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Effects of Newcastle Disease/Infectious Bronchitis Vaccine and Feeding Yeast Products on the Innate Immune System in the Proventriculus and Ileum of Broiler Chicks

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The aim of this study was to determine whether Newcastle disease/infectious bronchitis (ND/IB) vaccination and yeast product diet supplementation modulate the expression of innate immune molecules in the proventriculus and ileum of broiler chicks. One-day-old male broiler chicks were divided into four groups (V–Y– (control), V–Y+, V+Y–, and V+Y+ groups, where V and Y represent vaccination and yeast product supplementation, respectively). Chicks in the V+Y– and V+Y+ groups were immunized with the live ND/IB vaccine, whereas chicks in the V–Y– and V–Y+ groups were not. Chicks in the V–Y+ and V+Y+ groups received feed containing yeast products from day 4, whereas chicks in the V–Y– and V+Y– groups did not. The proventriculus and ileum were collected on day 7 to analyze the expression of seven Toll-like receptors (TLRs) and Dectin-1. In the proventriculus, compared with those of the V–Y– control group, the TLR7 and TLR21 expression levels were higher in the V+Y– group; however, there were no differences in the expression levels of any TLR or Dectin-1 in the ileum. There were also no differences in the expression of avian β-defensins and cathelicidin-1 in the proventriculus and ileum between the control and treatment groups. The expression of granzyme in cytotoxic cells and interleukin (IL)-1B was upregulated by ND/IB vaccination in the proventriculus. Supplementation with yeast products upregulated only granzyme expression in the ileum and downregulated IL-6 expression in the proventriculus in chicks immunized with the ND/IB vaccine. Thus, we concluded that ND/IB vaccination is effective at enhancing the innate immune system in the proventriculus of chicks, at least until day 7 post-hatching, whereas the effects of diet supplementation with yeast products may be limited, at least under the present study conditions.

Key words: chick gut, innate immunity, vaccination, yeast

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Introduction

The chicken gut is the primary organ through which pathogens invade the body. Chickens are most susceptible to invading pathogens during the first week post-hatching when the immune system may be functionally inefficient (Lowry et al., 2005). Recent studies have identified the expression of innate immune molecules in the chick gut that may be associated with local defenses against infection by pathogenic microbes (Terada et al., 2018, 2020a, 2020b). This innate immune system may be important for defense against infection at early life stages, considering that the adaptive immune system of chicks is not developed for the first a few weeks of life. Innate immune responses are initiated by the binding of microbe-associated molecular patterns to pattern recognition receptors, including Toll-like receptors (TLRs), leading to the synthesis of proinflammatory cytokines and antimicrobial peptides, as well as the activation of phagocytic and cytotoxic cells (Yoshimura, 2015).

Ten TLRs have been identified in chickens to date. Among these, TLR2, 4, 5, and 15 recognize bacterial lipopeptides and peptidoglycan, lipopolysaccharide, flagellin, and virulence-associated bacterial proteases, respectively. TLR3, 7, and 21 recognize double-stranded RNA, single-stranded RNA (ssRNA), and unmethylated CpG-DNA, respectively (Yoshimura, 2015). β-Glucan, a fungal cell wall component, is recognized by dendritic cell-associated c-type lectin-1 (Dectin-1) in cooperation with TLR2 to activate immune cells, including macrophages

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(Yadav and Schorey, 2006). A functional Dectin-1-like β-glucan receptor was identified in chicken heterophils and mononuclear cells (Nerren and Kogut, 2009).

Previous studies in different organisms, including invertebrates (Kurtz, 2005) and mammals (de Bree et al., 2018; Netea et al., 2011, 2020; van der Meer et al., 2015), suggested that vaccinations may induce trained innate immunity. The *Bacillus* Calmette-Guérin vaccine was reported to induce innate immune training by increasing histone modification and cytokine production in immune cells in response to agents unrelated to the vaccine antigen (Kleinnijenhuis et al., 2012, 2014). Recently, we reported that the proventriculus of 11-day-old chicks who had been inoculated with the Newcastle disease and infectious bronchitis (ND/IB) vaccine at one day old had higher expression levels of TLR21 compared with those of unvaccinated chicks, although no other TLR was affected (Yoshimura et al., 2022). Moreover, the expression levels of TLR7 and 21, but not TLR3, in the kidney were higher in ND/IB-vaccinated chicks than those in control chicks at day 3 of age, whereas no differences were found on day 10 (Shimizu et al., 2020). However, the influence of ND/IB vaccination on the intestinal innate immune system of chicks remains unknown.

