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Non Ruminant Nutrition

NON RUMINANT NUTRITION

Evaluation of equine rectal inoculum as representative of the microbial activities within the horse hindgut using a fully automated *in vitro* gas production technique system

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Abstract

The *in vitro* gas production technique (IVGPT) has been a valuable tool in ruminant nutrition research for decades and has more recently been used in horse nutrition studies to investigate fermentation activities of the equine hindgut though primarily using feces as inoculum. This study was conducted to evaluate the use of equine rectal content in the IVGPT system as a viable inoculum that can be considered representative of the activities throughout the equine hindgut. Additionally, the study was conducted to measure the effects on fermentation kinetics and end-product production using inoculum from horses fed supplemental levels of coated sodium butyrate in an IVGPT system. Eight warmblood horses were fed a diet consisting of haylage (1% DM intake based on ideal body weight [BW]) and a mash concentrate formulated to provide 2.5 g nonstructural carbohydrate (NSC)/kg BW per meal. The diet was intended to create a NSC challenge to the microbial populations of the hindgut. The horses were randomly assigned to treatment or control group and after a 1-wk diet-adaptation period, the treatment group received 0.4 g/kg BW per day of a coated sodium butyrate supplement, while the control group received a placebo (coating only). After a 3-wk treatment period, the animals were sacrificed and digesta from the cecum, left ventral colon, right dorsal colon, and the rectum were collected within 30 min postmortem and used as inocula for the IVGPT trial. Haylage and concentrates fed to the test animals were also used as substrates *in vitro*. Sodium butyrate supplementation was not significant for gas production parameters or VFA measured suggesting no effect of sodium butyrate supplementation on the extent or kinetics of gas production or microbial end-product production ($P \geq 0.073$). Differences in inocula were significant for organic matter corrected cumulative gas production ($P = 0.0001$), asymptotic gas production of the second phase (A2) ($P < 0.0001$); and maximal rate of OM degradation of the second phase (R_{max2}) ($P = 0.002$). Inocula had a significant effect on total VFA ($P = 0.0002$), butyrate (Bu) ($P = 0.015$), branched chain fatty acids ($P < 0.0001$), pH ($P < 0.0001$), and ammonia (NH_3) ($P = 0.0024$). In conclusion, based on observed results from this study, total tract digestibility may be overestimated if using rectal content inoculum to evaluate forage-based feeds in an IVGPT system.

Key words: digesta inocula, equine, intestinal compartments, *in vitro* gas production, microbial fermentation

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Abbreviations

A1,A2	asymptotic gas production for the first and second phase, respectively
B1,B2	switching characteristics for the first and second phase, respectively
BCAA	branched chain amino acid
BCFA	branched chain fatty acid
Bu	butyrate
C1,C2	halftime of gas production for the first and second phase, respectively
CBC	complete blood count
CE	cecum
CN	concentrate
GIT	gastrointestinal tract
H	haylage
IVGPT	*in vitro* gas production technique
I	inoculum
LVC	left ventral colon
NEm	net energy for maintenance
NSC	nonstructural carbohydrates
OMCV	organic matter corrected cumulative volume
PL	placebo (coating only)
RDC	right dorsal colon
RE	rectum
R_{max1}, R_{max2}	maximum rate of gas production for the first and second phase, respectively
SB	coated sodium butyrate
S	substrates provided to *in vitro* cultures
T	treatments fed to equine subjects
VREp	digestible crude protein value for horse

Introduction

The horse is a hindgut fermenter and its associated anatomical arrangement and function of the microbiota contribute to the horse's susceptibility to gastrointestinal tract (GIT) disorders such as colic, diarrhea, and laminitis (Garner et al., 1978; White, 1990; Reeves et al., 1996). Feeding starch-rich diets may put the horse at risk for these conditions (Clarke et al., 1990). The relative inaccessibility of the equine hindgut for various diagnostic tools impedes insight into the etiology and understanding of how nutritional strategies may trigger, modify, help, or even prevent these disorders. Therefore, researchers have employed *in vitro* techniques to study the impact of feed on the microbiota, fermentation end products, and GIT health of horses. One such model utilized is the *in vitro* gas production technique (IVGPT) and its use in equine studies has been documented (Murray et al., 2009, 2014). Because obtaining digesta from the GIT of the horse requires either animals surgically fitted with a cannula or access to slaughter horses, feces is the most common inoculum used in IVGPT studies. As a result, equivalences between total tract *in vivo* and fecal microbial fermentation activities must be assumed. Therefore, the current study was undertaken to compare the use of rectal content (RE) as inoculum in the IVGPT system (Desrousseaux et al., 2012 modified from Cone et al., 1996), to inocula from three additional sources such as cecum (CE), left ventral colon (LVC), and right dorsal colon (RDC). In addition, studies suggest exogenous supplementation of the VFA butyric acid (Bu) may benefit the GIT health of animals, particularly as preferred fuel for colonocytes (Wang et al., 2005; Biagi et al., 2007). Hence, the secondary objective of this study was to measure changes in fermentation kinetics and microbial activity of inocula from horses fed a high starch diet supplemented with a coated sodium butyrate.

