2021 Straus Dairy Farm Pilot Study Report

December 3, 2021
B.M. Roque, PhD

Project Setup

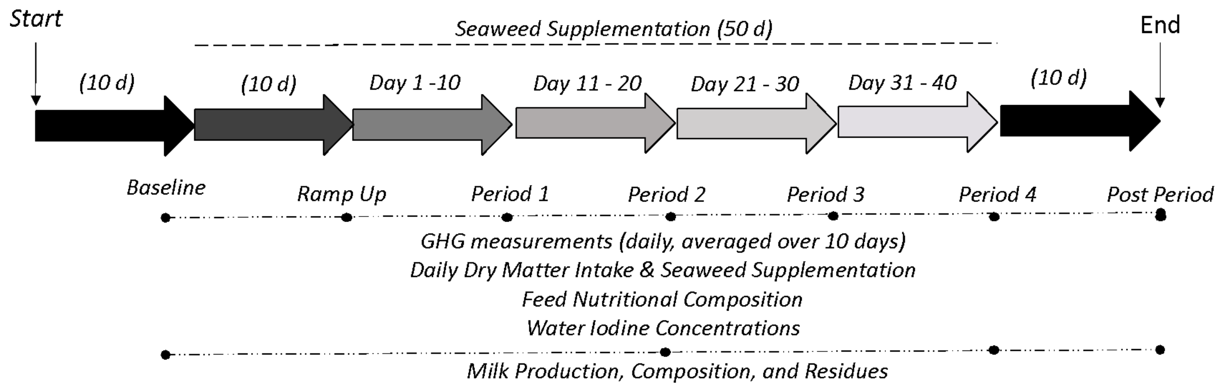
The 2021 Pilot Study was conducted at Straus Dairy Farm as a collaboration between Blue Ocean Barns and Straus Family Creamery. The objectives of this study were to 1) test the on-farm application and methane reduction potential of *Asparagopsis* seaweed, 2) quantify any changes in milk production and components from *Asparagopsis*-fed cows, 3) establish human safety margins of residue transfer from *Asparagopsis* material to milk. Forty-eight lactating, mixed breed cattle (Jersey-Holstein) were first blocked by milk production, days in milk, lactation stage, and dominant breed, then randomly assigned one of two treatments: control (basal diet) or treatment (basal diet + *Asparagopsis*). Both treatment groups were housed in the same barn. A dividing electric wire was placed between the two groups. Figure 1 demonstrates the experimental timeline used in this study. The first 10 days of the study were used as a baseline period for cows to learn to use the GreenFeed machines and adjust to the trial barn. The next 10 days were used as a ramp-up period during which *Asparagopsis* inclusion was slowly increased daily until an optimal level was established. Experimental periods 1 through 4 comprise the 40 days (10-day increments) *Asparagopsis* was fed at 3 kg dry matter (DM) mixed into the total mixed ration (0.125 kg/head/day). Average daily dry matter intake (DMI) ranged from 18.9 – 23.2 kg/head/day in the control group and from 17.6 – 20.5 kg/head/day in the treatment group. The *Asparagopsis* inclusion level was held at 3 kg DM, with an average inclusion of approximately 0.65% DM.

Data from all cows was averaged over each experimental period as follows:

- Baseline (July 24 – Aug 3)
- Ramp Up (Aug 4 – Aug 13)
- Period 1 (Aug 14 – Aug 23)
- Period 2 (Aug 24 – Sep 2)
- Period 3 (Sept 3 – Sept 12)

- Period 4 (Sept 13 – Sept 22)
- Post-Treatment Period (Sept 23 – Oct 2)

Figure 1. Visual depiction of the study's experimental timeline including measurement days, seaweed supplementation, and data measurement periods.



The following measurements were recorded:

- **Enteric Gas Production:** Enteric gas emissions were measured using one GreenFeed large animal gas measurement device (C-Lock Inc.) per treatment group. Cows were allowed to visit the GreenFeed up to five times per day and received eight drops of barley per visit (maximum of 40 drops per day). Each drop of barley equaled 27 g of additional feed and was included in daily dry matter intake. Criteria for data selection required each cow to visit at least two times per day and a GreenFeed measurement to be logged during each of the preselected timepoints (0001 – 0700, 0701 – 1200, 1201 – 1900, 1901 – 0000) to account for variations in methane production throughout the day. Data was first averaged within each timepoint, then averaged across timepoints to achieve an estimated methane production (g/day).

- **Milk Production:** Milk production data was collected during dairy herd assessments provided by Straus Dairy Farm DHIA testing on Aug 2, Aug 26, Sept 16, and Oct 2. These measurements were used as representative milk production samples for each cow during baseline, 2, 4, and post-treatment experimental periods, respectively.

- **Milk Components:** Milk fat and solid non-fat percentages were provided by Straus Dairy Farm DHIA testing in coordination with the milk production days. Additional sampling was conducted once per 10 days to determine, for each cow, average protein percentages as a proportion of solid non-fat. These measurements were used as representative milk production samples for each cow during baseline, 2, 4, and post-treatment experimental periods, respectively.

- **Feed Intake:** Straus Dairy Farm management provided daily group feeding amounts, and weigh backs when appropriate, on an as-fed and DM basis. Proximate analysis was conducted on each total mixed ration (TMR) via bunk grab samples using Cumberland Valley Analytical Services to determine nutritional composition. Bunk grab samples were taken once every 10 days (on the last day of each experimental period) and averaged across all time periods. Average barley intake (DM) from the GreenFeed machines was included in the determination of nutritional composition of daily feed intake.

- ***Asparagopsis* Intake:** *Asparagopsis* was mixed into the TMR immediately before distribution in the feed wagon and fed to the treatment group in a group bunk at 0.125 kg/head/day (DM). Proximate analysis was conducted on seaweed samples on the last day of each experimental period. To determine total intake and mineral additions to the treatment diet, the control diet nutritional composition was adjusted for the addition of seaweed. This provided average DM and iodine concentrations for the treatment group cows. Additional testing was conducted by Cumberland Valley Analytical Service for heavy metal and mineral composition and by California Department of Food and Agriculture (CDFA) for bromoform concentrations of the seaweed and TMR.

- **Water Intake:** Water intake was estimated based on Cardot et al. (2008) equation; FWI, (L/d) = 1.53 × DMI (kg/d) + 1.33 × MY (kg/d) + 0.89 × DM Feed (%) + 0.57 × Min Temp (°C) − 0.30 × RF (mm/d) − 25.65 where FWI = daily free water intake, DMI = daily dry matter intake, MY = milk yield, DM Feed = dry matter content of feed, Min Temp =

estimated minimum temperature in Marshall, CA, and RF = average rainfall in Marshall, CA. Water was also tested for iodine concentrations every 10 days and calculated in coordination with estimated FWI to determine total iodine water intake per cow per day. Additionally, water was tested for bromoform every 10 days. Water bromoform analyses were conducted by Dr. MaryAnn Drake at North Carolina State University.

