Effect of emulsifier and multicarbohydrase enzyme supplementation on performance and nutrient digestibility in broiler diets containing rapeseed meal

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ABSTRACT The aim of this study was to determine the effect of emulsifier and multicarbohydrase enzyme supplementation on performance, nutrient utilization, and apparent metabolizable energy—nitrogen (AME_N) value of broiler diets containing rapeseed meal (**RSM**) as well as their influence on the gut morphological structures, excretion of total and free sialic acid, and cecum concentration of short-chain fatty acids (**SCFAs**) in broiler chickens. A total of 384 male broiler chicks were assigned to four dietary treatments. The diet of the control treatment (**CON**) consisted of soybean, maize, and RSM (5% in starter, 7% in grower, 15% in finisher) with soybean and palm oils. The diets used for the experimental treatments were the control diet supplemented with an emulsifier (**EMU**), enzyme (**ENZ**), or both (**EMU + ENZ**). The duodenum (n = 10/treatment) and ileum (n = 10/treatment) digesta samples were assessed to determine nutrient digestibility: crude protein (**CP**), ether extract (**EE**), starch, Ca. Throughout the experimental period, EMU + ENZ treatment indicated the lowest total average feed intake and feed conversion ratio, with the highest average weight gain among the studied treatments ($P < 0.05$). The EMU + ENZ treatment also resulted in higher ($P < 0.05$): apparent prececal digestibility (**APD**) of CP, total tract neutral detergent fibre (**NDF**) degradation, apparent total tract digestibility (**ATTD**) of EE, villus height to crypt depth ratio ($P < 0.1$). The highest APD of EE was noted in the EMU treatment ($P < 0.05$). No significant differences were found in the AME_N values of the diets. A greater jejunum villi surface area was found in groups supplemented by enzyme compared to CON ($P < 0.05$). The EMU + ENZ treatment presented lower sialic acid excretion in the ileum and concentration of cecum SCFAs compared to the CON treatment ($P < 0.05$). The obtained results indicate that simultaneous usage of additives had beneficial effect on production parameters, nutrient digestibility, NDF degradation, as well as gut mucosa morphology. Based on the SCFAs concentration results, separate or simultaneous addition of emulsifier or/and enzyme did not provoke excessive fermentation activity of cecal bacteria.

Key words: broiler performance, carbohydrases, emulsifier, rapeseed meal, non-starch polysaccharide

INTRODUCTION

The global intensification of poultry production with limited resources of feed materials, together with increasing environmental pollution, has led to the urgent need to develop strategies for sustainable production. One of the reasonable solutions to overcome this challenge is to improve the utilization of genetic potential, proper estimation and fulfilment of animals' nutritional requirements. Some nutritional aspects that may be

considered are increasing the utilization of feed components, such as fat, and enhancing the decomposition of poorly digestible nonsoluble structures such as non-starch polysaccharides (**NSP**).

The gastrointestinal tract (**GIT**) of a broiler chicken represents a water environment where fatty acids (**FA**) are absorbed in the form of micelle (Ravindran et al., 2016). Monoglycerides and short-chain FA presented mainly in unsaturated oil fats, such as soybean oil (Sauvant et al., 2004), are passively absorbed from the intestinal lumen (Pond et al., 2005). However, saturated FA included, for instance, in palm oil, are poorly digested and absorbed by birds (Scott et al., 1982) and require solubilization first (Davenport, 1980). Chemical substances such as bile acid salts exhibit emulgating properties that enable micelle formation, as well as further decomposition of FA by lipase (Ravindran et al., 2016). However, fully developed secretion of both bile acid salts and lipase does not occur until around 14 d of a broiler's life (Krogdahl, 1985; Uni, et al. 1999). Therefore, emulsifier substances can be added to broiler diets to enhance fat digestibility in early age and improve birds' performance during the whole rearing period (Kaczmarek et al., 2015; Ravindran et al., 2016). Additionally, the common practice from the economical point of view is to blend saturated and unsaturated fats due to the natural emulsifying properties of unsaturated FA (Freeman, 1969; Wiseman and Lessire, 1987).

Literature data indicate that the digestibility of nutrients is negatively influenced by the presence of NSP in the diet (Salih et al., 1991; Lazaro et al., 2003). In diets containing rapeseed meal (**RSM**), NSP-degrading enzymes improve carbohydrate digestibility and eliminate potential nutrient-encapsulating effect of NSP. This has been proven by Meng et al. (2005b) and Rutkowski et al. (2012), who observed improvement in nutrient digestibility and birds' performance due to the presence of NSP-degrading enzymes in RSM-based diets. Interestingly, a previous study on broiler chickens by Kaczmarek et al. (2015) revealed that neutral detergent fibre (**NDF**) degradation in the wheat-corn and soybean meal (**SBM**) diet was increased after combined supplementation with emulsifier and carbohydrase. Initially, improvement in NDF degradation was considered as an indirect result of improving substrate-to-enzyme ratio after improvement in fat digestion by emulsifier. However, in vitro studies, as well as studies on ruminant animals, indicated that emulsifiers have a direct effect on the GIT microbiome (Ahn et al., 2009). It was established that non-ionic surfactant (**NIS**) enhance the secretion of bacterial enzymes responsible for the carbohydrates degradation (Lee et al., 2004), which are one of the NDF components. Kubis et al. (2020) analyzed the addition of xylanase and emulsifier to wheat-based diets with tallow, and their results partially explained those obtained by Kaczmarek (2015); in experimental diets supplemented simultaneously with both additives, a decrease in digesta viscosity was observed, which presumably resulted in a favorable shift of fermentation from the ileum to the cecum, where the activity of bacterial enzymes is greater. As the abovementioned studies involved the use of viscous grain, the response of the NSP enzyme was as expected. In the present study, a similar experimental model was used as in the work of Kubis et al. (2020). Nevertheless, the carbohydrate source used was RSM, which is considered as a nonviscous component, with negligible susceptibility to microbial fermentation in the lower GIT (Zdunczyk et al., 2015a). However, NSP of RSM still possesses properties which negatively influence accessibility and digestibility of nutrients; a possibility to encapsulating nutrients causing their losses (Raza et al., 2019). Therefore, response after enzyme may vary compared to the viscous grains, however, still may presents positive effect on nutrients availability (Francesch and Geraert, 2009).

