Effects of different starch sources on *Bacillus* spp. in intestinal tract and expression of intestinal development related genes of weanling piglets

Guo-Quan Han · Zhen-Tian Xiang · Bing Yu · Dai-Wen Chen · Hong-Wei Qi · Xiang-Bin Mao · Hong Chen · Qian Mao · Zhi-Qing Huang

Abstract The study was conducted to evaluate the effects of different starch sources on *Bacillus* spp. in intestinal tract and expression of intestinal development related genes of weanling piglets. Twenty-eight PIC male piglets were divided into four homogeneous groups according to initial body weight (similar birth and parity, weaned at 21 ± 1.5 days). Diets for the four treatments consisted of corn starch, wheat starch, tapioca starch and pea starch with the determined ratio for amylose to amylopectin of 0.21, 0.24, 0.12 and 0.52 respectively. Real-time quantitative polymerase chain reaction was applied to: (1) detect genomic DNA of *Bacillus* and to quantify the number of *Bacillus* in the intestinal tract chyme of piglets with the primers and probe which designed based on the 16S rRNA sequences of maximum species of *Bacillus* on GenBank; (2) measure the mRNA level of glucagon-like peptide 2 (GLP-2), insulin-like growth factors 1 (IGF-1) and epidermal growth factor (EGF) in duodenum, jejunum and ileum. Results showed that the number of *Baciilus* and the percentage based on all bacteria in the whole intestinal content of piglets fed pea starch was highest in all groups ($P < 0.05$). There was no significant differance on copy numbers for all bacteria and *Bacillus* in the whole intestinal tract of piglets between the corn starch group and wheat starch group ($P > 0.05$). In addition, the expression level of GLP-2, IGF-1 mRNA in jejunum and ileum of pea starch treatment (the high amylose/amylopectin ratio) were increased while the tapioca starch decreased their mRNA level significantly compared to other three treatments ($P < 0.05$). There was no significant difference for the mRNA level of EGF in each group. The present study revealed that high amylose/amylopectin ratio of starches significantly enhanced the numbers of *Bacillus* in all segments of intestine and the mRNA level of intestinal development related genes.

Keywords Starch · *Bacillus* · Real-time PCR · Weanling piglets · GLP-2 · IGF-1

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Introduction

The swine gastrointestinal tract (GIT) represents a dynamic ecosystem composing of a highly complex and diverse community of microaerophilic and anaerobic microbes that are involved in the fermentation of ingested feed and the components secreted by the host into the GIT [1, 2]. Regulating the composition and metabolic activity of the intestinal microbiota through the diets to improve gut metabolism and health is an increasing focus of nutritionists in the post-antibiotic era [1]. Recently, prebiotics and probiotics have been used as a strategy to manipulate the intestinal ecosystem for enhancing the growth performance and gut health of animals [3, 4]. Some *Bacillus* species have been as probiotics added to the swine feed for improvement in nutrient digestion and utilization in the latter part of the growing-finishing period. *Bacillus* supplementation in previous research may have been due to

several functions of *Bacillus* microorganisms, which have been identified as potent producers of extracellular degrading enzymes, including amylases, cellulases, lipases and proteases [5]. The role of *Bacillus* organisms is that it may increase porcine intestinal health through regulating immune system and protect from pathogenic challenges [6]. *Bacillus* is a genus of Gram-positive rod-shaped bacteria. *Bacillus* species can be obligate aerobes or facultative anaerobes, which has the vital role in the intestinal microecology balance by consuming intestinal oxygen, creating the anaerobic environment. Pieper et al. [1] reported that different amylose/amylopectin ratio starch not only result in changing microbial ecophysiology but also affect the susceptibility of the host to opportunistic pathogens.

Starch is mostly composed of the dry matter in certain corps, and it is the major energy-yielding ingredient in diets for both animal and human [7]. It is a polysaccharide, and consists of the polymers amylose and amylopectin [8]. The starches are classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) [9]. They have significant influence on the composition and activity of the intestine Microflora, tending to enhance bacteria associated with a healthy intestine and tending to reduce those potentially diseases [10].

