



# Impact of concentrations of camphor on the in vitro mixed ruminal microorganism fermentation from goats selected for consumption of low and high levels of *Juniperus* spp.-2

Darren S. Seidel,<sup>†</sup> John W. Walker,<sup>‡</sup> Jeffrey M. Musser,<sup>||</sup> Jeferson M. Lourenco,<sup>†,1</sup> Christina B. Welch,<sup>†,1</sup> Travis R. Whitney,<sup>‡</sup> and Todd R. Callaway<sup>†,1</sup>

<sup>†</sup>Department of Animal and Dairy Science, University of Georgia, Athens, GA 30602, USA

<sup>‡</sup>Texas A&M AgriLife Research, San Angelo, TX 76901, USA

<sup>||</sup>Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843, USA

<sup>1</sup>Corresponding author: [todd.callaway@uga.edu](mailto:todd.callaway@uga.edu)

## Abstract

The microbial population in the gastrointestinal tract of ruminant animals aids in the utilization of forages with high levels of secondary plant compounds. Two divergent bloodlines of meat goats have been selected by screening fecal samples with near-infrared reflectance spectroscopy to assess the goat's consumption of high or low levels of *Juniperus* sp. leaves containing several monoterpenes, including camphor. The mechanism by which these goats can consume greater concentrations of *Juniperus* spp. leaves than their counterparts is unclear, and therefore, this study was designed to determine if differences existed between the ruminal microbial populations of the low and high juniper-consuming bloodlines (LJC vs. HJC) by analyzing their ruminal microbiota and fermentation end products. In the present study, concentrations (0.00, 0.5, 0.99, 1.97, or 5.91 mM) of camphor were added to mixed ruminal microorganism fermentation. 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 that contained 30% fresh *Juniperus ashei* leaves for 21 d prior to ruminal fluid collection. In vitro fermentations used LJC and HJC, ruminal fluid inoculum added (33% v/v) to anoxic media in sealed Balch tubes. Camphor increased ( $P < 0.05$ ) total short-chain fatty acid (SCFA) concentrations for all but one experimental group. Between the main dietary and bloodline goat effects, the diet was significant for all SCFA results except butyrate. In contrast, bloodline was only significant for acetate and butyrate molar proportions. Rumen fluid from juniper-free-fed goats exhibited greater concentrations of *Ruminococcaceae*, whereas juniper-fed goats contained more *Coriobacteriaceae*. Results demonstrated that mixed ruminal microorganisms fermentations from HJC goats did not produce greater concentrations of SCFAs or have the ability to degrade camphor at a higher rate than did that from LJC goats. Results suggest that camphor tolerance from *J. ashei*, was related to hepatic catabolic mechanisms instead of ruminal microbial degradation; however, further in vivo work is warranted.

**Key words:** camphor, goat, juniper, microbial fermentation, microbiome

## INTRODUCTION

Juniper expansion in the western and southwestern United States has led to ecological disruption and threatens other vegetation ecosystems (Ansley and Rasmussen, 2005; Campbell and Taylor, 2007). Since the early developments of the livestock industry in west-central Texas, invasion, and increase of Pinchot's juniper (*Juniperus pinchottii* Sudw.) and Ashe juniper (*Juniperus ashei* J. Buchholz.) encroachment into previously dominated herbaceous vegetation has been a problem (Taylor et al., 1997; Dunson et al., 2007). Juniper plants contain high concentrations of secondary plant compounds, including alkaloids and terpenes, which impact the herbivore consumption (Riddle et al., 1996; Frost et al., 2008). Mechanical grubbing or chemical treatment techniques for juniper removal are expensive methods thus researchers have investigated other control options for minimizing juniper encroachment, such as prescribed burns (Taylor, 2006, 2008), selection of livestock to preferentially consume juniper (Markó et al., 2008; Waldron et al., 2009), and juniper processed as a forage source to include in feed rations (Whitney et al., 2014; Ishaq et al., 2017).

To limit juniper dissemination, researchers with Texas A&M AgriLife in San Angelo, TX began selecting divergent meat goat bloodlines (Spanish × Boer) that consumed high or low percentages of juniper in their diets starting in 2003. Fecal near-infrared spectroscopy (fNIRS) was used to estimate juniper consumption and breeding lines were established: high juniper consuming (HJC) goats and low juniper consuming (LJC) goats (Walker et al., 2007; Waldron et al., 2009). Despite the high phytochemicals present in all plant parts of *Juniperus* sp. limiting herbivory (Estell et al., 2014), there were no differences in body weight between LJC and HJC in a voluntary intake trial and only minor differences in ruminal short-chain fatty acid (SCFA) concentrations. It is readily understood that diet selection by ruminants is a learned response based on postingestive feedback mediated by morphological, physiological, and digestive processes of the animal (Foley et al., 1999).

The rumen microbial population can detoxify many organic compounds that are antinutritional factors (Kronberg and Walker, 1993; Domínguez-Bello, 1996; Duncan et al.,

Received February 20, 2022 Accepted July 21, 2022.

© The Author(s) 2022. Published by Oxford University Press on behalf of the American Society of Animal Science.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (<https://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact [journals.permissions@oup.com](mailto:journals.permissions@oup.com)

1997; Malecky et al., 2012), and the ability of the microbial ecosystem to detoxify compounds varies between populations of ruminants (Palmer et al., 2010). Therefore, our present objective was to utilize a subset of both HJC and LJC bloodline goats fed either a juniper-free (0) diet or a juniper-supplemented (30) diet containing 30% fresh leaves of Ashe Juniper in a 2 × 2 factorial design, and analyze differences in the microbial populations between the experimental groups. Following the 21-d preconditioning phase, rumen fluid was collected from a random subset of goats from each of the four bloodline-by-diet groups to determine if the addition of concentrations (0, 0.25, 0.49, 0.99, 1.97, or 5.91 mM) of a monoterpene (camphor) commonly found in *Juniperus* spp. altered the in vitro mixed rumen microbial fermentation. Furthermore, whole fresh ruminal fluid samples were collected to examine native microflora differences between experimental groups.

## 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. In addition, 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). Methods are also described in detail in the companion manuscript.

### Experimental Design

This experiment aimed to assess the differences in SCFA production by the mixed rumen microbes from two selected bloodlines of goats ( $n = 20$  total,  $n = 10$  [HJC] &  $n = 10$  [LJC]) in the presence of camphor in an anaerobic in vitro fermentation. Prior to ruminal fluid collection, each bloodline was fed a diet for 21 d containing 0% juniper (0) or a diet containing 30% fresh leaves from *J. ashei* Buch. (30) in a 2 × 2 factorial design (Table 1, diet composition). Rumen fluid was collected using esophageal tubing from each of the four bloodline-by-diet (D × B) treatment groups, which contained  $n = 4$  goats. Ruminal fluid samples from each D × B group were pooled using equal amounts into a 1 L thermos, resulting in D × B rumen fluid groups ( $n = 4$ ): LJC0, LJC30, HJC0,

and HJC30. The experimental design fitted a nested model in which rumen fluid from each group represented batches ( $n = 4$ , d.f. = 3). Each batch was influenced by the fixed effects of camphor concentration (0, 0.25, 0.49, 0.99, 1.97, or 5.91 mM) and time of incubation (0 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 test tube level.

### Study Logistics

The study site where goats were housed and fed was at the Texas A&M AgriLife Research Station in Sonora, TX located in Texas's western Edward Plateau region (30°15'N, 100°33'W). The two diet groups (0 and 30) were penned separately, but bloodline groups (LJC and HJC) were cmingled together. Upon collection of 250 mL of raw rumen fluid via esophageal tubing from each of four goats within each bloodline × diet 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, TX (31°33'N, 100°30'W) for further experimental processing. Following the fermentation, samples were frozen for at least 24 h at -20° C (Prates et al., 2010). For the microbiome analysis, two 15 mL vials from each B × D group were flash-frozen ( $\approx -196$  °C) for 45 s in liquid nitrogen, transported on ice to the laboratory, and stored at -20 °C.

