Original Article

Effect of feeding a *Saccharomyces cerevisiae* fermentation product on immune response to vaccines against Newcastle disease virus, Avian influenza virus, and the prevalence of chronic respiratory disease and coccidiosis in chickens raised in a low biosecurity production system

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Abstract

This study was carried out in a low biosecurity production system to explore the effects of a *Saccharomyces cerevisiae* fermentation product (SCFP) on immune responses to vaccination against Newcastle disease virus (NDV) and Avian influenza (H5N1) and the prevalence of common diseases in chickens including chronic respiratory disease (CRD) and coccidiosis. One hundred and ninety-two 1-day-old chickens were randomly assigned into control (a standard basal diet) and treatment (a standard basal diet containing 1.25 kg/MT of a *Saccharomyces cerevisiae* fermentation product). Immunity data on antibody titers to NDV and H5N1 were collected at 3 weeks and 10 weeks post-vaccination for H5N1 and at 1 week and 6 weeks post-vaccination for NDV. The seroprevalence of CRD was detected at 5 weeks of age by ELISA. The incidence of coccidiosis infection was evaluated by weekly oocyst counting starting from 2 weeks of age. Results showed that supplementation of SCFP did not affect the immune response to vaccination with NDV and H5N1 vaccines ($P > 0.05$). The seroprevalence of CRD infection was similar between treatment groups fed diets with and without SCFP supplementation, while the intensity of coccidiosis infection tended to be higher in the SCFP supplementation compared to the control. Overall, supplementation of the SCFP in the diet did not affect the immune response to NDV and H5N1 post-vaccination and the prevalence of CRD.

Keywords: coccidiosis, immune, infection, postbiotic, *Saccharomyces cerevisiae*

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Introduction

Inappropriate use of antibiotic growth promoters (AGPs) accelerates the presence of antimicrobial resistance (AMR), and increasing AMR is a grave global public health concern (Smith *et al.*, 2002). In this regard, the use of in-feed AGPs has been banned in Europe since 2006 (European Commission, 2003). In Vietnam, the government issued Decree 13/2020/ND-CP that banned the use of antimicrobials for disease prevention in mature terrestrial animals in March 2020 and planned a roadmap for a complete ban on the prophylactic use of antibiotics in young terrestrial animals by 2026 (Vietnamses Government, 2020). However, the global prohibition on the use of AGPs may increase the prevalence of economically important diseases in the future. Withdrawal of AMPs was reported to be linked to a decline in animal health, which includes a rise in diarrhea, weight loss, and death in early post-weaning pigs from *Escherichia coli*, *Lawsonia intracellularis*, and Clostridial necrotic enteritis in broilers (Casewell, 2003). Therefore, there is an urgent need to identify viable alternatives to in-feed antibiotics that can effectively compensate for the benefits lost due to the antibiotic ban.

Studies on feed additives as potential alternatives to antibiotics have been conducted on various products, such as prebiotics, probiotics, postbiotics or fermentation products, organic acids, and other feed additives. Among the alternatives, *Saccharomyces cerevisiae* fermentation products (SCFP) showed beneficial effects on performance, health, and immunity in various species, including swine and cattle (Abd EI-Ghany *et al*., 2022; Alugongo *et al*., 2017; Chou *et al*., 2017; Gao *et al*., 2009; Soren *et al*., 2024). However, information regarding the effect of SCFP on the prevalence of common diseases, including chronic respiratory disease (CRD) and coccidiosis and immunity in the chicken within the small-scale production systems with low to minimal biosecurity practices (sector 3 production system according to the classification of Food and Agriculture Organization) is lacking.

The goals of this study were to determine the effects of incorporating SCFP into the diet of crossbred broiler chickens from one day of age on common diseases and post-vaccination immune responses. The study was conducted in a sector 3 chicken production system in Vietnam, aiming to provide valuable insights into the potential benefits of SCFP supplementation for chickens in this particular setting.

Materials and Methods

Animals, housing, experimental design, diets: In this study, 192 one-day-old F1 (Ri x Luong Phuong) chickens (National Institute of Animal Sciences, Ha Noi, Vietnam) were used. The study was carried out in an open house without humidity or temperature controls at the farm of Hue University of Agriculture and Forestry, Hue City, Vietnam. It is classified as a sector 3 production system according to the Food and Agriculture Organization (FAO) classification. A thermal and humidity meter (Nakata, Japan) was used to record the temperature and humidity three times a day. The experiment was conducted from July 1st to September 22nd, 2023. The house temperature was kept between 31 and 35 °C during the incubation phase using a chicken brooder lamp and was gradually lower based on visual inspection of the condition of the chickens in the pens as the chickens grew older.

