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Original Research Article

An increase in corn resistant starch decreases protein fermentation and modulates gut microbiota during *in vitro* cultivation of pig large intestinal inocula

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ABSTRACT

High-protein diet could cause an increase in protein fermentation in the large intestine, leading to an increased production of potentially detrimental metabolites. We hypothesized that an increase in corn resistant starch content may attenuate the protein fermentation. The aim of this study was to evaluate the effect of resistant starch on protein fermentation by inocula from large intestine of pigs using *in vitro* cultivation. Fermentation patterns were analyzed during a 24-h incubation of cecal and colonic digesta with varying corn resistant starch contents, using casein protein as sole nitrogen source. The results showed that the concentration of short-chain fatty acids (SCFA) and cumulative gas production were significantly increased ($P < 0.05$), while ammonia–nitrogen (NH_3–N) and branched-chain fatty acids (BCFA), which indicated protein fermentation, decreased when the corn resistant starch levels increased ($P < 0.05$). The copies of total bacteria, *Bifidobacterium* and *Lactobacillus* were significantly increased with the increased corn resistant starch levels after incubation ($P < 0.05$). The copies of the *Bifidobacterium* and *Lactobacillus* in cecum were significantly higher than those in colon ($P < 0.05$). We conclude that the addition of corn resistant starch weakens the protein fermentation by influencing microbial population and reducing protein fermentation in the cecum and colon *in vitro*.

1. Introduction

Gut health is influenced by the composition of the microbial community and the end-products of bacterial metabolism of diet components (Klose et al., 2010; Sørensen et al., 2009). When dietary protein is consumed in high amounts, more dietary protein may reach to the colon, which results in increased protein fermentation products that include harmful nitrogenous metabolites (Magee et al., 2000; Mu et al., 2016). Protein fermentation products have been associated with toxic and proinflammatory impacts on the intestinal epithelium (Bertschinger et al., 1979; Hampson, 1994; Sørensen et al., 2009). For example, in animal models, protein fermentation metabolites such as ammonia, hydrogen sulfide, and nitrosamines have been shown to be cytotoxic and carcinogenic, and interfere with the cellular metabolism of other substrates (Sørensen et al., 2009). Previous studies showed that, compared with the maintenance diet, a high-protein and low-carbohydrate diet resulted in increased concentrations of phenylacetic acid, N-nitroso compounds and decreased proportion of butyrate in human feces (Magee et al., 2000). Our previous study showed that high-protein diet increased protein fermentation products associated with pro-inflammatory processes in the colonic epithelium of rats (Mu et al., 2016). Therefore, an increased protein fermentation is generally considered to be detrimental to gut health.

To balance the intestinal environment, resistant starch (RS) is used as a beneficial substrate. Resistant starch is defined as the portion of starch that escapes degradation and absorption in the small intestine and reaches the large intestine to be used as a

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fermentation substrate (Englyst et al., 1992). In the large intestine, RS is fermented by the microorganisms. Data from *in vitro* fermentation studies with human fecal inoculum suggest that starch fermentation may be beneficial to host because it favors the production of short-chain fatty acids (SCFA) (Weaver et al., 1992). Increased SCFA production, especially butyrate, usually exerts health benefits. Short-chain fatty acids can lower the pH in the colon and thus prevent the overgrowth of pathogenic bacteria (Roy et al., 2006). However, whether an increase in RS addition could ameliorate the protein fermentation *in vitro* remains unclear.

Several studies assumed that an increase RS would decrease the abundance of protein fermentation products and abundance of harmful bacteria in the porcine gut (Metzler-Zebeli et al., 2010; Pieper et al., 2009). However, little information exists about the effect of corn resistant starch (CRS) on intestinal microbial activity and protein fermentation by porcine large intestinal inocula *in vitro*, the site of highest microbial fermentation activity. The aim of this experiment, therefore, was to assess whether protein fermentation in cecum and colon of pigs could be reduced by increasing the amount of CRS.

