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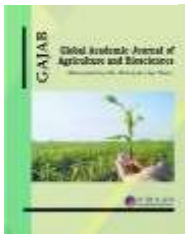

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Research Article

Immunological Response and Caecal Microflora of Brown-egg type Layers fed With a Novel Yeast *Pichia kudriavzevii*

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Abstract: The intestinal microflora influences the overall health and immune status of poultry birds. It is therefore obvious that the future success of poultry farming lies in the use of probiotics as growth promoters and health management agents. A total of 196 points of lay (12 weeks) hens ISA strain of pullets was used to investigate the effects of *Pichia kudriavzevii* on the immunological response and ceacal microflora of layer hens. The birds were randomly allotted to seven treatment groups of 9 replicates each. There were 3 birds per replicate, making a total of 27 birds per treatment in a completely randomized design. The birds were vaccinated with Lasota strain of New Castle Disease Vaccine (NCDV) at week 20 of diet supplementation. At the end of the experiment, the spleen of birds was collected for gene expression of some cytokines. The result of this study showed ($P<0.05$) and a sustained antibody titre against NCDV in all the treatment groups supplemented with strains of *P. kudriavzevii* as compared with the positive and negative control groups. Interferon-gamma and interleukin 10 gene expressions in the spleen was upregulated by the yeast strains with the highest IFN-γ concentration of 34.38 µg/mL recorded in diet supplemented with *P. kudriavzevii* MH458239. It was also observed that total bacteria count was significantly ($P<0.05$) reduced through the administration of these yeast strains. Conclusively, the use of *P. kudriavzevii* as a growth promoter in layer hen positively influenced their caecal microflora which consequently primed their immune response.

Keywords: *P. kudriavzevii*, Immunity, Cytokines, Antibody titre, NCDV, gene expression.

INTRODUCTION

Disease outbreak in large scale poultry farms usually leads to serious economic and financial losses. Prevention and control of such diseases have resulted in a rise in the use of antibiotics. Antibiotic therapy may not be a permanent solution as there is extensive documentation on the evolution of antimicrobial resistance and serious concern on their side effects [1, 2]. The immune status of birds is a crucial factor in the fight against disease outbreaks, and probiotics as growth promoters have been used to modulate the immune system of poultry birds [3, 4].

The two types of antigens that challenge the gut-associated lymphoid tissue (GALT) in birds include non-immune evoking innocuous antigens such as nutrients and antigens from intestinal or external pathogens that should evoke protective immune responses [5, 6]. Consequently, the activation of both humoral and cellular gut-associated immune systems was largely influenced by the gut microflora [7]. The short digestive tract

and fast passage of feed ingested in the upper intestine of birds usually result in incomplete digestion [8]. But the presence of different species of non-pathogenic microorganisms in their caecum help prolongs the retention time of digesta which leads to their further breakdown, pathogen exclusion, production of micronutrient and immune stimulation [9, 10].

Trino IB is an immune-modulatory agent comprising oleuropein and alpha-lipoic acid. It has been proven to be effective against viral infections even in animal models [11]. Based on no history of association with foodborne illnesses, many yeast species are recognized as safe by many regulatory authorities. Although most commercial probiotic feed supplements are bacterial in origin, previous studies have reported that dietary yeast products could improve intestinal immunity and prevent infections in poultry birds [12-14]. *Saccharomyces boulardii* is well-recognized probiotic yeast that is known for its ability to provide nutrients for the host inhibit pathogen growth, improve the immunity of intestinal mucosa, and activities of beneficial intestinal microflora [15]. *Pichia kudriavzevii* has been investigated for its ability to degrade phytate [16]. Ogunremi *et al*. [17] used it as a starter culture for the development of cereal-based functional food where it was also reported as a high phytase producer. Magnoli *et al*. [18] also reported that *Pichia kudriavzevii* could cushion the adverse effects of Aflatoxin B when added to feed of broiler chicks. Although *P. pastoris* was studied on its effect on feed efficiency and immune modulation, studies about the immunomodulatory abilities of *P. kudriavzevii* are yet to be explored, especially with the use of layer hens. This study was designed to investigate the immunological response of laying hens fed with *P. kudriavzevii*.

MATERIALS AND METHODS

Experimental site

The experiment was carried out at the layer house of the Teaching and Research Farm (Poultry Section), Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.

