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Probiotic *Bacillus* species and *Saccharomyces boulardii* improve performance, gut histology and immunity in broiler chickens

M. Manafi[1#], M. Hedayati[1] & S. Mirzaie[2]

[1] Department of Animal Science, Faculty of Agricultural Sciences, Malayer University, Malayer, Iran
[2] Department of Agriculture, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran

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Abstract

The aim of this study was to compare the effects of a new multispecies probiotic containing four *Bacillus* species and *Saccharomyces boulardii* (Microguard®) with a commercial probiotic (Protexin®) and a commonly used antibiotic in broilers. Six hundred one-day-old male Ross 308 broilers were randomized to six experimental treatments, with five replicates of 20 chicks each, for 42 days, receiving an *ad libitum* corn-soybean basal diet. Treatments were added to the basal diet and consisted of tetracycline as an antibiotic growth promoter (500 g/ton), three dosages of Microguard (50, 100 and150 g/ton) or Protexin (100 g/ton). The control group received the basal diet with no additive. The group fed with Microguard at 150 g/ton showed increased final bodyweight, weight gain, high density lipoprotein, triglyceride, and antibody titres against Newcastle disease (ND) and avian influenza (AI) levels. Improved feed conversion ratio, increased villus height, and villus highest crypt depth ratio, along with lower plasma gamma-glutamyl transpeptidase, alkaline phosphatase, alanine aminotransferase, were found in probiotic-supplemented broilers. Carcass yield, liver weights, breast muscle values, and abdominal fat weights were reduced in groups fed with 100 or 150 g/ton of Microguard. Caecal coliforms, Salmonella and *Escherichia coli* numbers decreased in groups fed with 100 or 150 g/ton of Microguard. These results show that Microguard at 150 g/ton is a promising probiotic to replace antibiotics in broiler feed as a growth-promoter while enhancing immune system responses and inducing beneficial modulations in the caecal microflora.

Keywords: Blood biochemistry, broiler chicks, carcass traits, performance, probiotic
[#] Corresponding author: manafim@malayeru.ac.ir

Introduction

The intensive conditions in which commercial broiler chicks are reared are stressful, particularly during the first few days of life, when the immune system and intestinal flora are still developing. The addition of beneficial probiotic microorganisms to the chicks' diet during this critical early period may improve the overall health status and performance during the entire production period (Shivaramaiah *et al*., 2011). The use of sub-therapeutic antibiotic treatments as growth promoters has increasingly been limited or banned in recent years because of the emergence of multiple drug-resistant bacteria, the potential contamination of the environment with antibiotics, and increasing awareness of antibiotic use in poultry products by health mindful consumers (Peric *et al*., 2010). Increasing consumer awareness and preference for poultry products that are free from chemical residues has resulted in intense, global efforts to identify environmentally friendly and healthy replacements to improve animal health and performance (Wolfenden *et al*., 2010). These alternative strategies include focusing on alternatives to antibiotic use such as improving the gut health of livestock.

Probiotics are defined as live microorganisms that have a beneficial effect on the health of their host when consumed in an adequate dose (Manafi, 2015). Multi-species probiotic preparations are thought to be more effective than single strain probiotics (Timmerman *et al*., 2004). Among the probiotic microbial species available, *Lactobacillus, Saccharomyces, Bacillus, Streptococcus,* and *Aspergillus* species have been reported to have a beneficial role in poultry nutrition (Lema *et al*., 2001; Tannock, 2001; Zhang *et al*., 2005; Chen *et al*., 2009, Manafi & Khosravinia, 2013). Many studies have shown that probiotics play an important role in correcting intestinal ecological imbalances and improving animal health (Fritts *et al*., 2000; Ehrmann

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et al., 2002; Nava *et al*., 2005; Torres-Rodriguez *et al*., 2007; Vicente *et al*., 2007; Vila *et al*., 2009; Wolfenden *et al*., 2010; Chuka *et al*., 2014).

