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Full Length Research Paper

Real-time TaqMan polymerase chain reaction to quantify the effects of different sources of dietary starch on *Bifidobacterium* in the intestinal tract of piglets

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Twenty-eight PIC male piglets (similar birth and parity, weaned at 21±1.5 days) were used to study the effect of the different sources of dietary starch on the number of *Bifidobacteria* in the digesta of the duodenum, jejunum, ileum, cecum and colon. Pigs were randomly assigned to one of four diets formulated with corn starch, wheat starch, tapioca or pea starch. The determined ratio of amylose to amylopectin for these starches was 0.21, 0.24, 0.12 and 0.52, respectively based on the 16s rRNA sequences of maximum species of *Bifidobacterium* from GenBank to design the primers and probe. TaqMan polymerase chain reaction was developed to quantify the number of *Bifidobacterium*. We used this assay to detect genomic DNA of *Bifidobacterium* in the intestinal tract digesta of piglets, including duodenum, jejunum, ileum, cecum and colon. Our results indicated that, developed new real-time quantitative PCR assays can be allowed for rapid, convenient, reproducible and steady quantification of the *Bifidobacterium* in the intestinal content of piglets. Additionally, the present study revealed that high amylose/amylopectin ratio of starches significantly enhanced the numbers of *Bifidobacterium* in all segments of intestine.

Key words: Starch, *Bifidobacterium*, Taq-man polymerase chain reaction (PCR), weaned pigs.

INTRODUCTION

Nowadays, weaned piglets undergo a transition from milk-based diet to adult-type plant-based diet. The transition combined with the stress of being transported to production farms is often followed by a period of low feed intake (Brooks et al., 2003). The combination of these stresses can lead to intestinal malfunctions, intestinal microbiota in particular, which is subsequently followed by a reduced growth performance, diarrhea and mortality (Mikkelsen et al., 2004a; Spreeuwenberg et al., 2001a).

The gastrointestinal tract (GIT) of pigs is harbored by dense and diverse bacteria (Pryde et al., 1999). Because there is a more stable microbial ecosystem when bacterial diversity is increased, microbial diversity has been proposed as an indicator of the intestinal stability and health (Zoetendal et al., 2004). The gut microbial communities are affected by various factors, including the diets (Duncan et al., 2007a), environment (Pluske et al., 2007) and so on. Diet changes are likely to have a direct influence to place stress on the stability of bacterium and affect the entire GIT eco-physiology in lifestyle (Spreeuwenberg et al., 2001b). Certain dietary non-digestible carbohydrates can allow specific changes in the composition or activity of the intestinal microbiota (Roberfroid, 2007) and selectively stimulate the growth of the health-promoting bacteria (*Bifidobacterium* and *Lactobacillus*) in the gastrointestinal tract (GIT) and potentially prevent or moderate intestinal infections (Ebersbach et al., 2010; Kaplan et al., 2000).

Indigenous *Bifidobacteria* are believed to play an important role for animal and human health and immune

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function (Orrhage et al., 2000) and it is considered to be the target organism due to their potential to inhibit the growth of pathogenic bacteria that may prevent intestinal disorders (Mikkelsen et al., 2004b). Yazawa et al. (1978) explained that, *Bifidobacteria* are able to suppress pathogenic bacteria (*Escherichia coli*) because they utilize oligo- and polysaccharides that other intestinal bacteria cannot use.

Starches are the main source of carbohydrates in mixed diets and the major source of energy for mono-gastric animal and human (Deng et al., 2009a). They are α-glucans and exist in two forms: amylopectin and amylose (Cummings et al., 1995). Starch are classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) (Englyst et al., 1999), where RDS and SDS are digested in proximal intestinal tract and the major digested products are absorbed in small intestine. However, the non-digestible starches (RS) together with a small quantity of digested products can reach the large bowel (Aspvan et al., 1996; Conlon et al., 2009). They have significant effects on the composition of the intestine microbiota, tending to increase bacteria associated with a healthy bowel and tending to decrease those potential diseases (Conlon et al., 2009). Pieper et al. (2009) reported that, the ratio of amylose to amylopectin and starch sources affected the abundance of *Bifidobacterium* spp. *in vitro*, in particular high-amylose maize starch granules. Consequently, the ratio of amylose to amylopectin of starch may enhance desirable intestinal bacteria and induce beneficial gut health. However, the underlying mechanism is still unclear.