- **Milk Residue:** A subset of milk samples was taken from 20 cows (10 cows per treatment group) on the last day of each experimental period and tested for milk iodine, bromide, and bromoform residues. Milk iodine and bromide concentrations were provided by Michigan State University Veterinary Diagnostics Laboratory and milk bromoform analyses were conducted by Dr. MaryAnn Drake at North Carolina State University.

- **Urine Residue:** Urine samples were taken from a subset of cows and tested for bromoform residues. Six samples were taken on Sept 3 (three from each treatment group). Fourteen samples were taken on Sept 12 (seven from each treatment group). Urine bromoform analyses were conducted by Dr. MaryAnn Drake at North Carolina State University.

Statistical Analysis

Individual cow was established as the experimental unit (n). At the start of the experiment, control and treatment groups consisted of 24 cows each. Collected data was first averaged within each 10-day experimental period, then a mixed-effects model and an appropriate repeated measures correlation structure were applied for statistical analysis. The mixed-effects model includes fixed effects of treatment, diet, interaction between treatment and diet, and random effect of cow. Repeated measurements of cows can introduce correlation bias into the data; therefore either an autoregressive AR(1) correlation structure (evenly spaced measurement periods such, as DMI & emissions) or a spatial correlation structure (uneven measurement periods, such as milk production and components) was applied to the model.

Outcomes

Nutritional Composition and Feed Intake: For this trial, freeze-dried *Asparagopsis taxiformis* gametophytes that had been wild harvested from the Azores (Portugal) in 2019 were incorporated in the feed provided to dairy cows in the treatment group. Nutritional composition of *Asparagopsis* seaweed used, barley grain from the GreenFeed machines, as well as control and treatment rations are shown in Table 1. Control and treatment TMR are fairly consistent, with the exception of mineral contributions. Both control and treatment groups were fed the same mineral pack, which included iodine and other minerals known to be present in *Asparagopsis*. Iodine is elevated in the treatment TMR, which is a direct consequence of *Asparagopsis* inclusion. The iodine concentration of the *Asparagopsis* used was approximately 2,467 mg/kg, and when applied at 0.125 kg/head/day without adjusting the iodine inclusion in the mineral pack, resulted in 24.1 mg/kg dietary iodine in the treatment TMR. According to the NRC's Mineral Tolerance of Animals Report (2005), the maximum tolerable limit for dietary iodine is 50 mg/kg dry matter intake, which is well above the 24.1 mg/kg iodine concentration of the treatment TMR. Furthermore, a heavy metal and extended mineral analysis was analyzed for the *Asparagopsis* seaweed used in the current study to determine if any compounds exceeded the NRC recommendations when *Asparagopsis* is applied at 0.125 kg/head/day (Table 2). Table 2 shows that, with regard to animal safety, *Asparagopsis* seaweed can be applied to cattle diets at 0.125 kg/head/day without reaching or exceeding maximum tolerable limits.

Table 3 shows average individual daily DMI for control and treatment cows. Average individual DMI was calculated by taking DMI of the group and dividing by the number of animals. When averaged across all four experimental periods, an 11.2% decrease in DMI (control: 21.8 kg/head/day; treatment: 19.4 kg/head/day) was demonstrated in the current study. Roque et al. (2019) and Stefenoni et al. (2021) also reported decreases in DMI of 10.8% and 7.11%, respectively, at similar inclusion rates (0.93% DM and 0.50% DM, respectively). Additionally, the largest DMI reductions in the current study came during the ramp up and first experimental period, after which DMI started to stabilize between 19 – 20.5 kg/day. This is a strong indication that there is an initial adaptation period during which cows adjust to *Asparagopsis* inclusion in their diets. An adaptation period had previously been suggested by Roque et al. (2019), which noted sorting behavior in *Asparagopsis*-fed cows over a 14-day experimental period.

<u>Milk Production and Components:</u> Milk yield (kg/day) was higher for control cows than treatment cows, even during the baseline collection period (Figure 2A). This initial difference between the control and treatment group held constant across experimental periods and the post-treatment period data collection points, and no statistical significance was found between control and treatment milk production. This is consistent with Roque et al. (2019), where no differences in milk production were found at the lower *Asparagopsis* inclusion level of 0.93% DM. However, Stefenoni et al. (2021) reported a slight decrease in milk production (2.6 kg/day) when using a 0.50% DM *Asparagopsis* inclusion rate. The treatment cows in the current study show a maintenance of milk production despite a decrease in DMI (Figure 2B), which indicates that there may be added nutritional benefits from *Asparagopsis* seaweed included in ruminant diets. While the results of the current study are promising for on-farm application, milk production needs to be carefully monitored to ensure steady production of milk. It is important to note that the first milk production measurement was conducted during experimental period 2, which may not have given the cows enough of an adaptation period to adjust their intake and maintain milk production.

Energy corrected milk (ECM) was also calculated to account for milk fat and protein energy content (Figure 2C). During experimental period 2, there appears to be a significant difference between control and treatment ECM (control: 25.0 kg; treatment: 20.2 kg, $P<0.05$). This significant difference is directly correlated to decreases in percent fat and protein during experimental period 2 (Figure 3A/B). In comparison, Roque et al. (2019) and Stefenoni et al. (2021) found no statistical differences in milk fat and SNF percentages between control and *Asparagopsis*-fed cows. However, both Roque et al. (2019) and Stefenoni et al. (2021) report numerically lower values for the treatment-fed cows. It is likely neither study had sufficient experimental units to determine statistical significance whereas the current study does (current study: 24 cows; Roque et al. (2019): 12 cows; Stefenoni et al. (2021): 20 cows). What is particularly interesting from the current study is that both milk fat and SNF percentages appear to recover to pre-treatment levels during experimental period 4, which extend to the post-treatment period as well. This result strongly supports the hypothesis that, while the initial introduction of *Asparagopsis* in dairy cow diets is disruptive to DMI as well as milk fat and SNF percentages (likely due to the changes in DMI), this effect quickly subsides and milk component values recover to normal levels in less than 40 days. No significant differences were found in somatic cell counts between control and treatment cows (Figure 3C). Somatic cell count is a major marker for increased stress and inflammation in dairy cows.

