

Dietary selenium supplementation enhances antiviral immunity in chickens challenged with low pathogenic avian influenza virus subtype H9N2

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ABSTRACT

Selenium supplementation in poultry feeds has been known to have beneficial effects on the bird health and performance; however antiviral effects of selenium have remained largely unknown. In this study, we have evaluated the effects of supplementation of chicken diets with organic (Selenium Enriched Yeast; SEY) and inorganic selenium (Sodium Selenite; SS) on low pathogenicity avian influenza virus (H9N2) shedding in the cloacal and oropharyngeal swab samples as well as examined the expression of immune related genes. Chickens were fed two doses (High- 0.30 mg/kg of feed; Low- 0.15 mg/kg of feed) of selenium supplementation for 2 weeks followed by low pathogenicity avian influenza virus challenge. Our results showed that the cloacal shedding of virus in all the selenium supplemented groups was significantly lower when compared to the non-supplemented control groups. In addition, the oropharyngeal shedding of virus in chickens fed with organic selenium supplementation was significantly lower than that in the chickens that received either inorganic selenium supplemented feed or controls. Furthermore, the expression of interferon stimulated genes (Viperin, OAS: 2'-5' oligoadenylate synthetase and MDA5: melanoma differentiation-associated gene) in the cecal tonsils was significantly elevated in the selenium treated groups when compared to controls. Additionally, a significantly higher transcription of interferon (IFN)- α , IFN- β and IFN- γ genes in the cecal tonsils and spleens of chickens receiving SEY-L and SS-H supplemented feed was also observed at post virus challenge time points compared to untreated controls. The results of this study demonstrated that supplementation of chicken diets with selenium, can enhance antiviral defense and thus, may have a beneficial effect in controlling viral infections in poultry.

1. Introduction

Many nutrients, including vitamins and minerals have been demonstrated to influence performance parameters and enhance immune responses to infectious agents as well as experimental antigens (Jose et al., 2017; Pan et al., 2018; El-Senousey et al., 2018). Among these nutrients, selenium which has a beneficial role on the immune system (Arthur et al., 2003) is of note. Selenium is an important micronutrient and is fed either in organic or inorganic forms in poultry rations, with organic selenium known to be more bioavailable (Delezie et al., 2014). The effect of different selenium sources on bird performance or the immune system has been investigated. For example, organic selenium

supplementation has been shown to enhance performance and antioxidant activity in broiler chickens during heat stress (Rao et al., 2016). Rajashree et al. (2014) found that supplementing broiler breeder diets with selenium, at the onset and during egg production, resulted in higher egg production and hatchability. Furthermore, the important role of selenium in the regulation of immune system functions including innate and adaptive responses has been studied (Turner and Finch, 1991; McKenzie et al., 2001; Arthur et al., 2003).

Immune enhancing activities of selenium are largely attributed to its role as an antioxidant. Selenium essentially regulates the function of glutathione peroxidase (GPx; Baker et al., 1993), an enzyme with antioxidant activity that neutralizes Reactive Oxygen Species (ROS);

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Bogdan et al., 2000), to reduce oxidative stress and protect the integrity of cells, including cells of the immune system (Bathige et al., 2015; González de Vega et al., 2017). It has been shown that dietary selenium levels are directly related to the level and activity of GPx (Cantor and Tarino, 1982; Penglase et al., 2010). In the context of viral or bacterial infections, selenium supplementation has been shown to influence T helper (Th1/ Th2) responses (Steinbrenner et al., 2015). Feed supplementation with selenium has also been shown to regulate the inflammation process, which underscores the important role of selenium in resolving infection via avoiding unwanted excessive pathology (Duntas, 2009). In this context, a challenge of selenium deficient mice with influenza virus has been shown to result in a severe infection with increased lung lesions (Beck, 2001).

Avian influenza (AI) is considered a major challenge to the poultry industry (Capua and Marangon, 2006). Despite significant losses due to mortality, as well as prevention and control effort costs, the current advances in science and technology have not been able to prevent or control AI outbreaks. Different types of vaccines have been used to confer immunity against avian influenza viruses (AIV) (Swayne, 2009). However, vaccines have had limited success partly due to the fact that AIV, like other influenza viruses undergoes antigenic shift and drift (Cattoli et al., 2011). Therefore, other strategies are required to enhance immunity against AIV. These strategies may include supplementation of feed with nutrients with immunostimulatory effects in order to enhance innate immune defenses against the virus as well as augmenting vaccine induced protective immune responses.