After completing the in vitro trial, all 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.

### Animals and Breeding Program

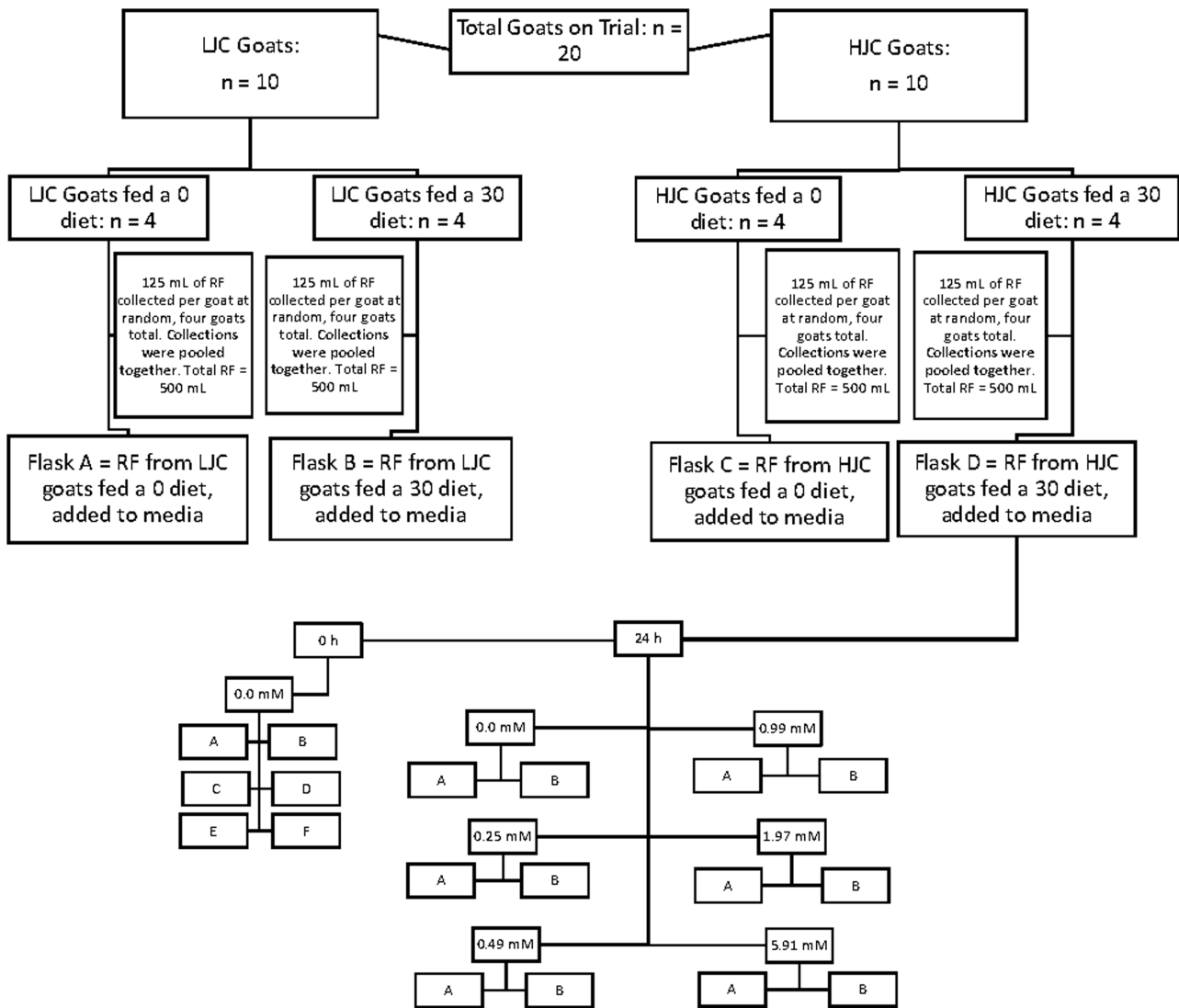
Animals in this study were intact Boer × Spanish males and half in each breeding group were born in 2015 and 2016. In 2003 goats' fNIRS calibrations (Campbell et al., 2007) were used to divide goats into high and low juniper consumers. From then forward, goats were bred to create divergent lines for high and low juniper consumption based on their expected

**Table 1.** Chemical analyses of diet components of the preconditioning juniper free (J0) and juniper-supplemented (J30) rations

Diet Type—DM Basis*	Juniper (5/1/18)	Juniper (5/6/18)	Juniper (5/8/18)	Juniper + Hay (5/1/18)	Juniper + Hay (5/6/18)	Juniper + Hay (5/8/18)	Hay†
Nutrient composition							
CP, %	10.5	9.8	10.8	11.7	9.9	9.4	13.7
ADE, %	32.7	37.7	32.4	33.4	38.2	37.9	36
aNDE, %	51	56.9	44.4	56.5	56.7	50.9	59.0
NFC, %	28.5	23.4	34.8	21.8	23.4	29.7	17.9
TDN, %	59.0	57.0	61.0	58.0	58.0	59.0	57.5
NEM, Mcal/lb	0.55	0.51	0.59	0.53	0.52	0.55	0.52
NEG, Mcal/lb	0.29	0.26	0.33	0.27	0.27	0.30	0.26
RFV	116	97	133	104	97	109	97

\*Ration Balancer analysis by Dairy One, 730 Warren Road, Ithaca, NY 14850. NEG, net energy gain; NEM, net energy maintenance; NFC, nonfiber carbohydrates; RFV, relative feed value.

†Average values for two separate hay collections during the preconditioning feeding phase.



**Figure 1.** Experimental design for assessing the end-product alterations during *in vitro* mixed ruminal microorganism fermentation when goat rumen fluid was dosed with different concentrations of camphor and incubated for 24 h. The only entire experimental breakdown is shown for the HJC goats fed a juniper-supplemented diet, all other B × D followed the same approach for each of the four B × D combinations. 0, goats fed a juniper free diet; 30, goats fed a juniper-supplemented diet; HJC, high juniper consuming goats; LJC, low juniper consuming goats; RF, rumen fluid.

breeding value (EBV) calculated from fNIRS determinations of percentage juniper in their diet and pedigree information. EBV and fNIRS determined the percent juniper in their diet just prior to the initiation of this study, and it was 13 and 50, respectively, for the HJC goats, and -14 and 16 for the LJC goats used in this study. All goats had similar initial and final body weights 52.24 ( $P = 0.87$ ) and 54.80 kg ( $P = 0.83$ ), respectively.

### Diet and Time of Feeding

Both HJC and LJC goats received one of two diets for 21 d prior to rumen fluid collection. Tifton 85 and late bloom alfalfa hay 1:1 wt:wt ratio were ground in a tub grinder. Small stems (<1 cm) and 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 previously ground hay mixture were combined 3:7 wt:wt dry weight basis, and were hammermilled with a wood and leaf chipper and stored at 5 °C until feed. Goats were fed twice daily at 0800 and 1600 CST.

