



Final report



Development of a microbiological and Brewers' Spent Grain (BSG) supplement

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Abstract

The project addresses the challenge of reducing greenhouse gas emissions and improving food security by developing a novel ruminant feed using Brewer's Spent Grain (BSG) and microalgae. By leveraging the abundant waste from breweries, this initiative supports a circular bioeconomy aimed at cutting methane emissions from ruminants while creating nutrient-rich feed for farmers.

Initial in vitro screening of 26 algae species, at an optimal inclusion rate of 0.5-1% dry matter, showed 8% reduction in methane production without impacting fermentation. However, RUSITEC trials, though demonstrating that algae could be included in a BSG and hay diet without negatively affecting microbial populations, failed to show any significant methane reduction, underscoring the need to continue screening of algae species that can achieve a greater methane reduction (>20%).

A 100-day lamb trial found that microalgae inclusion at 1% significantly increased polyunsaturated fatty acids (PUFA) in the liver and subcutaneous fat, enhancing meat quality without affecting animal performance. Additionally, two supply chain models were created to optimize the distribution of BSG and microalgae, reducing CO₂ emissions and transport costs while fulfilling 100% of the demand for these feedstocks in the production of nutrient-rich animal feed.

This project benefits breweries, farmers, and the broader society by reducing emissions, improving meat quality, and creating new revenue streams.

Executive Summary

Background

It's projected that by 2050 the global population will surpass 9 billion people, leading to a significant rise in the demand for high-quality protein. Agricultural production is expected to double to meet these global food demands. This has brought into question the sustainability of current practices within agricultural sectors. In recent years there has been concentrated efforts to mitigate direct emissions associated with ruminant production. Direct emissions are considered the greenhouse gasses (GHG) naturally produced by the animal, in particular enteric methane (CH₄). Research has shown that altering the diets of these animals can effectively mitigate methane production. In this context, algae supplements have emerged as a promising avenue for reducing methane emissions in ruminants. By incorporating nutrient-rich microalgae into ruminant feed, this project aims to develop a sustainable solution.

Beer production in Australia represents a major source of revenue, generating \$4.9 billion from 2022-2023 [52]. In 2022 it was estimated 17.45 million hectolitres (hL) of beer was produced. The resulting products of brewing are an aqueous solution (wort) that is further utilised for fermentation, insoluble undegraded solid residue known as brewers spent grain (BSG) and carbon dioxide (CO₂). BSG is the most abundant by-product generated, accounting for 85% of the total waste products produced. Various BSG reuse and recycling strategies have been proposed including use of BSG for human foods (i.e. source of flour) or biodegradable packaging. However, the most common reuse of BSG is as an animal feed additive. BSG is considered an unconventional lower quality concentrate due to possess low quantities of lipids (~8.4%), and starch (~5.3%) compared to other conventional concentrates commonly utilised for animal production (i.e. soybeans). However, by enhancing BSG with

microalgae, which is cultivated using CO₂ emissions from breweries, this project seeks to create a novel supply chain for ruminant feed supplements. This innovative approach not only repurposes brewery waste but also contributes to a circular bioeconomy, aligning with the goals of sustainability and environmental responsibility.

As part of the Emissions Avoidance Partnership (EAP), the project employs advanced techniques such as the rumen simulation technique (RUSITEC) to comprehensively evaluate the effects of the algal/BSG supplement. Through feeding trials conducted with animals, the project aims to validate its impact on various aspects including animal performance, carcass characteristics, meat quality, and the composition of the rumen microbiome. By addressing both environmental concerns and agricultural needs, this initiative represents a holistic approach towards sustainable livestock management.

Objectives

1. Investigating the effects of the chosen dietary concentrations of algal/BSG supplements and a control feed on microbial growth, fermentation, protein synthesis, and gas production using the rumen simulation technique (RUSITEC).
2. Examining the effects of four dietary treatments of algal/BSG supplements (control, low dose, optimal dose, and high dose) on animal performance responses, carcass characteristics, meat quality, and fatty acid profiles in sheep.
3. Investigating the effects of four dietary treatments of algal/BSG supplements (control, low dose, optimal dose, and high dose) on rumen fermentation, methane production, nutrient utilisation, and the rumen and faecal microbiota in steers.
4. Identifying further pathways: Preparing for the final phase of testing and development and developing pipelines for appropriate commercialisation.

The project successfully achieved its first two objectives. In Objective 1, we screened 26 microalgae species to study their effects on fermentation, gas production, and the rumen microbial population. This provided valuable insights into how different species and dosages impact these factors. The findings from Objective 1 were used to select species and dosages for Objective 2, which showed that adding microalgae to lamb diets slightly improved meat quality by increasing PUFAs in the liver ($P = 0.05$; borderline significance) and showing a non-significant trend for higher PUFAs in subcutaneous fat ($P = 0.09$; trend).

However, we were unable to complete Objective 3 because no microalga tested so far has significantly reduced methane production in vitro ($\geq 20\%$). As a result, the project was adjusted to continue screening more algae species to find one with stronger anti-methanogenic properties. Objective 4 was partially completed with the creation of a supply chain model, aiming to optimize the distribution network between breweries and cattle producers for feed.

Methodology

1. Batch assays were utilised to screen algal strains (both freshwater and marine) across a range of inclusion rates (0% - 10%), and within two types of diets, to evaluate the species and dosage effects on fermentation, and methane production over a 24h period.
2. A rumen simulation apparatus (RUSITEC) was utilised to evaluate the effect of the two most promising algae candidates, at various dosages (0%, 0.5%, and 1%) on fermentation, methane production, and microbial populations, over a 15-day period.

3. Subsequently, one freshwater and one marine algae species were chosen to investigate their impact on animal performance, carcass traits, meat quality, fatty acid composition, and microbial populations. A trial involving 60 lambs over 100 days at the Queensland Animal Science Precinct examined different treatment pellets containing one of the two algal species at three inclusion rates (0%, 0.5%, and 1%).
4. Furthermore, a supply chain analysis explored brewers' spent grain logistics for red meat producers nationwide, aiming to address current limitations and provide recommendations for improvement.

Key findings

The initial screening of 26 algal species did not identify a potent methane reducer ($\geq 20\%$). It was observed that algae inclusion at $\geq 5\%$ affected various fermentability parameters. Consequently, in subsequent RUSITEC experiments the inclusion rate of microalgae did not exceed 1%. The two best-performing microalgal species were selected for *in vitro* and *in vivo* studies. However, the *in vitro* studies indicated that the selected algal species at three inclusion rates (0.5, and 1% DM) did not significantly impact fermentation parameters and microbial diversity or richness. *In vivo* results showed that the pellets were equally palatable across all treatments, regardless of the type or number of algae used. Including microalgae in the diet did not affect animal performance or carcass traits. However, adding 1% microalgae had a dose-dependent effect, significantly increasing PUFA levels in the liver ($P = 0.05$) and showing a non-significant trend for higher PUFA levels in subcutaneous fat ($P = 0.09$). Finally, supply chain mapping resulted in the development of an optimised supply network across Australia to meet 100% supply-demands with minimal CO₂ emissions and transport costs.

Benefits to industry

The objective of this project was to identify a nutritious and bioactive feed supplement with potential for national-scale implementation and significant benefits. These benefits include reducing methane emissions, improving animal health and nutrition, potentially enhancing meat quality, and contributing to the Carbon Neutral by 2030 Australian Red Meat Industry initiative.

Future research and recommendations

For future research, more algal strains (both single and mixed species; wild vs mutant species) should be tested to find one that can reduce methane by 20% or more. Dosage optimization should also continue. Once a promising species is identified *in vitro*, *in vivo* feeding trials are needed to confirm its effect on methane emissions, animal performance, and meat quality. The next step would involve testing the feed in commercial settings, such as feedlots and grazing systems, while optimizing how the feed is delivered. This will help assess its economic and environmental impact on a larger scale.

Additionally, collaboration with industry stakeholders and public awareness efforts will be essential for successful implementation and adoption of algal/BSG supplements in the Australian red meat industry.

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1. Background

The escalating levels of greenhouse gas emissions pose a critical challenge to the stability of our planet's climate. Among these emissions, enteric methane produced from ruminant livestock are a notable contributor to agriculture's total emissions [1, 2]. In ruminant species, microbes (i.e. methanogens) facilitate the process of methanogenesis utilising enteric hydrogen (H₂) to convert carbon dioxide (CO₂) to methane (CH₄) as an end product of fermentation [3, 4]. Hence, enteric CH₄ mitigation strategies have commonly focused on manipulating ruminant diets aiming to alter the presence and function of targeted microbes, such as methanogens [5, 6]. In this regard, algae supplements have emerged as a promising solution, offering a sustainable approach to reducing methane emissions in ruminants [7-9]. The integration of these supplements into ruminant feed will develop a practical and eco-friendly solution that addresses both environmental and agricultural sustainability concerns.

As climate change continues to pose formidable challenges globally, there is a mounting call from consumers for more sustainable products. Considering this, companies are facing increasing pressure, both from governmental regulations and societal expectations, to take decisive action in reducing their carbon emissions and managing waste more effectively. A notable example illustrating this trend is the recent installation of a cutting-edge CO₂ collection facility at the manufacturing plant of Manildra Group Pty Ltd in Nowra, NSW [10]. This initiative represents a proactive response to the environmental concerns surrounding CO₂ emissions. By implementing innovative technologies to capture and repurpose waste CO₂, companies like Manildra Group are not only demonstrating their commitment to environmental stewardship but also actively contributing to efforts aimed at reducing greenhouse gas emissions. This proactive stance underscores the importance of corporate responsibility and sustainability in today's business landscape.

Moreover, initiatives like the CO₂ collection plant serve as a beacon of progress, inspiring other companies to follow suit and adopt similar measures to mitigate their environmental footprint. As the global community grapples with the urgent need to address climate change, such initiatives play a crucial role in fostering a more sustainable and resilient future for generations to come. In addition to large-scale initiatives like the CO₂ collection plant at Manildra Group Pty Ltd, smaller-scale endeavours such as Earthy Labs' [10, 11] carbon capture unit for craft breweries highlight a broader shift towards sustainability within the brewing industry. These initiatives represent a growing recognition among businesses of the need to address environmental challenges and reduce their carbon footprint. Given that Australia's 750 breweries collectively are calculated to emit approximately 5 million tonnes of CO₂ annually [12], the implementation of carbon capture and reuse technologies holds immense potential to mitigate environmental harm and contribute to overall sustainability efforts.

Another significant aspect of brewery waste management is the handling of brewers' spent grain (BSG), which amounts to around 500,000 tonnes per year in Australia [13]. Traditionally, BSG has been utilised as livestock feed due to its nutritional content, as it is high in fibre and protein [14]. However, there is an opportunity to further enhance its nutritional value by supplementing it with microalgae, which are rich in lipids and bioactive compounds [15]. This innovative approach not only maximises the utilisation of brewery waste but also has the potential to address another critical environmental concern: enteric methane emissions from ruminants. By incorporating microalgae into BSG, the feed can potentially mitigate methane production in ruminants, thus contributing to efforts aimed at reducing greenhouse gas emissions from the agriculture sector. Overall, the growth in initiatives like the Manildra Group Pty Ltd and Earthy Labs' carbon capture unit make the utilisation of microalgae to enhance BSG a feasible prospect.

By leveraging CO₂ emissions from breweries to cultivate microalgae, which can then be combined with BSG to create a nutritious ruminant feed, this project exemplifies a circular economy approach. Such initiatives not only benefit breweries and farmers but also contribute to broader environmental and social responsibility objectives. Australia's advancements in microalgal research, particularly in areas like waste treatment and nutraceutical production, underscore the country's potential in sustainable innovation. Moreover, the Australian red meat industry's ambitious goal to achieve Carbon Neutral by 2030 highlights the urgency of addressing methane emissions [16] for which innovative solutions like microalgae-based feed supplements could be instrumental.

The progress made in the previous UTS/Young Henry project, which involves capturing CO₂ emissions from breweries and utilising them to enhance on-site microalgae cultivation, represents a significant advancement in sustainable practices. Additionally, another project funded by the Australian Government Department of Business Innovation Connections Program (Young Henry Protected background IP) determined the optimal algal strains and blending ratios with BSG.

Through collaborative efforts and innovative research, the current project aims to optimise the algal/BSG supplement and evaluate its efficacy in reducing methane emissions in rumen. Commercial pathways, such as integration with manufacturing partners and licensing agreements with breweries, aim to scale up the adoption of the developed feed supplement, contributing to broader sustainability goals within the red meat industry and beyond.

This project aims to utilise CO₂ and BSG waste streams from breweries to formulate an algae-based high-nutrient supplement. The supplement aims to reduce the CO₂ footprint of breweries and, when fed to ruminants, reduce CH₄ emissions. The beneficiaries include Australian cattle farmers in collaboration with their local breweries, as it offers a decentralised supply chain of alternative, nutritious, and bioactive ruminant feed supplements that can reduce CH₄ emissions and or improve ruminant productivity and meat quality. Breweries stand to benefit from increased revenue streams and potential eligibility for future carbon pricing policies.

It is anticipated that if this feed product can be verified to be a nutritious and bioactive feed supplement, implementation on a national scale has the potential to: improve animal health benefits due to CH₄ reduction; and/or improved health/nutrition and possible improvements to meat quality, increase in VFA production and microbial efficiency [17], support maintenance of average daily gains [18], increase productivity characteristics [7, 19], and beneficial impact towards achieving the Carbon Neutral by 2030 Australian Red Meat Industry initiative.

2. Objectives

1. Investigating the effects of the chosen dietary concentrations of algal/BSG supplements and a control feed on microbial growth, fermentation, protein synthesis, and gas production using the rumen simulation technique (RUSITEC).
2. Examining the effects of four dietary treatments of algal/BSG supplements (control, low dose, optimal dose, and high dose) on animal performance responses, carcass characteristics, meat quality, and fatty acid profiles in sheep.
3. Investigating the effects of four dietary treatments of algal/BSG supplements (control, low dose, optimal dose, and high dose) on rumen fermentation, methane production, nutrient utilisation, and the rumen and faecal microbiota in steers.
4. Identifying further pathways: Preparing for the final phase of testing and development and developing pipelines for appropriate commercialisation.

We encountered several technical challenges that required changes to the original objectives, particularly Objective 3, as we couldn't find an algal species that reduced methane by $\geq 20\%$ during initial screening. As a result, MLA agreed to revise our project and redirected the original funding to screen additional algae strains using batch assays. High inclusion rates ($\geq 10\%$) negatively affected rumen fermentation, leading us to remove these higher dosages from later RUSITEC tests (Objective 1) and the sheep trial (Objective 2). Although we developed an initial supply chain model for Objective 4, optimizing the supply of BSG to cattle locations while minimizing CO₂ emissions and costs still requires further work. While we made progress in setting up for commercialization, refining the supply chain, balancing commercial and environmental factors, and revising project plans means more work is needed to fully achieve the project's goals. The challenges in finding an effective algae species and reducing methane *in vivo* are key factors for completing Objective 4.

Therefore, additional work is required to address these factors and ensure that the final phase of testing, development, and commercialisation is successful. While the calculations at this stage may seem rudimentary, they represent a crucial initial phase in outlining a distribution pipeline for an algal/BSG feed supplement that can fulfil the feed requirements of Australian cattle farmers. This process has enabled the identification of current limitations within the existing supply chain concerning volumes and capacities that are needed for a relatively small-scale feed scenario (~54 cattle farms; see Section 3.11). By doing so, it provides insight into how the proposed concept could effectively meet and support a modest feed demand. This initial supply chain assessment is essential before tackling the more complex challenges of meeting the needs of the red meat industry, which encompasses substantially more cattle and larger farm sizes. Nevertheless, despite encountering obstacles, adjustments were made to address these challenges, and progress was made in refining the supply chain model.

3. Methodology

3.1 Animal Ethics Approval

The *in vitro* work was conducted in accordance with University of Sydney Animal Ethics Committee (Approved Protocol number 2019/1491) and completed within laboratories at the University of Technology Sydney and University of Sydney. Whilst *in vivo* work was conducted at the Queensland Animal Science Precinct, in accordance with Animal Ethic Approval Certificates (2021/AE000762) obtained from the University of Queensland Animal Ethics Committee (Production and Companion Animals, AEC – PCA).

3.2 Project Design

The significant progress was already made through our industry partnership with Young Henrys Brewing Company Pty Ltd (protected background IP), facilitated by previous Government Innovation Grants (2019-2020, 2020-2022). This project encompassed the process of capturing CO₂ in a food and beverage environment, utilising it for algae cultivation, and identifying the most suitable algae strains. These efforts have culminated in the development of processing techniques and formulations for future stock feed, with potential anti-methanogenic effects. Any results obtained under these funding agreements are considered protected background intellectual property.

Following on from our comprehensive literature review on algal feed additives in ruminant feeds [9, 20, 21], a

high-throughput batch fermentation screening assay was used with aims to identify a microalga capable of significantly reducing (by $\geq 20\%$). The next steps followed the typical progression of ruminant feed evaluation. Before any modification/negotiation, the project design comprised of 3 distinct phases. Phase 1a of the project used the Rumen Simulation Technique (RUSITEC) to find the optimal dosage of the algal/BSG supplement. This was followed by Phase 1b, a sheep study to confirm the supplement's effects on animal productivity and meat quality *in vivo*. Phase 1c was planned to test the supplement's effects on rumen fermentation and methane reduction in cattle. After each phase, the project was to be reviewed and adjusted based on the “desirable-viable-feasible” approach. If Phase 1c was successful, the plan was to move to Phase 2—a 200-day feedlot cattle trial. However, Phase 1c was not conducted because no algal species reduced methane by the target of $\geq 20\%$ *in vitro*. After discussions with MLA and Young Henrys, resources were redirected to continue screening more algae species in Phase 1a, with a total of 26 species tested. The failure to complete Phase 1c also meant that the planned large-scale feedlot trial in Phase 2 was not pursued.

3.3 Microalgae Cultivation

A total of 26 microalgae species, comprising of 14 freshwater and 12 marine species, were selected from the UTS algae culture collection database for inclusion in the batch culture experiments. Freshwater microalgae were grown in freshwater MLA medium [22], whereas marine microalgae were grown in F/2 (seawater medium) [23]. All cultures were maintained at $\sim 20^\circ\text{C}$ and under light irradiance of $\sim 100 \mu\text{mol photons} / \text{m}^2 \text{ sec}$ in a 12:12h light:dark cycle. Before the onset of stationary phase, the biomass was harvested using centrifugation (3500 rpm, 5 mins). The biomass was washed with MilliQ water followed by drying for 24 – 48 h using a freeze drier (Martine Christ Alpha 2-4 LSC basic). The dried biomass was ground manually using a mortar and pestle to create a fine powder and stored at -20°C until used within the *in vitro* experiments.

3.4 Rumen Content Collection

In both the batch assays and RUSITEC, rumen contents were sourced from three non-lactating fistulated crossbreed Shorthorn \times Angus cows housed at the University of Sydney, NSW, Australia. These cows had *ad libitum* access to pasture, and rumen contents were collected approximately 3 hours after they were removed from morning grazing. For the batch culture assay, the liquid portion of rumen digesta was obtained by separating ruminal solid and liquid contents through filtration using a piece of stocking. Equal amounts of rumen liquor from all three cows were combined and stored in a preheated thermos container, then promptly transported to the laboratory for use. In the RUSITEC experiment, both the liquid and solid portions of rumen digesta were collected. Rumen liquid was obtained following the same protocol as for the batch culture inoculum. Additionally, solid proportions were collected in separate preheated thermos containers. After collection, solids from all three cows were combined before being used for RUSITEC inoculation.

3.5 High throughput Batch Screening

Under a previous Government Innovation Connections Grant (2020-2022, Young Henry's protected background IP), we utilised the Automatic Methane Potential Test System (AMPTS[®]) tool to assess methane production in a feed blend with algal substitution for typical dairy concentrate and maize silage. This tool, facilitated by our collaboration with the Centre for Technology in Water and Wastewater at the University of Technology Sydney, provides automated analysis, sampling, recording, and report generation. However, due to its low throughput, we transitioned to a more robust, high-throughput method. Through our partnership with the University of Sydney, we established a high-throughput *in vitro* ruminal fermentation assay at UTS, enabling screening of up

to 10 species per run in triplicate at four concentrations. This approach allowed us to screen a minimum of two independent runs of 18 microalgae species at concentrations ranging from 0% to 10% inclusion.

Microalgae species were chosen for the *in vitro* batch culture assay based on their availability. Batch culture rounds 5 and 6 were conducted (Table S1). In round 5, there were 5 species of microalgae screened within two basal diets (Table 1). Diet one consisted of 80% maize silage, and 20% dairy cow concentrate. Diet two consisted of 80% mixed hay (35% C4 grass hay, 30% oaten hay and 30% lucerne hay) and 20% brewers spent grain. During round 6, 13 species of microalgae were screened, within basal diet two (Table 1).

For basal diet one, maize silage and dairy cow concentrate were sourced from the Corstorphine Farm research site at the University of Sydney's Camden Campus, NSW, Australia. For basal diet two, barley distiller grains were obtained from Young Henry's Brewery, Newtown, NSW, Australia. After collection barley was dried and milled (1 mm sieve). The hay was obtained from the University of Queensland Gatton Campus, QLD, Australia, and the University of Sydney's Sheep Reproductive Unit, Cobbitty, NSW, Australia.

For batch culture round 5, the experimental setup involved four treatments, including a control diet (basal diet with 0% microalgae) and three diets with each microalga replacing the dairy concentrate at inclusion concentrations of 1%, 5%, and 10% in the substrate DM. Batch culture round 6 treatments consisted of a total of three treatments, including a control diet (basal diet+ 0% microalgae), and two diets with each of the microalga replacing the concentrate and forage proportion equally, at an inclusion rate of either 1%, or 5% in the substrate DM.

Table 1: Chemical analysis of the basal diets utilised for batch culture screening rounds one and two.

	% of dry matter					
	DM%	Crude protein (CP)	Neutral detergent fibre (NDF)	Ether extract (EE)	Non-fibrous carbohydrates (NFC)	Ash
Diet 1						
Maize Silage	90	5.4	41.1	3.5	45.5	4.5
Dairy cow concentrate	92	15.5	20.8	2.7	52.2	8.8
Diet 2						
Hay	87.1	10.0	50.9	1.0	30.7	7.4
Barley distiller grain	97.7	23.0	55.4	6.0	11.5	4.1

Abbrev; DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; EE, ether extract; NFC, non-fibrous carbohydrates; $NFC = 100 - CP - NDF - EE - Ash$].

Each treatment, comprising 500 mg of substrate with or without microalgal inclusion, was placed into Ankom F57 filter bags (ANKOM Technology, Macedon, New York). These filter bags were then transferred to 50 mL amber glass bottles and pre-incubated at 39°C overnight in an oven before inoculating with rumen. Following inoculation with 25 mL of rumen inoculum under a stream of 99.99% nitrogen gas, the bottles were sealed and placed into a shaking incubator set to 90 rpm at 39°C for 24 hours. Each treatment was examined in triplicate, with three additional bottles serving as negative controls, containing only 25 mL of rumen inoculum.

3.5.1 Sample Collection and Analysis

Before total gas collection, 20 mL sub-samples of gas were drawn and transferred into 12 mL flat-bottom evacuated exetainers (Labco Limited, Lampeter, UK) for methane (CH₄) analysis, with the gas chromatography

settings specified in Section 3.7. The remaining gas volume in the amber bottle, along with the 20 mL extracted for CH₄ analysis, was determined using a water displacement apparatus [24], providing the total gas (mL/d) produced during the experiment's duration. The pH of the fermenter media was measured using a pH meter (TPS pH-mV-Temp Meter, Model WP-80), calibrated to 39 °C. Additionally, 1.5 mL of inoculum was collected as sub-samples for the analysis of volatile fatty acids (VFAs), as detailed in Section 3.8. Following the incubation period, the F47 filter bags containing the feed substrate were removed from the bottles, and fermentation was halted by placing them in an ice bath. After thorough rinsing with distilled water, the bags were dried in an oven at 55 °C. Subsequently, the dried bags were weighed to calculate in vitro dry matter digestibility (IVDMD), using Equations 2 (1a and 1b):

$$IVDMD = [Digestible\ dry\ matter\ (g)] / (Total\ substrate\ dry\ matter\ incubated\ (g)) \times 100 \dots \text{eq. 1a}$$

$$Digestible\ dry\ matter\ (g) = Total\ substrate\ dry\ matter\ (g) - residues\ dry\ matter\ (g) \dots \text{eq. 1b}$$

3.6 RUSITEC – Round 1 and Round 2

Among all the algae species screened, the two most effective microalgae species – freshwater (Round 1) and marine (Round 2) - overall was chosen for RUSITEC experimentation. Each round of RUSITEC spanned a 45-day period, divided into three runs lasting 15 days each. Each run comprised 7 days of adaptation followed by 8 days of sampling. Within the experiment, three treatments were administered, with two replicates (or fermenters) per treatment in every RUSITEC run, employing a completely randomised design. The three treatments; a control diet (0% algae), and two treatments with either 0.5% or 1% microalgae supplementation. The basal diet, comprising 9-parts forage (Hay mix; including 35% C4 grass hay, 30% oaten hay, and 30% lucerne hay) and 1-part barley distiller grain, was enclosed in nylon bags (11cm × 6.5cm, pore size 50 µm). The BSG was sourced from the waste stream of Young Henry's Brewery in Newtown, NSW, Australia, dried in an oven at 55°C for 72 hours, and milled using a 1 mm sieve. The forage (Hay mix), acquired from the University of Queensland Gatton Campus, QLD, Australia, and the University of Sydney's Sheep Reproductive Unit, Cobbitty, NSW, Australia, was milled using a 4 mm sieve. Each nylon bag contained a total mass of 10-11g of dry matter and was stored at room temperature until incubation. A chemical analysis was conducted on the selected microalgae, forage, and BSG utilised in the RUSITEC rounds 1 and 2 (Table S2 & S3).