Probiotic *Bacillus* bacteria have been considered good candidates for feed additives because their aerobic and endospore-forming nature gives them the capacity to survive environmental stresses, including storage, transport, and feed pelleting processes (Setlow, 2006; Cartman *et al*., 2008; Wu *et al*., 2011). Probiotic yeasts such as *Saccharomyces* have also been shown to stimulate the immune system of chicks without decreasing growth performance (Bai *et al*., 2013).The inhibition of pathogenic microorganism growth by probiotics can potentially increase nutrient bioavailability and improve growth rate and feed efficiency (Manafi *et al*., 2016).

The authors aimed to investigate the effects of supplementing the diet of broiler chickens with a new probiotic containing *Bacillus* species, together with *S. boulardii,* on growth performance, carcass traits, intestinal histology, immune responses, and caecal pathogen number. Second, they aimed to identify the potential of this probiotic supplement to replace antibiotic growth promoters in the broiler diet.

Material and Methods

Six hundred one-day-old male Ross 308 broiler chicks were obtained from a commercial hatchery. Chicks were randomly allocated to six experimental treatments with five replicates of 20 chicks in each treatment group. Each replicate was assigned to a floor pen (2 m^2) and birds were raised on a 5-cm deep wood shaving litter with *ad libitum* access to feed and water for 42 days. The diet was formulated with corn and soybean to meet broiler nutrient requirements based on Ross 308 broiler nutrition specifications (2014) for starter (1 to 10 days), grower (11 to 28 days) and finisher (29 to 42 days) periods (Table 1).The experimental treatments consisted of Group 1 (control with no feed additive); Group 2 (tetracycline as growth promoter dose at 500 g/ton of feed); Group 3 (multi-species/multi-string probiotic containing four *Bacillus* spp. and *S. boulardii* (Microguard) at 50 g/ton); Group 4 (Microguard at 100 g/ton); Group 5 (Microguard at 150 g/ton); and Group 6 (a commercial probiotic (Protexin) at 100 g/ton).

Table 1 Composition of experimental diets used for broilers at different ages

Item	D 1 to 10	D 11 to 28	D 29 to 42
Ingredient (%)			
Corn	49.30	59.6	65.99
Wheat	5.58	5.00	5.00
Soybean meal	26.86	16.05	10.12
Corn gluten	10.00	11.48	11.50
Soybean oil	3.50	3.34	3.09
Limestone	1.45	1.23	1.00
Dicalcium phosphate	1.95	1.80	1.83
Salt	0.36	0.36	0.36
Vitamin premix*	0.25	0.25	0.25
Mineral premix**	0.25	0.25	0.25
DL-Methionine	0.52	0.58	0.57
Lysine	0.25	0.06	0.04
Total calculated value			
Metabolizable energy (kcal/kg)	3010	3150	3200
Crude protein (%)	23	20	18
Calcium (%)	1	0.9	0.9
Available phosphorus (%)	0.5	0.45	0.45

* Vitamin premix contained per kilogram: vitamin A, 8800000 IU; vitamin D_3, 2500000 IU; vitamin E, 1100 IU; vitamin K_3, 22g; vitamin B_1, 1.477 g; vitamin B_2, 4 g; vitamin B_3, 7.84 g; vitamin B_6, 2.462 g; vitamin B_{12}, 0.01 g; folic acid, 0.48 g; biotin, 0.15 g

** Mineral premix contained per kilogram: Mn, 29.76 g; Fe, 30 g; Zn, 25.87 g; Cu, 2.4 g; I, 0.347 g; Se, 0.08 g

The regional disease prevention programme for ND and AI included vaccination against ND with live attenuated vaccine on day 1 (spray) in the hatchery, day 7 (eye drop) (CEVAC® BI L containing the Hitchner B1 strain of ND virus in live freeze-dried form), and days 12 and 25 as clone-30 (HIPRAVIAR®) through drinking water. A combined oil emulsion-inactivated ND-AI vaccine (HIPRAVIAR) was also injected on day 7.

