Original Article

Effect of dietary chitosan oligosaccharide levels and molecular weight with deacetylation degree characteristics on peptide transporter 1 gene expression in weaning pig's small intestine

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Abstract

The present study aimed to assess the expression response of peptide transporter1 (PepT1) mRNA (SLC15A1) in weaning pig's small intestine to the effect of dietary chitosan oligosaccharide (COS) supplement level, molecular weight (MW) and deacetylation degree (DD) characteristics. On days 28 and 56 of the experiments, small intestine samples obtained from our previous two experiments on weaned pigs were used to study the PepT1 mRNA expression. In experiment 1, pigs received either a basal diet or a basal diet supplemented with 75, 150, or 225 mg/kg COS. In experiment 2, pigs received either a basal diet or a basal diet supplemented with 150 mg/kg of one of the following COS differing in MW and DD: 8kDa~90%DD, 65kDa~80%DD, or 65kDa~90%DD. Relative expression of porcine PepT1 mRNA in duodenal, jejunal, and ileal mucosa was determined by real-time quantitative PCR. On day 56 of the experiment, pigs supplemented with not only COS levels but COS characteristics as well altered PepT1 gene expression in weaning pigs' small intestine, which increased and decreased the expression of PepT1 mRNA in the jejunum and the duodenum ($P < 0.05$, respectively) when compared to the control pigs. Supplementing different levels and characteristics with a 28-day trial term of COS did not show any effect on the PepT1 mRNA expression. These data suggest that the regulation of PepT1 mRNA expression at the jejunum and the duodenum of weaning pigs depends on COS' level, characteristics, and long-term diet supplementation.

Keywords: chitosan oligosaccharide, molecular weight, peptide transporter 1 mRNA, small intestine, weaned pigs

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Introduction

Chitosan oligosaccharide (COS), as a degradation product of natural chitosan, is being studied for its potential applications as a dietary supplement in weaning pigs. The weaning-associated disorder or damage to small intestinal morphology and barrier function, which consequently reduces digestive and absorptive capacities (Yang *et al*., 2016), was ameliorated by COS supplementation (Yang *et al*., 2012; Xiong *et al*., 2015). Our earlier studies identified 150 mg/kg as an effective COS supplementation dose (Suthongsa *et al*., 2017), and 8 kDa as an effective molecular weight (MW) with ~90% as an effective degree of deacetylation (DD) (Thongsong *et al*., 2018) to promote nutrient absorption and digestibility efficacy, as well as to optimize protein utilization in weaned pigs during 56 days of the experiment. After oral administration, COS is not digested by gastrointestinal enzymes but is readily absorbed through the intestinal epithelium (Lodhi *et al*., 2014). Then, some previous studies have shown that COS has a beneficial effect mediating the transfer of major nutrients (Xu and Du 2003; Yin *et al*., 2010), including peptide molecules by increasing the transcellular and/or paracellular transport (Kotze *et al*., 1998). Intestinal peptide transporter 1 (PepT1) is one of the main determinants of pigs' development, growth, and metabolism (Klang *et al*., 2005; Gilbert *et al*., 2008; Nosworthy *et al*., 2013; Thongsong *et al*., 2019) because it contributes to maintaining the balance of homeostasis and proper function in the small intestine by regulating microRNA or protein expression along the crypt-villus axis (Zhang *et al*., 2016). PepT1; solute carrier family 15 member 1 (SLC15A1), located on the apical membrane of small intestinal enterocytes, is an important transporter for the oral absorption of therapeutic agents (Ganapathy, 2012). At weaning and later in the life of healthy livestock, including pigs, PepT1 mRNA is mostly expressed in the brush border membranes of the small intestine, especially the jejunum (Thongsong *et al*., 2016). Thus, PepT1 mRNA regulation is dynamically modulated by dietary factors and nutritional status (Gilbert *et al*., 2008). It may be speculated that PepT1 mRNA expression may be a participant in the regulation of intestinal transport function by COS in accordance with the published evidence (Kotze *et al*., 1998; Xu and Du, 2003; Yin *et al*., 2010). However, few studies have investigated how COS supplementation levels and characteristics with age affect PepT1 mRNA expression in the post-weaning pig small intestine.

