Selenium biofortified alfalfa hay fed in low quantities improves selenium status and glutathione peroxidase activity in transition dairy cows and their calves

Shana Jaaf, Brandon Batty, Angela Krueger, Charles T. Estill and Massimo Bionaz

SUPPLEMENTAL FILE

Supplemental Material and Methods

Animals, ration, and experimental design:

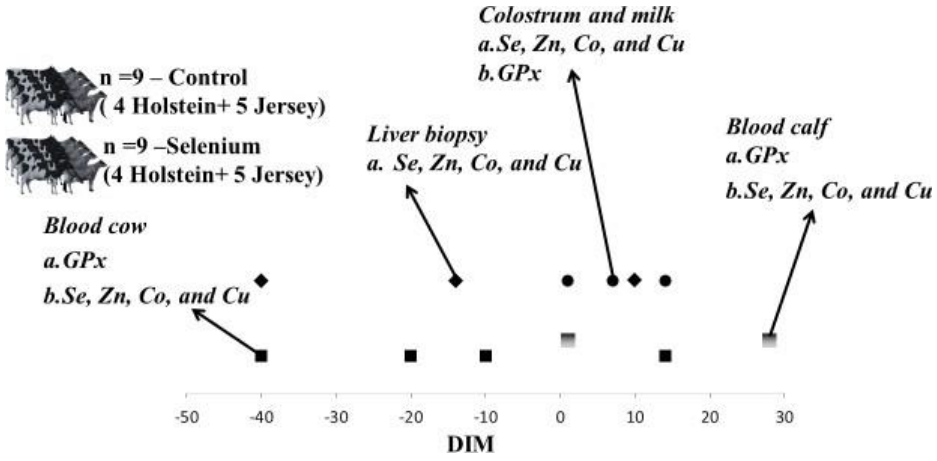
The overall experimental design is shown in Supplementary Figure S1. Three different batches of alfalfa hay from a prior experiment conducted by Hall et al. (2013) were used for the present study. Two alfalfa hay batches obtained from fields fertilized with 45 and 90 g sodium selenite/ha were mixed in equal amounts to obtain alfalfa with 3.25 mg Se/kg DM and used for the treatment group. As a control, we used alfalfa hay containing 0.43 mg Se/kg DM, which was obtained from a field that was not fertilized with sodium selenite. Chemical analysis results of the three hays, obtained from a commercial laboratory (Dairy One Forage Testing Laboratory, Ithaca, NY) are reported in Supplementary Table S1.

Ten Jersey and eight Holstein pregnant heifers from the Oregon State University Dairy Center were enrolled in the study. Around 45 days before expected parturition, the animals were moved into a pen equipped with free stalls bedded with mattress and sawdust, free access to water, and Calan gates. Due to the year-round herd calving, the low number of available animals, the use of Jerseys and Holsteins in both treatments, and the feeding period of around 56 days (40 days before two weeks post-partum), the entire experiment spanned nine months (from September 2017 to May 2018). Heifers were randomly assigned to two groups blocked by breed and expected time of calving. Starting from 40 days before expected calving, the heifers received either 1 kg DM/100 kg BW of Se-biofortified alfalfa hay (TRT; 5 Jersey and 4 Holstein heifers) or 1 kg of control hay (CTR; 5 Jersey and 4 Holstein heifers) mixed into their ration. All the heifers received the supplemental hay until 14 days in milk (DIM). One week before expected calving, animals were moved to a single calving pen where they kept receiving the lactation total mixed ration (TMR) and the alfalfa hay supplement. At 3 DIM, the animals were moved back into the Calan gate pen. Animals were milked twice a day, at 04:15 AM and 2:15 PM, and milk yield was recorded by the Afilab system (Afimilk, Israel). Heifers were monitored daily for health status, dry matter intake, milk yield, and weekly for body weight (BW).

