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The Effect of Copra Meal-Hydrolysateon The Broiler Chicken Microbiota

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Abstract Copra meal hydrolysate (CMH), which was β-mannanasehydrolyzed to defat copra meal. The effect of CMH and yeast-MOS on broiler chickens microbiota and their performance were investigated. Supplements of 1 g/kg of CMH and yeast-MOS (commercial diet) were tested, and a basal diet (non-supplement) as the control. The ileum and cecum were collected at 0, 21, 35, and 42 days for the growth of bacterial population which was determined by real-time PCR, and short chain fatty acids (SCFAs) were monitored. At 35 days, the CMH-supplemented diet exerted the greatest effect on *Lactobacillus* population in the ileum ($p<0.05$). Acetate was only detected in chickens that consumed a diet supplemented with CMH at 35 days ($p<0.05$). The 21-days of ileum and cecum samples from chickens that consumed the CMH supplemented diet exhibited a greater reduction in population of Enterobacteriaceae ($p<0.05$). The CMH-supplemented diet in 35-days, thechickens exerted the greatest effect on *Lactobacillus* population in the cecum with no significant differences compared to other groups. Supplementation and nonsupplementation had no effect on *Pseudomonas* concentrations in chicken ileum and cecum from day 0 to day 42. No Campylobacter and Bifidobacterium groups were detected.

Keywords: Broiler chickens, Copra meal hydrolysate**,** Microbiota**,** Real-time PCR, Short chain fatty acids

Introduction

The International Scientific Association of Probiotics and Prebiotics (ISAPP) define a dietary prebiotic as 'a selectively fermented ingredient that resulted in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health' (Gibson *et al.*, 2010).

Many researchers have established prebiotics for animal feed supplementation, includingmannooligosaccharides (MOSs) from the cell wall

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of *Saccharomyces cerevisiae* (Jones and Ballou, 1969; Spring *et al.*, 2015). MOSs from yeast cell walls are widely applied as an animal feed additive in poultry production**.** Yeast-MOS has also been shown to improve growth performance and increase the intestinal immunoglobulin secretion of broiler chickens (Hooge and Connolly, 2011; Iji *et al.*, 2001). Benites *et al.* (2008) evaluated the effects of dietary MOSs from commercial products. Birds fed Bio-Mos at 1.0/0.5/0.5 (starter/grower/finisher) kg/ton diets had significantly greater body weight (BW) at market ages compared with unsupplemented diets. Addition of MOSs significantly decreased aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in the blood. Mean total cholesterol was significantly lower in the 0.05% MOS-fed group (Yalçinkaya *et al.*, 2008).

Aside from yeast cell walls, MOSs can also be extracted from plant cell walls such as copra (Titapoka *et al.*, 2008; Ghosh *et al.*, 2015; Pangsri *et al.*, 2015a,b). Copra meal, or coconut residual cake, is the dried meal that remains as the main by-product after coconut milk extraction. Saittagaroon *et al.* (1983) reported the percentage proximate composition of copra meal on a dry weight basis as follows: carbohydrate (43-45%), crude protein (19-20%), crude fat (10-11%), and crude fiber (12%). The non-starch polysaccharide of copra meal exists as mannan (26%), galactomannan (61%), and cellulose (13%) (Balasubramaniam, 1976). The galactomannan in copra meal is composed of repeating β-(1,4)-mannose units and a few α-(1,6)-galactose units attached to a β-(1,4)-mannose backbone (Ghosh *et al.*, 2015; Hossain *et al.*, 1996). Copra meal cannot be utilized by monogastric animals and humans because the high level of mannan makes it resistant to the digestive enzymes in the gastrointestinal tract. However, the enzyme β-1,4-mannanase can digest complex mannan polysaccharides to form MOSs. Previously published data detailed the use of β-mannanase from *Bacillus circulans*NT 6.7 in the hydrolysis of copra meal. Copra meal hydrolysate (CMH) was assessed in pure culture and successfully promoted the growth of *Lactobacillus* bacteria. CMH did not support the growth of harmful bacteria such as*Shigelladysenteriae* DMST 1511, *Staphylococcus aureus* TISTR 029 and *Salmonella entericaserovarEnteritidis*DMST 17368 (Pangsri *et al.*, 2015a). Therefore, mannan-oligosaccharide from copra meal that could potentially be developed into prebiotics for animal feed supplement. Ibuki *et al.* (2014a) observed the effect of mannanase-hydrolysed copra meal (MCM) containing β-1,4-mannobiose (MNB) on growth performance and intestinal histology in broiler chickens. Dietary MCM also significantly increased the weights of body, breast muscle, and thighs in broiler chickens (*Gallus gallusdomesticus*), whereas the weights of abdominal adipose tissue and liver were not affected (Ibuki *et al.*,

2014b). The microflora in the gastrointestinal tracts of chicken play an important role in health and growth performance (Wielen *et al.*, 2002; Yeoman *et al.*, 2012).Nutrient absorption occurs in the final section of the small intestine (ileum) and the cecum is an important site of bacteria fermentation (Gong *et al.*, 2007; Józefiak *et al.*, 2004).

Objective: *in vivo* experiment was to investigate the effect of CMH on ileum and cecum chicken microbiotausing a real-time PCR technique.

Materials and methods

Animal care

The experimental procedure was approved by The Institutional Animal Care and Use Committee at Kasetsart University.

Diet and feed additives

Three experimental diets were set in the *in vivo* study.Two types of feed additives were used, and a copra meal-hydrolysate was prepared using 1.0% defatted-copra meal in 20 units/ml crude mannanase enzyme following the method described byPangsri *et al.* (2015a). Yeast mannooligosaccharide provided by Alltech, Inc., Thailand (Actigent^{TM}, Alltech Inc., Kentucky, USA) was used as the prebiotic reference.The conventional diets were provided as the basal diet (control) (Bangkok Animal Research Center Co., Ltd. (BARC), Thailand).

