The Use Of Fecal Inoculum To Determine The Rate And Extent Of *In Vitro* Fermentation For Cellulose, Beet Pulp, Citrus Pulp, And Citrus Pectin Across Three Lemur Species: *Varecia variegata*, *Eulemur fulvus*, And *Hapalemur griseus*

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In order to estimate fermentative capacity among lemur species, four fiber substrates were tested across three herbivorous species: *Eulemur fulvus, Hapalemur griseus*, and *Varecia variegata.* Substrates were cellulose (CE), beet pulp (BP), citrus pulp (CP), and citrus pectin (PE), which ranged in composition from completely insoluble fiber (CE) to completely soluble fiber (PE), respectively. Animals were offered the same diet for a thirty day adaptation period which consisted of 85% of the diet dry matter Mazuri Leafeater Mini Biscuit (#5672, Purina Mills Inc., St. Louis MO) and 15% of the diet dry matter a mixture of locally available produce. Following the adaptation period, feces collected from donor animals were used to inoculate fermentation tubes pre-filled with the fiber substrates and an anaerobic growth medium. Dry matter disappearance (DMD) and total short-chain fatty acid production were measured in tubes subjected to 6, 12, 24, or 48 h of fermentation. Production plateaus were not reached by 48 h for BP and CP substrates. The DMD of PE was higher at 48 h than other substrates ($p<.0001$). For all substrates, inoculum from *V. variegata* produced lower DMD values at 48 h than *E. fulvus* or *H. griseus*. *V. variegata* produced more total short chain fatty acids ($p<.005$) from PE at 6 and 12 h and from CP at 6 h when compared to the other species. Results provide some evidence for differences in fermentative capacity across the three species, and suggest that fiber solubility and fermentability should be addressed in assessing their nutritional management needs.

Key words: non-human primates; short-chain fatty acid production; *in vitro* fermentation systems; fiber

INTRODUCTION

Due to differences in cell wall makeup across types of plant tissue, foods available to free-ranging herbivores can vary in the type, amount, and variety of fiber. Most mammals whose diet is mainly leaf matter will possess adaptations that slow digesta passage and allow time for microbial processing of insoluble fiber, while species that are not reliant upon insoluble fiber as an energy source, for example some frugivores, may simply reap some benefit from breakdown of soluble fibers, even if passage of food is rapid. Animals that consume a generalist diet are likely to be capable of subsisting on a low-fiber diet but will possess sufficient modifications for some slowing of digesta passage to allow for microbial breakdown of insoluble fiber when advantageous.

Extensive research characterizing fiber utilization has been conducted on ruminants, pigs, dogs, cats, and horses [Van Soest, 1994; Sunvold et al., 1995a; Sunvold et al., 1995b; Sunvold et al., 1995c; Varel and Yen, 1997]. Due to the possible health benefits of fiber, fermentability of various fiber sources in the human diet has also been examined, however little work has been conducted with non-human primates [Titgemeyer et al., 1991; Bourquin et al., 1992, Bourquin et al., 1993].

Within the lemurs, primates that possess a ceco-colic fermentation chamber, species exhibit a variety of herbivorous feeding patterns that could result in less or more dependence upon microbial fermentation in the cecum and proximal colon. This project sought to estimate the fermentative capacity across three lemur species with differing dietary profiles, *Varecia variegata*, *Eulemur fulvus*, and *Hapalemur griseus*, through comparison of rate and extent of fermentation by resident microflora.

MATERIALS AND METHODS

Four fiber substrates, cellulose (CE), beet pulp (BP), citrus pulp (CP), and citrus pectin (PE), were tested on three lemur species (*H. griseus, V. variegata*, and *E. fulvus*). Substrates chosen differed in their relative amounts of insoluble and soluble fiber. The dry matter portion of CE was 95.2 $\pm$ 0.2% total dietary fiber (TDF) and 92.0 $\pm$ .04% insoluble fiber (IF) and PE was 72.9 $\pm$ 0.8% TDF and 6.3 $\pm$ .002% IF. The dry matter portion of the mixed substrate BP was 81.9 $\pm$ .014% TDF and 68.9 $\pm$ 0.2% IF and CP was 85.5 $\pm$ .01% TDF and 41.99 $\pm$ .001% IF.