During the dry and lactation period, heifers received *ad libitum* total mixed ration (TMR) formulated for dry and lactating cows, respectively. TMR was provided twice a day, approximately at 07:30 AM and 04:30 PM. Before feeding, TMR was mixed by hand with the chopped Se-biofortified alfalfa hay or the control alfalfa hay for each animal. Samples of TMR were collected once a month during the trial and preserved at -20□C until analysis. Except for Se, the chemical analysis of the TMR was performed by a commercial laboratory (Dairy One Forage Testing Laboratory, Ithaca, NY). The composition and chemical analysis of the TMRs, including the analysis for Se, are reported in Suppl. Table 2**.** Cows received an individual commercial Trace Mineral Salt Brick (cat#270220, American Stockman) without Se that was inserted into the Calan gate using a commercial plastic adaptor.

Calves born from the heifers enrolled in the experiment received colostrum from their mothers 1- 6 hours after birth (depending on whether the calf was born during a milking shift). They received 2.84

liters of colostrum for their first feeding and 1.89 liters of colostrum for their second feeding. Both feedings occurred within the first 24 hours after parturition. From day 2 to weaning all calves were fed raw cow milk collected from the bulk tank. Furthermore, *ad libitum* access to a starter concentrate was provided starting at 1-day post-birth to all calves. Blood samples were collected at 1 and 24-day post-birth. Calves were kept in individual hutches for the entire experimental period.



Suppl. Figure S1. Experimental design and samplings. The Selenium group received 1 kg/100 kg of body weight of Se-biofortified alfalfa (3.25 ppm of Se) from -40 to 14 day relative to parturition (or day in milk – DIM) while the Control group received alfalfa without biofortification (0.43 ppm of Se). GPx = measurement of glutathione peroxidase activity via a commercial kit. Microminerals were measured via ICP-MS.

Dry matter of the TMR

Dry matter of the TMR and residuals after feeding was measured weekly. Briefly, approx. 200 g of the sample was put in a glass container of known weight (Tare) and precisely weighed (W1) and put in the microwave at high power for 8 min. Then it was mixed by hand and put back in the microwave for 4 min at high power; sample weight was then recorded (W2).

The % dry matter was = (W2-Tare)/ (W1-Tare) 100.

Suppl. Table S1. Chemical characteristics of alfalfa hays used for the present experiment

	Control Hay	**Medium Se hay**	**High Se hay**
DM, %	88.6	89.6	88.9
Nutrient, DM basis			
CP, %	21.8	19.8	20.8
ADF, %	31.6	35.9	31.9
NDF, %	37.9	44.2	40.4
NFC, %	28.3	24.0	26.8
NEL, Mcal/kg	0.63	0.60	0.62
Relative Feed Value	158	128	148
Minerals, DM basis			
Ca, %	1.52	1.35	1.42
P, %	0.22	0.22	0.24
Mg, %	0.38	0.37	0.24
K, %	1.96	1.97	1.95
Na, %	0.092	0.094	0.097
S, %	0.34	0.31	0.33
Fe, ppm	258	723	305
Zn, ppm	17	16	17
Cu, ppm	11	10	10
Mn, ppm	42	51	46
Mo, ppm	< 0.1	< 0.1	< 0.1
Se, ppm	0.43	2.2	4.4

Suppl. Table S2. Composition and chemical characteristics of the total mixed rations used in the present experiment

Item	Dry cows	Lactating cows
Ingredient, %		
Alfalfa	-	13.6
Corn Silage	40	39.6
Grass Silage	45	26.1
Grass Hay	15	-
Corn/Barley	-	14.4
Soybean Meal	-	5.6
Mineral Supplement[1]	-	0.7
Dry Matter (DM)[2]	41.40± 3.68	50.00±3.96
NE_L; Mcal/kg of DM	1.54±0.00	1.67±0.01
Chemical composition, % DM		
Crude protein	9.00±0.14	18.3±0.99
NDF	52.3±0.8	32.5±4.0
ADF	30.1±0.3	19.6±3.3
Calcium	0.33±0.02	0.59±0.09
Phosphorus	0.24±0.01	0.42±0.08
Magnesium	0.19±0.01	0.25±0.03
Potassium	1.44±0.47	1.87±0.50
Sodium	0.08±0.07	0.06±0.01
Sulfur	0.14±0.01	0.22±0.01
Iron (ppm)	1021±238	536±133
Zinc (ppm)	28.5±0.7	44.0±21.2
Copper (ppm)	6.00±1.41	11.50±4.9
Manganese (ppm)	140.5±33.2	58.5±7.8
Molybdenum (ppm)	1.00±0.00	1.15±0.21
Selenium (ppm)	0.26±0.17	0.99±0.37

