Running Title: Rumen papillae morphology and gain and intake

**Rumen papillae morphology of beef steers relative to gain and feed intake and the
association of volatile fatty acids with *kallikrein* gene expression¹**

R. J. Kern^{*†}, A. K. Lindholm-Perry^{†,2}, H. C. Freetly[†], L. A. Kuehn[†],
D. C. Rule^{*}, and P. A. Ludden^{*}

^{*}Department of Animal Science, University of Wyoming, Laramie, WY, USA 82070

[†]USDA, ARS, U.S. Meat Animal Research Center, Clay Center, NE, USA 68933

² Address for correspondence: Rebecca Kern; Email: Rebecca.Kern@ARS.USDA.GOV;

Current address: USDA, ARS, U.S. Meat Animal Research Center, Clay Center, NE

ABSTRACT: Feed costs are the most expensive input in beef production. Improvement in the ability of beef cattle to convert feed into meat would lower feed inputs and reduce the cost of production. The rumen epithelium is responsible for absorption and metabolism of nutrients and microbial by-products, and may play a significant role in gain or feed intake. Our objective was to determine the relationships among rumen papillae morphology, gene expression, volatile fatty acid concentrations, and gain and feed intake. Average daily gain (ADG) and average daily feed intake (ADFI) were collected on a crossbred population of beef steers over three feeding trials. Based on feed intake and weight gain differences, 48 steers were selected for the project (16 from each feeding study). At harvest, rumen epithelial samples were taken from three locations in the rumen of each animal. The number of papillae on 1 cm² of epithelium was counted to determine density. Papillae (n = 30) from each sample were measured for length and width. The density, length, and width were combined to determine surface enlargement factor (SEF). None of the morphological characteristics of the papillae (length, width, density or SEF) were associated with feed intake or gain ($P \geq 0.10$). Ruminal fluid was collected from steers (n = 15) in the third trial for volatile fatty acid (VFA) analysis to determine if a relationship between VFA and ADG or ADFI existed. No differences in volatile fatty acid (VFA) concentrations were associated ($P \geq 0.17$) with ADFI or ADG. VFA variation was also evaluated for a relationship with *kallikrein* (*KLK*) genes since ruminal butyrate concentrations have previously been associated with the transcript abundance *KLK* genes. Additionally, qRT-PCR data showed that variation in the transcript abundance of *KLK6,8-10, and 13* associated with feed intake in our first trial of steers. Thus, we evaluated the transcript abundance of eight *KLK* genes in rumen

55 papillae from steers in the third trial ($n = 16$). Expression levels of *KLK5* correlated with
56 valerate concentrations in the rumen ($P < 0.05$), *KLK10* correlated with acetate
57 concentrations in the rumen ($P < 0.05$), *KLK12* correlated with acetate and butyrate
58 concentrations in the rumen ($P < 0.05$), and the expression levels of *KLK9* and *KLK10*
59 were associated with gain. While rumen papillae morphology was not associated with
60 beef steer gain or intake, our data suggests members of the kallikrein gene family have a
61 relationship with the VFA environment in the rumen, and also appear to play a role in the
62 highly correlated traits of ADG and ADFI.

63 **Keywords:** ADG, ADFI, kallikrein, papillae, rumen, volatile fatty acids

64

65 INTRODUCTION

66 Feed costs account for the largest fraction of the total cost to produce beef (Hill,
67 2012). Consequently, any improvement in feed utilization through gain or feed intake of
68 the beef animal will result in a decreased cost for producers. The rumen is a potential
69 location for variation of these traits among cattle due to its role in the fermentation of
70 feed and absorption of key nutrients. Rumen papillae have three major physiological
71 functions: 1) increase surface area for the absorption of nutrients which allows for an
72 increase in microbial attachment to the rumen wall, 2) promote metabolic activity by
73 allowing metabolites to pass from the rumen into the capillary bed of rumen epithelial,
74 and 3) immunological protection (Galfi et al., 1991). We hypothesized that variation in
75 rumen papillae morphology (papillae length, width, and/or density) may be associated
76 with the gain and/or feed intake of the animal.

99 cattle complied with the Guide for the Care and Use of Agricultural Animals in
100 Agricultural Research and Teaching (FASS, 2010).

