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Original Research

Sample-Handling Factors Affecting the Enumeration of Lactobacilli and Cellulolytic Bacteria in Equine Feces

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The objectives were to compare media types and evaluate the effects of fecal storage time and temperature on the enumeration of cellulolytic bacteria and lactobacilli from horses. Fecal samples were collected from horses (n = 3) and transported to the laboratory (100% CO_2, 37°C, 0.5 hours). The samples were assigned to one of four storage temperatures: initial (no storage), 37°C, room temperature (RT; 22°C–24°C), or 4°C. The initial samples were enumerated five times to assess repeatability. Feces stored at each temperature were used to enumerate cellulolytics (rich or defined media) and lactobacilli (Rogosa selective lactobacilli [SL] or MRS agar) over a 24-hour time course. All media were repeatable within media type. The cellulolytic media types produced similar results. After 2 hours, the cellulolytics lost >99% viability in RT and 4°C and 90% viability in 37°C ($P < .05$). By 24 hours of storage, 10^5-fold to 10^6-fold fewer cellulolytics were observed in 37°C and RT ($P < .05$). There were no detectable cellulolytics in 4°C at 24 hours. Viable counts on MRS were not reported due to insufficient selectivity. There were fewer lactobacilli in RT and 4°C after 2 hours ($P < .05$). There was no change in lactobacilli from 2 to 4 hours at 37°C storage, but there was an increase in lactobacilli after 8 hours ($P < .05$). These results demonstrate that storage time and temperature are important to consider when enumerating lactobacilli and cellulolytics from feces.

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1. Introduction

To gain a better understanding of microbial fermentation and functionality in the equine hindgut, microbial identification and quantification procedures have become increasingly important in equine nutrition research. Both culture-based and molecular methods (e.g., next generation sequencing, denaturing gradient gel electrophoresis, and real-time polymerase chain reaction) have been applied in human and animal research to better characterize complex microbial communities [1]. Enrichment culture is used to multiply microorganisms by allowing them to reproduce in culture media under controlled laboratory conditions [2]. Culture is one of the primary diagnostic methods for many infectious diseases and is necessary to identify bacterial species. When culture is used in combination with serial dilution, the number of particular bacteria in a sample can be determined. There are many protocols for the enumeration of bacteria which can vary based on selective and differential media type used and sample-handling methodologies. Differences in these media components as well as sample handling and culture conditions can change the type and number of bacteria that grow [2].

Enumeration and culture of pathogens is well established in veterinary medicine, but less is known about the

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factors affecting enumeration of normal flora. Cellulolytic bacteria (a guild or functional group) and lactobacilli (a taxonomic group) are both important mutualists in the equine hindgut. These bacteria are frequently enumerated in equine research studies as indicators of the health or stability of the hindgut microbial community [3–7]. The objectives of the present study were to: (1) compare media types used for the enumeration of lactobacilli and cellulolytic bacteria; (2) determine the repeatability of enumerations; and (3) evaluate the effects of fecal storage time and temperature on cellulolytic and lactobacilli enumerations.

2. Material and Methods

2.1. Media Composition

Lactobacilli were enumerated aerobically on two different commercially available media: Rogosa SL agar and Lactobacilli MRS agar (Becton Dickinson and Company; Franklin Lake, NJ). Both are based on the work of Rogosa et al [8,9]. Rogosa SL agar uses acetic acid at low pH as a selective agent. Both media types were prepared as per the manufacturer's directions.

Cellulolytic bacteria were enumerated in two different media types: a rich cellulolytic media (CRM) and a defined cellulolytic media (DCM) (Table S1). The CRM was an anaerobic broth that has been previously used for enumeration of cellulolytic bacteria from horse feces [6]. The composition was based on Julliand et al [10]. However, the cecal fluid was replaced with a fecal fluid addition. The fecal fluid addition was prepared by mixing (1 minute) equine feces with deionized water (1:1 m/m) in a blender. The solids were then removed by centrifugation, and the supernatant was sparged with CO_2 and sterilized before addition. The DCM was a buffered anaerobic liquid media that was based on Stack et al [11]. The growth substrate in both media types was cellulose strips (Whatman #1 filter paper, Whatman; Tonglu, China).

