Nutrient Analysis of Large Intestinal Digesta and Effects of Different Protein Levels on In Vitro Fermentation Characteristics of Cecal Microbiota in Duroc × Landrace × Yorkshire Finishing Pigs: A Postprint

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Abstract

This experiment aimed to investigate the effects of different protein levels on cecal microbial fermentation characteristics in Duroc × Landrace × Yorkshire finishing pigs using an in vitro method. Digesta from different segments of the large intestine were first collected from 10 Duroc × Landrace × Yorkshire finishing pigs to determine their main nutrient contents, thereby establishing the ratio of carbohydrates to crude protein to be added; an in vitro fermentation experiment was then conducted using cecal digesta from 3 pigs as inoculum and casein hydrolysate as the fermentation substrate. The protein levels in the experimental groups were 1.00, 1.75, and 2.50 mg/mL, with 4 replicates per group, and fermentation was carried out at 37 °C for 24 h; gas production, pH, and contents of ammonia nitrogen, microbial protein, and short-chain fatty acids were measured. The results showed: 1) The nutrient contents in porcine cecal digesta varied within a certain range, with the carbohydrate to crude protein ratio ranging from 1.86 to 3.24 and averaging 2.66. 2) With increasing protein levels, gas production increased significantly ($P<0.05$), and the contents of ammonia nitrogen, total short-chain fatty acids, branched-chain fatty acids, acetate, butyrate, valerate, isobutyrate, and isovalerate increased significantly ($P<0.05$). Therefore, the carbohydrate to crude protein ratio in the porcine large intestine varied within a certain range, while the capacity of cecal microorganisms to ferment protein increased with rising substrate protein levels.

Full Text

Nutrient Analysis of Chyme in Large Intestine of Duroc×Landrace×Yorkshire Finishing Pigs and Effects of Different Protein Levels on in vitro Fermentation Characteristics of Pig Caecal Microbiota

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Abstract

This study was conducted to estimate the effects of different protein levels on caecal microbial fermentation characteristics of Duroc×Landrace×Yorkshire finishing pigs *in vitro*. We firstly analyzed the main nutrient content of chyme collected from different extents of the large intestine of 10 pigs to determine the appropriate carbohydrate-to-crude protein ratio for subsequent fermentation experiments. Subsequently, *in vitro* fermentation was performed using caecal chyme from 3 pigs as inoculum and casein hydrolysate as the fermentation substrate. The experimental groups, each with 4 replicates, were designed with protein levels of 1.00, 1.75, and 2.50 mg/mL and fermented at 37 °C for 24 h. Gas production, pH, and contents of ammoniacal nitrogen, microbial protein, and short-chain fatty acids (SCFA) were measured. The results showed that: (1) nutrient contents in pig caecal chyme varied within certain ranges, with the carbohydrate-to-crude protein ratio ranging from 1.86 to 3.24 (average 2.66); (2) with increasing protein levels, gas production increased significantly ($P<0.05$), as did the contents of ammoniacal nitrogen, total SCFA, branched-chain fatty acids, acetate, butyrate, valerate, isobutyrate, and isovalerate ($P<0.05$). These findings indicate that while the carbohydrate-to-crude protein ratio in the porcine large intestine varies within a specific range, the fermentative capacity of caecal microbiota for protein strengthens as substrate protein levels increase.

Key words: large intestine of pigs; protein level; caecal microbiota; fermentation characteristics

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Introduction

The gastrointestinal tracts of humans and animals harbor vast and diverse microbial communities that are intimately linked to host nutrition, metabolism, and health. Although the small intestine of monogastric animals exhibits high

microbial diversity, microbial populations in the large intestine far exceed those in the small intestine. In growing-finishing pigs, the caecum, colon, and rectum represent the primary sites of microbial colonization [1]. While most ingested nutrients are digested and absorbed in the small intestine of monogastric animals like pigs, a substantial portion reaches the hindgut where it becomes available for microbial fermentation [2]. Microbial fermentation of carbohydrates in the caecum and colon generally produces short-chain fatty acids (SCFA) beneficial to host health, whereas protein fermentation primarily generates potentially harmful substances such as ammonia, phenols, and indoles [3]. The fermentation rate and metabolic output in the porcine hindgut are predominantly influenced by the levels of protein and carbohydrate present in the digesta [4].