[1] Wilbur-Ellis Feed, LLC, OR (Cat# 1187036). It contains (as %DM) 17.60-21.00 % Ca, 7% P, 8% Mg, 1.65% S, 20-24 ppm Se, 440,000 IU/Kg DM Vitamin A. In addition to the mineral provided via TMR cows also received an individual mineral block without Selenium (Cat#90013, Stockman Trace Mineralized & Salt Brick). The indicated content included a minimum guarantee of 98% NaCl, 4000 ppm Zn, 1600 ppm Fe, 1200 ppm Mn, 260 ppm Cu, 100 ppm I, and 40 ppm Co.

[2] The data are mean±SD of monthly TMR samples collected during the trial

Collection of blood and milk

Blood samples from cows and calves were collected from the jugular vein into Na-heparin tubes (Cat# 6102751; Becton Dickinson, Franklin Lakes, NJ) before the morning feeding to measure GPx activity. Blood samples were also collected into EDTA tubes (Cat# 455036, Greiner bio- one. Monroe, NC 28110, USA) to measure Se and other trace minerals. Colostrum and milk samples were collected during the morning milking in a 15-ml tube (Cat# 525-0400. VWR. USA) and immediately put on ice for transport to the laboratory. Plasma, serum, whole blood, colostrum, and milk samples were preserved at -20°C until analyses.

Liver tissue collection

Ultrasound (Ibex Pro, E.I. Medical., Loveland, CO) was used to determine the liver location at the right 10th intercostal space at the level of the greater trochanter. The biopsy area was clipped and scrubbed with 7.5% povidone-iodine (Cat# 055479, VetUS) and 70% ethanol. Following infiltration of a local anesthetic (lidocaine 2%, Cat# 002468, VetUS) and intravenous 10 mg xylazine sterile solution (20 mg/ml) (Cat# 4811, Akorn, Inc., Decatur, IL62522), a small incision (approx. 0.5-1.0cm) was made in the skin at the right 10th intercostal space. The biopsy instrument was inserted through the body wall, introduced into the liver parenchyma, and a liver sample was collected. Following the biopsy, the cutaneous incision was closed using wound stapler (Cat# 8535, USA). Liver samples were immediately put in 2 ml cryotubes (Cat# 10018-760, VWR), flash frozen in liquid nitrogen, and stored at -80°C until analysis. Before the biopsy and the day after the surgical intervention, the rectal temperature of the animals was measured to check for possible inflammation or health issue.

Measurement of Se and other trace minerals

Se, Zinc (Zn), Cobalt (Co), and Copper (Cu) were measured by using 0.5 ml of whole blood, colostrum, and milk. Samples were added into screw cap glass vials with 2 ml of home-distilled concentrated nitric acid, 1 ml hydrogen peroxide (Cat# 0000191085, Avantor, USA), and with 0.1 ml of indium (200 ppb) as an internal standard. Vials were capped and heated at 60□C for approx. 2 hours. Then, 2 ml of concentrated HNO3 and 0.5 ml of H2O2 were added. Samples were heated at 80□C until a clear digest was obtained, then they were uncovered and left to evaporate completely. The pellet was dissolved in 5 ml of 1% HNO3. Finally, 1 ml of the sample was diluted with 4 ml of 1% HNO3 before analysis.

