

Effects of probiotic and phytogetic products on performance, gut morphology and cecal microflora of broiler chickens

LIDIJA PERIĆ¹, NIKO MILOŠEVIĆ¹, DRAGAN ŽIKIĆ¹, SINIŠA BJEDOV¹, DRAGOLJUB CVETKOVIĆ², SINIŠA MARKOV², MICHAELA MOHNL³ and TOBIAS STEINER³

¹Faculty of Agriculture, University in Novi Sad, Republic of Serbia, ²Faculty of Technology, University in Novi Sad, Republic of Serbia, ³BIOMIN Holding GmbH, Herzogenburg, Austria

Abstract

The trial involved 684 Cobb 500 broiler chicks in order to investigate the effects of probiotics and phytogetic additives on performance, gut morphology and cecal microbial concentrations. Birds were assigned to three treatments: control feed, treatment with addition of probiotics in feed and water and treatment with addition of phytogetic blend in feed. The results showed a significant improvement ($P < 0.01$) in body weight gain and a numerical, but non significant ($P > 0.05$) improvement in feed conversion ratio by adding both probiotics and phytogetics in feed. There were no statistically significant differences among treatments regarding total aerobes, anaerobes, lactic acid bacteria, bifidobacteria, enterococci, and *Escherichia coli* populations ($P > 0.05$) in cecum of broilers. The gut morphology examination showed that probiotics had beneficial effect on jejunum morphology causing a significant ($P < 0.005$) increase in villus height and villus surface area compared to other two groups. On the other hand, phytogetic additive had no effect ($P > 0.05$) on villus height or villus surface area, but reduced the villus/crypt ratio ($P < 0.05$), which may indicate that the improved production results in the group with added phytogetics are not directly connected with changes in gut integrity, but with other physiological mechanisms.

Keywords: broiler, probiotics, phytogetics, intestinal microbiota, gut morphology

Zusammenfassung

Einfluss Probiotischer und Phytogener Additive auf Leistungsparameter sowie auf Morphologie und Mikroflora des Verdauungstraktes bei Broilern

Die Wirksamkeit probiotischer und phytogener Futteradditive wurde in einem Fütterungsversuch mit 684 Cobb 500 Broilern untersucht. Die Tiere wurden auf drei Behandlungen verteilt: (1) Kontrollfutter, (2) Applikation von Probiotika im Futter und Tränkwasser, und (3) Applikation eines phytoenen Additivs im Futter. Die Applikation der Additive bewirkte eine Erhöhung der Lebendmasse ($P < 0.05$), sowie eine numerisch reduzierte ($P > 0.05$) Futtermittelverwertung. Die Anzahl an Bakterien (gesamte aerobe und anaerobe Keime, Milchsäurebakterien, Bifidobakterien, Enterokokken, *Escherichia coli*) im Caecum wies keine Unterschiede ($P > 0.05$) zwischen den Behandlungen auf.

Die morphologischen Untersuchungen zeigten eine höhere Villuslänge, sowie eine vergrößerte Villusoberfläche im Jejunum am Ende des Versuches bei Applikation der Probiotika. Das Verhältnis der Villushöhe zur Kryptentiefe wurde durch den Einsatz des phyto-genen Additivs reduziert ($P < 0.05$). Dies deutet darauf hin, dass die verbesserte Leistung nicht auf einer Veränderung der Darmmorphologie beruht, sondern durch andere Faktoren bedingt ist.

