

Impact of camphor on the in vitro mixed ruminal microorganism fermentation from goats selected for consumption of low and high levels of *Juniperus* spp. 1

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ABSTRACT

Encroaching plant species in West Texas continues to significantly reduce livestock production capacity and cause an imbalance between plant and livestock ecology. *Juniperus ashei* and *Juniperus pinchotii* are encroaching species rarely used by browsing animals, mainly due to phytochemical defenses. *Juniperus* spp. contain large concentrations of monoterpenes in their essential oil profiles to deter herbivory. Since 2003, two divergent bloodlines of meat goats have been selected to consume low or high (LJC or HJC) amounts of juniper foliage, through screening fecal samples with near-infrared reflectance spectroscopy. However, it remains unclear whether HJC goats expressed a physiological ability to consume a greater amount of juniper or if they were colonized by a ruminal microbial population that could detoxify juniper phytochemicals. Therefore, this study aimed to investigate the impact of 0.00 and 1.97 mM of camphor on the mixed ruminal microorganism fermentation after 0, 1, 2, 4, 12, and 24 h of incubation. Five LJC and five HJC goats were fed a juniper-free diet ($n = 10$) and five LJC and five HJC goats ($n = 10$), were fed a diet containing 30% fresh *J. ashei* leaves for 21 days prior to ruminal fluid collection. In vitro fermentations used LJC and HJC, ruminal fluid inoculum was added (33% v/v) to anoxic media in sealed Balch tubes. Total short-chain fatty acid (SCFA) production and acetate to propionate ratio were increased ($P < 0.05$), but there was no effect on pH (6.56 ± 0.09). Goats that received the juniper-free diet had higher ($P < 0.05$) SCFA production than juniper-containing diets. There was no consistent difference in LJC and HJC microbial fermentation end products caused by the addition of 1.97 mM of camphor, and goats receiving a juniper-free diet consistently generated more SCFAs in the presence of 1.97 mM of camphor. Furthermore, bloodline differences in juniper consumption were likely related to physiological adaptation capacities within the animal and not a ruminal microbial detoxification advantage.

Key words: camphor, goat, in vitro, juniper, short-chain fatty acids

INTRODUCTION

In the Edwards Plateau region of west-central Texas, rangeland is subject to Pinchot's juniper (*Juniperus pinchotii* Sudw) and Ashe's juniper (*Juniperus ashei* J. Buchholz) encroachment, to which have negative impacts on livestock production as well as wildlife habitat (Dye et al., 1995; Whitney, 2017). Removal of Pinchot's and Ashe's juniper species can be expensive through the processes of mechanical grubbing of the plant and/or chemical treatment techniques. Additional control mechanisms include prescribed fires (Taylor, 2006; Taylor, 2008), using animal behavioral affinities as a browsed plant (Campbell et al., 2006; Markó et al., 2008), and collecting *Juniperus* spp. as roughage sources for inclusion in feed ingredients (Whitney et al., 2014; Stewart et al., 2015a; Ishaq et al., 2017).

Juniperus sp. contains secondary plant compounds (SPCs), particularly volatile oils (VOs) and tannins (Riddle et al., 1996; Adams et al., 2013; Adams, 2014), which limit their use as forages for livestock. The VO profiles of *Juniperus* spp. vary widely, and the monoterpene camphor varies from 26% to 50% of the total VO (Adams, 2010; Whitney and Muir, 2010).

Seasonality, maturity, and location in the plant of *Juniperus* spp. have been documented to impact concentrations of VO and other SPC (Owens et al., 1998; Whitney and Muir, 2010), making it challenging to compare rumen microbial tolerance or detoxification of *Juniperus* VO.

To improve the efficacy of using goats to control Pinchot's and Ashe Juniper encroachment, since 2003, researchers at the Texas A&M AgriLife Experiment Station in San Angelo, Texas, developed bloodlines of meat goats (Spanish × Boer) that consumed high or low amounts of *Juniper* in their diets. These divergent lines were developed using expected breeding values (EBVs) for high or low juniper consumption with the constraint that the inbreeding coefficient was less than 5%. EBVs were calculated using percentage juniper in the diet as determined by fecal near-infrared reflectance spectroscopy (fNIRS) and pedigree data (Campbell et al. 2007a).

Previous research indicated that following an intraruminal dose of camphor, serum levels of camphor were lower in high juniper consuming goats than in low juniper consuming goats (Campbell et al., 2010). However, that study could not determine if the difference was caused by biotransformation of

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camphor by rumen microflora or metabolism by the animal. Diet selection by ruminants is a learned response based on post-ingestive feedback, mediated by morphological, physiological, and digestive processes of the animal (Foley et al., 1999). The rumen can detoxify many organic compounds (Kronberg and Walker, 1993; Domínguez-Bello, 1996; Duncan et al., 1997; Malecky et al., 2012), and the ability to detoxify compounds varies between populations of ruminants and can be selected for by slow adaptation of the ruminal microbial population (Palmer et al., 2010). The objective of this study was to determine the effect of bloodline, that is, goats selectively bred for high or low juniper consumption, diet, and camphor at concentrations consumed by free-grazing goats on the production of short-chain fatty acids (SCFAs) and metabolism of camphor by mixed rumen microorganism fermentation.