2. Materials and methods

2.1. Piglets and sampling

Intestinal samples used in this study were derived from 5 healthy finishing Duroc × Landrace × Yorkshire pigs fed a corn and soybean meal based diet. Immediately after slaughtering, the large intestine was dissected and segmented with sterile threads, placed into vacuum bottles, and flushed with oxygen free CO_2. Briefly, the digesta was gently squeezed out into a sterile flask while continuous gassing with CO_2. Proper volume (at the ratio of 5 mL per 1 g mixed 5 digesta) of prewarmed sterile anaerobic phosphate-buffered saline (pH 7.4) was then injected continuously into the flask which contained the digesta. The solution was filtrated through 4 layers of sterile cheesecloth into a sterile serum bottle fitted with a butyl rubber stopper and an aluminum crimp seal. Bottles were then placed into a 37 °C water bath to provide inocula for the *in vitro* cultivation experiment.

For the *in vitro* cultivate experiment, basal medium was prepared as described by Williams et al. (2005) with modifications. An aliquot (5 mL) of inocula (derived from the cecum or colon) was subsequently injected into a pre-warmed medium containing CRS (2.5, 5.0 and 7.5 mg/mL). Negative controls contained all components except CRS. Positive controls contained CRS but no inoculum. Four bottles were used for each treatment. Bottles were then incubated at 37 °C for 24 h. Samples (3 mL) were taken from each bottle immediately after inoculation and at 6, 12 and 24 h after inoculation and then stored at −20 °C for the analysis of SCFA, ammonia−nitrogen (NH_3−N) and microbial crude protein (MCP). At the end of 24 h, pH was measured immediately and approximately 1 mL of samples was stored at −25 °C until use for the isolation of DNA and analysis of the bacterial population.

2.2. Determination of pH and fermentation end products

The gas production was measured using the pressure transducer technique (Theodorou et al., 1994). Culture medium pH was measured by a pH meter (Schott, Germany). The SCFA were determined by gas chromatography (Shimadzu, GC-14B, Japan) according to Mao et al. (2007). The NH_3−N concentration was measured by the indophenol method (Weatherburn, 1967). The MCP concentration was determined according to Makkar et al. (1982) by spectrophotometer (Thermo Fisher Scientific, NanoDrop 2000c, Wilmington, DE, USA).

2.3. DNA extraction

Total DNA was extracted from digesta samples by bead-beating for 3 min using a bead beater (MP. Biomedicals invine, California, USA), followed by phenol-chloroform extraction (Zoetendal et al., 1998). DNA was then precipitated with ethanol, and the pellets were re-suspended in 50 μL of TE (10 mmol/L Tris, 1 mmol/L EDTA [pH 8.0]). A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine DNA concentrations and electrophoresis in agarose gel 1.2% (wt/vol) containing ethidium bromide was used to check the quality of DNA.

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed using previously published primer sets (Table 1) on a StepOnePlus (Applied Biosystems, California, USA) with StepOne Software (version 2.2.2, Applied Biosystem). The real-time PCR was performed as described by Yang et al. (2014). Quantification of 16S rRNA gene copies in each sample was performed in triplicate, and the mean value was calculated. Standard curves were generated with 10-fold serial dilutions of the 16S rRNA genes amplified from the respective target strains. The number of genes copies were calculated from the appropriate standard curve based on the cycle number at the set threshold fluorescent intensity. Results were reported as lg16S rRNA gene copies per gram wet weight.

2.5. Statistics

Data were analyzed using generalized linear model procedures in SPSS (version 16.0) with CRS levels and segments and their interaction as sources of variation. When a significant interaction

Table 1
16S rDNA PCR primer sets used for real-time PCR in this study.

Primer	Orientation	Primer sequence (5′ to 3′)	Reference
Total bacteria	Forward	CGGTGAATACGTTCYCGG	Suzuki et al., 2000
	Reverse	GGWTACCTTGTTACGACT	
Bacteroidetes	Forward	GGTGTCGGCTTAAGTGCCAT	Rinttilä et al., 2004
	Reverse	CGGAYGTAAGGGCCGTGC	
Firmicutes	Forward	GGAGYATGTGGTTTAATTCGAAGCA	Guo et al., 2008
	Reverse	AGCTGACGACAACCATGCAC	
Bifidobacterium	Forward	CTCCTGGAAACGGGTGG	Matsuki et al., 2004
	Reverse	ACATCTATAGCCCTTCTTGTGG	
Lactobacillus	Forward	AGCAGTAGGGAATCTTCCA	Khafipour et al., 2009
	Reverse	ATTCCACCGCTACACATG	
Escherichia coli	Forward	CATGCCGCGTGTATGAAGAA	Huijsdens et al., 2002
	Reverse	CGGGTAACGTCAATGAGCAAA	

among CRS levels and segments was observed, data were reanalyzed as a one-way ANOVA with 6 levels followed by means separation using Tukey's honestly significant difference test. Differences at $P < 0.05$ were considered significant.