The aim of the present study was based on the 16s rRNA sequences of different species of *Bifidobacterium* from GenBank to design the primers and probe. TaqMan polymerase chain reaction was developed to quantify the number of *Bifidobacterium*. We used this assay to investigate the effects of different sources of dietary starch (CS; corn starch, WS; wheat starch, TS; tapioca starch and PS; pea starch) on the presence of *Bifidobacterium* in the intestinal tract digesta of piglets, including duodenum, jejunum, ileum, cecum and colon.

Nowadays, weaned piglets undergo a transition from milk-based diet to adult-type plant-based diet. The transition combined with the stress of being transported to production farms is often followed by a period of low feed intake.

MATERIALS AND METHODS

Animals and housing

Twenty-eight PIC male piglets (similar birth and parity), weaned at 21±1.5 days of age were used. They were fed in the Animal Center of Animal Nutrition Institute in Sichuan Agricultural University (Ya an, China). They were kept in special metabolism pens over 24 days (3 days pre-experimental and 21 days experimental periods) in a thermo regulated environment (ambient temperature 22°C and relative humidity 55%). They had *ad libitum* access to feed or water.

Experimental design

The experimental pigs were distributed into four blocks of seven piglets each according to body weight. Treatments were arranged simple factor with four sources of starch (corn starch, wheat starch, pea starch and tapioca starch). Experimental diets were formulated to meet NRC (1998) nutrient requirements for piglets weighing 5 to 10 kg. Ingredients and chemical composition of experimental diets are presented in Table 1. Antibiotic growth promoters were not included in the diets. Diets were balanced in essential amino acid concentration (lysine, methionine + cysteine and tryptophan) and had similar general energy (GE), crude protein (CP), starch (St) content, amylose content and amylopectin content.

Digesta sampling

At the end of the experiment, the pigs was held under general anesthesia and killed by an intracardiac injection of sodium pentobarbital (30 mg/kg BW) after 2 h feeding. Following euthanasia the abdominal cavity was opened from sternum to pubis to expose the gastrointestinal tract without damaging the wall of the digestive tract. The small intestine and large intestine were tied off respectively, and 5 to 10 cm sections of the duodenum, jejunum, ileum, cecum and colon were tied off.

The digesta of the duodenum, jejunum, ileum, cecum and colon were removed immediately and stored at -80°C until further analyses. The small intestine was stripped free of its mesentery and further divided into 3 sections: (1) the ileum from the ileal-cecal junction to 80 cm anterior to this junction; (2) the duodenum, 80 cm posterior to the gastro-duodenal sphincter; (3) the jejunum constituted the regions between the ileum and duodenum (Adeola et al., 2006).

Extraction of DNA from digesta

Genomic DNA from the *Bifidobacterium adolescentis* (ATCC15703) was extracted from the cultures with a TaKaRa minibest bacterial genomic DNA extraction kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. Bacterial DNA from the digesta samples was extracted using an E.Z.N.A.TM stool DNA isolation kit (Omega Bio-Tek, Doraville, CA) according to the manufacturer's specifications. The final elution volume was 100 µl and the concentration was determined by spectrophotometer (Beckman Coulter DU 800, Fullerton, CA).

Designing and validation of primers

Primers and probe (Table 2) were designed with primer express 3.0 (Perkin Elmer Applied Biosystem, San Francisco, California) for quantitative detection of particular *Bifidobacterium*. 16S rRNA sequences of maximum species of each genus encountered in the swine intestinal tract were downloaded from the GenBank database as well as EMBL and DDBJ. The sequence of the *Bifidobacterium* blocks of hyper variable regions was comprised with all other genera in order to avoid any non-specific amplification, the sequences of all the genera fetched from the database were submitted to DNAStar (MegAlign) programme (DNASTAR, Inc., Madison, WI). These sequences were then submitted to second round of alignment where the maximum number of species belonging one genus was aligned and the regions showing conservations were selected as *Bifidobacterium* genus-specific primers and probe. To further ensure that the oligonucleotide sequences were complementary pairing with the target genus only, they were checked with GenBank program BLAST (NCBI BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and RDP program Check-

Table1. Ingredients and nutrient levels in the experimental diets (%).