Schlüsselwörter: Broiler, Probiotika, Phyto-gene Additive, Intestinale Mikroflora, Darmmorphologie

Introduction

Use of antibiotics as growth promoters is no longer acceptable and therefore it is forbidden in EU countries. Because of that it was necessary to offer other feed additives as an alternative such as enzymes, organic or inorganic acids, plant oils, immunostimulators, microelements, probiotics and prebiotics. Probiotics beneficially affect the host animal by improving its intestinal balance (FULLER 1989). They create gut conditions that suppress harmful microorganisms and favor beneficial ones (LINE *et al.* 1998, MEAD 2000). They have been largely shown to reduce disease risk, possibly through a reduction in proliferation of pathogenic species, maintaining microbiota balance in the gut (LINE *et al.* 1998, MACFARLANE and CUMMINGS 1999, MOUNTZOURIS *et al.* 2007), boost immune function (ZULKIFLI *et al.* 2000, KABIR *et al.* 2004) and increase resistance to infection (HERICH *et al.* 1998, AYSIGI *et al.* 2005, REKIEL *et al.* 2007). Beyond the maintenance of health, they have been shown to improve the growth performance of poultry (JIN *et al.* 1997; LI *et al.* 2008) and to have an important influence on gut morphology of broiler chickens (UŠĆEBRKA *et al.* 2005).

Phytogenic feed additives are plant extracts derived from herbs or spices, which have beneficial effect on animal production and health. A large variety of the plants have properties which could potentially improve feed intake, digestion, feed conversion and body weight gain (LOVKOVA *et al.* 2001, WILLIAMS and LOSA 2001, ERTAS *et al.* 2005). The mode of action of these feed additives is not completely clear. They have antimicrobial, antiviral, antioxidant and many other biological activities (ERTAS *et al.* 2005, CROSS *et al.* 2007). They act as a digestibility enhancers, stimulating the secretion of endogenous digestive enzymes (WILLIAMS and LOSA 2001, LEE *et al.* 2003). These traits made phytogenic additives a promising group of growth promoters that are already being used in practice.

The objective of this study was to examine the effects of dietary supplementation of probiotics or phytogenic product in broiler diets compared to standard broiler feed on the growth performance, jejunal morphology and cecal microflora of broiler chicks.

Material and methods

Animals and diets

A total of 684 day-old Cobb 500 broilers were assigned to three treatments with six replicates. Each replicate consisted of 38 as-hatched birds per pen. Each floor pen

measured 2.5 m², to give a stocking density of 15 birds/m². Air temperature was adjusted in accordance to the technological demands. Lighting program provided 23 h of light +1 h of dark. Birds were vaccinated against Newcastle disease (NCD) and infectious bursal disease (IBD) as per commercial recommendations.

Feed and water supply were *ad libitum*. Feed was composed according to the technological demands of the concerned hybrid (Table 1) and it was the same for all groups, except added additives.

Table 1
Composition (%) of the basal diets
Zusammensetzung (%) der Basalrationen

Ingredient	Starter, 1-21 d	Grower, 22-35 d	Finisher, 36-42 d
Corn	47.50	55.24	61.70
Soybean meal	34.24	24.85	22.48
Full fat soybeans (extruded)	10.20	11.85	7.79
Vegetable oil	4.00	4.00	4.00
Limestone	1.30	1.18	1.09
Monocalcium phosphate	1.30	1.39	1.40
Salt	0.30	0.30	0.38
DL-methionine	0.16	0.19	0.16
Vitamin + mineral premix ¹	1.00	1.00	1.00
Total	100	100	100
Nutrients and energy level (calculated)			
Crude protein, %	22.80	20.00	18.00
ME MJ/kg	13.00	13.40	13.40
Lysine, %	1.31	1.10	0.96
Methionine, %	0.51	0.50	0.45
Calcium, %	0.90	0.85	0.80
Total P, %	0.70	0.70	0.67
Available P, %	0.44	0.44	0.41

¹Vit+mineral mixture provides per kg of diet: 12 000 IU of vitamin A; 5 000 IU of vitamin D3; 50 mg of vitamin E; 4 mg of vitamin K3; 4 mg of vitamin B1; 10 mg of vitamin B2; 6 mg of vitamin B6; 60 mg of nicotinamide; 2 mg of folic acid; 0.02 mg of vitamin B12; 0.2 mg of biotin; 400 mg of choline chloride; 15 mg of pantothenic acid; 2 mg of I. 40 mg Fe, 100 mg Zn, 20 mg Cu; 120 mg Mn, 0.3 mg Se

The experimental treatments consisted of control diet (treatment C), the control diet with addition of probiotic blend 1 in drinking water during first three days of life, providing 20 mg/bird/day and addition of probiotic blend 2 in feed in dose of 1.0 kg/t in starter phase, 0.5 kg/t in grower phase and 0.25 kg/t in finisher phase (treatment PB), and the control diet with addition of phytogetic blend in dose of 125 g/t for the whole period (treatment PHY).

