Effect of multi-enzymes in combination with a direct-fed microbial on performance and welfare parameters in broilers under commercial production settings

Y. Dersjant-Li,*[1] K. van de Belt,[†] J. D. van der Klis,[†] H. Kettunen,[‡] T. Rinttilä,[‡] and A. Awati*

Danisco Animal Nutrition, Market House, Ailesbury Court, High Street, Marlborough, Wiltshire, UK SN8 1AA; †Schothorst Feed Research B.V., PO Box 533, Lelystad, Netherlands 8200 AM; and ‡Alimetrics Ltd., Koskelontie 19B, Espoo, Finland FIN-02920

Primary Audience: Nutritionist, Poultry feed producer, Poultry farmers

SUMMARY

The objective of this study was to determine the response of broilers to the combination of multi-enzymes and direct-fed microbial (DFM) under commercial production settings. A total of 7,000 1-day-old male broilers (Ross 308) were distributed over 10 pens (700 broilers/pen). Two dietary treatments were tested using complete randomized design, including a control diet and a test diet with addition of multi-enzymes (xylanase, amylase, and protease (XAP)] and DFM (a combination of spores from 3 strains of *Bacillus amyloliquefaciens*). Pelleted diets were offered ad libitum in 3 phases and water was freely available. During starter and grower phases (0 to 21 d), the enzyme and DFM combination resulted in improved FE ($P < 0.05$). During the finisher phase, higher feed intake and BW gain ($P < 0.05$) were observed for the test group. Overall, there were significantly higher feed intake, BW gain, and lower water-to-feed ratio in test group compared to the control group. This was related to improved ($P < 0.05$) modified production efficiency factor which was calculated based on final BW, survival rate, feeding period, and mortality-weight-corrected FCR. The test group had improved litter quality and a reduced foot-pad lesion score compared to the control. In addition, there was a tendency ($P < 0.1$) of reducing *Clostridium perfringens* population in cecal digesta and higher lactic acid content in the ileal digesta, when expressed on an as-is basis, in the test group. In this study, we demonstrated that using a multi-enzymes and DFM combination in the diet for broilers can result in improved FE in starter/grower phases and animal welfare parameters, and lead to improved production efficiency under commercial settings.

Key words: multi-enzymes, direct-fed microbial, modified production efficiency factor, litter quality, foot pad lesions, *Clostridium perfringens*

2015 J. Appl. Poult. Res. 00:1–11
http://dx.doi.org/10.3382/japr/pfv003

DESCRIPTION OF PROBLEM

High FE and production efficiency is the key for successful poultry farming. Feed costs are by far the largest costs in animal production, contributing from 60 to 70% of production cost. The main ingredients' prices such as corn and soybean meal have tripled over the last 10 yr. This has led to a change in feed formulation towards increased usage of alternative feed

[1]Corresponding author: Yueming.dersjant-li@dupont.com

ingredients. As a consequence, diets may contain higher level of antinutritional factors including nonstarch polysaccharides, and have lower nutrient digestibility and FE. Consequently, there is an increased usage of feed enzymes in animal feed. It was estimated that in yr 2011 the feed worldwide enzyme market reached $550 million, resulting in a global market saving of $3 to $5 billion [1]. This represents a return of investment of 6:1 to 9:1, demonstrating a clear benefit of using feed enzymes. Phytase and carbohydrases are the most commonly used feed enzymes in poultry feed.

With the withdrawal of antimicrobial growth promoters in the European Union (**EU**) and the increased public concern on bacterial resistance to antibiotics jeopardizing antibiotic treatment of humans, the use of direct-fed microbial (**DFM**, or probiotics) in animal feed has increased. DFM are defined as "live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host" [2]. Maintaining a beneficial bacterial population in the intestine will improve "gut health" of the birds and reduce the gut maintenance cost [3, 4]. Based on the literature we read, DFM can be effective in improving immune response, intestinal health, and growth performance of broilers [3, 5].

An interaction between multi-enzymes and DFM has been reported in the literature [6]. Feed enzymes such as carbohydrase can improve the utilization of carbohydrates and provide "prebiotics" to beneficial bacteria, whereas protease can reduce the indigestible protein and reduce the substrate for pathogen bacteria. DFM can improve microbial balance and intestinal health, and provide an environment that may stimulate the activity of enzymes. In some studies, researchers have demonstrated the beneficial effect of using the combination of multi-enzymes and DFM in poultry [6–8]. The objective of this study is to verify the effect of a combination of DFM and multi-enzymes, on production efficiency and animal welfare parameters, such as litter quality and foot-pad lesion score, in broilers under commercial production settings.

MATERIALS AND METHODS

Animals, Facility, and Vaccination

The study was conducted in commercial settings that avoided unnecessary of discomfort of the animals. The Institutional Animal Care and Use Committee approved the study protocols. Seven thousand 1-day-old male broiler chickens (Ross 308, Aviagen Group) were distributed over 10 study units (700 birds per study unit). Chickens received infectious bronchitis vaccinations (Zoetis Belux, Zaventem, Belgium) at the hatchery. Birds were vaccinated against newcastle disease (Merial, Horsholm, Denmark) and infectious bursal disease (Intervet, Boxmeer, Netherlands) at 12 and 20 d age, respectively. Diets contained Maxiban (Elanco Animal Health, Houten, Netherlands) and Sacox (Huvepharma, Sofia, Bulgaria) as a coccidiostat in the starter and grower diets, whereas no coccidostat was used in the finisher (Table 1).

