JAPSC
Journal of Animal and Poultry Sciences, 2015, 4 (1): 1-9
Available online at http://www.JAPSC.com

Quantitative Real-Time PCR Analysis of the Caecal Bacteria Population of Broiler Chickens Fed with Corn-Soy Diet containing 20% of Palm Kernel Meal with or without Enzyme Supplementation

A. Sharmila[1], A. Kasim[1], H. M. Noor[2], M. F. Jahromi[3], and A. A. Samsudin[1*]

[1]Department of Animal Science, Faculty of Agriculture, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[2]Department of Pre-clinical Science, Faculty of Veterinary Medicine, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[3]Institute of Tropical Agriculture, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Abstract

In the present study, quantitative real-time polymerase chain reaction analysis was performed to enumerate the caecal bacterial population of broiler chickens fed with corn-soy based diet containing 20% of palm kernel-meal with or without enzyme supplementation. A total of seventy five day old male Cobb broiler chickswas used in the feeding trial receiving a corn-soy diet with20% of palm kernel meal with or without xylanase or cellulase supplementation at an amount of 200U/kg of feed respectively. On day 35, birds were slaughtered and the caecum content was aseptically collected for the bacterial quantification. Both xylanase and cellulase supplementation in the diet significantly reduced the population of total bacteria, enterococcus and Salmonella spp. bacteria in the caecal digesta of broiler chickens. Moreover, the population of lactic acid bacteria, enterobacteria and Escherichia coli in digesta of broiler chickens fed with enzyme supplementation was also significantly reduced compared to broiler chickens received no enzyme addition in the diet. However, eventhough the population of bifidobacteria analyzed in the caecal digesta of enzyme supplemented broiler chickens was reduced compared to non-supplemented broiler chickens, it is not significantly different. The result obtained suggested that xylanase and cellulase supplementation at an amount of 200 U/kg of feed reduced a number of pathogenic bacteria in the caecal particularly enterobacteria, Escherichia coli and Salmonella spp. bacteria.

Key words: Real-time PCR, Corn-soy based diet with 20% PKM, Caecal bacteria, Enzyme, Broiler chicken

*****Corresponding author: Tel: +0389474878; Fax: +0389432954
E-mail address: anjas@upm.edu.my

Introduction

Caecal of poultry is the main site for the fermentation of high fiber feed materials and it is populated by a large number of bacteria within the gastrointestinal tract (GIT) (Dunkley et al., 2007). The diversity and activities of Caecal bacteria could depend on several factors associated with the environment, the poultry, and dietary factors (Goa et al., 2007) including feed composition and additives such as exogenous enzymes, antibiotics and metabolites that have a strong influence on the intestinal microorganism (Engberg et al., 2000; Knarreborg et al., 2004; Geier et al., 2009; Józefiak et al., 2010).

Normally, the digestion of dietary non-starch polysaccharides (NSPs) feed by broiler chickens is not efficient since there is alack of endogenous enzyme produced in their GIT (Singh et al., 2012). The ingestion of NSPs feed materials will create a viscous environment in the intestinal lumen and eventually slowing the digesta passage rate, which then provide a conducive environment for the bacterial proliferation particularly pathogenic bacteria such as *Escherichia coli* and *Salmonella* spp. (Bedford and Coweison, 2012).

The application of exogenous enzyme in diets containing PKM have been widely practiced, in which most of the research focusing on their action on the digestibility of the feed ingredients (Sundu et al., 2005; 2004; Sekoni et al., 2008) and growth performance of the broiler chickens (Luis, 2002; Akpodiete et al., 2006). However, there is a lack of information on the effect of enzyme supplementation on altering the population and composition of microbial in the caeca. The intestinal microbiota population and composition can be examined in order to determine the degree of digestion by poultry, since microbial react to the nutrients left after the digestion had completed by the host.

