Application of Real-Time PCR for Quantifying Gastrointestinal Microbiota in Weaning Pigs Influenced by Dietary Feed Additive Supplementation

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Abstract

To apply real-time polymerase chain reaction (PCR) to detect and quantify the gastrointestinal microbiota modulated by dietary feed additives was the objective of this study. Sixty-four three-crossbred weaning piglets at 24 days were used and randomly assigned to be fed one of the experimental diets supplemented with different feed additives (0.2% inulin, 0.1% fructooligosaccharide and sub-therapeutic antibiotics), and compared with the non-supplemented group. Comparison of microbiota enumeration by conventional culture and real-time PCR methods in response to feed additives was investigated. The microbiota enumeration by conventional culture and real-time PCR methods showed corresponding responses of selected bacterial counts (Lactobacilli, Bifidobacteria, *Escherichia coli*) in feces and digesta from caecum and colon to different feed additives. The inulin-treated group tended to have the highest Lactobacilli number, while the sub-therapeutic antibiotics-treated group had significantly lower number of Lactobacilli and Bifidobacteria in the fecal and digesta samples ($p < 0.05$) and tended to have a reduction in the population of *Escherichia coli* in the digesta. However, higher numbers were detected by real-time PCR due to the count of DNA copies from both non-viable and viable bacteria. The sensitivity of real-time PCR of this study could detect as low as 10^2 copies of specific bacteria 16S rRNA gene. Besides, the optimized real-time PCR had the advantage of being less time-consuming and less labor-intensive, and allowing samples to be stored until analysis compared to the culture method. Therefore, real-time PCR could be used as an effective method to detect microbiota changing in the GI tract. Moreover, this technique can be applied in the future for evaluation of feed quality, which affects microbiota balance.

Introduction

The microbiota in gastrointestinal (GI) tract of pigs is composed of diverse and complex microbial population which is generally categorized into commensal and pathogenic groups. Microbiota, especially the commensal bacteria, relates to animal health and performance because it plays an important role such as in provision of the benefit to host, vitamin-like production, immune stimulation and inhibition of harmful bacteria. In contrast, the pathogenic bacteria are involved in the induction of infection and toxin production (Koutsos and Arias, 2006). Lactobacilli and Bifidobacteria are the major members of the beneficial group, while *Escherichia coli* (*E.coli*), *Salmonella enterica* or *Clostridium perfringens* represent the members of pathogenic microbiota (Samanta et al., 2013). Considering the high complexity of the GI microbiota, the *Lactobacillus* and *Bifidobacterium* populations serve as an index of a health-promoting microbiota (Murphy et al., 2013), while some strains of *E.coli* can be a cause of disease (Bok et al., 2013). Changing of microbiota population in pigs has been observed following weaning (Franklin et al., 2002). The balance of GI microbiota is disturbed in this period, for example, beneficial Lactobacilli decrease dramatically while potentially pathogenic bacteria such as coliforms and *E.coli* proliferate (Heo et al., 2012). However, some factors may also alterate the GI microbiota such as diet and environment (Konstantinov et al., 2004). Furthermore, supplementation of feed additives such as antibiotic, probiotic and prebiotic can modulate the microbial profile of the GI tract (Dibner and Richards, 2005). Prebiotic such as inulin and fructooligosaccharide (FOS) are the interesting choices to manipulate GI microbiota in the weaning transition of piglets. Their prebiotic properties to stimulate beneficial bacteria and reduce pathogenic microbes proliferation were proved in a previous study (Kolida et al., 2002). Inulin is widely found in many plant species (Baclay et al., 2010), while FOS can be produced by enzymatic process from inulin (Singh and Singh, 2010).

Animal nutritionists interested to study the microbiota balance have tried to develop ways to promote commensal bacteria growth (Dibner and Richards 2005). Thus, a suitable method to characterize the complex bacterial community present in the GI tract is important. The conventional culture method, which is widely used in the present, however has some limitations such as being laborious, prone to misinterpretation (Vaughan et al., 2000), and imprecise (Abu Bakar et al., 2010). Furthermore, only 10 to 40% of the GI microbiota population can be cultivated by the conventional culture method (Ott et al., 2004). These limitations can be overcome by using culture-independent molecular techniques based on sequence comparisons of nucleic acids (DNA and RNA) (Simpson et al., 2000). Real-time PCR is a powerful technique which has been successfully applied for detection and quantification of bacterial DNA in various samples (Phong et al., 2010). It presents the most sensitive and precise results when compared with other molecular methods (Klein, 2002). Moreover, this method is suitable for analysis in field condition because samples can be stored until analysis (Castillo et al., 2006). Therefore, to apply real-time PCR to detect and quantify the GI microbiota of weaning pigs which were modulated by dietary feed additives was the objective of this study.