In the present study, we evaluated the effect of supplementation of chicken diets with organic (Selenium Enriched Yeast: SEY) and inorganic (Sodium Selenite: SS) selenium on avian influenza virus shedding and expression of antiviral genes in tissues.

2. Materials and methods

2.1. Experimental design

Two experiments were conducted. In the first experiment, which was designed to assess viral shedding, 60 one day old SPF chickens were randomly divided into 6 treatments of 10 chickens/group, and placed in Horsfall units in a biosafety level II facility in the Isolation Unit at the University of Guelph. Treatments included diets supplemented with the following doses of selenium: a low dose of SEY (SEY-L; 0.15 mg/kg of feed), high dose of SEY (SEY-H; 0.30 mg/kg of feed), low dose of SS (SS-L; 0.15 mg/kg of feed), high dose of SS (SS-H; 0.30 mg/kg of feed), as well as two groups where chickens were fed unsupplemented diet and either challenged with H9N2 virus strain (A/turkey/Wisconsin/1/1966) by oculo-nasal route (Positive Control) or unchallenged (Negative Control). Ad-libitum access to selenium supplemented diets was provided from day one.

In the second experiment, which was conducted to evaluate the effect of selenium on antiviral gene expression, 150 one day old SPF chickens were randomly divided into 6 treatment groups of 25 chickens/group with the same treatments as in the first experiment. Feed and water were provided *ad libitum* from day one. At 14 days of age, groups 1 to 5 were challenged with 400 µl of 10⁷ TCID₅₀ of H9N2 strain. All animal experiments were approved by the Animal Care Committee, University of Guelph.

2.2. Virus propagation, titration and infection of chickens

Propagation of the virus was performed by injection of low pathogenicity H9N2 AIV into allantoic fluid of 10 day old SPF embryonated chicken eggs, as described previously (Szretter et al., 2006) with some modifications. Briefly, embryonated SPF chicken eggs (CFIA, Ottawa Laboratory, Nepean, ON, Canada) were incubated at 37 °C for 10 days followed by inoculation of each egg with 4HA units of H9N2. Eggs were further incubated for 72 h at 35 °C and monitored every 24 h and dead

embryos were discarded. At 72 h of incubation, embryonated eggs were kept at 4 °C overnight followed by the collection of allantoic fluid and centrifugation at 400 x g for 5 min. Allantoic fluid was pooled and stored at –80 °C until further use. Virus titre of allantoic fluid was determined using Tissue Culture Infectious Dose₅₀ (TCID₅₀) on Madin-Darby canine kidney (MDCK) cells (WHO, 2002). At 14 days of age, all birds except negative control were challenged with 400 µl of the suspension containing 1 × 10⁶ TCID₅₀/mL of the virus through the oculo-nasal route. At 3, 5 and 7 days post challenge, oropharyngeal and cloacal swab samples were collected. A virus titration assay using MDCK cells was performed to assess viral shedding from both oropharyngeal and cloacal swabs as described previously (WHO, 2002). Viral titre was expressed as TCID₅₀/mL of virus present in the supernatant of each well after 3 days of inoculation, and was calculated based on the formula by Reed–Muench (Szretter et al., 2006).

2.3. RNA extraction and complementary DNA (cDNA) synthesis

Six chickens per time point and treatment were euthanized at 14, 17, 19 and 21 days of age (days 0, 3, 5 and 7 days post infection; dpi), and tissue samples from the cecal tonsils and spleens were collected and preserved in RNAlater at –80 °C until extraction of RNA. Tissue samples were homogenized in Trizol for RNA extraction according to the manufacturer's instructions (Invitrogen Canada Inc., Burlington, Ontario, Canada). RNA quantity and quality were determined using the NanoDrop® ND-1000 spectrophotometry (NanoDrop Technologies, Wilmington, DE). cDNA synthesis was done by reverse transcription of 500 ng of total RNA using oligo (dT)12–18 primers and the Super Script™ First Strand Synthesis System was carried out in a total reaction volume of 20 µl according to manufacturer's instructions (Life Technologies Inc., Burlington, Ontario, Canada).

2.4. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was run in 384-well plates with 20 µL total reaction volume consisting of 5 µl of cDNA (1:10 dilution), 1 µl of each forward and reverse primers (5 µM each), and 13 µl of SYBR Green (Roche Diagnostic, Laval, QC, Canada). Primer sequences along with their respective annealing temperatures are provided in Table 1.

