

# Exposure of embryonating eggs to *Enterococcus faecalis* and *Escherichia coli* potentiates *E. coli* pathogenicity and increases mortality of neonatal chickens

Ruwani Karunaratna,\* Khawaja Ashfaque Ahmed,\* Kalhari Goonewardene,\* Thushari Gunawardana,\* Shanika Kurukulasuriya,\* Mengying Liu,\* Ashish Gupta,\* Shelly Popowich,\* Lisanework Ayalew,\* Betty Chow-Lockerbie,\* Philip Willson,<sup>†</sup> Musangu Ngeleka,<sup>‡</sup> and Susantha Gomis \*,<sup>1</sup>

\*Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B4 Canada; <sup>†</sup>Canadian Centre for Health and Safety in Agriculture, University of Saskatchewan, Saskatoon, Canada, SK S7N 5E5, Canada; and <sup>‡</sup>Prairie Diagnostic Services Inc., Saskatoon, SK S7N 5B4, Canada

**ABSTRACT** Enterococci and *Escherichia coli* are opportunistic pathogens of poultry and are associated with embryo and neonatal chick mortality. We have recently demonstrated that 56% of dead broiler chicken embryos in commercial hatcheries in western Canada were due to the coinfection of *Enterococcus* species and *E. coli*. The objective of this study was to investigate the host-pathogen interactions of *Enterococcus faecalis* and *E. coli* in developing chicken embryos. Embryonating eggs at 12 d of incubation were dipped in a solution of *E. faecalis* and/or *E. coli* for 30 s to expose the eggshell to study the migration and colonization of *E. faecalis* and *E. coli* in the internal organs of chicken embryos and subsequent neonatal chicken mortality following hatch. A multidrug-resistant *E. faecalis* isolate from a dead chicken embryo and an *E. faecalis* isolate from a case of yolk sac infection were able to colonize the inter-

nal organs of chicken embryos rapidly compared to an *E. faecalis* isolate from a healthy chicken without affecting viability or hatchability of embryos. Although *E. faecalis* colonized internal organs of chicken embryos, no evidence of inflammation of these organs nor the expression of virulence genes of *E. faecalis* was observed. Although *E. faecalis* and *E. coli* alone did not affect the viability of embryos, a significantly high neonatal chicken mortality (27%) was observed following exposure of embryos to both *E. faecalis* and *E. coli*. Upregulation of IL-1 and CXCR4 was evident 48 h before peak mortality of neonatal chickens; this could suggest a possible link of cytokine dysregulation to increased mortality in coinfecting neonatal chickens. However, further studies are warranted to investigate this issue vis-à-vis coinfection with *E. faecalis* and *E. coli* in chicken embryos and neonatal chickens.

**Key words:** *Enterococcus faecalis*, *Escherichia coli*, coinfection, chicken embryos, neonatal chicken

2022 Poultry Science 101:101983

<https://doi.org/10.1016/j.psj.2022.101983>

## INTRODUCTION

Bacterial infections of embryonating chicken eggs are frequently associated with embryo mortality and clinical disease in neonatal chicks (Berrang et al., 1999; Cortés et al., 2004; Olsen et al., 2012). However, the mechanism of such host-pathogen interactions is unclear. Bacterial colonization of chicken embryos starts early during embryo development, and the composition of bacterial species changes during the incubation of

fertile eggs (Ding et al., 2017). Although various infectious and non-infectious causes lead to chicken embryo mortality, bacterial infections are recognized as the leading cause of embryo and neonatal chick mortality (Al-Sadi et al., 2000; Kalita et al., 2013; Babaca, 2014). Chicken embryo mortality and yolk sac infections of neonatal chickens due to enterococci and *Escherichia coli* are the most common and economically important pathogens. These bacterial infections lead to acute and chronic diseases during the entire growth period of broiler chickens, including increased mortality due to septicemia in neonatal chickens, chronic joint diseases, poor performance, poor feed conversion efficiency, loss of uniformity of the flock, and downgrading and increased condemnations at processing (Jassim et al., 1996; Razmyar and Zamani, 2016).

Historically, *E. coli*-associated yolk sac infections accounted for most embryo death compared to

© 2022 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

**Abbreviations:** BA, Blood Agar; BHI, Brain Heart Infusion; MDR, Multidrug Resistant; SEM, Scanning Electron Microscope; SPF, Specific Pathogen Free; TEM, Transmission Electron Microscope

Received November 5, 2021.

Accepted May 29, 2022.

<sup>1</sup>Corresponding author: [susantha.gomis@usask.ca](mailto:susantha.gomis@usask.ca)

## MATERIALS AND METHODS

### Selection of *Enterococcus* and *E. coli* Isolates

*Enterococcus* species (Babaca, 2014). Avian pathogenic *E. coli* causes extraintestinal tract infections in neonatal broiler chickens, predominantly yolk sac infections, omphalitis, respiratory infections, swollen head syndrome, pericarditis, airsacculitis, perihepatitis, arthritis, and osteomyelitis, septicemia, and cellulitis (Melata, 2013). *Enterococcus* species are the most abundant inhabitant of the normal gastrointestinal flora of chickens (Devriese et al., 1991; Fertner et al., 2011). However, recently, *Enterococcus*-associated yolk sac infections in poultry have become an emerging problem in the poultry industry worldwide, including in Canada. *E. faecalis* has been associated with amyloid arthropathy in table-egg layers, and *E. cecorum*-associated osteomyelitis and spondylitis in broiler chickens and broiler breeder parents were the primary economically important pathogens in the poultry industry (Landman, 1999; Jung and Rautenschlein, 2014). *E. hirae* and *E. durans* have been associated with encephalomalacia and endocarditis in broiler breeders and young broiler chicks with high mortality (Abe et al., 2006).

Enterococci are opportunistic pathogens and cause nosocomial infections in humans, such as bacteremia, septicemia, valvular endocarditis, urinary tract infections, and intra-abdominal and pelvic infections (Moellering Jr, 1992). Among different *Enterococcus* species, *E. faecalis*, and *E. faecium* are responsible for the majority of infections in people (Noskin et al., 1995). Therapeutic failures associated with multidrug resistant (MDR) enterococci, such as vancomycin-resistant enterococci, cause high mortality among immunocompromised and debilitated patients (Noskin et al., 1995; Jean et al., 2001; Arias and Murray, 2012). In polymicrobial infections, synergistic interactions between various microorganisms are involved in causing diseases in the host (Hughes and Winter, 2016). Studies of wound infections in a mouse model suggest that *E. faecalis* promotes *E. coli* biofilm formation under low-iron availability, thus facilitating polymicrobial infections (Hughes and Winter, 2016).

We have recently reported that *Enterococcus* species, followed by *E. coli* were the predominant species isolated from dead chicken embryos in poultry hatcheries in western Canada (Karunaratna et al., 2017). Furthermore, we found that 56% of *E. coli* positive dead embryos had coinfection with *Enterococcus* species suggesting a potential synergism between *Enterococcus* species and *E. coli* that may increase embryo mortality (Karunaratna et al., 2017). A recent study reported that coinfection of chicken embryos with *Enterococcus* species and *E. coli* contributes to the development and increased severity of colibacillosis and enhanced embryo death (Walker et al., 2020a). Despite recent advances, whether *E. faecalis* and *E. coli* coinfections of chicken embryos could increase neonatal chicken mortality remains to be investigated. Therefore, the objective of this study was to mimic embryonic coinfections with *E. faecalis* and *E. coli* and examine their impact on posthatch mortality in neonatal chicks.

Three *E. faecalis* isolates were used in these experiments. The first isolate was recovered from the rectum of a healthy 32-wk-old broiler breeder chicken and was used as the control isolate. This isolate was resistant to tylosin. The second isolate was recovered from the yolk sac of an early dead embryo from a broiler hatchery (Karunaratna et al., 2017) and determined to be multi-drug resistant (MDR) to bacitracin, ceftiofur, erythromycin, lincomycin, neomycin, tetracycline, triple sulfa, and tylosin. The third isolate was recovered from the yolk sac of a 3-day-old neonatal broiler chicken that died of a yolk sac infection. This field isolate was resistant to lincomycin and tylosin.

A field isolate of *E. coli* from a turkey with septicemia was used as previously described. This isolate belongs to serogroup O2, is nonhemolytic, serum-resistant, produces aerobactin, K1 capsule, and Type 1 pili (Gunawardana et al., 2014).

### Preparation of *E. faecalis* and *E. coli* for Experimental Challenge

Each isolate of *E. faecalis* was streaked on 5% Columbia sheep blood agar (BA) (Oxoid, Nepean, Ontario, Canada) and incubated aerobically at 37°C for 24 h. A single colony was inoculated in 100 mL Todd Hewitt broth (THB) and incubated at 37°C for 12 to 13 h on a shaker. According to growth curve analysis, each *E. faecalis* isolate reached  $1 \times 10^9$  colony forming units (cfu)/mL at the end of the incubation period. Following incubation, a 1:100 dilution was made in THB, then incubated at 37°C for 4 h on a shaker to bring *E. faecalis* to the logarithmically growing phase at the concentration of  $1 \times 10^9$  cfu/mL.

*E. coli* was streaked on 5% BA (Oxoid, Nepean, Ontario, Canada) and incubated aerobically at 37°C for 24 h. A single colony of *E. coli* was inoculated in 100 mL of Luria broth (LB) (Difco LB broth Miller; Becton, Dickinson, and Company, Sparks, MD) in a 250 mL Erlenmeyer flask. The culture was grown at 37°C for 12 h, shaking at 150 rpm. This stationary phase culture contained approximately  $1 \times 10^9$  cfu/mL.

For *E. faecalis* and *E. coli* coinfection experiments, 1.5 L of THB containing *E. faecalis* at the required concentration was mixed with 1.5 L of THB containing *E. coli* with corresponding concentration according to each experiment. *E. faecalis* and *E. coli* culture preparations were cooled to 10°C and placed in 6 L plastic containers.

Viable bacterial counts of *E. faecalis* or *E. coli* were determined by plating serial dilutions of bacterial cultures in duplicate before and after each experiment on either m-Enterococcus agar or MacConkey agar (Oxoid, Nepean, Ontario, Canada) to enumerate *E. faecalis* or *E. coli*, respectively.

## Ethics Statement

The animal experiment was approved by the University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan and conducted following the guidelines of the Canadian Council on Animal Care.

## EXPERIMENTAL DESIGN

### Experiment A. *E. faecalis* infection, pathology and host cytokine gene expression in chicken embryos

The objectives of this experiment were to explore the migration of enterococci from the eggshell to internal organs, histopathology and electron microscopy of chicken embryos during incubation, expression of virulence factors of enterococci, and expression of host cytokines. The bacterial inoculation was performed using a well-established egg-dipping model (Sauter and Petersen, 1969, 1974; Mayes and Takeballi, 1983; Landman et al., 1999b; Jones et al., 2002; De Reu et al., 2006). An *E. faecalis* incubating egg infection model was used with few modifications (Landman et al., 1999a). Briefly, a temperature gradient was maintained between specific-pathogen-free (SPF) (Canadian Food Inspection Agency, Nepean, ON, Canada) incubating eggs at 12 d of incubation (37°C) and bacterial broth (10°C) to facilitate entry of *E. faecalis* into incubating eggs. Eggs were candled to determine embryo viability prior to *E. faecalis* infection. Viable eggs were divided into 5 groups (n = 60) as (1) nondip; (2) THB dip; (3) control *E. faecalis*; (4) MDR *E. faecalis*; and (5) field *E. faecalis*. Three liters of the logarithmically growing phase of *E. faecalis* at  $1 \times 10^9$  cfu/mL were prepared for each *E. faecalis* isolate and maintained at 10°C during the experiment. Eggs from each group were immersed in the respective bacterial solution for 30 s and held at room temperature to air dry. All eggs were placed in incubators until they hatched. Swab samples were taken from the outer shell, inner shell, shell membrane, amnion at 6 and 8 d postinfection; additional swabs were collected from the intestine, liver, lung, and yolk at 48 h, 6, and 8 d postinfection from 5 viable embryos per group at each time point. The presence of bacteria and bacterial counts were enumerated from the direct culture method. Tissue samples from the intestine and liver were collected from 3 live embryos at 6 d postinfection for total RNA extraction to detect the expression of host cytokines (IL-1 $\beta$  and IL-8) and chemokines (CXCR4 and MIP-1 $\alpha$ ). The expression levels of putative virulence genes of *E. faecalis* (*agg*, *gelE*, *cylM*, *cylB*, *cylA*, *efaAfs*, *cob*, and *ccf*) were studied using samples from the intestine, liver, and yolk collected at 48 h, 6, and 8 d postinfection. Yolk, liver, and lung samples were collected at 6, and 8 d postinfection for histopathology. Yolk samples were collected from 5 live embryos at 6 d postinfection for electron microscopy.