Dry matter (DM) content of the hay and barley was determined by drying samples in an oven at 55°C for 72 hours. The dried samples were subsequently ground through a 1 mm sieve and analysed for neutral detergent fibre (NDF) as per the method outlined by Van Soest et al., [25], modified for an Ankom 200/220 Fiber Analyser (Ankom Technol. Corp., Fairport, NY, United States). Furthermore, basal diet analysis was carried out in accordance with the Association of Official Analytical Chemist [AOAC [26]] for ash content (method 942.05), nitrogen (method 990.03), and crude protein (CP), which was calculated using a conversion factor of 6.25 × N (%). Crude fat analysis was conducted using method 920.29 with a slight modification for an Ankom Fat Analyzer (i.e., 60 minutes at 90°C using Petroleum ether; Ankom Technol. Corp., Fairport, NY, United States). Additionally, non-fibrous carbohydrate (NFC) was calculated according to the method outlined by Mertens [27], following equation (2):

$$NFC = 100 - CP - NDF - EE - Ash \dots \text{eq.2}$$

The RUSITEC apparatus comprises six fermentation vessels submerged in a water bath set to 39°C, each filled with 810 mL of ruminal liquids. Equipped with input and output ports for buffer infusion and effluent waste,

respectively, every vessel is continuously infused with McDougall's buffer at a dilution rate of 24 mL per hour. Within each vessel, a small container houses two nylon bags: one containing 70 g of wet weight rumen solids and the other holding the designated treatment according to the randomised design (control, 0.5% algae, 1% algae) on day 0. To simulate rumen contractions, an electric motor continuously moves the vessels up and down to mix particles and ruminal fluid. Bag replacement occurs every 24 hours, with the removal of the nylon bag that has been incubating for 48 hours. Thus, each vessel consistently houses two bags over the 15-day period. Only bags undergoing 48 hours of fermentation are considered in the dry matter digestibility (DMD) calculations; hence, bags collected on day 1 containing rumen solids and those collected on day 15 are excluded from the analysis. For further details on the RUSITEC methodology, refer to Teoh [28].

3.6.1 Sample Collection and Analysis

Daily gas production was collected in airtight bags connected via plastic tubing to effluent flasks. The total daily gas production (mL/day) was measured by connecting the airtight bags to plastic tubing attached to a drum-type gas meter [29], with gas expelled manually using pressure. Gas sample collection for CH₄ analysis occurred from day 7 to day 15. Before total gas measurement, 25 mL of gas was extracted from each bag using a syringe and discarded, followed by extraction and collection of another 25 mL in 12 mL evacuated exetainers (Labco Limited, Lampeter, UK). Once total gas production was recorded, the discarded gas volume (25 mL) was added to ensure accurate calculation of total gas production. A subsample (3 mL) was collected from the evacuated exetainers for CH₄ analysis using gas chromatography (GC-FID, Agilent model 7890A), as outlined in Section 3.7. Daily fluid pH was recorded using a pH meter (TPS pH-mV-Temp Meter, Model WP-80) calibrated at 39°C. After 48 hours of incubation, the remaining digested substrate in each nylon bag was used to determine dry matter digestibility according to the protocol outlined by Teoh [28]. The bags were washed, dried in an oven at 60°C, and weighed. The percentage of dry matter digestibility (DMD) was calculated according to Equation 2. Effluent waste was collected daily in 1 L flasks and measured using a 2 L measuring cylinder. Total effluent produced was expressed as mL/day. For each run (on days 7-15), the effluent flasks were submerged in ice to halt fermentation and microbial proliferation. Total effluent was measured using different measuring cylinders for each of the three treatments to prevent contamination between treatments. Subsamples of effluent were collected, with 1.5 mL added to centrifuge tubes for further analysis of volatile fatty acids (VFA), as outlined in Section 3.8.

3.6.2 Microbial sequencing and analysis using 16S rRNA gene

For each run-on days 5, 10 and 15 residual feed substrate and rumen liquid was collected from each fermenter. The feed substrate was utilised to determine the solid associated-microbes (SAM) and rumen liquid to determine liquid associated-microbes (LAM). The SAM and LAM samples were used for total DNA extraction using a standard QIAGEN DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), according to the manufacturing guidelines, following a slight modification, with 150 mg of SAM or LAM samples mixed with 800 µL CD1 buffer in a spin power tubes, to obtain supernatant quantities (~600 ul) required for the extraction protocol. DNA yield and purity were measured using a NanoDrop spectrophotometry (Thermo Fisher Scientific, Waltham, MA, United States). DNA extracts were then stored at -20°C. Approximately 20 µL of DNA was sent to Ramaciotti Centre for Genomics (University of New South Wales, 2052, Australia) for amplification of the 16s rRNA V4 hypervariable and the ITS1 region of fungi. The primers 515-F and 806-R were used to amplify the archaeal and bacterial 16s rRNA V4 hypervariable region. The primers ITS1F and ITS2 were used to amplify the ITS1 region of fungi. Gene amplicons were generated on an Illumina MiSeq instrument (Illumina, Inc., San Diego, CA, United States), using the MiSeq Reagent Kit v2 (500 cycles; Illumina, Inc.).

The 16S rRNA gene region sequences were processed using DADA2 v.1.8 (R v 4.2.2.). Briefly, the forward and

reverse reads were trimmed to 240 and 160bp, to obtain a quality score >30%. Pair reads were merged and then chimeras were removed. Taxonomy assignment was conducted for the remaining sequences, referred to as amplicon sequence variants (ASV) at 100% similarity rate using the trained Silva database (V138.1) and RDP naïve Bayesian classifier (V.18). Reads for the ITS1 fungal sequences were processed using DADA2 v.1.8. (R v 4.2.2.). Reads were quality-filtered using the default parameter in DADA2, where a minimum sequence length of 50bp was set. Quality filtered reads were merged, chimeras removed, and taxonomy assigned to the ITS1 sequences using the RDP naïve Bayesian classifier and the UNITE database. Data filtering and analysis occurred in MicrobiomeAnalyst (<https://www.microbiomeanalyst.ca/>). Data filtering resulted in the removal of low abundance and variance features. The filtering parameters were set to a minimum count of 2 in 10% of samples. Low variance features were removed if the standard deviation was below 3%. Additionally, 16s rRNA and ITS1 samples were subsampled to the minimum number of sequences per read. Alpha diversity indexes were then calculated including ASVs per sample (observed), Shannon diversity index, and inverse Simpson's diversity index. Bray-Curtis dissimilarities were calculated using MicrobiomeAnalyst and the effect of microalgae concentration was assessed using PERMANOVA. All 16S rRNA and ITS1 gene sequences will be submitted to the NCBI sequence read archive under the existing Bioproject: PRJNA1090247

3.7 Methane Analysis

In both the batch culture assay and RUSITEC experiments, CH₄ concentration was determined using gas chromatography (GC-FID, Agilent model 7890A), following the methodology outlined in previous studies [28, 30]. The flame ionisation detector (FID) was set to 60°C, with an air flow of 300 mL/min and makeup flow (N₂) at 30 mL/min. A capillary column (Restek Rt-Q-Bond, 30 m × 0.53 mm ID × 20 µm) was used, with helium (H₂) as the carrier gas at a flow rate of 30 mL/min. CH₄ concentration was expressed as CH₄ mL/g DM.

3.8 Volatile Fatty Acids

For both the batch culture assay and RUSITEC experiments, VFA samples were prepared following the protocol described by Teoh [28]. In summary, 1.5 mL of subsamples were added to centrifuge tubes containing 0.3 mL of metaphosphoric acid (20%, w/v) acid in a 5:1 ratio and stored at -20°C. Prior to analysis, VFA subsamples were centrifuged at 12,000 rpm for 2 minutes at 25°C, and then 1.2 mL of supernatant was combined with 0.2 mL of the internal standard, Crotonic acid (0.1 mM). Analysis of VFAs was performed using gas chromatography (GC-FID, Agilent model 7820A), following the methodology outlined in Forwood [30], and Teoh [28]. The FID was set at 250°C, with an air flow of 300 mL/min, makeup flow (N₂) at 30 mL/min, and a capillary column (DB-FFAP, 30 m × 0.32 mm ID × 1 µm). Hydrogen (H₂) served as the carrier gas at a flow rate of 30 mL/min. The VFA yield was calculated using the following equation 3:

$$VFA\ yield\ (mmol/d) = VFA\ concentration\ (mmol/L) \times\ effluent\ volume\ (L/d) \dots \dots eq. 3$$

3.9 Statistical Analysis

The *in vitro* batch culture data were analysed using PROC MIXED in SAS (SAS, Inc., 2024; SAS Online Doc 9.4), with treatment means (microalgae species, concentration, microalgae species × concentration interactions) considered as fixed effects. The random effects included run and the interaction between run, microalgae species, and concentration. Each incubation run (n=2) served as the experimental unit. Normality of the data was assessed using PROC UNIVARIATE in SAS (SAS, Inc., 2023; SAS Online Doc 9.4). When the type 3 tests of fixed effect for concentration yielded P ≤ 0.05, orthogonal polynomial contrasts were applied to evaluate linear

and quadratic responses to varying concentrations of microalgae species (0%, 1%, 5%, and 10% on a dry matter basis). Differences among means were determined using LSMEANS/DIFF, with significance declared for all data at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

The normal distribution of the data from RUSITEC was assessed using the univariate procedure in SAS (SAS, Inc., 2024; SAS Online Doc 9.4). Fermentation data were analysed using the mixed procedure in SAS, with individual fermenters ($n=6$ per treatment) considered as the experimental units [31]. The model included fixed effects of treatment, day, and treatment \times day interactions, with the day of sampling treated as a repeated measure for each fermenter. The covariance structure was selected based on the minimum values of Akaike's information criterion, with compound symmetry used for all parameters. Treatment means were compared using the least squares mean linear hypothesis test (LSMEANS/DIFF), with significance declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. The effect of treatment on microbial community structures was assessed using PERMANOVA and Bray–Curtis dissimilarities in MicrobiomeAnalyst. Statistical significance was determined at $P \leq 0.05$, with tendencies reported at $0.05 < P \leq 0.10$. Additionally, differences in the relative abundance of the top 10 most abundant bacterial taxa were analysed using the mixed procedure in SAS, incorporating fixed and random-effects parameters as described above. The linear discriminant analysis effect size (LEfSe) method was employed using MicrobiomeAnalyst to assess differences in bacterial diversity between treatments, with differential abundance assumed when the LDA score was > 2.0 and $P \leq 0.05$ [32].

3.10 Lamb Performance Trial

The study aimed to assess the effects of different dosages (0.5% vs 1%) and types of algae (freshwater vs marine) on animal performance, carcass traits, and meat quality. Initially, five pellet formulations were planned (Table S23). However, a manufacturing error by Riverina Stockfeed was discovered after the experiment. Treatment four, which was supposed to contain 0.5% marine algae, however it was mistakenly supplied with a mix of both freshwater and marine algae. Because of this error, treatment four was excluded from the analysis to avoid invalid results. As a result, only three treatments were analysed: the control (treatment one), 1% freshwater algae (treatment three), and 1% marine algae (treatment five).

3.10.1 Lamb Trial – overview

Microalgae offer a sustainable method for converting industry waste into biomass rich in proteins and polyunsaturated fatty acids (PUFAs). This study explored the impact of incorporating freshwater or marine microalgae as alternative protein sources in a pelletized finisher diet for lambs, focusing on performance, carcass traits, meat quality, and fatty acid profiles. Sixty Dorper lambs, each 7-month-old of 33.67 ± 2.4 kg were randomly assigned to one of five diets: control (T1), low dosage of species 1 (0.5%, T2), optimal dose of species 1 (1%, T3), low dosage of species 2 (0.5%, T4), and optimal dosage of species 2 (1%, T5). Diets were iso-nitrogenous, with feed deliveries and refusals recorded daily, and lambs weighed fortnightly until they reached ≥ 45 kg live weight. Liver and Longissimus Lumborum (LL) muscle samples were collected post-slaughter for fatty acid analysis. Due to a manufacturing error, treatment four contained both freshwater and marine microalgae, making it unsuitable for statistical analysis. Only treatments one, three, and five were analysed. Results showed no significant differences in lamb performance between treatments. However, lambs fed microalgae had higher PUFA concentrations in their liver ($P = 0.05$), with a trend towards increased PUFA in subcutaneous fat ($P = 0.09$). PUFA levels in the LL muscle remained unaffected. Incorporating microalgae at 1% improved liver and fat PUFA content, with no effect on overall lamb performance.

3.10.2 Materials and Methods

The experiment was conducted at the Queensland Animal Science Precinct (QASP) in Gatton, QLD, the study received approval from The University of Queensland Animal Ethics Committee (Project No. 2021/AE000762).

3.10.2.1 Pre-Trial Body weight and Temperament assessment

Upon purchase, lambs underwent weighing to evaluate their body weight, ensuring they met the required criteria (~25 kg) for the experimental trial. Additionally, temperament assessments were conducted during weighing to identify lambs with lower stress tolerance and interaction tolerance. This involved visually assessing lambs' behaviour in the weighing pen and scoring them on a scale of 1 to 5 based on their movements and reactions. Scoring was determined as follows, 1: calm, no movement; 2: calm, with occasional movements; 3: moderate movements; 4: abrupt episodic movements; 5: permanent episodic movements. A total of 60 suitable lambs were selected for the trial, organised into five groups of 12 lambs each. Lambs that did not meet the body weight requirements were excluded from the trial. Furthermore, lambs scoring a 5 in temperament assessment were excluded from the trial unless the reserve lamb count had not reached 10 after body weight assessments. In cases where 10 lambs were excluded based solely on body weight, those with high temperament scores were recorded and handled with extra care to minimise handler risk and ensure the well-being of sensitive lambs. The 10 reserve lambs were housed at the Queensland Animal Science Precinct (QASP) for the trial's duration, with housing arrangements determined by QASP. Continuous access to fresh, clean water was provided, and welfare monitoring was conducted throughout the trial using a QASP welfare monitoring score sheet, assessing both the experimental lambs and the reserve lambs.

3.10.2.2 Feeding Experimental Design and Animal Performance

A total of 60 suitable lambs were chosen for the trial, divided into five treatment groups, with 12 lambs per treatment. Additionally, 10 reserve lambs were housed at QASP for the trial's duration, with housing arrangements determined by QASP. The reserve lambs received a growing diet comprising Riverina feedlot pellets and high-quality hay to support a daily growth rate of 300 g/day. Health and condition monitoring of the reserve lambs were conducted throughout the trial using the QASP welfare monitoring score sheet. At the trial's conclusion, all 10 reserve lambs were transported to the local abattoir along with the 60 experimental lambs for scheduled euthanasia. Two species of microalgae (fresh and marine) were purchased and imported from Phytobloom (Necton, Portugal). Pellets were manufactured by Riverina stockfeed (<https://www.riverina.com.au/>) following industry standard pelletisation procedures. A total of 60, 7-month-old dorper lambs were chosen and blocked live weight (33.67 ± 2.4 kg) and randomly assigned to one of five experimental diets: control (T1), pellets containing low dosage of species 1 (0.5%, T2), pellets containing optimal dose of species 1 (1%, T3), pellets containing low dosage of species 2 (0.5% T4), or 5) pellets containing optimal dosage of species 2 (1%, T5). The chemical composition of all diets was determined by methods outline in section 3.11.3 and formulated to be balanced for crude protein (i.e. iso-nitrogenous) (Table 2). Pellets were formulated in consultation with Prof. Alex Chaves (University of Sydney) and Riverina Stockfeed (Queensland) to meet industry standards (Table S23).

Table 2: Treatment Chemical Composition

Feed	DM %		% of DM			
	CP	NDF	EE	ASH	NFC	

Cereal Hay	94.68 ± 0.26	5.92 ± 0.1	71.33 ± 2.34	7.2 ± 0.74	9.50 ± 0.47	6.06
Control	97.20 ± 0.74	15.27 ± 0.27	20.62 ± 0.58	5.67 ± 0.36	7.83 ± 0.26	50.6
Fresh, 0.5%	97.47 ± 0.36	15.11 ± 0.21	20.49 ± 0.86	5.77 ± 0.24	7.20 ± 0.21	51.4
Fresh, 1%	97.51 ± 0.28	15.39 ± 0.15	20.06 ± 1.08	6.3 ± 0.32	6.94 ± 0.42	51.3
Marine & Fresh, 0.5% ^a	97.83 ± 0.73	15.67 ± 0.30	21.10 ± 2.60	5.16 ± 0.46	7.40 ± 0.32	50.7
Marine, 1%	97.39 ± 0.42	15.56 ± 0.44	19.76 ± 0.60	5.87 ± 0.21	6.80 ± 0.26	52.0

Abbrev: DM; Dry matter, CP; Crude Protein, NDF; Neutral detergent fibre, EE; Ether Extract, NFC = Non-fibrous carbohydrates [NFC = 100 - (CP + NDF + EE + Ash)]. ^aUpon completion of the trial it was revealed treatment four included both fresh and marine microalgae due to a manufacturing error.

Alongside pellets, all lambs received high-quality hay daily, aiming for a targeted intake of 3.5% of body weight with a 10% allowance for refusals. Diets were formulated to support a daily weight gain of 300g/day. Lambs were housed indoors in individual pens and underwent a two-week diet adaptation period before the 3-month experimental phase commenced (~100 days). Lambs were housed indoors in individual pens (1.8m X 2 m), surfaced with wire mesh. There was *ad libitum* access to fresh clean water. Lambs were provided their full ration once daily at 0900h during the adaptation and experimental periods. Refusals were collected daily and DM intake (DMI) was calculated weekly, as the difference between feed offered and that refused. Weekly samples of each pellet were collected for dry matter analysis. Dry matter content was determined by drying pellets and hay samples for 24-h at 60°C. Samples were subsequently ground a 1 mm screen and stored at room temperature until analysis for chemical composition. Lamb live weights were recorded fortnightly prior to the morning feed. Average daily gain (ADG) was calculated as difference between final and initial liveweight gain divided by the number of days animals were on feed. Feed conversion efficiency (FCE) was calculated as the ratio between mean DMI and ADG (g of DMI/g of ADG).

3.10.2.3 Feed chemical composition

The dried and ground feed samples were analysed for neutral detergent fibre (NDF) using as described by Van Soest et al. [25] modified for an Ankom 200/220 Fiber Analyser (Ankom Technol. Corp., Fairport, NY, United States). The NDF analysis included amylase and sodium sulphite which is expressed inclusive of residual ash (aNDFom). Further basal diet analysis was performed according to the Association of Official Analytical Chemist [AOAC] [26] for Ash content (method 942.05), nitrogen (method 990.03) and crude protein (CP) calculated by 6.25 × nitrogen (%). Crude fat analysis followed method 920.29 with modification for an Ankom Fat Analyzer (60 minutes at 90°C using Petroleum ether; Ankom Technol. Corp., Fairport, NY, United States). Additionally, the calculation of non-fibrous carbohydrates (NFC) followed methods outlined by Mertens et al. [27], according to equation 2 in section 3.6.

3.10.3 Procedure Specification

3.10.3.1 Blood Sampling

Blood samples were collected twice throughout the trial from each of the 60 lambs by trained project/QASP staff members. The first collection occurred when the lambs average body weight reached 35 kg (approximately halfway through the trial), and the second at the slaughter weight (average 48 kg). As per the handling of sheep SOP (ATT 027), lambs were safely restrained to immobilise the head. Blood samples were collected from venepuncture of the jugular vein. Blood was collected in 10 mL lithium heparin vials, immediately after collection, vials were rotated to mix the blood and then stored on ice. Subsequently, vials were centrifuged,

with the remaining plasma collected and stored at -80 °C until the metabolomic analysis was conducted.

3.10.3.2 Slaughter and carcass characteristics

Following the completion of the experimental trial, the lambs were transported to a nearby commercial abattoir. Upon arrival, they were held in lairage overnight to allow them to acclimate to their new environment and reduce stress before slaughter. The next morning, the lambs were humanely slaughtered, processed, and dressed according to standard industry practices. Post-mortem, the liver was collected from each carcass, immediately weighed, and a sub-sample snap-frozen in liquid nitrogen for further metabolomic analysis. Individual hot carcass weights (HCW) were recorded, and the carcasses were placed in a refrigerated cool room (4°C) until transportation back to QASP for sectioning. A temperature logger was placed in the last processed carcass at the abattoir to monitor cooling procedures and prevent cold shortening. Due to logistical reasons, the carcasses remained in the refrigerated cool room at the abattoir for 5 days before being transported back to QASP to be broken down into meat cuts. Upon arrival, the cold carcass weight of each individual was recorded, and the cold dressing percentage calculated. The carcasses were then broken down by the QASP team butchers. Paired Longissimus lumborum (LL) muscles were excised from each carcass and stored overnight at 4°C. The following day, the LL was deboned, and pH were measured using a meat meter with a built-in temperature compensator. Fresh colour measurements were taken using a chromameter fitted with a standard xenon lamp, aperture, and observer settings (Figure S1, left panel).

3.10.3.3 Meat Quality

Drip Loss was calculated as the percentage difference in weight observed over a 24-hour period during which meat samples were suspended within inflated plastic bags at 4°C (Figure S1, right panel). This measurement is used to assess the amount of liquid (drip) released or lost from the meat samples during storage or refrigeration. Due to a technical fault in the cooking equipment cook loss was unable to be performed.

3.10.3.4 DNA Sampling

Rumen digesta was collected immediately after the lambs were slaughtered and stored on ice before being returned to QASP, where it was kept at -80 °C for DNA analysis. The extraction of solid-associated rumen microbes followed the steps outlined in section 3.6.2. Approximately 20 µL of DNA was then sent to the Ramaciotti Centre for Genomics at the University of New South Wales. To amplify the archaeal and bacterial 16S rRNA V4 hypervariable region, primers 515-F and 806-R were used, while primers ITS1F and ITS2 amplified the ITS1 region of fungi. Gene amplicons were generated using an Illumina MiSeq system with the MiSeq Reagent Kit v2 (500 cycles). The sequences for the 16S rRNA gene region were processed using DADA2 v.1.8 (R v 4.2.2). The forward and reverse reads were trimmed to 240 and 200 bp to ensure a quality score above 30%. Paired reads were merged, and chimeras were removed. Taxonomy assignment for the remaining sequences, called amplicon sequence variants (ASVs), was done at a 100% similarity rate using the RDP naïve Bayesian classifier and the trained Silva database (V138.1). For the ITS1 fungal sequences, the same DADA2 v.1.8 process was followed, with a minimum sequence length of 50 bp set for quality filtering. Merged reads had chimeras removed, and taxonomy was assigned using the RDP naïve Bayesian classifier and the UNITE database. All 16S rRNA and ITS1 gene sequences will be submitted to the NCBI sequence read archive under Bioproject PRJNA1090247. Alpha diversity indexes for both 16S rRNA and ITS1 sequences were calculated using R and Phyloseq v. 1.26.024, including the number of ASVs per sample, Shannon diversity index, and inverse Simpson's diversity index.

3.10.3.5 FAME Analysis

Post-slaughter, the liver and Longissimus lumborum muscle were collected, and the subcutaneous fat was separated from the muscle before freeze-drying for 48 hours. Fatty acids were then extracted from both the subcutaneous fat and muscle samples. The freeze-dried biomass was accurately weighed and extracted using 3 mL of a chloroform (2:1, v/v) mixture by vortexing and centrifuging. The supernatants were collected, and the residues were re-extracted three more times with 2 mL of a chloroform (1:1, v/v) mixture. The pooled supernatants were washed with 2 mL of saturated sodium chloride solution and allowed to settle. The lower organic phases were collected and evaporated under nitrogen gas to determine the total lipid content gravimetrically. The extracted lipids were then converted to fatty acid methyl esters (FAMES). Nonadecanoic acid (C19:0) served as the internal standard. The dried lipids were treated with 1 mL of 1% NaOH in methanol and heated for 15 minutes at 55 °C. Then, 2 mL of 5% methanolic HCl was added, and heating continued for another 15 minutes. After adding 1 mL of Milli-Q water, FAMES were extracted using hexane (3 × 1 mL) and evaporated under nitrogen gas. The FAMES were redissolved in 200 µL of hexane and stored at -20 °C in glass vials until analyzed by GC-MS. FAME analysis was performed using a GC-MS-QP2020 (Shimadzu Corporation) with an AOC-20is autosampler. The analysis utilized an SH-Rxi-5Sil MS fused silica capillary column (30.0 m x 0.25 mm x 0.25 µm) operating in electron impact mode at 70 eV. Helium served as the carrier gas at a constant flow of 1.0 mL/min, with an injection volume of 1 µL at an injector temperature of 280 °C and an ion source temperature of 230 °C. The oven temperature was programmed from 50 °C (held for 2 minutes) to 220 °C (4 °C/min) and then from 220 °C to 300 °C (60 °C/min) for 3 minutes. FAME peaks were identified by comparing their retention times to authentic standards and quantified using area normalization and response factors.

3.10.3.6 Sensory Analysis

A sensory analysis was conducted by the Department of Agriculture and Fisheries Queensland at the sensory analysis facility in Coopers Plains, QLD. After completing the lamb feeding trial (2021/AE000762), a subsample of meat was collected from lambs, vacuum-packed using a VS4500 FoodSaver from Sunbeam Products Inc., and frozen at -20°C until the sensory analysis. The day before the analysis, the frozen meat cuts were thawed overnight at 2°C and then stored at room temperature for about one hour before cooking. The loin was cooked (Figure S2) on a Silex clam-shell grill preheated to 200°C for 1-minute meat side down, then 2 minutes subcutaneous fat side down, before transferring to an oven preheated to 180°C. A meat temperature probe was inserted into the loin, and once the internal temperature reached 62°C, the loins were rested for 15 minutes at 50°C. This cooking time achieved a medium cook, reducing drying out compared to well-done cooking. Samples were prepared consistently to minimise bias. Participants in the sensory analysis were presented with three samples arranged in a triangle test format to determine if they could detect differences between control-fed and treatment-fed lamb samples (Figure S3). A total of 42 participants, untrained Australian lamb consumers, were asked to identify the different samples and describe any perceived differences. The nature of the analysis was disclosed to participants beforehand. The optimised protocol for the sensory analysis, including preparation of loins, placement under red light to eliminate colour as a factor, and participant feedback using a survey in the sensory/tasting room, is outlined in Figure S2.