Materials and Methods

The experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2014/103) and was in accordance with national and institutional guidelines for the care and use of animals.

Animals, diets, and housing

This experiment was conducted in conjunction with a histological and immunohistochemical evaluation of gut tissues from the same group of test animals (Wambacq et al., 2017). The animals used in these experiments included eight warmblood horses with an average body weight (BW) of 594 ± 56.6 kg (mean ± SD). These animals were each destined for slaughter and ranged in age between 5 and 16 yr. The horses' body condition scores varied between 4 and 6 on a scale of 1–9 (Henneke et al., 1983). Prior to inclusion in this experiment, the overall quality of health was clinically assessed and a basic complete blood count was performed. The horses had no history of gastrointestinal disease before starting this study. Prior to study commencement, all horses were dewormed using an oral gel preparation at 200 μg ivermectin and 1.5 mg praziquantel/kg BW (Equest Pramox, Fort Dodge Animal Health Benelux B.V., Weesp, the Netherlands). The horses were individually housed in stables (3 × 5 m^2) using wood shavings as bedding and were allowed outdoor access to a sand paddock a few hours three times per week. All horses were fed a diet consisting of haylage (1% DM intake based on ideal BW) and a mash concentrate (Cavalor Mash and Mix, Cavalor, Belgium) in order to provide 2.5 g nonstructural carbohydrate (NSC)/kg BW per meal (Table 1). The diet contained energy in excess required for maintenance (NRC, 2007) and was designed to provide surplus starch intake such that undigested starch would overflow to the hindgut inducing saccharolytic fermentation, thereby creating a mild NSC challenge to the microbial populations of the CE and large intestines. The diet met NRC (2007) vitamin and mineral requirements and was divided in two equal meals, offered at 0800 and 1700 h. Water was freely available at all times. The adaptation period was 1 wk prior to the experimental period which continued for 3 wk.

Experimental design

The horses were randomly assigned to treatment or control group. After a 1-wk diet-adaptation period, the treatment group received 0.4 g/kg BW per day of a coated sodium butyrate supplement (Excential Butycoat, Orffa Additives B.V., Werkendam, the Netherlands), while the control group received a placebo (coating only). The first group (Run1; 2 horses in treatment and 2 in control) participated in the fall. The second group (Run2; 2 horses in treatment and 2 in control) participated the following spring. The treatment period continued for 3 wk after which the animals were slaughtered. Approximately 30-min postslaughter the intestinal tract was presented for sampling.

Sample collection, inocula preparations, and in vitro incubations

Upon presentation of the intestinal tract the CE, LVC, RDC, and RE were ligated using zip ties to prevent backflow. Digesta and rectal samples (>300 g) were taken from each of the ligated compartments. The samples were prepared as described by Desrousseaux et al. (2012), which was based on a modified

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Table 1. Chemical composition of the dietary components

Component[1]	Haylage[2]	Concentrate[3]
DM, g/kg of product	860.2	878.8
Crude ash	59.3	63.0
Crude fiber	324.1	50.9
Crude protein	80.3	134.1
Crude fat	11.1	81.3
Sugar	137.1	60.5
Starch	0.0	351.2
DE, MJ/kg DM	8.3[4]	13.3[5]
NEm, MJ/kg DM	5.02[4]	8.39[5]
VREp	32[4]	100[5]

[1]Chemical composition expressed in g/kg DM unless otherwise indicated. DE = Digestible energy; NEm = Net energy value for horse (CVB, 2004); VRE*p* = Digestible crude protein value for horses (CVB, 2004).
[2]Analyzed by wet chemistry (NUTRIQUINE N.V., Drongen, Belgium).
[3]Concentrate ingredients (Cavalor Mash & Mix): flaked barley, wheat bran, linseed, oats, expanded barley, expanded corn, cane molasses, toasted soybeans, horse bean flakes, carrot pieces, calcium carbonate, leek, sodium chloride, soybean meal, fructooligosaccharides, sunflower meal, palm kernel expeller. Additives: 15,000 IU/kg vitamin A, 1,500 IU/kg vitamin D3, 150 mg/kg vitamin E, 3 mg/kg vitamin B1, 3 mg/kg vitamin B2, 100 µg/kg biotin, 80 mg/kg choline chloride, 40 mg/kg ferrous sulphate, 0.2 mg/kg calcium iodate, 0.1 mg/kg coated granulated cobalt(II) carbonate, 40 mg/kg cupric sulphate, 15 mg/kg dicopper chloride trihydroxide, 100 mg/kg manganese oxide, 15 mg/kg manganese chelate, 100 mg/kg zinc sulphate, 20.3 mg/kg zinc chloride hydroxide, 0.3 mg/kg sodium selenite, 0.2 mg/kg L-selenomethionine.
[4]Based on tabulated values of hays of similar quality (CVB; 2018).
[5]Declared calculated values by the manufacturer (Cavalor, Dalton, GA, USA).

