# Final report

## Effect of Asparagopsis extract in a canola oil carrier for long-fed Wagyu cattle

Project code: P.PSH.1353

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Date published: 10-Jul-2023

PUBLISHED BY
Meat & Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

This is an MLA Donor Company funded project.

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

# Abstract

An extract of the seaweed *Asparagopsis* in a canola carrier oil has previously been demonstrated in MLA funded research to achieve reductions in methane ($CH_4$) yield of 54.5 – 95.0 % when included in short-fed finisher rations at 17 – 35 mg bromoform/kg feed dry matter (DM) over an 81 d feeding period. This project aimed to understand the $CH_4$ mitigation achievable in long-fed Wagyu production systems, and the implications for cattle feedlot performance, and carcase grading, residues and trained sensory panel evaluation, in the longest feeding trial of an *Asparagopsis* product reported to-date. Eighty F1 Wagyu steers were fed Wagyu Grower and Finisher diets in 8 pens of 10 head for a 275-day feeding period. Four pens were fed a control diet, and four pens were fed the same diet with part of the oil ingredient replaced by Asp-Oil supplying 25 mg bromoform/kg DM. Asp-Oil reduced $CH_4$ production by 28 % from Control over the whole feeding period, along with a 22% decrease in methane yield (g/kg DMI). Asp-Oil reduced feed intake consistently without improvement in feed efficiency. This resulted in persistently reduced liveweight, liveweight gain, and a trend to reduced carcase weight. Due to the decline in liveweight, methane intensity (g/kg LW gain) did not differ between treatments. Other carcase grading traits and trained sensory panel attributes were not affected by Asp-Oil supplementation. Consistent with other research, there was no bromoform detectible in meat or offal. Canola oil stabilised bromoform over the duration of use in this study with no volatilisation evident in vegetable oil tanks at the feedlot. Results of Meat Standards Australia (untrained sensory panel data) will be available in May, 2024 via MLA Project L.EQT.2306.

# Executive summary


## Background

The Wagyu sector is an increasingly important high value sector to the Australian beef industry and ways to stabilise and decrease emissions of the sector will be important in the coming years. An extract of the seaweed Asparagopsis in a canola carrier oil (Asp-Oil) has previously been demonstrated in MLA funded research to achieve reductions in methane ($CH_4$) yield of 54.5 – 95.0 % when included in short-fed finisher rations at 17 – 35 mg bromoform ($CHBr_3$)/kg feed dry matter (DM) over an 81-day feeding period. It is not known how the higher roughage content of Wagyu diets may interact with CHBr3 inclusion rate to inhibit CH4 production and yield. To collect information on cattle and carcase performance, larger experiments, testing conditions more relevant to commercial management are required. While all research to date has shown that $CHBr_3$ does not transfer to meat or reduce meat quality, it is necessary to determine whether this holds with long-term feeding of Asparagopsis.

## Objectives

The objectives of this project were to determine the effect of an Asparagopsis extract in a canola oil carrier (Asp-Oil) on:

- Enteric methane production of long-fed wagyu feedlot cattle (using C-Lock Greenfeed units as the emissions monitoring technique)
- Animal health and performance metrics
- Carcase grading characteristics
- Concentrations of bromoform, iodide and bromide in carcase, fat, liver and kidney depots
- Trained sensory panel evaluation including tenderness and flavour standards designed for AACo's Westholme product.

## Methodology

Eighty F1 Wagyu steers were fed Wagyu Grower and Finisher diets in 8 pens of 10 head for a 275-day feeding period. Four pens were fed a control diet, and four pens were fed the same diet with part of the oil ingredient replaced by Asp-Oil supplying 25 mg bromoform/kg feed dry matter (DM). Feed intake was measured individually with an autofeeder; $CH_4$ production was measured with Greenfeed Emissions Monitors. Liveweight was measured regularly throughout the feeding period. At sampling, carcases were graded and samples of the meat and offal tested for residues. The meat was tested by a trained sensory evaluation panel for eating quality attributes.

