*The PHILIPPINE JOURNAL OF*

# Veterinary Medicine

Volume 61 No. 1 January - June 2024

Published by the College of Veterinary Medicine
University of the Philippines Los Baños

# The Philippine Journal of Veterinary Medicine

**Volume 61 No. 1 January - June 2024**

The Philippine Journal of Veterinary Medicine (ISSN 0031-7705 print; eISSN 2984-763X online) is a peer-reviewed international journal of basic, applied, and translational research in veterinary medicine and biomedical science. It is published semi-annually, for the periods January-June and July-December each year, by the College of Veterinary Medicine, University of the Philippines Los Baños. All articles are subjected to double-blind review.








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*Original Research*

# Comparative Gene Expression Analysis of Immune-Related Cytokines in *Riemerella anatipestifer* - stimulated Philippine Banaba Native Chicken and Native Duck Embryonic Fibroblasts

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Submitted: 13 Dec 2023 Revised: 7 Feb 2024 Accepted: 9 Feb 2024 Published: 14 Feb 2024

## Abstract

**Background:** Studies on Philippine Banaba native chickens and Philippine Mallard ducks were generally focused on their production and management. However, little is known on their immune responses to pathogens like *Riemerella anatipestifer (R. anatipestifer)*, one of the economically important infectious disease-causing pathogens in Philippine Banaba native chickens and Philippine Mallard ducks. **Methods:** Chicken and duck embryonic fibroblasts (CEFs and DEFs) isolated from 11-day old healthy embryos were stimulated with heat-inactivated *R. anatipestifer* for 4, 8 and 24 h. The cells were collected at each time point and subjected to qRT-PCR for gene expression analyses. **Results:** Comparative gene expression analyses of immune-related cytokines revealed that the ex- pression levels of IL-17A, IL-17F, IL-1ß and IL-6 were significantly upregulated in both CEFs and DEFs stimulated with heat-inactivated *R. anatipestifer* serotype 7. However, levels were higher in DEFs compared to the corresponding levels in heat-inactivated *R. anatipestifer* stimulated CEFs. On the other hand, IFN-IL-2, IL-4 and IL-10 expression levels of stimulated DEFs were also upregulated. In CEFs, it is interesting to note that IFN-γ level was significantly downregulated at 24 h while upregulated in DEFs. **Conclusion:** Results showed that the Philippine Banaba native chickens and Mallard ducks are also susceptible to *R. anatipestifer infection*.

## Keywords

*R. anatipestifer,* Philippine Banaba, Mallard duck, Cytokines, Immunity

## 1. Introduction

In the Philippines, chicken and duck industries are the first and second largest contributors to the poultry industry, respectively. Among these species, native chickens and native ducks have the ability to adapt, grow and reproduce regardless of unfavorable climatic conditions, however, they are less recognized, and little is known on the immune responses of these avian species to pathogens and may remain susceptible to a number of pathogens including *R. anatipestifer.* The Philippine Banaba native chickens and Mallard ducks might have different susceptibilities to *R. anatipestifer* infection. However, information

regarding this mechanism and their immunity to *R. anatipestifer* infection are still limited and poorly understood. Given these, research and monitoring are essential to understand the specific diseases to which the Philippine Banaba native chickens and Mallard ducks may exhibit resistance and susceptibility.

The avian immune system is a significant model for basic and comparative immunological studies. Cytokines are important mediators in the immune response of the avian species, wherein expression patterns and functions of these immune genes vary between species of birds. Immune system studies are presently more focused on studies related to adaptive immunity, which takes longer to develop, than innate immunity [1-2]. Moreover, considerable amount of information has been accumulated but the immune system in birds is still poorly understood. Different species of birds, particularly ducks and chickens, have different susceptibilities to *R. anatipestifer* infection and different generated immunological responses [3].

*R. anatipestifer* is a Gram-negative, non-motile bacterium belonging to the family Flavobacteriaceae and is responsible for the disease known as riemerellosis, duck septicemia or infectious serositis which causes meningitis, air sacculitis, caseous salpingitis, septicemia, and fibrinous exudates in the pericardial and hepatic cavities [4]. Riemerellosis is an economically important infectious disease in domestic ducks worldwide as well as variety of other avian species including chickens, turkeys, and geese [4-5]. The mortality rate typically ranges from 5% to 75%, depending on the virulence of the strain [4,6]. Currently, there are at least 21 identified serotypes [6-8]. There is no significant serologic cross-protection between serotypes [7-8], which makes vaccination control and treatment more difficult. *R. anatipestifer* infection causes a significant economic impact in the duck industry worldwide particularly in native breeds of chickens and ducks, not much progress has been re- ported in understanding the molecular mechanism of the host protective immunity against the infection [9-10].

