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Effect of carboxymethyl chito-oligosaccharide on cysteine absorption in intestinal porcine epithelial cells

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Abstract: Oxidative stress is associated with retarded growth and the initiation and progression of diseases in pigs. The carboxymethylation of chito-oligosaccharide (COS) can improve its antioxidant activity for such issues. Herein, an orthogonal experiment L9 (3^4) test design was used to optimise the preparation conditions of carboxymethyl (CM)-COS. Fourier-transform infrared analysis evidenced the carboxymethylation of COS, and an *in vitro* study indicated that the hydroxyl radical scavenging ability of CM-COS is superior to that of COS. CM-COS can also better promote the absorption of cysteine and increase the expression of the amino acid transport system $b^{0,+}$ in intestinal porcine epithelial cells. The results suggested that CM-COS can effectively resist oxidative damage by promoting cysteine absorption mediated by transport system $b^{0,+}$, which provides important information regarding the antioxidative damage application of COS and CM-COS in the pig farming industry.

Keywords: carboxymethyl modification; antioxidant activity; amino acid transport system; intestinal health

In pig farming, piglets are commonly subjected to various stressors, such as dramatic changes in their diet and environment, which have been shown to induce oxidative stress resulting from the overproduction of reactive oxygen species (ROS) (Engwa et al. 2022). Oxidative stress is known to be a mediator of damage to cell structures, including lipids, membrane proteins, and DNA (Luo et al. 2020), and is associated with the initiation and progression of some diseases (Lauridsen 2019; Lauridsen 2020). Weaning stress in piglets has been shown to induce oxidative damage, resulting in low feed intakes, intestinal dysfunction, and growth retardation (Engwa et al. 2022). Therefore, efforts to develop natural antioxidants which can effectively protect piglets from such oxidative stress are of particular interest since synthetic antioxidants have been reported to have adverse effects in some cases (Pisoschi and Pop 2015).

As a rich natural resource, chito-oligosaccharide (COS) originating from the chemical and enzymatic hydrolysis of poly-chitosan has attracted

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much attention due to its excellent biocompatibility, biodegradability, and biological activities (No and Prinyawiwatkul 2009). Thus far, COS has been widely used in the food, chemical, biomedical, and farming industries. Many studies have shown that COS has a decent antioxidant capacity, which is related to its amino (c-2), primary hydroxyl (c-3), and secondary hydroxyl (c-6) groups. A previous study indicated that COS benefits the intestinal microecology, morphology, function, and growth performance of weaned piglets, partly due to its antioxidative properties (Xiong et al. 2015). Dietary COS supplementation in sows protects sows from oxidative stress by increasing plasma antioxidants and blocking inflammatory responses, and promotes placental amino acid transport (Xie et al. 2016). Dietary COS supplementation of sows during late gestation and lactation can increase the blood glucose level of newborn piglets and their ability to maintain energy supply, thus increasing the growth rate of suckling piglets (Xie et al. 2015). Interestingly, Shariatinia (2018) reported that carboxymethyl (CM) modification of chitosan can increase some of its bioactive properties, including its antioxidant activity. Thus, we speculated that CM-COS could have a higher antioxidant activity than COS for preventing oxidative damage in piglets.

Previous studies have indicated that COS can improve the absorption rate of sulphur-containing amino acids (AAs), including methionine and cysteine (Cys) (Yang et al. 2016), which have been shown to be closely related to the antioxidant capability of the body (Djuric 2018). Cys absorbed by the gut is an important raw material for antioxidant substances [e.g. glutathione (GSH)] and antioxidant enzymes (e.g. GSH-Px) (Toz and Deger 2018) and has been implicated in protecting the morphology and function of the intestine from oxidative damage by regulating mitochondrial functions (Xiao et al. 2016), NF-kB and Nrf2 signalling pathways, etc. (Song et al. 2016). However, whether structurally modified COS can promote the absorption of Cys to have an antioxidant protective effect on piglets has not been reported.