The hypothesis of the present study was that the amino groups of COS or the sequential effect of slow diffusion of protein induced by gel formation between COS and gastric acid may regulate the gene expression of PepT1 in enterocyte cells. Therefore, our objective was to generate experimental data to set the basis for further research on the expression response of PepT1 mRNA in different sections of weaning pig's small intestine to the effect of dietary COS levels: 0, 75, 150, 225 mg/kg; and dietary COS characteristics: either low (8 kDa) or medium (65 kDa) MW with more than 80% DD.

Materials and Methods

Ethical approval: All procedures described in this study were approved by the Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University.

Characterizations of experimental COS: The different concentrations or types of COS used in this experiment were produced from shrimp shells by the Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand. In brief, the production of these COS was accomplished by a controlled enzymatic hydrolysis of chitosan. The resultant products were deacetylated in 50% (w/w) NaOH solution to ~80% or ~90% DD as determined by ultraviolet (UV) spectroscopy. The average MWs as determined by gel permeation chromatography (GPC) or size exclusion chromatography (SEC) were 7.47 kDa (Fig. 1A), 7.97 kDa (Fig. 1B), 65.70 kDa (Fig. 1C), and 65.37 kDa (Fig. 1D).

Animals and experimental treatments: The experimental design and the basal diet were similar to those described in Suthongsa *et al*. (2017) and Thongsong *et al*. (2018). In brief, the female piglets (Large White × Landrace × Duroc) were randomly divided into 2 experiments. In experiment 1, weaned pigs with an initial body weight (BW) of 5.68 ± 0.09 kg were randomly allocated into one of the four dietary treatment groups with 12 or 13 animals per treatment. Treatment groups differed only in their COS supplementation level: 1) Basal diet with no COS supplementation (Control); 2) 75 mg/kg of COS supplementation; 3) 150 mg/kg of COS supplementation; 4) 225 mg/kg of COS supplementation. COS used was 7.5 kDa and had more than 90% DD in all the treatment diets. In experiment 2, weaned pigs with an initial BW of 5.70 ± 0.30 kg were randomly allocated into one of the four dietary treatment groups with 12 animals per treatment. All treatments were fed with 1) no COS supplementation (Control) or supplemented with 150 mg/kg of COS differing in its MW and DD: 2) 8 kDa MW and ~90% DD (8 kDa~90%); 3) 65 kDa MW and ~80% DD (65 kDa~80%); and 4) 65 kDa MW and ~90% DD (65 kDa~90%). The experiments lasted 56 days. Pigs were individually housed and had ad libitum access to water and feed. The basal diet for post-weaning pigs was provided as a meal formulated.

Small intestinal tissue sampling and sample processing analysis: On days 28 and 56 of the experiment, four randomly selected pigs from each group were sedated (i.m. azaperone, 6 mg/kg BW) and euthanized (i.v. pentobarbital, 50 mg/kg BW) to collect small intestinal tissues for relative expression of PepT1 mRNA determination. To ensure consistency between pigs, the small intestine was removed and divided into three segments: duodenum, jejunum, and ileum. The duodenum extends from the pyloric sphincter to the junction with the jejunum. The jejunum and ileum were separated using an anatomical landmark, the ligament of Trietz. A 5 cm length was excised from the middle of each part. Each intestine segment was cut

longitudinally and washed with ice-cold 0.9% NaCl to remove luminal contents. The mucosa from each segment was scraped with a glass slide, placed in a vial containing RNA later solution (Qiagen, Hilden, Germany), and stored at -20°C for subsequent RNA extraction.