Materials and Methods

Animals and dietary treatments: Sixty-four three-crossbred weaning pigs (Large White x Landrace x Duroc) weighing about 8.40±0.82 kg were used in this study. They were randomly divided into four groups with four replicates, which consisted of two castrated males and two females per each replicate, and were kept in a pen in an open-air house where feed and water were provided *ad libitum.*

Two types of prebiotics, inulin and fructooligosaccharide (FOS), as well as sub-therapeutic antibiotics were used as feed additives for induction of the GI-microbiota alteration, while feed additive was not supplemented for the control group. One of the four experimental diets based on corn-soybean was randomly fed to each replicate of pigs for six weeks as follows:

Diet 1: Basal diet without supplementation as the control group

Diet 2: Basal diet supplemented with sub-therapeutic antibiotics composed of 300 ppm Amoxycillin 50%, 80 ppm Colistin 10% and 100 ppm Tiamulin 10%

Diet 3: Basal diet supplemented with 0.2% of inulin (Orafti® GR)

Diet 4: Basal diet supplemented with 0.1% of FOS (Orafti® P95)

The basal diet was formulated for two periods of growth (4-7 and 8-10 weeks of age) according to the recommendations of National Research Council: NRC (2012). The inulin and FOS products used in this study contained > 95% inulin with 2-60 (average ≥ 10) degree of polymerization (DP) and > 95% FOS with 2-8 DP, which represented long chain and short chain fructans, respectively.

Sample collection and data measurement: Fresh fecal samples were collected from the pigs (8 pigs per group) at 0, 3 and 6 weeks after feeding experimental diets (4, 7 and 10 weeks of age) for bacterial quantification. Furthermore, at the end of the experiment (10 weeks of age), eight pigs (two pigs per group) were euthanized by a lethal injection of Euthatal (pentobarbitone sodium BP). Digesta contents from caecum and colon were immediately collected for microbiota quantification, in which Lactobacilli and Bifidobacteria served as representatives of beneficial microbes and *Escherichia coli* as pathogenic microbes, by conventional culture and real-time PCR methods.

Conventional culture quantification: The selected bacterial groups were enumerated by the conventional culture method using selective medium composed of MRS agar (Difco, USA) for *Lactobacillus* spp. (Deman, et al., 1960)**,** LP-MRS agar (MRS agar supplemented

with lithium chloride and sodium propionate) for *Bifidobacterium* spp. (Lapierre et al., 1992) and EMB agar (Difco, USA) for *E. coli* (Morsea et al., 1956), respectively. Plates containing between 30 to 300 colonies were counted and were expressed as $\log_{10}$ colony forming units (CFU) per gram of contents.

Real-time PCR quantification: Standard bacterial strains used in this study including *Lactobacillus acidophilus* ATCC 4356, *Bifidobacterium bifidum* ATCC 29521 and *E. coli* ATCC 35218 were obtained from the American Type Culture Collection (ATCC; Manassas, USA.).

Total genomic DNA from the fecal and digesta samples was extracted and purified using QIAamp® DNA Stool Mini Kit (Qiagen Hilden, Germany), while DNA from the standard bacterial strains was extracted and purified using DNeasy Blood and Tissue Kit (Qiagen Hilden, Germany). The extraction procedures followed the manufacturer's instructions. Purified DNA was stored at -80° C until analysis.

The genus-specific 16S rRNA-targeted primer sets used for quantitative real-time PCR in this study are listed in Table 1. Specificity of the primers was tested by conventional PCR and also sequenced (SolGent Co. Ltd, Korea) to confirm targeted bacterial species before performing in real-time PCR assay.

Table 1   Group-specific 16S rRNA-targeted primers sets used for real-time PCR

Target bacteria	Primer	Sequence (5′-3′)	Length (base pair)	References
Lactobacillus spp.	LAA-f	CATCCAGTGCAAACCTAAGAG	286	Wang et al. (1996)
	LAA-r	GATCCGCTTGCCTTCGCA		
Bifidobacterium spp.	SB-F	CGCGTCCGGTGTGAAAG	126	Xiang et al. (2011)
	SB-R	CTTCCCGATATCTACACATTCCA		
E. coli	ECO-f	GACCTCGGTTTAGTTCACAGA	585	Wang et al. (1996)
	ECO-r	CACACGCTGACGCTGACCA		

Quantitative real-time PCR was performed with the Mastercycler® ep realplex (Eppendorf, Germany). The PCR mixtures were performed in a total volumn of 20 µl containing 10 µL of 1X master-mix (LightCycler® FastStart DNA Master PLUS SYBR Green I, Roche, Germany, which contained Taq DNA polymerase, buffer, $MgCl_2$, SYBR green I dye and mix of dNTP), 0.3 µM of each primer (3 µl), 50 ng of DNA template (2 µL) and PCR-grade water (2 µL). The concentration of $MgCl_2$ was 1.0 mM. The DNA amplification condition was initial denaturation at 95°C for 3 mins followed by 40 cycles with denaturation for 40 s at 95°C, annealing temperature at 61°C for 25 s, and extention for 1 min at 72°C (Jittaprasatsin, 2008). To determine the specificity of amplification, analysis of product melting curve was performed after the last cycle of amplification. Obtained products were also sequenced (SolGent Co.

Ltd, Korea) and analyzed by using BLAST database (http://www.ncbi.nlm.nih.gov/BLAST).

Standard curves used for the quantification of DNA sample were generated from standard dilution series of known concentration of PCR products from the 16S rRNA gene of standard bacterial strain. Genomic DNA from the standard bacterial strain was amplified by conventional PCR as a positive control while the PCR mixture solution without genomic DNA was used as negative control.

