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Detection of *Lactobacillus, Bacteroides* and *Clostridium perfringens* in the gastrointestinal contents of chicken fed different diets by real-time PCR

(Pengesanan *Lactobacillus, Bacteroides* dan *Clostridium perfringens* melalui PCR masa nyata di dalam usus ayam yang diberi makan pelbagai rangsum)

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Abstract

The rapid molecular method of real-time PCR assay was used to detect the presence of *Lactobacillus*, *Bacteroides* (including *Prevottella-Porphyromonas*) and *Clostridium perfringens* groups from the intestinal contents of 30 samples of chickens fed five different types of diet. All the samples were positive for *Lactobacillus* and *Bacteroides*. A total of 21 out of 30 samples were positive for *C. perfringens*. The DNA sequencing results confirmed the identity of the microorganisms as *Lactobacillus salivarius*, *Bacteroides vulgatus, Bacteroides caccae, Clostridium perfringens* and *Clostridium kluyveri*. These partial DNA sequences of 16S rRNA genes were deposited in the Gene Bank under accession numbers FJ151421, FJ211591, FJ375300, FJ182043 and FJ182042 respectively.

Introduction

The microbiota of the chicken gastrointestinal (GI) tract constitutes a complex ecosystem of microorganisms which is involved in the health and physiological functions of the host (Gibson and Roberfroid 1995). It plays an important role in the supply of nutrients and vitamins, providing colonization resistance against pathogenic bacteria and interacting with the host immune system and intestinal epithelium. The understanding of the GI microbiota and its interaction with the host is important as it can contribute to the provision of a healthy gut environment and the use of tropical herbal plant as prebiotics. Prebiotics are dietary components that can assist in the growth of beneficial microorganisms in the gut, which may lead to better health of the chicken (Guarner and Malagelada 2003b).

Gastrointestinal microbiota generally comprised of the genera *Bacteroides, Clostridium, Fusobacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Bifidobacterium, Escherichia* and *Lactobacillus* (Guarner and Malagelada 2003a). Some of these bacteria are friendly while others are opportunistic pathogens. For instance, *Bacteroides* in the GI tract assist in the digestion of food, producing valuable nutrients and energy

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required by the body. However, when the same bacteria are introduced into other parts of the body, they can cause or exacerbate abscesses and other infections.

In the case of *Clostridium*, most of them are saprophytes, with the exceptions of a few that are opportunistic pathogens, primarily *C. perfringens, C. difficile* and *C. tetani*. When *Clostridium* proliferates to high numbers in the small intestine, they produce extracellular toxins that cause necrotic enteritis in chicken, leading to increased mortality, impaired feed conversion and retarded growth rates (Kaldhusdal et al. 2001). *Lactobacillus* and *Bifidobacterium* on the other hand are generally considered as friendly bacteria, necessary for proper body functions (Guarner and Malagelada 2003a).

The GI microbiota is dominated by obligate anaerobes and therefore investigation by culture-dependent techniques is laborious, time consuming and prone to misinterpretation (Vaughan et al. 2000). It is evident that conventional culture-based methods provide an incomplete and biased picture of the biodiversity of the intestinal microbiota, as the majority of the GI tract bacterial species cannot be cultivated (Langendijk et al. 1995; Suau et al. 1999).

The use of polymerase chain reaction (PCR) for the detection of DNA was first published in 1985 (Saiki et al. 1985). PCR has markedly improved the diagnosis of microorganism by increasing the sensitivity compared with traditional techniques (Lacroix et al. 1996). PCR is more rapid than culture-dependent technique but its protocols are still time consuming and labour intensive (Kares et al. 2004), particularly when running the agarose gel for DNA detection.

Real-time PCR technique has enabled rapid detection of DNA and its quantification during the PCR reaction. It is a powerful advancement of the basic PCR technique and it has been successfully applied for the detection and quantitation of bacterial DNA in various environments such as faeces (Requena et al. 2002; Bélanger et al. 2003; Malinen et al. 2003), colonic tissue (Fujita et al. 2002), rumen (Tajima et al. 2001), gastric tissue (He et al. 2002) and periodontal samples (Asai et al. 2002). Real-time PCR using SYBR Green I chemistry is a rapid molecular method for detection of the pathogens in faecal samples and has an advantage of being very sensitive and precise for extensive quantitative evaluation of the gut microbiota (Rinttilä et al. 2004).