Therefore, in the present study, CM-COS was first prepared by optimising the modification conditions. Then, the *in vitro* antioxidant capabilities of COS and CM-COS were investigated, and the effects of CM-COS and COS on the absorption of Cys in pig intestinal epithelial cells, as well as possible implicated transport systems, were further determined. The present study aimed to gain insights into the antioxidative effects of COS and CM-COS on piglets and the involved mechanisms, which will help extend the application of COS and CM-COS in pig farming.

MATERIAL AND METHODS

Materials and reagents

COS with a molecular weight less than 2000 Da and deacetylation degree more than 90% was purchased from Dalian Meilun Biotechnology (China). Isopropanol, sodium hydroxide (NaOH), hydrochloric acid, anhydrous ethanol, and 30% hydrogen peroxide were purchased from Chongqing Chuandong Chemical (China). Monochloroacetic acid, 1640 medium, ferrous sulphate heptahydrate, and salicylic acid were purchased from Shanghai Macklin Biochemical Technology (China). Line 1 intestinal porcine epithelial cells (IPEC-1) were purchased from Shanghai Suer Biochemical Technology (China). L-Cys (≥ 98%, non-animal source) for cell cultures was purchased from Shanghai Aladdin Biochemical Technology (China), and Cys content detection kits were purchased from Beijing Solarbio Technology (China). Foetal bovine serum, pancreatic ferment, and penicillin-streptomycin were purchased from GIBCO (Waltham, MA, USA). All reagents used in this study were of analytical grade, and all solutions were prepared with distilled water.

Preparation of CM-COS

CM-COS was prepared based on a literature method (Chen and Park 2003) with slight modifications. Briefly, COS was dispersed in isopropanol alcohol, and a 30% NaOH solution and isopropanol solution of monochloroacetic acid were added in turn. After the reaction reached completion, the pH was adjusted to neutrality with 10% HCl. The amounts of added reagents and the duration of alkalization and carboxymethylation were selected as described in subsection 2.3. The product was washed with anhydrous ethanol three times and dissolved in a small amount of water. The precipitate was collected by centrifugation, and the white product was obtained through vacuum drying to a constant weight. The degree of substi-

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tution (DS) was estimated by potentiometric titration based on a literature method (Kong 2011) with slight modifications.

Optimisation of CM-COS preparation

An orthogonal experiment L9 (3^4) test design in preparation mode was used to optimise the CM-COS preparation conditions. The key parameters influencing the degree of CM-COS substitution were analysed, including the added amount of NaOH (A), added amount of monochloroacetic acid (B), alkalisation reaction period (C), and carboxymethylation reaction period (D) (Sabaa et al. 2009). There were three levels to be optimised for each factor. Nine preparations were carried out with factor A of 2, 4, and 6 g; factor B of 2, 3, and 4 g; factor C of 2, 7, and 12 h; and factor D of 4, 12, and 24 h [Table S1 in electronic supplementary material (ESM); for the supplementary material see the electronic version].

FT-IR spectroscopy

FT-IR spectra of COS and CM-COS were recorded on a Nicolet IR200 FT-IR Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) at a resolution of 4 cm^{-1} with 32 scans at room temperature. Each sample was vacuum-dried at 80 °C for 6 h and then cooled in a desiccator with colour-indicating silica. A pellet was formed from 2.5 mg of each sample and 250 mg of KBr. Data analysis was carried out using Omnic (Home Edition, Microsoft, Redmond, WA, USA).

In vitro determination of antioxidant properties

Using the salicylic acid colorimetric method (Wang et al. 2012), the hydroxyl free radical scavenging abilities of CM-COS and COS were measured to determine their *in vitro* antioxidant performances. The preparation of different sample concentrations was tested in advance. During measurements, the samples were added in turn according to Table S2 in ESM. After hydrogen peroxide was added, the samples were immediately placed into a water bath at 37 °C for 15 minutes. A colorimeter with a 1-cm path length sample cell was used to measure the absorbance at 510 nm, and distilled water was used to zero the instrument before measuring the light absorption value of each sample. Each sample was measured five times, and the results were averaged. The –OH scavenging rates of CM-COS and COS were calculated using the following formula:

$$\text{–OH scavenging rate (\%)} = [A_0 - (A_X - A_{X0})/A_0] \times 100 \quad (1)$$

where:

A_0 – absorbance of the blank control solution;

A_X – absorbance of the reaction system with the sample solution;

A_{X0} – background absorbance of the sample solution.