### Experiment B. *E. faecalis* and *E. coli* coinfection of chicken embryos and subsequent mortality and pathology in neonatal chickens

The objectives of this experiment were to explore the colonization of *E. faecalis* and *E. coli* in chicken embryos and subsequent mortality and pathology in neonatal chickens. The MDR *E. faecalis* isolate obtained from the yolk sac of an early dead embryo and the *E. coli* isolate described above were used in this experiment. Viable SPF eggs at 12 d of incubation were divided into 9 groups (n = 60) as (1) nondip; (2) THB dip; (3) *E. faecalis* ( $1 \times 10^9$  cfu/mL); (4) *E. coli* ( $1 \times 10^9$  cfu/mL); (5) *E. coli* ( $1 \times 10^6$  cfu/mL); (6) *E. coli* ( $1 \times 10^3$  cfu/mL); (7) *E. faecalis* and *E. coli* ( $1 \times 10^9$  and  $1 \times 10^3$  cfu/mL); (8) *E. faecalis* and *E. coli* ( $1 \times 10^9$  and  $1 \times 10^6$  cfu/mL); and (9) *E. faecalis* and *E. coli* ( $1 \times 10^9$  and  $1 \times 10^9$  cfu/mL). Incubating SPF eggs were exposed to a logarithmically growing phase of *E. faecalis*, and a stationary phase of *E. coli* as described in experiment A. Bacterial swabs were taken from the yolk at 48 hr, 6, and 8 d postinfection from 5 viable embryos per group at each time point. Following hatch, neonatal chicks from five groups [nondip, *E. faecalis* alone ( $1 \times 10^9$  cfu/mL), *E. coli* alone ( $1 \times 10^9$  cfu/mL), *E. faecalis* together with *E. coli* ( $1 \times 10^9$  and  $1 \times 10^3$  cfu/mL) and *E. faecalis* together with *E. coli* ( $1 \times 10^9$  and  $1 \times 10^6$  cfu/mL)] (n = 15/group) were monitored for clinical signs and mortality for one week following hatch. Tissues from the yolk sac, liver, lung, and heart from any dead chicks following hatch were collected for histopathology.

### Experiment C. *E. faecalis* and *E. coli* coinfection in chicken embryos and subsequent mortality and host cytokine gene expression in embryos and neonatal chickens

The objectives of this experiment were to explore host cytokine gene expression of chicken embryos and neonatal chickens and neonatal chicken mortality following exposure of *E. faecalis* and *E. coli* to chicken embryos. The MDR *E. faecalis* isolate and *E. coli* were used in this experiment as in experiment B. Viable SPF eggs were exposed to *E. faecalis* and *E. coli* as in experiment B, at 12 d of incubation (n = 100) as (1) nondip; (2) *E. faecalis* alone ( $1 \times 10^9$  cfu/mL); (3) *E. coli* alone ( $1 \times 10^6$  cfu/mL) and (4) *E. faecalis* and *E. coli* ( $1 \times 10^9$  and  $1 \times 10^6$  cfu/mL). Following hatch, neonatal chicks (n = 40/group) were monitored for two weeks for clinical signs and mortality. Tissue samples from the lungs were collected from 3 live embryos and 3 live chicks at 48 hr, 6, and 8 d postinfection, and 3 and 10 d posthatch to obtain total RNA for host cytokine (IL- $\beta$ ) and chemokine (CXCR4) gene expression.

## Bacterial Isolation and Identification from Embryos and Neonatal Chickens

Swab samples were plated on 5% Columbia sheep BA, m-Enterococcus and MacConkey agar and incubated at 37°C for 24 to 48 h. Semiquantitative analyses on plates were conducted on a scale from 0 to 4+ (Gunawardana et al., 2014). To study bacterial growth in enriched cultures, swabs were inoculated in 3 mL of THB and incubated overnight at 37°C in a shaking incubator. Bacterial isolates were identified using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) as previously described (van Veen et al., 2010). The antimicrobial susceptibility profile of 12 antimicrobials (i.e., ampicillin, bacitracin, chloramphenicol, enrofloxacin, erythromycin, florfenicol, gentamycin, neomycin, penicillin, spectinomycin, trimethoprim-sulfamethoxazole, and tylosin) were conducted using the disk diffusion method (Kirby Bauer), interpretation of results were done according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2013). Antibigrams of input and output pools of bacterial isolates were compared at each time point and organ (2 isolates/group/time point) to identify the bacteria recovered from embryos to confirm the causality.

## Histopathological Examination

Histopathology of the yolk sac, liver, and lungs was examined from 3 live embryos per group at 6 and 8 d postinfection from experiment A. The yolk sac, liver, lung, and heart from any dead chicks following hatch were collected from the experiment B. Tissue sections were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned in 5 µm, and stained with hematoxylin and eosin (H&E) and Gram stain.

## Scanning Electron Microscopy and Transmission Electron Microscopy

Electron microscopy of yolk sacs of embryos from all the groups was performed 6 d after the *E. faecalis* infection (experiment A). Tissue sections for transmission electron microscopy (TEM) were treated with 1% osmium tetroxide, then dehydrated through a graded ethanol series to propylene oxide and subsequently infiltrated with epoxy resin by a gradual exchange. The blocked specimen was polymerized at 60°C overnight. Blocks were sectioned to 90 nm on a Leica Ultracut ultramicrotome and mounted on 200 mesh copper grids. Images were collected using the Hitachi HT7700 TEM. Scanning electron microscopy (SEM) samples were paraffin-embedded and affixed to glass slides by baking at 60°C. Samples were dewaxed in xylene and rinsed in 100% ethanol, then sputter-coated with 10 nm of gold. Images were collected using the Hitachi SU8010.

## Molecular Screening of Putative Virulence Determinants of *E. faecalis*

Total genomic DNA from *E. faecalis* isolates was extracted using Qiagen kit–DNeasy. Blood and tissue kits were used according to the manufacturer's instructions. Extracted DNA sample concentrations were determined by spectrophotometry at the wavelength of 260 nm and 280 nm. Virulence gene targets and polymerase chain reaction (PCR) primers are listed in Table 1. Primers were designed as previously described (Özmen Toğay et al., 2010; Yildiz and Turkyilmaz, 2015). All PCR reactions were carried out in a final volume of 50 µL reaction mixture containing 100 ng of DNA, 10X PCR buffer, 3.5 mM MgCl<sub>2</sub>, 0.4 mM each of the four dNTPs (Fermentas, Rockville, Maryland, USA), 0.8 µM of each primer and 2 units of Taq DNA polymerase (Fermentas, Rockville, Maryland, USA). Samples were subjected to an initial cycle of denaturation (95°C for 5 min), annealing (at an appropriate temperature for 30 s; Table 1), and elongation (72°C for 1 min), followed by 35 cycles in the thermocycler. Reference strain *E. faecalis* ATCC 29212 was used as a positive control. The amplification products were analyzed by electrophoresis on 1.0% agarose gel at 100 V for 40 min in Tris-acetate-EDTA buffer and revealed in ethidium bromide (20 µg/mL).

## RT-qPCR Based Determination of Putative Virulence Gene Expression in *E. faecalis* During Embryo Infection

Intestine and liver samples were collected in 500 µL RNAlater from 3 live embryos per group at 48 h, 6, and 8 d postinfection from the experiment A. Total RNA was extracted using Qiagen RNeasy Mini Kit according to the manufacturer's instructions. cDNA was prepared

**Table 1.** Oligonucleotide primers used to amplify putative virulence determinants (*Experiment A: E. faecalis* infection, pathology, and host cytokine gene expression in chicken embryos).

Virulence gene	Primer(5-3)	Product size (bp)	Annealing temp (°C)
<i>Ragg</i> -F	AAGAAAAAGAAGTAGACCAAC	1553	55
<i>Ragg</i> -R	AAACGGCAAGACAAGTAAATA		
<i>RgelE</i> -F	ACCCCGTATCATTGGTTT	419	55
<i>RgelE</i> -R	ACGCATTGCTTTTCCATC		
<i>RcylM</i> -F	CTGATGGAAAGAAGATAGTAT	742	53
<i>RcylM</i> -R	TGAGTTGGTCTGATTACATTT		
<i>RcylB</i> -F	ATTCTACCTATGTTCTGTTA	843	53
<i>RcylB</i> -R	AATAAACTCTTCTTTCCAAC		
<i>RcylA</i> -F	TGGATGATAGTGATAGGAAGT	517	53
<i>RcylA</i> -R	TCTACAGTAAATCTTTCGTCA		
<i>RefA</i> <i>Afs</i> -F	GACAGACCCTCACGAATA	705	53
<i>RefA</i> <i>Afs</i> -R	AGTTCATCATGCTGTAGTA		
<i>Rcob</i> -F	AACATTCAGCAAACAAAGC	1405	53
<i>Rcob</i> -R	TTGTCATAAAGAGTGGTCAT		
<i>Rccf</i> -F	GGGAATTGAGTAGTGAAGAAG	543	53
<i>Rccf</i> -R	AGCCGCTAAAATCGGTTAAAT		

using QuantiTect Reverse Transcription kit according to the manufacturer's instructions using random primers. Amplification, detection, and real-time analysis were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Waltham, Massachusetts, USA). SYBR Green I (Applied Biosystems, Waltham, Massachusetts, USA) was used for the detection of the amplified product. Primers were selected for virulence gene expression level analysis as previously described (Shepard and Gilmore, 2002). Amplification was carried out in a total volume of 20  $\mu$ L containing 0.5  $\times$  SYBR Green master mix, and 2  $\mu$ L of 1:5 diluted cDNA. The reactions were cycled 40 times under the following parameters: 95 C for 5 min initial cycle of denaturation followed by 95°C for 5 min denaturation, 60°C for 20 s annealing, 72°C for 30 s extension steps. At the end of the PCR, the temperature was increased from 60 to 96°C at a rate of 0.5°C/min, and the fluorescence was measured every 5 s to construct the melting curve. 23S rRNA gene was used as the positive control and primers were used as previously reported (Shepard and Gilmore, 2002). A nontemplate control (NTC) was run with every assay, and all determinations were performed at least in duplicates to demonstrate reproducibility.

### Determination of Host Cytokine Gene Expression in Chicken Embryos Following *E. faecalis* and *E. coli* Infection

Total RNA extraction and cDNA synthesis were conducted as described above. Host cytokine gene expression was determined in the intestine and liver for experiment A and the lung for experiment C using real-time PCR (Mx3000P qPCR system, Agilent Technologies, Santa Clara, California, USA) and TaqMan probes. Host cytokine gene expression was conducted at 48 h, 6, and 8-d postinfection in embryos and 3 and 10 d following hatch in experiment C. The respective primer-probes and 18S rRNA amplification in the same tube (20  $\mu$ L

**Table 2.** Primers and probes for host cytokine gene expression detection by RT-qPCR (Experiment A: *E. faecalis* infection, pathology, and host cytokine gene expression in chicken embryos and Experiment C: *E. faecalis* and *E. coli* coinfection in chicken embryos and subsequent mortality and host cytokine gene expression in embryos and neonatal chickens).

Primers/probe	Sequence
IL-1 Forward	5-GCTCTACATGTCGTGTGTGATGAG-3
IL-1 Reverse	5-TGTCGATGTCCCGCATGA-3
IL-1 Probe	5-(FAM)-CCACACTGCAGCTGGAG-GAAGCC-(ZEN/IBFQ)-3
IL-8 Forward	5-GCCCTCCTCCTGGTTTCAG-3
IL-8 Reverse	5-TGGCACCAGCTCATT-3
IL-8 Probe	5-(FAM)-TCTTTACCAGCGTCC-TACCTTGCGACA-(ZEN/IBFQ)-3
MIP-1 Forward	5-GGCAGACTACTACGAGACCAACAG-3
MIP-1 Reverse	5-ACGGCCCTTCTGGTGAT-3
MIP-1 Probe	5-(FAM)-ACACAACACCAGCATGAGGG-CACTG-(ZEN/IBFQ)-3
CXCR-4 Forward	5-TGCTGCCTCAATCCAATTCTT-3
CXCR-4 Reverse	5-CAAGGCATTTTGTGCTGATGTT-3
CXCR-4 Probe	5-(FAM)-ACGCCCTTCTGGGTGC-CAAGTTC-(ZEN/IBFQ)-3

total volume) were performed using Prime Time-Gene Expression Master Mix (IDT, Redwood City, California, USA), 2  $\mu$ L of cDNA template, and primers and probes as described in Table 2.