Up to now, there have been few reports about the effects of different starch sources on *Bacillus* spp. in intestinal tract and expression of intestinal development related genes of weanling piglets. In this paper, in an effort to elucidate this problem, real-time quantitative PCR protocol was applied to quantify the absolute abundance of *Bacillus* species in the porcine intestinal chyme and expression of intestinal development related genes treated with different dietary starch source.

Materials and methods

Animals and management

A total of 28 PIC male piglets (similar birth and parity), weaned at 21 ± 1.5 days of age were used in this experiment. The piglets were housed in special metabolism pens (0.6×1.2 m) in a thermo regulated environment (ambient temperature 22°C and relative humidity 55%) in the Animal Center of Animal Nutrition Institute in Sichuan Agricultural University. Piglets were fed with the experimental diets for 24 days (3-d pre-experimental and 21-d experimental periods).

Experimental design and diets

The piglets were randomly assigned into four treatments (7 pigs/treatment) with four sources of starch (CS: corn starch, WS: wheat starch, TS: tapioca starch, PS: pea starch). The determined ratio of amylose to amylopectin for corn starch, wheat starch, tapioca starch and pea starch was 0.21, 0.24, 0.12 and 0.52 respectively. Experimental diets were formulated according to NRC (1998) nutrient requirement for piglets weighing 5 to 10 kg. The ingredients and nutrient levels of the four experiment diets were uniform except for the starch source. Diets contained neither antibiotics nor alternative antimicrobial substances.

Sample collection

At the end of the experiment, the chyme of the duodenum, jejunum, ileum and cecum and colon were removed immediately and stored at −80°C until further analysis. The intestinal mucosa from ileum, duodenum and jejunum were also collected and stored at −80° until use. All experimental procedures followed the actual law of animal protection which was approved by the Animal Care Advisory Committee of Sichuan Agricultural University.

DNA Extraction from chyme

Genomic DNA from *Bacillus subtilis* ATCC6633 was extracted with the TaKaRa minibest bacterial genomic DNA extraction kit (TaKaRa, Dalian, China) and bacterial DNA was isolated from the chyme samples using an E.Z.N.A.TM stool DNA isolation kit (Omega Bio-Tek, Doraville, USA) according to the manufacturer's instructions.

Design and validation of primers for Bacillus

Primers and probe (Table 1) for quantitative detection of particular *Bacillus* were designed accorrding to 16S rRNA sequences of maximum species of each genus encountered in the swine intestinal tract downloaded from the GenBank database as well as EMBL and DDBJ. 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). It was picked up that the *Bacillus* blocks of hyper variable regions comprised with all other genera. 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 *Bacillus* genus-specific primers and probes. In order to ensure furthermore 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-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 [11]. All the primers and probe were commercially synthesized from Invitrogen (Shanghai, China).

Reference strains, culture conditions and genome extraction

A total of thirty strains including *Bifidobacterium*, *Lactobacillus*, *Bacillus*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus* were used as reference strains in this study (Data not shown). The strains were cultured anaerobically or aerobically in respective culture supplemented with 1% glucose at 37°C for 12 to 48 h. Total genomic DNA from the different reference strains were extracted and purified by using the method described in related Kit Manual (E.Z.N.A. TM Bacterial DNA Kit, Omega Bio-Tek, Doraville, USA).

Standard curve generation

To quantify the copy numbers of *Bacillus* and all bacteria in test samples, two specific standard curves were generated by constructing the standard plasmids. In brief, DNA encoding *Bacillus* and all bacteria were respectively extracted from the standard stain of *Bacillus* (ATCC6633) and the test samples. PCR amplification was carried out using their specific primers YB-P1, YB-P2 and Eub338F, Eub518R (Table 1). After amplification, approximately 424 and 200 bp fragment

were obtained respectively. The transformation of purified PCR products, screening of transformed bacteria and extraction of the positive recombinant plasmids containing the inserts were conducted according to the standard procedure. Then the standard plasmids for *Bacillus* and all bacteria were constructed successfully.