101 ***Gain and Feed Intake Phenotypes***

102 Three cohorts of a total of 484 steers were evaluated for feed intake using the
103 Insentec feeding system (Marknesse, The Netherlands) and 16 steers with the furthest
104 variation higher or lower from the average of the entire cohort for gain and feed intake
105 phenotypes were harvested from each of the three cohorts for a total of 48 steers. The
106 first cohort began trials when the steers were 344 ± 48 d old (n=139), and were harvested
107 in the fall of 2012; the second cohort began trials when the steers were 377 ± 23 d old
108 (n=197), and were harvested in the spring of 2013; and the third cohort began trials when
109 the steers were 349 ± 17 d old (n=148), and were harvested in the fall of 2013. All three
110 cohorts of steers were finished during the feeding trials and had *ad libitum* access to a
111 ration consisting of: 57.35% dry-rolled corn, 30.00% wet distillers grains with solubles,
112 8.00% alfalfa hay, 4.25% steackmaker (Land O'Lakes Feed LLC, Gray Summit, MO), and
113 0.40% urea on a dry matter basis. A 100 g feed sample was collected daily and frozen.
114 Samples within a week were composited and dry matter content for weekly samples was
115 determined. Dry matter intake was calculated as the feed intake multiplied by the weekly
116 dry matter content corresponding to the feed intake. Body weights were measured on two
117 consecutive days at the beginning and end of the study and approximately every three
118 weeks throughout each trial period. Weights were regressed over days on study, and total
119 gain was calculated from the regression equation. The total cumulative dry matter intake
120 (DMI) over the trial period was the total DMI. Average daily DMI (also referred to as
121 ADFI) was total DMI divided by the number of days on study. At the end of each

122 feeding period, steers were ranked based on their standardized distance from the bivariate
123 mean (ADG and ADFI) assuming a bivariate normal distribution with a calculated
124 correlation between ADG and ADFI. Four steers with the greatest deviation within each
125 Cartesian quadrant were sampled. Sixteen steers were selected for harvest from each of
126 the 3 cohorts, and 12 total steers were studied from each phenotypic group. In the event
127 that a sire breed was over represented within a quadrant, a steer with the next highest
128 rank of a different breed was selected. The result was a 2x2 factorial arrangement
129 consisting of greater and less ADFI, and greater and less ADG. Animals with health
130 issues that might affect body weight (BW) gain or intake including lameness, rectal
131 prolapsed, bloat, or respiratory infections were not evaluated in this study. Average daily
132 gain and average daily feed intake phenotypes for each group are displayed on Table 1.

133 *Population*

134 A crossbred cattle population was used for this study in order to increase genetic
135 diversity to produce robust results across breeds. Over-representation of one breed in a
136 specific quadrant was avoided in order to achieve biological results that are consistent
137 across cattle breeds. Steers selected for this study came from the USMARC Continuous
138 Germplasm Evaluation Program (Kuehn et al., 2008). This program developed
139 populations of cattle with a high percentage of the following breeds: Angus, Beefmaster,
140 Bonsmara, Brahman, Brangus, Charolais, Chiangus, Gelbvieh, Hereford, Limousin,
141 Maine Anjou, MARCII, MARCIII, Red Angus, Salers, Santa Gertrudis, Shorthorn,
142 Simmental and South Devon. Each year heifers and cows were artificially inseminated
143 with semen from prominent industry bulls of their dominant breed. This program
144 resulted in offspring ranging from 50% to 75% of the same breed as their sire with the

exception of Angus and Hereford which ranged from 50% to 100% of the same breed as their sire. Breed compositions for all three cohorts by ADG and ADFI phenotype quadrant are shown on Table 2.

The breeds included in only the third cohort of animals which were used for VFA and *KLK* expression analysis included:

- High gain, high intake steers: 21.875% Angus, 12.500% Brangus, 3.125% Charolais, 12.500% Hereford, 12.500% MARCIII, 18.750% Salers, and 18.750% Santa Gertrudis.
- High gain, low intake steers: 25.000% Angus, 6.250% Chiangus, 31.250% Hereford, 3.125% MARCIII, 12.500% Salers, 18.750% Shorthorn, and 3.125% Simmental.
- Low gain, low intake steers: 4.688% Angus, 12.500% Brahman, 3.125% Charolais, 18.750% Chiangus, 31.250% Hereford, 14.063% Maine Anjou, 6.250% MARCII, 3.125% MARCIII, and 6.250% Unknown.
- Low gain, high intake steers: 18.750% Angus, 12.500% Beefmaster, 12.500% Chiangus, 18.750% Gelbvieh, 6.250% Hereford, 3.125% MARCIII, and 28.125% Simmental.

