro was monitored by intracellular "ATP-meter" generating a cell suicide signal with ATP temporarily threefold decreases, which was achieved by measuring the level of ATP in Hela cells (Izyumov et al., 2004). Consequently, my goals in this section are to rapidly update expanding the field and give a short comparison of apoptosis and necrosis followed by a detailed description (Table.1) (Kroemer et al., 1998; Lally et al., 1999).

Table 1.Comparison between apoptosis and necrosis (Kroemer et al., 1998; Lally et al., 1999).

<table>
<thead>
<tr>
<th>Difference</th>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cause</td>
<td>physiological cell death (apoptosis)</td>
<td>in some cases, accidental cell death (necrosis)</td>
</tr>
<tr>
<td>Area</td>
<td>Single</td>
<td>Large</td>
</tr>
<tr>
<td>The cell membrane</td>
<td>integrated</td>
<td>destroy</td>
</tr>
<tr>
<td>Organelles</td>
<td>no</td>
<td>welling disintegrating the endoplasmic reticulum</td>
</tr>
<tr>
<td>Cell volume</td>
<td>Small</td>
<td>Large</td>
</tr>
<tr>
<td>Apoptotic body</td>
<td>yes</td>
<td>No</td>
</tr>
<tr>
<td>DNA- Electrophoresis gel</td>
<td>Ladder-like</td>
<td>Smear-like</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>yes</td>
<td>No</td>
</tr>
<tr>
<td>Regulation process</td>
<td>Gene</td>
<td></td>
</tr>
<tr>
<td>The inflammatory</td>
<td>No</td>
<td>Yes- release the content</td>
</tr>
<tr>
<td>Intracellular ATP level</td>
<td>ATP-dependent</td>
<td>No ATP</td>
</tr>
<tr>
<td>Immunofluorescence staining</td>
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</tbody>
</table>
2.2.3. Pore-forming toxins

Caspase-mediated apoptosis in the vertebrate ovary
It has been demonstrated that extracellular and intracellular pathogens cause eukaryotic cell death due to the infection of host cells or the production of toxic products. In the past few years, the mechanism by which pathogenic microorganisms induce apoptosis or necrosis in host cells has been further revealed and the importance of eukaryotic programmed cell death has been highlighted (Majno and Joris, 1995; Lizard et al., 1999; Fink and Cookson, 2005). In many cases, numerous microbial pathogens regulate apoptosis which is important for pathogenesis within their hosts including the PFTs, Protein Synthesis Inhibitors, Superantigens and other mechanisms pathogenic elements (Moss et al., 1999). Characterization and comparison of the patterns of pathogen-induced host apoptosis, cell death versus necrosis is briefly summarized below as well as the interaction between virulent bacteria and apoptosis in order to understand pathogenesis under in apoptotic events.

Pore-forming toxins induce different types of cellular responses promoting bacterial survival
Similar to other PFTs, HlyA as hemolysin of Escherichia coli can promote host cell death via lysis, necrosis or apoptotic-like death resulting from the outbreak of RTX intoxication aggregated in a short time compared with canonical programmed cell death completely sustained for several days (Bhakdi et al., 1989; Wiles and Mulvey, 2013). In this section, we describe molecular mechanisms of bacteria-induced apoptosis, the interaction between RTX toxins and target cells, and the dynamic interference / rebalance of cellular processes during infection (Wiles and Mulvey, 2013; González-Bullón et al., 2019).

RTX molecular mechanisms of RTX bacteria-induced apoptosis
Gram-negative pathogens shared a functionally conserved structure among RTX toxin family containing lipases, cytolytic toxins and metalloproteases (Lally et al., 1999; Kloft et al., 2009). PFT plays a toxic role in cell death, primarily by altering the permeability of cell membrane and disrupting the integrity of plasma membrane through its pore-forming disruption and cytolytic activities, which contributes to the catastrophic damage process of normal host cells and promotes the pathogenesis (Kao et al., 2011; Wiles and Mulvey, 2013). Proteins secreted by PFTs have been reported to elicit multiple specific responses and trigger perturbations required biologically activation, while cells trigger signaling pathways to restore completeness of plasma
membrane and ionic homeostasis without protein synthesis (Gonzalez et al., 2011; Wiles and Mulvey, 2013). The novel investigation into various PFT functions during host-pathogen interactions modelling is ranging from bacterial adherence and cell-specific pore-forming to biofilm formation, as well as new findings of toxin-regulated host cell signal transduction disorder (Kloft et al., 2009; Gonzalez et al., 2011; Kao et al., 2011; Wiles and Mulvey, 2013). In this section, we provide a description of molecular mechanisms of bacteria-induced apoptosis, the interaction between RTX toxins and target cells, and the dynamic interference / rebalance of cellular processes during infection (Gonzalez et al., 2011; Wiles and Mulvey, 2013).

**Pathways**

Various apoptosis pathways have been outlined: The JNK- MAPK pathway include caspase-1 and MAPK p38, the NF-κB pathway related to immune response and chicken caspases 1, 2, 3, 6, 8 and 9 is activated by intrinsic and extrinsic pathways *in vitro* (Johnson and Bridgham, 2002; Kloft et al., 2009; Kao et al., 2011; Dhakal and Mulvey, 2012; Wiles and Mulvey, 2013). In 2011, global functional analysis of cellular responses to PFTs was finally confirmed by genome-wide RNA interference (RNAi) screening, which was used to identify 106 knockout genes. The knockout of the genes resulted in hypersensitivity to a PFT and protection of *Caenorhabditis elegans* from PFT attack (Kao et al., 2011). Interestingly, *in vivo* RNAi genome studies have found that the cellular processes involved in PFT defenses was enormous, demonstrating that the JNK/ MAPK pathway was a critical regulator of transcription-induced PFT defense and AP-1 was identified as the cellular element being used for fighting against PFT defense (Kao et al., 2011; Wiles and Mulvey, 2013).

The pore-forming toxins initiate permeabilization of host cells by altering Ca\(^{2+}\) and K\(^{+}\) fluxes and trigger a series of synergistic reactions, including extracellular and endocytic activities, which accelerate membrane renovate and removal of pores on the cell surface (Kloft et al., 2009; Wiles and Mulvey, 2013). In some cases, K\(^{+}\) efflux induces autophagy, and lipogenesis of caspase-1 / SREBP signaling axis, cell quiescence, and MAPK p38 in host cell response (Kao et al., 2011; Kloft et al., 2009). Czuprynski et al. have demonstrated that the *lkt* gene of *M. haemolytica* causes apoptosis of bovine lymphoblastoid cells (BL-3 cells) in caspase-9 dependent pathway, causing e.g disintegration of the mitochondrial membrane, the release of cytochrome c (cyt c) and membrane cracking (Atapattu and Czuprynski, 2005, 2007).
In a recent study, it was observed that HlyA inhibit various signal proteins in the host cell, including pro-survival and immunomodulatory proteins Akt and NF-κB, as well as many cytoskeletal regulators containing paxillin (Dhakal and Mulvey, 2012). To date, six caspase orthologs (chicken caspases 1, 2, 3, 6, 8 and 9) have been characterized in birds, at least in part, and these orthologs are expressed in caspase-mediated apoptosis of vertebrate ovarian cell (Figure 11) (Johnson and Bridgham, 2002). Particularly, after acting the initiator/effector caspase at the mitochondria, bacterial pathogens selectively prevent apoptosis and facilitate replication during cell survival by several pathways including protecting mitochondria, preventing CytoC release and inhibiting caspase activation during the host/pathogen interactions (Zychlinsky and Sansonetti, 1997; Johnson and Bridgham, 2002; Faherty and Maurelli, 2008).

2.2.4. G. anatis dependent apoptosis and necrosis

During the host/pathogen interactions, RTX toxin may be involved in the regulation of apoptosis and necrosis. In addition, bacterial PFTs puncture holes in the plasma membrane of cells, where the rupture of the membrane cracking facilitates the release of virulence factors (Frey, 2011; Kao et al., 2011). Different pathogenic bacteria, e.g. E. coli, Shigella and Salmonella excrete PFTs that may regulate or restrict the host’s apoptosis processes, which is conducive for circumventing the host’s anti-infection activity and increases pathogen replication (Fernandez-Prada et al., 1998; Moss et al., 1999; Robinson and Aw, 2016; Ashida et al., 2020).

Although the importance of GtxA-N in the process of immunogenic and leukotoxicity against G. anatis challenge in chicken tissues has been recognized recently, there is little information about the role of this GtxA-N in G. anatis infection in vitro. Various other genes in other pathogens, e.g. E. coli α-hemolysin5 (HlyA), Pasteurella multocida (NLRP3 inflammasome), Kingella kingae (RtxA), Actinobacillus pleuropneumoniae (ApxI), Bordetella pertussis (CyaA) and Mannheimia haemolytica (LktA) have been shown to exhibit apoptosis and cell death in their host (Suarez et al., 2012; Wiles and Mulvey, 2013; Ahmad et al., 2016; Hsu et al., 2016; Benz et al., 2019; Fang et al., 2019).

Similar to other PFTs, HlyA can promote host cell death via lysis, necrosis or apoptotic-like death, which are caused by cytotoxicity of RTX burst in a short time compared with classic programmed cell death completely sustained for several days (Bhakdi et al., 1989; Wiles and
Mulvey, 2013) (Figure 13). This is an example for studying the description of molecular mechanisms of bacteria-induced apoptosis, the interplay between RTX toxins and target cells, the dynamic interference and rebalances of cellular processes during (Gonzalez et al., 2011; Wiles and Mulvey, 2013).

Figure 13. Pathways that PFTs participate in affecting cell apoptosis and biological activity. Host cell response: Ca^{2+} influx induced by PFTs stimulates exocytic delivery of new membrane and the enzyme ASM to the cell surface. Accumulation of ceramide as a result of ASM activity favours subsequent endocytosis of the pore-ridden membrane (Wiles and Mulvey, 2013).

Like other RTX toxins in pathogenic species of Pasteurellaceae, the lkt toxin of M. haemolytica stimulates cell deaths in BL-3 cells, Apx toxins in A. pleuropneumonia induce necrosis, and toxin rtxA of V. vulnificus causes acute cell death. Studies of these genes have revealed that they culminated in a necrotic cell in a different way (Atapattu and Czuprynski, 2005; Kim et al., 2008).

G. anatis 12656-12, which is also one of the major strains of pathogenic bacteria belonging to family Pasteurellaceae, has a homologous toxin with hlyA secreted by E. coli (Kristensen et al., 2010). In egg-laying chickens, GtxA contributes significantly to G. anatis pathogenesis during infections. The importance of PFTs in G. anatis-induced apoptosis and in pathogenic diseases has not been elucidated yet. To examine the effect of GtxA in vivo in egg-laying chickens, lesions observed in egg-laying hens after experimental infection with a G. anatis WT strain and
its isogenic gtxA deletion mutant strain were compared in our first paper (**paper1**). Studies have revealed a higher number of apoptotic cells in birds receiving the *G. anatis* WT compared to bird infected with ΔgtxA mutant, indicating that GtxA contributes significantly to the pathogenesis of *G. anatis* during infections in egg-laying chickens. Therefore, the potential mechanisms by which *G. anatis* causes upper respiratory tract lesions may be through inducing apoptosis and necrosis of infected cells.

Zhang *et al.*, confirmed the initiation of apoptosis and necrosis by *G. anatis* using primary chicken oviduct epithelial cells (Zhang *et al.*, 2017). Relatively limited attention has been distributed to the function of GtxA included in the pathogenicity of *G. anatis* in *vitro*. It has been shown that pathogenic bacterial strains of *Pasteurellaceae* are usually associated with a variety of virulence factors, which are involved in immune dynamics following infection of avian macrophages (Taylor *et al.*, 2005; Wiles and Mulvey, 2013). One of the mechanisms by which *G. anatis* causes the damage of upper respiratory tract may be through inducing apoptosis and necrosis of infected cells. Notably, the GtxA protein has been demonstrated to induce a great leukotoxic impact on the avian macrophage cell-line HD11 (Kristensen *et al.*, 2011). It has been shown that the GtxA mutant not only lost its haemolytic and cytolytic activity but also cause attenuation of experimental infections in egg-laying hens. There is clear evidence that GtxA adds markedly to the pathogenesis of *G. anatis* upon experimental infection in its natural host. However, the role of the RTX-toxin GtxA in colonization and infection in avian macrophage is much less studied and remain mostly unclear.

Many molecules or genes required in the management or installation of apoptosis have been identified. These include the caspase family with executioner caspase-3 and the initiator caspase-9, the balance of anti-apoptotic and pro-apoptotic Bcl-2 family, p53 tumor suppressor gene, TNF/NGF receptor (Thornberry and Lazebnik, 1998; Chipuk *et al.*, 2010). However, limited knowledge has been obtained about GtxA-induced necrosis and apoptosis. In this study, HD1 1 cells were established as a cellular model to study *G. anatis* infection *in vitro*. For the first time, it was shown that GtxA clearly has the ability to cause host cell lysis through cell viability assay, qPCR assay, observed morphological changes, and flow cytometry (**paper2**). It is possible that the initial survival of the host cell by promoting *G. anatis*-induced cell death is achieved by regulating the anti-apoptotic bcl-2 response. However, whether it subsequently participates in cell division through cell perforation like in *E. coli* has to be further studied.
Moreover, the investigation a revealed that the GtxA mutant exhibited restricted ability to proliferate or survive within HD11 cells in contrast to the WT strain at 4, 6 and 24 h.p.i., reaching an obvious difference at 4 h.p.i. The analyses of the growth indicated that the GtxA gene has little influence on the basic metabolism of the *G. anatis* *in vitro*. These results showed that the GtxA influences the survival and/or replication of *G. anatis* in macrophages. In the adherence assay, *G. anatis* strain 12656-12 has a higher adhesion capacity than the other two minutes (30 and 90), after 60 minutes infection indicate that the corresponding receptors on the cell surface have reached saturation. It is corroborating the results published studies on primary chicken oviduct epithelial cells (PCOECs) reaching the peak of adherence slightly earlier (Qureshi *et al.*, 2000; Vaca *et al.*, 2011). Additionally, a diversity of adhesins, e.g. fimbriae clusters (F17-like fimbria) and capsular the polysaccharide gene (*cpsD*) have been reported to participate in the adherence process (Bager *et al.*, 2013a; Xu *et al.*, 2013). The amount of adhered mutant ∆gtxA strain was approximately 2 times less than that of adhered WT bacteria on pre-fixed macrophage HD 11 cells, which indicates that GtxA stimulates the invasion of macrophages. The macrophage HD 11 cell-line may be mediated by surface proteins feasibly acting as a barrier against colonization of *G. anatis* by covering or suppressing the expression of adherence-associated proteins such as fimbriae, and might be involved in bacterial cell attachment. This hypothesizes may be determined by phase-contrast microscopy in further studies.

