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Changes in bacterial communities from swine feces during continuous culture with starch☆

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ABSTRACT

Bacteria from swine feces were grown in continuous culture with starch as the sole carbohydrate in order to monitor changes during fermentation and to determine how similar fermenter communities were to each other. DNA extracted from fermenter samples was analyzed by denaturing gradient gel electrophoresis (DGGE). A significant decrease in diversity was observed, the Shannon–Weaver index dropped from 1.92 to 1.13 after 14 days of fermentation. Likewise, similarity of fermenter communities to those in the fecal inoculum also decreased over time. Both diversity and similarity to the inoculum decreased most rapidly in the first few days of fermentation, reflecting a period of adaptation. Sequencing of DGGE bands indicated that the same species were present in replicate fermenters. Most of these bacteria were placed in the *Clostridium coccoides*/*Eubacterium rectale* group (likely saccharolytic butyrate producers), a dominant bacterial group in the intestinal tract of pigs. DGGE proved useful to monitor swine fecal communities *in vitro* and indicated the selection and maintenance of native swine intestinal bacteria during continuous culture.

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1. Introduction

Continuous culture is a useful tool for the study of microbial populations from the gastrointestinal tract of humans [1–3] and animals ([4,5] pigs, [6] rumen). In humans and monogastric animals continuous culture systems generally aim to model the large intestine. *In vitro* fermentations of mixed bacterial populations are meant to model natural ecosystems yet are inherently different from the gastrointestinal environment. For example, absorption of fermentation products such as volatile fatty acids and secretion of antibodies triggered by the presence of immunogenic microorganisms in the gut can contribute to these differences. Instances of microbial population changes during adaptation to *in vitro* environments have been noted in continuous culture systems. Selection of bacteria over archaea and eukarya as well as shifts in populations of Methanobacteriaceae [7] and Fibrobacter [8] have been observed in continuous culture models of the rumen. An indication of how well an *in vitro* bacterial community matches the community in the ecosystem it is meant to model would be a useful descriptor of fermenter performance.

Denaturing gradient gel electrophoresis (DGGE) has been used to investigate populations of bacteria in human [9–11] and porcine [12–14] feces but has not been widely applied to the examination of continuous culture models of fecal communities. DGGE can be used to survey an entire community while still providing information regarding population changes of individual species. A five stage human intestinal continuous culture model system [15] used DGGE to demonstrate changes in microbial community structure among vessels representing the ascending, transverse, and descending colon over 27 days of operation. In their system, up to 15 days were required to reach microbial population stability in the last three vessels as measured by DGGE banding patterns. Zhu et al. [14] used DGGE with *in vitro* batch culture to evaluate changes in microbial community structure when pig feces were incubated with or without sugar beet pulp. DGGE profiles tended to cluster together based on whether fecal samples had been incubated with sugar beet pulp, although similarities across samples were less than 60% [14]. In this study replicate fermenters inoculated with mixed swine fecal bacteria were maintained, and community development monitored using DGGE, in order to measure how well continuous culture supported natural fecal communities and to determine whether pooled fecal inoculum resulted in greater similarity amongst bacteria in the fermenters.

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2. Materials and methods

2.1. Feces collection and fermentation

Freshly voided fecal samples from 10 pigs (100–125 kg females) were placed into Whirl-pak bags (Fisher Scientific, Hanover Park, IL), kept at 37 °C and transported to the lab within 60 min. A composite was mixed in an anaerobic chamber (Coy, Grass Lake, MI) using 15 g of each individual fecal sample. An inoculum slurry was made by blending the composite sample with anaerobic phosphate buffered saline (all microbiological media were obtained from Fisher Scientific, Pittsburgh, PA), 1:10 ratio (w/v), in a Waring blender (Torrington, CT).

BioFlo 110 fermenters (New Brunswick Scientific, Edison, NJ), with 300 ml anaerobic nutrient medium with starch as the sole carbohydrate (Table 1) previously added, received 375 ml of inoculum slurry. Starch was selected as the carbohydrate source because it is the primary carbohydrate in corn-soybean meal based diets and it is soluble in the nutrient medium. Fermenters were then allowed to reach a working volume of 700 ml by addition of nutrient medium at a dilution rate of 0.03 h^{-1}. The operating conditions of the fermenters were set to model conditions in the large intestine (cecum and colon) of the pig. Contents of the vessels were continuously mixed and sparged with nitrogen to maintain anaerobic conditions. Working volume was maintained by removal of contents with an outflow pump. Throughout the 14 days of operation, a temperature of 37 °C and pH of 6 were maintained. The experiment was replicated three times (A–C) using four fermenters (1–4).

2.2. Bacterial counts

Daily samples were taken from each fermenter at 24 h intervals. Samples were serially diluted (1 in 10) in half-strength peptone water to 10^{-8} dilution in the anaerobic chamber. Quadrants on agar plates (Wilkins–Chalgren agar for total anaerobes and nutrient agar for total aerobes) were spread with 20 μl of appropriate dilutions (10^{-10}–10^{-7} for total anaerobes and 10^{-7}–10^{-4} for total aerobes), in duplicate [16,17]. Colonies on plates were counted after incubation at 37 °C in the appropriate atmosphere, 24 h for total aerobes and 72 h for total anaerobes.