### Rumen Fluid Collection and Dosing

Ruminal fluid was added (33% v/v) from each bloodline × diet flask to anoxic media as described in [Cotta and Russell \(1982\)](#) and [Callaway and Martin \(1996\)](#). Media composition was 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  $CaCl_2 \cdot 2H_2O$ , 4000 mg of  $Na_2CO_3$ , and 600 mg/L of cysteine-HCl. Each inoculated ruminal fluid per media flask was equilibrated and mixed with  $O_2$ -free  $CO_2$  for 10 min 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, and 200 µL of camphor stock solution was added to each appropriate fermentation tube to achieve a final concentration of 1.97 mM camphor. Additional dosage levels were diluted or concentrated to represent different inoculation levels of camphor based on the 300 mg/L, 1.97 mM

concentration. The camphor concentration used in the study was calculated using the following assumptions: 45.36 kg BW goat (with a 10-L ruminal volume) that consumed 3% BW of a diet composed of 30% *J. ashei* J. Buchholz, Ashe Juniper leaves are approximately 2% VO (DM basis), and 44% (on average) 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 dose (200  $\mu$ L) of 95% ethanol. The individual in vitro fermentation tubes were incubated at 39 °C for 0 or 24 h and were frozen (–20 °C) immediately.

### 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 using similar methods described in Goetsch and Galyean (1983) and Lourenco et al. (2020). Briefly, 2 mL from each sample was centrifuged at 10,000  $\times$  g for 10 min, and then the supernatant was frozen (–20 °C), and 5 mL of raw sample was transferred to screw-thread vials, which were frozen before overnight shipment to Texas A&M University (College Station, TX) for camphor concentration analysis. For SCFA analysis, 1 mL of the supernatant was mixed with 0.2 mL of 25% (wt/vol) metaphosphoric acid, vortexed, and frozen overnight. Subsequently, samples were thawed for and centrifuged (10,000  $\times$  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  $\times$  0.32 mm  $\times$  0.25  $\mu$ m; Phenomenex Inc., Torrance, CA). The sample injection volume was 1.0  $\mu$ L, and helium was used as 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, isobutyrate, butyrate, iso-valerate, valerate, and caproic acids. Total SCFAs were calculated using the sum of acetate, propionate, butyrate, and valerate concentrations.

### Microbiome Analysis: DNA Extraction and Sequencing

Deoxyribonucleic acid (DNA) was extracted from the B  $\times$  D samples following the procedures described by Welch et al. (2020) with slight modifications. This procedure uses 250  $\mu$ L of the sample placed in 2-mL Lysing Matrix E tubes (MP Biomedicals LLC, Irvine, CA), which are homogenized using a QIAGEN vortex adapter (QIAGEN, Venlo, the Netherlands) to disrupt the cells. Enzymatic inhibition was achieved by using InhibitEX Buffer (QIAGEN, Venlo, the Netherlands), and DNA elution and purification were carried out using a spin column and a series of specialized buffers according to the manufacturer's specifications (QIAamp Fast DNA Stool Mini Kit; QIAGEN, Venlo, the Netherlands). Determination of DNA concentration and purity in the resulting eluate was performed spectrophotometrically using the Synergy LX Multi-Mode Microplate Reader in conjunction with the Take3 Micro-Volume Plate (BioTek Instruments Inc; Winooski, VT). Samples with a minimum volume of 100  $\mu$ L and 10 ng/ $\mu$ L of

DNA were stored at 4 °C until the following day. Samples that failed to meet these requirements were rejected and subjected to a new DNA extraction cycle.

Following DNA extraction, samples were taken to the Georgia Genomics and Bioinformatics Core (<https://dna.uga.edu>) for library preparation and 16S ribosomal ribonucleic acid gene sequencing. The library preparation step included polymerase chain reaction (PCR) replications using the forward: S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and reverse: S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') primer pairs (Klindworth et al., 2013), followed by a PCR clean-up using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN). A second PCR step was then carried out to attach Illumina's indices and sequencing adapters (Nextera XT Index Kit; Illumina Inc., San Diego, CA), followed by another PCR clean-up step using AMPure XP beads. Following this final library clean-up, the library was quantified using qPCR, and the nucleotides were sequenced using an Illumina MiSeq instrument and a MiSeq v3 reagent kit (Illumina Inc., San Diego, CA). A well-characterized bacteriophage PhiX genome (PhiX Control v3 Library; Illumina Inc., San Diego, CA) was used as a control for the sequencing runs.

The sequencing data were demultiplexed and converted to FASTQ files. Pair-end reads were set and merged using BBMerge Paired Read Merger v37.64 with default sensitivity and an expected insert size of 500 bp. The files were analyzed using QIIME pipeline v1.9.1 (Caporaso et al., 2010), quality-filtered according to the default values provided in QIIME's script "multiplpe\_split\_libraries\_fastq.py," merged into a single file, and converted into the FASTA format. Sequences were clustered based on operational taxonomic units (OTUs) at 97% similarity using the Uclust OTU picking method and the Greengenes database (gg\_13\_8\_otus). Samples that did not align to PyNASt were excluded from the analysis. Sequencing depth was set at 3,148 sequences per sample.

### Analysis of Postfermentation Camphor Concentrations

Experimental methodology used to quantify levels of camphor before and after the present in vitro trial is defined in Lee et al. (2012). Stock solution preparations were carried out with 100  $\mu$ g/mL of camphor (Sigma-Aldrich, St. Louis, MO) was dissolved in ethyl acetate and further diluted in solvent to 1  $\mu$ g/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 cartridges equipped with a 10-mL solvent reservoir was preconditioned with 5 mL of methanol and filled with 10 mL of deionized water wash. 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  $\mu$ g/mL) vs. 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 GLM procedure in SAS 9.4 (SAS Inst., Inc., Cary, NC), fitting a straight nested experimental design for all SCFA data. An analysis of variance (ANOVA) was used to compare all end-product data between nested design factors. The experimental unit of the in vitro portion of this study was Balch tubes representing each grouping of goats within their respective bloodline and diet combination, B × D (f); LJC0, LJC30, HJC0, and HJC30 ( $f = 4$ , d.f.=3). Total observations were 48 (d.f. = 47), where six dosage levels (0, 0.25, 0.49, 0.99, 1.97, or 5.91 mM of camphor) for 24 h at 39.0 °C. An additional GLM procedure was used for comparison of control samples (0 mM camphor) at 0 and 24 h of incubation. For the goat performance and microbiome data on the raw rumen fluid, 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 ANOVA was used to compare measured camphor concentrations between the different B × D groups at time zero to analyze postfermentation 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 version 10.0.1 was used for performing this particular analysis (Systat Software, Inc., San Jose, CA).

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. Although not statistically significant ( $P = 0.38$ ), there was a sizable numerical difference in average daily gain (kg/d) between goats fed a juniper-free diet (0.13 kg/d). Goats fed a diet containing juniper leaves (0.20 kg/d) for the 15-d intermediate time between weight collections between Periods 1 and 2 (Table 2). The same animal performance data for the experimental goat herd are presented in the companion paper (Seidel et al., unpublished data).

### Total SCFAs, pH, and SCFA Proportions of Controls

Concerning in vitro tubes without camphor dosages, Table 3 highlights comparisons of each B × D combination control results for pH, total SCFAs, acetate to propionate ratio (A:P), and molar proportions. Although the pH was different for the B × D interaction, the pH range of 6.45 to 6.65 is a normal associated pH range for goats on a forage diet (Castro-Costa et al., 2015). Total SCFA data for the control samples exhibited a decrease in diet ( $P = 0.04$ ) shown by the juniper-enhanced groups having a lower total SCFA concentration in the control samples as compared juniper-free feed groups. The juniper-enhanced diet groups had higher A:P ratios ( $P < 0.001$ ), 4.95, compared to the juniper-free diet, 4.34. Although there were significances associated with various factors of molar proportions of controls in Table 3, the numerical representation of these differences was not different enough to establish biological interest in the results. Therefore, the purpose of examining differences in control tubes was to establish limited alterations between incubation time points. Generally speaking, the only B × D combination that was altered significantly was the increase in SCFA in the LJC30 group.