The microbial contents of both feed additives Microguard (Zeus Biotech Ltd, Mysore, India) and Protexin® (Probiotics International Ltd, UK) are shown in Table 2. The rearing and feeding protocols employed in current study were approved by the bioethical committee of Malayer University under the Iranian guidelines of animal protection used for experimental and other scientific purposes.

Table 2 Probiotic microbes contained in Microguard® and Protexin® supplements

Supplement	Probiotic microbes	
Microguard®		
	Bacillus subtilis	*Bacillus mesentericus*
	Bacillus licheniformis	*Bacillus polymyxa*
	Bacillus megaterium	*Saccharomyces boulardii*
Protexin®		
	Lactobacillus acidophilus	*Enterococcus faecium*
	Lactobacillus rhamnosus	*Streptococcus thermophilus*
	Lactobacillus plantarum	*Aspergillus oryzae*
	Lactobacillus bulgaricus	*Candida pintolopesii*
	Lactobacillus bifidus	

The chicks in all treatment groups were weighed weekly to determine bodyweight and weight gain. Accordingly, cumulative feed intake was recorded weekly to calculate feed conversion ratio (FCR) by dividing the feed consumed by total bodyweight gain. One chicken from each replicate (five birds per experimental group) was randomly selected from each treatment group, stunned, and killed (euthanasia through cervical dislocation).The weights of the liver, spleen, heart, abdominal fat, breast muscle, thigh muscle, and eviscerated carcass weight and length of the intestine were measured using a digital pan balance with 0.001 g accuracy at the end of trial (42 days) and expressed as g/100 g BW.

At the end of the trial, the small intestine from one slaughtered bird from each replicate was removed aseptically. Five centimetre sections from the midpoint of the ileum were detached from Meckel's diverticulum, proximal to the ileo-caecal junction, and the digestive tract, including its contents, was collected aseptically. The removed gut sections were prepared and examined using Mott cell light microscopic images. The villus height, crypt depth, and number of goblet cells in 1 mm section were determined according to the method of Xu *et al*. (2003). Villus height was measured from the top of the crypt using the lamina propria of the villus. Crypt depth was the shortest vertical distance from the villus contact point to the mucus membrane.

Blood samples were collected from two chickens in each replicate at the end of the trial after being fasted for eight hours before venipuncture. Blood samples were kept in cotton-plugged test tubes in a slant position for two hours. After centrifuging EDTA-mixed blood at 3000 rpm for 10 min, plasma was isolated, and the samples were stored at -20 °C. The activity of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT), plasmahigh-density lipoprotein(HDL) and triglyceride concentrations were determined by spectrophotometric methods (Asadi *et al*., 2008; Bagherzadeh Kasmani *et al*., 2012) using commercial kits (Zist Chimi kits, Pars Azmoon kits Tehran, Iran).The Friedewald formula was used to estimate LDL concentration (Friedewald *et al*., 1972). Antibody titres against New castle disease (ND) and Avian influenza (AI) viruses were determined using the haemagglutination inhibition (HI) test on samples from two birds in each replicate at day 42.

One gram of caecal digesta of two birds in each replicate was homogenized separately in test tubes containing 9 ml phosphate-buffered saline and a series of ten times dilutions was prepared. Selective agar media was used to enumerate *E. coli*, total Coliform bacteria (Eosin Methylene Blue, MacConkey agar,

Quelab,UK), and Salmonella (*Salmonella-Shigella* Agar, Quelab, UK).The results were expressed as $\log_{10}$ colony forming units (CFU) per gram of caecal digesta, following the methods detailed by Li *et al*.(2009).

Experimental data were analysed in a randomized design, using the GLM procedure in SAS V9.3 (SAS Institute, 2007). Differences between means were compared with Duncan's multiple range test. Significance was defined as a *P*-value of <0.05.