As reported by Bedford and Coweison (2012) the use of exogenous enzymes seem to improve the nutritional value of broiler chicken's diet, facilitate the growth of beneficial gut microflora and consequently, improve the utilization of feeds by maximizing the conversion of dietary nutrients into broiler chickens meat. This statement was corroborated with a study conducted by Silva and Smithard (2002) where they found that feed enzyme supplementation can reduce the bacterial activity in the ileum by reducing the amount of nutrients available for microbial fermentation in the poultry gut whereas, Józefiak et al. (2010) observed that supplementation of xylanase and β-glucanase tended to decrease the numbers of coliform bacteria and also notably decrease the numbers of *Enterobacteriaceae*, giving beneficial property of using feed enzyme since these two types of bacteria are included as pathogens and zoonotic bacteria like *Salmonella* spp. This could have an important impact on gut health in broiler chickens, in particular with respect to the ceacal lumen reflux and possible migration of some of the bacterial species to upper parts of the GIT.

In the early 1990s, most of the work on the intestinal microflora enumeration and identification is carried out using the culture techniques (Bedford and Coweison, 2012). However, the advance in the new molecular techniques to enumerate the bacteria population of a given sample becomes desirable. The quantification of the Caecal bacterial population can be carried out through qPCR analysis in which this

method will be more sensitive and a faster mean of bacterial quantification compared to culture-based methods that rely on the ability of the bacteria to grow (Castillo et al., 2006).

Materials and methods

Enzyme

A commercial Xylanase (*Thermomyces lanuginosus* expressed in *Aspergillus oryzae*) and cellulase (from *Trichoderma reesi* ATCC 26291) from Sigma-Aldrich was used for this experiment. The xylanase and cellulase was added in each treatment diets to provide a guaranteed minimum of 200 units per kg of feed, at an inclusion rate of 80g and 200g per tonne respectively. The activity of cellulase enzyme was defined as the amount of a unit enzyme that will deliberate 1.0µmole of glucose from cellulose in one hour at pH 5.0 at 37˚C (two hour incubation time).

Feeding trial and sampling methods

A total of 75 day old male Cobb broiler chicks was used in this study. The chicks were weighed and randomly allocated to three dietary treatments: 1) corn-soy based diet with20% of PKM; 2) corn-soy based diet with20% of PKM with xylanase; 3) corn-soy based diet with20% of PKM with cellulase in a completely randomized design. The nutrient analysis of PKM is presented in Table 1. Each treatment consisted of five replicates with five birds per replicate. The feed and water were provided *ad libitum*. The experimental period was divided into starter (1 to 21 days) and finisher period (22 to 35 days). The compositions of the experimental diets are shown in Table 2. Birds were vaccinated against Newcastle disease, infectious bronchitis and infectious bursal disease as per recommended at day 3 and 14. MyVac 201 was used against Newcastle disease and infectious bronchitis, meanwhile MyVac IBD V877 was used against infectious bursal disease. Both vaccines used were purchased from pharmaceuticals Sdn. Bhd. Malaysia. All chicks were provided with 24 hour light for the first four days, followed by lighting at night for 21 days. The chickens were inspected daily and dead birds were removed. In the fifth week of the experiment, the final body weight of individual broilers as well as feed conversion ratio was recorded and all chickens from each pen were slaughtered. The contents of caecum were aseptically collected for caecal bacterial quantification.

Table 1. The nutrient composition of PKM

Ingredients	%
Dry matter (%)	90.86
Crude protein (CP) (%)	15.69
Crude fiber (CF) (%)	16.47
Ether extract	3.32
Ash (%)	4.53

Table 2. Ingredient composition and nutrient level (%) of the experimental diet

	Diets	
Ingredients(% use)	Starter (0-21 days)	Finisher (22-35 days)
Corn grain	40.73	46.35
Soybean meal	28.0	26.0
Fish meal	7.0	3.63
PKM	20.0	20.0
Crude palm oil	1.0	1.0
Dicalcium phosphate	1.39	0.38
Salt	0.35	0.35
Limestone	1.0	1.6
L-lysine	0.12	0.32
DL-methionine	0.16	0.11
Vitamin premix[1]	0.13	0.13
Mineral premix[2]	0.13	0.13
Nutrient analysis		
Dry matter %	86.41	88.77
Protein %	21.34	20.51
Energy Kcal/kg	3441.80	3541.1
Calcium %	0.96	0.9
Phosphorus %	0.57	0.49
Digestible amino acids (%)		
Lysine	1.47	1.41
Methionine	0.35	0.44