### Effects of Camphor on pH, Total SCFAs, and Acetate to Propionate Ratio

Final pH, the concentration of total SCFA, and A:P are shown in Table 4. Camphor concentrations impacted both total SCFA ( $P = 0.03$ ) and A:P ( $P = 0.005$ ). The SCFA data, all B × D groups exhibited an increase ( $P < 0.05$ ) from the control except for the LJC30 diet, which showed a decrease ( $P < 0.05$ ) from the control. Likewise, there was a total SCFA and A:P dietary effect, with increased ( $P < 0.05$ ) SCFA concentration for the goats fed a juniper-free diet and a subsequent decrease ( $P < 0.05$ ) in A:P for the same experimental group. However, there was a B × D interaction ( $P < 0.05$ ) for pH and SCFA concentrations, and there is no clear biochemical driver as to why this happened. As previously described in the results section for control comparisons, the pH data ranged from 6.45 to 6.57 and are well within the biological range of goat rumen fluid. Interestingly, there were no differences ( $P = 0.090$ ) for SCFA regarding bloodline effect.

A monoterpene effect ( $P < 0.05$ ) was detected for all molar proportions for acetate, propionate, and butyrate (Table 5). Acetate molar proportions increased ( $P < 0.05$ ), and propionate decreased ( $P < 0.05$ ) in the juniper-supplemented groups.

**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 Diet	LJC		HJC		SEM	P-value		
	0	30	0	30		Bloodline	Diet	B × D
<i>Item</i>								
Period 1 BW <sup>*</sup> , kg	50.46	52.16	51.82	55.22	2.91	0.60	0.55	0.84
Period 2 BW <sup>*</sup> , kg	51.94	55.11	54.09	58.06	3.34	0.60	0.46	0.93
ADG <sup>†</sup> , kg/d	0.10	0.20	0.15	0.19	0.05	0.76	0.38	0.69

Period 1 (4/23/2018) represents goat weights at the day 7 of the 21-d preconditioning diet, and Period 2 (5/8/2018) indicates goat weights the day of rumen fluid collection.

<sup>\*</sup>BW, body weight.

<sup>†</sup>ADG, average daily gain.

**Table 3.** pH, total short-chain fatty acid (SCFA) (mM), acetate to propionate ratio, and specific molar proportion of SCFA data from bloodline and diet combinations of goat rumen fluid control groups (0.00 mM camphor) and incubated for 0 or 24 h

Item	Treatment				SEM	P-value			
	LJC0	LJC30	HJC0	HJC30		B × D	Hour	B	D
pH					0.01	<0.001	<0.001	0.67	0.67
0 h	6.62	6.62	6.58	6.65					
24 h	6.56	6.45	6.47	6.54					
Total SCFA, mM					2.31	0.002	0.06	0.74	0.04
0 h	21.20	19.12	26.38	18.70					
24 h	20.97	29.96	32.14	16.31					
A:P					0.07	0.54	0.40	0.02	<0.001
0 h	4.33	4.84	4.47	5.09					
24 h	4.25	4.89	4.32	4.98					
Acetate, mmol/100 mmol					0.24	0.02	0.01	0.001	<0.001
0 h	74.46	76.39	76.18	76.81					
24 h	74.99	77.81	76.01	77.99					
Propionate, mmol/100 mmol					0.19	0.26	0.11	0.08	<0.001
0 h	17.20	15.77	17.05	15.15					
24 h	17.65	15.92	17.59	15.68					
Butyrate, mmol/100 mmol					0.08	<0.001	<0.001	<0.001	0.002
0 h	7.19	7.09	5.82	7.40					
24 h	6.39	5.71	5.58	5.88					

B × D, bloodline diet interaction.

Hour—time effect, all incubation time points nested within B × D group.

B, bloodline effect, LJC vs. HJC.

D, diet effect, J0 vs. J30.

A similar inverse dynamic between proportions of acetate and propionate was exhibited in the juniper-free fed goat groups. The molar proportion for butyrate increased in percentage as camphor concentrations increased. Although there was significance detected for the bloodline effect ( $P = 0.009$ ) of molar proportions of acetate, the numerical values remained relatively small.

### Postfermentation Camphor Concentration

Camphor concentrations in samples incubated for 24 h at 39 °C are shown in Figure 2. There were no differences ( $P > 0.05$ ) for the control group at camphor dosage levels of 0.25, 0.49, 0.99, and 1.97 mM. The only treatment differences are shown in the 5.91 mM dosage level of camphor. Contrary to hypothesized degradation potential, in the 5.91 mM inoculation group, the LJC0 diet had less ( $P < 0.05$ ) camphor than all other B × D groups. The average postfermentation output concentration of camphor for the LJC0 was 572.1 µg/mL vs. LJC30 (644.5 µg/mL), HJC0 (662.6 µg/mL), and HJC30 (652.1 µg/mL).

### Microbiome

As previously mentioned, sequencing depth was set at 3,148 sequences per sample to analyze the ruminal microbiota. Microbial richness (number of [OTUs] and Chao1) and microbial diversity are shown for each B × D group (Table 6). The juniper free- and juniper-added diets were distinctly different in their microbial populations. Overall, HJC goats had greater ruminal microbial richness and diversity. Faith's phylogenetic diversity index, a measure of both richness and

diversity, was greater from goats selected for HJC. Juniper addition to the diets increased phylogenetic diversity. The number of observed OTUs differed between bloodline and diet groups with HJC goats and goats fed a juniper diet exhibiting the highest number of unique OTUs. Conversely, there were no differences in microbial richness expressed as Chao1 index. Interestingly, the LJC bloodline fed a juniper-free diet exhibited the lowest level of alpha diversity in all output indexes.

Profiles of the microbial relative abundances for each B × D group at the phylum and family taxonomic levels (Figures 3 and 4). In addition, all table representations of taxonomic abundances are shown in Supplementary Tables 1–3. This is the first profiling of microbiome data for these respective goat bloodlines. The most abundant phyla within liquid fractions of goat ruminal fluid were Bacteroidetes and Firmicutes, and ranged from 47.47% to 65.52% and 25.98% to 43.31%, respectfully, which alone account for 87.48% to 91.50% of overall microbial relative abundance (Figure 3). The overall averages of each phylum were calculated across all B × D groups, and all percentages under 0.7% were added together and categorized as Phylum\_Other. Only juniper-fed goats exhibited increases in TM7 and Actinobacteria abundance at the phylum level, and no differences were identified between Euryarchaeota, Bacteroidetes, or Firmicutes.

At the family level, all unidentifiable classes and orders, phyla categorized previously as Phylum\_Other, and family averaged abundances across B × D groups less than 0.7% were combined into the Family\_Other percentage. In addition, all unidentifiable families with identified orders were

**Table 4.** Effect of camphor addition (0, 0.25, 0.49, 0.99, 1.97, or 5.91 mM) on pH, total short-chain fatty acid concentrations (mM), and the acetate to propionate ratio from separate in vitro mixed ruminal microorganism fermentations incubated for 24 h at 39 °C

Item	Treatment				SEM	P-value			
	LJC0	LJC30	HJC0	HJC30		B×D	MO	B	D
pH					0.02	<.001	0.182	0.467	0.715
0.00 mM	6.56	6.45	6.47	6.54					
0.25 mM	6.56	6.45	6.45	6.55					
0.49 mM	6.56	6.49	6.51	6.60					
0.99 mM	6.55	6.51	6.46	6.57					
1.97 mM	6.54	6.51	6.46	6.57					
5.91 mM	6.54	6.45	6.54	6.55					
Total SCFA, mM					2.90	0.009	0.028	0.090	<.001
0.00 mM	20.97	29.96	32.14	16.31					
0.25 mM	30.19	21.48	34.81	23.94					
0.49 mM	32.04	25.90	38.63	30.10					
0.99 mM	33.32	18.72	40.01	25.47					
1.97 mM	32.68	26.68	29.90	20.89					
5.91 mM	31.36	27.60	37.67	26.09					
A:P					0.07	0.791	0.005	0.470	<.001
0.00 mM	4.25	4.88	4.32	4.98					
0.25 mM	4.29	5.02	4.28	5.02					
0.49 mM	4.30	5.00	4.27	4.78					
0.99 mM	4.26	4.79	4.28	5.00					
1.97 mM	4.12	4.75	4.19	4.74					
5.91 mM	4.13	4.58	4.20	4.60					

B × D, bloodline diet interaction across time and MO combinations.