Ingredient	CS diet	PS diet	TS diet	PS diet
Corn starch (86%)	54.5			
Wheat starch (87%)		54.5		
Tapioca starch (86.5%)			54.5	
Pea starch (88%)				54.5
Decupled soybean meal	2	2	2	2
Soybean protein concentrate	17.83	17.83	17.83	17.83
Soybean meal	10	10	10	10
Whey	7.3	7.3	7.3	7.3
Fish meal	6	6	6	6
Calcium carbonate	0.55	0.55	0.55	0.55
Monocalcium phosphate	0.7	0.7	0.7	0.7
Sodium chloride	0.15	0.15	0.15	0.15
DL-Met (98%)	0.17	0.17	0.17	0.17
L-Thr (98.5%)	0.01	0.01	0.01	0.01
Chromium Oxide	0.4	0.4	0.4	0.4
Premix*	0.39	0.39	0.39	0.39
Total	100	100	100	100
Nutrient levels (%)				
Total starch	51.71	51.17	51.98	52.92
Amylose	8.81	9.76	5.42	17.26
Amylopectin	42.9	41.41	46.56	33.66
Amylose/Amylopectin ratio	0.21	0.24	0.12	0.52
Cross energy(KJ/g)	14.5	14.43	14.49	14.51
Crude protein	19.9	19.87	19.87	19.99
Lys	1.29	1.29	1.29	1.29
Met+Cys	0.74	0.74	0.74	0.74
Thr	0.81	0.81	0.81	0.81
Try	0.25	0.25	0.25	0.25
Crude fiber	1.36	1.37	1.37	1.35
Crude fat	2.6	2.53	2.58	2.62

*Supplied per kg diet: 6.0 mg of Cu as $CuSO_4 \cdot 5H_2O$, 100 mg of Fe as $FeSO_4 \cdot 7H_2O$, 4 mg Mn of as $MnSO_4 \cdot H_2O$, 100 mg of Zn as$ZnSO_4 \cdot H_2O$, 0.3 mg of Se as Na_2SeO_3, 0.3 mg of I as KI, 1000 mg of choline chloride (50%), 200 mg of sweeteners, 0.04% of vitamin premix.

Table 2. Sequences of oligonucleotide primers and probe.

Assay	Primer/probe name and sequence (5′-3′)	Product size(bp)	Annealing temperature (°C)	Reference
All bacteria	Eub338F, ACTCCTACGGGAGGCAGCAG Eub518R, ATTACCGCGGCTGCTGG	200	60	(Fierer et al., 2005)
Bifidobacterium	SB-P1, AGGGCTCGTAGGCGGTTCGTC SB-P2, CCCCACATCCAGCATCCA	264	56.8	This study
Bifidobacterium	SB-F, CGCGTCCGGTGTGAAAG SB-R, CTTCCCGATATCTACACATTCCA SB-P, (FMA) ATTCCACCGTTACACCGGGAA(BHQ-1)	126	60	This study

Table 3. Reference strains used in this study and quantitative real-time PCR test results of reference strains.

Reference strain	Real-time PCR test result*	
	SL-F,SL-R,SL-P	Eub338F, Eub518R
Bifidobacterium adolescentis ATCC15703(D)	+	+
Bifidobacterium animalis CICC6165(A)	+	+
Bifidobacterium suis SB08ZY(E)	+	+
Bifidobacterium suis SB09NJ01(E)	+	+
Bifidobacterium suis SB09NJ02(E)	+	+
Lactobacillus acidophilus CICC6005(A)	-	+
Lactobacillus plantarum CICC6009(A)	-	+
Lactobacillus suis SL0501(E)	-	+
Lactobacillus suis SL0502(E)	-	+
Lactobacillus suis SL0503(E)	-	+
Bacillus subtilis ATCC6633(D)	-	+
Bacillus cereus CMCC63302(D)	-	+
Bacillus subtilis Bs01J(E)	-	+
Bacillus subtilis Bs02J(E)	-	+
Bacillus pumilus Bp0106(E)	-	+
Escherichia coli ATCC8739(D)	-	+
Escherichia coli ATCC25922(D)	-	+
Entero-hemorrhagic Escherichia coli O157:H7(D)	-	+
Porcine pathogenic *Escherichia coli* E.c0401(F)	-	+
Porcine pathogenic *Escherichia coli* E.c0402(F)	-	+
Staphylococcus aureus ATCC25923(C)	-	+
Staphylococcus aureus ATCC6538(C)	-	+
Streptococcus hemolytic-β CMCC32210(D)	-	+

A, Purchased from China center of industrial culture collection; B, isolated from the attenuated live vaccine of *Salmonella choleraesuis*; C, saved in laboratory of veterinary pharmacology of Sichuan agricultural university; D, saved in laboratory of preventive veterinary medicine of Sichuan agricultural university; E, isolated from healthy pig intestine by laboratory of preventive veterinary medicine of Sichuan agricultural university; F, isolated from clinical samples by laboratory of preventive veterinary medicine of Sichuan agricultural university. *Amplification results with primers SL -F/SL-R and probe SL-P for validation specificity; *amplification results with primers Eub338F/ Eub518R for verification feasibility; '+' represents positive test results; '-' represents Negative test results.

