Enzymes and/or combination of organic acid and essential oils supplementation in pasture-fed free-range laying hens increased the digestibility of nutrients and non-starch polysaccharides

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ABSTRACT Pasture intake can be a major challenge for free-ranging hens. This study was conducted to examine pasture digestion and to manage its negative effects. A total of 300 ISA Brown laying hens were used to investigate the effect of time on range (T) in short-term (6 wk) and long-term (12 wk) of 2 range types (R) (gravel vs. pasture) and dietary supplements (F) (T1 = xylanase; T2 = xylanase/beta-glucanase/pectinase/protease; T3 = xylanase/benzoic acid/essential oils) on crude protein, crude fiber, acid detergent fiber, neutral detergent fiber, non-starch polysaccharides (NSP), calcium and phosphorus digestibility, pH of the crop, and ileum digesta viscosity and morphology. Hens exposed to the range for 12 wk had lower ($P < 0.05$) digestibility of crude protein, insoluble rhamnose, ribose, and lower ileal pH compared to hens that ranged for 6 wk. Hens ranging on pasture had lower digestibility ($P < 0.05$) of crude protein, acid detergent fiber, neutral detergent fiber, insoluble arabinose, and insoluble xylose, but higher digestibility ($P < 0.05$) of insoluble mannose and glucose compared to hens that ranged on gravel. Hens fed T2 and T3 had higher digestibility ($P < 0.05$) of CP, acid detergent fiber, and neutral detergent fiber compared to hens fed T1. Hens fed T2 had higher digestibility ($P < 0.05$) of free oligosaccharide arabinose and xylose than those fed T1 or T3 diets. A significant interaction between T × R was detected for crude fiber digestibility and villus height. Digestibility of crude fiber was reduced and villus height was increased in hens ranged on pasture for 12 wk compared to 6 wk. An interaction between R × F was observed on phosphorus and soluble NSP digestibility ($P < 0.05$). Hens fed T2 and T3 diets had lower digestibility of phosphorus and NSP on gravel than on pasture.

In conclusion, pasture consumption impaired the digestibility of nutrients. Supplementing free-range diets with a multi-enzyme or xylanase/benzoic acid/essential oil product reduced these negative effects and increased the ileal nutrient digestibility.

Key words: crude fiber, feed additives, viscosity, digestion, poultry

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INTRODUCTION

Free range poultry with access to pasture often consume grass at quantities resulting in diet dilution and reduced feed intake (Singh and Cowieson, 2013; Ruhnke et al., 2015). This uncontrolled ingestion of fibrous material from the range increases the quantity of non-starch polysaccharides (**NSP**) in the gastrointestinal tract (Svihus and Hetland, 2001). The plant origin NSP in a poultry diet can be classified as soluble and insoluble NSP based on water solubility (Smits and Annison, 1996; Choct, 1997). The water-binding capacity of soluble NSP is associated with increased viscosity in the intestine (Choct, 1997). This can limit enzyme–substrate interactions and ultimately reduce nutrient digestion and absorption. In addition, the presence of undigested nutrients in the gastrointestinal tract can also increase the microbial proliferation, which can be responsible for the production of bile acid deconjugating enzymes (Johnson and Gee, 1981; Feighner and Dashkevicz, 1987, 1988; Edwards et al., 1988; Ikegami et al., 1990; Choct, 1997; Langhout, 1998). The negative effects of overconsumed fiber are not only restricted to the physical barrier for digestion

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and absorption of nutrients, but also the sloughing of intestinal epithelial cells, which may modify the secretion of endogenous proteins, lipids, water, and electrolytes (Johnson and Gee, 1981; Angkanaporn et al., 1994). In contrast, insoluble NSP can have beneficial effects in the gastrointestinal tract, such as increasing the weight and size of the gizzard, pancreas, and liver, as well as increasing the intestinal villus height and subsequently surface area (Yoo et al., 2007; Praes et al., 2011). In addition, it has been estimated that each hen consume up to 60 g grass/day (Ruhnke et al., 2015). This consumption can be accompanied with a decreased passage rate which may result in compaction of the gastrointestinal tract (**GIT**). Therefore, grass consumption for longer periods especially in free-range laying hens ranging on long pasture may have adverse effects on health and performance. However, this aspect should be explored further. The negative effects of soluble NSP can be overcome by supplementing diets with exogenous NSP degrading enzymes. Dietary supplementation with carbohydrase has been reported to improve performance in egg laying hens and free-range broilers (Lázaro et al., 2003; Świątkiewicz and Koreleski, 2006; Buchanan et al., 2007). The improved performance in both layers and broilers is due to reduced chymus viscosity as a result of hydrolysis of soluble NSP and the release of nutrients leading to improved nutrient and energy utilization (Choct et al., 1996; Bedford and Schulze, 1998; Adeola and Bedford, 2004; Ali et al., 2005; Cowieson, 2005). Additionally, beta-glucans are polymers of glucose in the grass cell wall which can be hydrolyzed by β-glucanase activity on the β (1,4) D-glucan chains (Yoo et al., 2007). This allows other exogenous enzymes to access their substrates such as xylose-oligosaccharides, hemicelluloses, mannans, pectins, and increase the efficiency of endogenous enzymes as well (Huo et al., 1993; Cowieson, 2005; Yoo et al., 2007). It has been reported that dietary supplementation with β-glucanase and xylanase increased the NSP digestibility by 83.3% in broilers (Lázaro et al., 2003). A combination of enzymes including xylanase, β-glucanase, and hemicellulase reduced intestinal chymus viscosity, which resulted in improved digestibility of fat (4.4%) and AMEn (2.5%) in hens (Lázaro et al., 2003; Świątkiewicz and Koreleski, 2006). Subsequently, a significant (2.1%) increase in the egg production was observed (Lázaro et al., 2003).

Feeding organic acids (**OA**) such as acetic acid, malic acid, or benzoic acid have been anecdotally reported to reduce grass impaction in egg laying hens in Australia (Ruhnke et al., 2015). In general, OA can be associated with improved body weight gain, reduced feed intake, and subsequently improved feed conversion ratio (**FCR**) in broilers and pigs (Kluge et al., 2006; Józefiak et al., 2007). Similarly, a dietary supplementation with a combination of benzoic acid and essential oils (thymol $\geq$ 10%, eugenol $\geq$ 0.5%, piperine $\geq$ 0.05%) resulted in significantly higher body weight gain, improved FCR, a significantly higher number of lactic acid producing bacteria and lower coliform counts in the cecum of broilers (Giannenas et al., 2014). Increased numbers of lactic acid producing bacteria have been associated with reduced numbers of gastrointestinal pathogens (*Salmonella* Enteritidis), increased nutrient digestibility, and overall better performance of broilers (van der Wielen et al., 2002; Rehman et al., 2006). Crina Poultry Plus® (DSM Nutritional Products, Singapore) is a commercial preparation of benzoic acid, thymol, eugenol, and piperine that has been reported to enhance amylase activity, and increased the digestibility of crude protein (**CP**) and crude fiber (**CF**) (Weber et al., 2012).

The aim of this study was to reduce the negative effects of fiber intake as a result of pasture consumption in free-range hens through the addition of dietary carbohydrase (xylanase, beta-glucanase, proteases, hemicellulases, and pectinases), or a combination of benzoic acid and essential oils (thymol, eugenol, and piperine) on nutrient digestibility, digesta viscosity, and intestinal villus morphometry.

MATERIAL AND METHODS

Birds and Housing

All procedures used in this experiment were approved by the Animal Ethics Committee of the University of New England (AEC 15-009). A total of 300 ISA Brown laying hens were obtained from a commercial rearing farm at the age of 16 wk (flock uniformity of the 300 hens = 89.7%; mean body weight = 1.26 $\pm$ 0.126 kg) and randomly assigned to 30 experimental pens with 10 hens per pen. Half of the treatment groups had a range (adjacent to pens (n = 15)) covered with gravel (**G**), and for the other half (n = 15) the range was covered with pasture (**P**) of cultivated fescue grass (*Festuca arundinacea*), of young growth and 30 cm of height. Each pen was furnished with a perch of 2 m length, a gravity filled tube feeder (Blenheim indoor feeder, Osprey Ltd, Craven Arms, UK), and a bell drinker (BEC 75 hanging drinker, Osprey Ltd, Craven Arms, UK). Feed and water was provided ad libitum. Pens had partially slatted floors (9 m^2) and a 1 m^2 dust bathing area, covered with wood shavings. Hens were adapted to the housing conditions for 14 d prior to range access at the age of 18 wk. The range was available from 9:00 to 19:00 daily. The range was established with green pasture of 30 cm height (*Festuca arundinacea*) at the beginning of trial which was consumed over a period of 12 wk resulting in completely denuded ranges. The outdoor stocking density for the first 6 wk was 1.6 hens/m^2 which decreased to 0.8 hens/m^2 in the second time period after 50% of the hens were sacrificed for the first sampling period. In order to investigate the short and long-term effect of the dietary treatment, as well as the pasture exposure, hens were sampled at 6 and 12 wk after exposure to the range.