DNA concentrations of the standard plasmids were determined by spectrophotometer (Coulter DU 800, Beckman, USA). A series of 10-fold dilution (1×10^9 to 1×10^1 copies/μl) of the plasmids DNA for *Bacillus* and all bacteria were prepared and used to generate their respective standard curves with the the logarithm of Standard templates as the abscissa while the Ct as the ordinate. The copy numbers for *Bacillus* and all bacteria were calculated using the following formula: (DNA concentration in μg/μl $\times 6.0233 \times 10^{23}$ copies/mol)/(DNA size(bp) $\times 660 \times 10^6$).

Quantitative PCR conditions and specificity validation of primers

All primers and probe used in this study are presented in Table 1. Quantitative Real-time PCR was carried out in a 25 μl reaction volume which 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$_2$O and 1 μl DNA template for detecting *all Bacteria*. The reaction was

Table 1 Sequences of primers and probe for Bacillus and All bacteria

Assay	Names and sequences for primers/probe (5′-3′)	Product size(bp)	Annealing temp(°C)	Reference
All bacteria	Eub338F, ACTCCTACGGGAGGCAGCAG	200	60	[11]
	Eub518R, ATTACCGCGGCTGCTGG			
Bacillus	YB-P1, ACGCCGTAAACGATGAGT	424	60	This study
	YB-P2, GTGTGTAGCCCAGGTCATAA			
Bacillus	YB-F, GCAACGAGCGCAACCCTTGA	92	60	This study
	YB-R, TCATCCCCACCTTCCTCCGGT			
	YB-P, (FMA) CGGTTTGTCACCGGCAGTCACCT (BHQ-1)			

Table 2 Primers for intestinal development related genes

Gene	Primer sequences (5′-3′)	Product size (bp)	Annealing temp (°C)	Reference
β-actin	F: TCTGGCACCACACCTTCT	114	52.2	[13]
	R: TGATCTGGGTCATCTTCTCAC			
GLP-2	F: ACTCACAGGGCACGTTTACCA	149	56	This study
	R: AGGTCCCTTCAGCATGTCTCT			
EGF	F:ATCTCAGGAATGGGAGTCAACC	165	60	This study
	R:TCACTGGAGGATGGAATACAGC			
IGF-1	F: CTGAGGAGGCTGGAGATGTACT	137	58.5	This study
	R: CCTGAACTCCCTCTACTTGTGTTC			

carried out using the following reaction cycles: initial predenaturation at 95°C for 20 s followed by 40 consecutive cycles of denaturation at 95° for 5 s, annealing for 30 s at 60°, extension at 72° 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 *Bacillus* detection the 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 2 min; 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 primers YB-F and YB-R and probe YB-P for specificity validation while primers Eub338F and Eub518R were used for verification feasibility.

Real-time PCR for quantification of GLP-2, IGF-1 and EGF

Total RNA were extracted from intestinal mucosa using RNAzol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. Reverse transcription reactions were performed using PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) following the manufacturer's instructions. Real-Time PCR for quantification of GLP-2, IGF-1and EGF were perfomed using β-actin as the housingkeeping gene. Sequences of primers for GLP-2, IGF-1and EGF were shown in Table 2. The relative quantification of target gene transcripts in comparison to the reference gene transcript was calculated as described previously [12].

Statistical analysis

The datas were analyzed using SPSS12.0. All results were expressed as means ± SD. One-way ANOVA procedure was performed to assess the statistical significance between treatments. $P < 0.05$ was considered significant.

Results

Specific verification of quantitative PCR products and specificity of the PCR

Primers and probe designed with Primer Express 3.0 for quantitative detection of particular *Bacillus* were firstly verificated to be specific by corresponding software from website. Then conventional PCR were carried out using DNA extracted from thirty reference strains including *Bifidobacterium*, *Lactobacillus*, *Bacillus*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus* as the templates
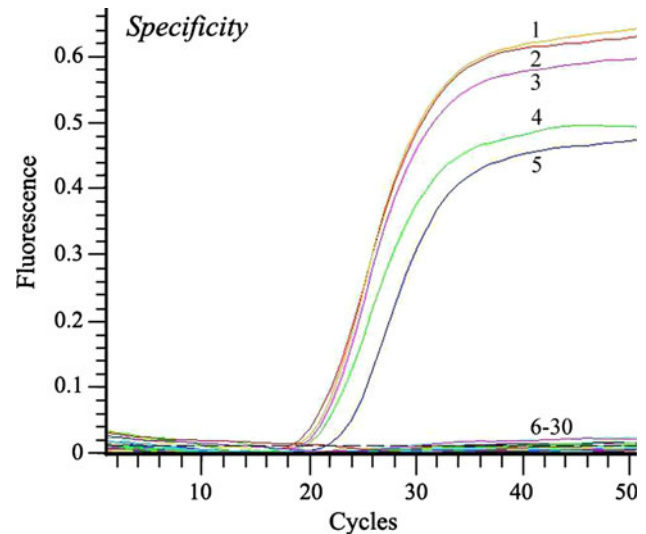