Amplified PCR products were purified with DNA Clean/Extraction Kit (Genemark Technology Co, Ltd.). The quality as well as concentration of DNA were measured by spectrophotometer (Nanodrop, ND1000; Thermo Scientific, Wilmington, DE) and amplicons were also examined for expected size by 1.5% agarose gel electrophoresis. Concentrations were converted to copy number of DNA by using the following formula (Xiang et al., 2011).

$$\text{Copy concentration (copies/µl)} = \frac{[\text{DNA concentration in µg/µl} \times 6.0233 \times 10^{23}]}{[\text{DNA size (base pair)} \times 660 \times 10^6]}$$

Then, the standard curves were prepared by making duplication of 10-fold dilutions of the target amplicon using 10^2-10^{10} copies number of the 16S rRNA gene. Results from the standard curves showed a graph between threshold cycle (C_t) values in each dilution and $\log_{10}$ of known concentration of target amplicons.

The C_t values from the duplicate of each amplicon were used for calculating copy number of 16S rRNA in the fecal and digesta samples. Standard curve equation described the relationship between C_t (threshold cycle) and x (log copy number).

Standard curves were generated for copy number of 16S rRNA calculation of each bacterial

species. Correlation coefficient (r) for the associated standard curves was 0.996, 0.990 and 0.998 for *Lactobacillus* spp., *Bifidobacterium* spp., and *E. coli*, respectively. PCR amplification efficiency calculated by using the equation: $E = 10^{(-1/\text{slope})}$ (Amann and Ludwig, 2000) were 0.92, 1.01 and 0.89, respectively.

The sensitivity of these assays could detect at 10^2 copies of specific bacterial 16S rRNA gene. The melting curve analysis was performed immediately after real-time PCR amplification (Eppendorf Mastercycler® ep realplex) for determining the specificity of primers and PCR condition and the agarose gel electrophoresis was performed to further confirm specific PCR products. There was only one specific PCR product for each

Lactobacillus spp., *Bifidobacterium* spp. and *E. coli*, confirmed by only one peak in melting curve analysis and one band in agarose gel electrophoresis. These results indicated that the used primers and PCR conditions were specific.

A completely randomized design (CRD) was set up for the experiments. The number of targeted bacteria was statistically analyzed by analysis of variance (ANOVA) following the method of Steel and Torries (1980) to determine the influence of dietary treatments on bacterial counts. Duncan's New Multiple Range Test (DMRT) was also performed to compare treatment means following the method of Duncan (1955). Differences were considered statistically significant when $p \leq 0.05$ and $p \leq 0.01$.

Results

To examine the application of real-time PCR to quantify the microbiota alteration, the target bacteria from feces as well as digesta from distal part of intestinal tract, which is the main area of microbiota inhibition, were enumerated by conventional culture and real-time PCR methods. The quantification by real-time PCR was expressed in terms of copy numbers of 16s rRNA/g of fresh matter, while the conventional culture showed the number in CFU/g of fresh matter.

The quantification of fecal targeted bacteria of pigs by conventional culture and real-time PCR is shown in Figs 1, 2 and 3, and the results from quantification in the digesta samples are shown in Figs 4, 5 and 6, respectively. Although the population of Lactobaciili, Bifidobacteria and *E. coli* at the beginning of experiment was not significantly different ($p > 0.05$), after the feeding of feed additives the microbial profiles changed.

The conventional culture quantification exhibited fecal Lactobacilli and Bifidobacteria significantly suppressed by the sub-therapeutic antibiotic supplementation compared with the other groups ($p < 0.05$), as well as Bifidobacteria counts in the digesta samples ($p < 0.05$). A tendency of the same responses was also found in the case of Lactobacilli counts in the digesta samples. Contrastly, the prebiotic application, especially in the case of 0.2% inulin, tended to modulate the microbiota by increasing the Lactobacilli population, which presented the highest number compared with the other groups in both fecal and digesta samples, whereas no significant effect of feed additive was found in the case of *E. coli* ($p > 0.05$). However, the number of *E.coli* continually decreased from 4 to 7 weeks of the experiment. This might be due to the excessive stress during early weaning period (4 weeks) leading to the overgrowth of *E.coli* in the GI tract of pig (Heo et al., 2012). However, Sugiharto et al. (2013) reported that the increase in age reduced *E.coli* growth in the GI tract.
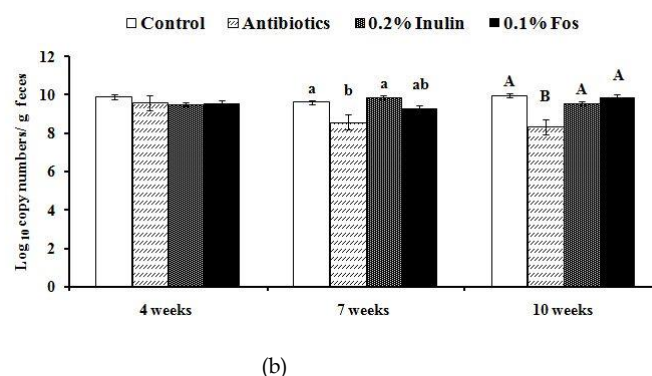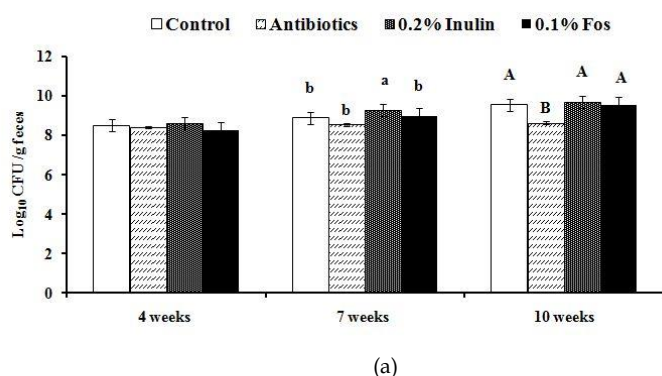

