RESEARCH ARTICLE Indian Journal of Animal Research, Volume 55 Issue 4: 451-456 (April 2021)

Probiotic Attributes of *Lactobacillus fermentum* NKN51 Isolated from Yak Cottage Cheese and the Impact of Its Feeding on Growth, Immunity, Caecal Microbiology and Jejunal Histology in the Starter Phase of Broiler Birds

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10.18805/ijar.B-3970

ABSTRACT

Background: The discovery of antibiotics was a great success in control of the pathogenic infections in living beings. In addition, it also played a significant role in upliftment of the meat industry with their use as the growth promoters in the food animals including poultry. However, massive and incorrect use of antibiotics has resulted in the adverse outcome of antibiotic resistance over the years. In order to limit the antibiotic resistance at industry level itself, exploration of the possible alternatives such as probiotics is the need of the hour to replace the antibiotics in poultry industry without compromising the productivity. Hence, the present study was conducted to explore the effects of a novel probiotic bacterium, *Lactobacillus fermentum* NKN51 on the production performance of the broilers with an aim to replace the antibiotics in poultry production.

Methods: In the present study, a total of 128 broiler birds were grouped randomly into four treatment groups which were fed basal diet (BD) along with the different levels of *L. fermentum* NKN51*, viz*.,T_1 (BD+10^7cfu/gm), T_2(BD+10^6 cfu/gm), T_3 (BD+10^5cfu/gm) and T_4 or control (BD only) for a period of 28 days so as to observe the effect of *Lactobacillus fermentum* NKN51 in the starter phase of the broiler birds in terms of growth, immunity, gut health and physiology.

Result: The result revealed that the group of broiler birds supplemented with *Lactobacillus fermentum* NKN51 at the level of 10^7cfu/gm of basal feed had significantly better growth performance, cell mediated and humoral immunity, gut health and jejunal histology in terms of villus height, VH:CD ratio and intestinal absorption capacity. Thus, *Lactobacillus fermentum* NKN51 was found as an ideal probiotic supplement exhibiting better potential in enhancing the efficiency of broiler birds in terms of improved productivity.

Key words: Broiler, Growth performance, Gut physiology, Immunity, Lactobacillus fermentum NKN51.

INTRODUCTION

Augmentation in the perception of society towards the scientific acquaintance has led the people to turn out to be more vigilant concerning their health aspects. Therefore, the demand for animal protein has also increased in recent years. Poultry meat, being the most widely accepted world wide with no religious taboos, is well known for its specific taste, flavor and low-fat virtues. After the discovery of antibiotics, the poultry industry flourished more with their use as growth promoters in poultry production. Since decades, the antibiotics have been an imperative part of poultry production as a growth promoter (AGPs) but the extravagant use of these has resulted in amplified trouble of antibiotic resistance (Furtula *et al.*, 2010; Forgetta *et al.,* 2012), which eventually influences living beings in terms of their health issues (Diarra *et al*., 2010). Thus, with the ultimate aim of reducing resistance development in bacterial pathogens, there is an urgent need to look for such alternatives, which can be utilized as growth-promoters in broilers in addition to the ability to control pathogenic infections in them.

Therefore, the exploration of the probiotic bacteria in the current scenario is the need of the hour for their further expedition as an alternative to antibiotics in the poultry industry. Among the probiotics, genus *Lactobacillus* is the most common group of probiotic bacteria to be used widely in the industry today. Although the probiotic properties of genus *Lactobacillus*, the natural gut inhabitants with GRAS status (Generally Recognized as Safe) are well known to researchers but even after being used extensively in the food industry, various inconsistent and adverse reports regarding the use of probiotic at some point of time are not uncommon, which may be as a result of lack of the

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How to cite this article: Geeta, Yadav, A.S., Pradhan, S., Rajoria, R., Kumar, M.A., Gopi, M., Navani, N.K. and Pathania, R. (2021). Probiotic Attributes of *Lactobacillus fermentum* NKN51 Isolated from Yak Cottage Cheese and the Impact of Its Feeding on Growth, Immunity, Caecal Microbiology and Jejunal Histology in the Starter Phase of Broiler Birds. Indian Journal of Animal Research. 55(4): 451-456. DOI: 10.18805/ijar.B-3970.

