Nuclear magnetic resonance-based metabolomics of blood plasma from dairy calves infected with the main causal agents of Bovine Respiratory Disease (BRD)

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Supplementary Methods S1. Pathogen preparation and challenge administration

Bacterial Challenge

The authors previously described this protocol in a recent publication[1]. In preparation for the controlled bacterial challenge, *M. haemolytica* isolate D153 was streaked onto BD Brain Heart Infusion (BHI) agar (BactoTM 237500) then incubated at 37 °C overnight. For the starter culture, a single colony was selected and inoculated in 5 mL BHI broth (DifcoTM 241830) and incubated overnight at 200 rpm at 37 °C in a shaker incubator, then diluted 1:100 and incubated again overnight. On the day of the challenge study, the culture was diluted again 1:100 and centrifugated at 200 rpm and 37 °C until Abs_{600} = 1.0, then bacteria were pelleted at 10,000 rpm for 10 minutes at 4 °C and then resuspended to a final concentration of 1.0 x 10^9cfu/30 mL in 0.9 % saline. The challenge inoculum was administered at 1.0 x 10^9 cfu for every 91 kg of calf body weight. The bacterial challenge was given via bronchoalveolar lavage (BAL) catheter. Briefly, each calf was physically restrained in a chute, and a halter and lead were used to position the calf's head straight up. Five mL of 2 % lidocaine was used as a local anesthetic and was squirted into one nostril, and the BAL tube was introduced through this nostril and gently advanced into the trachea until the end of the catheter wedged into a bronchus. The appropriate volume of challenge inoculum, based on body weight, was administered through the BAL catheter, followed immediately by 60 mL of sterile 0.9 % saline and 120 mL of air. Following the challenge procedure, a sub-sample of the challenge inoculum was used to prepare a quantitative culture assay on BHI agar and was checked at 24 - 48 h, and cfu were counted at 96 - 120 h.

Viral Challenge

Seven days prior to challenge MDBK (Madin-Darby bovine kidney), cells were seeded at a density of 40,000 cells / cm^2 into T-75 tissue culture flasks containing DMEM amended with 10 % fetal bovine serum and 2 mM Glutamax (Gibco). The cultures were maintained at 37 °C at 5 % CO_2, and cells were given approximately 4-6 h to adhere prior to infecting them with BRSV (GA-1, P5). To infect the cells, media was aspirated, and the cell monolayer in each flask was gently rinsed with 10 mL of PBS. Following removal of the PBS, 1 mL of virus suspension was added to each of the flasks, and 1 mL of complete cell culture media was added to an additional flask to provide an infection negative control. Flasks were gently rocked to ensure that the 1 mL of virus suspension or media alone covered the entire cell culture surface and were returned to the incubator for 30 minutes. After 30 minutes, 12 mL of fresh pre-warmed media was added to each flask and then returned to the incubator, where they remained in culture for 7 days. Cell monolayers were monitored visually at 4X daily from 72 h post-infection up to the day of challenge to track the formation and propagation of cytopathogenic effect (CPE) in the infected flasks. On the day of the challenge, the media from the BRSV infected flasks was pooled into a sterile 50 mL tube, mixed well and five 5 mL aliquots were prepared (1/calf). For the determination of viral titer, 24-well plates were seeded with MDBK cells utilizing the same technique described for the culture flasks. Using a serial dilution technique, 3 wells were treated with 10^{-1} of viral suspension, and serial dilutions from these wells were carried out to 10^{-15}. This was done for virus suspension collected prior to the challenge and was repeated with another aliquot of the virus that was exposed to the same conditions as the challenge aliquots. TCID50 plates were monitored for 13 days until no further change was observed, and the viral titer was calculated and determined to be TCID50 -5.20 for both pre-and post-challenge samples. For the challenge, each calf was haltered and allowed to stand or lie down in ventral recumbency while 5 mL of BRSV (GA-1, P5) was administered via nebulizer (DeVilbiss Pulmo-Neb) through a custom-made face mask. The challenge procedure took approximately 20 - 25 minutes per calf. Following the challenge, any calf exhibiting depression, elevated temperature, or respiratory effort was treated with dexamethasone (0.1 mg/kg). One calf exhibited signs of bacterial pneumonia and was also treated with florfenicol (40 mg/kg).

