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

Ruwani Karunaratha, Khawaja Ashfaqe Ahmed, Kalhari Goonewardene, Thushari Gunawardana, Shanika Kurukulasuriya, Mengying Liu, Ashish Gupta, Shelly Popowich, Lisanework Ayalew, Betty Chow-Lockerbie, Philip Willson, Musangu Ngeleka, and Susantha Gomis

*Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B4 Canada; 1Canadian Centre for Health and Safety in Agriculture, University of Saskatchewan, Saskatoon, Canada, SK S7N 5E5, Canada; and 1Prairie 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 egg-shell 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 internal 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 coinfected 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

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
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, perhepatitis, arthritis, and osteomyelitis, septicemia, and cellulitis (Mel-lata, 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 (Karunarathna 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 (Karunarathna 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.

**MATERIALS AND METHODS**

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

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 (Karunarathna et al., 2017) and determined to be multidrug 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 × 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-1β and IL-8) and chemokines (CXCR4 and MIP-1α). The expression levels of putative virulence genes of *E. faecalis* (agg, gccE, cylM, cylB, cylA, efaAf8, 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 × 10^9 cfu/mL); (4) *E. coli* (1 × 10^9 cfu/mL); (5) *E. coli* (1 × 10^6 cfu/mL); (6) *E. coli* (1 × 10^5 cfu/mL); (7) *E. faecalis* and *E. coli* (1 × 10^6 and 1 × 10^3 cfu/mL); (8) *E. faecalis* and *E. coli* (1 × 10^9 and 1 × 10^6 cfu/mL); and (9) *E. faecalis* and *E. coli* (1 × 10^9 and 1 × 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 × 10^9 cfu/mL), *E. coli* alone (1 × 10^9 cfu/mL), *E. faecalis* together with *E. coli* (1 × 10^9 and 1 × 10^3 cfu/mL) and *E. faecalis* together with *E. coli* (1 × 10^9 and 1 × 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 × 10^9 cfu/mL); (3) *E. coli* alone (1 × 10^6 cfu/mL) and (4) *E. faecalis* and *E. coli* (1 × 10^9 and 1 × 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-β) and chemokine (CXCR4) gene expression.
Bacterial Isolation and Identification from Embryos and Neonatal Chickens

Swab samples were collected on 5% Columbia sheep BA, m-Enterococcus and MacConkey agar and incubated at 37°C for 24 to 48 h. Semi-quantitative analyses on plates were conducted on a scale from 0 to + (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 tetracycline) were conducted using the disk diffusion method (Kirby Bauer), interpreted using CLSI guidelines (Clinical and Laboratory Standards Institute, 2013). Antibiotics 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 de-waxed 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 Togay et al., 2010; Yildiz and Turkylmaz, 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 MgCl2, 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 RNA later 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) |
| Ragg-F | AAGAAAAAGAATGACCCAA | 1553 | 55 |
| Ragg-R | AAACGGCAGAACTGTTAAATA | 419 | 55 |
| Rge-EF | ACCCGTGATCATCGTGGTTT | 742 | 53 |
| Rge-ER | ACCGTTGTTTTTCATC | 843 | 53 |
| RcM-F | CTTGAGGAAAAGATAGTA | 517 | 53 |
| RcM-R | TGGATGATAGTGATCGAAGT | 705 | 53 |
| RcAf-F | GACAGACCCCTCAGGA | 1405 | 53 |
| RcAf-R | TGGTATGATAGTGATCGAAGT | 543 | 53 |
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 μL containing 0.5 × SYBR Green master mix, and 2 μL 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 μL total volume) were performed using Prime Time-Gene Expression Master Mix (IDT, Redwood City, California, USA); 2 μL of cDNA template, and primers and probes as described in Table 2.

**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-GCTCTCATATGCTTGTTGTGATGAG-3                                               |
| IL-1 Reverse           | 5-TGTCGATGTCGGCGATGA-3                                                    |
| IL-1 Probe             | 5-(FAM)-CCACACTGCGATCTGGAG-GAAGC-ZEN/IBFQ-3                               |
| IL-8 Forward           | 5-GCCTTCCTCTGGCTTTTCAAG-3                                                 |
| IL-8 Reverse           | 5-TGGACCGCGAAGCTATT-3                                                     |
| IL-8 Probe             | 5-(FAM)-GTGTTTACCGCTGTTCACT-3                                             |
| MIP-1 Forward          | 5-GGCAAGATCTACGGAGA/CGAGCAGACACAG-3                                      |
| MIP-1 Reverse          | 5-ACGGCCTTCCCTGTGATGAT-3                                                  |
| MIP-1 Probe            | 5-(FAM)-ACACACACACAGCTAGGG-CACTG-ZEN/IBFQ-3                               |
| CXCR-4 Forward         | 5-TGCTGTCTCAATCTCAATTTT-3                                                 |
| CXCR-4 Reverse         | 5-CAAGGCAATTCTTGTGAGGTTGT-3                                               |
| CXCR-4 Probe           | 5-(FAM)-ACGCCCTTCTGGTGTCG-3                                               |