Chickens were randomly assigned to 2 treatment groups with 16 replicates of 6 chicks each. The control group (CON) chickens were fed a standard basal control broiler diet. The chickens in the treatment group were fed a standard basal control broiler diet containing 1.25 kg/MT of SCFP (Diamond V XPC™, Cedar Rapids, IA, USA). The diets of the experimental chickens were formulated to meet the animal requirements. Three distinct diets were created based on their three feeding phages for chickens (Supplemental Table 1). The feed was kept in plastic bags and marked with the date and treatment code after formulation. There were no antibiotic growth promoters in the diet. Chickens had an *ad libitum* supply of feed and water.

All animal procedures in this study were conducted following the Guidelines for the Use of Animals in Research and Welfare of Hue University. This animal experiment was approved by the Animal Ethics Committee, Hue University (Protocol #HUVN0028).

Vaccination and disease control: The vaccination schedule for the chickens in both the SCFP and control group is detailed in the test article information and schedule of events (Supplemental Table 2). Chickens in both control and treatment groups were vaccinated against Antibodies against Newcastle Disease Virus (NDV) at 7, 18, and 42 days of age (Vaccine Medivac ND-IB at 7 and 18 days of age; Medivac ND G7 Emulsion at 42 days of age). Chickens in both treatments were also vaccinated against Avian influenza virus (AIV; NAVET-VIFLUVAC, subtype H5N1) one time at 14 days of age. During the experimental period, no concurrent pharmacological regimens were administered.

Post-vaccination immune response evaluation: The immunology effect of SCFP on chickens was evaluated using an Enzyme-Linked Immunosorbent Assay (ELISA) test and a Hemagglutination-inhibition test (HI-test) on serum samples. Blood was collected from one chicken per pen, and the chicken was allowed to be clotted. Serum samples were separated and stored at -20 °C until analyses.

Antibodies against Newcastle Disease Virus were measured at one week (16 days of age) after the first dose of Medivac ND-IB and six weeks (85 days of age) after the dose of Medivac ND G7 Emulsion (third dose). The antibody titers against NDV were measured using a commercial ELISA kit (ID Screen® Newcastle Disease Indirect ELISA Kit, IDVet, Grabels, France), following the manufacturer's instructions.

Antibodies against Avian Influenza Virus (AIV) were measured at 4 weeks (41 days of age) and 10 weeks (85 days of age) after vaccination, using the HI test. The samples were sent to and analyzed at the Regional Animal Health Office No. 4 (Da Nang City, Vietnam). The HI test was performed according to the OIE Manual of Diagnostic Tests and Vaccines for

Terrestrial Animals (Arnold *et al*. 2018) and the WHO method (World Health Organization, 2011), as follows: In 96-well, V-bottom plates (25 µL/well), serum samples were inactivated at 56 °C for 45 min, then serially diluted 2-fold in phosphate-buffered saline (PBS). Avian Influenza Virus Antigen (H5N1 inactivated antigen, APHA Scientific, Addlestone, Surrey, UK) was added (4 hemagglutinin units per well in 25 µL) and incubated at room temperature for 60 min. After incubation, 50 µL/well of erythrocytes was added, and the plates were further incubated at room temperature for 60 min when inhibition of hemagglutination was determined by visual inspection. Serum HI titers equal to or greater than 1:16 were considered positive. For test vaccine groups, the Geometric Mean Titers (GMT) of HI-positive sera at each sampling time point were calculated.

Incidence of coccidiosis: Feces were collected for oocyst counting weekly from 2 weeks of age until the end of the study (12 weeks). Feces were collected and pooled for each pen. Aliquots of 2 g were transferred into 15 mL centrifuge tubes for oocyst quantification (3 tubes for each group). Oocysts per gram of feces (OPG) were counted using the fecal flotation method using the modified McMaster technique (Ho *et al*., 2021). Briefly, fecal samples (2 g/tube) were mixed thoroughly with 10 mL distilled water, followed by centrifugation at 4000 rpm for 5 min at room temperature using a centrifuge (Hettich Lab Technology™, Tuttlingen, Germany). The supernatant was then discarded, and 10 mL of a saturated sucrose solution was added to the tubes, mixed thoroughly, and centrifuged at 4000 rpm for 5 minutes at room temperature. The supernatant was transferred to other 15 mL centrifuge tubes and mixed well. A drop of the supernatant (10 µL) was placed on a glass slide and covered using cover glass, and the oocysts were counted using light microscopy (three drops per tube). OPG was calculated by the following: OPG = n x 500 (n is the average number of counted oocysts).

Chronic respiratory disease (CRD): Blood samples of three chickens per pen were collected for the CRD test at 5 weeks of age. The samples were collected from the wing veins, and the serum was prepared and subjected to CRD diagnostic using the Chicken *Mycoplasma Gallisepticum* Antibodies ELISA kit (Elabscience®, Houston, TX, USA), following the manufacturer's directions.