(a)	(b)

Figure 1   Quantification of fecal *Lactobacillus* spp. at different periods of growth by (a) conventional culture method and (b) real-time PCR method

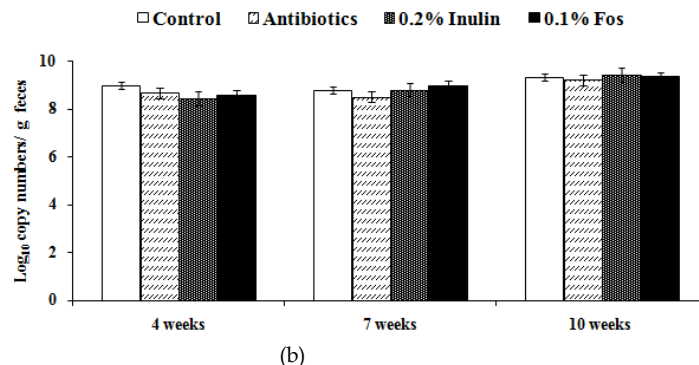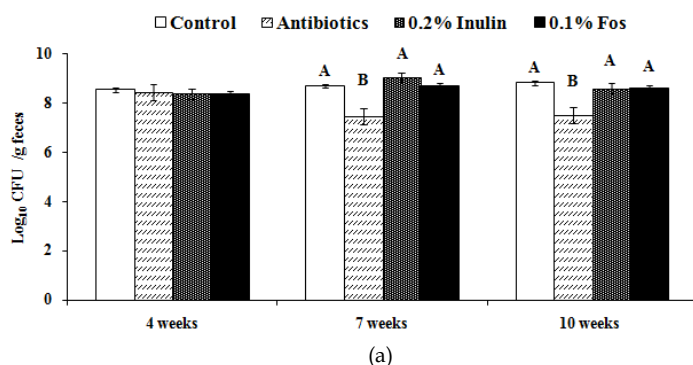|	
(a)	(b)

Figure 2   Quantification of fecal *Bifidobacterium* spp. at different periods of growth by (a) conventional culture method and (b) real-time PCR method
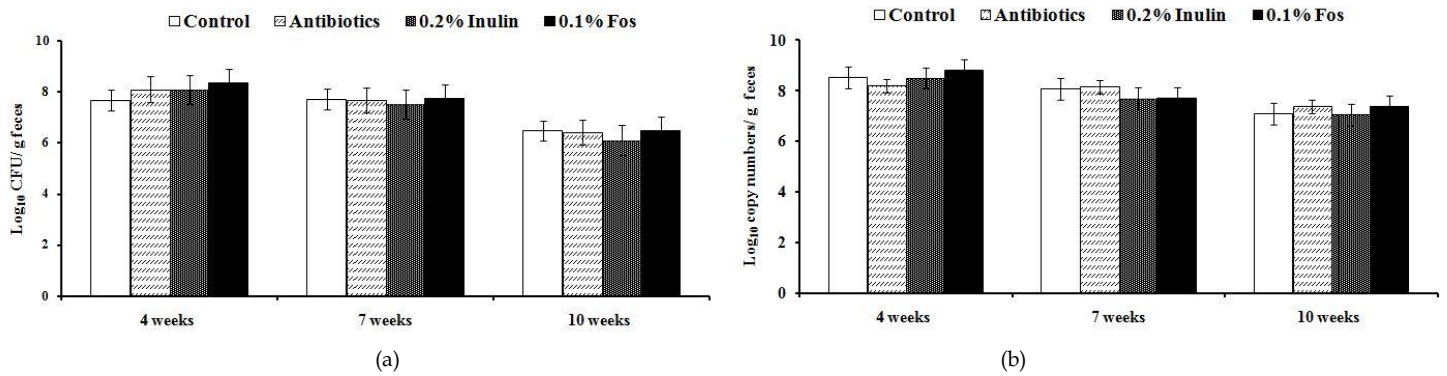
Figure 3   Quantification of fecal *E. coli* at different periods of growth by (a) conventional culture method and (b) real-time PCR method
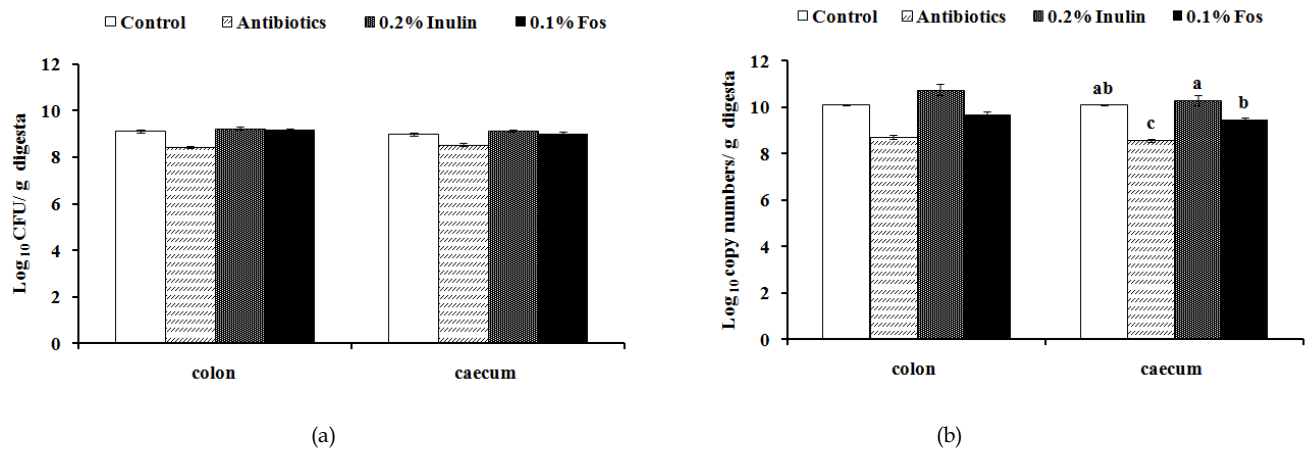

