The potential use of fermented seaweed in sustainable dairy diets

Evaluation of fermented seaweed plus rapeseed meal on the gas emissions and production characteristics of Dutch dairy cows

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Samenvatting NL – Robuuste dieren met een verminderde ecologische en klimaat impact zijn essentieel voor een duurzame toekomstige veehouderij. In de transitie naar een meer duurzame toekomst moet de melkveehouderij een aantal uitdagingen overwinnen. Fermentationexperts hebben een product ontwikkeld op basis van gefermenteerd raapzaadschroot en gefermenteerd zeewier (RS). Dit product kan zowel een alternatieve eiwitbron leveren, als ook een positieve bijdrage leveren aan diergezondheid en het verminderen van de enterische methaan uitstoot. Het doel van dit project was om de toevoeging van RS aan het rantsoen van lacterende melk koeien te evalueren op zijn potentie om de enterische methaan uitstoot te verlagen en zijn effect op de dierlijke productie karakteristieken. In deze studie zijn geen negatieve effecten gevonden op de dierlijke productie karakteristieken. Het RS product had ook geen effect op de enterische methaan emissie, of op de andere gemeten gas parameters.

Summary UK – Robust animals with a reduced ecological and climate impact are essential for future sustainable livestock farming. The dairy sector faces multiple challenges in their transition to a more sustainable practice. Fermentationexperts have developed a product based on fermented rapeseed meal and fermented seaweed (RS), that could both serve as an alternative protein source and supplement for improvement of animal health or reduction of enteric methane production. The goal of this project was to evaluate the addition of RS in the diet of lactating dairy cattle on it's potential to reduce enteric methane production and effects on animal production characteristics. No negative effects on the production characteristics of the dairy cattle were found. The RS product did also not reduce enteric methane emission, or influenced the other gas parameters.

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Summary

Robust animals with a reduced ecological and climate impact are essential for future sustainable livestock farming. The dairy sector faces multiple challenges in their transition to a more sustainable practice. Currently alternative protein sources for animal feed and the reduction of greenhouse gas emission by livestock are two important themes. Rapeseed meal is often considered as a local alternative for soybean meal. Various seaweed species show potential to reduce methane production, both in vitro and in vivo. Fermentationexperts have developed a product based on fermented rapeseed meal and fermented seaweed (RS), that could both serve as an alternative protein source for improvement of animal health and reduction of enteric methane production. The objective of this project was to determine the methane mitigation potential of RS and its effect on animal production characteristics when fed to lactating dairy cattle. The experiment was conducted from October 2021 until February 2022 at the animal research facilities for dairy cattle of Wageningen University and Research (Leeuwarden, the Netherlands). The experiment lasted in total 19 weeks, of which 1 week of adaptation to the barn, 2 weeks of covariate measurements and 16 weeks of experimental treatments. The experiment followed a completely randomized block design with two dietary treatments and 32 Holstein-Friesian dairy cows in total. The two dietary treatments consisted of a control diet without the fermented seaweed plus rapeseed meal supplement (Control) and the control diet in which a portion of the rapeseed meal was exchanged for RS. The RS treatment group had a 0.3kg lower DMI. No other effects of RS on the production characteristics, gas production or rumen fermentation related parameters were observed in the current study. Overall, it can be concluded that RS can be fed to dairy cattle as an alternative protein source without expecting negative effects on animal production characteristics. The inclusion rate of RS might potentially have been too low to have an effect on the gas emissions. In order to have an effect on the methane production, it is advised to explore a higher inclusion rate of RS in the diet or include a higher fermented seaweed content in the RS product.

1 Introduction

1.1 Background

Robust animals with a reduced ecological and climate impact are essential for future sustainable livestock farming. The dairy sector faces multiple challenges in their transition to a more sustainable practice. Current EU/Dutch policies focus both on protein (nitrogen) supply (i.e., mainly by reducing the import of (soy) protein and making more use of local protein sources) and the loss of nitrogen (protein; mainly in the form of ammonia; Beltran et al., 2021). The reduction of greenhouse gasses also plays a key role in this transition. The livestock supply chain emits approximately 7.1 GT of carbon dioxide (**CO$_2$**) equivalents (**CO$_2$-eq**) worldwide per year, of which 2.2 GT of CO$_2$-eq consists of enteric methane (**CH$_4$**) emitted by beef and dairy cattle (Gerber et al., 2013). Additionally, CH$_4$ has 28 times the global warming potential of CO$_2$ (IPCC, 2014).

Rapeseed meal is often considered as a local alternative for soybean meal, even if the environmental impact is not lower in every production system (Lehuger et al., 2009). Compared to other alternatives, the crude protein (**CP**) content, energy for lactation (according to the Dutch system; **VEM**) and intestinal digestible protein (**DVE**) of rapeseed meal is quite high, but always lower than that of soybean meal. In comparison, the general CP (g/kg), VEM and DVE of rapeseed meal are 339-383, 852-857 and 128-141 respectively while for soybean meal this is in general 469-489, 1013-1016, 238-245 (CVB, 2021). Rapeseed meal based diets are therefore often supplemented with other ingredients to meet the total energy requirements of the dairy cow.

Seaweeds, also called macroalgae, are marine based photosynthetic organisms with a plant-like structure and can be mostly found along shores with a hard substrate to which the seaweed can attach. Seaweeds are often divided into the three general groups of red (Rhodophyta), green (Chlorophyta) and brown (Phaeophyceae) seaweeds. In an *in vitro* setting, several seaweed species like *Ascophyllum nodosum* (brown), *Laminaria digitata* (brown), *Asparagopsis taxiformis* (red) *Chondrus crispus* (red) or extracts from *L. digitata* show potential to reduce CH$_4$ production when used as a supplement (Machado et al., 2014; Belanche et al., 2015; Kinley and Fredeen, 2015; Vissers et al., 2018). The red seaweed *A. taxiformis* can reduce enteric CH$_4$ emission of cattle *in vivo* up to 98%, with an inclusion rate of less than 0.5% in the feed on organic matter basis (Kinley et al., 2020). The seaweed *A. nodosum* also showed potential to reduce enteric CH$_4$ production *in vivo*, however this effect seems to be short due to potential adaptation, and only observed for CH$_4$ production (g/d) and not for CH$_4$ yield (g/kg dry matter intake) or CH$_4$ intensity (g/kg correct milk yield) (Antaya et al., 2019).

In general, fresh seaweed consists of more than 80-90% of water. After harvesting seaweeds quickly decompose due to all kinds of microbial activity (Enríquez et al., 1993) and fast preservation is needed. Drying is a common method, but unfavorable due to the high energy requirements and costs due to its high water content. Fermenting seaweed, a process in which certain acids or bacteria cultures are added and sealed air tight, is a potential method to preserve the seaweed for animal feed usage (Stévant et al., 2017; Yen et al., 2022). Internal experimental results at Fermentationexperts[1] showed that the addition of fermented seaweeds can reduce the methane production *in vitro* up to 20%. Fermentationexperts have developed a product based on fermented rapeseed meal and fermented seaweed (RS), that could both serve as an alternative protein source and supplement for improvement of animal health or reduction of enteric methane production.

The objective of this project was to determine the methane mitigation potential of RS and its effect on animal production characteristics when fed to lactating dairy cattle.

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[1] https://fermentationexperts.com/

2 Materials and Methods

2.1 Experimental design

The experiment was conducted from October 2021 until February 2022 at the animal research facilities of Wageningen University and Research (Leeuwarden, the Netherlands) and was in accordance with Dutch law on animal experiments and approved by the Central Authority for Scientific Procedures on Animals (CCD, The Hague, the Netherlands; 2020.D-0018.004). The experiment lasted in total 19 weeks, of which 1 week of adaptation to the barn (all cows received the same control diet), 2 weeks of covariate measurements (again all cows receiving the control diet) and 16 weeks of experimental treatments. The experiment followed a completely randomized block design with two dietary treatments and 32 Holstein-Friesian dairy cows, 8 primiparous and 24 multiparous cows. The two dietary treatments consisted of a control diet without the fermented seaweed plus rapeseed meal supplement (**Control**) and the control diet in which a portion of the rapeseed meal was exchanged for the fermented seaweed plus rapeseed meal (**RS**). Cows were blocked in pairs before the start of the trial according to parity (3.0 ± 1.50; mean ± standard deviation; **SD**), days in milk (**DIM**; 112 ± 19.5), and fat- and protein-corrected milk yield (**FPCM**; 34.4 ± 5.02 kg/d). At the end of the covariate period cows were re-blocked where necessary. Cows within a block were randomly assigned to one of the two treatments. The average DMI and FPCM of the Control and RS group during the covariate period was 23.3 ± 3.21 vs 22.8 ± 2.56 g/kg DMI and 34.6 ± 4.70 vs 34.0 ± 2.56 g/kg FPCM, respectively.

2.2 Diets, Feeding and Housing

All cows received the same partial mixed ration (**PMR**) during the adaptation and covariate period, which is the same as the Control diet. During the experimental period in the RS treatment 347.2 g dry matter (**DM**) of rapeseed meal was exchanged for 356.0 g DM of fermented seaweed plus rapeseed meal, with the aim to create an isonitrogenous PMR to the Control treatment. The PMR of the Control diet consisted of 54.7% grass silage, 22.3% maize silage, 7.7% rapeseed meal, 5.2% barley and 10.1% concentrate (DM basis). For the RS diet the PMR consisted of 55.0% grass silage, 22.1% maize silage, 6.0% rapeseed meal, 5.4% barley, 1.9% RS and 9.6% concentrate (DM basis). The chemical composition of the individual feed ingredients, GF bait and milking carrousel bait is presented in Table 1, and the chemical composition of the complete diet is presented in Table 2. Additional concentrate feed was provided through the Greenfeed (**GF**; C-Lock Inc., Rapid City, South Dakota, USA) system and milking carrousel as bait. The RS product consisted of *Ascophyllum nodosum*, harvested January 2020 in Norway, and *Saccharina lattisima*, harvested May 2020 at the Faroe Islands.

The PMR was automatically mixed three times per day with a Trioliet feed mixing robot (Triomatic HP 2300, Trioliet BV, Oldenzaal, the Netherlands) and distributed in Insentec feed bins (**FB**; RIC system, Hokofarm Group BV, Marknesse, the Netherlands) for automatic individual feed intake registration, as described in detail by van Gastelen et al. (2022). To avoid cross-contamination, a rinsing diet, which was not fed to the cows in the experiment, was mixed between mixing the different treatment diets. Cows were fed ad libitum, allowing 10% refusals.