2.5. Statistical analysis

The results for shedding of the virus (TCID₅₀/ml), as well as the expression of each gene relative to beta-actin (housekeeping gene) were

Table 1
Primer sequences used for real-time PCR.

Target Gene	Primer Sequence, 5'-3'	Annealing temp (°C)	Reference
Viperin	F: GGAGGCGGGAATGGAGAAAA R: CAGCTGGCCCTACAAATTCGC	60	Barjesteh et al., 2015
MDA5	F: GCAAAAACCGACTGAATGGG R: CGTAAATGCTGTCCACTAACGG	60	Barjesteh et al., 2015
IFN-α	F: ATCTGCTGCTCAGGCTCTTCT R: GGTGTTGCTGGTTCAGGATG	64	St. Paul et al., 2011
IFN-γ	F: AACTGACAAGTCAAAGCCGC R: AGTCGTTTCATCGGGAGCTTG	60	Brisbin et al., 2010
IFN-β	F: GCCTCCAGCTCCTTCCAGAAATACG R: TGGATCTGGTTGAGGAGGCTGT	64	Villanueva et al., 2011
OAS	F: 5'-AGA ACT GCA GAA GAA CTT TGT C-3' R: 5'-GCT TCA ACA TCT CCT TGT ACC-3'	60	Villanueva et al., 2011
β-Actin	F: CAACACAGTGTCTGTGGTGGTA R: ATCGTACTCTGCTTGCTGATCC	60	Shojadoost et al., 2017

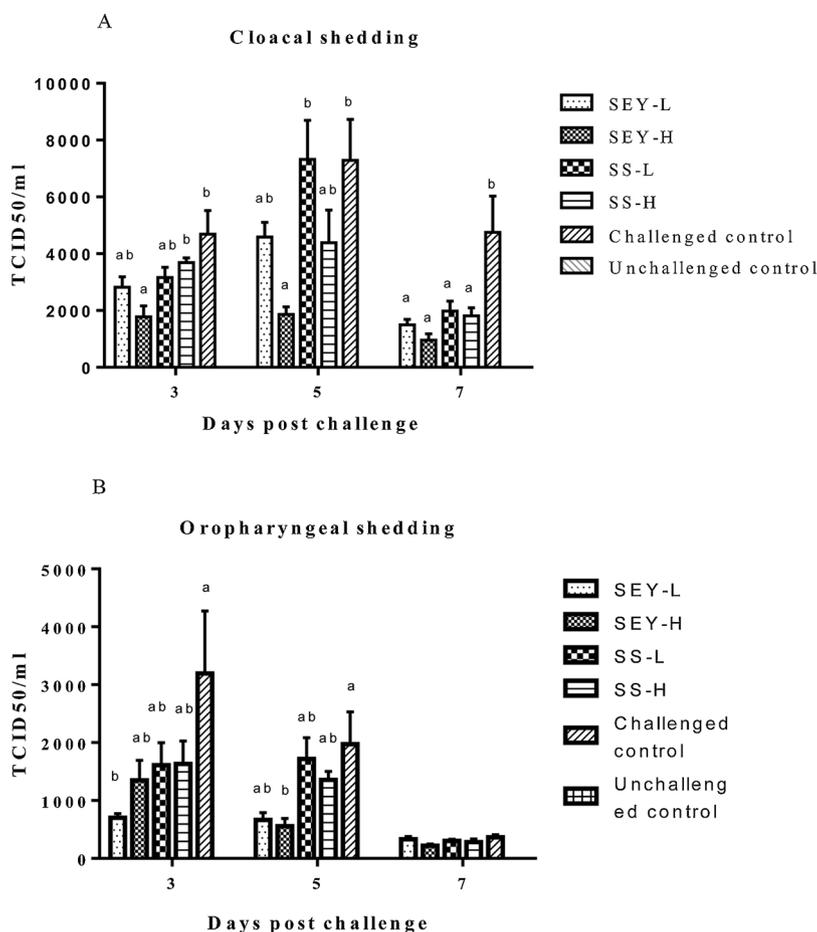


Fig. 1. Effect of in-feed selenium supplementation on cloacal (A) and oropharyngeal (B) shedding of low pathogenic avian influenza virus. Chickens were fed with a diet supplemented with selenium enriched yeast (SEY: 0.15 and 0.30 mg/kg) and sodium selenite (SS: 0.15 and 0.30 mg/kg) and were challenged with low pathogenic AIV (H9N2), at 14 days of age. Challenged and unchallenged control groups did not receive supplemented selenium. Cloacal and oropharyngeal samples were collected at 3, 5 and 7 dpi and the titer of the virus was measured by TCID₅₀ assay. The error bars indicate standard error of means of ten biological replicates (n = 10). Groups with different letters are significantly different (P < 0.05).