MO, monoterpene effect, control vs. camphor nested within B × D group.

B, bloodline effect, LJC vs. HJC.

D, diet effect, J0 vs. J30.

classified by their respective order association and label \_ Unassigned. Of the identifiable families, *Prevotellaceae* was the family with the greatest overall abundance, ranging from 15.14% for LJC0 to 36.17% for HJC0 (Figure 4), where abundance was higher for HJC goats. Interestingly, the second most identifiable family was *Ruminococcaceae* and showed a substantial increase in abundance in rumen fluid when goats received a juniper-free diet vs. a juniper-added diet. Besides *Prevotellaceae*, there was a B × D interaction for the families RF16, *Paraprevotellaceae*, and *Veillonellaceae*. Like *Ruminococcaceae*, there was only a dietary effect for the families *Coriobacteriaceae*, *Streptococcaceae*, and F16. Further identification of microbial abundance at the genus level (Supplementary Figure 1) only yielded two identifiable genera contributing a major respective percentage to the family level abundance data; *Prevotella* from the family *Prevotellaceae* and *Streptococcus* from the family *Streptococcaceae*. Both genera had the same treatment significance as the family counterparts.

## DISCUSSION

Camphor concentrations from 0.25 to 5.91 mM increased SCFA concentrations compared to controls in three of the four B × D experimental groups. Camphor concentrations used in this experiment (0.25 to 5.91 mM; 37.5 to 900 mg/L) were similar to previous values in small ruminant animals models fed *Juniperus* sp. without adverse health or growth performance

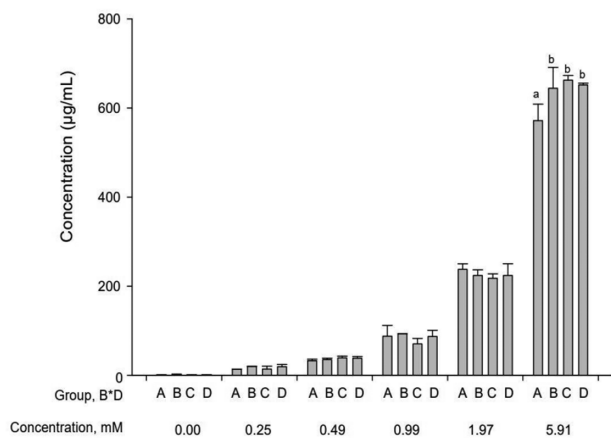
effects: 0.719 g to 3.6 g oil/d (Animut et al., 2004; Whitney et al., 2014). Camphor is a potential causative agent for feeding deterrence in snowshoe hares (Sinclair et al., 1988) and sheep (Estell et al., 1998). Our present study demonstrated that overall microbial end-product concentrations were not adversely affected by camphor. Campbell et al. (2010) suggested that selecting goats with higher juniper consumption preference may have been an inherent pharmacological tolerance for phytochemicals. Moreover, our findings suggest that the mechanism of tolerance is not related to microbial degradation activity or limited by decreases in SCFA concentrations that affect the energetic status of the host animal. SCFAs account for 60% to 70% of the energetic requirements for the maintenance of ruminants (Stewart et al., 1958; Bergman, 1990), and CO<sub>2</sub> account for 82% of carbon metabolism in ruminants (Hungate et al., 1961).

Camphor (and VO) impacts on the ruminal fermentation are critical to our understanding of the effect of high-phytochemical content forages in small ruminant diets. The rumen microbial consortium is a dynamic ecological system that can ferment complex and simple carbohydrates and amino acids, and the presence of this ruminal ecosystem provides an ecological niche for ruminant animals as browsers and grazers (Allison and Cook, 1981; Allison et al., 1992; Nelson et al., 1998; Andrew et al., 2000). The process of ruminal microbial fermentation is driven primarily by substrate availability and substrate fermentation potential (Murphy et al., 1982; Russell, 2002; Nam et al., 2009).

**Table 5.** Effect of camphor addition (0, 0.25, 0.49, 0.99, 1.97, or 5.91 mM) on acetate, propionate, and butyrate molar proportion of total short fatty acid concentrations (mM) from separate in vitro mixed ruminal microorganism fermentations incubated for 24 h

Item	Treatment				SEM	P-value			
	LJC0	LJC30	HJC0	HJC30		B×D	MO	B	D
Acetate, mmol/100 mmol					0.31	<.001	0.001	0.009	<.001
0.00 mM	74.99	77.81	76.01	77.99					
0.25 mM	75.08	78.19	75.83	78.02					
0.49 mM	75.10	78.18	75.82	77.05					
0.99 mM	75.04	77.23	75.85	77.93					
1.97 mM	74.30	77.21	75.40	76.72					
5.91 mM	73.99	76.44	75.22	75.96					
Propionate, mmol/100 mmol					0.19	0.254	0.010	0.979	<.001
0.00 mM	17.65	15.92	17.59	15.68					
0.25 mM	17.49	15.60	17.73	15.54					
0.49 mM	17.45	15.64	17.77	16.12					
0.99 mM	17.61	16.11	17.74	15.58					
1.97 mM	18.03	16.25	17.98	16.20					
5.91 mM	17.92	16.68	17.90	16.54					
Butyrate, mmol/100 mmol					0.12	<.001	<.001	<.001	0.421
0.00 mM	6.39	5.71	5.58	5.88					
0.25 mM	6.45	5.65	5.58	5.98					
0.49 mM	6.48	5.62	5.57	6.33					
0.99 mM	6.40	6.05	5.57	6.03					
1.97 mM	6.67	5.96	5.75	6.55					
5.91 mM	7.03	6.27	5.99	6.95					

B × D, bloodline diet interaction across time and MO combinations.  
 MO, monoterpene effect, control vs. camphor nested within B × D group.  
 B, bloodline effect, LJC vs. HJC.  
 D, diet effect, J0 vs. J30.



**Figure 2.** Camphor concentrations (µg/mL) remaining in mixed ruminal microorganism fermentations for each respective B × D and incubated for 24 h. <sup>A</sup> LJC0, <sup>B</sup> LJC30, <sup>C</sup> HJC0, <sup>D</sup> HJC30. <sup>a–g</sup> Within a concentration group, means containing a different letter differ ( $P < 0.05$ );  $n = 2$  for each B × D within concentration group.