Papillae Morphology Tissue Collection (All 3 cohorts)

At harvest, 10 cm² rumen epithelial samples were taken from three different locations of the rumen: the cranial sac, the cranial ventral sac, and the caudal ventral sac (Lesmeister et al., 2004). Samples were frozen at -20°C until analysis. The samples were then thawed for data collection. The density of the papillae was measured by counting the number of papillae on 1 cm² of epithelial tissue. Papillae (n = 30) were

168 randomly sampled and length and width were measured using digital calipers. No
169 measurements were taken for samples which were undeveloped and goose bump-like in
170 appearance (n = 6) and density was recorded as 0 papillae/cm². Surface enlargement
171 factor was calculated from the average length (L), average width (W), and density (D)
172 using the following equation (Jiang et al., 2003):

$$173 \quad \text{SEF} = (2 \times L \times W \times D) + 1$$

174 *Statistical Analysis (Papillae morphology)*

175 Response variables length, width, density and SEF were analyzed using a split plot
176 design with quadrant (greater intake, greater gain; greater intake, less gain; less intake,
177 greater gain; less intake, less gain), cohort (year × season) and sample site and its
178 interactions were tested on the overall residual term. Linear contrasts of quadrant were
179 used to detect effects of gain, intake and their interaction on response variables.

180 *Volatile Fatty Acid Analysis (Cohort 3 only)*

181 Ruminal fluid was strained through four layers of cheesecloth and flash frozen
182 from the third group of steers at harvest. Ruminal fluid samples were thawed and
183 centrifuged for 30 min at 10,000 × g. Two milligrams of 2-ethyl butyric acid were added
184 as the internal standard to 5 mL portions of supernatant which were then centrifuged for
185 10 min at 10,000 × g (Goetsch and Galyean, 1983). One sample did not have enough
186 volume to be measured resulting in analysis of 15 samples. Volatile fatty acid
187 concentrations were then determined for each animal using an Agilent 6890 gas
188 chromatograph with Agilent ChemStation software (Agilent Technologies, Santa Clara,
189 CA) equipped with a Supelco Nukol (Sigma-Aldrich Corp., St. Louis, MO), 15 m × 0.53
190 mm capillary column. Detection and injection temperatures were 250°C. The initial

191 temperature of the oven was 80°C and was increased at a rate of 8°C/min to 150°C. The
192 carrier gas used was hydrogen (H₂) with a flow rate of 20 mL/min. The resulting data
193 was adjusted to the amount of internal standard by dividing the 2 mg of ethyl butyrate
194 (EB) by the area under the peak associated with EB. This value was then multiplied by
195 the area under the peak associated with each VFA resulting in mg VFA. The mg VFA
196 was multiplied by the detector response factor to account for differences in molecular
197 weight, which was then divided by 5 mL to obtain the mg VFA/mL ruminal fluid. The
198 concentrations of VFA in mg/mL ruminal fluid were divided by the molecular weight of
199 the VFA to obtain VFA mM in ruminal fluid. Finally, the molar proportions of individual
200 VFA were calculated for each sample by dividing the mol/L concentration for each VFA
201 by the sum of all VFA resulting in the proportion VFA in each sample of ruminal fluid.

202 ***Tissue Collection for RNA Isolation (Cohorts 1 and 3)***

203 Papillae were clipped from the caudal ventral sac approximately 30-min post-
204 mortem, and flash frozen in liquid nitrogen. Samples were stored at -80°C until RNA
205 isolation. Papillae samples (50 to 100 ng) were homogenized in 1 mL of TriPure (Roche
206 Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA), and RNA was
207 isolated according to the manufacturer's protocol. A NanoDrop 8000 spectrophotometer
208 (NanoDropTechnologies Inc., Wilmington, DE, USA; data not shown) was used to check
209 quantity and quality of RNA with the 260:280 nm wavelength as the quality control with
210 all RNA having a ratio greater than or equal to 1.8. The Transcriptor First Strand cDNA
211 Synthesis Kit (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN,
212 USA) was employed to reverse transcribe the resulting RNA (2 µg) into cDNA according
213 to the manufacturer's protocol.