Pre-experiment preparations

The strains of *P. kudriavzevii* used in this work were isolated from *ogi*, a Nigerian traditional fermented food. The strains were coded as *P. kudriavzevii* MH458239 and *P. kudriavzevii* MH458240 [19]. Yeasts were kept on Yeast Peptone Dextrose agar (yeast extract 10g/l; peptone 20g/l; glucose 20g/l; agar 15g/l) for up to 2 weeks and in 10 % glycerol solution in case of long-term storage.

Test ingredients and preparation

An overnight culture of *P. kudriavzevii* grown in yeast extract broth was centrifuged at 4,000rpm for 10minutes and washed in normal saline to harvest the yeast cells. The pellets were suspended in normal saline and adjusted to a final concentration of 10^8 cfu/ml [20].

Experimental animals and management

A total of 196 points of lay Isa brown hens were allocated in a completely randomized design. Necessary vaccinations were administered, and the birds were weight-balanced as they were randomly divided into seven (7) treatment groups of 9 replicates each. There were 3 birds per replicate to make a total of 27 birds per treatment in a completely randomized design (CRD). A basal diet, "grower mash" was administered until 5 % egg production when the layer mash was introduced. Dietary yeasts were used at an inclusion level of 15 x 10^8 cfu/kg of feed while Trino IB (an Immune booster) was added at a concentration of 10ml/ kg of feed and served as the positive control. Treatment groups were as follows: Treatment 1 was the control (basal diet), Treatment 2 diet was supplemented with *P. kudriavzevii* MH458239; Treatment 3 diet was supplemented with *P. kudriavzevii* MH458240; treatment 4 diet was supplemented with *P. kudriavzevii* MH458239 and Trino IB; Treatment 5 diet was supplemented with *P. kudriavzevii* MH458240 and Trino IB, Treatment 6 diet was supplemented with both yeast strains while Treatment 7 was supplemented with Trino IB and this treatment served as the positive control. The gross composition of the experimental diet is shown in Table 2. Feed and clean water were given to the birds ad libitum. The experiment lasted for 28 weeks.

Microbial enumeration of faecal samples of experimental birds

The ability of tested yeast strains to pass through the gastrointestinal tract of birds and faecal bacterial load was determined by taking faecal samples from each treatment group on weeks 2 and 10 of diet supplementation for analysis. 1g of faeces was pooled from birds (3 birds per treatment), serially diluted and appropriate dilutions were enumerated on yeast extract agar plate supplemented with chloramphenicol (100mg/l).

Immune response determination

Immune response was measured through antibody titre against Newcastle disease and gene expression analysis of some anti-inflammatory and pro-inflammatory cytokines which include interleukin (IL) - 10, IL-12, and interferon-gamma.

Determination of Antibody Titre against Newcastle disease virus

Layer birds were vaccinated against Newcastle disease with a strain of New Castle disease vaccine (Lasota) and antibody titre in serum was measured on days 1, 7, and 14 post vaccinations. One bird from each replicate was randomly selected and blood was collected by neck venipuncture. Antibody titre against Newcastle disease virus (NDV) was detected by haemagglutination-inhibition test method [12].

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from individual spleen samples using the Trizol extraction method as described by the Trizol manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada). The quantity and purity of the RNA samples were measured by using NanoDrop spectroscopy (Thermo Scientific) with the ratio of absorbance at 260 nm and 280 nm. Reverse-Transcription was performed by using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mississauga, ON, Canada) according to the manufacturer's protocol [21].

Quantitative Real-time PCR

Primer sequences for IL-10, IL-12, and interferon-gamma (IFNγ), were designed using Genebank database sequences from the National Center for Biotechnology Information (Bethesda, MD) corresponding to each quantified gene. Quantitative real-time (qRT) PCR was performed using the Step One Thermo Cycler (Applied Biosystems) on a 48-well plate with 25 µL of total reaction volume. The iTaq Universal SYBR Green Supermix was used as the QRTPCR master mix and each reaction was run in duplicate. The PCR cycling protocol includes an initial denaturation step at 95°C, followed by amplification for 40 cycles at 95°C for 10 s, and 30 annealing steps at a temperature described in Table1.0 for each of the primer pairs, and extension at 72°C for 10 s [21].

STATISTICAL ANALYSIS

Data obtained were analyzed using one-way Analysis of Variance (ANOVA of SAS, 2003 version 9.3). Means were separated using the least significant difference test at 5% probability level.