Real-time-qPCR analysis: Relative expression of porcine PepT1 mRNA in duodenal, jejunal, and ileal mucosa was determined by real-time quantitative PCR. 18S ribosomal RNA (rRNA) was used as an internal control. The primer sets used for porcine PepT1 (GenBank accession number AY180903.1) was forward primer, 5′-AGC ATC TTC TTC ATC GTG GTC AA 3′, and reverse primer, 5′ GTC TTG AAC TTC CCC AGC CA-3′, and for 18S rRNA (GenBank accession number AF102857) was forward primer, 5′-CCG CGG TTC TAT TTT GTT GGT TTT-3′, and reverse primer, 5′-CGG GCC GGG TGA GGF TTC-3′. The target sizes of PepT1 and 18S rRNA were 206 and 399 base pairs, respectively. Total RNA from the mucosa of the duodenum, jejunum, and ileum was extracted using a commercial kit (Bio-Rad Laboratory, Hercules, USA) and reverse-transcribed into complementary DNA (cDNA) by using iScript Reverse transcription supermix included in the RT-qPCR kit (Bio-Rad Laboratory, Hercules, USA) in accordance with the manufacturer's instructions. Quantitative real-time PCR was carried out using an ABI 7300 Real-time PCR system (Applied Biosystems, USA). A SYBR Green PCR reagent kit (Bio-Rad Laboratory, Hercules, USA) was used to measure the expression of the target genes. The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, followed by annealing and extension at 58°C for 60 seconds. A melt curve was performed at the end to confirm the specificity of the resulting PCR products. Each sample was done in triplicate, and relative expression of PepT1 mRNA was calculated in relation to the 18S rRNA using the formula $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis: Results are presented as means ± standard errors (SE). Data were checked for normality. The experimental data for the relative expression level of PepT1 mRNA were analyzed by one-way analysis of variance (ANOVA) as a randomized complete block design by using the generalized linear model (GLM) procedure of SAS (2003). Statistical models included treatment categories of diet (level or characteristic) as main effects. Differences in means among treatment groups were separated by the Holm-Sidak test. Orthogonal polynomial contrasts were performed to find a linear or quadratic response to the inclusion level effect of COS. Statistical significance was taken at $P < 0.05$.

