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Research Article

Microbial Count and *AvBD10* Expressions in Ovaries and Oviducts of Kampung Unggul Balitbangtan (KUB)-1 Chickens Following Intravaginally CpG-ODN and *S.* Enteritidis

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ABSTRACT

Indonesia boasts diverse native chickens (*Gallus gallus domesticus*) known for more disease resistance in comparison to broiler chicken, and Kampung Unggul Balitbangtan (KUB)-1 is designated as Indonesia's superior breed. *Salmonella* Enteritidis (SE) is associated with salmonellosis, a foodborne illness that can be transmitted by transovarial, wherein colonisation in the oviduct ascends to the ovaries. However, studies mimicking transovarial salmonellosis via intravaginal treatment of chicken have been limited. Meanwhile, Cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) stimulation has been known to induce avian β-defensins (*AvBDs*). This *in vivo* study aimed to determine the effects of intravaginal CpG-ODN treatment and SE challenged on microbial count and *AvBD10* expression regarding the potential of intravaginally CpG-ODN to enhance innate immunity as an alternative approach against transovarial Salmonellosis. A total of 39 KUB-1 chickens were divided into four groups: T1 (CpG-ODN treatment), T2 (SE treatment), T3 (CpG-ODN treatment + challenged with SE), and C (Control). Observation was carried out from day 1 to day 4 post-intravaginal (PI). We found a significant increase in ovarian microbial count ($p \leq 0.05$). Notably, ovaries and oviducts remained uncontaminated post-SE challenge. Intravaginal CpG-ODN treatment significantly upregulated *AvBD10* in both ovaries ($p=0.016$) and oviducts ($p=0.023$). Therefore, KUB-1 chickens exhibit SE immunity, and intravaginal CpG-ODN administration holds promise for preventing transovarial Salmonellosis in laying hens.

INTRODUCTION

In several countries, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (SE) is a major cause of foodborne illnesses worldwide, often associated with chicken meat and egg consumption (Antunes et al. 2016; Midorikawa et al. 2020). Nonetheless, chicken egg is a popular and economical protein source with high biological values (Lopez et al. 2018). Chicken products, on average 1.11 million tons are consumed annually,

with Indonesia producing 1.48 million tons, are widely consumed (Wardhana et al. 2021). In the USA, 1.4 million annual cases of foodborne diseases cause are attributed to *Salmonella*, originating from chickens (Midorikawa et al. 2020). SE infections impact the poultry industry as infected chickens can spread the pathogen and pose a zoonotic risk through contaminated eggs. Notably, over half a billion eggs were withdrawn in the USA due to an SE contamination outbreak (Mon et al. 2020).

The Centers for Disease Control and Prevention (CDC) reported that *Salmonella* is a significant public health concern, causing an estimated 1.35 million infections, 26,500 hospitalisations, and 420 deaths annually in the USA. Most of these infections stem from contaminated food, with chicken being a major source, as roughly 1 in 25 packages at grocery stores are tainted with *Salmonella*. Gong et al. (2022) reported that in a global context, non-typhoidal *Salmonella* (NTS) leads to 93.8 million cases of gastroenteritis each year, resulting in 155,000 deaths. Investigation in Indonesia found high *Salmonella* contamination rates in chicken meat (85%), intestine contents (57.5%), and rinse water (52.5%) at traditional markets in Surabaya as reported by Yulistiani et al. (2019), meanwhile Walyani et al. (2019) reported in Subang, 75% isolates of *Salmonella* spp. which infects chickens were resistant to antibiotics with a 95% confidence interval (0%-35.4%), one of which was SE.

SE contamination on eggshells can occur horisontally from bacteria that originally inhabited the gut or from fecal contact during or after egglaying. Vertical transmission happens through direct contamination of yolk, albumen, or shell before laying, from reproductive organs infected with SE (Gantois et al. 2008). The interaction between pathogens like *Salmonella* and their host relies on a complex microbiota. Understanding the association between chicken microbiota and *Salmonella* is crucial to reducing its harm (Foley et al. 2013). Vertical transmission of SE contamination in eggs and the passage of chicken microbiota to chicks occur during egg formation in the reproductive tract (Ding et al. 2017). Microbiota from the parental oviduct, including bacterial species, is conveyed to the embryo through albumen, suggesting the potential for microbiota transfer during egg formation (Lee et al. 2019).

Cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) is an ODN with an unmethylated CpG motif found in bacterial DNA which is read as a "danger signal" by the immune system, so it can stimulate innate and adaptive immune responses in humans and various animal species (Li et al. 2017). In response to SE infection, oviductal epithelial cells constitutively expressed *AvBDs* (Ebers et al. 2009), and following stimulation with CpG-ODN, the expression of *AvBDs* is upregulated (Abdel-Mageed et al. 2011; Sonoda et al. 2013). The ability of reproductive tissues to synthesise *AvBDs* and their regulation in response to pathogens may play an essential role in innate immunity in the ovary and oviduct, establishing localised defenses within the host. Synthesised *AvBDs* notably fortify the defense mechanism of reproductive organs (Yoshimura et al. 2014) and offer protection against infection (Yoshimura 2015).

This study focuses on analysing partially and temporally microbial counts and *AvBD10* expressions in the ovaries and oviducts of Kampung Unggul Balitbangtan (KUB)-1 chickens, a superior Indonesian native strain. These chickens exhibit improved egg production and disease resistance due to six generations of selective breeding (Sartika 2014). The research investigates innate immune responses in ovaries and

oviducts using an intravaginal CpG-ODN treatment and SE challenge test with observations occurring at four-time points (day 1 to day 4) post -intravaginal (PI) treatment.

MATERIALS AND METHODS

Experimental design and animal groups

Experiments were performed under the ethical guidelines and regulations set forth by the Ethics Commission of Institut Teknologi Bandung, Indonesia, with the issuance of Decree No. 01/KEPHP-ITB/3-2019 by the provisions internationally accepted principles for laboratory animal use and care. The study involved 39 KUB-1 females, 60 weeks old, *Salmonella*-free, and divided into four groups: T1 (CpG-ODN treatment, n=12), T2 (SE treatment, n=12), T3 (CpG-ODN treatment + challenged with SE, n=12), and C (Control, n=3). CpG-ODN (5 µg/500 µl) from ODN 2007 Class B CpG oligonucleotide 5′-TCGTCGTTGTCGTTTTGTCGTT-3′ (InvivoGen) and SE $5x10^7$ CFU (Culti-Loops SE ATCC 13076™) were used. Ovary and oviduct samples were aseptically in a BSL2+ room from day 1 to day 4 PI and stored separately. *Salmonella* isolation and TPC followed *Office International des Epizooties* (OIE) protocols. *AvBD10* relative expression gene (RGE) was assessed through real-time qPCR using the Livak & Schmittgen (2001) $2^{-\Delta\Delta Ct}$ formula.

Salmonella isolation test and Total Plate Count (TPC) analysis

A *Salmonella* isolation test was conducted according to OIE (2018). The procedure involved pre-enrichment and enrichment, followed by selective medium culturing and *Salmonella* identification using biochemical assays, accompanied by a positive control. During pre-enrichment, a 25 g sample was homogenized with 225 ml of 0.1% Buffer Peptone Water (BPW). The mixture was incubated at 35°C for 24 hours (h). In the enrichment step, the pre-enrichment culture was inoculated into Tetra Thionate Broth (TTB) medium and Rappaport Vassiliadis (RV) medium, then incubated at specific temperatures for 24 h. From the enrichments, selected colonies were inoculated onto Xylose Lysine Deoxycholate Agar (XLDA), Hektoen Enteric Agar (HEA), and Bismuth Sulfite Agar (BSA), then incubated at 35°C for 24 h. *Salmonella* identification was performed by streaking suspected colonies onto Triple Sugar Iron Agar (TSIA) and Lysine Iron Agar (LIA), followed by 24 h incubation. Biochemical assays include urease, indole, Methyl Red-Voges Proskauer (MR-VP), citrate, and malonate tests.