214 ***Real-time PCR Gene Expression (Cohorts 1 and 3)***

215 Forward and reverse primers were designed to bridge an intron in each gene using Primer
216 3 (Table 3; Koressaar and Remm, 2007; Untergrasser et al., 2012), and 50 ng
217 complimentary cDNA was used to perform quantitative real-time polymerase chain
218 reaction (qRT-PCR) in triplicate using LightCycler 480 Sybr Green I Master mix
219 (Roche). The qRT-PCR reaction occurred under the following conditions: 95°C for 5
220 min followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, 72°C for 10 s, and a final
221 melting curve from 65° to 95°C. The threshold cycle (C_t) was determined for each gene
222 in each sample with GAPDH as the reference gene. Analysis of RT-qPCR data was
223 performed using the C_t of the target gene and the reference gene GAPDH to calculate the
224 $\Delta\Delta C_t$. Fold changes between samples were determined using the $2^{-\Delta\Delta C_t}$ method and the
225 log of the $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen, 2001).

226 ***Statistical Analysis (VFA and Gene Expression)***

227 *Kalikrein* gene expression and VFA concentrations from the third cohort were
228 analyzed for differences among quadrant. A one-way ANOVA was performed using the
229 mixed procedure of SAS. Additionally, expression of *KLKs* was correlated with ruminal
230 VFA proportions and concentrations. The GLM with MANOVA in SAS was used to
231 determine residual correlations while accounting for the divergent quadrant phenotypes
232 of the steers.

233 **RESULTS**

234 ***Papillae Morphology***

235 Rumen samples were collected from the cranial sac, cranial ventral sac, and
236 caudal sac from 48 steers. The resulting length, width, density, and SEF for all 48

237 animals are displayed in Table 4. None of the papillae characteristics measured were
238 associated ($P > 0.1$) with gain x feed intake quadrant. Morphological differences were
239 associated with sample site regardless of treatment group (Table 5). There was no
240 interaction ($P > 0.1$) between sample site and quadrant. Length ($P < 0.001$), density ($P =$
241 0.004), and SEF ($P < 0.001$) varied across sample site, with each variable being greater in
242 the cranial sac. Width was the only morphological characteristic not found to vary ($P =$
243 0.9 with sample site).

244 ***Volatile Fatty Acids***

245 The concentrations and proportions of VFA: acetate, butyrate, propionate,
246 isobutyrate, valerate and isovalerate in the rumen fluid of 15 2012 fall-born steers were
247 measured by gas chromatography and were analyzed for correlation with BW gain and
248 intake. Volatile fatty acid concentrations and proportions were not associated with ADG
249 and ADFI (Table 6-7).

250 ***Gene Expression***

251 RNA was isolated from the papillae of the same 2012 fall-born steers with VFA
252 collections to determine whether KLK expression correlated with ruminal VFA
253 concentrations and proportions. Expression levels of *KLK10* and *12* were negatively
254 correlated with acetate concentrations in the rumen ($P < 0.05$; Table 8), and *KLK12* was
255 negatively correlated with butyrate concentrations ($P = 0.005$; Table 8). Additionally,
256 *KLK5* was negatively correlated with the concentration and proportion of valerate present
257 in the rumen as well as the concentration of valerate ($P = 0.05$; Table 8 and Table 9). The
258 expression of *KLK9* and *10* increased with BW gain (quadrant $P \leq 0.04$, gain $P \leq 0.02$)

259 and displayed a trend toward association with intake as well (quadrant $P \leq 0.04$, gain $P \leq$
260 0.1; Table 10).

261 **DISCUSSION**

262 Morphological differences in rumen papillae due to diet induced transition
263 periods, via changing metabolizable energy content of the diet have been detected along
264 with variation in gene expression between a low energy diet and a high energy diet
265 (Steele et al., 2015). Variation in rumen papillae length, width, and density was observed
266 at the harvest of animals with divergent BW gain and feed intake phenotypes leading to
267 the hypothesis that these differences may be involved in the cattle gain or feed intake.
268 Appearance of rumen papillae samples varied from short and wide, to long and skinny,
269 while some were undeveloped and goose bump-like. The results of this study showed
270 that length, width, density, and SEF of papillae were not associated with divergent ADG
271 and ADFI phenotypes, and did not support our hypothesis that rumen papillae
272 morphology may be associated with gain or feed intake differences among beef steers. In
273 this sampling of animals, these findings suggest that the morphological characteristics of
274 rumen papillae do not affect feed intake or BW gain, suggesting further investigation into
275 metabolic pathways in the rumen epithelium that may play a role in the gain and intake
276 phenotypes as opposed to the morphology of the rumen papillae.