The dairy cows were housed as one group in a free stall barn with 32 cubicles with commercially available rubber mats and covered with sawdust as bedding material. One FB per 2 cows belonging to the same treatment was available, i.e., every cow had access to 8 different FB's containing her allocated diet. The assignment of the cows to the FB was established at the start of the experiment, and remained the same throughout the experiment. The FB were equipped with an automated identification system (monitor ID system based on transponders withing the collar of the dairy cows) to enable access. The experimental diets were equally distributed over the FB to avoid potential barn location effects. For each visit of a cow to the FB the start and end time as well as the start and end weight of the FB were recorded. The FB were calibrated with a standard weight on a weekly basis. Two GF systems were present in the barn and freely available for all cows. Cows had free access to clean drinking water throughout the experiment and were exposed to light

from 0500 to 2300h. Cows were milked twice daily at 0500h and 1500h at the milking carrousel (AutoRotor PerFormer, GEA Farm Technologies, Leeuwarden, the Netherlands), and a small amount of concentrate was offered.

Table 1 *Chemical composition (g/kg DM, unless otherwise stated) of the individual feed ingredients, GreenFeed bait (GF bait) and milking carrousel bait (MC bait).*

	GS	MS	RM	RS	Barley	Concentrate	GF bait	MC bait
Dry matter (g/kg product)	350	373	882	890	876	888	868	874
Ash	126	41	89	100	26	84	76	79
Nitrogen	32	11	60	60	19	27	23	26
Crude protein[a]	201	70	375	372	120	165	143	165
Crude fat	48	39	55	52	34	54	51	55
Starch	[b]	367	22	9	558	173	114	279
Sugar	34	[b]	105	46	25	71	102	67
NDF	396	342	268	241	229	390	377	292
ADF	259	203	201	175	79	219	225	159
ADL	17	17	76	76	11	48	17	43

GS = Grass silage; MS = Maize silage; RM = Rapeseed meal; GF = Greenfeed; MC = milking carousel.
[a]Crude protein is calculated as N × 6.25.
[b]Not determined.

Table 2 *Chemical composition (g/kg DM, unless otherwise stated) of the complete diet fed to cows in the different treatment groups.*

	Treatment	
	CON	RS
Dry matter (g/kg product)	525.3	526.8
Ash	92.4	92.6
Nitrogen	27.9	28.0
Crude protein[a]	174.5	174.8
Crude fat	47.0	46.9
Starch	138.7	138.5
Sugar	41.0	40.3
NDF	360.7	359.8
ADF	224.0	223.3
ADL	25.1	25.0

[a]Crude protein is calculated as N × 6.25.

2.3 Sample Collection and Measurements

Samples of all individual feed components and concentrate feed were taken weekly and stored at -20 ºC. These samples were subsequently pooled per 4 weeks, subsampled and stored at -20 ºC pending analysis. Milk samples were collected from all animals on Tuesday PM and Wednesday AM on a weekly basis. A milk sample (10 mL) of each milking event was collected in a tube containing sodium azide (5 µL) for preservation, stored no longer than one day at 4 ºC, and analysed on fat, protein, lactose and urea content. A weighted average daily milk composition was calculated from the milk composition and milk yield of both milking events.

Measurements of enteric CH_4, H_2 and CO_2 emissions were recorded using two GF systems as described in detail by van Gastelen et al. (2022) for the same barn as in the present experiment. The GF systems were calibrated at the start and during the experiment according to the manufacturer recommendations. Cows

were encouraged to visit the GF systems with a pelletized bait (Table 1). Average weight of the pellet cup drops was recorded on a weekly basis per system and used for the daily dry matter intake (**DMI**) calculations. Maximum intake of the GF bait allowed was based on the actual milk yield of the cow, and settings of the GF system were changed accordingly based on the average weight of the cup drop. If both cows in a block were <100 days in lactation then they received 2kg (product basis) of concentrate through both the Greenfeed and milking parlour and 4 kg of concentrate in the PMR. When both cows in a block are >100 days in lactation and the 2 week average FPCM of both cows was <36 kg, then both cows received 2kg of concentrate via the Greenfeed and milking parlour and 2kg in the PMR. Finally, when both cows in a block are >100 days in lactation and the 2 week average FPCM of both cows was <26kg, then both cows received 2kg of concentrate via the Greenfeed, 0 kg at the milking parlour and 2kg in the PMR. The settings allowed for a maximum of 6 visits per day, 9 cup drops per visit, 30 second interval per cup drop and minimum of 3 h between visits. Only GF gas emission data based on at least a 2 minute uninterrupted visit were used for further calculations (gas production, g/d; gas yield, g/kg DMI; gas intensity, g/kg milk or g/kg FPCM).

All animals were sampled for rumen fluid according to the oral stomach tubing (**OST**) method described by Muizelaar et al. (2020). In short, after the morning milking event all FB's were closed off for 3-4 hours before sampling. Animals were fixed in a standard industrial feeding fence with a headlock gate, after which the oral stomach tube was inserted. The first 200mL of rumen fluid was discarded before collecting the next 200 mL. The OST device was rinsed and flushed with water after each cow. After collection of the rumen fluid, the pH was measured and one 10 mL pipet tip was filled with rumen fluid and equally distributed over four 2 mL Eppendorf (Eppendorf AG, Hamburg, Germany) tubes for volatile fatty acid (**VFA**) analysis. Immediately after collection, the tubes were put on dry ice before storage in a -80 ºC freezer pending analysis. All animals were sampled in total 3 times. Sampling took place 3 days before the start of the experimental period, 8 weeks after the start of the experimental period and 16 weeks after the start of the experimental period.

2.4 Chemical Analysis

Feed ingredient samples were thawed at room temperature and dried until constant weight, grass and maize silage samples were freeze-dried until constant weight. Samples were ground to pass a 1-mm screen by using a rotor beater mill for both silages (Retsch SR300, Retsch GmBh, Haan, Germany) and an ultra-centrifugal mill for all other samples (Retsch ZM200, Retsch GmbH, Haan, Germany). The samples were analysed by using wet chemistry for DM, ash, crude fat (**CF**), starch (except for grass silage), reducing sugars (except for maize silage), neutral detergent fibre (**NDF**), acid detergent fibre (**ADF**) and acid detergent lignin (**ADL**) as described by Abrahamse et al. (2008). Crude protein (**CP**) was calculated as N × 6.25 for all feedstuffs, where N was determined following the Dumas method (NEN-EN-ISO 16634-1; International Organization for Standardization, 2008). The factor 6.25 may overestimate the CP content of all macroalgae in general (Biancarosa et al., 2017), resulting in a slightly overestimated CP content of the RS product. The seaweed species in the RS used in this study do not contain starch, but contain similar storage polysaccharides (laminarin) that could possibly be detected as starch (Rioux et al., 2010).

Milk samples were analysed for fat, protein, lactose and urea content by mid-infrared spectroscopy (ISO 9622) and somatic cell count by flow cytometry (Qlip BV, Zutphen, the Netherlands). Fat- and protein-corrected milk yield (**FPCM**) was calculated according to the equation FPCM (kg/d) = (0.337 + 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d) (CVB, 2018).

Rumen fluid samples for VFA analysis were centrifuged during 5 minutes at 20817 g at 4 ºC and diluted with phosphoric acid containing iso caproic acid as internal standard. The VFA's were separated by gas chromatography using HP-FFAP (30 m × 0.32 mm × 0.25 µm) from Agilent J&W (USA) as column and hydrogen as mobile phase and detected by FID. Quantification was based on a chemical standard solution after internal standard correction. For calibration of the machine, a standard mix of acetic acid (43.71 mM), propionic acid (26.78 mM), isobutyric acid (10.78 mM), isovaleric acid (9.06 mM), valeric acid (9.18 mM), 2-methyl valeric acid (15.89 mM), isocapronic acid (3.97 mM) and caproic acid (4.00 mM) was used.
The internal standard was 2-methyl valeric acid (31.78 mM), and was 1 on 1 diluted with the sample. Rumen fluid pH was measured immediately after sampling with a portable electronic pH meter, calibrated before each sampling event according to the manufacturer instructions.

2.5   Statistical Analysis

The final dataset consisted of 32 dairy cows. All parameters related to feed intake, milk production, milk composition and GF visits were averaged per cow per week. Rumen fluid pH and VFA were compared at sampling moment. In week 11 both Greenfeed systems suffered a network error resulting in less than 20 visits for all cows during the week. Therefore, gas emission related data from week 11 was deleted from the dataset and statistical analysis.

Data were subjected to a repeated measurements residual maximum likelihood (REML) analysis in Genstat (19th edition, VSN International, Hemel Hempstead, United Kingdom). Differences between treatment means were compared using the least squares means and the Fisher's LSD method for multiple comparisons when there was an interaction detected at $P \leq 0.05$. Treatment, experimental week, the interaction treatment × week and the baseline measurement from the covariate period were considered fixed effects. Blocking factors were considered random effects and a first-order autoregression term was estimated for timelag-dependent correlation of residual effects within cow. All results are reported as least squares means with significance of effects declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

3 Results

3.1 Dry Matter Intake, Feed efficiency and Greenfeed visits

No interaction effects of treatment × week or treatment effects were found for the Greenfeed visits per cow per week, DMI of the Greenfeed bait and DMI of the milking parlour bait (Table 3). The number of GreenFeed visits per cow per week as well as total DMI tended to be affected by a treatment × week interaction (P = 0.088 and P = 0.092, respectively). Feed Efficiency (kg FPCM/kg DMI; Figure 1) was affected by a treatment × week effect (P = 0.014), and was higher in week 4 for RS than Control. The DMI of the PMR (P = 0.045) and the total DMI (P = 0.04; Table 3) was significantly higher for the Control treatment (19.4 ± 0.14 and 22.7 ± 0.10 k/g, respectively) than the RS treatment (19.1 ± 0.14 and 22.4 k/g ± 0.10, respectively).