### Statistical Analysis

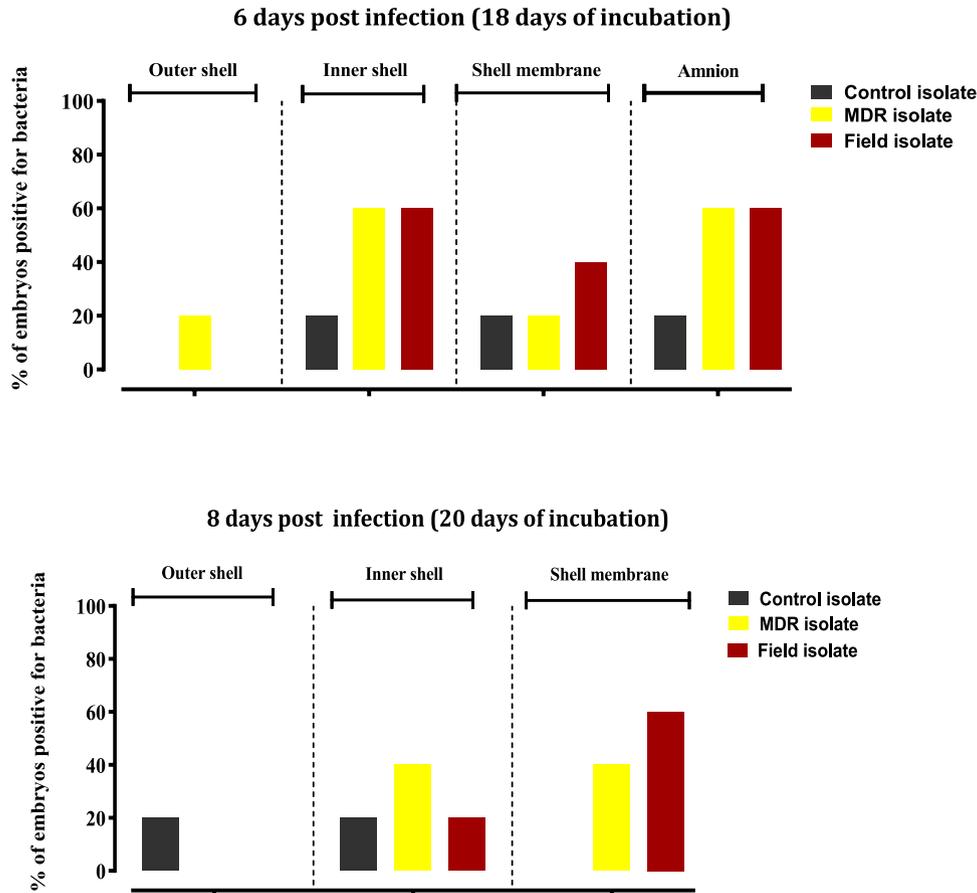
Fisher's exact test was performed to determine the significance of differences in hatchability and mortality among groups. One-way ANOVA was conducted to determine the difference in cytokine and chemokine gene expression among groups.

## RESULTS

### Experiment A. *E. faecalis* infection, pathology, and host cytokine gene expression in chicken embryos

***E. faecalis* egg-shell penetration, and colonization in internal organs of embryos** Hatchability and embryo mortality were conducted between days 12 and 21 of incubation. No significant difference in hatchability or embryo mortality was observed among groups of hatching eggs [hatchability = nondip (85%); THB dip (80%); control *E. faecalis* (79%); MDR *E. faecalis* (94%); and field *E. faecalis* (92%)] ( $P > 0.05$ ). At 6 d postinfection, the MDR *E. faecalis* isolate was recovered from the outer shell (20%), inner shell (60%), shell membrane (20%) and amnion (60%) of embryos. The field *E. faecalis* isolate was recovered from the inner shell (60%), shell membrane (40%) and amnion (60%) while control *E. faecalis* isolate was recovered from the inner shell (20%), shell membrane (20%) and amnion (20%) of embryos (Figure 1). At 8 d postinfection, the MDR *E. faecalis* isolate was recovered from the inner shell (40%) and shell membrane (40%) of incubating eggs. The field *E. faecalis* isolate was recovered from the inner shell (20%) and shell membrane (60%) while control *E. faecalis* isolate was recovered from the outer shell (20%) and inner shell (20%) of incubating eggs. Swabs were not collected from the amnion at 20 d following incubation since the amniotic fluid was not sufficient in the amniotic cavity on 20 d of incubation (Figure 1).

No *E. faecalis* was isolated from nondip and THB group at any time point. *E. faecalis* load and rate of *E. faecalis* colonization in internal organs following egg infection was demonstrated in Figure 2 by direct culture. At 48 h postinfection, neither of the MDR *E. faecalis* isolate, field or control isolates were recovered from any internal organs of embryos. At 6 d postinfection, the MDR isolate was recovered from the yolk (40%), liver (20%), intestine (20%), and lung (40%), while the control isolate was recovered only from the yolk (20%). The field isolate was not recovered from any internal organs. At 8 d postinfection, the MDR isolate was recovered from the yolk (20%), while



**Figure 1.** Isolation of *E. faecalis* from the surface of the eggshell, inside of the eggshell and shell membrane at 6 and 8 days following exposure of incubating eggs to *E. faecalis*. (Amnion was not collected at 20 days following incubation since amniotic fluid was not sufficient for bacterial isolations.) (*Experiment A: E. faecalis* infection, pathology and host cytokine gene expression in chicken embryos).

control *E. faecalis* isolate was not recovered from any internal organs. The field *E. faecalis* isolate was recovered from the yolk (40%), liver (20%), lung (40%), and intestines (20%). The antimicrobial susceptibility profile of the isolate remained unchanged before and after challenge at all time points.

### Histopathology and Electron Microscopy

Histopathologic examination of tissue sections of the yolk, lung, and liver revealed colonization of Gram-positive cocci in the yolk, lung, and liver (Figure 3). There was no evidence of inflammation in any of the tissues in any of the groups exposed to *E. faecalis*. TEM and SEM demonstrated colonization of *E. faecalis* and biofilm-like structures attached to epithelial cells of the yolk sac at 6 d following exposure of MDR *E. faecalis* isolate (Figure 4).

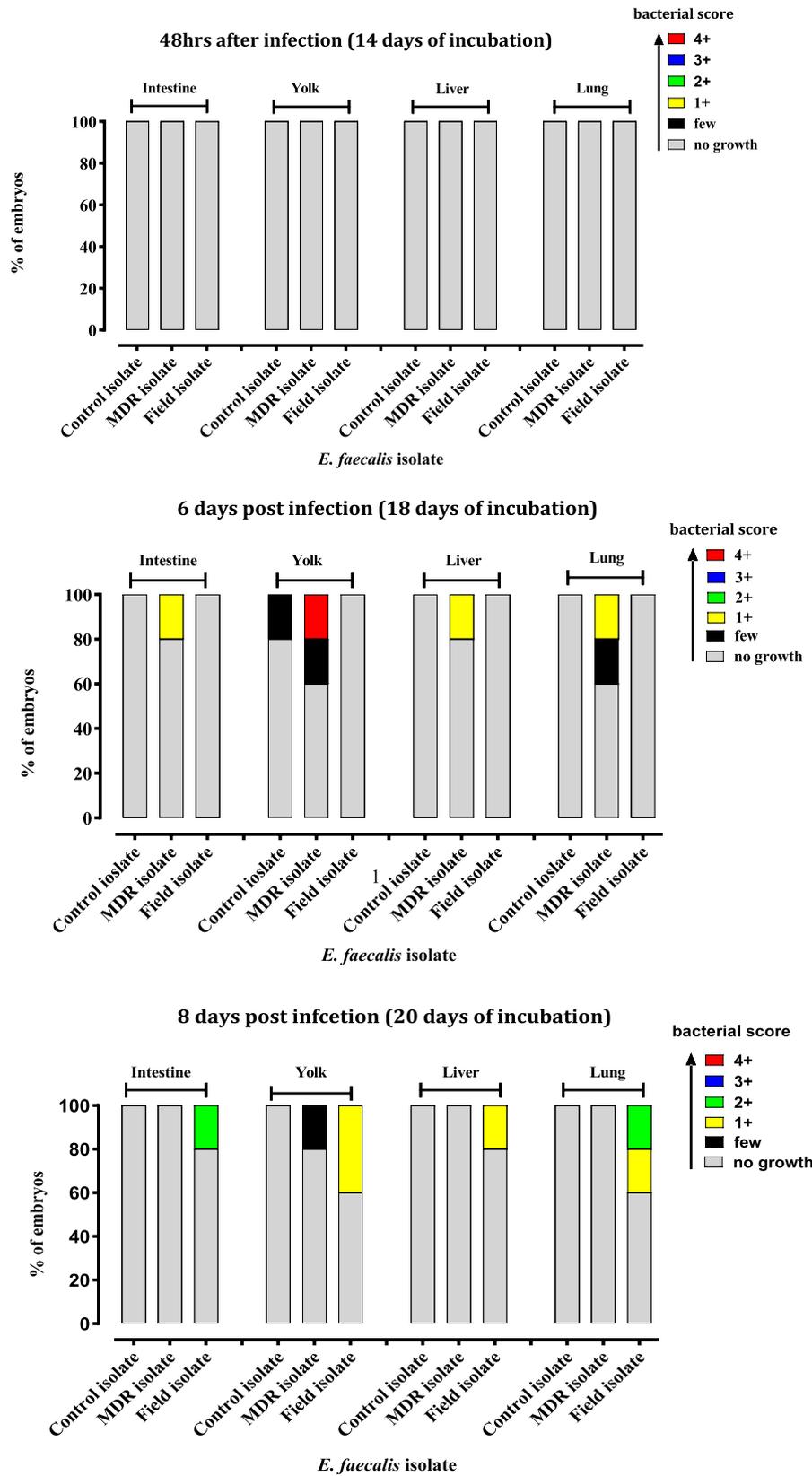
### Expression of Virulence Genes of *E. faecalis* in Chicken Embryos During Egg Incubation

All putative virulence genes (i.e., *agg*, *gelE*, *cylM*, *cylB*, *cylA*, *efaAfs*, *cob*, and *ccf*) were present in all three *E. faecalis* isolates used. The amplified

genomic DNA products of putative virulence genes of MDR *E. faecalis* isolate are shown in Figure 5. The presence of *E. faecalis* in the intestine, liver, and yolk sac from respective groups was confirmed by PCR amplification of *cpn60*. However, the virulence genes of *E. faecalis* were not expressed in any of the three *E. faecalis* isolates recovered from any internal organs from any of the groups during the experiment.