Table 1. Ingredient composition and nutrient concentrations of the diets.[1]

Ingredient composition (kg/100 kg)	T1 (Control)	T2 (Multi-enzyme)	T3 (OA & EO)
Wheat	66.12	66.01	66.05
Soybean meal	14.11	14.12	14.12
Limestone	6.04	6.04	6.04
Canola meal	3.92	3.95	3.93
Meat meal	3.76	3.76	3.76
Limestone grit	3.00	3.00	3.00
Canola oil	2.21	2.25	2.23
Sodium bicarbonate	0.20	0.20	0.20
Salt	0.17	0.17	0.17
D,L-methionine	0.16	0.16	0.16
Lysine-HCl	0.11	0.11	0.11
Free-range layer premix[1]	0.10	0.10	0.10
Choline chloride	0.05	0.05	0.05
L-threonine	0.03	0.03	0.03
Ronozyme WX CT[2,5]	0.01	0.00	0.01
Ronozyme Hi Phos 600 FYT[3,5]	0.01	0.01	0.01
Ronozyme Pro ACT[5]	0.00	0.02	0.00
Ronozyme Multigrain[4,5]	0.00	0.01	0.00
Ronozyme VP[5]	0.00	0.02	0.00
Crina Plus[5]	0.00	0.00	0.03
Total	100	100	100
Calculated nutrient concentration			
Metabolizable energy (MJ/kg)	11.63	11.63	11.63
Crude protein (g/kg)	173.70	173.70	173.70
Lysine (g/kg)	8.70	8.70	8.70
Methionine + cysteine (g/kg)	7.40	7.40	7.40
Threonine (g/kg)	6.30	6.30	6.30
Tryptophan (g/kg)	2.20	2.20	2.20
Crude fiber (g/kg)	29.00	29.00	29.00
Ether extract (g/kg)	42.70	43.00	42.90
Calcium (g/kg)	40.00	40.00	40.00
Available phosphorus (g/kg)	4.40	4.40	4.40

[1]Provided mg/kg of diet: Menadione 1.5; Thiamine 1.2; Riboflavin 3; Pyridoxine hydrochloride 2; Niacin 15; Pantothenic acid 6; Folic acid 0.35; Cyanocobalamin 0.01 Biotin 0.065; Ferrous iron 30; Zinc 50; Manganese 50; Copper 6.5; Selenium 0.1; Molybdenum 1; Cobalt 0.2; Provided miu/ton of diet: DL-α-Tocopheryl acetate 10; Retinol 6.5; Cholecalciferol 2.0.

[2]Xylanase activity (analyzed IU/kg) T1 = 132; T3 = 113,[3]Phytase activity (analyzed IU/kg) T1 = 944; T2 = 1054; T3 = 697,[4]Glucanase (analyzed IU/kg) T2 = 157.

[5]DSM Nutritional Products, Singapore.

Experimental Procedure

All 300 hens were randomly allocated to 6 treatments in a 2 × 2 × 3 factorial arrangement. Factors included range substrate (G on range adjacent to 15 pens or P on range adjacent to remaining 15 pens) and dietary supplements (T1 = xylanase, T2 = enzymes: xylanase/beta-glucanase/pectinase/protease, T3 = xylanase/benzoic acid/essential oils: thymol, eugenol and piperine). Subsequently, there were 15 pens on each range substrate and 10 pens on each dietary treatment. The experimental diets were fed for the entire duration of the experimental period. All hens were fed wheat-soybean meal-based diets, containing 0.2% titanium dioxide as an inert digestibility marker. Treatment 1 (T1) included the diet with 0.01% Ronozyme WX (DSM Nutritional Products, Singapore); T2 included the diet with 0.02% Ronozyme ProACT (DSM Nutritional Products, Singapore) plus 0.01% Ronozyme Multigrain (DSM Nutritional Products, Singapore) and 0.02% Ronozyme VP (DSM Nutritional Products). Treatment 3 (T3) included the diet with 0.01% Ronozyme WX (DSM Nutritional Products) and 0.03% Crina Poultry Plus® (benzoic acid, thymol, eugenol, and piperine) (DSM Nutritional Products). Diet composition and nutrient concentrations are presented in Table 1. In addition, time on the range (2 samplings with an interval of 6 wk) was used as an applied treatment as the pasture consumption changed over experimental time period (6 wk: short term, where the range was full of lush green pasture, or 12 wk: long term, where range was almost denuded).

Data Collection

Measurement of non-starch polysaccharides (NSPs). Experimental diets and pasture samples (pasture samples were collected from 5 different locations of each pen before hen placement) and analyzed for free oligosaccharides, soluble and insoluble NSP following the method as described by Englyst and Hudson (1996). Analyzed values are presented in Table 2. Fat was extracted using hexane and 80% ethanol. Samples were centrifuged and the supernatant was used for free oligosaccharides determination, and residues were

Table 2. Analyzed nutrient concentration and non-starch polysaccharides content of grass and diets.

g/kg lyophilisate	Grass	T1 (Xylanase)	T2 (Multi-enzyme)	T3 (OA & EO)
Crude protein	158.10	214.60	217.70	216.43
Crude fiber	205.20	26.83	28.24	26.83
Acid detergent fiber	286.70	28.78	29.23	27.65
Neutral detergent fiber	446.89	10.22	10.43	10.83
Calcium	4.56	45.55	43.77	48.10
Phosphorus	3.78	4.40	4.97	4.94
Free oligosaccharides				
Rhamnose	0.89	0.34	0.20	0.23
Fucose	0.07	0.00	0.00	0.00
Ribose	0.11	0.09	0.10	0.08
Arabinose	1.32	0.42	0.65	0.35
Xylose	1.63	0.33	0.32	0.33
Mannose	2.75	1.36	1.37	1.09
Galactose	5.94	7.16	7.32	7.96
Glucose	30.83	24.44	19.41	23.32
Non-starch polysaccharides	43.50	34.11	29.44	33.44
Soluble NSPs				
Rhamnose	0.42	0.00	0.00	0.05
Fucose	0.25	0.00	0.00	0.05
Ribose	0.47	0.10	0.18	0.15
Arabinose	2.33	2.99	3.22	3.47
Xylose	1.14	4.30	4.73	4.07
Mannose	3.68	2.19	1.95	2.02
Galactose	2.82	1.66	1.74	2.01
Glucose	3.26	1.91	1.98	2.06
Non-starch polysaccharides	12.98	11.70	12.33	12.30
Insoluble NSPs				
Rhamnose	1.75	0.18	0.19	0.19
Fucose	0.70	0.68	0.71	0.71
Ribose	1.04	0.29	0.26	0.30
Arabinose	43.17	20.81	19.00	15.45
Xylose	101.00	17.00	16.63	14.21
Mannose	7.06	0.11	0.10	0.10
Galactose	28.40	2.56	2.54	2.53
Glucose	264.44	1.08	0.73	0.72
Non-starch polysaccharides	399.72	33.87	31.61	26.44

further processed for the determination of soluble and insoluble NSP.

Free oligosaccharides were measured using the supernatants separated at the start of the process. These supernatants were evaporated in a vacuum concentrator (B-Braun Biotech Instruments GmbH, Melsungen, Germany). Hydrolysis of samples was completed by adding 3 mL of 1M H_2SO_4 and heating at 100°C for 2 h on a heating block with continuous string bars (Pierce-Reacti-therm, Rockford, IL). After centrifugation and cooling down to room temperature, supernatant was collected. An aliquot (supernatant) of 0.4 mL was mixed with 0.1 mL 28% ammonia before adding 50 μL of internal standard (allose and inositol each 4 mg/mL) and evaporated in a vacuum concentrator.