The study aimed to analyze the influence of carbohydrase, emulsifier, or a combination of both on performance, nutrient utilization, and apparent metabolizable energy—nitrogen (**AME$_N$**) value of maize-soybean-RSM-based broiler diets, and their influence of gut morphological structures, and short-chain fatty acids (**SCFAs**) concentration in chickens. Additionally, ileum excretion of total and free sialic acid, a product of mucin bacterial breakdown will be analyzed, as it is consider as a good indicator of endogenous losses (Cowieson et al., 2004).

We hypothesized that the combined use of emulsifier and carbohydrase would improve nutrient utilization as well as broiler chickens' performance to a greater extent than the individual use of enzyme or emulsifier.

MATERIALS AND METHODS

The study was carried out in the Experimental Station of Poznan University of Life Sciences in Gorzyn, Poland. All the experimental procedures were performed in accordance with the guidelines of the Local Ethical Committee for Experiments on Animals in Poznan regarding animal experimentation and animal care under study (European Union [EU] Directive 2010/63/EU for animal experiments). Approval for conducting the experiment was not required as the production methods and sample collection (excreta) had no negative impact on the birds' welfare and is not considered as experimental procedure carried out on each animal.

Experimental Design

A total of 384 Ross 308 male broiler chicks (1-day-old) were obtained from a local hatchery (DanHatch Poland, Wolsztyn, Poland). The birds were divided into 4 weight classes (each class differed by 0.5 grams) and randomly assigned to 4 dietary treatments (12 replications, 8 individuals; 2 birds from each weight class). All birds were reared in 1.2 × 0.8 m floor pens on wood-shaving litter. In the first week of the experiment, the birds were exposed to light for 24 h, and subsequently to 18 h of light and 6 h of darkness. The temperature in the pens was maintained at 32°C in the first week of the

experiment and then gradually reduced up to 23°C at the end of the third week. Fresh water and crumbled or pelleted feed were provided ad libitum.

Diets

The dietary treatments were divided into 3 phases; 1 to 11 d: starter, 12 to 25 d: grower, 26 to 42 d: finisher. Dietary treatments consisted of: the control (**CON**) diet (Table 1) based on maize, SBM, and RSM at 5%, 7%, and 15% (starter, grower, and finisher, respectively), and the control diet supplemented with emulsifier (**EMU**), enzyme (**ENZ**), or both (**EMU + ENZ**). The soybean and palm oils were the main fat sources of the diets, and were determined for FA content (Table 2). Quantum blue phytase (1,000 phytase units/kg; AB Vista Feed Ingredients, Marlborough, UK) was included in all diets. The emulsifier was made of glyceryl polyethylene glycol ricinoleate (E484, Bredol 683; AkzoNobel SC AB, Stenungsund, Sweden) and added to the EMU and ENZ + EMU treatment diets instead of maize at 0.015 g/kg of starter diet, 0.0173 g/kg of grower diet, and 0.0188 g/kg of finisher diet. Replacement of maize by emulsifier was justified by the greatest amount of this component in a formulated diet; levels of replacement were negligible and not consider as factor which may disrupt balance of a diet. The hydrophilic-to-lipophilic balance value of Bredol 683 was 9.5. The enzyme used at 0.12 g/kg in all diets was Superzyme OM (Canadian

Table 1. Composition and nutrient contents of the control (CON)[1] diet: starter, grower, finisher (as feed basis).

Ingredients, %	Starter 1−11 d	Grower 12−25 d	Finisher 26−42 d
Maize	53.6	58.9	52.2
Soybean meal	32.8	27.3	22.1
Rapeseed meal	5.00	7.00	15.0
Soybean oil	3.25	1.60	3.80
Palm oil	2.00	1.60	3.90
Monocalcium phosphate	0.68	0.56	0.49
Premix[2]	1.00	1.00	1.00
Limestone	0.38	0.31	0.16
DL - met	0.36	0.28	0.25
HCL - Lys	0.38	0.29	0.26
NaHCO$_3$	0.25	0.26	0.26
NaCl	0.17	0.10	0.10
L - Thr	0.14	0.09	0.08
TiO$_2$	-	0.30	0.30
L - trp	-	0.17	-
Quantum Blue	0.01	0.01	0.01
Analyzed nutrient content in % or otherwise noted			
Crude protein [%]	22.2	21.1	20.5
Gross energy [kcal/kg] (MJ/kg)	-	-	4,562 (19.1)
Ether extract [%]	8.63	6.71	10.2
TiO$_2$ [%]	-	-	0.32

[1]EMU, ENZ and EMU + ENZ diets were supplemented with emulsifier (0.015 g/kg of starter diet, 0.0173 g/kg of grower diet, and 0.0188 g/kg of finisher diets), enzyme (0.12 g/kg in all diets), or both simultaneously.