The probiotic blend 1 (PoultryStar w, BIOMIN GmbH) comprised probiotic bacteria isolated from the crop (*Lactobacillus reuteri*), jejunum (*Enterococcus faecium*), ileum (*Bifidobacterium animalis*), and cecum (*Pediococcus acidilactici* and *Lactobacillus salivarius*) of healthy adult chicken. The product had a total bacterial count, expressed as colony-forming units of 5·10¹² cfu/kg of product. Probiotic blend 2 (Biomin IMBO, BIOMIN GmbH) comprised probiotic bacteria *Enterococcus faecium* together with prebiotics fructooligosaccharides, cell wall fragments and phycophytic substances. Phytogetic blend (Biomin P.E.P. 125 poultry) was composed of essential oils derived from oregano, anis and citrus, and fructooligosaccharides.

Body weight and feed intake were monitored by pen at weekly intervals and mortality was recorded daily. Birds that died were noted and their bodyweight was used to adjust the feed conversion ratio accordingly. European Production Efficiency Factor (EPEF) was calculated according to the following formula:

$$\text{EPEF} = \frac{\text{bodyweight (g)} \times \text{survivalrate (\%)}}{\text{feed conversion} \times \text{duration of trial (days)}} \quad (1)$$

Methodology of bacterial enumeration

Conventional microbiological techniques using selective agar media were used for analysis. Cecum sample were homogenized in buffered peptone water and serial of decimal dilution were prepared. Selective agar media were used for enumeration of target bacterial groups - total aerobes (Nutrient Agar - Merck), total anaerobes (Wilkens-Chalgren Agar - HiMedia), lactic acid bacteria (MRS agar - HiMedia), *Bifidobacterium* spp. (Wilkens-Chalgren Agar with recommended supplements - HiMedia), *Escherichia coli* and total coliform bacteria (Chromocult Coliform Agar - Merck), enterococci (Chromocult Enterococci Agar - Merck) and *Clostridium* spp. (TSC Agar - HiMedia). Anaerobic milieu in the anaerobic jar for cultivation of obligatory and facultative anaerobes, was achieved using Anaerocult A (Merck). Anaerobiosis is indicated by the colour change of the Anaerotest A (Merck). Results were expressed as log₁₀ colony forming units per gram of cecum digesta (log cfu/g).

Methodology of gut morphology measurements

At the end of the starter period (21 days) and the end of the trial (42 days), 8 broilers from each group were slaughtered and small intestines were removed. Samples of jejunum were fixed in Bouin solution, and after histological procedure stained with hematoxylin and eosine. Villus height, villus width, crypt depth and thickness of tunica muscularis were determined using the light microscope and software for image analysis. Apparent villus surface area was estimated by trigonometry as described by IJI *et al.* (2001). A minimum of 15 measurements were made for each parameter per chicken.

Statistical analyses

Data are presented as means ± standard deviation. Data were analyzed by ANOVA using general linear models, followed by a LSD *post hoc* test to separate means, using StatSoft software (STATISTICA 7, 2005). Results were considered significant when $P < 0.05$.

Results

Performance

Body weight of the birds were controlled every week. The results (Table 2) showed a significant positive effect of probiotic and phytogenic treatments on body weight of broilers. The differences were established at the end of the starter period (3 weeks of age) and maintained until the end of the trial (6 weeks of age). The best result was achieved in

PHY group. These results indicate that the phytogetic product under investigation and probiotics have a growth promoting effect on broiler chickens. Probiotic group also showed a positive effect on feed conversion ratio (FCR) compared to the control group, but the differences were not significant ($P>0.05$). Mortality rates were not significantly affected by treatments ($P>0.05$). Broilers of PHY group achieved the best EPEF value.