Table 3 *Greenfeed visits, DMI and Feed Efficiency of lactating dairy cattle fed the Control or RS diet.*

	Treatment means			P-value		
	Control	**RS**	**SEM**	**Week**	**Treatment**	**Treatment × Week**
Greenfeed visits (number/cow/w)	43.1	42.1	1.17	<0.001	0.408	0.088
DMI Greenfeed bait (kg/d)	1.63	1.61	0.035	<0.001	0.447	0.278
DMI milking parlour bait (kg/d)	1.70	1.70	0.090	0.012	0.919	1.000
DMI of the PMR (kg/d)	19.4[a]	19.1[b]	0.14	<0.001	0.045	0.172
DMI total (kg/d)[1]	22.7[a]	22.4[b]	0.10	<0.001	0.040	0.092
Feed Efficiency (kg FPCM/kg DMI)	1.42	1.42	0.015	<0.001	0.492	0.014

[1] *DMI total is calculated as the sum of DMI Greenfeed bait, DMI milking parlour bait and DMI of the PMR.*

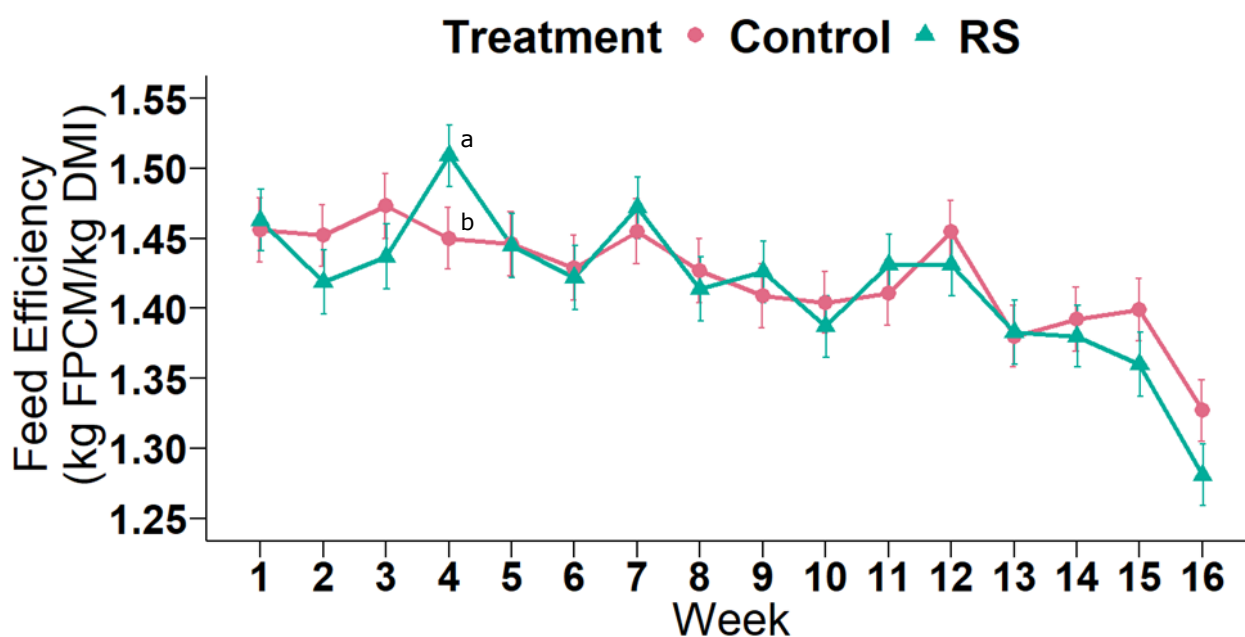[a,b] *Values with a different superscript indicate significant (P < 0.05) differences between the treatments.*

Figure 1 *The LSmeans ± SEM of the Feed Efficiency (kg FPCM/kg DMI) of lactating dairy cattle fed the Control or RS diet. [a-b] Values with a different superscript indicate a significant (P < 0.05) difference between the diets in the specific week indicated. Week is expressed relative to first week of feeding respective treatment diets.*

3.2   Milk production and milk composition

Milk fat content (*P* = 0.037; Table 4) and milk urea content (*P* < 0.001) were affected by a interaction effect of week × treatment. The milk fat content (%; Figure 2) was higher for Control than for RS in week 5. Milk urea content (mg/dL; Figure 3) was higher in weeks 2, 7 and 10 for Control than for RS and lower in week 4 for Control than for RS. There were no interaction effects of week × treatment or treatment effects for all the other parameters (Table 4). A trend for a higher milk fat yield (g/d, *P* = 0.062; Table 4) was found for the Control treatment (1330 ± 14.3) compared to the RS treatment (1306 ± 14.3).

Table 4 *Milk yield, fat- and protein corrected milk (FPCM) and milk composition of lactating dairy cattle fed the Control or RS diet.*

|	Treatment means			P-value		
	Control	RS	SEM	Week	Treatment	Week x Treatment
Milk yield (kg/d)	30.1	29.8	0.45	<0.001	0.749	0.436
FPCM yield (kg/d)	32.0	31.6	0.34	<0.001	0.165	0.302
Milk fat content (%)	4.47	4.44	0.042	<0.001	0.333	0.037
Milk protein content (%)	3.59	3.59	0.240	<0.001	0.861	0.185
Milk lactose content (%)	4.36	4.36	0.016	<0.001	0.765	0.184
Milk urea content (mg/dL)	21.5	20.8	0.46	<0.001	0.066	<0.001
Milk SCC (x1000 cells/mL)	133	159	32.4	0.248	0.505	0.983
Fat yield (g/d)	1330	1306	14.3	<0.001	0.062	0.212
Protein yield (g/d)	1076	1060	12.3	<0.001	0.350	0.260
Lactose yield (g/d)	1314	1301	20.6	<0.001	0.572	0.266
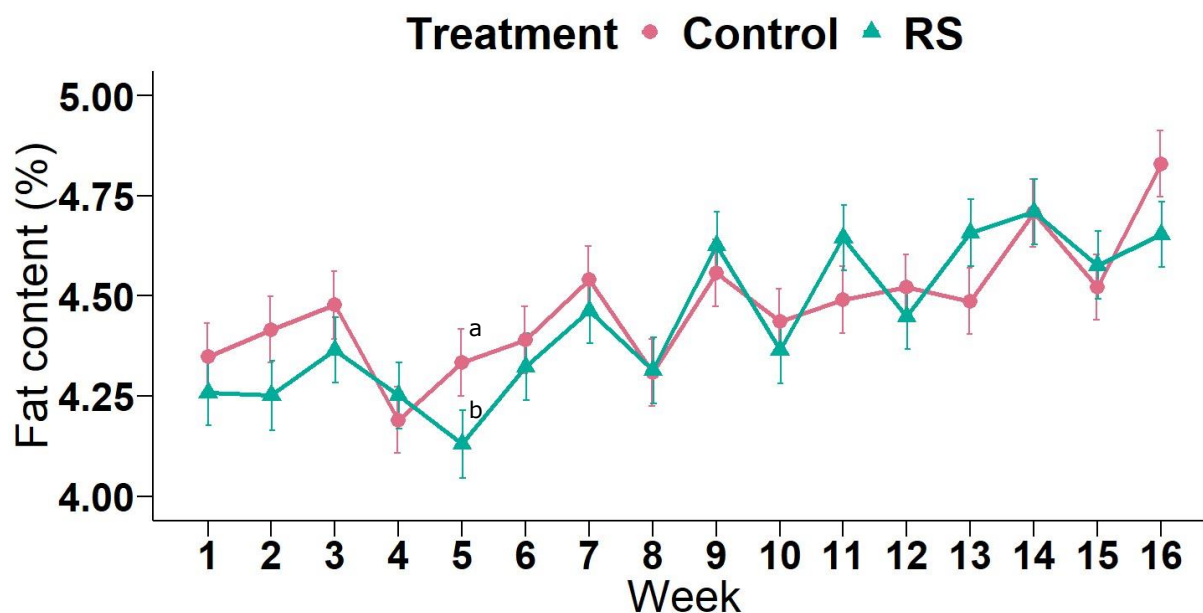
Figure 2 *The LSmeans ± SEM of the milk fat content (%) of lactating dairy cattle fed the Control or RS diet. [a-b] Values with a different superscript indicate a significant (P < 0.05) difference between the diets in the specific week indicated. Week is expressed relative to first week of feeding respective treatment diets.*
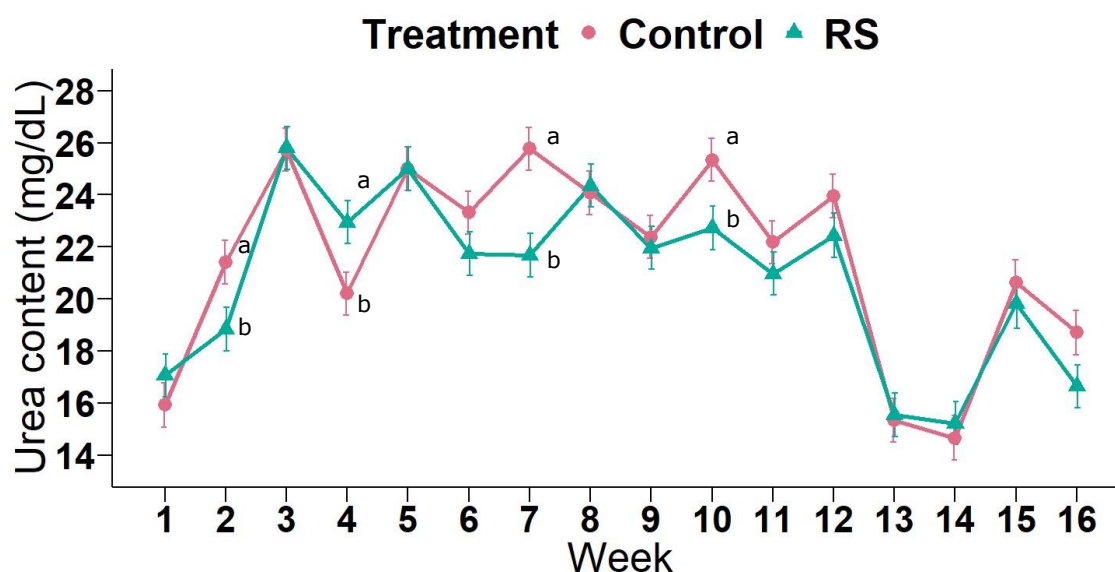