Methane Production in Treatment Cows: Percent reductions of per-period methane production (g/day) compared to their own baseline varied widely between individual cows in the treatment group, as demonstrated for 21 of 24 treatment cows in Figure 4 (three cows were excluded for lack of sufficient visits to the GreenFeed machine). Four cows obtained over 85% methane reductions during at least one experimental period during the trial, and an additional 11 cows (15 total) achieved more than a 50% reduction during at least one experimental period. When averaged across experimental periods, 12 cows achieved more than a 50% reduction in methane production (g/day) over the whole trial. Such variability is notable, but perhaps expected, given that cows were group fed and had greater opportunity for sorting through the TMR compared to a controlled study where animals are individually fed. However, it is noteworthy that at least half of the treatment group achieved more than 50% reductions in methane and that this reduction persisted throughout the 50-day seaweed-inclusion period (10-day ramp up and 40-day experimental period), especially considering that this is the longest period of time that dairy cows have been fed *Asparagopsis* seaweed.

Methane Production Between Treatment and Control Cows: An average of 52% methane emissions reduction (g/day) was shown across all experimental periods when comparing treatment methane production to control methane production (Figure 5A). Methane percent reductions shown in this study (52% at 0.65% DM inclusion rate) are even greater than the reductions found in Roque et al. (2019), where the lowest inclusion rate fed, 0.93% DM, resulted in a 24.6% reduction in methane production (g/day), and Stefenoni et al. (2021), where 0.5% DM inclusion resulted in methane reductions of between 55 – 80%. The variability between and within studies is largely due to the initial concentrations and persistence of bromoform within the *Asparagopsis* seaweed. Roque et al. (2019) reported bromoform concentrations of 1.32 mg/g DM and Stefenoni et al. (2021) reported bromoform concentrations as high as 10 mg/g DM, though the Stefenoni et al. (2021) *Asparagopsis* material declined in bromoform concentrations over a four-month period to 2 mg/g (an average 20% decrease per month). The *Asparagopsis* material used in the current study, started at 3.2 mg/g and slowly decreased to an average of 2.47 mg/g over a seven-month period (an average 3.3% decrease per month). A regression analysis was conducted on average methane production from control and treatment groups for the current study, Roque et al. (2019), and Stefenoni et al. (2021), and standardized based on mg of bromoform per kg of neutral detergent fiber intake (NDF) (Figure 6). Figure 6 shows a consistent negative linear relationship ($R^2 = 0.82$) between methane production (g/day) and bromoform concentrations (mg/kg NDF) across all three studies conducted in dairy cows. While bromoform concentrations varied across studies, this figure provides a rough estimate of the volume of methane reduced per unit of bromoform using the following regression equation,

$$y = 380 - 3.4x$$

In this equation, y represents the dependent variable (methane production, g/day), 380 represents the initial value of methane production (at 0 mg bromoform), 3.4 is the slope of the regression line, and x represents the independent variable (bromoform, mg/kg NDF).

<u>Methane Yield and Intensity:</u> Enteric methane production can vary depending on the type/breed of ruminant, environment, diet specifications, and productivity. Therefore, it is common to standardize the amount of methane produced per day either on a per unit of feed intake or per unit of productivity (such as meat or milk) basis. In the current study, methane production was standardized on both a per kg dry matter intake (DMI) (Methane yield, Figure 5B) and per kg of energy corrected milk (Methane intensity, Figure 5C) basis. Methane yield reductions reached an average 50% across the experimental periods. This result is notable, as it shows that methane reductions are maintained even when reduced intake is shown. In contrast, Roque et al. (2019) fed significantly more *Asparagopsis* as a percentage of feed intake (0.93% DM – 1.84% DM), which resulted in decreased dry matter intake (between 10.8% – 38%), meaning methane yield reductions were smaller (20.3% – 42.7%, respectively) than overall decreases in methane production (24.6% – 67.2%, respectively). The persistence in methane yield reductions in the current study provides evidence that methane reductions are not a direct consequence of reduced DMI. The differences between methane production and yield demonstrated in Roque et al. (2019) may be due to the shorter experimental period (14 days) than the current study (10-day ramp up and 40-day experimental period), where methane reductions were similar between production and yield.

Methane intensity in the current study was reduced by 44% between control and treatment, which is a smaller effect than both production and yield. This may be due to a slight decrease in milk components (such as fat and protein) during experimental period 2. Additionally, there were differences between overall milk yields between control and treatment even during the baseline period. The results seen in this study are consistent with both Roque et al. (2019) and Stefenoni et al. (2021), where slight numerical decreases in milk components were reported. However, the previous studies were conducted over a shorter period of time (Roque et al. (2019): 14 days; and Stefenoni et al (2021): 28 days). In the current study, milk components return to normal percentage levels after approximately 20 days, which may help maintain reductions in methane intensity over time.

Milk Residue Testing: Like many ocean-grown seaweeds, wild *Asparagopsis* is known to carry high concentrations of some heavy metals and minerals Therefore, when feeding *Asparagopsis* to dairy cows, it is crucial for residue testing to be carried out on the milk produced from these cows. Iodine concentrations of feed and water were estimated to determine total intake for control and treatment cows and used to calculate a percent excretion rate through the milk (Table 4). High concentrations of *Asparagopsis* iodine (3,200 ppm) led to elevated milk iodine residues in the treatment group (5.24 – 7.53 mg iodine per kg milk). Stefenoni et al. (2021) reported milk iodine concentrations of 2.97 mg/kg from cows fed 0.50% DM *Asparagopsis*, 1.5 to 2.5 times lower than what was demonstrated in the current study. The difference here is most likely due to lower iodine concentrations in the Stefenoni et al. (2021) *Asparagopsis* material, though actual concentrations were not reported. Tolerable upper intake limits of iodine set by the US Food and Nutrition Board for humans are between 0.2 and 1 mg per day, depending on age, gender, and lactation demographics. *Asparagopsis* fed to cows will need to be tested to ensure low iodine concentration. The *Asparagopsis* used in this study was wild harvested from Portugal and contains a much higher concentration of iodine than tank-based operations (3,200 vs 200 mg/kg). Supplementing cows with *Asparagopsis* with iodine content 16 times lower than material used in the current trial will dramatically reduce the issue of iodine transfer once available for commercial use. Additionally, milk bromide concentrations are elevated in cows receiving *Asparagopsis* (58 – 74 mg bromide per kg milk) compared to control cows (6 – 21 mg bromide per kg milk) (Table 5). In comparison, Stefenoni et al. (2021) reported bromide concentrations of 40.4 mg/kg in cows fed 0.50% DM *Asparagopsis*. The World Health Organization has set recommended bromide upper limit levels to be 4 mg per kg bodyweight for humans. More information is needed on the transfer of bromide to milk and the potential health implications associated with this.