Table 2
Impact of probiotic or phytogetic additives in broiler diets on the zootechnical performance of broilers
Einfluss probiotischer und phytogener Additive auf Leistungsparameter bei Broilern

Parameter	Treatment			Statistics	
	Control (C)	Probiotic (PB)	Phytogetic (PHY)	SE	P value
Body weight, g					
3 weeks	645 ± 43.7 ^A	688 ± 47.2 ^B	680 ± 54.8 ^B	2.94	<0.001**
6 weeks	2 135 ± 278 ^A	2 184 ± 241 ^B	2 210 ± 253 ^B	9.94	0.008**
Feed conversion ratio (FCR)					
3 weeks	1.52 ± 0.07	1.48 ± 0.02	1.46 ± 0.02	0.0028	0.2925
6 weeks	1.91 ± 0.06	1.89 ± 0.07	1.87 ± 0.10	0.0016	0.2204
Mortality rate, %					
3 weeks	1.74	2.61	2.17		
6 weeks	3.07	3.07	4.38		
European Production Efficiency Factor (EPEF)					
6 weeks	258	267	269		

^{A,B} Means within the same row with no common superscript differ significantly ($P<0.01$). ** $P<0.01$

Intestinal microflora

The composition of cecal bacterial population of chicken (total number of aerobes, anaerobes, lactic acid bacteria, bifidobacteria, enterococci, and *Escherichia coli*) at the end of the experiment is shown in Figure. Number of *Clostridium* spp. in all samples was lower than 2 log cfu/g and the results are not shown in the figure. Number of coliform bacteria in cecum was not significantly different between treatments ($P>0.05$) and was much lower (for 1-2 log cfu unit per gram) than the number of *Escherichia coli* in all cecal samples.

There were no statistically significant differences among treatments regarding total aerobes, anaerobes, lactic acid bacteria, bifidobacteria, enterococci, and *Escherichia coli* populations ($P>0.05$). Anaerobic bacteria, lactic acid bacteria and bifidobacteria were dominant bacterial species in cecal contents. Domination of *Escherichia coli* is a main reason of difficulties in enumeration and identification of coliform bacteria in intestinal samples. Notable reduction in *Escherichia coli* was found in both experimental groups (PB and PHY) compared to control, but the difference was not statistically significant mostly due to the high variation within groups (SD for PB group was 1.06 and for PHY group 1.20 LogCFU/g).

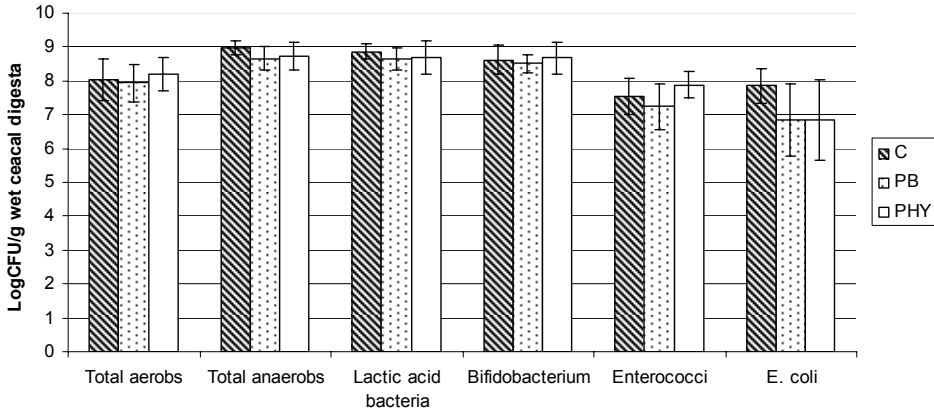


Figure
Number of different bacteria in ceecal contents
Anzahl (koloniebildende Einheiten) unterschiedlicher Bakterien im Caecum

Gut morphology

Significant differences ($P < 0.05$) in examined gut morphology parameters were found between groups at the age of 6 weeks but not at the age of 3 weeks (Table 3).