Although numerous studies have investigated the role of protein type and level entering the hindgut, the complex intestinal environment has left the precise effects of protein level on porcine hindgut fermentation patterns unclear [5]. Morita et al. [6] demonstrated in a mouse model that elevated protein levels promoted SCFA production, while Htoo et al. [7] reported that reducing dietary protein from 24% to 20% significantly decreased caecal SCFA concentrations. Conversely, Opapeju et al. [8] found that lowering protein levels did not affect SCFA content in the porcine hindgut. With the rapid expansion of China' s swine industry, environmental nitrogen pollution from livestock operations has become increasingly severe, with approximately 66% of nitrogen emissions originating from manure and urine [9]. Research has shown that reducing dietary protein while supplementing with crystalline amino acids can maintain growth performance while decreasing urinary nitrogen excretion [7]. Therefore, investigating the transformation mechanisms of protein in the porcine hindgut to improve feed protein utilization represents an effective strategy for promoting animal health and reducing nitrogen pollution. This study first determined the nutrient composition of digesta from different segments of the hindgut in normally fed Duroc×Landrace×Yorkshire finishing pigs, then examined the effects of varying protein levels on caecal microbial fermentation characteristics *in vitro* to provide insights into protein metabolism in the porcine hindgut.

1.1 Preparation of Substrate, Medium, and Inoculum

Substrate and medium: The protein substrate was casein hydrolysate (Beijing Solarbio Science & Technology Co., Ltd., C8210, total nitrogen content approximately 13.1%). The medium was prepared according to the method described by Dai et al. [10].

Inoculum preparation: Three healthy Duroc×Landrace×Yorkshire pigs weighing approximately 60 kg were selected and fed a basal diet (composition and nutrient levels shown in) with free access to water. After slaughter, the caecum was immediately isolated and transported to the laboratory. Equal amounts of fresh chyme were weighed, diluted 1:5 (w/v) with sterile phosphate

buffer (pH 7.4), shaken thoroughly, and filtered through four layers of sterile gauze. The filtrate was transferred to serum bottles, sealed, and placed in a 37 °C water bath for later use. All procedures were performed under continuous CO flushing to maintain anaerobic conditions.

1.2 Experimental Design

Chyme was initially collected from the caecum, proximal colon, and distal colon of 10 Duroc×Landrace×Yorkshire finishing pigs to determine moisture, crude protein, ether extract, and ash contents. Carbohydrate content (including crude fiber and nitrogen-free extract) was calculated using the formula: Carbohydrate (%) = 100 -[moisture (%) + crude protein (%) + ash (%) + ether extract (%)].

Based on these nutrient analyses, *in vitro* fermentation experiments were conducted with a negative control group (basal medium + inoculum) and experimental groups (basal medium + inoculum + substrate). The experimental groups were designed with protein levels of 1.00, 1.75, and 2.50 mg/mL, each with 4 replicates. Casein hydrolysate served as the sole nitrogen source in all experimental groups except the negative control.

1.3 Measurement of Fermentation Parameters

Gas production was measured according to Theodorou et al. [11]. SCFA content was determined using the method of Qin [12]. Microbial protein (MCP) content was measured following Makkar et al. [13]. Ammoniacal nitrogen content was determined according to Liang et al. [14].

1.4 Data Analysis

Experimental data were initially processed using Excel 2007, followed by one-way ANOVA using SPSS 17.0 statistical software. Data are presented as means ± standard deviation. Differences were considered significant at $P<0.05$ and highly significant at $P<0.01$.

2.1 Nutrient Composition of Porcine Hindgut Chyme

As shown in , nutrient contents in porcine hindgut chyme exhibited considerable variability. Crude protein content in caecal chyme ranged from 1.40% to 3.24% (mean 2.59%), while carbohydrate content ranged from 3.49% to 8.49% (mean 6.81%), yielding a carbohydrate-to-crude protein ratio of 1.86–3.24. Crude protein content was notably higher in the colon, with mean values of 4.05% and

4.96% in the proximal and distal colon, respectively. However, the carbohydrate-to-crude protein ratios in these segments were similar to those in the caecum, averaging 2.57 and 2.78, respectively. Based on these results, the carbohydrate-to-protein mass ratio (C/N) for *in vitro* fermentation was set at 1.60 (below 1.86) for the high protein level group, 4.00 (above 3.24) for the low protein level group, and 2.28 (within 1.86–3.24) for the medium protein level group.

2.2 Effects of Protein Level on *in vitro* Gas Production

As illustrated in [Figure 1: see original paper], gas production in all experimental groups increased gradually over time, with rapid fermentation occurring during the first 12 h. Gas production increased linearly (R^2=0.999) between 3 and 9 h, plateauing after 24 h. No significant differences in cumulative gas production were observed among the three groups at 3 and 6 h ($P>0.05$). At 9, 12, 18, and 24 h, cumulative gas production did not differ between low and medium protein level groups ($P>0.05$), but the high protein level group showed significantly greater gas production than the other two groups ($P<0.05$).

2.3 Effects of Protein Level on pH, Ammoniacal Nitrogen, and MCP Content

As presented in , ammoniacal nitrogen content increased highly significantly with rising protein levels ($P<0.01$), showing a linear relationship (R^2=0.998). MCP content also increased significantly with substrate protein level ($P<0.05$), with the high protein level group exhibiting significantly higher MCP than the low protein level group ($P<0.05$). Substrate protein level had no significant effect on pH ($P>0.05$).