[2]Provides per kg diet: vitamin A, 11250 IU; vitamin D, 2500 IU; vitamin E, 80 mg; vitamin K, 2.50 mg; vitamin B12, 0.02 mg; folic acid, 1.17 mg; choline, 379 mg; D - pantothenic acid, 12.5 mg; riboflavin, 7.0 m; niacin, 41.67 m; thiamin, 2.17 mg; D - biotin, 0.18 mg; pyridoxine, 4.0 mg; ethoxyquin, 0.09 mg; Mn, 73 mg as managnous oxide; Zn, 55 mg as zinc oxide; Fe, 45 mg as iron sulfate; Cu, 20 mg as copper sulfate; I, 0.62 mg as calcium iodide; Se, 0.3 mg as selenium potassium; salinomycin, 60 mg.

Table 2. Fatty acids composition (% of total fatty acids) of oils used in experimental diets.

Fatty acids, %	Soybean oil	Palm oil
Saturated fatty acids		
C12:0 Myristic acid	0.07	0.16
C14:0 Myristic acid	0.08	1.01
C16:0 Palmitic acid	10.0	42.9
C18:0 Stearic acid	5.50	4.78
Monounsaturated fatty acids		
C18:1cis9 Oleic acid	21.8	39.8
C18:1c11 Vaccenic acid	1.30	0.72
C20:1t Eicosenoic acid	0.47	0.39
Polyunsaturated fatty acids		
C18:2c9c12 Linoleic acid	51.7	9.30
C18:3c9c12 c15 α - Linolenic acid	7.53	0.22
C18:3n6 γ - Linolenic	0.19	0.15
C20:3n6 Homo - γ - Linolenic	0.58	0.10
C20:5n3 icosapentaenoic acid	0.23	0.12
SFA[1]	16.1	49.1
UFA[2]	83.9	50.9
MUFA[3]	23.1	40.9
PUFA[4]	60.4	9.95

[1]Saturated fatty acids.
[2]Unsaturated fatty acids.
[3]Monounsaturated fatty acids.
[4]Polyunsaturated fatty acids.

Bio-Systems Inc., Calgary, Canada) which is a multicarbohydrase with 2100 U cellulase, 300 U mannanase, 37.5 U galactanase, 750 U xylanase, 450 U glucanase, 1875 U amylase, and 150 U protease per kg of diet. All formulated diets met or exceeded the Aviagen nutrient recommendations for broiler chickens (AVIAGEN, 2014). All diets were crumbled (0−11 d) or pelleted (12−42 d) and were isoenergetic and isonitrogenous.

A horizontal mixer (Zuptor 300 MPW; Zuptor sp. zoo., Gostyń, Poland) was used to prepare diets. The mixing band was set at 27.4 rpm for 4 min for mixing. Maize was ground by a Skiold disk mill (SK2500; Skiold A/S, Sæby, Denmark). Minerals, vitamins, amino acids, enzymes, emulsifier, and fats were added directly to the mixer during mixing. The homogeneity of emulsifier and enzyme was assured by mixing a small amount of the emulsifier with oils, and the enzyme with basic feed as a premix before being added to the final feed. The finisher phase diets were supplemented with an internal marker, titanium dioxide (TiO$_2$), at 3.0 g/kg, to determine prececal digestibility as well as total and free sialic acid excretion.

Sample Collection

To determine growth performance, the average feed intake (**AFI**) and average gain (**AG**) were monitored (with pen as an experimental unit) after each nutrition phase (starter, grower, finisher), and the feed conversion ratio (**FCR**) was calculated. The birds were fasted for 4 h before weighing, as this is the minimum time proper to live weight measures due to the excretion of intestinal wastes without affecting the yielding (Veerkamp, 1986; Kim et al., 2007).

During the finisher phase (28−36 d), collection trays were installed in floor pens to obtain excreta samples (12

replications per treatment/per collection; one replication represented eight birds). The individuals were removed from pens and next, wire floor trays were placed above the collection tray. Contaminant-free excreta samples were collected after approximately 3 h. The samples were frozen, freeze-dried, and ground for further analyses.

A total of 100 individuals (25 from each treatment) were euthanized by electric stunning following the recommendations for euthanasia of experimental animals. The duodenum (n = 10/ treatment) and ileum (n = 10/ treatment) digesta samples were collected by gentle squeezing from randomly selected birds from each pen. The samples were frozen in liquid nitrogen to determine nutrient digestibility, and segments (2 cm) of the middle duodenum and middle ileum were used for histomorphometric measurements. All samples (except used for histomorphometric measurements) were stored at $-80°C$ for further analyses.

Chemical Analyses

Feed, digesta, and excreta samples were ground in a laboratory mill with a 1-mm sieve and were analyzed for dry matter (**DM**; method 2001.12), crude protein (**CP**; method 976.05), fat as ether extract (**EE**; method 920.39), and Ca (method 927.02) according to the guidelines of the AOAC International (AOAC, 2005). The gross energy (**GE**) of feed was determined using an adiabatic bomb calorimeter (KL-12Mn; Precyzja-Bit PPHU, Bydgoszcz, Poland) standardized with benzoic acid. The feed samples were analyzed for acid detergent fibre (expressed inclusive of residual ash) and NDF (assayed with a heat-stable amylase and expressed inclusive of residual ash) using the official methods 942.05 and 973.18, respectively (AOAC, 2005). Fat content was determined using a Soxtec System HT 1043 Extraction Unit (Foss Tecator, Denmark).

Analysis of the excreta samples (for GE, NDF, and N) and ileum digesta samples (for CP, Ca, starch, and EE) were carried out using the abovementioned methods. The FA concentrations in soybean oil and palm oil were determined by gas chromatography as described by Szczechowiak et al. (2016).