Probe (Details about RDP data and analytical functions can be found at http://rdp.cme.msu.edu/). Primers (Table 2) for all bacteria were obtained from the published work (Fierer et al., 2005). All the primers and probe were commercially synthesized from invitrogen (Shanghai, China).

Reference strains, culture conditions and genome extraction

The source of strain and reference strains used in this study were described in detail in Table 3. Briefly, five strains of *Bifidobacterium*, five strains of *Lactobacillus*, five strains of *Bacillus*, five strains of *E. coli*, two strains of *Staphylococcus aureus*, four strains of *Salmonella* and four strains of *Streptococcus* were used in this study. The strains were cultured anaerobically or aerobically in LB broth supplemented with 1% glucose at 37°C for 12 to 48 h. Total genomic DNA from the different reference strains was extracted and purified by using the method described in related kit manual (E.Z.N.A. TM Bacterial DNA kit, OMEGA Bio-Tek, USA).

Standard curve generation

To quantify the number of *Bifidobacterium* and all bacteria in test samples, the standard curves were produced by constructing two specific standard control plasmids. Two amplicons were produced first. The 264 bp fragment was amplified, DNA extracted from *B. adolescentis* (ATCC15703), using the primers (Table 2) SB-P1 and SB-P2 and the following conditions: 95°C 5 min, followed by 35 cycles of 95°C 30 s, 56.8°C for 30 s, 72°C for 20 s, with a final extension of 72°C for 10 min. The 200 bp fragment was amplified, DNA extracted from the test samples, using the primers (Table 2) Eub338F and Eub518R (Fierer et al., 2005) and the following conditions: 95°C 5 min, followed by 32 cycles of 95°C 30 s, 55°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 10 min. The amplified products were eluted from the agarose gel using TIANquick mini purification kit (TIANGEN, Beijing, China) and cloned into the pMD19-T vector (TaKaRa, Dalian, China). Plasmids DNA was purified using the E.Z. N.A TM plasmid miniprep kit (OMEGA Bio-Tek, USA). Clones were screened for the inserts using

the appropriate restriction enzymes and positive clones were sequenced. DNA concentration of the plasmids preparation was determined by spectrophotometer (Coulter DU 800, Beckman, USA) and the copy number calculated using the following formula: (DNA concentration in μg/μl × 6.0233 × 1023 copies/mol)/ (DNA size(bp) × 660 $\times 10^6$). A 10-fold dilution series of the plasmid DNA (1×10^8 to 1×10^1 copies/μl) was prepared and used to generate the standard curve. Target copy numbers for each reaction were calculated from the standard curves.

Quantitative PCR conditions and validation of primers specificity

All primers and probe used in this study are presented in Table 2. Real-time quantitative PCR was carried out with IQ5 real-time PCR detection System (Bio-Rad, CA, USA) using optical grade 96-well plates in a final volume of 25 μl. Reaction system was composed of 12.5 μl SYBR Premix Ex Taq (2×), 1 μl each of forward and reverse primers (100 nM), 9.5 μl ddH_2O and 1 μl DNA in each reaction for detecting all bacteria. The following cycling condition: 1 cycle of predenaturation at 95°C for 20 s; 40 cycles of denaturation at 95°C for 5 s; annealing at 60°C for 30 s and extension at 72°C for 50 s. Melting curve conditions were 95°C for 0 s, 55°C for 1 min and 95°C for 1 min (temperature change velocity: 0.5°C/s). For *Bifidobacterium* a PrimerScriptTM PCR kit (perfect real time) (TaKaRa, Dalian, China) was used with 100 nM of genus-specific primers and fluorescent probe. The reaction protocol was composed of 1 cycle of predenaturation at 95°C for 2min; 50 cycles of denaturation at 95°C for 15 s; annealing at 60°C for 30 s and extension at 72°C for 50 s. The genomic DNA of each reference strain was detected using quantitative real-time PCR with primers SB-F and SB-R and probe SL-P for validation specificity and with primers Eub338F and Eub518R for verification feasibility.