calculated and compared among groups in each time point, using one way ANOVA, Kruskal Wallis and Tukey's test with chickens as an experimental unit. GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA) was used for this purpose and P value less than 0.05 was considered significant.

3. Results

3.1. Virus shedding

To determine the efficacy of selenium as a feed supplement in reducing H9N2 AIV infection burden in chickens, shedding of the virus in cloacal and oropharyngeal samples of birds was analyzed at 3, 5 and 7 dpi. Chickens receiving SEY-H supplemented feed had significantly lower virus titers (P < 0.01) in their cloacal samples at all the time points when compared to untreated but H9N2 challenged birds (Fig. 1A). In addition, the oropharyngeal shedding of virus in SEY-H chickens was significantly lower (P < 0.01) at 5 dpi when compared to chickens that received SS-L/ SS-H supplemented feed or no treatment but only the virus challenge (Fig. 1B). Cloacal virus shedding at 7 dpi in all selenium treated groups was significantly lower when compared to the virus challenged control group; however, there was no significant difference among the supplemented groups. Furthermore, oropharyngeal shedding analysis showed that at 3 and 5 dpi, SEY-L supplemented feeding resulted in a significantly lower virus shedding compared to virus challenged control group (Fig. 1B).

3.2. Expression of immune system genes

To determine the effects of selenium supplementation in chicken feed on the immune system, we evaluated the expression of interferon

stimulated genes (ISG) and interferon (IFN) genes in cecal tonsil and spleen tissues. These genes included: Viperin, OAS (2'-5' oligoadenylate synthetase), MDA5 (melanoma differentiation-associated gene 5), interferon (IFN)- α , IFN- β and IFN- γ .

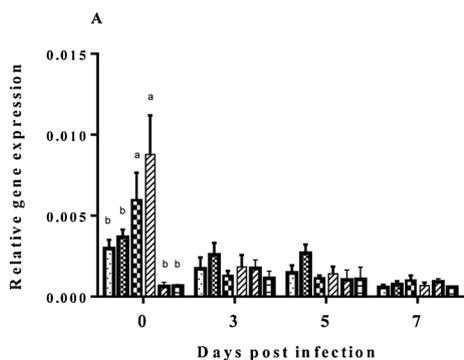
As depicted in Figs. 2A, 2C and 2E, while the chickens that received SS-L or SS-H supplemented feed had significantly higher (P < 0.0001) expression of viperin in their cecal tonsils at the pre-virus challenge time point (day 0) compared to the rest of the groups, the expression of MDA5 and OAS genes was significantly elevated (P < 0.0001) in all the selenium treated groups when compared to untreated controls. However, no changes in the expression of viperin, OAS or MDA5 genes were observed at 3, 5 or 7 dpi. Furthermore, in the spleen, there were no changes observed between the groups at any of the time-points examined (Figs. 2B, 2D and 2F).

In regard to the expression of interferon genes, there was a significantly higher transcription of IFN- α in the cecal tonsils of chickens receiving SEY-L supplemented feed at 5 dpi compared to those fed with SS-H supplemented diet (Fig. 3A). Furthermore, IFN- α gene expression in the cecal tonsils of these birds remained significantly higher at 7 dpi when compared to groups that received either SS-L supplemented feed or virus-alone challenge. However, no changes were observed in the expression of IFN- α gene between the groups at either 3 dpi in the cecal tonsils (Fig. 3A) or at any of the time points in the spleen (Fig. 3B). Furthermore, expression of IFN- β gene in the cecal tonsils of chickens fed with SS-H supplemented diet was found to be significantly higher at 3 dpi when compared to chickens receiving either SS-L or no selenium supplementation (Fig. 3C). However, no changes were observed in the expression of IFN- β gene between the groups at either later time-points dpi in the cecal tonsils (Fig. 3C) or at any of the time-points in the spleen (Fig. 3D).