Animals and diets

One hundred ten newly hatched male broiler chicks of a commercial strain (Arbor Acres Plus) were used in this study (Bangkok Animal Research Center Co., Ltd. (BARC), Thailand). Twenty 4-day-old birds were randomly selected, chickens were sacrificed by decapitation, and their ileal and cecal samples were collected.Each segment was pooled and transferred to a 15 ml centrifuge tube and immediately kept at -20°C for molecular analysis. The remaining 90 birds were randomly divided to 3 treatments. Each treatment comprised 3 replicates and 10 birds with an equal mean body weight in each replicate. The chickens were housed on solid floor pens covered with rice chaff.

Chickens in the control group were fed with the basal diet; a practical corn-soybean meal was formulated and used as the basal diet for each growing phase, whereas the chickens in the treatment group were fed the same basal diet

supplemented with 0.1% copra meal hydrolysate and 0.1% yeast-MOS (1 g supplemented/kg basal diet). The composition and calculated nutrient content of the basal diet for each growing phase are presented in Table 1. The experimental starter diets were administered from the first day of age until day 21, the grower diets were administered from day 21 until day 35, and the finisher diets were administered from day 35 until day 42. All the diets were processed with a conditioning temperature of 82°C and a 3-mm-diameter pellet size. During the first 12 days, feeds were provided to birds in crumble form and then in pellet form thereafter until completing the 42-day test period.

Table 1. Composition and nutrient contents of the experimental diets

Ingredient	**Starter % (0-21 days)**	**Grower % (21-35 days)**	**Finisher % (35-42 days)**
Corn	50.85	57.35	56.44
SBM 49% dehulled	32.80	21.26	18.88
Rice bran, S.E.	4.00	6.00	8.00
Full fat soybean	3.00	4.00	5.00
Rapeseed meal	2.00	3.00	3.00
Soybean oil	3.00	4.00	4.50
Monocalcium phosphate	1.64	1.65	1.50
Limestone 39.9%	1.20	1.30	1.35
Salt	0.36	0.25	0.28
Sodium bicarbonate	0.20	0.20	0.20
Choline chloride 60%	0.07	0.04	0.02
BS premix*	0.20	0.20	0.20
L-Lysine HCl	0.15	0.19	0.13
DL-Methionine	0.18	0.19	0.17
L-Threonine	0.00	0.02	0.03
Pellet binder	0.30	0.30	0.30
Sacox (Salinomycin 12%)	0.05	0.05	0.00
Total	100	100	100

* Premix Broiler Starter 1 kg/ton (Each kg contains): Vitamin A/D3 12,000,000 IU, Vitamin E 20.00 g, Vitamin K 2.45 g, Vitamin B1 1.90 g, Vitamin B2 4.99 g, Vitamin B6 1.94 g,Vitamin B12 0.02 g, Niacin 49.00 g, Cal-D-Pan 14.78 g, Biotin 0.05 g, Folic acid 0.98 g, Copper 9.00 g, Ferrous 38.75 g, Manganese 60.00 g, Zinc 45.00 g, Iodine 0.75 g, Selenium 1.00 g, Antioxidant 2.50 g.

Management and sampling

The experiment was conducted in a close-sided house with tunnel ventilation and evaporative cooling system using rice hulls as the bedding material. Each pen measured 1 m x 1 m and was equipped with a self-feeder and 3 nipple water drinkers. Feeds and water were provided *ad libitum*.Birds were

maintained under the lighting and management programs according to the Arbor Acres Plus broiler management manual. All birds were vaccinated for Newcastle and infectious bronchitis diseases at 7 days and Gumboro disease at 14 days of age.

The total pen feed consumption was recorded weekly. The body weight of birds was measured as a pen basis at starting and on days 21, 35 and 42. After body weight measurement at 21, 35 and 42 days, one bird from each pen with a body weight close to the pen average was selected. Chickens were randomly selected and were euthanized by decapitation, and ileum and ceca samples were collected. The gut samples were immediately stored in anaerobic boxes and preserved on ice until required for further analysis within 1-2 h post-collection.The gut samples were immediately stored in anaerobic boxes and preserved on ice until required for further analysis. Body weight gain, feed intake, feed conversion ratio and livability were calculated and were subjected to analysis of variance as randomized complete block design.

Short-chain fatty acid analysis

The samples collected from the ileum and cecum, were suspended in 900 µl of PBS buffer (PBS; 0.1 mol/liter, pH 7.4) (Oxoid, Basingstoke, United Kingdom) using Cytobrush® Plus (CooperSurgical, Berlin, Germany). The sample solution was then transferred to a 2-ml tube and centrifuged at 13,000 × *g* for 5 min. The supernatants were filtered through a 0.22 µm filter unit (Millipore, Cork, Ireland), and 20 µl was injected into an HPLC system (Waters 1525 Binary HPLC, Milford, Massachusetts, USA) equipped with a UV detector (Waters 2489 UV visible detector, Milford, Massachusetts, USA). The column used was the ion-exclusion Aminex HPX-87H (300 × 7.80 mm; Bio-Rad, California, USA), maintained at 50°C. The mobile phase was 5 mM H_2SO_4 in HPLC-grade water, and the flow rate was 0.6 ml/min. The samples were quantified using calibration curves of lactate, formate, acetate, propionate, and butyrate at concentrations of 1.0, 10, 20, 40, 80 and 100 mM. 2-Ethyl butyric acid (Sigma-Aldrich, United Kingdom) at a final concentration of 20 mM was the internal standard.