The residue, dried under nitrogen, was redissolved in 10 mL acetate buffer and heated at 100°C, while 2 blank tubes (contains 10 mL acetate buffer only) were also added at this stage. After 30 min, samples were subject to α-amylase degradation and incubated overnight after addition of amyloglucosidase. Samples were centrifuged and the supernatant was used for soluble NSP determination, while the residue was used for insoluble NSP determination.

Supernatants were washed using 80% ethanol and absolute ethanol. The precipitate was dried under nitrogen and redissolved in trifluoroacetic acid, heated for 1 h at 125°C, cooled down to room temperature, and internal standard (allose and inositol each 4 mg/mL) was added. Samples were dried and washed twice again and redissolved in water. Two standard tubes were added carrying the standard solution (a mixture of rhamnose, fucose, ribose, arabinose, xylose, allose, mannose, galactose, glucose, and inositol and each sugar weighing precisely 10 mg/mL solution). Samples were dried and acetylated.

The residue was washed twice with water and once with 2 mL acetone. After washing, the samples were dissolved in 1 mL of 12M H_2So_4 with constant stirring at 35°C and diluted in 11 mL of water and heated at 100°C for 2 h. Samples were centrifuged and an aliquot of 0.8 mL was mixed well with 0.20 mL 28% ammonium, and 50 μL of internal standard. The samples were dried and acetylated with soluble and free oligosaccharides samples.

One drop of 3M NH_4OH was added to all samples (soluble, insoluble NSP, and free oligosaccharide) mixed well and 0.3 mL freshly prepared $NaBH_4$ was

added before incubating at 40°C (Ratek, Instruments Pty Ltd, Boronia, VIC, Australia). Afterwards, 250 μL of glacial acetic acid was added to the samples used for soluble NSP determination while 200 μL in samples used for insoluble and free oligosaccharides determination. After cooling down to room temperature, 0.5 mL 1-methylimidazole, 5 mL acetic anhydride, 8 mL water, and 3 mL of dichloromethane were added. Samples were rested until phases were clear. Using a pipette, the bottom layer was carefully transferred into 8 mL tube, evaporated to dryness under nitrogen, and redissolved in 1.2 mL of ethyl acetate and 1.2 mL of water. Samples were centrifuged and top layer was carefully transferred using a pipette into separate vials before loading them onto the gas chromatograph (Varian CP 3800) for reading and compared to standard sugars (a mixture of rhamnose, fucose, ribose, arabinose, xylose, allose, mannose, galactose, glucose, and inositol and each sugar weighing precisely 10 mg/mL solution).

Apparent Ileal Nutrient Digestibility. At 6 and 12 wk, 5 hens per pen were sacrificed via stunning followed by cervical dislocation. The ileal digesta was collected from the distal two-thirds of the ileum (excluding the content localized in the distal 3 cm prior to the ileocecal junction) from all 5 hens and pooled per pen, according to the method of Kluth et al. (2005) and Rezvani et al. (2008). The digesta samples were freeze-dried and homogenized. Weende analysis was performed to determine dry matter, CP, CF, calcium, and phosphorus of the digesta, grass, and feed according to the methods of the Association of Official Analytical Chemists (2005). Excreta samples were collected for 3 consecutive days during week 6 and 12. The excreta samples were freeze-dried and ground using 0.5 mm screen cyclone mill. Alkane analysis was performed to evaluate feed dilution due to pasture consumption following the method described by Singh et al. (2016). Alkane content in grass, feed, and excreta samples was determined using a modification of Mayes et al. (1986) including gas chromatography. The identity of odd chain alkanes (C25 to C33) was determined from their retention times relative to the known standard. The area under the peak for each alkane was determined using an integrator (Model 3393A, Hewlett Packard, Palo Alto, CA), and peak areas were converted to amounts of alkane by reference to the internal standard C32. Fecal recovery of each alkane was calculated as a proportion of the ingested ingredient, which was recovered in the excreta. Diet proportions were estimated using a non-negative least squares procedure in the software "EatWhat" (Dove and Moore, 1995). In this study, 5 odd chained alkanes (C25, C27, C29, C31, and C33) were found in traceable concentrations and used for diet proportion estimates. The excreta concentration of individual alkanes was corrected allowing for incomplete recovery based on published values by Hameleers et al. (1996). Feed dilution calculation and correction of nutrient intake were made based on calculated feed and grass consumption. Titanium dioxide was measured in the feed and ileum digesta samples following the method as described by Short et al. (1996).

Nutrient digestibility including NSP was calculated using the following equation:

$$\begin{aligned}\text{Digestibility coefficients} &= 1 \\ &-\{(\text{TiO2 in feed} / \text{TiO2 in ileal digesta}) \\ &\times (\text{nutrient in ileal digesta} / \text{nutrient in feed})\}\end{aligned}$$

pH Measurement and Viscosity Determination. Ileum and crop content pH was measured using a spear tip piercing pH electrode (Sensorex, CA) following the method described by Morgan et al. (2016). Ileal contents pooled from 5 hens were analyzed for viscosity following the method as described by Rodrigues et al. (2017). Briefly, approximately 5 g of pooled digesta sample was centrifuged at 12,000 × g (Allegra-6R centrifuge, Beckman Instruments, Inc., Fullerton, CA) for 5 min immediately after sampling and viscosity was measured using a rheometer (Brookfield DV-III, Brookfield Engineering Laboratories, Inc., Middleboro, MA). Samples were analyzed in duplicate and averaged for statistical analysis.

Mucosal Morphometry of the Ileum. Mucosal morphometry was assessed in tissues obtained from 2 hens from each pen, after 6 and 12 wk of range access. Approximately 3 cm long sections of ileum tissue were obtained from the distal two-thirds of the ileum. These tissue samples were gently rinsed with saline water before storing in Bouin solution for 8 h. The samples were then transferred into storing cassettes (Livingstone Laboratory Supplies, Rosebery NSW, Australia) and preserved in 70% ethanol. The samples were processed using an automatic tissue processor (Leica TP 1020, Leica Biosystems GmbH, Heidelberger, Nussloch, Germany) and then embedded in paraplast in a single mold (Erdaw et al., 2017). Ten sections per tissue sample were collected; each section (6 μm) was trimmed using a microtome equipped with a feather blade (Feather Safety Razor Co, Ltd, Osaka, Japan). Five sections were used to assess morphometric changes in villus height by staining with Harris' hematoxylin and eosin solutions. The other 5 sections that were assessed to count goblet cells were deparaffinized and stained with Periodic Acid-Schiff and counterstained with hematoxylin solution (modified acc. to Gill III for microscopy) following the standard protocol of Merck (Frenches, NSW, Australia) and as described by Baptista et al. (2009). The slides with stained tissue were viewed on an Olympus BH-2 microscope (Leica Mikroskopie system, GmbH, Wetzlar, Germany) and digitized using video image software (Video Pro, Leading Edge, Bedford Park, Australia) following the method described by Iji et al. (2001). Villus height, crypt depth, apical and basal width, and mucosal depth were measured in each sample. The apparent

Table 3. Effect of time point, range type, and feed additives on ileum digestibility coefficients of nutrients, pH of crop and ileum, and digesta viscosity in free-range laying hens.[1]

g/kg lyophilisate	Time point (T)[2]		Range (R)[3]		Feed additives (F)[4]				*P-value*						
	6 wk	12 wk	Gravel	Pasture	T1	T2	T3	SEM[5]	T	R	F	T×R	T×F	R×F	T×R×F
Crude fiber	0.24	–0.27	0.12	–0.15	–0.19	0.10	0.37	0.061	<0.001	0.034	0.134	0.010	0.140	0.278	0.575
Crude protein	0.75	0.71	0.75	0.71	0.70[b]	0.75[a]	0.75[a]	0.007	0.011	0.019	0.012	0.344	0.734	0.524	0.203
Calcium	0.45	0.45	0.51	0.39	0.42	0.41	0.52	0.03	0.978	0.088	0.273	0.506	0.897	0.886	0.189
Phosphorus	0.47	0.33	0.35	0.46	0.34	0.45	0.42	0.001	0.001	0.006	0.063	0.368	0.573	0.037	0.968
Acid detergent fiber	0.41	0.33	0.46	0.29	0.27[a]	0.37[a,b]	0.48[b]	0.003	0.233	0.004	0.008	0.435	0.413	0.594	0.783
Neutral detergent fiber	–0.37	–0.20	0.01	–0.25	–40.9[a]	5.30[b]	–0.19[b]	0.006	0.175	0.039	0.005	0.096	0.081	0.359	0.993
pH crop	5.00	5.33	5.16	5.16	5.15	5.25	5.09	0.03	<0.001	0.962	0.112	0.108	0.011	0.469	0.577
pH Ileum	7.35	6.90	7.17	7.09	7.13	7.18	7.08	0.03	<0.001	0.146	0.373	0.382	0.154	0.356	0.209
Viscosity(mPas)	3.36	4.29	3.72	3.78	3.77	3.63	3.83	1.10	0.005	0.476	0.392	0.476	0.204	0.061	0.001

Significant interactions are illustrated in Figure 1A–D.

