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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

Xiangyu He, Weiwei Sun, Ting Ge, Chunlong Mu, Weiyun Zhu*

Jiangsu Key Laboratory of Gastrointestinal Nutrition and Animal Health, Laboratory of Gastrointestinal Microbiology, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

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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

* Corresponding author.
E-mail address: zhuweiyun@njau.edu.cn (W. Zhu).

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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 lg 16S 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	

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