Fig. 1 Specificity of PCR amplification. 1–5: PCR amplification curves for *Bacillus*; 6–30: PCR amplification curves for *Lactobacillus, Bifidobacterium, salmonella, Escherichia coli, Staphylococcus aureus* and *Streptococcus*

for verifying the specificity of the primers and the specificity of the PCR. Results showed that a band of approximately 92 bp was obtained after PCR amplification (data not shown) when used DNA extracted from *Bacillus* as the template while there were no specific amplification for other reference strains (Fig. 1) with the primers YB-F, YB-R, YB-P. For the primers Eub338F, Eub518R, there were specific amplification for all the reference strains. The above results demonstrated that there were 100% specificity for the primers (Table 1) and could be used in the following study.

Quantitative PCR standard curve and reproducibility

The standard curves with the logarithm of Standard templates as the abscissa while the Ct as the ordinate were obtained in this study. There was good linear relation between the copy numbers and the Ct value when the copy numbers within $1 \times 10^9 \sim 1 \times 10^4$ copies/μl. The regression equation was: $Y = -2.991X + 51.76$ (Y represented the threshold cycle (C_t), X represented the $Log_{10}^{(copy\ number\ of\ 16S\ rDNA)}$). The correlation coefficient of the standard curves were 0.999 and the amplification efficiencies calculated using the equation: $E = 10^{[-1/slope]}$ [14] were both above 115.94%, which indicated that the crossing threshold values for the standard curves were within an acceptable range. To verificate the validity of the standard curves, it was further confirmed by reproducibility test. Four different concentrations $(1.5 \times 10^9 - 1.5 \times 10^6$ copies/μl) of the standard plasmids DNA were applied to the PCR as template in triplicate to verify the reproducibility between experiments. The results showed that the coefficient of variation was statistically low, at <1.5%.

The threshold cycle for each concentration ranged from 1.5×10^9 copies/μl to 1.5×10^6 copies/μl and was different between 0.1 and 0.3 cycles, which indicated that the assay was highly reproducible. DNA copies of samples were calculated using the above regression equation.

Calculation of copy numbers for Bacillus and all bacteria

Real-time PCR analysis was performed to determine the copy numbers of *Bacillus* and all bacteria in the content of intestine of all piglets. Quantitative test results for the copy numbers of *Baciilus* and all bacteria in the chyme treated with different starch were presented in Figs. 2 and 3. Results showed that there was no obvious difference ($P > 0.05$) on the copy numbers of all bacteria in the chyme of proximal intestine (duodenum, jejunum and ileum) for the different treatments (Fig. 2). However, the copy numbers of all bacteria in the cecal and colon content of piglets fed PS was significantly lower than other treatments ($P < 0.05$) while there was no difference ($P > 0.05$) among other three treatments. It can be seen from Fig. 3 that the copy numbers of *Baciilus* in the whole intestinal tract were affected significantly. The copy numbers of *Baciilus* in the chyme of the whole intestinal tract (duodenum, jejunum, ileum, cecum and colon) for treatment with PS was the highest in all groups ($P < 0.05$) while there was no difference ($P > 0.05$) among other three treatments. Throughout the entire digestive tract, the copy numbers of all bacteria and *Bacillus* were lowest in the chyme of duodenum and gradually increasing from proximal intestine to distal intestine.
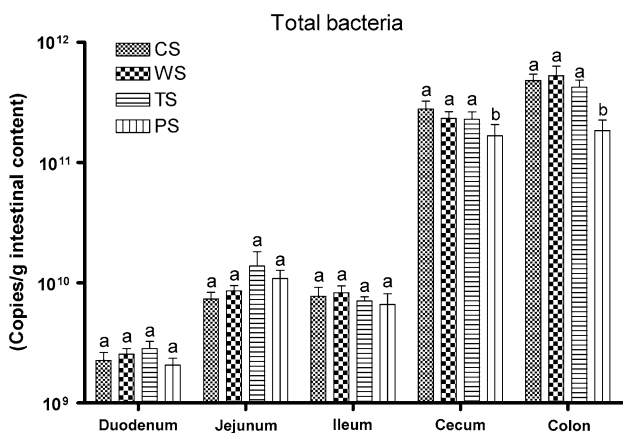