Our previous studies have shown that ducks are more susceptible to *R. anatipestifer* infection compared to chickens and inflammatory cytokines such as IL-17 cytokine family and other Th17 immune-related cytokines are associated with the infection [10-11]. Moreover, this hypothesis further confirmed in another related study wherein there was an increased survival rate after berberine (BBR) and 3,3'diindolyl- methane (DIM) treatments, anti-inflammatory agents, in *R. anatipestifer*-infected ducks [12-13].

Several studies mostly focused on the host immunity and pathogenesis of *R. anatipestifer* which includes identifying virulence factors [14-15], immunogenic proteins [6,16-17], mutant strains for live or attenuated vaccine development [18-20], and various immunoreactive proteins [21]. Numerous investigations mostly concentrated on the pathophysiology and host immunity of *R. anatipestifer* which includes virulence factors [14-5], immunogenic proteins [6,16-17], mutant strains that might make excellent live or attenuated vaccines [18-20], and different immunoreactive proteins [21]. *R. anatipestifer* infections have been controlled by antibiotics. However, alternative control methods are still necessary due to the emergence of drug-resistant strains and the increasing public concern regarding drug residues in poultry-related products. Moreover, due to the lack of information on bacterial-host interactions and on host protective immunity, the control of *R. anatipestifer* infection has only been partially successful.

Hence, this study investigated the relative contributions of immune-related cytokines in *R. anatipestifer*-stimulated chicken and duck embryonic fibroblasts to further elucidate and better understand the molecular mechanisms mediating bacterial pathogenesis, bacterial-host interactions and immune responses of this detrimental poultry disease.

## 2. Materials and Methods

### 2.1 Philippine Banaba Native Chicken and Mallard Duck Embryonated Eggs

Chicken and duck embryonated eggs of 11 days were obtained from the Bureau of Animal Industry - National Swine and Poultry Research and Development Center (BAI- NSPRDC) in Tiaong, Quezon. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (UPLB-2023-011a).

## 2.2 *Riemerella anatipestifer* Serotype 7 Preparation

*R. anatipestifer* serotype 7 which was isolated and serotyped in Changwon, Gyeongnam Province, South Korea and Chonbuk National University, respectively. It was a kind gift from Prof. Wongi Min of Gyeongsang National University, South Korea. The isolate was cultured on tryptic soy broth (TSB) for 24 h and placed in a cryopreservation tube with 50% glycerol for long term storage at -80°C. Prior to cell stimulation, the bacterium was cultured in TSB for 24 h and pure culture isolation was done using nutrient agar (NA). The identity of the bacterial isolate was confirmed by polymerase chain reaction (PCR) assay using the following primers: forward 5'-GTATTGAAAGCTCTGGCGG-3' and reverse 5'-TCGCTGAGTCTCTGAACCC-3'. Subcultures of *R. anatipestifer* serotypes 7 were prepared by placing isolates in a 250 mL nutrient broth (NB) followed by centrifugation at 2000 rpm for 10 to 15 min. After which, the isolates were resuspended with phosphate buffered saline (PBS) which were then subsequently submerged in a water bath set at 100°C for 5 min. Confirmation of the heat-inactivation of the bacteria were performed by plating in NA plates to make sure all isolates were inactivated prior to cell stimulation.

## 2.3 Isolation and Culture of Embryonic Fibroblasts

The viability of the chicken and duck embryos was checked through candling. Only viable 11-day old healthy embryos were utilized in this study. Embryonic fibroblasts were isolated aseptically from chicken and duck embryonated eggs. Briefly, the whole eggs were disinfected with 70% ethanol using a sterile swab. With the use of sterile scissors and forceps, the shells of the eggs at the top were removed then the contents including the embryo were placed in a sterile petri dish. The embryo was sacrificed by rapid decapitation; extremities and underdeveloped organs such as stomach, spleen, liver, heart and lungs were removed from the body of the embryo. After which, rest of the embryo were washed with PBS, placed in a new sterile petri dish with trypsin-EDTA culture medium. The tissues were homogenized and were then filtered through a cell strainer (70 µm) (SPL, Korea). The filtrate was then centrifuged three times at 2000 rpm at 4°C for 10 min. Resuspension of the cell pellets was done in T-175 flasks containing 1% antibiotic-antimycotic solution (10,000 U/mL) and 10% fetal bovine solution (FBS) which were added to 60 mL Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, USA). The isolated embryonic fibroblasts were cultured and incubated at 41°C with 5% $CO_2$ until 80%-90% cell confluency.

## 2.4 Stimulation of Embryonic Fibroblasts

Following a resuspension of the CEFs and DEFs cell cultures to a 6-well plate at a density of 5 x $10^6$ cells/mL in duplicate, the cells were stimulated with heat-inactivated *R. anatipestifer* serotype 7 (1 x $10^6$ CFU/mL). For 4, 8, and 24 h, the stimulated cells were incubated at 41°C with 5% carbon dioxide ($CO_2$).