Milk bromoform concentrations were analyzed for each cow during experimental days 0 and 40 (Table 6). On experimental day 0, milk from most cows showed no bromoform, using a detection limit of 0.01 ug/L and quantification limit of 0.03 ug/L. Milk from three cows in the treatment group had detectable (> 0.01ug/L) amounts of bromoform, though only one sample was quantified (0.131 ug/L) during baseline, when no *Asparagopsis* had yet been included in the diet. Bromoform has also been seen in control milk in Roque et al. (2019) and Stefenoni et al. (2021) at concentrations of 0.11 ug/L and 16.5 ug/L, respectively. This is important to note because bromoform is commonly found in treated drinking water and it is likely that the animals' drinking source is a major contributor to milk bromoform levels. Water bromoform concentrations for the current study were also quantified for control water troughs (average: 3.02

ug/L; range: 0.44 – 10.3 ug/L) and treatment water troughs (average: 3.01 ug/L; range: 0.66 – 9.83 ug/L). On experimental day 40 (last day of the experiment), milk samples from six cows in the control group contained bromoform, with an average concentration of 0.53 ug/L and a range of 0.14 – 1.11 ug/L (one sample was excluded; see Table 6 footnotes). Milk samples from 16 cows in the treatment group contained bromoform, with an average concentration of 0.78 ug/L Decand a range of 0.13 – 2.42 ug/L. Results from the current study and previous studies show that bromoform can be found in milk even with no *Asparagopsis* added to the feed and that average concentrations of milk bromoform do not vary significantly between control and *Asparagopsis*-fed cows. However, it does appear that there may be an increase in the instances of bromoform found in the milk (six control vs. 16 treatment cows on experimental day 40). The data show that *Asparagopsis* seaweed does not contribute to elevated bromoform concentrations in milk. However, the increase in instances of bromoform detection requires further investigation. Additionally, the bromoform concentrations found are well below the recommendations set by CDC (low safe: 1 ug/L; high safe: 10 ug/L), EPA (80 ug/L), and WHO (100 ug/L) (Figure 7) for drinking water, meaning that neither milk from control nor *Asparagopsis*-fed cows poses safety concerns for human consumption.

Urine Residue Testing: Bromoform concentration in urine was not statistically different between control and treatment groups. On average, 4.88 ug/L was measured in the control group compared with 5.78 ug/L in the treatment group (Table 7). The average bromoform level in treatment group urine dropped from 6.09 ug/L at the Day 20 sampling to 5.63 ug/L at Day 30 (Table 8), supporting a hypothesis that no bioaccumulation was occurring in the urine.

Project Limitations

This study encountered limitations that should be noted and considered when evaluating the results.

The initial setup of the project included both training cows to use the GreenFeed machines and collecting baseline measurements. Some cows take longer to learn to use the GreenFeed machine at least twice daily and at each of the four prescribed intervals at least once during the 10-day baseline period. Therefore, due to time constraints, baseline measurements were not able to be recorded for all 48 cows in the trial. Additional to this, there was one cow in the treatment group that refused to use the GreenFeed during the entire trial. Employing a “training/testing period” before the baseline measurement period begins in the future will help mitigate the loss of measurements due to untrained cows. The use of GreenFeeds on-farm has many benefits, such as portability and voluntary measurements from cows, though these machines require weekly attention from trained individuals. One GreenFeed bait cup continuously jammed throughout the study and, as a consequence, multiple days were lost for the group using that machine.

Data collection for milk was originally set to occur twice per day; however, due to issues with RFID readers in the milking parlor and milking meters that were not calibrated, the data available for daily milk production was not accurate enough for use in the study. Including daily milk production could help identify the exact point at which *Asparagopsi*s-supplemented cows start to recover from the initial adaptation period. Recording of technology-enabled daily milk weights should be included in protocols moving forward. Additionally, milk samples were taken once per 10 days for milk components and residue testing; however, the milk samplers at the farm were unfortunately not in working order. Straus Dairy Farm staff took samples by hand before attaching teats to the milking machine and, therefore, these samples may not be representative of the entire milking. This may have introduced error in milk components such as fat and protein and could have also had an impact on residue concentrations.

The barn used at the Straus Dairy Farm for the study was split down the middle by a hot wire, which separated the two groups. However, there was only one feed bunk with no blocker between the control and treatment group. This may have introduced errors if some cows were able to access the other treatment’s feed. Additionally, on September 14 – 15 (period 4) there appears to have been an accidental regrouping of cows (thought to be caused by a power outage which inactivated the hot wire divider). Therefore, these two days were not included in the averaging of period 4. During the September 14 – 15 mix-up, two cows from each treatment group were accidentally replaced into the wrong group, and therefore have been removed from period 4 (control: 3657, 3671; treatment: 3675, 3601). Period 4 control n = 22, treatment n = 21.

Summary of Outcomes

This project has demonstrated on-farm applicability of feeding *Asparagopsis* seaweed to reduce enteric methane emissions by over 50% while maintaining milk production over the course of 40 experimental days. There was a brief period of acclimation during which dry matter intake, milk fat, and solid non-fat percentages decreased slightly in experimental period 2; however, the values for these variables quickly returned to normal ranges after acclimation to the change in diet. Iodine concentrations in the seaweed were higher than the research team expected and consequently resulted in higher milk iodine residues; however, current cultivation of *Asparagopsis* seaweed yields a 16-fold lower iodine concentration for commercial *Asparagopsis* seaweed compared to the wild-harvested *Asparagopsis* used in this study. Milk bromoform concentrations were not elevated between control and *Asparagopsis*-fed groups; however, there was an increase in the presence of milk bromoform in samples during experimental day 40 (six control vs. 16 treatment cows). The bromoform concentration in all samples, both control and treatment, are at or below the low end of the CDC safe range for drinking water.

What's Next

Next steps include additional on-farm projects. Use of cultivated *Asparagopsis* seaweed on farm will serve to solidify methane reductions, milk production and component persistence and to determine milk residue scenarios.

Table 1. Nutritional composition of diets, seaweed, and barley grain.

DM %	**Base**	**CTL**	**TRT**	**Seaweed**	**Grain**
Crude Protein	17.8	17.7	17.7	16.2	12.4
Acid Detergent Fiber	24.7	24.4	24.4	14.3	8.3
Neutral Detergent Fiber	33.4	33.2	33.2	29.7	21.4
Lignin	5.09	5.0	5.0	2.63	2.39
Starch	15.3	16.2	16.0	0.55	57.4
Crude Fat	5.37	5.31	5.3	0.66	2.68
Total Digestible Nutrients	67.2	67.4	67.2	30.0	78.1
Calcium	0.90	0.89	0.9	2.60	0.22
Phosphorus	0.41	0.41	0.4	0.20	0.40
Magnesium	0.28	0.27	0.3	1.11	0.18
Potassium	2.37	2.33	2.3	1.97	0.65
Sodium	0.21	0.21	0.30	8.53	0.06
mg/kg DMI					
Iron	875	859.4	884.5	4249	115
Manganese	74.3	73.4	73.6	86.3	29.5
Zinc	46.3	46.3	46.2	19.0	47.0
Copper	14.5	14.4	14.4	9.67	9.33
Iodine	2.42	2.50	24.1	3218	6.30
Bromoform	Not Detected	5.45	18.5	2467	No data

CTL = Control Diet = Base (97.95% DMI) + Grain (2.05% DMI).
TRT = Treatment Diet = Base (97.58% DMI) + Grain (1.77% DMI) + *Asparagopsis* (0.650% DMI).
Grain = avg consumption for control = 0.44kg DM & treatment = 0.340kg DM, ranged between 0.00 – 0.94kg DM.