Figure 3 *The LSmeans ± SEM of the milk urea content (%) of lactating dairy cattle fed the Control or RS diet. [a-b] Values with a different superscript indicate a significant (P < 0.05) difference between the diets in the specific week indicated. Week is expressed relative to first week of feeding respective treatment diets.*
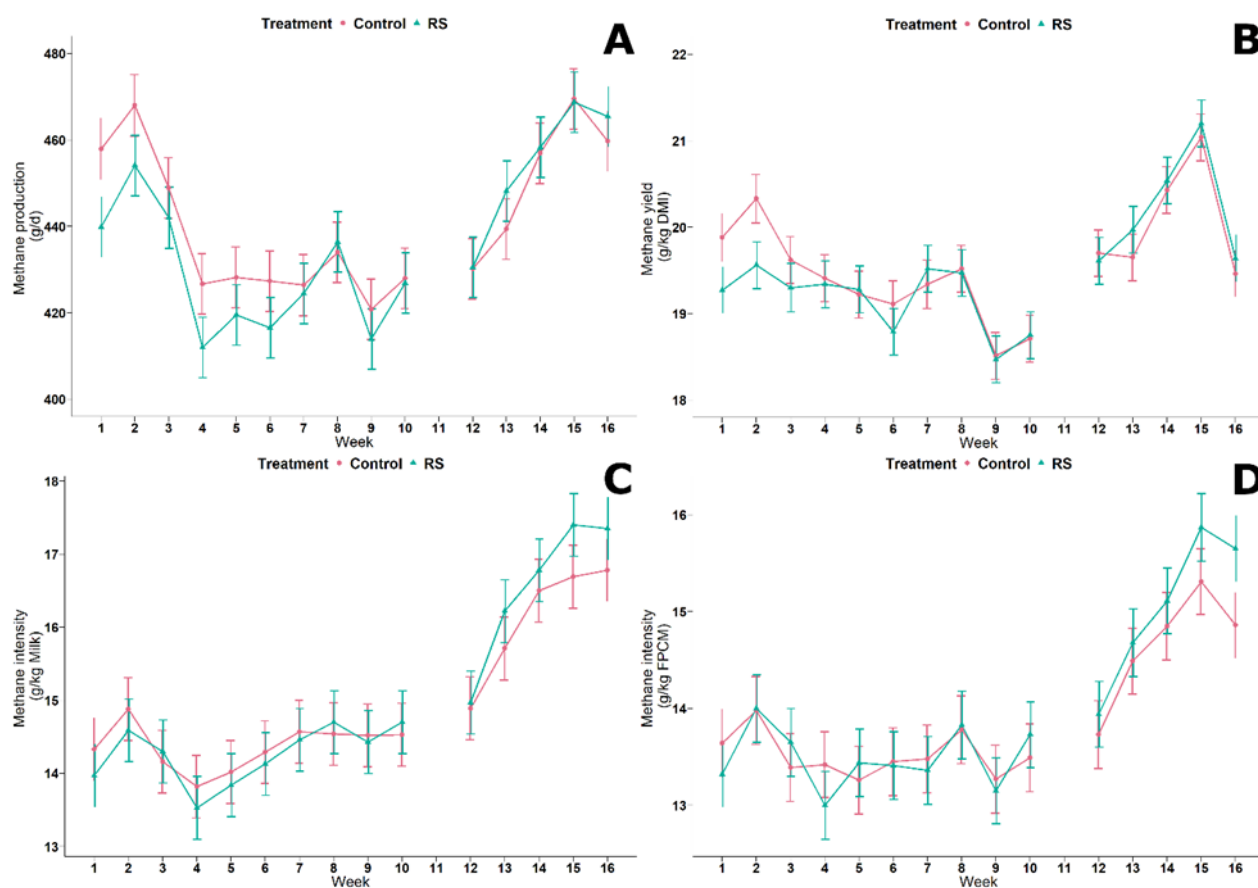
Figure 4 *The LSmeans ± SEM of the methane production (g/d; **A**), methane yield (g/kg DMI; **B**), methane intensity (g/kg Milk; **C**) and methane intensity (g/kg FPCM; **D**) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*

3.3 Gaseous exchange

There was no interaction effect of week × treatment, or a treatment effect for any of the CO_2, CH_4 or H_2 related data (Table 5). All emissions were affected by a week effect. Figure 4 gives an overview of the CH_4 production (g/d), yield (g/kg DMI), intensity (g/kg milk) and intensity (g/kg FPCM) over the course of the experimental period.

Table 5 *Carbon dioxide, methane and hydrogen emissions of lactating dairy cattle fed the Control or RS diet.*

	Treatment means			P-value		
	Control	RS	SEM	Week	Treatment	Week x Treatment
CO_2 emissions						
Production (g/d)	14536	14433	89.3	<0.001	0.469	0.624
Yield (g/kg of DMI)	644	646	3.5	<0.001	0.491	0.604
Intensity (g/kg milk)	494	492	11.1	<0.001	0.933	0.314
Intensity (g/kg FPCM)	459	460	8.0	<0.001	0.636	0.460
CH_4 emissions						
Production (g/d)	442	437	4.5	<0.001	0.365	0.550
Yield (g/kg of DMI)	19.6	19.5	0.15	<0.001	0.550	0.790
Intensity (g/kg milk)	15.0	15.0	0.35	<0.001	0.815	0.437
Intensity (g/kg FPCM)	13.9	14.0	0.26	<0.001	0.539	0.296
H_2 emissions						
Production (g/d)	1.30	1.28	0.025	<0.001	0.668	0.248
Yield (g/kg of DMI)	0.058	0.058	0.0011	<0.001	0.809	0.498
Intensity (g/kg milk)	0.044	0.044	0.0014	<0.001	0.934	0.332
Intensity (g/kg FPCM)	0.041	0.041	0.0009	<0.001	0.633	0.170

3.4 VFA and pH

No interaction effects of treatment × week or treatment effects were found for the pH, total VFA or individual VFA's in the rumen fluid (Table 6).

Table 6 *Volatile Fatty Acid (**VFA**) and pH in rumen fluid of lactating dairy cattle fed the Control or RS diet.*

	Treatment means			P-value		
	Control	RS	SEM	Week	Treatment	Sample x Treatment
pH	6.98	7.03	0.034	<0.001	0.227	0.113
Total VFA (mM)	77	76	2.2	<0.001	0.605	0.438
VFA (% of total VFA)						
Acetate (A)	67.7	67.4	0.24	<0.001	0.337	0.372
Propionate (P)	19.0	16.0	0.27	<0.001	0.542	0.191
Butyrate	11.9	11.9	0.18	<0.001	0.81	0.282
Isobutyrate	0.76	0.76	0.016	<0.001	0.903	0.269
Valerate	0.96	0.99	0.018	<0.001	0.202	0.879
Isovalerate	0.93	0.94	0.040	<0.001	0.863	0.112
A:P ratio	3.9	3.9	0.07	<0.001	0.545	0.148

4 Discussion and Conclusion

Research with seaweeds and ruminants is mostly restricted to two seaweed species, the brown macro algae *Ascophyllum nodosum* and the red macro algae *Asparagopsis taxiformis*. Almost no *in vivo* studies with other brown, green or red seaweed species have been published in peer reviewed scientific articles. The main brown seaweed component of RS is on some key points different from *A. nodosum*. These differences might limit the comparability of the results.

4.1 Feed intake and milk production characteristics

In the present study the total DMI of the RS group was 300 gram lower than the Control (equal to 1.3% of DMI, which is very low), which was mainly due to the difference in PMR intake. In studies with the brown seaweed *A. nodosum*, the DMI tended to increase when the seaweed was fed up to a difference of 1.2 kg/d, but were not significantly different (Antaya et al., 2015, 2019; Silva et al., 2022). In these studies a different seaweed species was used, *A. nodosum*, which was dried and directly added as a supplement (57-170 g/d, DM basis) and not fermented and mixed with another feed product as in the current study. Also when fed a mixture of 91% *A. nodosum* and 9% *Laminaria digitata* the DMI of lactating dairy cattle did not differ from the control (Newton et al., 2021). There was an interaction effect for the Feed Efficiency, which was higher for RS than for Control, but only in week 4. This was caused by a 0.9 kg drop in DMI for the RS group and a constant milk yield of both groups. This effect was not persistent and did not increase or decrease in the period before or after week 4. Both DMI of the Control and RS lowered in week 4, after a grass silage change in week 3. The drop in DMI for RS was larger than for the Control, explaining the interaction effect at week 4.

Milk yield and FPCM were not affected by an interaction effect or treatment, which was in accordance with literature feeding *A. nodosum* or a mixture of *A. nodosum* and *L. digitata* to dairy cattle (Antaya et al., 2015, 2019; Newton et al., 2021; Silva et al., 2022). For milk fat content there was an interaction effect, which was higher for the Control than for RS, but only in week 5. Milk urea content was affected by an interaction effect in weeks 2, 4, 7 and 10. This effect was inconsistent and impersistent over time. In weeks 2, 7 and 10 the Control was higher than RS, while in week 4 RS was higher than the Control. The interaction effects are also not consistent with grass (week 3 and 13) or maize (week 15) silage changes in the ration, since diet is one of the many factors influencing the urea content (Spek et al., 2013).

4.2 Gas production and rumen fluid characteristics

There was no treatment or interaction effects on the gas production parameters for any of the treatments. These results are similar to Antaya et al. (2019), which used *A. nodosum*, however in the study of Antaya et al. (2019) the seaweed reduced CH_4 production in the first period but not in the second and third period. In week 11 a sensor in one of the Greenfeed systems was deemed defect, which resulted in insufficient amount of good visits for all of the animals. In week 13 an increase in CH_4 (figure 4A) and H_2 production (Supplementary Figure 15A) and a decrease in CO_2 production (Supplementary Figure 14A) can be detected, which stabilised from week 14 onwards. This change in gas emission pattern coincides with a change in grass silage. In week 13 a grass silage with higher NDF content was fed than in weeks 3-12. A higher fibre content is often related to an increased CH_4 and H_2 production (Hristov et al., 2013), which is reflected by the grass silage change in the present study.

No interaction or treatment effects were found for the pH and VFA related parameters. In the study of Silva et al. (2022) ruminal total VFA decreased linearly with increasing amounts of *A. nodosum* fed to lactating dairy cows, pH did not differ and no explanation could be given. Feeding *A. nodosum* to rams did not alter total VFA and pH, but did affect the molar proportions of individual VFA (Zhou et al., 2018). In both Silva et al. (2022) and Zhou et al. (2018) the molar proportion of butyrate tended to decrease with increasing amounts of *A. nodosum*. In contrast, in the current studies no effects on the individual VFA's were observed.

This might be explained by the different brown seaweed species used in RS, or the lower inclusion of the seaweed in RS and subsequently in the diet. Based on the relationship between rumen pH and total VFA, established by Dijkstra et al. (2012), a rumen fluid pH of 6.3-6.4 was expected based on the measured total VFA in the current study. In general the ruminal VFA concentration increases and pH decreases after a meal. In the current study the cows experienced a fasting period of a couple of hours after morning milking till sampling was finished. Additionally during the night time cows tend to eat less due to the lack of fresh feed availability. Samples for VFA and pH analysis are often taken shortly before a meal without fasting, or 1-4 hours after feeding (Muizelaar et al., 2020). The sampling moment and sampling technique (OST) in the current study might explain the general low total VFA and higher pH than expected.