Results

There were significant differences in BW among birds in different experimental treatment groups at 28 and 42 days old (Table 3). Broilers fed with feed supplemented with Microguard at 100 g/ton and 150 g/ton showed higher bodyweight than other groups (*P* <0.05). The BW of all probiotic-supplemented groups was higher (*P* <0.05) than the tetracycline-supplemented group at day 42. The BW of the control group was lower than all other treatment groups at day 42.The BW of the broiler chicks at one day old was not significantly different at the beginning of the experiment. Broilers receiving Microguard as 100 g/ton or 150 g/ton showed increased (*P* <0.05) overall weight gain during the whole experimental period compared with treatment groups that did not receive Microguard (*P* <0.05). The weight gain of the broilers was similar between groups from day 0 up to day 28. There were significant differences in overall feed intake and FCR among experimental treatment groups. Broilers that consumed Microguard at 150 g/ton had the highest feed consumption and the lowest FCR. All probiotic treatment groups showed a lower FCR than the tetracycline-supplemented group by day 42. All treatments showed a significantly (*P* <0.05) lower overall FCR compared with the control group.

Table 3 Effects of application of Microguard and Protexin on performance of broilers

Treatment	Body weight (g)			Weight gain (g)		Feed intake(g)		Feed conversion ratio	
	Days			Days		Days		Days	
	0	28	42	0 - 28	0 - 42	0 - 28	0 - 42	0 - 28	0 - 42
Control	42	1050^{c}	2510^{d}	790	2468^{c}	1310	4355^{ab}	1.65	1.73^{a}
Tetracycline (500 g/ton)	42.15	1060^{c}	2580^{c}	795	2537.85^{bc}	1295	4360^{a}	1.62	1.68^{b}
Microguard® (50 g/ton)	42.10	1100^{b}	2600^{b}	830	2557.9^{b}	1250	4350^{b}	1.50	1.67^{c}
Microguard® (100 g/ton)	41.98	1110^{a}	2640^{a}	835	2598.02^{ab}	1250	4355^{ab}	1.49	1.64^{d}
Microguard® (150 g/ton)	42.12	1115^{a}	2650^{a}	835	2607.88^{a}	1245	4360^{a}	1.49	1.64^{d}
Protexin® (100 g/ton)	42.18	1090^{b}	2610^{b}	818	2567.82^{b}	1250	4355^{ab}	1.52	1.66^{c}
SEM	0.03	112.69	186.57	46.72	160.73	71.46	327.95	0.03	0.064
P-value	0.1248	0.0243	0.0153	0.1247	0.0029	0.1893	0.0279	0.1872	0.0396

$^{a, b, c}$ Means in the same column with different superscripts show significant differences (*P* <0.05)

Table 4 shows the effects of probiotics on the carcass traits of broilers at 42 days old. Carcass yield and the relative organ weights of breast muscle, thigh, liver, and abdominal fat in broilers supplemented with antibiotic or probiotics were significantly lower than the control group. Neither Microguard- nor Protexin-fed groups showed different thigh muscle weight compared with the control group. However, tetracycline treatment decreased (*P* <0.05) the thigh muscle weight compared with the other treatment groups.

The experimental treatments had a significant effect on villus height, crypt depth, goblet cell number, and villus height/crypt depth ratio in the ileum at day 42 (Table 5).The Protexin® group showed the highest villi height, while the Microguard feed group at 100 g/ton had the shortest villi height among the probiotic-supplemented groups. Villi height was greater in all probiotic-supplemented groups compared with the tetracycline group, while all treatment groups showed greater villi height than the control group. Broilers in the Microguard 150 g/ton group had the highest villus/crypt ratio. However, the differences in the villus/crypt ratio were not significant between treatments of Microguard (100 g/ton), tetracycline, and control. Both probiotic and antibiotic treatments reduced the number of goblet cells when compared with the control group.

