A Gradient Centrifugation Separation Method for Microorganisms from Porcine Gastrointestinal Contents and Feces (Postprint)

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Abstract

This study was designed to investigate a gradient separation method for microorganisms from pig gastrointestinal contents and feces. The experiment first diluted pig gastrointestinal contents and fecal samples with separation buffer to determine the optimal dilution ratio, followed by gradient centrifugation to obtain microbial cells from the gastrointestinal contents and fecal samples. Using this method, the optimal dilution ratio range for stomach, jejunum, and ileum content samples with separation buffer was determined to be 8.0%~12.0%, with sample weights of 3.2~4.8 g; for cecum, colon contents, and fecal samples with separation buffer, the optimal dilution ratio range was 2.0%~4.0%, with sample weights of 0.8~1.6 g. Microorganisms were separated from gastrointestinal contents and feces of 30 kg pigs fed three dietary protein levels (12.0%, 15.0%, and 18.0%), and the microbial amino acid composition in ileal contents and feces, as well as microbial diversity in gastrointestinal contents and feces, were analyzed. The results demonstrated that most amino acids in microbial cells from ileal contents and feces exhibited significant responses ($P<0.05$) to the nutritional patterns of different dietary protein levels, and PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) bands of microorganisms from gastrointestinal contents and fecal samples were abundant and distinct. These results indicate that this method features high separation efficiency, yields high microbial dry weight, and also has the characteristics of low cost, simple operation, and good reproducibility, providing a methodological approach for further investigation of the physiological functions of intestinal microorganisms.

Full Text

A Gradient Centrifugation Method for Separating Microorganisms from Porcine Gastrointestinal Contents and Feces

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Abstract

This study aimed to develop a gradient centrifugation method for separating microorganisms from porcine gastrointestinal contents and feces. The experimental procedure involved initial dilution of gastrointestinal and fecal samples with separation buffer to determine optimal dilution ratios, followed by gradient centrifugation to harvest microbial biomass. The optimal dilution ratio range for stomach, jejunum, and ileum contents was 8.0%–12.0% (sample weights of 3.2–4.8 g), while for cecum, colon contents, and feces, the optimal range was 2.0%–4.0% (sample weights of 0.8–1.6 g). The method was applied to 30 kg pigs fed diets containing three protein levels (12.0%, 15.0%, and 18.0%) to analyze microbial amino acid composition in ileal contents and feces, as well as microbial diversity throughout the gastrointestinal tract. Results demonstrated that most amino acids in microbial biomass from ileal contents and feces responded significantly to different dietary protein levels ($P<0.05$). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) produced rich, clear bands for microorganisms separated from gastrointestinal contents and feces. This method achieves high separation efficiency, yields substantial dry cell weight, and offers advantages of low cost, operational simplicity, and good reproducibility, providing a robust approach for in-depth investigation of intestinal microbial physiological functions.

Keywords: swine; gastrointestinal contents; feces; microorganism; gradient centrifugation

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The intestinal tract of animals harbors a vast and diverse microbial community, with populations reaching 10^{13}–10^{1} cells that constitute a complex ecosystem. These gut microorganisms play critical roles in host energy metabolism, nutrient absorption, and physiological and immune functions. With the rapid development of molecular biotechnology, numerous techniques have emerged for studying gut microbiota, including fingerprinting technologies such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), fluorescence in situ hybridization, and real-time quantitative PCR. However, efficient microbial separation from animal gastrointestinal contents and feces remains a prerequisite for these analytical techniques, yet no standardized protocols currently exist for microbial isolation from gastrointestinal contents and feces. Therefore, this study aimed to determine optimal ratios of gastrointestinal and fecal materials to separation buffer and establish a gradient centrifugation method for microbial separation from porcine gastrointestinal contents and feces, thereby providing a methodological foundation for further research on microbial nitrogen metabolism, endogenous nitrogen and amino acid composition, microbial flora, and fecal nitrogen and amino acid excretion.

1.1 Sample Collection

Eighteen healthy 30 kg Duroc × Landrace × Yorkshire growing pigs were randomly allocated to three groups (n=6). Each group received a corn-soybean meal diet containing one of three protein levels (12.0%, 15.0%, or 18.0%) for 30 days. Pigs were housed individually with ad libitum access to feed and water. At the conclusion of the trial, all six pigs per group were slaughtered, and approximately 50 g each of stomach, mid-jejunum, mid-ileum, cecum, and mid-colon contents were collected aseptically under anaerobic conditions. Anal feces were also collected from each group for microbial isolation.