The levels of TiO_2 marker in the finisher diet and excreta were determined as described by Short et al. (1996) in samples prepared as proposed by Myers et al. (2004). The AOAC colorimetric method 996.11 (AOAC, 2005) based on a Megazyme total starch determination kit was performed to determine starch content in ileum digesta samples.

Histomorphometric measurements of ileum and jejunum were made as described by Konieczka et al. (2018). Briefly, fixed tissue samples were dehydrated and embedded in paraffin wax. Transverse sections of prepared samples were cut on a microtome with a thickness of 4.5 μm, and the sections were stained with hematoxylin and eosin. For further analyses, images of samples were taken using a light microscope (Olympus BX51 microscope; Olympus Corp., Tokyo, Japan) with CellD Imaging Software (Olympus Soft Imaging Solutions, Münster, Germany). The following parameters were measured in the sections: villus height (**VH**)—from the tip of the villus to the villus−crypt junction; crypt depth (**CD**)—from the crypt mouth to base; lamina muscularis mucosae thickness; villus width—at the midline of the villus; and villus surface area. The VH/CD ratio was calculated.

The content of total and free sialic acids in excreta was determined using the procedure of Jourdian et al. (1971). The isolation of crude mucin from excreta was performed as described by Lien et al. (1996), Briefly, approximately 0.6 g freeze-dried excreta at 4°C was combined with 5 mL sodium chloride (0.15 mol/L) containing 0.2 M/L sodium azide. The homogenized samples were centrifuged at 12,000 g for 30 min. The supernatant was moved to second test tube and reused in second centrifugation (12,000 g for 30 min) prepared due to the proper removal of insoluble material. Then, the supernatant was pipetted into a preweighed test tube and cooled in an ice bath. Ice-cold ethanol (60%) was added to the supernatant, and the samples were cooled in an ice bath. Then, the samples were precipitated overnight at 20°C. After centrifugation at 1,400 g for 10 min, crude mucin was solubilized in 2 mL distilled water. Next, 0.1 mL of 0.04 M periodic acid solution was added to 0.5 mL of crude mucin preparation. The solution was mixed and placed in an ice bath for 20 min. Afterward 1.25 mL of resorcinol reagent was added to the solution. The solution was mixed, placed in an ice bath for 5 min, heated for 15 min (100°C), cooled, and filled with tert-butyl alcohol (1.25 mL). A single-phase solution was generated by vigorous mixing. Then, the tubes were placed in a water bath for 3 min (37°C) to stabilize the color, and cooled to the room temperature. The absorbance of the solution was read at 630 nm using a Media Spectrophotometer (Marcel Lamidey S.A., Châtillon, France). Total and free sialic acid in excreta was expressed in μmol/g of TiO_2.

The SCFA concentration in the cecum digesta was determined by gas chromatography following the method of Konieczka et al. (2019). An HP 5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) with a flame-ionization detector, a Supelco Nukol fused silica capillary column (30 m × 0.25 mm internal diameter, film 0.25 mm), and helium as the carrier gas was used for separation. The concentrations of individual SCFAs were determined using an internal standard (isocaproic acid).

Calculations and Statistical Analyses

The values of APD and AME_N of the diets were calculated in relation to the TiO_2 ratio of the nutrient content of the feed, digesta, or excreta. The nutrient digestibility was calculated using the following equation:

$$\text{Digestibility} = \{1 - [(TiO_{2\text{diet}}/TiO_{2\text{digesta}}) \times (\text{component}_{\text{digesta}}/\text{component}_{\text{diet}})]\},$$

where the content of TiO_2 and that of the dietary components are given as grams per kilogram.

The AME_N of diets was calculated using the following equation:

$$AME_N \ [MJ/kg]$$
$$= \ GE_{excreta} - \ [GE_{exreta} \ x \ (TiO_{2diet}/TiO_{2exreta})]$$
$$- 0.0344 \ x\{N_{diet} - \ [N_{excreta} \ x \ (TiO_{2diet}/TiO_{2excreta})]\},$$

where GE represents the gross energy [MJ/kg], N represents nitrogen, and TiO_2 represents the dietary marker. AME was corrected to a zero-nitrogen balance with 34.4 MJ/kg N retained as described by Hill and Anderson (1958).

The experiment was conducted as a completely randomized design. Each pen represented one experimental unit for performance results and apparent total tract digestibility (**ATTD**) and AME_N, and individual birds for APD, duodenum and ileum morphometry, and sialic acid excretion. The significance level was set at $P < 0.05$. All data were calculated using the analysis of variance of the general linear model procedure under the R environment (R Development Core Team, 2014) with the Agricola package (de Mendiburu, 2014) using the following model:

$$Y_i = \ \mu \ + \ \alpha_i \ + \ \varepsilon_i$$

where Yi is the measured dependent variable, μ is the overall mean, α_i is the effect of additive, and ε_i is the random error.

RESULTS

Chemical Composition and Performance

The FA composition of the oils used in the diets is presented in Table 2. The determined FA profile of the feed showed the differences between soybean oil and palm oil. In palm oil, SFAs constituted almost 50%, while in soybean oil their content did not exceed 16.5%. The predominant SFAs in palm oil were palmitic acid (42%) and stearic acid (4.78%). Soybean oil mainly consisted of polyunsaturated FA (60%), and the dominant unsaturated FA was linoleic acid (517 g/kg).

The mortality in the experiment was not found to be related to dietary treatments used, and averaged approximately 4% (15 birds). Throughout the experimental period, the lowest AFI was observed in the

EMU + ENZ treatment ($P < 0.05$). AG significantly differed only in the starter and finisher phases of nutrition, with the greatest values observed in ENZ and EMU + ENZ treatments. No significant differences in AG were noted among the treatments during the experiment; however, the experimental treatments showed numerically greater AG values than the CON treatment. The CON treatment showed the highest FCR, while the lowest value was recorded in the EMU + ENZ treatment. The detailed results of the analysis of AFI, AG, and FCR among the dietary treatment treatments are presented in Table 3.