The broilers were housed in 10 floor pens with fresh wood shavings as bedding material. Each pen had 47.5 m^2 floor space and contained 700 birds. The ambient temperature was gradually decreased from 35°C at arrival to 18°C at 42 d age. Light was continuously on during the first day. The next day a schedule of 22L:2D was used. During the remaining experimental period a schedule of 8L:4D and 10L:2D was used.

Study Design and Measurement

Study design, diets, and animals. The trial was performed as a complete randomized design and comprised 2 dietary treatments with 5 replicate pens each. Pen was the study unit. The study diets included a control diet and a test diet containing multi-enzymes [endo-xylanase from *Trichoderma reesei*, alpha-amylase from *Bacillus licheniformis*, and serine protease from *Bacillus subtilis* (**XAP**), and a direct-fed microbial containing a combination of spores from 3 strains of *Bacillus amyloliquefaciens*]. The control diet was based on wheat, soybean meal, and corn and the composition is presented in Table 1. The basal diet was formulated with lower energy and digestible amino acids compared to

breeder recommendations, which was due to the expected contribution of energy and digestible amino acids from exogenous enzyme (phytase and the multi-enzymes) supplementation. Starter diets were provided from 0 to 10 d age, grower diets from 10 to 21 d age, and finisher diets from 21 to 42 d age. All diets were fed as pellets (pelleting temperature below 80°C). Birds had unrestricted access to feed and drinking water.

Sampling and measurement. A composite sample per diet was taken during the feed packaging process. Basal diets were analyzed for moisture [9], ash [10], CP [11], crude fat [12], crude fiber [13], starch [14], phosphorus [15], and calcium [16].

Performance parameters measurement. Average weight of 1-day-old broilers was measured at arrival by randomly weighing 180 birds. Average BW per pen was daily measured by automatic weighing plateaus. At the end of the study the final BW was measured by weighing all birds per pen. BW gain was reported from the 0 to 10, 10 to 21, 21 to 42, 0 to 21, and 0 to 42 d ranges. Feed intake was measured per pen for the same period as for BW gain and was corrected for mortality. All animals were daily monitored for abnormalities, such as abnormal behavior, clinical signs of illness, and mortality. Animals in poor condition, and unlikely to recover or survive, were euthanized.

FCR was calculated based on body weight gain **(BWG)** (corrected for mortality weight) and feed intake from the 0 to 10, 10 to 21, 21 to 42, and 0 to 42 d ranges. Mortality weight was

Table 1. The ingredients and nutrient composition of the control diets

	Starter		Grower		Finisher	
Ingredients, % as-is	0 to 10 d		10 to 21 d		21 to 42 d	
Wheat	25.00		40.30		49.52	
Corn	34.78		24.50		14.50	
Soybean meal	26.00		24.99		23.90	
Rapeseed meal	4.00		4.00		4.00	
Corn gluten meal	3.00		-		-	
Soybean oil	1.50		1.50		1.12	
Animal fat	0.00		0.88		4.17	
Mono calcium phosphate	0.76		0.13		0.06	
Limestone	1.70		1.29		0.75	
Salt	0.14		0.30		0.31	
Sodium bicarbonate	0.43		0.00		0.00	
Premix starter[1]	1.00					
Premix grower[2]			0.5			
Premix finisher[3]					0.6	
Premix Sacox[4]	-		0.50			
Premix Maxiban[5]	0.50		-			
Lysine premix (65%)	0.27		0.19		0.19	
Methionine premix (55%)	0.31		0.30		0.27	
Threonine premix (40%)	0.11		0.12		0.11	
Test product premix[6]	0.50		0.50		0.50	
Nutrients	Calculated	Analyzed	Calculated	Analyzed	Calculated	Analyzed
AME (kcal/kg)	2,897		2,955		3,075	
Dry matter (g/kg)	876	896	873	894	875	877
Ash (g/kg)	56	51	46	43	44	45
Crude fiber (g/kg)	28	29	29	29	29	32
CP (g/kg)	218	221	203	205	195	202
Crude fat (acid hydrolyzed) (g/kg)	44	47	49	52	75	74
Starch (g/kg)	374	391	393	398	381	372
D-Lys (g/kg)	10.8		10.0		9.5	
D-Met (g/kg)	4.8		4.4		4.1	
D-M+C (g/kg)	7.9		7.3		6.9	
D-Thr (g/kg)	7.0		6.5		6.2	
D-Trp (g/kg)	2.1		2.1		2.1	
D-Val (g/kg)	8.6		8.0		7.6	
D- (g/kg)	7.8		7.2		6.9	

Table 1. *continued*

	Starter		Grower		Finisher	
Ca (g/kg)	8.9	8.8	6.3	6.6	5.8	6.5
Cl (g/kg)	1.9		2.7		2.7	
K (g/kg)	8.7		8.7		8.4	
Na (g/kg)	1.9		1.3		1.4	
P (g/kg)	5.6	5.7	4.1	4.5	3.8	4.2
Ca (g/kg)	8.8		6.3		5.8	
Available P (g/kg)	3.0		1.6		1.4	

[1]Starter premix supplies per kg diet: vitamin A, 12,000 IU; vitamin D3, 2,900 IU; vitamin E, 90 mg; vitamin K3, 2.0 mg; vitamin B1, 2.0 mg; vitamin B2, 8.5 mg; vitamin B6, 4.5 mg; vitamin B12, 20 mcg; Niacin, 50 mg; D-pantothenic acid, 15.0 mg; choline, 445 mg; folic acid, 1.25 mg; biotin, 275 mcg; Fe, 80 mg (as $FeSO_4.H_2O$); Cu, 12 mg (as $CuSO_4.5H_2O$); Mn, 85 mg (as MnO); Zn, 60 mg (as $ZnSO4.H_2O$); Co, 0.4 mg (as $CoSO_4.7H_2O$); I, 0.8 mg (as KI); Se, 0.15 mg (as Na_2SeO_3).