method from Cone et al. (1996). In summary, the samples were transferred to and sealed in containers previously flushed with CO_2. Immediately afterward, the samples were placed on ice and transported to the laboratory within 6 h. Individual animals' digesta and rectal content samples from corresponding compartments (300 g each) were combined with 1.2 L of a CO_2-saturated bicarbonate-phosphate buffer solution, prewarmed to 39 °C, maintained under a constant CO_2 flow and blended for 1 min (Tefal Performa blender, France). The buffer solution was composed of a bicarbonate-phosphate buffer solution that included (g/L): 8.75 g $NaHCO_3$, 1.00 g NH_4CO_3, 1.43 g Na_2HPO_4, 1.55 g KH_2PO_4, 0.15 g $MgSO_4{\cdot}7H_2O$, 0.52 g Na_2S, 0.017 g $CaCl_2{\cdot}2H_2O$, 0.015 g $MnCl_2{\cdot}4H_2O$, 0.002 g $CoCl_3{\cdot}6H_2O$, 0.012 g $FeCl_3{\cdot}6H_2O$, and 0.125 g resazurin (Cone et al., 1996). The buffered digesta/rectal mixtures were filtered through two layers of cheesecloth, and placed into flasks continuously flushed with CO_2 to ensure anaerobic conditions. During inoculation the buffered digesta/rectal mixtures were continuously mixed using a magnetic stirrer and maintained at 39 °C.

Prior to inoculation, samples of the same concentrate and roughage fed to the donor animals were taken (approximately 500 mg air dried material) and weighed into fermentation bottles (250 mL, Schott, Mainz, Germany). Immediately prior to inoculation, each bottle containing substrate was placed in a shaking water bath, prewarmed to 39 °C and flushed with CO_2 under a gentle flow for approximately 1 min. Next, 60 mL of buffered digesta or rectal inoculum was delivered into each fermentation bottle. Bottles were returned immediately into the shaking water bath and connected to a fully automated gas production measurement system (Cone et al., 1996), and gas production was recorded for 72 h. Postincubation, pH was measured, and fermentation fluids were sampled (750 µL) for VFA and NH_3 and stored at −20 °C for further analysis as described by Huyen et al. (2016). The inocula order was randomized for treatment and intestine compartment. Per treatment-inocula-substrate combination three replicate bottles were prepared resulting in a total of 96 bottles. In addition, blanks (60 mL buffered digesta inoculum without substrate) were included in 120 mL serum bottles and crimp cap sealed with rubber stoppers, gas production was measured manually using the pressure transducer technique (Theodorou et al., 1994). The gas production in blanks (Mean ± SD) were 13 ± 2.3 mL (CE), 11 ± 7.6 mL (LVC), 19 ± 2.2 mL (RDC), and 21 ± 0.9 mL (RE) for run 1, and 7 ± 2.0 mL (CE), 9 ± 5.1 mL (LVC), 14 ± 2.0 mL (RDC), and 11 ± 2.3 mL (RE) for run 2. Blanks were not used for corrections as discussed by Cone et al. (1997) and Williams et al. (2005). In the absence of an energy source, the microbial fermentation in blank bottles will differ from those in the presence of a substrate because the gas and VFA produced originate mainly from microbial protein turnover.

In vitro fermentation kinetics

Gas production curves were fitted using a biphasic modified Michaelis–Menten equation as described by Groot et al. (1996) by nonlinear least squares regression procedure (SAS, version 9.3; SAS Inst. Inc., Cary, NC; Eq. (1)):

$$Y = A_1/[1 + (C_1/t)^{B_1}] + A_2/[1 + (C_2/t)^{B_2}], \quad (1)$$

where Y is the cumulative gas volume per unit OM (OMCV; mL/g OM incubated); A_i is the asymptotic gas production (mL/g OM); B_i is the switching characteristic of the curve; C_i is the time at which 50% of the asymptotic gas production (halftime, h) was reached, *t* is the time (h), with *i* indicating the first or second phase. The maximum rate of gas production ($R_{max(i)}$) and the time at which it occurs ($T_{RM(i)}$) were calculated for both phases (i = 1, 2) according to Yang et al. (2005) (Eqs. 2 and 3):

$$R_{max(i)} = A_i \times (C_i^{B_i}) \times B_i \times [T_{RM}{}^{(-B_i-1)}]/\left[1 + (C_i^{B_i}) \times [T_{RM}{}^{(-B_i)}]\right]^2, \quad (2)$$

$$T_{RM(i)} = C_i \times \left[[(B_i - 1)/(B_i + 1)]^{(1/B_i)}\right]. \quad (3)$$