4.3 Conclusion

The objective of this project was to determine the methane mitigation potential of RS and its effect on animal production characteristics when fed to lactating dairy cattle. The RS treatment group had a 0.3kg lower DMI. No other effects of RS on the production characteristics, gas production or rumen fermentation related parameters were observed in the current study. Overall, it can be concluded that RS can be fed to dairy cattle as an alternative protein source without expecting negative effects on animal production characteristics. The inclusion rate of RS might potentially have been too low to have an effect on the gas emissions. In order to have an effect on the methane production, it is advised to explore a higher inclusion rate of RS in the diet or include a higher fermented seaweed content in the RS product.
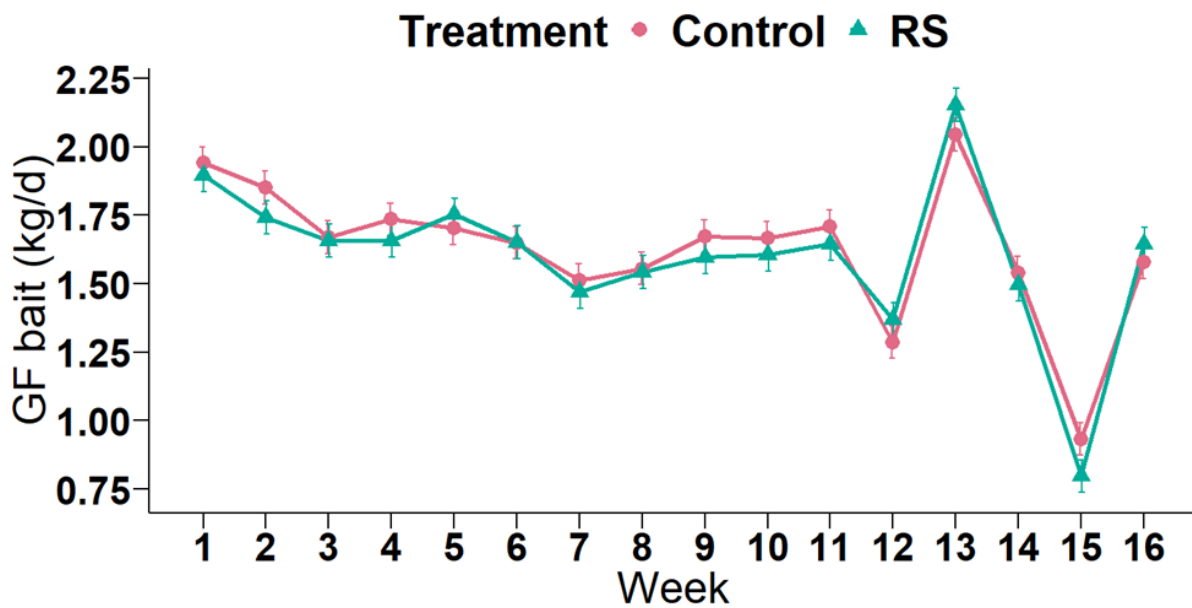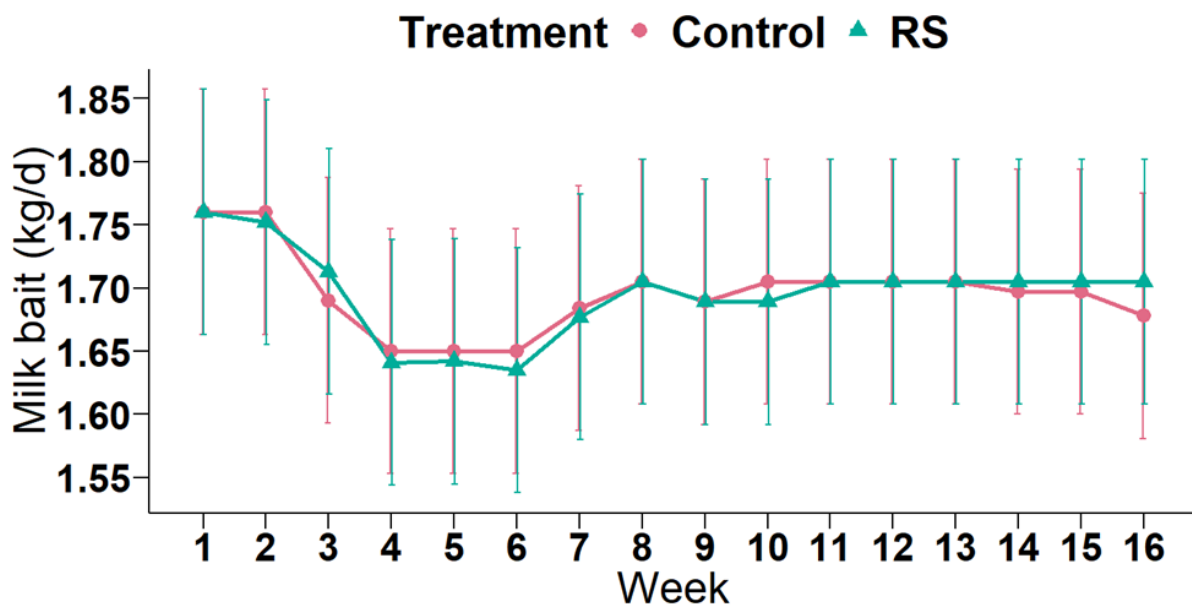
5   Supplementary files
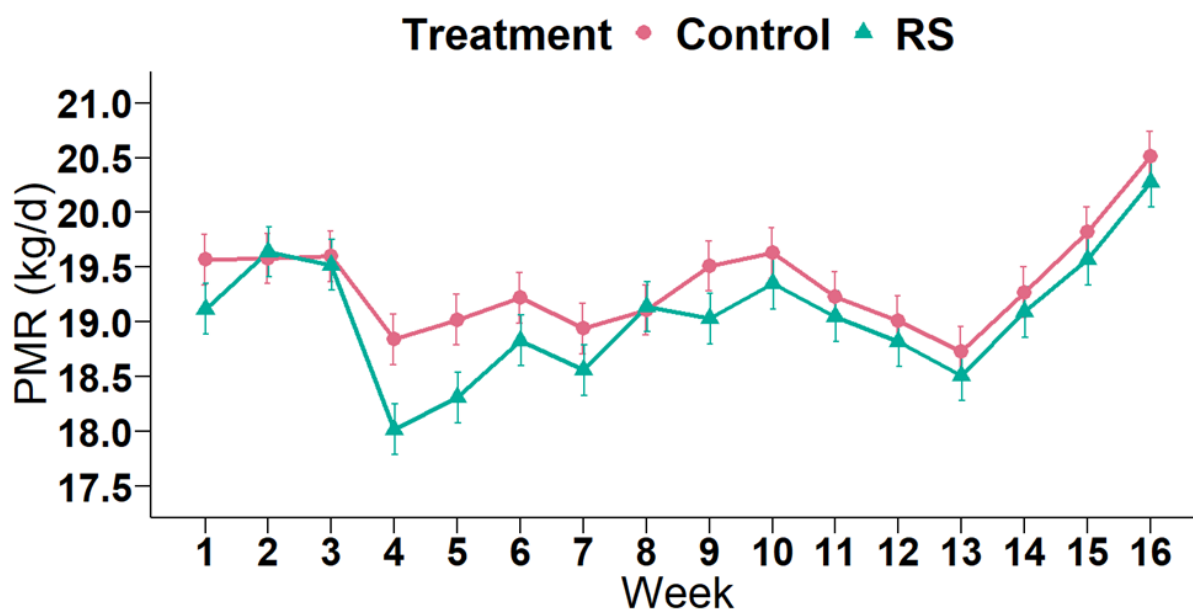
Supplementary Figure 1 *The LSmeans ± SEM of the DMI of the PMR (kg/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*
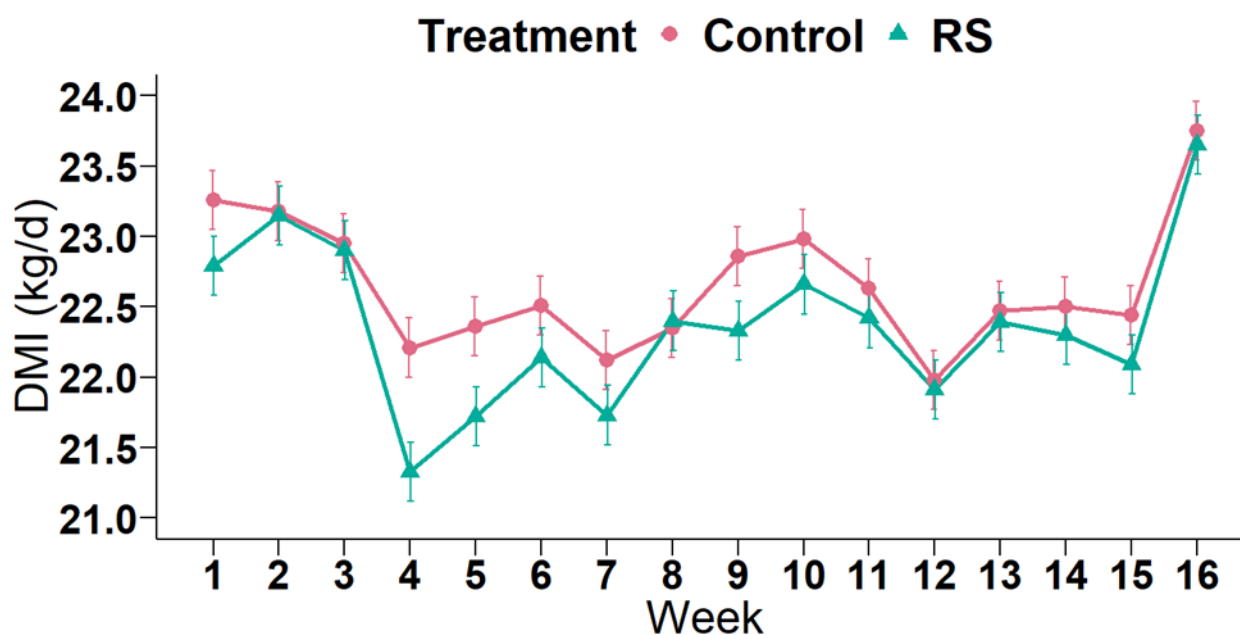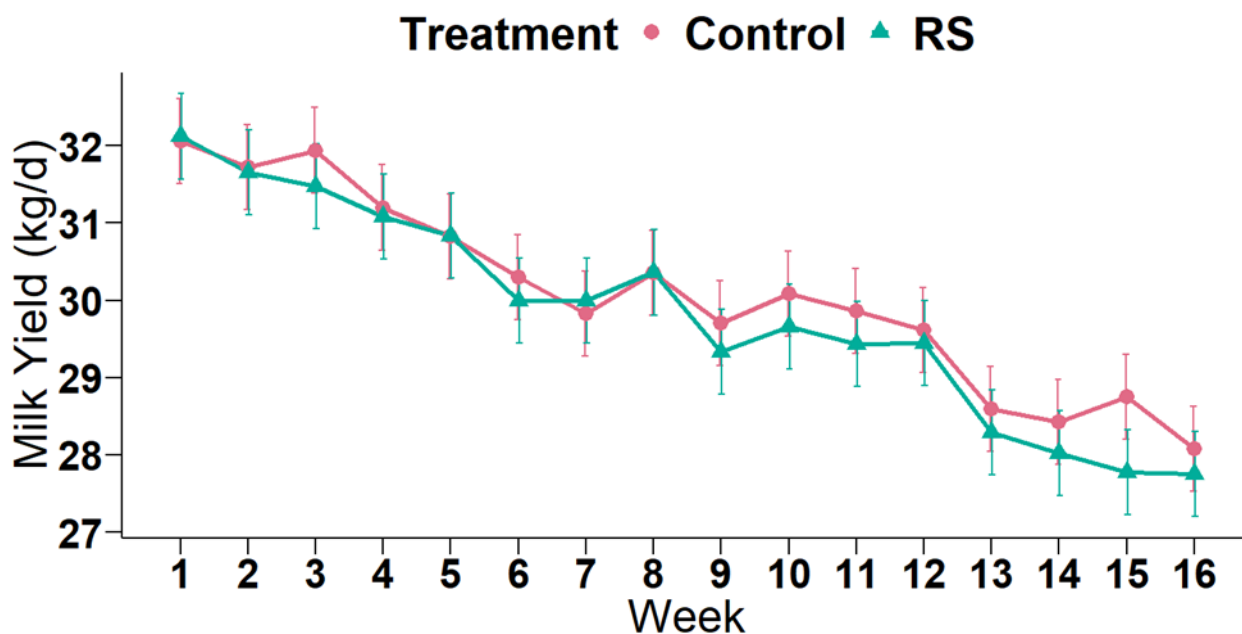