Recent reports have demonstrated that supplementation with yeast or yeast cell wall extracts improves growth performance, cecal microbial composition, and digestibility of nutrients, and reduces the inflammatory status in the intestine of broilers (Khalid et al., 2021; Pascual et al., 2020). Yeast products also support pro- and anti-inflammatory cytokine production via T helper type 1 and 2 cell-associated pathways (Yitbarek et al., 2013), and upregulate the cell-mediated immune response to Newcastle disease virus vaccine (Bi et al., 2022). β-Glucan was reported to increase phagocytosis, bactericidal activity, and oxidative burst in chicks (Lowry et al., 2005). β-Glucan treatment could also induce trained innate immunity in primary chicken monocytes (Verwoolde et al., 2020). A study in humans also suggested that β-glucan induces trained immunity by enriching histone modification at the promoter regions of proinflammatory genes in human monocytes (Domínguez-Andrés et al., 2020). Although these reports suggest that yeast-related substances could affect immune cell function, it remains unknown whether the innate immune system in the chick gut is affected by feeding yeast products.

To address these questions, the aim of this study was to determine whether ND/IB vaccination and/or yeast product feeding affect the expression of innate immune molecules in the proventriculus and ileum of chicks. We hypothesized that ND/IB vaccination may affect the expression of TLRs, and thus the immune response caused by yeast products administered after vaccination may differ between vaccinated and non-vaccinated chicks. To test this hypothesis, vaccination was performed on day 0 (hatching day), and yeast product feeding was initiated on day 4 post-hatching. The examined innate immune molecules were pattern recognition receptors (TLRs and Dectin-1); antimicrobial peptides such as avian β-defensins (AvBDs) and cathelicidin-1 (Cath1); and cytotoxic cell markers such as B-NK [a natural killer (NK) cell receptor (Nii et al., 2014)], granzyme (a cytotoxic molecule), and proinflammatory cytokines [interleukin (IL)-1β and IL-6].

Materials and Methods

Experimental design

One-day-old male broiler chicks (day 0) were divided into four groups with 6 chicks per group: V–Y– (control), V–Y+, V+Y–, and V+Y+, where V represents vaccination and Y represents yeast product feeding. Chicks in the V+Y+ and V+Y– groups were inoculated with the ND/IB vaccine, whereas chicks in the V–Y– and V–Y+ groups were not. Chicks in the V–Y+ and V+Y+ groups received yeast products from day 4, whereas those in the V–Y– and V+Y– groups did not. All chicks were euthanized on day 7 for the expression analysis of innate immune molecules. This study was approved by the Hiroshima University Animal Research Committee (no. C15-16).

Experimental birds and vaccine inoculation

Male broiler chicks (Chunky) were obtained by incubating fertilized eggs purchased from a local hatchery (Fukuda Breeder, Okayama, Japan) and their sex was determined by feather sexing. One-day-old male chicks of the V+Y+ and V+Y– groups were given the mixed live ND/IB vaccine (Poulvac COMBI, Kyoritsu Seiyaku Co., Tokyo, Japan) containing the ND virus B1 strain and IB virus H120 strain using a nasal drip; chicks in the V–V– and V–Y+ groups were given sterile phosphate-buffered saline (PBS) instead of vaccination. The chicks were maintained in a brooding room with electric heaters under 23 h light and 1 h dark conditions, with free access to a commercial starter diet (Nichiwa Sangyo Co. Ltd., Hyogo, Japan) and water. From the age of 4 days post-hatching, chicks in the V–Y+ and V+Y+ groups were fed a starter diet containing 250 mg/kg of yeast product (ASCOGEN; CHEMOFORMA AG, Augst, Switzerland), consisting of 17% inactivated yeast (*Saccharomyces cerevisiae*), 82% of yeast extracts (the soluble fraction of yeast, excluding the insoluble fractions consisting of the cell wall and proteins), 1% colza oil, and 5000 mg/kg of clinoptilolite. On day 7, the chicks were euthanized using carbon dioxide and the middle part of the proventriculus and ileum tissues were collected. The tissues included the mucosa and smooth muscle layers, which were placed in RNA later (Thermo Fisher Scientific Co., Waltham, MA, USA) and stored at −80°C until the analysis of gene expression levels of immune molecules. Tissues of the proventriculus and ileum were fixed in 10% (v/v) formalin in PBS and used for histological analysis.

Real-time polymerase chain reaction (PCR) analysis for immune molecules

RNA isolation and cDNA preparation

Total RNA was extracted from the collected tissues using Sepasol RNA I Super (Nacalai Tesque, Inc., Kyoto, Japan), according to the manufacturer's instructions, and dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid). The total RNA concentration was measured us-

ing a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). The samples were incubated with RQ1 RNase-free DNase (Promega Co., Madison, WI, USA) on a programmable thermal controller (PTC-100; MJ Research, Waltham, MA, USA) according to the manufacturer's instructions. Total RNA samples were reverse-transcribed using Rever-Tra Ace (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The obtained complementary DNA (cDNA) samples were stored at −80°C until use.

