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SUPPLEMENTARY TABLES AND FIGURES FOR THE FOLLOWING PAPER PUBLISHED
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**Milk metabolites and fatty acids as noninvasive biomarkers of metabolic status and energy
balance in early lactation cows.**

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INTERPRETIVE SUMMARY

Milk metabolites and fatty acids as noninvasive biomarkers of metabolic status and energy balance in early lactation cows, by Pires *et al.* (2021). After calving, feed intake is not sufficient to meet lactation requirements and dairy cows experience some degree of negative energy balance. Concentrations of milk metabolites and fatty acids were modified during early lactation, a period of spontaneous nutritional deficit for dairy cows, and in response to experimental nutrient restriction. Milk metabolites and fatty acids are potential noninvasive indicators of energy status of early lactation dairy cows.

Supplemental Table 1. Single linear regressions between concentrations of selected milk metabolites and NAGase activity measured in p.m. and a.m. milkings. Early lactation cows were allowed ad libitum intake of a lactation TMR. Cows were milked twice daily at approximately 0900 and 1600. Samples collected at 8, 15, 21, 23, 35, 42 and 49 ± 3 DIM (mean ± SD; 15 to 17 cows per time point), and at 24, 25 and 26 ± 3 DIM (mean ± SD; 8 cows per time point).

Regression dependent variable ¹	n	Intercept			Slope			Model		
		Estimate	95% CI	SE	Estimate	95% CI	SE	RMSE	R ²	P-value
a.m. Milk BHB (Log10(μM))	139	0.75	0.53 to 0.96	0.11	0.50	0.39 to 0.61	0.06	0.15	0.36	< 0.001
a.m. Milk Glucose (Log10(μM))	137	1.41	1.02 to 1.81	0.20	0.46	0.30 to 0.62	0.08	0.18	0.19	< 0.001
a.m. Milk Glucose-6P (Log10(μM))	139	0.42	0.11 to 0.72	0.15	0.78	0.65 to 0.92	0.07	0.15	0.49	0.05
a.m. Milk Isocitrate (Log10(μM))	139	0.21	0.01 to 0.41	0.10	0.89	0.79 to 0.98	0.05	0.10	0.71	< 0.001
a.m. Milk Galactose (Log10(μM))	138	0.89	0.59 to 1.19	0.15	0.65	0.53 to 0.77	0.06	0.09	0.47	< 0.001
a.m. Milk Glutamate (μM)	138	113.64	78.23 to 149.05	17.90	0.59	0.49 to 0.68	0.05	68.44	0.52	< 0.001
a.m. Milk Uric acid (μM)	139	41.34	25.84 to 56.83	7.84	0.71	0.58 to 0.83	0.06	19.38	0.47	< 0.001
a.m. Milk Creatinine (μM)	139	35.68	19.2 to 52.20	8.35	0.84	0.77 to 0.91	0.04	15.40	0.79	< 0.001
a.m. NAGase (Log10(U/L))	139	0.022	0.007 to 0.051	0.02	0.83	0.76 to 0.90	0.04	0.10	0.80	< 0.001

¹ BHB = β-hydroxybutyrate; Glucose-6P = glucose-6-phosphate; NAGase = N-acetyl-β-D-glucosaminidase activity.

² Single linear regressions between AM and PM concentrations were explored using the GLM procedure, where a.m. concentration was coded as dependent variable and p.m. as independent variable. Data were log10 transformed for BHB, glucose, isocitrate, glucose-6P, galactose, and NAGase activity

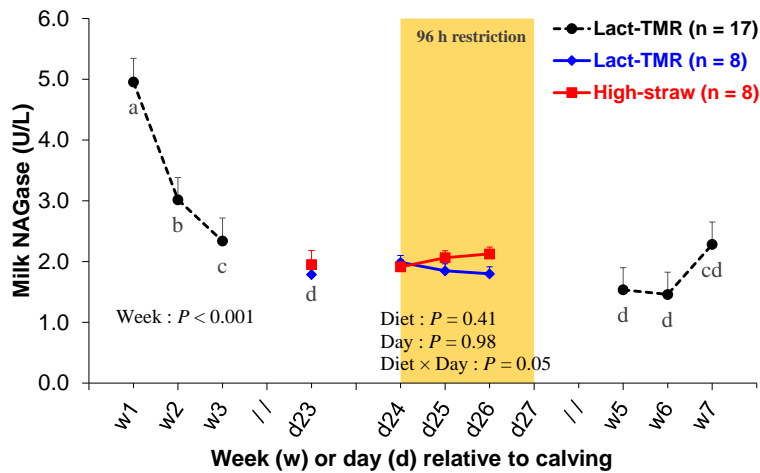
Supplemental Table 2. Single linear regressions between energy balance as dependent variable, selected milk and plasma metabolites and insulin, and milk fatty acids (FA)¹.