[1]Vitamin premix provided per tones of diets: vitamin A 50.00 MIU; vitamin D3 10.00 MIU; vitamin E 75.00g; vitamin K3 20.00g; vitamin B1 10.00g; vitamin B2 20.00g; vitamin B6 20.00g; vitamin 12 0.10g; calcium D-pantothenate 60.00g; nicotinic acid 5.00g; folic acid 5.00g; biotin 235.00g.

[2]Mineral premix provided per tones of diets: selenium 0.20g; iron 80.00g; zinc 80.00g; copper 15.00g; potassium chloride 4.00g; magnesium oxide 0.60g; sodium bicarbonate 1.50g; iodine 1.00g and cobalt 1.25g

Caecal sample preparation

Samples of caecal digesta were obtained from 35 day olds male Cobb broiler chickens. The caecal digesta of birds received on corn-soy based diet with 20% PKM with or without xylanase and cellulase were immediately and aseptically collected following slaughtered. The caecal digesta were kept frozen at -20˚C before was used for quantification of total bacteria, lactic acid bacteria (LAB), bifidobacteria, enterococcus, enterobacteria, *E.coli* and *Salmonella* spp. bacteria.

Genomic Caecal DNA extraction

The DNA was isolated from the Caecal samples using QIAamp DNA stool kit (Qiagen, Hilden, Germany). The DNA of caecal samples were extracted followed the protocol provided by the manufacturer. Approximately 200mg of caecal digesta of broilers fed with corn–soy based diet with 20% PKM-based diet with or without xylanase and cellulase supplementation was transferred into 1.5mL of sterile microcentrifuge tube. One mL of ASL lysis buffer provided in the kit was then added to the Caecal sample to disrupt the cell and were homogenized using a vortex and centrifuged. The supernatant was removed and the InhibitEX tablet was then added. Later, DNA was precipitated by adding 200µL of ethanol, captured in QIAamp spin

column, washed by 500µL of AW1 buffers and followed by buffer AW2. Finally, the DNA was eluted in 200µL elution buffer. The extracted DNA was kept in -20˚C for qPCR analysis.

Analysis of Caecal bacteria by quantitative real-time PCR

The populations of total bacteria, LAB, enterococcus, enterobacteria, *E. coli*, bifidobacteria and *Salmonella* spp. of Caecal digesta were analyzed by qPCR. Genomic DNA from caecal digesta was used as templates for PCR amplification. Absolute quantification of caecal bacteria was achieved by using standard curves constructed by amplification of known amount of target bacteria DNA. The qPCR master mix was prepared on a total volume of 25µL using the QuantiFast® SYBR® Green PCR kit (Qiagen Inc., Valencia, USA) consisted of 12.5µL of 2 × SYBR Green Master Mix, 1µL of 10µM forward primer, 1µL of 10µM reverse primer, 2µL of DNA samples and 8.5µL of nuclease-free water for each reaction. Each sample was analyzed with four replication reactions. The targeted caecal bacteria groups, primer sequences, annealing temperature and literature references in this study are detailed in Table 3.The qPCR assay was performed with BioRad CFX96 real-time PCR system (BioRad, USA) using optical grade plates as follows: the qPCR cycling conditions comprised an initial denaturation at 94˚C for 5 min, followed by 40 cycles of denaturation at 94˚C for 20s, primer annealing at 50˚C for *E.coli*, 55˚C total bacteria and *Salmonella* spp., 58˚C for LAB, 60˚C for bifidobacteria and enterobacteria for 30s for respectively, and extension at 72˚C for 20s (Navidshad et al., 2012).

Table 3. The sequence of primers used targeting total bacteria, LAB, Bifidobacteria, Enterobacteria, *E.coli* and *Salmonella* spp.