3. Results

3.1. In vitro gas production

As shown in Fig. 1, the gas production were increased with the increasing levels of CRS. Gas production in colon group was significantly higher than that in cecum group at same level of CRS ($P < 0.05$).

3.2. pH, NH_3–N, microbial crude protein, and short-chain fatty acids

The final pH reduced with increasing CRS levels, but all values were ranging from 6.52 to 6.97 throughout the incubation (Table 2). Interactions between CRS levels and segment content were found for pH, NH_3–N at 12 h, MCP at 6 and 12 h ($P < 0.05$). The NH_3–N and MCP concentrations decreased with the increased RS levels at incubation of 6, 12 and 24 h ($P < 0.05$). The NH_3–N concentration increased in cecum compared with colon at 6 h after incubation ($P < 0.05$). The NH_3–N and MCP concentrations decreased in cecum compared with colon at 12 h after incubation ($P < 0.05$). The MCP concentration decreased in cecum compared with colon at 6 h after incubation ($P < 0.05$).

Interactions between CRS levels and segment content were significant for total SCFA and propionate concentrations ($P < 0.05$) at 12 h after incubation (Table 3). The total SCFA, acetate, propionate and butyrate concentrations increased with the increased CRS levels at 12 and 24 h after incubation ($P < 0.05$). The concentrations of total BCFA and isovalerate decreased with the increased CRS levels at 12 and 24 h after incubation ($P < 0.05$). The isobutyrate concentration decreased with the increased CRS levels at 12 and 24 h after incubation ($P < 0.05$). The concentrations of total SCFA, acetate, propionate and butyrate decreased in colon compared with cecum at 6 h after incubation ($P < 0.05$). The concentrations of BCFA and isovalerate increased in colon compared with cecum at 12 h after incubation ($P < 0.05$). The concentration of butyrate and isobutyrate increased in colon compared with cecum at 24 h after incubation ($P < 0.05$).

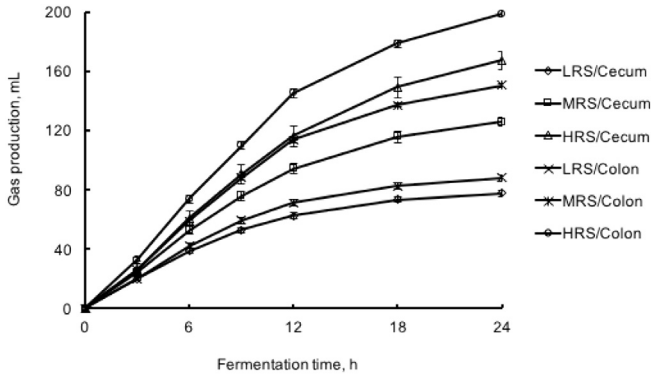

Fig. 1. Dynamic change curves of gas production of different corn resistant starch level after 24 h *in vitro* fermentation. Error bars indicate standard error. The LRS, MRS and HRS after the link represented for low resistant starch (LRS), middle resistant starch (MRS) and high resistant starch (HRS), respectively.

3.3. Real-time PCR

As shown in Table 4, interactions between CRS levels and segment content were significant for copies of *Bifidobacterium* ($P < 0.05$). The copies of total bacteria, *Bifidobacterium* and *Lactobacillus* were significantly increased by the increased CRS levels after incubation ($P < 0.05$). The copies of *Escherichia coli* was significantly decreased by the increased CRS levels after incubation ($P < 0.05$). The copies of the *Bifidobacterium* and *Lactobacillus* in cecum were significantly higher than those in colon ($P < 0.05$).

4. Discussion

By adding different levels of CRS using the *in vitro* fermentation system, the present study investigated the effect of CRS on protein fermentation characteristic in the large intestine of pigs. We found that CRS addition increased gas production and SCFA, while decreased NH_3–N and MCP concentration. In addition, CRS addition also changed microbiota composition.