Real-time PCR analysis

Real-time PCR was performed using the AriaMix Real-time PCR System (Agilent Technologies Japan, Ltd., Tokyo, Japan), as described previously (Yoshimura et al., 2022). The reaction mixture (10 μL) consisted of 1 μL cDNA, 1× Brilliant III SYBR Green QPCR Mix (Agilent Technologies Japan, Ltd.), 0.25 μM of each primer, and water. Primer sequences used in this study are listed in Supplementary Table S1. The amplification protocol was 50 cycles at 95°C for 5 s and 10 s at the annealing temperature corresponding to each primer (Table S1). To calculate the relative levels of gene expression in each sample, real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method, and expression levels of the target genes were normalized against the expression level of the housekeeping gene *RPS17* (Livak and Schmittgen 2001).

Identification of IB vaccine virus in the gut

We confirmed that the IB virus in the vaccine reached the gut by identifying viral RNA. Three male chicks were administered a nasal drop of the NB/IB vaccine at one day old, and their proventriculi and ilea were collected 24 h later, as described above. Total RNA was isolated and reverse-transcribed, and PCR amplification was performed using the real-time PCR system described above (annealing at 50°C for 10 s; 60 cycles). PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing ethidium bromide (0.5 μg/mL).

Histology of the proventriculus and ileum tissues

The proventriculus and ileum tissues were fixed in 10% (v/v) formalin in PBS and processed into paraffin sections (4 μm in thickness). The sections were stained with hematoxylin and eosin and covered after dehydration. They were then examined under a light microscope.

Statistical analysis

The significance of differences in gene expression levels of immune molecules between the control group (V–Y–) and the groups administered with vaccination and/or yeast (V–Y+, V+Y–, and V+Y+) was examined using Dunnett's test. Statistical significance was set at $P < 0.05$.

Results

Figure 1 shows the PCR products of the IB virus in the proventriculus and ileum of chicks 24 h after inoculation with the ND/IB vaccine. Amplification products of the IB virus were identified in both the proventriculus and ileum of the vaccinated chicks, confirming that the IB virus in the vaccine had reached these segments of the gut.

Figures 2 and 3 show the effects of ND/IB vaccination and yeast supplementation on the expression of TLRs and Dectin-1 in the proventriculus and ileum, respectively. In the proventriculus, the expression levels of TLR7 and TLR21 were higher in the group vaccinated without yeast supplementation (V+Y–) than those in the control group (V–Y–), whereas there was no difference between the control and V–Y + or V+Y + groups (Fig. 2E and 2G). Expression levels of other TLRs and Dectin-1 in the treatment groups (V–Y+, V+Y–, and V+Y+) did not show significant differences compared with those of the control group (V–Y–) (Fig. 2A–D, F, and H). In contrast, in the ileum, the expression levels of all TLRs and Dectin-1 did not differ between the control group (V–Y–) and the groups administered the vaccine and/or yeast products (V–Y+, V+Y–, and V+Y+) (Fig. 3).

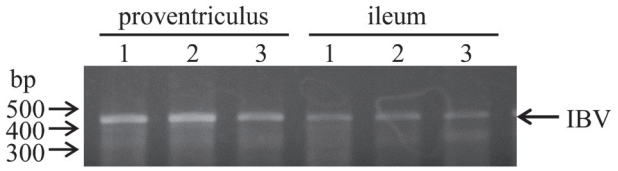

Fig. 1. **Reverse transcription-polymerase chain reaction (RT-PCR) products of the infectious bronchitis virus (IBV) in the proventriculus and ileum of 2-day-old chicks (n = 3, lanes 1–3) inoculated with Newcastle disease and IB vaccine (ND/IB) at one day old.** The dense arrow (IBV) at the right side shows the position of the bands of specific PCR products.

The effects of ND/IB vaccination and yeast products on AvBD and Cath1 expression in the proventriculus are shown in Fig. 4. The expression levels of AvBDs (Fig. 4A–E) and Cath1 (Fig. 4F) were not different between the control group (V–Y–) and the groups administered the vaccine and/or yeast supplementation (V–Y+, V+Y–, V+Y+). In the ileum, there were also no significant differences in the expression of AvBDs and Cath1 between the control (V–Y–) and treatment groups (Fig. S1).

Figure 5 shows the effects of ND/IB vaccination and yeast supplementation on the expression of cytotoxic leukocyte-related genes and pro-inflammatory cytokines in the proventriculus. Although the expression of B-NK, an NK cell receptor, was unaffected by vaccination or yeast (Fig. 5A), the granzyme expression level was higher in the vaccinated without yeast supplementation group (V+Y–) than that in the control group (V–Y–) (Fig. 5B). The expression level of IL-1B was also higher in the group vaccinated without yeast supplementation (V+Y–) (Fig. 5C), whereas the IL-6 expression was lower in the group administered both the vaccine and yeast products (V+Y+) than those of the control group (V–Y–) (Fig. 5D).