Regression independent variable	Period	n	Intercept			Slope			Model ¹			
			Estimate	95% CI	SE	Estimate	95% CI	SE	R ²	P-value	Intercept differ	Slope differ
Milk Glucose (μM)	WOL	84	-34.85	-47.27 to -22.44	6.24	0.056	0.029 to 0.084	0.14	0.17	<0.001	<0.001	0.03
	Challenge	64	-80.90	-102.25 to -59.56	10.68	0.126	0.063 to 0.189	0.315	0.20	<0.001		
Milk Glucose-6-P (μM)	All dataset	147	6.30	-8.83 to 21.43	7.65	-0.191	-0.276 to -0.107	0.043	0.12	<0.001	ns	ns
Milk Galactose (μM)	All dataset	148	-47.73	-68.40 to -27.07	10.46	0.061	0.074 to 0.114	0.027	0.03	0.03	ns	ns
Milk Glutamate (μM)	WOL	84	-30.09	-51.54 to -8.65	10.78	0.056	-0.007 to 0.178	0.031	0.04	0.08	<0.001	0.02
	Challenge	64	-96.03	-127.90 to -64.15	15.95	0.198	0.085 to 0.312	0.057	0.16	<0.001		
Milk Uric acid (μM)	All dataset	148	-63.23	-92.20 to -34.27	14.66	0.310	0.079 to 0.540	0.117	0.05	0.01	ns	ns
Milk Creatinine (μM)	All dataset	148	100.64	54.35 to 146.92	23.42	-0.554	-0.756 to -0.352	0.102	0.17	<0.001	ns	ns
Plasma insulin (μIU/mL)	WOL	82	-34.15	-48.57 to -19.73	7.24	1.02	0.41 to 1.64	0.31	0.12	0.001	<0.001	0.02
	Challenge	68	-85.34	-108.11 to -62.58	11.40	2.63	1.32 to 3.94	0.65	0.20	<0.001		
Plasma urea (mM)	WOL	82	-24.68	-49.38 to 0.02	12.41	2.88	-2.51 to 8.27	2.71	0.01	0.29	<0.01	<0.001
	Challenge	68	41.73	4.37 to 79.08	18.71	-17.34	-24.68 to -10.00	3.68	0.25	<0.001		
Σ C10:0 to C15:0 (g/100 g of FA) ²	WOL	85	-72.79	-94.06 to -51.52	10.69	3.06	2.01 to 4.10	0.52	0.29	<0.001	<0.001	<0.01
	Challenge	64	-136.60	-163.92 to -109.29	13.66	5.80	4.19 to 7.42	0.81	0.45	<0.001		
C18:0 (g/100 g of FA)	WOL	85	53.50	26.82 to 80.18	13.42	-6.47	-9.06 to -3.88	1.3	0.23	<0.001	<0.01	<0.01
	Challenge	64	137.55	72.40 to 202.70	32.59	-14.83	-20.12 to -9.54	2.65	0.34	<0.001		
Σ OBCFA ³ (g/100 g of FA)	WOL	85	-26.77	-60.60 to 7.06	17.01	4.31	-5.43 to 14.06	4.90	0.01	0.38	<0.001	<0.01
	Challenge	64	-163.20	-246.57 to -79.83	41.71	35.77	11.10 to 60.44	12.34	0.12	<0.01		
Σ OBCFA <C16 ⁴ (g/100 g of FA)	WOL	85	-37.29	-56.39 to -18.19	9.60	14.45	3.96 to 24.94	5.28	0.08	<0.001	<0.001	<0.001
	Challenge	64	-126.40	-158.67 to -94.13	16.14	54.91	34.50 to 75.33	10.21	0.32	<0.001		
Σ OBCFA >C16 ⁵ (g/100 g of FA)	WOL	85	59.85	22.75 to 96.94	18.65	-42.88	-64.81 to -20.94	11.03	0.15	<0.001	0.07	0.02
	Challenge	64	133.29	51.97 to 214.60	40.68	-96.05	-139.95 to -52.16	21.96	0.24	<0.001		