Table S1. Selected blood plasma samples for BRSV and *M. haemolytica* infections ^{1}H-NMR metabolomic profiling. Each plasma sample, on average, contained 43 ± 8 of the selected metabolites.

Sample ID	CHALLENGE	Calf's ID	Day	Category	# Identified Metabolites
1	MH	C_6	D1	INFECTED	49
2	MH	C_6	D2	INFECTED	45
3	MH	C_6	D3	INFECTED	43
4	MH	C_7	D13	INFECTED	51
5	MH	C_7	D15	INFECTED	48
6	MH	C_7	D17	INFECTED	51
7	MH	C_8	D1	INFECTED	43
8	MH	C_8	D2	INFECTED	46
9	MH	C_8	D19	INFECTED	42
10	MH	C_9	D1	INFECTED	33
11	MH	C_9	D2	INFECTED	46
12	MH	C_9	D3	INFECTED	38
13	MH	C_10	D1	INFECTED	40
14	MH	C_10	D2	INFECTED	40
15	MH	C_10	D3	INFECTED	50
16	BRSV	C_1	D7	INFECTED	26
17	BRSV	C_1	D9	INFECTED	28
18	BRSV	C_1	D11	INFECTED	49
19	BRSV	C_1	D13	INFECTED	32
20	BRSV	C_2	D7	INFECTED	47
21	BRSV	C_2	D3	INFECTED	49
22	BRSV	C_2	D4	INFECTED	22
23	BRSV	C_2	D5	INFECTED	42
24	BRSV	C_3	D7	INFECTED	47
25	BRSV	C_3	D9	INFECTED	43
26	BRSV	C_3	D11	INFECTED	39
27	BRSV	C_3	D13	INFECTED	45
28	BRSV	C_4	D7	INFECTED	40
29	BRSV	C_4	D9	INFECTED	42
30	BRSV	C_4	D11	INFECTED	52
31	BRSV	C_4	D13	INFECTED	46
32	BRSV	C_5	D7	INFECTED	44
33	BRSV	C_5	D9	INFECTED	45
34	BRSV	C_5	D11	INFECTED	45
35	BRSV	C_5	D13	INFECTED	48
36	MH	C_6	D-3	BASELINE	48
37	MH	C_6	D-1	BASELINE	58

Sample ID	CHALLENGE	Calf's ID	Day	Category	# Identified Metabolites
38	MH	C_6	D0	BASELINE	43
39	MH	C_7	D-2	BASELINE	44
40	MH	C_7	D-3	BASELINE	46
41	MH	C_7	D0	BASELINE	49
42	MH	C_8	D-2	BASELINE	32
43	MH	C_8	D-1	BASELINE	28
44	MH	C_8	D0	BASELINE	36
45	MH	C_9	D-2	BASELINE	43
46	MH	C_9	D-1	BASELINE	37
47	MH	C_9	D0	BASELINE	49
48	MH	C_10	D-2	BASELINE	42
49	MH	C_10	D-1	BASELINE	56
50	MH	C_10	D0	BASELINE	25
51	BRSV	C_1	D-3	BASELINE	53
52	BRSV	C_1	D-2	BASELINE	35
53	BRSV	C_1	D-1	BASELINE	32
54	BRSV	C_1	D0	BASELINE	48
55	BRSV	C_2	D-3	BASELINE	47
56	BRSV	C_2	D-2	BASELINE	43
57	BRSV	C_2	D-1	BASELINE	51
58	BRSV	C_2	D0	BASELINE	47
59	BRSV	C_3	D-3	BASELINE	56
60	BRSV	C_3	D-2	BASELINE	49
61	BRSV	C_3	D-1	BASELINE	40
62	BRSV	C_3	D0	BASELINE	37
63	BRSV	C_4	D-3	BASELINE	49
64	BRSV	C_4	D-2	BASELINE	48
65	BRSV	C_4	D-1	BASELINE	44
66	BRSV	C_4	D0	BASELINE	47
67	BRSV	C_5	D-3	BASELINE	33
68	BRSV	C_5	D-2	BASELINE	54
69	BRSV	C_5	D-1	BASELINE	41
70	BRSV	C_5	D0	BASELINE	49

Table S2. Selected ^{1}H-NMR metabolites (n = 72) and their chemical classification. Reviewed from the Bovine Metabolome Database (BMDB)[2], the Livestock Metabolome Database (LMDB)[3], and the Human Metabolome Database (HMDB)[4].