Target bacteria	Sequence 5'-3'	Annealing temperature (˚C)	References
Total bacteria	**F**-CGGCAACGAGCGCAACCC **R**_CCATTGTAGCACGTGTGTTAGCC	55	Jahromiet al. (2013)
LAB	**F**-CATCCAGTGCAAACCTAAGAG **R**_GATCCGCTTGCCTTCGCA	58	Wanget al. (1996)
Enterococcus	**F**-CCCTTATTGTTAGTTGCCATCATT **R**_ACTCGTTGTACTTCCCATTGT	60	Navidshadet al. (2012)
Bifidobacteria	**F**-GGGTGGTAATGCCGGATG **R**_TAAGCCATGGACTTTCACACC	60	Bartosch et al. (2005)
Enterobacteria	**F**-CATTGACGTTACCCGCAGAAGAAGC **R**_CTCTACGAGACTCAAGCTTGC	60	Navidshadet al. (2012)
E.coli	**F**-GTGTGATATCTACCCGCTTCGC **R**_AGAACGCTTTGTGGTTAATCAGGA	50	Frahm and Obst (2003)
Salmonella spp.	**F**-TCGTCATTCCATTACCTACC **R**_AAACGTTGAAAAACTGAGGA	55	Nam et al. (2005)

Statistical analysis

The effect of enzyme supplementation on the Caecal bacteria population was subjected to General Linear Model (GLM) procedures of SAS® 9.0 (SAS Institute) according to the following general model:

$Y_{ij} = \mu + \alpha_i + \delta_{ij}$

Where Y_{ij} is the observed dependent variable; μ is the overall mean; α_i is the effect of enzyme; and δ_{ij} is the random error.

Results

In this experiment mean mortality rates was recorded at 7% and was not related to the dietary treatment. The cumulative feed conversion ratio (CFCR) between unsupplemented and enzyme supplemented broiler chickens is not significant (P>0.05). The CFCR for control, xylanase and cellulase supplemented broiler chickens are 2.2, 2.1 and 2.1 respectively. However, the individual final body weight of broiler chickens fed cellulase supplemented corn-soy based diet with 20% PKM was the lowest which is 1367.81 kg, whilst in control and cellulase supplemented group are 1436.8 kg and 1495.25 kg respectively.

The effect of xylanase and cellulase supplementation in corn-soy based diet with 20% PKM on the number of caecal bacteria of broiler chickens fed with or without xylanase and cellulase is shown in Table 4.

The supplementation of Xylanase and Cellulase in the diets significantly reduced (P>0.05) the population of total bacteria and Enterococcus, while the LAB, Enterobacteria and *E.coli* was highly reduced (P<0.01) compared to broiler chickens received non-supplemented diet. However, the population of Bifidobacteria of caecaldigesta was not affected by the NSPases supplementation.

The reduction in the number of enterobacteria and *E.coli* ofcaecaldigesta was higher in xylanase supplemented group which value are 43.57% and 34.06% compared to cellulase supplemented group at 30.33% and 24.63% respectively. Although, statistically the count of bifidobacteria of caecaldigesta in enzyme supplemented groups was not significant (P>0.05) compared to broiler chickens fed with non-supplemented diet, there was a reduction in the population almost to 6.87% in xylanase group and 22.28% in cellulase group respectively.

Table 4. Effect of Xylanase and Cellulase supplementation on cell counts of Caecal bacteria (log^{10} of copy number/g DNA extract)

	Treatment			
Log^{10} copy no/ml DNA extract	Control	Xylanase-supplemented	Cellulase-supplemented	P
Total bacteria	9.30±0.56^a	7.98±0.08^b	7.86±0.21^b	*
LAB	6.22±0.29^a	4.98±0.04^b	5.03±0.16^b	**
Enterococcus	5.44±0.44^a	3.07±0.43^b	3.79±0.67^b	*
Enterobacteria	5.01±0.19^a	4.05±0.12^b	4.24±0.11^b	**
Escherichia coli	4.67±0.16^a	3.08±0.31^b	3.52±0.22^b	**
Bifidobacteria	5.97±0.31	5.56±0.20	4.64±0.83	NS
Salmonella spp.	2.05±0.10	1.75±0.472	1.33±0.55	*

*Significantly different at 5% level (P<0.05), **Significantly different at 1% level (P<0.01),
$^{a, b, and c:}$ Means with different letter within a row differed significantly.
NS: Not Significant.