After an initial diet transition period, all animals were fed a diet consisting of Mazuri Leafeater Mini Biscuit (#5672, Purina Mills, St. Louis, MO) at 85% of the diet dry matter and locally available produce at 15% of dry matter for 30 days before the collection period began. Feces, collected from four donor animals per species, were then collected twice weekly over a three-week period and used to inoculate fermentation tubes filled with one of each of the four fiber substrates. Samples were collected only once from each animal. Fresh samples were collected in the early morning, immediately sealed in a waterproof plastic bag,

and placed in a water-filled (37°C) thermos for transport to the laboratory. At the laboratory, samples were diluted 1:10 in an anaerobic dilution solution and blended for 15 seconds. The solution was filtered through four layers of cheesecloth and the filtrate was kept sealed under CO_2 until 1-mL aliquots were used to inoculate 50-mL centrifuge tubes pre-filled with 310 mg of fiber substrate and 30-mL of anaerobic growth medium [Sunvold et al., 1995a]. The tubes, run in triplicate, were flushed with CO_2, capped with one-way release valve stoppers, and subjected to a 6, 12, 24, or 48 hour fermentation period at 37°C.

At the end of each time period, the designated tubes were removed from the incubator and the pH was taken. Four millilieter portions were then removed and prepared for short chain fatty acid (SCFA) analysis. The remaining solution was used to determine dry matter disappearance (DMD). First 100 mL of 95% ethanol was added and the solution was allowed to precipitate for one hour. Samples were then filtered through ash-free filter paper under a mild vacuum and rinsed sequentially with 78% ethanol, 95% ethanol, and acetone. After samples had been dried in a forced air oven at 105°C for 6 h, they were weighed and DMD was calculated.

Data were analyzed as a repeated measures design using the MIXED model procedure of SAS (SAS Statistical Software, Cary, NC). Species, time, substrate, and all interactions were tested. Day and day x species x substrate interaction were used as random effects in the model. Time was used as the repeated statement. Least Significant Differences was used to determine differences between individual means.

RESULTS

All animals remained healthy, maintained consistent daily dietary intakes, and produced normal feces throughout the experiment. For DMD, species x time ($P < .025$) and substrate x time ($P < .0001$) interactions were significant. At the 48 hour time period, the DMD from all fermentation tubes inoculated by *V. variegata* (50 $\pm$ 2.2%, mean $\pm$ SEM) was less than for those inoculated by *E. fulvus* (57.18 $\pm$ 2.2%). The DMD from fermentation tubes inoculated by *H. griseus* (54.94 $\pm$ 2.1%) did not differ from the other two species. The DMD of PE was greatest at 48 h (85.03 $\pm$ 2.1%), followed by BP (65.5 $\pm$ 2.1%) and CP (59.10 $\pm$ 2.1%), while CE disappearance was much less (7.6 $\pm$ 2.1%). Both CP and BP substrates had not reached a plateau by 48 h, while PE had reached a plateau by 24 h of fermentation.

For total SCFA production, the species x substrate x time interaction was significant ($P < .005$). No species differences were observed for CE or BP substrate tubes, but for CP, fecal inoculum from *V. variegata* produced more total SCFA at the 6-hour time period than did the other two species (see Table 1).

Varecia variegata also produced higher amounts of total SCFA at the 6 and 12 hour time periods for PE than either *E. fulvus* or *H. griseus*. These early differences for PE and CP were not evident at the 24 and 48-hour times, and total amounts of SCFA produced by 48 h were similar across the three species.

The pH of the medium following fermentation did not differ across species, however there was a significant substrate x time interaction (Table 2; $P < .0001$). For PE, CP and BP, pH values dropped most between the 6 and 12-hour times. At the 24 and 48-hour time periods, pH values were lowest for PE, intermediate for BP and CP, and highest for CE.