277 We did not detect an association between rumen VFAs and BW gain or intake
278 phenotypes, possibly due to small sample size ($n = 15$). Previous literature suggests there
279 may be a relationship between increased ruminal butyrate and valerate mM
280 concentrations and low residual feed intake (RFI), which is an index that takes into
281 account both ADG and ADFI (Guan et al., 2008). This suggests that increasing butyrate

282 concentrations in the rumen may decrease ADFI or increase ADG. A transgenic mouse
283 study showed that colonic butyrate protects against diet-induced obesity, which is a trait
284 affected by an animal's ADG and ADFI, through the regulation of gut hormone release
285 (Lin et al., 2012). Butyrate has also been shown to affect several biologically active
286 molecules in the gastrointestinal tract including Glucagon-like peptide 2 (GLP-2), which
287 is involved in insulin signaling and subsequent regulation of BW gain, and Insulin-like
288 growth factor (IGF-1; Shen et al., 2004; Gorka et al., 2009). Insulin-like growth factor
289 has been correlated with residual feed intake and used to select for improved feed
290 efficiency with mixed results (Moore et al., 2009). While our phenotypes are not directly
291 comparable with the studies cited above, ADG and ADFI are factors included in the RFI
292 equation and the obesity phenotype, and with the known biological activity of butyrate
293 further investigation is required to determine whether these biological phenotypes are
294 related to rumen VFAs and to evaluate the potential pathways involved. Additionally,
295 butyrate has also been identified as a signaling molecule involved in transcriptional
296 regulation of genes involved in the cell cycle and apoptosis (Li and Li, 2006). A
297 microarray study of rumen papillae from butyrate supplemented dairy cows identified
298 genes involved with non-specific host defense, remodeling and adaptation, and immune
299 response that were affected by additional butyrate and suggests butyrate plays a role in
300 the immunological protection function of the rumen (Dionissopoulos et al., 2013).

301 The transcript abundance of the *KLK* gene family was previously associated with
302 feed intake in rumen papillae of the first cohort included in this papillae morphology
303 study (supplemental Table 1). In this study, the third cohort of steers displayed increased
304 relative expression of *KLK9* and *10* associated with an increase in gain suggesting that

305 *KLK* genes are involved with the ADG phenotype as indicated in previous studies
306 (Lkhagvadorj et al., 2010; Mori et al., 2008; Veerkamp et al., 2012). Negative
307 correlations were observed between *KLK5*, *10* and *12* with ruminal concentrations of
308 VFAs, agreeing with previous literature showing a negative correlation between *KLK10*
309 and *12* and butyrate concentrations in the rumen of Holstein cows (Baldwin et al., 2012).
310 It seems likely that the expression level of the *KLK* gene family is in response to VFA
311 concentrations; however, the mechanism of this biological response is unclear. Our
312 findings suggest that a relationship exists between the *kallikrein* gene family and ruminal
313 VFAs and that these factors may affect ADG in beef cattle.

314 In summary, morphological characteristics of the rumen epithelium had no
315 association with ADG or ADFI and no relationship with VFAs. Papillae change with diet,
316 and our study evaluated steers fed the same diet; therefore, perhaps it should not be
317 surprising that we detected no difference in papillae characteristics. However, this study
318 supports that a relationship exists between the expression of *KLK* genes and ruminal VFA
319 concentrations and between *KLK* transcript abundance and ADG phenotypes. In
320 conclusion, we suggest that the role of the rumen papillae in ADG and ADFI phenotypes
321 is likely to be metabolic in nature within the same diet.

322 CONFLICT OF INTEREST: None.

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332

333 **LITERATURE CITED**

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Table 1. Average daily gain and average daily feed intake phenotypes by group across cohorts.