As described in the previous studies, mutants lacking GtxA have reduced toxicity in *in vivo* (Kristensen *et al.*, 2011). Lower intracellular production of the ∆GtxA mutant strain was observed in macrophages at 4, 6 h.p.i. compared with WT, but similar counts were observed at 2 h.p.i, indicating that ∆GtxA mutant strain has a lower capacity to survive the macrophage. These results confirms the finding of the transcription of GtxA was extinguished during stationary phase in overnight cultures (Kristensen *et al.*, 2011). Since the growth phase is affected by transcriptional regulation, the subsequent bacterial degradation and pronounced cell death can be quantified using LDH cytotoxicity assay in the future.

Moreover, the present study provides evidence the mutant GtxA adhered less than WT to cultivated chicken macrophages *in vitro*. These data suggest that GtxA is dramatically important for pathogenic characteristics of *G. anatis* strain 12656-12 in the process of invasion into macrophages and intracellular growth. The production of apoptotic genes such as caspase-3, -8, -
9 and Bcl-2 in the spleen and ovary were examined using real-time PCR, to reveal the possible role of GtxA in *G. anatis*-induced inflammatory response and apoptosis in the spleen and ovary of chickens. The results are suggesting that the GtxA mutant had an anti-inflammatory effect in the spleen. The GtxA mutant has the potential ability to delay the infection by decreasing the bacterial production by inducing cytokines. In conclusion, we show that the *G. anatis* GtxA deletion mutant is attenuated upon experimental infection.
Chapter 2 Introduction
Chapter 2 Objectives

The overall aim of this Ph.D project was to explore how GtxA induce distinct types of immune-responses during the pathogenesis of \textit{G. anatis} \textit{in vivo} and \textit{in vitro}. The following two hypotheses was investigated in this work.

**Hypothesis 1:** GtxA participates in mediating different immune response \textit{in vivo}. Our hypothesis is that GtxA play a key role in the interaction between \textit{G. anatis} and the chicken reproductive tract during a natural infection.

**Hypothesis 2:** GtxA specifically induce host cellular changes during interaction with chicken macrophage-like HD11 cells and thereby affects the host immune function.

To test the proposed hypotheses, the following objectives were investigated:

**Objective 1:** To evaluate the role of GtxA at induction of an immune response in the chicken following experimental infection. This work included (paper I):

- Comparison of lesions following post-mortem examination of chickens experimentally infected with a GtxA expressing wildtype \textit{G. anatis} and its isogenic gtxA deletion mutant, respectively.
- Determination of the host innate and adaptive immune responses following experimental infection
- Analysis of expression levels of chicken apoptosis genes during \textit{in vivo} experiments.
- Assessment of the systemic anti-GtxA antibody responses by ELISA

**Objective 2:** To understand the host-pathogen interactions between \textit{G. anatis} and chicken macrophage-like HD11 cells and evaluate the specific impact of GtxA on the cellular changes \textit{in vitro}. This work included (paper II):

- Comparing the effect of GtxA on the adherence and intracellular survival of \textit{G. anatis} in HD11 macrophages.
- Characterize the different types of HD11 cellular immune responses \textit{in vitro}
- Assessment of the role of the GtxA toxin on HD11 cell death

These Objectives were addressed and the following sections including results, discussion, conclusions and future perspectives are presented in the manuscripts.
Chapter 3 Results and discussion
Chapter 3 Results and discussion

The virulence determinant, GtxA, which is in charge of G. anatis’ haemolytic and leukotoxic activity has previously been suggested as a primary virulence factor (Kristensen et al., 2011; Johnson et al., 2013). Currently, however, very little is known about the mechanisms that determine G. anatis host or cell specificity, although some researchers have shown that GtxA play an important role in the pathogenesis of G. anatis in the natural host (Kristensen et al., 2010; Kristensen et al., 2011; Johnson et al., 2013). Increasing evidence indicate that the recombinant protein GtxA-N has immunogenic and protective capacity in a G. anatis challenge model (Bager et al., 2014; Pedersen et al., 2015; Pors et al., 2016; Persson et al., 2018). Still, the specific role of GtxA in the pathogenesis in vivo is sparsely understood. A truncated version of GtxA, lacking the N-terminal part lost its haemolytic and cytolytic activity. It is widely reported that bacterial interaction with immune cells e.g. avian macrophages is an effective way to assess specific protein-host interactions during a systemic infection in the natural host (Beeckman et al., 2007; Kristensen et al., 2010; Pedersen et al., 2015). To understand better the host-pathogen interactions between G. anatis and different chicken tissue types, and evaluate the impact of GtxA on cultured macrophages, we used an avian pathogenic G. anatis (12656-12) wild-type strain and its isogenic gtxA deletion mutant (ΔgtxA) for inoculations in egg-laying hens and for co-cultivation with the chicken macrophage-like cell line HD11, respectively. The investigations provided the basis for a series of experiments in this project, and a further theoretical basis for the function of GtxA, which may help prevent future infections where GtxA is involved.

From previous in vivo studies investigating RTX deficient bacterial strains, a significant decline in virulence as shown by lower mortality, morbidity and diminished lesions, respectively, has been reported, as exemplified by examples include the pathogens Mannheimia haemolytica, Actinobacillus pleuropneumoniae in Pasteurellaceae family and E. coli, respectively (Ludwig et al., 1996; Kamp et al., 1997; Frey, 2011). To assess the importance of GtxA-host interactions during avian G. anatis infection, an in vivo model was used to evaluate and compare lesions caused by a G. anatis wild type and its isogenic GtxA deletion mutant, G. anatis ΔgtxA. We evaluated the effect of the G. anatis ΔgtxA in pathogenicity of avian pathogenic G. anatis survival in infected chickens; and determined the innate and adaptive immune responses locally and systemically; and specifically investigated the ability of GtxA at induced apoptosis in vivo (Manuscript I). A total of 24 egg-laying hens raised under high biosecurity standards were randomly assigned to one of three
groups and injected into the peritoneal cavity with *G. anatis* 12656-12 (wildtype), *G. anatis* ΔgtxA (gtxA deletion mutant of 12656-12) or sterile BHI broth, respectively (Table 2). Subsequently the chickens were euthanized and subjected to post mortem examination after two or six days, respectively.

Table 2. Design of study. A total of 24 birds were inoculated with *G. anatis* ΔgtxA (16 chickens), *G. anatis* 12656-12 (4 chickens) or BHI (4 chickens).

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>Dose/CFU</th>
<th>2 days PI</th>
<th>6 days PI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. anatis</em> ΔgtxA</td>
<td>ΔgtxA</td>
<td>4.9x10^8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><em>G. anatis</em> wildtype</td>
<td>12656-12</td>
<td>5.0x10^8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>BHI</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

3.1 Preparation and handling of different materials taken from the chicken

For the *in vivo* assessments, the gross pathology and the re-isolation rates of *G. anatis* after experimental infection was assessed. Tissue samples from different organs were selected and characterized by semi-quantitative grading system for histological lesions. In addition, samples from the spleen and ovary were stored in RNA later and prepared for subsequent cytokine and apoptosis gene expression quantification. For further investigation of the role of RTX toxins in the pathogenesis, ovary and oviduct fixed in formalin were evaluated using standard histological procedures. Serum sampling from all birds was obtained from *vena brachialis* to allow quantification of GtxA-specific antibodies by ELISA at 0, 2, 6 days inoculation, respectively. For the *in vitro* assays aliquots of avian macrophage (HD11 cells) suspensions were incubated in RPMI medium and used for subsequent experiments including multiplicity of infection (MOI) assays, invasion assays, qPCR assays and flow cytometry assays, respectively.

3.2 Summary of results

The results from the *in vivo* experiments showed that 75% (3/4) of the chickens infected with *G. anatis* WT had severe lesions whereas 62.5% (10/16) chickens receiving *G. anatis* ΔgtxA only had mild or no clinical signs and very limited or absent lesions from the postmortem examinations. The gross lesions and histopathology results indicated that a functional gtxA gene was highly correlated with virulence and ability to induce lesions (Figure 2A, B). As an example there was heavy
multifocal infiltration of inflammatory cells including granuloma formation and necrosis and several clusters of bacterial colonization in the oviduct and ovary in the WT infected chickens (Figure 2D), compared to little or no infiltration of lymphocytic cells and no follicular degeneration or purulent oophoritis was observed in the oviduct of chickens from the ΔgtxA group. Except for the partial hyperemia in the vascular system of the ovarian interstitial tissue no lesions were observed here (Figure 2C). Based on the above it appears likely that GtxA was involved in activating heterophilic cell, which has previously been shown during the immediate inflammatory response against G. anatis (Bojesen et al., 2004).

Figure 2 Detailed histopathological and HE-straining results of ovaries from 29-week laying hens infected with G. anatis ΔgtxA mutant or G. anatis wild-type (WT) strain respectively. A Post-inoculation with G. anatis ΔgtxA. Oophoritis accompanied by vascular congestion and mild edema. B Post-inoculation with G. anatis wildtype. Diffuse purulent oophoritis and folliculitis with ruptured follicles. C HE-straining of ovary in the G. anatis ΔgtxA infection group. Focal inflammation and vascular congestion, accompanied by infiltration of fewer inflammatory cells, heterophils and monocytes. D HE-straining of ovary in the G. anatis WT infection group. In purulent oophoritis, a large number of inflammatory cells infiltrate, and granulomas form in the follicles (Tang et al., 2020).
The histological lesion scores of organs from hens infected by the *G. anatis* Δ*gtxA* mutant strain were significantly lower than hens infected by the GtxA-producing strain (Figure 3). The latter corresponded to results obtained in previous *in vivo* studies (Bojesen *et al.*, 2004; Pedersen *et al.*, 2015; Pors *et al.*, 2016). The virulence of related bacteria including *A. pleuropneumoniae* was negatively affected during *in vivo* infections of pigs when expression of the *ApxIVA/ApxI* gene was impaired, and experimental infection with other RTX-toxins deficient bacterial strains also had a similar sharp decline in lesions when compared to their isogenic WT (Kamp *et al.*, 1997; Tatum *et al.*, 1998; Frey and Kuhnert, 2002; Liu *et al.*, 2009) (*Table 1*). Our results were in accordance with previous observations GtxA expressing *G. anatis* strains in the chicken infection model, however, this was the first time when the specific role of GtxA was investigated in the natural host.

### 3.3 Re-isolation of *G. anatis*

The experimental hens were already colonized with *G. anatis* in the intestinal tract at initiation of the *in vivo* investigations and that complicates the subsequent assessments of lesions during diagnostic procedures (Neubauer *et al.*, 2009; Wang *et al.*, 2018). In order to check whether the cloacal isolates were different to those recovered from the internal organs the bacterial strains isolated from cloacal mucosa and tissue lesions from eleven individual birds were obtained and characterized by pulsed-field gel electrophoresis (PFGE). The results of the phenotypic bacteriological examination showed that the re-isolated bacteria from the cloaca in the *G. anatis* WT and MT groups were hemolytic, had grey, semi-transparent colonies as expected. Whereas the colonies obtained from the internal organs in the Δ*gtxA* group lacked the hemolytic zone (Christensen *et al.*, 2003; Kristensen *et al.*, 2010).

Moreover, our genotyping results showed that isolates from lesions had similar PFGE profiles, but all isolates from cloaca had different banding patterns, which supported the fact that there was no overlap between the two bacterial populations (Figure 4).
3.4 Host immune response

It is generally believed that neutralizing antibodies represent an effective mean to prevent diseases caused by pathogenic species in *Pasteurellaceae* (Frey and Kuhnert, 2002; Johnson et al., 2011). Moreover, Frey already mentioned in the review that specific RTX toxins play a diverse role in host specificity and are actively involved in the immune responses against several bacteria such as *Actinobacillus pleuropneumoniae* (Frey and Kuhnert, 2002). Hence, we assessed the presence of GtxA-specific antibodies by characterizing serial threefold dilutions of chicken serum obtained at 0, 2 and 6 days after chickens were inoculated with *G. anatis* and used this for ELISA experiments. Our results showed that the levels of GtxA specific antibodies were unchanged during the infection period (Figure 5, Table 3) and GtxA specific antibodies were at or below the background level in all infected groups (Pedersen et al., 2015; Pors et al., 2016). At the same time, the histology results indicated that, compared with the severe pathological changes such as granuloma formation and a large number of inflammatory cells in the *G. anatis* WT group, the *G. anatis ΔgtxA* group only had focal oophoritis very limited cellular infiltration and inflammatory reaction (Figure 2A and C). Moreover, our results indicated that hens infected with *G. anatis ΔgtxA* only had very minor pathological changes in the oviduct, which was supported by a low pro-inflammatory response. In other words, GtxA had a major impact but was not the only bacterial factor responsible for changes found in the chicken reproductive tract.
Table 3. Gross pathology and re-isolation rates of *G. anatis* from different organs following experimental infection with a wild-type strain (*G. anatis* 12656-12) (4 chickens) or its isogenic gtxA deletion mutant (ΔgtxA) (16 chickens) (Tang *et al.*, 2020).

<table>
<thead>
<tr>
<th></th>
<th>Peritoneum</th>
<th>Ovary</th>
<th>Oviduct</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. anatis</em></td>
<td>Purulent peritonitis</td>
<td>Re-isolation of <em>G. anatis</em></td>
<td>Purulent oophoritis</td>
</tr>
<tr>
<td>ΔgtxA</td>
<td>2/16 (13%)</td>
<td>2/16 (13%)</td>
<td>5/16 (31%)</td>
</tr>
<tr>
<td>Wildtype</td>
<td>3/4 (75%)</td>
<td>3/4 (75%)</td>
<td>3/4 (75%)</td>
</tr>
</tbody>
</table>

The reduced virulence of the *G. anatis* ΔgtxA mutant indicating clearly that GtxA had an important contribution to the severity of host-specific infections. As previously described in 3.2, the gross lesions and histopathology in the *G. anatis* WT group were similar to observations in previous studies (Bojesen *et al.*, 2004; Pedersen *et al.*, 2015; Pors *et al.*, 2016). Previous reports have clearly showed that GtxA-N can induce high specific titers yet in the current hens no stimulation appeared to have taken place (Pedersen *et al.*, 2015; Pors *et al.*, 2016).

To evaluate more specifically how GtxA affects different parts of the immune system more in-depth studies were needed. Therefore, RNA was extracted from ovary and spleen tissue in the infected chickens. Five microgram RNA was reverse-transcribed into cDNA, which was stored for subsequent experiments. To examine the role of GtxA during interaction with the immune system we examined differential immune gene expression (Table 1) based on the 3-step amplification by RT-qPCR (Bager *et al.*, 2013). The levels of gene expression were compared using adjusted inflammatory cytokine mRNA concentrations according to the formulas below (Hangalapura *et al.*, 2006).