Supplementary Figure 2 *The LSmeans ± SEM of the total DMI (kg/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*
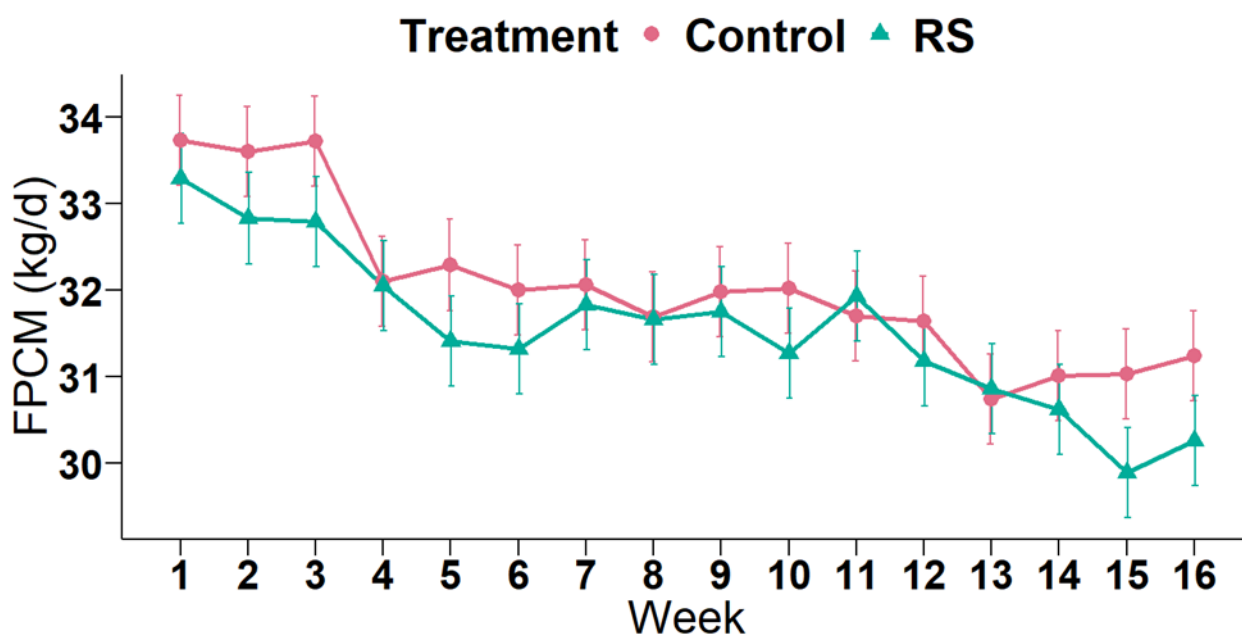
Supplementary Figure 3 *The LSmeans ± SEM of the DMI of the Greenfeed bait (kg/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment.* diets.
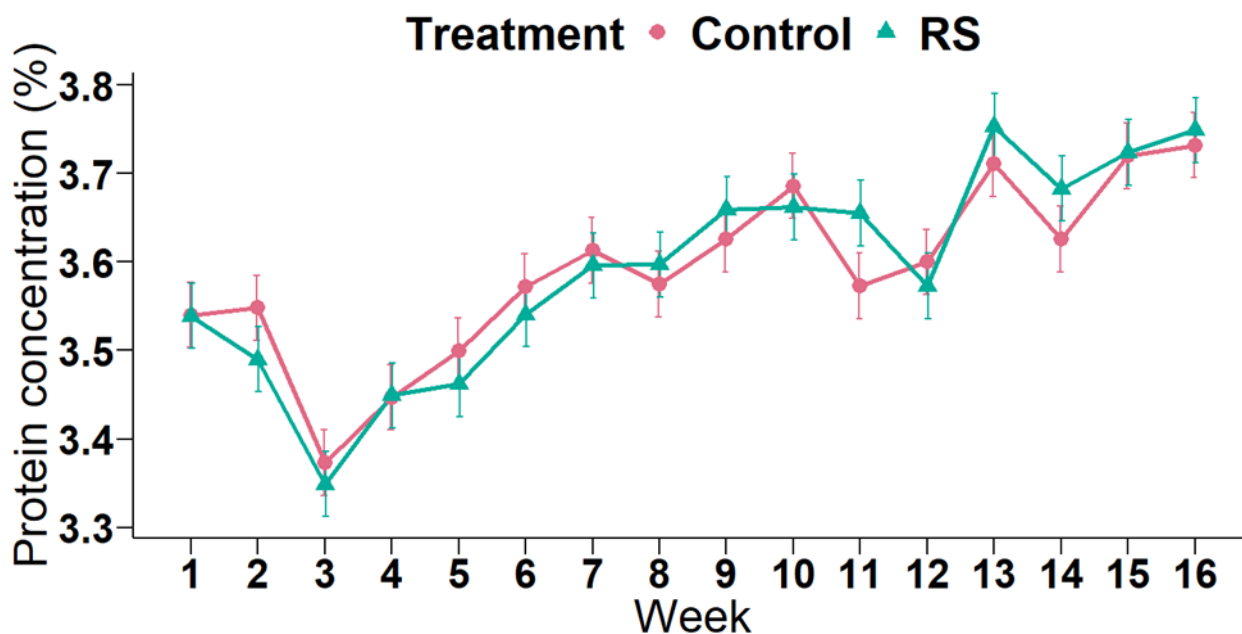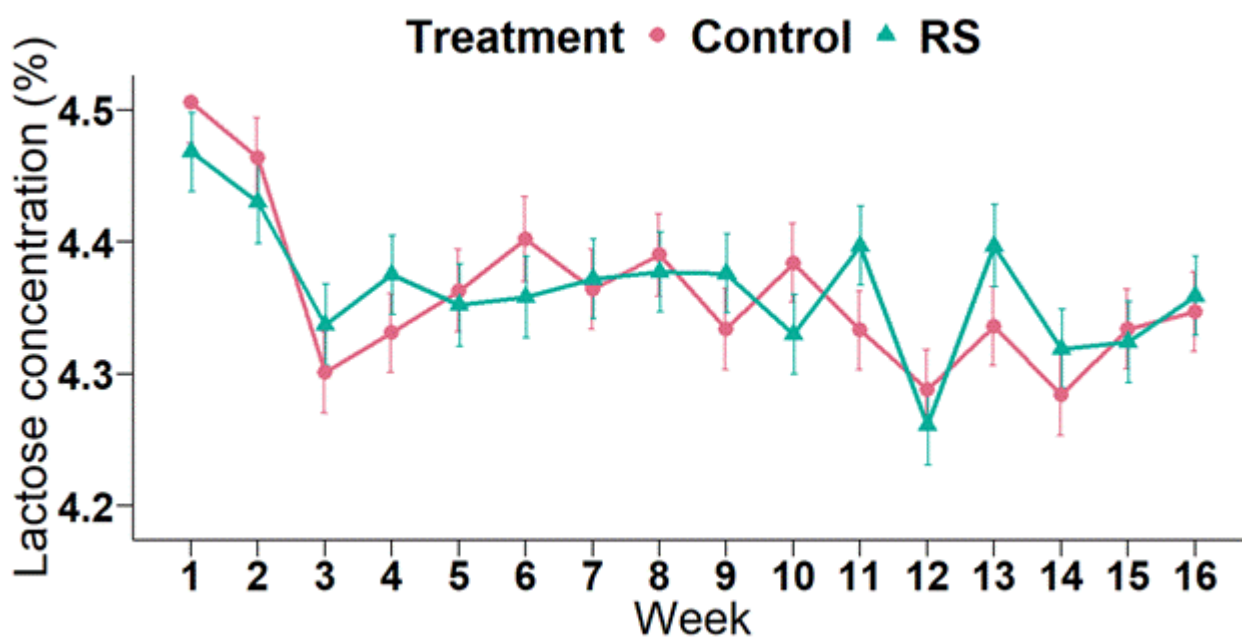

Supplementary Figure 4 *The LSmeans ± SEM of the DMI of the milking parlor bait (kg/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*