Cohort ¹	Treatment ² Group	Average Daily Gain				Average Daily Feed Intake			
		Mean	Minimum	Maximum	Standard Deviation	Mean	Minimum	Maximum	Standard Deviation
1	A	2.26	2.09	2.36	0.116	11.40	10.88	11.91	0.520
	B	2.21	2.13	2.31	0.076	8.87	8.40	9.46	0.555
	C	1.54	1.43	1.70	0.122	8.15	7.74	8.83	0.518
	D	1.68	1.53	1.90	0.154	10.67	10.17	11.01	0.356
2	A	2.09	1.93	2.30	0.173	14.04	12.60	14.88	1.005
	B	1.73	1.59	1.82	0.107	9.50	8.74	10.20	0.626
	C	0.91	0.87	1.00	0.062	8.61	7.51	9.37	0.824
	D	1.31	1.16	1.53	0.173	12.42	11.60	13.61	0.916
3	A	2.23	1.93	2.43	0.217	14.40	12.09	16.53	2.411
	B	1.98	1.90	2.07	0.081	9.08	7.51	9.98	1.112
	C	1.61	1.33	1.88	0.283	8.84	8.52	9.43	0.403
	D	1.48	1.02	1.84	0.340	13.51	12.06	14.93	1.504

¹Cohort : 1 = Fall of 2012; 2 = Spring of 2013; 3 = Fall of 2013.

²Treatments: A = High Gain-High Intake; B = High Gain-Low Intake; C = Low Gain-Low Intake; D = Low Gain-High Intake.

450 **Table 2.** Breed composition proportions of the sample population (n = 48)

Breed	High Gain High Intake	High Gain Low Intake	Low Gain Low Intake	Low Gain High Intake
Angus	0.203	0.229	0.078	0.276
Beefmaster	0.083	0.042	0.000	0.042
Bonsmara	0.000	0.000	0.000	0.021
Brahman	0.000	0.000	0.104	0.000
Brangus	0.083	0.042	0.042	0.042
Charolais	0.094	0.042	0.167	0.083
Chiangus	0.000	0.063	0.063	0.042
Gelbvieh	0.010	0.000	0.031	0.063
Hereford	0.083	0.167	0.188	0.021
Limousin	0.000	0.021	0.063	0.000
Maine Anjou	0.047	0.042	0.047	0.016
MARCII ¹	0.000	0.000	0.042	0.042
MARCI ²	0.063	0.031	0.021	0.021
Red Angus	0.042	0.031	0.073	0.010
Salers	0.063	0.104	0.021	0.000
Santa Gertrudis	0.104	0.000	0.000	0.000
Shorthorn	0.063	0.104	0.021	0.125
Simmental	0.042	0.042	0.000	0.156
South Devon	0.000	0.042	0.000	0.000
Unknown	0.021	0.000	0.042	0.042

451
452 ¹ MARCII is composed of Hereford, Angus, Gelbvieh, and Simmental.

453
454 ² MARCI is composed of Pinzgauer, Red Poll, Hereford, and Angus.

Table 3. Primer sequences with amplification length and accession number of gene sequence used to design the primer

Gene	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Length (bp)	GenBank accession no.
<i>KLK10</i>	kallikrein-related peptidase 10	TCTTCAATGGCCTTTCGTTC	AGGTGGTCATCCCCGACT	180	NM_001075890
<i>KLK12</i>	kallikrein-related peptidase 12	GTTCTCACGGCTGCTCACT	GATCTGTTCGTCAGTCCA	180	NM_001193190
<i>KLK13</i>	kallikrein-related peptidase 13	ACCCCAAATGGGTCCTCAC	GAGCCACCTCCCTCACCT	180	XM_002695143
<i>KLK5</i>	kallikrein-related peptidase 5	CTGCGCTGTTGGTGAGATT	GCCAAGACGGACTCTGAAAA	180	NM_001206280
<i>KLK6</i>	kallikrein-related peptidase 6	GTCTCATTCACCCGCTGT	TGGAAAACTCCCTTTGCTG	180	NM_001101140
<i>KLK7</i>	kallikrein-related peptidase 7	GTGATCCTGCTGCTGACCTT	GACACGGGACTCCATCAATAA	180	NM_001075256
<i>KLK8</i>	kallikrein-related peptidase 8	CGGGGGTGTCTCATAGAGG	CCTTTTCTTCAGGCTGTGC	180	NM_001193189
<i>KLK9</i>	kallikrein-related peptidase 9	CCTCACCCACCTCTTCTGTG	CATTTCAGAGGTGGTGCTC	180	NM_001205984
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	GCGTGAACCAACGAGAAGTAT	CGTGGACAGTGGTCATAAGT	137	NM_001034034

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Table 4. Morphological characteristics of rumen papillae (n = 48)