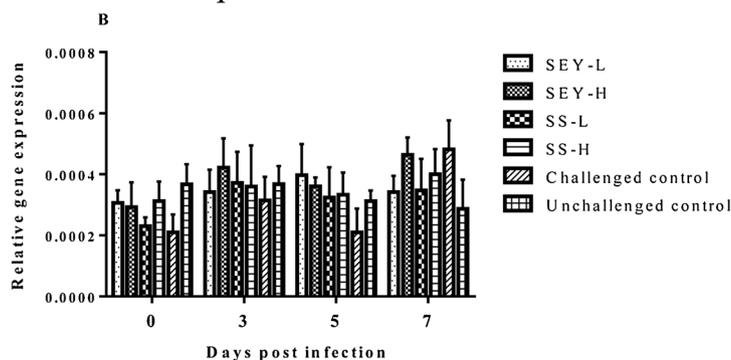
Significant (P < 0.05) changes in the IFN- γ expression were

Viperin

Cecal tonsil

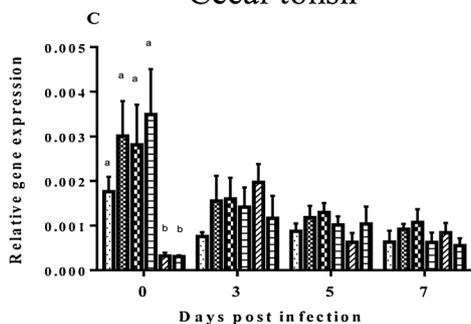


Spleen

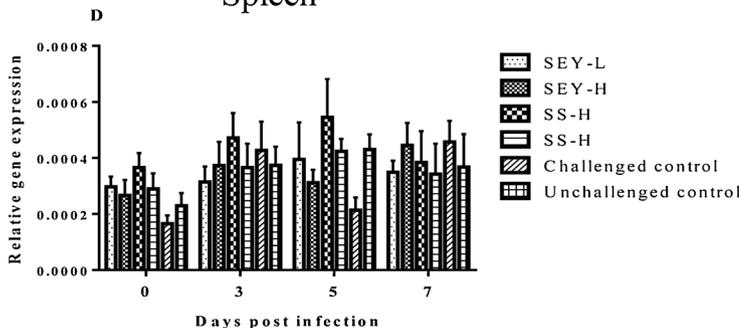


MDA5

Cecal tonsil

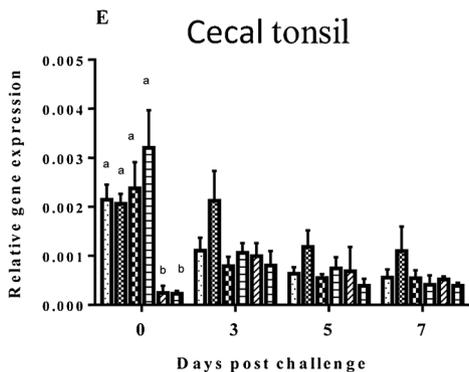


Spleen



OAS

Cecal tonsil



Spleen

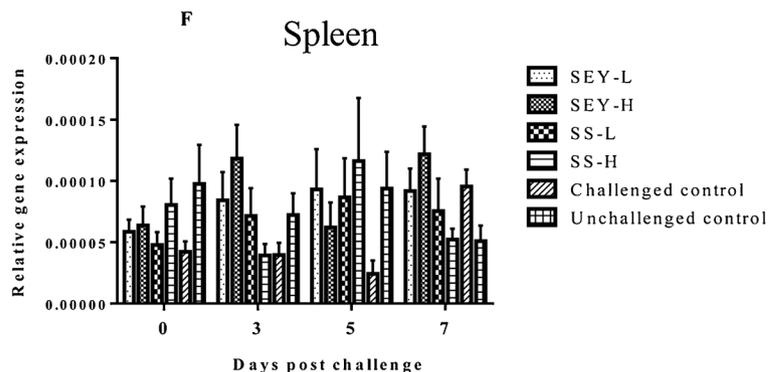


Fig. 2. Relative Viperin, OAS, MDA5 gene expression in lymphoid tissues of chickens fed with selenium supplemented diet. Chickens were fed with a diet supplemented with selenium enriched yeast (SEY: 0.15 and 0.30 mg/kg) and sodium selenite (SS: 0.15 and 0.30 mg/kg) and were challenged with low pathogenic AIV (H9N2), at 14 days of age. Challenged and unchallenged control groups did not receive selenium. Cecal tonsils and spleens were collected at 0, 3, 5, and 7 dpi and viperin (A, B), MDA5 (C, D) and OAS (E, F) gene expression was quantified using real-time PCR technique. The results are shown as relative to β -actin. The error bars indicate standard error of means of 6 biological replicates (n = 6). Groups with different letters are significantly different (P < 0.05).

observed in spleen but not in the cecal tonsils. To this end, chickens fed with SEY-L had significantly elevated splenic IFN- γ transcription when compared to virus only challenged controls (Fig. 3F).

