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

Bo Tang, Susanne E. Pors, Bodil M. Kristensen, Ragnhild Bager J. Skjerning, Rikke H. Olsen and Anders M. Bojesen

Abstract
GtxA, a leukotoxic RTX-toxin, has been proposed as 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.
**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 to obtain single colonies, which were stored at 80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were stored at 80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were stored at 80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were stored at 80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were stored at 80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were stored at 80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were stored at 80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were stored at 80 °C and cultivated overnight on BA in a sealed plastic bag to obtain single colonies, which were stored at 80 °C and cultivate...
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 RNAlater (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 18 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 Sall (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 Serotec) diluted 1:4000 in diluting buffer were added to each well and the plates incubated for 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 RNaseasy 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, caspase-3, caspase-8, caspase-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:

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

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

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

| Name  | Primer | Primer sequence(5′–3′)       | Accession | Conc. (mM) |
|-------|--------|-------------------------------|-----------|------------|
| bcl-2 | Forward| GATGACCGAGTACCTGAACG         | NM205339  | 0.2        |
|       | Reverse| CAGGAGAATCGAACAAAGGC         |           |            |
| bax   | Forward| GCTCCTCATCGCAGCTGCTCAT       | XM422067  | 0.4        |
|       | Reverse| CTTTGTGCTGGAAGCAGAAGA        |           |            |
| caspase-8 | Forward| TGTCCTCTTGAAGACTGAAG        | AYO57940  | 0.4        |
|       | Reverse| CCTACTGCTGCCTCTCATTCC        |           |            |
| caspase-9 | Forward| CGAAGAGACGAACGACGACG        | AYO57940  | 0.2        |
|       | Reverse| CGCAGGCTTCTCTCAGCAT          |           |            |
| caspase-3 | Forward| TGGCCCTCTTGAACGTGAAG         | AYO57940  | 0.4        |
|       | Reverse| TCCACTGCTGCTGCTCAATTCC       |           |            |
| IL-6  | Forward| GCTGTGCGGCTTCCGA            | AJ250838  | 0.4        |
|       | Reverse| GTAGGTTGCTGAAAGCGACAG        |           |            |
| IL-4  | Forward| AACATCGGTCGACTCTCTGAAG     | AJ621735  | 0.4        |
|       | Reverse| TCTGCTAGAAACTTCTCATTGAA     |           |            |
| IL-10 | Forward| CGTCTTCTTGAGCCTCTGAAG      | J621614   | 0.4        |
|       | Reverse| CGTCGGCTTGCTTTCTTGATG        |           |            |
| TNF-α | Forward| GCCCTTCTTGTAACGAGT           | NM_204267 | 0.2        |
|       | Reverse| ACACGACGACGACGTCAGC         |           |            |
| β-actin| Forward| CGCGCTCTATGAGGCTCGC         | LO8165   | 0.4        |
|       | Reverse| CTCTGGCTGTGGTCGTGGA          |           |            |

### 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)

| *G. anatis* | Peritoneum | Ovary | Oviduct |
|-------------|------------|-------|--------|
|             | Purulent peritonitis | Re-isolation of *G. anatis* | Purulent oophoritis | Re-isolation of *G. anatis* | Salpingitis | Re-isolation of *G. anatis* |
| ΔgtxA       | 2/16 (13%) | 5/16 (31%) | 0/16 (0%) | 7/16 (44%) |
| Wild-type   | 3/4 (75%)  | 3/4 (75%)  | 3/4 (75%)  | 3/4 (75%)  |
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](image_url)
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 ΔgtxA 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 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 ΔgtxA (Figure 4A), whereas significantly lower expression of IL-4 and TNF-α, respectively, was found in the ΔgtxA group compared to the WT group. At 6 days pi, expression of IL-4 remained significantly lower in the ΔgtxA 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 ΔgtxA 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].
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** The relative expression levels of apoptosis genes in ovary tissue at 2 days and 6 days post-infection of gtxA mutant compared with WT strain. Error bars are S.E. for each treatment group. The figure shows the gene expression profile of apoptosis-related (Bcl-2, caspase-8, caspase-3, caspase-9) differed significantly (*P < 0.05, **P < 0.01) between the WT strain and ΔgtxA mutant groups, respectively.
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* unability 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

![Figure 7](image_url)
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 (intrinsc 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 defence 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 inoculum. The Tda56-12 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.