[2]Grower premix supplies per kg diet: vitamin A, 12,000 IU; vitamin D3, 2,400 IU; vitamin E, 50 mg; vitamin K3, 1.5 mg; vitamin B1, 2.0 mg; vitamin B2, 7.5 mg; vitamin B6, 3.5 mg; vitamin B12, 20 mcg; Niacin, 35 mg; D-pantothenic acid, 12 mg; choline, 345 mg; folic acid, 1.0 mg; biotin, 0.2 mg; Fe, 80 mg (as $FeSO_4.H_2O$); Cu, 12 mg (as $CuSO_4.5H_2O$); Mn, 85 mg (as MnO); Zn, 60 mg (as $ZnSO4.H_2O$); Co, 0.4 mg (as $CoSO_4.7H_2O$); I, 0.8 mg (as KI); Se, 0.15 mg (as Na_2SeO_3).

[3]Finisher premix supplies per kg diet: Ca, 1.68 g; vitamin A, 15,000 IU; vitamin D3, 4,200 IU; vitamin E, 36 IU; vitamin K3, 3.6 mg; vitamin B1, 3.6 mg; vitamin B 9 mg; panthotenic acid, 18 mg; niacin, 60 mg; biotin, 180 mcg; vitamin B12, 24 mcg; folic acid, 1.2 mg; vitamin B6, 4.8 mg; choline 60 mg; Betaine, 120 mg; Fe, 72 mg (as $FeSO_4.H_2O$); Cu, 18 mg (as $CuSO_4.5H_2O$); Zn, 120 mg (as $ZnSO4.H_2O$); Mn, 96 mg (as MnO); I, 2.4 mg (as KI); Se, 0.3 mg (as Na_2SeO_3); Co, 300 mcg.

[4]Premix Sacox supplies per kg diet: Salinomycine sodium, 60 mg (Huvepharma, Bulagaria).

[5]Premix Maxiban supplies per kg diet: Vitamin A, 2,000 IU; vitamin D3, 500 IU; vitamin E, 10 IU; vitamin B2, 1 mg; panthotenic acid, 1 mg; niacin, 15 mg; folic acid, 0.25 mg; biotin, 75 mcg; choline 100 mg; Cu, 3 mg; Mn, 20 mg; Zn, 18 mg; Nicarbazin, 50 mg; Narasin, 50 mg (Elanco Animal Health, US).

[6]It is a premix based on corn with phytase (Phyzyme XP, 500 FTU/kg diet) in control diet, with addition of test materials in the test diet. Test product provides 2,000 U/kg xylanase, 200 U/kg amylase, and 5,000 U/kg protease (Axtra XAP, Danisco Animal Nutrition/DuPont) and 75,000 CFU/g B. *amyloliquefaciens* (Danisco Animal Nutrition/DuPont). Feed enzymes recovery was tested and met the target levels.

estimated as 80% of the average BW (measured by the weighing plateau) in that pen on the day when the bird was removed, using the following equation:

$$\sum_{i=1}^{n}(0.8 \times (\text{number of mortality})_i \times (\text{average weight of living broilers})_i)$$

where i is the day of mortality.

FE can be influenced by age or BW of animals. In the comparative study, standardizing data will allow more accurate comparison of treatments. In the equations provided by Patricio et al. [17], corrected FCR adjusted based on live weight at 2.5 kg can be calculated by adjusting 2.9 to 3.1 points per 100 g BW difference. In this study, BW-corrected feed conversion (FCR_c) was calculated by 3 points reduction in FCR for every 100 g BW increase vs. control.

$$FCR_c = FCR - \frac{BW_{test} - BW_{com}}{100} \times 0.03$$

where BW_{test}: BW of birds in the test group; BW_{con}: BW of birds in the control group.

Production efficiency factor (PEF) was used to evaluate the live-bird performance of flocks. PEF is calculated based on live weight, survival rate, age of the birds, and FCR [18]. As in this study the FCR was corrected for mortality weight (FCR_{mc}), a modified production efficiency factor (MPEF) [19] was calculated:

$$MPEF = \frac{\text{Liveweight(kg)} \times \text{liveability(\%)}}{\text{Age at slaughter(days)} \times FCR_{mc}} \times 100$$

Carcass quality. On d 42, 40 randomly selected birds per pen were individually weighed, marked, and delivered to a slaughter house. At the slaughter house, the birds were electrically stunned, exsanquinated, defeathered, and eviscerated. Carcass, breast meat, and abdominal fat weight were determined. Carcass percentage was calculated relative to live weight, and breast meat and abdominal fat weight were calculated as percentage of the carcass weight.

Litter score and litter composition. Litter quality was scored visually on d 21 and 41 [20]. Litter was scored on a scale of 0 to 10 in which a score of 0 corresponds with low litter quality (wet) and score 10 corresponds with high litter quality (dry and friable), as described below:

0: The whole floor surface is caked from bottom to top of the layer
1: The whole floor surface is caked. Some parts are caked from bottom to top; some parts are only caked on the upper layer
2: The whole floor space is caked but the lower layer is friable
3: >75% of the floor surface is caked
4: >50% of the floor surface is caked
5: Litter under the waterline is caked from top to bottom
6: Upper layer of the litter under the waterline is caked
7: Litter under the waterline is wet but still friable
8: Litter under the waterline is damp
9: The litter is not fresh anymore but still friable and dry everywhere in the pen
10: Fresh litter

On d 41, from every pen 3 litter samples (in a diagonal, one at the drinking line, one at the feeding line and one in between) were taken. These 3 samples were pooled for analysis of moisture, CP, phosphorus, calcium, and soluble phosphorus.