Submitted: 17-12-2019 **Accepted:** 12-04-2020 **Online:** 28-09-2020

information at genetic level itself (Sharma *et al*., 2018). Thus, at present, it becomes elemental to have a detailed study regarding the use of the respective probiotic bacteria with some known genetic properties before introducing into an animal model. *L. fermentum,* a promising probiotic bacterium, has undergone various research studies and is found to be bestowed with the various functional attributes which can be utilized for further application from a scientific point of view. *Lactobacillus fermentum* NKN51, a novel probiotic, isolated from Yak cottage cheese by IIT, Roorkee, India is reported to have a novel phytase, PhyLf of class PTP that can enhance the bioaccessibility of mineral ions. Its resistance to oxidative environment and gastric milieu along with the capability of releasing micronutrients are distinctive attributes of PhyLf which depicts a valid point for its further use in ameliorating the nutritional value of cereals and animal feed (Sharma *et al*., 2018). The present study was thus conducted to explore the effects of *Lactobacillus fermentum* NKN51 on performance parameters of slow-growing broilers including growth, immunity and gut physiology in their starter phase so as to address the wellbeing of the poultry birds with improved gut physiological conditions leading to better utilization of nutrients for maximum gains without the use of antibiotics.

MATERIALS AND METHODS

The present study was conducted at the division of Post-Harvest Technology, ICAR-Central Avian Research Institute, Izatnagar in the year 2019 with due consent from the Institute's Animal Ethics Committee (IAEC). A total of one hundred and twenty-eight day-old broiler chicks (n=128) of CARIBRO-VISHAL were procured from broiler farm, ICAR-Central Avian Research Institute, India, which were allotted randomly in four experimental groups in a completely randomized design (CRD). Each treatment group included 4 replicates with 8 chicks in each making a total of 32 chicks per treatment group. The broiler birds were raised under the standardized conditions of a deep litter system using paddy husk as a litter material with a floor space provision of 0.5-1 square ft per bird. Initially, the birds were provided with 23 hrs light with the light intensity of 3-4 foot-candle which was then reduced up to 16-18 hours with the light intensity of 0.5-1 foot-candle. On the first day, the brooding temperature was maintained around 95 ^{o}F which was then reduced 3-5 ^{o}F weekly till it reached 70 ^{o}F. The birds were reared for a period of 28 days to assess the effect of feeding of *L. fermentum* NKN51 in slow-growing broilers in the early phase of life (pre-starter and starter period). The culture of *Lactobacillus fermentum* NKN51 was isolated from Yak cottage cheese samples of Himachal Pradesh by the Indian Institute of Technology, Roorkee, India and was revitalized with the use of MRS broth and agar. Each of the four treatment groups were fed different levels of *Lactobacillus fermentum* along with the basal feed *i.e*. T_1 (basal feed + *L. fermentum* @ 10^7 cfu/gm of feed), T_2 (basal feed + *L. fermentum* @ 10^6 cfu/gm of feed), T_3 (basal feed + *L. fermentum* @ 10^5 cfu/gm of feed) and T_4 (basal feed only). The T_4 was served as the control group. The components of the basal diet have been summarized in Table 1 (Mandal *et al*., 2013). During the experimental period of 28 days, growth performance parameters including weekly body weight, feed intake and feed conversion ratio (FCR) were evaluated. The *in-vivo* cell-mediated immunity response was evaluated as per the methodology of Cheng and Lamont (1988**)** on 25th day post-hatch (n=8) as a response to phyto-haemagglutinin type P (PHA-P), whereas the humoral immunity was evaluated by using the method of Siegel and Gross (1980) with slight modification by injecting 1 ml of 1% sheep RBC suspension intravenously at 22nd day post-hatch in broilers (n=8). On the 28th day (5 days post-immunization), 2 ml of blood was collected from the jugular vein, after which, immune serum was harvested and determination of antibody titer was done by haemagglutination test (HA) in which, the reciprocal of the highest dilution showing clear agglutination was taken as the end titer which was expressed as $\log_2$. Further, the gut microbiology was conducted as per the methodology of Tatini *et al*. (1984), where samples (n=8) of the caecal contents were serially diluted in 0.85% sterile saline solution to evaluate the *Lactobacillus*, total plate and *E. coli* counts using lactobacilli MRS agar, plate count agar and EMB agar, respectively. The colony counts were noted from the countable dilutions and were expressed as $\log_{10}$ value of colony forming units per gram of intestinal contents ($\log_{10}$ cfu/g). In addition to these parameters, jejunal histomorphometry was also done for each of the treatment groups (n=8) to measure villus height, villus width, crypt depth, crypt width, villus height-crypt depth ratio and intestinal absorptive surface area. The formula used for calculating the mean absorptive surface area as follows (Kisielinski *et al*., 2002).