[a-b]Means in each row for each factor (indicating main effects) with different superscripts differ significantly ($P < 0.05$).

[1]Means of 5 replicates with 5 birds per replicate and 25 birds per treatment (n = 150) at each time point.

[2]6 wk = hens had access to the range for the duration of 6 wk, 12 wk = hens had access to the range for the duration of 12 wk.

[3]Gravel = gravel on range, Pasture = *Festuca arundinacea* cultivated on range.

[4]T1 = Xylanase: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %) & Ronozyme Hi Phos 600 FYT (0.006 %).

T2 = Multi-enzyme: Wheat soy based diet supplemented with Ronozyme Hi Phos 600 FYT (0.006 %), Ronozyme Multigrain (0.01 %), Ronozyme ProACT (0.02 %) & Ronozyme VP (0.02 %).

T3 = Xylanase/Organic acid & essential oils: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %), Ronozyme Hi Phos 600 FYT (0.006 %) & Crina Poultry Plus (0.03 %).

[5]SEM = Standard error of mean.

villus surface area was calculated using the following equation:

$$\text{Apparent villus surface area} = (\text{apical width} + \text{basal width}/2) \times \text{villus height}$$

Statistical Analysis. Grubbs' test was used to detect outliers (Barnett and Lewis, 1994). Normal distribution of data was tested using the Kolmogorov-Smirnov test. Data obtained were analyzed using a 2 × 2 × 3 factorial arrangement considering range, diet, and time point (IBM SPSS Statistics version 2.2, SPSS Inc., Chicago, IL). General linear model was used to determine interactions between the factors. When means were significantly different, Tukey's post hoc multiple comparison tests were conducted to differentiate between them. Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Apparent Ileal Nutrient Digestibility

The analyzed values of nutrient contents of feed and grass samples are given in Table 2. The impact of range substrate, feed additives, and range duration on nutrient digestibility is presented in Table 3. CP digestibility was lower in the hens that had been on the range for 12 wk compared to those that had been on the range for 6 wk ($P = 0.011$). Hens ranged on pasture range had significantly reduced digestibility of CP, acid detergent fiber (**ADF**), and neutral detergent fiber (**NDF**) compared to hens that ranged on gravel. Hens fed T2 (multi-enzyme) and T3 (xylanase, OA & EO) diets showed higher digestibility of CP ($P = 0.012$), ADF ($P = 0.008$), and NDF ($P = 0.005$) compared to hens fed T1 diet. A significant interaction between T × R was observed on CF digestibility. Interactions are illustrated in Figure 1. Ranging hens on pasture for 12 wk had significantly reduced CF digestibility compared to hens ranged on pasture for only 6 wk or hens ranged on gravel for 6 or 12 wk. A significant interaction between R × F was observed on phosphorus digestibility. Phosphorus digestibility was significantly improved in hens fed on a multi-enzyme diet or xylanase, OA & EO and ranged on pasture compared to hens fed on xylanase supplemented diets (T1) or hens ranged on gravel (Figure 1B). Feed intake, FCR, and egg production did not differ significantly in hens fed with the different diets, as previously reported (Iqbal et al., 2018). This indicates that the lower digestibility in hens with access to pasture was not severe enough to have an impact on performance. The reason that the digestibility of nutrients was reduced in hens ranged on pasture became obvious when necropsy was performed: hens ranged on pasture had their intestines filled with long grass, while the intestines of hens ranged on gravel were filled with feed (Figure 2A–D). It is well known that the amount of fiber in a diet reduces the digestibility and this has been quantified in monogastrics where increasing cellulose quantities from 0 to 20% reduced organic matter digestibility from 86 to 68% (Brown, 1989). These results of digestibilities with negative values are not surprising as higher dietary fiber in particular soluble and insoluble NSP in free-range laying hens can impact intestinal digestion and absorption processes by increasing the ileum digesta viscosity, slowing down the passage rate, and impairing enzyme and substrate interactions (Choct, 1997; Svihus and Hetland, 2001). Hens fed the xylanase diet in the current study had higher viscosity of digesta compared to hens fed on other dietary treatment. This higher digesta viscosity is associated with higher retention times

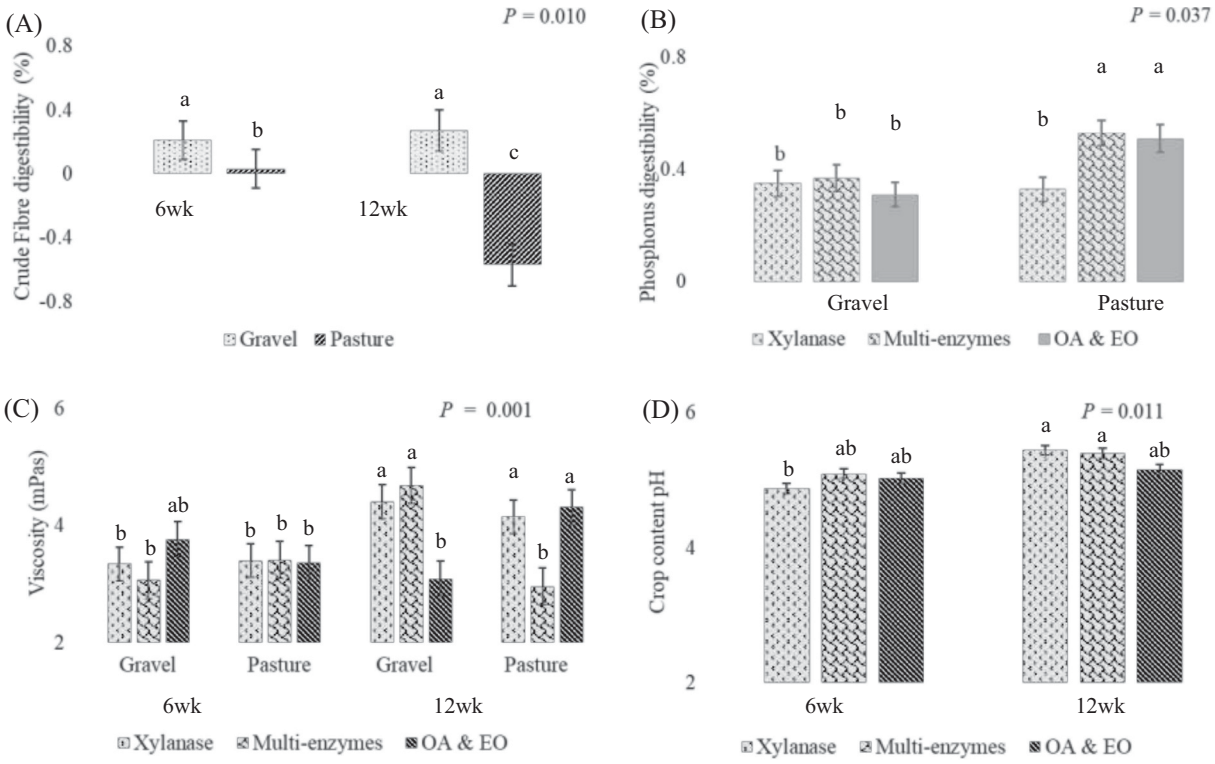

Figure 1. Effect of interaction between time point × range on (A) crude fiber (CF) digestibility, between range × feed on (B) phosphorus digestibility and (C) ileum digesta viscosity (D) pH of crop content.[a,b,c] Indicate that recorded values differ significantly. Error bars indicated the standard error of mean ± average value of 5 replicates with 5 hens per replicate at each time point.