## 2.5 Cell Viability

The cell viability of the stimulated embryonic fibroblasts using 0.4% trypan blue was deter- mined at 4, 8, and 24 h. Using a clean hemocytometer and a pipette, 100 µL of cell suspension was loaded at the edge of each hemocytometer chamber for counting at 100x magnification using an inverted microscope. Colorless and bright cells were identified and counted as viable cells, while blue and non-refractile cells were deemed as non-viable cells. The number of viable and non-viable cells from all four corner squares from both chambers of the hemocytometer grid was recorded.

## 2.6 Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

The cultures of CEFs and DEFs were collected at 4, 8, and 24 h post stimulation with three replicates and were placed in RNA later solution. Total RNA from embryonic fibroblasts were extracted with RNeasy extraction mini kit (Qiagen, Germany) and the RNA concentration of each sample was determined using a nano spectrophotometer. Using one-step qPCR kit (Promega, USA), cDNA synthesis was performed through a PCR thermal cycler (Thermo Fisher, USA) with a random hexamer primer, reverse transcriptase and chicken and duck cytokine primers for the expression analyses of the target cytokine genes (Supplementary Table 1). Briefly, 1 µL of cDNA, 10 µL of SYBR Green PCR Master Mix, and 0.2 µM of specific cytokine

primers were combined to create a 20 µL total reaction volume. The initial denaturation step lasted for 2 min at 95°C.

There were 40 cycles of 15 s at 95°C and 60 s at 60°C during the amplification process. Gene expression levels were measured using the comparative $^{-\Delta\Delta}$Ct method, normalizing the results with the ß-actin gene as a reference [22].

## Statistical Analysis

Student's *t*-test using SPSS ® (IBM, USA) statistical software was used to analyze the data. It was deemed statistically significant when a $P < 0.05$. Data in this study was expressed as the mean values ± standard error (SE) from two independent experiments.

## 3. Results

### 3.1 Effect of heat-inactivated *R. anatipestifer* on cell viability

Trypan blue (0.4%) exclusion assay was conducted to determine the effect of heat-inactivated *R. anatipestifer* on the viability of CEFs and DEFs (Fig. 1). DEFs and CEFs were isolated from 11-day-old healthy chicken and duck embryos, cultured and stimulated with heat-inactivated *R. anatipestifer* for 4, 8 and 24 h. For DEFs stimulated with *R. anatipestifer,* cell viability at 4, 8, and 24 *h* showed 90.91%, 86.49% and 88%, respectively as compared to the unstimulated and cultured DEFs with 86.67%, 89.47% and 88.76% cell viability at 4, 8, and 24 h, respectively. On the other hand, CEFs stimulated with *R. anatipestifer* showed 91.49%, 90.12% and 87.67% cell viability at 4, 8 and 24 h, respectively as compared to the unstimulated and cultured CEFs with 93.02%, 90.91% and 82.35% cell viability at 4, 8, and 24 h, respectively. Results showed that heat-inactivated *R. anatipestifer* did not significantly affect cell viability of both DEFs and CEFs.

### 3.2 Th-17 cytokines are Upregulated during *R. anatipestifer* Stimulation in DEFs and CEFs

Expression profiles of IL-17A, IL-17F, IL-1ß and IL-6 were determined in DEFs and CEFs stimulated with *R. anatipestifer* serotype 7 using quantitative RT-PCR. In *R. anatipestifer*-stimulated DEFs, mRNA expression levels IL-17A, IL-17F, IL-1ß and IL-6 were generally upregulated at all time points (4, 8 and 24 h) (Fig. 2). Compared to unstimulated cultured (or control) DEFs, IL-17A transcript expression levels showed a slight increased by 1.10- ($p < 0.05$), 1.28-, and 1.68-fold at 4, 8 and 24 h post inoculation, respectively (Fig. 2A). Levels of IL-17F were also slightly upregulated by 0.47-, 1.23- and 0.36-fold at 4, 8 and 24 h compared with the unstimulated cultured DEFs (Fig. 2B). Moreover, it is important to note that the levels at 8 and 24 h post inoculation were significantly upregulated ($p < 0.01$). The mRNA transcript levels of both IL-1ß and IL-6 were significantly upregulated at all time points. IL-6 expression levels increased by 4.60- ($p < 0.01$), 9.85- ($p < 0.05$) and 6.37-fold ($p < 0.05$) at 4, 8 and 24 h, respectively (Fig. 2C). The same pattern was also observed with the expression levels of IL-1ß with higher levels compared with the IL-17A, IL-17F and IL-6 corresponding levels. Compared with the unstimulated cultured DEFs, IL-1ß expression levels greatly and significantly upregulated by 15.36- ($p < 0.01$), 34.58- ($p < 0.01$) and 22.11-fold ($p < 0.05$) at 4, 8 and 24 h, respectively (Fig. 2D). On the other hand, *R. anatipestifer*-stimulated CEFs also showed a slight increase in the expression of IL-17A with 0.63- ($p < 0.05$), 0.48- ($p < 0.05$) and 0.63-fold at 4, 8 and 24 h, respectively as compared with the unstimulated cultured CEFs (Fig. 3A). Although the expression levels of IL-17F were upregulated by 1.08-, 0.23-fold at 4 and 8 h, the levels were