Foot pad lesions. On d 21 and 41 left-foot pads of 20 broilers per study unit were scored. The scoring classes were according to the Bristol foot-burn scale [21]. The average score per broiler per pen was calculated.

Intestinal microbial and short-chain fatty acid analysis. On d 41, 2 randomly chosen birds per pen were euthanized by intracardiac injection with T61 [22]. The birds had free access to water and feed until they were removed from the pen. Immediately after euthanasia, the abdominal cavity was opened and the intestines were removed from the abdominal cavity. The cecal lobes and the small intestine sections were sampled. The small intestine section was about 10 to 15 cm length after leaving 8 to 10 cm from the ileal-cecal valve. The samples were stored frozen at −80°C before being analyzed.

Microbes in the digesta samples were analyzed for total eubacteria, *Lactobacillus* spp., *Bifidobacterium* spp., Clostridial Cluster XIVa and IV, *Bacteroides* spp., *Streptococcus* spp., *Enterococcus* spp., *Clostridium perfringens*, *Salmonella* spp., and *Escherichia coli*. For these analyses, samples were subjected to quantitative bacterial lysis and DNA purification essentially as described for chicken intestinal samples [23]. Quantitative real-time PCR (qPCR) and the primers specific for the abovementioned bacterial species, genera, or groups were used for the analysis as described previously [24–26] with slight modifications.

Short-chain fatty acids (**SCFA**) were analyzed from ileal and caecal digesta samples as free acids by gas chromatography, using pivalic acid as an internal standard as described previously [27]. The acids measured were acetic, propionic, butyric, isobutyric, 2-methylbutyric, valeric, isovaleric, and lactic acid.

Statistics

Data were subjected to one-way ANOVA using the JMP 10.0 software [28] and treatment means were compared by Student's t-test. Each pen was one study unit for performance and carcass quality data analysis. For microbial analysis each bird was used as one study unit. A treatment effect with $P \leq 0.05$ was considered statistically significant, whereas $0.05 < P \leq 0.10$ was considered a near-significant trend.

RESULTS AND DISCUSSION

Performance and Production Efficiency

Starter and grower phase. The average initial BW of the birds was 41.9 g. In starter phase

(1 to 10 d), no significant differences were found on feed intake and weight gain between the 2 treatment groups. However, FCR was lower ($P = 0.04$) in the test group compared to the control group in the same phase. Although in the starter phase the difference in calorie conversion ratio (kcal/kg BW gain) between the test group and the control group did not reach a significant level, during the 0 to 21 d period FCR and calorie conversion ratio were lower ($P = 0.02$) in the test group compared to control (Table 2). The effect of supplementation of the combination of multi-enzymes and DFM was related to the age of broilers. During starter and grower phase (0 to 21 d), the significant reduction in FCR might imply that this combination improved digestibility of nutrients and reduced maintenance cost, resulting in increased FE. This study was done with European-type wheat-based diets. Similar results were reported in corn- and soybean-meal-based diets. Murugesan et al. [7] observed that in broilers fed corn- and soybean-meal-based diets, FCR was reduced by supplementation of multi-enzymes (XAP) or DFM (3 *Bacillus* strains) during 0 to 21 d age, and the effect of the combination was higher than that of the additives used individually. The combination of enzymes and DFM showed an additive response for FCR which suggests independent mechanisms involved in increasing energy utilization. Romero et al. [29] determined the effect of XAP and DFM (3 *Bacillus* strains) and their combination on energy and nutrient digestibility in broilers at 21 d age. Both supplements improved ileal digestible energy (DE) and AME_n. Enzymes improved ileal protein and fat digestibility at 21 d, whereas starch digestibility was affected by both DFMs and enzymes, and exhibited an interaction. The DFM+XAP treatment increased ileal digestible energy to a larger extend than the supplements used alone. This was explained by the improvement in digestibility of starch, fat, protein, and non-starch polysaccharides **(NSP)**. It was suggested that the enzymes and DFM may have complementary effects on nutrient digestibility in broilers.

Finisher phase and overall period. Supplementation of the combination of DFM and enzymes increased feed intake ($P = 0.004$) and BW gain ($P = 0.047$) compared to control. Water intake was not significantly influenced by dietary treatments; however, the test group had lower water to feed ratio ($P = 0.04$). FCR and calorie conversion were not significantly different among treatments. Based on the results of 0 to 42 d, enzyme and DFM supplementation to the control diet increased feed consumption ($P = 0.003$), improved BW gain ($P = 0.04$) and reduced water to feed ratio ($P = 0.02$). FCR, calorie conversion, and mortality were not significantly influenced by dietary treatments. MPEF was higher ($P = 0.03$) in test group compared to control (326 vs. 305, Table 2).