as it slows down the passage rate, impairing enzyme and substrate interactions, thus negatively affecting nutrient digestibility, and thickness of the unstirred water layer adjacent to mucosa, which decreases nutrient absorption (Smits, 1996). Although CP, Ca, and P digestibility values are not negative, CP digestibility values in hens fed on T2 or T3 diets (0.75 and 0.75) were reduced compared to results of CP digestibility values (84%) in broilers fed on corn-soy based diet fortified with similar complex of enzymes (Meng and Slominski, 2005). This reduced digestibility may also be related with difference in substrate, strain of birds, as well as age, as has been reviewed by Aftab (2012). Nevertheless, these values is an indication of impaired digestibility which can also be explained by predicting the enzyme supplementation at a higher dietary rate in free-range layers. As pasture consumption increases the dietary CF concentration, enzyme supplementation at recommended level may not be sufficient to breakdown and digest all the dietary fiber, resulting in negative digestibility values. Supplementing carbohydrase enzymes 5 times more than recommended levels or 500 g/ton instead of 250 g/ton of feed showed improved AMEn and ileal protein digestibility (Kocher et al., 2002). In addition, ileum digesta instead of excreta should be used for alkane analysis in future studies to reduce any ambiguity in feed dilution calculations.

In the present study, hens consumed the entire grass present after 12 wk of range access, resulting in a denuded range. CF digestibility was negative in hens ranged on pasture for 12 wk compared to hens ranged on pasture for 6 wk (Figure 1A). As discussed, these negative values of CF digestibility suggest the accumulation of pasture CF in the GIT over time and indicate the limited capability of hens at digesting grazed pasture. Similar negative digestibility values of pentosans have been reported in broilers and pigs (Pettersson and Åman, 1989; Cadogan and Choct, 2015). This accretion of highly viscous material would also explain the lower nutrient digestibility observed with increasing ranging duration as well as lower nutrient digestibility values in hens ranged on pasture compared to hens ranged on gravel.

However, improved digestibility of CP, NDF, and ADF in hens fed on a T3 diet (OA & EO) can be related to the effects of this blend of OA/EO on pH and modification in intestinal microbial count (Giannenas et al., 2014). Improved digestion of free sugar and soluble NSP by chemical and microbial actions in broilers, cockerel, ducks, and rats has been reported in the absence of carbohydrase enzymes (Carré et al., 1990, 1995). Also, combination of OA and EO has been reported to increase the digestive enzyme activities of both pancreas and intestinal mucosa which could be another explanation of better nutrient digestibility (Wenk, 2003; Jang et al., 2004). Further research is warranted to investigate the underlying cause of improved digestion by OA & EO. Additionally, the T3 diet was also supplemented with xylanase along with OA & EO and higher CF digestibility due to this dietary treatment may be

Figure 2. Gastrointestinal content of the crop (A), gizzard (B), and intestine (D) samples from hens ranged on pasture demonstrates the high amount of fiber retained. In comparison, the gizzard content of a hen ranged on gravel (C) demonstrated high concentrate food only.

related to increased fiber hydrolysis. However, higher digestion in hens fed on T2 diet was obvious as it was supplemented with a combination of enzymes which are better in digesting most of the glucans, pentosans, and other substrates which became available after grass cell wall degradation.

Ileum Digesta Viscosity and pH of Crop and Ileum Content

The results of pH of crop and ileum content as well as ileum digesta viscosity are shown in Table 3. Ileum pH was lower after 12 wk of range access compared to 6 wk of range access ($P < 0.001$). A significant interaction between T × F was observed on crop pH; crop pH was significantly lower in hens fed on T1 diet compared to T2 and T3 after 6 wk of range access or T1 and T2 fed hens after 12 wk of range access compared to T3 fed hens (Figure 1D). A significant interaction of T × R × F was observed on ileum digesta viscosity. Ileum digesta viscosity was only higher in hens fed on T1 and T2 diet and ranged on gravel for 12 wk or in hens fed on T1 or T3 diet and ranged on pasture for 12 wk compared to 6 wk (Figure 1C). The observed lower pH in crop content may be linked with the fermentation process, resulting in higher proliferation of beneficial microbes and higher production of volatile fatty acids (Olukosi and Dono, 2014). The lower pH in GIT has been recognized to act as a strong antimicrobial activity and may have health benefits (Ricke, 2003; Davidson et al., 2013). The water holding property of the soluble NSP tends to increase viscosity in the ileum of free-range laying hens. This increased viscosity was decreased in hens fed on T2 diet and ranged at pasture for 6 wk or 12 wk suggesting the depolymerization of soluble NSP by fortifying the diets with a combination of enzymes. It further revealed that this combination of enzymes is efficient in breaking down the complex structure of soluble NSP allowing for decreased chymus viscosity (Pettersson and Åman, 1989).

Non-Starch Polysaccharides Digestibility

Tables 4–6 illustrate the effect of range type (R), feed additives (F), and the amount of time on the range (T) on digestibility of soluble NSP, insoluble NSP, and free oligosaccharides. Figures 3 and 4 illustrate the interaction effects on free oligosaccharides, soluble NSP, and insoluble NSP digestibility.

Free Oligosaccharide NSP Digestibility

Digestibility of free arabinose was significantly increased in hens fed the T2 diet, compared to T1 and

Table 4. Effect of time point, range type, and feed additives on ileum digestibility coefficients of free oligosaccharides in free-range laying hens.[1]

g/kg lyophilisate	Time point (T)[2]		Range (R)[3]		Feed additives (F)[4]				*P-value*						
	6 wk	12 wk	Gravel	Pasture	T1	T2	T3	SEM[5]	T	R	F	T×R	T×F	R×F	T×R×F
Rhamnose	0.62	–0.91	–0.97	0.69	0.38	–0.08	–0.73	0.321	0.023	0.015	0.384	0.018	0.360	0.347	0.380
Ribose	0.73	0.77	0.81	0.70	0.78	0.75	0.72	0.035	0.611	0.120	0.781	0.296	0.124	0.900	0.765
Arabinose	–0.38	–0.17	–0.33	–0.23	–0.69[b]	0.45[a]	–0.60[b]	0.104	0.321	0.650	<0.001	0.271	0.145	0.958	0.924
Xylose	–1.19	–1.59	–1.81	–0.97	–1.78	–0.35	–2.05	0.260	0.455	0.119	0.017	0.871	0.921	0.241	0.040
Galactose	0.47	0.37	0.35	0.48	0.15	0.56	0.54	0.088	0.592	0.469	0.128	0.086	0.187	0.377	0.320
Glucose	0.84	0.85	0.85	0.85	0.84	0.85	0.86	0.011	0.685	0.974	0.729	0.918	0.660	0.605	0.561
Non-starch polysaccharides	0.66	0.68	0.68	0.65	0.64	0.69	0.67	0.021	0.633	0.530	0.552	0.714	0.431	0.789	0.826

Significant interactions are illustrated in Figure 2A and B.
[a,b]Means in each row for each factor (indicating main effects) with different superscripts differ significantly ($P < 0.05$).
[1]Means of 5 replicates with 5 birds per replicate and 25 birds per treatment (n = 150) at each time point.
[2]6 wk = hens had access to the range for the duration of 6 wk, 12 wk = hens had access to the range for the duration of 12 wk.
[3]Gravel = gravel on range, Pasture = *Festuca arundinacea* cultivated on range.
[4]T1 = Xylanase: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %) & Ronozyme Hi Phos 600 FYT (0.006 %).
T2 = Multi-enzyme: Wheat soy based diet supplemented with Ronozyme Hi Phos 600 FYT (0.006 %), Ronozyme Multigrain (0.01 %), Ronozyme ProACT (0.02 %) & Ronozyme VP (0.02 %).
T3 = Xylanase/Organic acid & essential oils: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %), Ronozyme Hi Phos 600 FYT (0.006 %) & Crina Poultry Plus (0.03 %).
[5]SEM = Standard error of mean.