Supplementary Figure 5 *The LSmeans ± SEM of the milk yield (kg/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*
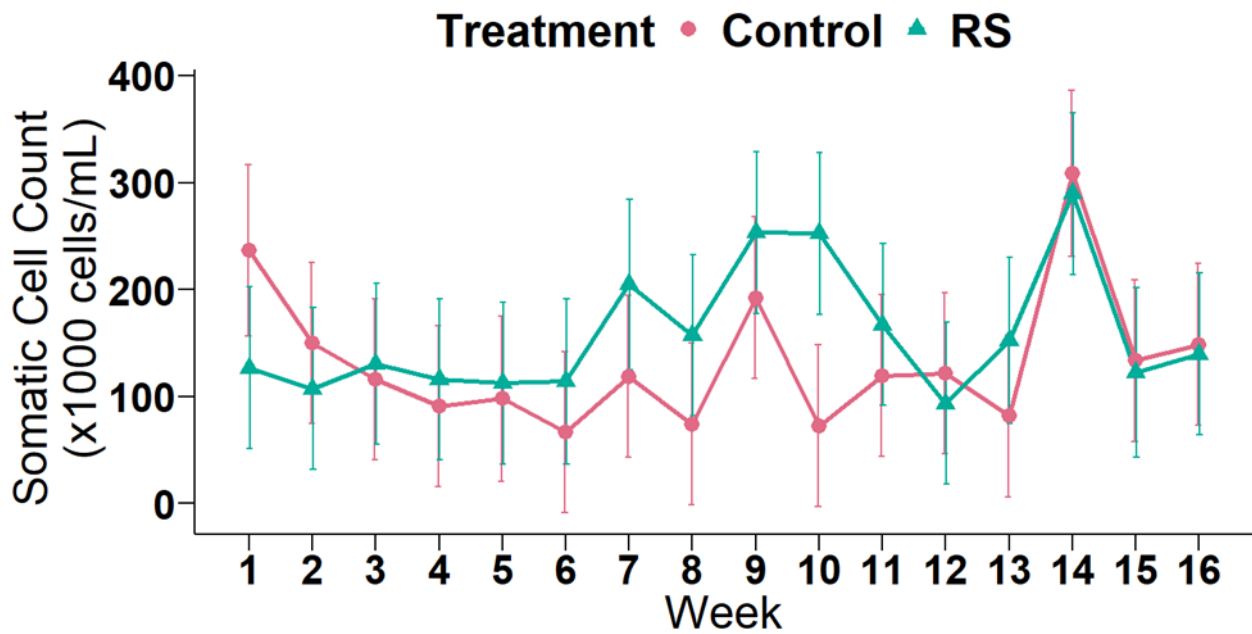

Supplementary Figure 6 *The LSmeans ± SEM of the Fat and Protein Corrected Milk (FPCM; kg/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*
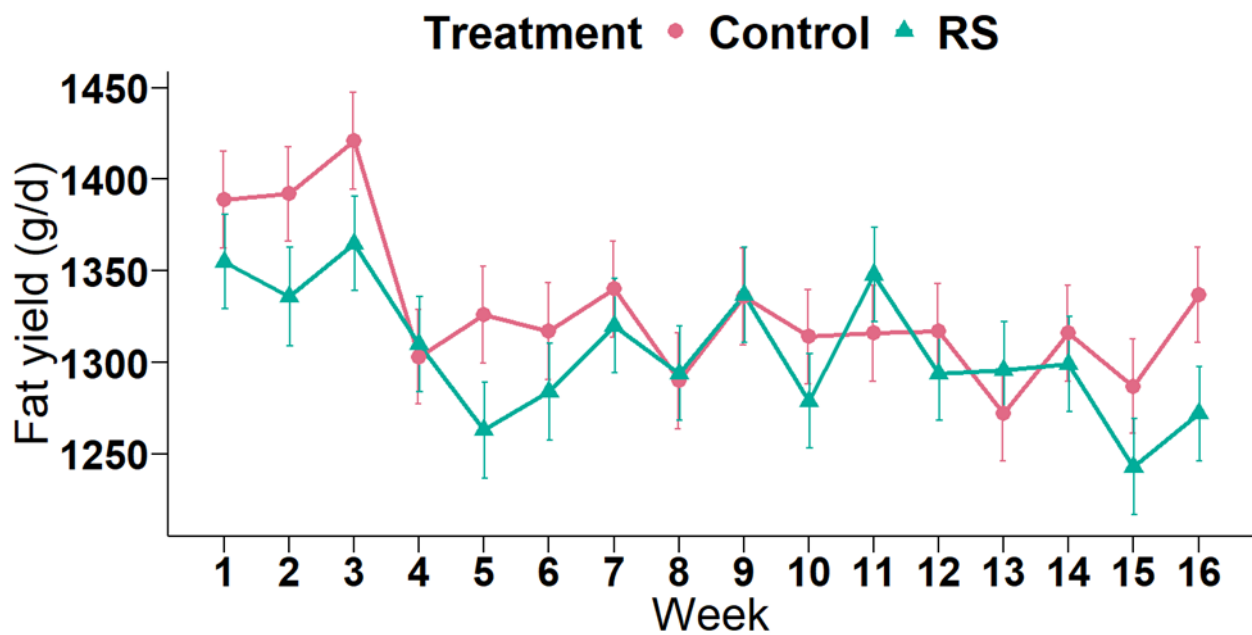
Supplementary Figure 7 *The LSmeans ± SEM of the milk protein concentration (%) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*
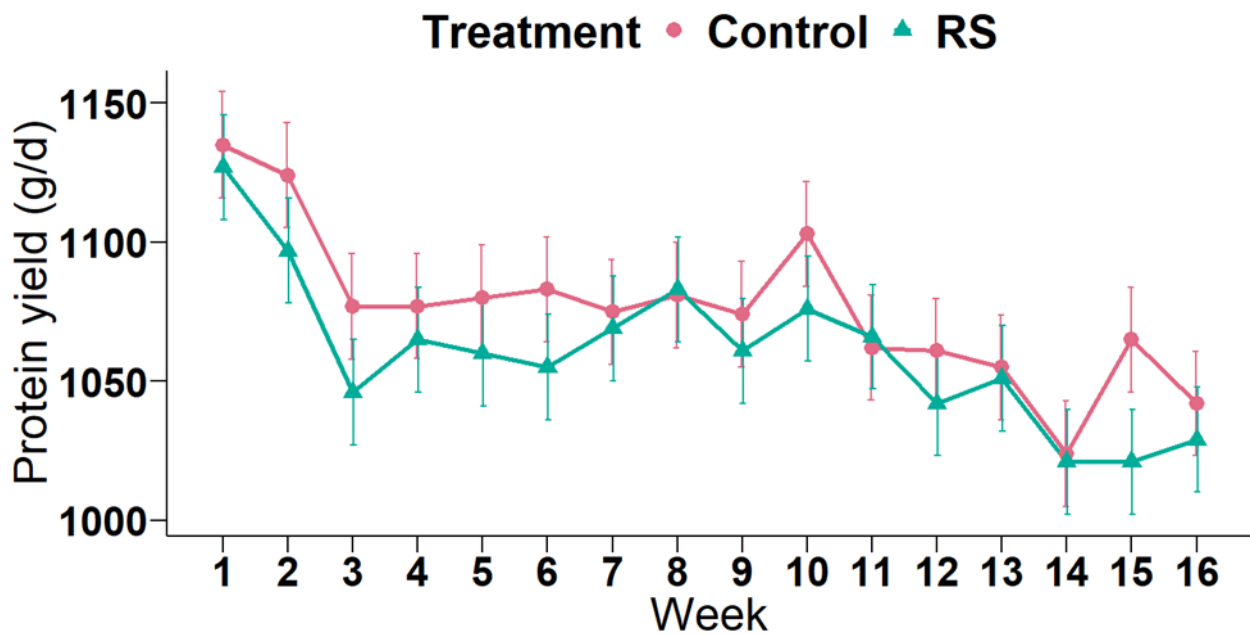

Supplementary Figure 8 *The LSmeans ± SEM of the milk lactose concentration (%) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*
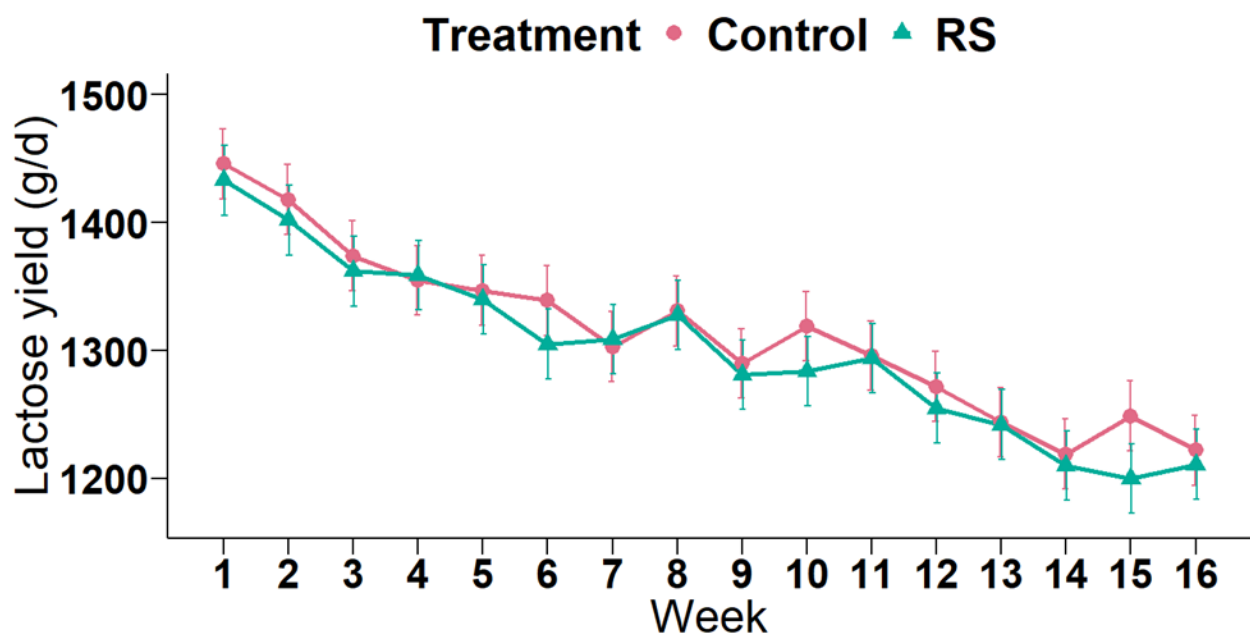
Supplementary Figure 9 *The LSmeans ± SEM of the somatic cell count (x1000 cells/mL) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*
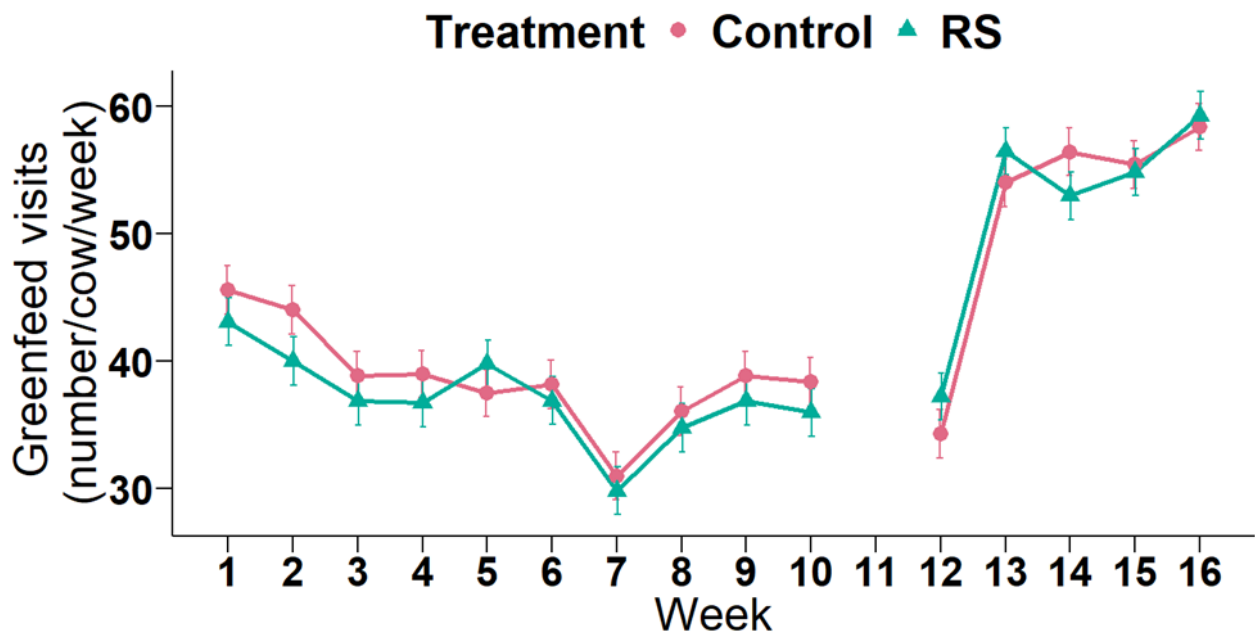