The objective of this study was therefore to optimize the real-time PCR assay for the detection and DNA sequencing identification of selected microorganisms isolated from the ileum of chickens fed different types of diet. The microorganisms screened were *Bacteroides* (including *Prevotella* and *Porphyromonas*), *Clostridium perfringens* and *Lactobacillus* groups. This selection was to cater for the presence of good bacteria, namely *Lactobacillus* and opportunistic pathogens, namely *Bacteroides* and *Clostridium*.

Materials and methods

A total of 180 Cobb 500 broilers were allocated to five dietary treatments (Group A to E) with six replicates. Each replicate had six chickens and were raised in cages with *ad libitum* access to feed and water. Chickens in Group A were fed a commercial starter and grower diet. Chickens in Group B–E were fed with starter diets and grower diets as shown in *Table 1*.

At 42 days of age, six chickens from each group were sacrificed and their ileal contents were collected and kept at –20 °C until further analysis.

DNA extraction

DNA was extracted from chicken ileal contents using QIAamp DNA stool kit (Qiagen Inc., Hilden, Germany). The extraction was carried out in accordance with the manufacturers' instructions, except for lysozyme which was added before the use of inhibitEX tablets. Briefly, about 0.2 g of ileal materials were cut using a scalpel

Table 1. Group A to E dietary composition

Group	Starter diet	Grower diet
A	Commercial starter diet	Commercial grower diet
B	58% corn, 28% soybean meal	60% corn, 27% soybean meal
C	43% corn, 13% soybean meal, 30% fermented PKC	44% corn, 14% soybean meal, 30% fermented PKC
D	60% feed rice, 30% soybean meal	60% feed rice, 23% soybean meal
E	29% corn, 21% soybean meal, 30% untreated PKC	24% corn, 16% soybean meal, 30% untreated PKC

and placed in 2 ml tubes on ice. The buffer ASL (Qiagen) was immediately added to the sample and vortexed until homogenized.

The samples were then heated at 70 ºC for 5 min. The samples were centrifuged at 14,000 rpm (Spectrafuge 16M, Labnet, In., Inc., USA) for 1 min. The supernatant was then pipetted into 2 ml tube and 140 µl of 10 mg/ml solution of lysozyme (Sigma-Aldrich, St. Louis, MO, USA) in Tris-EDTA buffer (10:1 mM), pH 8, was added to each extraction tube and the samples were incubated at 37 ºC for 30 min.

InhibitEX (Qiagen) tablet was added into each tube, vortexed and centrifuged at full speed. The supernatant was then pipetted into 1.5 ml tube to which was added 15 µl proteinase K together with 200 µl buffer AL to digest the protein. After incubation at 70 ºC for 10 min, 200 µl absolute ethanol was added into each tube and mixed by vortexing. The supernatant was then applied onto the QIAamp spin column and centrifuged at 14,000 rpm for 1 min. This was then followed by two washes of the spin column with buffer AW1 and AW2.

Finally, the DNA was eluted in 200 µl buffer AE (Qiagen) and stabilized by the addition of 4 µl (40 mg/ml) bovine serum albumin (Sigma) and 2 µl (1 mg/ml) Ribonuclease-A (Sigma). All DNA samples were stored at –20 ºC for further processing.

Primer design

Three sets of primer designated BR, BF, CPR, CPF, LR and LF as described by Rinttilä et al. (2004) were used in the real-time PCR. These primers set are designed to amplify different regions of 16S rDNA of intestinal bacterial group. The reverse primer BR, 5'-CGG ACG TAA GGG CCG TGC-3' and forward primer BF, 5'-GGT GTC GGC TTA AGT GCC AT-3' were used to amplify 140 bp of *Bacteriodes-Prevotella-Porphyromonas*.

The set of primers CPR (reverse primer), 5'-TAT GCG GTA TTA ATC TCC CTT T-3' and CPF (forward primer), 5'-ATG CAA GTC GAG CGA TG-3' were used to amplify 120 bp of *Clostridium perfringens* gp and finally, the primers of LR (reverse primer), 5'-CAC CGC TAC ACA TGG AG-3' and LF (forward primer), 5'-AGC AGT AGG GAA TCT TCC A-3' were used to amplify 341 bp of *Lactobacillus* gp.