Cell culture, treatment and Cys content determination

IPEC-1 cells were grown in 1640 media supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin, and 5 mmol/l Cys, then they were cultured at 37 °C in a 5% CO2 incubator. The cells in the CM-COS and COS treatment groups were treated with 50, 100, and 300 μg/ml CM-COS and COS after reaching 80% confluence (Ji et al. 2016). At 30, 150, 390, and 1 080 min, the cells were harvested and stored at –80 °C. These treatments were repeated three times. The Cys content in the cell culture medium was determined using a Cys assay kit BC0180 (Beijing Solarbio Technology Co., Ltd, Beijing, China).

Real-time PCR

The total RNA of the IPEC-1 cells was isolated using Trizol reagent (Invitrogen, Waltham, MA, USA) and quantitatively measured by UV-Vis spectroscopy (DU800; Beckman Coulter, Fullerton, CA, USA). According to the manufacturer's instructions, the RNA from each sample was reverse-transcribed using a reverse transcriptional kit (TaKaRa, Kusatsu, Japan). The mRNA of the target gene was quantitatively expressed by SYBR Premix Ex Taq II and ROX reagent (TaKaRa, Kusatsu, Japan) in an ABI 7900HT detection system (Applied Biosystems, Waltham, MA, USA).

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The real-time PCR primer sequences are shown in Table S3 in ESM. The melting temperature of all primers according to the design is approximately 60 °C. The cycle conditions were as follows: predenaturation, 95 °C, 30 s, one cycle; PCR reaction, 95 °C, 5 s, 60 °C, 30 s, 40 cycles; dissolution, 95 °C, 15 s, 60 °C, 1 min, 95 °C, 15 s. The relative quantitative $2^{-\Delta\Delta CT}$ method was used to analyse the relative fold gene expression of the samples. The difference between the cycle threshold (CT) of the target gene and of the internal reference gene is defined as ΔCT, and the difference in ΔCT mean values between each group and the control group is defined as ΔΔCT. Finally, $2^{-\Delta\Delta CT}$ was used to calculate the relative gene expressions.

Statistical analysis

SPSS v19.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Differences were tested by one-way analysis of variance (ANOVA) and Duncan multivariate comparison with $P < 0.05$ considered statistically significant. The data from the orthogonal test were subjected to range analysis, and the Cys contents were analysed using the GLM procedure.

RESULTS

Optimisation of CM-COS preparation arameters

The results of the orthogonal test and extreme difference analysis are presented in Table 1, which shows that the maximum DS of the prepared CM-COS was 1.03. However, the best preparation conditions could not be selected based only on the maximum DS. Thus, a further orthogonal analysis was warranted. The K and R values were calculated and are listed in Table 1. The results showed that the influence of the factors on the mean DS of the compound decreased in the order B > C > D > A according to the R values. Thus, the added amount of monochloroacetic acid was the most important determinant of the DS.

As shown in Table 1, the K value of factor B increased with the added amount of monochloroacetic acid, indicating the increased range of K. Therefore, DS was measured with the increasing added amount of monochloroacetic acid (5, 6, 7, 8, 9, and 10 g) without changing the other secondary factors (Figure 1). The DS of CM-COS gradually increased with the increasing added amount of monochloroacetic acid within the set range until reaching a maximum

Table 1. The result of orthogonal experiment L9 (3^4)