A TPC analysis, following the OIE 2018 guidelines, involved homogenising a 25 g sample with 225 ml of 0.1% BPW. The resulting suspension was transferred to a sterile tube for a 10^{-1} dilution, followed by a 1 ml sample transfer into 9 ml of 0.1% BPW for further dilutions up to 10^{-6}. From each dilution, 1 ml of suspension was plated in Duplo on Petri dishes. Subsequently, 15-20 ml of 45°C plate count agar (PCA) was added to each plate and mixed. The PCA was allowed to solidify and incubated upside down at 36°C for 24-48 h. Colonies were counted with a colony counter for each dilution series. Petri dishes containing 25-250 colonies were selected for measurement and interpretation of results.

Real-time qPCR analysis of *AvBD10* expressions

RNA extraction from the ovary and oviduct tissue utilised the Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions. Subsequent cDNA synthesis employed the Cyclescript RT Premix Synthesis Kit (Bioneer) following the manufacturer's protocols. The

resulting cDNA was mixed with primers and SYBR Green in the SensiFAST SYBR Lo-Rox Kit (Bioline), according to the manufacturer's guidelines. Applied Biosystems™ 7500 Fast Real-Time PCR Systems for qPCR and used NFW without cDNA as a negative control in the qPCR reaction to ensure that there is no contamination or error in the reaction. Total reaction volume in qPCR = 20 µl (10 µl SYBR, 0.8 µl forward primer, 0.8R µl reverse primer, and 4.4 µl NFW). Real-time qPCR condition involved an initial polymerase activation step at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 5 seconds (s), and annealing/extension at 58°C for 30 s. The treatment groups (T1, T2, and T3) were compared to the Control (C) group. Cycle Threshold (CT) values were determined in six replicates and quantifications were normalised using Beta-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as housekeeping genes. Table 1 for primer details.

Data analysis and statistics

The *Salmonella* isolation test involves a series of culture steps to analyse results. Tissue samples are subjected to TPC analysis to quantify microbial presence, with a contamination threshold of $1x10^6$ CFU/gram, based on OIE. The *Salmonella* contamination threshold value, set by the Indonesian Ministry of Agriculture, is non-detectable in 25 g samples. For statistical analysis, One-way ANOVA and Duncan's test were employed in SPSS25 with significance $p \leq 0.05$. Data normality was confirmed through the Shapiro-Wilk test (Sig.>0.05). Graphical representation was created using GraphPad Prism 9.4.0. The data set comprised the mean ± SEM from day 1 to day 4 PI.

RESULTS AND DISCUSSION

Intravaginal SE significantly increased microbial count, but there was no microbial contamination in the ovaries of KUB-1 chickens

On day 4 PI, intravaginal SE did not induce clinical signs in KUB-1 chickens. This could be attributed to age, as adult poultry show milder Salmonellosis symptoms per OIE 2018. Host-pathogen interactions, age, stress, and health, emphasised by Foley et al. (2013), play key roles, while Wessels et al. (2021), note morbidity/mortality influenced by age, breed, nutrition, and flock management. We chose 60-week-old chickens known for their prolific egg-laying abilities and Lee et al. (2019) findings that bacterial species significantly increased after sexual maturation, and the microbiota in adult chicken oviducts remained consistent throughout the reproductive tract. Additionally, Wigley et al. (2014) reported that *Salmonella* infection in the reproductive tract primarily occurs after chickens reach reproductive maturity, making them more susceptible to SE challenges during egg-laying.

Observations were limited to 4 days PI as Rehman et al. (2021) noted a four-day duration for the innate immune response. Meanwhile,

Table 1. Primer of *AvBD10* and housekeeping genes.