The effects of vaccination and yeast feeding on the expression of genes encoding cytotoxic leukocyte-related molecules and proinflammatory cytokines in the ileum are shown in Fig. 6. The expression level of granzyme was higher in the group administered yeast products without vaccination (V–Y+) than that in the control group (V–Y–), whereas there was no difference in granzyme levels between the vaccinated groups, with or without

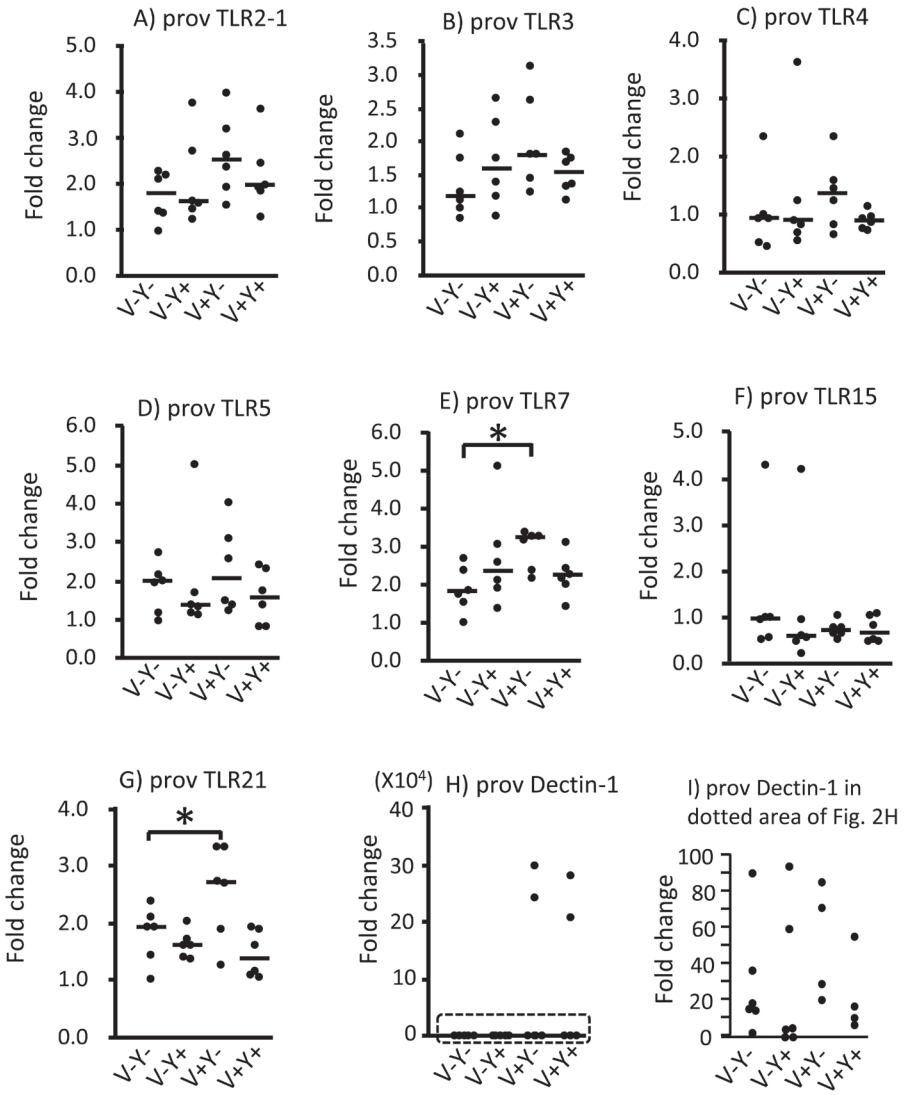

Fig. 2. **Effects of Newcastle disease and infectious bronchitis (ND/IB) vaccination and yeast supplementation on the expression of TLRs and Dectin-1 in the proventriculus of chicks.** Chicks in the V+Y– and V+Y+ groups were immunized with live ND/IB vaccine, whereas chicks of the V–Y– and V–Y+ groups were not. Chicks of the V–Y+ and V+Y+ groups received yeast product in their diet from day 4, whereas those in the V–Y– and V+Y– groups did not. Gene expression levels were analyzed at day 7. The dots indicate the fold changes in gene expression levels in each chick, and the solid bars represent the median value within each treatment. Asterisks indicate significant differences between the control (V–Y–) and treated (V–Y+, V+Y–, and V+Y+) groups as determined by Dunnett's test (*$P < 0.05$).

yeast supplementation (V+Y– and V+Y+), and the control group (V–Y–) (Fig. 6B). There were also no significant differences in the expression levels of B-NK, IL-1B, and IL-6 between the control group (V–Y–) and the three treatment groups (V–Y+, V+Y–, and V+Y+) (Fig. 6A, 6C and 6D).