Statistical analyses: For ELISA titers and CRD measurement, individual birds were used as the experimental unit. The cage was the experimental unit for coccidiosis. Various descriptive statistics were applied. The geometric mean was applied to interval data, such as titers. The Kruskal-Wallis Test was used to determine the difference between the treatment and control. Frequency and geometric mean are shown for coccidiosis data. Differences were considered significant with $P < 0.05$. Minitab Statistics software 16.0. were used for data analysis.

Result

Effects of feeding a Saccharomyces cerevisiae fermentation product on post-vaccination immune response in chickens: Effects of feeding a *Saccharomyces cerevisiae* fermentation product on HI antibody titers three and ten weeks after Avian Influenza vaccination are presented in Tables 1 and 2, respectively. Both treatment and control elicited specific anti-AIV antibodies, and only minor differences between these two groups were observed (Fig.1 and Tables 1 and 2). Unexpectedly, the antibody levels obtained after the HI test of control chickens seem higher than that of the treatment group. Three weeks after the vaccination, the geometric mean titer (GMT) of the HI Anti-AIV antibodies was 41.50 in the control while it was 32.00 in the treatment. This trend was the same at 10 weeks post-vaccination; the GMT was 61.29 in the control group and 53.82 in the treatment groups, which was slightly increased. The HI response was strong, and 87.50% of chickens in the control group and 81.25% of chickens in the treatment group were HI positive (Table 1). Ten weeks post-vaccination, the HI-positive rate increased to 93.75% and 87.50% in the control and treatment groups, respectively (Table 2). In addition, the HI titers as high as 256 and 512 were detected after immunization for 3 weeks and 10 weeks, respectively. However, the significant differences in specific anti-AIV antibody titers between chickens fed a diet with and without SCFP after 3 and 10 weeks of vaccination were not observed ($P > 0.05$). Notably, the box-and-whisker plots (Fig. 1) showed that the antibody level of chickens five weeks after avian influenza (H5N1) vaccination in the treatment group seemed to be stimulated rather than those in the control group, but this trend was not statistically significant ($P > 0.05$).

Effects of feeding a Saccharomyces cerevisiae fermentation product on Newcastle disease virus-specific antibodies post-vaccination in chickens: The antibody titers against Newcastle disease virus (NDV) in serum of F1 (Ri x Luong Phuong) chickens, fed with or without SCFP after vaccination are shown in Fig. 2. At one week after immunized with Newcastle disease virus vaccine, the titers obtained through ELISA appeared higher in the treatment group compared to the control group. The log10 OD value for samples from chickens in the control group ranged from 2.317 to 3.860 (mean = 3.482), while the $\log_{10}$ OD value for samples from chickens in the treatment group ranged from 2.426 to 4,283 (mean = 3.511). Antibody titer at 6 weeks after the last dose of vaccination in both treatments was increased significantly ($P < 0.001$) compared to one-week post-vaccination. However, there are no significant differences ($P>0.05$) in antibody titers between the control and treatment.

Effect of feeding a Saccharomyces cerevisiae fermentation product on coccidiosis infection in chickens: The results of coccidiosis infection are presented in Table 3. Results showed that chickens in both groups were not infected with coccidiosis in the first 4 weeks of age. The infection rate gradually increased when chickens were older than 5 weeks in both control and treatment groups. There were peaks of coccidial oocyst shedding in the feces of control and

treatment chickens during the experiment. In general, later peaks had greater numbers of oocyst than prior peaks in both control and treatment chickens. Chickens in the control group had peaks of their oocyst excretion on weeks 6, 8, and 12 (4 x 10^3, 4.5 x 10^3, and 54.0 x 10^3 oocysts per gram of droppings, respectively). The chickens in the treatment showed later peaks of oocyst excretion on weeks 9, 11, and 12 (8.0 x 10^3, 61.2 x 10^3, and 135.5 x 10^3 oocysts per gram of droppings, respectively).

Effects of feeding a Saccharomyces cerevisiae fermentation product on chronic respiratory disease infection in chickens: The effects of SCFP on chronic respiratory disease infection in chickens are given in Fig. 3 and Table 4. The seroprevalence of chronic respiratory disease infection in experimental chickens was recorded at 5 weeks of age by the indirect ELISA method. The ELISA for detection of *Mycoplasma gallisepticum* (MG) results had a mean OD of 0.4195 (range 0.196–1.4715) for the control group and 0.421 (0.269–1.0585) for the treatment group. Out of 92 blood samples tested, 41 (OD ranging from 0.3405–1.4715) and 39 (OD ranging from 0.33–1.0585) samples positive for MG were diagnosed in the control and treatment chicken groups, respectively. The seroprevalence in the treatment group was 81.3%, whereas the seroprevalence in the control group was 85.4%. The effect of dietary SCFP on chronic respiratory disease infection was not statistically significant (Table 4 and Figure 3). Additionally, no clinical signs related to CRD, such as cough, tail bobbing when breathing, emaciation, rales, sneezes, or open-mouthed breathing, were observed during the experimental period (data not shown).