### Host Cytokine and Chemokine Gene Expression in Chicken Embryos Following *E. faecalis* Infection

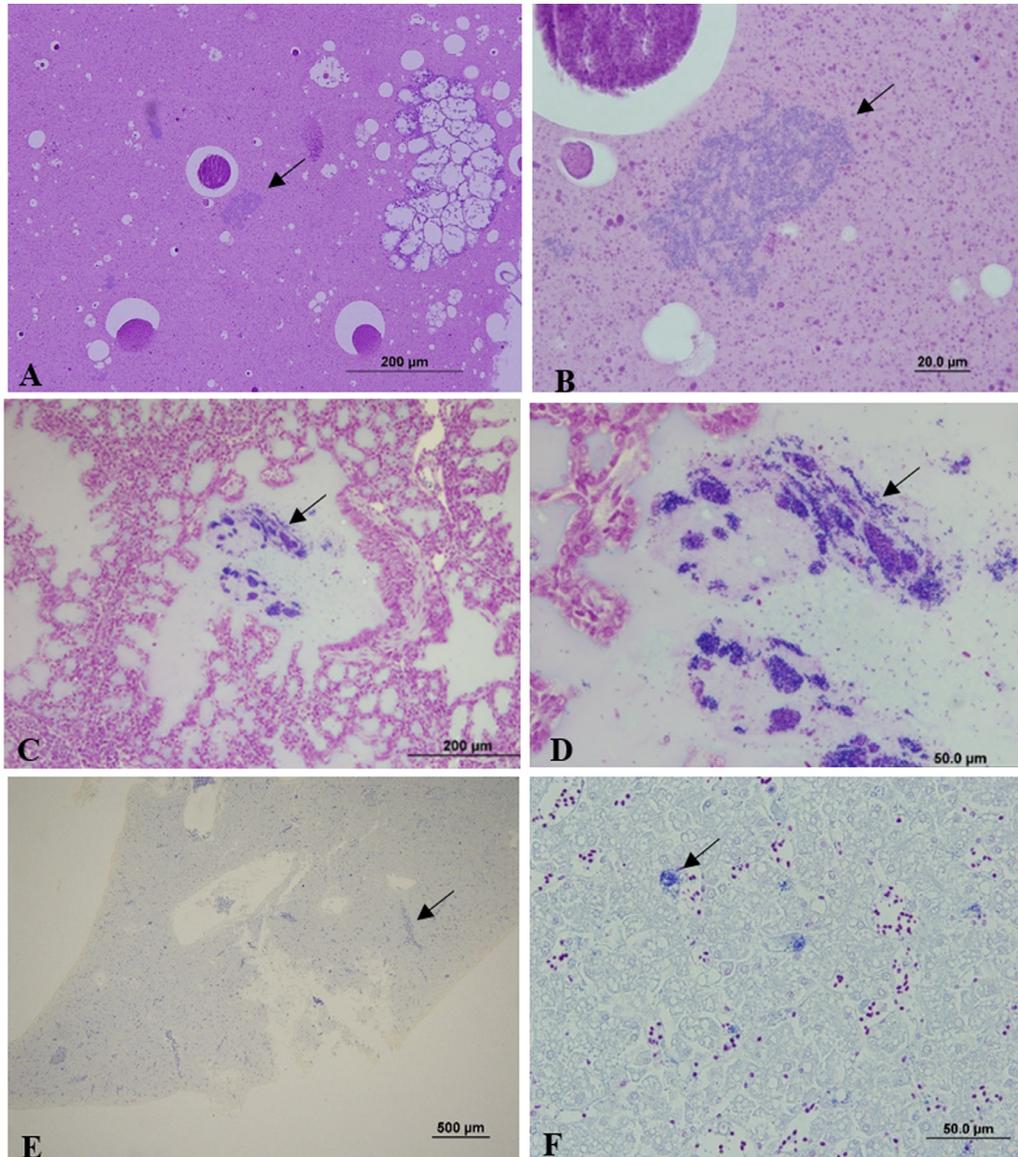
The housekeeping gene, 18S rRNA, was stable across all the time points and all samples tested. Upregulation of IL-1 $\beta$ , IL-8, CXCR4, and MIP-1 $\alpha$  was significantly high in the intestines of embryos infected with MDR *E. faecalis* isolate at 6 d post-infection ( $P < 0.01$ ). In contrast, no significant upregulation or downregulation of IL-1 $\beta$ , IL-8, CXCR4, and MIP-1 $\alpha$  were noted in the intestine of chicken embryos infected with either field *E. faecalis* isolate or control *E. faecalis* isolate ( $P > 0.05$ ) (Figure 6). Upregulation of IL-1 $\beta$  and CXCR4 was significantly high in the liver of embryos infected with MDR



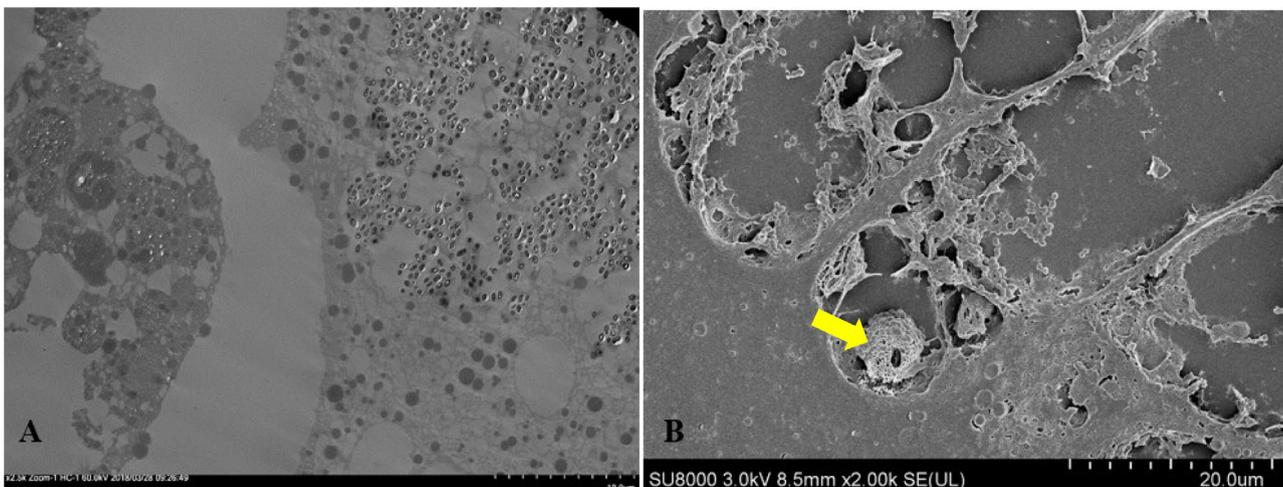
**Figure 2.** Isolation of *E. faecalis* from the yolk, intestine, liver and lung following 48 h, 6, and 8 d after exposure of incubating eggs to *E. faecalis*. (*E. faecalis* isolation was conducted in m-Enterococcus agar). Rate of systemic colonization of MDR *E. faecalis* was higher compared to other *E. faecalis* isolates. The field isolate of *E. faecalis* was detected predominantly at 8 days postinfection from all organs. (*Experiment A: E. faecalis* infection, pathology and host cytokine gene expression in chicken embryos).

*E. faecalis* isolate at 6 d postinfection ( $P < 0.05$ ). Upregulation of CXCR4 was significantly high in the liver of embryos infected with field *E. faecalis* isolate at 6 d postinfection ( $P < 0.05$ ). In contrast, no

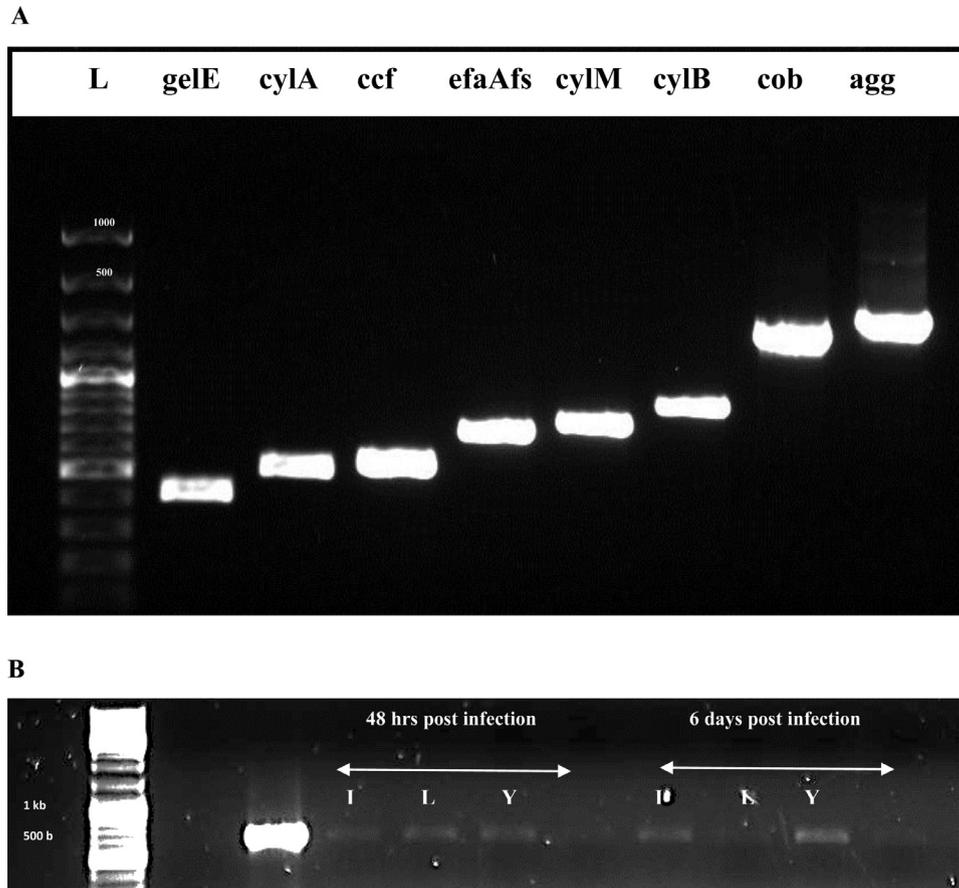
significant upregulation or downregulation of IL-1 $\beta$ , IL-8, CXCR4, and MIP-1 $\alpha$  were noted in the liver of chicken embryos infected with control *E. faecalis* isolate ( $P > 0.05$ ) (Figure 7).



**Figure 3.** Gram and H&E stained sections of yolk, lung and liver 6 days following infection with MDR *E. faecalis*. Aggregates of gram positive cocci (black arrows) in the yolk, lung and liver. (A; x20 and B; x100= yolk; C; x20 and D; x60 = lung; E; x20 and F; x60 = liver) (*Experiment A: E. faecalis* infection, pathology and host cytokine gene expression in chicken embryos).



**Figure 4.** Electron microscopic demonstration of MDR *E. faecalis* in the yolk sac 6 days following exposure to *E. faecalis*. TEM image (A) = ( $\times 2,000$ ,  $\times 8,000$  magnification) illustrates heavy colonization of *E. faecalis* in the yolk sac. SEM image (B) = ( $\times 2,000$ ,  $\times 8,000$  magnification) illustrates biofilm like structure of *E. faecalis* attached to epithelial cells of yolk sac (Yellow arrow). (*Experiment A: E. faecalis* infection, pathology and host cytokine gene expression in chicken embryos).



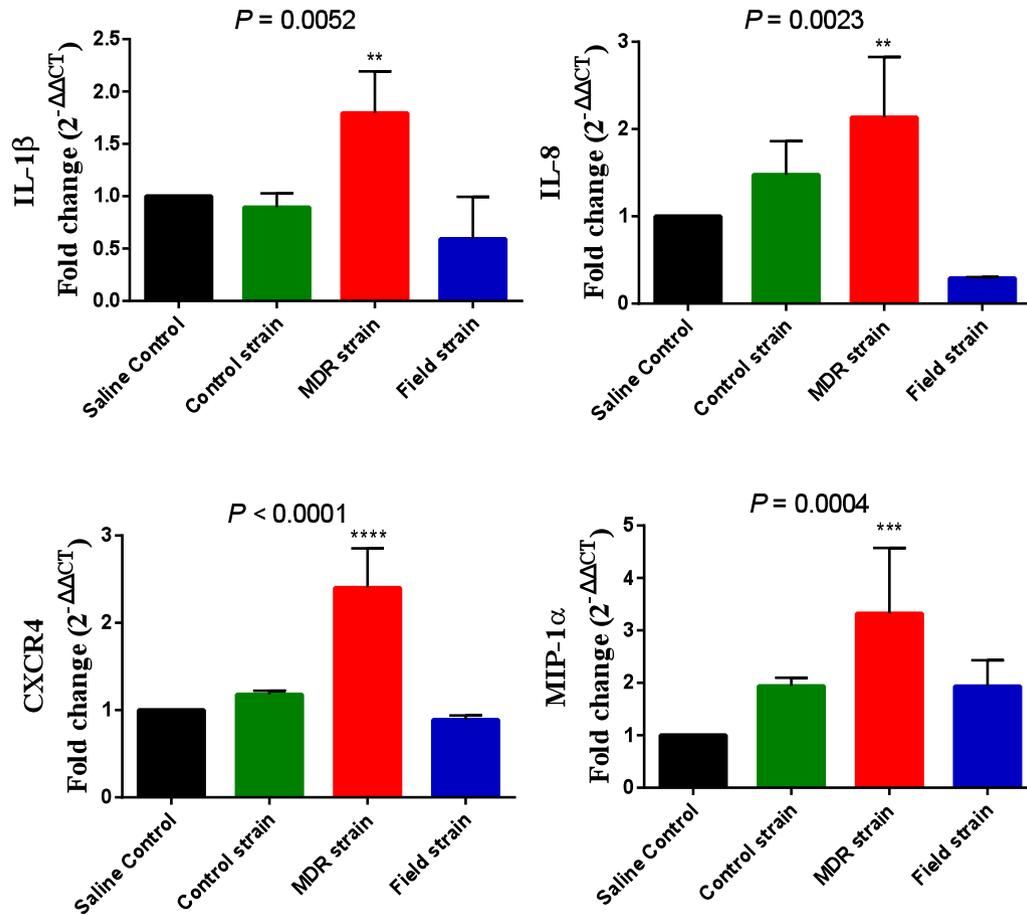
**Figure 5.** (A) Amplified genomic DNA products of putative virulence genes of MDR *E. faecalis* isolate. (B) Amplified genomic DNA products of putative virulence genes of MDR *E. faecalis* isolate (A). Amplified *cpn60* gene target from cDNA obtained from intestine (I), liver (L), and yolk (Y) at 48 h and 6 d post infection. (*Experiment A: E. faecalis* infection, pathology and host cytokine gene expression in chicken embryos).