## Results/key findings

Feeding Asp-Oil at 25 mg/kg DM reduced $CH_4$ production by 28 % from Control over the whole feeding period, along with a 22% decrease in methane yield (g/kg DMI). Asp-Oil reduced feed intake consistently (by 7.93 % overall) without improvement in feed efficiency. This resulted in persistently reduced liveweight gain (by 9.38 % overall), and a trend to reduce carcase weight by 15.1 kg. Due to the decline in liveweight, methane intensity (g/kg LW gain) did not differ between treatments. Other carcase grading traits were not affected by Asp-Oil supplementation, and there was no effect on trained sensory panel attributes. Consistent with other research, there was no bromoform detectible in meat or offal. Canola oil stabilised bromoform over the duration of use in this study with no volatilisation evident in vegetable oil tanks at the feedlot.

## Benefits to industry

The research project has been one of the first commercialisation trials of *Asparagopsis* products conducted under Australian feedlot industry-relevant conditions, and is the largest-scale, and longest feeding experiments conducted to-date on *Asparagopsis* products. As such, it provides essential data to support the business case for adoption of Asp-Oil into long-fed feedlot programs, in terms of cattle productivity, and $CH_4$ abatement. It has highlighted that further research is required to understand the reasons for feed intake depression caused by Asp-Oil, and management strategies which could overcome this, so that Asp-Oil can be widely adopted with confidence.

## Future research and recommendations

Further research is required to refine feeding protocols for Asp-Oil supplements to overcome intake depression in Wagyu cattle. This may not be unique to *Asparagopsis*, and should consider not only dose titration and adaptation protocols, but potentially, co-feeding of hydrogen sinks or products that promote hydrogen utilisation in the rumen together with $CH_4$ inhibitors. Results of Meat Standards Australia (untrained sensory panel data) will be available in May, 2024 via MLA Project L.EQT.2306.

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# 1. Background

This project aims to determine the effect of Asparagopsis extract in a canola oil carrier for long-fed Wagyu cattle on enteric methane emissions. This includes evaluation of the product's effect on methane production, feedlot performance, animal health, carcase traits, food safety and eating quality.

Solutions to decrease methane emissions of beef cattle are important to align to CN30 initiative as outlined in the Red Meat 2030 strategic plan. The Wagyu sector is an increasingly important high value sector to the Australian beef industry and ways to stabilise and decrease emissions of the sector will be important in the coming years. No research has yet occurred on methane emissions of Wagyu cattle fed Australian feedlot diets. Wagyu cattle fed over long feeding periods (300 days +), typically have lower rates of average daily gain (0.9 to 1.1kg/d) and lower feed efficiency (F:G DM basis in range of 9.0 to 10:1) than shorter grain-fed market categories. Wagyu diets typically have increased roughage (in range of 15 to 20% of dry matter as roughage) than conventional feedlot finisher diets (7-10% roughage), theoretically predisposing them to higher levels of enteric methane production per unit of weight.

*Asparagopsis* contains a variety of halogenated compounds that inhibit methane production in the rumen. The primary candidate is bromoform, a volatile compound that currently requires freeze drying to ensure its stability. In high concentrate feedlot finisher diets, methane inhibition may occur up to 98% when supplied with 25mg/kg dry matter of bromoform active from freeze dried (FD) *Asparagopsis* (Kinley *et al.* 2020). FD *Asparagopsis* inhibition of methane in higher roughage North American grower diets may approach 60 % based on recent Californian research (Roque *et al.* 2021).

The economic and energetic costs, and nutritional, logistical and stability questions surrounding feeding *Asparagopsis* biomass in freeze-dried (FD) form have been potentially overcome by the development of an oil-based *Asparagopsis* product (Asp-Oil): SeaForest, a Tasmanian seaweed producer has recently produced an Asparagopsis extract in a canola oil carrier with low levels of iodine. When included in a short-fed finisher ration, Asp-Oil resulted in reductions in $CH_4$ production of 97.8 % when fed to steers in respiration chambers at 35 mg $CHBr_3$/kg feed dry matter (DM) and 64.4 % when fed at 17 mg $CHBr_3$/kg DM. Methane yield was similarly decreased by 95.0 % and 54.5 %, respectively (MLA Final Report P.PSH.1351, Cowley *et al.* (2023)).