**Adjusted cytokine quantity for each Ct sample:** \[(40 - \text{mean cytokine Ct sample}) \times \text{cytokine slope}\]/\text{Difference factor sample - β-actin slope}

**Difference factor for each Ct sample:** \[\frac{\text{mean Ct value for β-actin gene of individual sample}}{\text{mean Ct value for β-actin gene of all samples}}\]

Previous studies have indicated that inflammatory responses were induced by wild-type bacteria *G. anatis* in primary chicken oviduct epithelial cells (Zhang *et al.*, 2017). Based on that it is not surprising that the expression of IL-4 and TNF-α mRNA changed significantly in the wild-type
group (Figure 6 and 7). It could be speculated that GtxA may significantly contribute to Th2-like response by stimulation of IL-4 and TNF-α, which has been previously shown to assist the chicken host defense against bacteria. Considering that increased IL-4 expression in the ovary at two-time points, while a relatively lower level in the spleen at 2 days pi, could indicate that ovary could be the main target for *G. anatis* (Figures 6 and 7). It is believed that the spleen is not only a major contact point between the host immune system and invading pathogens but also represent a critical tissue for clearance of pathogens. It seems that *G. anatis* may first enter the circulatory system and then like other pathogens may be partially killed within the spleen, causing a slightly delayed response. From previous results, the pro-inflammatory cytokine IL-6 has been proposed involved in the recruitment of immune cells to the site of infection in chicken (Nawab et al., 2019). However, after testing of pro-inflammatory cytokines IL-6 and anti-inflammatory cytokine IL-10, no significant difference was observed in the ovary or the spleen tissues at 2 or 6 days pi between WT strain and *G. anatis A*gtxA group in our work (Figure 6 and 7). These results indicated that GtxA may regulate the adaptive rather than innate immune response in the *in vivo* challenge model.

**Figure 6** Changes of the cytokine mRNA expression levels in the ovary. Cytokine mRNA was isolated from culture-positive ovary tissue at 2 days pi (A) and 6 days pi (B). As values are subtracted from the negative endpoint, higher values represent higher levels of cytokine mRNA levels. Error bars are S.E. for each treatment group (*P< 0.05, **P< 0.01) between WT (white bars) and *A*gtxA mutant groups (black bars) (Tang,Pors et al. 2020).

**Figure 7** Changes of the cytokine mRNA expression levels in the spleen. Cytokine mRNA was isolated from culture-positive spleen tissue at 2 days pi (A) and 6 days pi (B). Error bars are S.E. for each treatment group (*P< 0.05, **P< 0.01) between WT (white bars) and *A*gtxA mutant groups (black bars).
A secreted virulence factor that mediates cytokine response was reported by Beuscher et al., who revealed that suppression of TNF-α production by YopB helped *Y. enterocolitica* at evading antibacterial host defense factors (Beuscher et al., 1995). On the contrary, our results have shown that TNF-α production in the spleen was significantly higher in the ΔgtxA group, while expression of IL-4 remained at a relatively lower level (Figure 6B) indicating that the ΔgtxA mutant could regulate the acute phase reaction by promoting of TNF-α production. Moreover, these results indicate that ΔgtxA mutant could interfere with humoral immunity by suppressing secretion of IL-4 in poultry (Chtanova et al., 2001). In addition, the pathological results mentioned in 3.2 provide indications that ΔgtxA mutant induces less cellular infiltration and inflammatory reaction (Figures 2A and 2C), which suggest that ΔgtxA mutant could take part in a weaker host response possibly through partial destruction of the Th2-like pathway. If so this is in good agreement with a previous report stating that a live attenuated mutant of *Salmonella Montevideo* triggered ascending trends of the immunological profiles against *Salmonella Montevideo* infection by inducing INF-α, and IL-12 cytokines (Lalsiamthara and Lee, 2017). It was shown that interleukin-10 negatively triggered a Th1 cytokine response in the host, when infected with a AvrA mutant (Duell et al., 2012; Arsenault et al., 2016). In our investigation there was no change in the expression of IL-10 and IFN-γ in WT infected group, which indicated the presence of a Th2-like response. This study is the first to describe the role of GtxA in the immune response caused by *G. anatis* infections in laying hens and paper 2 provides further insight into the immune responses in vitro.

### 3.5 Host apoptosis response

Regulation of programmed cell death or apoptosis takes part in the development and homeostasis of multicellular organisms (Fan et al., 2005; Elmore, 2007). Bacterial pathogens may engage or circumvent the host's apoptotic processes e.g. pore-forming toxins induce apoptosis to limit the inflammation, which has been shown in E. coli through a calcium-dependent mechanism (Czuprynski and Welch, 1995; Fernandez-Prada et al., 1998; Moss et al., 1999). In a previous in vivo study, the results of the TUNEL assay showed that G. anatis dramatically increased the percentage of apoptosis in the organ, but the specific mechanisms were not well documented, although pore-forming toxin were suggested to be involved in the process of the pathogenesis. In order to reveal the possible mechanism of G. anatis-induced apoptosis in vivo, the mRNA expression of apoptosis-related genes including caspase-3, -8, -9, and Bcl-2 was examined in the spleen and ovary tissues from chickens. Previous reports have described Actinobacillus leukotoxin
and other RTX toxins e.g. Staphylococcus aureus (alpha) and E. coli (Hly) able of inducing apoptosis at lower concentrations. This presumably by inducing intracellular Ca2+ oscillations by formation of small pores in target cell’s plasma membrane, which subsequently activated the regulators of the intrinsic apoptotic pathways (Jonas et al., 1994; Lally et al., 1999; Oxhamre et al., 2005; Benz, 2020). Our results showed a lowered expression of caspase -3, -8, -9, bax and bcl-2 in the spleen tissue at 2 days pi of the ΔgtxA mutant group compared to the WT group (Figure 8), while the production of TNF-α was increased (Figure 7A). Considering of a possible dose-response relationship during the cell-death program, it could be speculated that some WT bacterial cells made it to the spleen, whereas almost none reached to the spleen in ΔgtxA mutant group.

On the contrary, in ovarian tissue, a slight up-regulation of pro-apoptosis caspase-8,-9 and bax was induced by ΔgtxA mutant at 2 days, while the inhibition of apoptosis caused by increased bcl-2 expression appeared at 6 days (Figure 9). The results were consistent with the observation in the ΔgtxA mutant group which has no macroscopic or microscopic lesions in the ovary tissue.
despite the presence of bacteria in this organ (Table 3). These observations indicated that the ΔgtxA mutant might modulate the apoptosis response and prolong bacteria survival in the host.

As stated in section 3.4, differential inflammatory responses were observed in the ovary tissue following exposure to the wildtype or mutant infected groups, respectively. Induction of cell death could be a mechanism utilized by G. anatis to escape the host defenses by forming large pores and quickly dissolve the antibacterial effect of phagocytic cells (Kristensen et al., 2010). Previous research reported that RTX toxins produced by other bacteria were involved in destroying a primitive defense to permit successful bacterial replication by preventing or delaying apoptosis of host cells, such as Salmonella enterica, serovar Typhimurium and some Shigella species involved in mediating pro-inflammatory death pathway in the chicken (Lally et al., 1999; Boise and Collins, 2001; Kristensen et al., 2010; Arsenault et al., 2016). In conclusion, it could be speculated that GtxA may participate in apoptosis suppression at an early stage in the
ovary to promote *G. anatis* multiplication, which appears to be a preferred locus of this bacterium. In a further study, we hypothesized that GtxA toxin of *G. anatis* mediates apoptosis through a calcium-dependent mechanism like *E. coli* or are involved in mediating the pro-inflammatory death pathway as described above. The hypothesis could be confirmed by investigating how TNF-α is induced by non-GtxA expressing bacterial cell and its roles in the regulation of apoptosis in cells exposed to *G. anatis*.

### 3.6 Adhesion and invasion characteristics of *G. anatis* strains to macrophage cells *in vitro*

In addition to the results detailed in sections 3.2 to 3.5, these experiments were intended to understand the role of *G. anatis* and GtxA on development of lesions in the natural host, and further revealed the possible role of GtxA in *G. anatis*-induced inflammatory responses and apoptosis in the spleen and ovary of chickens combined with previous studies (Jordan *et al.*, 2005; Neubauer *et al.*, 2009). In general the host-pathogen interface triggers a complex molecular crosstalk, resulting in innate host defense and subversion of cellular processes (Beachey, 1981; Soto *et al.*, 2016). There is clear evidence that GtxA contributes to the pathogenesis of *G. anatis* upon experimental infection in its natural host, but the role of the RTX-toxin GtxA in the specific process of attachment and interaction with avian immune cells, here macrophages, were much less studied and remained uncertain. In our study, modulation of antimicrobial activity, cell invasion and intracellular survival in avian macrophage HD11 cells were examined to describe adhesion and invasion characteristics of *G. anatis* strains to macrophage cells *in vitro*.

#### 3.6.1 Establishment of an Appropriate Multiplicity of Infection (MOI)

In order to obtain an appropriate bacterial concentration range for subsequent adhesion and invasion tests, suspensions of cultured *G. anatis* 12656-12 in three different MOIs (10:1, 50:1 and 100:1) was used for preliminary MOI assays. Our results showed that the adhesion level of *G. anatis* wild-type peaked at 60 min following exposure to HD11 macrophage, which may be due to the saturation of the corresponding receptors on the cell surface (Figure 10). Moreover, the result was similar to the recent findings that chicken primary oviduct epithelial cells (PCOECs) reached the adhesion peak at an early stage (Zhang *et al.*, 2017). Several adherence proteins of *G. anatis* including GtxA toxins, F17-like fimbria and secreted surface proteins has
been proposed important for the adhesion process (Bager et al., 2013a; Persson et al., 2018), so it was not surprising that GtxA could play an important role during that part of the pathogenesis. In addition, the amount of attached ΔgtxA mutant bacteria was nearly two fold less than that of G. anatis WT on the same pretreated HD11 cells (Figure 11).

**Figure 10** The adhesion kinetics of G. anatis 12656-12 strain to HD11 cells after 30, 60 and 90 min incubation at three different multiplicities of infection (MOI). Significant differences were labelled (p ≤ 0.05)* and (p ≤ 0.001)*** (Tang and Bojesen, 2020).

**Figure 11** The intracellular viability of G. anatis 12656-12 and G. anatis ΔgtxA in the chicken HD11 cells at 2, 4, 6, 8, 10, 12 and 24 h were tested by the gentamicin protective assay. The ability of the G. anatis ΔgtxA mutant to enter and survive in the HD11 cells was compared to its G. anatis 12656-12 parent strain. Bacterial counts (CFU/cell) were obtained and compared from lysed HD11 cells over time following addition of gentamicin **(p ≤ 0.01)*** (p ≤ 0.001) (Tang and Bojesen, 2020).

At 4 and 6 h PI, the intracellular number of the ΔgtxA mutant strain was lower than that of G. anatis WT and remained at a relatively lower level after 6 h PI (Figure 11). Most likely the dead host cells released intracellular bacteria to the outside, and then got killed by gentamicin in the medium.

These results suggested that GtxA contributes significantly to the severity of infections and cytotoxicity of HD11 cells. The results not only indicate that G. anatis wild-type has a higher capacity of adherence and invasion during the initial processes of exposure *in vitro*, but also suggests that GtxA contributes significantly to the severity of host cell death.

### 3.6.2 Bacterial Growth and macrophage viability assay

In order to determine if GtxA expression affected the growth of G. anatis, a bacterial growth assay was made for both the G. anatis WT and its ΔgtxA mutant, respectively. The results
showed that the growth curves of the WT and ΔgtxA mutant strains were highly similar, indicating that deletion of gtxA does not influence significantly the growth of G. anatis (Figure 12). When exposing HD11 cells to the GtxA mutant and the wildtype the number of viable HD11 cells was 91.3% and 78.10% at 2 PI, and 90% and 66.73% at 4 PI, respectively (Figure 13).

Figure 12 Growth curves of the G. anatis wild-type (WT) strain and the ΔgtxA mutant strain. Growth was assessed by measurement of the OD600 at 2, 4, 6, 8, 10 and 12 h. The graph is representative of three independent experiments (Tang and Bojesen, 2020).

Figure 13 The effect of GtxA on macrophage viability after incubation of wild-type strain and its ΔgtxA mutant strain. Data shown represent means ± SEM from three independent experiments *** (p ≤ 0.001)(Tang and Bojesen, 2020).

3.7 The immune response in Macrophage-Like HD11 Cells

Pro-inflammatory cytokine expression was previously investigated by exposing primary chicken oviduct epithelial cells to pathogenic G. anatis in immune cells (Zhang et al., 2017). As described in 3.4, recent studies have revealed that the GtxA of G. anatis 12656-12 stimulated innate and partial adaptive cellular immune response in the ovary and spleen of infected chickens. To study these effects in further detail we investigated the role of GtxA cytotoxicity after exposure to macrophage cells. Specifically, we quantified the expression of IL-1β, IL-6, IL-10, and TNF-α cytokines in macrophage HD11 infected with G. anatis WT and ΔgtxA, respectively, using qPCR. The results indicated that IL-10 expression was down-regulation the cell exposed to the G. anatis ΔgtxA mutant compared with the WT group (Figure 14). This was in accordance with cytokine expression levels observed in the spleen from the in vivo experiments (Figure 7).
Figure 14 Real-time relative quantification of cytokine expressions. Quantification of cytokine mRNA levels in RNA extracted from HD11 macrophage cells infected with WT (white stripe bars) and ΔgtxA mutant (diagonal stripe bars) or subjected to non-infected macrophage (gry bars), respectively (Figure 14). Error bars are S.E. for three treatment group. Asterisks indicate significance from the uninfected control cells (* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001) (Tang and Bojesen, 2020).

The results indicated that GtxA primarily induces an anti-inflammatory response through IL-10 expression in macrophages. Simultaneously, a significantly increased expression of IL-1β was observed in the ΔgtxA mutant group. This was accompanied by a large TNF-α release (Figure 14), which was partially consistent within our in vivo results (paper I) and in accordance with a pro-inflammatory reaction (IL-6, TNF-α) in PCOECs as reported previously (Zhang et al., 2017). In contrast to IL-10 as a signal of macrophage deactivation, it is believed that TNF-α is able of triggering avian macrophage activation (Giansanti et al., 2006). All in all the data indicate that GtxA may trigger an altered host response including inhibition of pro-inflammatory cytokines, which may allow the bacterium to survive inside the host.