Histological images of the tissue structures of the proventriculus and ileum of chicks treated with or without the vaccine and yeast are shown in Fig. S2. In the control group (V–Y–), mucosal folds and surface proventriculus glands were formed and lymphocytes accumulated in the lamina propria of the surface layer of the proventriculus (Fig. S2A). The deep proventricular glands were lined by a single-cell layer that developed in the deep layer (Fig. S2B). Villi and crypts developed in the ileal mucosa (Fig. S2C). These structures were commonly observed in the treatment groups administered the vaccine and/or yeast products (V–Y+, V+Y–, and V+Y+). Tissue inflammation and disintegration were not observed in any chick of the four groups (Fig. S2).

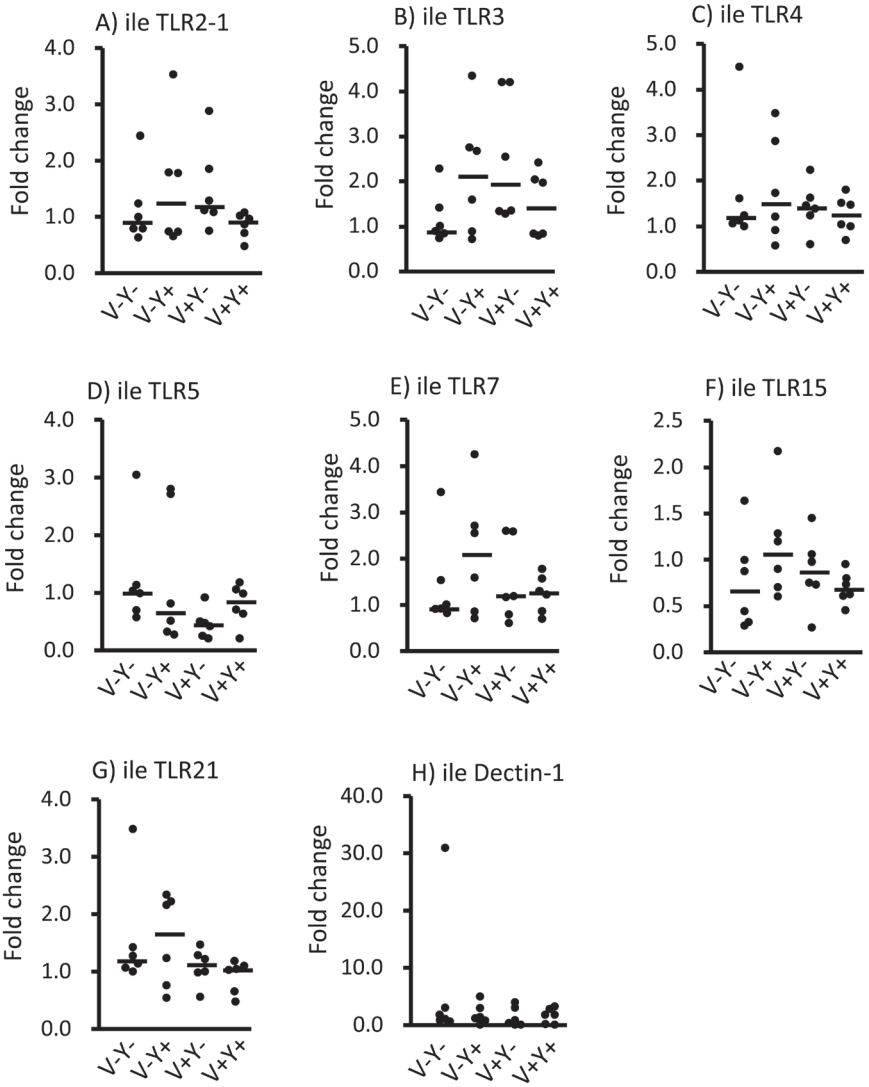

Fig. 3. **Effects of Newcastle disease and infectious bronchitis (ND/IB) vaccination and yeast supplementation on the expression of TLRs and Dectin-1 in the proventriculus of chicks.** See the legend to Figure 2 for the description of the chick groups (V–Y–, V–Y+, V+Y–, and V+Y+). The dots indicate the fold changes in gene expression levels in each chick, and the solid bars represent the mean value within each treatment.