Supplementary Figure 10 *The LSmeans ± SEM of the fat yield (g/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*

Supplementary Figure 11 *The LSmeans ± SEM of the protein yield (g/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*

Supplementary Figure 12 *The LSmeans ± SEM of the lactose yield (g/d) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*
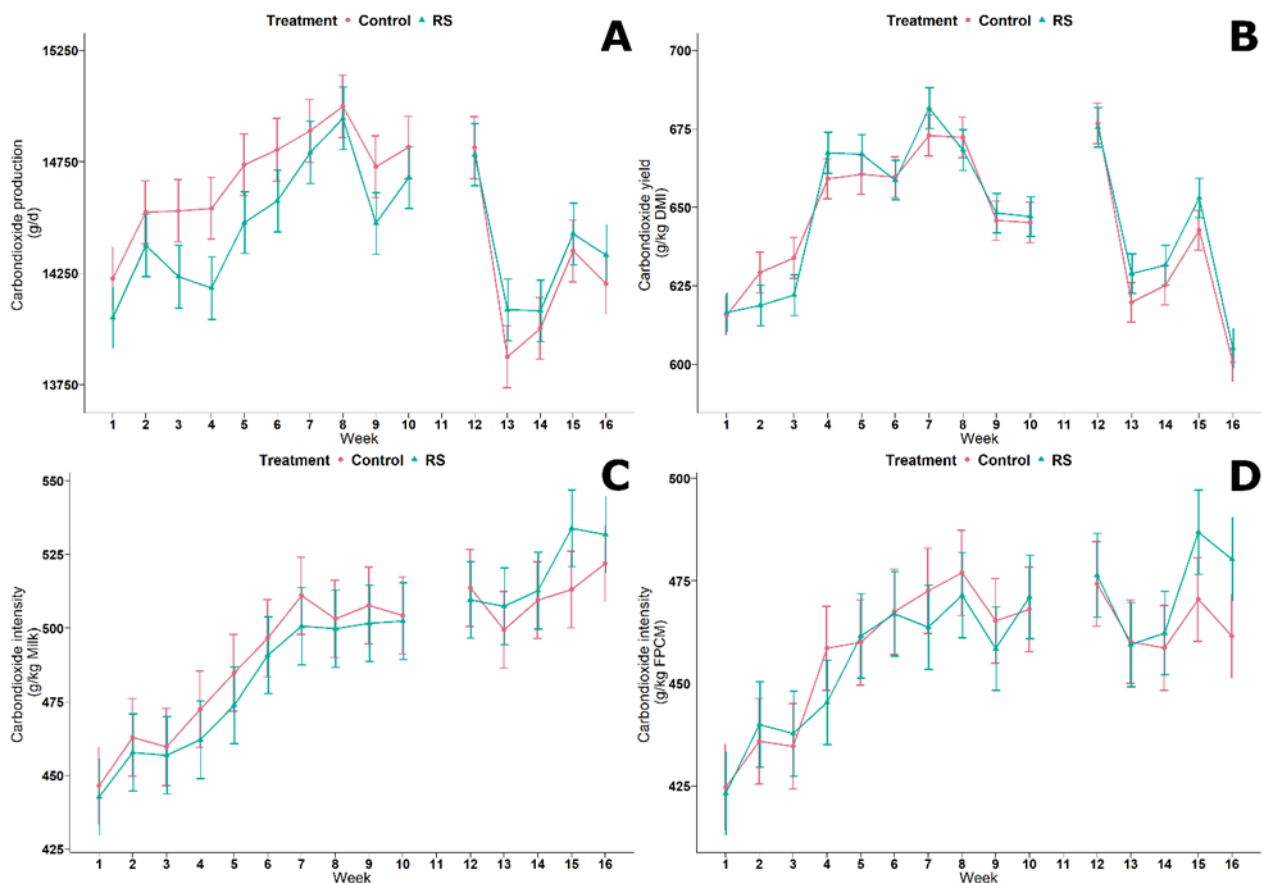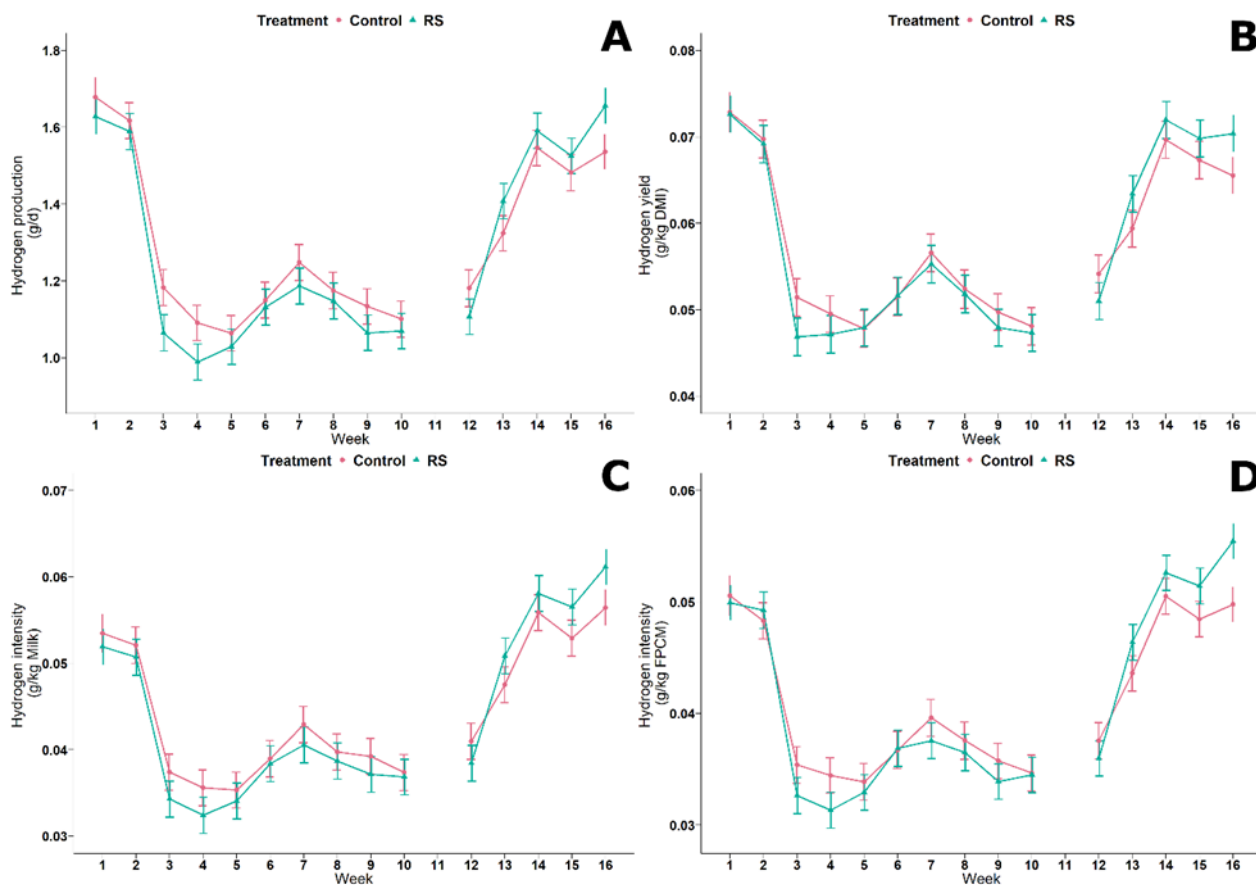
Supplementary Figure 13 *The LSmeans ± SEM of the Greenfeed visits (number/cow/week) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*

Supplementary Figure 14 *The LSmeans ± SEM of the carbon dioxide production (g/d; **A**), carbon dioxide yield (g/kg DMI; **B**), carbon dioxide intensity (g/kg Milk; **C**) and carbon dioxide intensity (g/kg FPCM; **D**) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*

Supplementary Figure 15 *The LSmeans ± SEM of the hydrogen production (g/d; **A**), hydrogen yield (g/kg DMI; **B**), hydrogen intensity (g/kg Milk; **C**) and hydrogen intensity (g/kg FPCM; **D**) of lactating dairy cattle fed the Control or RS diet. Week is expressed relative to first week of feeding respective treatment diets.*

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