Table 1 Effect of feeding a *Saccharomyces cerevisiae* fermentation product on HI antibody titers of F1 (Ri x Luong Phuong) broilers three weeks after Avian Influenza vaccination.

Antibody Titer	Total		Control[1]		Treatment[2]	
	n	%	n	%	n	%
Negative	5	15.63	2	12.50	3	18.75
1/16	4	12.50	2	12.50	2	12.50
1/32	4	12.50	3	18.75	1	6.25
1/64	7	21.88	2	12.50	5	31.25
1/128	8	25.00	5	31.25	3	18.75
1/256	4	12.50	2	12.50	2	12.50
Total positive	27	84.38	14	87.50	13	81.25
Total	32	100	16	100	16	100
log2GMT	5.19		5.38		5.00	
GMT[3]	36.44		41.50		32.00	
P-value[4]			0.45			

[1]Control = Basal diet; [2]Treatment = Basal diet containing 1.25 kg/MT (2.5 lb/t) of Diamond V XPC™; [3]GMT = Geometric mean titer. [4]*P*-value = The difference in specific anti-AIV antibody titers between the control and treatment was analyzed on a log2 scale and calculated by the Kruskal-Wallis Test.

Table 2 Effect of feeding a *Saccharomyces cerevisiae* fermentation product on HI antibody titers of F1 (Ri x Luong Phuong) broilers ten weeks after Avian Influenza vaccination.

Antibody Titer	Total		Control[1]		Treatment[2]	
	n	%	n	%	n	%
Negative	3	9.38	1	6.25	2	12.50
1/16	4	12.50	3	18.75	1	6.25
1/32	4	12.50	0	0.00	4	25.00
1/64	5	15.63	3	18.75	2	12.50
1/128	12	37.50	8	50.00	4	25.00
1/256	2	6.25	0	0.00	2	12.50
Total positive	2	6.25	1	6.25	1	6.25
Total	29	90.63	15	93.75	14	87.50
log2GMT	32	100	16	100	16	100
GMT[3]	5.84		5.94		5.75	
P-value[4]			0.93			

[1]Control = Basal diet; [2]Treatment = Basal diet containing 1.25 kg/MT (2.5 lb/t) of Diamond V XPC™; [3]GMT = Geometric mean titer. [4]*P*-value = The difference in specific anti-AIV antibody titers between the control and treatment was analyzed on a log2 scale and calculated by the Kruskal-Wallis Test.

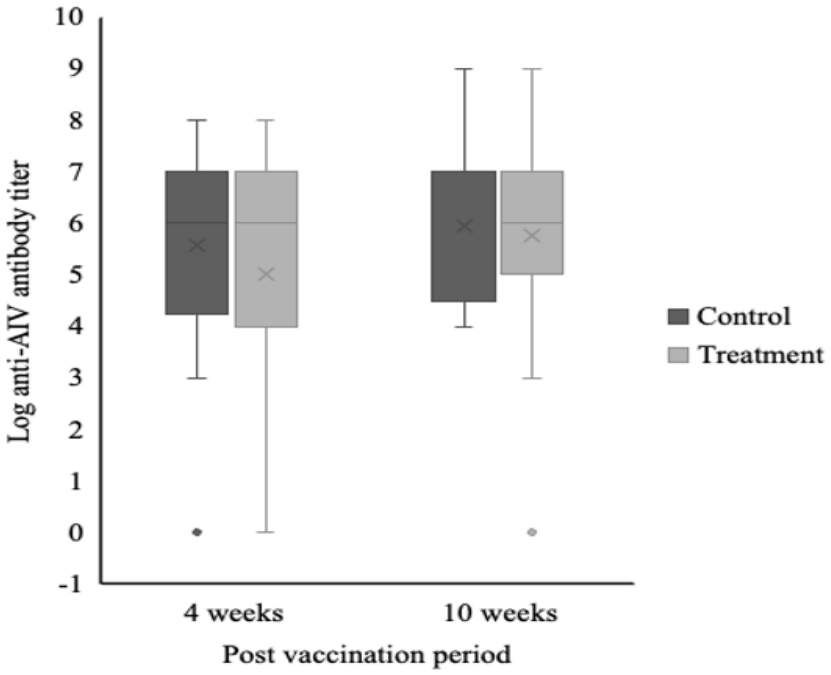

Figure 1 Anti-AIV antibody titer 4 and 10 weeks after vaccination against Avian influenza virus in chickens fed a diet with and without a *Saccharomyces cerevisiae* fermentation product. A log2 scale was used on the y-axis to minimize the data dispersion. Box-and-whisker plots represent the median of log2 antibodies in the hemagglutination inhibition test for detecting avian influenza virus antibodies in the serum of treatment and control chicken groups. Boxes indicate interquartile ranges with median values as horizontal lines inside the box. Whiskers indicate ranges. × markers represent the mean values of the samples. All data points from 2 experimental groups of sample size n=16 in each group and samples collected at 2 time points are plotted. *P*-value: 0.94.