Discussion

The current study examined whether ND/IB vaccination and yeast supplementation in feed affected the expression of innate immune molecules in the proventriculus and ileum of chicks. The expression of seven TLRs and Dectin-1 was identified in both the proventriculus and ileum, suggesting that pattern molecules of gram-positive and gram-negative bacteria, microbial RNA, and unmethylated CpG DNA can initiate the innate immune response. The expression of Dectin-1 suggests that β-glucan in the yeast cell wall could be recognized in the chick gut, as Dectin-1 recognizes β-glucan in combination with TLR2 in chickens and mammals (Kalia et al., 2021; Nerren and Kogut, 2009; Taylor et al., 2007).

The current study showed that TLR7 and TLR21 expression in the proventriculus was increased in the ND/IB-vaccinated group compared with that in the non-vaccinated control group at 7 days old (Fig. 2E, 2G). We previously reported that the expression of TLR21 in the proventriculus of chicks immunized with ND/IB vaccine at one day old was higher than that in non-immunized chicks when examined at 11 days post-hatching, although the expression of other TLRs, including TLR7, did not show differences between vaccinated and non-vaccinated chicks (Yoshimura et al., 2022). Thus, we assumed that the expression of TLR7 and

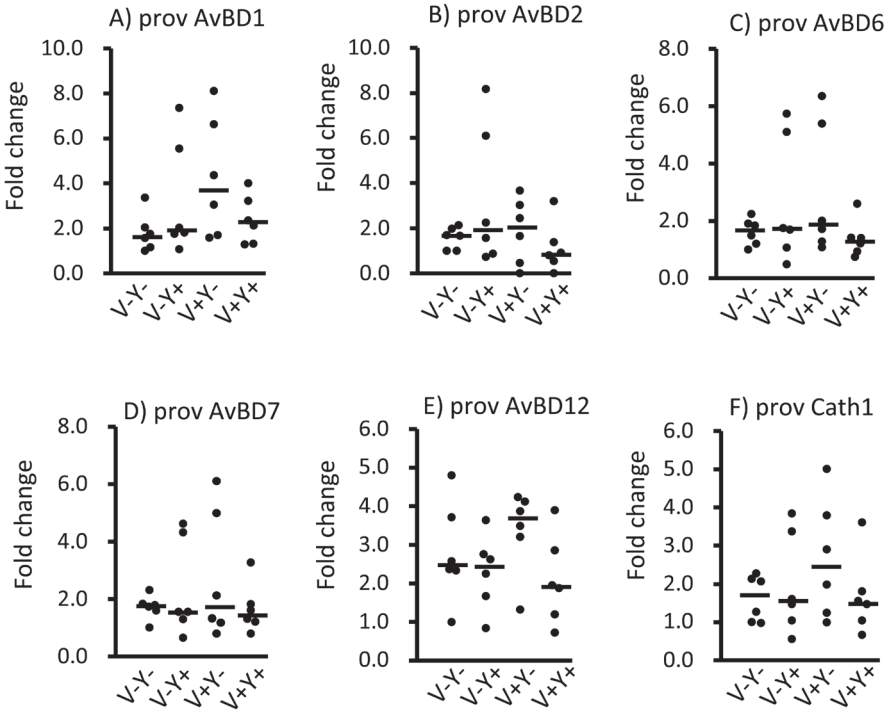

Fig. 4. **Effects of Newcastle disease and infectious bronchitis (ND/IB) vaccination and yeast supplementation on the gene expression of antimicrobial peptides (avian β-defensin, AvBDs; cathelicidin-1, Cath-1) in the proventriculus of chicks.** See the legend to Figure 2 for the description of the chick groups (V–Y–, V–Y+, V+Y–, and V+Y+). The dots indicate the fold changes in gene expression levels in each chick, and the solid bars represent the mean value within each treatment.

TLR21 in the proventriculus was upregulated by ND/IB vaccination for seven days. The expression of TLR7 may decline with growth thereafter, whereas higher expression of TLR21 is maintained even on day 11. The upregulation of these TLRs may be due to innate immune training induced by vaccination, as reported in different organisms, including invertebrates (Kurtz, 2005) and mammals (de Bree et al., 2018; Netea et al., 2011, 2020; van der Meer et al, 2015). Enhanced TLR7 and TLR21 expression may improve the ability to recognize microbial ssRNA and unmethylated CpG DNA, which initiates the innate immune response. In contrast, although the genomic RNA of the IB virus in the vaccine was identified in both the proventriculus and ileum (Fig. 1), upregulated expression of TLRs by vaccination was not observed in the ileum (Fig. 3). The exact reason why vaccination did not affect the expression of TLRs in the ileum remains unknown; however, we assume that the number of viruses reaching the ileum might be smaller than that reaching the proventriculus because some viruses may be killed by the gastric juice and other factors before arriving at the ileum.