Corn resistant starch changed fermentation pattern by porcine large intestinal inocula. Gas production was enhanced by CRS addition, especially in the later stage of incubation. The positive relationship between RS content and gas production has already been shown in several researches (Annison and Topping, 1994; Faisant et al., 1993). Our results also showed that adding CRS could cause a reduction in pH and an increase in SCFA concentration in the cecum and colon. An increase in concentration of SCFA appears the major reason to pH reduction, which is considered beneficial to the host for inhibiting potential pathogen growth (Magee et al., 2000; Roy et al., 2006). Therefore, the increased pH may partially contribute to the beneficial function of CRS.

Short-chain fatty acids are mainly produced from carbohydrate fermentation by microbiota in the large intestine. In the present study, increasing CRS enhanced *in vitro* fermentation, as evidenced by increased concentrations of total SCFA, acetate, propionate and butyrate in cecal and colonic incubation of 12 and 24 h. Thus, the addition of CRS could change fermentation pattern, which may be

Table 2
pH, ammonia–nitrogen (NH_3–N) and microbial crude protein (MCP) in the different levels corn resistant starch of cecum and colon *in vitro* fermentation.

Item	Time, h	Cecum			Colon			SEM	*P*-value		
		Low	Middle	High	Low	Middle	High		RS levels	Segment	RS levels × Segment
pH	24	6.88^{e}	6.73^{c}	6.52^{a}	6.97^{f}	6.77^{d}	6.64^{b}	0.03	<0.05	<0.05	<0.05
NH_3–N, mmol/L	6	4.11^{c}	3.68^{bc}	3.27^{b}	3.53^{bc}	2.67^{a}	2.32^{a}	0.08	<0.05	<0.05	0.33
	12	10.55^{d}	6.16^{b}	2.98^{a}	9.73^{d}	7.29^{c}	6.06^{b}	0.13	<0.05	<0.05	<0.05
	24	12.70^{b}	8.58^{a}	7.30^{a}	11.66^{b}	7.46^{a}	7.08^{a}	0.27	<0.05	0.63	0.50
MCP, g/mL	6	41.96^{ab}	75.94^{bc}	36.67^{a}	127.54^{d}	85.17^{cd}	64.43^{abc}	4.76	<0.05	<0.05	<0.05
	12	189.91^{d}	110.96^{b}	53.62^{a}	175.16^{d}	131.27^{c}	109.15^{b}	2.34	<0.05	<0.05	<0.05
	24	228.59^{b}	154.46^{a}	131.40^{a}	209.82^{b}	134.28^{a}	127.42^{a}	4.85	<0.05	0.62	0.51

RS = resistant starch.
$^{a-f}$Means within a row with different superscripts are different ($P < 0.05$).

Table 3
Concentrations (mmol/L) of short-chain fatty acids (SCFA) in the different levels corn resistant starch of cecum and colon *in vitro* fermentation.