3.11.3.7 Metabolomic Analysis

Metabolites from blood plasma, freeze-dried liver, and rumen digesta were extracted using 750 µL of a 100% methanol extraction solution, which contained three internal standards: 100 µmol/L DL-valine-d8, 60 µmol/L stearic acid-d3, and 60 µmol/L 5-α-cholestane, for every 50 mg of sample. An additional extraction with 750 µL

of 50% methanol was performed. Samples were incubated for 20 minutes at 5 °C and 1250 rpm in a thermomixer, followed by centrifugation for 3 minutes at 20,800 g. The supernatant was collected and pooled, then 150 µL was evaporated to dryness in a vacuum evaporator for 3 hours at room temperature. To derivatize the samples, 20 µL of 20 mg/mL methoxylamine in pyridine was added, and the samples were incubated at 37 °C for 2 hours at 750 rpm. Next, 20 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide was added, and samples were incubated at 37 °C for another 30 minutes. After resting at room temperature for 1 hour, 1 µL of the samples was injected for GC-MS analysis using a GCMS-QP2020 (Shimadzu Corporation). The analysis employed an SH-Rxi-5Sil MS fused silica capillary column (30.0 m x 0.25 mm x 0.25 µm) in electron impact mode at 70 eV. Helium served as the carrier gas at a flow rate of 1.0 mL/min, with an injector temperature of 280 °C and an ion source temperature of 230 °C. The oven temperature gradient started at 70 °C for 1 minute and increased to 325 °C at 7 °C per minute. The scan range was set to m/z 50–600, with samples analyzed in a randomized order to minimize experimental bias. Raw GC-MS data were converted to CDF format using GCMS solution software (v.4.0, Shimadzu) and imported into XCMS (v.3.2, Galaxy Project Metabolomics). XCMS facilitated non-linear retention time alignment, matched filtration, peak detection, and matching. The bandwidth for grouping was set to 10, producing a peak list of features (ions, retention time, intensity). Area normalization was performed using the total peak area of internal standards, and the normalized features were subjected to statistical analysis using MetaboAnalyst 6.0 software.

3.10.4 Statistical Analysis (updated to accommodate manufacturing error)

Due to a manufacturing error in treatment four, it was excluded from statistical analysis. Therefore, only three treatments were analyzed: treatment one (control), treatment three (1% freshwater algae), and treatment five (1% marine algae). Data were analyzed as a completely randomized design using mixed model procedures (PROC MIXED; SAS Institute Inc.), considering treatment means (microalgae species, dosage, and their interactions) as fixed effects, while individual lambs within each treatment were treated as a random effect (n=12). Mean dry matter intake (DMI) was calculated over all weeks, and data normality was assessed using PROC UNIVARIATE in SAS. Differences among means were determined using LSMEANS/DIFF, with significance set at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Sensory data were collected using EyeQuestion® (version 5.0.7.8). The Critical Number of Correct Responses for a Triangle Test (ISO, 2004) determined significant differences in sensory attributes between control and test samples at $P \leq 0.05$. Alpha diversity indices were analyzed using the MIXED procedure in SAS 9.4, with treatment means as fixed effects and lambs as random effects. The effect of diet on microbial community structures was assessed using PERMANOVA (adonis2 function) and Bray–Curtis dissimilarities in R (vegan 2.5-630). Statistical significance for MIXED analyses was declared at $P < 0.05$, and tendencies at $0.05 < P \leq 0.10$. Univariate analysis of plasma, rumen, and liver metabolome data was conducted using MetaboAnalyst 6.0. Data were normalized with a two-factor normalization method and Pareto scaled to minimize variability and false discovery. Principal component analyses (PCA) were performed to assess the separation of differentially expressed metabolites by treatment effect. Fold change analyses were conducted for each sample type, using thresholds of fold change ≥ 1.2 and FDR P-value = 0.05 to determine statistical significance.

3.11 Supply Chain Mapping

This project is designed to address two critical environmental challenges simultaneously: reducing CO₂ emissions from breweries and mitigating methane emissions from cattle. By harnessing CO₂ and brewery waste streams to produce an algae-based high-nutrient supplement, the project aims to create a sustainable solution that benefits both industries.

The first step involves the development of the algae-based supplement, which requires understanding the optimal cultivation conditions, nutrient requirements, and processing techniques. Once developed, breweries can integrate the production of this supplement into their operations, thereby reducing their CO₂ footprint by utilising captured CO₂ and waste streams. On the other end, red meat producers stand to benefit from the utilisation of this supplement in cattle feed. By incorporating the algae-based supplement into the diet of cattle, methane emissions can be effectively reduced. This not only aligns with sustainability goals but also addresses a significant environmental concern associated with livestock farming. To ensure the success and scalability of this initiative, a comprehensive supply chain analysis is necessary. This analysis involves assessing various logistical factors, including the volume of raw materials required for supplement production, the number and locations of participating breweries, and the transportation routes from the breweries to the end-users, such as farms and processing facilities. Furthermore, economic feasibility and cost-benefit analysis are crucial aspects of the supply chain analysis. It's essential to evaluate the financial implications for both breweries and red meat producers, considering factors such as production costs, transportation expenses, and potential revenue streams. This analysis will help identify opportunities to optimise the supply chain and maximise the benefits for all stakeholders involved. Overall, by integrating sustainability principles into both the brewing and livestock industries, this project aims to demonstrate the potential for cross-sector collaboration in addressing environmental challenges and promoting a more sustainable future.

An adoption plan (Tables S4 and S5) was prepared according to the provided MLA template, assessing the project output for utilisation, and mapping the extension activities across MLA Adoption Pathway. A supply chain stakeholder - Dr Sanjoy Paul (Program Director and Senior Lecturer in the UTS Business School) was identified and consulted with the feedback (Table S6), and supply chain was mapped (Table S7) for volumes, costs, time for transport and quality attributes for each section of the supply chain with a focus on identifying the potential issues end-users may have using the output.

3.11.1 Australian Breweries outputs and CO₂ emissions

The brewing industry in Australia is thriving, with a significant number of independent brewers contributing to its growth. As of 2022, there are 348 brewers across the country, with the highest growth observed among smaller breweries producing less than 300,000 litres and larger ones exceeding 5 megalitres annually ([33], Table S8). Despite the varying production scales, the estimated total CO₂ emissions from all Australian breweries during the fermentation stage are substantial, ranging from 1325 to 5951 tons per annum (Table S9).

One of the by-products of the brewing process is Brewers' Spent Grain (BSG), which holds considerable value for cattle farms as animal feed. However, the current process of collecting and utilising BSG involves significant effort from farmers, including understanding its feed value and integrating it into daily feeding routines (Table S10 & Table S11). Given its potential as a valuable resource, efforts to streamline the collection and distribution process are essential. Australia's agricultural sector, particularly livestock farming, significantly contributes to the country's total greenhouse gas emissions, with methane emissions from livestock being a major concern. Dairy farms, which account for approximately 5055 establishments with 1.3 million cows, present a more manageable option for trailing the use of algae-based supplements due to their relatively smaller size and concentrated distribution. In contrast, there are approximately 20,300 beef cattle farms across Australia, with varying sizes and distribution patterns (Table S12). While beef cattle farming plays a significant role in the industry, the larger number of dairy farms and their strategic locations, particularly in Southeast Queensland and along the South and South East coasts, make them ideal candidates for initial trials and product adoption. Given the moisture content of BSG, establishing close relationships between breweries and farms is crucial for daily collection and utilisation. Therefore, careful consideration of the geographical distribution of both

breweries and farms is essential to identify suitable locations for trials and to determine early adopters (Table S12). Overall, integrating algae-based supplements into the feed supply chain presents an opportunity to address environmental challenges associated with both brewing and livestock farming industries. Streamlining the collection and utilisation of BSG, particularly in dairy farms, can contribute to reducing greenhouse gas emissions and promoting sustainable practices in agriculture.

3.11.2 Proposed product utilisation and management

Utilising the feed budgeting tool provided by Dairy Australia (Table S13), a feeding scenario for a typical dairy farm was constructed. In this scenario, the proposed feed supplement, comprising 10-20% inclusion of BSG and 1-10% inclusion of algae, was integrated into the feeding regime as a donated product. Considering current hay and grain prices, this integration could result in significant cost savings. Based on the calculations, the incorporation of the algal/BSG feed as a donated item could potentially lead to savings of up to \$18,675 per month or \$224,100 annually.

These savings would be realised through the partial replacement of purchased feed with the supplemented feed product, demonstrating the economic benefits of integrating sustainable feed alternatives into dairy farm operations. To meet the feed demand for one typical dairy farm, the brewer would need to produce between 0.83-8.30 tons of algae per month. For a 1% algae inclusion, approximately 55 m³ of algae would be required to supply a one-month feed budget (based on the assumption highlighted in Table S14). This process could potentially sequester up to 1.5 tons of CO₂. However, the efficiency of algae in up taking carbon is not 100%, varying depending on factors such as species, growth system, location, and season. Therefore, excess CO₂ supply to the system would be necessary, with variable losses to the atmosphere that need to be considered in the proposed system. To meet the monthly feed demand of a typical dairy farm with BSG supplementation at 10%, approximately 8300 kg of spent grain would be required per month (Table S14). This would necessitate a brewer with a production capacity of at least 300K L beer per annum (Table S9), assuming no drying of the spent grain.

Given that many independent brewers produce less than 300K beer per annum, collaboration between smaller breweries or the establishment of local processing facilities may be necessary to meet this demand efficiently. Additionally, utilising 15% of the CO₂ generated by a brewer of this capacity could help produce the required algae for the monthly feed budget of one dairy farm. The main objective is to optimise the supply chain network, ensuring the required BSG is supplied to the cattle farms while minimizing supply chain costs and CO₂ emissions (from production and transportation) to the environment. All the associated costs and CO₂ emissions are mentioned in Table S15.

4. Results

4.1 Batch Culture Screening

4.1.1 First stage of batch culture screening

In a second Government Innovation Connections Grant (Young Henry Pty Ltd Protected background IP; 2020-2022), the AMPTS II methane potential analysis tool was utilised to assess methane production of a typical feed blend with 5% algal substitution for a typical dairy concentrate and maize silage substrate. Preliminary trials involving 5 different microalgae species at 5% substrate substitution revealed differences among the species. Three species (Species 7, 19 & 4) demonstrated a decrease in CH₄ gas production.

Species 19 exhibited the greatest numeric reduction in CH₄, up to 10%, followed by species 7 with a 7% reduction, with minor variation between replicates. Numeric reductions in total volatile fatty acids and increases in pH were observed for all species compared to the control, indicating negative fermentation effects. However, algal species' impact on fermentation parameters was not significant ($P = 0.140$). These findings emphasise the need to explore varying algae concentrations for a dose-response relationship and expand the range of microalgal species studied.

Due to the low throughput of the AMPTS II system, a more robust, high-throughput method was adopted. Through collaboration with the University of Sydney, a high-throughput *in vitro* ruminal fermentation assay was established at UTS. This assay allowed screening of up to 10 species per run in triplicate at 4 concentrations. It was then used to evaluate a minimum of 2 independent runs of 18 microalgal species at 4 concentrations (0%, 1%, 5%, and 10% inclusion). Across these species, methane reduction varied from 1% (for freshwater species) to 29% (for marine species).

Marine microalgae species (Table S16) did not significantly affect fermentation parameters analysed ($P > 0.180$), but tendencies ($P \leq 0.095$) showed that two species (17 and 14) led to greater reductions in CH₄ production per gram of dry matter and per day. Increasing microalgae concentration raised pH ($P < 0.01$). CH₄ concentration (expressed as CH₄%, CH₄ mL/d, and CH₄ mL/gDM) declined with higher microalgae concentrations, notably decreasing at 1% inclusion compared to 0% ($P \leq 0.004$). However, tendencies indicated that higher marine microalgae concentrations tended to decrease total gas production (mL/d; $P=0.095$) and *in vitro* dry matter digestibility (IVDMD) ($P = 0.091$).

Freshwater microalgae (Table S17) significantly increased media pH ($P < 0.001$), and higher microalgae concentrations led to elevated pH levels ($P < 0.001$). Total gas production was affected by freshwater microalgae species ($P \leq 0.024$), with Species 4 producing the highest volume. However, total gas production decreased with increasing microalgae concentration ($P < 0.001$).

Microalgae species did not impact CH₄ parameters ($P \geq 0.440$), but higher microalgae concentrations reduced CH₄ production ($P \leq 0.004$). At 1% inclusion rate, all freshwater species decreased CH₄ production compared to 0%. Additionally, increasing microalgae concentration tended to increase *in vitro* dry matter digestibility (IVDMD) ($P = 0.094$).

Marine microalgae species did not affect total VFA concentration ($P=0.217$), but increasing their concentration led to a decrease ($P = 0.007$) in total VFA concentration (Table S18). Certain species showed a tendency ($P = 0.058$) to reduce the percentage of propionate acid, with Species 15 notably causing the biggest reduction ($P = 0.030$) in % of butyrate. Freshwater Species 10 had the smallest total VFA concentration ($P = <0.001$) compared to other species (Table S19). Variability was observed in acetate concentration among microalgae species ($P < 0.001$). Species 10 maintained propionate acid proportions relative to other freshwater species despite a lower total VFA concentration (Table S19).

Increased concentrations of freshwater algae reduced % of propionate acid of total VFA ($P < 0.001$) and increased BCVFA percentage ($P < 0.022$) and acetate or propionate ratio ($P < 0.001$). Thirteen freshwater and five marine microalgae species were analysed to identify suitable candidates for subsequent evaluation with BSG and RUSITEC trials.

The study found no species effect on CH₄ production for both freshwater and marine microalgae in batch culture, yet marine algae exhibited significant reductions in VFA propionate. An optimal inclusion concentration

of 1% DM was determined for freshwater microalgae, maintaining total gas production, IVDMD, and total VFA while reducing CH₄ production. A species matrix was developed to rank species based on desirable traits, with Species 10 chosen for further evaluation due to its robustness, biomass productivity, and CO₂ tolerance. Assessment of Species 10 showed no significant effects on gas parameters, but algal concentration did affect media pH ($P = 0.021$), with a decrease observed in pH in samples containing algae.

4.1.2 Second stage of batch culture screening

See Table S1 for a summary of species screened in each run.

4.1.3 Round 5 Screening

Concentration effects on CH₄ production were initially assessed using maize silage and dairy concentrate (Table S1, Rounds 1-3). Doses were limited to 1% due to negative fermentation effects at higher doses ($\geq 5\%$). Transitioning to a hay mix with brewer's spent grain (BSG) in round 4 showed no effects fermentation parameter at 1% inclusion. The subsequent run (Round 5) tested five selected species in both basal feeds to assess their ability to reduce CH₄ irrespective of diet.

Table 3 illustrates the impact of incubating five microalgae species at varying concentrations on *in vitro* fermentation characteristics within a basal diet of maize silage and dairy cow concentrate. Consistent with previous batch culture findings, there was a significant increase in pH with higher microalgae concentrations ($P < 0.001$), while no adverse effects on pH were observed due to species. Increasing algae concentration resulted in decreased total gas (mL) ($P < 0.017$), mirroring previous batch culture outcomes.

Although there was a numerical trend of decreasing CH₄% with increasing algae concentration, it was not deemed significant. However, both CH₄ (mL/d) and CH₄ mL/g DM showed a significant decrease with increasing algae concentration ($P < 0.014$ and $-P < 0.051$ respectively). IVDMD remained unaffected by algae inclusion.

Table 4 represents the impact of incubating five microalgae species at varying concentrations on *in vitro* VFA production within a basal diet of maize silage and dairy cow concentrate. Increasing microalgae inclusion from 0 to 5% reduced the percentage of Butyrate by 9.48% ($P = 0.012$). The inclusion of microalgae did not significantly any other measured VFA parameters.

Table 3: The effect of incubating five different microalgae species at various concentrations on *in vitro* fermentation characteristics in a basal diet consisting of Maize Silage and Dairy Cow Concentrate.

	pH	Total Gas, mL/d	CH ₄ , %	CH ₄ , mL/d	CH ₄ , mL/gDM	IVDMD
Species						
2	6.39	40.2	10.20b	4.12	10.55	63.6
4	6.39	40.43	10.28ab	4.16	10.67	65.72
12	6.37	40.53	10.21ab	4.16	10.68	64.33
19	6.42	39.08	10.32ab	4.05	10.38	71.05

7	6.40	40.55	10.99a	4.47	11.46	70.39
SEM Species	0.016	0.981	0.358	0.234	0.597	2.791
Conc.						
0%	6.31c	42.45a	10.90a	4.64a	11.60a	71.72
1%	6.40b	39.32b	10.40ab	4.12b	10.35b	64.57
5%	6.46a	38.58b	10.14b	3.92b	10.12b	64.93
10%	6.42ab	40.2ab	10.15b	4.09b	10.91ab	66.85
SEM Conc.	0.015	0.894	0.338	0.222	0.566	2.531
P-values						
Species	0.461	0.754	0.215	0.440	0.438	0.182
Conc.	<.001	0.017	0.108	0.014	0.051	0.155
Species × Conc.	0.347	0.856	0.912	0.440	0.828	0.984

Abbrev; SEM; standard error of the mean, CH₄; methane, IVDMD; In vitro dry matter digestibility, Conc; concentration.

Table 4: The effect of incubating five different microalgae species at various concentrations on in vitro VFAs in a basal diet consisting of Maize Silage and Dairy Cow Concentrate.

	Total VFA (mM)	% of Total VFA				
		Acetate (A)	Propionate (P)	Butyrate	BCVFA	Ratio A:P
Species						
2	86.72	54.43	27.64	15.25ab	2.70	1.99
4	86.34	54.90	26.87	15.40a	2.83	2.05
12	85.07	54.07	27.37	15.70a	2.86	1.94
19	84.53	56.43	26.34	14.29b	2.94	2.15
7	86.05	55.39	26.63	14.92ab	3.06	2.09
SEM Species	2.353	0.673	0.590	0.595	0.303	0.055
Conc.						
0%	87.18	54.05	27.36	15.93a	2.67	1.99
1%	85.84	55.32	27.05	14.93b	2.88	2.05
5%	83.67	55.62	26.87	14.42b	2.93	2.06
10%	86.29	55.19	26.60	15.17ab	3.03	2.08
SEM Conc.	2.149	0.602	0.528	0.578	0.300	0.049
P-values						
Species	0.945	0.154	0.533	0.052	0.183	0.108
Conc.	0.619	0.301	0.784	0.012	0.057	0.550
Species × Conc.	0.945	0.932	0.976	0.557	0.299	0.891

Abbrev; SEM; standard error of the mean, Conc., concentration, VFA; Volatile fatty acids, BCVFA, Branched chain volatile fatty acids, Ratio A:P, Acetate to propionate ratio.

Table 5 presents the impact of incubating five microalgae species at various concentrations on in vitro fermentation characteristics in a basal diet of hay mixture and brewer's spent grain (BSG). There was a

significant concentration effect on pH, indicating an increase with higher microalgae concentrations ($P < 0.012$). Additionally, increasing concentration led to a decrease in total gas production (mL/d), signifying reduced fermentation efficiency ($P < 0.013$). However, no concentration effects were observed on CH₄ parameters. Notably, the assessment of five species in the hay and BSG diet revealed a significant species effect on all CH₄ production parameters (CH₄ %: $P < 0.006$, CH₄ mL/d: $P < 0.012$; CH₄ mL/g DM; $P < 0.011$), along with an effect on IVDMD ($P < 0.026$). Furthermore, a species concentration interaction effect was observed on CH₄ %, where inclusion of Species 2 at 5% reduced CH₄% by 11.8% (Table S20). However, inclusion of microalgae at $\geq 5\%$ also reduced fermentation efficiency (Table S20).

Table 6 displays the impact of incubating five microalgae species at various concentrations on *in vitro* VFA production in a basal diet of hay and BSG. Increasing microalgae concentration in the hay + BSG diet from 0 to 1% increased BCVFA by 11.85% ($P = 0.017$). The inclusion of microalgae did not significantly any other measured VFA parameters.

Round 5 batch culture results revealed a dosage effect when microalgae were included in the maize and DCC diet, whilst there was a significant species effect evident in the Hay + BSG diet. These results convey both species and dosage selection are important considerations when utilising microalgae in various basal diets.

Table 5: The effect five different microalgae species at various concentrations on *in vitro* fermentation characteristics in a basal diet consisting of Hay and Barley Spent Grain.

	pH	Total Gas mL/d	CH ₄ , %	CH ₄ , mL/d	CH ₄ , mL/gDM	IVDMD
Species						
2	6.6	34.87	10.82c	3.80c	9.74c	57.13c
4	6.6	35.25	11.22bc	3.96bc	10.15bc	58.38bc
12	6.57	36.52	11.53ab	4.22ab	10.81ab	67.40a
19	6.59	34.14	11.36bc	3.90bc	9.96bc	66.64ab
7	6.57	37.03	12.07a	4.47a	11.49a	68.11a
SEM Species	0.015	0.803	0.408	0.18	0.463	4.207
Conc.						
0%	6.55b	37.43a	11.32	4.24a	10.58	66.53
1%	6.59a	35.15bc	11.52	4.07ab	10.24	61.36
5%	6.57ab	35.94ab	11.36	4.09ab	10.57	62.89
10%	6.61a	33.73c	11.4	3.88b	10.33	63.34
SEM Conc.	0.013	0.718	0.397	0.171	0.437	4.011
P-values						
Species	0.385	0.105	0.006	0.012	0.011	0.026
Conc.	0.012	0.013	0.873	0.211	0.811	0.545
Species × Conc.	0.071	0.245	0.045	0.062	0.055	0.933

Abbrev; SEM; standard error of the mean, CH₄; methane, IVDMD; *In vitro* dry matter digestibility, Conc; concentration.

Table 6: The effect five different microalgae species at various concentrations on *in vitro* VFA production in a basal diet consisting of Hay and Barley Spent Grain.

Species	Total VFA (mM)	% of Total VFA			
		Acetate (A)	Propionate (P)	Butyrate	BCVFA Ratio A:P

2	79.20	66.15	21.15	9.99ab	2.59	3.14
4	78.72	66.39	20.94	9.88abc	2.76	3.19
12	81.63	66.21	20.81	10.25a	2.74	3.19
19	78.04	67.16	20.90	9.30c	2.65	3.22
7	81.02	66.79	20.93	9.46bc	2.82	3.20
SEM Species	1.564	0.891	0.545	0.365	0.778	0.124
Conc.						
0%	78.50	66.54	21.47	9.49	2.53b	3.10
1%	78.26	66.59	20.80	9.78	2.83a	3.22
5%	81.65	66.59	20.76	9.93	2.69ab	3.23
10%	80.48	66.44	20.76	9.91	2.81a	3.21
SEM Conc.	1.398	0.883	0.528	0.355	0.071	0.122
P-values						
Species	0.431	0.069	0.947	0.018	0.235	0.908
Conc.	0.256	0.965	0.203	0.310	0.017	0.319
Species × Conc.	0.327	0.210	0.861	0.264	0.713	0.748

Abbrev; SEM; standard error of the mean, Conc., concentration, VFA; Volatile fatty acids, BCVFA, Branched chain volatile fatty acids, Ratio A:P, Acetate to propionate ratio.

4.1.4 Round 6

A total of 13 microalgae species at multiple concentrations were investigated on fermentation parameters (Table 7). Due to no significant species and concentration interactions observed for the measured fermentation parameters, the results are presented as single effects. There was a species-specific effect on media pH with Species 7 inclusion increasing the media pH compared to Species 4 inclusion ($P = 0.003$, Table 7). There was a species-specific effect on CH₄ parameters (presented as % and mL/gDM). For instance, the inclusion of Species 20 resulted in the lowest percentage of CH₄ (11.87%) compared to Species 24 (13.11%) and Species 7 (13.01%) (Table 7). The inclusion of Species 17 and 26 resulted in the lowest production of CH₄ on a mL/ g DM basis compared to the inclusion of Species 7,12,23, and 24 ($P = 0.005$, Table 7). There was no species-specific effect on the percentage of dry mater digestibility (DMD).

An increase in algal concentration increased pH (Table 7, $P < 0.021$). The incorporation of microalgae at a 1% DM resulted in a 6% increase in total gas production compared to the control (Table 7, $P = 0.002$). A dosage effect was evident on CH₄ production (presented as %). The inclusion of microalgae at 1% (DM) reduced CH₄ % by 8.72% (Table 7, $P < 0.001$). There was a tendency for microalgae inclusion at 1% and 5% to reduce CH₄ mL/g DM by 3.12% and 4.25%, respectively (Table 7, $P = 0.08$). Additionally, DMD increased with increasing concentrations of microalgae (Table 7, $P < 0.001$).