### **Experiment B. *E. faecalis* and *E. coli* coinfection of chicken embryos and subsequent mortality and pathology in neonatal chickens**

***E. faecalis* or *E. coli* isolation from the yolk of chicken embryos exposed to *E. faecalis* and/or *E. coli*** There was no significant difference in hatchability or embryo mortality among groups of chicken embryos exposed to *E. faecalis* and *E. coli*, THB or the group not exposed to bacteria [hatchability = nondip (71%); THB dip (62%); *E. faecalis* ( $1 \times 10^9$  cfu/mL) (65%); *E. coli* ( $1 \times 10^9$  cfu/mL) (85%); *E. coli* ( $1 \times 10^6$  cfu/mL) (82%); *E. coli* ( $1 \times 10^3$  cfu/mL) (58%); *E. faecalis* and *E. coli* ( $1 \times 10^9$  and  $1 \times 10^3$  cfu/mL) (95%); *E. faecalis* and *E. coli* ( $1 \times 10^9$  and  $1 \times 10^6$  cfu/mL) (83%); and *E. faecalis* and *E. coli* ( $1 \times 10^9$  and  $1 \times 10^9$  cfu/mL) (81%)] ( $P > 0.05$ ). Colonization of *E. faecalis* and/or *E. coli* in the yolk at different time points during the incubation period is shown in Figure 8. At 48 h postinfection, *E. coli* was isolated from 20% ( $1 \times 10^6$  *E. coli* cfu/mL), 20% ( $1 \times 10^9$  *E. coli* cfu/mL), 20% ( $1 \times 10^9$  *E. faecalis* and  $1 \times 10^6$  *E. coli* cfu/mL), and 20% ( $1 \times 10^9$  *E. faecalis* and  $1 \times 10^9$  *E. coli* cfu/mL) of embryos. *E. faecalis* was isolated from 40% ( $1 \times 10^9$

*E. faecalis* and  $1 \times 10^6$  *E. coli* cfu/mL) of embryos. At 6 d postinfection, no *E. coli* was isolated from *E. coli* alone groups or in combination with *E. faecalis*. *E. faecalis* was isolated from 60% ( $1 \times 10^9$  *E. faecalis* cfu/mL) and 20% ( $1 \times 10^9$  *E. faecalis* and  $1 \times 10^6$  cfu/mL) of embryos. At 8 d postinfection, *E. coli* was isolated from 20% ( $1 \times 10^3$  *E. coli* cfu/mL), 100% ( $1 \times 10^6$  *E. coli* cfu/mL), 20% ( $1 \times 10^9$  *E. coli* cfu/mL), 20% ( $1 \times 10^9$  *E. faecalis* and  $1 \times 10^6$  *E. coli* cfu/mL) and 40% ( $1 \times 10^9$  *E. faecalis* and  $1 \times 10^9$  *E. coli* cfu/mL) of embryos. *E. faecalis* was isolated from 20% ( $1 \times 10^9$  *E. faecalis* and  $1 \times 10^6$  *E. coli* cfu/mL) and 20% ( $1 \times 10^9$  *E. faecalis* and  $1 \times 10^9$  *E. coli* cfu/mL) of embryos. No bacteria were isolated from groups not exposed to bacteria at any time point.

Percentage of bacterial recovery from the shell membrane, amnion, and yolk resulting from enrichment culture were shown in Table 3. The group of embryos exposed to  $1 \times 10^9$  *E. faecalis* cfu/mL had 0%, 40%, and 20% *E. faecalis* recovery rate from the shell membrane at 48 h, 6 d, and 8 d respectively. Similarly, the rate of recovery of *E. faecalis* from the yolk was 0%, 80%, and 80% at 48 h, 6 d, and 8 d, respectively, while rate of *E. faecalis* recovery from the amnion was 0% and 40% at 48 h and 6 d, respectively.



**Figure 6.** Cytokine and chemokine gene expression in the intestines at 6 d postinfection with *E. faecalis*. Upregulation of IL- $\beta$ , IL-8, CXCR4, and MIP-1 $\alpha$  was significantly high in the intestine of embryos infected with MDR *E. faecalis* at 6 d postinfection ( $P < 0.01$ ) ( $n = 3$ ). (*Experiment A: E. faecalis* infection, pathology and host cytokine gene expression in chicken embryos).

The embryos exposed to  $1 \times 10^3$  *E. coli* cfu/mL had no bacterial recovery from the shell membrane, amnion, and yolk from any time point except 20% *E. coli* recovery was obtained from the yolk at 8 d. The group of embryos exposed to  $1 \times 10^6$  *E. coli* cfu/mL had 0%, 40%, and 80% *E. coli* recovery rate from the shell membrane at 48 h, 6 d, and 8 d respectively. Similarly, rate of recovery of *E. coli* from the yolk was 20%, 20%, and 100% at 48 h, 6 d, and 8 d, respectively while rate of *E. coli* recovery from the amnion was 20% and 20% at 48 h and 6 d, respectively. The group of embryos exposed to  $1 \times 10^9$  *E. coli* cfu/mL had 0%, 20%, and 40% *E. coli* recovery rates from the shell membrane at 48 h, 6 d, and 8 d, respectively. Similarly, rates of recovery of *E. coli* from the yolk were 20%, 20%, and 60% at 48 h, 6 d, and 8 d, respectively, while rates of *E. coli* recovery from the amnion were 20% and 0% at 48 h and 6 d, respectively.

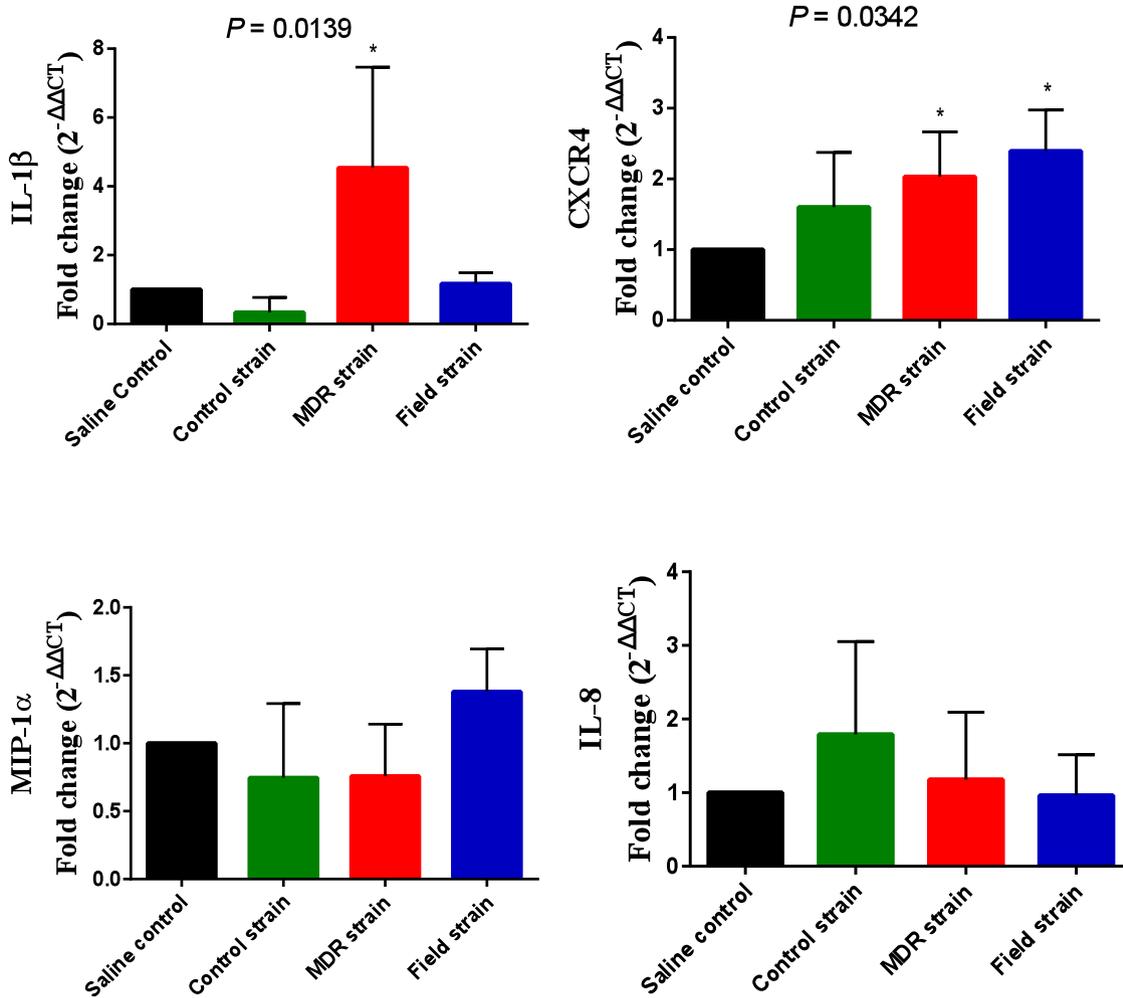
The embryos coinfecting with  $1 \times 10^9$  *E. faecalis* and  $1 \times 10^3$  *E. coli* cfu/mL had 40%, 60% and 60% *E. faecalis* and 0%, 0%, and 20% *E. coli* recovery from the shell membrane at 48 h, 6 d, and 8 d, respectively. Similarly, rates of recovery of bacteria from the yolk were 40%, 60% and 60% *E. faecalis* and 0%, 0%, and 0% *E. coli* at 48 h, 6 d, and 8 d, respectively, while rates of bacterial recovery from the amnion were 40% and 80% *E. faecalis* and 0% and 0% *E. coli* at 48 h and 6 d, respectively.

The embryos coinfecting with  $1 \times 10^9$  *E. faecalis* and  $1 \times 10^6$  *E. coli* cfu/mL had 20%, 0%, and 0% *E. faecalis* and 0%, 0%, and 20% *E. coli* recovery from the shell membrane at 48 h, 6 d, and 8 d, respectively. Similarly, rates of recovery of bacteria from the yolk were 20%, 40%, and 60% *E. faecalis* and 20%, 20%, and 80% *E. coli* at 48 h, 6 d, and 8 d, respectively. The rate of bacterial recovery from the amnion was 20%, and 0% *E. faecalis* and 20% and 0% *E. coli* at 48 h and 6 d, respectively.

The embryos coinfecting with  $1 \times 10^9$  *E. faecalis* and  $1 \times 10^9$  *E. coli* cfu/mL had 20%, 20%, and 40% *E. faecalis* and 20%, 20%, and 40% *E. coli* recovery from the shell membrane at 48 h, 6 d, and 8 d, respectively. Similarly, rates of recovery of bacteria from the yolk were 20%, 80%, and 20% *E. faecalis* and 20%, 60%, and 20% *E. coli* at 48 h, 6 d and 8 d, respectively, while rates of bacterial recovery from the amnion were 20% and 20% *E. faecalis* and 20% and 40% *E. coli* at 48 h and 6 d, respectively.

### Neonatal Chick Mortality

The highest neonatal chick mortality of 13.33% (2 of 15) was observed in the group of chicken embryos exposed to  $1 \times 10^9$  cfu/mL *E. faecalis* with  $1 \times 10^6$  cfu/mL *E. coli* (Figure 9). The second highest neonatal chick



**Figure 7.** Cytokine and chemokine gene expression in the liver at 6 d postinfection with *E. faecalis*. Upregulation of IL- $\beta$  and CXCR4 were significantly high in the liver of embryos infected with MDR *E. faecalis* at 6 d postinfection ( $P < 0.05$ ) ( $n = 3$ ). (Experiment A: *E. faecalis* infection, pathology and host cytokine gene expression in chicken embryos).

mortality of 6.67% (1 of 15) was observed in the group of chicken embryos exposed to  $1 \times 10^9$  cfu/mL *E. faecalis* with  $1 \times 10^3$  cfu/mL *E. coli*. Neonatal chick mortality of 6.67% (1 of 15) was also observed in the group of chicken embryos exposed to  $1 \times 10^9$  cfu/mL *E. coli*. No clinical signs or mortality was observed in any of the other groups of chicken embryos exposed to *E. faecalis* or *E. coli*.