Acknowledgements
Katrine Madisen 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.

Funding
This work was supported by the Faculty of Health and Medical Sciences at the University of Copenhagen and The Chinese Scholarship Council.

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.

Received: 5 November 2019 Accepted: 31 January 2020

Published online: 11 March 2020

References
1. Bojesen AM, Nielsen OL, Christensen JP, Bisgaard M (2004) Genetic diversity and associated pathology of Gallibacterium anatis infected in chickens. Avian Pathol 33:145–152
2. Paudel S, Liebhart D, Hess M, Hess C (2011) Assessing pathogenicity of Gallibacterium anatis in a natural infection model: the respiratory and reproductive tracts of chickens are targets for bacterial colonization. Avian Pathol 42:527–533
3. Neubauer C, De Souza-Pilz M, Liebhart D, Hess M, Hess C (2013) Assessing pathogenicity of Gallibacterium anatis isolates in laying birds with reproductive disorders. Avian Pathol 42:443–449
4. Milne C (1991) Studies into incidence of Pasteurella haemolytica infections and their relevance to hens, with particular reference to diseases of the egg-laying apparatus. Monash Vet Med 46:545–549
5. Neubauer C, De Souza-Pilz M, Bojesen AM, Bisgaard M, Hess M (2009) Tissue distribution of haemolytic Gallibacterium anatis isolates in laying birds with reproductive disorders. Avian Pathol 38:1–7
6. Wang C, Pors SE, Christensen JP, Bojesen AM, Thøfner ICN (2019) Reproduction and coinfection of Gallibacterium anatis in poultry. Vet Res 41:57
7. Kawasaki Y, Zychlinsky A (1999) The induction of apoptosis by bacterial pathogens. Ann Rev Microbiol 53:155–187
8. Nawab A, An L, Wu J, Li G, Liu W, Zhao Y, Wu Q, Xiao M (2019) Chicken toll-like receptors and their significance in immune response and disease resistance. Int Rev Immunol 38:284–306
9. Zhang XP, Lu CJ, Li YT, Yang X, Wang XW, Chang HT, Liu HY, Chen L, Zhao L, Wang CQ (2017) In vitro adherence and invasion of primary chicken oviduct epithelial cells by Gallibacterium anatis. Vet Microbiol 203:136–142
10. Wang C, Pors SE, Christensen JP, Bojesen AM, Thøfner ICN (2019) Comparative analysis of resident Gallibacterium anatis isolates in laying birds with reproductive disorders. Avian Pathol 42:443–449
11. Kristensen BM, Frees D, Bojesen AM (2011) Expression and secretion of the RTX-toxin GtxA among members of the genus Gallibacterium. Vet Microbiol 153:116–123
12. Johnson TJ, Danzeisen JL, Trampel D, Nolan LK, Seemann T, Bager RJ, Bojesen AM (2013) Genome analysis and phylogenetic relatedness of Gallibacterium anatis strains from poultry. PLoS One 8:e54844
13. Bager R, Kudrikiené E, Da Peadade I, Seemann T, Nielsen TK, Pors SE, Mattsansson AH, Boyce JD, Adler B, Bojesen AM (2014) In silico prediction of Gallibacterium anatis pan-immunogens. Vet Res 45:80
14. Pors SE, Skjerning RB, Flachs EM, Bojesen AM (2016) Recombinant proteins from Gallibacterium anatis induces partial protection against heterologous challenge in egg-laying hens. Vet Res 47:36
15. Ozoa A, Isole N, Yoshimura Y (2009) Expression of Toll-like receptors (TLRs) and TLR4 response to lipopolysaccharide in hen oviduct. Vet Immunol Immunopathol 127:259–268
16. Michailidis G, Theodoridis A, Avdi M (2010) Transcriptional profiling of Toll-like receptors in chicken embryos and in the ovary during sexual maturation and in response to Salmonella Enteritidis infection. Anim Reprod Sci 122:294–302