Table 7: The effect of incubating microalgae species at various concentrations on in vitro fermentation characteristics in a basal diet consisting of Hay and Barley Spent Grain.

	pH	Total gas (mL/gDM)	CH ₄ %	CH ₄ mL/gDM	DMD %
Species					
4	6.44d	77.5188	12.00cd	9.24bcd	45.99
12	6.47cd	76.7	12.65abcd	9.67ab	45.62
20	6.50abc	74.42	11.87d	9.12bcd	45.27
21	6.50abc	74.31	12.45abcd	9.23bcd	44.79

17	6.51abc	72.21	12.18bcd	8.81cd	44.21
22	6.49bcd	76.37	12.74abc	9.50bcd	45.34
23	6.50abc	76.55	12.87ab	9.82ab	44.82
24	6.47cd	77.39	13.11a	10.38a	45.53
25	6.51abc	74.15	12.51abcd	9.25bcd	43.52
19	6.50bc	75.06	12.59abc	9.43bc	44.6
7	6.54a	75.55	13.01a	9.89ab	44.1
26	6.53ab	72.5	12.00cd	8.72d	45.07
10	6.54ab	71.59	12.96ab	9.29bcd	43.94
SEM	0.018	1.642	0.291	0.274	0.881
Conc.					
0 %	6.49b	72.80b	13.31a	9.65a	42.83c
1 %	6.50ab	77.17a	12.15b	9.35ab	45.13b
5 %	6.51a	74.88b	12.14b	9.24b	46.53a
SEM	0.011	0.826	0.146	0.132	0.467
P-Values					
Species	0.003	0.19	0.028	0.005	0.738
Conc.	0.021	0.002	<0.001	0.088	<0.001
Species x Conc	0.313	0.83	0.22	0.121	0.296

Abbrev; SEM; standard error of the mean, CH₄; methane, IVDMD; *In vitro* dry matter digestibility, Conc; concentration.

4.2 RUSITEC

4.2.1 Round 1 – Freshwater Microalgal species

The analysis of data obtained from *in vitro* batch cultures aimed to identify the optimal concentration and species of microalgae for utilisation in RUSITEC fermentation. This determination was based on finding concentrations that reduced CH₄ production while maintaining other fermentation parameters. Microalgae selection criteria included their ability to reduce CH₄ emissions, minimal impact on total gas production and *in vitro* dry matter digestibility (IVDMD), increased percentage of propionate, and desirable cultivation attributes such as high yields (dry matter). Emphasis was placed on cultivation attributes and production yield only when they did not significantly affect CH₄ production.

Freshwater Species 10 was used for the first round of RUSITEC. The *in vitro* fermentation utilising the RUSITEC apparatus revealed addition of Species 10 at 0.5% and 1% concentration (on DM basis) with 10% BSG had no effect ($P \geq 0.61$) on any fermentation parameters (Table 8).

Table 8: The effect of freshwater (Species 10) on fermentation parameters utilising a RUSITEC apparatus.

	Species 10, % of DM			SEM	P-values		
	0	0.50	1		Treat.	Day	Day × Treat.
Effluent, mL/d	572.28	580.93	576.80	22.520	0.983	0.065	0.496
Gas, mL/d	948.97	1058.23	1058.41	106.750	0.714	0.015	0.923
CH ₄ , %	4.55	4.48	4.21	0.254	0.610	0.027	0.991
CH ₄ , mL/d	43.17	46.92	43.82	0.254	0.779	0.001	0.838
DMD, %	42.22	41.67	42.87	1.971	0.911	<.0001	0.908
pH	7.04	7.03	7.02	0.030	0.950	<.0001	1.000

VFA yield, mmol/d							
Total VFA	9.34	9.25	9.21	1.007	0.995	0.125	0.570
Acetate (A)	4.82	4.71	4.71	0.513	0.985	0.249	0.568
Propionate (P)	2.42	2.39	2.36	0.242	0.982	0.076	0.610
Butyrate	0.90	0.92	0.93	0.121	0.981	0.258	0.631
BCVFA	0.49	0.50	0.50	0.047	0.983	<.0001	0.498
Ratio A:P	1.99	1.97	2.00	0.028	0.715	<.0001	0.338

Abbrev; SEM; standard error of the mean, Conc., concentration, VFA; Volatile fatty acids, BCVFA, Branched chain volatile fatty acids, Ratio A:P, Acetate to propionate ratio, Treat; treatment (Species 10, % of DM).

Supplementation of the selected microalga (Species 10) at either 0.5% or 1% (DM) did not significantly affect the most abundant taxa of the rumen microbial community for both solid-associated microbes (SAM) (Table 9, $P > 0.05$) and liquid associated microbes (LAM) (Table 10, $P > 0.05$). The microbial richness and diversity of both SAM (Table 11, $P > 0.05$) and LAM samples (Table 12, $P > 0.05$) were not significantly affected by Species 10 inclusion at either 0.5% or 1% (DM).

The Bray-Curtis dissimilarity analysis indicated no significant variance in bacterial community structure between treatments for both SAM and LAM samples ($P < 0.05$, as shown in Supplementary Figure S4 and Figure S5, respectively). However, at the genus level, *Selenomonas* was found to be differentially abundant between treatments in SAM samples ($P = 0.035$, LDA = 2.52, refer to Supplementary Table S21). In LAM samples, only *Methanimicrococcus* exhibited differential abundance between treatments at the genus level ($P = 0.029$, LDA = 2.11, refer to Supplementary Table S22).

Table 9: Effect of 0.5% or 1% of freshwater Species 10 on top ten taxa’s relative abundance in SAM samples on days 5, 10 and 15 of the RUSITEC experiment, with a mixed hay and BSG diet.

Genus	Species 10, % of DM				Day				P-values		
	0	0.5	1	SEM	5	10	15	SEM	Treat.	Day	Treat. x Day
Fibrobacter	6.34	5.85	6.93	0.667	9.12a	5.11b	4.90b	0.667	0.52	<.0001	0.95
Lactobacillus	11.60	10.23	10.65	1.194	12.44	9.58	10.46	1.19	0.71	0.23	0.97
Megasphaera	14.87	14.78	13.71	0.913	15.24	14.15	13.97	0.91	0.61	0.57	0.96
Not_Assigned	4.14	4.23	4.26	0.310	3.80	4.54	4.28	0.31	0.96	0.23	0.97
Others	20.81	21.80	20.91	0.958	22.43	21.63	19.46	0.96	0.72	0.09	0.98
Prevotella_1	5.43	5.12	4.64	0.673	4.75	5.47	4.97	0.67	0.71	0.74	0.91
Prevotella_7	11.42	12.59	12.98	1.193	4.84b	15.41a	16.74a	1.19	0.63	<0.001	0.99
Prevotellaceae_YAB2003_group	4.29	4.71	4.80	0.851	4.04	4.87	4.89	0.851	0.91	0.72	1.00
Rikenellaceae_RC9_gut_group	3.94	3.50	3.62	0.460	3.60	4.10	3.35	0.460	0.78	0.51	0.98
Streptococcus	8.11	7.38	6.94	0.804	7.28	6.29	8.85	0.804	0.58	0.09	0.86
Treponema_2	9.05	9.81	10.57	1.186	12.48a	8.84b	8.11b	1.186	0.67	0.03	1.00

Abbrev; SEM; standard error of the mean, Treat; treatment (Species 10, % of DM).

Table 10: Effect of 0.5% or 1% of freshwater Species 10 on top ten taxa’s relative abundance in LAM samples on day 15 of the RUSITEC experiment, with a mixed hay and BSG diet.

Species 10, % of DM

Genus	0	0.5	1	SEM	P-value
Christensenellaceae_R_7_group	3.96	3.78	4.19	0.507	0.85
Fibrobacter	2.35	2.31	2.07	0.723	0.96
Flexilinea	2.43	1.52	2.61	1.027	0.73
Not_Assigned	27.58	29.55	25.30	2.873	0.59
Others	26.00	23.48	28.31	1.535	0.12
Prevotella_1	4.44	4.84	5.79	1.447	0.80
Prevotellaceae_UCG_004	2.64	4.01	3.84	0.890	0.51
Rikenellaceae_RC9_gut_group	22.22	22.82	19.49	2.636	0.64
Ruminococcaceae_NK4A214_group	2.44	2.53	2.86	0.246	0.47
Streptococcus	2.79	2.66	2.92	0.891	0.98
Treponema_2	3.15	2.51	2.63	0.447	0.57

Abbrev; SEM; standard error of the mean, Treat; treatment (Species 10, % of DM).

Table 11: Effect of freshwater species (10) on SAM bacterial and archaeal richness and diversity in a RUSITEC system, with a mixed hay and BSG diet.

Indices	Species 10, % of DM				Day				P-values		
	0	0.5	1	SEM	5	10	15	SEM	Treat.	Day	Treat. x Day
No. ASVs	103.94	99.89	101.78	1.977	102.06	104.00	99.56	1.571	0.405	0.070	0.613
Simpson	1.10	1.10	1.10	0.007	1.10	1.10	1.11	0.005	0.895	0.470	0.946
Shannon	2.90	2.91	2.90	0.041	2.93	2.93	2.86	0.039	0.987	0.308	0.997
Fisher	11.52	11.17	11.29	0.234	11.40	11.47	11.10	0.184	0.599	0.210	0.638

Abbrev; SEM; standard error of the mean, Treat; treatment (Species 10, % of DM), No; number, ASV; amplicon sequence variant.

Table 12: Effect of freshwater Species 10 on LAM bacterial and archaeal richness and diversity in a RUSITEC system, with a mixed hay and BSG diet.

Indices	Species 10, % of DM				P-values	
	0	0.5	1	SEM	Treat.	
No. ASVs	118.83	114.67	115.67	4.082	0.762	
Simpson	1.11	1.11	1.09	0.014	0.385	
Shannon	3.23	3.17	3.32	0.067	0.307	
Fisher	14.66	14.66	14.87	0.408	0.920	

Abbrev; SEM; standard error of the mean, Treat; treatment (algae concentration within the diet), No; number, ASV; amplicon sequence variant.

4.2.2 Round 2 – Marine Microalgal species

The *in vitro* fermentation utilising the RUSITEC apparatus revealed addition of the selected marine microalgae at 0.5% and 1% concentration (on DM basis) did not affect gas or effluent production, pH, or DMD% (Table 13, $P > 0.05$). Hence, the marine microalgae included at 0.5% or 1% in the diet did not negatively affect fermentation. Our study demonstrated the inclusion of marine species 17 at 1% increased the percentage and yield (mmol/d) of caproate ($P = 0.042$, $P = 0.041$, respectively), with no effect on total VFAs. Methane production (presented as CH₄ % and CH₄ mL/d) numerically decreased with increasing concentration of microalgae inclusions; however, this was not deemed statistically significant (Table 13). Consequently, these results support the marine species of microalgae did not inhibit fermentation.

Table 13: The effect of marine microalgae species (17) on fermentation utilising a RUSITEC apparatus

	Marine Species 17, % of DM				P-values		
	0	0.50	1	SEM	Treat.	Day	Day × Treat.
pH	6.98	6.99	7.01	0.013	0.229	0.012	0.390
Effluent, mL/d	425.25	423.10	434.09	27.90	0.957	0.005	0.996
Gas, mL/d	634.89	662.78	687.25	47.07	0.739	0.004	0.677
CH ₄ , %	4.45	4.33	3.84	0.265	0.251	<.0001	0.910
CH ₄ , mL/d	28.35	28.48	26.70	2.791	0.882	0.0002	0.216
DMD, %	33.94	34.40	33.46	0.733	0.662	0.182	0.391
Total VFA	34.91	33.95	32.22	1.375	0.386	0.078	0.751
% of Total VFAs							
Acetate	57.03	57.52	56.77	0.372	0.377	0.010	0.086
Propionate	28.97	28.34	29.13	0.479	0.475	0.633	0.120
Butyrate	6.94	7.57	7.14	0.305	0.350	0.156	0.645
Valeric	2.93	2.63	2.73	0.206	0.595	0.046	0.005
Caproate	0.37b	0.40ab	0.51a	0.039	0.042	0.375	0.307
BCVFA	3.75	3.47	3.60	0.193	0.601	0.018	0.006
Ratio A:P	1.97	2.04	1.95	0.041	0.325	0.366	0.831
Total VFA yield (mmol/d)	14.44	14.02	13.76	0.557	0.690	0.336	0.692
Acetate	8.22	8.06	7.84	0.332	0.719	0.205	0.624
Propionate	4.20	3.97	4.02	0.172	0.617	0.210	0.453
Butyrate	0.99	1.05	0.98	0.040	0.376	0.041	0.936
Valeric	0.41	0.36	0.37	0.031	0.527	0.277	0.217
Caproate	0.05b	0.05b	0.07a	0.005	0.041	0.062	0.598
BCVFA	0.526	0.478	0.489	0.032	0.539	0.139	0.313

Abbrev; SEM; standard error of the mean, Conc., concentration, VFA; Volatile fatty acids, BCVFA, Branched chain volatile fatty acids, Ratio A:P, Acetate to propionate ratio, Treat; Treatment (Marine Species 17, % of DM).

4.2.2.1 Effect of Species 17 on the Rumen Bacterial and Archaeal Microbiota

Supplementation of Species 17 at either 0.5% or 1% (DM) did not affect the most abundant genera in the rumen microbial community for solid-associated microbes (SAM) ($P \geq 0.39$) and liquid-associated microbes (LAM) ($P \geq 0.27$). Additionally, microbial richness and diversity in both SAM ($P \geq 0.83$) and LAM ($P \geq 0.25$) samples were unaffected by treatment. Bray-Curtis dissimilarities analysis showed no significant differences in bacterial community structure between treatments for both SAM ($P = 0.85$) and LAM ($P = 0.69$) samples (see Figure 1).

Table 14: Effect of 0.5% or 1% of Species 17 on top ten bacterial and archaeal taxa's relative abundance in solid-associated microbe (SAM) samples on days 5, 10 and 15 of the RUSITEC experiment, with a mixed hay and BSG diet.

Genus	Marine Species 17, % of DM				Day			P-values			
	0	0.5	1	SEM	5	10	15	SEM	Treat.	Day	Treat. x Day
Prevotella_1	22.31	21.11	23.79	2.00	28.45a	19.31b	19.46b	2.00	0.641	0.003	0.837
Fibrobacter	18.05	18.38	16.44	1.615	13.18b	19.14a	20.56a	1.615	0.664	0.005	0.891
Others	15.60	15.54	16.58	0.546	15.87	16.53	15.31	0.546	1.150	1.250	0.260
Treponema_2	9.03	8.17	7.86	0.716	7.34	8.48	9.24	0.716	0.492	0.181	0.892
Prevotellaceae_YAB2003	8.22	8.42	9.53	1.237	6.80	10.43	8.94	1.237	0.727	0.125	0.588

_group											
Lactobacillus	7.70	8.49	6.86	1.204	7.88	7.49	7.69	1.204	0.634	0.975	0.542
Megasphaera	5.75	6.35	3.82	1.364	6.06	3.96	5.89	1.364	0.398	0.487	0.649
Oribacterium	5.22	5.45	5.84	0.338	6.01a	5.71ab	4.80b	0.338	0.429	0.039	0.654
Rikenellaceae_RC9_gut_g	3.40	3.08	3.57	0.327	3.49	3.75	2.80	0.327	0.565	0.112	0.859
roup											
Streptococcus	3.25	3.66	4.29	0.389	3.29	3.70	4.21	0.389	0.171	0.259	0.893
Pseudobutyrvibrio	1.47	1.34	1.30	0.192	1.52	1.50	1.10	0.192	0.822	0.234	0.961

Abbrev; SEM; standard error of the mean, Treat; treatment (Marine Species 17, % of DM).

Table 15: Effect of 0.5% or 1% of Species 17 on top ten bacterial and archaeal taxa’s relative abundance in liquid-associated microbe (LAM) samples on day 15 of the RUSITEC experiment, with a mixed hay and BSG diet.

Genus	Marine Species 17, % of DM			SEM	P-value
	0	0.5	1		
Others	29.34	29.78	33.61	1.603	0.150
Prevotella_1	17.66	15.31	15.17	2.629	0.757
Rikenellaceae_RC9_gut_group	13.84	16.95	17.39	1.740	0.319
Fibrobacter	9.21	10.21	7.92	1.678	0.634
Treponema_2	6.97	6.64	5.56	0.675	0.329
Prevotellaceae_YAB2003_group	5.36	4.28	2.52	1.136	0.268
Christensenellaceae_R_7_group	4.71	5.67	7.30	1.570	0.514
Streptococcus	3.91	2.92	3.32	0.448	0.308
Oribacterium	3.49	3.09	2.71	0.590	0.658
Lactobacillus	3.26	2.44	2.31	0.528	0.412
Campylobacter	2.25	2.72	2.27	0.479	0.738

Abbrev; SEM; standard error of the mean, Treat; treatment (Marine Species 17, % of DM).

Table 16: Effect of species 17 on bacterial and archaeal solid-associated microbes (SAM) richness and diversity in a RUSITEC system, with a mixed hay and BSG diet.

Indices	Marine Species 17, % of DM				Day				P-values		
	0	0.5	1	SEM	5	10	15	SEM	Treat.	Day	Treat. × Day
No. ASVs	79.5	80.7	79.72	1.51	74.89a	82.33b	82.72b	1.51	0.831	0.001	0.396
Simpson	0.86	0.87	0.86	0.010	0.85	0.88	0.87	0.010	0.971	0.081	0.924
Shannon	2.59	2.60	2.60	0.048	2.54	2.66	2.58	0.048	0.985	0.197	0.918
Fisher	9.29	9.34	9.24	0.171	8.73a	9.53b	9.62b	0.171	0.932	0.001	0.449

Abbrev; No. ASV; number of amplicon sequence variants, SEM; standard error of the mean, Treat; treatment (Marine Species 17, % of DM).

Table 17: Effect of species 17 on bacterial and archaeal liquid-associated microbes (LAM) richness and diversity in a RUSITEC system, with a mixed hay and BSG diet.

Indices	Marine Species 17, % of DM			SEM	P-values
	0	0.5	1		
					Treat.

No. ASVs	94	92.17	100.5	3.569	0.254
Simpson	0.91	0.91	0.92	0.007	0.982
Shannon	3.13	3.13	3.19	0.06	0.700
Fisher	12.37	12.36	13.3	0.434	0.250

Abbrev; No. ASV; number of amplicon sequence variants, SEM; standard error of the mean, Treat; treatment (Marine Species 17, % of DM).

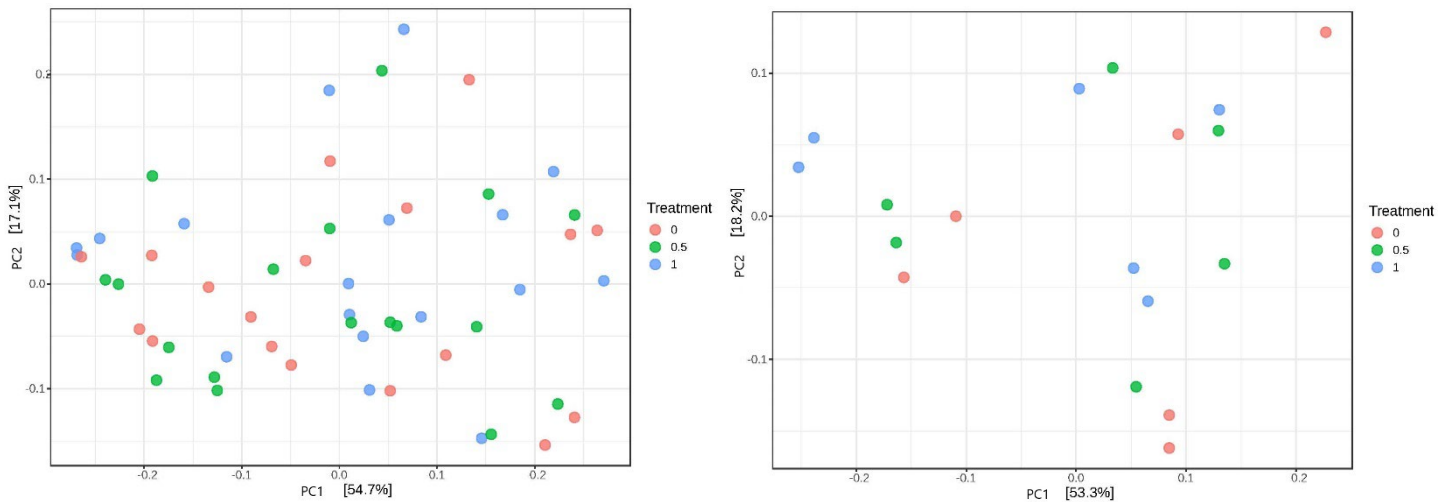


Figure 1: Principal coordinates plot (PCoA) of the Bray-Curtis dissimilarities for the bacterial solid-associated microbe (SAM) samples (Left) and for the bacterial liquid-associated microbe (LAM) samples (Right) by microalgae concentration. The percentage of variation explained by each principal coordinate are indicated on the axes.

4.2.2.2 Effect of Species 17 on the Rumen Fungal Microbiota

Supplementation of Species 17 at either 0.5% or 1% (DM) did not significantly affect the most abundant genera in the rumen fungal community for solid-associated microbes (SAM) ($P > 0.05$) and liquid-associated microbes (LAM) ($P \geq 0.45$). However, there were tendencies indicating that the inclusion of microalgae at 0.5% increased the abundance of solid-associated *Vishniacozyma* ($P = 0.082$) and *Sterigmatomyces* ($P = 0.061$) by 63.2% and 73.3%, respectively (Table 18). Treatment did not impact the microbial richness and diversity of both fungal SAM ($P \geq 0.67$) and LAM samples ($P \geq 0.96$). Bray-Curtis dissimilarities analysis showed no significant variance in community structure between treatments for both SAM ($P = 0.833$) and LAM samples ($P > 0.94$) (see Figure 2).

Table 18: Effect of 0.5% or 1% of Species 17 on top ten fungal taxa's relative abundance in solid-associated microbe (SAM) samples on days 5, 10 and 15 of the RUSITEC experiment, with a mixed hay and BSG diet.

Genus	Marine Species 17, % of DM				Day				P-values		
	0	0.5	1	SEM	5	10	15	SEM	Treat	Day	Treat x Day
Caecomyces	18.20	19.72	19.74	2.110	27.98a	13.44b	16.24b	2.110	0.851	<.001	0.976
Pecoramyces	44.70	35.81	38.67	4.743	34.31	38.74	46.13	4.743	0.408	0.216	0.878
Wallemia	19.94	25.24	22.84	3.250	22.81ab	29.47a	15.74b	3.250	0.518	0.017	0.697
Neocallimastix	8.24	9.61	9.01	2.492	3.27b	6.70b	16.89b	2.492	0.927	<.001	0.629
Wallemiaceae_gen_Incertae_sedis	5.04	6.61	6.34	1.146	4.84b	9.29a	3.86b	1.146	0.587	0.004	0.510
Orpinomyces	1.80	0.84	0.90	0.561	3.51a	0.00b	0.02b	0.561	0.404	<.001	0.494
Others	1.22	1.18	1.22	0.184	1.58a	1.42a	0.67b	0.184	0.985	<.001	0.526

Neocallimastigomyces_gen_Incertae_sedis	0.35	0.24	0.57	0.173	1.05a	0.11b	0.003b	0.173	0.396	<.001	0.211
Vishniacozyma	0.19	0.31	0.19	0.044	0.23ab	0.32a	0.15b	0.044	0.082	0.031	0.410
Filobasidium	0.17	0.17	0.26	0.047	0.21ab	0.30a	0.09b	0.047	0.287	0.013	0.332
Sterigmatomyces	0.15	0.26	0.26	0.036	0.21	0.21	0.25	0.036	0.061	0.612	0.885

Abbrev; SEM; standard error of the mean, Treat; treatment (Marine Species 17, % of DM).

Table 19: Effect of 0.5% or 1% of Species 17 on top ten fungal taxa's relative abundance in solid-associated microbe (SAM) samples on days 5, 10 and 15 of the RUSITEC experiment, with a mixed hay and BSG diet.

Genus	Marine Species 17, % of DM				P-value
	0	0.5	1	SEM	Treat.
Pecoramyces	62.35	53.45	58.86	8.06	0.739
Caecomyces	9.84	14.43	11.35	2.92	0.541
Neocallimastix	9.34	16.33	10.27	6.36	0.706
Wallemia	8.60	8.09	10.67	3.08	0.824
Others	4.58	2.64	3.30	1.09	0.456
Hannaella	1.09	0.87	0.98	0.32	0.893
Wallemiaceae_gen_Incertae_sedis	1.75	1.97	2.04	0.40	0.870
Preussia	0.75	0.77	0.82	0.15	0.952
Rhodotorula	0.70	0.67	0.66	0.21	0.993
Nigrospora	0.60	0.46	0.59	0.22	0.868
Cystobasidium	0.40	0.33	0.46	0.14	0.799

Abbrev; SEM; standard error of the mean, Treat; treatment (Marine Species 17, % of DM).

Table 20: Effect of species 17 on fungal solid-associated microbes (SAM) richness and diversity in a RUSITEC system, with a mixed hay and BSG diet.

Indices	Marine Species 17, % of DM				Day				P-values		
	0	0.5	1	SEM	5	10	15	SEM	Treat	Day	Treat × Day
No. ASVs	17.22	16.11	17.06	0.955	19.28	17.44	13.67	0.955	0.677	0.001	0.418
Simpson	0.65	0.66	0.66	0.023	0.68	0.65	0.63	0.023	0.970	0.310	0.955
Shannon	1.36	1.36	1.36	0.056	1.45	1.35	1.28	0.056	0.998	0.117	0.936
Fisher	2.31	2.15	2.29	0.146	2.64	2.34	1.77	0.146	0.678	0.001	0.415

Abbrev; No. ASV; number of amplicon sequence variants, SEM; standard error of the mean, Treat; treatment (Marine Species 17, % of DM).

Table 21: Effect of species 17 on bacterial and archaeal liquid-associated microbes (LAM) richness and diversity in a RUSITEC system, with a mixed hay and BSG diet.

Indices	Marine Species 17, % of DM				P-values
	0	0.5	1	SEM	Treat.
No. ASVs	22.33	22.83	22.67	1.988	0.984
Simpson	0.55	0.55	0.57	0.066	0.976

Shannon	1.34	1.28	1.32	0.164	0.964
Fisher	3.22	3.28	3.26	0.328	0.99

Abbrev; No. ASV; number of amplicon sequence variants, SEM; standard error of the mean, Treat; treatment (Marine Species 17, % of DM).