Table 5. Effect of time point, range type, and feed additives on ileum digestibility coefficients of soluble non-starch polysaccharides in free-range laying hens.[1]

g/kg lyophilisate	Time point (T)[2]		Range (R)[3]		Feed additives (F)[4]				*P-value*						
	6 wk	12 wk	Gravel	Pasture	T1	T2	T3	SEM[5]	T	R	F	T×R	T×F	R×F	T×R×F
Ribose	–0.10	–0.03	–0.06	–0.06	–0.51[b]	0.33[a]	–0.01[a]	0.070	0.632	0.997	<0.001	0.216	0.646	0.177	0.354
Arabinose	0.16	0.22	0.24	0.14	0.08	0.31	0.19	0.057	0.617	0.381	0.305	0.233	0.964	0.020	0.114
Xylose	0.29	0.42	0.42	0.30	0.36	0.45	0.26	0.006	0.292	0.325	0.435	0.205	0.941	0.071	0.239
Mannose	0.79	0.62	0.71	0.70	0.73	0.70	0.68	0.013	<0.001	0.621	0.456	0.558	0.422	0.105	0.036
Galactose	0.10	–0.4	0.28	0.30	–0.13	0.14	0.08	0.046	0.155	0.934	0.066	0.892	0.972	0.026	0.167
Glucose	0.59	0.28	0.46	0.41	0.035	0.57	0.38	0.041	<0.001	0.580	0.082	0.683	0.803	0.049	0.148
Non-starch polysaccharides	0.33	0.30	0.35	0.28	0.26	0.41	0.27	0.043	0.736	0.487	0.292	0.400	0.987	0.023	0.130

Significant interactions are illustrated in Figures 2D and 3A–D.
[a-b]Means in each row for each factor (indicating main effects) with different superscripts differ significantly ($P < 0.05$).
[1]Means of 5 replicates with 5 birds per replicate and 25 birds per treatment (n = 150) at each time point.
[2]6 wk = hens had access to the range for the duration of 6 wk, 12 wk = hens had access to the range for the duration of 12 wk.
[3]Gravel = gravel on range, Pasture = *Festuca arundinacea* cultivated on range.
[4]T1 = Xylanase: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %) & Ronozyme Hi Phos 600 FYT (0.006 %).
T2 = Multi-enzyme: Wheat soy based diet supplemented with Ronozyme Hi Phos 600 FYT (0.006 %), Ronozyme Multigrain (0.01 %), Ronozyme ProACT (0.02 %) & Ronozyme VP (0.02 %).
T3 = Xylanase/Organic acid & essential oils: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %), Ronozyme Hi Phos 600 FYT (0.006 %) & Crina Poultry Plus (0.03 %).
[5]SEM = Standard error of mean.

T3 (Table 4). A significant interaction between T × R was observed on rhamnose digestibility as illustrated in Figure 3A, hens ranged on gravel for 12 wk had significantly reduced digestibility of rhamnose compared to hens that ranged for 6 wk on gravel, or hens ranging on pasture for either 6 or 12 wk. A significant interaction between T × R × F was observed on xylose digestibility; hens fed on T2 diet and ranged on pasture either for 6 or 12 wk had a higher digestibility of xylose compared to those fed T1 or T3 diet and ranged on pasture for any duration (Figure 3B).

Soluble NSP Digestibility

Soluble ribose digestibility was significantly improved in hens fed the T2 diet compared to those fed on the T1 or T3 diet (Table 5). The soluble, arabinose, galactose, glucose, and total NSP digestibility were significantly affected by the interaction between R × F. Hens ranged on pasture and fed the T2 and T3 diet compared to T1 diet had higher digestibility of arabinose, galactose, glucose, and total NSP (Figure 4A–C). Soluble mannose digestibility was significantly affected by an interaction between T × R × F; mannose digestibility was lower only in hens fed on T1 and T2 diet and ranged on gravel for 12 wk or fed on T1, T2, and T3 diet and ranged on pasture for 12 wk (Figure 3D).

Insoluble NSP Digestibility

Digestibility of rhamnose and ribose was significantly lower in hens having access to range for 12 wk (Table 6) compared to 6 wk. Hens ranged on pasture had a lower digestibility of arabinose ($P = 0.005$) and higher digestibility of mannose ($P = 0.002$) and glucose ($P < 0.001$) compared to hens ranged on gravel. An interaction between T × R × F ($P = 0.014$) was observed on xylose digestibility; xylose digestibility was significantly

Table 6. Effect of time point, range type, and feed additives on ileum digestibility coefficients of insoluble non-starch polysaccharides in free-range laying hens.[1]

g/kg lyophilisate	Time point[2](T)		Range[3](R)		Feed additives[4](F)				*P-value*						
	6 wk	12 wk	Gravel	Pasture	T1	T2	T3	SEM[5]	T	R	F	T×R	T×F	R×F	T×R×F
Rhamnose	–0.83	–0.21	–0.59	–0.05	–0.75	–0.50	–0.44	0.098	0.011	0.404	0.839	0.598	0.502	0.575	0.358
Fucose	0.21	0.35	0.32	0.21	0.27	0.26	0.28	0.030	0.076	0.154	0.920	0.315	0.487	0.473	0.571
Ribose	0.67	0.77	0.73	0.70	0.70	0.26	0.28	0.019	0.035	0.707	0.247	0.522	0.300	0.728	0.984
Arabinose	0.24	0.36	0.42	0.14	0.36	0.38	0.15	0.042	0.376	0.005	0.063	0.506	0.627	0.451	0.071
Xylose	0.07	0.17	0.30	–0.11	0.16	0.24	–0.04	0.064	0.940	0.006	0.189	0.252	0.178	0.304	0.014
Mannose	–4.49	–3.45	–5.25	–2.53	–3.78	–4.00	–4.20	0.437	0.145	0.002	0.699	0.242	0.661	0.740	0.343
Galactose	–1.94	–1.22	–1.41	–1.86	–1.71	–1.66	–1.50	0.228	0.222	0.432	0.950	0.645	0.655	0.852	0.418
Glucose	–4.43	–6.73	–8.85	–1.43	–4.27	–6.04	–5.81	0.617	0.499	<0.001	0.141	0.893	0.162	0.700	0.664
Non-starch polysaccharides	–0.17	–0.099	–0.09	–0.19	–0.05	–0.03	–0.30	0.082	0.788	0.731	0.335	0.478	0.257	0.757	0.061

Significant interactions are illustrated in Figure 2C.

[a-b]Means in each row for each factor (indicating main effects) with different superscripts differ significantly ($P < 0.05$).

[1]Means of 5 replicates with 5 birds per replicate and 25 birds per treatment (n = 150) at each time point.

[2]6 wk = hens had access to the range for the duration of 6 wk, 12 wk = hens had access to the range for the duration of 12 wk.

[3]Gravel = gravel on range, Pasture = *Festuca arundinacea* cultivated on range.

[4]T1 = Xylanase: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %) & Ronozyme Hi Phos 600 FYT (0.006 %).

T2 = Multi-enzyme: Wheat soy based diet supplemented with Ronozyme Hi Phos 600 FYT (0.006 %), Ronozyme Multigrain (0.01 %), Ronozyme ProACT (0.02 %) & Ronozyme VP (0.02 %).

T3 = Xylanase/Organic acid & essential oils: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %), Ronozyme Hi Phos 600 FYT (0.006 %) & Crina Poultry Plus (0.03 %).

[5]SEM = Standard error of mean.