Test No.	Factors				Degrees of substitution (DS)	Mean DS
	A	B	C	D		
1	1	1	1	1	0.43, 0.48, 0.48	0.46
2	1	2	2	2	0.61, 0.61, 0.62	0.61
3	1	3	3	3	1.00, 1.07, 1.01	1.03
4	2	1	2	3	0.56, 0.55, 0.56	0.56
5	2	2	3	1	0.58, 0.61, 0.62	0.60
6	2	3	1	2	0.83, 0.86, 0.87	0.85
7	3	1	3	2	0.66, 0.65, 0.64	0.65
8	3	2	1	3	0.63, 0.66, 0.61	0.63
9	3	3	2	1	0.88, 0.86, 0.86	0.87
K1	2.10	1.67	1.94	1.93		
K2	2.01	1.84	2.04	2.11		
K3	2.15	2.75	2.28	2.22		
Optimal levels	A3	B3	C3	D3		
R	0.14	1.08	0.34	0.29		
Order	B > C > D > A					

A = the addition amount of NaOH; B = the addition amount of monochloroacetic acid; C = time of alkalization reaction; D = time of carboxymethylation

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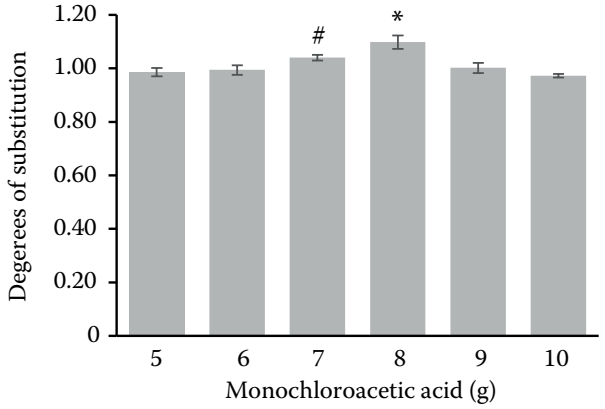

Figure 1. Effects of chloroacetic acid dosage on the degree of substitution of carboxymethyl chitosan oligosaccharide
Data were expressed as mean ± SD (n = 3). No special symbols in data superscripts or the same special symbols mean insignificant difference (P > 0.05), while different special symbols mean significant difference (P < 0.05)

of 1.10 at 8 g, which was significantly higher than that of the other added amounts (P < 0.05). The DS of CM-COS decreased with added amounts of monochloroacetic acid above 8 g. Thus, the results indicated that 6 g of NaOH, 8 g of monochloroacetic acid, 12 h of alkalisation, and 24 h of carboxymethylation were the optimum parameters for preparing CM-COS, and the DS reached 1.10.

FT-IR analysis

As shown in Figure 2, the FT-IR absorption bands of COS were observed at 3 100–3 400 (O–H stretching and N–H stretching), 1 576 (N–H bending),

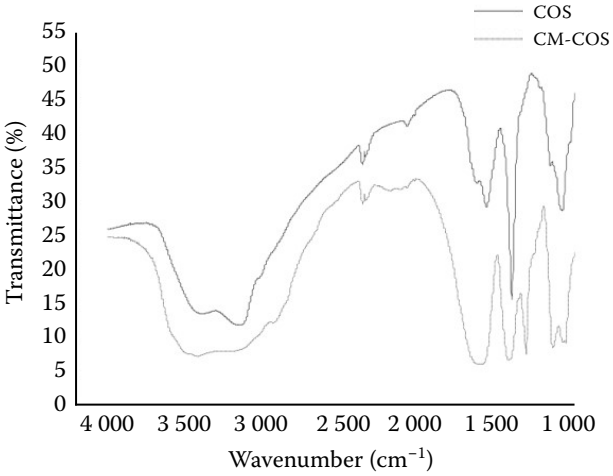

Figure 2. Infrared spectrum characterization of chitooligosaccharide (COS) and carboxymethyl (CM)-COS

and 1 402 cm^{-1} (C–N stretching). The absorption bands of CM-COS were observed at 3 100–3 400 (O–H stretching and N–H stretching), 1 599 (C=O of –COO– antisymmetric stretching), 1 405 (C–N stretching), and 1 072 cm^{-1} (C–O stretching). Compared with the COS spectrum, the new absorption band corresponding to –COO– in the CM-COS spectrum is a strong indication that a high degree of carboxymethylation occurred on the hydroxyl groups of COS.