Fig. 3 The copy numbers of *Bacillus* in the intestinal segments content of piglets. The same letter in figure represents there was no significant difference at 5% level ($P < 0.05$). The different letter in figure represents there was significant difference at 5% level ($P > 0.05$)
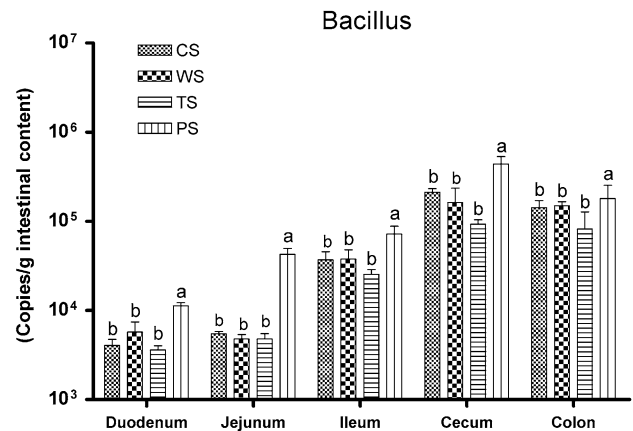
The proportion of Bacillus based on all bacteria

Results for the proportion of *bacillus* based on all bacteria in the chyme treated with different starch were presented in Fig. 4. Different starch source affected obviously the proportion of *Bacillus* (based on all bacteria) in the chyme of intestinal tract. The proportion of *Bacillus* in the chyme of the whole intestinal tract (duodenum, jejunum, ileum, cecum and colon) for PS treatment elevated significantly compared with other treatments ($P < 0.05$) while there was no difference among other three treatments ($P > 0.05$). In addition, the proportion of *Bacillus* in the chyme of the whole intestinal tract for TS treatment was the lowest.
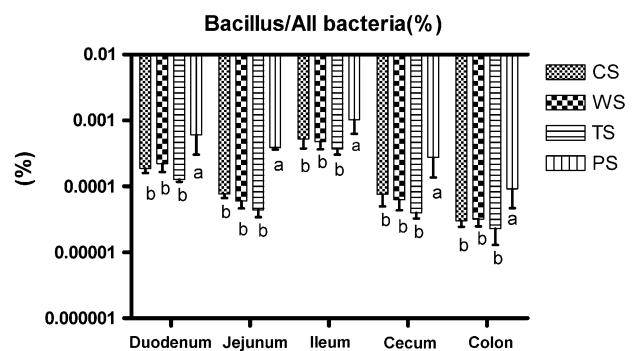

Fig. 2 The copy numbers of all bacteria in the intestinal segments content of piglets. The same letter in figure represents there was no significant difference at 5% level ($P < 0.05$). The different letter in figure represents there was significant difference at 5% level ($P > 0.05$)

Fig. 4 The percentage of *Bacillus* (based on all bacteria) in the intestinal segments content of piglets. The same letter in figure represents there was no significant difference at 5% level ($P < 0.05$). The different letter in figure represents there was significant difference at 5% level ($P > 0.05$)

mRNA level of intestinal development related genes GLP-2, IGF-1and EGF

Results for the mRNA level of intestinal development related genes treated with different starch sources were shown in Fig. 5. As was shown in Fig. 5, different starch source affected obviously the mRNA level of intestinal development related genes. For the expression level of GLP-2, IGF-1 in jejunum and ileum, the pea starch treatment (the high amylose/amylopectin ratio) increased their mRNA level significantly compared to other three treatments ($P < 0.05$) while the tapioca starch decreased their mRNA level significantly ($P < 0.05$). As to EGF, there was no significant difference for the mRNA level of it in each group ($P > 0.05$).