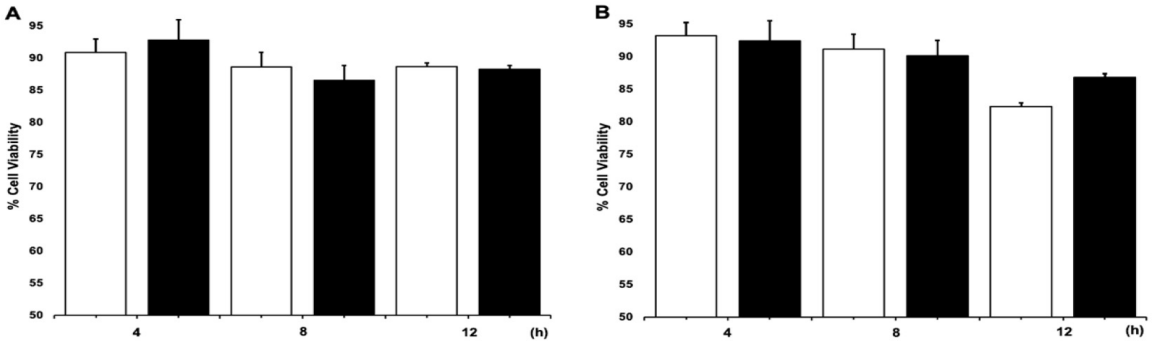


**Fig. 1.** Viability of embryonic fibroblasts stimulated with heat-inactivated R. anatipestifer serotype 7. Heat-inactivated *R. anatipestifer* serotype 7 ($1 \times 10^6$) was used to stimulate the embryonic fibroblasts of ducks (A) and chickens (B) for 4, 8, and 24 h. Cell viability was determined using trypan blue exclusion assay. The percentage (%) of the control (unstimulated/cultured cells) and a representative from two independent experiments was used to express the viability of the cells.

not significant. In addition, expression level at 24 h was unchanged (Fig. 3B). The expression levels of IL-6 were significantly upregulated with 1.12- ($p < 0.05$), 1.45- ($p < 0.05$), and 2.32-fold ($p < 0.001$), at 4, 8 and 24 h, respectively (Fig. 3C) while mRNA transcript levels of IL- 1ß were also significantly upregulated except at 8 h post stimulation. The levels were increased by 3.57- ($p < 0.01$), 3.98- and 12.89-fold ($p < 0.001$), respectively (Fig. 3D).