The results of the current study are in agreement with literature, where DFM and enzyme combinations improved FE and growth performance [6, 30] of broilers. Momtazan et al. [30] observed that there was an interaction between an enzyme complex containing β-glucanase, α-amylase, cellulase, protease, and lipase, and a DFM for BW, FCR, and the relative weight of the duodenum in broilers. It was concluded that the combination of the enzyme complex and DFM can improve the performance more than either supplement used on its own. Walsh et al. [6] observed an interaction between XAP and DFM (3 *Bacillus* strains) for BW gain of broilers during 0 to 42 d. Both enzymes and DFM improved BW gain in broilers compared to a control treatment. The results from this commercial setting trial have further confirmed the positive effect of the enzymes and DFM combination on growth performance of broilers.

Although the difference in calorie conversion ratio did not reach a significant level between the treatments for overall 0 to 42 d results, a slight saving of about 100 kcal energy per kilogram live weight gain was observed with addition of the XAP+DFM combination. This is suggested to have resulted in the improved ($P < 0.05$) MPEF in the treatment group compared to the control (326 vs. 305, 7%), resulting in a beneficial effect of the use of enzymes and DFM in commercial broiler production. Similar results have been observed in another commercial-scale study, where addition of DFM (combination of 3 *bacillus* strains) to a corn/soybean-meal-based diet containing mixed enzymes improved MPEF by 2.5% [19].

Slaughter yield. Carcass yield (%), breast meat, and abdominal fat as percentage of carcass were not affected by the treatments (Table 2).

Table 2. BW gain, feed and water intake, FCR and production efficiency, mortality rate, and slaughter yield of broilers in response to dietary supplementation of XAP[1] and DFM[2] in broilers fed study diets for 42 d

	Control	Test	SEM	P
d 0 to 10				
BW gain (g)	204	207	3.26	0.56
Feed intake (g)	316	302	4.91	0.13
FCR	1.55	1.46	0.02	0.04
Water intake (mL)	591	579	12.1	0.52
Water-to-feed ratio	1.83	1.95	0.03	0.06
Calorie consumption, kcal	917	862	15.0	0.10
Calorie conversion, kcal/kg BW gain	4,493	4,301	86.4	0.23
Mortality %	1.6	1.2	0.22	0.20
d 0 to 21				
BW gain (g)	781	774	5.13	0.41
Feed intake (g)	1,103	1,071	10.6	0.07
FCR	1.41	1.38	0.01	0.02
FCR_c[3]	1.41	1.38	0.01	0.07
Water intake (mL)	2,190	2,114	33.1	0.15
Water-to-feed ratio	1.98	1.97	0.02	0.62
Calorie consumption, kcal	3,241	3,148	31.2	0.07
Calorie conversion, kcal/kg BW gain	4,149	4,067	25.7	0.02
Mortality %	1.89	1.86	0.24	0.93
d 21 to 42				
BW gain (g)	1,547	1,659	20.7	0.047
Feed intake (g)	2,960	3,095	23.4	0.004
FCR	1.91	1.87	0.02	0.26
Water intake (mL)	5,289	5,353	69.3	0.45
Water-to-feed ratio	1.79	1.73	0.01	0.04
Calorie consumption, kcal	9,102	9,516	71.9	0.004
Calorie conversion, kcal/kg BW gain	5,887	5,737	63.3	0.26
Mortality %	2.2	1.9	0.24	0.30
d 0 to 42				
BW gain (g)	2,328	2,433	19.7	0.04
Feed intake (g)	4,063	4,166	29.3	0.003
FCR	1.75	1.71	0.01	0.22
FCR_c	1.75	1.65	0.01	0.45
Water intake (mL)	7,479	7,468	80.2	0.92
Water-to-feed ratio	1.84	1.79	0.01	0.02
Calorie consumption, kcal	12,343	12,664	89.1	0.003
Calorie conversion, kcal/kg BW gain	5,303	5,205	42.4	0.23
Mortality %	4.11	3.71	0.43	0.60
MPEF[4]	305	326	5.63	0.03
Slaughter yield				
Carcass (%)	68.6	68.9	0.32	0.52
Filet (% carcass)	28.9	28.8	0.17	0.49
Abdominal fat (% carcass)	0.88	0.96	0.04	0.16

[1]XAP: multi-enzyme containing xylanase, amylase, and protease.

[2]DFM: direct-fed microbial containing a 3-strain combination of *Bacillus*.

[3]FCR_c: BW-corrected FCR, corrected for 3 points by each 100 g BW difference between test and control group.

[4]MPEF: modified production efficiency factor.

Table 3. Effect of the XAP[1] and DFM[2] combination on litter quality, litter composition, and foot-pad lesion score in broilers fed study diets for 42 d

	Control	Test	SEM	P
Litter quality				
d 21	5.3	6.5	0.41	0.03
d 41	1.8	2.6	0.39	0.24
Litter composition, %				
Moisture	54.5	49.7	1.31	0.01
CP, DM basis	31.1	30.96	3.61	0.69
Phosphorus, DM basis	6.9	7.1	0.16	0.38
Calcium, DM basis	13.58	12.62	0.34	0.06
Soluble phosphorus, DM basis	2.32	1.84	0.19	0.04
Foot-pad lesion score				
d 21	1.73	1.94	0.17	0.06
d 41	2.47	2.06	0.14	0.04

[1]XAP: multi-enzyme containing xylanase, amylase and protease.
[2]DFM: direct-fed microbial containing a 3-strain combination of *Bacillus*.

Litter quality and foot pad lesion score. Supplementation of the multi-enzymes and DFM combination improved litter quality at d 21 ($P = 0.026$) and dry matter content in the litter at d 41 ($P = 0.006$). In addition, calcium content in the litter on a dry matter basis resulted in a nearly significant reduction in the test group ($P = 0.057$). Total CP and phosphorus content in the litter on dry matter basis were not significantly affected by the treatments; however, soluble phosphorus was reduced ($P = 0.04$) in the test group compared to control. Foot-pad lesion score was lower in the test group compared to the control group at d 41 ($P = 0.04$) (Table 3).