Discussion

As is known to all that starch is the main carbohydrate source of animal diets and the main energy source needed by animals. The digestive function and physiological effect of different corn starches were various due to their respective composition and structure. Generally, amylose and amylopectin were the main forms. The nutritional value of starch depends on the ratio of amylose/amylopectin to a great extent [15]. In this experiment, to compare the effects of different starch sources on *Bacillus* spp. in intestinal tract and expression of intestinal development related genes of weanling piglets, the corn starch (CS), wheat starch (WS), tapioca starch (TS), pea starch (PS) were selected as the only starch source of diets. The feed components and nutrient composition (GE, CP, St, lysine, methionine + cysteine and tryptophan contents) of the four experiment diets were uniform except for the starch source which had the different ratio of amylose/amylopectin. In addition, there was no difference for the feed processing and management condition for piglets, so the difference

between the treatments for *Bacillus* spp. in intestinal tract and expression of intestinal development related genes could be attributed to the starch source.

In recent years, real-time PCR is becoming the most suitable method in molecular biology for the detection and quantification of mRNA and was widely used by researchers [16, 17]. It has also become a potentially powerful method to quantify the population of gastrointestinal tract microbial [18, 19]. Moreover, with the development of biotechnology, the specific primer-probe combination is becoming an available method for detecting the counts of intestinal bacterial species [19, 20]. In order to study the copy numbers of *Bacillus* in the chyme of intestinal tract of piglets, an fluorescence quantitative PCR protocol was developed, optimized and applied in the present study. Results of comparison analysis on the sequences of the Primers and probe used in the study using the bioinformatics software and database indicated that there was high specificity for the primers and probe. To further conform their′s specificity, conventional PCR were carried out using DNA extracted from reference strains as the templates. PCR results verified that there were 100% specificity for the primers and probe and could be used for detection *Bacillus* and all bacteria. To quantify the copy numbers of *Bacillus* and all bacteria in the chyme of intestinal tract of weanling piglets treated with different starch sources, the standard curve with the the logarithm of Standard templates as the abscissa while the Ct as the ordinate was obtained which had good linear relation between the copy numbers and the Ct value when the copy numbers within $1 \times 10^9 \sim 1 \times 10^4$ copies/μl, the correlation coefficient was 0.999 and the amplification efficiencies was 115.94%. Besides, the reproducibility test showed that the crossing threshold values for the standard curve were within an acceptable range. So the standard curve we obtained could be used for quantitation of the copy numbers of *Bacillus*.

The applications of *Bacillus* species as probiotics have been developed in farming and aquaculture as alternatives
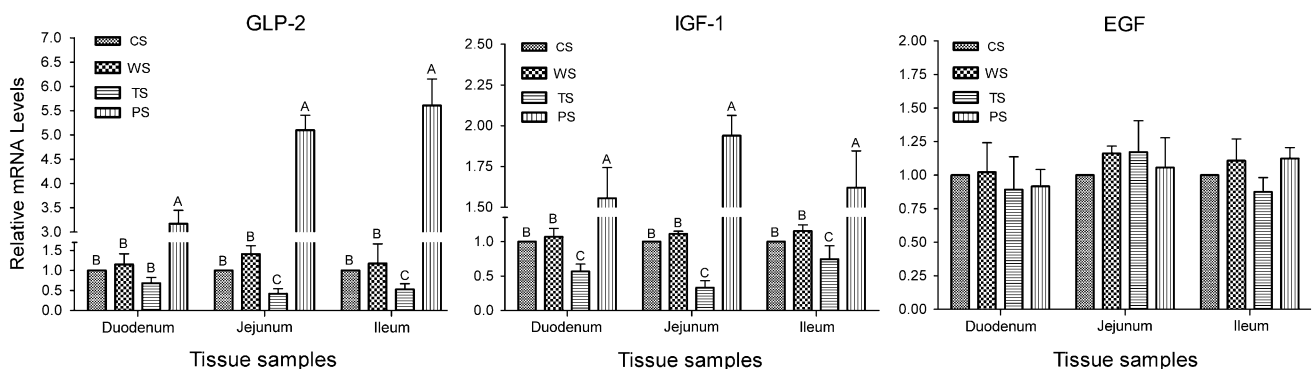