17. Nawab A, An L, Wu J, Li G, Liu W, Zhao Y, Wu Q, Xiao M (2019) Chicken toll-like receptors and their significance in immune response and disease resistance. Int Rev Immunol 38:284–306
18. Zhang XP, Lu CJ, Li YT, Yang X, Wang XW, Chang HT, Liu HY, Chen L, Zhao L, Wang CQ (2017) In vitro adherence and invasion of primary chicken oviduct epithelial cells by Gallibacterium anatis. Vet Microbiol 203:136–142
19. Weinaurach Y, Zychlinsky A (1999) The induction of apoptosis by bacterial pathogens. Ann Rev Microbiol 53:155–187
20. Fan TJ, Han LH, Cong RS, Liang J (2005) Caspase family proteases and apoptosis. Acta Biochim Biophys Sin 37:719–727
21. Elmore S (2007) Apoptosis: a review of programmed cell death. Tox Path 35:495–516
22. Czyzynski CJ, Welch RA (1995) Biological effects of RTX toxins: the possible role of lipopolysaccharides. Trends Microbiol 3:480–483
23. Fernandez-Prada C, Tall BD, Elliott SE, Hoover DL, Nataro JP, Venkatesan MA (1998) Hemolysin-positive enterocaggregative and cell-detaching Enterichia coli strains cause oncosis of human monocyte-derived macrophages and apoptosis of murine J774 cells. Infect Immun 66:3918–3924
24. Moss JE, Aliprantsis AO, Zychlinsky A (1999) The regulation of apoptosis by microbial pathogens. Int Rev Cytol 187:203–259
25. Bojesen AM, Vazquez ME, Robles F, Gonzalez C, Soriano EV, Olsen JE, Christensen H (2007) Specific identification of Gallibacterium by a PCR using primers targeting the 16S rRNA and 23S rRNA genes. Vet Microbiol 123:262–268
26. Bojesen AM, Torp Dahl M, Christensen H, Olsen JE, Bisgaard M (2003) Genetic diversity of Gallibacterium anatis isolates from different chicken flocks. J Clin Microbiol 41:2737–2740
27. Pedersen LJ, Pors SE, Bager Skjerning RI, Nielsen SS, Bojesen AM (2015) Immunogenic and protective efficacy of recombinant protein GtxA-N against Gallibacterium anatis challenge in chickens. Avian Pathol 44:386–391
28. Christensen H, Bisgaard M, Bojesen AM, Muters R, Olsen JE (2003) Genetic relationships among avian isolates classified as Pasteurella haemolytica, ‘Actinobacillus sapignitidis’ or Pasteurella anatis with proposal of Gallibacterium anatis gen. nov., comb. nov. and description of additional genomospecies within Gallibacterium gen. nov. Int J Syst Evol Microbiol 53:275–287
29. Hangalapura BN, Kaiser MG, van der Poel JF, Parmentier HK, Lamont SJ (2006) Cold stress equally enhances in vivo pro-inflammatory cytokine gene expression in chicken lines divergently selected for antibody responses. Dev Comp Immunol 30:503–511
30. Pors SE, Hansen MS, Christensen H, Jensen HE, Petersen A, Bisgaard M (2011) Genetic diversity and associated pathology of Pasteurella multo-cida isolated from porcine pneumonia. Vet Microbiol 150:354–361
31. Reimer D, Frey J, Jansen R, Veit HP, Inzana TJ (1995) Molecular investigation of the role of Apol and Apoll in the virulence of Actinobacillus pleuropneumoniae serotype 5. Microb Pathog 18:197–209
32. Kamp EM, Stockhofe-Zurwieden N, Van Leengoed L, Smits MA (1997) Endobronchial inoculation with Apx toxins of Actinobacillus pleuropneumoniae leads to pleuropneumonia in pigs. Infect Immun 65:4350–4354

33. Liu J, Chen X, Guo Y, Chen Y, Fu S, Bei W, Chen H (2009) In vivo induced RTX toxin ApxIVA is essential for the full virulence of Actinobacillus pleuropneumoniae. Vet Microbiol 137:282–289