MATERIALS AND METHODS

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Additionally, all experimental procedures and protocols were verified and approved by Texas A&M University's Office of Animal Care and Use (Texas A&M AUP 2018-012A).

Animal Selection and Feeding

Goats in this study were intact Boer × Spanish males, and half in each bloodline group were born in 2015 or 2016. In 2003 fNIRS calibrations (Campbell et al., 2007a) were used to divide goats into separate lines of high and low juniper consumers. Subsequently, goats were bred to create divergent lines for high and low juniper consumption based on their EBV calculated from fNIRS determinations of percentage juniper in their diet and pedigree information. EBV and fNIRS determined percent of juniper in each group's diet just prior to the initiation of this study were 13 and 50%, respectively, for the high juniper consuming goats (HJC) goats, and -14 and 16% for the low juniper consuming goats (LJC) goats used in this study.

Both HJC and LJC goats received one of two diets for 21 days prior to the collection of rumen fluid. Tifton 85 and late bloom alfalfa hay 1:1 wt:wt ratio were ground in a tub grinder and small stems (< 1 cm). The leaves of *J. ashei* were harvested prior to the beginning of the study and stored at 5

°C to minimize the volatilization of terpenoids. Juniper and the previously ground hay mixture were combined at 3:7 wt:wt dry weight basis was hammermilled with a wood and leaf chipper and stored at 5 °C until fed. Goats were fed twice daily at 0800 and 1600 Central Standard time. A chemical analysis of the diets is in Table 1. The ruminal microbial fermentation is driven primarily by substrate availability and fermentability (Murphy et al., 1982; Russell, 2002; Nam et al., 2009) therefore the goats in this study were not fasted before ruminal fluid collection, and ruminal fluid was collected within 2 h of feeding to ensure adequate (and realistic) substrate (e.g., carbohydrate and ammonia) availability in the mixed ruminal microorganism fermentation.

Experimental Design

The purpose of this experiment was to assess the differences in SCFA production by the mixed rumen microorganism fermentation in an anaerobic in vitro fermentation from two selected bloodlines of low juniper consuming (LJC) and high juniper consuming (HJC) goats ($n = 8$ HJC and $n = 8$ LJC) fed two biologically relevant levels of Ashe's juniper in a 2 × 2 factorial design (Table 1, diet composition). Prior to ruminal fluid collection, four of the goats in each bloodline were fed a diet for 21 d containing 0% juniper (0) and the other four, a diet containing 30% fresh leaves (30) from *J. ashei* J. Buchholz. Rumen fluid was collected using esophageal tubing from each of the four bloodline × diet (B × D) treatment groups ($n = 4$ goats/group). Equal aliquots of ruminal fluid samples from each bloodline × diet group were pooled into a 1-L thermos, resulting in four B × D rumen fluid groups: LJC0, LJC30, HJC0, and HJC30. The experimental design was a nested model in which rumen fluid from each group represented batches ($n = 4$, $df = 3$). Each batch was influenced by the fixed effects of camphor concentration (0 or 1.97 mM of camphor) and time of incubation (0, 1, 2, 4, 12, or 24 h) in duplicate for each camphor × time combination, resulting in experimental units defined as the individual anaerobic in vitro Balch tube. For further experimental design clarifications, Figure 1 shows the rumen collection for each B × D group and processing for the in vitro portion down to the Balch tube level.

Ruminal Fluid Collection

Goats were housed at the Texas A&M AgriLife Research Station in Sonora, Texas, located in the western Edwards

Table 1. Chemical analyses of diet components of the preconditioning juniper free (0) and juniper enhanced (30) rations

Diet type—DM basis ¹	Juniper	Juniper	Juniper	Juniper + Hay	Juniper + Hay	Juniper + Hay	Hay	Hay
	(May 1, 2018)	(May 6, 2018)	(May 8, 2018)	(May 1, 2018)	(May 6, 2018)	(May 8, 2018)		
Nutrient composition								
CP, %	10.5	9.8	10.8	11.7	9.9	9.4	13.7	12.5
ADF, %	32.7	37.7	32.4	33.4	38.2	37.9	36.0	41.0
aNDF, %	51.0	56.9	44.4	56.5	56.7	50.9	58.2	59.8
NFC, %	28.5	23.4	34.8	21.8	23.4	29.7	18.0	17.7
NEM, Mcal/lb	0.55	0.51	0.59	0.53	0.52	0.55	0.52	0.51
NEG, Mcal/lb	0.29	0.26	0.33	0.27	0.27	0.30	0.26	0.26

¹Ration Balancer analysis by Dairy One, 730 Warren Road, Ithaca, NY 14850.

ADF, acid detergent fiber; aNDF, apparent NDF; CP, crude protein; DM, dry matter; NFC, non-fiber carbohydrates; NEM, net energy maintenance; NEG, net energy gain.