Liver samples were freeze-dried (model # HR9000-AL, Harvest Right). The dried liver was precisely weighed and placed into screw cap glass vials. One ml of HNO3 with 0.1 ml of indium as an internal standard was added in four consecutive bouts of 0.25 ml to the sample and allowed reacting. Samples were covered and heated overnight at 60□C. Two ml of H2O2 was added to the samples in 0.5 ml increments. Vials were capped and heated overnight at 60□C, then uncapped and allowed complete evaporation in a fume hood. The pellet was re-dissolved in 8 ml of 1% HNO3. Following this, 1.6 ml of solution was diluted with 3.3 ml of 1% of HNO3 before analysis. The same protocol was used for the Se extraction from the TMR samples with the exception that freeze-dried samples were ground using a SPEX 6700 freezer Mill (Model#6700-115. Industries Edison, N.J., USA) before extraction.

Trace minerals were measured using an Elemental X-series 2 Inductively coupled plasma mass spectrometry (Cat# 01957C, Thermo Scientific USA) with a collision cell as previously described (Pérez-

Rodríguez et al., 2017; Memon et al., 2007). Briefly, samples were free aspirated through a Teflon nebulizer into a Peltier cooled cyclonic spray chamber. Analytes were measured in 3 runs of 120 sweeps per run with peak dwell times of 10 ms. Intensity fluctuations throughout the run were normalized using Indium (Cat# 13846, Alfa Aesar Specpure). Final data were corrected using a 5-point standard curve for each element using purified standards for ICP-MS available from the Keck Collaboratory for all microminerals except Se, which was purchased (Cat# 82026- 056, VWR Chemicals, USA).

Glutathione peroxidase activity

GPx activity was also assessed using a commercial kit (cat# 703102-480, Cayman Chemical, USA) in erythrocyte lysate from whole blood samples of heifers and calves collected in Na- heparin tubes (Cat# 6102751, 10 ml; Becton Dickinson, Franklin Lakes, NJ) at -40, -20, 7, and 14 DIM. Blood samples were centrifuged in a Beckman Coulter centrifuge (Cat# 392187, Allegra X-22R Centrifuge, Beckman Coulter, INC) using 50 ml tubes (Cat#525-0402, VWR, USA) at 3000×*g* for 15 minutes at 4°C. The plasma was collected in 1.7 ml Eppendorf (Cat# 22-281, Genesee Scientific) and stored at -80°C until analysis. The buffy layer was discarded, and four volumes of ice-cold distiller water were added, followed by centrifugation at 10,000×*g* for 15 min at 4°C. The supernatant (erythrocyte lysate) was collected in 1.7 ml Eppendorf (Cat# 22-281, Genesee Scientific) and frozen at -80°C until analysis. The GPx assay was run in duplicate in a 96 well plate (Cat#400012). The absorbance was read at 340 nm using Molecular Devices SpectraMax Plus Microplate Spectrophotometer 384.

Supplementary Results

Suppl. Table S3. Se balance estimated in Jersey and Holstein heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition.