Identity	Primer	Length	Accession No.
AvBD10	Forward: TGTTAAACTGCTGTGCCAAGATTC Reverse: TGTTGCTGGTACAAGGGCAAT	98 bp	NM_001001609.1
GAPDH	Forward: AGCCATTCCTCCACCTTTGA Reverse: CAACAAAGGGTCCTGCTTCC	190 bp	NM_204305.1
ACTB	Forward: ATGAAGCCCAGAGCAAAAGA Reverse: GGGGTGTTGAAGGTCTCAAA	244 bp	NM_205518.1

Miyamoto et al. (1998) studied SE levels in the cloaca at 7 days PI due to intravaginal SE, while Pudjiatmoko et al. (2014) highlighted 4–5 days *Salmonella* incubation period with 3-5 weeks of symptoms in chickens. Despite SE treatment (T2, T3), *Salmonella* was not present in the ovaries and oviducts of KUB-1 chickens, but positive controls showed *Salmonella* presence. Oludairo et al. (2022) described *Salmonella* as a Gram-negative rod-shaped bacterium, non-spore-forming, oxidase, indole, urease, lactose, sucrose negative, facultative anaerobic, and motile. In our study, based on OIE for the *Salmonella* test, SE exhibited characteristic features, including pink/black colonies on XLDA, green/blue on HEA, and black/gray on BSA, negative *Salmonella* for indole, urease, MR-VP, citrate, malonate, and a motile bacteria. Day 1 PI *Salmonella* isolation and biochemical assays did not reveal colonies in ovaries and oviducts. Okamura et al. (2001) found no *Salmonella* in ovary cultures on day 2 PI and day 3 PI. Ojima et al. (2021) observed no *Salmonella* colonies in ovaries/oviducts on day 4 PI.

In this study, we investigated the impact of SE $5x10^7$ CFU intravaginally inoculation in T2 and T3 groups on microbial contamination in ovaries and oviducts. Previous research (Chatterjee & Abraham 2018) established the concept of microbial contamination, encompassing unwanted microbes such as *Salmonella* sp., *Pseudomonas*, *Listeria monocytogenes*, *Shigella flexneri*, *Vibrio cholerae*, *Bacillus* sp., and *Campylobacter jejuni*. Such contamination could arise from clinical infections caused by these microbes. Microbial contamination assessment involved TPC derived from cultures on NA medium, quantifying colony numbers. Tissue was deemed contaminated if TPC exceeded $1x10^6$ CFU/g. Observing TPC across T1, T2, T3, and C groups from day 1 to day 4 PI revealed that 2.56% ovaries and 12.8% oviducts surpassed the threshold value (data not shown).

A partial analysis of TPC values was visualised in Figure 1. The results indicated an increase in microbial counts in T1, T2, and T3. However, only the ovaries in T2 showed a significant increase ($p \leq 0.05$). Interestingly, despite being the first site encountered by SE after intravaginal inoculation, the oviduct did not exhibit a significant increase. This phenomenon aligns with Kaspers et al. (2014), who proposed that in birds, lacking lymph nodes, epithelial cells of the oviduct can detect pathogens and mount an active immune response to fend off invasion. Furthermore, Wigley (2014) elucidated that *Salmonella* infection elicits a local innate immune reaction, notably the secretion of the antimicrobial peptide AvBD throughout the reproductive tract, particularly in the vagina and uterus.

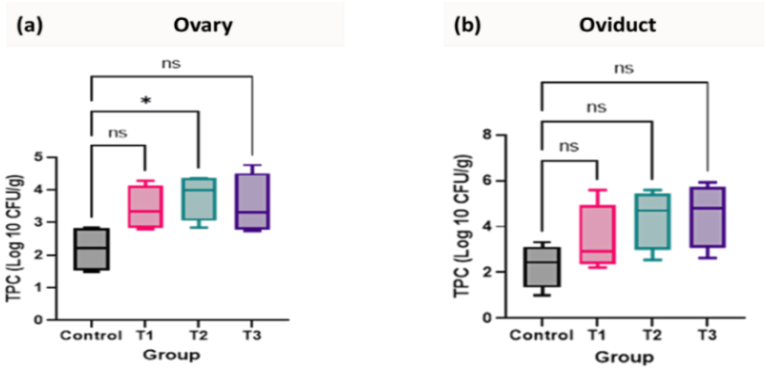

Figure 1 . Microbial count in the ovary (a) and oviduct (b) of KUB-1 chickens at day 1 to day 4 PI through TPC, in partial analysis, and each bar represents the mean ± SEM.
Sign * $p \leq 0.05$, ns = not significant.