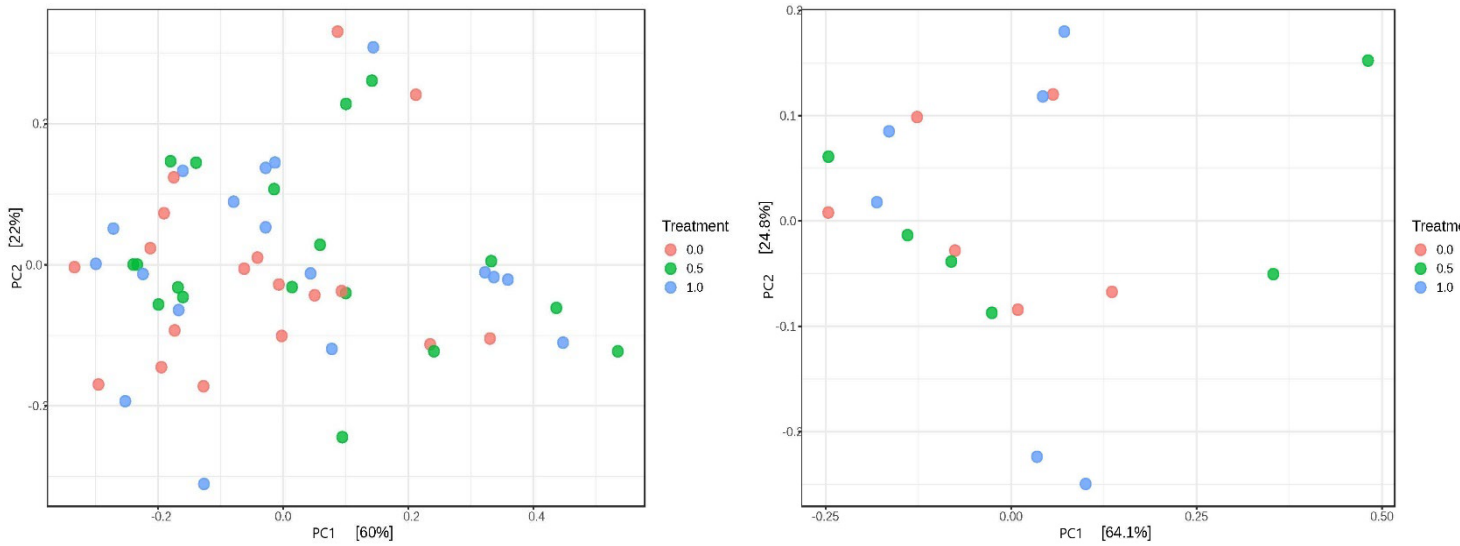


Figure 2: Principal coordinates plot (PCoA) of the Bray-Curtis dissimilarities for the fungal solid-associated microbe (SAM) samples (Left) and for fungal liquid-associated microbe (LAM) samples (Right) by microalgae concentration. The percentage of variation explained by each principal coordinate are indicated on the axes.

4.3 Lamb Trial

4.3.1 Animal Performance

There were no significant species × concentration interactions observed for any of the performance parameters tested; therefore, the results are presented as single effects. The inclusion of fresh or marine microalgae showed no effect on the measured performance parameters (Table 22, $P > 0.05$).

Table 22: Effect of species and concentration on sheep performance parameters.

Parameter	Species		Dosage, %		SEM	P-Value		
	Fresh	Marine	0	1		Species	Dosage	Treatment
Final Live Weight, kg	48.16	48.34	48.43	48.07	0.5	0.810	0.620	0.810
Pellet Intake, g/day	705.93	709.23	710.83	704.33	8.05	0.770	0.570	0.770
Hay Intake, g/day	391.25	389.05	386.72	393.58	14.17	0.910	0.730	0.910
DMI, g/day	1097.1	1098.2	1097.47	1097.83	14.36	0.960	0.990	0.960
ADG, g/day	160.93	156.46	159.34	158.14	5.33	0.560	0.870	0.560
FCE, kg intake/kg gain	6.95	7.17	6.96	7.15	0.22	0.470	0.530	0.470

Abbrev: DMI; Dry matter Intake, ADG; Average daily gain, FCE: Feed conversion efficiency (kg intake/ kg average daily gain).

4.3.2 Carcass and Meat Quality

There were no significant species × concentration interactions observed for any of the carcass or meat quality parameters tested; therefore, the results are presented as single effects. The inclusion of either fresh or marine microalgae had no effect on the tested carcass quality parameters (Table 23, $P > 0.05$). Additionally, no dosage effect was observed for carcass quality characteristics, 0% or 1% microalgae inclusion resulted in similar values (Table 23, $P > 0.05$). Liver weight measurements were obtained to provide preliminary insights into microalgae's effect on lipid metabolism within the liver. However, microalgae inclusion, regardless of species, had no effect on liver weights (Table 23, $P > 0.05$). Ultimate pH was not significantly affected for any of the muscle types tested (Table 23, $P > 0.05$). Due to a technical malfunction with the cook loss equipment, only drip loss was able to be obtained. The inclusion of either type of microalgae (fresh vs marine) at 1% had no effect on drip loss percentage for each muscle. Additionally, colour parameters (L^* , a^* , b^* , hue, and chroma) were not affected by the inclusion of either fresh or marine microalgae at 1% (Table 23, $P > 0.05$).

Table 23: Effect of species and concentration on carcass and meat quality

Parameter	Species		Dosage, %		SEM	P-Value		
	Fresh	Marine	0	1		Species	Dosage	Treatment
Carcass Characteristics								
Hot carcass weight, kg	23.29	23.54	23.33	23.50	0.24	0.463	0.616	0.463
Cold carcass weight, kg	22.66	22.96	22.76	22.87	0.24	0.388	0.759	0.388
Liver weight, g	753.49	738.88	745.33	747.03	17.12	0.549	0.945	0.549
Cold dressing percentage, %	47.14	47.49	47.00	47.63	0.44	0.577	0.323	0.577
<i>Longissimus lumborum</i> (LL)								
Drip Loss (%)	3.44	3.29	3.27	3.46	0.29	0.706	0.644	0.706
Ultimate pH (U)	5.61	5.61	5.60	5.61	0.04	0.934	0.852	0.934
Lightness (L^*)	29.07	28.51	28.96	28.62	0.51	0.435	0.636	0.435
Redness (a^*)	12.34	12.27	12.10	12.50	0.38	0.902	0.467	0.902
Yellowness (b^*)	4.81	4.75	4.74	4.82	0.28	0.873	0.848	0.873
Hue	20.82	20.54	20.60	20.76	0.64	0.755	0.856	0.755
Chroma	13.34	13.26	13.20	13.41	0.46	0.900	0.746	0.900
<i>Biceps femoris</i> (BF)								
Drip Loss (%)	5.11	4.76	4.70	5.17	0.34	0.461	0.333	0.461
Ultimate pH (U)	5.56	5.56	5.58	5.55	0.04	0.956	0.545	0.956
Lightness (L^*)	28.06	27.50	27.73	27.83	0.52	0.896	0.445	0.445
Redness (a^*)	12.98	12.96	12.72	13.22	0.37	0.968	0.339	0.968
Yellowness (b^*)	4.99	4.98	4.85	5.12	0.26	0.970	0.479	0.970
Hue	20.69	20.66	20.43	20.92	0.57	0.972	0.547	0.972
Chroma	13.92	13.89	13.62	14.19	0.44	0.965	0.361	0.965
<i>Semimebranosus</i> (SM)								
Drip Loss (%)	3.55	3.12	3.17	3.50	0.21	0.156	0.274	0.156
Ultimate pH (U)	5.58	5.58	5.59	5.57	0.03	0.969	0.598	0.969
Lightness (L^*)	29.86	29.81	29.64	30.03	0.41	0.920	0.502	0.920
Redness (a^*)	13.41	13.30	13.29	13.42	0.31	0.763	0.801	0.801
Yellowness (b^*)	5.16	5.22	5.11	5.27	0.25	0.875	0.632	0.875
Hue	20.80	21.11	20.63	21.28	0.57	0.429	0.703	0.703
Chroma	14.38	14.30	14.26	14.43	0.37	0.879	0.741	0.879

Abbrev: SEM; Standard error of the means.

4.3.3 Fatty Acids Profiling

4.3.3.1 Treatments

There was no significant effect observed for the species x dosage interaction (treatment), results are presented as single effects. A dosage effect was observed with inclusion of either fresh or marine microalgae inclusion at 1% DM decreasing the concentration of SFAs 12:0, 22:0 and 23:0, MUFAs, t9-18:1 and n9-24:1 (Table 24, $P \leq 0.05$). There was no difference between treatments in the total concentration of any of the major fatty acid groups including SFA, MUFA and PUFA.

Table 24: Fatty acid profile of treatment pellets.

Fatty acid, mg/100g	Species		Dosage		SEM	P-value		
	Fresh	Marine	0	1%		Species	Dosage	Treatment
Σ SFA	625.18	607.19	654.60	577.77	25.09	0.640	0.100	0.640
12:0	1.76	1.85	2.38	1.24	0.25	0.807	0.034	0.807
14:0	6.56	6.97	7.06	6.46	0.60	0.654	0.516	0.654
15:0	2.42	2.40	2.58	2.24	0.19	0.934	0.284	0.934
16:0	409.91	396.91	432.49	374.32	20.06	0.671	0.110	0.671
17:0	4.11	3.72	3.97	3.85	0.27	0.361	0.761	0.361
18:0	80.58	77.43	86.04	71.96	4.40	0.639	0.086	0.639
19:0	106.70	105.18	106.20	105.69	1.65	0.550	0.837	0.550
21:0	1.49	1.55	1.39	1.65	0.09	0.633	0.108	0.633
22:0	10.18	9.83	10.94	9.07	0.36	0.527	0.021	0.527
23:0	1.47469	1.35942	1.54311	1.291	0.06	0.267	0.048	0.267
Σ MUFA	405.81	381.87	433.87	353.81	26.04	0.550	0.100	0.550
c9-16:1	11.12	14.43	8.02	17.53	2.09	0.032	0.325	0.325
c9-18:1	318.42	299.23	343.03	274.61	19.69	0.529	0.070	0.529
t9-18:1	63.94	56.74	69.88	50.80	3.96	0.268	0.027	0.268
n9-20:1	8.70	8.29	8.87	8.12	0.39	0.504	0.245	0.504
n9-22:1	1.84	1.61	1.98	1.46	0.17	0.388	0.096	0.388
n9-24:1	1.79	1.57	2.07	1.29	0.17	0.410	0.034	0.410
Σ PUFA	488.24	469.79	537.17	420.86	52.93	0.820	0.200	0.820
t9, t12-18:2	488.24	469.79	537.17	420.86	52.93	0.820	0.200	0.820

Abbrev: Σ SFA; sum of saturated fatty acids (12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 21:0, 22:0, 23:0), Σ MUFA: sum of monosaturated fatty acids (c9-16:1, c9-18:1, t9-18:1, n9-20:1, n9-22:1, n9-24:1), Σ PUFA; sum of polyunsaturated fatty acids (t9, t12-18:2), SEM; standard error of the mean.

4.3.3.2 Longissimus Lumborum Muscle

There was no significant effect observed for the species x dosage interaction (treatment), results are presented as single effects. The inclusion of fresh and marine microalgae did not affect the concentration or composition of SFA, MUFA or PUFAs (Table 25).

Table 25: Fatty acid profile (mg/100g) of the *Longissimus lumborum* muscle of algae fed and control lambs.

Fatty acid, mg/100g	<i>M. longissimus lumborum</i>				SEM	P-value		
	Fresh	Marine	0%	1%		Species	Dosage	Treatment
Σ SFA	2937.86	2917.87	2923.97	2931.76	136.83	0.918	0.968	0.918
10:00	4.34	3.78	3.37	4.75	0.51	0.448	0.064	0.448
12:00	8.82	7.90	8.65	8.07	1.29	0.618	0.749	0.618
13:00	0.61	0.56	0.69	0.48	0.09	0.655	0.096	0.655
14:00	194.64	186.08	199.16	181.56	18.51	0.750	0.510	0.750
15:0 ^a	28.34	28.58	32.01	24.90	66.67	0.715	0.214	0.715
	3.20	3.25	3.32	3.13	0.10			
16:00	1361.48	1354.56	1342.06	1373.99	62.30	0.938	0.719	0.938
17:00	124.48	125.81	135.79	114.51	10.88	0.932	0.174	0.932
18:00	1106.17	1101.81	1094.68	1113.30	46.95	0.948	0.781	0.948
19:00	101.62	101.71	100.46	102.87	0.78	0.941	0.034	0.941
20:00	7.20	6.87	6.91	7.17	0.76	0.758	0.810	0.758
21:00	0.15	0.20	0.18	0.16	0.05	0.498	0.773	0.498
Σ MUFA	1417.91	1411.81	1379.39	1450.33	57.32	0.940	0.387	0.940
n5-14:1	4.23	3.76	4.46	3.53	0.66	0.619	0.325	0.619
c9-16:1	110.58	108.64	111.28	107.95	8.93	0.879	0.793	0.879
c9-18:1	1264.00	1259.80	1227.20	1296.61	49.41	0.952	0.326	0.952
t9-18:1 ^a	34.86	35.06	32.35	37.57	20.72	0.988	0.089	0.988
	3.51	3.51	3.43	3.59	0.06			
n9-20:1	4.23	4.54	4.10	4.67	0.35	0.543	0.261	0.543
Σ PUFA	790.81	811.27	804.58	797.51	23.28	0.540	0.831	0.540
t9, t12-18:2	473.26	491.68	483.77	481.16	14.47	0.373	0.899	0.373
n6-20:4	242.29	243.49	247.66	238.12	10.15	0.934	0.510	0.934
n3-20:5 (EPA)	53.70	53.76	53.49	53.97	3.58	0.990	0.920	0.990
n3-20:3	12.46	13.16	12.86	12.76	0.60	0.412	0.907	0.412
n6-20:2	1.02	1.13	1.17	0.98	0.10	0.440	0.200	0.440
n3-22:6 (DHA)	10.32	10.29	10.08	10.53	0.92	0.981	0.734	0.981
EPA + DHA	61.79	61.82	59.12	64.50	4.10	1.000	0.360	1.000
Σ n-3	74.25	74.98	71.97	77.26	4.17	0.900	0.380	0.900
Σ n-6	243.31	244.62	248.83	239.09	10.19	0.928	0.503	0.928
Σ n-6/ Σ n-3	3.51	3.51	3.76	3.26	0.29	0.985	0.224	0.985

Abbrev: Σ SFA; sum of saturated fatty acids (10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 21:0), Σ MUFA; sum of monosaturated fatty acids (n5-14:1, c9-16:1, c9-18:1, t9-18:1, n9-20:1), Σ PUFA; sum of polyunsaturated fatty acids (t9, t12-18:2, n6-20:4, n3-20:5, n3-20:3, n6-20:2, n3-22:6), EPA; Eicosapentaenoic acid, DHA, docosahexanoic acid; Σ n-3; sum of omega-3 fatty acids (n3-20:5, n3-20:3, n3-22:6), Σ n-6; sum of omega-6 fatty acids (n6-20:4, n6-20:2), Σ n-6/ Σ n-3; ratio of omega-6 to omega-3 fatty acid totals, SEM; standard error of the mean. ^a Data was not normally distributed, log change data presented under treatment means.

4.3.3.3 *Longissimus Lumborum* Subcutaneous Fat

Total SFA were similar among both dosages of algae (Table 26, $P > 0.05$), despite a dosage effect where inclusion

of algae at 1% DM increased concentrations of 10:0, 14:0 and 20:0 (Table 26). Similarly, MUFA were unaffected by the inclusion of microalgae despite an increase in the concentrations of n5-14:1, c9-16:1, and n9-2 (Table 26) at 1% inclusion of algae. A dosage effect was evident with the inclusion in of fresh or marine microalgae at 1% DM increasing the total PUFAs from 852.32 mg/100 g to 975.21 mg/100 g (Table 26, $P = 0.088$). This is due to an increase in the concentration of n6-20:4, n3-20:3 and n6-20:2 ($P \leq 0.05$). This resulted in an increase in the sum of omega-6s ($P = 0.043$) and tendency to increase the sum of Omega-3 ($P = 0.059$). However, the ratio of $\Sigma n-6/\Sigma n-3$ was similar ($P \geq 0.905$) for all LL subcutaneous fat samples, regardless of treatment (Table 26).

Table 26: Fatty acid profile (mg/100g) of the *Longissimus lumborum* subcutaneous fat of algae fed and control lambs.

Fatty acid, mg/100g	<i>S.F. longissimus lumborum</i>				SEM	P-value		
	Fresh	Marine	0%	1%		Species	Dosage	Treatment
ΣSFA	12843.18	12808.59	12477.64	13174.13	511.46	0.962	0.341	0.962
10:0	81.60	81.07	71.71	90.96	5.74	0.948	0.023	0.948
12:0	20.28	19.11	17.30	22.09	23.72	0.960	0.908	0.988
14:0	671.26	680.53	567.55	784.25	43.49	0.881	<0.01	0.881
15:0	184.15	176.26	175.26	185.15	9.60	0.564	0.471	0.564
16:0	5669.65	5654.94	5421.87	5902.72	230.79	0.148	0.964	0.964
17:0	1093.85	1047.14	1121.79	1019.21	58.86	0.578	0.225	0.578
18:0	5045.24	5075.80	5037.11	5083.93	234.36	0.927	0.888	0.927
19:0	61.42	61.30	60.87	61.85	0.62	0.895	0.276	0.895
20:0	49.28	43.94	40.87	52.35	4.06	0.357	0.052	0.357
$\Sigma MUFA$	5069.93	5040.35	5041.96	5068.32	202.57	0.918	0.927	0.918
n5-14:1	11.01	10.76	7.77	14.00	1.34	0.896	<0.01	0.896
n5-15:1 ^a	16.02	12.22	15.14	13.10	40.10	0.900	0.654	0.900
	1.99	2.04	2.10	1.93	0.26			
c9-16:1	340.23	317.57	279.52	378.29	23.14	0.493	<0.01	0.493
c9-18:1	4680.65	4680.21	4722.62	4638.25	191.50	0.999	0.757	0.999
n9-20:1	22.02	19.58	16.92	24.68	1.81	0.348	<0.01	0.348
$\Sigma PUFA$	942.57	884.96	852.32	975.21	49.71	0.417	0.088	0.417
t9, t12-18:2	885.23	834.12	805.28	914.07	46.93	0.446	0.109	0.446
n6-20:4	49.28	43.94	40.87	52.35	4.06	0.357	0.052	0.357
n3-20:3	6.14	5.21	4.80	6.55	0.64	0.307	0.059	0.307
n6-20:2	1.98	1.76	1.49	2.25	0.20	0.454	0.012	0.454
$\Sigma n-3$	6.14	5.21	4.80	6.55	0.64	0.307	0.059	0.307
$\Sigma n-6$	51.20	45.63	42.23	54.59	4.19	0.353	0.043	0.353
$\Sigma n-6/\Sigma n-3$	9.89	9.98	9.85	10.02	0.94	0.944	0.905	0.944

Abbrev: ΣSFA ; sum of saturated fatty acids (10:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0), $\Sigma MUFA$; sum of monosaturated fatty acids (n5-14:1, n5-15:1, c9-16:1, c9-18:1, t9-18:1, n9-20:1), $\Sigma PUFA$; sum of polyunsaturated fatty acids (t9, t12-18:2, n6-20:4, n3-20:3, n6-20:2), $\Sigma n-3$; sum of omega-3 fatty acids (n3-20:3), $\Sigma n-6$; sum of omega-6 fatty acids (n6-20:4, n6-20:2), $\Sigma n-6/\Sigma n-3$; ratio of omega-6 to omega-3 fatty acid totals, S.F.; subcutaneous fat, SEM; standard error of the mean. ^a Data was not normally distributed, log change data presented under treatment means.

4.3.3.4 Liver

Results are reported as single effect. Species of microalgae did significantly affect the concentration or composition of fatty acids in the liver (Table 27). However, a dosage effect was evident with the concentration of 20:0 tending to be higher in liver from samples fed either 1% fresh or marine microalgae (Table 27; $P \geq 0.085$).

Both total SFA and MUFA were similar for all treatments, despite a dosage effect were the concentration of t9-18:1 tended to decrease in the liver of algae fed lambs compared to the control (Table 27; $P = 0.354$). A dosage effect was evident with the liver of lambs fed either algae at 1% having an average of 3346.10mg/100g of PUFAs compared to 2859.10mg/100g compared to the control (Table 27; $P = 0.053$). This is due to an increase in concentration of n6-20:2, n3-20:3, n3-20:5 (EPA) and n3-22:6 (DHA) (Table 27; $P \leq 0.024$). Consequently, the sum of EPA and DHA increased by 202.98mg/100g in the liver algae fed lambs compared to the control (Table 27). Additionally, this resulted in an increase in the $\Sigma n-3$ and lower $\Sigma n-6/\Sigma n-3$ (Table 27; $P \leq 0.01$).

Table 27: Fatty acid profile (mg/100g) of the Longissimus lumborum liver of algae fed and control lambs.

Fatty acid, mg/100g	Liver				SEM	P-value		
	Fresh	Marine	0%	1%		Species	Dosage	Treatment
Σ SFA	3987.97	4164.39	4055.00	4097.36	133.11	0.823	0.354	0.354
10:0	6.67	6.74	7.09	6.32	0.69	0.944	0.442	0.944
13:0	1.14	1.08	1.13	1.09	0.10	0.705	0.799	0.705
14:0	108.37	107.36	104.56	111.17	8.09	0.931	0.567	0.931
15:0	44.08	45.97	45.01	45.04	2.39	0.581	0.993	0.581
16:0	1624.96	1712.84	1660.20	1677.60	65.88	0.351	0.853	0.351
17:0	171.87	172.70	177.18	167.39	8.65	0.946	0.428	0.946
18:0	1925.12	2010.35	1953.49	1981.99	59.93	0.320	0.738	0.320
19:0	101.32	102.58	102.05	101.85	0.78	0.257	0.856	0.257
20:0	4.44	4.77	4.30	4.91	0.25	0.360	0.085	0.360
Σ MUFA	1781.00	1816.66	1829.20	1768.46	87.40	0.774	0.626	0.774
n5-14:1	1.56	1.45	1.44	1.57	0.17	0.677	0.590	0.677
c9-16:1	127.62	125.49	126.14	126.97	8.78	0.865	0.947	0.865
c9-18:1	1364.38	1416.68	1405.17	1375.89	70.24	0.601	0.770	0.601
t9-18:1	276.83	264.04	286.76	254.11	12.56	0.475	0.073	0.475
n9-20:1	10.64	9.06	9.71	10.00	0.77	0.155	0.791	0.155
Σ PUFA	3169.61	3035.88	2859.40	3346.10	172.60	0.587	0.053	0.587
t9, t12-18:2	997.93	1027.52	947.17	1078.28	55.79	0.104	0.709	0.709
n6-18:3	20.53	20.50	18.61	22.42	1.77	0.990	0.137	0.990
n6-20:2	5.35	4.66	4.18	5.83	0.50	0.340	0.024	0.340
n3-20:3	82.78	77.36	71.20	88.94	4.72	0.421	0.011	0.421
n6-20:4	1351.37	1245.39	1233.68	1363.09	78.51	0.345	0.250	0.345
n3-20:5 (EPA)	287.86	294.97	258.07	324.76	18.28	0.785	0.013	0.785
n3-22:6 (DHA)	423.80	365.47	326.48	462.79	31.64	0.199	<0.01	0.199
EPA + DHA	711.66	660.44	584.56	787.54	46.31	0.439	<0.01	0.439
$\Sigma n-3$	794.44	737.80	655.75	876.49	50.18	0.429	<0.01	0.429
$\Sigma n-6$	1377.25	1270.56	1256.47	1391.34	79.92	0.351	0.239	0.351
$\Sigma n-6/\Sigma n-3$	1.78	1.76	1.92	1.63	0.04	0.682	<0.01	0.682

Abbrev: Σ SFA; sum of saturated fatty acids (10:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0), Σ MUFA; sum of monosaturated fatty acids (n5-14:1, c9-16:1, c9-18:1, t9-18:1, n9-20:1), Σ PUFA; sum of polyunsaturated fatty acids (t9, t12-18:2, n6-18:3, n6-20:2, n3-20:3, n6-20:4, n3-20:5, n3-22:6), EPA; Eicosapentaenoic acid, DHA, docosahexanoic acid; $\Sigma n-3$; sum of omega-3 fatty acids (n3-20:3, n3-20:5, n3-22:6), $\Sigma n-6$; sum of omega-6 fatty acids (n6-18:3, n6-20:2, n6-20:4), $\Sigma n-6/\Sigma n-3$; ratio of omega-6 to omega-3 fatty acid totals, SEM; standard error of the mean.

4.3.4 Sensory Analysis

Results of the triangles tests indicate that there was a perceivable difference between the control sample and samples from the marine algae treatment (Table 28). There was no perceivable difference between the control and the freshwater algae treatment (Table 28). Flavor and texture were the primary reasons for consumer choice in tests three and four (Table 29).

Table 28: Triangle test responses (total number of responses n=42).

Test	Responses		
	Correct	Incorrect	P-Value
Test 1 (Control vs. Fresh)	17	25	0.205
Test 2 (Control vs. Marine)	30	12	0.001

Table 29: Percentage (%) of consumers who indicated flavour, aroma or other as the reason for their choice in the triangle test, as well as the percentage who could not detect a difference and indicated 'guess' as their response.

Test	Flavor (%)	Aroma (%)	Other (%)	Guess (%)
Test 1 (Control vs. Fresh)	42.68	11.9	38.1	7.14
Test 2 (Control vs. Marine)	38.1	9.52	45.24	7.14

4.4 Rumen microbiome and metabolites of lambs

4.4.1 Ruminal Bacterial and Archaeal Structure and Diversity

Results are reported as single effects, as there was no significant species x dosage interaction on the most abundant bacterial and archaeal at the genus level of the rumen microbial community for solid-associated microbes (SAM) (Table 30; $P > 0.05$). However, there was a dosage effect where the inclusion of either microalgae at 1% increased the relative abundance of *Methanobrevibacter* by 1.7- fold ($P = 0.019$), and a tendency to increase *Olsenella* by 3.03-fold ($P = 0.084$). The microbial richness and diversity of SAM (Table 31, $P > 0.05$) were not affected treatment. Bray-Curtis dis-similarities analysis revealed no variance in the bacterial and archaeal community structure between treatments (Figure 3, $P = 0.270$).