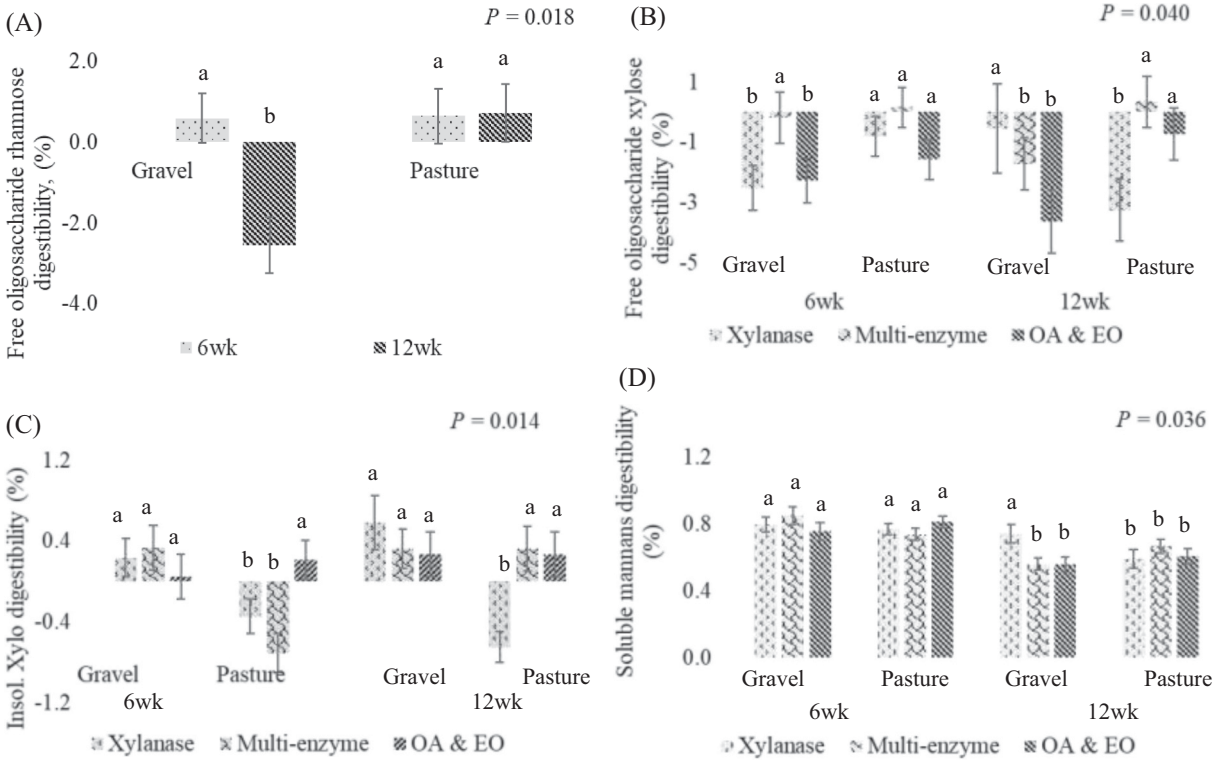

Figure 3. Effect of interaction between time point × range on (A) free oligosaccharides rhamnose digestibility and between time point × range × feed on (B) free oligosaccharides xylose digestibility, (C) insoluble xylose digestibility, and (D) soluble mannans digestibility. [a,b,c] Indicate that recorded values differ significantly. Error bars indicated the standard error of mean ± average value of 5 replicates with 5 hens per replicate at each time point.

lower in hens fed on T1 and T2 diet and ranged on pasture for 6 wk or fed T1 diet and ranged on pasture for 12 wk (Figure 3C).

Higher free oligosaccharide arabinose, and xylose digestibilities and soluble ribose, arabinose, galactose, glucose, and total NSP digestibilities in hens fed on diet fortified with combination of enzymes suggested the efficiency of these enzymes in deoplymerization of CF complex structure. However, enzyme activity is important and enzyme dose-rate concentrations in the free-range require a revaluation based on higher dietary fiber and higher amount of substrate availability in

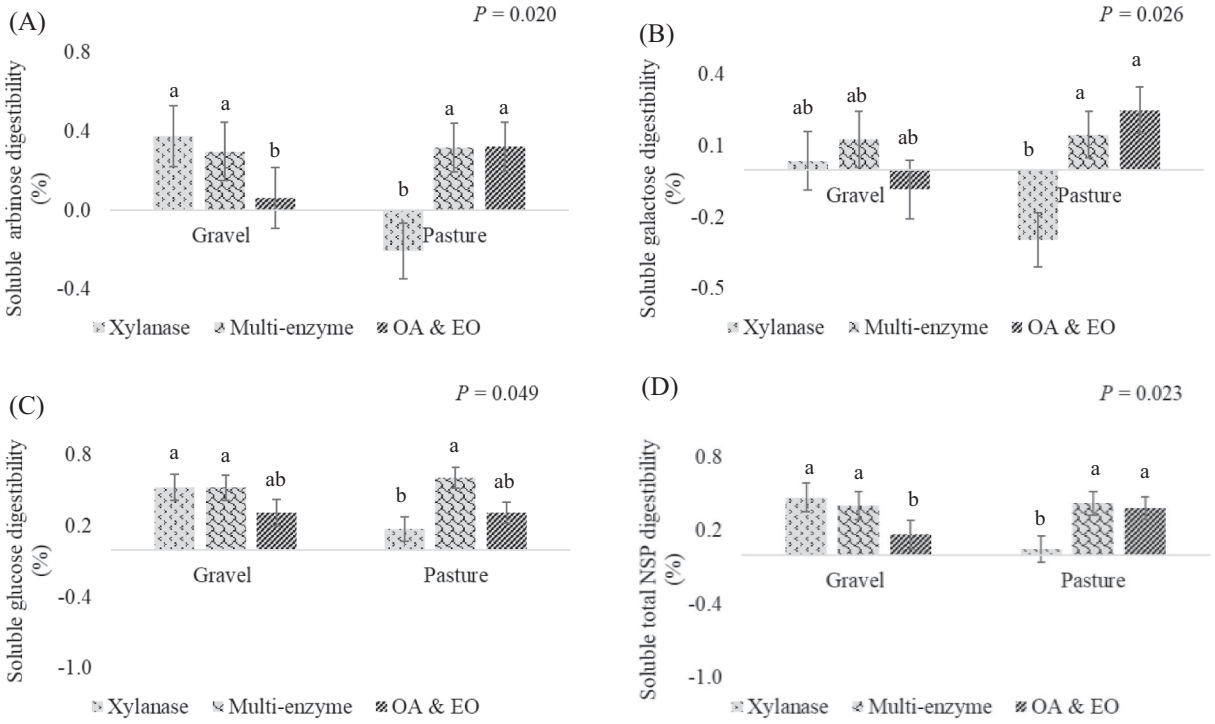

Figure 4. Effect of interaction between range × feed on (A) soluble arabinose, (B) galactose, (C) soluble glucose, and (D) total NSPs digestibility. [a,b,c] Indicate that recorded values differ significantly. Error bars indicated the standard error of mean ± average value of 5 replicates with 5 hens per replicate at each time point.

free-range laying hen farming (Kocher et al., 2002; Aftab, 2012). The observed decrease in the ileal viscosity also suggests that the fortification with the combination of carbohydrase enzymes is useful in the breakdown of soluble NSP complex matrix allowing for decreased chymus viscosity (Pettersson and Åman, 1989). A similar increased digestibility of pectic polysaccharides in in-vitro assays have been reported due to the addition of protease and carbohydrase (Slominski and Campbell, 1990; Simbaya et al., 1996). Additionally, an increase in insoluble NSP digestibility has been reported in broilers fed on soy-based diet supplemented with hemicellulase, pectinase, and β-glucanase (Kocher et al., 2002). A similar increase in NSP solubilization has been reported in in-vitro assay and with the supplementation of endoxylanase and arabinofuranosidase in broilers (Ravn et al., 2017, 2018).

Morphometric Modifications in Ileum Epithelium and Changes in Goblet Cell Count

The effects of range type, feed additives, and amount of time on the range of morphometric factors and goblet cell counts in the ileum tissues are illustrated in Table 7. A significant effect of interaction between T × R × F was observed on mucosal height and villus height. Hens fed on T2 (multi-enzymes) diet and ranged on pasture for 6 wk had lower mucosal height and villus height compared to those ranged on pasture for 12 wk or fed on other diets (Figure 5B and C). Crypt depth was significantly affected by an interaction between T × R. Pasture consumption in hens ranged for 6 wk resulted in lower crypt height compared to hens ranged on the pasture for 12 wk. However, crypt length was decreased significantly in hens ranged on gravel for 12 wk compared to 6 wk (Figure 5A). Similarly, a significant interaction effect between R × F was observed on goblet cell count; hens fed on T3 diets and ranged on gravel had less goblet cell compared to the hens ranged on pasture. While goblet cell counts were reduced in hens fed on T2 diet and ranged on pasture compared to T2 fed hens ranged on gravel (Figure 5D).