The absence of significant microbial contamination in the ovaries and oviducts of KUB-1 chickens, as indicated by the TPC value below the threshold, suggests the potential involvement of non-harmful microbes in pathogen control. El-Saadony et al. (2022) highlighted the intricate association between chicken microbes and *Salmonella*, emphasising their role in pathogen limitation. Intravaginally introduced *Salmonella* encounters epithelial and immune cell protection and competes with local microbes, as noted by Foley et al. (2013). *Pseudomonas veronii*, per Montes et al. (2016) and Canchignia et al. (2017), safeguards chicken oviducts against bacterial infections. Lee et al. (2019) identified dominant bacteria like *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* in the chicken oviduct. Among these, *Pseudomonas*, *Lactobacillus*, *Megamonas*, and *Bacteroides* prevail in adult chickens' oviducts, with similar abundance and diversity in purebred and SPF chickens, with the phylogenetic diversity values representing species richness, and the Shannon index representing alpha diversity, were similar between the two chicken breeds, with H = 0.001923, p-value = 0.965022, and q-value = 0.965022. The TPC results of microbial count in our study indicate good sanitation and hygienic practices, aligning with Mpundu et al. (2019), who attributed bacterial contamination to unhygienic practices and exposure risks.

Figure 2 illustrates the temporal analysis of microbial count fluctuations from day 1 to day 4 PI. Notably, T2 exhibited a decreasing trend in both ovaries and oviducts. This suggests the presence of commensal microbes that may compete with SE. Kogut & Arsenault (2017) highlighted that SE competes with commensals for colonisation and survival, while Lopez et al. (2018) identified significant poultry product contaminants like *Pseudomonas*, *Brochothrix*, *Salmonella*, and others. Investigation of contaminating microbes was limited to *Salmonella*, as it was to determine whether intravaginally SE had contaminated the ovaries and oviducts; and whether it increased the number of microbes in T2 and T3. While the result did not observe colony growth of *Salmonella* in T2 and T3, TPC analysis did reveal a slight increase in microbial counts, albeit below the contamination threshold. Therefore, non-pathogenic microbes and host defense molecules may have influenced the results.

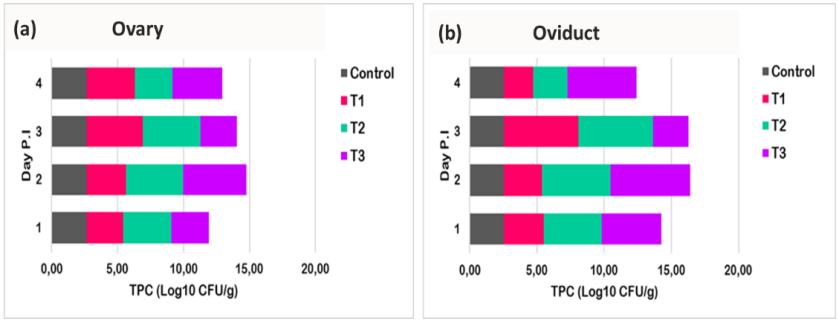

Figure 2. Microbial count in the ovary (a) and oviduct (b) of KUB-1 chickens through TPC, in temporal analysis, from day 1 to day 4 PI.

The host and its microbiota collaborate to prevent pathogen invasion as reported by Rogers et al. (2021), a defense mechanism against SE invasion. Microbiota-produced short-chain fatty acids selectively filter out bacteria unable to maintain pH balance. This, combined with epithelial hypoxia restricting respiratory electron acceptors, hinders *Salmonella*'s resource access. Facultative anaerobic microbes like *Escherichia coli*, *Streptococcus danieliae*, and *Staphylococcus xylosus* compete to strengthen this defense against *Salmonella* infections. Although

Salmonella contamination does not alter egg content, color, smell, and consistency, vigilance for human health remains crucial. Meanwhile, Ahmed et al. (2010) and Li et al. (2023) reported that the effect of the ovary and oviduct dysfunction disorders if exposed to SE for a long is oophoritis and salpingitis. In laying hens, salpingitis is characterized by a decrease in egg production, no or short peak laying time, poor quality of egg shells, and increased production of thin, soft, and sandy eggshells. This poses economic losses as the poultry industry relies heavily on bird reproductive health.