2.4 Effects of Protein Level on *in vitro* SCFA Content

As shown in , total SCFA and acetate contents in the medium and high protein level groups were significantly higher than in the low protein level group ($P<0.05$). Butyrate, valerate, branched-chain fatty acids, isobutyrate, and isovalerate contents increased progressively with protein level, with significant differences among all groups ($P<0.05$). Protein level had no significant effect on propionate content ($P>0.05$).

3.1 Effects of Protein Level on Fermentation Characteristics

pH serves as a comprehensive indicator of substrate fermentation status. In this study, pH ranged from 6.80 to 6.83, which is suitable for porcine caecal

microbial growth. Gas production reflects microbial substrate utilization and fermentation characteristics, with the rate of gas production indicating microbial utilization speed. The slow gas production during the initial 3 h likely resulted from microbial adaptation to the substrate and the time required for degradation, which slowed microbial growth. The subsequent rapid increase in gas production indicated accelerated microbial growth, probably because both casein hydrolysate and glucose used in the fermentation were water-soluble and readily degradable. The increase in gas production with protein level suggests that elevated protein promotes microbial fermentation. Since gas originates from SCFA generation during substrate fermentation, both the rate and cumulative volume of gas production reflect overall microbial activity and SCFA production [15].

SCFA represent important end products of microbial fermentation in the hindgut of monogastric animals. While SCFA are primarily produced through carbohydrate fermentation, protein also serves as a significant fermentation substrate [16], with over 90% of SCFA being rapidly absorbed [17] to provide energy for intestinal epithelial cells and other tissues. The significantly lower SCFA content in the low protein level group observed in this study likely occurred because nitrogen became the limiting factor for microbial growth under conditions of adequate carbon availability. Morita et al. [6] found in mice that increasing dietary levels of digestion-resistant protein elevated total caecal SCFA content. Similarly, Getachew et al. [18] observed in *in vitro* studies that gas production and SCFA content increased with nitrogen level. These findings align with our results, demonstrating that nitrogen level is a crucial factor affecting microbial fermentation and that increasing nitrogen availability may enhance SCFA production. Liu et al. [19] reported that elevated dietary protein increased acetate content in caecal and colonic digesta, which our results corroborate. Acetate can serve as a precursor for lipid synthesis or as an energy source for muscle tissue and promotes gluconeogenesis, thereby providing energy for the animal. Branched-chain fatty acids are exclusively produced through fermentation of branched-chain amino acids [16] and can serve as markers of protein fermentation. The observed increase in isobutyrate and isovalerate with protein level indicates enhanced microbial protein utilization. Butyrate is the preferred energy source for intestinal epithelial cells, and Walker et al. [20] found in human fecal *in vitro* fermentations that increased protein levels elevated butyrate content, consistent with our findings. These results demonstrate that using peptides as a nitrogen source, increasing nitrogen levels under equivalent carbon conditions can significantly increase butyrate production.

3.2 Nitrogen Utilization Characteristics of Caecal Microbiota at Different Protein Levels

Nitrogen is an essential nutrient for microbial growth. The utilization of proteins and peptides by intestinal microbiota is a complex process, with most microbes preferentially using amino acids and ammonia as nitrogen sources, while some can grow using proteins or peptides [5]. Using casein hydrolysate as the nitrogen source in this study, ammoniacal nitrogen content reflected the combined capacity for protein degradation and ammonia utilization by microbes. Previous studies have shown that ammoniacal nitrogen content in intestinal digesta and feces increases with protein intake [3,21], which our results confirm, indicating that microbial protein degradation intensifies with rising protein levels. High ammonia concentrations in the animal gut can disrupt normal energy metabolism in intestinal epithelial cells, increase paracellular permeability, and impair mucosal barrier function [22], which is considered a primary cause of diarrhea in piglets fed high-protein diets.

Microbial growth activity in the rumen is influenced by multiple factors including fermentable carbohydrates, amino acids, nucleic acids, peptides, ammoniacal nitrogen, and minerals, with carbohydrates and nitrogen being the primary nutrients required. *In vitro* fermentation systems are similar to the rumen in this regard. Hristov et al. [23] found that *in vitro* rumen microbial protein synthesis rate was linearly and positively correlated with nitrogen level and protein degradation rate, consistent with our results. The increase in microbial protein content indicates that elevated protein levels promoted microbial growth and ammonia utilization, which benefits intestinal health while reducing nitrogen in manure and urine, thereby decreasing nitrogen pollution.

Conclusions

1. The carbohydrate-to-crude protein ratio in the hindgut of Duroc×Landrace×Yorkshire finishing pigs varies within a specific range.
2. Under *in vitro* conditions, increasing nitrogen levels can enhance SCFA production by caecal microbiota.
3. Microbial fermentation of protein intensifies with increasing substrate protein levels, though this also elevates ammoniacal nitrogen content.

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Note: Figure translations are in progress. See original paper for figures.