Table 3
Effect of probiotic or phytogenic additives in broiler diets on the jejunum morphology of broilers
Einfluss probiotischer und phytogener Additive auf morphologische Parameter des Jejunums bei Broilern

Parameter	Treatment			Statistics	
	Control (C)	Probiotic (PB)	Phytogenic (PHY)	SE	P value
3 weeks of age					
Villus height, μm	1181.68	1095.10	1064.30	78.4	0.191
Crypt depth, μm	232.66	245.43	219.30	37.2	0.564
Villus/Crypt ratio	5.08	4.46	4.85	0.96	0.753
Surface area, mm^2	0.105	0.124	0.119	0.01	0.838
Villus width, μm	125.66	114.85	119.07	16.7	0.976
Tunica muscularis, μm	163.48	158.33	145.67	24.8	0.653
6 weeks of age					
Villus height, μm	1133.10 ^b	1474.50 ^a	1249.4 ^b	85.1	0.003**
Crypt depth, μm	245.81 ^b	281.95 ^b	334.61 ^a	52.9	0.015*
Villus/Crypt ratio	4.69 ^b	5.32 ^b	3.83 ^a	0.95	0.004**
Surface area, mm^2	0.12 ^b	0.176 ^a	0.147 ^b	0.04	0.001**
Villus width, μm	104.79	113.86	112.61	15.11	0.148
Tunica muscularis, μm	170.52	181.48	163.92	26.31	0.846

^{a,b} Means within the same row with no common superscript differ significantly ($P < 0.05$), * $P < 0.05$, ** $P < 0.01$

The results showed that probiotics had a beneficial effect on jejunum morphology at 6 weeks of broilers age, causing a significant increase ($P < 0.005$) in villus height and villus surface area compared to the other groups, which might be connected with increased production results (Table 2). On the other hand, the phytogenic additive had no effect ($P > 0.05$) on villus height or villus surface area, but reduced ($P < 0.005$) the villus/crypt ratio. That indicates that the improvement in performance parameters achieved in PHY

group is not directly connected with changes in gut morphology, but may be due to other mechanisms.

Discussion

The beneficial effects of probiotic treatment on broiler performance achieved in this trial are in agreement with a large number of studies which have shown positive effects of using different strains and combinations of probiotics (JIN *et al.* 1998, ZULKIFLI *et al.* 2000, KABIR *et al.* 2004). LI *et al.* (2008) reported that average daily weight gain was significantly increased and the FCR significantly decreased by application of *B. subtilis* in feed for broilers, either solely or in combination with other prebiotics. On the other hand there are research studies reporting the lack of effect of probiotics on broiler performance (WATKINS and KRATZER 1984, MAIOLINO *et al.* 1992). In order to give the scientific explanation of the different results achieved by using probiotics as growth promoters in broiler feed, most of the authors concluded that the efficacy of probiotic application depends on many factors including species composition of probiotics, administration levels, application methods, overall diet composition, bird age and environmental factors (JIN *et al.* 1997, 1998, PATTERSON and BRUKHOLDER 2003).

The similar conclusion can be drawn for the addition of phytogetic additives in broiler feed. Many experiments investigating the effects of herbs, plant extracts and essential oils on broiler performance yielded contradicting results. Some of the authors reported a significant positive effect of phytogetics on broiler performance (LOVKOVA *et al.* 2001, WILLIAMS and LOSA 2001, ERTAS *et al.* 2005, CROSS *et al.* 2007) while in other trials different phytogetic additives and essential oils did not affect body weight gain, feed intake or feed efficiency in broilers (CROSS *et al.* 2002, DEMIR *et al.* 2003, OCAK *et al.* 2008). It has been suggested that the different results of the trials are due to differences in the composition of phytogetic preparations, different methods used to extract the essential oils from the herbs and the level of the application in feed (CROSS *et al.* 2007).