Figure 4   Quantification of *Lactobaillus* spp. in digesta sample of pigs at the end of   experiment (10 weeks of age) by (a) conventional culture method and (b) real-time PCR method
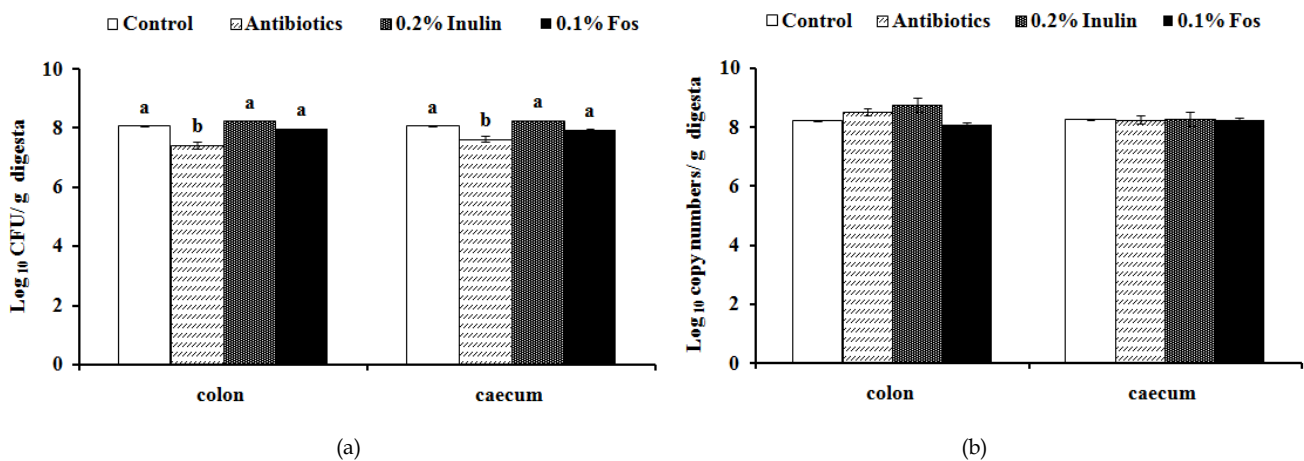

Figure 5   Quantification of *Bifidobacterium* spp. in digesta sample of pigs at the end of experiment (10 weeks of age) by (a) conventional culture method and (b) real-time PCR method
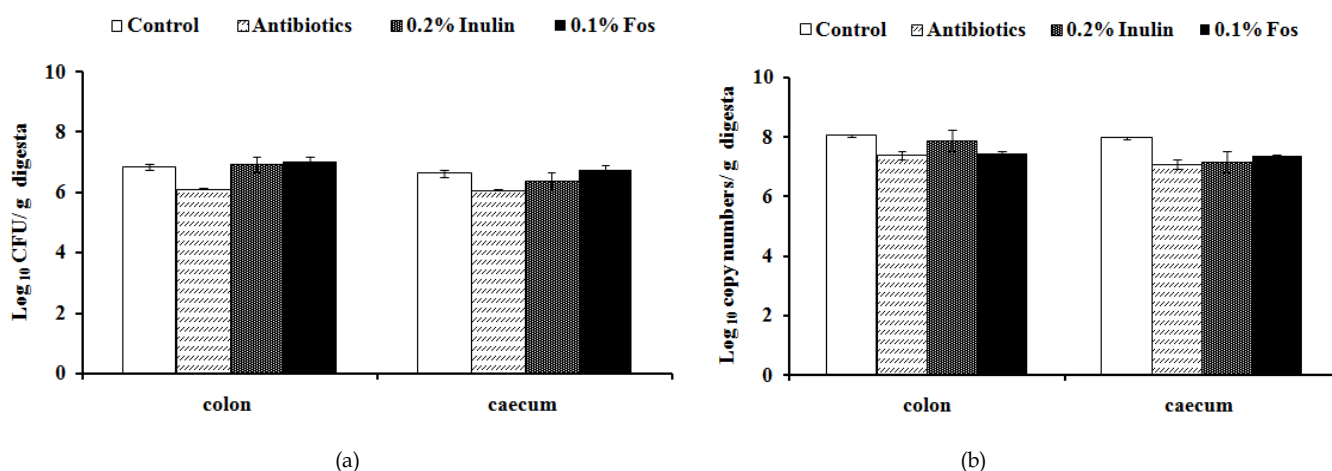
Figure 6   Quantification of *E. coli* in digesta sample of pigs at the end of experiment (10 weeks of age) by (a) conventional culture method and (b) real-time PCR method

The results from real-time PCR measurement were in agreement with those from the conventional culture method. The supplementation with sub-therapeutic antibiotics also provided significantly less copy number of 16S rRNA gene of Lactobacilli in the feces and caecum ($p < 0.05$), while Bifidobacteria and *E. coli* copy numbers were not significantly affected by the dietary feed additives ($p > 0.05$). There was a tendency of the same responses to 0.2% inulin

supplementation; the highest counts of Lactobacilli and Bifidobacteria were found, especially in the digesta samples.