An interesting result observed in this study was the lower water-to-feed ratio due to the supplementation of the enzymes and DFM. This is in agreement with some literature studies, where addition of NSP enzymes reduced water intake [31–33]. Van der Klis et al. [34, 35] reported that high viscous diets can increase water intake of broilers as high intestinal viscosity reduced sodium absorption from the lumen and thereby lowered absorption of water, resulting in a higher water intake. In the current study, the lower water-to-feed ratio may be explained by addition of NSP degrading enzymes that reduced the viscosity of digesta and consequently reduced water intake. The latter was associated with the high litter dry matter content, improved litter quality score, and consequently reduced foot-pad lesion score in the treatment group. In a study in turkeys, it was observed that dietary supplementation of XAP enzymes in combination with DFM (3 *Bacillus* strains) significantly reduced fecal moisture by 4.2% [36]. Wet litter is identified as one of the main causes for foot pad dermatitis in broilers and turkeys [37, 38]. Therefore, the reduced foot-pad lesion score may be partially explained by improved litter quality in the current study. In addition, the reduced foot-pad dermatitis may be also associated with the lower *C. perfringens* counts in the digesta, as both *C. perfringens* and *C. septicum* infection were associated with foot-pad dermatitis [39].

Supplementation of XAP and DFM may have an impact on mineral utilization. as indicated by decreased calcium and soluble P content in litter on DM basis. The control diet contained only phytase enzyme and the test diet contained phytase, XAP, and DFM. There might be a synergic effect between phytase and XAP in the study treatment, as Van der Klis et al. [40] demonstrated that Ca and P digestibility was improved adding endoxylanase to wheat-based diets coinciding with reduced chyme viscosity. A literature review [41] suggested that supplementation of a combination of phytase and carbohydrases to corn-, wheat-, or barley-based diets for poultry can be more beneficial with regard to nutrient utilization than supplementation of the enzymes individually as demonstrated in many literature studies.

Microbial profile and SCFA. Commercial settings of the study restricted the number of

Table 4. Effect of XAP[1] and DFM[2] combination on microbial profile and SCFA[3] content expressed on dry matter basis in broilers fed study diets for 42 d

		Ileal		Cecal	
	Target gene	Control	Test	Control	Test
Dry matter (DM), %	-	16.6	17.3	14.4	13.7
Gene copies/g DM					
Total eubacteria	16S rRNA	3.40×10^{11}	6.84×10^{11}*	2.45×10^{13}	2.02×10^{13}
Lactobacillus spp.	16S rRNA	2.07×10^{11}	2.61×10^{11}	5.27×10^{12}	4.70×10^{12}
Bifidobacterium spp.	16S rRNA	NA	NA	1.00×10^{11}	3.58×10^{11}
Clostridial cluster IV	16S rRNA	NA	NA	7.98×10^{12}	6.03×10^{12}
Clostridial cluster XIVa	16S rRNA	3.35×10^{9}	9.57×10^{8}	6.28×10^{12}	5.66×10^{12}
Bacteroides spp.	16S rRNA	5.26×10^{6}	4.00×10^{6}	4.59×10^{11}	7.40×10^{11}
Streptococcus spp.	16S rRNA	5.26×10^{10}	1.06×10^{11}	NA	NA
Enterococcus spp.	16S rRNA	7.32×10^{8}	5.73×10^{8}	NA	NA
Clostridium perfringens	Phospholipase C	2.18×10^{8}	1.66×10^{7}	5.53×10^{9}	6.86×10^{8}
Salmonella sp.	Nuclease	3.46×10^{4}	ND	5.50×10^{5}	9.53×10^{5}
Escherichia coli	16S rRNA	4.66×10^{6}	4.40×10^{6}	8.77×10^{9}	$6.92 \times ^{9}$
Gene copies/g as-is					
Clostridium perfringens	Phospholipase C	3.54×10^{7}	2.76×10^{6}	7.6×10^{8}*	7.4×10^{7}*
			μmol/g DM		
Lactic acid	-	351	494	72.7	96.7
Acetic acid	-	19.1	18.1	367	370
Propionic acid	-	1.12*	0.083*	35.5	40.2
Isobutyric acid	-	3.5	4.9	11.3	10.1
Butyric acid	-	0.4	0.2	102	88
Total SCFA[3]	-	378	521	600	614
Total volatile fatty acids	-	26.6	26.4	528	518
			μmol/g as-is		
Total SCFA[3]		60.6*	89.6*	10	11.1
Lactic acid		56.4*	85.0*	83.6	81.2

[1]XAP: multi-enzyme containing xylanase, amylase and protease.

[2]DFM: direct-fed microbial containing a 3-strain combination of *Bacillus*.

[3]SCFA: short-chain fatty acids.

*$P < 0.1$.