In vitro determination of antioxidant properties

The scavenging abilities of hydroxyl radicals by COS and CM-COS at different concentrations are given in Table 2. Both COS and CM-COS showed significant hydroxyl radical scavenging activities. When the sample concentration was 6 mg/ml, the hydroxyl radical clearance rates of COS and CM-COS reached maximum values of 95.7% and 99.89%, respectively. The hydroxyl radical clearance rate of CM-COS was significantly higher than that of COS within the mass concentration range selected in this study (P < 0.05). The sample concentrations when 50% of the hydroxyl radicals were removed, defined as EC50, for COS and CM-COS were calculated as 1.76 and 1.11 mg/ml, respectively. These results indicated that the hydroxyl radical scavenging ability of CM-COS is superior to that of COS.

Determination of Cys content

Supplementing with COS (Table 3) and CM-COS (Table 4) reduced the Cys contents in the IPEC-1 culture media (P < 0.05). When the concentration of both COS and CM-COS was 100 μg/ml, the Cys contents in the cell culture media were the lowest (Tables 3 and 4), indicating that the absorption of Cys was the strongest in IPEC-1 at this concentration. To further compare the difference between COS and CM-COS in promoting Cys absorption in IPEC-1, 100 μg/ml COS and CM-COS were used to treat IPEC-1 at the same time. The results showed that the Cys content in the culture medium of the CM-COS treatment group was significantly lower than that of the COS treatment group (P < 0.05), which indicated that CM-COS can better promote the absorption of Cys. Additionally, treatment type

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Table 2. Ability of COS and CM-COS to scavenge hydroxyl free radicals at different concentrations (mean ± SD, n = 5)

Group	0 mg/ml	2 mg/ml	4 mg/ml	5 mg/ml	6 mg/ml	8 mg/ml	10 mg/ml
COS	0	74.61 ± 0.37	82.06 ± 0.21	91.12 ± 0.17	95.7 ± 0.46	88.63 ± 0.44	86.4 ± 0.32
CM-COS	0	87.61* ± 0.5	97.14* ± 0.29	99.46* ± 0.29	99.89* ± 0.3	99.38* ± 0.21	98.06* ± 0.65

*In the same column, asterisks indicate statistical differences compared to COS group (P < 0.05)

Table 3. Effect of COS dose on Cys content in the cell culture medium (umol/ml) (mean ± SD, n = 3)

Time	Control	COS50	COS100	COS300	P-value		
					T	M	T × M
0 min	14.85 ± 2.25	15.15 ± 1.03	15.75 ± 0.51	16.04 ± 1.36			
30 min	26.16 ± 1.79	8.30 ± 0.90	9.50 ± 0.51	7.71 ± 0.51			
150 min	33.90 ± 2.73	7.71 ± 1.36	5.33 ± 1.03	11.28 ± 0.52	0.000	0.000	0.000
390 min	23.78 ± 2.25	7.41 ± 1.79	10.68 ± 2.25	8.90 ± 1.36			
1 080 min	32.41 ± 1.54	63.37 ± 2.57	65.45 ± 0.90	67.24 ± 1.55			

M = effects of measure time; T = effects of treatment; T × M = effects of interaction between treatment and measure time

Table 4. Effect of CM-COS dose on Cys content in the cell culture medium (umol/ml) (mean ± SD, n = 3)

Time	Control	CM-COS50	CM-COS100	CM-COS300	P-value		
					T	M	T × M
0 min	17.53 ± 1.03	19.32 ± 0.51	21.10 ± 2.06	19.91 ± 0.89			
30 min	23.18 ± 0.51	11.28 ± 2.58	15.15 ± 2.25	20.21 ± 0.51			
150 min	33.90 ± 0.52	7.41 ± 1.55	2.01 ± 0.07	7.71 ± 1.03	0.000	0.000	0.000
390 min	20.21 ± 1.37	4.73 ± 0.90	24.08 ± 0.52	3.54 ± 0.51			
1 080 min	27.95 ± 3.22	16.64 ± 2.87	12.77 ± 1.55	16.64 ± 0.51			

M = effects of measure time; T = effects of treatment; T × M = effects of interaction between treatment and measure time

(COS or CM-COS) and measurement time interact to affect the Cys content in the cell culture media (P < 0.01) (Tables 3–5).