Table-1: Primer sequences used for real-time quantitative PCR

Gene	Primer sequence (5'-3')	Base pair	Annealing temp(°C)	genBank accession number
IFN-γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	264	60	X99774
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	103	55	AJ621614
1L-12	F: CTGAAGGTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAGGTT	217	64	NM213588

KEY: IFN-**γ –**Interferon-gamma
IL –interleukin
F- Forward
R- Reverse

Table-2: Gross composition of the experimental diet

Ingredients	Kg
Maize	47
Soya bean meal	15.4
Wheat offal	14.05
Fishmeal	3
Palm kernel cake	8
Limestone	8.5
Bonemeal	3
Salt	0.3
Lysine	0.25
Methionine	0.25
Premix	0.25
Total	**100**

RESULTS

Effects of dietary supplementation with *P. kudriavzevii* on faecal microflora

There was a steady increase in the bacterial load of the birds from the second week of feeding the birds with the supplemented feed (Figure 1). However, a gradual reduction in bacterial load was observed as the feeding proceeded beyond 10 weeks. There was no observable bacterial growth in the faecal samples collected from birds fed with the diet that was supplemented with Trino IB alone. Layer bird's diet supplemented with *P. kudriavzevii* MH458240 plus Trino IB had the least bacterial count (6.9 logcfu/g) at week 10.

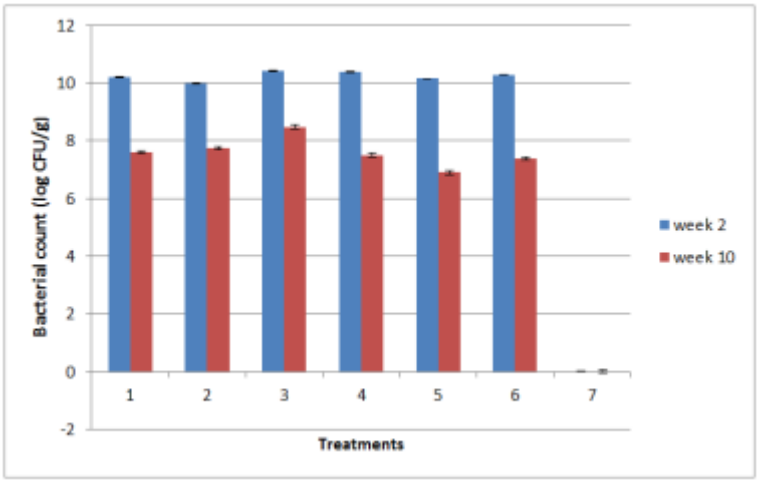

Fig-1: Effect of diet supplementation with *P. kudriavzevii* on the bacterial count of layer birds

Legend
T1 –control; **T2**- MH458239; **T3**- MH458240; **T4** – MH458239+Trino IB;
T5 – MH458240+ Trino IB; **T6** – MH458239+MH458240; **T7** – Trino IB

The ability of potential probiotic yeast (*P. kudriavzevii*) to pass through the layer birds' gastrointestinal tract was monitored (Figure 2). There was no yeast growth observed in the faecal samples of the experimental birds at the early stages of the experiment. However, after 10 weeks of feeding the birds with the supplemented diets, there was a heavy growth of yeast cells in their feacal samples. Diet supplementation with *P. kudriavzevii* MH458239 recorded a significant (P<0.05) higher yeast count (8.48 log cfu/g), than diet supplementation with *P. kudriavzevii* MH458240 (7.78 log cfu/g). The Addition of Trino IB in the diet significantly (P<0.05) elicited heavy growth of the yeast strains in the feacal samples of the experimental birds (10.48 log cfu/g and 10.18 log cfu/g for T4 and T5 respectively) at the early stages of the experiment. However, at week 10, the Trino IB effect was lost as there was no observable yeast growth recorded at this stage.