Real-time PCR

Real-time PCR amplifications were performed using Mx3005P QPCR system (Stratagene, USA). All real-time PCR experiments were carried out in triplicate using Mx3000 P optical grade PCR strip tubes and caps (Stratagene, USA). The efficiency of PCR amplification was optimized for each primer pair, using Brilliant SYBR Green QPCR master mix (Stratagene, USA).

The amplification reactions were carried out in a total volume of 25 µl. The reaction mixture consisted of 12.5 µl of 2x master mix, 1 µM of each primer, 2 µl of template and top-up with PCR-grade water to the total volume of 25 µl. The optimal annealing temperature (Tm) for *Bacteroides-Prevotella-Porphyromonas, Clostridium perfringens* and *Lactobacillus* gp were 68, 55 and 58 ºC respectively.

The amplification involved one cycle at 95 ºC for 10 min for initial denaturation followed by 30 cycles of denaturation at 95 ºC for 30 s, primer annealing at the optimal Tm for 1 min, extension at 72 ºC for 1 min. For the dissociation curve, the reactions were incubated for 1 min at 95 ºC, ramping down 5 ºC below the Tm and ramping up the temperature to 95 ºC, with fluorescence collection at 0.2 ºC intervals.

PCR purification

The real-time PCR products were purified using QIAquick PCR purification kit (Qiagen Inc., Hilden, Germany). The purification was carried out in accordance with the instructions of the manufacturer. Briefly, five volume of buffer PB (Qiagen) was added to one volume of real-time PCR samples and mixed. The samples were then applied to the QIAquick columns and centrifuged at full speed for 1 min. After discarding the flow-through, the QIAquick columns were washed with 0.75 ml buffer PE (Qiagen). The DNA was finally eluted in 50 µl buffer EB.

DNA sequencing and analysis

The purified DNA product was sequenced commercially using the ABI 3730XL sequencing system (AIT Biotechnology, Pte. Ltd. Singapore). The sequence chromatograph was edited, assembled and analysed using Chromas LITE program (version 2.01) and BioEdit Sequence Alignment Editor (version 7.0.9.0). Assembled 16S rDNA sequences from selected isolates were compared with 16S rDNA sequences available in GenBank databases by using the standard nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information, Bethesda, Md.). The isolates were identified as most closely related to the reference species (sequence obtained from Genbank) if its 16S rDNA sequence demonstrated the highest relatedness and had over 98% identical bases compared to the respective reference sequence.

Results and discussion

The targeted microbes were detected and identified using the optimized methodology developed in the present study. The real-time PCR standard curves were examined using 10-fold serial dilutions of target DNA extracted from ileal content of chickens. Standard curves had correlation coefficient values of 0.920 for *Clostridium*, 0.959 for *Lactobacillus* and 0.984 for *Bacteroides*. The PCR efficiencies for the individual assays were 82.9% for *Bacteriodes*, 91.8% for *Clostridium* and 97% for *Lactobacillus* and the slopes were –3.813, –3.536 and –3.396 respectively.

Melting curve analysis was carried out in each real-time PCR assay to distinguish the fluorescent signal obtained from the specific amplification product from artifacts like primer-dimers. The melting temperatures (T_m) of the desired PCR amplicons varied between 81 °C and 83 °C, whereas primer-dimers with considerably lower T_m values were observed with no-template and negative controls as well as with lower template concentrations in certain assays.

Bacteroides-Prevotella-Porphyromonas and *Lactobacillus* gp were detected in all ileal contents of chickens fed with five different dietary treatments. A total of 21 out of 30 samples of intestinal contents were found positive with *Clostridium perfringens* gp. From the total 21 positive samples, five samples were from group A and four samples each from group B, C, D and E. It appeared that bacterium *Clostridium* was present in 83% of the ileal content of chickens fed Diet A compared to only 67% in the other diets.

The exact reason for the increase in *Clostridium* in Diet A is difficult to ascertain in this study but could be associated to the ingredients used as they were based on a commercial ration. All the other diets (B to E) were prepared in house for the study. It is

also possible that the different feed additives used in diet A compared to the other diets may also have contributed to a 16% increase in the incidence of *Clostridium* in the gut.