Quantitative real-time PCR analysis of Cys transport gene

Real-time PCR analysis was used to determine the mRNA levels of the $b^{0,+}AT$ and *ASCT2* genes in IPEC-1. As shown in Figure 3, compared with the control group, the mRNA levels of the $b^{0,+}AT$ gene in IPEC-1 were significantly higher with 100 μg/ml CM-COS supplement (P < 0.05) as compared with COS supplement. Furthermore, the mRNA level of the $b^{0,+}AT$ gene in the CM-COS supplement group was significantly higher than that in the COS group (P < 0.05). The mRNA levels of the *ASCT2* gene in the COS and CM-COS groups were not significantly different from those in the control group.

Table 5. Comparative studies of CM-COS and COS on Cys content in the cell culture medium (umol/ml) (mean ± SD, n = 3)

Time	Control	COS100	CM-COS100	P-value		
				T	M	T × M
0 min	18.13 ± 0.90	20.21 ± 0.51	18.42 ± 1.86			
30 min	21.69 ± 1.55	14.85 ± 0.52	11.58 ± 0.52			
150 min	29.44 ± 1.86	8.30 ± 1.55	5.63 ± 0.90	0.000	0.000	0.000
390 min	21.10 ± 1.86	21.40 ± 1.86	21.70 ± 0.90			
1 080 min	24.67 ± 1.36	27.05 ± 2.68	16.04 ± 0.51			

M = effects of measure time; T = effects of treatment; T × M = effects of interaction between treatment and measure time

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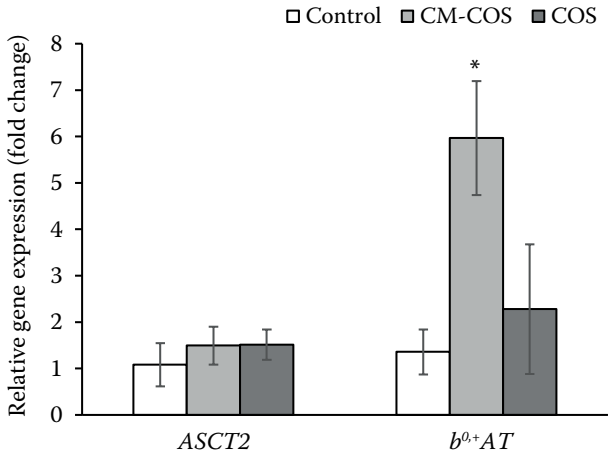

Figure 3. The mRNA level of *ASCT2* and $b^{0,+}AT$
Data were expressed as mean ± SD (n = 3). No special symbols in data superscripts or the same special symbols mean insignificant difference (P > 0.05), while different special symbols mean significant difference (P < 0.05)

DISCUSSION

The carboxymethylation modification of COS can change its bioactivity (Narayanan et al. 2014; Shariatinia 2018), and the activity of CM-COS is related to the DS (Sun et al. 2008). Previous studies showed that the synthesis of CM-COS proceeded mainly by the reaction of COS with monochloroacetic acid (No and Prinyawiwatkul 2009). However, thus far, neither standard operation parameters nor consistent results have been reported for the CM-COS preparation (Sun et al. 2008; Wahid et al. 2016; Yu et al. 2021). The solvent used to dissolve COS, times and added amounts of reagents for the alkalisation and carboxymethylation reaction systems, and reaction temperature have generally been considered to be the most important factors (Sabaa et al. 2009). Thus, to optimise the operating conditions and obtain CM-COS with a high DS, an orthogonal experiment L9 (3^4) test design in preparation mode was used in the present study. Using the optimised parameters, we obtained CM-COS with a DS of 1.1, which is nearly equal to that DS of 1.09 reported by Kong (2011). FT-IR spectroscopy, an effective tool for estimating structural information regarding functional groups, was used to further investigate the functional groups on the prepared CM-COS. The present FT-IR analysis showed that the characteristic bands of the prepared CM-COS were similar to those in the literature (Kong 2011; Olanipekun et al. 2021), indicating that CM-COS was successfully prepared.