Additionally, the quantification of *Lactobacillus* spp., *Bifidobacterium* spp. and *E. coli* number in both fecal and digesta samples by real-time PCR primarily showed similar patterns to the conventional culture quantification by showing correlations as shown in Figs 7, 8 and 9, respectively.
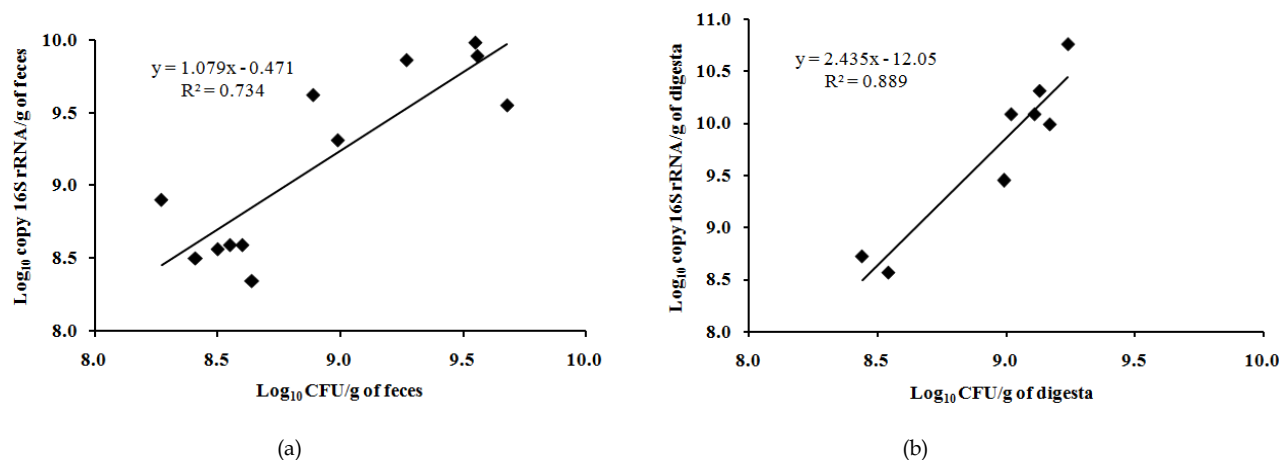

Figure 7   Correlation between number of *Lactobacillus* spp. measured by real-time PCR as Log_{10} copy 16S rRNA/ g of fresh matter and by conventional culture as Log_{10} CFU/g of fresh matter (a) from feces (b) from digesta samples

Discussion

The effect of feed additives on the alteration of microbiota was significantly expressed in the early stage after weaning (at 7 weeks of age) of pigs because they suffer from excessive stress which disturbed the balance of GI microbiota (Lalles et al., 2007). Antibiotics can suppress the proliferation of overall species of intestinal bacteria (Jensen, 1998; Gaskins et al., 2002). In contrast, other feed additives such as probiotic and prebiotic provide beneficial effects by supporting the growth of beneficial microbiota (Gibson and Roberfroid, 1995; de Lange et al., 2010). The effects of inulin which stimulated the growth of beneficial Lactobacilli and Bifidobacteria in this experiment confirmed the prebiotic property on GI microbiota

reported by previous studies (Nemcova et al., 1999; Klein Gebbing et al., 2001).

The results from this experiment demonstrated that the quantitative real-time PCR was suitable for applying for rapid quantification of selected bacteria in the complex microbial community from pig samples. However, the specificity of used primers and optimized PCR condition are important for accurate and reliable results by real-time PCR analysis. The corrected PCR products of each targeted bacterial DNA from this study were confirmed by sequencing. This method can be used to enumerate microbiota in 1 day, while the conventional culture method requires at least 2-3 days. This method is also convenient for detection anaerobic bacteria such as Bifidobacteria.

The 16S rRNA gene plays a role in culture-independent study of GI microbiota composition (Penders et al., 2005). The bacterial genome contains at least one copy of 16S rRNA genes, but varies in each species such as with 5.71, 3.57 and 7.00 copies per genome in Lactobacilli, Bifidobacteria and *E.coli*, respectively (Loredana, 2010; Pei et al., 2010). Therefore, the copy number of 16S rRNA enumerated by real-time PCR represented the number of bacterial cells in samples. However, the calculation of 16S rRNA genes to the number of bacterial cells in this experiment omitted the variation in *rrn* operon (group of genes encoding rRNA) and different copy numbers of 16S rRNA per genome of each target bacteria. Previous reports suggested that *rrn* operon number was a minor consideration in real-time PCR quantification (Lyons et al., 2000; Ott et al., 2004).