Fig. 5 mRNA level of intestinal development related genes treated with different starch sources. The same letter in figure represents there was no significant difference at 5% level ($P < 0.05$). The different letter in figure represents there was significant difference at 5% level ($P > 0.05$)

to antibiotics. Certain *Bacillus* species execute a positive stimulation of immune system and promote the synthesis of antimicrobials, thereby potentially prevent or moderate intestine inflection. Certain dietary non-digestible carbohydrates can allow specific changes in the composition or activity of the intestinal microbiota [21] and selectively stimulate the growth of the health-promoting bacteria (*Bifidobacterium* and *Lactobacilli*) in the intestine [22, 23]. Results of our study revealed that the copy numbers of *Baciilus* in the whole intestinal tract were affected significantly and treatment with PS (high ratio of amylose/amylopectin) was highest ($P < 0.05$). Throughout the entire digestive tract, the copy numbers of all bacteria and *Bacillus* were lowest in the chyme of duodenum and gradually increasing from proximal intestine to distal intestine. This may be relative to the anti-digestibility of the PS. It has been reported that resistant starch has strong anti-digestibility, but it could be used by microorganism in colon and generate much short-chain fatty acid which was capable of maintaining a low acid conditions and promote the proliferation of beneficial bacteria [24, 25]. Another study also found that pigs fed potato starch had an increased concentration of short-chain fatty acid in the large intestine with increasing amount of RS. The possible mechanism was that resistant starch could act as the fermentation substrate for bacteria in large intestine and thereby promote the growth of bacteria. We also found that the proportion (based on all bacteria) of *Bacillus* in the chyme of the whole intestinal tract for PS treatment elevated significantly compared with other treatments ($P < 0.05$) while there was no difference ($P > 0.05$) among other three treatments. This variation may be due to the different of the amylose/amylopectin ratio starch. It has been shown that the higher the amylose content, the more difficult the starch is to degrade [26]. The digestibility of starch is generally inverse proportion to its amylose content [27]. There is a higher amylose contents in the pea starch diets, and the previous result showed that it was a slowly digestible progress in the porcine GIT [28]. One explanation could be that high amylose/amylopectin ratio starch can promote the growth of indigenous *Bacillus* spp.

When it comes to the intestinal development related genes, our results indicated that the mRNA level of GLP-2, IGF-1 in jejunum and ileum were increased in the pea starch treatment (the high amylose/amylopectin ratio) while the tapioca starch decreased their mRNA level significantly. GLP-2 is supposed to play an important role in the regulation of the size and absorptive capacity of the gut. It was reported that GLP-2 increased mucosal thickness in mice [29] and mucosal weight, activity of jejunal maltase-glucoamylase, sucrase-isomaltase mRNA abundance of Neonatal Piglets [30]. Insulin-like growth factor-1 (IGF-1) is an important regulator of gastrointestinal tract and plays a fundamental role in postnatal mammalian growth, development, and metabolism [31]. In particular, orally administered IGF-I is beneficial for the integrity and function of the small intestine [32]. So we suggested that the the pea starch may promote the development of gastrointestinal tract by enhancing the expression level of GLP-2, IGF-1.

In conclusion, we developed a new real-time Taq-man PCR assays that allowed us for rapid, convenient, reproducible, and steady quantification of the *Bacillus* group in the intestinal content of piglets. Additionally, the present study revealed that high amylose/amylopectin ratio of starches not only significantly enhanced the numbers of *Bacillus* and the percentage of its (based on all bacteria) in chyme of the all intestine segment, but also the mRNA level of the intestinal development related genes of GLP-2, IGF-1. That was to say, high amylose/amylopectin ratio of starches may be more beneficial for the healthy intestinal tract.

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