Johnston et al. (2012) reported cases of salpingitis and oophoritis resulting from SE contamination of eggs, wherein bacteria from the cloaca can migrate to the oviduct and ovaries. This process is facilitated by SE fimbriae adhesins that aid in mucosal adhesion, and immune alterations may play a role in infection. Microbial counts were observed in the ovaries and oviducts in the control group, revealing a TPC of ≤ 5 log10 CFU/g. In contrast to the previous study (Miyamoto et al. 1998) intravaginal SE inoculation in adult White Leghorn resulted in a microbial count of 5.7 log10 CFU/g in the control group before SE inoculation. This indicates that the initial microbial count in KUB-1 chicken oviducts was lower than in White Leghorn chickens.

This research was initiated due to a government program providing low-income households with local chickens (KUB-1) for food production. According to Oludairo et al. (2022), Salmonellosis in chickens results in significant public health concerns. The disease is primarily transmitted through animal-derived foods and direct contact with animals, contributing to a wide range of health issues, including enteritis, septicemia, abortion, and meningitis, leading to global morbidity and mortality. Most *Salmonella* strains are pathogenic and can invade and survive within host cells, posing a significant health risk. Additionally, a study by Sartika & Iskandar (2019) demonstrated that KUB-1 chickens exhibit better resistance to avian influenza (AI) than broiler chickens, as indicated by the *Mx* gene. As a zoonotic disease, Salmonellosis control in Indonesia is given priority based on the Decree of the Minister of Agriculture of the Republic of Indonesia No. 237/Kpts/PK.400/M/3/2019. The internationally recognized strategy for preventing foodborne illnesses involves controlling *Salmonella* in poultry farming (Pulido-Landínez 2019), therefore it is essential to explore alternative approaches to combat salmonellosis. Moreover, considering colonisation of *Salmonella* is also influenced by the immune system (Foley et al. 2013), we analysed *AvBD10* expression levels to explore innate immunity variations.

Intravaginal CpG-ODN treatment significantly increased *AvBD10* RGE in the ovaries and oviducts of KUB-1 chickens

Partial analysis of *AvBD10* levels from day 1 to day 4 PI, shown in Figure 3, *AvBD10* RGE increased in T1 oviducts ($p \leq 0.05$), while not significantly in ovaries. There was a significant upregulated *AvBD10* in both ovaries ($p \leq 0.01$) and oviducts ($p \leq 0.05$) of T3. This is consistent with a previous study by Abdel-Mageed et al. (2011). However, a study by Sonoda et al. (2013) using 400-day-old White Leghorn chickens for vaginal cell culture found that *AvBD10* expression was detectable even without stimulation and could be regulated by LPS. In addition, CpG-ODN from microbes, through interaction with TLR21, upregulated *IL1B* and *IL6*, which in turn, induced *AvBD1* and *AvBD3* to combat vaginal infections. This study also revealed upregulated *AvBD10* in the ovaries and oviducts following CpG-ODN stimulation and SE challenge, suggesting activation of *AvBD10* in reproductive tissues, as one of 14

AvBDs that serve as antimicrobial molecules in the body's defense against SE, particularly those caused by LPS in Gram-negative bacteria.

In T2, upregulated *AvBD10* levels in both ovaries and oviducts, although it is not significant. Notably, the findings contrast with a previous study (Abdel-Mageed et al. 2014) that downregulated *AvBD10* in the oviduct cell cultures after CpG-ODN stimulation and challenged with LPS. However, their use of 0.1-10 µg/ml CpG-ODN in adult White Leghorn chickens and our study of 5 µg/0.5 ml intravaginal CpG-ODN in adult KUB-1 chickens might account for these differences. Upregulated *AvBD10* after SE infection in KUB-1 chicken aligns with previous research (Anastasiadou et al. 2013) which observed increased *AvBD10* expression in the vagina post-infection. While the upregulated *AvBD10* was not significant in the T2 group, it was noteworthy.