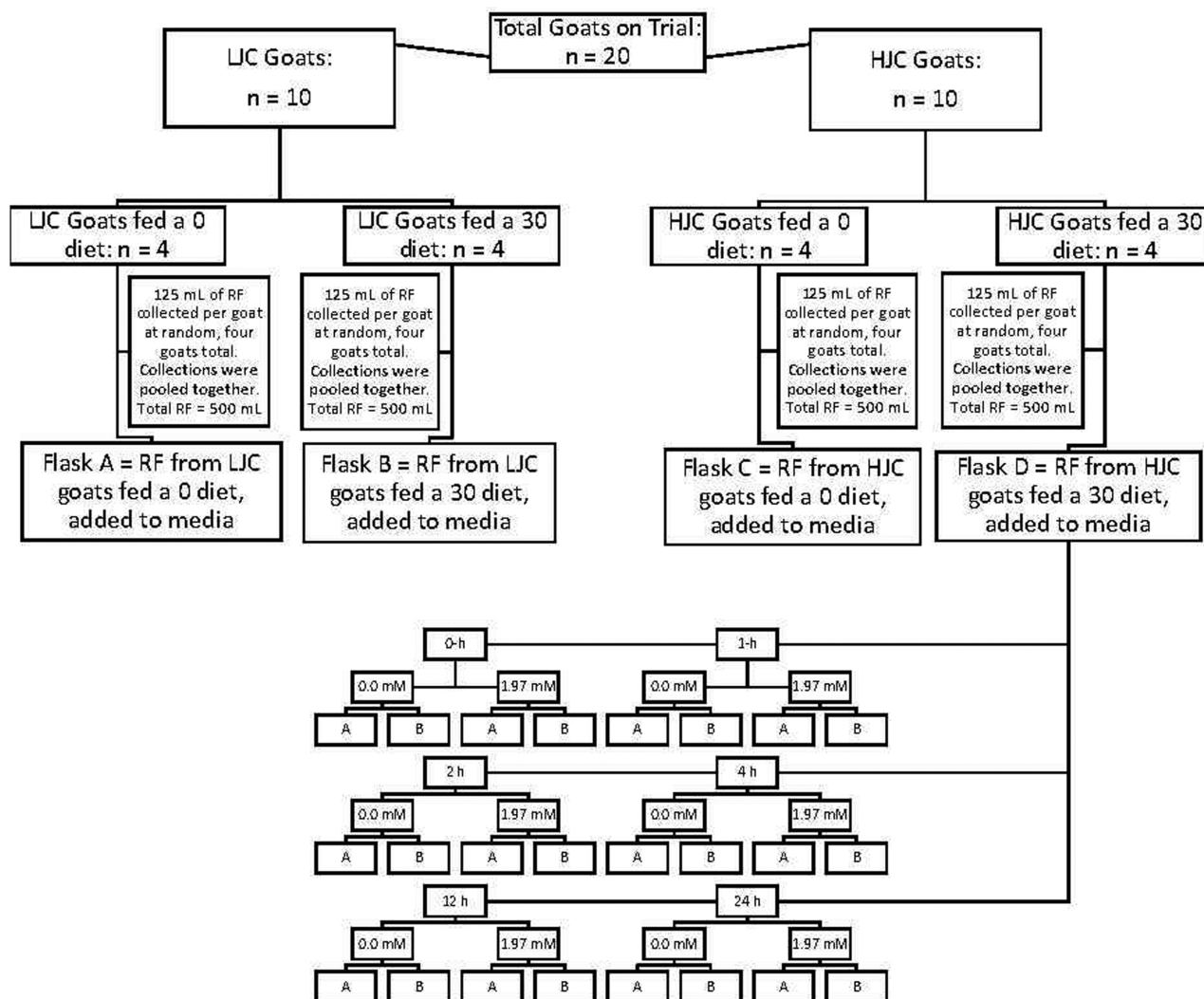


Figure 1. Experimental design for assessing the end-product alterations in mixed ruminal microorganism in vitro fermentation when goat rumen fluid was dosed with a single concentration of camphor and incubated for 1, 2, 4, 6, 12, or 24 h. The entire experimental approach is shown only for HJC goats fed a juniper enhanced diet, all other B × D groups were examined in the same manner. LJC, low juniper consuming goats; HJC, high juniper consuming goats; 0, goats fed a juniper free diet; 30, goats fed a juniper enhanced diet; RF, rumen fluid.

Plateau region of Texas (30°15' N, 100°33' W). Two diet groups (0 and 30) were penned separately, but bloodline groups (LJC and HJC) were comingled. Upon collection of 125 mL of raw rumen fluid via esophageal tubing from each of four goats within each B × D treatment group, rumen fluid samples from each B × D group were pooled into four separate 1-L thermos containers and transported to the Ruminant Nutrition Lab at the Texas A&M AgriLife Research Station in San Angelo, Texas (31°33' N, 100°30' W) for processing, and frozen for at least 24 h at -20 °C (Prates et al., 2010). After the initial freezing period, samples were packaged and shipped overnight on wet ice to the Animal and Dairy Science Department at the University of Georgia in Athens, Georgia (33°56' N, 83°22' W) for data collection and processing.