Digestibility

The values of AME_N of the diets and APD of CP, EE, Ca, and starch are presented in Table 4. No differences in the AME_N values were found among the treatments. The EMU + ENZ treatment resulted in the highest APD of CP and starch ($P < 0.05$) as well as the highest total tract NDF degradation. Interestingly, the total tract NDF degradation of the CON treatment was significantly higher than that of the EMU and ENZ treatments. The lowest ($P < 0.05$) APD of EE was found in the CON and ENZ treatments, whereas the highest value was recorded in the EMU treatment. Considering the APD of Ca, only the EMU + ENZ treatment showed a statistically higher value compared to the CON treatment ($P < 0.05$); however, the value did not differ from the EMU treatment. A similar trend was observed for the ATTD of EE at 24 d. No differences among treatments were found for the ATTD of EE at 42 d.

Gut Morphology

Supplementation with additives increased the jejunum VSA ($P < 0.05$); however, the EMU treatment did not show any difference compared to the CON treatment. A difference ($P < 0.1$) in the VW and VH of jejunum was observed only in the EMU + ENZ treatment compared to the CON treatment (greater VH and lower VW). The highest VH/CD ratio was noted in the EMU + ENZ treatment ($P < 0.1$). No differences were observed in jejunum CD and lamina muscularis mucosae thickness among the treatments. Similarly, no statistically significant differences or trends were found for the abovementioned parameters of ileum mucosa structures

Table 3. Performance of broiler chickens fed: control diet (CON), control diet supplemented with emulsifier (EMU), control diet supplemented with enzyme (ENZ), control diet supplemented with emulsifier, and enzyme (EMU+ENZ).

day	AFI (g/bird)				AG (g/bird)				FCR[1] (kg/kg)			
	1−11	12−25	26−42	1−42	1−11	12−25	26−42	1−42	1−11	12−25	26−42	1−42
CON	244[a]	1,409[a]	2,458[a]	4,110[a]	212[b]	973	1,493[b]	2,678[b]	1.15[a]	1.45	1.82[a]	1.60[a]
EMU	224[ab]	1,387[ab]	2,391[ab]	4,002[bc]	218[a]	990	1,603[b]	2,711[b]	1.03[b]	1.41	1.74[b]	1.54[b]
ENZ	233[ab]	1,388[ab]	2,433[ab]	4,054[ab]	220[a]	987	1,660[a]	2,766[ab]	1.06[ab]	1.41	1.70[b]	1.52[b]
EMU + ENZ	218[b]	1,371[b]	2,365[b]	3,954[c]	223[a]	981	1,694[a]	2,798[a]	0.97[b]	1.40	1.60[c]	1.46[c]
SEM	3.71	5.66	13.1	17.5	1.18	5.38	10.0	11.6	0.019	0.009	0.015	0.010
P - value	0.041	0.026	0.041	0.006	< 0.001	0.65	< 0.001	< 0.001	0.002	0.055	< 0.001	< 0.001

[a]−[c]Means in a column not sharing a common letter are significantly different ($P \leq 0.05$).
[1]Feed conversion ratio.

Table 4. Apparent metabolizable energy corrected to N equilibrium (AME$_N$), apparent pre-cecal digestibility (APD) of crude protein (CP), ether extract (EE) on 28th day, Ca, starch, EE apparent total tract digestibility (ATTD) on 35th day and neutral detergent fibre (NDF) total tract degradation in broiler chickens fed control diet (CON), control diet supplemented with emulsifier (EMU), control diet supplemented with enzyme (ENZ), control diet supplemented with emulsifier and enzyme (EMU+ENZ).

Treatment	AME$_N$, kcal/kg (MJ/kg) at 28 d	at 35 d	APD at 35th day CP	EE	Ca	Starch	ATTD of EE 28 d	35 d	Total tract NDF degradation
CON	2,985 (12.5)	2,913 (12.2)	71.8[b]	85.1[c]	15.3[b]	93.3[b]	80.3[b]	90.7	40.7[b]
EMU	2,866 (12.0)	2,913 (12.2)	68.3[c]	90.7[a]	22.2[ab]	94.7[b]	82.6[ab]	92.2	34.9[c]
ENZ	3,009 (12.6)	2,890 (12.1)	70.7[bc]	84.9[c]	13.6[b]	94.8[b]	81.7[b]	93.6	35.1[c]
EMU + ENZ	3,105 (13.0)	2,842 (11.9)	79.7[a]	87.2[b]	29.1[a]	97.5[a]	84.8[a]	91.3	49.2[a]
SEM	0.163	0.151	0.9	0.4	1.734	0.463	0.5	0.9	1.2
P- value	0.126	0.879	< 0.001	< 0.001	0.002	0.006	0.003	0.661	< 0.001

[a–c]Means in a column not sharing a common letter are significantly different ($P \leq 0.05$).

(data not shown). The detailed results of the analysis of jejunum morphology among the dietary treatment treatments are presented in Table 5.

Sialic Acid Excretion

The studied treatments showed no differences in total and free sialic acid excretion on d 24. At 42 d, both total and free sialic acid excretion were the lowest in the EMU + ENZ treatment ($P < 0.05$); however, no differences compared to the ENZ treatment were observed. The detailed results of the analysis of sialic acid excretion among the dietary treatment treatments are presented in Table 6.