	Control		Selenium			P-value[1]					
	Holstein	*Jersey*	*Holstein*	*Jersey*	*SEM*	*TRT*	*Breed*	*T*	*T×B*	*TxTRT*	*T×B×T*
% experimental hay in the ration (DM)											
Overall	36.2	33.7	35.9	34.7	*2.10*	0.85	0.36	<0.01	0.76	0.35	0.96
Dry	37.3	34.1	36.7	35.3	*1.95*	0.86	0.23	0.22	0.64	0.05	0.41
Lactation	34.1	32.9	34.3	33.4	*3.60*	0.91	0.76	0.01	0.97	0.01	0.98
Se fed (mg/d)											
Overall	5.80	4.96	19.5	16.0	*0.50*	<0.01	<0.01	<0.01	0.01	0.76	0.61
Dry	3.76	3.32	17.2	14.3	*0.47*	<0.01	<0.01	0.11	0.02	<0.01	0.29
Lactation	10.4	8.6	24.7	19.8	*0.93*	<0.01	<0.01	<0.01	0.11	0.93	0.15
mg Se/kg DM											
Overall	0.47	0.47	1.53	1.50	*0.03*	<0.01	0.58	<0.01	0.61	<0.01	0.30
Dry	0.32	0.32	1.43	1.37	*0.04*	<0.01	0.40	0.01	0.41	0.05	0.34
Lactation	0.78	0.78	1.77	1.80	*0.05*	<0.01	0.76	0.48	0.69	0.03	0.29
µg Se/BW[2]											
Overall	10.7	10.7	35.3	34.2	*1.25*	<0.01	0.65	<0.01	0.66	0.55	0.87
Dry	6.55	6.85	30.6	29.9	*1.10*	<0.01	0.87	0.04	0.64	<0.01	0.54
Lactation	20.1	19.3	45.9	43.9	*2.40*	<0.01	0.54	<0.01	0.80	0.89	0.08
Se balance (fed-losses; mg/d)											
NRC[3]											
Overall	2.14	1.81	15.7	12.8	*0.36*	<0.01	<0.01	<0.01	0.01	0.64	0.68
Dry	0.22	0.23	13.5	11.1	*0.37*	<0.01	<0.01	<0.01	<0.01	0.01	0.47
Lactation	6.43	5.32	20.4	16.5	*0.65*	<0.01	<0.01	0.01	0.04	0.96	0.16
Estimated[4]											
Overall	2.07	1.70	8.18	6.65	*0.20*	<0.01	<0.01	<0.01	<0.01	0.26	0.48
Dry	1.37	1.21	7.35	6.13	*0.20*	<0.01	<0.01	0.10	0.01	<0.01	0.30
Lactation	3.63	2.80	10.0	7.82	*0.33*	<0.01	<0.01	0.14	0.05	0.53	0.23

[1]TRT=effect of supplementing 1% BW of Se-biofortified hay (i.e., Selenium) vs. control hay; T×B= TRT × Breed interaction; T×B×T = TRT × Breed × Time interaction

[2]µg Se/BW=All mg Se intake/BW*1000 (from mg to µg) *0.5 (assuming 50% absorption)

[3]Balance of Se calculated using the requirements according to NRC 2001(i.e. 0.3 mg/kg DM)

[4]Balance of Se calculated using the requirements calculated using the urine losses (6.1% of Se intake in CTR and 13.1% of Se intake in cows receiving forage with high Se), feces losses (56.0% of Se intake in CTR and 43.8% of Se intake in cows receiving forage with high Se), and milk as calculated using our data or Se requirement for conceptus as indicated by NRC (0.055 mg/d) (Séboussi et al., 2016).

Suppl. Table S4. Se from hay, estimated Se absorbed, and estimated Se requirements in Jersey and Holstein heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior to expected parturition.

I	Contro		Selenium			P-*value*[1]					
	Holstein	***Jersey***	***Holstein***	***Jersey***	***SEM***	***TRT***	***Breed***	***T***	***TxB***	***TxTRT***	***TxBxT***
Se from Hay mg/d											
Overall	2.03	1.79	16.39	13.68	*0.31*	<0.01	<0.01	0.95	<0.01	0.62	0.98
Dry	2.04	1.79	16.25	13.66	*0.36*	<0.01	<0.01	0.93	<0.01	0.65	0.92
Lactation	2.03	1.78	16.72	13.73	*0.31*	<0.01	<0.01	0.90	<0.01	0.62	0.89
Se absorbed mg/d											
Overall	2.55	2.18	10.97	9.01	*0.26*	<0.01	<0.01	<0.01	<0.01	<0.01	0.36
Dry	1.65	1.46	9.66	8.06	*0.26*	<0.01	<0.01	0.08	0.01	<0.01	0.32
Lactation	4.57	3.77	13.86	11.13	*0.44*	<0.01	<0.01	<0.01	0.04	0.91	0.14
NRC[2] mg/d											
Overall	3.69	3.14	3.83	3.24	*0.20*	0.54	0.01	<0.01	0.92	0.78	0.69
Dry	3.58	3.09	3.67	3.20	*0.20*	0.62	0.03	0.04	0.96	0.26	0.18
Lactation	3.94	3.25	4.20	3.34	*0.29*	0.54	0.01	0.01	0.76	0.63	0.68
Estimated[3] mg/d											
Overall	3.73	3.25	11.34	9.38	*0.31*	<0.01	<0.01	<0.01	0.02	0.93	0.80
Dry	2.39	2.12	9.84	8.21	*0.27*	<0.01	<0.01	0.11	0.02	<0.01	0.28
Lactation	6.75	5.77	14.65	11.97	*0.64*	<0.01	0.01	<0.01	0.18	0.95	0.19