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 availability in the mixed ruminal microorganism fermentations without exogenous addition of substrate (e.g., glucose, starch, or cellulose). Antinutritional compounds in plants can be degraded by ruminal microbes, as best exemplified by detoxification of

the free amino acid mimosine in *Leucaena leucocephala* by the addition of *Synergistes jonesii* as a probiotic (Allison et al., 1990), *Streptococcus caprinus* sp. nov. growth in media containing at least 2.5% w/v tannic acid extracted from *Acacia aneura* (Brooker et al., 1994), and degradation of oxalic acid by goats (Duncan et al., 1997). Although the mode of action of camphor degradation is not fully understood, HJC goats had greater terpene clearance rates than low consumption goats intraruminally dosed with a terpene (49.5% camphor) cocktail (Campbell et al., 2010). Host hepatic phase I and II enzymes account for dietary terpene clearance when concentrations reach a threshold level (Torregrossa and Dearing, 2009). We assessed whether the ability of HJC goats to consume more juniper was related to an inherent difference in their ruminal microbial population composition or to a change in enzymatic activity of the goat.

Camphor's impact on microbial populations was not the same across all B × D groups because diet is a major determinant of ruminal bacteria population (Russell, 2002; Henderson et al., 2015). Consumption affinity and animal behavior are potential factors impacting microbiome shifts. However, it is important to note that the HJC0 goats exhibited the highest average total SCFA concentrations at all camphor concentrations, and this inocula had the highest percentage of *Prevotella* (Supplementary Table 3). *Prevotella* sp. has broad substrate utilization capabilities (Russell, 2002; Shabat et al., 2016), which is a valuable microbial advantage when substrate availability is limited.

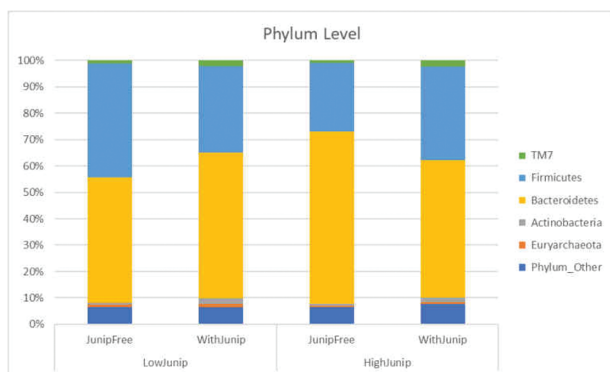


**Table 6.** Alpha diversity indexes of bacterial richness (OTUs and Chao1), diversity (FaithsPD and Shannon), and evenness

Item	Low Juniper Consuming		High Juniper Consuming		SEM
	Juniper free	Juniper added	Juniper free	Juniper added	
*OTUs	911.10	954.05	949.95	1034.60	17.33
Chao1	2067.97	2446.57	2409.33	2737.65	95.73
†FaithsPD	67.16	69.42	68.95	76.17	1.31
Shannon	7.98	8.51	8.36	8.63	0.09

\*OTUs, number of operational taxonomic units.

†FaithsPD, faith's phylogenetic diversity.

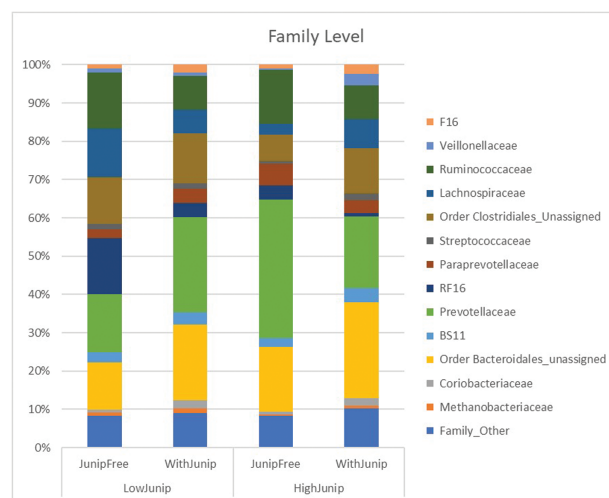
**Figure 3.** Bloodline-by-diet profiles of meat goat showing taxonomic differences in abundance at the phylum level of experimental groups.

Greater bacterial richness and diversity in the HJC goats likely results in having more catabolic genes present, representing a greater diversity of metabolic pathways and more relevant output metabolites (Shabat et al., 2016). This may provide greater biochemical diversity to degrade and utilize a wider array of nutrients that would otherwise be toxic or underutilized.

Volatile oils less frequently impact gram negative bacterial species. Thus, ruminants fed a diet containing more volatile oils are thought to have a higher abundance of gram-negative bacteria (Patra and Yu, 2015). Findings indicate the population of the gram-negative *Prevotellaceae* was greater in the HJC goats, corroborates findings of increased *Prevotella* populations in the presence of volatile oils (Patra and Yu, 2015).

Patra and Yu (2015) found that *Ruminococcaceae* were lower when volatile oils were fed, which contradicts our findings of *Ruminococcaceae* being the highest in juniper free diets. Many members of *Ruminococcaceae* are connected with ruminal fiber catabolism (Koike and Kobayashi, 2009). Decreased *Ruminococcaceae* in the presence of volatile oils are present is thought to be a result of the decrease in fiber degradation that occurs with the inclusion of volatile oils (Patra and Yu, 2012) since this bacterial family is made up of several cellulolytic and hemicellulolytic species (Helaszek and White, 1991; Biddle et al., 2013).

Similar overall results for SCFA concentrations have been indicated in a similar in vitro fermentation system using a goat ruminal fluid inoculum (Whitney et al., 2011). The two experimental goat bloodlines used in this study have been previously investigated, which found numerically higher ruminal

**Figure 4.** Bloodline by diet profiles of meat goat showing taxonomic differences in abundance at the family-level of experimental groups.

SCFA concentrations in HJC goats fed a juniper-containing diet vs. LJC goats exhibiting an increased adaption to juniper diets (Stewart et al., 2019). We hypothesized that HJC goats fed a preconditioning diet containing juniper (HJC30) would have the most robust and adapted rumen microbial populations when fed plant secondary compounds (e.g., camphor) found in *J. ashei* and *J. pinchottii*. However, the present data do not support that hypothesis, as bloodline was did not impact SCFA production, and the HJC30 diet had the lowest average SCFA produced in in vitro fermentations. Results suggested that diet type was a greater influence on SCFA production than “camphor tolerance” when examining ruminal in vitro fermentations.

## CONCLUSION

A distinct difference was observed between primary treatment group influences for goat rumen fluid dosed with camphor in vitro. Diet was more consistently impactful on SCFA concentrations from juniper-free diets producing greater levels of SCFAs. The reduction in SCFA concentration for the juniper-fed goats, however, does not alone suggest changes in animal performance. There were no differences in body weight or average daily gain for all experimental periods, and juniper-fed goats were fed small stems (<1 cm) and leaves of *J. ashei* for 21 d at 30% dry matter daily intake, equating to approximately 1.97 mM (300 mg/L) camphor daily. Therefore, the in vitro results demonstrate that the inclusion of camphor did not suggest HJC goats had greater levels of SCFAs, other phytochemicals than camphor, or induced hepatic metabolism within the animal may be more impactful in the development of phytochemical tolerance. However, to fully understand camphor's direct role in impacting rumen fermentation end products, experimental dosage levels must be assessed in live animals to elucidate the impact on consumption patterns and animal performance.

## Funding

D.S.S. and C.B.W. assistantships were supported by the National Science Foundation: Grant No. DGE-1545433.

## Conflict of Interest

Authors declare no direct or indirect financial interests involved in the results of this publication.