DNA extraction

Genomic DNA was extracted from each sample by using a combination of bead-beating procedures as described previously by Sakamoto *et al.* (2011) with modifications to lyse cells and using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) to purify the DNA. One milliliter of culture

samples obtained from each bottle at each sampling time point was centrifuged at 15,000 rpm for 5 min. The pellet was washed twice in 1 ml filtered sterilized phosphate buffered saline (PBS; 0.1 mol/liter, pH 7.4) (Oxoid, Basingstoke, United Kingdom) and resuspended in 900 µl of PBS buffer. The sample solution was then transferred to 2-ml screw-capped tubes containing 0.3 g of sterile zirconium/silica beads (0.1 mm in diameter, Biospec Product, Inc., Bartlesville, OK, USA) and 300 µl of phenol-chloroform-isoamyl alcohol (25:24:1); then, the mixture solutions were lysed by shaking at 2,700 rpm for 180 s using a minibead beater (Biospec Products, Inc., Bartlesville, OK, USA). The extracted DNA was collected by centrifugation at 13,000 rpm for 5 min and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Finally, the DNA pellet was resuspended in TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 8.0), and the DNA concentration was determined by the NanoDrop 1,000 microphotometer (Nanodrop Technologies, Wilmington, DE, USA). The DNA samples were stored at -20°C and used as template DNA in the real-time PCR analysis.

Real-time PCR analysis

Construction of standard DNA plasmids

The targeted groups and all specific primers sequences used are listed in Table 2. The standard curves of total bacteria, *Lactobacillus* group, *Campylobacter*, *Acinetobacter*, *Pseudomonus*, *Bifidobacterium*, *Bacteriod–Prevotell-Porphyromonas*group, *Clostridium coccoides –Eubacterium rectal* group, *Clostridium perfringens*group, *Enterobacteriaceae*, *Faecalibacteriumprausnitzii – Subdoligranulumvariabile* and *Enterococcus* sp. were constructed using the real-time specific primers to amplify the genomic DNA of *Escherichia coli* TISTR 527, *Lactobacillus salivarius*KUB-AC21*, Campylobacter jejuni* ATCC 33291, *Acinetobactercalcoaceticus*TISTR 360, *Pseudomonas* sp. TISTR 1249, *Bifidobacteriumbifidum* JCM 1255, *Bacteroidesfragilis* ATCC 25285, *Ruminococcusprodutus*JGD 07421, *Clostridium perfrigens* ATCC 13124, *Salmonella Typhimurium*TISTR 292,*Faecalibacteriumprausnitzii*DSM 17677 and *Enterococcus* sp. TISTR 927, respectively (Nakphaichit *et al.*, 2011). The PCR products of *Lactobacillus salivarius*KUB-AC21*, Campylobacter jejuni* ATCC 33291, *Acinetobactercalcoaceticus*TISTR 360, *Pseudomonas* sp. TISTR 1249, *Bifidobacteriumbifidum* JCM 1255, *Salmonella Typhimurium*TISTR 292 and *Enterococcus* sp. TISTR 927 were cloned separately into pGEM-T Easy vector according to the manufacturer's instructions (Promega, Madison, USA).

The PCR products of *Bacteroidesfragilis* ATCC 25285, *Ruminococcusprodutus*JGD 07421 and *Clostridium perfrigens* ATCC 13124 were cloned into the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedure(Nakphaichit *et al.*, 2011). The recombinant plasmids of *Escherichia coli* TISTR 527 were constructed following the method of La-ongkham *et al.* (2015) and the recombinant plasmids of *Faecalibacteriumprausnitzii*DSM 17677 were constructed following Ruengsomwong *et al.* (2014).

Real-time PCR

The PCR amplification and detection were performed using the LightCycler®480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). Each reaction mixture was performed with a final volume of 20 µl in a 96-well PCR plate, consisted of 10 µlof 2× LightCycler®480 SYBR Green I master mix (Roche Applied Science, Mannheim, Germany), 2 µl of a specific primer mix (5 µM each), 6 µl of PCR-grade water and 2 µl of DNA template (50-100 ng from each sample),whereas the negative controls (where the template DNA was replaced with PCR-grade water) and standards were run in duplicate. The amplification program consisted of one cycle of 95°C for 5 min for initial denaturation followed by 45 cycles of denaturation at 95°C for 10 sec, the primer-specific annealing temperature (Table 2) for 10 sec and extension at 72°C for 4-17 sec (the extension time was calculated by dividing the target amplicon size by 25 according to Roche's recommendation).

Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 21.0; SPSS, Inc.). Univariate analysis of variance (ANOVA) and post hoc Tukey's tests were used to determine significant differences according to the substrate used on the bacterial group population and SCFA production. The differences were considered significant at $P<0.05$.The differences between bacterial numbers and SCFA production at 0, 21, 35, and 42 day-old chickens of each substrate fermentation were checked for significance by paired t-test.