**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**

**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%) of embryos. The MDR 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
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. U.pregulation of IL-1β, IL-8, CXCR4, and MIP-1α 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-1β, IL-8, CXCR4, and MIP-1α 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-1β and CXCR4 was significantly high in the liver of embryos infected with MDR

---

**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).
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-1β, IL-8, CXCR4, and MIP-1α 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) = (x 2,000, x 8,000 magnification) illustrates heavy colonization of *E. faecalis* in the yolk sac. SEM image (B) = (x 2,000, x 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*).
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 × 10^9 cfu/mL) (65%); E. coli (1 × 10^9 cfu/mL) (85%); E. coli (1 × 10^6 cfu/mL) (82%); E. coli (1 × 10^3 cfu/mL) (58%); E. faecalis and E. coli (1 × 10^9 and 1 × 10^6 cfu/mL) (95%); E. faecalis and E. coli (1 × 10^9 and 1 × 10^6 cfu/mL) (83%); and E. faecalis and E. coli (1 × 10^9 and 1 × 10^6 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 × 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 × 10^9 E. faecalis cfu/mL) and 20% (1 × 10^9 E. faecalis and 1 × 10^6 cfu/mL) of embryos. At 8 d postinfection, E. coli was isolated from 20% (1 × 10^3 E. coli cfu/mL), 100% (1 × 10^6 E. coli cfu/mL), 20% (1 × 10^9 E. coli cfu/mL), 20% (1 × 10^9 E. faecalis and 1 × 10^6 E. coli cfu/mL) and 40% (1 × 10^9 E. faecalis and 1 × 10^6 E. coli cfu/mL) of embryos. E. faecalis was isolated from 20% (1 × 10^9 E. faecalis and 1 × 10^6 E. coli cfu/mL) and 20% (1 × 10^9 E. faecalis and 1 × 10^6 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 × 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.
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 from the shell membrane at 48 h, 6 d, and 8 d, respectively. Similarly, rates of recovery of bacteria from the yolk were 20%, 20%, 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 amnion were 20% and 0% at 48 h and 6 d, respectively.

The embryos coinfect 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%, 20%, and 40% E. faecalis and 0%, 0%, and 0% 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
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 coinfecte 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.
posthatch in the group coinfeclted 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* coinfeclted group on 10 to 11 d posthatch had pericarditis, airsacculitis, perihepatitis, peritonitis, or yolk sacculitis.

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

| Group-time point | Shell membrane | Amnion | Yolk |
|------------------|----------------|--------|------|
|                  | *E. faecalis (%)* | *E. coli (%)* | *E. faecalis (%)* | *E. coli (%)* | *E. faecalis (%)* | *E. coli (%)* |
| *E. faecalis* $10^9$ CFU/mL – 48 h | 0 | 0 | 0 | 0 |
| *E. faecalis* $10^6$ CFU/mL – 6 d | 40 | 40 | 0 | 0 |
| *E. coli* $10^6$ CFU/mL – 8 d | 20 | 20 | 0 | 0 |
| *E. coli* $10^5$ CFU/mL – 48 h | 0 | 0 | 0 | 0 |
| *E. coli* $10^5$ CFU/mL – 6 d | 0 | 0 | 0 | 0 |
| *E. coli* $10^5$ CFU/mL – 8 d | 0 | 0 | 0 | 0 |
| *E. coli* $10^6$ CFU/mL – 48 h | 0 | 0 | 0 | 0 |
| *E. coli* $10^6$ CFU/mL – 6 d | 0 | 0 | 0 | 0 |
| *E. coli* $10^6$ CFU/mL – 8 d | 0 | 0 | 0 | 0 |
| *E. coli* $10^9$ CFU/mL – 6 d | 0 | 0 | 0 | 0 |
| *E. coli* $10^9$ CFU/mL – 8 d | 0 | 0 | 0 | 0 |
| *E. coli* $10^9$ CFU/mL – 48 h | 0 | 0 | 0 | 0 |
| *E. coli* $10^9$ CFU/mL – 6 d | 0 | 0 | 0 | 0 |
| *E. coli* $10^9$ CFU/mL – 8 d | 0 | 0 | 0 | 0 |

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*).

![Graph](image)

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-1β was significantly high in chicken embryos at 48 h postinfection with *E. coli* alone group ($P < 0.01$). Similarly, expression of IL-1β was significantly high at 8 d postchallenge (prior to hatch) in *E. faecalis* alone group ($P < 0.01$) and in the group coinfected with *E. faecalis* and *E. coli* ($P < 0.0001$). No significant upregulation or downregulation of IL-1β was noted in any other time points with *E. faecalis*, *E. coli*, or coinfected 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 coinfected 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 coinfected 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 (Giovanardi 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...
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 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-1β, IL-8, CXCR4, and MIP-1α 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-1β cytokine expression following infection. A recent study supports our data that reported that the pathogenic *E. faecalis* strain could upregulate IL-1β by activating NLRP3 inflammasome (Ran et al., 2021). In our study,
we found upregulation of both IL-1β and CXCR4 following MDR *E. faecalis* infection. A study has reported that IL-1β 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

![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).](image-url)

---

**E. FAECALIS POTENTIATES PATHOGENICITY OF E. COLI**

---

[15]
activation by preventing the NF-κB 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 MP-1α in our study is also linked to the NF-κB signaling dysregulations.