## LITERATURE CITED

- Adams, R. P., J. P. Muir, C. A. Taylor, and T. R. Whitney. 2013. Differences in chemical composition between browsed and non-browsed *Juniperus ashei* Buch. Trees. *Biochem. Syst. Ecol.* 46:73–78. doi:10.1016/j.bse.2012.09.020.
- Allison, M. J., and H. M. Cook. 1981. Oxalate degradation by microbes of the large bowel of herbivores: the effect of dietary oxalate. *Science*. 212:675–676. doi:10.1126/science.7221555.
- Allison, M. J., A. C. Hammond, and R. J. Jones. 1990. Detection of ruminal bacteria that degrade toxic dihydroxypyridine compounds produced from mimosine. *Appl. Environ. Microbiol.* 56:590–594. doi:10.1128/aem.56.3.590-594.1990.
- Allison, M. J., W. R. Mayberry, C. S. McSweeney, and D. A. Stahl. 1992. *Synergistes jonesii*, gen. nov., sp. nov.: a rumen bacterium that degrades toxic pyridinediols. *Syst. Appl. Microbiol.* 15:522–529. doi:10.1016/s0723-2020(11)80111-6.
- Andrew, W. M., M. J. Williams, M. J. Allison, S. W. Coleman, J. Chase, C. G. Chambliss, R. S. Kalmbacher, and W. E. Kunkle. 2000. Detection of *Synergistes jonesii* in cattle and sheep feces. *Amer. Forage Grassland Conf. Proc.* 9:165–168.
- Animut, G., A. Goetsch, R. Estell, R. Merkel, L. Dawson, T. Sahl, and R. Puchala. 2004. Effects of methods of exposure to Eastern red cedar foliage on cedar consumption by Boer crossbred wether goats. *Small Rumin. Res.* 54:197–212. doi:10.1016/j.smallrumres.2003.11.006.
- Ansley, R. J., and G. A. Rasmussen. 2005. Managing native invasive juniper species using fire. *Weed Technol.* 19:517–522. <https://www.jstor.org/stable/3989470>
- Bergman, E. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70:567–590. doi:10.1152/physrev.1990.70.2.567
- Biddle, A., L. Stewart, J. Blanchard, and S. Leschine. 2013. Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity*. 5:627–640. doi:10.3390/d5030627.
- Brooker, J., L. O'donovan, I. Skene, K. Clarke, L. Blackall, and P. Muslera. 1994. *Streptococcus caprinus* sp. nov., a tannin-resistant ruminal bacterium from feral goats. *Lett. Appl. Microbiol.* 18:313–318. doi:10.1111/j.1472-765x.1994.tb00877.x.
- Callaway, T. R., and S. A. Martin. 1996. Effects of cellobiose and monensin on in vitro fermentation of organic acids by mixed ruminal bacteria. *J. Dairy Sci.* 80:1126–1135. doi:10.3168/jds.S0022-0302(97)76039-9
- Campbell, E., B. Engdahl, C. Lupton, C. Scott, C. Taylor, Jr, D. Waldron, J. Walker, and W. Whitworth. 2007. The use of fecal NIRS to identify levels of consumption of the toxic shrub juniper in a selective breeding program with goats. In: *Poisonous plants: global research and solutions*. Wallington, Oxon, United Kingdom: CABI Publishing; p. 386–393.
- Campbell, E. J., R. A. Frost, T. K. Mosley, J. C. Mosley, C. J. Lupton, C. A. Taylor, J. W. Walker, D. F. Waldron, and J. Musser. 2010. Pharmacokinetics differences in exposure to camphor after intraruminal dosing in selectively bred lines of goats. *J. Anim. Sci.* 88:2620–2626. doi:10.2527/jas.2009-2585.
- Campbell, E., and C. Taylor, Jr. 2007. Monoterpene production in redberry juniper foliage following fire. Panter, K. E., Wierenga, T. L., and Pfister, J. A. (eds) *Rangel. Ecol. Manag.* 60:104–109. doi:10.2111/04-160R2.1
- Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, and J. I. Gordon. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7:335–336. doi:10.1038/nmeth.f.303
- Castro-Costa, A., A. Salama, X. Moll, J. Aguiló, and G. Caja. 2015. Using wireless rumen sensors for evaluating the effects of diet and ambient temperature in nonlactating dairy goats. *J. Dairy Sci.* 98:4646–4658. doi:10.3168/jds.2014-8819.
- Cotta, M. A., and J. B. Russell. 1982. Effect of peptides and amino acids on efficiency of rumen bacterial protein synthesis in continuous culture. *J. Dairy Sci.* 65:226–234. doi:10.2527/2003.811329x
- Domínguez-Bello, M. 1996. Detoxification in the rumen. 45(1):23–327.
- Duncan, A. J., P. Frutos, and S. A. Young. 1997. Rates of oxalic acid degradation in the rumen of sheep and goats in response to different levels of oxalic acid administration. *Anim. Sci.* 65:451–455. doi:10.1017/s135772980000864x.
- Dunson, W. T., M. A. Carr, T. R. Callaway, C. A. Taylor, Jr, C. B. Scott, and E. S. Campbell. 2007. Rumen function and the ability of goats to consume redberry juniper (*Juniperus pinchottii*) [electronic resource]. In: K.E. Panter, T.L. Wierenga, J.A. Pfister, editors. *Poisonous plants: global research and solutions*. Wallingford, Oxfordshire; Cambridge, MA: CABI International. p. 377–385.
- Estell, R., E. Fredrickson, M. Tellez, K. Havstad, W. Shupe, D. Anderson, and M. Remmenga. 1998. Effects of volatile compounds on consumption of alfalfa pellets by sheep. *J. Anim. Sci.* 76:228–233. doi:10.1007/s10886-014-0389-1
- Estell, R., S. Utsumi, A. Cibils, and D. Anderson. 2014. Is differential use of *Juniperus monosperma* by small ruminants driven by terpenoid concentration? *J. Chem. Ecol.* 40:285–293.
- Foley, W., G. Iason, and C. McArthur. 1999. Role of plant secondary metabolites in the nutritional ecology of mammalian herbivores: how far have we come in 25 years? In: Vth International Symposium on the Nutrition of Herbivores. American Society of Animal Science, Savoy, IL, p. 130–209.
- Frost, R. A., K. L. Launchbaugh, and C. A. Taylor, Jr. 2008. Age and body condition of goats influence consumption of juniper and monoterpene-treated feed. *Rangel. Ecol. Manag.* 61:48–54. doi:10.2111/06-160R1.1
- Goetsch, A., and M. Galyean. 1983. Influence of feeding frequency on passage of fluid and particulate markers in steers fed a concentrate diet. *Can. J. Anim. Sci.* 63:727–730. doi: 10.4141/cjas83-084
- Helaszek, C. T., and B. A. White. 1991. Cellobiose uptake and metabolism by *Ruminococcus flavefaciens*. *Appl. Environ. Microbiol.* 57:64–68. doi:10.1128/aem.57.1.64-68.1991.
- Henderson, G., F. Cox, S. Ganesh, A. Jonker, W. Young, L. Abecia, E. Angarita, P. Aravena, G. N. Arenas, and C. Ariza. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi:10.1038/srep14567
- Hungate, R., R. A. Mah, and M. Simesen. 1961. Rates of production of individual volatile fatty acids in the rumen of lactating cows. *Appl. Microbiol.* 9:554–561. doi: 10.1128/am.9.6.554-561.1961
- Ishaq, S. L., C. J. Yeoman, and T. Whitney. 2017. Ground *Juniperus pinchottii* and urea in supplements fed to Rambouillet ewe lambs Part 2: ewe lamb rumen microbial communities. *J. Anim. Sci.* 95:4587–4599. doi:10.2527/jas2017.1731
- Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, and F. O. Glöckner. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi:10.1093/nar/gks808
- Koike, S., and Y. Kobayashi. 2009. Fibrolytic rumen bacteria: their ecology and functions. *Asian-Australas. J. Anim. Sci.* 22:131–138. doi:10.5713/ajas.2009.r.01.
- Kronberg, S. L., and J. W. Walker. 1993. Ruminal metabolism of leafy spurge in sheep and goats: a potential explanation for differential foraging on spurge by sheep, goats, and cattle. *J. Chem. Ecol.* 19:2007–2017. doi:10.1007/BF00983803.
- Lee, K. -M., S. Y. Dai, T. J. Herrman, and J. M. Musser. 2012. A gas chromatography–mass spectrometry assay to quantify camphor extracted from goat serum. *J. Chromatogr. B* 905:133–136. doi:10.1016/j.jchromb.2012.07.039