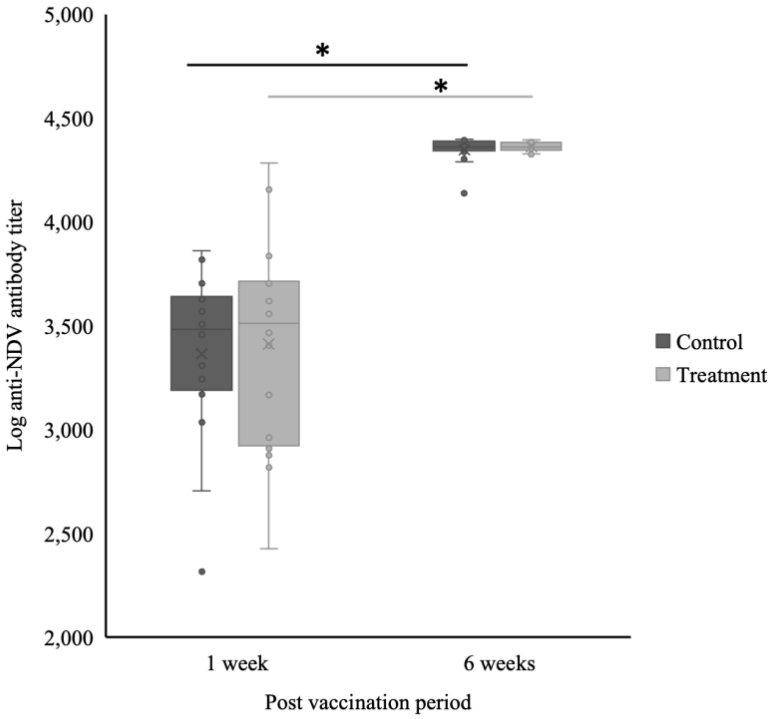

Figure 2 Anti-NDV antibody titer at 1 week post the first dose and at 6 weeks post the third dose of vaccination against Newcastle Disease Virus in chickens fed a diet with and without a *Saccharomyces cerevisiae* fermentation product. A log10 scale was used on the y-axis to minimize the data dispersion. Box-and-whisker plots representing the median of log10 antibodies titer of ELISA for detecting NDV antibodies in serum of treatment and control chicken groups. Boxes indicate interquartile ranges, with median values shown as horizontal lines inside the box. Whiskers indicate ranges. × markers represent the mean values of the samples. *, $P < 0.001$.

Table 3 Effects of feeding a *Saccharomyces cerevisiae* fermentation product on coccidiosis infection rate and oocyst per gram of dropping (OPG) counts.

Week old	Attribute	Control[1]	Treatment[2]
2	Infection rate (%, n=16)	0	0
	OPG[3] (x10[3])	0	0
3	Infection rate (%, n=16)	0	0
	OPG (x10[3])	0	0
4	Infection rate (%, n=16)	0	0
	OPG (x10[3])	0	0
5	Infection rate (%, n=16)	6.25	6.25
	OPG (x10[3])	0.1 ± 0.2	0.6 ± 2.6
6	Infection rate (%, n=16)	18.75	6.25
	OPG (x10[3])	4.0 ± 11.0	0.2 ± 0.7
7	Infection rate (%, n=16)	43.75	50.00
	OPG (x10[3])	0.3 ± 0.6	0.5 ± 1.1
8	Infection rate (%, n=16)	87.50	87.50
	OPG (x10[3])	4.5 ± 10.2	2.0 ± 2.8
9	Infection rate (%, n=16)	37.50	75.50
	OPG (x10[3])	1.0 ± 2.2	8.0 ± 28.4
10	Infection rate (%, n=16)	31.25	81.25
	OPG (x10[3])	0.7 ± 1.8	13.8 ± 28.3
11	Infected rate (%, n=16)	81.25	93.75
	OPG (x10[3])	2.7 ± 4.1	61.2 ± 120
12	Infection rate (%, n=16)	100	93.75
	OPG (x10[3])	54.0 ± 145.4	135.5 ± 427.1

[1]Control = Basal diet; [2]Treatment = Basal diet containing 1.25 kg/MT of Diamond V XPC™; [3]OPG = Oocyst per gram feces.

Table 4 Sero-prevalence of Chronic Respiratory Disease at five weeks of age for chicken fed a diet with and without a *Saccharomyces cerevisiae* fermentation product.