NA: not analyzed; ND: not detected.

replicates per treatment. Considering often observed higher variation in quantitative microbial population analysis, differences between the treatment groups on microbial counts, and the SCFA content did not reach statistical significance, when expressed on a dry matter basis (Table 4). However, it is still noteworthy that there was a clear tendency of treatment effect ($P < 0.1$). In ileal digesta, test group showed higher DM content and increased microbial density ($P < 0.1$). *C. perfringens* counts were lower in both ileal (1.66×10^7 vs. 2.18×10^8) and cecal digesta (6.86×10^8 vs. 5.53×10^9) compared to control (Table 4). When expressed on as is basis, tendency was observed for the test group to have lower number of *C. perfringens* ($P < 0.1$) in cecal digesta, and higher total concentration of SCFAs and lactic acid ($P < 0.1$) in ileal digesta when compared to the control group. It may be speculated that combination of multi-enzymes with DFM might have a positive effect on microbial balance and microbial metabolic end-products in the small intestine, by stimulating beneficial bacteria population such as lactobacilli (indicated by numerically higher lactic acid content in both ileal and cecal digesta) and reducing pathogen bacteria colonization such as *C perfringens*. This could be related to the increased total SCFA and lactic acid production and might imply an improved intestinal health of birds. Madisen et al. [42] reported that inclusion of a DFM (3-strain *Bacillus*) shifted the gastrointestinal lactic acid producing bacteria population toward enhanced *Lactobacillus spp.* populations and fewer *Enterococcus* during the starter phase of production in turkeys. In layers, Murugesan et al. [8] observed that colonization of *Campylobacter spp*. in the colon was reduced with the addition of

DFM. It was suggested that energy utilization was increased with exogenous enzymes and enzymes + DFM, while the DFM increased gut barrier function and lowered pathogen colonization. Thus addition of the combination of these additives may improve gut health and energy utilization of the birds.

CONCLUSION AND APPLICATIONS

1) Supplementation of multi-enzymes containing XAP in combination with a 3-strain combination of *Bacillus* (DFM) improved FE in the starter and grower phases, and increased feed intake and weight gain in finisher phase in broilers.
2) Use of XAP and DFM combination resulted in increased MPEF.
3) XAP and DFM combination improved litter quality and reduced foot-pad lesion scores.
4) Based in this study, we suggest that the combination of XAP and DFM may contribute to improved animal welfare condition, and feed and production efficiency in broilers under commercial settings.

REFERENCES AND NOTES

1. Adeola, O., and A. J. Cowieson. 2011. Board-invited review: Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. J. Anim. Sci. 89:3189–3218.

2. FAO/WHO. 2002. Guidelines for the evaluation of probiotics in food. Report of a joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) working group on drafting guidelines for the evaluation of probiotics in food. World Health Organization, Geneva, Switerland. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

3. Lee, K. W., H. S. Lillehoj, and G. R. Siragusa. 2010. Review: Direct-fed microbials and their impact on the intestinal microflora and immune system of chickens. J. Poult. Sci. 47:106–114.

4. Kiarie, E., L. F. Romero, and C. M. Nyachoti. 2013. The role of added feed enzymes in promoting gut health in swine and poultry. Nutr. Res. Rev. 26:71–88.

5. Lutful Kabir, S. M. 2009. The role of probiotics in the poultry industry. Int. J. Mol. Sci. 10:3531–3546.

6. Walsh, M. C., L. F. Romero, S. E. Indrakumar, and V. Ravindran. 2013. Influence of combinations of a direct-fed microbial and exogenous enzymes on the growth performance and feed efficiency of broilers. Poult. Sci. 92(E-Suppl. 1):87.

7. Murugesan, G. R., and M. E. Persia. 2013a. Effects of exogenous enzymes and direct-fed microbials on performance, energy utilization, and body composition of broiler chicks. Poult. Sci. 92(E-Suppl. 1):10.

8. Murugesan, G. R., I. V. Wesley, J. Remus, P. W. Plumstead, and M. E. Persia. 2013b. Effects of exogenous enzymes and direct-fed microbials supplementation on first-cycle laying hen performance, energy digestibility, gut integrity and pathogen colonization. Poult. Sci. 92(E-Suppl. 1):11.

9. NEN-ISO 6496:1999. 1999. Animal feeding stuffs—Determination of moisture and other volatile matter content. Technical committee ISO/TC 34, agricultural food products, subcommittee SC 10. ISO, Geneva, Switzerland.

10. NEN-ISO 5984:2003. 2003. Methods of Analysis for Feeding Stuffs. Determination of Crude Ash. Netherlands Normalization Institute, ISO, Geneva, Switzerland.

11. NEN-EN-ISO 16634:2008. 2008. Animal Feeding Stuff–Determination of Nitrogen Content Using Dumas Combustion. ISO, Geneva, Switzerland.

12. NEN-ISO 6492:1999. 1999. Animal Feedstuffs–Determination of Fat Content. ISO, Geneva, Switzerland.

13. NEN-ISO 6865:2001. 2001. Animal Feeding Stuffs–Determination of Crude Fibre Content–Method with Intermediate Filtration. ISO, Geneva, Switzerland.

14. NEN-ISO 15914:2005. 2005. Animal Feeding Stuffs–Enzymatic Determination of Total Starch Content. Netherlands Normalization Institute. ISO, Geneva, Switzerland.

15. NEN-ISO 6491:1999. 1999. Animal Feeding Stuffs–Determination of Phosphorus Content–Spectrometric Method. ISO, Geneva, Switzerland.

16. NEN-EN-ISO 6869:2001. 2001. Animal Feeding Stuffs - Determination of the Contents of Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Sodium and Zinc–Method Using Atomic Absorption Spectrometry. Netherlands Normalization Institute. ISO, Geneva, Switzerland.

17. Patricio, I. S., A. A. Mendes, A. A. Ramos, and D. F. Pereira. 2012. Overview on the performance of Brazilian broilers (1990 to 2009). Brazilian J. Poult. Sci. 14:233–304.