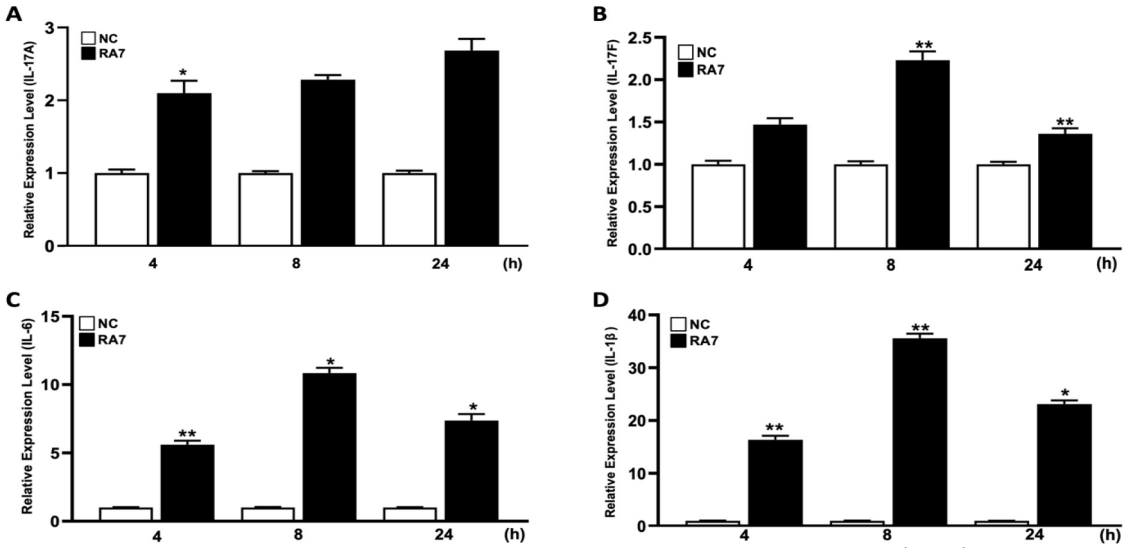


**Fig. 2.** mRNA expression profiles of Th-17 related cytokines in duck embryonic fibroblasts (DEFs). The transcript levels of IL-17A (A), IL-17F (B), IL-6 (C), and IL-1ß (D) were normalized with ß-actin expression levels and showed relative to the expression levels of unstimulated/cultured duck embryonic fibroblasts (NC). Results are presented as the mean ± SE values from 2 independent experiments performed in duplicates. $^{**}P < 0.01$ and $^{*}P < 0.05$. RA7: *R. anatipestifer*-stimulated embryonic fibroblasts; h: hours.

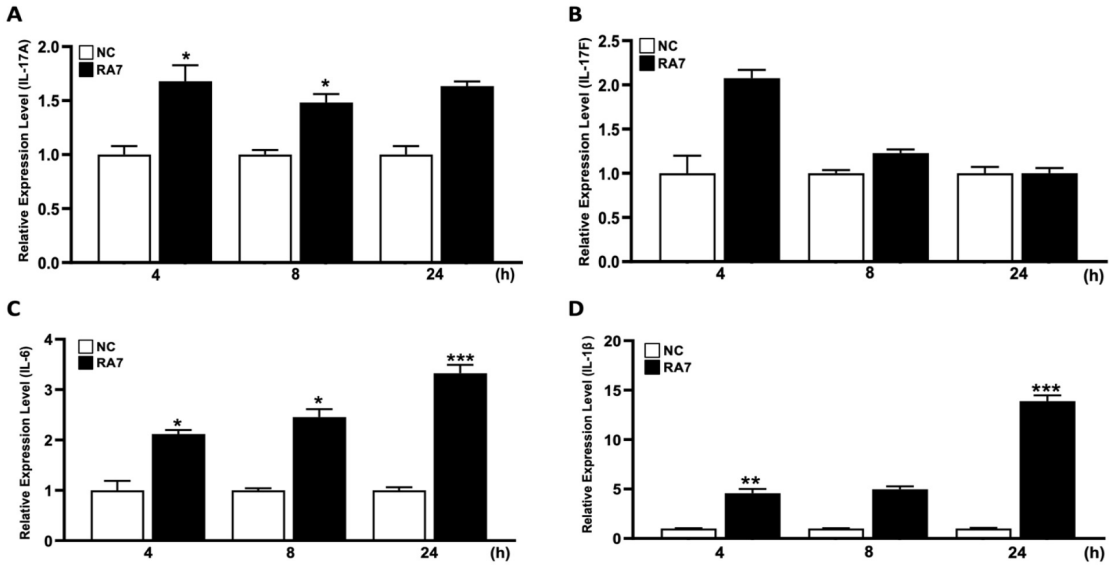


**Fig. 3.** mRNA expression profiles of Th-17 related cytokines in chicken embryonic fibroblasts (CEFs). The transcript expression levels of IL-17A (A), IL-17F (B), IL-6 (C), and IL-1ß (D) were normalized with ß-actin levels and showed relative to the expression levels of unstimulated/cultured duck embryonic fibroblasts (NC). Results are pre- sented as the mean ± SE values from 2 independent experiments performed in duplicates. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$. RA7: *R. anatipestifer*-stimulated embryonic fibroblasts; h: hours.

### 3.3 Th-1 and Th-2 Expression Profiles in *R. anatipestifer*-stimulated DEFs and CEFs

In this study, the mRNA expression pro- files of Th-1 and Th-2 related cytokines such as IL-2, IFN-γ, IL-4, and IL-10 were also investigated in *R. anatipestifer*- stimulated DEFs and CEFs using quantitative RT-PCR. mRNA transcript levels of IL-2 in stimulated DEFs were generally in- creased at all time points (4,8 and 24 h) by 1.05- ($p < 0.001$), 0.60- and 0.86-fold, respectively (Fig.4A) while levels of IFN-γ were also upregulated by 0.77- ($p < 0.001$), 0.05- and 0.73-fold at 4, 8 and 24 h, respectively (Fig. 4B) as compared to the un- stimulated cultured DEFs. In addition, IL-4 and IL-10 levels were also generally upregulated and with the same pattern of increase. Compared to the control, IL-4 levels were increased by 1.90- ($p < 0.01$), 1.43- and 2.87-fold ($p < 0.01$) at 4, 8 and 24 h, respectively (Fig. 4C). There were 2.90- ($p < 0.05$), 1.84- ($p < 0.01$) and 3.14-fold changes ($p < 0.05$) in the mRNA expression levels of IL-10 at 4, 8 and 24 h, respectively (Fig. 4D).