Item	Time, h	Cecum			Colon			SEM	*P*-value		
		Low	Middle	High	Low	Middle	High		RS levels	Segment	RS levels × Segment
Total	6	22.40^{b}	27.14^{b}	26.34^{b}	12.37^{a}	9.69^{a}	8.67^{a}	1.80	0.93	<0.05	0.17
SCFA	12	31.77^{a}	45.94^{ab}	57.23^{ab}	34.22^{ab}	53.59^{b}	52.64^{ab}	2.89	<0.05	0.33	<0.0 5
	24	41.17^{a}	57.34^{b}	83.63^{c}	42.33^{a}	61.74^{b}	81.39^{c}	3.71	<0.05	0.69	0.61
BCFA	6	0.24	0.38	0.23	0.26	0.24	0.20	0.02	0.47	0.57	0.17
	12	1.72^{c}	0.97^{ab}	0.71^{a}	1.79^{c}	1.66^{c}	1.56^{bc}	0.12	<0.05	<0.05	0.44
	24	5.27^{bc}	3.66^{a}	4.40^{ab}	5.79^{c}	4.05^{a}	4.56^{ab}	0.20	<0.05	0.60	0.94
Acetate	6	12.52^{b}	13.50^{b}	13.90^{b}	6.07^{a}	4.98^{a}	4.32^{a}	0.95	0.98	<0.05	0.35
	12	14.37^{a}	20.57^{b}	24.17^{c}	15.89^{a}	24.36^{c}	21.86^{bc}	0.91	<0.05	0.45	0.07
	24	17.79^{a}	26.97^{b}	37.38^{c}	16.82^{a}	27.89^{b}	34.85^{c}	1.72	<0.05	0.51	0.63
Propionate	6	7.76^{b}	10.07^{c}	9.67^{c}	3.80^{a}	3.30^{a}	2.95^{a}	0.67	0.62	<0.05	0.13
	12	11.10^{a}	19.72^{ab}	27.01^{ab}	11.13^{a}	20.78^{ab}	22.38	1.45	<0.05	0.17	<0.05
	24	11.43^{a}	20.09^{b}	32.56^{c}	12.28^{a}	21.24^{b}	30.69^{c}	1.76	<0.05	0.60	0.28
Butyrate	6	1.32^{ab}	1.99^{c}	1.80^{b}	1.04^{a}	0.91^{a}	0.96^{a}	0.11	0.34	<0.05	0.07
	12	3.28^{a}	3.83^{ab}	4.53^{ab}	3.65^{ab}	4.97^{ab}	5.37^{b}	0.28	<0.05	0.05	0.93
	24	3.80^{a}	4.58^{ab}	6.57^{c}	5.17^{b}	6.43^{c}	9.01^{d}	0.39	<0.05	<0.05	0.51
Isobutyrate	6	0.04	0.01	0.04	0.03	0.02	0.06	0.01	0.56	0.84	0.97
	12	0.40	0.21	0.18	0.26	0.31	0.27	0.02	0.28	0.79	0.13
	24	1.25^{ab}	0.97^{c}	1.08^{bc}	1.31^{a}	1.15^{abc}	1.13^{abc}	0.03	<0.05	<0.05	0.64
Isovalerate	6	0.20	0.34	0.19	0.23	0.22	0.14	0.02	0.15	0.42	0.13
	12	1.32^{a}	0.76^{bc}	0.53^{c}	1.53^{a}	1.35^{a}	1.28^{ab}	0.10	<0.05	<0.05	0.70
	24	4.02^{ab}	2.70^{c}	3.32^{bc}	4.48^{a}	2.90^{bc}	3.43^{abc}	0.18	<0.05	0.86	0.98

RS = resistant starch; BCFA = branched-chain fatty acids.
$^{a-d}$Means within a row with different superscripts are different ($P < 0.05$).

Table 4
Copy numbers (lg [copies/g]) of total bacteria, Firmicutes, Bacteroidetes, *Bifidobacterium* and *Lactobacillus* in different levels corn resistant starch of cecum and colon *in vitro* fermentation.

Item	Cecum			Colon			SEM	*P*-value		
	Low	Middle	High	Low	Middle	High		RS levels	Segment	RS levels × Segment
Total bacteria	11.29^{a}	11.33^{a}	11.73^{bc}	11.41^{ab}	11.37^{ab}	11.95^{c}	0.05	0.001	0.187	0.757
Firmicutes	10.59	10.72	11.30	10.92	10.76	11.38	0.11	0.072	0.504	0.854
Bacteroidetes	9.38	9.53	9.59	9.62	9.90	9.71	0.09	0.612	0.208	0.860
Bifidobacterium	5.92^{bc}	6.05^{c}	6.74^{d}	5.47^{a}	5.51^{a}	5.68^{ab}	0.04	0.000	0.000	0.022
Lactobacillus	9.13^{ab}	9.01^{a}	9.51^{b}	8.88^{a}	8.78^{a}	9.03^{a}	0.06	0.046	0.014	0.613
Escherichia coli	10.05^{ab}	10.69^{c}	10.22^{ab}	10.39^{bc}	10.40^{bc}	9.84^{a}	0.06	0.011	0.338	0.051

RS = resistant starch.
$^{a-c}$Means within a row with different superscripts are different ($P < 0.05$).

beneficial to nutrient digestion. This is in agreement with previous study that a diet high in CRS could significantly increase SCFA concentration in cecal and colonic of pigs compared with the control group (Haenen et al., 2013). Bird et al. (2007) also found that addition of high-amylose maize starch in diet significantly increased the total SCFA and decreased pH compared with the control. Our experiment also found that adding CRS increased butyrate concentrations in cecal and colonic incubation of 12 and 24 h. It has been observed that SCFA and butyrate concentrations were significantly greater in cecum and colon of RS-fed pigs (Haenen et al., 2013). In the pig colon, potato resistant starch could also increase butyrate production (Fang et al., 2014). These results confirmed the butyrogenic effect of RS. However, in the present study, an increase in CRS content did not change the concentrations of acetate, propionate, and butyrate in the cecal and colonic incubation at 6 h. It is possible that at the early fermentation stage, the added RS may be used for other pathways such as lactate and succinate production, but not acetogenesis and butyrogenesis. But, this needs further investigation in future research.