The results also indicate that HD11 cells can be employed as biologically relevant model for study of immune activating antigens of relevance to commercial vaccine applications and animal protection.
3.8 The cell death in Macrophage-Like HD11 Cells

Wiles & Mulvey stated that bacterial pore-forming toxins e.g. HlyA secreted by uropathogenic E. coli caused cell damage by poking holes in plasma membrane resulting in lysis, necrosis or apoptosis according to different toxin concentration and duration of exposure to the target host cell (Wiles and Mulvey, 2013). Interestingly, G. anatis induced apoptosis and necrosis in primary chicken oviduct epithelial cells as Zhang’s described (Zhang et al., 2017). Zhang and co-workers reported a dramatic number if apoptotic cells, visualized with the TUNEL assay, in birds receiving the G. anatis wild-type. In addition, an increased proportion of apoptotic cells were investigated in the spleen infected G. anatis 12656-12 strain that initially caused anti-inflammatory response in vivo trials. If the apoptosis and necrosis observed in macrophages HD11 infected by G. anatis wild-type correlates to the severe salpingitis, oophoritis and and upper respiratory tract injury this may likely also relate to the GtxA induced cell death observed. Therefore, we hypothesized that role of GtxA play a central role in regulating cell death in vitro as has been reported for several other RTX toxins of Pasteurellaceae bacteria e.g. HlyA, ApxI A/ LtxA and LktA (Table. 1) (Faherty and Maurelli, 2008; Frey, 2011). More experiments were required to support the hypothesis outlined above and understand more completely role of GtxA during interaction with HD11 cells. That could include cell viability assay, morphological changes, qPCR assays and flow cytometry.

After testing of the five apoptosis genes caspase -3, -8, -9, bax and bcl-2 in macrophage HD11 cells exposed to G. anatis strain and its isogenic ΔgtxA mutant (Figure 15), we discovered that, contrary to a lower level of the executioner caspase -3, HD11 cells exposed to GtxA had an increase in the expression of the initiator caspase -9, which was up-regulated at 2 h PI followed by a sharp decline at 6 hours PI. Based on a previous report, Shigella flexneri might block apoptosis by targeting the activated form of caspase-9 resulting in membrane damage and Mannheimia haemolytica LktA have also been shown to induce apoptosis in BL-3 cells in a caspase-9-dependent manner (Atapattu and Czuprynski, 2007; Faherty and Maurelli, 2008). The results suggested that GtxA may participate in the regulation of cell apoptosis leading to cell membrane collapse through the caspase-9-dependent mitochondrial pathway (Figure 15). In contrast to this, several anti-apoptotic strategies including prevention or partial delay of apoptosis of host cells through repair of damaged mitochondria, limiting cytochrome c release and suspending caspase activation have been proposed to allow bacteria to multiply and escape.
We suspect that \textit{G. anatis} may have the potential to deal with antibacterial activity performed by HD11 cells, assuming that at least part of it can be achieved by preventing caspase activation at different points during the apoptotic pathway. Considering the characteristics of Bcl-2 preventing cell death as an original defense mechanism against viral infection, our data showed that compared with the WT group, the production of the anti-apoptotic Bcl-2 protein in the \textit{\Delta gtxA} mutant group was at large down-regulated at 2 h PI rather than after 6 hours, which indicated that in the early stages of \textit{G. anatis} infection, GtxA may proactively prevent or delay cell damage through promoting an anti-apoptotic response in the host.

\textbf{Figure 15} Real-time quantitative PCR analysis of apoptosis genes in the macrophage. The relative abundance of mRNA was isolated from HD11 cells stimulated WT strain and \textit{\Delta gtxA} mutant strain to un-stimulated medium and then examined by using quantitative real-time PCR analysis, respectively. Values represent the mean ± SEM. Different asterisks indicate differences from the uninfected control cells (* \( p \leq 0.05 \), ** \( p \leq 0.01 \), and *** \( p \leq 0.001 \))(Tang and Bojesen, 2020).
Our flow cytometry analyses showed that compared with the non-infected control group (17.2% vs 80.6%) at 2 h PI, the ΔgtxA group had less dead cells (19.2%) than the WT group (26.9%) indicating that GtxA has the ability to induce cell death in HD11 cells (Figure 16A). At 6 h the trend of inhibiting cell death in the ΔgtxA group the fraction of dead cells was greatly lower (22.1%) than in the WT group (36.2%), again suggesting that GtxA is a major factor in lesions induced by *G. anatis* (Figure 16B).

The ΔgtxA mutant initially seemed to promote host cell’s survival by reducing *G. anatis*-induced cell death. Several researchers have mentioned that RTX toxins can bind to β2-integrin and disrupt the cell homeostasis at high toxin concentrations, which may include induction of cell lysis and necrosis, or toxin-mediated disturbance eventually destroying the integrity of cells exposed to lower toxin concentrations, which may result in triggering of a rapid signal cascade promoting activation of apoptosis-like cell death (Tatum *et al.*, 1998; Desagher and Martinou, 2000; Welch, 2001; Wiles and Mulvey, 2013). These findings are, at least partly, similar to our results where the ΔgtxA mutant infections *in vivo* and *in vitro* induce a very different host cellular behavior including some aspects of pro-survival protection, which may be related to toxin concentration and exposure time, although the specific mechanisms needs to be studied further.
Figure 16 Flow cytometry analysis of the role of GtxA in the apoptosis and necrosis of HD11 cells infected by *G. anatis*. The HD11 cells infected with cultured and stained with Annexin V FITC/ Propidium iodide (Pi). (A) Compared with unexposed HD11 cells, flow cytometric analysis was performed on HD11 cells exposed to *G. anatis* WT or *G. anatis ΔgtxA* mutant strain at 2 h. (B) Compared with unexposed HD11 cells, flow cytometric analysis was performed on HD11 cells exposed to *G. anatis* WT or *G. anatis ΔgtxA* mutant strain at 6 h. Representative flow plots of each group were analyzed for cell death at different times shown in A and B (Tang and Bojesen, 2020).
Chapter 4 Conclusions and future perspectives
Chapter 4 Conclusions and perspectives

4.1 Conclusion

The aim of this project was to investigate the effect of GtxA in the pathogenesis of *G. anatis* during infections in laying hens and macrophage HD11 cells, respectively. With the ultimate goal of increasing our insight into the underlying pathogenic mechanisms in laying hens and HD11 cells, the current work aimed at understanding whether GtxA is involved in regulating the host's inflammatory and apoptotic response against *G. anatis* during infection *in vivo* and *in vitro*. The following conclusions have been drawn with regards to these objectives as described below:

a) To evaluate the effect of the *G. anatis* *AgtxA* in pathogenesis of avian pathogenic *G. anatis* in chickens we investigated the following main topics:

1. To evaluate and compare lesions found in egg-laying hens after experimental infection with *G. anatis* GtxA wild type and its isogenic *G. anatis* *AgtxA*. The layer hens infected with *G. anatis* *AgtxA* had significantly fewer lesions and microscopic changes than those infected with *G. anatis* WT, indicating that GtxA plays an important role in the severity of lesions in an biologically relevant *in vivo* model.
2. To test whether *AgtxA* has ability to interfere with the host immune response. We demonstrated that GtxA is involved in stimulating both the innate and parts of the adaptive cellular immune system, especially in the regulation of the acute cytokine-mediated Th2-like response against *G. anatis* infection in the ovary tissue.
3. To identify whether GtxA is involved in rehabilitating the activity of immune and apoptosis response in the spleen and ovary of chickens. We found GtxA has an alteration effect on the apoptotic response of splenic tissue infected by *G. anatis* in the spleen tissue.

b) To determine innate and adaptive activation for Th1 and Th2 responses and determine local cellular changes in macrophage we investigated the following topics

1. To determine the role of GtxA in relation to adhesion and invasion to macrophage cells *in vitro* we found that *G. anatis* wildtype had a higher capacity of adherence and invasion in initial processes of exposure to HD11 cells, indicating that GtxA contributes significantly to the severity of infections in immune cells.
2. We also demonstrated that GtxA seems to inhibit the inflammatory host response through induction of IL-10 expression and lowered expression of TNF-α, while the ΔgtxA mutant promoted a pro-inflammatory response through induced expression of IL-1β, IL-6 and TNF-α. Although there are still several outstanding questions regarding the role of GtxA in the pathogenesis of G. anatis, our results provide further understanding of the role of GtxA in the interaction with the avian immune system.

c) To investigate if GtxA plays a role at inducing apoptosis in macrophage-like HD11 cells.
1. To determine the production of apoptosis genes in macrophage cells infected with WT and G. anatis ΔgtxA mutant in vitro. We found that the levels of anti-apoptotic bcl-2 gene in the ΔgtxA mutant group was mainly down-regulated at 2 h PI rather than after 6 hours, which indicating that in the early stages of G. anatis infection, GtxA may proactively prevent or delay cell damage through promoting anti-apoptotic responses in the host at an early stage.

2. To further examine whether G. anatis ΔgtxA mutant is participate in reducing pathogenicity of G. anatis as in vivo experiments. Flow cytometry analysis showed the ΔgtxA group had less dead cells and more surviving cells than the WT group respectively, which demonstrating that the ΔgtxA mutant can attenuate dead cells caused by G. anatis in HD11 cells. Consistent with previous in vivo experiments in spleen, these results further indicate that ΔgtxA mutant initially seems to promote host cell’s survival by reducing G. anatis-induced cell death. It can be speculated that GtxA of G.anatis be involved in the formation of large pores and cell lysis by allowing bacteria to escape spleen defenses, prolonging bacterial survival and increasing the severity of the disease. Whether it is similar to the mechanism of E. coli in cell perforation, and the pre-survival protection may be related to concentration, and exposure time needs to be further studied.

In conclusion, GtxA contributes significantly to the severity of infections in vivo and vitro. GtxA plays an important role in mediating a Th2-like immune response against G. anatis infection. The toxin also appeared to play a major role in the pathogenesis, especially during the early phases of attachment and invasion of avian host immune cells. These observations play a particularly important role for future research aiming at optimizing a multivalent vaccine against Gallibacterium anatis.
4.2 Future perspectives

Our experimental observations from *in vitro* and *in vivo* investigations provide a good initial framework for assessment of GtxA’s ability to inhibit apoptosis in chicken macrophages infected with *G. anatis*, however a clear limitation of our investigations concerns the potential role of GtxA at assisting *G. anatis* in escaping the immune system by inhibition of apoptosis. An alternative approach based on Immunofluorescence Staining and the TUNEL Assay may allow further insight into these aspects and help to fully characterize the intracellular fate of *G. anatis* in the cells infected with *G. anatis*. We demonstrated that GtxA especially is involved in stimulating in the regulation of the acute cytokine-mediated Th2-like response against *G. anatis* infection in the ovary tissue. To further assess how *G. anatis ΔgtxA* caused an inflammatory response we would evaluate the magnitude of NF-κB pathway activation and the background for the observed reduction in anti-inflammatory mediator expression in the ovary as our next step. Furthermore, we find it highly relevant to determine whether production of GtxA is induced by the initial cell contact between *G. anatis* and the macrophages or occur after bacterial internalization in the cells. Future studies could elucidate whether the production of GtxA is induced once *G. anatis* is internalized by macrophages of avian origin or not, *e.g.* by a Cytochalasin D assay where macrophage cells would be pretreated with Cytochalasin D to inhibit *G. anatis* uptake to investigate which pathway governs the internalization.

In a previous investigation on bacteria-host interactions, our studies indicated that immune suppression was induced by *G. anatis* GtxA in macrophage HD11 cells. To understand better the specific fate of GtxA during host cell interaction we would like to characterize the intracellular trafficking of *G. anatis* in macrophage HD11 incubated with FITC-labeled bacteria and observe the bacterial cells after conjugation with a Goat anti-Chicken IgY (H+L) secondary antibody and immunofluorescence microscopy (Esser *et al.*, 1995; Kim *et al.*, 2003; Reddy *et al.*, 2019). This may provide hints about GtxA’s possible participation in the programmed chicken cell death and further understanding of the toxin is involved in regulating cellular immune system against microbial infections.

Precious research has shown that *M. haemolytica* induced apoptosis in BL-3 cells in a caspase-9-dependent manner through internalization of LktA, which resulted in outer mitochondrial membrane damage (Atapattu and Czuprynski, 2005; Atapattu *et al.*, 2007). We hypothesize that
GtxA might prevent apoptosis and thereby serve as a survival opportunity because it enables the bacteria to replicate inside host cells. That is why the inhibitor efficacy assay could be used to examine the function of GtxA at activating the caspase-9-dependent apoptotic pathway that blocks apoptosis and promotes *G. anatis* replication in *G. anatis*-treated macrophage cells. These investigations may allow us to understand better the role of GtxA in the activation of certain apoptotic pathways and offer novel insights into the mechanisms of *G. anatis*-induced apoptosis in immune cells.
Chapter 5 References
Chapter 5 References


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Chapter 6 Manuscripts
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Manuscript I

GtxA is a virulence factor that promotes a Th2-like response during Gallibacterium anatis infection in laying hens

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Abstract
GtxA, a leukotoxic RTX-toxin, has been proposed a main virulence factor of Gallibacterium anatis. To evaluate the impact of GtxA during infection, we experimentally infected laying hens with a G. anatis wild-type (WT) strain and its isogenic gtxA deletion mutant (ΔgtxA), respectively, and monitored the birds during a 6 day period. Birds inoculated with ΔgtxA had significantly reduced gross lesions and microscopic changes compared to the birds inoculated with the WT strain. To assess the host response further, we quantified the expression of pro-inflammatory cytokines and apoptosis genes by RT-qPCR. In the ovarian tissue, the expression levels of IL-4 and TNF-α were significantly lower in the ΔgtxA group compared to the WT group, while IL-6 and IL-10 levels appeared similar in the two groups. In the spleen tissue of ΔgtxA infected chickens, IL-4 expression was also lower compared to the WT infected chickens. The results indicated that GtxA plays a key role in an acute cytokine-mediated Th2-like response against G. anatis infection in the ovary tissue. The pro-inflammatory response in the ovary tissue of birds inoculated with ΔgtxA mutant was thus significantly lower than the wild-type response. This was, at least partly, supported by the apoptosis gene expression levels, which were significantly higher in the ΔgtxA mutant compared to the wild-type infected chickens. In conclusion, GtxA clearly plays an important role in the pathogenesis of G. anatis infections in laying hens. Further investigations into the specific factors regulating the host response is however needed to provide a more complete understanding of the bacteria-host interaction.

Introduction
Gallibacterium anatis is a member of the Pasteurellaceae family colonizing the upper respiratory tract and lower genital tract of chickens [1]. Experimental infection studies have indicated that G. anatis has a pathogenic potential with a special affinity for the reproductive tract [2–4], and accumulating evidence points at G. anatis having an important role as a cause of salpingitis and peritonitis in laying hens worldwide, significantly compromising animal welfare and decreasing egg-yield [5–7].