Statistical analysis

The dates were analyzed using SPSS12.0. A one-way ANOVA procedure was carried out for all data. All results were expressed as means ± SD. The results were statistically analyzed using least significant difference test. $P < 0.05$ was considered significant.

RESULTS

Specific verification of quantitative PCR products

The primers of quantitative PCR were used for conventional PCR with *B. acidophilus* ATCC15703 (D) DNA templates for verifying the specific amplification. Results showed that, the PCR produced an intense band with the expected 126 bp (data not shown), which indicated 100% specificity.

Quantitative PCR standard curve

Real-time quantitative PCR is the ability to quantitate bacterial abundance in various complex environmental samples. The correlation coefficient for the associated standard curve was 0.998 and PCR efficiency was 111.4%. Amplification efficiencies, calculated using the equation: $E = 10^{[-1/slope]}$ (AmannLudwig et al., 1995), indicating that, the crossing threshold values for the standards fell within an acceptable range. The numbers of DNA copies for detecting samples was calculated by using the following equation: $Y= -3.075X + 47.94$ (where, Y is the threshold cycle (C_t) and X is the Log_{10} (copy number of 16S rDNA)) (Figure 1).

Reproducibility

Four different known concentrations of DNA (1.2×10^9-1.2×10^6 copies/μl) were amplified by performing the assay described earlier in triplicate for verifying the reproducibility between experiments. The results showed that, the assay was highly reproducible, because the coefficient of variation was statistically low, at < 1.5%. The threshold cycle for each concentration ranged from 1.2×10^9 copies/μl to 1.2×10^6 copies/μl and was different between 0.1 and 0.3 cycles (Figure 2).

Specificity of the PCR

All thirty bacterial strains were used to evaluate the specificity of the real-time quantitative PCR, which that indicated only *Bifidobacterium* genomic strains can show positive results. However, there was no amplification with other bacteria group (Figure 3).

Starch composition of experimental diets

Total starch content was basically similar among corn, wheat, tapioca and pea treatment. The ratio of amylose and amylopectin was 0.21, 0.24, 0.12 and 0.52, respectively (Table 1).

Enumeration of *Bifidobacterium* group and all bacteria

Real-time PCR analysis was performed to determine the numbers of *Bifidobacterium* and all bacteria in the intestine content of all piglets. The copy numbers of all bacteria in the digesta of proximal intestine were not affected by the different dietary treatment (Table 4). However, the copy numbers of all bacteria in the cecal and colon content of piglets fed PS was significantly lower than all other treatment ($P < 0.05$) (Table 4). The copy numbers all bacteria and *Bifidobacterium* was lowest for the duodenum and increased towards the colon (Table 4). The numbers of *Bifidobacterium* was the highest in all groups ($P < 0.05$) (Table 4). However, there was a tendency for lower numbers of *Bifidobacterium* in all segments of the intestinal tract of piglets fed TS. The copy numbers of all bacteria and *Bifidobacterium* in all segments of the intestinal tract of piglets was not affected between the CS and WS group.