		Positive	Negative
Control	n	41	7
	%	85.40	14.60
Treatment	n	39	9
	%	81.30	18.80

[1]Control = Basal diet; [2]Treatment = Basal diet containing 1.25 kg/MT (2.5 lb/t) of Diamond V XPC™

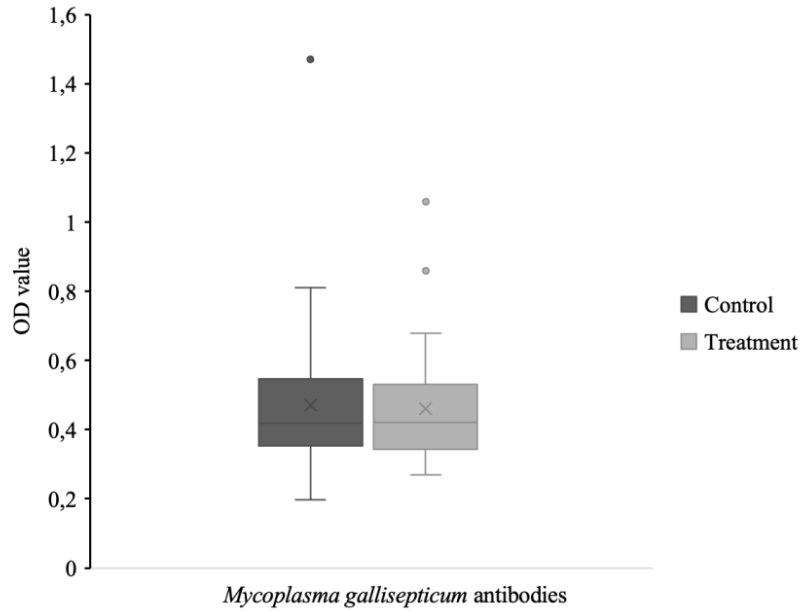

Figure 3 Box-and-whisker plot of absorbance values of indirect enzyme-linked immunosorbent assay for detection of *Mycoplasma gallisepticum* antibodies in serum of chicken fed with or without *Saccharomyces cerevisiae* fermentation product. Boxes indicate interquartile ranges, with median values shown as horizontal lines inside the box. Whiskers indicate ranges. × markers represent the mean values of the samples. All data points from 2 experiment groups of sample size n = 48 in each group are plotted. Outliers are indicated as small circles. OD = optical density. *P*-value = 0.87.

Discussion

Saccharomyces cerevisiae fermentation product is a postbiotic that is produced when *Saccharomyces cerevisiae* is fermented anaerobically. It may contain media used during the fermentation process, leftover yeast cells, and fragments of yeast cell walls (Wilson *et al*., 2022). Previous studies have shown that feeding SCFP to animals has changed their immunological functions (Cortés-Coronado *et al*., 2017; Deters *et al*., 2018; Wilson *et al*., 2022). Our study demonstrated that dietary supplementation with SCFP in chickens did not accelerate the establishment of an NDV-specific and AIV-specific humoral immune response when compared with non-supplemented chickens (Figure 1 and Figure 2). This result is in agreement with other researchers who reported no significant increase in immune response while feeding chickens with the postbiotic. The research of Silva *et al.* (2009) revealed that yeast extract supplementation did not provoke the anti-viral antibodies to NDV. Conversely, *Saccharomyces cerevisiae* cell wall (yeast cell wall), a postbiotic product, was reported to show a positive effect on immune response against Newcastle disease in chickens (Ghosh *et al*., 2012). Research conducted by Cortés-Coronado *et al*. (2017) reported that treatments with the postbiotic compound induced anti-viral antibodies to NDV post-vaccination. Abd El-Ghany *et al*. (2022) investigated the effect of a postbiotic produced by stabilized non-viable *Lactobacilli* on the immunity of chicken and determined that the humoral immunity against AIV showed a significant difference between postbiotic-treated and non-treated chickens. Ismael *et al*. (2022) also found that supplementing with SCFP and xylanase to the low-energy broiler diet improved antibody titer in response to NDV vaccines in comparison to the control. Notably, the chickens in the experiment of Ismael *et al*. (2022) were fed a diet with 0.625 kg/ton of SCFP, whereas our chickens received a diet with 1.25 kg/ton of SCFP. Cortés-Coronado *et al*. (2017) did the experiment with 4 dietary levels of yeast-fermented product (0, 400, 800, and 1,600 ppm) and found that only the 800ppm level enhances the antibody titer against NDV. Interestingly, the NDV-specific Ab titers were lowest at the highest dietary yeast-fermented product (Cortés-Coronado *et al*., 2017). Although it is unclear why the high inclusion level of the yeast ingredient resulted in lower antibody titers, it seems that an accurate level of the yeast provision is critical for a successful immune response enhancement. Therefore, further studies may be needed to identify an appropriate level of SCFP that can be added to the diet to boost chicken immunity.