Importantly, CRS reduced protein fermentation by the cecal and colonic inocula, as evidenced by the decrease of BCFA and NH_3–N. The BCFA, such as iso-butyrate, 2-methyl-butyrate and iso-valerate, originate from the deamination of valine, isoleucine and leucine (Macfarlane and Macfarlane, 1995). In the current study, BCFA (including isobutyrate and isovalerate) concentrations significantly decreased when the CRS was added in incubation after 12 and 24 h, suggesting that the CRS changed fermentation pattern and decreased protein fermentation. It is in agreement with previous studies that long-term intake of raw potato starch decreased BCFA concentration and its proportion in the porcine colon (Zhou et al., 2016). Therefore, the increase of CRS level may reduce the protein availability for bacterial fermentation, thus reducing BCFA concentrations.

Ammonia–nitrogen is also a major end-product of protein fermentation. Our result showed that increasing CRS levels could reduce the NH_3–N concentration. Dietary inclusion of fermentable carbohydrates (wheat bran and sugar beet pulp) is reported to reduce large intestinal formation of metabolites derived from fermentable proteins, such as NH_3–N (Pieper et al., 2012). Long-term intake of raw potato starch diet decreased ammonia–nitrogen when compared with the corn starch diet (Zhou et al., 2016). In the present study, the difference in NH_3–N concentration may be related to CRS concentration and degradability, which needs further research. Protein fermentation products have been associated with toxic and proinflammatory impacts on the intestinal epithelium (Blaut and Clavel, 2007; Geypens et al., 1997; McGarr et al., 2005). These results imply that CRS may be protective to the epithelium by reducing NH_3–N production, which can be further validated *in vivo*. Our results also showed that an increased CRS levels could reduce the MCP concentration, while increased the

number of total bacteria. Generally, MCP represents the quantity of nitrogen incorporated into the bacteria. In these fermentation systems, since the nitrogen source was the same, an increased CRS led to the relative decrease of available nitrogen. Although high CRS may stimulate the bacterial growth, the nitrogen utilization ability may be limited due to the relative limitation of nitrogen source.

Furthermore, CRS affects microbial quantities in the cecum and colon. *Bifidobacterium* spp. and *Lactobacillus* spp. are assumed to have beneficial effects on health. Several members of *Bifidobacterium* and *Lactobacillus* genus are commercially applied as probiotics (Mitsuoka, 1990; Vaughan and Mollet, 1999). In this study, the copies of total bacteria, *Bifidobacterium* and *Lactobacillus* were significantly increased by the increased CRS levels after incubation. It is in agreement with previously studies that addition RS increased amounts of *Lactobacillus* species and *Bifidobacterium* species in the cecal contents of rats (Sybille et al., 2013). Keenan et al. (2013) also found that RS from high amylose maize increases gut bacteria, such as lactic acid producing bacteria, *Lactobacillus* species, *Bifidobacterium* species and Clostridial cluster IV, in large intestinal of ovariectomized rats. In addition, we also found that copies of the *Bifidobacterium* and *Lactobacillus* in cecum were higher than those in colon. It suggested that addition CRS may be more suitable for *Bifidobacterium* and *Lactobacillus* bacteria growth in cecum than colon. The increase of the generally beneficial microbes by CRS *in vitro* further support its practical use in porcine production.

5. Conclusion

In conclusion, an increased addition of CRS may influence the protein fermentation *in vitro*, which was characterized as an increase in SCFA, a decreased in protein-fermenting products and *E. coli*, and an increase in *Bifidobacterium*. Protein-fermenting products decreased with the CRS addition both in the cecum and colon. These results provide essential evidence for the use of CRS to reduce ammonia production and ameliorate protein fermentation in pigs.

Conflict of interest

The authors declare no competing financial interest.

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