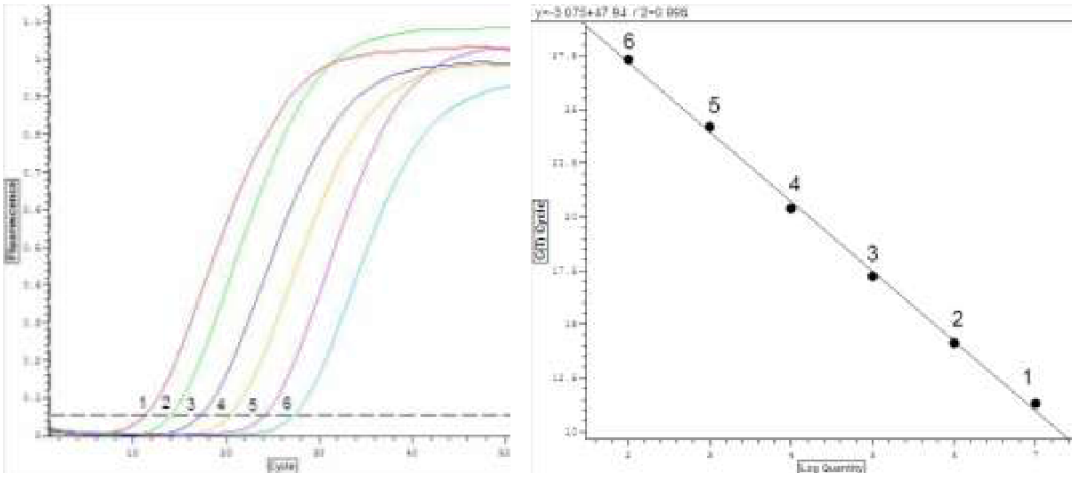

Figure 1. Linear relationship between threshold cycles and the copy number of 1 6S rDNA from *Bifidobacterium*. The standard curve was developed by using the standard DNA template with 10 fold serial dilutions. 1: 1 ×10^9 copies/μl; 2: 1 × 10^8 copies/μl; 3: 1 × 10^7 copies/μl; 4: 1 × 10^6 copies/μl; 5: 1 × 10^5 copies/μl; 6: 1 × 10^4 copies/μl. Linear regression (R^2 =0.998) results in an equation of Y= -3.075X + 47.94.

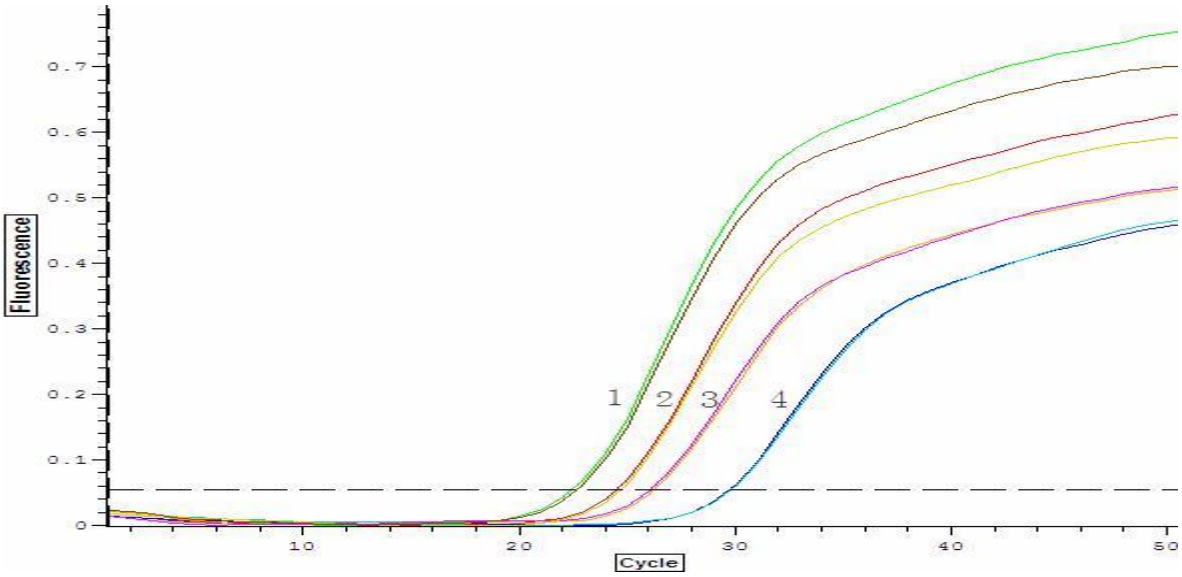

Figure 2. The values of two repeats of four different were showed to prove the reproducibility of developed real-time quantitative PCR.

The percentage of *Bifidobacterium* based on all bacteria

The percentage of *Bifidobacterium* (based on all bacteria) in all intestinal segments content were affected among dietary treatments (Table 4). However, there was no effect between the CS group and WS group. The percentage of *Bifidobacterium* (based on bacteria) in PS group was the highest among all treatments ($P < 0.05$) (Table 4). Meanwhile, the percentage of *Bifidobacterium* (based on bacteria) in PS group was the lowest among all the treatments ($P < 0.05$) (Table 4).

DISCUSSION

This experiment was conducted to evaluate the influence of dietary starch (different amylose/amylopectin ratio) on microbial populations of duodenum, jejunum, ileum, cecum and colon in piglets. In this study, the GE, CP, St, lysine, methionine + cysteine and tryptophan contents were all similar in the four experimental diets with the different starch sources, while there were differences in the amylose/amylopectin ratio of the starch.