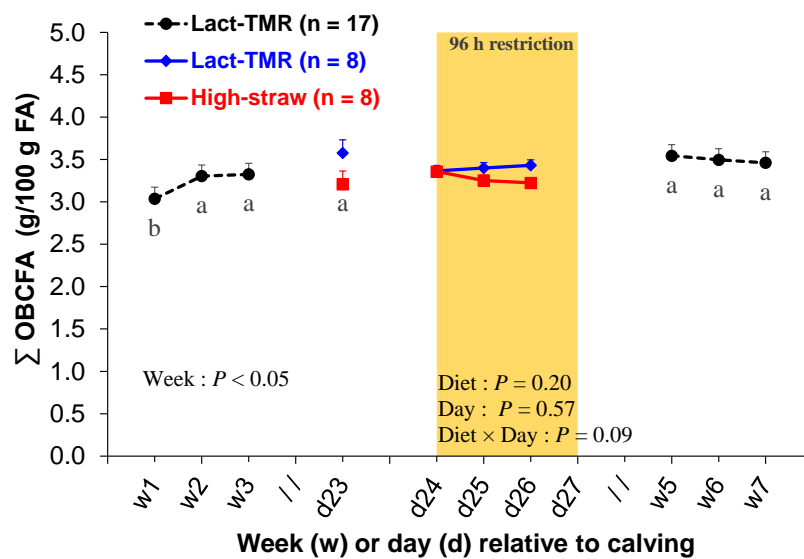
¹ Seventeen cows were studied during the first 7 wk of lactation while allowed ad libitum intake of a lactation diet (WOL; samples collected at 8, 15, 21, 23, 35, 42 and 49 ± 3 DIM; mean ± SD). A subset of 8 cows underwent 4 days of nutrient restriction from 24 to 27 ± 3 DIM by receiving a ration composed of 48% of straw, whereas 8 cows maintained on the lactation diet served as controls (Challenge; samples collected at 23, 24, 25 and 26 ± 3 DIM; mean ± SD). Differences in regression intercept and slope between the two datasets (i.e., WOL vs. nutritional challenge period) were tested by including the effect of dataset and the interaction between dataset and independent variable in the model using GLM procedure of SAS.

²Sum of FA with 10 to 15 carbons. ³ Sum odd and branched chain FA. ⁴ Sum of OBCFA with carbon chain shorter than 16. ⁵ Sum of OBCFA with carbon chain greater than 16.

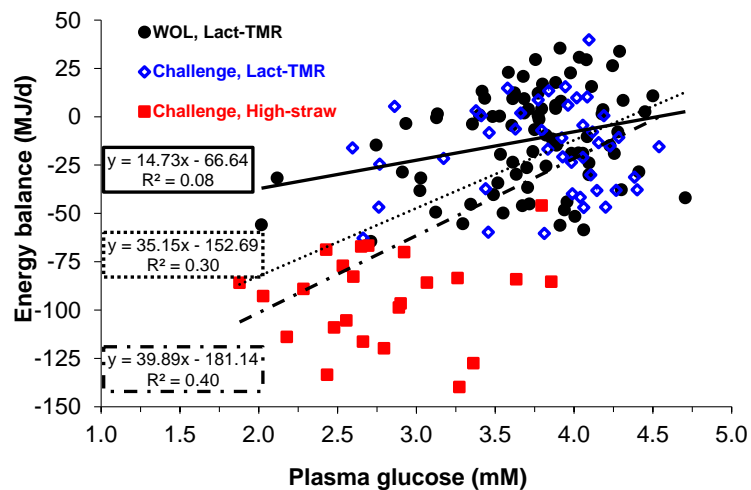
Supplemental Figure 1. Milk NAGase activity in early lactation multiparous Holstein cows. Cows were studied during the first 7 weeks of lactation while allowed ad libitum intake of a lactation diet (Lact-TMR; $n = 17$). A subset of 8 cows underwent 4 days of nutrient restriction (High-straw, $n = 8$) from 24 to 27 ± 3 DIM (mean \pm SD) by receiving a ration composed of 48% of straw (DM-basis). Eight cows maintained on the lactation diet were sampled during the same period to serve as controls. Data were analyzed separately to assess effects of wk of lactation (samples collected at 8, 15, 21, 23, 35, 42 and 49 ± 3 DIM; mean \pm SD) and nutritional challenge (samples collected at 23, 24, 25 and 26 ± 3 DIM; mean \pm SD). Values are LSM \pm SEM. Time points not sharing a common alphabetic superscript differ in wk of lactation dataset ($P \leq 0.05$). Milk NAGase activity at 23 DIM was a significant covariate for the analysis of nutritional challenge dataset and is represented separately as mean and SEM.