Table 4 Effects application of Microguard and Protexin on carcass traits of broilers

Treatment	Relative organ weight (g/100 g BW)				
	Eviscerated carcass	Thigh	Breast	Liver	Abdominal fat
Control	95[a]	22[a]	35[a]	3.7[a]	1.2[a]
Tetracycline (500 g/ton)	83[c]	19[b]	31[b]	2.9[d]	0.96[b]
Microguard® (50 g/ton)	81[d]	22.5[a]	28.5[c]	3.1[c]	0.8[c]
Microguard® (100 g/ton)	83[c]	22[a]	28[c]	2.8[d]	0.7[c]
Microguard® (150 g/ton)	85.5[b]	23[a]	27[d]	3.1[c]	0.97[b]
Protexin® (100 g/ton)	85[b]	21.5[a]	28.5[c]	3.3[b]	093[bc]
SEM	0.485	0.071	0.083	0.001	0.001
P-value	0.0171	0.0341	0.0419	0.0015	0.0121

[a, b, c] Means in the same column with different superscripts show significant differences (P <0.05)

Table 5 Effects of application of Microguard and Protexin on the intestinal histological parameters of broilers

Treatment	Villi height (µm)	Crypt depth (µm)	Villi height: crypt depth	Number of Goblet cells
Control	3.13[d]	0.88[d]	3.55[c]	9.00[a]
Tetracycline (500 g/ton)	3.63[c]	0.96[c]	3.78[c]	8.33[bc]
Microguard® (50 g/ton)	4.06[ab]	0.90[c]	4.51[b]	8.66[b]
Microguard® (100 g/ton)	3.93[b]	1.12[a]	3.50[c]	8.12[c]
Microguard® (150 g/ton)	4.03[ab]	0.77[e]	5.23[a]	7.23[d]
Protexin® (100 g/ton)	4.33[a]	1.04[b]	4.16[b]	8.00[c]
SEM	0.18	0.03	0.26	0.78
P-value	0.0361	0.0241	0.0415	0.0377

[a, b, c] Means in the same column with different superscripts show significant differences (P <0.05)

The effects of various treatment groups on broiler blood biochemistry parameters at six weeks old are shown in Table 6. Plasma GGT and ALP activities were higher in the treatment groups compared with the control group. The dietary supplementation of Microguard at 100 and 150 g/ton significantly reduced GGT and ALT values compared with the other groups. However, ALT was higher in broilers that received Microguard at 50 and 150 g/ton of feed. The Microguard (100 g/ton) treatment increased HDL concentration. The lowest LDL concentration was observed in the antibiotic treatment group while Microguard at 50 g/ton level and Protexin were showed the highest LDL concentration. Microguard at 50 and 150 g/ton feed as well as Protexin groups were observed to have higher triglyceride concentrations compared with the antibiotic and control groups.

Titres of antibodies produced against ND and (AI) viruses on day 42 are shown in Table 7. Antibody titres against ND were significantly higher in the Microguard100 g/ton group, compared with the other dietary treatments. In addition, broilers fed with Microguard at 150 g/ton showed highest antibody titres against AI (P <0.05) when compared with other dietary treatments.

The caecal microflora in broilers at day 42 of all treatment groups showed significantly fewer coliforms, *E. coli,* and Salmonella compared with the control group (P <0.05) (Table 8). Supplementation with Microguard at 150 g/ton of feed resulted in a significant (P <0.05) reduction in coliforms compared with all other dietary treatments.

Discussion

In this study the authors show that supplementing the diet of broiler chicks with Microguard or Protexin probiotics, or with tetracycline at a subtherapeutic dose, significantly improved the overall growth

performance compared with the control group. Among the various treatments, Microguard at 150 g/ton of feed was the most effective at increasing final bodyweight, weight gain, and feed intake of the broiler chickens. The current results confirm previous studies that reported that dietary supplementation with *B. subtilis* or yeast cell-wall components improved the growth rate, feed consumption, and feed efficiency in chickens (Santoso *et al*., 2001; Karaoglu & Durdag, 2005). The improvement in final bodyweight may be due to the probiotic supplementation resulting in bacterial antagonism, competition for colonization sites, competition for nutrients, reduction in toxic compounds, modulation of immune system, or increased digestibility of diet leading to improved nutrient absorption (Applegate *et al*., 2010). Here the authors show for the first time that the use of Microguard was effective at improving growth performance.