Table 2. Heavy metal and mineral composition of seaweed fed to treatment group.

	Maximum Tolerable Limits	Dietary DM @ 19kg/day	Seaweed	Seaweed Intake @ 0.125kg/day
	mg/kg DMI	**max mg per day**	**mg/kg**	**mg per day**
Aluminum	1000	19000	4093	512
Antimony	No data	No data	<5	<0.6
Arsenic	30	570	15.0	1.88
Barium	No data	No data	10.7	1.34
Boron	150	2850	109	13.6
Cadmium	10	190	0.56	0.07
Calcium	15000	285000	24480	3060
Chromium	100	1900	84.2	10.5
Cobalt	25	475	3.5	0.44
Copper	40	760	6.2	0.78
Iodine	50	950	3218	402
Iron	500	9500	5251	656
Lead	100	1900	1.3	<0.3
Magnesium	6000	114000	10121	1265
Manganese	2000	38000	99.5	12.4
Mercury	2	38	0	0
Molybdenum	5	95	2.3	0.29
Phosphorus	7000	133000	1694	212
Potassium	20000	380000	16042	2005
Selenium	5	95	0.30	0.04
Sodium Chloride	84742	1610094	71571	8946
Sulfur	3000	57000	30657	3832
Thallium	No data	No data	<10.0	<1.2
Zinc	500	9500	14.4	1.8

Table 3. Average daily dry matter intake[1] (kg) for control (CTL) and *Asparagopsis*-fed (TRT) cows.

	CTL	TRT	P
Baseline	19.0	19.0	ns
Ramp Up	23.2	18.8	***
Day 1 - 10	22.2	17.6	***
Day 11 - 20	22.2	20.4	***
Day 21 - 30	21.7	20.4	***
Day 31 - 40	21.1	19.0	***
Post Period	21.3	20.5	***

*P < 0.10, **P < 0.05, ***P < 0.01

[1]Daily dry matter intake was calculated by total dry matter fed to each group of cows divided by the number of cows in each treatment group (control = 24, treatment = 24). Daily barley intake DM was also included for each cow's daily intake. Seaweed was also included as part of the treatment cow daily intake (0.125 kg DM).

Table 4. Feed and water iodine intake levels (mg/day), milk iodine residue (mg/kg), and percent iodine excretion through milk for control (CTL) and *Asparagopsis*-fed (TRT) groups based on a subset of 10 cows per group.

	Feed Iodine			Water Iodine			Milk Iodine			Iodine % Excretion		
	CTL	TRT	P	CTL	TRT	P	CTL	TRT	P	CTL	TRT	P
Baseline	29.3	30.2	ns	20.1	21.1	ns	0.19	0.21	ns	12.4	13.4	ns
Period 2	62.9	453	***	33.5	27.4	ns	0.48	5.25	***	11.5	24.0	ns
Period 4	47.8	413	***	45.7	40.8	ns	1.35	7.53	***	37.1	38.8	ns
Post Period	40	39.1	ns	26.4	22.3	ns	1.08	1.27	ns	47.3	54.0	ns

Table 5. Milk bromide residue (mg/kg) for control (CTL) and *Asparagopsis*-fed (TRT) groups based on a subset of 10 cows per group.

	Bromide		
	CTL	TRT	P
Baseline	2.08	2	ns
Day 1 - 10	6.05	58.36	***
Day 11 - 20	12.37	75.35	***
Day 21 - 30	11.61	70.67	***
Day 31 - 40	9.45	74.79	***
Post Period	21.17	12.63	ns

*P < 0.10, **P < 0.05, ***P < 0.01

Table 6. Milk bromoform residues for control (CTL) and *Asparagopsis*-fed (TRT) cows during experimental days 0 and 40.

CTL Cow ID	Experimental Day	Bromoform ug/L	TRT Cow ID	Experimental Period	Bromofor m ug/L
2418	Day 0	ND	3221	Day 0	ND
	Day 40	ND		Day 40	0.225
3278	Day 0	ND	3326	Day 0	ND
	Day 40	ND		Day 40	ND
3291	Day 0	ND	3328	Day 0	ND
	Day 40	ND		Day 40	1.059
3292	Day 0	ND	3359	Day 0	ND
	Day 40	ND		Day 40	0.935
3369	Day 0	ND	3498	Day 0	ND
	Day 40	0.144		Day 40	0.312
3441	Day 0	ND	3508	Day 0	ND
	Day 40	ND		Day 40	1.993
3486	Day 0	ND	3535	Day 0	ND
	Day 40	ND		Day 40	0.756
3490	Day 0	ND	3546	Day 0	ND
	Day 40	ND		Day 40	0.815
3509	Day 0	ND	3620	Day 0	ND
	Day 40	0.327		Day 40	2.419
3617	Day 0	ND	3648	Day 0	ND
	Day 40	ND		Day 40	0.615
3652	Day 0	ND	3656	Day 0	ND
	Day 40	9.316^{+}		Day 40	0.721
3662	Day 0	ND	3665	Day 0	ND
	Day 40	ND		Day 40	0.914
3765	Day 0	ND	3753	Day 0	ND
	Day 40	ND		Day 40	0.537
3766	Day 0	ND	3761	Day 0	ND*
	Day 40	ND		Day 40	0.724
3780	Day 0	ND	3777	Day 0	ND
	Day 40	1.105		Day 40	0.927
3784	Day 0	ND	3783	Day 0	ND
	Day 40	ND		Day 40	0.351
3792	Day 0	ND	3809	Day 0	ND
	Day 40	ND		Day 40	0.514

3818	Day 0 Day 40	ND 0.188	3813	Day 0 Day 40	ND* 0.651
3820	Day 0 Day 40	ND ND	3828	Day 0 Day 40	0.131 0.716
3601**	Day 0 Day 40	ND ND	3835	Day 0 Day 40	ND 0.22
3675**	Day 0 Day 40	ND 0.88			
Bromoform 6 cows	Average 0.53	Range 0.14 – 1.11	Bromoform 16 cows	Average 0.78	Range 0.13 - 2.42

ND* = Milk bromoform was below Limit of Quantification (0.03), however was above Limit of Detection (0.01).
** = Cows were originally placed in TRT group, but were switched on day 20 to CTL group.
+ = CTL cow 3652 milk sample on Day 40 contained 9.316 micrograms bromoform per L milk and was considered to be an outlier. This sample was not used for average or range for CTL group.