Trait	Treatment ¹ Group Least Squares Means					<i>P</i> -values			
	A	B	C	D	Standard Error	Quadrant ²	Gain	Intake	Gain × Intake
Density (papillae / cm ²)	31.86	32.78	34.64	32.94	2.472	0.882	0.555	0.876	0.600
Length (mm)(n = 1440)	6.66	7.34	7.94	6.90	0.510	0.319	0.412	0.730	0.101
Width (mm) (n = 1440)	2.75	3.14	2.83	2.75	0.173	0.322	0.373	0.357	0.174
SEF	1473.14	1820.72	1636.02	1645.06	157.55	0.494	0.968	0.264	0.289

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458 ¹Treatments: A = High Gain-High Intake; B = High Gain-Low Intake; C = Low Gain-Low Intake; D = Low Gain-High
 459 Intake.

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461 ²Test the null hypothesis that quadrants are equal.

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Table 5. Morphological characteristics of rumen papillae by sample site (n = 48)

Trait	Sample Site Means			Standard Error	<i>P</i> -values
	Cranial Sac	Cranial Ventral Sac	Caudal Ventral Sac		
Density (papillae / cm ²)	35.48 ^a	28.35 ^b	35.33 ^a	1.85	0.0041
Length (mm)(n = 1440)	8.99 ^a	6.18 ^b	6.46 ^b	0.404	<0.0001
Width (mm) (n = 1440)	2.90	2.85	2.86	0.126	0.940
SEF ¹	2088.79 ^a	1318.80 ^b	1523.62 ^b	124.16	<0.0001

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465 ^{a-b}Means within a row with different subscripts differ (*P* < 0.05).

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467 ¹SEF = Surface Enlargement Factor

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Table 6. Group means and *P*-values from concentrations of volatile fatty acids in ruminal fluid (n = 15)

VFA	Treatment ¹ Group LS Means (Concentrations mM)					<i>P</i> -values			
	A	B	C	D	Standard Error	Quadrant ²	Gain	Intake	Gain × Intake
Acetate	87.79	80.49	60.05	89.44	10	0.19	0.29	0.34	0.10
Propionate	63.35	61.72	40.55	52.85	20	0.73	0.33	0.73	0.85
Butyrate	15.74	14.87	17.82	24.47	5	0.49	0.28	0.49	0.31
Valerate	4.03	3.21	2.83	2.73	0.8	0.72	0.34	0.62	0.79
Isovalerate	3.62	3.50	3.18	3.42	0.7	0.98	0.78	0.88	0.84
Isobutyrate	1.44	1.57	1.36	1.33	0.3	0.63	0.63	0.94	0.77

¹Treatments: A = High Gain-High Intake; B = High Gain-Low Intake; C = Low Gain-Low Intake; D = Low Gain-High Intake.

²Test the null hypothesis that quadrants are equal.

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Table 7. Group means and *P*-values from molar proportions of volatile fatty acids in ruminal fluid (n = 15)

VFA	Treatment ¹ Group Means (mol/mol)				<i>P</i> -values				
	A	B	C	D	Standard Error	Quadrant ²	Gain	Intake	Gain × Intake
Acetate	0.51	0.50	0.51	0.51	0.059	0.99	0.78	0.90	0.89
Propionate	0.35	0.36	0.29	0.30	0.080	0.73	0.30	0.82	0.94
Butyrate	0.09	0.09	0.13	0.14	0.027	0.17 ^a	0.03 ^a	0.87	0.68
Valerate	0.02	0.02	0.02	0.02	0.004	0.46	0.38	0.17	0.83
Isovalerate	0.02	0.02	0.03	0.02	0.007	0.44	0.47	0.36	0.27
Isobutyrate	0.008	0.01	0.01	0.008	0.004	0.45	0.53	0.47	0.21

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 478 ¹Treatments: A = High Gain-High Intake; B = High Gain-Low Intake; C = Low Gain-Low Intake;
 479 D = Low Gain-High Intake.

480 ²Test the null hypothesis that quadrants are equal.

481 ^aWeakly trending toward being statistically significant in association with BW gain.