In Vitro Fermentation

Ruminal fluid was added from each B × D flask was added (33% v/v) to an anoxic media for mixed ruminal microorganism fermentation (Cotta and Russell, 1982; Callaway and Martin, 1996). Media composition was (per liter): 292 mg of

K_2HPO_4 , 240 mg of KH_2PO_4 , 480 mg of $(NH_4)_2SO_4$, 480 mg of $NaCl$, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4,000 mg of Na_2CO_3 , and 600 mg of cysteine-HCl. Each inoculated ruminal fluid/media flask was equilibrated and mixed with O_2 -free CO_2 for ten minutes prior to anaerobic transfer (10 mL) to O_2 -free CO_2 flushed Balch tubes which were subsequently sealed with butyl rubber stoppers and aluminum crimps. A stock solution that contained 0.3 g of camphor (MilliporeSigma, St. Louis, MO, (±)-Camphor, ≥95.0%) was dissolved in 20 mL of 95% ethanol; 200 μL of camphor stock solution was added to each appropriate fermentation tube to achieve a final concentration of 1.97 mM camphor. The camphor concentration used in the study represented a 45.4-kg goat (with a 10-L ruminal volume) consuming 3% body weight (BW) of a 30% *J. ashei* J. Buchholz diet whose leaves contain approximately 2% VO (dry matter [DM] basis), and 44% of the VO was reported as camphor (Riddle et al., 1996; Owens et al., 1998; Adams et al., 2013). All tubes received either camphor or an equivalent positive control (200 μL) of 95% ethanol. The individual in vitro fermentation tubes was

incubated at 39 °C for 0, 1, 2, 4, 12, or 24 h and was frozen (−20 °C) immediately at each time point.

Sample Analysis

Samples were thawed for at least 45 min to equilibrate to room temperature and vortexed before pH was measured (Orion pH meter). Rumen samples were analyzed for SCFA analysis according to [Goetsch and Galyean \(1983\)](#) and [Smith et al. \(2010\)](#). Briefly, 2 mL from each sample was centrifuged at 10,000 × g for 10 min, and then the supernatant was frozen (−20 °C). Five mL of raw sample for post-fermentation camphor concentration analysis was transferred to screw-thread vials, frozen, and shipped overnight shipment to Texas A&M University (College Station, TX) for post-fermentation camphor concentration analysis. For SCFA analysis, 1 mL of the supernatant was mixed with 0.2 mL of 25% (wt/vol) meta-phosphoric acid, vortexed, and frozen overnight. Subsequently, samples were thawed and centrifuged (10,000 × g, 10 min), before 1 mL of supernatant was transferred to screw-thread vials that contained 2 mL of 95% ethyl acetate, vortexed and allowed to settle for at least 5 min. The upper layer was transferred (1 mL) into gas chromatography vials for SCFA analysis using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation Kyoto, Japan) equipped with a flame ionization detector and capillary column (Zebron ZB-FFAP; 30 m × 0.32 mm × 0.25 μm; Phenomenex Inc., Torrance, CA, USA). The sample injection volume was 1.0 μL, and helium was the carrier gas. The starting temperature of the column was set at 110 °C and gradually increased to 200 °C, the injector temperature was set at 250 °C, and the detector temperature was set at 350 °C. The output variables recorded were; acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, and caproic acids. Total SCFAs were calculated using the sum of acetate, propionate, butyrate, and valerate concentrations.

Camphor Concentrations

Experimental methodology used to quantify levels of camphor before and after the in vitro trial is defined in [Lee et al. \(2012\)](#). Stock solution preparation was carried out with 100 μg/mL of camphor (Sigma-Aldrich, St. Louis, MO) dissolved in ethyl acetate, diluted in solvent to 1 μg/mL, and used for the calibration standards, precision and accuracy analysis, and spiking samples. Solid-phase extraction (SPE) cartridges, 500 mg C18, were obtained from Biotage (Biotage, Charlotte, NC). A C18 SPE cartridge equipped with a 10-mL solvent reservoir was pre-conditioned with 5 mL of methanol and filled with 10 mL of deionized water wash. The conditioned SPE was loaded with 4 mL of camphor sample, placed under vacuum pressure (20 Pa), and followed with a 10 mL water wash. The SPE was dried (10 min) under vacuum and eluted with ethyl acetate. The elute from each SPE column was injected into the gas chromatography-mass spectrometry using a Combi PAL autosampler (Agilent Technologies, Santa Clara, CA). The ratio of the peak area of camphor to internal standards (0.01, 0.05, 0.1, 0.5, 1.0, and 5.0 μg/mL) versus camphor concentration was plotted to obtain a standard curve using equal-weighted linear regression analysis. The camphor in each sample was determined using the standard curve's slope, and the sensitivity of the method was assessed by determining the limit of detection and the

limit of quantification with a signal-to-noise of 3 and 10, respectively.