Interactive effects of T × F ($P = 0.030$) on crypt height indicated that pasture consumption for the duration of 6 wk significantly reduced the crypt height compared to crypt height in hens fed pasture for 12 wk (Figure 5A). We can speculate that these results are a consequence of lower pasture availability after 12 wk of range exposure. Decreased availability significantly reduced the abrasive effects of fiber on the intestinal epithelium thus increasing the crypt height (Dierick et al., 1989; Jin et al., 1994). In agreement with these findings, previous research suggested approximately 15% reduction in villus length in the jejunum and ileum with the oral administration of cellulose in pre-weaning pigs (Jin, 1992). In addition, abrasive effects of fiber on intestinal morphology and cellulose induced jejunum hypoplasia have been reported in rats (Cassidy et al., 1981; Tasman-Jones et al., 1982; Thomson, 1982; Cassidy et al., 1986). In line with this explanation, enzyme addition in hens

Table 7. Effect of time point, range type, and feed additives on morphometric changes and goblet cells counts in the ileum of free-range laying hens.[1]

	Time point[2](T)		Range[3](R)		Feed additives[4](F)				*P-value*						
	6 wk	12 wk	Gravel	Pasture	T1	T2	T3	SEM[5]	T	R	F	T×R	T×F	R×F	T×R×F
Mucosal height (μm×10^3)	1.045	1.071	1.031	1.085	1.029	1.111	1.026	0.02	0.549	0.216	0.176	0.638	0.350	0.517	0.019
Crypt height (μm×10^3)	0.184	0.182	0.185	0.181	0.180	0.189	0.181	0.04	0.871	0.691	0.739	0.030	0.530	0.579	0.065
Villus height (μm×10^3)	0.861	0.890	0.843	0.907	0.851	0.921	0.854	0.03	0.453	0.091	0.250	0.938	0.573	0.589	0.022
Basal villus width (μm×10^3)	0.030	0.031	0.030	0.031	0.029	0.032	0.031	0.01	0.437	0.425	0.337	0.822	0.539	0.172	0.304
Apical villus width (μm×10^3)	0.029	0.031	0.030	0.030	0.028	0.029	0.032	0.01	0.311	0.936	0.303	0.683	0.784	0.061	0.531
Apparent villus surface area (μm^2×10^3)	25.91	28.25	25.69	28.47	25.38	28.69	27.16	2.50	0.257	0.178	0.445	0.974	0.975	0.318	0.125
Goblet cells (count/ μm)	0.149	0.152	0.148	0.153	0.170	0.152	0.129	0.01	0.803	0.703	0.021	0.723	0.419	0.038	0.658

*Indicates that recorded values differ significantly ($P < 0.05$); Significant interactions are illustrated in Figure 4A–D.

[1]Means of 5 replicates with 2 birds per replicate and 10 birds per treatment (n = 60) at each time point.

[2]6 wk = hens had access to the range for the duration of 6 wk, 12 wk = hens had access to the range for the duration of 12 wk.

[3]Gravel = gravel on range, Pasture = *Festuca arundinacea* cultivated on range.

[4]T1 = Xylanase: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %) & Ronozyme Hi Phos 600 FYT (0.006 %).

T2 = Multi-enzyme: Wheat soy based diet supplemented with Ronozyme Hi Phos 600 FYT (0.006 %), Ronozyme Multigrain (0.01 %), Ronozyme ProACT (0.02 %) & Ronozyme VP (0.02 %).

T3 = Xylanase/Organic acid & essential oils: Wheat soy based diet supplemented with Ronozyme WX CT (0.01 %), Ronozyme Hi Phos 600 FYT (0.006 %) & Crina Poultry Plus (0.03 %).

[5]SEM = Standard error of mean.

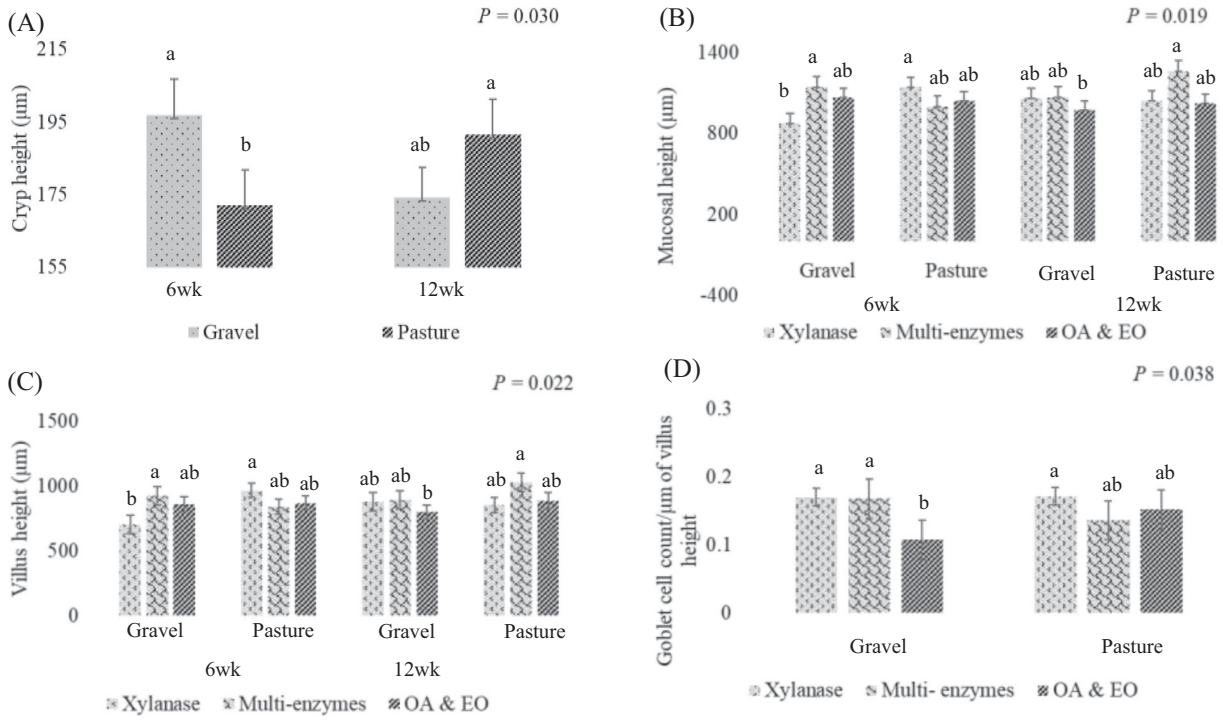

Figure 5. Effect of interaction between time point × range on (A) crypt height, between time point × range × feed on (B) mucosal height, (C) villus height, and between R × F on (D) goblet cell count.[a,b,c] Indicate that recorded values differ significantly. Error bars indicated the standard error of mean ± average value of 5 replicates with 5 hens per replicate at each time point.

ranged on pasture for 12 wk resulted in increased villus and mucosal height (Figure 5B and C) compared with 6 wk ranging on pasture. It has been demonstrated that negative effects of fiber on villus morphometry in laying hens could be reversed with xylanase supplementation to wheat-based diets (Jaroni et al., 1999; Wu et al., 2004). The abrasive effect of dietary fiber can result in an increased mucous production by goblet cell, and increased the mucin layer between lumen content and brush border protecting the enterocytes from mechanical damage (Langhout et al., 1999). In the present study, we observed significantly lower goblet

cell count in hens fed T2 and T3 diet when housed on pasture or T3 when housed on gravel (Figure 5D). These results indicated the reduced need of enterocyte protection due to reduced exposure to abrasive effects of CF. These findings are in agreement with previous research of increased goblet cell count and higher mucin production with the supplementation of carboxymethyl cellulose in pigs and broilers (Smits et al., 2000; Piel et al., 2005). Based on these results, enzyme addition has increased fiber digestibility and reduced its negative effects, resulting in lower goblet cell count in enzyme fed pasture ranged hens in the current study.

CONCLUSION

Exposure of free-range laying hens to pasture significantly impaired nutrient digestibility, and these negative effects worsened over time. Dietary supplementation with a combination of enzymes or combination of organic acids and essential oils resulted in partial depolymerization of complex NSP structure. This NSP degradation reduced the ileum viscosity, increased the nutrient digestibility, and reduced the abrasive effects on epithelium morphometry indicated by higher villus height and less goblet cell count. Future research should aim to investigate the underlying mechanism of improved nutrient digestibility when supplementing a blend of organic acids and essential oils. In addition, enzyme supplementation at higher levels should be considered allowing for higher dietary NSP in free-range laying hens to balance enzyme–substrate interaction.

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