Bifidobacterium is considered to be a beneficial component of the intestinal microbiota which can establish an

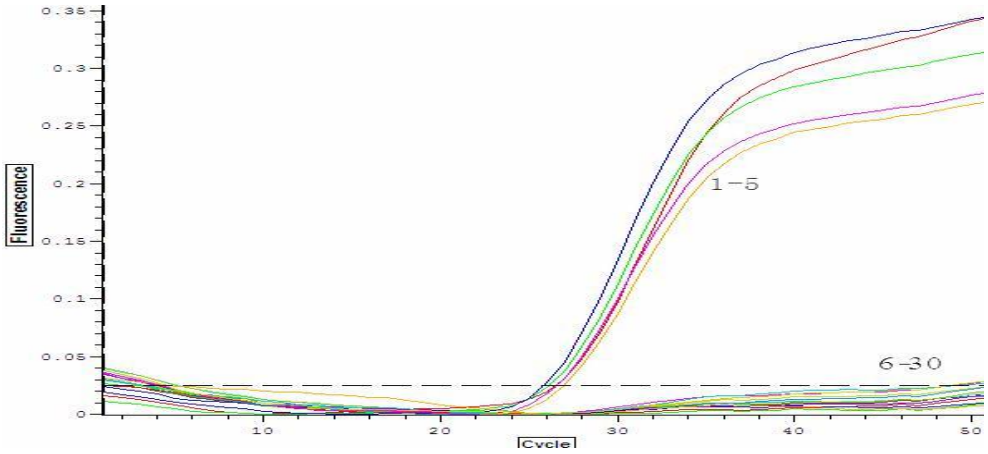

Figure 3. 1-5 are curves of *Bifidobacterium*; 6-30 are curves of *Lactobacillus, bacillus, salmonella, E. coli*, *S. aureus* and *Streptococcus* (the strains were shown in Table 3).

Table 4. THE copy numbers of all bacteria, *Bifidobacterium* and the percentage of *Bifidobacterium* (based on all bacteria) in the all intestinal segments content of piglets among different dietary starch sources treatments.

Parameter	Corn starch	Wheat starch	Tapioca starch	Pea starch
All Bacteria				
Duodenum	(2.26±0.74)×10^{9}	(2.55±0.56)×10^{9}	(2.83±0.86)×10^{9}	(2.07±0.59)×10^{9}
Jejunum	(7.35±2.04)×10^{9}	(8.58±1.81)×10^{9}	(1.38±0.88)×10^{10}	(1.09±0.37)×10^{10}
Ileum	(7.74±2.93)×10^{9}	(8.28±2.29)×10^{9}	(7.06±1.19)×10^{9}	(6.60±3.20)×10^{9}
Cecum	(2.79±0.90a)×10^{11}	(2.33±0.63a)×10^{11}	(2.29±0.69a)×10^{11}	(1.67±0.81b)×10^{11}
Colon	(4.83±1.20a)×10^{11}	(5.28±2.12a)×10^{11}	(4.23±1.27a)×10^{11}	(1.84b±0.82)×10^{11}
Bifidobacterium				
Duodenum	(5.49±0.69b)×10^{6}	(6.03±0.57b)×10^{6}	(3.52±0.76c)×10^{6}	(8.04±1.38a)×10^{6}
Jejunum	(1.53±0.42b)×10^{7}	(1.64±0.65b)×10^{7}	(4.14±0.59b)×10^{6}	(3.85±1.78a)×10^{7}
Ileum	(8.72±3.08b)×10^{6}	(1.04±0.22b)×10^{7}	(3.44±0.93c)×10^{6}	(2.32±0.31a)×10^{7}
Cecum	(5.91±2.26b)×10^{8}	(5.45±1.17b)×10^{8}	(2.86±1.05c)×10^{8}	(1.23±0.15a)×10^{9}
Colon	(3.76±1.21b)×10^{8}	(3.89±0.89b)×10^{8}	(7.56±2.80b)×10^{7}	(1.24±0.53a)×10^{9}
B/A (%)*				
Duodenum	0.26±0.08b	0.25±0.05b	0.13±0.01c	0.41±0.07a
Jejunum	0.22±0.09b	0.19±0.08b	0.04±0.02c	0.35±0.09a
Ileum	0.12±0.02b	0.14±0.05b	0.05±0.02c	0.36±0.05a
Cecum	0.21±0.06b	0.24±0.05b	0.13±0.04c	0.74±0.05a
Colon	0.08±0.03b	0.09±0.04b	0.02±0.01c	0.69±0.17a

The dates are expressed as mean values ± the standard deviation for all samples. $^{a, b, c}$ means with different superscripts in the row differed significantly ($P < 0.05$). * B/A (%): the percentage of *Bifidobacterium* (based on all bacteria).

efficient barrier to the invasion and colonization of the gut by putrefactive and pathogenic bacteria and produces a range of metabolic substrates such as short chain fatty acids, especially butyrate. It is used by the host and stimulates the immune system in a non-inflammatory manner (Crittenden et al., 1999; Lene Lind et al., 2004; Trevisi et al., 2008). In the present study, real-time TaqMan polymerase chain reaction assays is developed targeting the 16S rRNA gene to quantify *Bifidobacterium* groups in the intestinal tract content of weaned-piglets and thirty bacterial strains were used to assess the specificity of the PCR. Positive results were only observed in *Bifidobacterium* genomic strains, while there was no amplification with other spp. (Figure 3).