Table 7. Average and standard deviation of bromoform residues in urine, by treatment group.

	Average bromoform concentration (ug/L)	**Std. dev. of bromoform concentration (ug/L)**
Control	4.88	2.76
Treatment	5.78	3.58

Table 8. Average bromoform residues in urine, by treatment group and sampling date.

Average bromoform concentration (ug/L)	**9/3/2021**	**9/12/2021**
Control	5.42	4.65
Treatment	6.09	5.63

Figure 2. Average daily milk production (kg/day) [A], milk production persistence compared to baseline values over time (%) [B], and energy corrected milk (kg/day) [C] for control (gray) and treatment (blue) cows during each experimental period. Significant differences are noted as *(P <0.10), **(P<0.05), and ***(P<0.01).

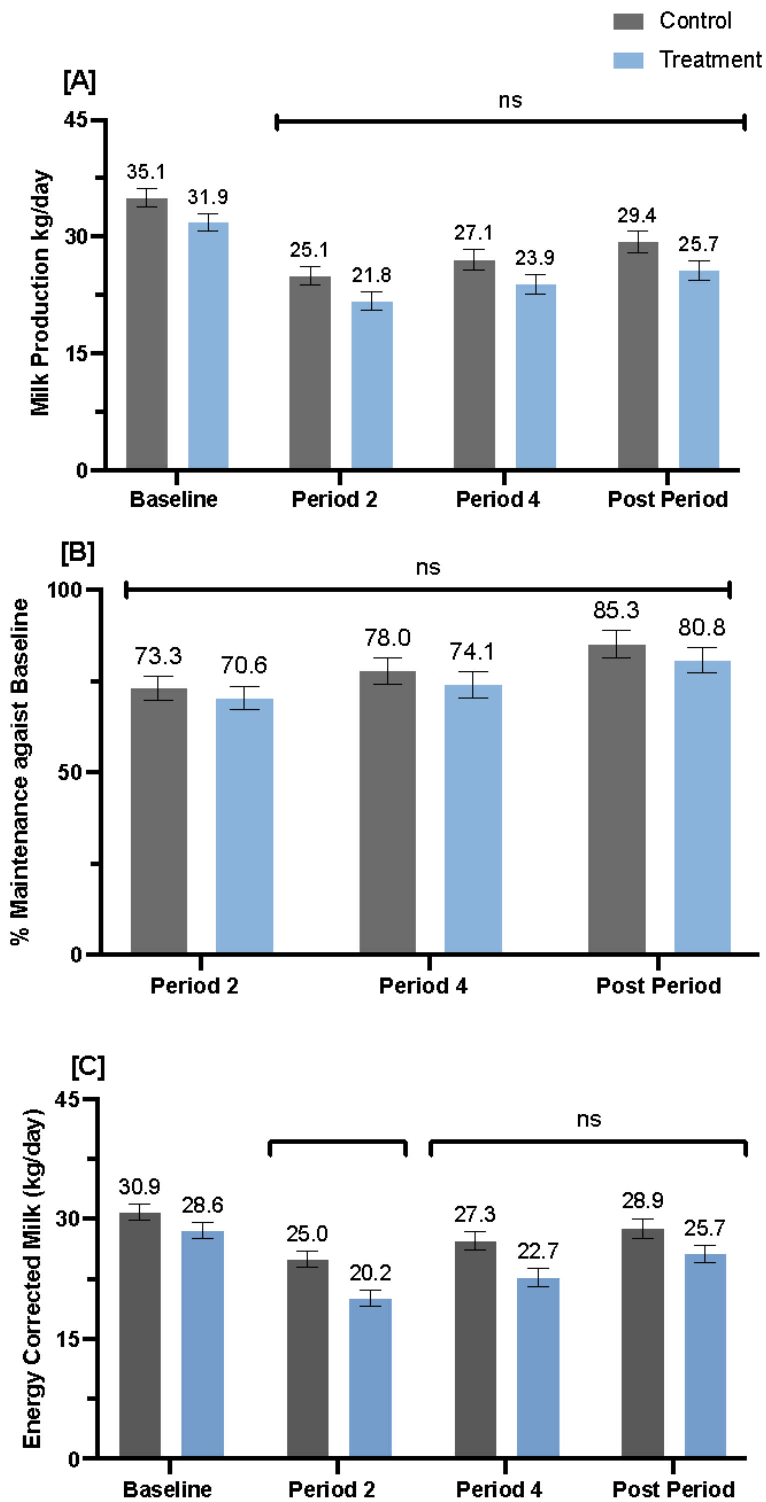


Figure 3. Average milk fat (%) [A], solid non-fat (%) [B], and intensity (x10^3/mL) [C] for control (gray) and treatment (blue) cows during each experimental period. Significant differences are noted as *(P <0.10), **(P<0.05), and ***(P<0.01).