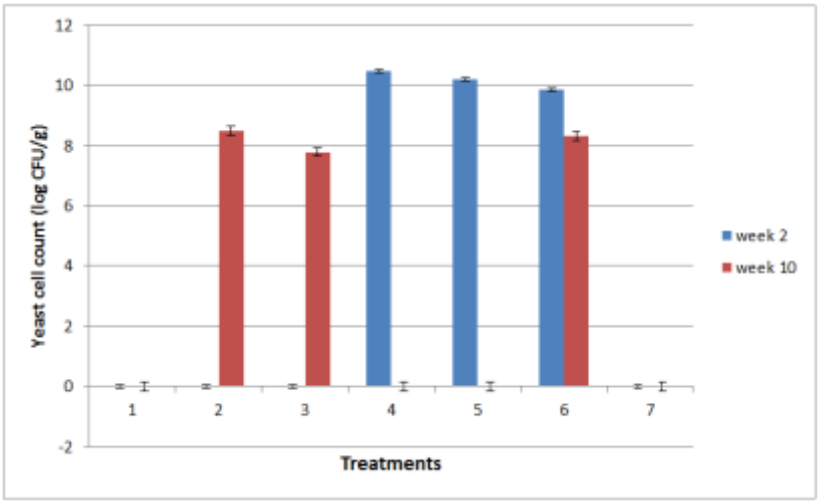

Fig-2: Ability of *P. kudriavzevii* to pass through the gastrointestinal tract of layer birds

Legend
T1 –control; **T2**- MH458239; **T3**- MH458240; **T4** – MH458239+Trino IB;
T5 – MH458240+ Trino IB; **T6** – MH458239+MH458240; **T7** – Trino IB

Figure 3 shows the coliform counts of faecal samples of the experimental birds. At week 2 of diet supplementation, treatment supplemented with *P. kudriavzevii* MH458239 plus Trino IB (T4) had the highest coliform count of 10.40 logcfu/g. Next to it was treatment supplemented with *P. kudriavzevii* MH458240 plus Trino IB (T5) with a coliform count of 10.23 logcfu/g. The treatment supplemented with Trino IB alone and treatment supplemented with *P. kudriavzevii* MH458240 had no observable coliform

colony. Treatment supplemented with both yeast strains (T6) had a coliform count of 9.86 logcfu/g of the faecal sample. At week 10, treatment supplemented with *P. kudriavzevii* MH458240 plus Trino IB (T5) had the highest coliform count of 9.19 logcfu/g. There was no observable coliform colony seen in faecal samples of treatments supplemented with each of the yeast strains and the combination of both.

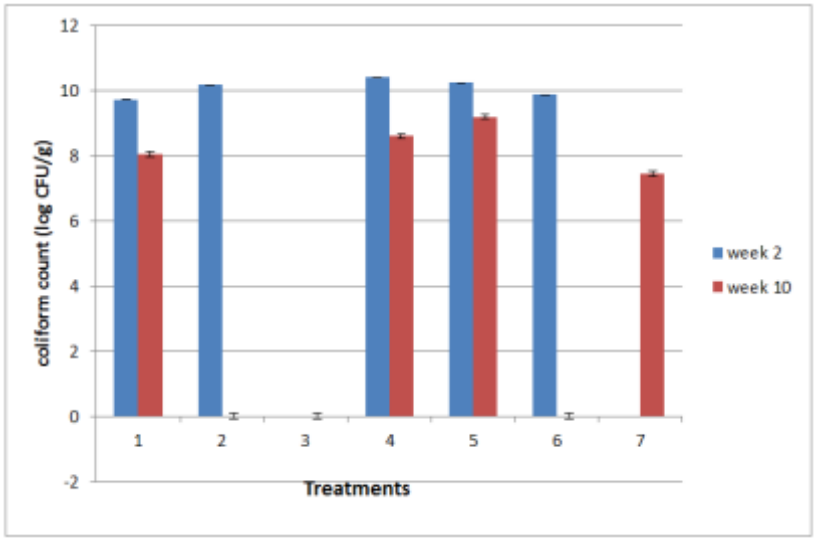

Fig-3: Effect of diet supplementation with *P. kudriavzevii* on the coliform count of layer birds

Legend
T1 –control; **T2**- MH458239; **T3**- MH458240; **T4** – MH458239+Trino IB;
T5 – MH458240+ Trino IB; **T6** – MH458239+MH458240; **T7** – Trino IB

Effects of dietary administration of *P. kudriavzevii* on immune Response of layer birds

Supplementation of *P. kudriavzevii* strains in the feed improved the immune response of the birds to Newcastle disease virus vaccination. The immune response of the birds to Newcastle disease virus significantly (P< 0.05) improved as the period of feeding increased (Figure 4). Supplementation with *P. kudriavzevii* MH458240 (T3) resulted in a sustained antibody titre throughout the 14 days of the experimental period in the layer birds (7.19±0.07 on day 1, significantly increased (P<0.05) to 11.0793±0.01 on day 7 and 17.9673±0.06 on day 14 post- vaccination). A Similar observation was recorded with supplementation with *P. kudriavzevii* MH458239 plus Trino IB (T4) and *P. kudriavzevii* MH458240 plus Trino IB (T5).