^{1}H-NMR Metabolite	ID	Chemical classification
2-Aminoadipate	1	Alpha amino acid
2-Aminobutyrate	2	L-alpha-amino acid
2-Hydroxybutyrate	3	Alpha hydroxy acids and derivatives
2-Hydroxyisobutyrate	4	Alpha hydroxy acids and derivatives
2-Hydroxyvalerate	5	Hydroxy fatty acid
2-Oxoglutarate	6	Gamma-keto acids and derivatives
3-Hydroxy-3-methylglutarate	7	Dicarboxylic acids and derivatives
3-Hydroxybutyrate	8	Beta hydroxy acids and derivatives
3-Hydroxyisobutyrate	9	Beta hydroxy acids and derivatives
4-Aminobutyrate	10	Gamma amino acids and derivatives
4-Pyridoxate	11	Pyridinecarboxylic acid
5-Aminolevulinate	12	Delta amino acids and derivatives
Acetamide	13	Acetamide
Acetate	14	Carboxylic acid
Acetoacetate	15	Short-chain keto acids and derivatives
Acetone	16	Ketone
Agmatine	17	Guanidine
Alanine	18	Alanine and derivatives
Allantoin	19	Imidazole
Arabinitol	20	Sugar alcohol
Betaine	21	Alpha-amino acids and derivatives
Cadaverine	22	Monoalkylamine
Carnitine	23	Carnitine
Citrulline	24	L-alpha-amino acid
Creatine	25	Alpha-amino acids and derivatives
Creatine phosphate	26	Alpha-amino acids and derivatives
Creatinine	27	Alpha-amino acids and derivatives
Cysteine	28	Cysteine and derivatives
D-Threitol	29	Sugar alcohol
Dimethyl sulfone	30	Sulfone
Dimethylamine	31	Dialkylamine
Ethanol	32	Primary alcohol
Formate	33	Carboxylic acid
Galactitol	34	Sugar alcohol
Glucose	35	Hexose
Glutamate	36	Glutamic acid and derivatives
Glutamine	37	L-alpha-amino acid
Glycine	38	Alpha-amino acid
Guanidoacetate	39	Alpha-amino acids and derivatives
Histamine	40	2-arylethylamine
Histidine	41	Histidine and derivatives
Isobutyrate	42	Carboxylic acid
Isoleucine	43	Isoleucine and derivatives

^{1}H-NMR Metabolite	ID	Chemical classification
Isovalerate	44	Methyl-branched fatty acid
Lactate	45	Alpha hydroxy acids and derivatives
Leucine	46	Leucine and derivatives
Malonate	47	Dicarboxylic acids and derivatives
Methanol	48	Primary alcohol
Methionine	49	Methionine and derivatives
Methylamine	50	Monoalkylamine
N,N-Dimethylglycine	51	Alpha-amino acids and derivatives
N-Acetylglutamate	52	Glutamic acid and derivatives
N-Acetylglycine	53	N-acyl-alpha amino acid
N-Isovaleroylglycine	54	N-acyl-alpha amino acid
N-Methylhydantoin	55	Imidazoline
O-Acetylcholine	56	Acyl choline
Phenylalanine	57	Phenylalanine and derivatives
Propionate	58	Carboxylic acid
Pyruvate	59	Alpha-keto acids and derivatives
Saccharopine	60	Glutamic acid and derivatives
Sarcosine	61	Alpha-amino acid
Succinate	62	Dicarboxylic acids and derivatives
Succinylacetone	63	Medium-chain keto acids and derivatives
Taurine	64	Organosulfonic acid
Threonate	65	Sugar acids and derivatives
Trimethylamine	66	Trialkylamine
Trimethylamine N-oxide	67	Trialkyl amine oxide
Tyrosine	68	Tyrosine and derivatives
Urea	69	Urea
Valine	70	Valine and derivatives
cis-Aconitate	71	Tricarboxylic acids and derivatives
π-Methylhistidine	72	Histidine and derivatives