Table 2. Specific primers for the target bacteria

Target	Primer	Sequence (5′–3′)	Size	References
Total bacteria	Q-968F	WACGCGARGAACCTTACC	442	(Nakayama, 2010)
	Q-1390R	TGACGGGCGGTGWGTAC		
Lactobacillus group	LbF	AGCAGTAGGGAATCTTCCA	341	(Walter *et al.*, 2001)
	LBR	CACCGCTACACATGGAG		(Heilig *et al.*, 2002)
Campylobacter spp.	Cam-F	GGATGACACTTTTCGGAG	246	(Rinttilä *et al.*, 2004)
	Cam-R	AATTCCATCTGCCTCTCC		
Acinetobacter	Ac-F	TTTAAGCGAGGAGGAGG	240	(Vanbroekhoven *et al.*, 2004)
	Ac-R	ATTCTACCATCCTCTCCC		
Pseudomonas	Pse-F	GGCGACGATCCGTAAC	180/440	(Khan and Yadav, 2004)
	Pse-R	CCTTCCTCCCAACTT		
Bifidobacterium spp.	Bif-F	TCGCGTC(C/T)GGTGTGAAAG	243	(Rinttilä *et al.*, 2004)
	Bif-R	CCACATCCAGC(A/G)TCCAC		
Bacteroides–Prevotella-Porphyromonas	BacP-F	GGTGTCGGCTTAAGTGCCAT	140	(Rinttilä *et al.*, 2004)
	BacP-R	CGGA(C/T)GTAAGGGCCGTGC		
C. coccoides–E. rectal group	Clos-F	CGGTACCTGACTAAGAAGC	429	(Rinttilä *et al.*, 2004)
	Clos-R	AGTTT(C/T)ATTCTTGCGAACG		
C. perfringens group	Perf-F	ATGCAAGTCGAGCGA(G/T)G	120	(Rinttilä *et al.*, 2004)
	Perf-R	TATGCGGTATTAATCT(C/T)CCTTT		
Enterobacteriaceae	En-F	CATTGACGTTACCCGCAGAAGAAGC	195	(Bartosch *et al.*, 2004)
	En-R	CTCTACGAGACTCAAGCTTGC		
Faecalibacteriumprausnitzii-Subdoligranulumvariabile	F_Faec	ACCATGAGAGCCGGGGGG	100	(Lund *et al.*, 2010)
	PROK1492R-T	GGTTACCTTGTTACGACTT		
Enterococcus spp.	Ent-F	CCCTTATTGTTAGTTGCCATCATT	144	(Rinttilä *et al.*, 2004)
	Ent-R	ACTCGTTGTACTTCCCATTGT		

Results

Growth performance

The effects of CMH on broiler performance are shown in Table 3. When considering the starter and finisher periods together (day 0 to day 42 of age), the body weight gain was significantly higher with the control diet treatment than with the yeast-MOS and CMH treatments (P<0.05). Feed intake in the CMH treatment group was not significant higher than in the yeast-MOS and control groups. The feed conversion ratios (FCR) in all treatments were not significantly different, but the CMH treatment group was better than the control diet group. The highest livability rate from 0 to 42 days of age was in the CMH treatment group (100%).

Table 3. Effect of copra meal hydrolysate and yeast-MOS onbroiler performance[1] (0-42 days of age)

Treatment			Initial body weigh	Final weigh gain	Body weigh gain	Feed intake	Feed conversio ratio[2]	Livabilit
Grou	Diet	Supplementatio (%)	(g)	(g)	(g)	(g)		(%)
1	Diet	Copra meal	43	3441 [b]	3398 [b]	5304	1.561	100.00
2	Diet	Yeast-MOS	43	3371 [b]	3328 [b]	5243	1.575	96.67
3	Contro	-	43	3564 [a]	3521 [a]	5389	1.531	96.67
P-value				0.016	0.016	0.252	0.1564	0.4444
Pooled SEM				26.54	26.65	52.19	0.128	1.925
C.V.%				1.33	1.35	1.70	1.43	3.41

[a,b] The means within columns with no common superscript differ significantly (P<0.05).
[1] Male broilers (Arbor Acres plus).
[2] Feed conversion ratio corrected for mortality and culls.

Bacterial enumeration in ileum chicken intestine

Result showedthe microbial populations at 0, 21, 35 and 42 day of age in broiler chickens. Twenty 4-day-old ileum samples were pooled and were used as the baseline (0 day) for bacterial enumeration (Tables 4, 5, 6 and 7).The *Pseudomonas* population in all substrate tests exhibited a slight decrease in the chicken ileum from day 0 to day 42. Neither supplementation nor

nonsupplementation affected the *Pseudomonas* concentration.No *Campylobacter* and *Bifidobacterium* groups were detected in any sample in this experiment.

In 21-day-old chickens,the total bacteria were significantly higher in the ileums of the control group than in the ileums from the CMH and yeast-MOS supplemented diet. There were no statistically significant differences in the population changes of *Lactobacillus*, *Enterococcus* sp. and *Acinetobacter* between the chickens in the supplement diet group and those in the control diet group ($P<0.05$) at 21-days. *Bacteroides*was significantly ($P<0.01$) decreased with CMH and yeast-MOS treatments (4.32±0.06 and 4.36±0.22 log copy number of gene/g ileum, respectively) compared with the control treatment (8.98±1.36 log copy number of gene/g ileum). *Bacteroides, Enterobacteriaceae*, *Faecalibacteriumprausnitzii*, *Clostridium coccoides*, and *Clostridium perfringens* were significantly decreased populations with the CMH and yeast-MOS supplemented diets compared with the control diet ($P<0.01$).

By day 35, the highest total bacteriaachieved were in the ileums from chickens that consumed the CMH supplement diet ($P<0.05$). The CMH supplemented diet yielded the greatest effect on the *Lactobacilllus* population in the ileumpart, while yeast-MOS was the second highest with respect to the *Lactobacilllus* population.Therewas a significant difference between groups ($P<0.05$) compared with the control diet. Thirty-five-day-old ileums from chickens that consumed the CMH supplemented diet showed lower levels of *Acinetobacter, Bacteroides, Enterobacteriaceae*, *Faecalibacteriumprausnitzii*, *Clostridium coccoides*, and *Enterococcus* sp.populations compared with the ileums from the yeast-MOS supplemented and control diet groups, but without statistically significant differences between diet groups ($P>0.05$). No statistically significant difference between the three diet groups was observed; the level of the *Pseudomonas*population at all time points remained stable compared with the 0-day-old ileum samples. No significant difference was detected in the *Clostridium perfringens*population with all treatments tests in 35-day and 42-day chickens.