Statistical analyses

The average of three replicate bottles per treatment-inocula-substrate combination within run was considered the experimental unit and used for statistical analyses. Data were analyzed by ANOVA using the MIXED model procedure in SAS (version 9.3; SAS Inst. Inc., Cary, NC) using the model (Eq. 4):

$$Y_{ijklm} = \mu + T_i + S_j + I_k + (T \times S)_{ij} + (T \times I)_{ik} + (S \times I)_{jk} + (T \times S \times I)_{ijk} + P(H)_{lm} + \varepsilon_{ijklm},$$

where Y_{ijklm} is the variable to be tested; µ is the overall mean; T_i is the fixed effect of treatment (i = 2); S_j is the fixed effect of substrate (j = 2); I_k is the fixed effect of inocula (k = 4); $(T \times S)_{ij}$, $(T \times I)_{ik}$, $(S \times I)_{jk}$ is the two-way interaction terms; $(T \times S \times I)_{ijk}$ is the three-way interaction term; $P(H)_{lm}$ is the random effect of horse (m = 8) nested within period (l = 2); and ε_{ijklm} is the error term. Differences among main effects were analyzed using the Tukey–Kramer's multiple pairwise comparison procedure in the LSMEANS statement. Post-hoc analyses were performed to determine differences between the sets of treatment × substrate × inocula using Tukey multiple pairwise comparison test.

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Table 2. Measured gas production parameters

Parameters	T	S	I: CE	LVC	RDC	RE	SE[1]
OMCV, mL/g	SB	CN	243.7	273.8	325.3	289.3	18.62
	PL	CN	269.3	264.3	280.4	291.4	
	SB	H	149.3	224.6	242.4	231.0	
	PL	H	170.0	184.3	204.0	240.5	
A1, mL/g	SB	CN	169.9	171.0	183.1	176.2	21.05
	PL	CN	194.2	158.4	164.4	131.6	
	SB	H	116.3	143.8	160.1	146.6	
	PL	H	125.8	139.0	134.9	122.5	
C1, h	SB	CN	2.9	2.2	2.3	2.1	0.90
	PL	CN	2.3	1.9	1.8	1.3	
	SB	H	2.3	2.7	4.3	2.5	
	PL	H	1.3	1.7	1.6	1.8	
A2, mL/g	SB	CN	81.3	106.1	148.0	118.0	15.55
	PL	CN	83.8	109.3	120.5	162.6	
	SB	H	40.4	88.0	111.2	105.3	
	PL	H	56.7	51.0	78.6	138.5	
C2, h	SB	CN	12.8	7.2	8.9	8.0	4.67
	PL	CN	11.2	8.7	7.6	7.0	
	SB	H	22.3	17.7	16.4	18.0	
	PL	H	10.7	25.2	17.9	19.4	
R_{max1}, mL/h	SB	CN	40.5	64.3	60.6	57.6	16.98
	PL	CN	82.6	66.4	71.9	73.2	
	SB	H	54.1	68.8	60.0	84.5	
	PL	H	80.9	78.2	74.3	100.5	
R_{max2}, mL/h	SB	CN	9.1	24.0	30.9	29.8	2.98
	PL	CN	15.7	20.7	23.3	27.7	
	SB	H	4.3	7.3	10.7	6.8	
	PL	H	14.8	3.3	5.6	8.0	

P-values[3,2]

	T	S	I	T×S	T×I	S×I
OMCV	0.466	<0.0001	0.0001	0.760	0.050	0.360
A1	0.263	0.004	0.747	0.933	0.364	0.483
C1	0.073	0.702	0.841	0.379	0.863	0.605
A2	0.503	0.015	<0.0001	0.472	0.011	0.735
C2	0.384	0.062	0.459	0.935	0.342	0.502
R_{max1}	0.239	0.250	0.645	0.938	0.585	0.557
R_{max2}	0.870	<0.0001	0.002	0.433	0.004	0.0001

[1]Pooled standard error.
[2]P-values for main effects and two-way interactions.
[3]P-values for three-way interactions are ≥ 0.522 and are therefore not presented in the table.

Differences between pairs were declared significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

Results

Gas production kinetics

Treatment was not significant for any gas production parameters measured (Table 2) indicating there was no effect of sodium butyrate treatment on the extent or kinetics of gas production ($P \geq 0.073$). Significant differences between substrates (haylage vs. concentrate) were observed for most gas production parameters including: OMCV ($P < 0.0001$), A1 ($P = 0.004$), A2 ($P = 0.0001$), A2 ($P < 0.0001$), and R_{max2} ($P = 0.002$). Three-way interactions between treatment, substrate, and inoculum were not significant for any gas production parameter ($P \geq 0.571$).

Figure 1 illustrates the corrected cumulative gas volume per unit of organic matter (OMCV) from four inocula: CE, LVC, RDC, and RE each cultured with one of two substrates: concentrate (CN) or haylage (H). During the first hours of *in vitro* incubation, representing the first phase, no difference in gas production was measured. Differences in gas production between inocula become significant during the second phase. This effect can be visualized at 4 h in regard to haylage and at 7 h in regard to concentrate as indicated in Figure 1.