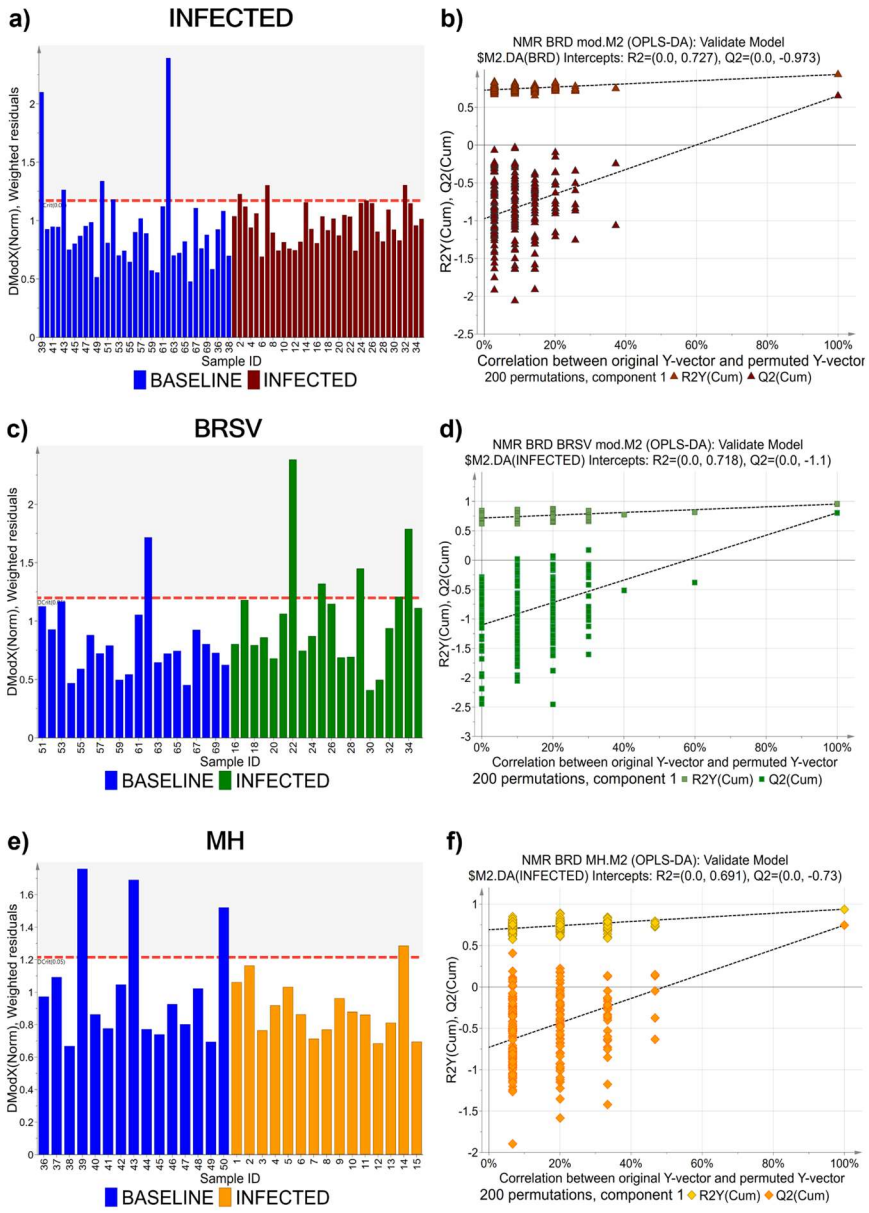

Figure S1. OPLS-DA Distance to model plots and Permutation analysis from the viral and bacterial challenge as well as the BRD database. Observations well above the red line are significantly dissimilar from the others; however, they are not considered outliers if they are not outside the confidence ellipse. (**a**) Baseline vs. Infected distance to model plot. Three samples from the Infected category and four from the Baseline can be seen as dissimilar to the others. (**b**) Baseline vs. Infected permutation analysis plot. (**c**) Baseline vs. BRSV distance to model plot. Four samples from the Infected category and one from the Baseline are found to be different. (**d**) Baseline vs. BRSV permutation analysis plot. (**e**) Baseline vs. *M. haemolytica* (MH) distance to model plot. One sample from the infected category and four from the Baseline can be seen above the red line. (**f**) Baseline vs. *M. haemolytica* permutation analysis plot.

References

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