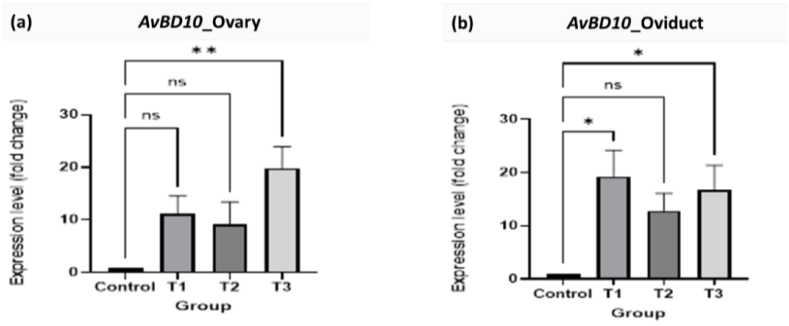

Figure 3. The relative expression of *AvBD10* levels in the ovary (a) and oviduct (b) of KUB-1 chickens at day 1 to day 4 PI in partial analysis through real-time qPCR normalised by *ACTB* and *GAPDH*, analyzed with One-way ANOVA and each bar represents the mean ± SEM. Sign * $p \leq 0.05$; ** $p \leq 0.01$.

In contrast, the T3 group displayed significant upregulated *AvBD10* in both ovaries ($p \leq 0.01$) and oviducts ($p \leq 0.05$). Meanwhile, in a study by Mowbray et al. (2018), AvBD10 with a charge of ≤+4, displayed less potent antimicrobial activity in broiler chicks 21 days old. Bacterial time-kill testing with 0.5 – 12µM recombinant peptide (r) AvBD10 revealed its strong antimicrobial efficacy against fecal *Enterococcus*. This finding supports the evidence of transovarial transmission of Salmonellosis, starting from fecal contamination in the cloaca and progressing to the oviducts and ovaries, as previously reported by Gantois et al. (2009), and this study mimics the transovarial transmission. In another investigation by Goonewardene et al. (2021), CpG-ODN was administered to day-old broiler chicks, challenged with a lethal dose of *E. Coli* altered the metabolic landscape, leading to enhanced antimicrobial immunity in neonatal chicks. Those receiving CpG-ODN had significantly lower bacterial counts ($p<0.05$) compared to the control group. In our investigation involving 60-week-old chickens, also observed enhanced antimicrobial immunity, particularly *AvBD10*. Given the differences in age and microbial populations in the ovaries and oviducts of older chickens compared to day-old chicks, we sought to determine whether the microbial count in these reproductive organs could be categorized as microbial contamination.

The increase in *AvBD10* levels suggests potential immunological memory from previous exposure, reflecting an effective immune response, which aligns with an earlier report (Bultman 2017). Additionally, Van der Mer et al. (2015) highlighted that trained immunity can confer protection against subsequent infection, enhancing immune defense. Moreover, Rusek et al. (2018) outlined that innate immunity exhibits immunological memory akin to adaptive immunity,

bolstering its resistance to subsequent infections. This memory is manifested through trained innate immunity, induced by exposure to infectious or non-infectious agents. This concept holds promise for proactive disease prevention. The study focused on stimulating innate immune memory in chickens using SE bacteria as an infectious agent and CpG-ODN as a non-infectious trigger for *AvBD10* modulation.

Temporal analysis of *AvBD10* levels from day 1 to day 4 PI, shown in Figure 4, revealed noteworthy patterns. In T1, an increase of 21-fold and 26-fold occurred on day 3 PI in the ovary and oviduct respectively, persisting till day 4 PI with a peak increase of 28.3-fold. This suggests that intravaginal CpG-ODN induced *AvBD10* in KUB-1 chicken's ovaries and oviducts. Divergent fluctuations were observed in T2 between the ovary and oviduct. In the oviduct, levels ascended from day 1 to day 4 PI, while the opposite was seen in the ovary. Specifically, the ovary of T2 exhibited a 19-fold increase by day 4 PI, and the oviduct showed a 20-fold increase by day 1 PI. This indicates the involvement of *AvBD10* in the innate immune system against SE, which might differ from its interaction with commensal microbes. Notably, as reported in previous studies (Michailidis et al. 2012) *AvBD* expression in ovaries, and Yoshimura (2015) demonstrated gradually upregulated *AvBD10* in ovaries. In T3, *AvBD10* levels peaked significantly, increasing by 29.5-fold in the ovary and 25-fold in the oviduct on day 3PI. Subsequent fluctuations on day 4 PI suggested decreasing *AvBD10* post-microbial clearance, consistent with a previous report (Van der Meer et al. 2015) indicating a rapid decline of innate immunity after infection resolution. Additionally, Sonoda et al. (2013) supported the role of *AvBD* in eliminating microbes in vaginal tissue during infections. In the oviduct of T3, an increase of 21-fold followed by a decline to 11-fold could be attributed modulation of *AvBD10* by CpG-ODN, promoting an immune response against SE. This aligns with a previous report (Yoshimura 2015), that *Salmonella* infection upregulates *AvBDs* in the oviduct.