### Gross and Histopathological Lesions of Neonatal Chickens that Died Following Hatch

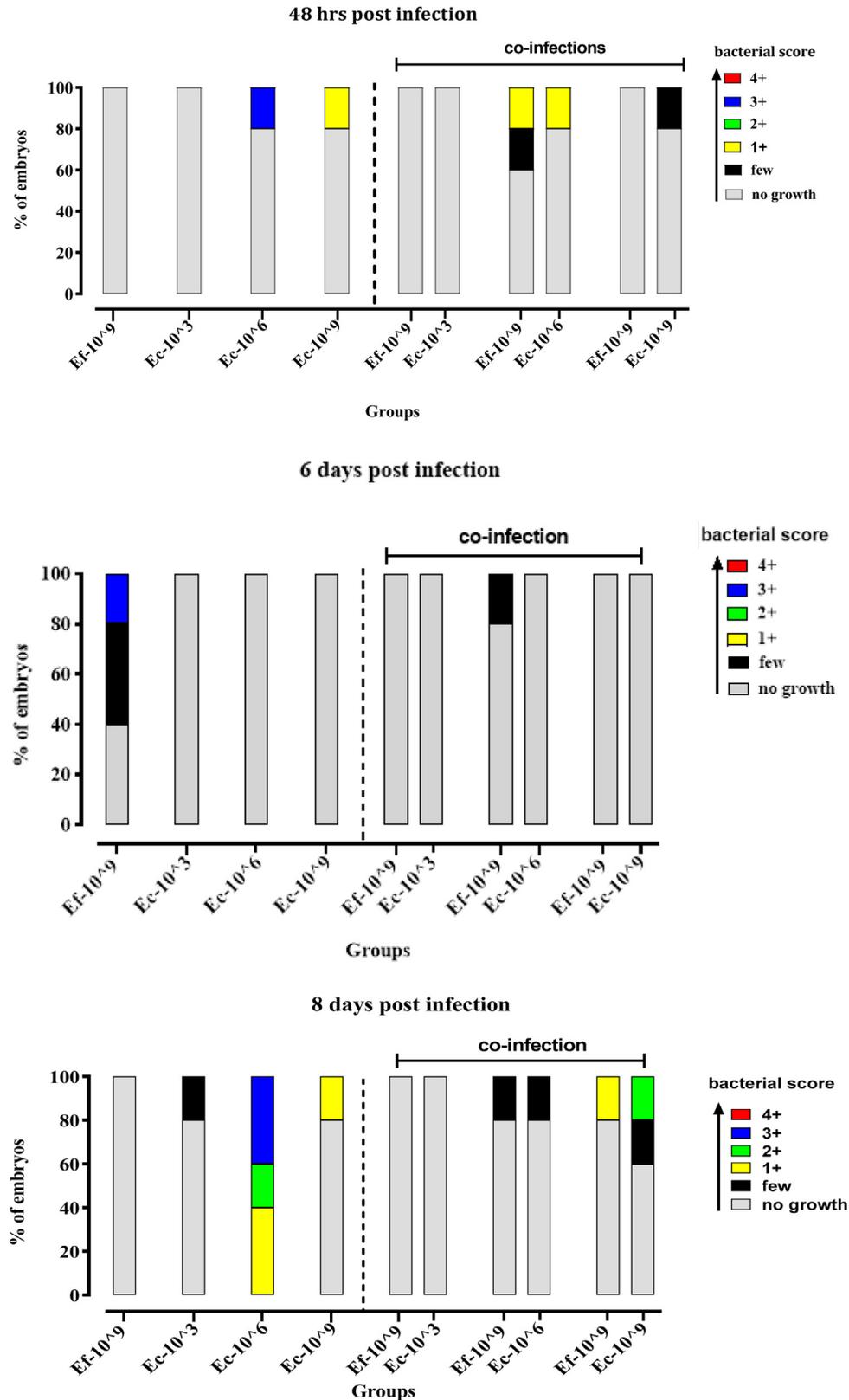
Macroscopic and microscopic examination of dead chicks revealed yolk sacculitis, pericarditis, and perihepatitis. The liver stained with Gram stain revealed Gram-negative rods representing *E. coli* in hepatic sinusoids. Epicarditis, pericarditis, myocarditis, along with infiltration of heterophils and macrophages were prominent in the heart. Gram-negative rods were prominent in the heart, and blood vessels of the lungs. Yolk sac membranes were thick and congested and multifocal areas had infiltration of heterophils and macrophages around necrotic debris. Gram-positive cocci and Gram-

negative rods were present in the yolk sac of 2 of 3 dead birds. *E. faecalis* and *E. coli* were isolated from tissue with inflammation.

### Experiment C. *E. faecalis* and *E. coli* coinfection in chicken embryos and subsequent mortality and host cytokine gene expression in embryos and neonatal chickens

#### Neonatal Chick Mortality

There was no significant difference in hatchability or embryo mortality among groups of chicken embryos exposed to *E. faecalis* and *E. coli* or the group not exposed to bacteria [hatchability = nondip (64%); *E. faecalis* alone ( $1 \times 10^9$  CFU/mL) (59%); *E. coli* alone ( $1 \times 10^6$  CFU/mL) (55%) and *E. faecalis* and *E. coli* ( $1 \times 10^9$  and  $1 \times 10^6$  CFU/mL) (65%)] ( $P > 0.05$ ). After hatching, the highest cumulative mortality of 27.5% was observed in the group coinfecting with *E. faecalis* and *E. coli* ( $P < 0.01$ ). The cumulative mortality of groups infected with *E. coli* alone, *E. faecalis* alone, and nondipped were 17.5%, 2.5%, and 5%, respectively. The highest mortality of 12.82% was observed at 2 d



**Figure 8.** Isolation of *E. faecalis* (Ef) and *E. coli* (Ec) from the yolk at 48 h, 6, and 8 d following exposure of incubating eggs to different doses of *E. faecalis*, *E. coli* or co infection with *E. faecalis* and *E. coli*. (Experiment B: *E. faecalis* and *E. coli* coinfection in chicken embryos and subsequent mortality and pathology in neonatal chickens).

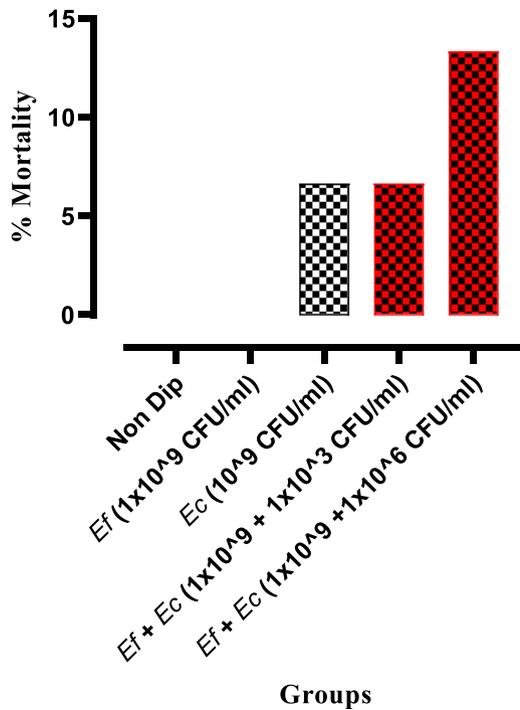
posthatch in the group coinfecting with *E. faecalis* and *E. coli* (Figure 10). No macroscopic lesions were found in any of the dead birds observed until 9 d posthatch. Dead birds in groups infected

with *E. coli* alone or *E. faecalis* and *E. coli* coinfecting group on 10 to 11 d posthatch had pericarditis, airsacculitis, perihepatitis, peritonitis, or yolk sacculitis.

**Table 3.** Isolation percentages of *E. faecalis* and/or *E. coli* from chicken embryos in enrichment culture.

Group-time point	Shell membrane		Amnion		Yolk	
	<i>E. faecalis</i> (%)	<i>E. coli</i> (%)	<i>E. faecalis</i> (%)	<i>E. coli</i> (%)	<i>E. faecalis</i> (%)	<i>E. coli</i> (%)
<i>E. faecalis</i> 10 <sup>9</sup> CFU/mL – 48 h	0		0		0	
<i>E. faecalis</i> 10 <sup>9</sup> CFU/mL – 6 d	40		40		80	
<i>E. faecalis</i> 10 <sup>9</sup> CFU/mL – 8 d	20				80	
<i>E. coli</i> 10 <sup>3</sup> CFU/mL – 48 h		0		0		0
<i>E. coli</i> 10 <sup>3</sup> CFU/mL – 6 d		0		0		0
<i>E. coli</i> 10 <sup>3</sup> CFU/mL – 8 d		0				20
<i>E. coli</i> 10 <sup>6</sup> CFU/mL – 48 h		0		20		20
<i>E. coli</i> 10 <sup>6</sup> CFU/mL – 6 d		40		20		20
<i>E. coli</i> 10 <sup>6</sup> CFU/mL – 8 d		80				100
<i>E. coli</i> 10 <sup>9</sup> CFU/mL – 48 h		0		20		20
<i>E. coli</i> 10 <sup>9</sup> CFU/mL – 6 d		20		0		20
<i>E. coli</i> 10 <sup>9</sup> CFU/mL – 8 d		40				60
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>3</sup> ) – 48 h	40	0	40	0	40	0
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>3</sup> ) – 6 d	60	0	80	0	60	0
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>3</sup> ) – 8 d	60	20			60	0
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>6</sup> ) – 48hrs	20	0	20	20	20	20
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>6</sup> ) – 6 d	0	0	0	0	40	20
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>6</sup> ) – 8 d	0	20			60	80
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>9</sup> ) – 48 h	20	20	20	20	20	20
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>9</sup> ) – 6d	20	20	20	40	80	60
<i>E. faecalis</i> + <i>E. coli</i> (10 <sup>9</sup> /10 <sup>9</sup> ) – 8 d	40	40			20	20

Enrichment cultures were obtained from shell membrane, amnion and yolk sac at different time points following exposure to *E. faecalis* and/or *E. coli*. The empty boxes indicate no isolation was attempted. (*Experiment B: E. faecalis* and *E. coli* coinfection in chicken embryos and subsequent mortality and pathology in neonatal).



**Figure 9.** Cumulative neonatal chick mortality during day 0–7 posthatch. Ef; *E. faecalis*, Ec; *E. coli* (*Experiment B: E. faecalis* and *E. coli* coinfection in chicken embryos and subsequent mortality and pathology in neonatal).

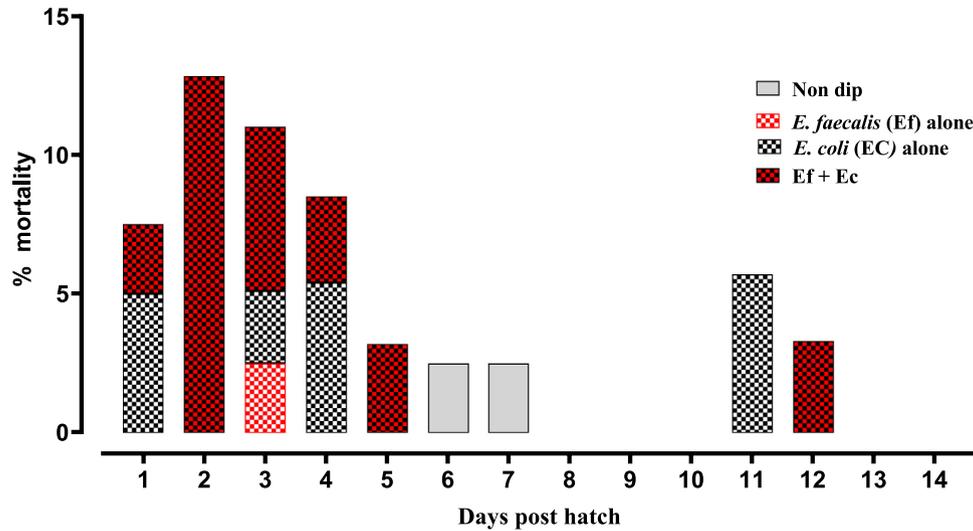
### Host Cytokine and Chemokine Gene Expression in Chicken Embryos and Neonatal Chicks Following *E. faecalis* and *E. coli* Infection

Upregulation of IL-1 $\beta$  was significantly high in chicken embryos at 48 h postinfection with *E. coli* alone group ( $P < 0.01$ ). Similarly, expression of IL-1 $\beta$  was significantly high at 8 d postchallenge (prior to hatch) in

*E. faecalis* alone group ( $P < 0.01$ ) and in the group coinfecting with *E. faecalis* and *E. coli* ( $P < 0.0001$ ). No significant upregulation or downregulation of IL-1 $\beta$  was noted in any other time points with *E. faecalis*, *E. coli*, or coinfecting groups (Figure 11). Upregulation of CXCR4 was significantly high at 8 d postchallenge in *E. faecalis* alone group ( $P < 0.05$ ) and in the group coinfecting with the *E. faecalis* and *E. coli* ( $P < 0.0001$ ). No significant upregulation or downregulation of CXCR4 was noted in any other time points with *E. faecalis*, *E. coli*, or coinfecting groups (Figure 11).