Statistical Analyses

Statistical analyses were performed using the general linear model (GLM) procedure in SAS 9.4 (SAS Inst., Inc., Cary, NC) fitting a straight nested experimental design for all SCFA data. The experimental unit of the in vitro portion of this study was Balch tubes representing each B × D (f) combination; LJC0, LJC30, HJC0, and HJC30 (f = 4, df = 3). Each B × D had 24 represented analysis points, total observations were 96 (df = 95), where 12 samples from each flask were replicated twice (r). Between samples alone have the associated degrees of freedom: (f·s) − 1 = df = 47. Between samples within flasks (f·(s − 1) = df = 44) models included fixed effects of camphor concentration (c = 2; 0.00 or 1.97 mM, f·(c − 1) = df = 4), time of incubation (t = 6; 0, 1, 2, 4, 12, or 24 h, f·(t − 1) = df = 20), camphor × time interaction (f·(c − 1)·(t − 1) = df = 20), and the respective residual (f·(r − 1) = df = 48). For the goat performance data, a GLM procedure in SAS 9.4 was used for a 2 × 2 factorial design with two factors (Bloodline and Diet), and two levels for each factor.

A one-way Analysis of Variance (ANOVA) was used to compare measured camphor concentrations between the different B × D groups at time zero to analysis post-fermentation camphor concentrations. Two-way ANOVA was used to compare measured camphor concentrations between the different B × D groups and at different times over the experimental time course. The Holm-Sidak method was used for all pairwise multiple comparison procedures. Sigma Plot (v. 10.0.1) was used for this analysis (Systat Software, Inc., San Jose, CA, USA).

All results were considered significant at $P \leq 0.05$.

Results

Animal Performance

All goats had similar initial and final weights ($P > 0.42$), 52.2 and 54.3 kg, respectively ([Table 2](#)).

Total SCFAs and pH

Final pH, total SCFA, and acetate to propionate (A:P) ratio from each mixed ruminal microorganism fermentation are

Table 2. Animal performance data for two bloodlines of goats fed two different diets; each B × D data point is an average of $n = 4$ goats

Bloodline	LJC		HJC		P			
	0	30	0	30	SEM	Bloodline	Diet	B × D
Item								
Period 1								
BW, kg	50.5	52.1	51.8	55.2	2.91	0.60	0.55	0.84
Period 2								
BW, kg	52.0	55.1	54.1	58.1	3.34	0.60	0.46	0.93
ADG, kg/d	0.10	0.20	0.15	0.19	0.05	0.76	0.38	0.69

Period 1 (April 23, 2018) represents goat weights on day 7 of the 21d pre-conditioning diet, and Period 2 (May 8, 2018) indicates goat weights on the day of rumen fluid collection. ADG, average daily gain; BW, body weight.

Table 3. Effects of 0 or 1.97 mM camphor (CNT or CAM, respectively) on pH, total short-chain fatty acid, and acetate to propionate ratio concentrations (mM) from separate in vitro mixed ruminal microorganism fermentations incubated for 0, 1, 2, 4, 12, or 24 h.

Item	Treatment								P					
	LJC0		LJC30		HJC0		HJC30		SEM	B×D	MO	Hour	B	D
	CNT	CAM	CNT	CAM	CNT	CAM	CNT	CAM						
pH														
0 H	6.62		6.62		6.58		6.65							
24 H	6.50	6.50	6.50	6.50	6.46	6.47	6.61	6.57						
Total SCFA, mM									0.62	<0.001	0.28	<0.001	0.02	<0.001
0 H	21.20		19.12		26.38		18.70							
1 H	24.53	23.93	23.04	22.79	31.67	32.61	29.91	28.10						
2 H	34.99	35.67	30.91	29.26	28.10	28.50	20.74	22.25						
4 H	27.15	19.82	26.88	24.41	34.48	21.49	22.00	20.80						
12 H	31.51	34.08	29.02	26.70	30.84	30.84	20.39	16.10						
24 H	30.84	32.61	29.14	30.02	26.83	28.97	27.45	20.88						
A:P									0.14	0.02	0.05	<0.001	<0.001	<0.001
0 H	4.33		4.84		4.47		5.09							
1 H	4.24	4.36	4.92	4.92	4.47	4.51	7.36	7.65						
2 H	5.62	5.65	6.84	7.92	4.62	4.61	5.03	5.03						
4 H	4.16	3.71	8.63	6.36	4.38	4.54	5.10	5.44						
12 H	4.11	6.18	8.03	6.39	4.68	4.67	5.61	5.64						
24 H	4.28	6.00	7.84	6.65	4.57	4.33	4.86	5.16						

B × D, bloodline diet interaction across time and MO combinations; MO, monoterpene effect, control versus Camphor nested within B × D group; Hour, time effect, all incubation time points nested within B × D group; B, bloodline effect, LJC versus HJC; D, diet effect, 0 versus 30.

presented in Table 3. Regardless of bloodline by diet influence, there was little difference in pH in any bloodline by diet or time points. There was a B × D interaction ($P < 0.001$) for total SCFA caused by similar concentrations of SCFA for HJC0 and LJC0, while SCFA concentration had a greater reduction (23%) in HJ30 compared to LJC30 (8%). Camphor addition did not change total SCFA concentration across all incubation time points compared to controls ($P = 0.28$). Unsurprisingly, time impacted ($P < 0.001$) SCFA concentrations across B × D groups. Diet also impacted SCFA concentrations with J0 goats having greater ($P < 0.001$) concentrations than J30 goats (28.53 mM vs. 24.02 mM). An interaction ($P = 0.02$) between bloodline and diets for the A:P ratio. Diet had a greater effect on LJC compared to HJC goats. The addition of juniper to the diet increased the A:P ratio more in LJC than HJC. LJC goats had higher ($P < 0.001$) A:P ratios than HJC, and goats on juniper-added diets had higher ($P < 0.001$) A:P ratios than goats on juniper-free diets.