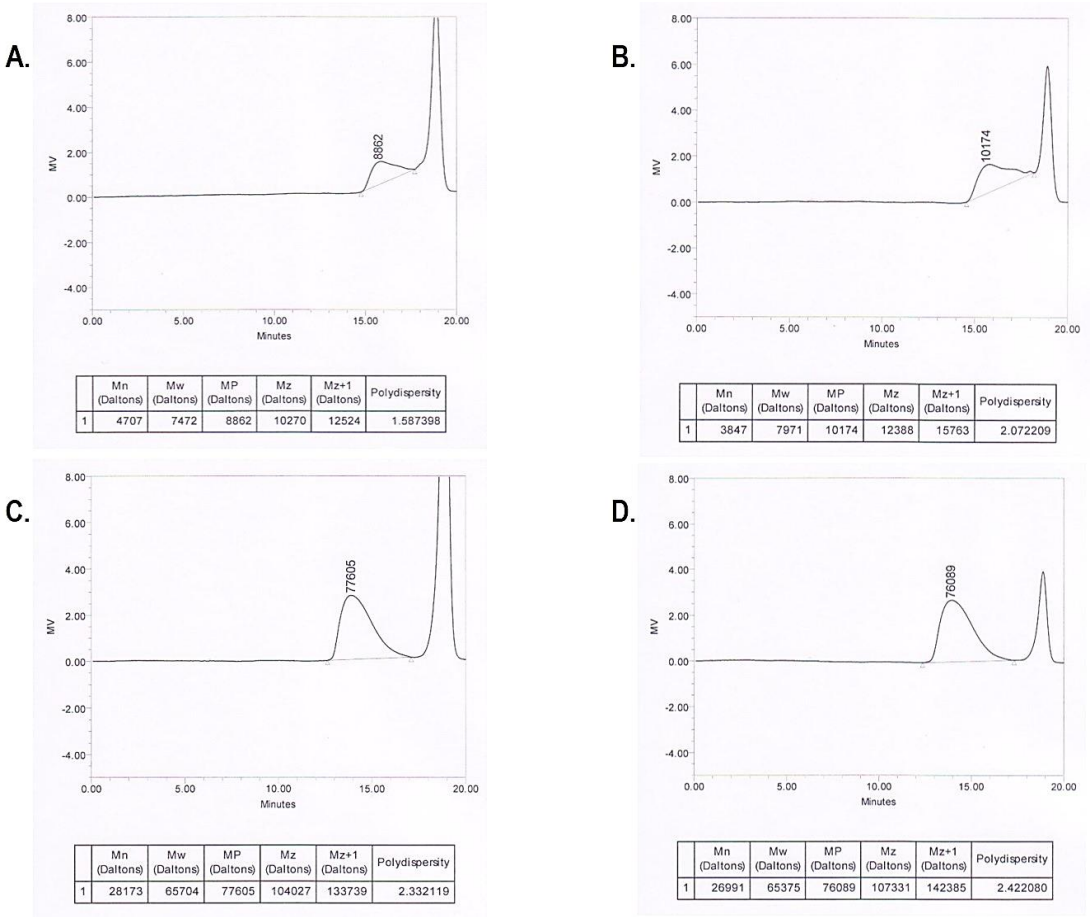

Figure 1 Gel permeation chromatography (GPC) curve was about 7.5 kDa (A), 8 kDa (B) or 65 kDa (C and D) of the experimental chitosan oligosaccharides (COS) used and characterization reports for each COS sample analysis; number average molecular weight (Mn), weight average molecular weight (Mw), molecular weight of the highest peak (Mp), higher average molecular weight (Mz, Mz+1) and polydispersity index, were determined.

Result

On day 28 of experiment 1, no treatment effect of dietary COS level was observed on the relative expression of PepT1 mRNA in any small intestine segment of weaning pigs (Fig. 2A). In contrast, on day 56 of the experiment, there was treatment effect of dietary COS levels on the relative expression of PepT1 mRNA in the duodenum ($P = 0.008$) and jejunum ($P < 0.001$) of weaning pigs (Fig. 2B). When compared to pigs fed the control diet, dietary supplementation with increasing COS levels (75 and 150 mg/kg) showed up-regulation of the PepT1 mRNA expression at the jejunum ($P < 0.05$), and down-regulation of its expression with dietary COS-supplemented levels (150 and 225 mg/kg) was found at the duodenum ($P < 0.05$) (Fig. 2B). On this day of the experiment, quadratic increase ($P < 0.001$) in the jejunum and linear decrease ($P = 0.001$) in the duodenum for the relative expression of PepT1 mRNA were observed with increasing COS supplementation.

On day 28 of the experiment 2, no treatment effect of dietary COS characteristic was observed on the relative expression of PepT1 mRNA in any small intestine segment of weaning pigs (Fig. 3A). Subsequently, on day 56 of the experiment, there was treatment effect of dietary COS characteristics on the relative expression of PepT1 mRNA in the duodenum ($P = 0.041$), jejunum ($P = 0.016$) and ileum ($P = 0.005$) of weaning pigs (Fig. 3B). However, when compared to weaning pigs fed the control diet, in the duodenum of pigs, there was a significant decrease in the relative expression of PepT1 mRNA of both 8 kDa~90% and 65 kDa~80% COS groups ($P < 0.05$, respectively). Pigs fed a structural property of COS with 65 kDa~90% group showed a remarkable increase in expression of PepT1 mRNA in the jejunum as compared to pigs fed the control diet ($P < 0.05$). In the ileum of pigs fed different dietary COS characteristics, there was no difference in relative expression of PepT1 mRNA when compared to pigs fed the control diet ($P > 0.05$). Among COS-supplemented groups, the ileum of pigs fed with the 65 kDa~90% COS diet showed a significant increase in the expression of PepT1 mRNA compared to both 8 kDa~90% and 65 kDa~80% COS groups ($P < 0.05$, respectively).