The copy number of 16S rRNA quantified by real-time PCR in this study showed the nearby values of microbial counts from weaning pigs reported by previous studies with 9-10 log copy numbers of *Lactobacillus* spp./g of feces (Castillo et al., 2007), 8-9 log copy numbers of *Bifidobacterium* spp./g of feces

(Xiang et al., 2011) and 7.5- 10 log copy numbers of *E. coli*/ g. of feces (Modesto et al., 2011). However, the copy numbers of 16S rRNA of bacterial population enumerated by the real-time PCR method mostly showed a higher number than CFU from the conventional culture quantitation. Differential range varied from 7.60% in the case of prebiotic-supplemention to 11.5% in the sub-therapeutic antibiotics supplemented group. These results are in agreement with previous reports which demonstrated that the higher quantification in terms of 16S rRNA by real-time PCR than CFU counts differed about 1 or even 2 log units (Huijsdens et al., 2002; Nadkarni et al., 2002). This situation is related to the multiplicity of 16S rRNA gene copies per genome of prokaryotes (Fogel et al., 1999). Additionally, real-time PCR could detect viable cells which were not cultivated by conventional culture or free DNA from dead cell bacteria (Castillo et al., 2006). Apajalahti et al. (2003) found that between 17 and 34% of total bacteria in fecal samples were nonviable and thus permanently disappeared by the culture method.
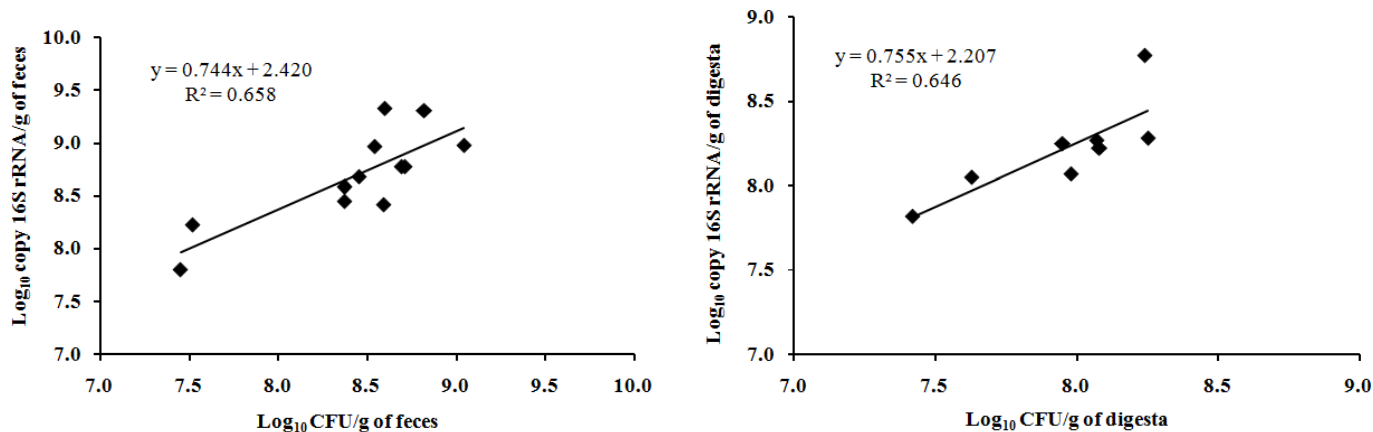

Figure 8 Correlation between number of *Bifidobacterium* spp. measured by real-time PCR as Log_{10} copy 16S rRNA/ g of fresh matter and by conventional culture as Log_{10} CFU/g of fresh matter (a) from feces (b) from digesta samples
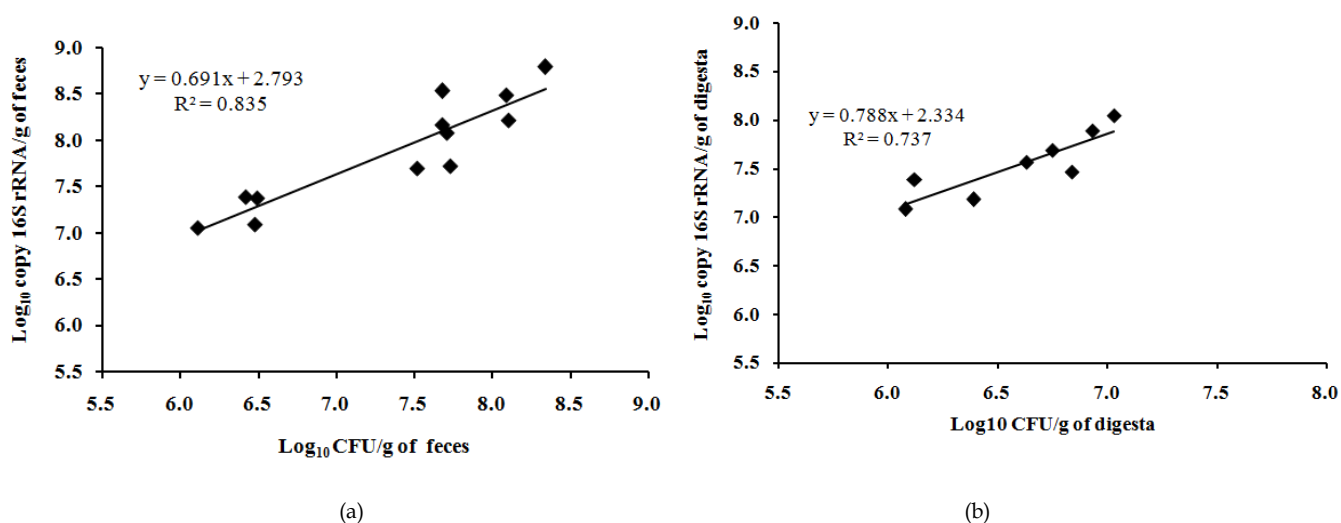