Coccidiosis is a protozoa disease causing diarrhea, weight loss, and decreased production in poultry (Ho *et al*., 2019). In Vietnam, the prevalence of coccidia infection in chickens is very high, especially in chickens raised in low hygiene conditions (Hoan *et al*., 2014; Hung *et al*., 2021). Ho *et al*. (2019) reported that the pathogenicity of *Eimeria* species isolated from chickens in Thua Thien Hue province, where the experiment was conducted, had high virulence in the chickens. In this study, feces were collected for oocyst counting weekly from 2 weeks of age until the end of the study to evaluate the effects of SCFP supplementation on coccidiosis. Results showed that chickens were severely infected with coccidiosis in both control and treatment. Normally, chickens are severely infected with coccidia at about 2-4 weeks of age. However, in this study, chickens began to be infected with coccidiosis after 5 weeks of age and became more and more severe until the end of the experiment at 12 weeks of age in both control and treatment. This may be because the experiment was conducted in an area that had not raised chickens for a long time, so the environment was free of coccidia oocysts in the first weeks. Then, during the process of taking care of chickens, the chickens were gradually infected with coccidia from neighboring areas because of the low biosecurity (sector 3) of the farm where the experiment was conducted.

Chickens in the control and treatment groups showed several peaks of oocyst discharge in feces during the experiment. Commonly, the oocyst is detected in the feces of chickens from 6-14 days post-infection, and oocyst excretion reaches the peak at 8 days post-infection (Ho *et al*., 2019). The number of oocysts excreted in feces, and the peak of oocyst excretion depends on the time and number of oocysts ingested. In this study, the chickens were naturally infected; therefore, there are differences in the amount and peak of oocyst excretion between treatments. The results of oocyst per gram of dropping (OPG) in this study align with previous research examining the efficacy of *Saccharomyces cerevisiae* fermentation product on coccidia-challenged laying hens (Lensing *et al*., 2012). They also reported that chickens supplemented with SCFP had a later oocyst excretion and a larger number of oocyst shedding compared to the control.

In the present study, SCFP did not significantly affect the seroprevalence of chronic respiratory disease (CRD) infection in chickens (Table 4). The obtained results agree with the results of Elliott *et al*. (2020), who studied the influences of dietary SCFP in chickens challenged with *M. gallisepticum* at 12 weeks of age and found that dietary treatment with SCFP in challenged chickens did not significantly affect ELISA titers. Importantly, Elliott *et al*. (2020) and our study added the same concentration of SCFP in the chicken diet; however, we studied totally different chicken breeds. They studied the Hy-Line W-36 commercial layers while F_1 (Ri x Luong Phuong) chickens were used in our study. Therefore, it seems that the chicken breed might not be a factor that affects the effectiveness of SCFP on the prevalence of CRD in chickens.

Seroprevalence of CRD infection was high in our experimental chickens in both treatments, although CRD clinical signs were not observed (Table 4). Chronic respiratory disease (CRD) caused by *Mycoplasma gallisepticum* is a frequent respiratory infection in chickens and is considered to cause great economic losses within the poultry industry (Ferguson-Noel *et al*., 2020; World Organisation for Animal Health, 2023; Yadav *et al*., 2022). CRD is highly prevalent and globally distributed in chicken farms (Yadav *et al*., 2022; World Organization for Animal Health, 2023). In Vietnam, CRD has been reported to circulate and is one of the 25 common chicken infectious diseases in the region (Choisy *et al*., 2019;

Van *et al*., 2020). The sources and time points of *M. gallisepticum* infection were unclear in our study. Notably, *Mycoplasma* infections can spread through infected eggs (Ferguson-Noel *et al*., 2020; Yadav *et al*., 2022). That may lead to the early infection of *M. gallisepticum* in young chickens and proceed to the chronic stage, although we thoroughly disinfected the barn before conducting the experiment. It should also be noted that our experiment was carried out in a sector 3 production system, which is a low biosecurity system. Moreover, in our experiment, antibiotics were not used for disease prevention, and the chickens were not vaccinated against *M. gallisepticum*. Therefore, the infections from the environment could not be prevented even though we used the SCFP as an immune booster in the treatments.

This is the first report on the addition of SCFP to the diet for chickens raised in open-farm settings, which is a common, low-biosecurity method of raising poultry in developing countries. Under such a production system, antibiotics are frequently used to prevent common infections, including coccidiosis, CRD, etc... In this study, SCFP was used in the hopes that it could act as an antibiotic alternative for disease prevention since earlier research revealed that supplementing SCFP would boost immunity and minimize infections (Abd El-Ghany *et al*., 2022; Chou *et al*., 2017; Elliott *et al*., 2020; Ghosh *et al*., 2012; Soren *et al*., 2024). Unexpectedly, our results indicated that supplementing SCFP in the diet did not affect chicken immune response after vaccination against NDV and AIV. It's interesting to note that the majority of research examining SCFP's potential as a substitute for antibiotics was carried out in well-managed farming environments, where environmental infections have less of an impact on chickens (Elliott *et al*., 2020; Soren *et al*., 2024). In contrast, chickens in this study were naturally exposed to a number of rather serious respiratory and digestive tract diseases, including coccidiosis infection (Table 3) and CRD (Table 4). Therefore, additional research is required to validate the theories suggesting that the exposure of chickens to infections naturally in open-farming systems may impact how SCFP affects the immune response.