OMCV was significantly ($P < 0.0001$) lower in cultures incubated with haylage compared with cultures incubated with concentrate regardless of inoculum or treatment, 205.8 vs. 279.7 mL/g respectively. Further, a significant difference ($P = 0.0001$) in OMCV between inoculum sources, regardless of treatment or substrate, was measured. Figure 2 provides an alternative comparison of OMCV fermentation rates from the various inocula considering substrate. Here the data revealed significant differences in OMCV rates from cultures inoculated with CE compared with RDC (160.0 vs. 223.2, respectively) and in cultures inoculated with RDC compared with RE (223.2 vs. 235.8,

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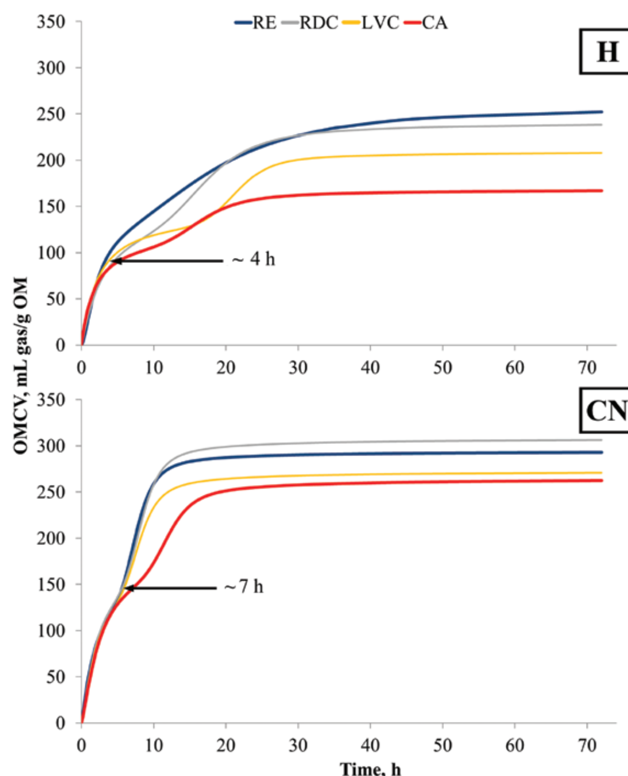

Figure 1. Gas production curves from *in vitro* fermentation using 4 different inocula sources and two substrates (H and CN) utilizing a biphasic curve-fitting model (Groot et al., 1996) as measured with a fully automated system (Cone et al., 1996). Inoculum sources include CE, LVC, RDC, and RE. Substrates were H and CN.

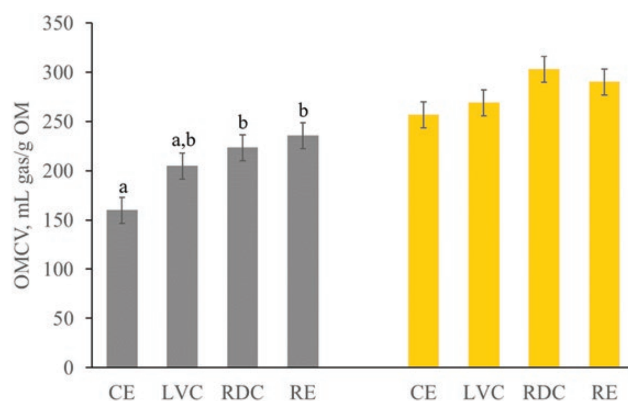

Figure 2. Comparison of measured OMCV rates of *in vitro* fermentation from within substrate groups, haylage (gray bars) or concentrate (yellow bars) utilizing four different inocula and regardless of treatment. Inoculum sources include CE, LVC, RDC, RE. [a,b]$P \le 0.017$.

respectively) when fermenting haylage ($P \le 0.036$). Interestingly, no differences in OMCV rates were measured between cultures fermenting concentrates regardless of inoculum ($P \ge 0.744$).

End products

Treatment was not significant for any end product parameter (Tables 3 and 4). Substrate had a significant effect ($P \le 0.004$) on almost all end product parameters except for Bu ($P = 0.134$), branched chain fatty acids (BCFA) ($P = 0.678$), and ammonia (NH_3) ($P = 0.087$). Inocula had a significant effect on total VFA ($P = 0.0002$), Bu ($P = 0.015$), BCFA ($P < 0.0001$), pH ($P < 0.0001$), and NH_3 ($P = 0.0024$). “Horse”, nested within each run, was considered a random variable. Interaction between treatment × substrate × inoculum had no effect on any end product parameters measured.