Table 4. Changes in the bacterial population of the intestinal samples (log copy number of gene/g ileum or cecum) from 0-day-old chickens

Target bacteria	Bacterial population (log copy number of gene /g)					
	Ileum			Cecum		
	Control diet	CMH	Yeast-MOS	Control diet	CMH	Yeast-MOS
Total bacteria	7.88± 0.20 [a]	7.88±0.20 [a]	7.88±0.20 [a]	8.77±0.20 [a]	8.77±0.20 [a]	8.77±0.20 [a]
*Lactobacillus*group	4.08±0.23 [a]	4.08±0.23[a]	4.08±0.23 [a]	3.73±0.04 [a]	3.73±0.04 [a]	3.73±0.04 [a]
Acinetobacter	6.93±0.26[a]	6.93±0.26[a]	6.93±0.26 [a]	6.39±0.10 [a]	6.39±0.10 [a]	6.39±0.10 [a]
Pseudomonas	4.21±0.08[a]	4.21±0.08[a]	4.21±0.08 [a]	3.98±0.09 [a]	3.98±0.09 [a]	3.98±0.09 [a]
Bacteroides – Prevotella-Porphyromonas	6.37±0.58 [a]	6.37±0.58 [a]	6.37±0.58 [a]	7.29±1.50[a]	7.29±1.50[a]	7.29±1.50[a]
C. coccoides–E. rectal group	3.46±0.63 [a]	3.46±0.63 [a]	3.46±0.63 [a]	2.84±0.77 [a]	2.84±0.77 [a]	2.84±0.77 [a]
C. perfringens group	7.48±0.26 [a]	7.48±0.26 [a]	7.48±0.26 [a]	8.37±0.07 [a]	8.37±0.07 [a]	8.37±0.07 [a]
Enterobacteriaceae	8.38±0.18 [a]	8.38±0.18 [a]	8.38±0.18 [a]	8.38±0.18 [a]	8.38±0.18 [a]	8.38±0.18 [a]
Faecalibacteriumprausnitzii	2.07±0.50 [a]	2.07±0.50 [a]	2.07±0.50 [a]	3.24±1.08 [a]	3.24±1.08 [a]	3.24±1.08 [a]
Enterococcus spp.	8.63±0.23 [a]	8.63±0.23 [a]	8.63±0.23 [a]	9.08±0.13 [a]	9.08±0.13 [a]	9.08±0.13 [a]

Values are expressed as means bacterial population ± standard deviations (n=3). [a, b, c] Difference letters indicate significant differences between treatment at the same time point (a > b > c, $P< 0.05$).

*, ** significant with respect to the control diet group at the same time point (*, $P<0.05$; **, $P<0.01$).

Table 5. Changes in the bacterial population of the intestinal samples (log copy number of gene/g ileum or cecum) from 21-day-old chickens

Target bacteria	Bacterial population (log copy number of gene /g)					
	Ileum			Cecum		
	Control diet	CMH	Yeast-MOS	Control diet	CMH	Yeast-MOS
Total bacteria	9.68±0.15 [a]	6.33±0.80 [b**]	6.18±0.89 [b**]	9.22±0.36 [a]	9.03±1.27 [a]	9.79±0.30 [a]
*Lactobacillus*group	6.31±1.05 [a]	5.92±1.82 [a]	5.72±1.64 [a]	5.94±1.06 [a]	5.56±1.58 [a]	6.54±1.12 [a]
Acinetobacter	7.00±0.38 [a]	7.42±0.05 [a]	7.45±0.12 [a]	6.73±0.81 [a]	6.58±0.82 [a]	6.41±0.41 [a]
Pseudomonas	3.93±0.61 [a]	3.84±0.08 [a]	4.17±0.15 [a]	4.00±0.35 [a]	3.27±0.83 [a]	3.82±0.27 [a]
Bacteroides – Prevotella-Porphyromonas	8.98 ± 1.36 [a]	4.32 ± 0.06 [b**]	4.36 ± 0.22 [b**]	8.67±1.33 [a]	7.81±2.75 [a]	9.54±0.89 [a]
C. coccoides–E. rectal group	9.71±0.37 [a]	5.16±0.70 [b**]	5.09±0.21 [b**]	9.19±0.32 [a]	9.19±1.14 [a]	9.57±0.42 [a]
C. perfringens group	5.67±0.04 [a]	4.91±0.07 [b*]	4.71±0.43 [b**]	5.30±0.27 [a]	5.31±0.35 [a]	5.92±0.86 [a]
Enterobacteriaceae	7.91±0.82 [a]	4.34±0.18 [b**]	4.57±0.15 [b**]	7.91±0.81 [a]	4.34±0.18 [b]	4.57±0.15 [b]
Faecalibacteriumprausnitzii	9.80±0.06 [a]	4.47±0.81 [b**]	4.08±0.24 [b**]	9.54±0.11 [a]	8.64±1.15 [a**]	9.92±0.20 [a**]
Enterococcus spp.	7.66±0.11 [a]	7.31±0.57 [a]	7.17±0.12 [a]	7.46±0.07 [a]	7.12±1.06 [a]	7.96±0.71 [a]

Values are expressed as means bacterial population ± standard deviations (n=3). [a, b, c] Difference letters indicate significant differences between treatment at the same time point (a > b > c, $P< 0.05$).

*, ** significant with respect to the control diet group at the same time point (*, $P<0.05$; **, $P<0.01$).

Bacterial enumeration in the cecum chicken intestine

Twenty 4-day-old cecum samples were pooled and used as the baseline (0-day) for bacterial enumeration.The *Pseudomonas* population in all substrate tests exhibited a slight decrease in the chicken cecum from day 0 to day 42. Supplementation and nonsupplementation were observed to have no effect on the *Pseudomonas* concentration.