Table 30: Effect including fresh or marine microalgae at 1% in lamb feedlot pellets on top ten bacterial and archaeal genus relative abundance.

	Species		Dosage		SEM	P-values		
	Fresh	Marine	0%	1%		Species	Dosage	Species x Dosage
Prevotella	47.28	50.33	43.84	53.77	4.22	0.103	0.612	0.612
Sharpea	14.32	11.68	15.45	10.55	4.00	0.644	0.391	0.644
Fibrobacter ^a	13.63	13.03	17.05	9.62	0.41	0.960	0.440	0.960
	1.78	1.81	2.016	1.57				
Prevotella_7	4.89	5.32	6.94	3.28	3.33	0.927	0.442	0.927
Olsenella	4.14	3.31	1.85	5.6	1.5	0.699	0.084	0.699
Treponema	3.94	5.38	4.56	4.76	0.89	0.260	0.879	0.260
Ruminococcus	3.51	2.64	3.36	2.79	0.75	0.415	0.594	0.415
Succiniclasticum	3.31	3.98	3.43	3.87	0.97	0.632	0.75	0.632

Methanobrevibacter	2.66	2.12	1.72	3.05	0.39	0.333	0.019	0.333
Prevotellaceae_YAB2003	2.32	2.2	1.81	2.71	1.05	0.940	0.546	0.940

Abbrv: SEM; standard error of the mean. ^a Data was not normally distributed, log change data presented under treatment means.

Table 31: Effect of fresh and marine microalgae on lamb’s bacterial and archaeal solid-associated microbes’ richness and diversity.

Index	Species		Dosage, %		SEM	P-values		
	Fresh	Marine	0	1		Species	Dosage	Species x Dosage
No. ASVs	269.97	309.04	270.67	308.35	27.284	0.317	0.334	0.317
Inverse Simpson	14.56	15.82	13.21	17.17	2.042	0.665	0.177	0.665
Shannon	3.35	3.44	3.22	3.58	0.152	0.669	0.101	0.669
Fisher	42.05	49.35	42.26	49.14	5.121	0.319	0.348	0.319

Abbrv: No. ASV; number of amplicon sequence variants, SEM; standard error of the mean.

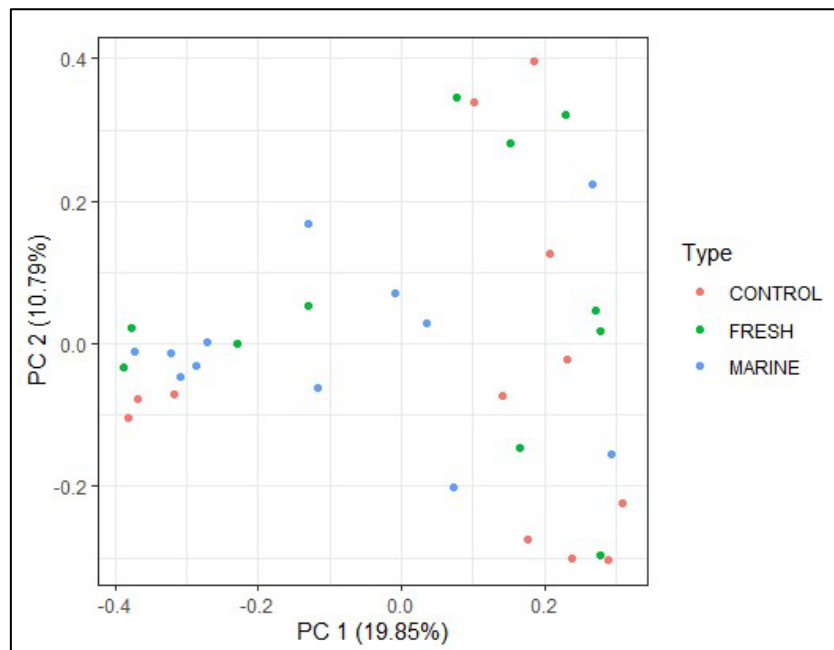


Figure 3: Principal coordinates plot (PCoA) of the Bray-Curtis dissimilarities for the 16 s RNA Bacterial and Archaeal solid-associated microbe by microalgae species. The percentage of variation explained by each principal coordinate are indicated on the axes. PFRMANOVA Analysis: $P = 0.270$.

4.4.2 Ruminal Fungi Structure and Diversity

Due to no significant effect of species x dosage interactions, results are presented as single effects. Species had no effect on the relative abundance of the top ten taxa. However, there was a dosage effect on *Rhodotorula* where inclusion of either algae at 1% resulted in a 4.2-fold increase (Table 32), and a tendency to reduce *Wallemia* ($P = 0.069$). Fungal richness and diversity of SAM (Table 33, $P > 0.05$) were not affected treatment. Bray-Curtis dis-similarities analysis revealed no variance in the fungal community structure between treatments (Figure 3, $P = 0.280$).

Table 32: Effect including fresh or marine microalgae at 1% in lamb feedlot pellets on top ten fungal genus relative abundance.

	Species		Dosage			P-values		
	Fresh	Marine	0%	1%	SEM	Species	Dosage	Species x Dosage
Wallemia	26.22	29.28	33.34	22.16	4.24	0.61	0.07	0.61
Neocallimastix ^a	18.18	11.99	10.91	19.27	0.42	0.27	0.30	0.27
	2.30	1.63	2.30	1.65				
Sporobolomyces	13.34	15.84	17.31	11.87	2.40	0.47	0.12	0.47
Caecomyces ^a	11.84	12.79	6.23	18.40	0.43	0.81	0.26	0.81
	2.03	1.88	1.60	2.30				
Alternaria ^a	6.09	6.90	8.03	4.95	0.25	0.24	0.36	0.24
	1.46	1.89	1.85	1.51				
Pecoramyces	5.78	4.65	2.97	7.46	3.15	0.80	0.32	0.80
Symmetrospora	5.17	7.35	7.70	4.82	1.54	0.32	0.19	0.32
Buwchfawromyces ^a	25.46	0.00	50.76	0.00	1.81	1.00	0.17	1.00
	0.36	0.00	2.51	0.00				
Cladosporium ^a	3.86	4.28	3.52	4.62	0.29	0.58	0.17	0.58
	1.073	1.305	0.90	1.15				
Rhodotorula	1.54	6.45	1.54	6.45	1.05	0.09	0.00	0.09

Abbrv: SEM; standard error of the mean ^a Data was not normally distributed, log change data presented under treatment means.

Table 33: Effect of fresh and marine microalgae on lamb's fungal solid-associated microbes' richness and diversity.

Indices	Species		Dosage			P-values		
	Fresh	Marine	0%	1%	SEM	Species	Dosage	Species x Dosage
No. ASV ^a	42.47	42.29	47.58	37.18	0.21	0.68	0.20	0.68
	3.47	3.59	3.60	3.22				
Inverse Simpson	8.33	8.63	8.38	8.58	1.06	0.85	0.90	0.85
Shannon	2.47	2.45	2.61	2.32	0.17	0.94	0.25	0.94
Fisher	9.63	9.70	11.01	8.32	1.47	0.97	0.20	0.97

Abbrv: No. ASV; number of amplicon sequence variants, SEM; standard error of the mean. ^a Data was not normally distributed, log change data presented under treatment means.

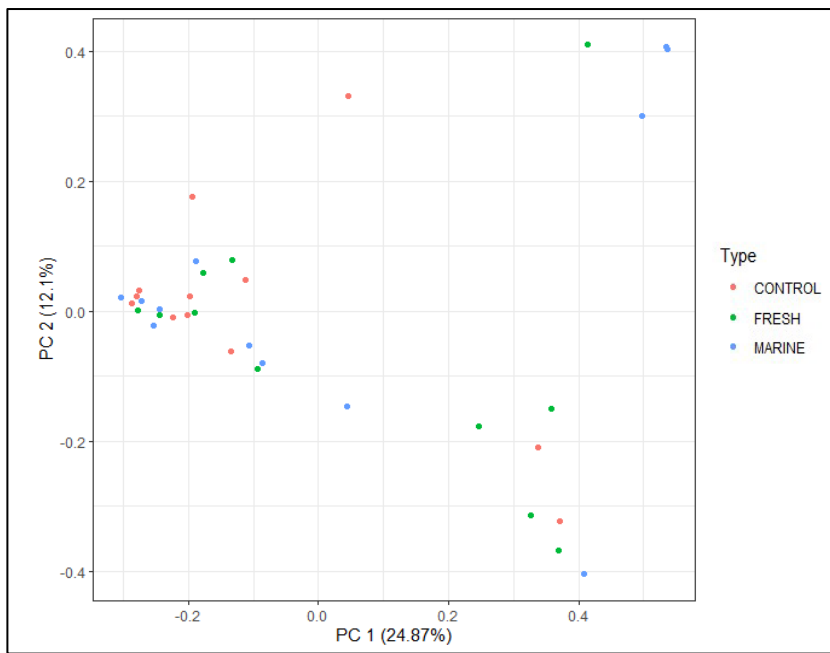


Figure 4: Principal coordinates plot (PCoA) of the Bray-Curtis dissimilarities for the ITS1 Fungal solid-associated microbe by microalgae species. The percentage of variation explained by each principal coordinate are indicated on the axes. PERMANOVA Analysis: $P = 0.280$.

4.4.3 Metabolome profiles

Overall, 171 metabolites were detected in liver, 140 in the rumen and 149 in the blood plasma. There was no effect on the metabolome of the rumen (Figure S6). The PCA analysis of the liver sample showed tendency for separation between the control and fresh microalgae fed lambs liver (Figure 5, $P = 0.095$). There was no effect on the metabolome of the plasma samples (Figure 5).

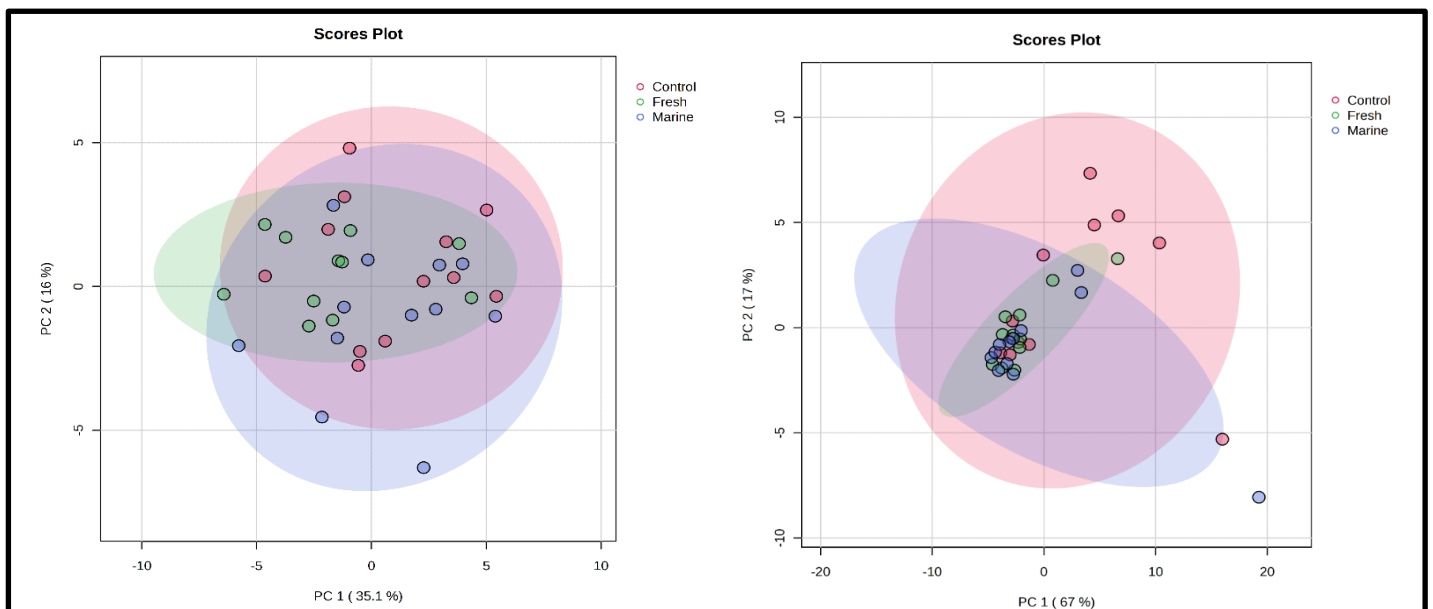


Figure 5: Principal component analysis of all metabolites for the liver (left) and plasma (right), where each data point represents one lamb. The percentage of variation explained by each principal coordinate are indicated on the axes. PERMANOVA Analysis: $P = 0.095$ and $p = 0.175$.

For the liver samples as there was a tendency for clustering between control and fresh samples, a subsequent PCA plot revealed a trend for clustering of freshwater fed samples from the control samples along the second principal component with the differentially expressed metabolites, explaining 15.3% of the variance between samples (Figure 6, $P = 0.081$). An orthogonal partial least squares discriminant analysis (OPLS-DA), conducted on the total liver metabolites validated the diet-associated separation of metabolites by $n = 100$ permutations, which resulted in a $P < 0.01$ ($Q^2 = 0.35$; $R^2 = 0.95$; Figure S7). However, a log fold analysis was conducted revealing there were 12 differentially expressed metabolites based on t-test raw p-values, however when the p-value was adjusted for FDR, no metabolites were statistically significant ($P > 0.05$).

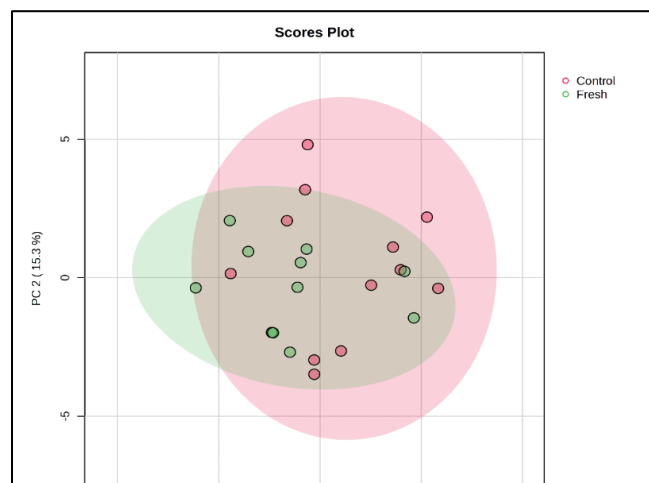


Figure 6: Principal component analysis of all metabolites, where each data point represents one lamb. The percentage of variation explained by each principal coordinate are indicated on the axes. PERMANOVA Analysis: $P = 0.081$.

5. Supply Chain Mapping

This project aims to utilise CO_2 and BSG waste streams from breweries to create an algae-based high-nutrient supplement. This supplement is intended to reduce both the CO_2 footprint of breweries and methane emissions from ruminant cattle when fed. To facilitate production and distribution, a supply chain analysis is essential, considering factors such as raw material volumes, brewery locations, farm distribution, processing facilities, and cost implications for end-users (breweries and farmers). There are 348 brewers and 54 cattle farms across Australia in this problem. The brewers produce a by-product called BSG, a valuable resource for cattle farms as it can be used as animal feed. The main objective is to optimise the supply chain network, ensuring the required BSG is supplied to the cattle farms while minimizing supply chain costs and CO_2 emissions (from production and transportation) to the environment (see Methodology Section 3.11). The overall supply chain network of the brewers and farms is depicted in Figure 8.



Figure 7: Overall supply chain network of the brewers and farms across Australia

5.1 Supply chain across Australia without algae treatment

We employed the advanced 'anyLogistix software' to optimise the supply chain network. The objective was to minimise transportation costs and CO₂ emissions stemming from production and transportation processes while meeting the demands of cattle farms. After running the network optimisation, we ensured that the brewers' capacity seamlessly met the cattle farms' requirements. The model was designed to enable the delivery of BSG from brewers in any region to farms in any other region, with the model handling the decision-making process. For a visual representation of the optimised supply chain involving the brewers and farms, please refer to Figure 9. To understand the allocation of demand from brewers to farms, see Table A1 in Appendix A (submitted as a separate file). Table A2 in Appendix A demonstrates how the farms' demand is smoothly fulfilled, while Table A3 provides information on transportation costs and overall CO₂ emissions. The total transportation cost amounts to AU\$ 97,862.09 and the CO₂ emissions generated throughout the process total 2,035,169.50 tonne (of which from production CO₂ emits 2,029,601.30 tonne and from transportation CO₂ emits 5568.20 tonne). In this scenario, the minimum number of brewers is necessary to deliver the required BSG to the farms due to the high CO₂ emissions generated during production. It is important to mention that since BSG is a freely available product, we consider the production cost to be zero at this stage.



Figure 8: Supply chain network of the brewers and farms across Australia after network optimisation (Without algae treatment).

5.2 Supply chain across Australia region to region and without algae treatment

We have developed a new model that only allows for the transfer of products from brewers within the same territory to farms within the same territory. A diagram of this optimised SC network is shown in Figure 10, and more information on how demand is allocated from brewers to farms can be found in Table B1 in Appendix B (submitted as a separate file). Table B2 in Appendix B explains how farms' need is met, while Table B3 provides details on transportation costs and the total amount of CO₂ emissions. Unfortunately, this model cannot meet all of the farms' demands because it only allows for products to come from the same region. Therefore, we have decided not to consider this model further. The total CO₂ emission for this model is 3,922,364.09 tonne which is bit higher, and the transportation cost is AUD \$49,798.20.



Figure 9: Supply chain network of the brewers and farms across Australia after network optimisation (Region to region and without algae treatment)

5.3 Supply chain across Australia with algae treatment

In this scenario, we have added algae treatment costs. The algae treatment cost is 127.2 AU\$/tonne-BSG. And for algae treatment, CO₂ emission from production reduces to 50%. Now that means CO₂ emission from production becomes 2748.38 gCO₂/tonne-BSG. Please refer to Figure 11 for the visual representation of the optimised supply chain network after implementing algae treatment. To understand the allocation of demand from brewers to farms, see Table C1 in Appendix C (submitted as a separate file). Table C2 in Appendix C demonstrates how the farms’ demand is smoothly fulfilled, while Table C3 provides information on production cost, transportation costs and overall CO₂ emissions. The total transportation cost amounts to AU\$ 65,241.39 and the CO₂ emissions generated throughout the process total 166,416.32 tonnes (of which from production CO₂ emits 148,033.68 tonne and from transportation CO₂ emits 18,382.64 tonne). And total production cost for BSG is now AU\$ 6,665,537.00.



Figure 10: Supply chain network of the brewers and farms across Australia after network optimisation (With algae treatment)

In this scenario, the production cost for algae and the reduction of CO₂ emissions resulting from this production have been considered. Consequently, a higher number of brewers are used to process the BSG and deliver it to customers in the nearest zones, thereby reducing transportation costs and CO₂ emissions from transportation. With CO₂ emissions being cut by 50% due to algae treatment, the overall CO₂ emissions are significantly reduced.

However, the production cost is higher compared to processes without algae treatment. In summary, this scenario demonstrates that while the implementation of algae treatment increases production costs, these costs are offset by reductions in transportation costs and total CO₂ emissions to the environment. In general, this model demonstrates the most favourable outcome. Please refer to the separate excel file for Appendices A, B and C.

6 Discussion and Conclusions

A key goal in ruminant nutrition is to manipulate ruminal fermentation to mitigate CH₄ emissions while enhancing nutrient utilisation. Incorporating microalgae into ruminant diets is increasingly researched as a strategy to achieve these aims. In this study, we evaluated 26 algal species effect on rumen fermentation and methane production. Subsequently, two species were then selected for use in an *in vivo* trial, assessing the effect of marine and fresh water algae on lamb performance, meat quality, rumen microbial diversity and metabolome.

Batch culture results suggested that including microalgae at low dosages (1% DM) improved feed fermentability, whilst higher inclusion rates ($\geq 5\%$) reduced fermentation. The inclusion of microalgae at 1% increased total gas production (Table 7), whilst increasing concentrations reduce total gas and total VFAs (Tables 3, 5 and 7). *In vitro* gas production provides insight into feed and fermentation kinetics [34]. Similarly, Salehian et al. [35] reported low inclusion rates of microalgae (1g/100g DM), increased the total volume of gas produced. It was suggested an increase in gas production at lower algal concentrations may be due to the utilisation of nutrients without the accumulation of anti-microbial factors that can negatively affect the ruminal microbial community [35]. Microalgae can contain antioxidative, anti-microbial or cytotoxic compounds [36, 37]. Gomaa [38], and Elghandour [39] reported that high inclusion rates of microalgae *in vivo* reduced feed intake and fiber digestibility. Additionally, microalgae inclusion at 1% increased IVDMD. An increase in DMD suggests that ruminants can easily digest and utilise microalgae intra-cellular nutrients [34]. Hence, the batch culture results support the inclusion of microalgae at low concentrations (1% DM), enables utilisation of microalgae nutrients without negatively impacting fermentation. Consequently, we determined the optimal inclusion rate to be 1% dry matter (DM) for inclusion of microalgae species. This rate maintained total gas production, *in vitro* dry matter digestibility (IVDMD), and total volatile fatty acid (VFA) concentration while decreasing CH₄ production.

Batch screening round five revealed significant dosage effects within maize and dairy concentrate diets, including increased pH and decreased total gas and CH₄ production (Table 3) at 1% inclusion. Similar effects were observed in hay and BSG diets, with a decrease in pH and total gas at 1% microalgae inclusion (Table 5). However, a species-specific effect on CH₄ production was not always observed (Table 3, S16 and S17). This finding contradicts numerous sources which support certain species are more effective at inhibiting CH₄ production than others [40-43]. However, in subsequent Round 6 batch screenings, it was demonstrated that the inclusion of marine Species 17 and 26 resulted in the lowest production of CH₄ on a mL/ g DM basis compared to the inclusion of Specie 12 and 7 (Freshwater) and Species 23 and 24 (Marine). It is well supported in literature there are large variations in chemical composition between types of microalgae (freshwater vs marine) [15, 36, 44]. For instance, freshwater species typically contained lower concentrations of fatty acids compared to marine species (i.e. 269 g/kg vs 481 g/kg)[45]. Various studies correlate mitigation ability with microalgae's chemical composition, particularly the type and concentration of fatty acids [41, 46, 47]. Our findings agree with multiple sources that report on the effectiveness of specific species in inhibiting CH₄ production [40-43, 48, 49]. For instance, marine microalgae have been shown to reduce CH₄ by up to 80%, while freshwater species have been shown to reduce CH₄ production by between 13-60% [34, 50]. It's important to

note that microalgae have poor reproducibility in terms of anti-methanogenic abilities. For example, Species 4 utilised in this study has been previously reported to reduce methane by up to 69% [8, 34, 48], while also being reported to increase CH₄ production [43]. While this study conveyed a species-specific effect on methane production, further research is required to gain a comprehensive understanding of factors affecting the reproducibility of anti-methanogenic ability *in vitro*.

Due to the data generated by the batch culture assay, the RUSITEC experiment utilized a dosage range of 0%, 0.5% and 1% inclusion (DM). This range was determined as 1% inclusion reduced CH₄ without negatively affecting the other fermentation parameters measured (i.e. total gas production and pH). The selection of a species was based on each microalga's impact on fermentation and methane production and on whether it possessed desirable cultivation qualities (i.e. robustness, biomass productivity and CO₂ tolerance).

The selection of species for use in round one RUSITEC, was based on results from batch culture rounds 1,2 and 3. As no species-specific effect was evident for methane production, species effect on total gas and VFA concentration were considered. As gas production reflects differences in feedstuff nutritional value and fermentability [34] species with low total gas production were eliminated. Inclusion of freshwater species resulted in an increase in the proportion of propionic acid. This is in agreement with Anele [34], who reported that freshwater species were able to improve molar proportions of propionic acid and reduce CH₄ production. Although Species 3 showed high total gas, VFA, and IVDMD, its small cell size made practical cultivation challenging. Hence, freshwater Species 10, with a larger cell size, was chosen for RUSITEC (Round-1) its ability to maintain high total gas production and IVDMD despite lower VFA production.

The selection of a species for round two RUSITEC was based on findings generated by round 5 and 6 batch cultures. The selection of a species was based on each microalga's impact on fermentation and methane production and on whether it possessed desirable cultivation qualities (i.e. robustness, biomass productivity and CO₂ tolerance). The batch screening revealed the inclusion of Species 17 resulted in the lowest value of methane compared to other species screened without affecting total gas production and media pH. Additionally, there has been an increase in the commercial use of Species 17 as a nutraceutical due to its nutritional composition [45, 51], indicating its suitability for large-scale cultivation.

The variability between batch screening underscores the need to analyse effects on fermentation over extending periods of time (>24hrs), emphasizing the need to evaluate species using a RUSITEC apparatus. To the best of our knowledge, this is the first time a fresh and marine microalga has been assessed for its effect on ruminal fermentation and microbial structures using a RUSITEC system fed a forage-based diet. Evaluation of freshwater Species 10 in RUSITEC (Round-1; Table 8) at 0, 0.5, and 1% inclusion of hay and BSG basal feed didn't affect CH₄ production. However, pH, total gas, and VFA proportions were within the expected range for a forage-based diet [28, 43]. RUSITEC results indicate microalgae are fermentable and don't inhibit nutrient digestion by ruminant microbes at 1% max inclusion. Microbial profiling of RUSITEC Round-1 (freshwater Species 10; Tables 9-12) analysed Solid-associated microbe (SAM) and Liquid-associated microbe (LAM) samples. Bacteria linked with solid digesta account for 50-75% of total ruminal microbial population [52]. Profiling revealed a significant difference between positive control (Day 0 rumen fluid and solids) and BSG diet (0% treatment), attributed to diet adaptations rather than microalgae treatment. BSG feeding positively influenced microbial diversity. However, no significant difference in microbial richness and diversity was found among algae treatments (0%, 0.5%, and 1% DM) for RUSITEC Round-1.