The expression of TLRs did not differ between the yeast product-supplemented (V–Y+ and V+Y+) and control (V–Y–) groups in both the proventriculus and ileum (Figs. 2 and 3). Furthermore, although the expression levels of TLR7 and TLR21 were elevated by ND/IB vaccination without yeast supplementation (V+Y–) in the proventriculus (Fig. 2E and 2G), this modulatory effect of vaccination was not observed when the diet was supplemented with yeast products (V+Y+) (Fig. 2E and 2G). Thus, it is likely that the yeast supplementation used in this study did not modulate the expression of TLRs in these gut segments. Rather, the yeast products may have reduced the effects of vaccination to induce TLR7 and TLR21 expression.

The current study revealed that five AvBDs and Cath1 were expressed in the proventriculus and ileum (Figs. 4 and S1), supporting previous reports (Mohammed et al., 2015; Terada et al, 2018, 2020c). We previously reported that ND/IB vaccination did not affect the gene expression of these antimicrobial peptides in the proventriculus, although the levels of some AvBD peptides increased following vaccination (Yoshimura et al., 2022). In the current study, we did not detect changes in the content of antimicrobial peptides; however, it is likely that the gene expression of antimicrobial peptides is not affected by vaccination and yeast supplementation in the proventriculus and ileum.

The expression of B-NK, a receptor of NK cells, was not affected by vaccination or yeast supplementation (Figs. 5A and 6A); however, granzyme expression was stimulated by vaccination alone (V+Y–) in the proventriculus (Fig. 5B) and was elevated by yeast supplementation alone (V–Y+) in the ileum (Fig. 6B). Thus, it is possible that the recruitment of NK cells may not

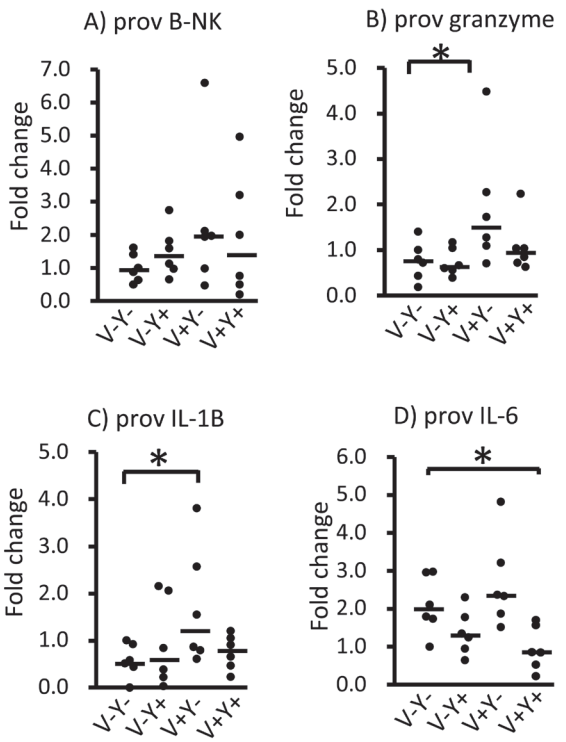

Fig. 5. **Effects of Newcastle disease and infectious bronchitis (ND/IB) vaccination and yeast supplementation on the gene expression of cytotoxic leukocyte-related molecules and proinflammatory cytokines in the proventriculus.** See the legend to Figure 2 for the description of the chick groups (V–Y–, V–Y+, V+Y–, and V+Y+). The dots indicate the fold changes in gene expression levels in each chick, and the solid bars represent the mean value within each treatment. Asterisks indicate significant differences between the control (V–Y–) and treated (V–Y+, V+Y–, and V+Y+) groups as determined by Dunnett's test (*P < 0.05).