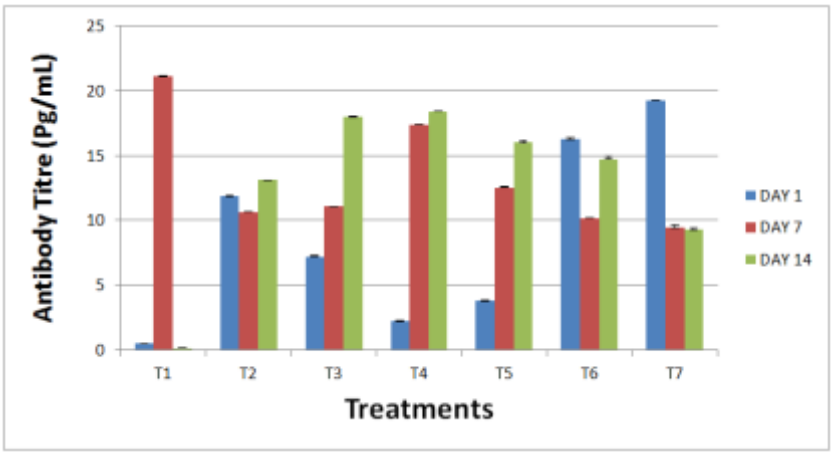

Fig-4: Effect of *P. kudriavzevii* supplementation on antibody titre to Newcastle disease virus in layer birds

Legend:
T1 –control; **T2**- MH458239; **T3**- MH458240; **T4** – MH458239+Trino IB;
T5 – MH458240+ Trino IB; **T6** – MH458239+MH458240; **T7** – Trino IB

The two strains of *P. kudriavzevii* upregulated the expression of cytokine genes expression. Interferon gamma gene expression was upregulated by the two yeast strains with the highest (34.38 µg/mL) recorded in diet supplemented with *P. kudriavzevii* MH458239 (Table 3). The expression of the gene was, however, downregulated by the application of a mixed culture of both strains of *P. kudriavzevii* and the administration of Trino IB. Expression of interleukin 10 (IL 10) genes in the spleen of layer birds was also upregulated by the two yeast strains. The highest release of IL 10 was recorded with the spleen of birds whose diet was supplemented with *P. kudriavzevii* MH458240 plus Trino IB (34.95 µg/mL). Supplementation with Trino IB alone down-regulated the release of interleukin 10 in the spleen of layer birds. Supplementation with *P. kudriavzevii* MH458239 plus Trino IB (T4) and a combination of both strains (T6) inhibited the expression of IL-10 gene in the spleen of experimental birds. There was a significant increase (P<0.05) in the expression of interleukin 12 genes when the diet was supplemented with a mixed culture of both strains of *P. kudriavzevii* (34.31 µg/mL).

Table-3: Concentration of cytokine released by the spleen of layer birds fed with *P. kudriavzevii*

Treatments	IFN-γ	Cytokine(µg/mL) IL-10	IL-12	SEM
Control	32.65[a]	32.50[a]	33.30[a]	1.390
MH458239	34.38[a]	34.20[a]	33.94[a]	1.244
MH458240	33.14[a]	34.40[a]	33.11[a]	1.244
MH458239+Trino IB	33.50	-	30.86	1.523
MH458240+Trino IB	33.50[a]	34.95[a]	33.22[a]	1.244
MH458239+MH458240	20.24[b]	-	34.31[b]	1.523
Trino IB	29.11[c]	28.86[c]	31.20[c]	1.244

[a-c]Means within a row with different superscripts are significantly different (P<0.05)