1TRT=effect of supplementing 1% BW of Se-biofortified hay (i.e., Selenium) vs. control hay; T×B= TRT × Breed interaction; T×B×T = TRT × Breed × Time interaction

2Requirement according to NRC 0.3 mg/kg DM

3Se requirement calculated using the urine losses (6.1% of Se intake in CTR and 13.1% of Se intake in cows receiving forage with high Se), feces losses (56.0% of Se intake in CTR and 43.8% of Se intake in cows receiving forage with high Se), and milk as calculated using our data plus the Se requirement for conceptus as indicated by NRC (0.055 mg/d) (Séboussi et al., 2016),

Suppl. Table 5. The overall concentration of Se and other trace minerals in whole blood, milk, and liver of Jersey and Holstein heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving. Reported is also the level of the microminerals in whole blood of their offspring during the first 24 days after birth.

Micromineral	Control		Selenium			P-value[1]			
	Holstein	*Jersey*	*Holstein*	*Jersey*	*SEM*	*TRT*	*Breed*	*TxB*	*TxBxT*
Whole blood cows, ng/ml									
Se	122.8	98.3	171.0	175.5	*10.30*	<.01	0.40	0.31	0.62
Co	3.64	1.92	1.64	1.44	*0.91*	0.22	0.34	0.45	0.34
Cu	1276.5	887.7	812.4	745.5	*147.8*	0.60	0.80	0.28	0.74
Zn	1154.8	840.3	1466.2	849.7	*267.5*	0.32	0.02	0.35	0.50
Whole blood calves, ng/ml									
Se	158.9	163.5	196.3	234.7	*13.22*	<0.01	0.19	0.29	0.43
Co	2.78	1.55	1.69	1.61	*0.29*	0.13	0.05	0.10	0.29
Cu	668.6	679.8	769.1	727.5	*70.90*	0.36	0.84	0.74	0.24
Zn	1170.8	1022.1	1229.4	994.7	*110.9*	0.87	0.09	0.66	0.52
Liver cows, µg/g									
Se	0.74	0.51	1.34	1.13	*14.25*	<0.01	0.05	0.91	0.15
Co	0.78	0.39	0.49	0.44	0.01	<0.01	<0.01	<0.01	0.18
Cu	35.83	46.34	42.32	28.48	*88.39*	0.58	0.87	0.24	0.12
Zn	73.25	55.74	63.85	49.47	*13.58*	0.62	0.32	0.92	0.85
Colostrum/Milk, ng/ml									
Se	19.78	25.26	42.55	54.14	*4.48*	<0.01	0.08	0.53	0.71
Co	7.39	9.39	6.87	14.20	*4.34*	0.65	0.33	0.58	0.63
Cu	259.2	273.1	258.1	380.9	*68.7*	0.48	0.37	0.47	0.84
Zn	2971.6	3201.8	2982.3	2843.9	*413.1*	0.69	0.91	0.67	0.99
Se transfer into calves[2], %	8.05	9.90	6.32	6.02	*0.87*	*<0.01*	*0.42*	*0.26*	

[1]TRT=effect of supplementing 1% BW of Se-biofortified hay (i.e., Selenium) vs. control hay; T×B= TRT × Breed interaction; T×B×T = TRT × Breed × Time interaction

[2]Efficiency of transferred Se into calves %= Se concentration in whole blood of calf (mg/L) × 9.71% of BW of the calf (kg) / Se concentration in whole blood of cows (mg/L) × 9.71% of BW of a cow (kg) modified from (ref).

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