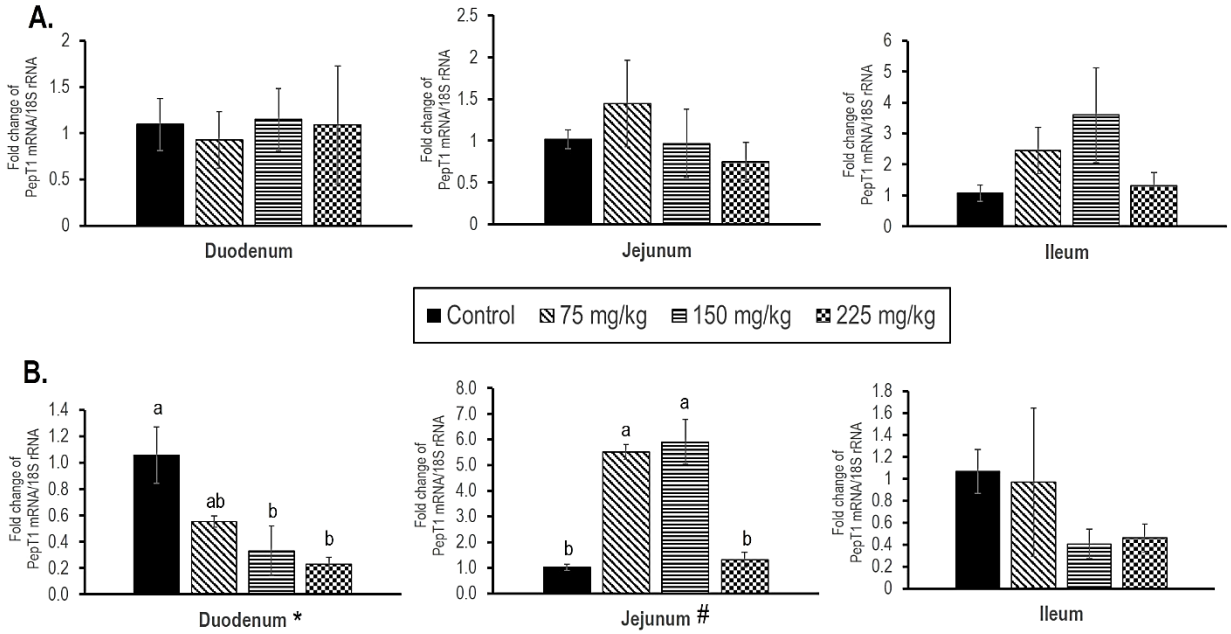

Figure 2 Effect of feeding different supplemental levels of chitosan oligosaccharide (COS) with same molecular weight (7.5 kDa of MW) and degree of deacetylation (more than 90% DD) in post-weaning pig diets on relative mRNA expression of the peptide transporter 1 (PepT1) along longitudinal axis of small intestine at 28 (A) and 56 (B) days of experiment. All samples were normalized using 18S rRNA expression as an internal control in each real-time reverse transcription polymerase chain reaction (RT-PCR). Relative levels of PepT1 mRNA (fold of control) were analyzed by the $2^{-\Delta\Delta Ct}$ method. Values are means (n = 4) with their standard errors represented by vertical bars. Means without a common letter differ, $P < 0.05$.
*Linear effect ($P = 0.001$) of COS inclusion level.
Quadratic effect ($P < 0.001$) of COS inclusion level.