4. Discussion

Selenium has been used experimentally and commercially as a feed

supplement and has been shown to improve bird health and performance. However, the antiviral activity of selenium is not well studied. In the present study, the effects of supplementation of chicken diets with organic (SEY) and inorganic selenium (SS) on the avian H9N2 low pathogenicity virus shedding and the induction of immune system genes were evaluated. We found that selenium supplementation resulted in reduced virus shedding and enhanced expression of ISG and

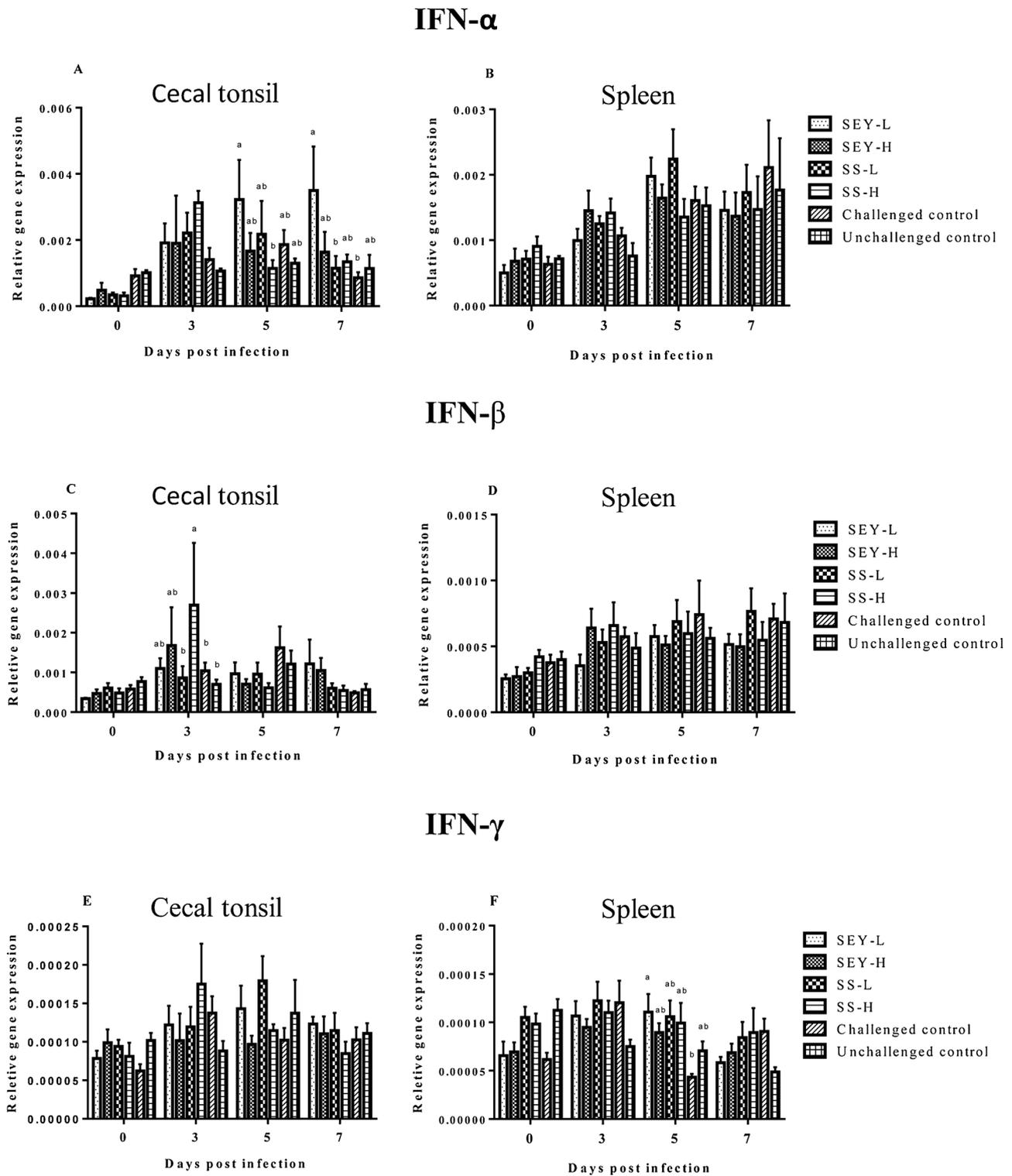


Fig. 3. Relative IFN- α , IFN- β and IFN- γ gene expression in lymphoid tissues of chickens fed with selenium supplemented diet. Chickens were fed with a diet supplemented with selenium enriched yeast (SEY: 0.15 and 0.30 mg/kg) and sodium selenite (SS: 0.15 and 0.30 mg/kg) and were challenged with low pathogenic AIV (H9N2), at 14 days of age. Challenged and unchallenged control groups did not receive selenium. Cecal tonsils and spleens were collected at 0, 3, 5, and 7 dpi and IFN- α (A, B), IFN- β (C, D) and IFN- γ (E, F) gene expression was quantified using real-time PCR technique. The results are shown as relative to β -actin. The error bars indicate standard error of means of 6 biological replicates (n = 6). Groups with different letters are significantly different (P < 0.05).

IFN genes.

Successful control of AIV is not just limited to preventing the disease in birds but more importantly, to curtail virus shedding. This is because AIV that replicates in mucosa and is shed through feces as well as small air droplets, can pose a great threat to other susceptible birds within the

flock and also those in the neighboring flocks (Jonges et al., 2015). Therefore, reducing AIV shedding could potentially prevent the spread of the virus restricting the size of AI outbreaks. To this end, we observed that the chickens given both SEY and SS supplemented feed had significantly reduced virus shedding as determined by virus titers in their

cloacal samples at 7 dpi compared to untreated virus challenged control chickens. In particular, organic SEY fed birds shed significantly less virus cloacally (3, 5 and 7dpi) as well as oropharangeally (3 and 5dpi) compared to untreated chickens. These observations suggest an efficacious role for selenium as a beneficial feed supplement in reducing AI virus shedding in chickens. In this context, it is noteworthy that the extent and severity of some viral diseases including AI are directly related to the selenium