The mRNA expression levels of IL-2, IFN-γ, and IL-10 in CEFs were also investigated to determine their expression profiles after *R. anatipestifer* stimulation. It is noteworthy that the expression levels of these cytokines were generally unchanged or slightly increased at 8 h post stimulation only. IL-2 expression levels were upregulated by 2.00- ($p < 0.05$), and 1.85-fold ($p < 0.05$) at 4 and 24 h, respectively but downregulated at 8 h (Fig. 5A). Moreover, IFN-□ levels were unchanged at 4 and 8 h post stimulation. However, expression level at 24 h was significantly downregulated by 0.39-fold ($p < 0.05$) (Fig. 5B). IL-10 levels were significantly increased by 0.65-fold at 8 h and 0.28- fold at 24 h while level at 4 h was unchanged (Fig. 5C).

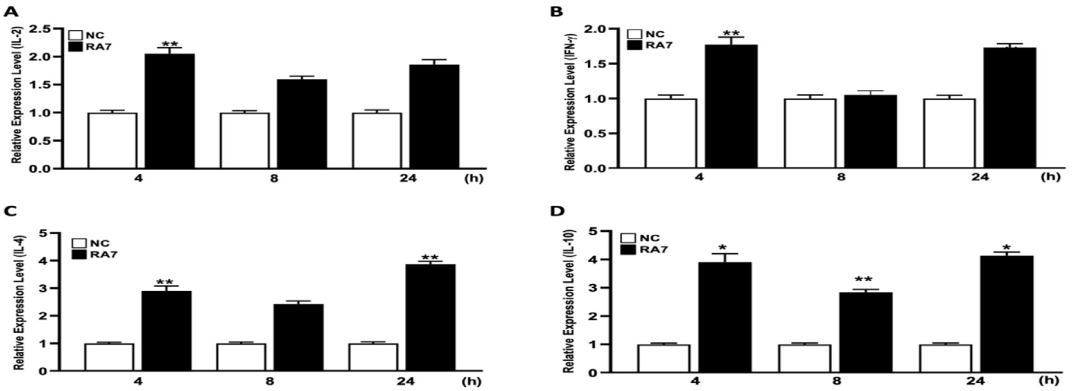


**Fig. 4.** mRNA expression profiles of Th-1 and Th-2 related cytokines in duck embryonic fibroblasts (DEFs). The transcript expression levels of IL-2 (A), IFN-γ (B), IL-4 (C), and IL-10 (D) were normalized with β-actin levels and showed relative to the expression levels of unstimulated/cultured duck embryonic fibroblasts (NC). Results are presented as the mean ± SE values from 2 independent experiments performed in duplicates. $^{**}P < 0.01$ and $^{*}P < 0.05$. RA7: *R. anatipestifer*-stimulated embryonic fibroblasts; h: hours.

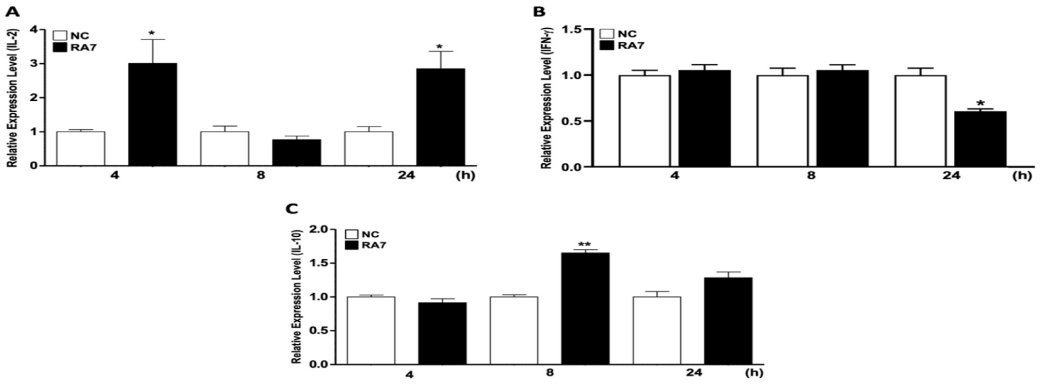


**Fig. 5.** mRNA expression profiles of Th-1 and Th-2 related cytokines in chicken embryonic fibroblasts (CEFs). The transcript expression levels of IL-2 (A), IFN-γ (B), and IL-10 (C) were normalized with β-actin levels and showed relative to the expression levels of unstimulated/cultured duck embryonic fibroblasts (NC). Results are presented as the mean ± SE values from 2 independent experiments performed in duplicates. $^{**}P < 0.01$ and $^{*}P < 0.05$. RA7: *R. anatipestifer*-stimulated embryonic fibroblasts; h: hours.