Volatile Fatty Acids Production

Acetate production was different amongst bloodline ($P = 0.004$) and diet ($P < 0.001$) but no differences caused by camphor ($P = 0.21$) were observed. The LJC goat inoculum had greater acetate concentrations ($P = 0.004$) than HJC goats (21.31 mM vs. 19.83 mM, respectively). Fermentations inoculated from J0-fed goats produced more ($P < 0.001$) acetate than goats fed J30. Propionate concentration was different between B × D ($P < 0.001$) and by incubation time ($P = 0.03$) but camphor (1.97 mM) caused a slight decrease ($P = 0.05$). Propionate production was not different ($P = 0.40$)

in tubes containing inocula from LJC and HJC goats, but diet differed ($P < 0.001$), with juniper-free diets producing more propionate. Butyrate was different ($P < 0.001$) between B × D groups and was different over time ($P = 0.03$), but the difference caused by camphor addition to the in vitro fermentations was minimal ($P = 0.09$), with camphor addition reducing butyrate production. There was no difference ($P = 0.22$) between goat bloodlines but the diet was different ($P < 0.001$) between ruminal fluid fermentations from J0 and J30-fed goats. The average of each duplicate sample used for the in vitro acetate, propionate, and butyrate results are profiled in Table 4.

Molar proportions of acetate, propionate, and butyrate per 100 mM of total SCFAs were also examined (Table 5). B × D ($P < 0.001$), time of incubation ($P < 0.001$), and camphor addition ($P = 0.02$) all altered the acetate proportion produced by the in vitro fermentations. Camphor addition to the in vitro mixed ruminal microorganism fermentation increased ($P = 0.02$) the molar proportion of acetate (Control [CNT] = 78.08 mmol/100 mmol vs. Camphor [CAM] = 78.17 mmol/100 mmol). Fermentations inoculated from LJC goats produced a greater proportion ($P = 0.01$) of acetate than HJC goats, whereas HJC produced a greater ($P < 0.001$) proportion of propionate. Fermentations inoculated with ruminal fluid from J30 fed goats had greater ($P < 0.001$) acetate proportions and J0-fed goat inoculum resulted in a greater ($P < 0.001$) production of propionate. Inclusion of camphor in fermentations increased ($P = 0.01$) proportion of propionate generated. Finally, proportions of butyrate production were impacted by B × D treatments ($P < 0.001$) and time of incubation ($P < 0.001$), and camphor addition increased

Table 4. Acetate, propionate, and butyrate concentrations (mM) from in vitro mixed ruminal microorganism fermentations containing 0 or 1.97 mM of camphor (CNT or CAM, respectively) and incubated for 0, 1, 2, 4, 12, or 24 h

Item	Treatment								P					
	LJC0		LJC30		HJC0		HJC30		SEM	B×D	MO	Hour	B	D
	CNT	CAM	CNT	CAM	CNT	CAM	CNT	CAM						
Acetate, mM									0.48	<.001	0.21	<.001	0.004	<0.001
0 H	15.79		14.61		20.08		14.36							
1 H	18.18	17.81	17.69	17.51	24.14	24.92	24.81	23.45						
2 H	27.76	28.32	25.46	24.60	21.62	21.91	15.98	17.14						
4 H	20.14	14.30	23.03	19.68	26.22	16.46	17.05	16.24						
12 H	23.44	27.66	24.69	21.56	23.89	23.87	16.24	12.83						
24 H	23.20	26.36	24.67	24.52	20.69	21.96	21.26	16.35						
Propionate, mM									0.12	0.02	0.05	0.03	0.4	<0.001
0 H	3.65		3.02		4.50		2.84							
1 H	4.29	4.11	3.60	3.56	5.40	5.53	3.38	3.07						
2 H	4.94	5.01	3.73	3.19	4.68	4.76	3.18	3.41						
4 H	4.84	3.81	2.67	3.26	5.98	3.62	3.35	3.01						
12 H	5.70	4.50	3.07	3.65	5.11	5.12	2.89	2.28						
24 H	5.41	4.38	3.17	3.93	4.53	5.10	4.37	3.17						
Butyrate, mM									0.04	0.039	0.086	0.025	0.216	<.001
0 H	1.53		1.36		1.54		1.38							
1 H	1.76	1.69	1.56	1.55	1.84	1.87	1.60	1.47						
2 H	2.00	2.03	1.57	1.35	1.56	1.59	1.46	1.58						
4 H	1.87	1.47	1.08	1.34	1.98	1.21	1.48	1.34						
12 H	2.05	1.67	1.13	1.35	1.59	1.61	1.61	0.92						
24 H	1.94	1.63	1.18	1.44	1.40	1.65	1.69	1.27						

B × D, bloodline diet interaction across time and MO combinations; MO, monoterpene effect, control vs. camphor nested within B × D group; Hour, time effect, all incubation time points nested within B × D group; B, bloodline effect, LJC versus HJC; D, diet effect, 0 versus 30. CNT, control; CAM, camphor.