DISCUSSION

Generally, the extent of fermentation, as evidenced by both total SCFA production and DMD for a given substrate was similar across species at the 48-hour fermentation times. Despite the absence of a plateau for the two mixed substrates CP and BP, the similarities across species for all time periods suggest no differences would be evident at plateau. Results at later time periods should be considered with respect to published transit time values for the different species. *H. griseus* is the only species for which values come close to 48 h, with values ranging from 18.2 h to 39.3 h, depending on marker type (Cabre-Vert and Feistner, 1995; Overdorff and Rasmussen, 1997). Published values for *V. variegata* and *E. fulvus* are considerably shorter at 2.3 to 5.2 h and 2.8 to 4.6 h, respectively [Ganzhorn, 1986; Cabre-Vert and Feistner, 1995; Edwards and Ullrey, 1999]. Therefore the extent of fermentation has little meaning if the *in vitro* fermentation time is not considered with regard to the *in vivo* transit time.

Two of the substrates used in this study were pure (CE and PE), while BP and CP were a mixture of fiber types. These mixed substrates, both byproducts of feedstuff processing, are more indicative of the fibrous portion of a potential feedstuff, so are useful in discussion of fermentative capacity of fiber within a diet. All species fermented the BP substrate, with a higher proportion of IF, similarly, while for CP, species differences were evident. This suggests that differences in fermentative capacity may have more to do with the SF content for these species; however further work with more mixed substrates is necessary.

Perhaps the most biologically significant result obtained was the higher SCFA production for *V. variegata* compared to the other two species for PE at the 6 and 12-hour fermentation times. *V. variegata* also showed a greater capacity for fermentation of CP at 6 h of fermentation, suggesting the capability to more rapidly process the fiber present in this mixed substrate. At later time periods, however, *V. variegata* did not differ from *H. griseus* and *E. fulvus* for either of these substrates.

Use of fecal inoculum in an *in vitro* fermentation system is a relatively low cost and low risk method of assessing fermentative capacity across a variety of species and substrate types. Animals can remain in normal housing situations and sample collection is simple. However, as these data indicate, results must be interpreted with respect to *in vivo* measurements such as digestibility or transit

time when available, in order to determine the true biological value of SCFA production by gastrointestinal microflora. These data do suggest differences in fermentative capacity among lemur species, and therefore fiber type should be considered along with quantity when designing appropriate captive diets.

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TABLE 1. Total short chain fatty acid production, in mmol/gram of original substrate, by species for cellulose, beet pulp, citrus pulp, and citrus pectin at 6, 12, 24, and 48 h of fermentation

Substrate	Hours	*Hapelumer griseus*	*Eulemur fulvus*	*Varecia variegata*	P <[1]
Cellulose (CE)					
	6	ND[2]	ND	ND	
	12	.39[3]	.41	.64	
	24	.67	.63	.83	
	48	1.11	.76	.91	
Beet Pulp (BP)					
	6	.32	.62	1.08	
	12	1.72	2.03	2.19	
	24	4.71	4.87	4.82	
	48	6.18	6.50	6.17	
Citrus Pulp (CP)					
	6	.61	1.03	1.87	**
	12	3.42	3.46	3.77	
	24	4.52	5.12	5.24	
	48	5.70	5.91	5.83	
Citrus Pectin (PE)					
	6	.26	.40	1.02	**
	12	1.80	1.67	3.65	**
	24	5.85	5.73	5.80	
	48	5.81	5.64	5.76	

[1]Asterisks indicate $P < .01$ for the species x time effect within a substrate.
[2]ND=not determined.
[3]Pooled SEM is .22.

TABLE 2. pH values obtained for all substrates at 6, 12, 24, and 48 hours of fermentation

Substrate	6 h	12 h	24 h	48 h	P <[1]
Cellulose (CE)	ND[2]	7.33[3]	7.54	7.68	.025
Beet Pulp (BP)	6.92	6.49	6.25	6.19	.001
Citrus Pulp (CP)	6.66	6.19	6.18	6.23	.0001
Citrus Pectin (PE)	7.05	6.17	5.34	5.57	.01

[1] P-value indicates significance level for the time effect within a substrate.
[2] ND=not determined.
[3] Pooled SEM is .072.