1.2 Buffer Preparation

Separation buffer was prepared by dissolving 0.85 g NaCl in 1000 mL double-distilled water, adding 1 mL Tween-80 after complete dissolution, and autoclaving at 121°C for 20 minutes.

1.3 Separation Procedure

The microbial separation workflow is illustrated in [Figure 1: see original paper]. The detailed protocol was as follows: (1) Following slaughter of a 30 kg pig, gastrointestinal contents and feces were collected aseptically and stored at 4°C until processing. (2) Pre-weighed samples (within the specified weight range) of stomach, jejunum, ileum, cecum, colon contents, or feces were placed in sterile 50 mL centrifuge tubes (Tube A) and centrifuged at 4°C, 10,000 r/min for 5 minutes, after which the supernatant was discarded. (3) Forty milliliters of separation buffer were added to Tube A, vortexed vigorously for 2–3 minutes, and centrifuged at 4°C, 1,500 r/min for 5 minutes. (4) Tube A was carefully removed without vigorous shaking, and the supernatant was transferred to a corresponding sterile 50 mL centrifuge tube (Tube B). Tube B was then centrifuged at 4°C, 10,000 r/min for 5 minutes, and the supernatant was discarded. (5) Forty milliliters of separation buffer were added to Tube A, vortexed vigorously for 2–3 minutes to completely resuspend the pellet, and centrifuged at 4°C, 1,500 r/min for 5 minutes. The supernatant was transferred to Tube B, which was centrifuged at 4°C, 10,000 r/min for 5 minutes, and the supernatant was discarded. (6) Steps 3 and 5 were each repeated once. (7) After final supernatant removal, the pellet was transferred to a sterile 2 mL centrifuge tube and stored at -70°C pending analysis.

1.4 Amino Acid Analysis of Microbial Biomass

Microbial pellets from feces and ileal contents (0.8 mL) were measured into 15 mm × 150 mm test tubes, and 0.8 mL of 6 M HCl was added. After mixing, the tube neck was narrowed to 4–6 mm using an alcohol lamp, vacuum-extracted for 10 minutes, and sealed. Samples were hydrolyzed in a sand bath at 110±10°C for 22 hours, cooled to room temperature, and centrifuged. One milliliter of filtrate was evaporated to dryness in a 50 mL beaker at 60°C, reconstituted with 1 mL of 0.02 M HCl, filtered through a 0.22 m membrane, and analyzed using an automatic amino acid analyzer.

1.5 PCR-DGGE Analysis

Microbial DNA was immediately extracted from separated biomass obtained at different dilution ratios. The V3 variable region of bacterial 16S rRNA was amplified using primers 357F-GC-clamp, 357F, and 518R. The GC clamp sequence was 5' -CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3' , the 357F primer sequence was 5' -CCT ACG GGA GGC AGC AG-3' , and the 518R primer sequence was 5' -ATT ACC GCG GCT GCT GG-3' . Electrophoresis was performed using a D-code universal DGGE system (Bio-Rad) with 1×TAE buffer (pH 8.0). After sample loading, the electrophoresis temperature was raised to 60°C and run at 100 V for 19 hours. Gels were silver-stained, photographed, and images were saved.

1.6 Statistical Analysis

Data were processed using Excel 2007 and analyzed by one-way ANOVA using SAS 9.1.3 software. Duncan' s multiple comparison test was used for inter-group significance analysis. Results are expressed as means with standard error of the mean (SEM).

2.1 Dilution Ratios, Sample Weights, Separation Efficiency, and Dry Cell Weight Content

The dilution ratios, sample weights, separation efficiency, and dry cell weight content for different intestinal contents and fecal samples are presented in , , and . For stomach, jejunum, and ileum contents, separation efficiency and dry cell weight content increased as sample weights decreased from 5.6 g to 3.2 g and dilution ratios decreased from 14% to 8%. Stomach contents showed consistently high separation efficiency (>99%) across all conditions, with dry cell weight content exceeding 1.63 g/kg. For jejunum and ileum contents at dilution ratios 12%, separation efficiency reached 98.0%–99.9%, with dry cell weight contents of 2.00 g/kg and 2.50 g/kg, respectively. For cecum, colon contents, and feces, decreasing dilution ratios from 8% to 2% and sample weights from 3.2 g to 0.8 g progressively increased separation efficiency and dry cell weight content. At dilution ratios 6%, cecal microbial separation efficiency exceeded 97% with dry cell weight content 7.04 g/kg; colonic microbial separation efficiency exceeded

95% with dry cell weight content 15.29 g/kg; and fecal microbial separation efficiency exceeded 98% with dry cell weight content 20.32 g/kg.