Although some knowledge has been generated on specific factors important in the pathogenesis of G. anatis infections, several questions remain [8]. "Repeats in Toxins" (RTX-toxins) are potent cytolytic toxins produced by many Gram-negative bacteria including a wide range of species within the Pasteurellaceae family [9]. A G. anatis specific RTX-toxin (GtxA; Gallibacterium toxin) was identified by Kristensen et al. [10]. Gallibacterium anatis strains lacking parts of gtxA lost their haemolytic and cytolytic activity [11, 12]. Furthermore, GtxA has been shown to be immunogenic, and thus a promising vaccine candidate [13], which was partly confirmed by a study showing that the three recombinant proteins, GtxA-N, GtxA-C, and FlfA, could induce protection against G.
anatis in an in vivo challenge model [14]. Several questions however remain regarding the role of GtxA in the pathogenesis of G. anatis [10].

The innate immune system utilizes pathogen-associated molecular patterns, such as Toll-like receptors (TLRs), which are expressed in chicken reproductive organs [15, 16] and play an essential role in the host defense mainly through immune recognition by the chicken [17]. Subsequently, a series of downstream cytokines, such as interferons (IFN) and interleukins (IL) are induced via cellular signaling pathways. Pathogenic G. anatis adhering to primary chicken oviduct epithelial cells have been shown to induce a strong inflammatory response and secretion of various cytokines, such as IL-6, TNF-α, and IFN-γ, which may lead to cell damage [18]. To what extent pathogenic G. anatis induces inflammatory cytokines in vivo has however not been reported.

Regulation of programmed cell death or apoptosis also plays an important role during bacteria-host interactions [19–21]. Many bacterial pathogens including Escherichia coli and Salmonella excrete pore-forming toxins that induce apoptosis, which may allow the pathogen to engage or circumvent the host’s efforts at limiting infections [22–24]. The importance of pore-forming toxins in G. anatis-induced apoptosis and their role in the pathogenesis has however not yet been clarified.

To investigate the role of GtxA during experimental infection in the natural host, we aimed at comparing lesions in laying hens infected with a G. anatis wild-type (WT) strain and its isogenic gtxA deletion mutant strain, which is unable to express GtxA. To assess the specific impact of GtxA on selected host immune factors, expression levels of genes encoding apoptosis and pro-inflammatory cytokines were assessed.

Materials and methods
Experimental animals and housing facilities
Twenty-four Lohmann Brown layer hens, 29-week old, were purchased from a commercial breeder with high biosecurity standards. At arrival, a swab from the cloacal mucosa was obtained from each chicken and immediately streaked on blood agar (BA) plate (Blood agar base, Difco, Heidelberg, Germany added 5% citrated bovine blood) The plates were incubated in a sealed plastic bag at 37 °C for 18 h. All plates were examined and presumptive G. anatis colonies were confirmed by PCR [25]. One colony of confirmed G. anatis growth from each bird was picked and prepared for storage at −80 °C for later use. The hens were kept under free indoor housing conditions with controlled ventilation, humidity and temperature, and were provided with fresh water and feed ad libitum. The hens were allowed to acclimatize for 1 week prior to the trial. All work with the chickens was carried out with the approval of the Danish Animal Experiments Inspectorate (license number: 2012-15-2934-00339).

Bacterial strains and experimental infection
A GtxA deletion mutant, G. anatis ΔgtxA, constructed as previously reported by Kristensen et al. [10] and its virulent parent wild-type (WT) strain G. anatis (strain no. 12656-12) were used for the inoculations. The WT strain was originally isolated from a bird with septicaemia. The strain has been characterized in detail by phenotypic and genotypic methods and has previously been used for in vivo studies of pathogenicity [2, 26]. Both strains were stored at −80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were subsequently incubated in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with agitation at 37 °C overnight. Prior to inoculation, an overnight culture of each strain was added to fresh BHI (in dilution ratio 1:2) followed by incubation at 37 °C for approximately three hours to reach the late log-phase and a concentration of approximately 10^8 CFU/mL. The bacterial concentration in each inoculum was verified by plate counts of tenfold serial dilutions of inocula on BA plates in duplicate. For the infection study, birds were randomly assigned to one of three groups (Table 1). The G. anatis ΔgtxA infection group was inoculated with a single dose of 4.9 × 10^8 CFU (in 1 mL BHI). The G. anatis WT infection group received a single dose of 5 × 10^8 CFU (in 1 mL BHI). The inocula were injected into the peritoneal cavity, just ventral for the spine and caudal to the last rib, using a 25 G cannula with a 30 mm-length. Chickens in the control group served as uninfected controls and were inoculated with 1 mL of sterile BHI. The unequal distribution of chickens in the gtxA mutant and wild-type groups (16 vs 4, respectively) was based on several previous studies done with the G. anatis WT (strain 12656-12), which made infections with this strain highly predictable and thus allowed fewer birds to be included [2, 14, 27].

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>Dose/CFU</th>
<th>Chickens examined post-inoculation (pi)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 days pi</td>
<td>6 days pi</td>
<td></td>
</tr>
<tr>
<td>G. anatis ΔgtxA</td>
<td>4.9 × 10^8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>G. anatis wild-type</td>
<td>5.0 × 10^8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>−</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1 Design of the study. A total of 24 birds were inoculated with G. anatis ΔgtxA (16 chickens), G. anatis 12656-12 (4 chickens) or BHI (4 chickens).
Post-mortem examination
The chickens were euthanized and subjected to post-mortem examination at either 2 or 6 days post-infection (pi) (Table 1). Recording of gross lesions and tissue sampling for histology was done for selected organs including the spleen, liver, ovary and oviduct. Tissue samples from the spleen and ovary were stored in RNA later (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Bacterial isolates from the spleen, liver, ovary and salpinx were obtained by streaking tissue swabs onto BA. Isolates of the G. anatis WT strain were identified as G. anatis when greyish, semi-transparent colonies surrounded by a haemolytic zone of 1 to 2 mm and had phenotypic characters resembling those previously reported [28]. Gallibacterium anatis ΔgtxA showed identical characteristics but lacked a haemolytic zone [10]. Tissue samples from the ovary and oviduct were fixed in 10% neutral buffered formalin for 24 h, processed by standard histological procedures through graded concentrations of ethanol and xylene, and finally embedded in paraffin wax, sectioned 3–5 µm thick, stained with haematoxylin and eosin, and evaluated by light microscopy (Olympus BX45). Samples from all birds were examined for histological lesions together with the controls and the inflammatory reactions, including cellular infiltration, edema, exudation and necrosis, were evaluated according to extent and distribution. The lesions were differentiated using semi-quantitative grading system where scored 0 (no lesions), + (few scattered lesions), ++ (moderate number lesions), and +++ (severe diffuse lesions).

Pulsed-field gel electrophoresis (PFGE)
To verify that the re-isolated G. anatis from lesions were identical to the inoculum and different from the resident cloacal strains, pairs of G. anatis obtained from the cloaca and from lesions from eleven birds were characterized by pulsed-field gel electrophoresis (PFGE). The isolates had been kept at -80 °C in BHI broth with 10% glycerol and were plated on BA. Inoculated plates were incubated at 37 °C in sealed plastic bags for 24 h. From pure cultures, a single colony typical of G. anatis was picked and incubated overnight in 10 mL BHI broth at 37 °C with shaking. The preparation of bacterial DNA and separation of fragments was done as described [30] and using SalI (R0114, New England BioLabs) and XbaI (R0145, New England BioLabs) as restriction enzymes.

Serum sampling and enzyme-linked immunosorbent assay (ELISA)
GtxA specific antibody levels were quantified in serum samples by ELISA. Blood, 1–2 mL, was taken from vena brachialis from all birds at the day of inoculation (Day 0) and at 2 and 6 days after inoculation, respectively, and left at 4 °C overnight. Serum was collected after centrifugation at 1800 g for 10 min and stored at -20 °C. Two microtiter plate wells (Nunc-Immuno™ MicroWell™ 96-Well Plates, Thermo Scientific) were coated overnight at 4 °C with 0.5 µg GtxA recombinant protein diluted in carbonate-bicarbonate buffer (pH 9.6) (Sigma-Aldrich). Each well was then washed; this and all subsequent washing steps consisted of three washes in 350 µL wash buffer (PBS + 0.05% Tween 20) [13]. The wells were blocked for 2 h at room temperature in 200 µL blocking solution (PBS containing 0.05% Tween 20 and 2% bovine serum albumin (BSA)) and washed. The antibody titers were assayed by serial threefold dilutions of chicken serum ranging from 1:200 to 1:48 600. All dilutions were prepared in triplicates in a dilution buffer (PBS containing 0.05% Tween 20 and 0.1% BSA), 100 µL was added to each well and plates were incubated for 1 h at 37 °C. For each assay, 12 control wells were included, which contained pure dilution buffer; secondary antibody was added to 6 of these wells as a measure of background, and the other 6 wells remained blank as a negative control for the ELISA. Following incubation, the wells were washed and 100 µL polyclonal goat anti-chicken IgG (Fc): HRP (AbD Sero tec) diluted 1:4000 in diluting buffer were added to each well and the plates incubated for a 1 h at 37 °C and then washed. To detect binding, 100 µL of 3,3’,5,5’-Tetramethylbenzidine liquid substrate (Sigma) were added to each well. The plates were incubated for 2 min and then the reaction was stopped by addition of 100 µL 1 M HCl. The absorbance was read immediately at 450 in a PowerWave XS spectrophotometry (BioTek Instruments).

Differential gene expression analysis by Real-Time Quantitative PCR (RT-qPCR)
To examine the mRNA expression profiles in the spleen and ovary tissue samples obtained during necropsy and placed in RNA later and stored according to the manufacturer’s instructions (Merck Life Science A/S, Søborg, Denmark). For RNA extraction a total of 20 mg tissue from each organ was placed in 1.5 mL tube containing beads. Each sample was then homogenized in 1 mL lysate buffer RLT using a Vortex adapter (Mo Bio, Carlsbad, CA, USA) and centrifuged at 1800 g. After homogenization and centrifugation for 5 min, the upper phase of total RNA was collected and purified with an RNeasy mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. The concentration and purity of RNA were determined by spectrophotometer (Nanodrop 1000, Thermo Scientific). 5 µg of RNA extracted from each tissues sample was reverse-transcribed into cDNA using M-MLV reverse transcriptase kit (Invitrogen) primed with Invitrogen’s protocol. The amount of cDNA
corresponding to 20 ng of reverse-transcribed RNA was amplified by RT-qPCR, using specific primers (Tables 2 and 3). RT-qPCR was performed in 25 μL volumes in 96-well microplates using FastStart Essential DNA Green Master Mix (Roche, Germany). The 3-step amplification and signal detection were performed using a LightCycler R® 96 (Roche) with an initial pre-incubation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 56 °C for the 30 s and 72 °C for 30 s. The β-actin gene was used as a housekeeping gene, to correct for differences in template RNA levels between samples during the experiment. Each RT-qPCR experiment thus included triplicates of 16 test samples, one no-template-control, and a log10 dilution series. The mean Ct value was used for subsequent calculations. Expression of the IL-4, IL-6, IL-10, TNF-α, casp-3, casp-8, casp-9, bax and bcl-2 genes was quantified according to Hangalapura et al. [29] (Table 2). First, the Difference Factor for each sample was calculated by dividing the mean Ct value for the β-actin gene of each individual sample by the mean Ct value for the β-actin gene of all samples. Secondly, the adjusted cytokine mRNA amount per sample was calculated using the following formulae:

\[
\text{Difference factor for each Ct sample:} \\
\frac{\text{mean Ct value for } \beta-\text{actin gene of individual sample}}{\text{mean Ct value for } \beta-\text{actin gene of all samples}}
\]

\[
\text{Adjusted cytokine quantity for each Ct sample:} \\
\frac{\text{mean cytokine Ct sample}}{\beta-\text{actin slope}} \times \beta-\text{actin slope}
\]

Table 2 List of the primers used in RT-qPCR analysis of mRNA expression of selected host genes

<table>
<thead>
<tr>
<th>Name</th>
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Table 3 Gross pathology and re-isolation rates of G. anatis from different organs following experimental infection with a wild-type strain (G. anatis 12656-12) (4 chickens) or its isogenic gtxA deletion mutant (ΔgtxA) (16 chickens)

<table>
<thead>
<tr>
<th>G. anatis</th>
<th>Peritoneum</th>
<th>Re-isolation of G. anatis</th>
<th>Ovary</th>
<th>Re-isolation of G. anatis</th>
<th>Oviduct</th>
<th>Re-isolation of G. anatis</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔgtxA</td>
<td>2/16 (13%)</td>
<td>5/16 (31%)</td>
<td>0/16 (0%)</td>
<td>7/16 (44%)</td>
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<tr>
<td>Wild-type</td>
<td>3/4 (75%)</td>
<td>3/4 (75%)</td>
<td>3/4 (75%)</td>
<td>3/4 (75%)</td>
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</tbody>
</table>
Statistics
Fisher’s Exact test was used for comparison of differences in the extent of the gross lesions and bacterial re-isolation rates. ANOVA was used to compare antibody-titers. The Kruskal–Wallis test was used for comparisons of histological lesion scores. Differences in gene expression levels were compared by Students’ t-test using GraphPad Prism 7® for Windows (GraphPad, San Diego, USA). P-values ≤ 0.05 were considered statistically significant.

Results
Pathology induced by G. anatis WT strain and its isogenetic ΔgtxA mutant
No clinical signs of infection were seen after the bacterial inoculations. At the necropsy, ten out of the 16 birds having received G. anatis ΔgtxA demonstrated mild macroscopic lesions whereas the remaining chickens had no lesions. Of the chickens infected with G. anatis WT, three had severe lesions and one had no lesions. No lesions were found in the birds in the BHI inoculated control group. The number of organs with lesions in birds from the ΔgtxA group, was significantly lower compared to the number of organs with lesions in WT group (P = 0.003). No difference was found between number of lesions when comparing birds examined at 2 or 6 days pi from the ΔgtxA group (P = 0.2). The histological lesions in both the ΔgtxA and WT infection groups supported the observations noted from the gross lesions (Figure 1). No histological lesions were found in birds without gross lesions. In the ΔgtxA group, few focal infiltrations of inflammatory cells, predominantly lymphocytes, were found in the oviduct and in one bird a necrotic focus was found. The ovarian stromal tissue was edematous with blood in the vascular system. Additionally, only light infiltration of inflammatory cells and degeneration of follicles were seen in birds with purulent oophoritis (Figure 1C). In the group infected with the WT strain, multifocal

Figure 1 Gross lesions, histopathology and apoptotic cells found in chickens infected with G. anatis ΔgtxA mutant or G. anatis wild-type (WT) strain. Ovaries from 29-week old laying hens. Two days post-inoculation with either G. anatis ΔgtxA or G. anatis WT. A G. anatis ΔgtxA infection group. Oophoritis with vascular engorgement and slight edema. B G. anatis wild-type. Diffuse purulent oophoritis and folliculitis with ruptures follicles. C Hematoxylin and eosin (HE)-staining of the follicle in ovarian tissue from the G. anatis ΔgtxA infection group. Focal oophoritis with the presence of inflammatory cells in the stroma and vascular engorgement. Infiltrates of few heterophilic granulocytes and mononuclear cells. D HE-staining of the ovary of the G. anatis WT infection group. Purulent oophoritis with heavy infiltration of inflammatory cells and granuloma formation (black arrows) in the follicle.
infiltration with inflammatory cells was present in the oviduct and several foci with bacterial colonization were also evident. In the ovary, infiltration with inflammatory cells including lymphocytes and heterophils was identified. In some areas, granuloma formation surrounded by epithelioid cells and necrosis was found (Figure 1D). A significant difference in histological lesion scores of both ovarian ($P < 0.001$) and oviduct tissues ($P < 0.001$) was found when comparing the two groups (Figure 2).