Figure 9 Correlation between number of *E. coli* measured by real-time PCR as Log_{10} copy 16S rRNA/ g of fresh matter and by conventional culture as Log_{10} CFU/g of fresh matter (a) from feces (b) from digesta samples

Therefore, some considerations of real-time PCR such as quality and yield of the extracted DNA should be taken into account for precise assessment.

In conclusion, dietary feed additives affect the population of GI microbiota depending on their activities. Prebiotics, especially 0.2% inulin, improved the balance of microbiota by increasing beneficial bacteria, while sub-therapeutic antibiotics controlled GI microbiota with growth suppression. Quantitative real-time PCR is a practical method to quantify the microbial population of GI tract due to its convenience, rapidity and reliability. Although the higher copy number of 16S rRNA enumeration was found, the responses of each bacteria to different feed additives were the same as those quantified by the conventional culture method. Therefore, quantitative real-time PCR can be a tool for study in many different works of animal nutrition such as changes in bacterial population which is influenced by age, diet and health status, making it an interesting alternative for animal nutritionists to evaluate the quality of feed or feed ingredients which can affect microbiota.

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บทคัดย่อ

การประยุกต์ใช้เทคนิค Real-time PCR สำหรับนับจำนวนจุลินทรีย์ในระบบทางเดินอาหารของลูกสุกรหย่านมที่ได้รับอิทธิพลจากสารเสริมในอาหาร

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การศึกษานี้มีวัตถุประสงค์เพื่อประยุกต์ใช้เทคนิค Real-time PCR สำหรับตรวจสอบและนับจำนวนจุลินทรีย์ประจำถิ่นในระบบทางเดินอาหารอันเป็นผลเนื่องมาจากการได้รับสารเสริมในอาหารสัตว์ โดยใช้ลูกสุกรหย่านมสามสายจำนวน 64 ตัว เลี้ยงด้วยอาหารทดลองสูตรพื้นฐานที่ผสมสารเสริม (อินนูลิน 0.2%, ฟรุคโตโอลิโกแซคคาไรด์ 0.1%, และยาปฏิชีวนะในระดับต่ำ) เปรียบเทียบกับกลุ่มที่ไม่ได้รับสารเสริม และวัดผลโดยการนับจำนวนจุลินทรีย์บนอาหารเลี้ยงเชื้อเปรียบเทียบกับวิธี Real-time PCR ผลการทดลองจากการนับจำนวนจุลินทรีย์โดยทั้งสองวิธีการ พบความสอดคล้องในการตอบสนองของจำนวนจุลินทรีย์ (Lactobacilli, Bifidobacteria, *Escherichia coli*) จากมูลและของเหลวในลำไส้ใหญ่ของสุกรส่วน caecum และ colon ต่อสารเสริมในอาหารแต่ละชนิด การเสริมอินนูลินในอาหารมีแนวโน้มทำให้เชื้อ Lactobacilli มีปริมาณสูงที่สุดเมื่อเทียบกับกลุ่มทดลองอื่น ในขณะที่การเสริมยาปฏิชีวนะในระดับต่ำทำให้ปริมาณของ Lactobacilli และ Bifidobacteria ที่พบในมูลและของเหลวจากลำไส้ใหญ่มีปริมาณลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) และมีแนวโน้มส่งผลให้ปริมาณเชื้อ *Escherichia coli* จากของเหลวในลำไส้ใหญ่ลดลง อย่างไรก็ตามจำนวนจุลินทรีย์ที่นับได้โดยใช้ real-time PCR มีค่าสูงกว่าการนับบนอาหารเลี้ยงเชื้อ เนื่องจากวิธีดังกล่าวสามารถนับจำนวน DNA ได้จากทั้งเซลล์ที่มีและไม่มีชีวิต ค่า sensitivity ของ real-time PCR จากการทดลองนี้สามารถใช้ตรวจวัดจำนวนจุลินทรีย์จากยีนเป้าหมาย 16S rRNA ได้ที่ระดับ 10^2 copies นอกจากนี้การกำหนดสภาวะการทำงานที่เหมาะสมของเครื่อง real-time PCR ทำให้วิธีการนี้มีข้อดีกว่าวิธีการเพาะบนอาหารเลี้ยงเชื้อคือช่วยลดเวลาและแรงงาน รวมถึงยังสามารถเก็บตัวอย่างไว้ได้เพื่อทำการวิเคราะห์ภายหลัง ดังนั้น real-time PCR จึงเป็นวิธีการที่มีประสิทธิภาพสำหรับประยุกต์ใช้ในการตรวจสอบคุณภาพอาหารสัตว์ซึ่งส่งผลต่อการเปลี่ยนแปลงสมดุลของจุลินทรีย์ในระบบทางเดินอาหาร

คำสำคัญ: สารเสริมในอาหารสัตว์ การนับจำนวนจุลินทรีย์ประจำถิ่น สุกร real-time PCR

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