Discussion

The microbial populated in the gastrointestinal tract (GIT) of broiler chickens is not well documented in comparison to the microbiota of rumen, probably due to the supposition that they do not involved in the feed digestion and utilization, growth performance as well as their health. The present study was carried out to study the effect of supplementing Xylanase and cellulase in corn-soy based diet with PKM on the caecal bacteria population of broiler chickens. Overall the species of caecal bacteria in this study was not reflected the diversity of microbial inhibiting in the caecum of broiler chickens. However, the purpose of quantifying caecal bacteria was to determine wether Xylanase and cellulase supplementation in corn-soy based diet with 20% PKM could reduce the population of the pathogenic bacteria in the caecum of broiler chickens.

The data presented in this study show that both xylanase and cellulase supplementation in the diet significantly reduced ($P<0.05$) most of the caecal bacteria population observed except bifidobacteria compared to broiler chickens received only the diet. In the present study, the population of enterobacteria and enterococcus in caecal digesta of broiler chickens fed the diet supplemented with xylanase were reduced and is corroborated with data presented by Dänicke et al. (1998) and Khattak et al. (2006). Xylanase supplementation in wheat and rye-based diet in a study conducted by Khattak et al. (2006) reduced the population of these two groups of bacteria. However, the population of LAB observed is increased, and this result is not similar with the present study where the population of LAB in caecal digesta of xylanase supplemented broiler chickens was reduced. The contradictory result obtained may be related to the different dietary ingredients used in this study. In contrast, Nian et al. (2011) observed that the supplementation of xylanase in corn/soy based diet had increased the count of LAB and bifidobacteria in caecal contents of broiler chickens whilst Engberg et al. (2004) found xylanase supplementation has no effect on bifidobacteria, and *E.coli* of caecal digesta. However, the reduction of *E.coli* population in the current study is corroborated with a data presented by Rosin et al. (2007); that reported enzyme supplementation in wheat and corn-based diet reduce the number of *E.coli* in the caecum of broiler chickens.

There is a complex interaction between the intestinal microflora with the host animal and feed ingredients of the diets, where the population and composition of intestinal microbiota is strongly related and influence by these two factors (Khattak et al., 2006). This theory is supported by Józefiak et al. (2010), where in a study conducted previously showed that the type of exogenous enzyme supplementation as well type of feed ingredients used may affect the microbiota population in the caeca of broiler chickens, and also be effective in reducing potentially pathogenic bacteria such as enterobacteria population.

The mode of action of exogenous enzyme includes providing and delivering more nutrients to the host and producing nutrients specifically for certain of bacteria population in animal gut (Metzler et al., 2005). Bedford (2002) has grouped the mode of enzymes action on intestinal microflora into two phases which involved the ileal and caecal phase. The quantity of bacteria in the ileum is reduced through the increase in the rate of digestion and utilization of host animal by enzymes hence; limit the amounts of nutrients available

for them. The poorly absorbed sugar released in the ileum might escape to the caeca which can be then utilize by beneficial bacteria producing VFAs that can help controlling the caecal enterobacteria population particularly *E.coli* and *Salmonella* spp.

Conclusion

The reduction in the population of caecal bacteria of broiler chickens fed a corn-soy based diet with 20% PKM supplemented with Xylanase and Cellulase was observed in the present study. The Xylanase and cellulase supplementation was found to be beneficial in reducing the number of pathogenic bacteria observed especially *E.coli* and *Salmonell*a spp. that can potentially cause foodborne disease to human. Besides that, these enzymes can be used to replace the used of antibiotic in broiler chickens feed to promote their health as well as reducing the bacterial contamination to the environment excreted via their faeces.

Acknowledgements

Sharmila, A. was a recipient of MyBrain15 Scholarship from the Malaysia Ministry of Higher Education. We would like to thank Malaysian Agro-Biotechnological Institute (ABI), for letting us use their laboratory facilities such real-time PCR machine.

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