levels in the soil. For example, during the highly pathogenic AI outbreaks during 1983–84 and 1994–95 in North America, the levels of selenium were found to be low in the affected areas (Swayne et al., 1998). Furthermore, experiments using the mouse model have shown that feeding a diet containing higher levels of selenium reduces pathogenicity of influenza virus and the resulting lung lesions (Beck et al., 2003). In the present study, we used a low pathogenicity virus and it would be interesting to determine the effects of selenium on pathogenicity and shedding of highly pathogenic viruses in the future.

An important observation of the present study was that supplementation of chicken feed with the organic form of selenium (SEY) was more effective at reducing virus shedding compared to the inorganic selenium (SS) supplemented diet. This may be due to the fact that SEY is more bioavailable than SS and that it may also have a more potent immunostimulatory activity in terms of augmenting the functions of the immune system cells through reducing the cellular oxidation status (Delezie et al., 2014). This concept is further supported by the findings of Peretz et al. (1991) and McKenzie et al. (2008) who found that SEY supplementation was associated with an increased lymphocyte proliferation with augmented IL-2 receptor expression as well as reduced damage to immune system cells by free radicals. Similar observations regarding the superior activities of enriched organic dietary selenium have also been reported in broilers and turkeys. For example, while feeding young turkeys with organic selenium supplemented feeds enhanced plasma GPx activity (Cantor and Tarino, 1982), feeding breeder turkeys with a diet supplemented with organic selenium resulted in reduced rate of oxidation in eggs as well as in the tissues of newly hatched poults (Jankowski et al., 2011). Similarly, it was observed that broiler chicks fed on a diet containing organic selenium had improved performance and enhanced antioxidant activity as determined by reduced lipid peroxidase and increased superoxide dismutase activities (Rao et al., 2016). Furthermore, Rajashree et al. (2014) showed that parent broiler breeder feeding with organic selenium diets led to increased egg productivity, number of settable eggs and hatchability as well as reduced oxidation activity in hatching eggs. Considering these lines of evidence, our findings add on a new dimension to the beneficial effects of dietary selenium supplementation in terms of augmenting antiviral immunity in poultry.