Re-isolation of *G. anatis*
Resident cloacal *G. anatis* was isolated from 17 of the 24 birds prior to the experimental infections. At the necropsy, *G. anatis* was re-isolated in pure culture from all gross lesions in the infected groups. Additionally, *G. anatis* was isolated from the oviduct mucosa of hens of the Δ*gtxA* infected group even in cases where no gross lesions were evident (Table 3). No bacteria were isolated from the internal organs of birds in the control group. To compare the isolates obtained from lesions and from the cloacal mucosa, pairs of isolates were characterized from eleven individual birds. All eleven isolates associated with lesions had identical band patterns corresponding to the inoculated strains, whereas all of the cloacal isolates clearly had different PFGE profiles (Additional file 1).

The titer of GtxA specific antibodies was quantified at the time of inoculation and found at background levels in both groups (Figure 3).

Gene differential expression analysis by real-time quantitative PCR
To assess the ability of GtxA in mounting a host response during the *G. anatis* infection, the expression levels of cytokines IL-4, IL-6 IL-10 and TNF-α were quantified in the ovary and spleen tissues. No differences in the expression of the pro-inflammatory cytokines IL-6 and IL-10 were found in the ovary at 2 days pi between WT strain and Δ*gtxA* (Figure 4A), whereas significantly lower expression of IL-4 and TNF-α, respectively, was found in the Δ*gtxA* group compared to the WT group. At 6 days pi, expression of IL-4 remained significantly lower in the Δ*gtxA* group compared to the WT strain group (Figure 4B), while the ovarian expression of IL-6, TNF-α and IL-10 did not differ between the two infection groups (Figure 4B).

In the spleen, the TNF-α expression was significantly higher in the Δ*gtxA* infected hens compared to the WT group at 2 days pi, while for IL-4, IL-6 and IL-10 no statistically significant difference was found between the two groups (Figure 5A). At 6 days pi, the expression of
IL-4 and IL-10 in the spleen tissue was significantly lower in the ΔgtxA group compared to the WT group, while TNF-α expression was significantly higher in the ΔgtxA group (Figure 5B). In all tissue samples from WT group and the ΔgtxA group the level of IFN-γ was not detectable (data not shown).

**Apoptosis-related gene expression in the ovary**

The expression of selected apoptosis genes was evaluated in the ovarian tissue where the expression of **bax** was significantly increased in the ΔgtxA infection group compared to the WT group at 2 days pi (Figure 6). At 6 days pi, the expression the apoptosis genes was not different between the ΔgtxA and WT group, respectively (Figure 6).

**Apoptosis-related gene expression in the spleen**

The expression of apoptosis genes in the spleen tissue was assessed at 2 days and 6 days pi (Figure 7). The expression of **bax, bcl-2, casp-3, casp-8 and casp-9** were all numerically lower at 2 days pi in the gtxA mutant group compared with WT group, yet this was only significant for **casp-3 and casp-9** (Figure 7). At 6 days pi, no statistically significant difference in the expression was observed between the ΔgtxA infected group and the WT group (Figure 7).

**Discussion**

The results of the present study showed that the virulence of *G. anatis* was severely decreased in vivo when expression of the gtxA gene was impaired. Loss of virulence was indicated by a significantly lower lesion score in birds infected with the ΔgtxA strain compared to those infected with the GtxA-producing strain (Figures 1 and 2). The lesions induced by the *G. anatis* WT strain corresponded closely with observations made from previous in vivo studies [2, 14, 27], and thus justified the limited number of chickens used in the WT group. One WT infected hen had no apparent lesions and the inoculum had seemingly been deposited and trapped in a lump of abdominal fat. The observed differences in severity of lesions are in accordance with in vivo studies investigating strains of *Actinobacillus pleuropneumoniae* lacking the ability to produce different RTX-toxins either due to inactivation or deletion of the toxin genes, which all showed a sharp decline in virulence including lower mortality, morbidity and diminished lesions in pigs [31–33]. Experimental infection with a lktA (a leukotoxin gene)
deleted mutant of Mannheimia haemolytica also led to a decrease in clinical signs and lung lesions in calves when compared to the isogenic WT strain [34]. Hence our findings support previous observations of RTX-toxins produced by other members of Pasteurellaceae and stress the profound role of GtxA in the pathogenesis of G. anatis.

Most RTX-toxins seem to enforce a strong leukotoxic effect and thereby debilitating the host immune response, which seems particularly aimed at specific host cells and thereby a likely cause of the host-specific pathogenicity commonly observed among Pasteurellaceae bacteria [9, 35]. In the present study, the inflammatory response promoted by the ΔgtxA mutant was significantly reduced whereas the WT induced severe purulent oophoritis with a high number of heterophilic granulocytes present and granuloma formation, indicating that GtxA plays an important role at activating heterophils during the immediate inflammatory response against G. anatis. Previous reports have indicated a similar role of the heterophils during other bacterial infections [36–38].

The highly decreased cellular infiltration and inflammatory reaction found in the tissues of the birds infected with the ΔgtxA mutant (Figures 1A and C) indicated less tissue destruction in the absence of GtxA, subsequently leading to a weaker host response. Virulence factors, including RTX-toxins, of other species of Pasteurellaceae have been shown to boost a strong pro-inflammatory response in vitro [36] and in vivo [2] and the lack of GtxA expression might similarly,

![Figure 6](image_url)
lead to a diminished inflammatory response. This is, at least partly, supported by the re-isolation of the ΔgtxA mutant from the oviduct without a concurrent inflammatory response. However, it also suggests that G. anatis inability to produce GtxA withstands the capacity to effectively colonize the chicken oviduct and thereby points at other bacterial factors e.g. the F17-like fimbria that may take part in this process [12, 40, 41].

Due to the widespread occurrence of G. anatis among laying hens, it was not surprising that G. anatis could be isolated in the cloacae of most of the chickens entering the experiment. However, the strains isolated from the lesions associated with the experimental infections all showed to be identical to the strains used for the inoculation and different from the G. anatis isolates from the cloaca (Additional file 1). The factors permitting a commensal lifestyle of G. anatis are not well understood, but the present results indicate that the bird’s immune system generally have not been stimulated to initiate an adaptive response in healthy carriers as the GtxA specific titers were at or below the background level, as previously reported [14, 27]. Overall, the G. anatis ΔgtxA mutant was strongly attenuated upon experimental infection in its natural host suggesting that GtxA contributes significantly to the severity of infections in chickens.

Previous studies have clearly indicated GtxA to be a virulence factor while having the ability to induce protective immunity against G. anatis [27]. However, the specific host immune responses, here represented by cytokine and apoptosis gene expression profiles, in poultry have not previously been reported. In the current study we investigated pro-inflammatory cytokines involved in systemic inflammation and stimulation of the acute phase reaction [29, 36, 41]. We observed a high level of IL-4 and TNF-α mRNA expression in the ovary at 2 days and 6 days pi in the WT group, but a relatively low level in the
spleen tissue at 2 days pi (Figures 4 and 5). The results are consistent with those reported by Zhang et al. [18], who revealed that *G. anatis* could induce marked inflammatory responses in primary chicken oviduct epithelial cells. The results indicate that GtxA may be involved in the stimulation of an IL-4 and TNF-α and promotes Th2-like response particularly in the ovary tissue, which previously has been suggested a main target for *G. anatis* [39].

As a pro-inflammatory cytokine, IL-6 is involved in the recruitment of immune cells, including lymphocytes and circulating monocytes, to the site of infection [17]. In our study, there were no significant differences in the IL-6 mRNA expression when comparing the WT and the ΔgtxA mutant in the ovary or the spleen tissues, respectively (Figures 4 and 5). It may be that in chickens, GtxA modulates the adaptive rather than innate immune response.

The regulation of the Th1-Th2 cytokine ratio plays an important role in balancing the host immune response [42, 43]. Our results indicated that GtxA expression primarily initiated a Th2-like response, as indicated by the increased IL-4 expression at 2 days and 6 days in the ovary tissue and similar but slightly delayed response (only at day 6) in the spleen tissue. On the contrary, in the ΔgtxA infected group, TNF-α was highly expressed, particularly in the spleen tissue at 2 days and 6 days whereas the level of IL-4 was low. These results indicate that the ΔgtxA mutant is able to inhibit or simply unable to induce secretion of IL-4. IL-4 is produced by Th2 cells that take part in the regulation of humoral immunity in poultry [44]. The cytokine expression levels are in good accordance with our pathological findings, showing a highly decreased cellular infiltration and inflammatory reaction in the tissues of birds infected with ΔgtxA (Figures 1A and 1C). The results also indicated that the ΔgtxA mutant induced less tissue and cellular destruction, subsequently leading to a weaker host response possibly through partial impairment of the Th2-like pathway. IL-10 is an anti-inflammatory cytokine [45, 46], negatively affecting the expression of Th1 cytokines [41]. We found that tissues from the WT infected group also had increased expression of IL-4 and partly IL-10 but not IFN-γ (which was below the detection level in all samples examined), supporting the Th2 bias suggested.

We hypothesized that GtxA was involved in regulating the apoptotic response in the host. Two major forms of apoptosis are commonly recognized, one involving activation of *casp*-8 (external induction) through the “death receptor” in the plasma membrane and the other involving disruption of the host cell homeostasis (intrinsic induction) by *casp*-9 regulation [47]. Both initiator caspases can recruit the executioner *casp*-3, which degrades cellular targets during apoptosis [47]. RTX toxins, such as *Staphylococcus aureus* toxin (alpha) and *Actinobacillus* leukotoxin, at low concentrations, have been suggested to cause the formation of small pores in the host cell plasma membrane allowing influx of Ca^{2+}, which activates the intrinsic apoptotic pathways [48–50]. In our study, *casp*-3, -8, -9, *bax* and *bcl*-2 expression was significantly lower in the spleen tissue at 2 days pi in the chickens challenged with the ΔgtxA mutant compared to the WT (Figure 7), while the level of TNF-α was elevated (Figure 5A). This may reflect a dose–response relation where some but only a limited number of WT bacterial cells reached the spleen as opposed to the ΔgtxA mutant where very few if any bacterial cells made it to the spleen. On the contrary, in the ovary tissue, ΔgtxA induced higher levels of some of the pro-apoptosis genes (*casp*-8, -9 and *bax*) at 2 days, whereas apoptosis suppression, induced by increased *bcl*-2 expression, seemed apparent at 6 days (Figure 6B). The results are consistent with the fact that we observed only mild or no macro- and microscopic lesions in the ovary of the ΔgtxA infected chickens despite presence of bacteria in 10 out of 16 birds (Table 3). Based on these observations the ΔgtxA mutant seem able of inducing an apoptotic host response, which may allow prolonged survival in the host. At a high exposure of ovary tissue to the WT and GtxA a clear inflammatory response was observed. GtxA presumably forms large pores and rapid cytolysis of phagocytic cells [10], allowing the bacteria to escape the host defenses, which may prolong bacterial survival and increase the severity of the disease. The possible RTX toxin-induced pro-inflammatory death pathway, is a mechanism employed by *Salmonella enterica*, serovar Typhimurium and some *Shigella* species by initiating *casp*-1 expression through the lymphocyte function-associated antigen-1 (LFA-1) pathway [50–52]. Further research is however needed on how TNF-α is induced by non-GtxA expressing bacterial cell and how that may be involved in the regulation of apoptosis in cells exposed to *G. anatis*. Host cells may exploit the apoptosis process as a primitive defense mechanism against bacterial infections. On the other hand, several bacteria seem to have evolved mechanisms to prevent or delay apoptosis of host cells to permit successful bacterial replication [45]. GtxA may thus be involved in apoptosis suppression to facilitate *G. anatis* multiplication in the ovary at an early stage, which is considered a preferred site of this bacterium [39].

This study is the first investigation to assign a specific role of GtxA in the outcome of an infection of the natural host. It was documented that GtxA is important for the severity of lesions in an biologically relevant in vivo experiment and that GtxA contributes to stimulating both the innate and parts of the adaptive cellular immune system through primarily a Th2 response. Finally, our results indicated GtxA to be involved in the induction of apoptosis-related genes in spleen tissue.
Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13567-020-00764-2.

Additional file 1. Pulsed-Field Gel Electrophoresis typing of resident G. anatis isolates and strains used in the inocolum. The T3E65-12 wild-type (WT) strain and gtxA mutant (M) strains. Cloacal isolates (C) and isolates obtained post-inoculation from organs with lesions (L). WT, M and L strains have identical genotypes whereas the cloacal isolates belong to a genetically different group.

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Katrine Madsen is acknowledged for her technical assistance.

Authors’ contributions
BT, SEP, RJS, BMK and AMB designed the experiments, conducted the analyses, and drafted the manuscript; BT, SEP, RJS, RHO, BMK, AMB conducted the study and supervised all analyses; BT and AMB critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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References

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Manuscript II

Immune Suppression Induced by *Gallibacterium anatis* GtxA During Interaction with Chicken Macrophage-Like HD11 Cells

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Article

Immune Suppression Induced by Gallibacterium anatis GtxA During Interaction with Chicken Macrophage-Like HD11 Cells

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Abstract: The RTX toxin GtxA expressed by Gallibacterium anatis biovar haemolytica has been proposed a major virulence factor during disease manifestations in the natural host, the chicken. To better understand the role of GtxA in the pathogenesis of G. anatis, we compared the GtxA expressing wildtype strain with its isogenic ∆gtxA mutant that was unable to express GtxA during exposure to chicken macrophage-like HD11 cells. From adhesion and invasion assays, we showed that GtxA appears to promote adhesion and invasion of HD11 cells. By using quantitative RT-PCR, we also demonstrated that the G. anatis expressing GtxA induced a mainly anti-inflammatory (IL-10) host cell response as opposed to the pro-inflammatory (IL-1β, IL-6 and TNF-α) response induced by the GtxA deletion mutant. Interestingly, the results, at least partly, resemble recent responses observed from spleen tissue of chickens infected with the same two bacterial strains. The effect of the GtxA toxin on the type of cell death was less clear. While GtxA clearly induced cell death, our efforts to characterize whether this was due to primarily necrosis or apoptosis through expression analysis of a broad range of apoptosis genes did not reveal clear answers.