The probiotic treatment and phytogetic feed additives implemented in this trial did not have a significant effect on the investigated bacterial groups in the gut. As for phytogetic additives, similar observations were obtained by CROSS *et al.* (2007). The authors reported that dietary inclusion of five culinary herbs or their essential oils had no effect on the intestinal microflora populations. The results regarding probiotics are numerous and quite inconsistent. Using the same combination of probiotic bacteria (probiotic blend 1) as investigated in this trial, which were unlike to this trial applied during the whole experiment via the feed, MOUNTZOURIS *et al.* (2007) obtained no significant differences between the control and probiotic treatments regarding total anaerobes, total aerobes, coliforms and *Bacteroides spp.* in cecum of broilers, but found significantly higher concentrations of *Bifidobacterium spp.*, *Lactobacillus spp.*, and gram-positive cocci. Numerous reports indicated that addition of probiotics in feed, either solely or in combination with other feed additives like prebiotics, could regulate the intestinal microflora in order to increase the concentration of the beneficial bacteria such as *Lactobacillus ssp.* and *Streptococcus ssp.* and inhibit the reproduction of harmful bacteria in the gut (LINE *et al.* 1998, FRITS *et al.* 2000, LI *et al.* 2008). Other studies using

different probiotics have shown no significant influence on the gut microbial composition (JIN *et al.* 1998, Priyakarange *et al.* 2003). The general suggestion is that molecular approaches identifying bacterial populations and changes in microbial community are needed to enhance our understanding of intestinal microbial ecology, including the influence of probiotics (APAJALAHTI *et al.* 1998, ZHU *et al.* 2002).

Regarding the gut morphology traits, the results of this trial showed a positive effect of probiotics on villi height and villi surface compared to the control and PHY groups. Thus, it can be speculated that increased integrity of the gastrointestinal tract associated with a higher surface area of the villi, resulted in improved production results. It is suggested that probiotic cultures could reduce both the damage of enterocytes and the need for cell renewal in the gut. Obtained results confirmed the fact exposed by many researchers that the gastrointestinal tract can adapt and react morphologically to external factors related to dietary changes, i.e. addition of probiotics (HUISMAN *et al.* 1990, VAN DER KLIS and VAN DER VOORST 1993). These changes were represented by elongated villi and a higher villi/crypt ratio, which indicate a lower rate of enterocyte-cell migration from the crypt to the villus. It was reported that the intestine can change its surface by growing to length, and/or by increasing or decreasing the height of its villi (IJ *et al.* 2001, UŠĆEBRKA *et al.* 2005, ŽIKIĆ *et al.* 2008).

The phytogetic blend used in this trial did not affect villi height, but caused the highest crypt depth and lowest villus/crypt ratio. OČAK *et al.* (2008) reported that adding dry peppermint or thyme leaves as a growth promoters in broiler feed had no effect on the relative weights of the whole gut, pancreas and addible inner organs. That indicates that the improved production results obtained in broilers fed the phytogetic additive are not directly connected with the improved gut morphology, but with other mechanisms. It was reported that active principles of herbs, essential oils or plant extracts may act as digestibility enhancers, balancing the gut microbial ecosystem and stimulating the secretion of endogenous digestive enzymes and thus improving growth performance in poultry (LOVKOVA *et al.* 2001, WILLIAMS and LOSA 2001, CROSS *et al.* 2007).

Although most of the studies carried out in this field have shown positive effects of probiotics and phytogetic products on broiler performance, further research is needed to establish the optimal application of such additives including their optimal dosage level in the feed in order to obtain maximum effects.

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Corresponding author:

LIDIJA PERIĆ

email: lidija@polj.uns.ac.rs

Department of Animal Science, Faculty of Agriculture, Trg Dositeja Obradovića 8, 21000 Novi Sad, Republic of Serbia
