

Supplemental Methods

DNA microbe RT-qPCR

Extracted DNA was standardized to 8 ng/μL for RT-PCR. qPCR was performed in a MicroAmp Optical 384-Well Reaction Plate (Cat. #4309849, Applied Biosystems). Within each well, 4 μL of diluted DNA combined with 6 μL of a mixture composed of 5 μL 1×SYBR Green master mix (Cat. #4309155, Applied Biosystems), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL of DNase/RNase-free water were added. To test the relative expression level, three replicates and a 6-point standard curve plus the nontemplate control (NTC) were run for each sample. qPCR was conducted in QuantStudio 7 real-time PCR machine (Applied Biosystems) following the conditions: 95 °C for 5 min, followed by 40 cycles of 1 s at 95 °C (denaturation) and 30 s annealing at 60 °C. The threshold cycle (Ct) and quantity data were analyzed and transformed using the standard curve with the QuantStudio Software (version v1.7.2, Applied Biosystems, CA). Relative abundance of bacterial species was calculated by bacterial species CT value minus the geomean of 2 universal eubacterial CT values (Table S1).

Metabolomics analysis

Samples of ruminal fluid were delivered to the Metabolomics Unit of the High-Throughput DNA Sequencing and Genotyping Unit of the W. M. Keck Biotechnology Center at University of Illinois, Urbana-Champaign for metabolomics analysis. Ruminal samples (200 μL) were dried in a vacuum concentrator (SpeedVac) prior to Gas chromatography–mass spectrometry (GC-MS) Metabolite Profiling using aliquots derivatized with 100 μL methoxyamine hydrochloride (40 mg/mL) for 60 min at 50 °C and then with 100 μL N-Methyl-N-trimethylsilyl-trifluoroacetamide at 70 °C for 120 min, followed by 2-h incubation at room temperature. Twenty μL of the internal standard (hentriacontanoic acid, 1 mg/mL) were added to each sample prior to derivatization. Targeted metabolite profiling analysis was performed using an Agilent GC/MS system (Agilent Inc, CA, USA) consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 MSD, and an HP 7683B auto-sampler, as previously described previously (Curtis-Quick et al., 2021). Gas chromatography was performed on a ZB-5MS (60 m × 0.32 mm I.D. and 0.25 μm film thickness) capillary column (Phenomenex Inc, CA, USA). The inlet and MS interface temperatures were 250 °C, and the ion source temperature was adjusted to 230 °C. An aliquot of 1 μL was injected at a split ratio of 10:1. The helium carrier gas was kept at a constant flow rate of 2 mL/min. The temperature program was 5 min isothermal heating at 70 °C, followed by an oven temperature increase of 5 °C per min to 310 °C and a final 10 min at 310 °C. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy at a scan range of m/z 30–800.

RNA extraction

Approximately 50 mg of tissue was homogenized with 1 mL Qiazol (Qiagen, Hilden, Germany). Samples were centrifuged for 10 min at 12,000 × g at 4°C, and the supernatant was collected and held at room temperature for 5 min. Two-hundred μL chloroform was added, shaken by hand, incubated at room temperature for 3 min, and centrifuged for 15 min at 12,000 × g at 4°C. The supernatant was collected and mixed with 750 μL ethanol. The samples were centrifuged at 12,000 rpm for 15 sec in the miRNeasy mini spin column to collect the pellet. Three-hundred fifty μL RWT buffer was used to wash the sample, followed by centrifugation at 12,000 rpm for 15 s. Eighty μL DNase I digestion mix (DNase I: RDD buffer = 1:7) (Qiagen, Hilden, Germany)

was added to the spin column to degrade DNA. Three-hundred fifty µL RWT buffer was used to wash the sample, followed by centrifugation at 12,000 rpm for 15 s. Five-hundred µL RPE buffer was used to wash the sample twice for 15 seconds, followed by centrifugation at 12,000 rpm for 2 min. The miRNeasy mini spin column was placed in a new 2 mL collection tube, and centrifuged at 15,000 rpm for 1 min. The miRNeasy mini spin column was transferred to a new 1.5 mL collection tube, and 50 µL of RNase-free water was added directly on to mini spin column membrane. After incubating for 1 min, the column was centrifuged for 1 min at 12,000 rpm to elute RNA. Total RNA quantification was determined using a Nanodrop ND-1000 (NanoDrop Technologies, Rockland, DE). The RNA was diluted to 100 ng/µL with DNase/RNase-free water. The purity and integrity of extracted RNA were evaluated using an Agilent Bioanalyzer at the ROY J. CARVER Biotechnology Center, University of Illinois, Urbana-Champaign.

RNA RT-qPCR

qPCR was performed in a MicroAmp Optical 384-Well Reaction Plate (Cat. #4309849, Applied Biosystems). Within each well, 4 µL of diluted cDNA combined with 6 µL of a mixture composed of 5 µL 1×SYBR Green master mix (Cat. #4309155, Applied Biosystems), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL of DNase/RNase-free water were added. To test the relative expression level, three replicates and a 7-point standard curve plus the nontemplate control (NTC) were run for each sample. qPCR was conducted in QuantStudio 7 real-time PCR machine (Applied Biosystems) following the conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s, then 60 °C for 1min. The threshold cycle (Ct) and quantity data were analyzed and transformed using the standard curve with the QuantStudio Software (version v1.7.2, Applied Biosystems, CA). Quantity data were normalized by dividing the geometric mean of the three Internal Control Genes (ICG): *UXT*, *GAPDH*, and *RPS9*. Each gene was run in triplicate. Primers are listed in Table S2.