Similarly, round-2 RUSITEC revealed that the addition of the selected marine microalgae at 0.5% and 1% concentration (on DM basis) did not affect gas or effluent production, pH, or DMD (Table 13). It has been proposed that the carbohydrate fraction of microalgae may be less fermentable than commonly utilised feeds such as soybean meal and alfalfa hay [53]. However, it has been reported that including microalgae in foraged-based diets can increase microbial fermentation efficiency [48]. Likewise, the results presented in this study support the inclusion of Species 17 did not reduce the fermentability of the diet as fermentation parameters were maintained the same as the control diet.

It is suggested that the presence of PUFAs has an inhibitory effect on methanogens [40, 41, 49]. Hence, it is hypothesised that including species rich in FAs will inhibit CH₄ production. For instance, multiple studies have reported lower CH₄ produced when marine microalga rich in PUFAs, specifically Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) were included in the diet [43, 49, 54]. However, our results conflict with this assumption, as the inclusion of the marine species containing EPA had no significant effect on methane production. Similarly, Anele et al. [34] demonstrated that species with a low lipid content reduced methane like species with high lipid content, proposing anti-methanogenic ability does not solely rely on the presence and quantity of fatty acids. Additionally, microalgal species can contain high concentrations of sulfate or sulphur. It has been hypothesised that increasing sulfate in feed, within safe limits (< 4% DM), can assist sulfate-reducing bacteria in the rumen to outcompete methanogens for hydrogen sink, thereby reducing methanogenesis [55, 56].

RUSITEC round two demonstrated that the inclusion of the selected marine microalgae at 1% increased caproate percentage and yield (mmol/d) (Table 13). It is suggested that an increase in hydrogen (H₂) favours the fermentation pathways that consume H₂, such as pathways that produce valerate and caproate [57]. Terry et al. [58] recently demonstrated using a RUSITEC apparatus that the red seaweed *Asparagopsis taxiformis* at 2% inclusion decreased CH₄ production and increased the production of H₂, and the molar proportion of caproate. However, including *A. taxiformis* also decreased the molar proportions of acetate, propionate, and total VFAs production. Whilst our study demonstrated microalgae increased the concentration of caproate there was no effect on total VFAs or methane production.

In both RUSITEC rounds, microalgae inclusion did not alter the relative abundance of the top ten predominant primary bacteria and archaea in both SAM and LAM samples. No significant differences were observed in microbial richness and diversity among the 0%, 0.5%, and 1% algae treatments. Studies indicate that more efficient ruminants produce less methane and have higher abundance of key ruminal microbes like Prevotella, Succinivibrio, Treponema, Fibrobacter, and Ruminococcus [59, 60]. Microalgae supplementation has been linked to increased Prevotella spp. prevalence and propionate production [60]. In vivo microalgae inclusion in ruminant diets has also reduced methanogen presence, including Methanomassiliicoccales, Methanobrevibacter spp., Methanosphaera stadmanae, and Methanobacterium formicum in goat rumens [19]. However, RUSITEC Round 1 (using freshwater Species 10) showed no significant effects of microalgae inclusion on the top 10 taxa's relative abundance in both LAM and SAM samples (Tables 9-12). Whilst in round 2, including marine microalgae (Species 17) at 0.5% increased the relative abundance of solid associated fungi from the genus *Vishniacozyma* and *Sterigmatomyces* (Table 9). Both genera of fungi are yeast, with species from the *Vishniacozyma* genus (*Vishniacozyma victoriae*) being identified in cow's milk [61] and *Sterigmatomyces* found in the small-intestinal epithelium of beef cattle [62]. Similarly, Teoh et al. [28] demonstrated using a RUSITEC apparatus a significant increase in OUTs classified as *Vishniacozyma victoriae* with biochar supplementation (800 mg/d). However, despite biochar supplementation causing significant changes to fungal OUT abundance, treatment did not affect the measured fermentation parameters.

Whilst no microalgae screened thus far has been identified as a potent CH₄ reducer (i.e. > 20% reduction) demonstrating improved animal growth, and feed conversion efficiency can also reduce total emission per animal. Several species, including *Chlorella vulgaris* [7, 63-68], and *Schizochytrium* spp. [19, 69, 70], as well as commercially available microalgal products like DHA-Gold and Forplus (*Auramtiochytrium limacinum*), have been investigated as ruminant feed additives [71-74]. These investigations have shown that including microalgae can enhance organic matter digestibility and increase concentrations of alpha-linolenic acid, EPA (omega-3), and MUFAs + PUFAs in goat rumens [65]. Some in vivo studies involving *Spirulina* sp. [66] have shown positive impacts on animal productivity parameters. Although these studies didn't directly assess CH₄ emissions, they indicated that supplementation increased immunity, daily gain, and reduced the time required to reach desirable body weights for slaughtered lambs, thereby minimizing greenhouse gas (GHG) emissions per animal unit. As in vitro data is not directly representative of expected performance and health outcomes, *in vivo* studies are essential for assessing the suitability of an algae and BSG additive.

The lamb performance trial showed no effect on overall growth performance and carcass characteristics of lambs fed feedlot pellets containing either fresh (Species 4) or marine (species 17) microalgae. This is likely due to the similarity in diet composition despite algae species and similar DMI (Table 22; av. 1.1kg/day). Likewise, Meale et al 2015 [75] reported similar feed intake values (av.1.08kg/day) when supplementing marine algae (*Schizochytrium*) to Canadian Arcott ewe and ram lambs. Additionally, a recent study by Gadzama et al., 2024 [76] reported no effect on performance of doper cross merino lambs when supplemented with either 0.5% or 1% freshwater algae in the form of a paste. Consequently, due to the similarity in final live weights, carcass hot and cold weights were not affected by treatment. Meat quality parameters were also unaffected by microalgae inclusion (Table 23). Meat colour assessments can provide insights into muscle composition, including the amount of intramuscular fat (IMF). Higher IMF concentrations, especially after blooming, can influence colour parameters due to increased light scattering caused by the presence of fat droplets. [77]. Whilst the presence of IMF plays a role in meat quality, it is important to understand algae's effect on lipid metabolism and how this impacts the fatty acid profile of ruminant's edible fats.

Rumen microbes have the capacity to hydrolyse lipids and rapidly biohydrogenate unsaturated fatty acids [78]. There has been an increase focus in developing rumen-protected sources of PUFAs specifically omega-3s, in efforts to increase omega-3 to omega-6 ratios in meat [75, 78, 79]. Given the liver's critical role in lipid metabolism and the regulation of lipid distribution to extra-hepatic tissues, liver weight and fatty acid profiles were analysed [80]. Despite no variance in the weight of lamb livers there was a dose-dependent response where the inclusion of microalgae increased the concentration of PUFAs. Specifically, the sum of EPA and DHA increased, contributing to higher concentration of total omega-3s. This increase resulted in a reduction in the omega-6 to omega-3 ratio. A similar trend was evident with elevated concentrations of PUFA in the subcutaneous fat of fresh or marine algae fed lambs (Table 26; $P = 0.088$). Additionally, the sum of both omega-3 and omega-6 levels were elevated in the subcutaneous fat of algae fed lambs ($P = 0.059$ and $P = 0.043$, respectively). The elevated PUFA concentrations within the liver and subcutaneous fat support the concept that microalgae could serve as a source of rumen-protected PUFAs, consequently reducing the biohydrogenation of PUFAs in the rumen [78]. Interestingly this trend was not observed for muscle samples where the concentration and composition of SFA, MUFA and PUFAs were similar despite diet (Table 25). Previous studies have reported the ability of microalgae to increase the concentration of PUFA in either the subcutaneous adipose tissue or muscle of lambs [71, 81]. However, these studies both utilised a higher concentration of algae within the diet (~2% DM), hence there is the need to investigate the effect of increasing the concentration of microalgae within feedlot pellets.

Previous, studies have reported an increase in PUFA, specifically EPA and DHA in meat can increase lipid oxidation resulting in a change to the flavour and aroma of meat [82, 83]. Despite the similarities between the FA profile of muscle samples the sensory analysis revealed consumers were able to detect a notable difference between marine microalgae fed lamb and control fed lamb meat (Table 28). Interestingly, majority of correct selections were based on flavour and texture attributes, with the control samples reported to be both milder in flavour and chewier (Table 29; Flavour and Other). However, a recent study by Vitor et al., [84] revealed that the supplementation of the marine microalgae, provided as either oil, spray-dried, or freeze-dried biomass to lambs, had no effect on various meat sensory attributes (i.e. odour intensity, succulence, tenderness, flavour intensity, and flavour acceptance), despite a significant increase in Omega-3 levels in the longissimus lumborum muscle.

In ruminants the diverse microbial population produced metabolites used for microbial growth, animal maintenance and muscle production [85]. Metabolites are considered important markers for identification of cell process that influence an organism's functioning [86]. By analysing various sample types metabolomic profile you can gain a comprehensive overview of these processes. For instance, blood plasma can give insight into performance and yield parameters, whilst liver metabolites give an indication of interactions between diet and nutrient utilisation [86]. Our study showed the inclusion of microalgae in lamb feedlot pellets had minimal impact on the rumen and blood plasma metabolome, likely because algae inclusion in the feedlot pellets it did not significantly influence animal performance, and yield parameters. Whilst the fatty acid results suggest lipid metabolism was influence by microalgae inclusion there was no effect on the liver metabolome profile, with the log-change analysis revealing no variation in metabolite expression between treatments.

Incorporation of Brewery waste in ruminant diets can facilitate the development of a bioeconomy from brewers to farms. A supply chain analysis was conducted to investigate the logistics of BSG transportation between suppliers and customers, identifying current limitations and making recommendations for supplying BSG to red meat producers nationwide (Figures 8-11). While these calculations are preliminary, they are crucial for proposing a distribution pipeline for an algal/BSG feed supplement to meet the feed demands of Australian cattle farmers. This analysis helps identify limitations within the supply chain, particularly in terms of volumes and capacities needed for smaller feed scenarios, such as a typical-sized dairy farm, before addressing the challenges of meeting the demands of the larger red meat industry.

In conclusion, the approach for assessing the impact of microalgae and BSG on productivity parameters, potentially influencing greenhouse gas emissions indirectly. However, there is a significant knowledge gap concerning the effects of microalgae on fermentation and productivity in various ruminant species, impeding their widespread use as feed additives.

5.1 Key Findings

1. Evaluated a total of 26 (freshwater and marine) algal species using two basal diet formulations for fermentation parameters.
2. None of the species exhibited significant (>20%) reduction in CH₄ emissions.
3. RUSITEC Rounds 1 and 2, using freshwater and marine algal species respectively, showed no significant difference in CH₄ reduction when included at 0.5% or 1% on a dry matter basis.
4. Round 2 results were consistent with Round 1, indicating species alteration does not affect fermentation parameters.
5. Successfully selected freshwater and marine microalgae species for a 100-day lamb trial.

6. Pellet intake by lambs, with or without algae, remained similar, ensuring consistency in meat analysis.
7. There was a dose effect, where inclusion of either microalgae at 1% increased the concentration of PUFA within lamb livers and subcutaneous fat.

5.2 Benefits to industry

The proposed initiative holds promise for the Australian red meat industry, particularly cattle farmers partnering with local breweries. It offers a decentralised supply chain for an innovative ruminant feed supplement derived from brewery waste, aiming to reduce CH₄ emissions in livestock. This collaboration benefits both parties, as breweries can concurrently decrease CO₂ emissions and waste streams. Recent research highlights the potential of microalgal biomass in reducing CH₄ emissions, enhancing meat quality, and boosting livestock growth performance. By tapping into brewery waste, this initiative could open new revenue streams and qualify breweries for carbon pricing incentives, aligning with the industry's goal of achieving carbon neutrality by 2030. If proven effective and bioactive, widespread adoption of this supplement could significantly improve animal health, enhance meat quality, and propel the red meat industry towards its sustainability targets.

7 Future Research and Recommendations

1. For future research efforts in the red meat industry, there is a critical need to delve deeper into the screening and evaluation of algal strains, both individually and in combination with other species, to identify more potent methane-reducing species. This process should aim to optimise dosage to maximise efficacy while ensuring safety and feasibility.
2. Implementing high-throughput screening of algal strains is crucial to expedite the identification of potent methane-reducing species for application in the livestock industry. By utilising automated systems and advanced technologies, researchers can significantly increase screening output, allowing for the evaluation of more than **2000 algal strains/combinations per quarter**. This accelerated screening process will not only enhance the efficiency of strain selection but also enable the identification of novel strains with promising methane reduction capabilities. Moreover, it will facilitate the exploration of diverse combinations of algal species, potentially uncovering synergistic effects that could further enhance methane mitigation strategies in red meat production.
3. Additionally, high-throughput screening will provide an opportunity to identify the methane-reducing capability of algal strains at the **molecular level**. This approach will enable researchers to delve deeper into the mechanisms underlying methane reduction, providing valuable insights into the specific pathways and **biochemical processes involved**. By understanding the molecular basis of methane reduction in different algal strains, researchers can more effectively target and optimise breeding or genetic engineering efforts to enhance methane reduction capabilities. This molecular-level understanding will also contribute to a more comprehensive assessment of algal strains, allowing for the identification of a diverse range of methane-reducing species within a reduced timeframe, while providing invaluable knowledge for further research and development in this field.
4. Conducting long-term feeding trials using steers represents a crucial next step to assess the sustained effects of algal supplements on methane emissions, animal health, and product quality. These trials should encompass comprehensive monitoring over extended periods to capture any potential fluctuations or trends in methane emissions and animal performance.
5. Furthermore, it is imperative to conduct thorough economic and **environmental impact assessments** to evaluate the feasibility of implementing algal/BSG supplements on a national scale within the red meat industry. These assessments should consider factors such as cost-effectiveness, resource utilisation, and

potential environmental benefits, including reductions in greenhouse gas emissions and waste streams.

6. Collaboration with industry stakeholders, including cattle farmers, breweries, and feed manufacturers, will be instrumental in guiding research efforts and ensuring the practical applicability of any findings. Engaging in collaborative ventures can facilitate access to resources, expertise, and funding, thereby enhancing the likelihood of successful implementation and adoption of algal/BSG supplements.
7. Moreover, public awareness efforts should be prioritised to educate stakeholders and consumers about the benefits and implications of utilising algal/BSG supplements in livestock production. This proactive approach can help garner support, address potential concerns, and foster widespread acceptance and adoption of sustainable practices within the red meat industry.

Declaration – Peer review journal

The methodology (Section 3.0), results (Section 4.0), and conclusions (Section 5.0) presented in this report are intended for publication in manuscript form, pending discussions with industry partners. Measures will be implemented to safeguard intellectual property throughout the publication process. The findings will be disseminated across a minimum of three to four manuscripts, targeting journals such as Algal Research, Bioresource Technology, and Animal Nutrition Science, chosen for their relevance to the research. Anticipated completion dates for manuscript submissions are tentatively set to align with the finalisation of the analyses, estimated in 2025.

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Supplementary Files

Table S1: Summary run conducted, dosages, method, number of species screened, and basal diet utilised. Protected background IP Innovation Connection Grant.

Round No.	% Conc.	Assay	Species	Basal Feed	Runs	Reps per run
1	0, 5	AMPTS II system	3	Maize silage and dairy concentrate	2	3
2	0, 5	AMPTS II system	3	Maize silage and dairy concentrate	2	3
3	0, 1, 5, 10	Batch	18	Maize silage and dairy concentrate	2	3
4	0,1,5	RUSITEC	1	Hay mix with BSG	3	2
5	0, 1, 5, 10	Batch	5	Maize silage and dairy concentrate; Hay mix with BSG	2	3
6	0, 1, 5	Batch	12	Hay mix with BSG	2	3
7	0,1,5	RUSITEC	1	Hay mix with BSG	3	2

Table S2: Chemical analysis of microalgae (Species 10, Freshwater), barley distiller grain, and hay utilised for RUSITEC fermentation.

	DM%	% of dry matter				
		Crude protein (CP)	Neutral detergent fiber (NDF)	Ether extract (EE)	Non-fibrous carbohydrates (NFC)	Ash
Algae (Freshwater)	91.1	37.0	0.4	1.5	55.5	5.6
Forage (Hay Mix)	87.1	10.0	50.9	1.0	30.7	7.4
Barley distiller grain	97.7	23.0	55.4	6.0	11.5	4.1

Abbrev; DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; EE, ether extract; NFC, non-fibrous carbohydrates; $NFC = 100 - CP - NDF - EE - Ash$].

Table S3: Chemical analysis of microalgae (Species 17, Marine), barley distiller grain, and hay utilised for RUSITEC fermentation.

	DM%	% of dry matter				
		Crude protein (CP)	Neutral detergent fiber (NDF)	Ether extract (EE)	Non-fibrous carbohydrates (NFC)	Ash
Algae (Marine)	98.1	35.0	3.2	19.1	26.0	16.7
Forage (Hay Mix)	87.1	10.0	50.9	1.0	30.7	7.4
Barley distiller grain	97.7	23.0	55.4	6.0	11.5	4.1

Abbrev; DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; EE, ether extract; NFC, non-fibrous carbohydrates; $NFC = 100 - CP - NDF - EE - Ash$].

Figure S1: Colour assessment using a chromameter (left) and drip loss measurements (right).



Figure S2: Sensory Analysis Method Optimisation

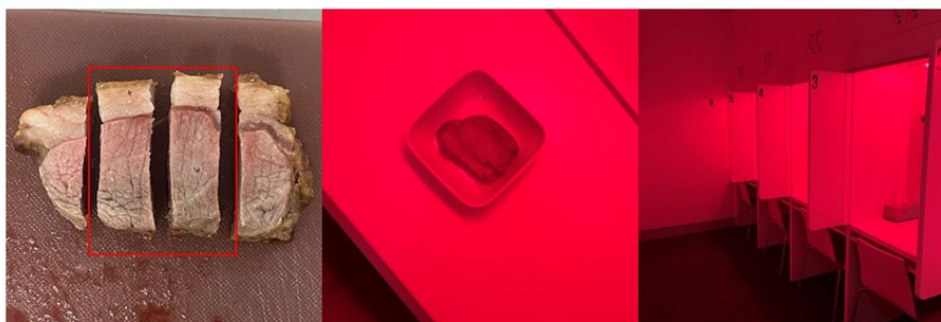


Figure S3: Triangle test for sensory analysis



Table S4: Assessing project outputs for utilisation

Project Output	End-User	How will the end-user use the output?	What are the potential issues that the end-user may have in using the output?	How will end-users be engaged & their feedback sought on how easy the outputs are to use in their business and where they see benefits?
Algal/BSG supplement	Farmer	Producers will purchase the feed supplement for use in their business e.g. feed their livestock	<ul style="list-style-type: none"> • Cost • Availability (i.e. will it be reliable) • Storage (infrastructure requirements); is it wet/dry and how does this affect shelf life and usage • Modifying feeding regime to incorporate the supplement • Ability to modify feeding regime based on availability if there are issues with the supply • Amount feed they can accept per delivery or on any one day • Labour requirements to collect and distribute feed (i.e. 1 person daily to fill feeder) • Benefit from purely economic perspective (weight gain, milk production) 	<ul style="list-style-type: none"> • Conduct a trial with select set of producers • Develop and conduct a workshop for the trial cohort to discuss the feed value and how to integrate into their feeding regime • Develop a Feed Budgeting Spreadsheet tool similar to that provided by Dairy Australia (how to use this spreadsheet discussed during workshop) • Share preliminary findings with a more diverse group of producers and ask for their feedback on what it would take for them to implement the practice. • Facilitate a discussion about where they would see a benefit and if there was enough benefit there for them. • Establish a small group of producers or committee to review developments and provide feedback on usage

		<ul style="list-style-type: none"> Transition from donated feed to one which now has a cost 	
Algal/BSG supplement	Brewery	<ul style="list-style-type: none"> What is the benefit financially for short term, as well as long term consideration i.e. potential carbon tariffs? How would it effect existing relationships if donated grain now has a price? How do you grow/mix (i.e. prepare concentrated grain/algal mixture that farmers dilute on farm?) Labour requirements Bioreactor design and space requirements 	<ul style="list-style-type: none"> Discussion with large enterprise industries motivate engagement/influence from top-down through sustainability incentives Must determine and convey purely economic benefit but must also convey that the project is funded under CN30 for the red meat industry which is aligned with society's expectations and industries' expectations to create a sustainable cattle industry

Table S5: Extension activities mapped across MLA adoption pathway

Project Output;	End-user	Awareness raising activities	When	Short-term training activities	When	Long-term practice changes activities	When
Algal/BSG supplement	Farmer	Prepare a calendar of schedule events (for the livestock community) in order to discuss various aspects of the proposed idea. Could be led by an established committee (i.e. producers, universities, invested stakeholders, interested stakeholders) and this allows a review to be conducted on the developments and provide feedback on usage/progress/concerns etc.	Scheduled quarterly as outlined in communications plan	Workshops, electronic learning modules, training days which are scheduled as part of the calendar of events	Schedule monthly as outlined in communications plan	Short courses which offer coaching activities, peer to peer learning, support from advisors Annual lectures similar to that offered by CSIRO for FutureFeed, i.e. in 2021 they offered a lecture presenting the story behind the innovation, the science behind it and what is required to realise the full impact of FutureFeed.	Scheduled annually as outlined in communications plan
Algal/BSG supplement	Brewery	As above					

Table S6: Feedback from consultation with supply chain stakeholders

End-User	How the end-user will use the output?	Potential issues that end-user may have in using the output?
Farmer	Producers will purchase the feed supplement for use in their business e.g. feed their livestock	Cost and benefit from purely economic perspective (weight grain, milk production) Availability (i.e. will it be reliable) Storage (infrastructure requirements); is it wet/dry and how does this affect shelf life and usage Modifying feeding regime to incorporate the supplement – understanding dry matter content based on moisture content Ability to modify feeding regime based on availability if there are issues with the supply Amount feed they can accept per delivery or on any one day Labour requirements to collect and distribute feed (i.e. 1 person daily to fill feeder) Transition from donated feed to one which now has a cost
Brewery	Producers the feed for selling	What is the benefit financially for the short term as well as, long term (i.e. potential carbon tariffs)? How would it effect existing relationships if donated grain now has a price? How do you grow/mix (i.e. prepare concentrated grain/algal mixture that farmers dilute on farm?) Labour requirements – does it require additional staffing Bioreactor design and space requirements; limited space available at breweries

Table S7: Supply chain mapping for a pre-prepared or supplement which is delivered to the feedlot and fed out on site and key questions to be addressed

No.	Supply Chain Process	Process Detail	Quality Attributes	Key Considerations
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1.	Raw Materials	Grain and Algae	Reliable and consistent supply to meet fluctuating demand of consumers Key Question: What is the demand? What is the final moisture content? Will farmers prefer the algae mixed with some spent grain and at what percentage?	What volumes are needed to meet supply? Scenario: 1. How much algae and grain required per month to supply one-month feed demand for one farm? Wet and Dry Scenario 2. How much CO ₂ is utilised to growth this amount of algae 3. How much space would a brewery need to grow this number of algae? 4. How many farms (dairy and cattle) in Australia 5. What is typical farm size (dairy and cattle) 6. How many brewery's in Aus and what is their annual beer production; what is the typical size for independent? 7. What is required to upscale to meet the demand for the Australian red cattle industry?
2.	Manufacturing	Harvesting and Processing	Efficient to run (low energy consumption) Key Question: what infrastructure is required in order to produce enough product (in the right form) to meet demand. What pre-processing of algae is required?	Cost: 1. What is the cost and space required for infrastructure (wet and dry scenario); for small scale (one farm, one brewery and, for upscaling to meet the needs of the Australian red meat industry 2. What is the cost for the equipment (wet and dry scenario) 3. What is the cost of running and maintenance? (wet and dry scenario) 4. Does this involve a local centralised processing facility?
3.	Quality Assurance	Monitoring	Free from contaminate, not spoiled with minimal batch-batch variation; % algae must be within a SD of +/- xx Key Question: What is our key attribute or quality of our feed that must be maintained?	Safety: 1. How is this assessed (i.e. commercial laboratory, routine test) 2. How often is this monitored (daily, monthly, quarterly, each batch before shipping) 3. What is monitored (contaminates, nutrient profile etc) 4. What is the cost?
4.	Packaging	How is it packaged for storage and transit	Resealable, non-leakage and secure, easy to open, empty, clean and stack Key Question: In what form in this produce sold to be distributed? Does it facilitate ease of use?	End User: 1. Can end-user easily integrate into feed handling systems (wet and dry scenario) 2. Will their current handling systems accommodate supplement, do they have the necessary building, storage requirements? (wet and dry scenario) 3. What is the cost/benefit (wet and dry scenario)
6.	Shipping/Transport	From manufacturer to farm	Timely and reliable Key question: how will this be transported and where will it be transported to (distance) and does it involve any, intermediate storage facilities before reaching end user.	Transport 1. What is the maximum distance you would want to transport? (Wet and Dry scenario) 2. What is the Frequency of deliveries? (Wet and Dry scenario)
5.	Storage	Bulk storages supplement and systems for delivering to feed bunks	Use existing, silos, bunks, sheds i.e. compatible with handling equipment (augers and conveyors), grain processing, feed mixing and other standard delivery operations Key Question: Can this product integrate into existing storage and processing equipment like any other typical buy-in feed ingredient to facilitate ease of usage? What is shelf life and under what conditions	Shelf life 3. What is Shelf live under various expected storage conditions (Wet and Dry scenario) 1. How much 'stock' would be typically in reserve at processing facility and on farm? (Dry scenario only)

Table S8: Number of breweries as of 2023 (Independent Brewers Association 2022)

Location	Number of Breweries
Queensland	67
New South Wales	100
Australian Capital Territory	3
Victoria	89
Tasmania	18
South Australia	38
Western Australia	28
Northern Territory	4