($P = 0.02$) butyrate proportions. Butyrate production as a molar proportion was not different between bloodlines ($P = 0.47$) or diets ($P = 0.14$).

The post-fermentation camphor concentration results of tubes incubated for 1, 2, 4, 12, and 24 h and dosed with 1.97 mM of camphor are presented in [Figure 2](#). For the low juniper consuming goats, the juniper free-fed group showed significant differences in camphor concentration at the 4 and 24 h time points, and the juniper-enhanced diet showed differences for only the 2 and 12-hour incubation. For the high juniper consuming goats, the juniper free-fed goats had significantly less camphor during 4 and 24 h of incubation compared to incubation time points 1 and 2 h, whereas juniper enhanced fed HJC goats had significantly less camphor concentrations in incubation time points 4 and 24 compared to all remaining time points.

DISCUSSION

The present study was designed to evaluate in vitro camphor disappearance and impact on the mixed rumen microorganism fermentation collected from two divergent goat bloodlines, which were fed specific diets. Feeding of hay along with juniper leaves was utilized to remove differences inherent in percentage juniper in the diet of different goat bloodlines when grazing pasture that would impact ruminal

microbial populations. Exogenous addition of substrate (e.g., glucose, starch, or cellulose) introduces an artificial selection pressure to the in vitro fermentations that could mask potential differences between bloodlines and diets.

The ruminal microbial population is a dynamic biochemical reservoir with the capacity to degrade many different components found in the diet of grazing ruminants, including anti-nutritional factors such as tannins, oxalate, and toxic amino acids (Allison and Cook, 1981; Allison et al., 1992; Nelson et al., 1998; Andrew et al., 2000). Terpenes are anti-nutritional factors that can be degraded by ruminal microbes (Allison et al., 1990; Brooker et al., 1994; Duncan et al., 1997), which detoxify feedstuffs by preventing the absorption of the anti-nutritional compound and resultant side effects such as lipid vacuolation or hepatic cellular necrosis (Straka, 2001). Goats selected for the ability to consume high levels of terpenes in juniper (HJC) showed a higher clearance rate of camphor than LJC goats when dosed intra-ruminally (Campbell et al., 2007b), but the mode of action of this difference in clearance rate was not apparent. The ability of mixed rumen microbial populations to detoxify or degrade camphor (and other terpenes), thus protecting the host animal from their deleterious effects, was unknown. However, it has been reported that changes in the host hepatic phase I and II enzymes could account for much of the differential clearance of the dietary terpenes (Torregrossa and Dearing, 2009).

Table 5. Acetate, propionate, and butyrate molar proportion of total short-chain fatty acids concentrations (mM) produced by in vitro mixed ruminal microorganism fermentations containing 0 or 1.97 mM of camphor (CNT or CAM, respectively) and incubated for 0, 1, 2, 4, 12, or 24 h

Item	Treatment								P					
	LJC0		LJC30		HJC0		HJC30		SEM	B×D	MO	Hour	B	D
	CNT	CAM	CNT	CAM	CNT	CAM	CNT	CAM						
Acetate, mmol/100 mmol									0.34	<0.001	0.02	<0.001	0.01	<0.001
0 H	74.46		76.38		76.19		76.86							
1 H	74.12	74.52	76.80	76.86	76.22	76.42	82.82	83.45						
2 H	79.32	79.41	82.40	84.27	76.93	76.86	77.04	77.02						
4 H	74.17	71.90	85.67	80.43	75.99	76.55	77.44	78.56						
12 H	74.41	81.21	85.12	80.85	77.49	77.42	79.66	79.68						
24 H	75.21	80.75	84.73	81.96	77.12	75.86	77.45	78.30						
Propionate, mmol/100 mmol									0.24	0.13	0.01	<0.001	<0.001	<0.001
0 H	17.20		15.77		17.05		15.15							
1 H	17.47	17.12	15.62	15.63	17.07	16.95	11.36	10.91						
2 H	14.11	14.06	12.05	10.74	16.65	16.69	15.32	15.32						
4 H	17.83	19.48	9.93	13.49	17.36	16.87	15.28	14.45						
12 H	18.10	13.16	10.61	13.62	16.56	16.58	14.19	14.13						
24 H	17.56	13.47	10.83	12.86	16.88	17.58	15.92	15.17						
Butyrate, mmol/100 mmol									0.09	<0.001	0.02	<0.001	0.47	0.14
0 H	7.19		7.09		5.82		7.40							
1 H	7.18	7.06	6.78	6.82	5.81	5.74	5.38	5.21						
2 H	5.71	5.69	5.06	4.55	5.54	5.57	7.06	7.08						
4 H	6.95	7.46	4.01	5.52	5.75	5.67	6.72	6.44						
12 H	6.52	4.89	3.89	5.04	5.16	5.22	5.70	5.71						
24 H	6.29	5.01	4.04	4.71	5.23	5.69	6.15	6.07						

B × D, bloodline diet interaction across time and MO combinations; MO, monoterpene effect, control versus camphor nested within B × D group; hour, time effect, all incubation time points nested within B × D group; B, bloodline effect, LJC versus HJC; D, diet effect, 0 versus 30.