In 21-day-old chickens, *Enterobacteriaceae* was significantly ($P<0.05$) decreased in the CMH treatment and yeast-MOS supplemented diets (4.34±0.18 and 4.57±0.15 log copy number of gene/g cecum, respectively) compared with the control treatment (7.91±0.18 log copy number of gene/g cecum). The results showed that chickens that consumed the supplemented diet had greater reductions in the populations of*Enterobacteriaceae*.Cecum samples from all treatments at 21-day showed lower levels of *Clostridium perfringens*and*Enterococcus* sp.populationsthan in cecum samples from all diet groups at day 0, but no statistically significant differences between diet groups ($P<0.05$) were observed.

The CMH-supplemented diet in 35-day chickens exerted the greatest effect on the *Lactobacilllus* population in the cecum but no significant differences compared to other groups. In the cecum samples from 35-day-old chickens, there were no statistically significant differences in the population changes of *Clostridium coccoides* and *Faecalibacteriumprausnitzii* between chickens consuming the supplement diet and the control diet group ($P<0.05$). Thirty-five-day old ceca from chickens that consumed the CMH supplement showed lower levels of *Acinetobacter, Enterobacteriaceae*, *Clostridium perfringens* and *Enterococcus* sp.populations than did the ceca from the yeast-MOS supplement and control diet group, but there were no statistically significant differences between diet groups ($P<0.05$).

In the 42-day chicken cecum, there were no significant differences between diet groups; all groups of bacteria did not grow in every substrate test, and the supplemented diet did not promote or inhibit the bacterial growth.

Table 6. Changes in the bacterial population of the intestinal samples (log copy number of gene/g ileum or cecum) from 35-day-old chickens

Target bacteria	Bacterial population (log copy number of gene /g)					
	Ileum			Cecum		
	Control diet	CMH	Yeast-MOS	Control diet	CMH	Yeast-MOS
Total bacteria	6.51±0.18 ^{b}	7.78±0.32 ^{a**}	7.12±0.35 ^{ab}	9.18±0.27 ^{a}	9.23±0.13 ^{a}	9.37±0.18 ^{a*}
*Lactobacillus*group	6.62±0.53 ^{b}	8.09±0.38 ^{a*}	7.54±0.53 ^{ab}	7.68±0.58 ^{a}	8.03±0.17 ^{a}	7.64±0.43 ^{a}
Acinetobacter	7.16±0.53^{a}	6.59±0.77 ^{a}	7.52±0.40 ^{a}	6.43±0.78 ^{a}	6.03±0.65 ^{a}	6.77±0.30 ^{a}
Pseudomonas	3.96±0.29 ^{a}	4.00±0.05 ^{a}	3.78±0.04 ^{a}	3.73±0.05^{a}	3.65±0.06 ^{a}	3.32±0.37 ^{a}
Bacteroides – Prevotella-Porphyromonas	4.34±0.39 ^{a}	4.07±0.61 ^{a}	4.53±0.33 ^{a}	8.87±0.19 ^{b}	9.20±0.42 ^{ab}	9.63±0.15 ^{a}
C. coccoides–E. rectal group	5.72±0.48 ^{a}	4.98±0.57 ^{a}	5.40±0.46 ^{a}	9.31±0.33 ^{a}	9.35±1.18 ^{a}	9.29±0.16 ^{a}
C. perfringens group	4.59±0.67 ^{a}	4.59±0.35 ^{a}	4.97±0.50 ^{a}	5.14±0.21 ^{a}	4.91±0.26 ^{a}	5.34±0.19 ^{a}
Enterobacteriaceae	6.05±0.28 ^{a}	5.05±1.46 ^{a}	5.66±0.44 ^{a}	6.05±0.28 ^{a}	5.05±1.46 ^{a}	5.66±0.74 ^{a}
Faecalibacteriumprausnitzii	5.29±0.55 ^{a}	4.58±0.54 ^{a}	4.57±0.52 ^{a}	9.37±0.38 ^{a}	9.42±0.07 ^{a}	9.39±0.31 ^{a}
Enterococcus spp.	7.84±0.06 ^{a}	7.18±0.84 ^{a}	7.54±0.49 ^{a}	8.18±0.08 ^{a}	7.75±0.52 ^{a}	8.16±0.16 ^{a}

Values are expressed as means bacterial population ± standard deviations (n=3). $^{a, b, c}$ Difference letters indicate significant differences between treatment at the same time point (a > b > c, $P< 0.05$).

*, ** significant with respect to the control diet group at the same time point (*, $P<0.05$; **, $P<0.01$).

Table 7. Changes in the bacterial population of the intestinal samples (log copy number of gene/g ileum or cecum) from 42-day-old chickens