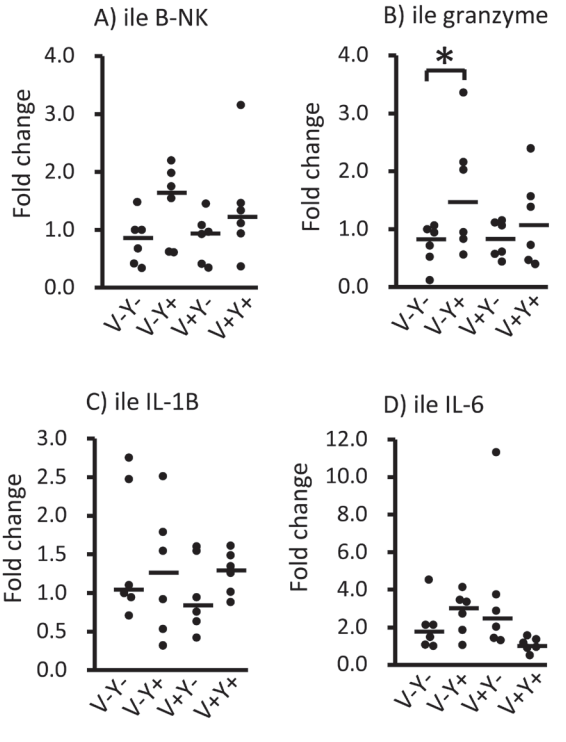

Fig. 6. **Effects of Newcastle disease and infectious bronchitis (ND/IB) vaccination and yeast supplementation on the gene expression of cytotoxic leukocyte-related molecules and proinflammatory cytokines in the ileum.** See the legend to Figure 2 for the description of the chick groups (V–Y–, V–Y+, V+Y–, and V+Y+). The dots indicate the fold changes in gene expression levels in each chick, and the solid bars represent the mean value within each treatment. Asterisks indicate significant differences between the control (V–Y–) and treated (V–Y+, V+Y–, and V+Y+) groups as determined by Dunnett's test (*P < 0.05).

be affected by vaccination and yeast supplementation, whereas granzyme synthesis in cytotoxic cells is stimulated by ND/IB vaccination in the proventriculus and by yeast supplementation in the ileum. This difference in granzyme expression between the proventriculus and ileum may be due to the amount and duration of antigens in the two gut segments. Namely, we assumed that the number of live vaccine viruses arriving at the proventriculus was greater than that arriving at the ileum, although the yeast was maintained for a longer time in the ileum. An increase in granzyme synthesis may enhance the potential of the defense system against pathogenic microbes via cytotoxic cells in the mucosa. However, it is likely that the stimulatory effects of yeast supplementation on granzyme expression were suppressed by vaccination in the ileum (V+Y+ in Fig. 5B). Although the mechanism by which granzyme expression is not upregulated by yeast supplementation in vaccinated chicks remains to be elucidated, we assume that the cellular functions regulated by the interaction of pattern recognition receptors and yeast molecules might be downregulated by vaccination.

The expression of IL-1B was upregulated by vaccination without yeast supplementation (V+Y–), whereas the expression level of IL-6 was reduced by vaccination and yeast supplementation (V+Y+) in the proventriculus (Fig. 5C and 5D). Although both molecules are multifunctional proinflammatory cytokines, changes in their expression at the observed levels are unlikely to cause inflammation and disintegration of the tissues, given that no inflammatory signs were identified by histology (Fig. S2). As IL-6 expression appears to be suppressed by yeast products, future studies are needed to examine whether yeast products are useful in regulating IL-mediated tissue inflammation and disorders. The effects of yeast feeding on the expression of interferons that may be induced by vaccination with live viruses also remain to be evaluated in future studies. Although it has been reported that yeast-derived macromolecules support pro- and anti-inflammatory cytokine production for cell-mediated immune responses

in the chick cecal tonsils (Yitbarek et al., 2013), supplementation with yeast products was unlikely to induce cytokines under the conditions used in this study.

The challenge of monocytes with β-glucan leads to the enrichment of acetylation and methylation in histones at the proinflammatory and anti-inflammatory regions (Domínguez-Andrés et al, 2020), which may lead to the induction of innate immune training. However, since yeast supplementation did not upregulate the expression of immune molecules, except for granzyme expression in the ileum, the effects of the yeast products used in this study to modulate the innate immune system in the chick gut may be limited. Further studies considering differences in the amount of yeast products and feeding period are needed to determine the efficacy of yeast products.

Therefore, we suggest that ND/IB vaccination modulates the innate immune system by upregulating the expression of TLR7 and TLR21, granzyme of cytotoxic cells, and IL-1B in the proventriculus on day 7 post-hatching, whereas this effect does not appear in the ileum. The effects of yeast feeding on the immune system may be limited because yeast supplementation upregulated only the expression of granzyme in the ileum and downregulated IL-6 expression in the proventriculus of male chicks immunized with the ND/IB vaccine. Although a recent report suggested that the yeast cell wall upregulated the cell-mediated immune response to ND vaccine (Bi et al., 2022), our results did not support this previous finding given that the expression of immune molecules was not upregulated by yeast feeding in the proventriculus or ileum of chicks administered ND/IB vaccination. Thus, we further conclude that ND/IB vaccination is effective in enhancing the innate immune system in the proventriculus of chicks, at least until day 7 post-hatching, and the effects of diet supplementation with yeast products on the innate immune system may be limited, at least under the conditions of this study. Further studies are required to confirm the effects of different doses of yeast products on innate immunity of the gut.

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Author contributions

YY designed the study and contributed to the animal treatments, histological studies, and drafting of the manuscript. TN performed the RT-qPCR analyses. NI designed the ASCOGEN. All the authors contributed to the discussion and preparation of the manuscript.

Declaration of competing interests

The authors declare that they have no conflicts of interest.

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