Although we have seen in our experiments that MDR Enterococcus faecalis isolate and field Enterococcus faecalis isolate had a higher colonization rate in internal organs compared to control Enterococcus faecalis isolate from a healthy chicken, all Enterococcus faecalis isolates were able to penetrate eggshell and colonize internal organs of chicken embryos, thus indicating the ability of Enterococcus faecalis to infect chicken embryos following fecal contamination of eggshells irrespective of genetic differences of Enterococcus faecalis isolates. Although we have not seen embryo mortality associated with Enterococcus faecalis in this study, we have recently demonstrated that 56% of dead chicken embryos had coinfection of Enterococcus species including Enterococcus faecalis with Escherichia coli in a field study conducted in poultry hatcheries in western Canada (Karunarathna et al., 2017). It is likely that certain virulent Enterococcus faecalis isolates together with Escherichia coli potentially cause chicken embryo mortality. A recent study using a broiler chicken embryo lethal assay coinfection of embryonated eggs with avian pathogenic Escherichia coli with Enterococcus faecalis resulted in increased mortality of chicken embryos compared to embryonated eggs infected with Enterococcus faecalis alone or Escherichia 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 Enterococcus faecalis with Escherichia coli leading to colonization of yolk with Enterococcus faecalis and Escherichia coli, particularly close to hatch. Enterococcus faecalis and Escherichia 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 Enterococcus faecalis or Escherichia coli. Although no significant colonization or mortality was observed in infected embryos, increased mortality of neonatal chickens was observed in the group coinfected with Enterococcus faecalis and Escherichia coli compared to the groups infected with either Enterococcus faecalis or Escherichia coli alone. Based on our data, we hypothesized that Enterococcus faecalis might have supported the pathogenicity of Escherichia coli during the coinfection of embryos with Enterococcus faecalis and Escherichia coli.

Therefore, a third experiment was designed to look for a possible mechanism for the increased mortality of neonatal chicks after coinfection with Enterococcus faecalis and Escherichia coli. The third experiment found significantly high mortality of neonatal chickens in the group exposed to Enterococcus faecalis and Escherichia 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 Enterococcus faecalis and Escherichia 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 (Tune et al., 2015). Therefore, our findings could suggest that increased mortality of neonatal chickens in the group that were coinfected with Enterococcus faecalis and Escherichia 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 Enterococcus faecalis can penetrate the eggshell, evade immune barriers in the egg, and colonize systemically. The coinfection of embryonated eggs with Enterococcus faecalis and Escherichia 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 Enterococcus faecalis and Escherichia 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. Iraq 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. Isaías, 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 Microbiología 46:12–16.

De Reu, K., K. Grijpjerdt, 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.

De Vriese, L., J. Hommez, R. Wijfels, and F. Haesebrouck. 1991. Commissio communication of embryonic mortality in chickens. Poult. Sci. 70:411–412.

Jassim, E., M. Grossman, W. Koops, and R. Luykx. 2014. Protection of neonatal broiler chickens from naturally fermented Turkish foods. J. Appl. Microbiol. 117:1525–1528.

Nozoki, S., A. Celebi Keskin, L. Acik, and A. Temiz. 2010. Virulence genes, antibiotic resistance and plasmid profiles of Enterococcus faecalis and Enterococcus fæcium from naturally fermented Turkish foods. J. Appl. Microbiol. 109:1084–1092.

Hughes, E. R., and S. E. Winter. 2016. Enterococcus faecalis: E. coli’s Siderophore-Inducing Sidekick. Cell Host Microbe 20:471–484.

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, M. E. 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.

Karunarathna, R., S. Popowich, M. Wawryk, B. Chow-Lockerbie, and 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.

Jung, A., and S. Raunstenschleien. 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.

Karunarathna, 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. Gryus. 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. Gryus. 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.

Moelling, R. C. Jr. 1992. Emergence of Enterococcus as a significant pathogen. Clin. Infect. Dis. 14:1173–1176.

Neskin, 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.

Ozmen Toğay, S., A. Celebi Keskin, L. Acik, and A. Temiz. 2010. Virulence genes, antibiotic resistance and plasmid profiles of Enterococcus faecalis and Enterococcus fæcium 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. 1996. 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 CXCL 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. 85:1–8.

Tunc, T., F. Cekmez, M. Cetinkaya, T. Kalayci, K. Fidanci, M. Saldır, O. Babacan, E. Sari, G. Erdem, T. Cayci, M. Kül, 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. Avian Dis. 54:1–16.