In conclusion, supplementation of the *Saccharomyces cerevisiae* fermentation product to the diet did not affect the immune response post-vaccination of NDV and H5N1 ($P > 0.05$). Moreover, the effects of SCFP supplementation on two common diseases in chickens showed that the seroprevalence of CRD infection was similar either with or without supplementation of SCFP, while the intensity of coccidiosis infection tended to be higher in the supplementation treatment compared to the control. Overall, supplementation of the *Saccharomyces cerevisiae* fermentation product in the chicken diet did not affect the immune response to NDV and H5N1 post-vaccination and the prevalence of CRD.

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Supplementary

Supplement Table 1 Ingredients (% as fed) and nutrient composition of the basal diet based on three feeding phages for experimental chickens.

Ingredients	Starter (0-21 days old)	Grower (22-41 days old)	Finisher (42-85 days old)
Corn	54,80	59.35	61,78
$CaCO_3$ (Calcium Carbonate)	1.40	1.30	1.20
DDGS (corn)	5.00	6,80	7.00
Dicalcium Phosphate	1.20	1.30	1.20
L-Lysine	0.39	0.40	0.26
L-Methionine	0.40	0.40	0.26
NaCl (Salt)	0.20	0.20	0.20
Rice bran	5.00	3.60	4.20
Soybean meal	29.00	24.00	21.00
Threonine	0.24	0.23	0.13
Vegetable oil	2.13	2.18	2.53
Vitamins and Minerals[1]	0.25	0.25	0.25
Total	100	100	100
Nutrient composition			
Dry Matter, %	88.36	88.38	88.39
Calcium, %	0.89	0.87	0.81
Crude Fiber, %	4.46	4.32	4.25
Crude Protein, %	19.94	18.41	17.03
Metabolizable Energy, Kcal/kg	2999.74	3050.60	3100.46
Phosphorus (easily digestible), %	0.29	0.30	0.28
Phosphorus, %	0.56	0.57	0.54
SID Lysine, %	1.20	1.15	0.95
SID Methionine+Cysteine, %	0.90	0.90	0.74
SID Threonine, %	0.80	0.77	0.64
SID Tryptophan, %	0.20	0.18	0.16

1Vitamin A (minimum): 3.200.000 IU/kg; vitamin E (minimum): 12.000 mg/kg; vitamin B1 (minimum): 400 mg/kg; vitamin D3 (minimum): 1.000.000 IU/kg; vitamin K3 (minimum): 660 mg/kg); vitamin B3 (minimum): 12.000 mg/kg; Fe (minimum - maximum): 10.200-13.800 mg/kg; Zn (minimum - maximum): 25.500-34.500 mg/kg; Cu (minimum - maximum): 3.200-4.800 mg/kg; Mn (minimum - maximum): 25.500-34.500 mg/kg.

Supplement Table 2 Vaccination and schedule of events.

Days of age	Activity
Hatchery	F1 (Ri x Luong Phuong) broilers were vaccinated for Marek's disease
1	Treatment diets began (Starter), F1 (Ri x Luong Phuong) broilers were fed an available feed as normally used in practice.
7	F1 (Ri x Luong Phuong) broilers were vaccinated with Medivac ND-IB 1st
11	F1 (Ri x Luong Phuong) broilers were vaccinated with Medivac pox 1000 and Gumboro A 500 1st
14	F1 (Ri x Luong Phuong) broilers were vaccinated with Avian influenza H5N1 (NAVET-VIFLUVAC, subtype H5N1), and feces were collected for oocyst counting.
16	Blood samples were drawn for evaluation of NDV titers
18	F1 (Ri x Luong Phuong) broilers were vaccinated with Medivac ND-IB 2nd
21	F1 (Ri x Luong Phuong) broilers' feces were collected for oocyst counting.
28	F1 (Ri x Luong Phuong) broilers were vaccinated with Medivac ILT 1000, and feces were collected for oocyst counting.
35	Blood samples were drawn for chronic respiratory disease (CRD) tests, and feces were collected for oocyst counting.
41	Blood samples were drawn for evaluation of H5N1 titers.
42	F1 (Ri x Luong Phuong) broilers were vaccinated with Medivac ND G7 Emulsion, and feces were collected for oocyst counting.
49	Boilers feces were collected for oocyst counting.
56	F1 (Ri x Luong Phuong) broilers' feces were collected for oocyst counting.
63	Boilers feces were collected for oocyst counting.
70	F1 (Ri x Luong Phuong) broilers' feces were collected for oocyst counting.
77	F1 (Ri x Luong Phuong) broilers feces were collected for oocyst counting
85	F1 (Ri x Luong Phuong) broilers' feces were collected for oocyst counting, and blood samples were drawn for NDV and H5N1.