## 4. Discussion

The avian immune system has been shown to function following the same principles as the mammalian immune system, however, it is still poorly understood as the studies for the avian immune system has lagged its mammalian counterparts [23]. Recent *in vitro* and *in vivo* studies revealed the varying susceptibility of chickens and ducks during *R. anatipestifer* infection, as shown by the differences in their cytokine expression profiles [11, 24] and mortality rate; i.e. 8% mortality rate in chickens [25] in contrast to 75% mortality rate in ducks [26]. However, the immune response of native chickens and ducks, which presumably been reported to be generally resistant to diseases [27], especially against *R. anatipestifer* infection has never been explored. With the minimal understanding of the pathogenesis of this bacterial infection, and the minimal cross-protection between serotypes provided by vaccinations, studies attempting to elucidate the inflammatory cascade would be of great help in mitigating the losses that this disease could cause [28]. The differential expression of Th1-, Th2-, and Th17-related cytokines are critical to the efficacy of the protective immune response mounted against *R. anatipestifer* infection. Therefore, in this study, DEFs and CEFs harvested from Philippine Banaba native chickens and Mallard ducks, respectively, were used to determine the cell viability against *R. anatipestifer* infection and the early immune responses of the adaptive immune system of these two native avian species in the Philippines.

Results showed a high percent cell viability in CEFs and DEFs stimulated with *R. anatipestifer* which indicates that the viability of these cells was not greatly affected after *R. anatipestifer* stimulation. Notably, at 24 hpi, the percent cell viability of transfected DEFs and CEFs was similar or higher than the control group, respectively. This validates the efficacy of the process of heat-inactivation of bacteria that is commonly done in transfection studies prior to bacterial inoculation to observe cellular response [29]. Moreover, since primary culture was used, the relatively lower percent cell viability of DEFs and CEFs in negative control than the heat-inactivated *R. anatipestifer*-stimulated cells might be attributed to senescence and other factors involved such as pH, temperature, and culture medium that might affect cell growth [30]. Nevertheless, results showed that cell viability of CEFs and DEFs were all above 80% which signifies that despite the stimulation with *R. anatipestifer*, viable fibroblasts were still present in the culture to produce the cytokines of interest in this study and thus, the heat-inactivated *R. anatipestifer* did not significantly affect the cell viability of both cells.

Th17 immune responses have been implicated in the pathogenesis of many diseases [31]. Recent studies have shown that Th17 specifically IL-17A is highly implicated in the disease progression of *R. anatipestifer* in ducks [3] while Th2 immune response, as manifested by the upregulation of IL-4, is observed to confer protection in less susceptible birds such as chickens [32]. In this study, Th17 cytokines such as IL-17A, IL-17F, IL-6, and IL-1ß were generally upregulated in *R. anatipestifer*-stimulated DEFs and CEFs. Of these, IL-1β had the highest peak expression. IL-1β is a potent proinflammatory cytokine, exerting protective activity against various bacterial, viral, and fungal infections and is involved in several pathologic manifestations such as in cancer, and neurodegenerative disease [33-34]. Additionally, IL- 1β has been reported to upregulate IL-6 levels in bacterial infections, followed by subsequent C-reactive production in hepatocytes which pro- motes phagocytosis of bacteria during the early immune response [35]. Moreover, it is also involved in several functions such as neutrophil chemotaxis, T cell production and activation, B cell production and activation, and Th17 cell differentiation [25,34]. Furthermore, IL-1β can induce the synthesis of IL-2, which controls T cell activation and proliferation thereby enhancing cell-mediated immunity. It is interesting to note, however, that DEFs expression levels of Th17- related cytokines were considerably higher as compared with the corresponding levels in CEFs stimulated with *R. anatipestifer*. It is also important to note that the cytokine expression profiles observed in *R. anatipestifer*-stimulated DEFs from Philippine Mallard ducks showed similar trends as with previous studies that employed different cells such as $CD4^+$ or Naïve T-helper cells [36]. Specifically, the current data is essentially consistent with our earlier study [3], which found that Th17 cytokines were also markedly elevated in *R. anatipestifer*-stimulated duck splenic lymphocytes and *R.*

*anatipestifer*-infected ducks as compared with chicken splenic lymphocytes stimulated with *R. anatipestifer* and *R. anatipestifer*-infected chickens. Additionally, the results of the recent study by Cammayo-Fletcher et al. (2023) [25], showing absence or unchanged expression levels of IL- 17A and IL-17F in *R. anatipestifer*-stimulated chicken splenic lymphocytes, while IL-6 and IL- 1□ expression levels were upregulated. This could also mean that even during embryonic development, Th17-related cytokines are released and thus can elicit a cascade of inflammatory pathways that may cause pathogenesis as early as oviparity.