Table S9: Litres of beer/annum, estimated CO₂ production, and BSG produced

Typical output (L beer/annum)	No. Breweries	Min Ton CO ₂	Max Ton CO ₂	Max. BSG per month (kg/month)
0-99999	212	0	3	1667
100K – 299K	43	3	10	4983
300K – 699K	3	10	24	11650
700K -999K	24	24	34	16650
1M – 5M	7	34	170	83333

5M – 40 M	2	170	1359	666667
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Table S10: Capacity of brewers in territories of Australia

Territory	Capacity (Tonne - BSG)
QLD	8532.550757
NSW	17065.10151
ACT	449.0816188
VIC	13771.83631
TAS	2844.183586
SA	4939.897807
WA	4490.816188
NT	449.0816188
Total	52542.5494

Table S11: Demand of BSG in farms and territories of Australia

Name of Farms	State	Demand (Tonne - BSG)
Cape York	QLD	47.1100006
Cooperative Management Area	QLD	76.407475
Northern Gulf	QLD	560.227131
Southern Gulf	QLD	807.4448152
Terain NRM	QLD	99.5730032
North Queensland Dry Tropics	QLD	895.603097
Desert Channels	QLD	972.9220872
Reef Catchements	QLD	84.2915082
Fitzroy Basin	QLD	1808.373064
South West Qld	QLD	443.3267398
Burnett Mary	QLD	550.4476908
Queensland Murray Darling Basin	QLD	725.3804998
Condamine	QLD	36.4133124
South East Qld	QLD	215.1706156
North Coast	NSW	272.4778342
Northern Tablelands	NSW	477.9335036
North West NSW	NSW	489.4442494
Western	NSW	125.8220612
Central West	NSW	355.3877376
Hunter	NSW	274.590371
Central Tablelands	NSW	228.7322706
Greater Sydney	NSW	24.862437
South East NSW	NSW	277.8566338
Riverina	NSW	378.8313066
Murray	NSW	268.7816114
ACT	ACT	2.8355862
North East	VIC	278.14829
East Gippsland	VIC	77.7052376
Goulburn Broken	VIC	361.807757
West Gippsland	VIC	525.0005082
Mallee	VIC	18.9805842
Wimmera	VIC	39.3599716
Glenelg Hopkins	VIC	605.4223764
North Central	VIC	171.600619

Corangamite	VIC	313.2781718
Port Philip and Western Port	VIC	171.7181414
North	TAS	262.7406734
South	TAS	30.459083
Cradel Coast	TAS	265.8005554
SA Arid Lands	SA	99.8015986
Eyre Peninsula	SA	6.7518052
Northern and Yorke	SA	29.0588466
Kangaroo Island	SA	10.8306924
Adelaide and Mount Lofty Ranges	SA	45.8366024
SA Murray Darling Basin	SA	100.3225668
South East	SA	457.6895536
South Coast	WA	218.6862552
South West	WA	233.944819
Peel-Harvey	WA	74.5937604
Perth	WA	1.4410826
Wheatbelt	WA	42.046505
Northern Agricultural	WA	78.589522
Rangelands	WA	878.5953126
Northern Territory	NT	1237.555301
Total		17138.01283

Table S12: Location of Australia breweries, dairy farms and cattle regions

	Number of Breweries [87]	No. of Dairy Farms [88]	No. of Cattle Regions (head of cattle, Million) [89]
QLD	47	327	14 (12.2)
NSW	99	534	12 (4.7)
ACT	3	0	0 (0)
VIC	83	3462	10 (3.8)
TAS	13	391	3 (0.7)
SA	26	206	7 (1.0)
WA	20	135	7 (2.0)
NT	3	0	1 (2.1)

Table S13: Average dry matter intake (assuming 11 MJ/KG dry matter (DM)) scenario for on typical dairy farm per month with estimated savings using donated feed product

Feed Scenario	Monthly (Ton)	Daily (Kg)	Estimated Savings (\$)	Estimated Cost
DM feed budget	83	11.3		
BSG (10-20% inclusion)	8.30 - 16.60	1.14-2.27	2490-16600	@300/ton
Algae (1-10% Inclusion)	0.83-8.30	0.11-1.14	207-2075	@250/ton
			2697-18675	

Table S14: Algal Carbon utilisation*

Variable	Assumption	Reference
Feed Demand	Dairy farm	[90]
BSG/month (10%)	8300 kg	
Algae/month (1%)	830 kg	
Carbon Output Brewery	3 - 1359 Ton	
Algal Carbon (d.w.)	50 %	[91]
CO ₂ per algae	1.83 kg/kg	[91]

Carbon Transfer Efficiency	25-90%	e.g. 25% open ponds, 79-90% closed system
Days in operation	300	
Algae growth rate	0.5 kg/m ³ /d	[92]
Algal productivity kg/m ³ /yr	150 kg/m ³ /yr	
Algae/month (30 days)	15kg/m ³ /month	
Size system for one-month feed	55m ³	
CO ₂ fixed	1.5 Ton	

Table S15: Associated costs and CO₂ emissions

Other Costs and CO ₂ Emission	Value
CO ₂ emission from production	5496.75 gCO ₂ /tonne-BSG
CO ₂ emission from transportation	52.7 gCO ₂ /km
Production cost of algae per BSG	127.2 AU\$/tonne-BSG

Table S16: The effect of incubating marine microalgae species at different concentrations on in vitro fermentation characteristics

	pH	Gas, mL/d	Gas, mL/gDM	CH ₄ , %	CH ₄ , mL/d	CH ₄ , mL/gDM	IVDMD
Species							
16	6.36	40.27	80.29	11.95	4.82	9.62	53.38
18	6.35	39.41	78.40	11.44	4.51	8.96	52.58
17	6.37	38.89	77.61	11.08	4.34	8.65	52.71
14	6.37	38.75	76.75	11.30	4.41	8.60	51.64
15	6.38	39.81	79.20	11.77	4.70	9.29	52.11
SEM Treat.	0.060	2.550	4.825	0.443	0.454	0.840	0.695
Conc.							
0%	6.30c	40.35	80.43	12.31a	4.99a	9.95a	52.94
1%	6.37b	40.05	79.84	11.35b	4.55b	9.07b	51.6
5%	6.38b	39.09	77.7	11.24b	4.41b	8.71b	51.81
10%	6.41a	38.21	75.83	11.13b	4.27b	8.37b	53.59
SEM Conc.	0.060	2.531	4.787	0.425	0.450	0.831	0.627
P-values							
Treatment	0.516	0.523	0.42	0.181	0.095	0.077	0.452
Conc.	<.0001	0.095	0.056	0.004	0.003	0.001	0.091
Treatment × Conc.	0.287	0.949	0.91	0.61	0.714	0.43	0.155

Abbrev; SEM; standard error of the mean, CH₄; methane, IVDMD; In vitro dry matter digestibility, Conc; concentration.

Table S17: The effect of incubating freshwater microalgae species at different concentrations on *in vitro* fermentation characteristics. Protected background IP Innovation Connection Grant.

	pH	Gas, mL/d	Gas, mL/g DM	CH ₄ , %	CH ₄ , mL/d	CH ₄ , mL/g DM	IVDMD
Species							
1	6.32ef	39.11ac	77.96 ac	11.04	4.17	8.32	53.10
2	6.35bcde	39.31ab	78.51ab	10.86	4.44	8.85	53.43
11	6.32ef	39.39ab	78.65ab	11.72	4.79	9.57	53.38
3	6.35bce	39.52ab	78.84ab	11.90	4.88	9.72	54.60
12	6.36bce	38.81abcd	77.48abc	11.01	4.44	8.87	52.73
4	6.32df	39.70a	79.27ab	11.23	4.63	9.25	54.32
5	6.36abcd	37.39bde	75.01bde	11.79	4.18	8.38	50.38
6	6.36bce	39.30ab	78.36ab	11.72	4.58	9.12	53.01
13	6.41a	37.75cde	75.31cde	11.28	4.44	8.85	52.05
7	6.33cde	38.72abcd	77.27cde	11.36	4.58	9.13	53.65
8	6.38ab	38.73abcd	77.13abcd	11.60	4.65	9.26	52.94
9	6.36abcd	36.56d	72.83d	11.69	4.14	8.26	54.49
10	6.34bcde	38.46abc	76.68abc	11.31	4.20	8.38	53.63
SEM Species	0.036	1.871	3.682	0.538	0.384	0.759	0.919
Conc.							
0%	6.30d	39.79a	79.43a	11.98a	4.83a	9.64a	52.62
1%	6.33c	39.64a	79.05a	11.45b	4.53b	9.03b	52.85
5%	6.37b	38.19b	76.31b	11.19b	4.37bc	8.72bc	53.27
10%	6.40a	37.07c	73.92c	11.08b	4.16c	8.29c	54.10
SEM Conc.	0.034	1.786	3.502	0.403	0.338	0.666	0.564
P-values							
Species	<.0001	0.018	0.024	0.439	0.589	0.598	0.067
Conc.	<.0001	<.0001	<.0001	0.004	<.0001	<.0001	0.094
Species × Conc.	0.031	0.063	0.121	0.994	0.497	0.536	0.427

Abbrev; SEM; standard error of the mean, CH₄; methane, IVDMD; *In vitro* dry matter digestibility, Conc; concentration.

Table S18: The effect of incubating marine microalgae species at different concentrations on volatile fatty acids (VFA).

	Total VFA (mMol)	Acetate	Propionate	Butyrate	BCVFA	Propionate %	Acetate %	Butyrate %	BCVFA %	Ratio A:P
Species										
16	106	62.58	24.45	12.37a	3.99	23.09	59.23	11.48	3.73	2.58
18	107.85	63.48	25.01	12.47a	4.2	23.22	59.05	11.27	3.96	2.55
17	104.57	61.33	24.3	12.43a	3.85	23.34	58.76	11.72	3.62	2.53
14	109.7	64.44	25.45	12.86a	4.15	23.23	58.95	11.5	3.77	2.55
15	101.15	59.04	22.38	10.57b	4.69	21.95	58.52	10.05	4.76	2.68
SEM Treat	15.032	8.331	2.868	3.148	0.872	0.729	0.57	1.414	0.406	0.067
Conc.										
0%	113.88a	66.95a	26.87a	13.47a	3.79	23.49	59.05	11.53	3.45	2.53
1%	103.56b	60.32b	23.68b	11.59b	4.58	22.91	58.32	10.87	4.47	2.55
5%	104.05b	61.05b	23.89b	12.06b	4.02	23.02	58.81	11.39	3.8	2.57
10%	101.93b	60.38b	22.83b	11.44b	4.31	22.45	59.41	11.03	4.13	2.66
SEM Conc	14.987	8.31	2.843	3.141	0.86	0.712	0.542	1.404	0.373	0.065
P-value										
Species	0.217	0.075	0.1495	0.007	0.474	0.058	0.771	0.03	0.201	0.098
Conc.	0.007	0.002	0.0076	0.003	0.286	0.164	0.199	0.45	0.165	0.091
Species × Conc.	0.92	0.867	0.9321	0.858	0.935	0.942	0.771	0.854	0.889	0.798

Abbrev; SEM; standard error of the mean, Conc., concentration, VFA; Volatile fatty acids, BCVFA, Branched chain volatile fatty acids, Ratio A:P, Acetate to propionate ratio.

Table S19: The effect of incubating freshwater microalgae at different concentrations on volatile fatty acid production (VFA). Protected background IP Innovation Connection Grant.

Species	Total VFA (mM)	% of Total VFA				
		Acetate (A)	Propionate (P)	Butyrate	BCVFA	Ratio A:P
1	98.68bc	58.53	24.46	11.19	2.85	2.39
2	109.84ab	57.84	25.29	9.80	3.87	2.29
11	109.24ab	58.17	25.05	10.05	3.56	2.32
3	110.96ab	57.80	25.41	9.36	4.28	2.27
12	108.26a	58.18	25.12	9.86	3.74	2.32
4	103.51bc	58.22	25.06	9.90	3.72	2.32
5	89.45de	59.26	24.02	10.87	2.90	2.47
6	107.09abc	58.36	24.67	10.10	3.54	2.37
13	103.30bc	58.59	24.55	10.14	3.52	2.39
7	100.82cd	58.82	24.85	9.76	3.49	2.37
8	108.09abc	58.03	24.67	10.35	3.73	2.35
9	90.48de	58.52	23.99	11.00	3.42	2.44
10	87.44e	58.92	23.99	10.27	3.81	2.46
SEM Species	17.921	0.650	0.825	0.688	0.612	0.092
Conc.						
0%	107.23a	58.14	25.41a	10.23	3.15b	2.29c
1%	101.30b	58.19	24.7b	10.32	3.62a	2.36b
5%	100.83b	58.42	24.51bc	10.23	3.69a	2.38ab
10%	98.99b	58.86	24.18c	10.04	3.83a	2.43a
SEM Conc.	17.678	0.510	0.770	0.624	0.518	0.082
P-values						
Species	<0.001	0.541	0.181	0.143	0.362	0.246
Conc.	0.001	0.056	<0.001	0.564	0.022	<0.001
Species × Conc.	0.459	0.992	0.981	0.845	0.995	0.989

Abbrev; SEM; standard error of the mean, Conc., concentration, VFA; Volatile fatty acids, BCVFA, Branched chain volatile fatty acids, Ratio A:P, Acetate to propionate ratio.

Table S20: Species X Concentration effect on CH₄% of microalgae included in a maize and dairy cow concentrate diet.

Species	Algae %	CH ₄ %
Control	0	11.32bc
2	1	11.13bcd
	5	9.98d
	10	10.83bcd
	10	10.83bcd
4	1	11.39bc
	5	11.18bcd
	10	10.98bcd
	10	10.98bcd
12	1	11.35bc
	5	11.86b
	10	11.58bc
	10	11.58bc
19	1	12.00b
	5	11.75b
	10	10.37cd
	10	10.37cd
7	1	11.73b
	5	12.0b
	10	13.27a
	10	13.27a
SEM		0.543

P-value	0.045
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Figure S4: Principal coordinates plot (PCoA) of the Bray-Curtis dissimilarities for the bacterial solid-associated microbe (SAM) samples by Species 10 concentration. The percentage of variation explained by each principal coordinate are indicated on the axes.

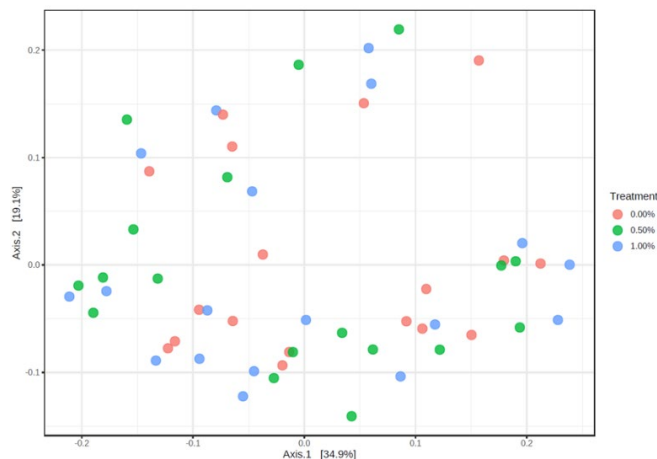


Figure S5: Principal coordinates plot (PCoA) of the Bray-Curtis dissimilarities for the bacterial Liquid-associated microbe (LAM) samples by Species 10 concentration. The percentage of variation explained by each principal coordinate are indicated on the axes.

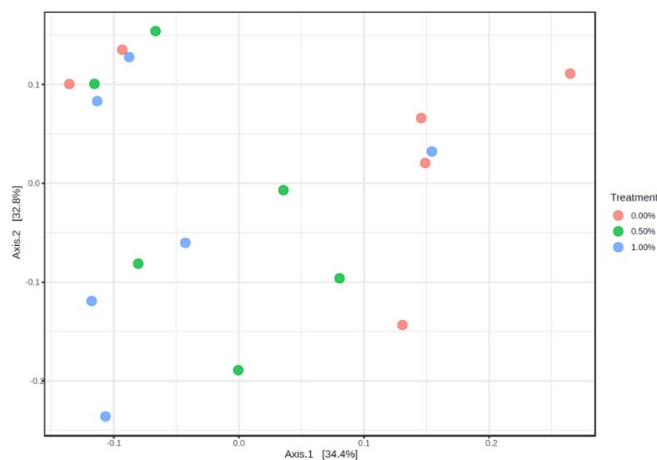


Table S21: Linear discriminant analysis of solid associated microbe (SAM) samples of Species 10 in RUSITEC round 1

Genus	0%	0.50%	1%	LDAscore	Pvalues	FDR
Selenomonas_1	2431.60	1764.60	1952.70	2.52	0.03	1.00
Campylobacter	101.78	62.67	70.78	1.31	0.04	1.00
Moryella	4.83	22.44	10.33	0.99	0.10	1.00
Staphylococcus	42.28	33.39	27.33	0.93	0.11	1.00
Fibrobacter	6121.30	4975.60	6474.40	2.88	0.13	1.00
Lachnoclostridium_5	14.83	5.28	11.89	0.76	0.13	1.00
U29_B03	12.61	13.78	16.72	0.49	0.17	1.00
Schwartzia	1107.90	882.00	1109.40	2.06	0.18	1.00

Weissella	0.56	0.83	2.83	0.33	0.23	1.00
Anaerovibrio	102.28	74.22	80.28	1.18	0.23	1.00
Dialister	158.00	294.94	287.94	1.84	0.25	1.00
Anaerobiospirillum	190.72	141.28	143.39	1.41	0.28	1.00

Table S22: Linear discriminant analysis effects size of liquid associated microbe (LAM) samples of Species 10 in RUSITEC round 1

Genus	0%	0.50%	1%	LDAscore	Pvalues	FDR
Anaerofustis	54.33	28.83	41.50	1.14	0.03	0.98
Methanimicrococcus	486.33	228.17	253.17	2.11	0.03	0.98
Alcanivorax	3.50	0.00	0.00	0.44	0.03	0.98
Syntrophococcus	21.17	9.33	26.67	0.99	0.06	0.98
Lachnospiraceae_AC2044_group	4.33	0.00	1.00	0.50	0.10	0.98
Lachnoclostridium_5	0.00	5.33	0.00	0.56	0.12	0.98
Succiniclasticum	215.83	178.67	161.67	1.45	0.13	0.98
Family_XIII_AD3011_group	372.67	256.33	375.83	1.78	0.13	0.98
Rikenellaceae_RC9_gut_group	10488.00	8526.30	7193.70	3.22	0.14	0.98
Methanobrevibacter	1019.00	663.67	974.00	2.25	0.15	0.98

Table S23: Pellet Formulation for lamb performance trial

	Treatment				
	T1	T2	T3	T4*	T5
Ingredients, %					
Barley	28.97	9.49	21.08	9.00	16.38
Sorghum	13.30	30.38	20.23	30.35	23.57
Wheat	25.20	25.43	25.24	25.29	25.27
Millrun	5.17	7.77	6.11	9.26	9.95
Canola meal	4.17	2.88	2.55	2.88	0.00
Soybean meal	1.81	1.97	2.10	1.92	2.71
Recycled vegetable oil	1.15	1.01	1.00	0.95	0.91
Molasses	1.84	2.01	1.97	1.01	1.59
Salt	1.28	1.21	1.25	1.26	1.31
Dried distillers' grain	14.96	15.04	14.97	15.15	15.02
Ammonium chloride	0.45	0.45	0.43	0.46	0.45
Acid buffer	1.18	1.03	1.20	1.04	1.00
Vitamin Premix ¹	0.10	0.10	0.10	0.10	0.10
Mineral Premix ²	0.20	0.20	0.20	0.20	0.20
Bovatec	0.02	0.02	0.02	0.02	0.02
Ammonium Sulphate	0.05	0.10	0.10	0.10	0.10
Calcium carbonate	0.00	0.15	0.15	0.25	0.10
Magnesium oxide	0.15	0.20	0.20	0.20	0.20
Freshwater Algae ³	0.00	0.56	1.10	0.20	0.00
Marine Algae ³	0.00	0.00	0.00	0.35	1.11

¹Vitamin premix = Riverina stock feed vitamin mix (0.1%, Vitamin A; 6,700 IU/kg, Vitamin D3; 800IU/kg, Vitamin E; 25mg/kg). ²Mineral Premix = Riverina stock feed mineral premix (0.1%), Toxin binder (0.1%). ³Microalgae supplied from Phytobloom, Necton. *Upon

completion of the trial it was revealed treatment four includes both fresh and marine microalgae due to a manufacturing error.

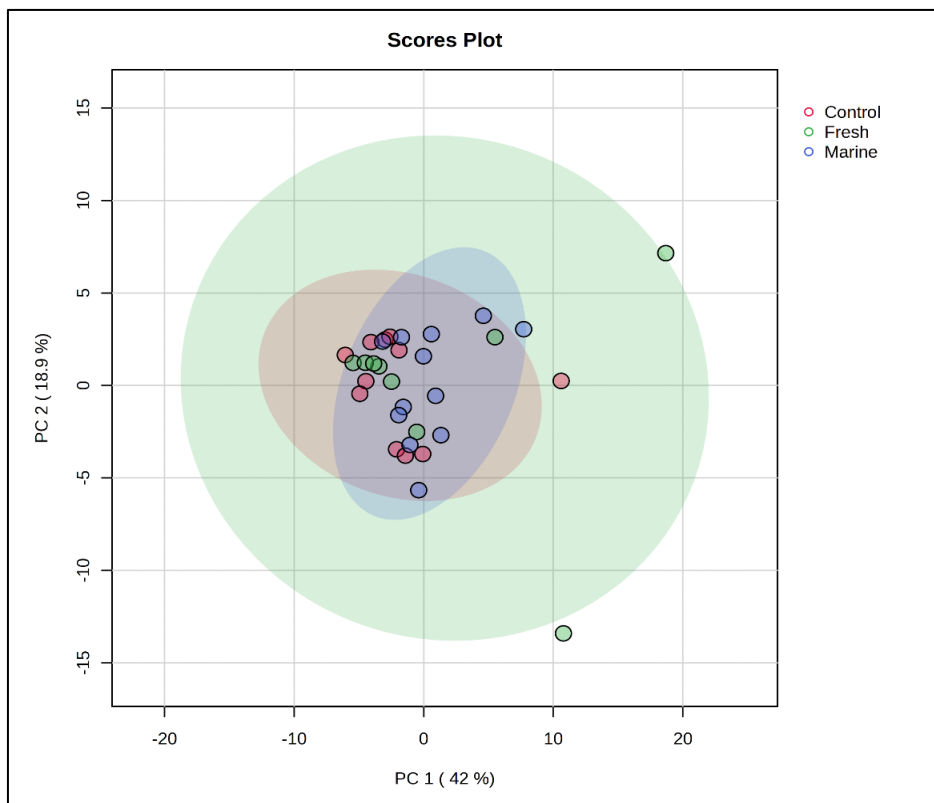


Figure S6: Principal component analysis of all metabolites for the rumen digesta, where each data point represents one lamb. The percentage of variation explained by each principal coordinate are indicated on the axes. PERMANOVA Analysis: $P = 0.629$.

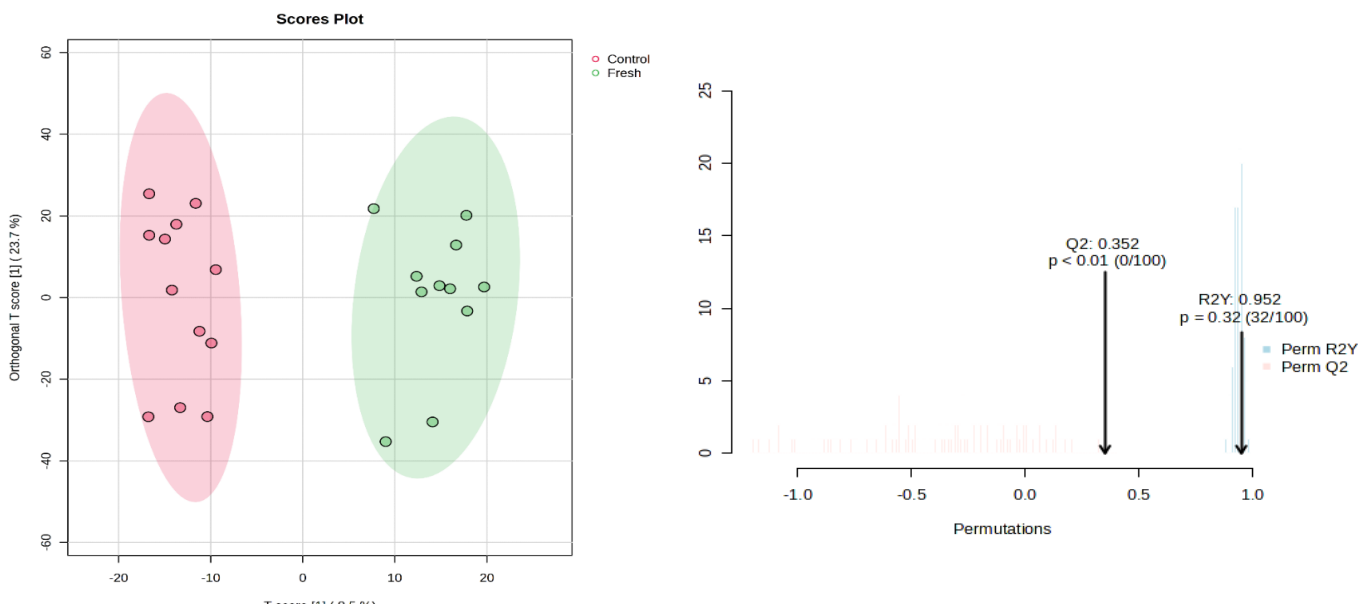


Figure S7: Orthogonal partial least squares discriminant analysis (OPLS-DA), conducted on the total liver metabolites for control and fresh fed lambs validates the diet-associated separation of metabolites.