Selenium is an essential dietary element with antioxidant roles in immune functions; however, there is little information available as to how this element acts at the molecular level in host immunity and inflammation (Shrimali et al., 2008). It is known that selenium is incorporated into the amino acid selenocysteine, which in turn is inserted into selenoproteins. The selenoproteins within immune system cells perform antioxidant functions, carry out protein folding and promote certain cell signaling events required in cellular activation (Huang et al., 2012). Cells of the immune system express several selenoproteins of which GPx isoenzymes 1 and 4 have been shown to have the highest expression levels, particularly in T cells and macrophages (Steinbrenner et al., 2015). In this regard, it is important to note that the level of cellular GPx activity has been shown to be directly associated with the amount of available dietary selenium and that additional dietary selenium supplementation results in enhanced GPx activity which in turn augments IFN- γ mediated T cell responses during infections (Broome et al., 2004). Several studies have demonstrated the beneficial effects of dietary selenium in immunity to viruses including influenza, HIV and Cocksackie viruses (Gill and Walker, 2008). For example, infection with influenza virus in mice is shown to decrease GPx activity resulting in an

increased oxidative stress in T cells leading to impaired immune response and reduced virus clearance (Beck et al., 2001). It has also been found that Selenium deficient mice and rats infected with influenza virus had macrophages, NK cells and CD8 + T cells with impaired functions (Ferencik and Ebringer, 2003; Hoffmann and Berry, 2008). Furthermore, Jaspers et al (2007) used an in vitro system of differentiated human bronchial epithelial cells to suggest that selenium has a significant impact on the maintenance of epithelial cell integrity and thus, host defense against influenza virus infection (Jaspers et al., 2007). Considering the observation in the present study that selenium supplementation could reduce low pathogenic avian influenza virus shedding in chickens and although the exact mechanisms of selenium mediated enhancement of immune function are not fully known, it seems reasonable to suggest that selenium can exert its effect by altering the redox status of the cells or by meeting the increased requirements for selenoproteins of the activated immune system cells that contribute to an effective antiviral defense (Gill and Walker, 2008).

An important antiviral defense mechanism includes efficient production of interferons and induction of ISGs that help curtail virus replication and reduce virus shedding. Considering the evidence that indicate dietary selenium supplementation improves immune system cell functions and that these cells will possess better interferon production ability, we further sought to measure the expression of interferons and ISGs (OAS, MDA5 and Viperin) in lymphoid tissues, the cecal tonsils and spleen, at pre- and post-virus challenge stages. It was found that selenium supplemented chickens had significantly higher expression of IFN- α , β and γ in these tissues following to virus challenge compared to untreated controls. It was also observed that the expression of OAS, MDA5 and Viperin genes in the cecal tonsils was significantly elevated in the selenium treated groups at pre-challenge stage when compared to untreated controls. It is of note that while MDA5 is an important virus sensing receptor, OAS and Viperin have been shown to assist in preventing budding and release of influenza virus particles from the infected cells (Chakrabarti et al., 2011; Tan et al., 2012). One of our findings was also that the induction of ISG expression preceded the expression of interferon genes, although it is generally observed that interferons induce ISG expression (Helbig and Beard, 2014), which was suggestive of a mechanism of ISG expression independent of IFN. This concept is supported by a previous study that used an in-vitro T cell culture to show that Interferon regulatory factor-3 (IRF-3), a ubiquitously expressed protein early on during viral infection, can directly stimulate expression of certain ISGs, including OAS, in an IFN-independent manner (Grandvaux et al., 2002). Similarly, IFN-independent expression of MDA5 has also been reported (Iwasaki, 2012). To this end, we have recently reported a significant expression of OAS and Viperin genes expression prior to the expression of type-I IFN in tracheal organ cultures in response to treatment by TLR ligands (Barjesteh et al., 2016). Nevertheless, taken together, our observations from these gene expression experiments suggest that selenium supplementation of poultry diets can result in augmented expression of interferon and ISG genes in the cells of the lymphoid tissues. Furthermore, it can be speculated that the selenium incorporation towards the synthesis of selenoproteins can likely render an antioxidant status in the immune cells and hence augment their antiviral responses including interferon and ISG production. Therefore, in summary, our results demonstrate that selenium supplementation of chicken diets increases the expression of antiviral response genes which could lead to reduction of virus shedding from infected birds.

Conflict of interest

Authors declare no conflict of interest.

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