Target bacteria	Bacterial population (log copy number of gene /g)					
	Ileum			Cecum		
	Control diet	CMH	Yeast-MOS	Control diet	CMH	Yeast-MOS
Total bacteria	6.71±0.90 [a]	6.19±0.58 [a]	6.70±0.72 [a]	9.11±0.09 [a]	8.97±0.26 [a]	9.00±0.17 [a]
*Lactobacillus*group	7.07±1.14 [a]	6.81±0.51 [a]	6.47±1.25 [a]	7.94±0.29 [a]	7.78±0.20 [a]	7.81±0.42 [a]
Acinetobacter	6.86±0.58 [a]	6.44±0.75 [a]	7.18±0.23 [a]	5.41±0.90 [a]	6.62±0.32 [a]	6.26±1.06 [a]
Pseudomonas	3.69±0.06 [a]	3.71±0.26 [a]	3.83±0.16 [a]	3.29±0.60 [a]	3.79±0.36 [a]	4.23±0.17 [a]
Bacteroides – Prevotella-Porphyromonas	3.95±0.36 [a]	3.83±0.28 [a]	4.16±0.32 [a]	9.61±0.01 [a]	9.26±0.18 [a]	9.43±0.44 [a]
C. coccoides–E. rectal group	4.61±0.28 [a]	5.14±0.59 [a]	5.68±0.64 [a]	9.16±0.06 [a]	9.09±0.25 [a]	9.04±0.26 [a]
C. perfringens group	4.55±0.72 [a]	4.37±0.07 [a]	4.83±0.32 [a]	5.25±0.68 [a]	5.55±0.37 [a]	5.00±0.17 [a]
Enterobacteriaceae	5.01±0.62 [a]	4.64±0.52 [a]	4.54±0.25 [a]	5.01±0.62 [a]	4.64±0.52 [a]	4.54±0.25 [a]
Faecalibacteriumprausnitzii	4.14±0.52 [a]	4.75±0.32 [a]	5.03±0.68 [a]	9.27±0.20 [a]	9.30±0.11 [a]	9.21±0.21 [a]
Enterococcus spp.	7.63±0.17 [a]	7.34±0.23 [a]	7.89±0.49 [a]	8.22±0.21 [a]	8.04±0.55 [a]	8.44±0.12 [a]

Values are expressed as means bacterial population ± standard deviations (n=3). [a, b, c] Difference letters indicate significant differences between treatment at the same time point (a > b > c, $P< 0.05$).
*, ** significant with respect to the control diet group at the same time point (*, $P<0.05$; **, $P<0.01$).

Short-chain fatty acid analyses in the ileum

Result showedthe concentrations of short chain fatty acids (SCFAs) during 0, 21, 35, and 42 day in broiler chickens. Twenty 4-day-old ileum samples were pooled and were used as the baseline (0-day) for SCFA analysis. Lactate was only found in 0 day ileum samples (139.95±4.25 mM). Acetate, formate and butyrate were not found in the ileum at 0-day (Table 8).

The concentration of lactate and formate were increased after 21-day. The control diet group presented the highest lactate and formate concentration, while there were no significant differences between the CMH and yeast-MOS supplements. No significant differences in butyrate concentrations were found between the three diet groups ($P<0.05$). Acetate was not detected in the ileum samples from the three diet groups from day 0 to day 21.

Acetate was only detected in chickens that consumed a diet supplemented with CMH at 35-day ($P<0.01$). Ileum samples from chickens consuming the CMH supplement presented the highest lactate, formate and acetate concentrations (454.04±309.56 mM),(288.56±88.78 mM) and (932.56±395.28 mM, respectively) compared with the yeast-MOS supplement and control diet.

In 42-day-old chickens, the control diet group presented the highest lactate concentration (445.13±335.27 mM), while there were no significant differences between the CMH (224.18±30.34 mM) and yeast-MOS supplements (301.97±95.73 mM).The ileum samples from chickens that consumed the yeast-MOSs supplemented diet presented the highest formate, acetate and butyrate concentrations, whereas the CMH supplemented group presented higher levels than did the control diet group.

Table 8. Concentrations of short chain fatty acids produced during 0, 21, 35, and 42 days in the ileal samples

SCFA	Time (day)	SCFA concentration (mM)		
		Control diet	CMH	Yeast-MOS
Acetate	0	0±0 [a]	0±0 [a]	0±0 [a]
	21	0±0 [a]	0±0 [a]	0±0 [a]
	35	0±0 [b]	932.56±395.28 [a**]	0±0 [b]
	42	445.16±159.55 [a]	772.84±401.40 [a]	805.66±423.38 [a]
Butyrate	0	0±0 [a]	0±0 [a]	0±0 [a]
	21	133.85±15.52 [a]	132.06±7.86 [a]	120.85±7.15 [a]
	35	120.71±23.00 [a]	122.63±7.74 [a]	132.67±3.71 [a]
	42	136.37±8.70 [a]	143.01±31.68 [a]	170.11±51.01 [a]
Lactate	0	139.95±4.25 [a]	139.95±4.25 [a]	139.95±4.25 [a]
	21	398.00±173.50 [a]	214.66±11.76 [a]	198.02±34.34 [a]
	35	248.66±25.72 [a]	454.04±309.56 [a]	276.47±63.92 [a]
	42	445.13±335.27 [a]	224.18±30.34 [a]	301.97±95.73 [a]
Formate	0	0±0 [a]	0±0 [a]	0±0 [a]
	21	194.43±180.16 [a]	112.68±25.69 [a]	104.61±25.61 [a]
	35	197.72±107.55 [a]	288.56±88.78 [a]	167.17±81.83 [a]
	42	172.50±98.36 [a]	200.72±61.16 [a]	281.73±150.08 [a]

Values are expressed as means SCFA concentration ± standard deviations (n=3).
[a, b, c] Difference letters indicate significant differences between treatment at the same time point (a > b > c, $P< 0.05$).
*, ** significant with respect to the control diet group at the same time point (*, $P<0.05$; **, $P<0.01$).

Short-chain fatty acid analyses in the cecum

It showed the concentrations of short chain fatty acids (SCFAs) during 0, 21, 35 and 42 day in broiler chickens. At 0-day, the cecum samples only exhibited lactate and formate, at 132.57±23.56 mM and 121.25±4.50 mM, respectively, Chickens consuming the control diet were only detected lactate in cecum samples at 0 day and were not found from day 21 to day 42 (Table 9).

The concentrations of lactate, formate, butyrate and acetate were increased after 21 day of growth. Cecum samples from chickens consuming the CMH supplement presented the highest lactate and formate concentrations (289.78±289.78 mM and 409.99±115.39 mM, respectively) compared with the yeast-MOS supplement and control diet.

At day 35, The cecum samples from the control diet group presented the highest formate, butyrate and acetate concentrations, and the yeast-MOS supplemented diet was the second highest concentration; however, there were no significant differences among the supplements.