Relative mRNA Abundance

The Ct value, slope, and R² data were analyzed by QuantStudio Software (version v1.7.2, Applied Biosystems, CA) (Table S3). The efficiency of qPCR amplification for each gene was calculated using the standard curve method ($\text{Efficiency} = 10^{(-1/\text{slope})}$). Relative mRNA abundance among measured genes was calculated by: $1/(\text{Efficiency of tested gene} \times \text{Median of } \Delta\text{Ct of tested gene in all samples})$. The ΔCt of tested genes for each sample was calculated by: $\text{Ct value of tested gene in each tested sample} - \text{geometric mean Ct of 3 ICG in each tested sample}$.

Table S1 Species-specific primers for quantification of target ruminal bacterial species

Target bacterial species	Primer sequence (5` - 3`)	
<i>Clostridium spp.</i> (C122)	F ¹ :	AAAGGAAGATTAATACCGCATAA
	R ² :	ATCTTGCGACCGTACTCCCC
<i>Selenomonas ruminantium</i>	F:	CAATAAGCATTCCGCCTGGG
	R:	TTCACTCAATGTCAAGCCCTGG
<i>Lactobacillus spp.</i> (C25)	F:	GAGGCAGCAGTAGGGAATCTT
	R:	GGCCAGTTACTACCTCTATCCTTCTT
<i>E scherichia coli</i> (EC42405)	F:	CATGCCGCGTGTATGAAGA
	R:	GGGTAACGTCAATGAGCAAAG
<i>Fibrobacter succinogenes</i>	F:	GCGGGTAGCAAACAGGATTAGA
	R:	CCCCCGGACACCCAGTAT
<i>Megaspheara elsdenii</i>	F:	AGATGGGGACAACAGCTGGA
	R:	CGAAAGCTCCGAAGAGCCT
<i>Butyrivibrio fibrisolvens</i>	F:	ACACACCGCCCCGTACCA
	R:	TCCTTACGGTTGGGTCACAGA
<i>Succinimonas amylolytica</i>	F:	CGTTGGGCGGTCAATTTGAAAC
	R:	CCTGAGCGTCAGTTACTATCCAGA
<i>Bacteroides spp.</i> (BF25)	F:	GAGAGGAAGGTCCCCCA
	R:	CGCTACTTGGCTGGTTCA
<i>Succinivibrio dextrinosolvens</i>	F:	TAGGAGCTTGTGCGATAGTATGG
	R:	CTCACTATGTCAAGGTCAGGTAAGG
<i>Rumicoccus flavefaciens</i>	F:	CGAACGGAGATAATTTGAGTTTACTTAGG
	R:	CGGTCTCTGTATGTTATGAGGTATTACC
<i>Rumicoccus albus</i>	F:	CCCTAAAAGCAGTCTTAGTTCG
	R:	CCTCCTTGCGGTTAGAACA
<i>Streptococcus bovis</i>	F:	TTCTTAGAGATAGGAAGTTTCTTCGG
	R:	ATGATGGCAACTAACAATAGGGGT
<i>Bifidobacteria spp.</i> (N124)	F:	CGCGTCYGGTGTGAAA
	R:	CCCCACATCCAGCATCC
<i>Prevotella bryantii</i>	F:	AGCGCAGGCCGTTTGG
	R:	GCTTCCTGTGCACTCAAGTCTGAC
<i>Eubacterium ruminantium</i>	F:	CTCCCGAGACTGAGGAAGCTTG
	R:	GTCCATCTCACACCACCGGA
Bacteria general 1	F:	GGATTAGATACCCTGGTAGT
	R:	CACGACACGAGCTGACG
Bacteria general 2	F:	GTGSTGCAYGGYTGTCGTCA
	R:	ACGTCRTCCMCACCTTCCTC

¹F = forward primer
²R= reverse primer

Table S2 GenBank accession number and sequence of primers for Bos taurus used to analyze gene expression.

Gene	Source	Sequence (5' - 3')
<i>TLR2</i>	XM_015475330.1	F ¹ : TGATGCTGCCATTCTGATTC R ² : GCCACTCCAGGTAGGTCTTG
<i>TNF</i>	NM_173966.3	F: CAAGTAACAAGCCGGTAGCC R: AGATGAGGTAAAGCCCGTCA

¹ Forward; ² Reverse
TNF, Tumor necrosis factor; *TLR2*, Toll like receptor 2

Table S3 Median Ct, Median ΔCt Slope, coefficient of determination of the standard curve (R2), and efficiency of amplification.

Gene	Median Ct ¹	Median ΔCt ²	Slope ³	(R ²) ⁴	Efficiency ⁵
<i>TLR2</i>	27.9908	7.8149	-3.098	0.964	2.1027
<i>TNF</i>	28.9976	8.8217	-3.086	0.893	2.1087

¹ The median was calculated considering all samples.
² The median of ΔCt was calculated as [Ct gene – geometrical mean of Ct internal controls] for all samples.
³ Slope of the standard curve.
⁴ R2 stands for the coefficient of determination of the standard curve.
⁵ Efficiency was calculated as $[10^{(-1 / \text{Slope})}]$.