34. Tatum FM, Briggs RE, Sreevatsan SS, Zehr ES, Hsuan SL, Whiteley LO, Ames TR, Maheswaran SK (1998) Construction of an isogenic leukotoxin deletion mutant of Pasteurella haemolytica serotype 1: characterization and virulence. Microb Pathog 24:37–46

35. Frey J, Kuhnert P (2002) RTX toxins in Pasteurellaceae. Int J Med Microbiol 292:149–158

36. Chen ZW, Chien MS, Chang NY, Chen TH, Wu CM, Huang C, Lee WC, Hsuan SL (2011) Mechanisms underlying Actinobacillus pleuropneumoniae exotoxin Apxl induced expression of IL-1β, IL-8 and TNF-α in porcine alveolar macrophages. Vet Res 42:25

37. Bojesen AM, Petersen KD, Nielsen OL, Christensen JP, Bisgaard M (2004) Pasteurella multocida infection in heterophil-depleted chickens. Avian Dis 48:463–470

38. Nakamura K, Maeda M, Imada Y, Imada T, Sato K (1985) Pathology of spontaneous colibacillosis in a broiler flock. Vet Pathol 22:592–597

39. Bager RJ, Nesta B, Pors SE, Soriani M, Serino L, Boyce JD, Adler B, Bojesen AM (2014) Chaperone-usher fimbriae in a diverse selection of Gallibacterium genomes. BMC Genomics 15:1093

40. Kudirkienė E, Bager RJ, Johnson TJ, Bojesen AM (2014) Chaperone-usher fimbriae in a diverse selection of Gallibacterium genomes. BMC Genomics 15:1093

41. Iqbal M, Phibbin VJ, Withanage G, Wigney P, Beal MK, Goodchild MJ, Barrow P, McConnell I, Maskell DJ, Young J (2005) Identification and functional characterization of chicken toll-like receptor 5 reveals a fundamental role in the biology of infection with Salmonella enterica serovar Typhimurium. Infect Immun 73:2344–2350

42. Mosmann TR, Coffman RL (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Ann Rev Immunol 7:145–173

43. Wu QJ, Zheng XC, Wang T, Zhang TY (2018) Effects of oridonin on immune cells, Th1/Th2 balance and the expression of BLys in the spleens of broiler chickens challenged with Salmonella pullorum. Res Vet Sci 119:262–267

44. Chtanova T, Mackay CR (2001) T cell effector subsets: extending the Th1/Th2 paradigm. Adv Immunol 78:233–266

45. Arsenault RJ, Genovese KJ, He H, Wu H, Neish AS, Kogut MH (2015) Wild-type and mutant AntA—Salmonella induce broadly similar immune pathways in the chicken ceca with key differences in signaling intermediates and inflammation. Poul Sci 95:354–363

46. Duell BL, Tan CK, Carey AJ, Wu F, Cripps AW, Ulett GC (2012) Recent insights into microbial triggers of interleukin-10 production in the host and the impact on infectious disease pathogenesis. FEMS Immunol Med Microbiol 64:295–313

47. Thumbikar P, Dileepan T, Kannan MS, Maheswaran SK (2005) Mechanisms underlying Mannheimia haemolytica leukotoxin-induced oncrosis and apoptosis of bovine alveolar macrophages. Microb Pathol 38:161–172

48. Khelef N, Zychlinsky A, Guiso N (1993) Bordetella pertussis induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. Infect Immun 61:4064–4071

49. Jonas D, Walew C, Berger T, Liebetrau M, Palmer M, Bhakdi S (1994) Novel path to apoptosis: small transmembrane pores created by staphylococcal alpha-toxin in T lymphocytes evoke internucleosomal DNA degradation. Infect Immun 62:1304–1312

50. Lally ET, Hill RB, Kieba IR, Korostoff J (1999) The interaction between RTX toxins and target cells. Trends Microbiol 7:356–361

51. Hibi H, Moss JE, Hersh D, Chen Y, Arandel J, Banerjee S, Flavell RA, Yuan J, Sansonetti PJ, Zychlinsky A (1998) Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB. J Biol Chem 273:32895–32900

52. Boise LH, Collins CM (2001) Salmonella-induced cell death: apoptosis, necrosis or programmed cell death? Trends Microbiol 9:64–67

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.