DISCUSSION

Results obtained in this study showed that supplementation of layer birds' diet with *P. kudriavzevii* reduced the total bacteria count in their caecum. This result agrees with Ozsoy *et al*. [22] who evaluated the reciprocal effects of probiotic yeast supplementation on the regulation of host animal's intestinal microflora and reported a similar result. Line *et al*. [23] and Nava *et al*. [1] established the inhibitory effect of yeast on intestinal pathogens. Another study by Hassanein and Soliman [24], reported that various inclusion levels of yeast supplementation of layer birds' diet significantly reduced the population of total bacteria in their gut. Further credence to the result recorded in this study is given by Laegreid and Bauery [25], who stated that many pathogenic bacteria exhibit a binding specific affinity for the sugar mannose, and live yeast cells contain mannose in their cell wall. The mannose in the yeast cell wall allows yeast cells to act as a trap for pathogenic bacteria and they are passed out in the birds' faeces, thereby reducing pathogens' colonization and multiplication in the birds' gut. A contrary result, however, was obtained by Wang *et al*. [26] reported that yeast supplementation increased total bacteria count in the intestine of layer birds.

The immune system protects the body from invasion by pathogens and foreign bodies. A change in antibody titre is usually used as a parameter for the measurement of immune responses of birds especially the humoral immunity to foreign invasion [27]. New castle disease is one of the most fatal diseases of poultry birds which usually cause a very high mortality rate resulting in economic losses [4]. Results obtained in this study showed that dietary supplementation of layer bird's diets with *P. kudriavzevii* produced a higher and a sustained antibody titre against new castle disease virus (Figure 4). This suggests that supplementing birds' diet with this yeast culture could help to prevent disease outbreaks when the birds are under challenged conditions because the yeast would have primed the immune system for a quick response as the situation warrants. The results obtained in this study are in agreement with Gao *et al*. [12] who reported increased antibody titres to NDV when broilers' diet was supplemented with yeast culture. Hatab *et al*. [28] also reported higher antibody levels against NDV when layer birds' diet was supplemented with *Bacillus subtilis* and *Enterococcus faecium*. However, in contrast to this result, Silva *et al*. [29] reported that there was no significant difference in the antibody response against Newcastle disease virus in broiler chickens fed with Yeast cell wall-supplemented diet.

Cytokines are considered as the major factor for immune cell communication and immunomodulatory agents, regulating both innate

and adaptive immune response to antigens and infectious agents [30]. In this study, the expression of IFN-γ, IL-10, and IL-12 in the spleen of the experimental birds was measured (Table 2). IFN- γ, and IL-12 are pro-inflammatory cytokines while IL-10 is an anti-inflammatory cytokine. IFN- γ regulates acquired immunity and high levels have been associated with protective immune responses to some parasitic infections [31]. Diets supplemented with both strains of *P. kudriavzevii* upregulated the expression of IFN- γ, and IL-10. This implies that *P. kudriavzevii* supports the production of both pro- and anti-inflammatory immune responses, thus suggesting that supplementation of layers' diet with *P. kudriavzevii* could produce an immune-stimulatory effect that will enhance resistance to pathogen invasion and therefore curb disease outbreak in poultry birds. This result agrees with that of Yitbarek *et al*. [32] who reported that the inclusion of yeast-derived products in broilers diet upregulated the expression of both pro-inflammatory and anti-inflammatory cytokines. Upregulation of IL-10 in layer birds fed with live yeast cells might be due to the anti-inflammatory properties of the β-glucan of yeast cell wall. It was reported by Jawhara *et al*. [33] that β-glucan from yeast cell wall increased IL-10 production in a dextran sulphate sodium-induced mice model. Alizadeh *et al*. [34] also reported an increment in the gene expression of anti-inflammatory cytokines when broiler chicks were fed with yeast-derived products. However, the findings of Cox *et al*. [35] and Munyaka *et al*. [36] who reported that supplementation of broiler diet with products derived from yeast down -regulated the expression of pro-and anti-inflammatory cytokines contradicts the results of this experiment.

It was also observed in this study that there was no significant difference ($P<0.05$) in the expression of IL-12 in the treatments that received each of the strains of *P. kudriavzevii*. This agrees with the results of Alizadeh *et al*. [34] who investigated the effect of yeast-derived products on gene expression pattern recognition receptors and cytokines in broiler chickens. They reported no significant difference in gene expression of IL-12 among their treatments.

In conclusion, supplementation of layer hen diet with *P. kudriavzevii* could increase their immunity against infectious diseases such as new castle disease virus as well as improve the microflora in their gastrointestinal tract.

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