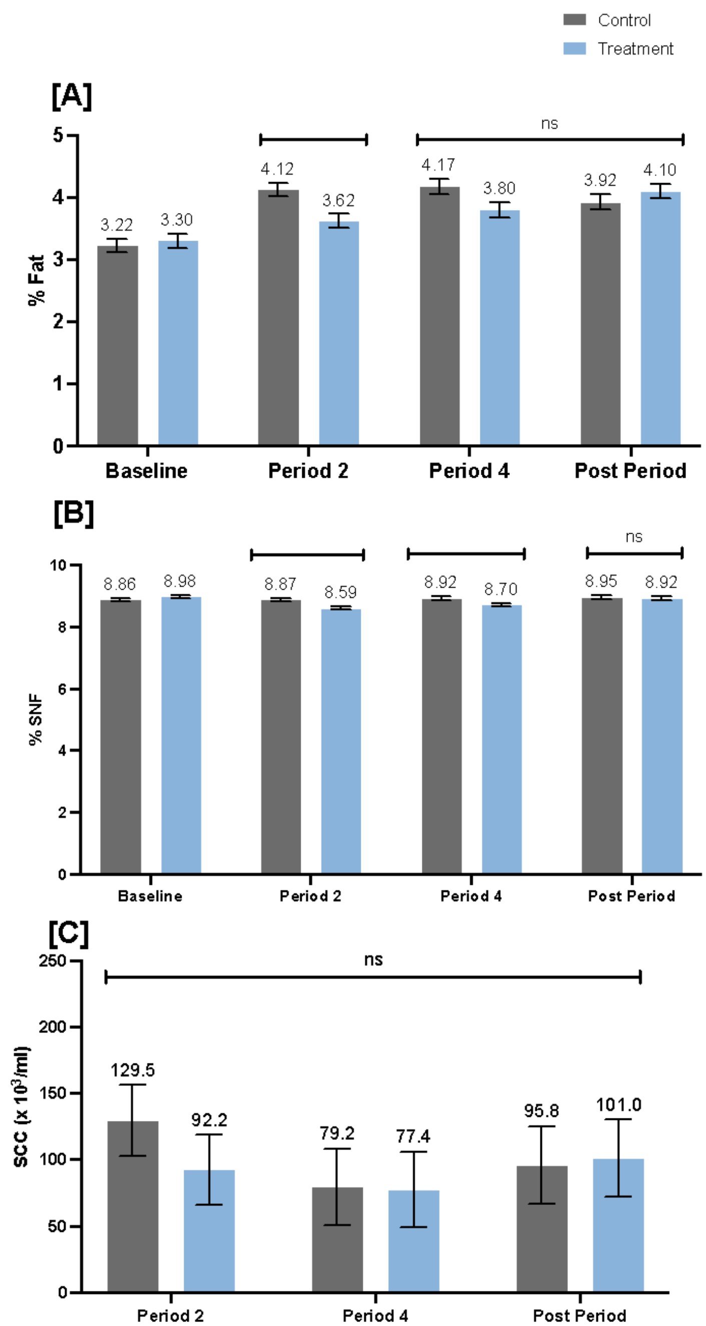


Figure 4. Average percent methane production (g/day) reductions for each treatment cow compared to their own baseline measurements. Twenty-one cows are shown here; three were removed due to insufficient data.

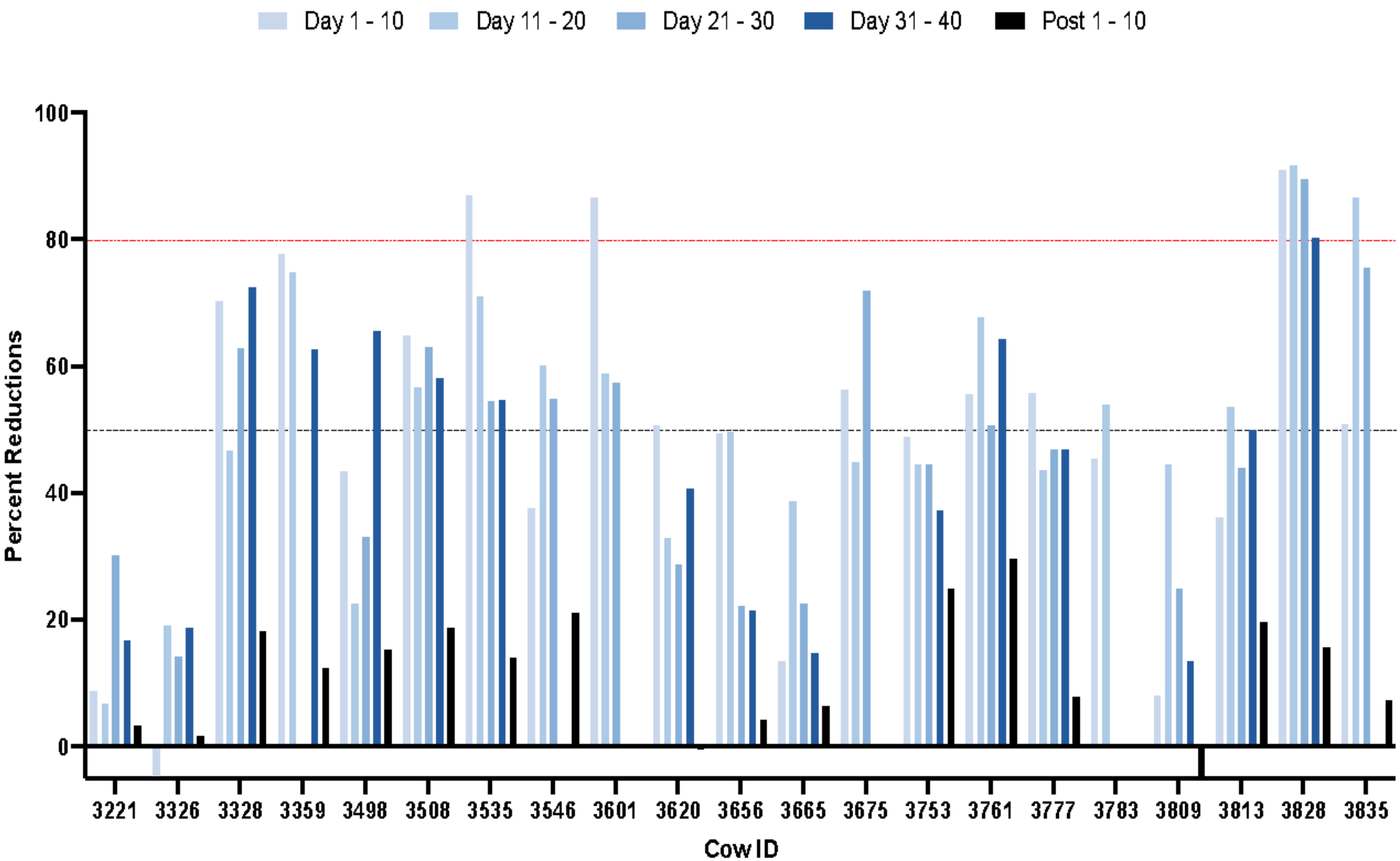


Figure 5. Average daily methane production (g/day) [A], yield (g CH_4/kg dry matter intake) [B], and intensity (g CH_4 / kg energy corrected milk) [C] for control (gray) and treatment (blue) cows during each experimental period. Significant differences are noted as *(P <0.10), **(P<0.05), and ***(P<0.01).