2.3. PCR, DGGE and DNA sequencing

Fermenters were sampled on days 0, 3, 6, 9, 12, 13 and 14 and samples stored at −20 °C prior to DNA extraction. DNA from fecal and fermenter samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, using 95 °C for the initial lysis step [18]. Extracted DNA was amplified by PCR; 100 μl reactions contained 1× Qiagen PCR buffer, 1.25 U of *Taq* polymerase (Qiagen), 0.25 mM of each dNTP (Amresco, Solon, OH), 25 pmol of each primer and 80 ng of template DNA. The primers, targeted to the V6–V8 regions of 16S rDNA, were F-968-GC (5′ **CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AA CGC GAA GAA CCT TAC) (Invitrogen, Carlsbad, CA) and R-1401 (5′ CGG TGT GTA CAA GAC CC) (Integrated DNA Technologies, Coralville, IA) [19]. A Robocycler model 96 Gradient Cycler (Stratagene, La Jolla, CA) was used for amplification using the following conditions: 5 min at 94 °C, 35 cycles consisting of 94 °C for 1 min, 56 °C for 1 min, 68 °C for 1 min with a final elongation step of 7 min at 68 °C. Prior to running on DGGE gels, PCR products were cleaned using QIAquick PCR Purification Kits (Qiagen).

A model 475 Gradient Delivery System (BioRad, Hercules, CA) was used to form gels with 35–50% urea–formamide denaturing gradients (100% denaturant is defined as 7 M urea and 40% formamide). Gels were 6% polyacrylamide (37.5:1 acrylamide–bisacrylamide) in 0.5× Tris-acetate-EDTA (TAE) buffer (pH 8.0). Gels were allowed to polymerize 4 h before use. Denaturing gradient gel electrophoresis was performed using the BioRad DCode™ Universal Mutation Detection System (BioRad) with 10-μl aliquots of PCR products (plus 10 μl loading buffer) electrophoresed at 85 V for 16 h in 0.5× TAE buffer at a constant temperature of 60 °C. Gels were stained with SYBR Gold (Invitrogen) for 40 min according to the manufacturer's instructions and photographed with a FluorS Imager (BioRad).

Samples from pairs of replicate fermenters, within a repetition of the experiment, were electrophoresed on the same gels. Pairings were: F1 with F2, F2 with F3, F3 with F4 and F4 with F1, for each repetition (A–C) for a total of 12 gels. Quantity One (Discovery Series, BioRad) software was used for band analysis. Band intensities were determined after background subtraction. Dice's coefficients [20], with weighting based on band intensities, were calculated and a similarity matrix made for each of the 12 gels. Dice's similarity coefficients for each matrix position were combined to get an averaged, overall matrix.

Ecological diversity indices were used to describe fermenter community similarity to the initial inoculum, within a repetition. The Shannon–Weaver index (H') [21] was calculated using the formula $H' = -\sum p_i \ln(p_i)$, where p_i is the band intensity of the ith band divided by the sum of all band intensities in a DGGE lane. Species evenness (E) was calculated as $E = H'/\ln S$. The number of bands detected in a DGGE lane was used as a measure of the number of species present (species richness, S) and band intensity was used as an estimate of the relative population size of each species. Changes in species diversity over time in the fermenters were evaluated using the two-tailed t-test [21] (SAS, version 8.2, SAS Institute, Cary, NC); differences were considered significant at $P \leq 0.05$.

In order to identify the species represented by DGGE analysis, DNA from bands was sequenced. Bands were selected to represent the range of migration patterns on the gels and several bands that migrated to the same position in different lanes were checked for similarity. Prior to sequencing, DGGE bands were excised on a UV light table and stored in 50 μl of water at 4 °C for 24 h in order to diffuse DNA from the gel into the water. Subsequently, 5 μl was used to re-amplify the DNA using the same primers and reaction conditions as previously described except that the forward primer without the GC clamp was used. PCR products were cleaned as previously described and sent to the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA). There the DNA (approximately 400 bp) was sequenced using ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA).

Table 1
Composition of anaerobic nutrient medium for continuous culture of swine feces.

Component	Amount/L
Peptone water	2.0 g
Yeast extract	2.0 g
Bile salts	0.5 g
Corn starch	10.0 g
Hemin[a]	0.05 g
Tween 80	2.0 ml
Vitamin K_1	10.0 μl
Cysteine·HCl	0.5 g
$NaHCO_3$	2.0 g
NaCl	0.1 g
K_2HPO_4	0.04 g
KH_2PO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.01 g
$CaCl_2 \cdot 6H_2O$	0.01 g

[a] Hemin was dissolved in 1 M NaOH prior to addition.