$$M = (\text{villus W} \times \text{villus L}) + (\text{villus W}/2 + \text{crypt W}/2)^2 - (\text{villus W}/2)^2 / (\text{villus W}/2 + \text{crypt W}/2)^2$$

Where,

M = intestinal absorptive surface area, villus W = mean width of villi, villus L = mean length of villi and crypt W = mean width of crypts.

The data obtained for each of the parameters were subjected to test of significance by analysis of variance (one way ANOVA) as per the methodology of Snedecor and Cochran (1994) using IBM-SPSS software package. The statistical significance was expressed as $P<0.05$ using Duncan's multiple comparison tests to compare means.

RESULTS AND DISCUSSION

The key indicator for the wellbeing of broilers can certainly be attributed to their growth performance since their performance is straightly associated with their feed utilization efficiency. The novel findings in this experiment revealed that the supplementation of *Lactobacillus fermentum* NKN51 @ 10^7 cfu/gm *i.e*. T_1 in the feed of slow broiler chicks resulted in significant ($P<0.05$) improvement in growth performance

Table 1: Composition of the basal diet for broilers used in the experiment.

Ingredients (%)	Pre-starter diet (0-14 days)	Starter diet (14-21 days)	Finisher diet (>21 days)
Maize	49.53	51	57.41
Soybean	38	36.35	30.3
Rapeseed meal	3	3	3
Roasted guar	3	3	3
Oil	3.1	3.61	3.4
Limestone	0.915	0.715	0.715
Di-calcium phosphate	1.7	1.6	1.5
Salt	0.3	0.3	0.3
DL-Methionine	0.14	0.11	0.06
TM. Premix-1[a]	0.1	0.1	0.1
Vit Premix-2[b]	0.15	0.15	0.15
B complex[c]	0.015	0.015	0.015
Choline chloride	0.05	0.05	0.05
Total	100	100	100
Nutrient composition			
ME (Kcal/kg)	3000	3050	3100
Crude protein (%)	22.00	21.5	19.5
Energy: protein ratio	135.77	141.96	159.02
Calcium (%)	1.01	0.91	0.86
Available phosphorus (%)	0.44	0.42	0.39
Lysine (%)	1.22	1.18	1.03
Methionine (%)	0.52	0.48	0.41

Note: [a]TM Premix-1: Each g of mineral mixture contained 200 mg ferrous sulphate ($F_eSO_4.7H_2O$), 20 mg copper sulphate ($CuSO_4.5H_2O$), 200 mg manganese sulphate ($MnSO_4.H_2O$), 150 mg zinc sulphate ($ZnSO_4.7H_2O$), 1 mg potassium iodide (KI).

[b]Vit Premix-2: Each g of vitamin A, B2, D3 and K (Spectromix, Ranboxy) provided vitamin A (retinol) 540 mg, vitamin B2 (riboflavin) 50 mg, vitamin D3 (cholecalciferol) 400 mg, vitamin K (menadione) 10 mg.

[c]B complex: Each g of B complex provided vitamin B1 (thiamine) 2 mg, folic acid 10 mg, pyridoxine HCl 4 mg cyanocobalamin 10 µg, nicotinamide 12 mg.

Table 2: Effect of different levels of *Lactobacillus fermentum* NKN51 supplementation on weekly body weight (grams) in broilers (Mean±SE).