Keywords: macrophage; invasion; Gallibacterium anatis; intracellular survival; ∆gtxA mutant

Key Contribution: We clearly demonstrate that GtxA has a primary role as a bacterial virulence factor responsible for cellular changes in chicken macrophages.

1. Introduction

Gallibacterium anatis (G. anatis) is a genus in the Pasteurellaceae family [1,2]. The haemolytic biovar of G. anatis [3,4] has been recognized as a common part of the microbiota of the upper respiratory tract and the lower genital tract in healthy chickens [5–7]. The bacterium has, however, also been proposed as a major cause of infectious salpingitis and peritonitis in egg-laying chickens [8–10], where it leads to decreased egg production, in creased mortality and lowered animal welfare. Recently, significant progress has been made in the knowledge of G. anatis epidemiology and pathology in its natural host, the chicken [7,11]. However, factors associated with the interaction with host immune cells like avian macrophages remain poorly understood [12].

A crucial step in bacteria–host interactions is attachment to the host surfaces allowing colonization [11,12]. For G. anatis, colonization of mucosal surfaces may go undetected by the host immune system [13]. Yet, the interactions may activate a complex cascade of molecular crosstalk at the host–pathogen interface and lead to a diverse set of downstream processes, including modulation of the innate host defenses [11,12,14,15]. Here, avian macrophages play a significant role in the initial defense against microbial infections [16–18].
Gallibacterium anatis produces several virulence factors, including cytotoxins like the RTX-like toxin GtxA [19,20], fimbriae [21], outer membrane vesicles [22], capsule [23] and other virulence determinants [12]. RTX toxins, like GtxA, have the ability to induce pores in the plasma membrane of host cells, which eventually can lead to necrosis or apoptosis of the target cells [24,25]. The related RTX toxin HlyA, which is secreted by pathogenic variants of E. coli, can promote host cell death via lysis, necrosis or apoptosis, depending on the target host cell, toxin concentration and duration of exposure [25,26].

GtxA shares several structural features with HlyA [24,25], and has been shown to induce a strong leukotoxic effect in vitro when exposed to avian macrophage-like HD-11 cells [27]. Induction of apoptosis and necrosis by G. anatis has also been demonstrated in primary chicken oviduct epithelial cells by Zhang and co-workers [28]. From in vivo trials in chickens, we recently reported that a GtxA expressing G. anatis strain (12656-12) induced increased levels of pro-inflammatory cytokines in ovarian tissue and a primarily anti-inflammatory response in spleen tissue, when compared to its GtxA devoid mutant. Here, an increased fraction of apoptotic cells was present in chickens infected with the GtxA expressing G. anatis wildtype compared to its isogenic GtxA mutant [20]. Based on that, GtxA appeared to be an important factor in the pathogenesis of G. anatis during infection of its natural host. To understand better the role of GtxA at the subcellular level, the aim of the present investigation was to study specific steps in the pathogenesis when chicken macrophage-like HD11 cells were exposed to a GtxA expressing G. anatis strain and its isogenic gtxA deletion mutant (ΔgtxA), respectively. Specifically, we aimed at investigating the molecular events taking place during the early phases of attachment and invasion of avian host immune cells.

2. Results

2.1. Establishment of an Appropriate Multiplicity of Infection (MOI)

To establish a useful MOI, a preliminary adherence assay with G. anatis 12656-12 was done using the following MOIs: 10:1 (0.7 × 10⁷ CFU/mL), 50:1 (3.5 × 10⁷ CFU/mL), and 100:1 (0.7 × 10⁸ CFU/mL) bacteria per cell, respectively. During the initial 60 min, there was a positive correlation between adherence and time (Figure 1). At all MOIs tested, an approximately 3–4 fold higher for MOI 10:1 compared to MOI 50:1 and 100:1 (p ≤ 0.01) in three independent assays, respectively. The adhesion-level was overall highest after incubation for 60 min for MOI 10:1 (p ≤ 0.05). In the subsequent assays, a MOI of 10:1 was used for all bacterial strains.

![Figure 1](image-url)  
**Figure 1.** Kinetics of adherence of G. anatis 12656-12 strain to HD11 cells after 30, 60 and 90 min of incubation at three different multiplicities of infection (MOIs). Significant differences were labeled (p ≤ 0.05) * and (p ≤ 0.001) ***.
2.2. Bacterial Invasion and Intracellular Survival

The ability of the ΔgtxA mutant to enter HD11 cells was analyzed using a gentamicin protection assay to determine the invasion and intracellular capacity. The results showed significantly lower invasion ratio (0.09 to 0.13) of ΔgtxA mutant compared to the parent strain G. anatis 12656-12 (p ≤ 0.05) (Figure 2). The invasion ratios of the ΔgtxA mutant were nearly two-fold lower than that of WT bacteria on pre-fixed HD11 cells (p ≤ 0.01), indicating partial impairment of invasion of the ΔgtxA mutant at least during the first six hours of exposure.

Figure 2. Gentamicin protection assay to determine intracellular survival ability of G. anatis 12656-12 and G. anatis ΔgtxA in the chicken HD11 cells over time. The ability of the G. anatis ΔgtxA mutant to enter and survive in the HD11 cells was compared to its G. anatis 12656-12 parent strain. Bacterial counts (CFU/cell) were obtained and compared from lysed HD11 cells at 2, 4, 6, 8, 10, 12 and 24 h following addition of gentamicin ** (p ≤ 0.01) *** (p ≤ 0.001).

To determine whether the expression of gtxA was required for invasion and intracellular survival, we compared the number of CFU of G. anatis WT and its isogenic ΔgtxA mutant strain recovered at 2, 4, 6, and 24 h PI, respectively (Figure 2). At four and six hrs PI, the number of WT bacteria invading the HD11 monolayer was significantly increased compared to the ΔgtxA mutant strain (p < 0.001). At 2 h PI, there was no difference found between the two strains. The number of intracellular G. anatis wild-type bacteria seemed to peak at 4 h and 6 h PI and then sharply decline. No G. anatis WT cells were detected intracellularly at 24 h PI.

2.3. Bacterial Growth

Bacterial growth according to time was compared between G. anatis WT and ΔgtxA. Both G. anatis WT and the ΔgtxA mutant reached exponential and stationary phases at 2 h and 6 h after initiation, respectively (Figure 3). There was no significant difference between the growth curves of the G. anatis WT and ΔgtxA mutant strains, suggesting that deletion of GtxA does not influence significantly the growth of G. anatis (Figure 3).
2.4. Viability of HD11 Cells Following Bacterial Exposure

The fraction of viable HD11 cells was measured following exposure to *G. anatis* WT and the ∆gtxA mutant, respectively. There was a significant reduction in the percentage of viable cells exposed to the WT and mutant, respectively (Figure 4). At 2 h PI, 97.9% and 78.1% viability were detected following infection with *G. anatis* WT and the ∆gtxA mutant, respectively. This was reduced to about 91.3% and 66.7% after 4 hrs incubation with *G. anatis* WT and the ∆gtxA mutant, respectively.

**Figure 3.** Growth curves of the *G. anatis* wild-type (WT) strain and the ∆gtxA mutant strain. Growth was assessed by measurement of the OD$_{600}$ at 2, 4, 6, 8, 10 and 12 h. The graph is representative of three independent experiments.

2.5. Real-Time Relative Quantification of Cytokine Expressions

The qRT-PCR analysis was performed to determine which cytokine genes were expressed in HD11 cells following exposure to the *G. anatis* WT and the ∆gtxA mutant strains. The cytokines were chosen to examine the difference between the two types of strains in their ability to elicit an immune response. The results of IL-1β, IL-6, IL-10, and TNF-α mRNA expression in HD11 cells infected with two different strains are displayed in Figure 5. The expression of IL-1β and IL-6 mRNA was significantly increased in HD11 cells infected with *G. anatis* ∆gtxA mutant, compared to the *G. anatis* WT at 2 PI. Moreover, the IL-10 mRNA expression was significantly down-regulated in HD11 cells treated with the *G. anatis* ∆gtxA mutant, compared with the WT at 2 PI. At 2 h PI no significant difference was observed in the TNF-α mRNA expression between the two bacterial strains. However, the TNF-α expression increased significantly in HD11 cells exposed to the ∆gtxA mutant, compared to the WT at

**Figure 4.** Effect of *G. anatis* infection on macrophage viability. HD11 cell viability assay following infection with the wild-type strain (*G. anatis* 12656-12) and *G. anatis* ∆gtxA mutant. Data shown represent means ± SEM from three independent experiments *** (p ≤ 0.001).
6 h PI. These results indicate that GtxA expression suppressed the induction of IL-1β, IL-6 and TNF-α, and induced expression of IL-10 in HD11 cells during the initial phases of exposure.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Real-time relative quantification of cytokine expressions. Cytokine expressions were measured on RNA isolated from HD11 cells in vitro. The relative gene expression levels were calculated as a ratio of stimulated WT and ΔgtxA to un-stimulated (non-infected control), respectively. Data were log2 transformed. Values shown are averages and SEM of three independent experiments. Asterisks indicate significance from the uninfected control cells (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

2.6. Real Time Relative Quantification of Apoptosis Gene Expression

To understand the reasons for the higher survival capacity of the ΔgtxA mutant in HD11 cells, and to study the role of GtxA in the pathogenesis of avian pathogenic G. anatis in vitro, we used qRT-PCR to investigate the expression of selected apoptosis genes including caspase-3, -8, -9 and the pro-apoptosis bax or anti-apoptosis bcl-2 genes in chicken HD11 cells (Figure 6). Expression of caspase-3 and bax was down-regulated at 2 and particularly at 6 h PI in both the ΔgtxA and the WT group compared with control group, respectively (Figure 6). At six hours PI, the expression of caspase-8 appeared down-regulated in both the ΔgtxA and the WT group, compared to the control. Caspase-9 was initially up-regulated in the WT group only but down-regulated in the same group at 6 h PI. Bcl-2 was down-regulated in the ΔgtxA mutant at 2 h, whereas both the mutant and the WT exposed cells expressed a five-fold lower level of bcl-2 at 6 h compared to the control.
Figure 6. Real-time relative quantification of apoptosis gene expression. Apoptosis gene expression was measured on RNA isolated from HD11 cells in vitro. The relative gene expression levels were calculated as a ratio of stimulated WT and ΔgtxA to un-stimulated (non-infected control), respectively. Data were log2 transformed. Values shown are averages and SEM of three independent experiments. Asterisks indicate significance from the uninfected control cells (* \( p \leq 0.05 \), ** \( p \leq 0.01 \), and *** \( p \leq 0.001 \)).

2.7. Flow Cytometric Analysis of HD11 Cell Death

The proportion of live and dead cells exposed to WT and ΔgtxA strains was investigated by flow cytometry using annexin V/PI double staining at 2 h and 6 h PI, respectively. As shown in
Figure 7A, at 2 h PI, the fraction of live HD11 cells was lower in the WT exposed cells (70.4%) than in the ∆gtxA exposed cells (79.8%) and the control group (80.6%), respectively. These results clearly indicate that GtxA has the ability to induce G. anatis-induced cell death in HD11 cells. At 6 h PI the fraction of dead cells exposed to G. anatis WT had increased further to 36.2%, compared with 16.8% in the non-infected control group. However, in the ∆gtxA exposed group the fraction of death cells was 22.1% (Figure 7B), which further supported that the deletion of gtxA lowered the G. anatis-induced cell death.

**Figure 7.** Effect of GtxA on apoptosis and necrosis in G. anatis-infected HD11 cells. The HD11 cells were stained with annexin V FITC/Propidium iodide (Pi) and analyzed by flow cytometry. (A) Flow cytometric analysis of non-exposed HD11 cells compared to cells exposed to the WT or the ∆gtxA mutant strain at 2 h PI (B) Flow cytometric analysis of HD11 cells infected with WT and ∆gtxA mutant strain at 6 h PI. Cells were stained with FITC-Annexin V and Pi. The lower inserted box: the living cells and early apoptotic cells. The upper inserted box: late apoptotic cells and necrotic cells.

### 3. Discussion

Our aim was to characterize the role of GtxA during the initial processes of the pathogenesis. In our assays aiming at adherence and invasion, the highest adherence capacity of the G. anatis wildtype was reached at 60 min PI, which could suggest that the corresponding receptors on the HD11 cell surfaces got saturated thereafter. This result was consistent with previously published results from use of primary chicken oviduct epithelial cells (PCOECs), although the peak of adherence in that investigation was reached slightly earlier [28]. Several adhesins, such as F17-like fimbria, capsular polysaccharides and secreted surface proteins, have been suggested as being involved in the adherence process [22,29–34]. The number of adhered ∆gtxA mutant bacteria was nearly two-fold lower than that of G. anatis WT on pre-fixed HD11 cells (Figure 2). This indicates that GtxA is involved in the attachment of HD11 cells, and at least partly explains the initial impairment of virulence observed for the ∆gtxA mutant.
As described previously, the $\Delta gtxA$ mutant was severely attenuated in experimentally infected chickens [27]. In the current investigation, we found lower HD11 intracellular counts of the $\Delta gtxA$ mutant strain at 4, 6 h PI compared to the $G.\ anatis$ WT. Subsequently, a higher relative number of intracellular $\Delta gtxA$ mutant bacteria were counted as practically no WT bacterial cells were recovered after 6 h PI (Figure 2). These results indicate that the WT adhered and invaded at a high level during the initial phase of exposure. Later, the infected HD11 cells may have suffered from the toxic effect of the GtxA toxin, which seemed to leave a large fraction of the exposed HD11 cells apoptotic and/or necrotic (Figure 6). Disintegration of the HD11 cell may have exposed the intracellularly-based $G.\ anatis$ to the gentamicin in the surrounding medium and explain the low number of viable bacteria and HD11 cells later than 6 h PI.