Supplemental Figure 2. Milk concentrations sum odd and branched chain fatty acids (Σ OBCFA) in early lactation multiparous of Holstein cows. Cows were studied during the first 7 weeks of lactation while allowed ad libitum intake of a lactation TMR (Lact-TMR; $n = 17$). A subset of 8 cows underwent 4 d of nutrient restriction (High-straw, $n = 8$) from 24 to 27 ± 3 DIM (mean \pm SD) by receiving a ration composed of 48% of straw (DM-basis). Eight cows maintained on the lactation TMR were sampled during the same period to serve as controls. Data were analyzed separately to assess effects of wk of lactation (samples collected at 8, 15, 21, 23, 35, 42 and 49 ± 3 DIM; mean \pm SD) and nutritional challenge (samples collected at 23, 24, 25 and 26 ± 3 DIM; mean \pm SD). Values are LSM \pm SEM. Time points not sharing a common alphabetic superscript differ in wk of lactation dataset ($P \leq 0.05$). Milk Σ OBCFA at 23 DIM was a significant covariate for the analysis of nutritional challenge dataset and is represented separately as mean and SEM.



Supplemental Figure 3. Simple regression between energy balance and plasma glucose concentrations in early lactation dairy cows. Seventeen cows were studied during the first 7 weeks of lactation while allowed ad libitum intake of a lactation TMR (●; WOL, Lact-TMR; samples collected at 8, 15, 21, 23, 35, 42 and 49 ± 3 DIM; mean ± SD). A subset of 8 cows underwent 4 d of nutrient restriction from 24 to 27 ± 3 DIM (mean ± SD) by receiving a ration composed of 48% of straw (■; Challenge, High-straw; samples collected at 23, 24, 25 and 26 ± 3 DIM; mean ± SD), and 8 cows maintained on the lactation TMR were sampled during the same period to serve as controls (◇; Challenge, Lact-TMR). Regression for WOL data (i.e., samples collected at 8, 15, 21, 23, 35, 42 and 49 ± 3 DIM) is represented with a continuous line (—); regression for nutritional challenge period (i.e., samples collected at 23, 24, 25 and 26 ± 3 DIM) using both restricted and control cows is represented with a dash-dot line (- · - · - ·); regression using the whole dataset is represented with a dotted line (·····).



Supplemental Figure 4. Single linear regressions between plasma nonesterified fatty acids (NEFA) and milk isocitrate (A), sum of FA with 6 to 15 carbons (Σ C6:0 to C15:0; B) and *cis*-9 C18:1 (C) concentrations in early lactation dairy cows. Seventeen cows were studied during the first 7 wk of lactation while allowed ad libitum intake of a lactation TMR (●; WOL, Lact-TMR; samples collected at 8, 15, 21, 23, 35, 42 and 49 ± 3 DIM; mean \pm SD). A subset of 8 cows underwent 4 d of nutrient restriction from 24 to 27 ± 3 DIM (mean \pm SD) by receiving a ration composed of 48% of straw (■; Challenge, High-straw; samples collected at 23, 24, 25 and 26 ± 3 DIM; mean \pm SD), and 8 cows maintained on the lactation TMR were sampled during the same period to serve as controls (◇; Challenge, Lact-TMR).