Treatment groups	Zero-day	I week	II week	III week	IV week
T_1	44.68±0.598[a]	172.44±1.62[b]	456.82±3.26[c]	756.77±4.91[b]	1108.44±6.14[c]
T_2	44.97±0.765[a]	170.54±1.51[b]	443.95±2.88[b]	745.95±6.96[ab]	1065.09±6.09[b]
T_3	42.86±0.679[a]	164.77±1.59[a]	434.81±2.38[a]	743.14±4.74[ab]	1033.61±4.78[a]
Control	43.99±0.780[a]	163.55±1.28[a]	435.51±1.88[a]	740.12±3.31[a]	1033.83±6.54[a]
P value	0.161	0.000	0.000	0.120	0.000

Note: T_1= Basal diet + *L.fermentum* @10^7cfu/gm of feed, T_2 =Basal diet + *L.fermentum* @10^6cfu/gm of feed, T_3= Basal diet + *L.fermentum* @10^5cfu/gm of feed, Control= Basal diet only. Mean ± SE with different superscripts column-wise differ significantly (P< 0.05).

parameters including body weight (Table 2) and feed conversion ratio (Table 3). These findings are in agreement with the previous studies (Walkunde *et al*., 2011; Bai *et al*., 2013; Zhang and Kim, 2014; Li *et al*., 2019).

One of the most imperative attributes of probiotic bacteria is considered to be its effect on the immune response. In the present study, the birds of the treatment groups fed probiotics @ 10^7 cfu/gm *i.e*. T_1 had significantly (P<0.05) better cell-mediated and humoral immunity as compared to that of the control group (Table 4). These results are in agreement with the results of Fathi *et al*. (2017), describing that the dietary supplementation of probiotics imparted the positive response towards the serum immunoglobulin M (IgM) and cell-mediated immunity as compared to that of the control, however, the immunoglobulin A (IgA) and immunoglobulin Y (IgY) were also improved but not significantly. The satisfactory immune response of dietary probiotics in broilers was also supported by other recent studies too (Seidavi *et al*., 2017). According to earlier studies, there is a close association between the microbiota of the gut and the intestinal immune system (Gabriel *et al*., 2006; Applegate *et al*., 2010). In another study conducted by Park *et al*. (2018), it was stated that the genome of *L. fermentum* constitutes candidate genes involved in the immune mechanism. The oral supplementation of *L. fermentum* can augment innate and adaptive immune cell generation, trafficking and function in the host (Westfall *et al*., 2018).

With the supplementation of *Lactobacillus fermentum* NKN51 in broiler feeding, the total plate and *E.coli* counts were found to be reduced significantly (P<0.05) as compared to that of the control group fed basal diet only, however, the *Lactobacillus* count was significantly (P<0.05) higher in probiotic fed broiler groups (Table 5). These results are in

Table 3: Effect of different levels of *Lactobacillus fermentum* NKN51 supplementation on feed conversion ratio and feed intake in broilers (Mean±SE).

Treatment groups	I week	II week	III week	IV week
		Feed conversion ratio		
T_1	1.00±0.038^a	1.48±0.015^a	1.60±0.012^a	1.77±0.037^a
T_2	1.00±0.003^a	1.54±0.024^{ab}	1.60±0.032^a	1.96±0.035^b
T_3	1.00±0.012^a	1.56±0.019^b	1.59±0.012^a	2.16±0.015^c
Control group	1.03±0.017^a	1.60±0.019^b	1.60±0.007^a	2.31±0.017^d
P value	0.696	0.009	0.970	0.000
		Feed intake (Grams)		
T_1	128.17±5.68^a	420.99±2.55^a	481.86±2.53^a	624.28±6.75^a
T_2	125.81±1.21^a	421.50±4.89^a	484.59±4.64^a	626.42±1.34^a
T_3	123.03±1.65^a	422.22±3.95^a	492.47±3.63^a	627.91±1.25^a
Control group	123.75±2.4^a	435.50±4.46^b	490.28±2.91^a	630.74±12.1^a
P value	0.689	0.076	0.176	0.928

Mean ± SE with different superscripts column-wise differ significantly (P< 0.05).

Table 4: Effect of different levels of *Lactobacillus fermentum* NKN51 supplementation on cell-mediated and humoral immunity in broilers (Mean±SE).