At higher doses of 100 g/ton or 150 g/ton in the diet Microguard also improved the broilers feed efficiency ratio, indicating an improved conversion of dietary energy to weight gain. This is in agreement with previous studies that observed benefits of probiotic supplementation on broiler performance and increased feed efficiency (Panda *et al*., 2005; Mountzouris *et al*., 2007; Toghyani *et al*., 2011; Cao *et al*., 2013; Manafi *et al*., 2016). Bai *et al.* (2013) reported average daily weight gain and feed efficiency significantly improved from days 1 to 21, but not from days 22 to 42 of life, in chickens fed probiotic *L. fermentum* and *S. cerevisiae*. In contrast, probiotic supplemented with *S. cerevisiae* (from 0.25 to 0.75%) showed no effect on the growth rate of broilers during the first 21 days of life.

Table 6 Effects of application of Microguard and Protexin on the blood parameters of broilers

Treatment	Blood parameters					
	GGT (IU/L)	ALT (IU/L)	ALP (IU/L)	HDL (mg/dl)	LDL (mg/dl)	Triglyceride (mg/dl)
Control	54.66^{c}	40.33^{b}	78.66^{ab}	52.31^{a}	1.27^{c}	24.87^{a}
Tetracycline (500 g/ton)	55.33^{c}	37.66^{c}	80.66^{a}	47.54^{ab}	1.22^{c}	21.93^{b}
Microguard® (50 g/ton)	64.00^{a}	45.00^{a}	76.00^{b}	45.06^{b}	1.79^{a}	22.93^{ab}
Microguard® (100 g/ton)	59.66^{b}	40.33^{b}	79.33^{a}	44.59^{b}	1.21^{c}	18.34^{c}
Microguard® (150 g/ton)	63.33^{a}	39.00^{bc}	72.00^{c}	41.67^{c}	1.81^{a}	20.42^{b}
Protexin® (100 g/ton)	64.66^{a}	45.33^{a}	75.33^{b}	42.52^{bc}	1.64^{b}	22.89^{ab}
SEM	2.11	1.17	1.31	1.76	0.04	1.95
P-value	0.0432	0.0216	0.0254	0.0439	0.0359	0.0365

$^{a, b, c}$ Means in the same column with different superscripts show significant differences (P <0.05)
GGT: gamma-glutamyltransferase; ALT: alanine transaminase; ALP: alkaline phosphatase; HDL: high-density lipoprotein; LDL: low-density lipoprotein

Table 7 Effects of application of Microguard and Protexin on antibody titres against Newcastle disease (ND) and Avian Influenza (AI) viruses

Treatment	Anti-NDV[1]titre (log2)	Anti-AIV[2]titre (log2)
Control	5.00^{bc}	4.20^{b}
Tetracycline (500 g/ton)	4.83^{c}	4.33^{b}
Microguard® (50 g/ton)	4.47^{d}	3.63^{cd}
Microguard® (100 g/ton)	5.50^{a}	3.53^{d}
Microguard® (150 g/ton)	5.20^{b}	4.76^{a}
Protexin® (100 g/ton)	4.90^{c}	3.73^{c}
SEM	0.95	0.11
P-value	0.0379	0.0001

$^{a, b, c}$ Means in the same column with different superscripts show significant differences (*P* <0.05)
[1]Newcastle disease virus
[2]Avian influenza virus

Table 8 Effect of application of Microguard and Protexin on coliforms, *E. coli,* and Salmonella in caecal contents of broilers

Treatment	Bacterial count (log_{10} CFU/g)		
	Coliform bacteria	***E. coli***	***Salmonella***
Control	3.06[a]	3.56[a]	3.43[a]
Tetracycline (500 g/ton)	1.73[bc]	0.13[c]	0.56[d]
Microguard® (50 g/ton)	2.16[b]	1.23[b]	3.10[b]
Microguard® (100 g/ton)	2.13[b]	1.16[b]	2.56[c]
Microguard® (150 g/ton)	1.06[c]	1.10[b]	2.36[c]
Protexin® (100 g/ton)	2.12[b]	1.05[b]	2.36[c]
SEM	0.45	0.05	0.39
P-value	0.0267	0.0197	0.0336