Cecum SCFA Concentration

Only the EMU + ENZ treatment showed a lower concentration of acetic acid and total SCFAs concentration in the cecum compared to the CON treatment. The EMU treatment and the ENZ treatment did not differ in acetate concentration compared to the CON and EMU + ENZ treatments. No differences were observed in the concentrations of propionic, butyric, isobutyric, valeric, and isovaleric acids among the treatments. The detailed results of the analysis of SCFAs concentration among the dietary treatment treatments are presented in Table 7.

Table 5. Villus height (VH), crypt cepth (CD), villus width (VW), lamina muscularis mucosae thickness (MT), villus surface area (VSA) of duodenum and ileum mucosa in broiler chickens fed control diet (CON), control diet supplemented with emulsifier (EMU), control diet supplemented with enzyme (ENZ), control diet supplemented with emulsifier and enzyme (EMU+ENZ).

Treatment	VH	CD	VW (μm)	MT	VSA (mm^2)	VH/CD
CON	1,169[B]	178	150[B]	169	0.566[b]	6.62[B]
EMU	1,320[AB]	196	160[AB]	148	0.687[ab]	6.81[B]
ENZ	1,349[AB]	203	160[AB]	150	0.704[a]	6.67[B]
EMU + ENZ	1,453[A]	177	173[A]	135	0.812[a]	8.46[A]
SEM	40.2	4.88	3.17	4.92	0.026	0.287
P- value	0.086	0.15	0.078	0.104	0.007	0.059

[a–c]Means in a column not sharing a common letter are significantly different ($P \leq 0.05$).
[A–C]Means in a column not sharing a common letter tend to differ ($P \leq 0.1$).

DISCUSSION

During the first week of a broiler's life, the secretion of lipase and bile salts is negligible (Sklan, 2001), which affects fat digestion. Thus, it has been proposed that broiler diets can be supplemented with additives to improve birds' performance. In this study, birds fed with experimental diets containing additives showed differences in AFI and FCR, which is in agreement with the results of previous studies (Aftab, 2009; de Vries et al., 2014; Kaczmarek et al., 2014). The authors of these studies speculated that improvement in FI and consequently in FCR after the addition of enzyme and/or emulsifier was related to better feed utilization. The digestibility results obtained in the present study partially support this hypothesis. After the addition of emulsifier, fat digestibility was found to be improved in the experimental treatments (APD, $P < 0.05$; ATTD on d 24, $P < 0.05$). Presumably, less feed was needed to fulfill the caloric requirements of broilers (Mathlouthi et al., 2002), which as a result lowered AFI. Additionally, improved fat digestibility enable physical access of other nutrients to the digestive enzymes, make them more prone to degradation and absorption (Danicke et al., 1999). Enhanced AFI observed after the use of additives (even in the starter phase) is inconsistent with the results of several previous studies (Cowieson et al., 2010; Alzawqari, 2011; Guerreiro et al., 2011; de Vries et al., 2014; Ahmadi, 2016). The contradictory results in these studies may also be related to the use of different types of emulsifiers (Abbas et al., 2016), for instance; desiccated ox bile or emulsifier consisted of milk derived casein. Considering that suboptimal diets were used in the present study, in which the fat levels for broilers exceeded above 5% and included saturated fats (palm oil), deterioration in FCR was expected in the CON treatment. Previously, Zaefarian et al. (2015) showed that, compared to unsaturated fats, the addition of saturated fats caused a reduction in broilers efficiency (tallow vs. soybean oil), as a result of deterioration in fat utilization. In the present study, the EMU + ENZ treatment presented the most preferable FCR in association with the lowest AFI and the highest AG during the whole period of experiment. As was mentioned, capacity to absorb and digest is impoverished in newly hatched chicks due to the immature GIT (Ravindran and

Table 6. Excretion of sialic acid (μmol/g of TiO$_2$) in ileum of digesta in broiler chickens fed control diet (CON), control diet supplemented with emulsifier (EMU), control diet supplemented with enzyme (ENZ), control diet supplemented with emulsifier and enzyme (EMU+ENZ).

	28 d		42 d	
Treatments	Total SA/1 gTiO$_2$	Free SA/1 gTiO$_2$	Total SA/1 gTiO$_2$	Free SA/1 gTiO$_2$
CON	514	550	678[a]	688[a]
EMU	570	561	705[a]	709[a]
ENZ	546	537	607[ab]	604[ab]
EMU + ENZ	422	449	516[b]	515[b]
SEM	0.622	0.822	25.0	24.9
P - value	0.104	0.303	0.026	0.017

[a–c]Means in a column not sharing a common letter are significantly different ($P \leq 0.05$).

Abdollahi, 2021). Thus, in condition of a diet consisting poorly digested saturated fat oil addition (palm oil), after emulsifier supplementation in the starter phase (1 −11 d), significant enhancement among FCR occurred, which also was reflected among increased AG. After neuralgic after-hatching period, approximately till second week of broiler's life, GIT become well-developed (Ravindran and Abdollahi, 2021). Maturation of broiler's GIT was reflected by no changes among AG or FCR during grower phase (12−25 d). Results of improved AG in the groups with enzyme are in contradictory with the previous studies where additives were supplemented separately (Aftab, 2009; de Vries et al., 2014; Kaczmarek et al., 2014).