The result of the present study indicates that feeding the chickens with different diets encourage the growth of different microbes such as the higher proportion of *Clostridium* in chickens fed Diet A. It is evident that *Bacteroides* and *Lactobacillus* are generally found in the ileum of chicken irrespective of dietary components.

Lactobacillus is a group of friendly gram-positive anaerobic bacteria necessary for normal gut health. It helps in metabolizing foods, absorbing nutrients, and preventing the colonization of pathogenic bacteria. On the other hand, higher numbers of *Clostridium* and *Bacteroides* in the body are detrimental as these may cause infection when the host immune system is suppressed, for instance under heat stress (Davis 1973).

It has been reported that chickens fed with conventional corn-soybean meal diets showed significantly higher body weight gains and better feed conversion efficiency compared to those fed diets with 30% PKC expeller (Thayalini et al. 2008). This may be due to the fact that 73% of the total carbohydrate (64%) of PKC (including lignin) is in the form of non-starch polysaccharides (Knudsen 1997).

Non-starch polysaccharides are poorly utilized by monogastric animals and are therefore considered as anti-nutritional components in the diet (Fengler et al. 1988). For instance, the addition of NSP to the diet of weaning piglets increased intestinal viscosity, altered the structure of the intestine and favoured the proliferation of pathogenic (McDonald et al. 2001) and haemolytic *Eschericia coli* (Hopwood et al. 2002). Many attempts have been made to improve the digestibility of feed ingredients high in NSP such as the use of in-feed enzymes and antibiotics.

In the present study, the fermented PKC was inoculated with *Aspergillus niger* to improve its nutritive value through degradation enzymes. It appears that the feeding of fermented or unfermented PKC did not alter the incidence of *Clostridium perfringens* and *Bacteroides* in the chicken gut. This indicates that PKC can be incorporated into chicken diets without adversely altering its gut microbiota. However this finding has to be investigated further on a large scale study.

The DNA sequencing analysis results in the present study identified and confirmed that the real-time PCR amplified DNA are 16S rDNA gene of *Lactobacillus salivarius*, *Bacteroides vulgatus, Bacteroides caccae, Clostridium perfringens and Clostridium kluyveri*.

The isolates were designated as LS1/08MARDI, B1/08LRMARDI, B2/08LRMARDI, CP2/08LRMARDI and CP1/08LRMARDI respectively. Partial sequences of 302 bp of *Lactobacillus salivarius*, 88 bp of *Bacteroides vulgatus,* 109 bp of *Bacteroides caccae,* 107 bp of *Clostridium perfringens* and 108 bp of *Clostridium kluyveri* of their 16S rRNA genes were deposited in the Gene Bank under accession numbers FJ151421, FJ211591, FJ375300, FJ182043 and FJ182042 respectively.

Conclusion

Real-time PCR is a promising molecular tool for the rapid detection of bacterial DNA from chicken intestinal contents. In the present study, real-time PCR assay was successfully developed to detect and identify variations in specific groups of *Lactobacillus, Bacteroides* and *Clostridium perfringens* from the intestinal contents of chickens fed different diets. However, further study need to be done on quantification using real-time PCR to survey the influence of diet on the composition of beneficial and non-beneficial gut microbes.

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Abstrak

Kaedah pantas pengasaian tindak balas rantaian polymerase (PCR) masa nyata telah digunakan untuk mengesan kehadiran *Lactobacillus*, *Bacteroides* (termasuk *Prevottella-Porphyromonas*) dan *Clostridium perfringens* di dalam 30 sampel kandungan usus ayam yang diberi lima rawatan makanan yang berbeza. Kesemua sampel didapati positif terhadap *Lactobacillus* dan *Bacteroides*. Sejumlah 21 daripada 30 sampel pula didapati positif terhadap *C. perfringens*. Keputusan penjujukan DNA telah mengesahkan identiti mikroorganisma sebagai *Lactobacillus salivarius*, *Bacteroides vulgatus, Bacteroides caccae, Clostridium perfringens* dan *Clostridium kluyveri*. Penjujukan DNA 16S rRNA gen-gen tersebut telah didaftar di dalam 'Gene Bank' masing-masing dengan nombor FJ151421, FJ211591, FJ375300, FJ182043 dan FJ182042.

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