2.2. Animals, Fecal Collection, and Bacteria Enumeration

Horses were selected from the University of Kentucky, Department of Animal and Food Sciences herd at Maine Chance Farm, Lexington, Kentucky. The University of Kentucky Institutional Animal Care and Use Committee approved all animal procedures. The feces donors were three mature Thoroughbred mares maintained on pasture. Replicate experiments ($n = 3$) were performed on three separate days using the feces from one horse on each day.

Feces were collected by catch sampling in a plastic bag before contact with the ground. Each sample was thoroughly mixed by hand. The fresh samples ($\sim$1 g tube^{-1}) were placed in 16 preweighed, sterile Hungate tubes. The stoppers were replaced, and the tubes were purged of air with CO_2 via a tuberculin needle. The Hungate tubes were maintained at 37°C and transported to the laboratory within 0.5 hours of collection. On arrival at the laboratory, the fecal samples were assigned to one of four treatments including: control (initial; $n = 1$), 37°C storage ($n = 5$), room temperature (RT; 22°C–24°C) storage ($n = 5$), or 4°C storage ($n = 5$). The initial sample was enumerated immediately on arrival at the laboratory. A sample from each storage temperature was enumerated after 2, 4, 8, 12, and 24 hours of incubation. At each sampling time, the designated fecal samples were weighed, suspended in sterile, anaerobic phosphate-buffered saline (PBS; 1:10 wt/wt), and mixed with a vortex until the suspension was homogenous (1 minute). The fecal suspensions were then serially diluted (10-fold wt/wt, PBS) in an anaerobic chamber (Coy, Grass Lake, MI; 95% CO_2, 5% H_2). The initial sample was used to inoculate five replicates of CRM, DCM, Lactobacilli MRS agar, and Rogosa SL agar to assess repeatability within each media type. At all other sampling points, each individual dilution series was used to inoculate one set of each media type. Liquid cellulolytic media were inoculated (1 mL) with a tuberculin syringe, and incubations were carried out at 37°C for 10 days. Growth was evaluated daily for 10 days by dissolution of cellulose and microscopy. The final dilution exhibiting dissolution of cellulose by the 10th day was recorded as the viable number. In addition, the five replicate initial tubes per dilution were used to determine the most probable number of cellulolytic bacteria using the tables of McCrady [12]. The solid lactobacilli media were inoculated (0.2 mL) with a sterile spreader. Plates with 30 to 300 colonies were counted after aerobic incubation (37°C, 3 days).

2.3. Statistical Analyses

Repeatability experiment enumeration results are presented as true means ± the standard error. To determine the effects of storage time and temperature on bacterial enumeration, enumeration data were first normalized by $\log_{10}$ transformations before statistical analyses. The data were analyzed as a repeated measures design using the MIXED procedure of SAS (version 9.3, SAS Inst. Inc; Cary, NY). To determine the effects of storage temperature and storage time within media type, the model included storage temperature, storage time, and the interaction between these variables (storage temperature × storage time) as fixed effects, whereas the horse was included as a random effect. When there was a significant storage temperature × storage time interaction, means were separated using the Fisher's protected least significant difference test with significance set at $P < .05$. To determine the effects of media type (CRM vs. DCM) within storage temperature, the model included media type, storage time, and the interaction between these variables (media type × storage time) as fixed effects, whereas the horse was included as a random effect. When there was a significant media type × storage time interaction, means were separated using the Fisher's protected least significant difference test with significance set at $P < .05$.

3. Results

3.1. Repeatability and Most Probable Number

Repeatability was determined by diluting a single sample (1 g) from three horses and performing five enrichments on each media type per sample. When the CRM and DCM were used to enumerate a single diluted sample five times, the results were highly repeatable (Table 1). In

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