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Table 8. Correlation coefficients and corresponding *P*-values of *KLK* gene expression and VFA concentrations in ruminal fluid (n = 15)

	<i>KLK5</i>		<i>KLK6</i>		<i>KLK7</i>		<i>KLK8</i>		<i>KLK9</i>		<i>KLK10</i>		<i>KLK12</i>		<i>KLK13</i>	
	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>
Acetate	-0.15	0.72	-0.13	0.77	0.55	0.15	-0.20	0.63	-0.54	0.16	-0.70	0.05 ^a	-0.87	0.005 ^a	-0.66	0.08
Propionate	-0.57	0.14	0.22	0.61	0.06	0.89	-0.33	0.43	-0.07	0.88	-0.57	0.14	-0.11	0.79	-0.50	0.21
Butyrate	-0.20	0.64	-0.36	0.38	-0.28	0.50	-0.64	0.09	-0.48	0.23	-0.48	0.22	-0.87	0.005 ^a	-0.32	0.44
Valerate	-0.71	0.049 ^a	-0.18	0.66	-0.18	0.66	-0.59	0.12	-0.12	0.78	-0.56	0.15	-0.37	0.36	-0.50	0.20
Isovalerate	0.11	0.79	-0.16	0.70	0.48	0.23	-0.03	0.95	-0.30	0.48	-0.34	0.42	-0.50	0.21	-0.32	0.44
Isobutyrate	0.23	0.59	-0.11	0.80	0.49	0.22	0.24	0.57	-0.06	0.89	0.02	0.97	-0.28	0.50	0.01	0.97

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485 ^aStatistically significant ($P \leq 0.05$).

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Table 9. Correlation coefficients and corresponding *P*-values of *KLK* gene expression and VFA proportions in ruminal fluid (n = 15)

	<i>KLK5</i>		<i>KLK6</i>		<i>KLK7</i>		<i>KLK8</i>		<i>KLK9</i>		<i>KLK10</i>		<i>KLK12</i>		<i>KLK13</i>	
	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>
Acetate	0.56	0.15	0.08	0.85	0.31	0.46	0.48	0.23	-0.02	0.97	0.42	0.31	-0.01	0.99	0.34	0.40
Propionate	-0.47	0.24	0.22	0.60	-0.13	0.76	-0.26	0.54	0.09	0.83	-0.34	0.41	0.22	0.60	-0.34	0.42
Butyrate	0.07	0.86	-0.38	0.36	-0.35	0.39	-0.36	0.39	-0.25	0.55	-0.00	0.99	-0.61	0.11	0.15	0.72
Valerate	-0.71	0.05 ^a	-0.32	0.44	-0.43	0.29	-0.57	0.14	0.09	0.84	-0.28	0.50	-0.09	0.83	-0.30	0.47
Isovalerate	0.41	0.31	-0.12	0.78	0.34	0.41	0.30	0.46	0.00	0.99	0.22	0.60	-0.03	0.94	0.18	0.67
Isobutyrate	0.46	0.25	-0.05	0.90	0.29	0.48	0.48	0.23	0.19	0.66	0.48	0.23	0.15	0.72	0.44	0.28

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488 ^aStatistically significant ($P \leq 0.05$).

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Table 10. Group means and *P*-values from *KLK* gene expressions (n = 16)

Gene	Treatment ¹ Group LS Means (relative quantification)				<i>P</i> -values				
	A	B	C	D	Standard Error	Quadrant ²	Gain	Intake	Gain × Intake
<i>KLK5</i>	0.31	0.14	-0.48	-0.15	0.4	0.39	0.14	0.81	0.47
<i>KLK6</i>	-0.88	-0.96	-1.43	-0.92	0.52	0.87	0.64	0.69	0.58
<i>KLK7</i>	0.70	1.07	0.99	-0.04	0.48	0.29	0.37	0.46	0.14
<i>KLK8</i>	0.07	0.26	-0.91	-0.16	0.34	0.13	0.06	0.19	0.42
<i>KLK9</i>	-0.15	0.43	-0.94	-0.47	0.27	0.03 ^a	0.01 ^a	0.08	0.82
<i>KLK10</i>	-0.38	-0.01	-0.92	-0.59	0.20	0.04 ^a	0.02 ^a	0.10	0.92
<i>KLK12</i>	-0.45	-0.39	-1.31	-0.99	0.32	0.19	0.04	0.57	0.71
<i>KLK13</i>	-0.21	-0.31	-0.93	-0.67	0.25	0.20	0.05	0.74	0.47

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491 ¹Treatments: A = High Gain-High Intake; B = High Gain-Low Intake; C = Low Gain-
 492 Low Intake; D = Low Gain-High Intake.

493

494 ²Test the null hypothesis that quadrants are equal.

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496 ^aStatistically significant (*P* < 0.05).

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