2.2 Amino Acid Composition of Microbial Biomass from Ileal Contents and Feces

The amino acid composition of microbial biomass from ileal contents and feces is shown in and . Microbial biomass contained 17 amino acids. In fecal microbes, aspartic acid (Asp), serine (Ser), alanine (Ala), proline (Pro), cysteine (Cys), valine (Val), isoleucine (Ile), leucine (Leu), lysine (Lys), histidine (His), and arginine (Arg) showed significant responses to different dietary protein levels ($P<0.05$), while glutamic acid (Glu), glycine (Gly), tyrosine (Tyr), threonine (Thr), methionine (Met), and phenylalanine (Phe) did not ($P>0.05$). In ileal microbes, Asp, Ser, Glu, Gly, Ala, Pro, Thr, Cys, Val, Met, Ile, Leu, Phe, His, and Arg responded significantly to dietary protein levels ($P<0.05$), whereas only Tyr and Lys showed no significant response ($P>0.05$).

2.3 PCR-DGGE Results at Different Dilution Ratios

PCR-DGGE profiles of microbial communities from stomach, jejunum, ileum, cecum, colon contents, and feces at various dilution ratios are shown in [Figure 2: see original paper]. Microbial genomic DNA was extracted from separated biomass and amplified with bacterial universal primers for PCR-DGGE analysis. Clear, rich DGGE bands were detected for all sample types across different dilution ratios, demonstrating good reproducibility.

3 Discussion

Conventional methods for isolating and culturing intestinal microbes rely on various media or broths followed by biochemical purification for analysis of microbial composition, abundance, and characteristics. However, these approaches have significant limitations: many intestinal microbes are difficult to culture, most anaerobic microorganisms remain unidentified and undescribed, and in vitro cultured microbes exhibit physiological differences from their in vivo counterparts. Additionally, the cumbersome operational procedures prevent comprehensive acquisition of gastrointestinal microbiota information. Some researchers have established regression equations between optical density (OD) values and dry cell weight by measuring OD of different bacterial dilutions and correlating them with plate counts and dry weight measurements, enabling real-time determination of dry cell weight. Although this method improves accuracy and efficiency compared with traditional plate counting or turbidimetry, the vast diversity of microbes in gastrointestinal contents and feces and their varying culture requirements limit its application. This study employed a gradient centrifugation method to separate microorganisms from porcine gastrointestinal contents and feces. At dilution ratios 12%, separation efficiency for stomach, jejunum, and ileum contents exceeded 98%; at dilution ratios 4%, separation efficiency for cecum, colon contents, and feces also exceeded 98%. Under appropriate dilution ratios, satisfactory dry cell weights were obtained from all sample types. DGGE analysis of separated microbes from all gastrointestinal segments and feces yielded clear, rich band patterns. Furthermore, using Tween-saline buffer for sample pretreatment followed by gradient centrifugation effectively reduced chemical reagent usage. Prior separation of microbes from contents or feces not only eliminates interference from undigested food residues, digestive enzymes, mucus, and polysaccharides (particularly in herbivores) during microbial cell wall disruption and high-quality DNA extraction, but also minimizes residual chemicals in extracted DNA that can result from excessive reagent use.

This study also analyzed amino acid composition of ileal and fecal microbes from pigs fed three protein levels. Microbial biomass contained 17 amino acids, with differential responses to dietary protein levels between fecal and ileal microbes. In fecal microbes, Asp, Ser, Ala, Pro, Cys, Val, Ile, Leu, Lys, His, and Arg showed more pronounced responses to dietary protein levels, while ileal microbes showed significant responses in Asp, Ser, Glu, Gly, Ala, Pro, Thr, Cys, Val, Met, Ile, Leu, Phe, His, and Arg. Additionally, this separation method can be applied to determine intracellular enzyme activities and decarboxylase activities of porcine intestinal microbes. These findings demonstrate that microbes isolated using this method are suitable for multiple analytical applications.

4 Conclusions

1. The gradient centrifugation method established in this study for separating microorganisms from porcine gastrointestinal contents and feces achieves high separation efficiency, yields substantial dry cell weight, and offers advantages of low cost, good reproducibility, and operational simplicity.
2. This method provides an effective and efficient approach for further investigation of gastrointestinal microbial functions.

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