[a, b, c] Means in the same column with different superscripts show significant differences (P <0.05)

In agreement with the current results other studies showed improved performance and increased feed efficiency in broilers after 21 days old (Stanley *et al*., 2004; Karaoglu & Durdag, 2005; Zhang *et al*., 2005; Gao *et al*., 2008).While the authors observed a trend towards improved feed efficiency during the first 28 days, this did not become significantly different between groups until measured over the first 42 days.

The current results showed that supplementation of the diet with probiotics decreased carcass yield, liver weight, abdominal fat, and breast muscle weight of broilers, with no influence on thigh weight. Such reductions are commonly due to improved protein and carbohydrate utilization. Pelicano *et al*. (2003) reported similar findings with probiotic-treated broilers showing lower carcass yields than control birds. In contrast, Kabir *et al*. (2004) and Denli *et al*. (2003) reported greater carcass yield, greater breast muscle, and no change in liver weight or abdominal fat weight in probiotic-fed birds at six weeks old, while Moreira *et al*. (2001) found no differences in carcass yield between probiotic-fed birds and control birds. Similar to the current findings, Loddi *et al*. (2000) and Falaki *et al*. (2011) found probiotic treatment had no effect on thigh muscle weight, while Pelicano *et al*. (2003) observed higher thigh yield in broilers receiving probiotics. Considered together, these earlier studies show that the use of probiotics as growth promoters in broiler feed resulted in conflicting outcomes on carcass yield. The varied outcomes in feed efficiency and carcass yield between the present and previous studies is potentially because of variations in the probiotic species, their viability, dosage, application routes, overall diet composition, bird age, and environmental factors (Awad *et al*., 2009; Flint & Garner, 2009; Peric *et al*., 2010).

The increased villi height and villi height/crypt depth ratio and reduced goblet cell numbers that the authors observed in the intestine of broilers fed Microguard at 50 or 150 g/ton of feed or Protexin at 100 g/ton feed confirm the results of previous research. Peric *et al*. (2010) reported that administration of probiotics increased the villi height and villi surface area of the jejunum in broiler chickens at six weeks old. Greater jejunal villus height in 21 and 28 days old broilers fed probiotic *E. faecium* was demonstrated in a study by Cao *et al.* (2013). Increases in villi height and villus height/crypt depth ratio is related to increases in epithelial cell turnover (Samanya & Yamauchi, 2002; Awad *et al*., 2009; Onlood *et al*., 2015). Pelicano *et al*. (2005) reported that the addition of probiotics to the diet could increase microbial fermentation, resulting in the production of beneficial organic acids, which could inhibit the growth of pathogenic micro-organisms and stimulate increases in the number of villi. Increased villi height is potentially associated with enhanced digestive and absorptive functions of the intestine owing to the larger surface area and higher expression of brush border enzymes (Pluske *et al*., 1996), which could lead to an increase in the absorption of nutrients (Pelicano *et al*., 2005). The increased villi height and villi height/crypt depth ratio in probiotic-supplemented broilers in the present study is potentially responsible for the improved weight gain and feed efficiency through increased digestion and absorption of feed.

In broilers supplemented with Microguard, the authors showed that GGT and ALP were reduced compared with the control group. Plasma concentrations of enzymes ALP, ALT, and GGT are sensitive and specific measures of hepatic function or liver injury (Fernandez *et al*., 1994; Abbès *et al*., 2006). Probiotics may improve liver health although the mechanisms through which probiotics may act on the liver are still under investigation. One potential benefit is through the prevention of lipopolysaccharide uptake from the gut

(Gratz *et al*., 2010). Surprisingly, the authors found that the inclusion of probiotics resulted in an increase in plasma ALT level. In contrast, Chuka (2014) found no effects on ALT and ALP levels of a probiotic containing *S. cerevisiae*. The reason for the increased ALT in the present study remains unclear. However, the reduced GGT and ALP are indicative of improved liver health in probiotic-supplemented broilers.