Figure 3 illustrates a comparison of BCFA and NH_3 production among inocula sources regardless of treatment or substrate ($P \le 0.046$ and $P \le 0.036$, respectively). Further, a congruent pattern of BCFA and NH_3 production resulting from each inoculum source was observed (Figure 3). Accordingly, as branched chain amino acids (BCAA) are utilized by the microbes, the resulting BCFA will be produced while NH_3 will accumulate as a result of the deamination process accompanying BCAA metabolism. These results suggest typical and expected fermentation kinetics were measured in this IVGPT trial.

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Table 3. Concentrations of total and individual VFA via *in vitro* fermentation of haylage or concentrate from inoculum of the CE, RDC, LVC, and RE

			I				
Item	T	S	CE	LVC	RDC	RE	SE[1]
Total VFA, mM	SB	CN	86.4	110.7	118.3	93.3	11.72
	PL	CN	88.1	95.9	102.3	87.0	
	SB	H	61.6	88.8	100.7	79.0	
	PL	H	66.2	82.5	77.2	81.0	
Acetate, mmol/100 mmol	SB	CN	60.4	57.3	58.1	56.7	2.62
	PL	CN	57.0	56.9	56.2	49.1	
	SB	H	61.9	59.3	58.6	58.4	
	PL	H	62.6	61.1	59.4	60.8	
Propionate, mmol/100 mmol	SB	CN	30.1	29.9	28.5	30.5	2.5
	PL	CN	32.9	32.6	31.7	36.4	
	SB	H	29.3	28.7	27.9	29.6	
	PL	H	27.0	28.2	29.3	27.8	
Butyrate, mmol/100 mmol	SB	CN	2.8	4.8	4.0	4.1	1.41
	PL	CN	3.2	3.9	3.6	4.5	
	SB	H	3.1	4.6	3.9	3.6	
	PL	H	3.0	3.7	2.9	3.1	
			P-values[2,3]				
	T	S	I	T×S	T×I	S×I	
Total VFA	0.591	<0.0001	0.0002	0.677	0.144	0.597	
Acetate	0.610	0.003	0.124	0.059	0.801	0.546	
Propionate	0.542	0.004	0.659	0.035	0.892	0.732	
Butyrate	0.844	0.134	0.015	0.332	0.452	0.589	

[1]Pooled standard error.
[2]P-values for main effects and two-way interactions.
[3]P-values for three way-interactions are ≥ 0.656 and are therefore not presented in the table.

Discussion

The first objective of this study was to investigate the suitability of feces as a representative inoculum of the hindgut constituents of the equine GIT when employing the IVGPT. Inoculum source had a significant effect on OMCV and A2 gas production parameters. Murray et al. (2014) also observed significant effects on asymptotic gas production from various inocula. In that particular study, the data were curve fitted according to a monophasic model vs. a biphasic model utilized in this study. Further, Murray et al. (2014) found higher values for asymptotic gas production than the values measured in this study. One explanation for this difference lies with the different methodology utilized Murray et al. (2014) vs. this study. Murray et al. (2014) employed the Theodorou et al. (1994) method, thereby creating less diluted inocula compared with the Desrousseaux et al. (2012) methodology (based on a modified method from Cone et al. (1996) applied in this study. The difference in dilutions resulted in longer lag-times (slower fermentation rates) in the current study as fewer organisms per culture were present compared with the shorter lag-times observed by Murray et al. (2014). Additionally, Murray et al. (2014) used different forage-based substrates for the incubation of inocula, specifically freeze–dried grass, high-temperature dried grass, and unmolassed sugar beet pulp. Lastly, Murray et al. (2014) used inocula collected from a single animal as compared with eight animals as utilized in this trial.

The distinct shift in fermentation rates observed between OMCV rates in cultures utilizing concentrates compared with haylage is not unexpected. When comparing cereal grain-based vs. forage-based substrates (high starch vs. low starch), quicker fermentation rates of cereal grains is expected in both *in vivo* and *in vitro* trials (Goodson et al., 1988; Murray et al., 2006). The maximum cumulative gas volume per unit OM is attained relatively quick in phase 2 of fermentation as concentrates are fermented, but continued to increase gradually from haylage fermentation. In fact, when inocula from the rectum (RE) was cultured with haylage (Figure 1) OMCV rates continued to rise to similar rates from cultures incubated with concentrates. Only OMCV rates of the fermentation of concentrates from LVC inoculum was different from the fermentation of haylage from RE inoculum (235.8 vs. 268.9). Hence, when using IVGPT to evaluate feedstuff digestibility in horses it may be important to appreciate this characteristic of RE inoculum as compared with other compartments of the GI tract. Specifically, microbes from the CE, LVC, and RDC appear to ferment forage-based feedstuffs more slowly over time compared with those from fecal material. An over-estimation of total tract digestibility may be calculated if using only RE inoculum to evaluate forage-based feeds in an IVGPT system, in particular if fermentation is halted between 12 and 24 h. Further, when the data are reported in a pair-wise comparison (Figure 2), evaluating inocula source regardless of treatment, it is interesting to note the distinct difference between fermentation rates of haylage in the CE and RDC inocula sources. This suggests, as previously reported by Goodson et al. (1988), the CE may act primarily as an organ of inoculation and less as a site of significant fermentation. This is most relevant during forage fermentation. Further, it might be important to consider that forage-based feedstuffs fermented by inocula from the RE and the RDC appear to exhibit