18. Shane, S. What's your 'PEF'? Live Production Management. http://www.datapoul.co.uk/wp-content/uploads/ProductionEfficiencyFactor.pdf.

19. Dersjant-Li, Y., A. Awati, C. Kromm, and C. Evans. 2014. A direct fed microbial containing a combination of three-strain *Bacillus* sp. can be used as an alternative to feed antibiotic growth promoters in broiler production. J. Appl. Anim. Nutr. 2(e11):1–6.

20. European Union. 2007. Council directive 2007/43/EC of 28 June 2007 laying down minimum rules for the protection of chickens kept for meat production, pp. 19–28.

21. Welfare Quality. 2009. Welfare Quality assessment protocol for poultry (broilers, laying hens). Welfare Quality Consortium, Lelystad, Netherlands.

22. T61 (0.1 ml/kg Body Weight). Intervet Nederland BV, Boxmeer, Netherlands

23. Apajalahti, J. H. A., L. K. Särkilahti, B. R. Mäki, J. P. Heikkinen, P. H. Nurminen, and W. E. Holben. 1998. Effective recovery of bacterial DNA and percent-guanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. Appl. Environ. Microbiol. 64:4084–4088.

24. Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter. 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257–266.

25. Rinttilä, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in fecal samples by real-time PCR. J. Appl. Microbiol. 97:1166–1177.

26. Rinttilä, T., A. Lyra, L. Krogius-Kurikka, and A. Palva. 2011. Real-time PCR analysis of enteric pathogens from fecal samples of irritable bowel syndrome subjects. Gut Pathogens. 3:1–9.

27. Holben, W. E., P. Williams, M. Gilbert, M. Saarinen, L. K. Särkilahti, and J. H. Apajalahti. 2002. Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma phylotype* in farmed and wild salmon. Microbiol. Ecol. 44:175–185.

28. SAS Institute. 2012. JMP 10. Discovering JMP, SAS Institute, Cary, NC.

29. Romero, L. F., S. E. Indrakumar, and V. Ravindran. 2013. Influence of combinations of a direct-fed microbial and exogenous enzymes on nutrient digestibility in broilers at 11 and 21 days of age. Poult. Sci. 92(E-Suppl. 1):88.

30. Momtazan, R., H. Moravej, M. Zaghari, and H. R. Taheri. 2011. A note on the effects of a combination of an enzyme complex and probiotic in the diet on performance of broiler chickens. Irish J. Agr. Food Res. 50:249–254.

31. Shirzadi, H., H. Moravej, and M. Shivazad. 2009. Comparison of the effects of different kinds of NSP enzymes on the performance, water intake, litter moisture and jejunal digesta viscosity of broilers fed barley-based diet. J. Food Agr. Environ. 7:615–619.

32. Engberg, R. M., M. S. Hedemann, S. Steenfeldt, and B. B. Jensen. 2004. Influence of whole wheat and xylanse on broiler performance and microbial composition and activity in the digestive tract. Poult. Sci. 83:925–938.

33. Garcia, M., R. Lazaro, M. A. Latorre, M. I. Gracia, and G. G. Mateos. 2008. Influence of enzyme supplementation and heat processing of barley on digestive traits and productive performance of broilers. Poult. Sci. 87:940–948.

34. Klis van der, J. D., A. Voorst van, and C. Cruyningen van. 1993. Effect of a soluble polysaccharide (carboxy methyl cellulose) on the physico-chemical condition in the gastrointestinal tract of broilers. Br. Poult. Sci. 34:971–983.

35. Klis van der, J. D., M. W. A. Verstegen, and A. Voorst van. 1993. Effect of a soluble polysaccharide (carboxy methyl cellulose) on the absorption of minerals from the gastrointestinal tract of broilers. Br. Poult. Sci. 34:985–997.

36. Ayoola, A. A., R. D. Malheiros, L. V. F. M. Carvalho, S. Indrakumar, L. F. Romero, and P. R. Ferket. 2013. The effect of supplementing corn-DDGS diet with exogenous enzymes and direct-fed microbials on nutrient digestibility in turkey poults. Poult. Sci. 92(E-Suppl. 1):36.

37. Mayne, R. K. 2005. A review of the etiology and possible causative factors of foot pad dermatitis in growing turkeys and broilers. World's Poult. Sci. J. 61:256–267.

38. Shepherd, E. M., and B. D. Fairchild. 2010. Invited review: Footpad dermatitis in poultry. Poult. Sci. 89:2043–2051.

39. Li, G. X., H. S. Lillehoj, K. W. Lee, S. I. Jang, P. Marc, C. G. Gay, G. D. Ritter, D. A. Bautista, K. Phillips, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. An outbreak of gangrenous dermatitis in commercial broiler chickens. Avian Path. 39:247–253.

40. Klis van der, J. D., C. Kwakernaak, and W. De Wit. 1995. Effects of endoxylanase addition to wheat-based diets on physico-chemical chyme conditions and mineral absorption in broilers. Anim. Feed Sci. Technol. 51:15–27.

41. Woyengo, T. A., and C. M. Nyachoti. 2011. Review: Supplementation of phytase and carbohydrases to diets for poultry. Can. J. Anim. Sci. 91:177–192.

42. Madisen, A. J., J. C. Remus, S. Dunham, E. Davis, and D. Petri. 2013. A commercial study comparing the effects of antibiotic and direct-fed microbial supplementation on gut lactic acid bacteria populations in turkeys. Poult. Sci. 92(E-Suppl. 1):10.