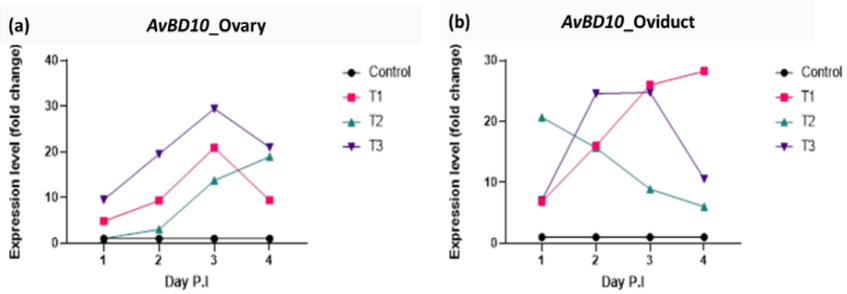

Figure 4. The relative expression of *AvBD10* levels in the ovary (a) and oviduct (b) of KUB-1 chickens from day 1 to day 4 PI in temporal analysis through real-time qPCR normalised by *ACTB* and *GAPDH*. Each plot represents the mean ± SEM.

Animal experiment with three representative chickens from the Control (C) group from day 1 to day 4 PI, each with a gene expression level of 1, as a valid reference for being compared with the 12 chickens in each Treatment (T) group. Reduction in the number of productive laying hens just in the C group as recommended by the Ethics Commission in line with the 3R principle of animal welfare. Additionally, Rieu et al. (2009) suggest conducting qPCR experiments with a minimum of three independent biological replicates for each treatment and at least two technical replicates for each qPCR biological replicate.

This study investigated the effects of CpG-ODN on productive-laying hens, both when administered alone and in combination with an

SE challenge. In the group stimulated by CpG-ODN and challenged with SE, notable upregulated *AvBD10*, supporting its role as a host defense molecule. This is consistent with Sonoda et al. (2013), indicating that *AvBD10* promotes an immune response through activation of its receptor, TLR21. As emphasised by Grzymajlo (2022), the *in vivo* experiment provides more robust insights into host protection factors, thereby this study can provide a basis for further investigation exploring interactions between CpG-ODN and the other 13 *AvBDs* in chickens, contributing to the development of host protection molecules.

CONCLUSIONS

The *AvBD10* was significantly induced by intravaginally CpG-ODN ($p \leq 0.05$) and there was no microbial contamination in the ovaries and oviducts of KUB-1 chickens after being challenged with *S.* Enteritidis. This indicates its ability to induce *AvBD10*, which can be developed as an alternative to preventing foodborne disease caused by transovarial salmonellosis. Further, these *in vivo* studies can be developed on other *AvBDs* in chickens.

AUTHOR CONTRIBUTION

Conceptualisation, S.H.S., E.A.G.R. and R.R.B.S.; methodology, R.R.B.S.; software, R.R.B.S.; validation, R.R.B.S.; formal analysis, R.R.B.S., S.E.P., R.S., and D.N.W.; investigation, R.R.B.S.; resources, R.R.B.S.; data curation, R.R.B.S.; writing—original draft preparation, R.R.B.S.; writing—review and editing, R.R.B.S.; supervision, S.H.S. and E.A.G.R.; project administration, R.R.B.S.; funding acquisition, R.R.B.S.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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