In a recent investigation, a highly virulent $G.\ anatis$ strain was shown to induce production of pro-inflammatory cytokines during exposure to primary chicken oviduct epithelial cells [28]. In the current study, we compared the cytokine mRNA expression levels in HD11 cells infected with the $G.\ anatis$ WT and $\Delta gtxA$ mutant using qRT-PCR. The previous report suggested an increased expression of IL-6, TNF-α, and IFN-γ by the PCOECs, dictating induction of a pro-inflammatory reaction [28]. We investigated if GtxA had a primarily pro-inflammatory (IL-1β, IL-6) or anti-inflammatory (IL-10) response, respectively, following exposure to HD11 cells. Our data showed that the expression of IL-1β was highly up-regulated while the expression of IL-10 was down-regulated in HD11 cells exposed to the $G.\ anatis\ \Delta gtxA$ mutant group. These results are, at least partly, consistent with the results from our previously reported in vivo investigation where the expression of IL-10 was up-regulated in the spleen while the TNF-α expression was suppressed following infection with the GtxA expressing WT [20]. TNF-α is generally considered to have an inverse relation to IL-10, where IL-10 promotes macrophage deactivation as opposed to TNF-α, which usually is regarded pro-inflammatory and macrophage activating. The effect of GtxA thus appeared to dampen the pro-inflammatory host reaction. The somewhat similar results obtained from HD11 cells (in vitro) and spleen tissue (in vivo) indicate that the HD11 cells may be used as a biologically relevant proxy prior to investigations of virulence factors or immune-activating (e.g., vaccine prototypes) in animals and support the 3R (replace/reduce/refine) animal protection initiative.

We hypothesized that one of the mechanisms by which $G.\ anatis$ causes oophoritis and upper respiratory tract lesions may be through induction of apoptosis and necrosis of affected host cells. We aimed to test if the observed inflammatory response and cell death generated by HD11 cell was dependent on GtxA expression. The ability of bacterial pathogens to promote or inhibit apoptosis in eukaryotic cells is an emerging theme in the study of bacterial pathogenesis [35]. Several approaches enable bacterial pathogens of replicating in host cells while preventing apoptosis by repairing the mitochondrial perturbations, destroying cytochrome c release or preventing caspase activation [35].

Although GtxA has been shown to be vital for $G.\ anatis$-induced leukotoxicity and a major virulence factor during infections in chickens [20], several questions regarding the specific roles of GtxA remain. RTX toxins, in cluding the Escherichia coli α-hemolysin (HlyA), Actinobacillus pleuropneumoniae (Apxl A), Actinobacillus actinomycetemcomitans (LtxA) and Mannheimia haemolytica (LktA) [24], have all been shown to induce apoptosis and cell death in a host-specific manner. Generally, RTX toxins bind to β2-integrins and induce changes associated with necrosis and cell lysis at high toxin concentrations, whereas cells exposed to lower toxin concentrations trigger signalling cascades promoting apoptosis-like cell death [36]. The molecular mechanisms leading to RTX-toxin cell death are complex and appear to affect cell homeostasis by destructing the host cell membrane integrity through formation and fusion of trans-membrane pores leading to cytolysis at high toxin concentrations [25]. At lower concentrations the toxin-mediated perturbations can either promote short-term activation of signaling cascades, resulting in rapid apoptosis-like cell death or programmed cell death destroying host cell integrity during a course of days [25,37–39]. Receptor-mediated activation of caspase-8 and mitochondrial activation of caspase-9 represent two common pathways at induction of apoptosis [38,40,41]. Subsequent effector caspases, like caspase-3, -6 and -7, are likewise hallmarks of apoptotic cell death [40].
In the current work, we found that HD11 cells expressed a significantly lower level of caspase-3 at 2 h PI following exposure to GtxA expressing *G. anatis*, while caspase-9 was up-regulated. At 6 h PI, a highly significant decrease in the expression of caspase-9 was observed, indicating that GtxA induced apoptosis is regulated in a caspase-9 dependent manner [41]. *G. anatis* thus appears to have the ability to escape antimicrobial activity asserted by HD11 cells, at least partly, by preventing caspase activation at different points along the apoptotic pathway. These findings are somewhat consistent with previous reports on *S. flexneri*, which might block apoptosis by targeting the already activated form of caspase-9 or the inactive form of caspase-3 thereby preventing caspase-3 activation [35]. We also characterized the expression of the anti-apoptosis or pro-survival *bcl-2* gene, which was dramatically down-regulated in the △gtxA exposed group, compared to the WT at 2 h PI. At 6 h PI, both the WT and the △gtxA exposed groups expressed significantly lower amounts of *bcl-2* mRNA than the control group. Again, the results indicate that GtxA may actively prevent host cell destruction by promoting a pro-survival response during the early phases of host cell exposure to GtxA.

4. Conclusions

We aimed at providing further insight into the role of GtxA during interaction with the avian immune system, here represented by the macrophage-like HD11 cell line. Although several pieces in the puzzle are still lacking, we believe the first contours of the pathogenesis, including both bacterial and host factors, respectively, have become apparent. While GtxA clearly has the ability to cause host cell lysis, GtxA also appears able of dampening the inflammatory host response based on an initial over-expression of IL-10 and a corresponding low-level expression of TNF-α. On the contrary, the △gtxA mutant induced a clear pro-inflammatory response, here represented by a very high expression of IL-1β, IL-6 and TNF-α. Furthermore, although less clear, GtxA initially seemed to promote partial host cell survival through an anti-apoptotic *bcl-2* response, while at a later stage that was less apparent. In conclusion, GtxA appears to be a main factor to control in order to mitigate the negative effects of a *G. anatis* biovar *haemolytica* on the host immune system and thus remain an obvious vaccine target.

5. Materials and Methods

5.1. Bacterial Strains and Growth Condition

The wild-type strain *Gallibacterium anatis* 12656-12 (*G. anatis* 12656-12) is a gentamicin-susceptible (MIC < 1 mg/L) haemolytica strain derived from the liver of a chicken with septicaemia [7]. Avian pathogenic strain *G. anatis* 12656-12 wild-type (WT) and *G. anatis* △gtxA mutant were cultivated on brain heart infusion (BHI) (Oxoid, Basingstoke, UK) agar supplemented with 5% citrated bovine blood in a closed plastic bag [27]. Single colonies were incubated on BHI in an orbital shaking incubator overnight at 37 °C. The overnight cultures were transferred to a fresh BHI and cultured for 2 h to reach an exponential growth phase of a highly invasive phenotype. Prior to use, the bacteria were pelleted by centrifugation, resuspended in phosphate buffered saline (PBS) to OD₆₀₀ = 0.2 and adjusted at a final concentration of approximately 2.5 × 10⁸ colony-forming unit (CFU)/mL. The bacterial concentration in each inoculum was verified by plate counts on BHI plates in duplicate.

5.2. Cell Lines and Culture Conditions

The MC29 bone marrow-transformed chicken macrophage-like cell line HD11 [42] was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium added GlutaMAX-1, 25 mM HEPES (Gibco, Carlsbad, CA, USA), 2.5% chicken serum, 10% heat-inactivated foetal bovine serum (ThermoFischer, Gibco, South America) and 25µg/mL gentamicin. The HD11 cells were incubated at 37 °C in an atmosphere of 5% CO₂. Cell concentrations were adjusted, and aliquots of cells (4 × 10⁶ cells/mL) were kept on ice until use. Prior exposure to bacteria, the culture medium of each sample was replaced with RPMI without antibiotics. The HD11 cell suspension was seeded into each well at 100 µL/well for
96-well plates and 600 µL/well for 24-well plates and allowed to grow to approximately 85% confluence before used for assays. The 96-well plates were used for multiplicity of infection (MOI) assay, whereas the 24-well plates were used for the invasion assays. For the qRT-PCR and apoptosis, assays cells were seeded at 4 × 10^6 cells/mL in 12-well plates. The entire experiment was performed in triplicate on two independent occasions.

5.3. Establishment of an Appropriate Multiplicity of Infection (MOI)

Preliminary MOI assays were performed to assess the infectivity of *G. anatis* in HD11 cells and determine an optimal concentration range of bacteria to be used in the subsequent adhesion and invasion assays. Incubation of *G. anatis* 12656-12 (WT and ΔgtxA) was performed in BHI to reach a final concentration of approximately 2.5 × 10^8 CFU/mL. Subsequently, the bacterial preparations were washed three times with PBS and resuspended in RPMI culture medium without antibiotics. After 1 h incubation period, monolayers of HD11 cells incubated in 24-well plates were added to bacterial suspensions of three different MOIs: 10:1 (0.7 × 10^7 CFU/mL), 50:1 (3.5 × 10^7 CFU/mL) and 100:1 (0.7 × 10^8 CFU/mL) bacteria per HD11 cell [28]. Subsequently, at 30, 60 and 90 min, samples of 100 µL infected HD11 cells were washed three times in pre-warmed PBS to remove the non-adherent bacteria. The remainder of HD11 cells were incubated with RPMI culture medium containing gentamicin (100 mg/mL) for one hour, and subsequently cells were lysed with 1 mL of 0.1% Triton X-100 solution (Sigma, Søborg, Denmark) [43]. After homogenization, a 100 µL aliquot of lysed cell suspension was diluted into 900 mL PBS enabling 10-fold serial dilutions to be inoculated on BHI agar plates for quantification of bacterial colonies. The number of colony-forming units (CFUs) after 24 h of incubation was determined in triplicate.

5.4. Cell Invasion and Intracellular Survival Assay

Following optimization of the HD11 and *G. anatis*, co-culture 24-well cell culture plates containing approximately 0.7 × 10^6 HD11 cells/well were incubated for 1 h prior to infection. Bacterial suspensions (2.5 × 10^8 CFU/mL) were prepared as described above and inoculated for 1 h into each well at 37 °C in a 5% CO_2 atmosphere. To quantify the invading bacteria, extracellular bacteria were removed by washing the HD11 cells three times and subsequently adding RPMI medium containing gentamicin (100 mg/mL) for one hour, and subsequently cells were lysed with 1 mL of 0.1% Triton X-100 solution. After homogenization, a 100 µL aliquot of lysed cell suspension was diluted into 900 mL PBS enabling 10-fold serial dilutions to be inoculated on BHI agar plates for quantification of bacterial colonies. The number of colony-forming units (CFUs) after 24 h of incubation was determined in triplicate.

5.5. Trypan Blue Exclusion Assay

Trypan blue viability measurement was performed by standard method [14,44,45]. The 24-well plates, containing 0.7 × 10^6 cell/well, were infected with *G. anatis* 12656-12 as invasion assay described above. Cellular counts were done after incubation with fresh culture media supplemented with gentamicin (100 mg/mL) after 1, 2, 4, 6, 8, 12 or 24 h, respectively. After gentamicin treatment, infected cells were washed three times with PBS and lysed by trypsin-EDTA following incubation for 10 min. One hundred microliter cell suspensions were mixed with an equal volume of 0.4% trypan blue solution (Sigma). The suspension was loaded into a Neubauer hemocytometer and scored with an inverted light microscope (Helmut Hund GmbH 6330; Wetzlar, Germany) at low magnification. The percentage of viable cells was determined by dividing the number of live infected cells by a number of live non-infected cells [14,44].
5.6. Growth Assay

Growth curve profiles were constructed to determine the significance of GtxA on G. anatis growth. The growth of the G. anatis WT and ΔgtxA mutant strains were compared by inoculating in BHI medium with shaking at 220 rpm and culturing at 37 °C for 12 h. Bacterial growth was estimated by OD$_{600}$ performed three times at 1 h intervals.

5.7. Flow Cytometric Analysis of HD11 Cell Death

To identify the percentage of HD11 cells undergoing cell death in vitro, the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) was used. Infection of HD11 cell cultures was performed with G. anatis WT and ΔgtxA during 2 h and 6 h, respectively. In parallel, an uninfected HD11 group served as the negative control. From either group in six-well tissue culture plates, material was harvested by adding 0.25% trypsin and washing three times with PBS. The cells were stained with 5 μL FITC-conjugated annexin V (Annexin V-FITC) and 3 μL Propidium iodide (Pi) in a volume of 100 mL on ice for 30 min. Subsequently, the mixture was incubated at room temperature for 20 min in the dark. The FITC and Pi fluorescence was measured by flow cytometry (BD Accuri™C6, Beckman Coulter, Indianapolis, IN, USA) within an hour. The fractions of live and dead HD-11 cells were quantified and compared two- and six-hours post infection, respectively.

5.8. Real-Time Quantitative RT-PCR

To assess the mRNA expression profile of the HD11 cells, samples were stored in RNALater solution (Qiagen, Hilden, Germany) at −80 °C prior to RNA purification. The six-well plates containing $4 \times 10^8$cells/well were infected with G. anatis 12656-12 WT and ΔgtxA as previously described. In parallel, an uninfected HD11 group served as the negative control. Samples from each of the three groups were obtained after 2 h and 6 h, respectively, extracted and homogenized in 1 mL RTL buffer individually. Subsequent to homogenization and centrifugation for 1 min, the upper phase containing RNA was collected and purified using the RNaseq mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. The concentration and purity of RNA were determined by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Five ug of RNA extracted from each sample were immediately reverse-transcribed into cDNA using M-MLV reverse transcriptase kit (Invitrogen) according to the manufacturer’s protocol. An amount of cDNA corresponding to 20 ng of reverse-transcribed RNA was amplified by qRT-PCR, using specific primers obtained from the NCBI database and synthesized at Germany listed in Tables 1 and 2. The cDNA product was used as template in 25 μL PCR reactions in 96-well microplates using FastStart Essential DNA Green Master Mix (Roche, Penzberg, Germany). The three-step amplification and signal detection were performed using a LightCycler ® 96 (Roche) with an initial preincubation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s, respectively. Apoptosis and cytokines mRNA expression levels in HD11 cells were quantified using qRT-PCR. β-actin expression was used as an internal control to normalize the quantification of target genes. The relative quantification of apoptosis gene-specific expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with chicken β-actin [46]. Quantification of cytokine gene expression of IL-6, IL-1β, IL-10, TNF-α and β-actin genes was calculated using the same formula. All qRT-PCR reactions were performed in triplicate.

<p>| Table 1. List of primers used in qRT-PCR analysis of mRNA expression of the apoptotic proteins. |
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Table 2. List of primers used in qRT-PCR analysis of mRNA expression of the cytokines.

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5.9. Statistical Analysis

All data were expressed as a mean and a standard error of the mean (SEM) from three independent experiments performed in triplicate. To compare the relative gene expression levels, we used the $2^{-\Delta\Delta\text{ct}}$ method [46]. The statistical analyses were conducted using Student’s t-test in GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). A p-value $\leq 0.05$ was considered significant, while a p-value $\leq 0.01$ and $p \leq 0.001$ was considered moderate and highly significant, respectively.

Author Contributions: Conceptualization, B.T. and A.M.B.; methodology, B.T. and A.M.B.; formal analysis, B.T.; data curation, B.T. and A.M.B.; writing—original draft preparation, B.T.; writing—review and editing, A.M.B.; supervision, A.M.B.; project administration, A.M.B.; funding acquisition, B.T. and A.M.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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