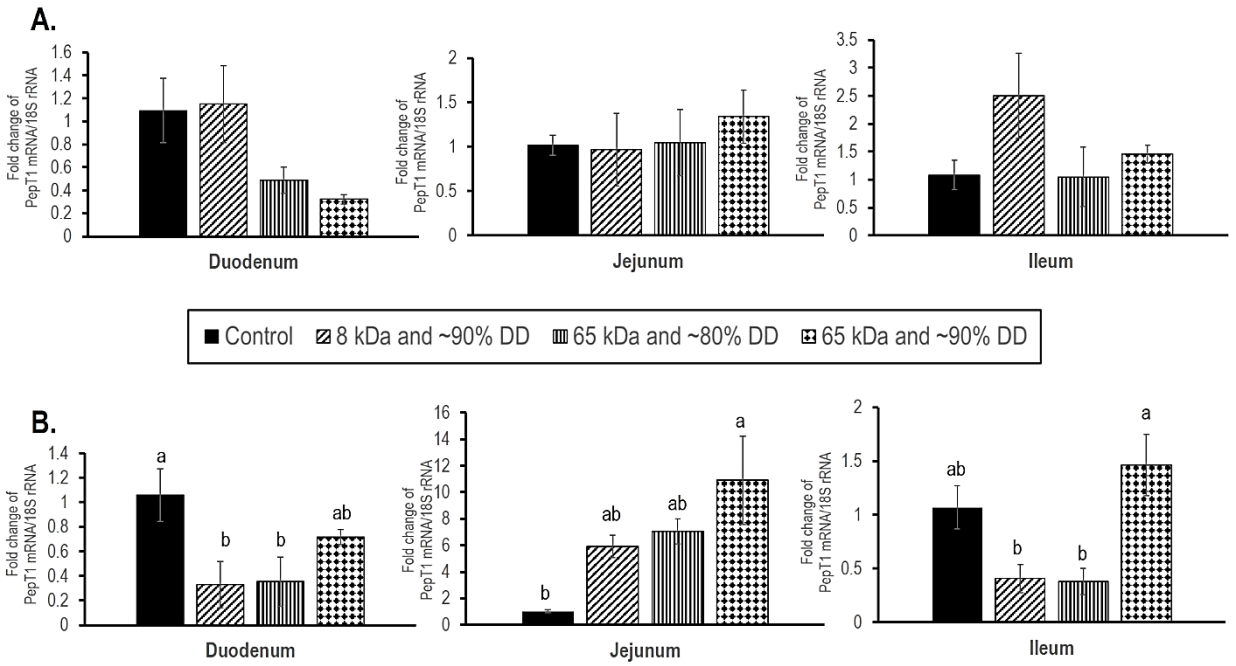

Figure 3 Effect of feeding low or medium molecular weight (MW) and more than 80% degree of deacetylation (DD) with same level (150 mg/kg) chitosan oligosaccharide (COS) supplementation in post-weaning pig diets on relative gene expression of the peptide transporter 1 (PepT1) along longitudinal axis of small intestine at 28 (A) and 56 (B) days of experiment. All samples were normalized using 18S rRNA expression as an internal control in each real-time reverse transcription polymerase chain reaction (RT-PCR). Relative levels of PepT1 mRNA (fold of control) were analyzed by the $2^{-\Delta\Delta Ct}$ method. Values are means (n = 4) with their standard errors represented by vertical bars. Means without a common letter differ, $P < 0.05$.

Discussion

The results found in the present study show a beneficial COS effect on PepT1 mRNA expression in different sections of the weaning pig's small intestine, although there were different effects depending on the age of the pigs and sampling points. The increased protein digestibility observed in Suthongsa *et al*. (2017) and Thongsong *et al*. (2018) could be related to the increased PepT1 mRNA expression observed in the present study and might be improved utilization of dietary protein to fulfill the nutrient needs for post-weaning pigs' health and growth (Gilbert *et al*., 2008). Similarly, Bauer *et al*. (2011) and Zhang *et al*. (2013) observed that dietary protein, peptides, and amino acids influence PepT1 expression and activity both in vitro and in vivo. Although PepT1 gene regulation has been researched extensively, there is very limited data in pigs on the effect of dietary COS-supplementation as a PepT1 gene and protein regulator. In the present study, on day 28 of the experiment, the relative mRNA level of PepT1 along the longitudinal axis of the small intestine was not influenced by different supplemental levels of COS with same MW (7.5 kDa) and DD (more than 90%), and different supplemental MW and DD characteristics of COS with same level (150 mg/kg). This agrees with Suthongsa *et al*. (2017) and Thongsong *et al*. (2018), who found no effect of different COS supplement levels and characteristics with a short-term trial (28 days) on body weight gain, gain per feed ratio (G: F), and blood urea nitrogen (BUN) in weaned pigs. Nonetheless, in the present study, the relative mRNA level of PepT1 in the small intestine was influenced by different supplemental levels and characteristics of COS with a long-term trial (56 days). Thus, the relative mRNA level of PepT1 in the weaning pigs' jejunum was upregulated by increasing COS levels, particularly with 75 and 150 mg/kg, and by an effective characteristic of COS with 65 kDa~90% when compared to pigs fed the control diet. The reason might be that with increasing the level of COS supplementation, the digestibility of crude protein improved quadratically (Suthongsa *et al*., 2017). Additionally, increasing the level of dietary supplementation with COS was reported to have a negative effect on average daily gain (ADG) and crude protein digestibility (Yan and Kim, 2011) as well as on small intestinal morphology (Yang *et al*., 2012). With regard to the different supplemental MW and DD characteristics of COS, the upregulation of PepT1 mRNA in the jejunum of pigs fed 65 kDa~90% experimental COS observed in the present study might be explaining previous findings of our research group in which medium MW (65 kDa) with ~80% and ~90% DD COS groups could affect ADG and BUN by reducing ADFI in weaned pigs. However, pigs fed dietary supplementation with 65 kDa of COS and ~90% DD characteristic could enhance apparent digestibility of crude protein and villus height at the jejunum (increased absorption area) better than those fed 65 kDa of COS with ~80% DD on day 56 of the experiment (Thongsong *et al*., 2018). Further, the reason might be that higher MW (65 kDa compared to 8 kDa) of COS increased the viscosity of the gastrointestinal tract content due to a gel complex formation, consequently reducing feed intake and slowing the diffusion of nutrients (including proteins and peptides), thus resulting in a highly effective increase in nutrient absorption. Finally, this phenomenon caused a shift in partitioning nutrients to