- Lourenco, J. M., T. J. Kieran, D. S. Seidel, T. C. Glenn, M. F. d. Silveira, T. R. Callaway, and R. L. Stewart Jr. 2020. Comparison of the ruminal and fecal microbiotas in beef calves supplemented or not with concentrate. *PLoS One* 15(4):e0231533.
- Malecky, M., H. Albarello, and L. P. Broudiscou. 2012. Degradation of terpenes and terpenoids from Mediterranean rangelands by mixed rumen bacteria in vitro. *Animal*. 6:612–616. doi:10.1017/S1751731111001947.
- Markó, G., V. Gyuricza, J. Bernáth, and V. Altbacker. 2008. Essential oil yield and composition reflect browsing damage of junipers. *J. Chem. Ecol.* 34:1545–1552. doi:10.1007/s10886-008-9557-5.
- Murphy, M. R., R. L. Baldwin, and L. J. Koong. 1982. Estimation of stoichiometric parameters for rumen fermentation of roughage and concentrate diets. *J. Anim. Sci.* 55:411–421. doi:10.2527/jas1982.552411x.
- Nam, I. S., P. C. Garnsworthy, and J. H. Ahn. 2009. Effects of freeze-dried citrus peel on feed preservation, aflatoxin contamination and in vitro ruminal fermentation. *Asian-Australas. J. Anim. Sci.* 22:674–680. doi:10.5713/ajas.2009.80419
- Nelson, K. E., M. L. Thonney, T. K. Woolston, S. H. Zinder, and A. N. Pell. 1998. Phenotypic and phylogenetic characterization of ruminal tannin-tolerant bacteria. *Appl. Environ. Microbiol.* 64:3824–3830. doi:10.1128/AEM.64.10.3824-3830.1998.
- Owens, M. K., C.-D. Lin, C. A. Taylor, and S. G. Whisenant. 1998. Seasonal patterns of plant flammability and monoterpenoid content in *Juniperus ashei*. *J. Chem. Ecol.* 24(12):2115–2129. doi:10.1023/a:1020793811615
- Palmer, B., R. J. Jones, S. Poathong, and J. Chobtang. 2010. Within-country variation in the ability of ruminants to degrade DHP following the ingestion of *Leucaena leucocephala*—a Thailand experience. *Trop. Anim. Health Prod.* 42:161–164. doi:10.1007/s11250-009-9398-4
- Patra, A. K., and Z. Yu. 2012. Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. *Appl. Environ. Microbiol.* 78:4271–4280. doi: 10.1128/AEM.00309-12
- Patra, A. K., and Z. Yu. 2015. Essential oils affect populations of some rumen bacteria in vitro as revealed by microarray (RumenBactArray) analysis. *Front. Microbiol.* 6:297. doi: 10.1128/AEM.00309-12
- Prates, A., J. A. de Oliveira, L. Abecia, and M. Fondevila. 2010. Effects of preservation procedures of rumen inoculum on in vitro microbial diversity and fermentation. *Anim. Feed Sci. Technol.* 155:186–193. doi:10.1016/j.anifeedsci.2009.12.005.
- Riddle, R. R., C. Taylor, M. Kothmann, and J. Huston. 1996. Volatile oil contents of ashe and redberry juniper and its relationship to preference by Angora and Spanish goats. *Rangel. Ecol. Manag./J. Range Manag. Arch.* 49:35–41. doi:10.2307/4002722
- Russell, J. B. 2002. *Rumen microbiology and its role in ruminant nutrition*. Ithaca, NY.
- Shabat, S. K. B., G. Sasson, A. Doron-Faigenboim, T. Durman, S. Yaacoby, M. E. Berg Miller, B. A. White, N. Shterzer, and I. Mizrahi. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10(12):2958–2972. doi: 10.1038/ismej.2016.62
- Sinclair, A., M. K. Jogle, and R. J. Andersen. 1988. Camphor from juvenile white spruce as an antifeedant for snowshoe hares. *J. Chem. Ecol.* 14:1505–1514. doi: 10.1007/BF01012422
- Stewart, W., D. G. Stewart, and L. Schultz. 1958. Rates of volatile fatty acid production in the bovine rumen. *J. Anim. Sci.* 17:723–736. doi:10.2527/jas1958.173723x
- Stewart, W., T. Whitney, E. Scholljegerdes, D. Waldron, J. Walker, R. Adams, and J. Musser. 2019. Ruminal fermentation characteristics of goats selectively bred to have greater consumption of juniper foliage. *Anim. Feed Sci. Technol.* 256:114240. doi:10.1016/j.anifeedsci.2019.114240.
- Taylor, C. A. 2006. *Targeted grazing to manage fire risk*. Englewood, CO: Am. Sheep Ind. Assoc.
- Taylor, C. A. 2008. *Ecological consequences of using prescribed fire and herbivory to manage Juniperus encroachment, Western North American Juniperus Communities*. Springer. New York, NY. p. 239–252.
- Taylor, C., K. Launchbaugh, E. Huston, E. Straka, and R. Pritz. 1997. Improving the efficacy of goating for biological juniper management. In: *Juniper Symposium Proceedings*. San Angelo, Tex. Texas Agr. Exp. Sta. p. 5.17–15.22.
- Torregrossa, A. -M., and M. D. Dearing. 2009. Nutritional toxicology of mammals: regulated intake of plant secondary compounds. *Funct. Ecol.* 23:48–56. doi:10.1111/j.1365-2435.2008.01523.x.
- Waldron, D., C. Taylor, Jr, J. Walker, E. Campbell, C. Lupton, T. Willingham, and S. Landau. 2009. Heritability of juniper consumption in goats. *J. Anim. Sci.* 87:491–495. doi: 10.2527/jas.2008-1140
- Walker, J., E. Campbell, C. Lupton, C. Taylor, Jr, D. Waldron, and S. Landau. 2007. Effects of breed, sex, and age on the variation and ability of fecal near-infrared reflectance spectra to predict the composition of goat diets. *J. Anim. Sci.* 85:518–526. doi: 10.2527/jas.2006-202
- Welch, C. B., J. M. Lourenco, D. B. Davis, T. R. Krause, M. N. Carmichael, M. J. Rothrock, T. D. Pringle, and T. R. Callaway. 2020. The impact of feed efficiency selection on the ruminal, cecal, and fecal microbiomes of Angus steers from a commercial feedlot. *J. Anim. Sci.* 98:skaa230. doi:10.1093/jas/skaa230
- Whitney, T., C. Lupton, J. Muir, R. Adams, and W. Stewart. 2014. Effects of using ground redberry juniper and dried distillers grains with solubles in lamb feedlot diets: growth, blood serum, fecal, and wool characteristics. *J. Anim. Sci.* 92:1119–1132. doi: 10.2527/jas.2013-7007
- Whitney, T. R., A. E. Lee, D. R. Klein, C. B. Scott, T. M. Craig, and J. P. Muir. 2011. A modified in vitro larvae migration inhibition assay using rumen fluid to evaluate *Haemonchus contortus* viability. *Vet. Parasitol.* 176:217–225. doi:10.1016/j.vetpar.2010.10.050.