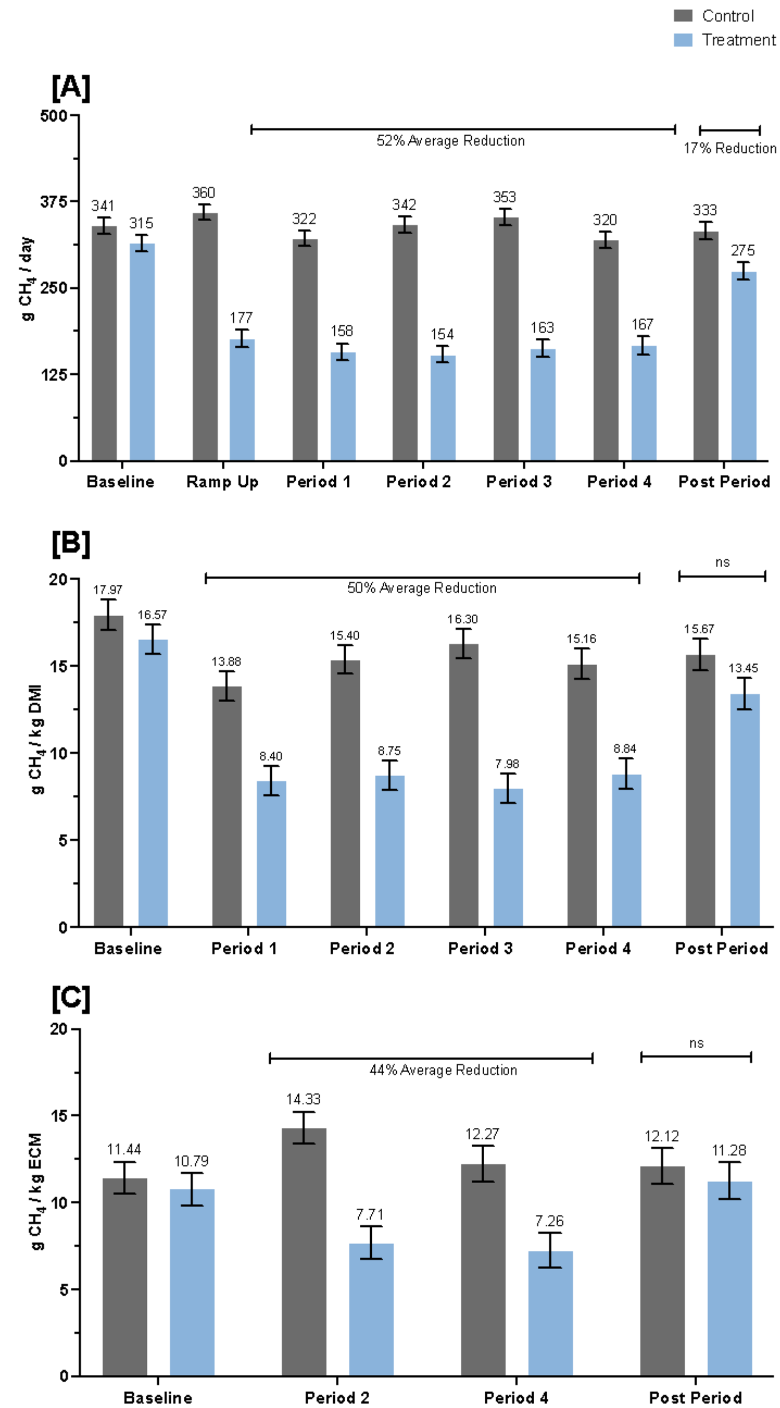


Figure 6. Methane Production (g/day) by Normalized Bromoform (mg/kg NDF)

Data points taken from a total of 9 bromoform inclusion rates across 3 dairy in vivo studies

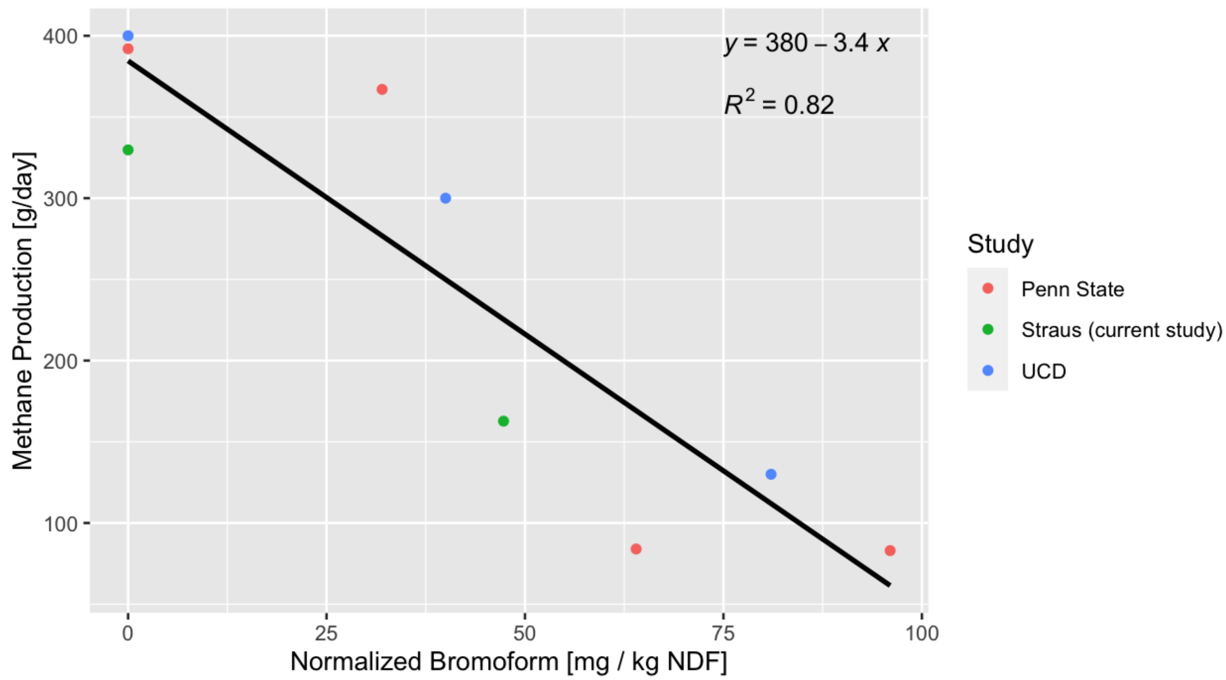


Figure 7. Graphical view of water sample and milk bromoform concentrations for experimental days 0 (baseline), 10, 20, 30, and 40 for control and *Asparagopsis*-fed (treatment) cows.

References

Cardot, V., Le Roux, Y., Jurjanz, S. (2008). Drinking behavior of lactating dairy cows and prediction of their water intake. *Journal of Dairy Science*, 91(6), 2257–2264. https://doi.org/10.3168/jds.2007-0204

National Research Council, Division on Earth and Life Studies, Board on Agriculture and Natural Resources, Committee on Minerals and Toxic Substances in Diets and Water for Animals. (2005). *Mineral Tolerance of Animals: Second Revised Edition, 2005*. National Academies Press.

Roque, B. M., Salwen, J. K., Kinley, R., Kebreab, E. (2019). Inclusion of *Asparagopsis armata* in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. *Journal of Cleaner Production*, 234, 132–138. https://doi.org/10.1016/j.jclepro.2019.06.193

Stefenoni, H. A., Räisänen, S. E., Cueva, S. F., Wasson, D. E., Lage, C. F. A., Melgar, A., Fetter, M. E., Smith, P., Hennessy, M., Vecchiarelli, B., Bender, J., Pitta, D., Cantrell, C. L., Yarish, C., Hristov, A. N. (2021). Effects of the macroalga *Asparagopsis taxiformis* and oregano leaves on methane emission, rumen fermentation, and lactational performance of dairy cows. *Journal of Dairy Science*, 104(4), 4157–4173. https://doi.org/10.3168/jds.2020-19686