This finding clearly indicates the positive mutual effect of both additives on nutrient utilization which was additionally reflected by the greatest APD of CP and starch, and total tract NDF degradation. Similarly, the ATTD of EE and APD of EE were greater in the experimental treatments compared to the CON treatment. However, the highest APD of EE was observed in the EMU treatment, which is in agreement with the results of Guerreiro et al. (2011). This indicates that enzyme addition may be contributed to lowering the positive influence of emulsifier on fat digestibility. On the one hand, after carbohydrase addition, some of the NSP

decomposition products of RSM may negatively influence fat digestion. Cell wall polysaccharides of RSM is strongly bounded by ester bonds or hydrogen bridges and even after degradation may consist of residues unable to further degradation, for instance xyloglucan and cellulose, or residues originating from pectic polysaccharides; rhamnosyl, arabinosyl, uronyl (Pustjens et al., 2013; 2014). On the other hand, Jia et al. (2012) suggested that the response to the addition of multicarbohydrase may vary due to the NSP profile, indicated high content of oligosaccharides and pectic polysaccharides increased AME$_N$ value for broilers. However, the AME$_N$ value in the current study did not differ among the treatments nor did the ATTD of EE on d 35. Additionally, a slight difference observed between the ATTD and APD of EE suggested that the jejunum and the upper ileum are the major sites of fat digestion and absorption in poultry species (Tancharoenrat et al., 2014). It is well accepted that fat absorption is negligible in the lower segments of the GIT in monogastric animals. This partially explains the lack of differences in ATTD of EE at the end of the experiment (d 35). However, differences in ATTD of EE on d 28 occurred in groups supplemented by emulsifier. Presumably, emulsifier by improving fat digestibility, diminished physical covering of nutrients by fat and consequently ensured their enhanced exposure to the digestive and exogenous enzymes (Danicke et al., 1999). This conclusion is also supported by the highest numerical ATTD of EE value on 28 d in the group with both additives. The ATTD of EE on d 28 was lower than that on d 35. It can be speculated that due to the underdeveloped synthesis of bile salts after hatching (Krogdahl, 1985), the EE digestibility is compromised in the early days of a bird's life.

The highest APD of CP and starch indicates the simultaneous action of both additives, while enzyme addition alone did not cause any differences, which agrees with the results of Kocher et al. (2000) and de Vries et al. (2014). Nutrient absorption is precisely related to intestinal villi development. In this study, the villus development was found to be enhanced after the use of additives, which is in agreement with the results of Alzawqari et al. (2011) who observed favorable villus morphology after emulsifier addition to the diet (desiccated ox bile; 0.25% and 0.50% of the diet). The addition

Table 7. Total short-chain fatty acid (SCFA) concentration in cecum digesta in broiler chickens fed control diet (CON), control diet supplemented with emulsifier (EMU), control diet supplemented with enzyme (ENZ), control diet supplemented with emulsifier and enzyme (EMU+ENZ).

Treatment	C2[1]	C3[2]	C4i[3]	C4[4]	C5i[5]	C5[6]	Total
CON	38.8[a]	3.55	0.55	9.21	0.64	0.70	52.8[a]
EMU	37.5[ab]	3.61	0.51	9.48	0.60	0.72	52.4[a]
ENZ	35.6[ab]	3.86	0.52	7.26	0.65	0.58	48.5[ab]
EMU + ENZ	33.4[b]	3.01	0.53	7.81	0.59	0.68	46.0[b]
SEM	0.750	0.159	0.023	0.529	0.050	0.022	1.08
P - value	0.013	0.341	0.829	0.422	0.840	0.311	0.035

[a–c]Means in a column not sharing a common letter are significantly different ($P \leq 0.05$).
[1]Acetic acid.
[2]Propionic acid.
[3]Isobutyric acid.
[4]Butyric acid.
[5]Isovaleric acid.
[6]Valeric acid.

of emulsifier and enzyme may improve the condition of the mucosa due to the enhanced flow in digesta, enabling better dispersal of endogenous bile acids. Bile acids are known for ensuring proper conditions for villus development, for instance; limit endotoxin absorption (Sheen-Chen et al., 2002), or even have the possibility to physical renewal of the damaged mucosa (Kamiya et al., 2004). On the other hand, previous studies revealed that the use of emulsifier (lysolecithin) led to increased deposition of collagen in the villi resulted in increased strength and height of villus and enhanced nutrient absorption due to its incorporation itself into epithelial cells (Wendel, 2000; Mandalari et al., 2009; Brautigan et al., 2017). Improved intestinal mucosa condition increases the villus surface, resulting in better nutrient absorption (Gopinger et al., 2014), which is reflected by the highest APD of CP and starch in the EMU + ENZ treatment.

Inclusion of a higher amount of saturated fats in broiler diets is expected to decrease the availability of minerals, diminishing Ca digestibility due to the formation of insoluble soaps in the gut lumen (Selle et al., 2009; Tancharoenrat and Ravidran, 2014). In this study, the APD of Ca was the highest in the EMU + ENZ treatment; however, the difference was not statistically significant in comparison to the EMU treatment. It can be assumed that emulsifier usage prevents soap formation by enhancing fat retention. Considering the combined use of emulsifier and enzyme, the mode of action is probably similar to that related to the APD of CP: efficient fat decomposition after emulsifier usage enhanced the exposure of nutrients to endogenous and exogenous enzymes and improved micelle formation, making the nutrients more prone to digestion and absorption (Cho et al., 2012). The lowest level of sialic acid in the EMU + ENZ treatment additionally confirmed this hypothesis. In broilers, the epithelium of the GIT is covered in mucins which act as a substrate for intestinal bacteria. Upon breakdown by bacterial enzymes, mucins are converted into sialic acid, which appears as residues in excreta and thus can be used to assess approximate total mucin production and also as an indicator of endogenous losses (Cowieson et al., 2004). Rapeseed NSP has an encapsulating effect ability; it may incorporate starch, protein and other nutrients into its cell wall. This negative effect unable endogenous enzymes to reach substrates, thus, impeding digestion and causing nutrient losses (Raza et al., 2019). However, the combined use of both additives in this study mitigated this effect. Francesch and Geraert (2009) also observed increased Ca retention, which was manifested as higher bone mineralization and Ca percentage of DM after multienzyme addition in their study.