There was no difference in lactate concentrations in the cecum samples from 42- day chickens consuming the CMH supplemented diet compared with the yeast-MOS supplemented diet group. Cecum samples from 42-day chickens consuming the CMH supplemented diet presented the highest butyrate and acetate concentration, whereas there were no significant differences between the CMH and yeast-MOS supplemented diets.Moreover, cecum samples from chickens consuming the control diet had the lowest formate, acetate and butyrate concentrations.

Table 9. Concentrations of short chain fatty acids produced during 0, 21, 35, and 42 days in the cecal samples

SCFA	Time (day)	SCFA concentration (mM)		
		Control diet	CMH	Yeast-MOS
Acetate	0	0±0 [a]	0±0 [a]	0±0 [a]
	21	595.65±125.03 [a]	456.87±249.36 [ab]	0±0 [b*]
	35	744.45±199.96 [a]	591.97±438.96 [a]	613.48±159.97[b]
	42	587.08±108.89 [a]	839.35±232.47 [a]	793.00±179.30 [a]
Butyrate	0	0±0 [a]	0±0 [a]	0±0 [a]
	21	285.30±83.05 [a]	278.81±18.65 [a]	258.77±75.72 [b]
	35	285.10±39.84 [a]	246.02±51.93 [a]	242.68±11.08 [a]
	42	277.92±34.75 [a]	314.79±79.49 [a]	299.58±28.49 [a]
Lactate	0	132.57±23.56 [a]	132.57±23.56 [a]	132.57±23.56 [a]
	21	0±0 [a]	289.78±282.78 [a]	106.20±24.43 [a]
	35	0±0 [b]	113.46±2.57 [a]	124.43±14.44 [a]
	42	0±0 [b]	151.62±22.26 [a]	151.40±6.09 [a]
Formate	0	121.25±4.50 [a]	121.25±4.50 [a]	121.25±4.50 [a]
	21	274.01±52.74 [a]	409.99±115.39 [a]	295.08±27.99 [a]
	35	397.25±184.56 [a]	278.78±165.20 [a]	325.35±101.06[b]
	42	228.46±76.90 [b]	468.99±125.70 [ab]	502.72±81.30 [a]

Values are expressed as means SCFA concentration ± standard deviations (n=3).
[a, b, c] Difference letters indicate significant differences between treatment at the same time point (a > b > c, $P< 0.05$).
*, ** significant with respect to the control diet group at the same time point (*, $P<0.05$; **, $P<0.01$).

Discussion

The microbial populations were enumerated by real-time PCR and concentrations of short chain fatty acids (SCFAs) were determined by HPLC.

The ileum of 35-day chickens that consumed the CMH diet presented the lowest *Acinetobacter, Bacteroides, Enterobacteriaceae*, *Faecalibacteriumprausnitzii*, *and Clostridium coccoides*populations. And SCFAs in 35-day chicken ileum presented the highest lactate, formate, and acetate concentrations. Surprisingly, acetate was only found in 35-day chickens fed on the CMH supplemented diet and highest lactate which relate to the greatest effect on the *Lactobacillus* population in the 35-day chickens ileum. Higher SCFA concentrations may relate to adecrease in pathogenic bacteria. Therefore, these results suggested that acetate may suppress *Acinetobacter, Enterobacteriaceae*, *Clostridium coccoides*, and *Clostridium perfringens*(Rinttilä and Apajalahti, 2013; Wang and Gibson, 1993).

One interesting result was that SCFAs in the ceca of 21-day chickens that consumed the CMH supplement presented the highest lactate and formate concentrations. However, the yeast-MOS supplemented diet exerted the greatest effect on the *Lactobacillus* population in the cecum. The *Lactobacillus* group and *Enterococcus* spp. were related to lactate production (Duncan *et al*., 2004; Rinttilä and Apajalahti, 2013) but this study *Lactobacillus* group and *Enterococcus* spp. were related to lactate production. This was most likely the result of lactate produced by other bacteria such as the genus Clostridium, the *Eubacteriumrectale* group, and *Faecalibacteriumprausnitzii*(Salminen *et al*., 1998). Enterobacteriaceae populations were lowest in ceca of 21-day chickens that consumed CMH supplemented diets, with ceca of 35-day chickens showing the lowest populations of *Acinetobacter*,*Enterobacteriaceae*, and *Clostridium perfringens* which are common pathogenic bacteria in the poultry industry (Rinttilä and Apajalahti, 2013).

Another important finding was that lactate and formate were only found in 0-day cecum samples; subsequently, lactate was not detected in ceca from chickens consuming the control diets at all time points. This may be because, as mentioned in previous reports, each bacterial genus produces different types of fermentation products as substrates for the fermentation of intermediate compounds in the pathways of other bacteria species (Chaia and Oliver, 2003; Hashizume *et al*., 2003; Salminen *et al*., 1998).

In this study, increasing the*Lactobacillus* populations in ceca samples from day 0 to day 42 did not induce the production of lactate, with lower concentrations after day 21. This was most likely because lactate was employed by lactate-utilizing bacteria (Duncan *et al*., 2004; Rinttilä and Apajalahti, 2013).

In conclusion, the microbial population of broiler chickens fed CMH presented the greatest effect on *Lactobacillus* populations in the ileum and cecumat day 35 with low levels of *Acinetobacter, Enterobacteriaceae*, and

Clostridium perfringens. Positive changes in the bacteria community in the CMH supplemented group were similar to those recorded from commercial prebiotics. Theresearch finding presented an initial report of the effect of CMH on broiler chicken microbiota as an initial evaluation of its prebiotic potential. CMH had a desirable effect on the microbiota. Further experiments with respect to chicken performance should be considered.

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