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Table 4. Concentration of total BCFA, ammonia, pH, and acetate to propionate ratio resulting from *in vitro* fermentation of hay or concentrate from inoculum of the CE, LVC, RDC, or RE

			I				
Item	T	S	CE	LVC	RDC	RE	SE[1]
BCFA, mmol/100mmol	SB	CN	2.2	3.2	4.6	3.4	0.30
	PL	CN	2.4	3.0	3.9	3.7	
	SB	H	2.5	3.1	4.4	3.4	
	PL	H	3.0	3.1	4.1	3.4	
Ammonia, mg/L	SB	CN	349.7	485.7	743.2	481.2	80.40
	PL	CN	431.7	459.1	551.7	561.0	
	SB	H	254.9	574.9	494.3	504.0	
	PL	H	354.7	398.3	479.8	460.1	
pH	SB	CN	6.70	6.58	6.62	6.60	0.041
	PL	CN	6.72	6.67	6.62	6.58	
	SB	H	6.87	6.72	6.69	6.70	
	PL	H	6.89	6.78	6.81	6.71	
Acetate/Propionate	SB	CN	2.1	2.0	2.1	1.9	0.25
	PL	CN	1.8	1.8	1.8	1.6	
	SB	H	2.2	2.1	2.1	2.0	
	PL	H	2.6	2.2	2.1	2.2	
			P-values[2,3]				
	T	S	I	T×S	T×I	S×I	
BCFA	0.861	0.678	<0.0001	0.502	0.192	0.531	
NH_3	0.630	0.087	0.002	0.800	0.225	0.442	
pH	0.265	<0.0001	<0.0001	0.424	0.370	0.766	
Acetate/Propionate	0.934	0.001	0.252	0.021[b]	0.861	0.792	

[1]Pooled standard error.
[2]P-values for main effects and two-way interactions.
[3]P-values for three way-interactions are ≥ 0.429 and are therefore not presented in the table.

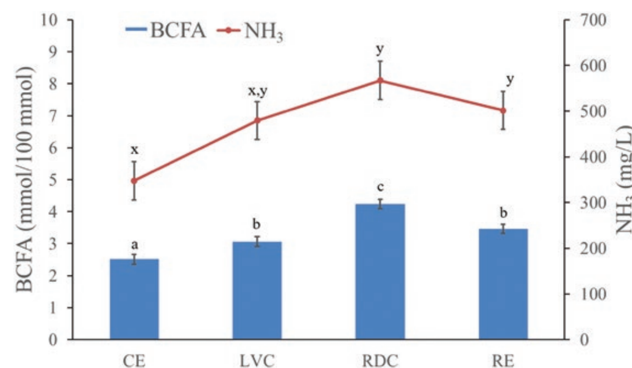

Figure 3. Comparison of BCFA production and ammonia production in cultures inoculated with four different sources: CE, LVC, RDC, RE, regardless of substrate or treatment. [a,b,c]Inocula with different superscripts are significantly different P ≤ 0.046. [x,y]Inocula with different superscripts are significantly different P ≤ 0.036.

significantly different fermentation kinetics, compared to CE, regardless of substrate as illustrated in Figure 2. Interestingly, in a study conducted by Lowman (1998) greater gas production values were observed when cecal fluid was incubated with oat feed compared with feces incubated with oat feed. However, no difference was measured when these inocula were incubated with sugar beet pulp. Furthermore, there were no differences between OMCV rates of concentrate fermentation between any inocula evaluated in this trial (Figure 2). One possible explanation no differences were observed between inocula may be due to the high-starch diets subjects received prior to digesta collection. As previously described, microbes from the GIT of these equine subjects had been exposed to high-starch diets for 3 wk prior to use in the IVGPT system so likely a shift toward more NSC-fermenting organisms had already occurred. In fact, these bacterial populations would have been naïve to an all haylage diet which may also explain the significant differences measured between CE vs. RDC (160.0 vs. 223.2) and RDC vs. RE (223.2 vs. 235.8) OMCV rates when cultured with haylage.

Additionally, results from total VFA measurements were consistent with differing fermentation kinetics between inocula sources. Fermentation by CE inoculum, regardless of substrate, yielded a lower concentration of total VFA compared with LVC and RDC, 75.6, 94.5, and 99.6 mM, respectively ($P = 0.0032$ and $P = 0.0002$). Lastly, fermentation of substrate by RE inoculum resulted in lower total VFA compared with fermentation from

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RDC inoculum, 85.1 vs. 99.6 mM, respectively ($P = 0.035$). According to Warren and Kivipelto (2005), fecal inoculum should have a greater ability to degrade structural carbohydrate compared with cecal fluid due to the higher number of microorganisms present in feces material. Results from this study were unable to support this observation as no differences were measured in either end-product production (total VFA) or OMCV rates when comparing fermentation of haylage with inocula from the CE and the RE, 75.6 vs. 85.1 mM and 160.0 vs. 235.8 mg/h, respectively.