gene expression responses (Bauer *et al*., 2011). The upregulation of PepT1 mRNA may be a mechanism to take advantage of the abundant peptides and amino acid forms from diet present in the intestinal lumen and efficiently transport them from the small intestinal lumen into the enterocyte (Qiu *et al*., 2016). Similarly, Spanier (2014) found that transcriptional and functional regulation of PepT1 was upregulated in response to a high protein level and/or a high-quality protein diet.

Furthermore, pigs fed dietary supplementation with 150 or 225 mg/kg COS levels, and 8kDa~90% or 65kDa~80% COS characteristics could down-regulate PepT1 mRNA in the duodenum of pigs. Similarly, in pigs fed diets containing different experiment COS levels or characteristics as compared to pigs fed the control diet for 56 days of the experiment, there was no effect on expression of PepT1 mRNA in the ileum. The causes of this result are unclear. Then, the increased expression of PepT1 mRNA at the jejunum caused by dietary COS-supplementation observed in experiment 1 and 2 might contribute to maintain or enhance nutrient absorption and might also indicate that a physiological response to maximize the absorption of peptides occurs in the jejunum to compensate for the reduced feed intake (Zhang *et al*., 2016), and to improve the whole-body protein metabolism in weaning pigs (Thongsong *et al*., 2018). Research has demonstrated enhanced ileal protein digestibility, enhanced jejunal adsorption capacity, and increased intestinal cell division, thus indicating the potential role of dietary COS-supplementation in raising the efficiency of the intestinal function after weaning (Suthongsa *et al*., 2017; Thongsong *et al*., 2018). We suggest that optimal dietary COS-supplementation may influence some digestive enzymes or their activities, and major nutrient transports by altering their transporters. However, availability of transportable substrates, concentrations of peptide-bound, and presence of other components in the intestinal lumen should also be considered in the expression of PepT1 mRNA (Gilbert *et al*. 2008) because substrates in the intestinal lumen can regulate transporters (Ganapathy 2012). From both the pig and biomedical industries' perspectives, there is a pressing need for further investigation, not only of the mechanism involved in PepT1 gene regulation and its protein abundance, but also to determine whether other genes and proteins related to nutrient absorption are regulated by dietary COS- supplementation.

In conclusion, the present study provides a starting point for future research aimed at determining the dietary COS relevance on PepT1 mRNA expression. On day 56 of both experiments, the upregulation of PepT1 mRNA in the jejunum was observed with supplemental COS levels (75 and 150 mg/kg), and a COS characteristic (65 kDa~90%) led us to speculate that dietary COS levels and characteristics may play an important role in designing nutritional strategies to influence long-term PepT1 mRNA expression in weaned pigs. However, some mechanisms underlying changes in PepT1 mRNA expression are still unclear. With understanding regarding the relationships between COS biofunctions and its levels or its MW and DD characteristics, our information in the previous studies indicated that optimal COS supplemented level of either low or medium MW with more than 80% DD might be more beneficial for the intestinal tract after long-term diet supplementation.

Conflict of interest: The authors declare no potential conflict of interest.

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