## DISCUSSION

The incidences of *E. coli* and *Enterococcus* species associated mortality in broiler chicken embryos and neonatal chickens have increased in the broiler chicken industry worldwide, including in western Canada, in recent years (Karunarathna et al., 2017). *E. faecalis* was isolated from the yolk sac of the majority of dead embryos, followed by *E. coli*, in a study conducted in Canada (Karunarathna et al., 2017). Bacterial contamination of fertile eggs of broiler breeder parents occurs throughout the production cycle, starting at broiler breeder farms, during egg storage, and incubation in commercial hatcheries. Vertical transmission of certain bacterial species from the hen's reproductive tract to their progeny is hypothesized in several studies, particularly with avian pathogenic *E. coli* isolated from neonatal broilers with omphalitis and yolk sac infections (Giovannardi et al., 2005). A study demonstrating that *E. faecalis* exposure via egg albumen led to arthritis in their progeny indicated possible vertical transmission (Landman et al., 1999a). Although it has been reported that chicken embryo mortality and yolk sac infections due to *E. faecalis* and *E. coli* were commonplace in the



**Figure 10.** Neonatal chick mortality 1–2 wk posthatch. Neonatal chicks following coinfection of incubating eggs with *E. faecalis* and *E. coli* had cumulative mortality of 27.5%. Of 27.5% cumulative mortality in the group coinfecting with *E. faecalis* with *E. coli* had a mortality of 12.82% on 2 d following hatch. (*Experiment C: E. faecalis* and *E. coli* coinfection in chicken embryos and subsequent mortality and host cytokine gene expression in embryos and neonatal chickens).

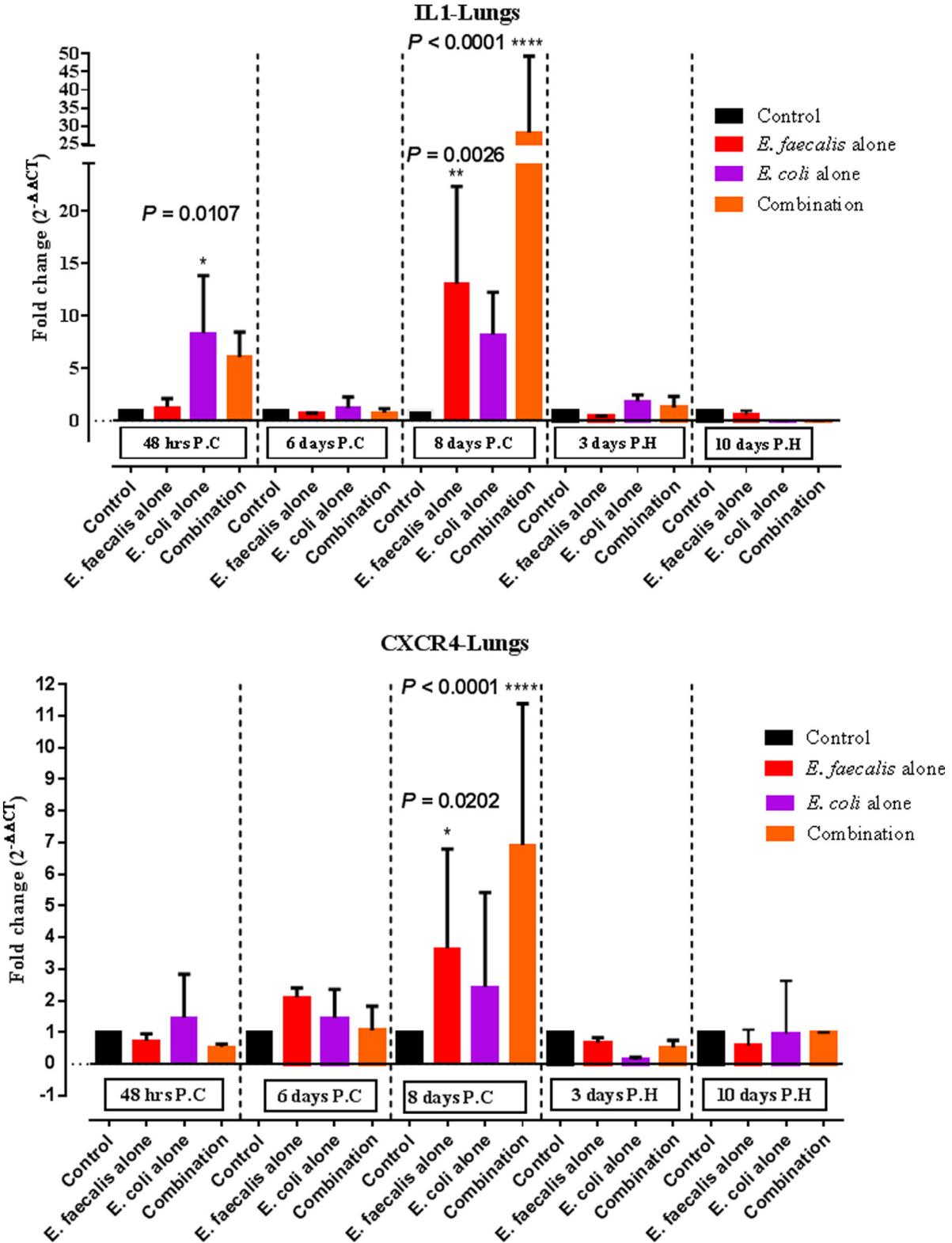
commercial poultry industry, the role of *E. faecalis* on *E. coli* associated yolk sac infections in chicken embryos and neonatal chickens is not clear. This study aimed to explore the impact of embryonic coinfections with *E. faecalis* and *E. coli* on the mortality of embryos and neonatal chickens.

We have conducted 3 experiments to demonstrate host-pathogen interactions of *E. faecalis* and *E. coli* in chicken embryos and neonatal chickens by exposing embryonated eggs to *E. faecalis* and *E. coli*. The first experiment was to explore the rate of *E. faecalis* migration and colonization in chicken embryos, cytokine and chemokine responses, and investigate the *E. faecalis*-associated pathology of embryos at microscopic and electron microscopic levels. The second experiment was to explore bacterial migration and colonization rates in chicken embryos following coinfection of chicken embryos with *E. faecalis* and *E. coli* and subsequent neonatal chicken mortality. The third experiment was to measure cytokine and chemokine responses of embryos and neonatal chickens and neonatal chicken mortality following coinfection of chicken embryos with *E. faecalis* and *E. coli*. To the best of our knowledge, this is the first study to demonstrate the role of *E. faecalis* on *E. coli*-associated pathology in chicken embryos and neonatal chickens by exposing *E. faecalis* and *E. coli* on eggshells of embryonated SPF eggs.

In the first experiment, we demonstrated that *E. faecalis* was able to penetrate through the eggshell to the inner shell, shell membrane and amnion during the incubation period without causing a negative effect on hatchability. The *E. faecalis* load and the rate of bacterial colonization were higher with the MDR *E. faecalis* isolate from a dead embryo and with the field *E. faecalis* isolate from a case of yolk sac infection compared to the control *E. faecalis* isolate from a healthy adult broiler breeder chicken. Similarly, colonization of *E. faecalis* in the liver, intestine, yolk, and lung was higher with the MDR *E. faecalis* isolate and with the field *E. faecalis*

isolate compared to the control *E. faecalis* isolate from a healthy chicken, particularly 6 d and 8 d following infection. Although *E. faecalis* was isolated and the presence of *E. faecalis* was confirmed by histopathology in the yolk, liver and lung, no inflammation was noted on histopathology around *E. faecalis* colonized areas in any of those internal organs 6 d following *E. faecalis* exposure in embryonated eggs. Furthermore, biofilm-like structures were noted in the yolk with the MDR *E. faecalis* isolate 6 d following exposure of embryonated eggs to *E. faecalis*. This may be a mechanism used by *E. faecalis* to evade the immune system of chicken embryos. In our study, the MDR *E. faecalis* isolate had virulence factors associated genes that play roles in adhesion, colonization, and cell damage, as evidenced by PCR amplification of these genes in vitro cultured bacteria. We could detect very low amplification of *cpn60* genes in vivo following *E. faecalis* infection; however, we failed to detect the expression of virulence-related genes in vivo in embryonated eggs. It is quite possible that the levels of expression of these genes may be low enough to get detected by the conventional PCR, or we missed the time to collect the samples when these genes were transiently expressed (mRNA) by the bacteria. Therefore, our study can not rule out the role of these virulence factors in bacterial pathogenesis.

Increased levels of IL-1 $\beta$ , IL-8, CXCR4, and MIP-1 $\alpha$  gene expression were noted in the liver or intestines of embryos 6 d following exposure with MDR *E. faecalis* isolate ( $P < 0.01$ ). In contrast, CXCR4 was increased in the liver with field *E. faecalis* isolate ( $P < 0.05$ ). No cytokine or chemokine mRNA expression was detected in the liver or intestines with control *E. faecalis* isolate. Our data indicate that the pathogenic but not the non-pathogenic strains of *E. faecalis* may stimulate IL-1 $\beta$  cytokine expression following infection. A recent study supports our data that reported that the pathogenic *E. faecalis* strain could upregulate IL-1 $\beta$  by activating NLRP3 inflammasome (Ran et al., 2021). In our study,



**Figure 11.** Cytokine gene expression in lungs at different time points following infection with *E. faecalis*, *E. coli* or coinfection of *E. faecalis* and *E. coli*. P.C; Postchallenge, P. H; Posthatch. (n = 3). No significant difference among groups for IL-1 and CXCR4 expression in lungs ( $P > 0.05$ ) but there was an upward trend in IL-1 and CXCR4 at 8 days postinfection. (Experiment C: *E. faecalis* and *E. coli* coinfection in chicken embryos and subsequent mortality and host cytokine gene expression in embryos and neonatal chickens).

we found upregulation of both IL-1 $\beta$  and CXCR4 following MDR *E. faecalis* infection. A study has reported that IL-1 $\beta$  can upregulate CXCR4 (Sun et al., 2015); we hypothesize it may also be the case here, but it remains to be determined experimentally. Interestingly, our study did not see mRNA expression of IL-8 and MIP-1,

whose expression largely depends on NF-kappaB activation. It is possible that *E. faecalis* down-regulates some proinflammatory responses of the host to escape from the immune system. It has been demonstrated in a mouse model of catheter-associated urinary tract infection that *E. faecalis* was able to subvert macrophage

activation by preventing the NF- $\kappa$ B signaling pathway, which controls the transcription of genes responsible for immune regulation and proinflammatory cytokines and chemokines that regulate recruitment and activation of immune cells (Tien et al., 2017). Future studies will investigate if our observation of no upregulation of host IL-8 and MIP-1  $\alpha$  in our study is also linked to the NF- $\kappa$ B signaling dysregulations.

Although we have seen in our experiments that MDR *E. faecalis* isolate and field *E. faecalis* isolate had a higher colonization rate in internal organs compared to control *E. faecalis* isolate from a healthy chicken, all *E. faecalis* isolates were able to penetrate eggshell and colonize internal organs of chicken embryos, thus indicating the ability of *E. faecalis* to infect chicken embryos following fecal contamination of eggshells irrespective of genetic differences of *E. faecalis* isolates. Although we have not seen embryo mortality associated with *E. faecalis* in this study, we have recently demonstrated that 56% of dead broiler chicken embryos have coinfection of *Enterococcus* species including *E. faecalis* with *E. coli* in a field study conducted in poultry hatcheries in western Canada (Karunaratna et al., 2017). It is likely that certain virulent *E. faecalis* isolates together with *E. coli* potentially cause chicken embryo mortality. A recent study using a broiler chicken embryo lethal assay coinfection of embryonated eggs with avian pathogenic *E. coli* with *E. faecalis* resulted in increased mortality of chicken embryos compared to embryonated eggs infected with *E. faecalis* alone or *E. coli* alone (Walker et al., 2020b). The differences in the method of bacterial infection could explain the discrepancy between our and this recent study. In our study, we have used eggshell bacteria application to facilitate embryonic infection (here, the number of bacteria penetrating eggshells will be in low number). In contrast, Walker et al. (Walker et al., 2020b) directly injected a substantially large number of bacteria into embryonated eggs to simulate the bacterial infection of embryos.