The current results show that supplementation with Microguard or Protexin increased triglyceride levels compared with other groups. Reductions in total cholesterol and LDL, with no changes in HDL or triglyceride concentrations, were reported by Cho *et al*. (2015). Similar observations were obtained by Alkhalf *et al*. (2010) in which chicken fed diets containing various levels of probiotics showed a decrease in blood cholesterol concentration. However, little information is available on the dosage of probiotics needed to exert hypolipidemic effects in birds. In addition, the effect of probiotics on cholesterol and LDL might depend on a variety of factors, including baseline level of biochemical parameters, treatment duration, and probiotic strains.

In terms of the humoral immune response, the present study showed a positive effect of Microguard probiotic on antibody production against NDV and AIV. Kabir *et al*. (2004) previously reported higher antibody titres against NDV and infectious bursal disease virus (IBDV) in broilers treated with Protexin probiotic). Khaksefidi & Ghoorchi (2006) reported that antibody titres against sheep red blood cells (SRBC) as a foreign antigen were significantly higher in broilers fed with a *B. subtilis* containing probiotic. Supplementation with a probiotic containing *Lactobacillus* showed no influence on cell mediated immune response, but did increase the humoral immune responses (Panda *et al*., 2005).In addition, Haghighi *et al*. (2005) demonstrated that administration of probiotics containing *L. acidophilus*, *B. bifidum,* and *S. faecalis* enhanced serum antibodies against SRBC in broiler chickens. The current study shows that supplementation with Microguard® probiotics improves immune responses to vaccination against common diseases affecting broilers.

Probiotics had a significant effect on caecal bacterial composition, resulting in reductions in number of coliforms, Salmonella, and *E. coli* in broilers supplemented with Microguard. Probiotic preparations belonging to single or multispecies of *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* had a potential effect on modulation of the intestinal microflora and pathogen inhibition (Kabir, 2009). This could be owing to the production of antimicrobial substances such as bacteriocins and lactic acid, and the adherence and co-aggregation of probiotic bacteria to the mucosa, forming a barrier that prevents colonization by pathogens (Patterson & Burkholder, 2003; Leser *et al*., 2008). The antimicrobial activity of *Bacillus* spp. similar to those used in the present study, against Clostridium and Salmonella and immune-modulating effects in the gut were reported (Teo & Tan, 2005; Scharek *et al*., 2007; Lim & Kim, 2008). However, other studies found no significant effects of some probiotics containing *L. reuteri*, *E. faecium*, *B. animalis,* and *P. acidilactici* on caecal populations of coliforms and *E. coli* of broilers (Mountzouris *et al*., 2007; Peric *et al*., 2010). The reductions in the number of coliforms, Salmonella, and *E. coli* in treated broilers in the present study could reduce the need for antibiotic treatment in probiotic-supplemented broilers.

Conclusions

The present study provides evidence that Microguard is a promising feed supplement that can act as a growth promoter, improve the morphology of the gut and immune responses to vaccination, and reduce pathogens in the caecal microflora of broiler chickens. Microguard, as a multispecies probiotic product, has the potential to exert synergic beneficial effects through its inclusion of *Bacillus* and *Saccharomyces* probiotics. The authors conclude that the addition of Microguard in the feed of broilers at a concentration of 150 g/ton is a new probiotic supplement that can be used in broiler production as a replacement for antibiotic growth promoters for the production of antibiotic-free chicken products.

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Authors' Contributions

The study was planned and conducted by MM. The entire trial observations were done by MH. The data collection was done by MM and some laboratory work by SM. Statistical analysis was carried out by MM and manuscript preparation was done by MM and SM.

Conflict of Interest Declaration

Authors have no conflict of interests to declare.

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