The hydroxyl radical is the most reactive ROS and can cause serious damage to adjacent biomolecules (Treml and Smejkal 2016). The hydroxyl radical clearance rate is an important indicator reflecting the antioxidant capacity of compounds. The present results indicated that the hydroxyl free radical scavenging ability for COS and CM-COS is as expected since it has been shown that COS and its derivatives have a strong reduction ability and can remove hydroxyl radicals and superoxide anions (Mi et al. 2020).

Additionally, we found that CM-COS had a higher antioxidant capacity than COS, which is consistent with a previous report that the antioxidant capacity of *O*-carboxymethyl chitosan was higher than that of chitosan (Shariatinia 2018). However, reported comparisons of the *in vitro* antioxidation capacities of COS and CM-COS are limited, as more studies have focused on the effect of introducing other functional groups on the antioxidant capacity of chitosan or CM-chitosan (Mi et al. 2020; Xu et al. 2021). These studies indicated that introducing different functional groups changed the difference in antioxidation capability between chitosan and CM-chitosan. Even though our *in vitro* study indicated that COS and CM-COS had antioxidation capacities, where that of CM-COS was higher, the presence of similar differences *in vivo* needs further investigation.

The intestinal health of piglets is easily disturbed by oxidative stress due to the abundance of mitochondria producing many ROS during electron transport (Lauridsen 2019; Lauridsen 2020). COS plays an antioxidant role mainly by strengthening the antioxidant defence system, including the levels of antioxidants (e.g. GSH) and activities of antioxidant enzymes (e.g. GSH-Px) in the body (Toz and Deger 2018). One *in vivo* rodent study with intravenous infusion of Cys indicated that an important metabolic fate of Cys in the gut is incorporation into GSH (Malmezat et al. 2000), which shows that the intestinal absorption of Cys is a key factor in enhancing the antioxidation system. Therefore, to investigate whether COS and CM-COS can enhance their antioxidant capacity by enhancing the uptake of Cys, we examined the Cys content in the cell culture medium of IPEC-1 cells. In the present study, it was observed that 100 μg/ml COS and CM-COS could maximise the absorption of AAs 150 min after IPEC-1 treatment. Thus, to compare CM-COS and

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COS in terms of promoting intestinal Cys absorption, COS and CM-COS at 100 μg/ml were used to treat IPEC-1. Our results indicated that CM-COS can better promote the absorption of Cys in IPEC-1 than COS. The absorption of AAs is associated with many transport systems which have been identified in mammals based on their independence and specificity towards AAs. Of these, the amino acid transport system $b^{0,+}AT$ and alanine-serine-cysteine transport system *ASCT2* have been reported to mediate the uptake of Cys (Wang et al. 2009; Colas et al. 2015; Scalise et al. 2015). However, in the present study, only the expression of AA transporter system $b^{0,+}AT$ was increased by COS and CM-COS, suggesting that this increase is how COS and CM-COS promote the absorption of Cys in IPEC-1. Furthermore, the significantly higher expression level of $b^{0,+}AT$ in the CM-COS treatment group than in the COS group may partially explain the higher Cys absorption level of the former.

To summarise, our results showed that CM-COS has an excellent antioxidant capacity and that CM-COS and COS can promote the absorption of Cys by enhancing the expression of the Cys transport vector $b^{0,+}AT$ gene in IPEC-1. This suggests that CM-COS and COS have *in vivo* antioxidative effects which are partially attributable to an increase in Cys absorption mediated by the AA transport vector $b^{0,+}$ system. Furthermore, CM-COS has a stronger antioxidant activity than COS. These findings support the further investigation of COS and CM-COS as the antioxidative damage agents in the pig farming industry.

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Conflict of interest

The authors declare no conflict of interest.

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