In the second experiment, we demonstrated coinfection of embryonated SPF eggs with *E. faecalis* with *E. coli* leading to colonization of yolk with *E. faecalis* and *E. coli*, particularly close to hatch. *E. faecalis* and *E. coli* colonization was measured by both direct and enrichment culture methods in yolk, shell membrane and amnion. Both culture methods confirmed that, on average, less than 50% of embryos were colonized with *E. faecalis* or *E. coli*. Although no significant colonization or mortality was observed in infected embryos, increased mortality of neonatal chickens was observed in the group coinfecting with *E. faecalis* and *E. coli* compared to the groups infected with either *E. faecalis* or *E. coli* alone. Based on our data, we hypothesized that *E. faecalis* might have supported the pathogenicity of *E. coli* during the coinfection of embryos with *E. faecalis* and *E. coli*.

Therefore, a third experiment was designed to look for a possible mechanism for the increased mortality of neonatal chicks after coinfection with *E. faecalis* and *E. coli*. The third experiment found significantly high

mortality of neonatal chickens in the group exposed to *E. faecalis* and *E. coli* during embryonic life. Moreover, in the third experiment, we found substantially high IL-1 and CXCR4 mRNA expression in the lung, particularly a day before hatch. Birds that died of *E. faecalis* and *E. coli* were septicemic and had severe pericarditis, airsacculitis, or polyserositis. The enhanced expression of CXCR4 has been linked with neonatal sepsis in humans and used as a biomarker for neonatal sepsis (Tunc et al., 2015). Therefore, our findings could suggest that increased mortality of neonatal chickens in the group that were coinfecting with *E. faecalis* and *E. coli* could be due to an excessive cytokine IL-1 and chemokine receptor CXCR4 resulting in sepsis, contributing to high mortality of neonatal chickens 24 h following hatch.

In summary, our findings suggest that *E. faecalis* can penetrate the eggshell, evade immune barriers in the egg, and colonize systemically. The coinfection of embryonated eggs with *E. faecalis* and *E. coli* leads to enhanced pathogenicity resulting in increased mortality of neonatal chickens. However, more studies are required to investigate the role of the cytokine IL-1 and chemokine receptor CXCR4 in the resulting septicemia and bacterial pathogenesis during *E. faecalis* and *E. coli* coinfection in chickens.

## ACKNOWLEDGMENTS

The authors greatly appreciate the assistance of animal care technicians at the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan for their service. Financial support for this research was provided by grants from the Alberta Agriculture and Forestry, Alberta, Canada (Grant # 2018F141R), and the Natural Sciences and Engineering Research Council of Canada (NSERC-Discovery 420261).

## DISCLOSURES

The authors declare no conflicts of interest.

## REFERENCES

- Abe, Y., K. Nakamura, M. Yamada, and Y. Yamamoto. 2006. Encephalomalacia with *Enterococcus durans* infection in the brain stem and cerebral hemisphere in chicks in Japan. *Avian Dis.* 50:139–141.
- Al-Sadi, H., H. Basher, and H. Ismail. 2000. Bacteriologic and pathologic studies on dead-in-shell chicken embryos. *Iraqi J. Vet. Sci.* 13:297–307.
- Arias, C. A., and B. E. Murray. 2012. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat. Rev. Microbiol.* 10:266–278.
- Babaca, Z. 2014. Epidemiological and bacteriological studies on dead-in-shell embryos. *J. Vet. Sci. Technol.* 5:2.
- Berrang, M., N. Cox, J. Frank, and R. Buhr. 1999. Bacterial penetration of the eggshell and shell membranes of the chicken hatching egg: a review. *J. Appl. Poult. Res.* 8:499–504.
- Clinical and Laboratory Standards Institute. 2013. VET01-S2 Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 2nd informational supplement. Clinical and Laboratory Standard Institute, Wayne, PA.

- Cortés, C. R., G. T. Isafas, C. L. Cuello, J. M. V. Flores, R. C. Anderson, and C. E. Campos. 2004. Bacterial isolation rate from fertile eggs, hatching eggs, and neonatal broilers with yolk sac infection. *Revista Latinoamericana de Microbiologia* 46:12–16.
- De Reu, K., K. Grijspeerd, W. Messens, M. Heyndrickx, M. Uyttendaele, J. Debevere, and L. Herman. 2006. Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella enteritidis*. *Int. J. Food Microbiol.* 112:253–260.
- Devriese, L., J. Hommez, R. Wijfels, and F. Haesebrouck. 1991. Composition of the enterococcal and streptococcal intestinal flora of poultry. *J. Appl. Microbiol.* 71:46–50.
- Ding, J., R. Dai, L. Yang, C. He, K. Xu, S. Liu, W. Zhao, L. Xiao, L. Luo, and Y. Zhang. 2017. Inheritance and establishment of gut microbiota in chickens. *Front. Microbiol.* 8:1967.
- Fertner, M. E., R. H. Olsen, M. Bisgaard, and H. Christensen. 2011. Transmission and genetic diversity of *Enterococcus faecalis* among layer chickens during hatch. *Acta Veterinaria Scandinavica* 53:56.
- Giovanardi, D., E. Campagnari, L. S. Ruffoni, P. Pesente, G. Ortali, and V. Furlattini. 2005. Avian pathogenic *Escherichia coli* transmission from broiler breeders to their progeny in an integrated poultry production chain. *Avian Pathol.* 34:313–318.
- Gunawardana, T., M. Foldvari, T. Zachar, S. Popowich, B. Chow-Lockerbie, M. V. Ivanova, S. Tikoo, S. Kurukulasuriya, P. Willson, and S. Gomis. 2014. Protection of neonatal broiler chickens following in ovo delivery of oligodeoxynucleotides containing CpG motifs (CpG-ODN) formulated with carbon nanotubes or liposomes. *Avian Dis.* 59:31–37.
- Hughes, E. R., and S. E. Winter. 2016. *Enterococcus faecalis*: *E. coli*'s Siderophore-Inducing Sidekick. *Cell Host Microbe* 20:411–412.
- Jassim, E., M. Grossman, W. Koops, and R. Luykx. 1996. Multiphasic analysis of embryonic mortality in chickens. *Poult. Sci.* 75:464–471.
- Jean, S. S., C. T. Fang, H. K. Wang, P. Hsue, S. C. Chang, and K. T. Luh. 2001. Invasive infections due to vancomycin-resistant enterococci in adult patients. *J. Microbiol. Immunol. Infect. = Wei mian yu gan ran za zhi* 34:281–286.
- Jones, D. R., K. E. Anderson, P. A. Curtis, and F. T. Jones. 2002. Microbial contamination in inoculated shell eggs: I. Effects of layer strain and hen age. *Poult. Sci.* 81:715–720.
- Jung, A., and S. Rautenschlein. 2014. Comprehensive report of an *Enterococcus cecorum* infection in a broiler flock in Northern Germany. *BMC Vet. Res.* 10:311.
- Kalita, N., N. Pathak, M. Ahmed, and G. Saikia. 2013. Various causes related to dead-in-shell embryos of crossbred (PB-2 x Indigenous) chicken egg. *Vet. World* 6:774–777.
- Karunaratna, R., S. Popowich, M. Wawryk, B. Chow-Lockerbie, K. A. Ahmed, C. Yu, M. Liu, K. Goonewardene, T. Gunawardana, and S. Kurukulasuriya. 2017. Increased incidence of enterococcal infection in nonviable broiler chicken embryos in Western Canadian hatcheries as detected by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. *Avian Dis.* 61:472–480.
- Landman, W. J. M. 1999. Amyloid arthropathy in chickens: (Summary of thesis, Utrecht University, faculty of veterinary medicine, 1998). *Vet. Q.* 21:78–82.
- Landman, W., D. Mekkes, R. Chamanza, P. Doornenbal, and E. Gruys. 1999a. Arthropathic and amyloidogenic *Enterococcus faecalis* infections in brown layers: a study on infection routes. *Avian Pathol.* 28:545–557.
- Landman, W. J., D. R. Mekkes, R. Chamanza, P. Doornenbal, and E. Gruys. 1999b. Arthropathic and amyloidogenic *Enterococcus faecalis* infections in brown layers: a study on infection routes. *Avian Pathol.* 28:545–557.
- Mayes, F. J., and M. A. Takeballi. 1983. Microbial contamination of the hen's egg: a review. *J. Food Prot.* 46:1092–1098.
- Mellata, M. 2013. Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. *Foodborne Pathogens Dis.* 10:916–932.
- Moellering, R. C. Jr. 1992. Emergence of *Enterococcus* as a significant pathogen. *Clin. Infect. Dis.* 14:1173–1176.
- Noskin, G. A., L. R. Peterson, and J. R. Warren. 1995. *Enterococcus faecium* and *Enterococcus faecalis* bacteremia: acquisition and outcome. *Clin. Infect. Dis.* 20:296–301.
- Olsen, R. H., C. Frantzen, H. Christensen, and M. Bisgaard. 2012. An investigation on first-week mortality in layers. *Avian Dis.* 56:51–57.
- Özmen Toğay, S., A. Celebi Keskin, L. Açıık, and A. Temiz. 2010. Virulence genes, antibiotic resistance and plasmid profiles of *Enterococcus faecalis* and *Enterococcus faecium* from naturally fermented Turkish foods. *J. Appl. Microbiol.* 109:1084–1092.
- Ran, S., J. Huang, B. Liu, S. Gu, W. Jiang, and J. Liang. 2021. *Enterococcus Faecalis* activates NLRP3 inflammasomes leading to increased interleukin-1 beta secretion and pyroptosis of THP-1 macrophages. *Microb. Pathog.* 154:104761.
- Razmyar, J., and A. H. Zamani. 2016. An outbreak of yolk sac infection and dead-in-shell mortality in common canary (*Serinus canaria*) caused by *Klebsiella pneumoniae*. *Iranian J. Vet. Res.* 17:141.
- Sauter, E. A., and C. F. Petersen. 1969. The effect of egg shell quality on penetration by *Pseudomonas fluorescens*. *Poult. Sci.* 48:1525–1528.
- Sauter, E. A., and C. F. Petersen. 1974. The effect of egg shell quality on penetration by various salmonellae. *Poult. Sci.* 53:2159–2162.
- Shepard, B. D., and M. S. Gilmore. 2002. Differential expression of virulence-related genes in *Enterococcus faecalis* in response to biological cues in serum and urine. *Infect. Immun.* 70:4344–4352.
- Sun, Y., D. Zhu, G. Wang, D. Wang, H. Zhou, X. Liu, M. Jiang, L. Liao, Z. Zhou, and J. Hu. 2015. Pro-Inflammatory cytokine IL-1beta up-regulates CXC chemokine receptor 4 via Notch and ERK signaling pathways in tongue squamous cell carcinoma. *PLoS One* 10:e0132677.
- Tien, B. Y. Q., H. M. S. Goh, K. K. L. Chong, S. Bhaduri-Tagore, S. Holec, R. Dress, F. Ginhoux, M. A. Ingersoll, R. B. Williams, and K. A. Kline. 2017. *Enterococcus faecalis* promotes innate immune suppression and polymicrobial catheter-associated urinary tract infection. *Infection Immun.* 85:1–14.
- Tunc, T., F. Cekmez, M. Cetinkaya, T. Kalayci, K. Fidanci, M. Saldır, O. Babacan, E. Sari, G. Erdem, T. Cayci, M. Kul, and S. Kavuncuoglu. 2015. Diagnostic value of elevated CXCR4 and CXCL12 in neonatal sepsis. *J. Maternal-Fetal Neonatal Med.* 28:356–361.
- van Veen, S. Q., E. Claas, and E. J. Kuijper. 2010. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. *J. Clin. Microbiol.* 48:900–907.
- Walker, G. K., M. M. Suyemoto, S. Gall, L. Chen, S. Thakur, and L. B. Borst. 2020a. The role of *Enterococcus faecalis* during coinfection with avian pathogenic *Escherichia coli* in avian colibacillosis. *Avian Pathol.* 49:589–599.
- Walker, G. K., M. M. Suyemoto, S. Gall, L. Chen, S. Thakur, and L. B. Borst. 2020b. The role of *Enterococcus faecalis* during coinfection with avian pathogenic *Escherichia coli* in avian colibacillosis. *Avian Pathol.* 49:589–599.
- Yildiz, O., and S. Turkyilmaz. 2015. Investigation of virulence genes of *Enterococcus faecalis* strains isolated from mastitic bovine milk. *Israel J. Vet. Med.* 70:4.