The balanced regulation of Th1 and Th2 immune response has been attributed to the degree of susceptibility against an infection, as shown by the upregulation of both pro-inflammatory and anti-inflammatory cytokines [37]. In this study, IFN-γ was significantly down- regulated at 24-hpi, following the significant upregulation of IL-10 at 8-hpi in *R. anatipestifer*-stimulated CEFs. Such findings contrast with mRNA expression of profiles in DEFs stimulated with heat-inactivated *R. anatipestifer* which revealed significant upregulation of IL-10 and IL-4 at all time points and significant upregulation of IL-2 at 4-hpi and IFN-γ at 4-hpi and 24-hpi. These findings suggest differences in immune response against RA infection. The inverse mRNA expression levels of IL-10 and IFN- at 8-hpi and 24-hpi, respectively, revealed an antagonistic relationship between the Th1 and Th2 immune response [38]. While the significant upregulation of IL-1β and IL-2, particularly IL-1β, at 24-hpi suggest a different regulatory pathway independent of Th1 immune response, as IFN-γ, the Th1-skewing cytokine, being significantly downregulated. This agrees with the connection of IL-1β in activating the Th17-immune response while suppressing IFN-γ levels. These data further support the findings of Fernandez *et al.* (2016) [3], revealing strong Th2 immune response rendering chickens less susceptible against *R. anatipestifer* infection. Despite the significant downregulation of IFN-γ following significant upregulation of IL-10, IL-4 is still interesting to be investigated, as it is implicated in the reduced susceptibility in chickens [3] in addition to the immunosuppressive activity of IL-10 towards IL-4 production [39]. In this study, however, it suggests that there is restricted cell-mediated immunity due to the strong Th2 immune response. Given the facultative intracellular nature of *R. anatipestifer*, such fluctuations in cytokine expression fits the picture. While Th1 response activating the cell-mediated immunity to tackle the intracellular bacteria, the Th2 response activates the humoral response to tackle the extracellular bacteria [39] in addition to surge of Th17 pro-inflammatory cytokines to facilitate elimination [34]. Furthermore, fibroblasts have been reported in the cytokine network orchestrating the regulation of Th1, Th2, and Th17 immune responses which are responsible for eliminating non-intracellular pathogens and intracellular pathogens, and promoting inflammation, respectively [40]. These studies further confirm the link between fibroblast and the immune system. Taken together, these findings parallel the findings in the mRNA expression profile of cytokines in T-cell populations [3,24] revealing that ducks and chickens display different susceptibility to *R. anatipestifer* infection, and that ducks are more susceptible as compared to chickens. More importantly, native chickens and native ducks which presumably believed to be relatively resistant to diseases are also susceptible to *R. anatipestifer*.

## 5. Conclusions

In conclusion, the mRNA expression profiles of cytokines measured in this study revealed that there is an increased Th17 immune response in native ducks stimulated with *R. anatipestifer* and elevated Th2 response in native chickens at oviparity despite the partially developed immune system. These results suggest that Th17-related cytokines play a role in the activation of the immune system during the initial phases of pathogen invasion in native ducks. On the other hand, the presence of balanced regulation of Th1 and Th2 immune response as early as oviparity in native chickens, suggest reduced susceptibility against *R. anatipestifer* infection. However, further studies, particularly *in vivo* experiments, must be done to correlate the mRNA expression profiles of cytokines to the pathology that may be observed in both native chickens and native ducks. Overall, the Philippine Banaba native chickens and Mallard ducks are also susceptible to *R. anatipestifer* infection and have different susceptibilities to *R. anatipestifer* infection.

## Availability of Data and Materials

Readers can access all datasets that support conclusions.

## Author Contributions

Conceptualization, C.P.F.C. and M.J.M.D.; Methodology & Investigation, C.P.F.C., M.J.M.D., S.E.A.S., G.K.B.M.T., K.E.M.U., K.V.K., J.J.R.F. R.C.R.L.; Writing – Original Draft, C.P.F.C, G.K.B.M.T., K.E.M.U., K.V.K., J.J.R.F., and R.C.R.L.: Writing – Review & Editing, C.P.F.C, M.J.M.D., and J. J. D. T.; Funding Acquisition, C.P.F.C.; Resources, S.E.A.S., and J.J.D.T.; Supervision, C.P.F.C., M.J.M.D., and S.E.A.S.

## Ethics Approval and Consent to Participate

All the pertinent international, national, and/or institutional guidelines for the care and use of animals were adhered to during the conduct of this study. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (UPLB-2023-011a).

## Acknowledgment

The authors would like to thank Prof. Wongi Min and Prof. Woo Hyun Kim for the avian cytokine primers and *Riemerella anatipestifer* serotype 7 culture that were utilized in this study. The authors would also like to thank Dr. Rochelle A. Flores for the technical expertise provided during the conduct of the study.

## Funding

This work was funded by the UP System Enhanced Creative Work and Research Grant (ECWRG-2021-1-11R).

## Conflict of Interest

The authors declare no conflict of interest.

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