Fluorescent quantitative has become a potential powerful method to quantify the population of gastrointestinal tract microbial due to its convenience, rapidity, reproducibility and accuracy (Deng et al., 2008; Deng et al., 2007). In recent studies, real-time quantitative PCR has been shown to be an available tool for quantifying bacterial abundance in many different kinds of complex environmental samples down to the genus and species level (Skovhus et al., 2004; WellinghausenFrost et al., 2001; Wilks et al., 2006). Real-time quantitative PCR provides information on the relative and absolute abundance of a genus and species in complex environmental samples. Such detailed information can often be difficult to obtain and investigate with conventional cell enumeration methods such as most probable number and direct cell counting methods (Skovhus et al., 2004). The specific primer-probe combination is an available alternative for detecting the counts of intestinal bacterial species (Dario De Medici et al., 2003; Deng et al., 2007). Four different known concentrations of DNA (1.2×10^9-1.2×10^6 copies/µl) were amplified by performing the assay described earlier in triplicate. Analysis of these values proved that the assay was reproducible. Meanwhile, the detected results showed that the coefficient of variation was statistically low, at < 1.5%. The main advantage of fluorescent quantitative PCR is the ability to quantitate unknown samples. In summary, a new real-time PCR assays was developed that allowed for rapid, convenient, reproducible and steady quantification of the *Bifidobacterium* group in the intestinal content of piglets.

The gastrointestinal tract of pigs is colonized by a densely diverse bacterium and the intestinal microbiota has important influence on animal health and growth performance (Leser et al., 2000; Moore et al., 1987). Meanwhile, the gut microbial communities are affected by various factors, such as the diets and environment (Duncan et al., 2007b; Pluske et al., 2007). Generally, *Bifidobacterium* can hydrolyze such polysaccharides, as starch and cellulose and so on. Starches are the main source of carbohydrates and energy for monogastric animal and human (Deng et al., 2009b). Here, high amylose/amylopectin ratio starch (PS) can increase the number of *Bifidobacterium* and the percentage of its (based on all bacteria) intestinal digesta of piglets. We also showed that, the low amylose/amylopectin ration starch (TS) can decrease the percentage of *Bifidobacterium* (based on all bacteria). Feeding high amylose starch can enhance the population of *Bifidobacterium* and short chain fatty acids in the colon and fecal content of mammals (Brown et al., 1997; Chang et al., 2006; Wang et al., 2002). Chang et al. (2006) found that high amylose starch diet resulted in increased *Bifidobacterium* growth compared with the low amylose starch diets in cecal contents of rats.

Brown et al. (1997) reported that, feeding high amylose starch to pigs increased fecal *Bifidobacterium* population compared with feeding low amylose starch. Pieper et al. (2009) reported that, the ratio of amylose to amylopectin and starch sources affected the abundance of *Bifidobacterium* spp. *in vitro* model of porcine gastrointestinal tract. Those results are in agreement with the present study *in vivo* of porcine intestinal tract. Another results in this study suggested that, the high amylose/amylopectin ratio PS can increase the *Bifidobacterium* numbers not only in the distal intestinal digesta but also in the proximate digesta. However, the high amylose/ amylopectin ratio PS decreased the number of all bacteria in the cecal and colon chymus of piglets, while the copy numbers of all bacteria in the digesta of proximal intestine were not affected by the different dietary treatment. These changes need a further study.

In conclusion, we developed a new real-time Taq-man PCR assays that allowed for rapid, convenient, reproducible and steady quantification of the *Bifidobacterium* group in the intestinal content of piglets. Additionally, the present study revealed that high amylose/amylopectin ratio of starches significantly enhanced the numbers of *Bifidobacterium* in digesta of the all intestine segment. The new methodology used in this study provides an important tool for studying the influence of different starch diet on the endogenic *Bifidobacterium* numbers in gastrointestinal tract of pigs.

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