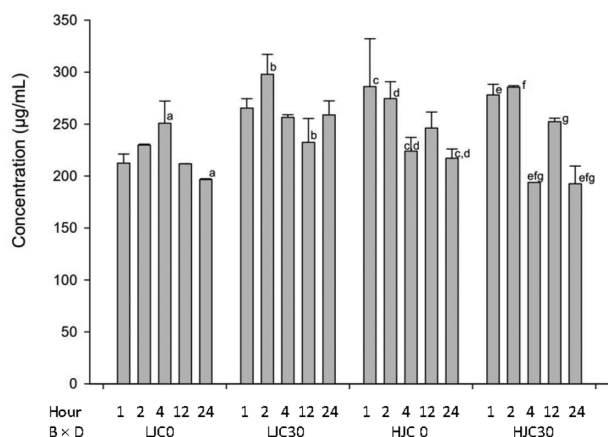


Figure 2. Concentrations (µg/mL) of tubes containing camphor for each respective bloodline by diet combination in the in vitro mixed ruminal microorganism fermentation. Tubes not dosed with 1.97 mM of camphor exhibited the following concentration averages (µg/mL); LJC0 = 0.9, LJC30 = 6.0, HJC0 = 1.0, HJC30 = 5.2. ^{a-e}Within a B × D group, means containing a common letter differ ($P < 0.05$); $n = 2$ for each time point within B × D group.

Thus, it was apparent we must understand if the ability of HJC goats to consume higher levels of juniper was related to an inherent difference in their ruminal microbial population

composition/activity or by a difference in the enzymatic production by the goat. In addition to detoxification, it is also important to understand the impact of camphor (and VO) on ruminal fermentation end products because SCFA are the primary energy source for the ruminant animal.

The experimental dose of camphor used in the in vitro mixed ruminal microorganism fermentation (1.97 mM or 300 mg/L) was similar to previous studies that fed *Juniperus* sp. to small ruminants ranging from 0.719 g to 3.6 g oil/d (Whitney et al., 2014) without adverse health or growth performance effects. Previous in vitro studies with terpene inoculations utilized from 1 to 5,000 mg/L to observe antimicrobial impacts of terpenes (Castillejos et al., 2006), 3 to 3,000 mg/L (Busquet et al., 2006) 31.2 to 312 mg/L (Busquet et al., 2005), and 1 to 6 mM (Macheboeuf et al., 2008).

In vitro studies using goat ruminal fluid and a similar in vitro fermentation system produced similar results (Whitney et al., 2011). Conversely, the LJC and HJC goat bloodlines have been investigated in in vivo studies with juniper-enhanced feed and found numerically greater SCFA concentrations (Stewart et al., 2019). SCFA concentrations increased over time when the substrate was available for rumen microbial fermentation; however, since substrate was not added to the present in vitro fermentation, endogenous substrates carried over with the ruminal fluid inoculum did impact microbial fermentation

end products. We hypothesized that HJC goats fed a pre-conditioning diet containing juniper (HJC30) would have the most robust and adaptive rumen microbial populations when exposed to plant secondary compounds (e.g., camphor) found in *J. ashei* and *Juniperus pinchotti*. However, that hypothesis was not supported by our data, the HJC30 group with added camphor had the lowest total SCFA concentrations from the ruminal microbial fermentation, and conversely, the HJC0 diet fermentations without added camphor had the highest SCFA concentrations in the entire study.

Similar investigations to our study have been conducted using VO blends both in vivo (McIntosh et al., 2003; Benchaar et al., 2007) and in vitro (Cardozo et al., 2005; Busquet et al., 2006; Macheboeuf et al., 2008). Further studies investigated the individual active compounds within VOs (Evans and Martin, 2000; Castillejos et al., 2006; Castillejos et al., 2008; Macheboeuf et al., 2008), but individual investigations into the activity of monoterpenes found in high proportions in *J. ashei* and *J. pinchotii* is limited. Terpene compositions and concentrations within *Juniperus* sp. plants vary tremendously (Whitney and Muir, 2010; Adams et al., 2013; Stewart et al., 2015b). Stewart et al. (2015b) found the VO profile of *J. ashei* was comprised of 65 distinct compounds, and 34% of the total VO was camphor and were greater in immature vs. mature plants.

CONCLUSION

The study's specific objectives were to identify differences in SCFA production when inoculum from specific bloodlines of meat goats were fed diets free of or containing camphor-containing *Juniperus* plants and examine the impacts of monoterpene addition in in vitro fermentation of mixed ruminal microorganisms collected from the different bloodlines of goats. Findings indicated a difference between goat bloodlines regarding to in vitro microbial tolerance of camphor. However, even the most camphor degradation selected ruminal microbial population (HJC fed the J30 diet) had the lowest production of SCFAs. Results suggest that detoxification and tolerance to camphor found in *J. ashei* and *J. pinchotii* were more likely related to hepatic mechanisms in the animal instead of a highly active ruminal microorganism population.

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Conflict of interest statement

None declared.

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