Differences in total VFA concentrations between inoculum sources were observed. Similar to the *in vivo* analysis by Hintz et al. (1971) who reported differences in total VFA, butyrate and isovalerate concentrations from cecal fluid compared with ventral colonic fluid. This follows the consequential differences measured in OMCV and A2 and R_{max2} between inocula compartments. That is, different cumulative gas production profiles correlate with corresponding total VFA productions between compartments.

In this study, significant differences in fermentation kinetics and end-products between inocula sources have been measured. However, multiple studies (Murray et al., 2005a, 2005b, 2006; Hastie et al., 2008) have reported on the suitability of equine feces as a source for IVGPT. Feces of ruminants are often used as an inoculum instead of rumen fluid for *in vitro* gas production studies (Mauricio et al., 1998, 2001; Akhter et al., 1999). An advantage of equine feces over ruminant feces exists because horses are hindgut fermenters with little to no postfermentative digestion and absorption of microbial cells, which does take place in ruminants (Lowman et al., 1999). Even though a significant effect of inocula on particular gas production and end-product parameters has been measured, this may not explicitly rule out feces as a suitable inoculum compared with cecal or colonic fluids for *in vitro* gas productions studies. However, due to the significant differences between inocula measured in this and other studies, RE inocula were not representative of all processes occurring in the different compartments of the equine hindgut. Furthermore, differences between the four compartments also indicate that cecal digesta is not singularly representative as a sole indicator of microbial fermentation within the equine hindgut. The clear advantage of using feces or rectal content as an inoculum over cecal or colonic chyme is the ease and less-invasive nature of collecting contents from the RE compared with chyme from intact organs.

The secondary objective of this study was to measure, *in vitro*, the effects of a coated sodium butyrate product on microbial activity when supplemented to equine diets. Supplementation of sodium butyrate in livestock diets has demonstrated numerous health benefits including stimulating rumen development in calves and enhancing the growth and intestinal morphology of weanling pigs (Lu et al., 2008; Górka et al., 2011). Furthermore, sodium butyrate supplementation has positively impacted gut microbiota thereby improving gastrointestinal functions (Hu and Guo, 2007; Zhou et al., 2017). In this current study, no effect of coated sodium butyrate supplementation was observed on any parameters assessing microbial activity via IVGPT. During the course of this trial, considerable quantities of undigested, coated supplement were observed in the feces of test subjects from both treatment and placebo groups. Hence, if the coating prevented access of microbiota to the active ingredient, sodium butyrate, this might explain the lack of measurable effects. However, while Wambacq and coworkers (Ghent University, Ghent, Belgium, personal communication) also observed the appearance of coating particles found throughout the digestive tract, they postulated this "does not necessarily indicate that the release of butyric acid happened gradually, since the degradation of coating could take longer than the absorption of butyrate". Additionally, the amount of sodium butyrate fed to the treatment animals may not have been optimal. Sodium butyrate was found to have significant effects on *in vitro* evaluation of intestinal microbiota of weanling pigs and on *in vivo* rumen function characteristics of weanling lambs fed sodium butyrate at rates ranging from 1 to 4 g/kg intake (Biagi et al., 2007; Soltani et al., 2017). The current study provided the equine treatment subjects with coated sodium butyrate at a rate of approximately 18 g/kg intake. Additional studies performed with rabbits, also hindgut fermenters, determined the amount of supplemented sodium butyrate was very specific before differences could be measured (Carraro et al., 2005; Ribeiro et al., 2012; Hassanin et al., 2015). Perhaps a similar phenomenon might apply to horses. Future studies evaluating effects of feeding coated sodium butyrate on fermentation parameters of equine microbial populations should consider greater rates of intake and further investigation into the microencapsulation coating which would ensure greater quantities of active sodium butyrate product will be released and available in the hindgut.

Conclusion

Results indicated sodium butyrate treatment had no effect on fermentation kinetics or microbial end-product production when measured in a fully automated IVGPT system. Moreover, rectal content was not a consistent, representative inoculum source for mimicking microbial activity throughout the entire equine hindgut. Also, this study revealed that no single inoculum measured would be fully representative of all processes in the equine hindgut. However, if donor horses are consuming a high starch diet and the inoculum used in an IVGPT is cultured with a grain-based substrate then any of the four inoculum sources employed in this trial might be suitably representative of each other when evaluating fermentation kinetics and end-products. Finally, future studies focusing on evaluating the coating technology of sodium butyrate are necessary to improve the mechanistic knowledge-base of such time-released supplements in the equine GIT.

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Conflict of interest statement

The authors declare no conflict of interest.

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