Parameters	T_1	T_2	T_3	Control	P value
Cell mediated immunity (PHA-P method) (mm)	1.12±0.091^c	0.94±0.075^{bc}	0.72±0.048^{ab}	0.60±0.123^a	0.001
Humoral immunity (Log_2 value)	3.08±0.049^c	3.00±0.000^{bc}	2.90±0.055^b	2.75±0.055^a	0.002

Means (±SE) in the same row with different superscripts differ significantly (P<0.05).

Table 5: Effect of different levels of *Lactobacillus fermentum* NKN51 supplementation on gut microbiology in broilers (Mean±SE).

Treatment groups	*Lactobacillus* count (Log_{10} value)	Total plate count (Log_{10} value)	*E. coli* count (Log_{10} value)
T_1	9.87±0.014^d	8.37±0.027^a	6.58±0.071^a
T_2	8.85±0.023^c	8.45±0.035^{ab}	6.96±0.034^b
T_3	8.06±0.024^b	8.53±0.026^b	6.95±0.068^b
Control	7.66±0.034^a	9.81±0.056^c	7.82±0.030^c
P value	0.000	0.000	0.000

Mean ± SE with different superscripts column-wise differ significantly (P< 0.05).

Table 6: Effect of different levels of *Lactobacillus fermentum* NKN51 supplementation on jejunal histomorphology of broilers (Mean±SE).

Treatment groups	Villus height (VW) (µm)	Villus width (VH)(µm)	Crypt width (CW) (µm)	Crypt depth (CD) (µm)	VH: CD ratio	Intestinal absorptive surface area (µm)^2
T_1	1393.79±36.14^c	170.08±4.34^c	50.30±1.11^a	129.39±4.03^a	10.97±0.45^c	19.66±0.74^c
T_2	1200.67±25.19^b	159.28±5.08^{bc}	50.43±1.04^a	158.56±4.82^b	7.74±0.35^b	17.02±0.49^b
T_3	1175.58±26.64^b	156.06±3.67^b	51.80±1.07^a	160.01±5.59^b	7.53±0.32^b	16.49±0.53^{ab}
Control	1004.45±24.02^a	138.10±4.44^a	49.51±1.03^a	183.73±4.00^c	5.50±0.15^a	15.19±0.38^a
P value	0.000	0.000	0.500	0.000	0.000	0.000

Mean ± SE with different superscripts column-wise differ significantly (P< 0.05).

agreement with the results of Smulikowska *et al*. (2005) who documented that a probiotic in diets decreased the fecal microflora of broiler chickens. A comparable conclusion was presented by Gunal *et al*. (2006) explaining that the counts of these microbes were affected ($P<0.05$) by dietary treatments such as probiotic mixture. The decrease in the microbial count recovered from the faecal matter of chickens fed with *Lactobacillus* may have been attributable to the lactic acid production or production of antimicrobial substances and these were accredited to the concept of competitive exclusion of pathogens. Further, Yun *et al*. (2017) also concluded that faecal *Lactobacillus* count was found significantly higher in broilers fed probiotic as compared to control; however, there was no significant difference in the *E. coli* counts. In contrast, Cengiz *et al*. (2015) proclaimed that the dietary probiotic supplementation in broilers did not affect the population of gut microflora.

Further, supplementation of *L. fermentum* in broiler feed significantly ($P<0.05$) improved the villus height, villus width and villus height-crypt depth ratio thus enhancing the nutrient absorption capacity of the intestine (Table 6), which is in agreement with the earlier research findings (Al-Baadani *et al.,* 2016: Ding *et al*., 2019). Increased villus height and villus width contributed towards the better intestinal absorptive surface area in the *L. fermentum* fed groups exhibiting maximum nutrient absorption in groups fed *L. fermentum* @ 10^7cfu/gm of basal feed.

CONCLUSION

Thus, the data obtained from the present study revealed that the incorporation of *Lactobacillus fermentum* NKN51, a novel probiotic in broiler diet @ 10^7cfu/gm of feed can impart the better weight gain, improved feed efficiency, enriched immune status including both cell-mediated as well as humoral immunity along with the boosted gut health assuring reduced level of microbes and better nutrients absorption capacity of intestine.

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