In the present study, the total tract NDF degradation was determined in all the treatments. Generally, analyses of NDF include only the content of nonsoluble fractions of cell wall, such as cellulose, hemicellulose, and lignin, including insoluble NSP, and do not include water-soluble components, which results in the underestimation of the actual total content of NSP (Van Soest et al., 1991). However, soluble NSP components mostly include pectin and β-glucans, which were not considered as the main NSP components in the present study. Meng et al. (2005b) reported that the water-soluble NSP of corn, SBM, and canola meal constitutes only a minority of the total NSP (around 10%). Thus, the total tract NDF degradation may still be considered to represent the NSP content similar to the actual values. In addition to decreasing viscosity, enzymes decomposing NSP dissolving the cell wall matrix of NSP, where nutrients may be incorporated, thus, limit the NSP nutrient-encapsulating ability (Slominski, 2011). Nutrients released from cell walls by the enzyme are more accessible to all digestive enzymes (Choct, 2006; Francesch and Geraert, 2009), as well as to emulsifier in the case of the present study. On the other side, as mentioned above, emulsifier addition probably ensure physical access of NSP enzyme to the substrate due to the enhanced fat digestibility (Danicke et al., 1999). In line with this study, Meng and Slominski (2005) in their in vitro study noticed an improvement in NSP degradation in soybean-maize diets after supplementation with multicarbohydrase (each enzyme added at 0.01 g/g of substrate). Moreover, emulsifier usage presumably has a positive influence on enzyme properties. It has been shown that NIS alter substrate structures by swelling and cracking them (Kim et al., 2006), which leads to enhanced water- and enzyme-holding capacities (Goto et al., 2003) and increased vulnerability to enzymatic attack (Kamande et al., 2000; Kim et al., 2006; Ahn et al., 2009). Additionally, NIS have the ability to stabilize enzymes and reduce enzyme denaturation during the hydrolysis of cellulose by alter its' ultrastructure and make it more prone and accessible to degradation, additionally leading to prolonged enzyme activity (Kim et al., 2006; Ahn et al., 2009). This positive interaction between surfactant and enzyme is not obvious as in general surfactants may cause conformational changes of the protein causing in decreased enzymatic activity (Holmberg, 2018). However, nonionic surfactants (as emulsifier) are consider more gentle in interaction with enzyme as they have no ability to electrostatic attraction (Holmberg, 2018).

Although NDF degradation was enhanced in the EMU + ENZ treatment, some of its' undigested components may still be observed. Based on study of Pustjens et al., (2013; 2014) probable components of NDF unable to further degradation were NSP compounds: xyloglucan, cellulose, or pectic residues. These residues may be considered as a microbiome breakdown substrate. Microbial fermentation mostly occurs in the cecum, where the microorganisms take part in the regulation of intestinal epithelial development (Van der Wielen et al., 2000). In the present study, the addition of enzyme and emulsifier caused a reduction in SCFA (acetic acid) concentration, which, due to the SCFA properties may be consider as negative change. Following Van der Wielen et al. (2000), higher fermentation and presence of SCFA in broiler's ceaca, especially acetic acid, ensure lower pH providing conditions inhibiting

pathogenic bacteria development. However, in the current study bacterial colonization was not altered (data not shown). Rather non-viscous component, such as RSM (Slominski, 2011; Raza et al., 2019) will not evoke an increase in viscosity in the ileum compared to most of the NSP in grains (Zdunczyk et al., 2015b); thus, undigested nutrients will pass to the lower part of the GIT. The enzyme added to the diets may have degraded the NSP of RSM to a substrate fraction that is not preferred by cecum microbiota for fermentation (Slominski and Campbell, 1990), and emulsifier seems to enhance this degradation process. This may be connected with a high content of insoluble polysaccharides and lignin in RSM, known as rather unsusceptible to microbial fermentation in the lower GIT (Bach Knudsen, 1997). Additionally, polyphenolic compounds presented in RSM can act as growth-reducing agents, thus, may diminished gastrointestinal microbiota proliferation (Amarowicz et al., 2001; Negi and Jayaprakasha, 2001).

Thus, the obtained results indicate the mutual effect of emulsifier and enzyme on production parameters and nutrient digestibility in birds fed diets with RSM, as well as on the jejunum morphology, total and free sialic acid excretion, and total cecum SCFA concentration. Overall, both additives did not provoke excessive, compare to the unsupplemented diets, fermentation in the ceca, did not increase production of putrefactive SCFAs as did not increase internal losses of the nutrients by the host. It seems that simultaneous addition of emulsifier and carbohydrases has more beneficial effect on the degradation of NDF than their usage alone, which may be a result of enhancement in substrate accessibility after improved fat digestion.

In conclusion, simultaneously supplemented emulsifier and multicarbohydrase provided favorable conditions for mucosa development, resulting in better nutrient absorption. Enhanced nutrient digestibility diminished endogenous losses, thus improved performance parameters. Studies on diets containing different type of structural carbohydrates and fats are necessary to understand the mode of action of dietary supplementation of enzyme and/or emulsifier. Also, differentiation in time of emulsifier supplementation may be needed to assess dependence of broilers age and effective emulsifier usage. In addition, more detailed analyses explaining changes in structural NSP and their impact on GIT are needed.

ACKNOWLEDGMENTS

DISCLOSURES

The authors declare that they have no competing interests. The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

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