The business case for adoption of *Asparagopsis* will depend not only on methane inhibition, but also effects on cattle performance, carcase value and feed cost. Most published research on feeding *Asparagopsis* to beef cattle has been conducted with small numbers of cattle (~20 total) in individual pens, and the longest feeding period tested to date is 147 days (Roque *et al.* 2021). To collect information on cattle and carcase performance, larger experiments, testing conditions more relevant to commercial management are required. While all research to date has shown that $CHBr_3$ does not transfer to meat or reduce meat quality, it is necessary to determine whether this holds with long-term feeding of *Asparagopsis.* This project will generate data for the wider red meat industry in long-fed wagyu cattle to build confidence and stimulate adoption of the oil product if favourable efficacy, animal performance, safety and trained sensory panel evaluation is determined.

The results of the research will help inform decision making processes on scaling up the use of Asparagopsis across beef cattle businesses and to provide the red-meat industry with new information on the application of the technology in commercial operations.

# 2. Objectives

The objectives of this project were to determine the effect of an *Asparagopsis* extract in a canola oil carrier (Asp-Oil) on:

- Enteric methane production of long-fed wagyu feedlot cattle (using C-Lock Greenfeed units as the emissions monitoring technique)
- Animal health and performance metrics
- Carcase grading characteristics
- Concentrations of bromoform, iodide and bromide in carcase, fat, liver and kidney depots
- Trained sensory panel evaluation including tenderness and flavour standards designed for AACo's Westholme product

These objectives were tested in a replicated feedlot trial using individual autofeeders and Greenfeed Emissions Monitoring units, with 80 F1 Wagyu steers. An additional untrained consumer analysis of eating quality will be reported separately in L.EQT.2306 Effect of red *Asparagopsis* oil on the eating quality of long-fed cattle by May, 2024.

# 3. Methodology

## 3.1 Experimental Design

The effect of Asp-Oil on Wagyu cattle emissions and feedlot performance was tested in a randomised block design with 2 dietary treatments (Asp-Oil or Control) and 4 initial cattle liveweight blocks, using 80 F1 Wagyu x *Bos indicus* steers, for 275 days. All procedures were approved by the University of New England Animal Ethics Committee (Approval number ARA22-014).

The experimental design was a Randomised Complete Block Design with 2 treatments and 4 blocks per treatment, blocked in 4 blocks of 2 pens (location x weight), running North to South. Each pen contained 10 AACo F1 Wagyu steers. The treatment diets were a modification of the AACo 300-day Wagyu feeding program without (Control) and with *Asparagopsis* oil (Asp-Oil) replacing part of the canola oil component of the total mixed ration (TMR).

## 3.2 Animals and husbandry

Five hundred Wagyu steers (sourced from 3 AACo breeding properties) were backgrounded at Goonoo Feedlot (Qld). All cattle were inducted into the feedlot into low density pens for 43 days and onto a silage-based ration. Only animals that were eating the ration in these pens were considered for the trial. Eighty steers were selected from the group of 500. On d –7 before experimental adaptation commenced, the 80 head were transported to the University of New England's Tullimba Research Feedlot, near Armidale NSW, and placed into two large pens in the feedlot. They were fed a pre-starter ration *ad libitum* for 3 days while re-hydrating and equalizing gut fill. On d -3, they were inducted, weighed and treated with an oral anthelmintic ("Flukazole C plus Selenium": Virbac, Milperra NSW Australia). At induction, the cattle were stratified into 4 weight blocks, each of 20 steers. Then, the 20 cattle in each block were randomly assigned to one of the two experimental diets: Control or *Asparagopsis*-oil supplemented (n = 10 animals per treatment). From d -3 to 18 day on feed, treatments were housed adjacently in two 40-head pens (one per treatment) and fed in a single bunk per pen, until commencing the grower ration. This was to facilitate

feeding of a high roughage transition diets that would fail to flow through auto-feeders in the experimental pens.

On d 18, the cattle completed the adaptation to the treatment diets, were re-drafted into their experimental group and pens (10-head per pen), and commenced the experimental period. The 4 blocks of paired Control and Asp-Oil treatments were randomly located within a contiguous group of 8 pens in the feedlot, and the location of each treatment within a contiguous blocked pair of treatments was also randomised, generating a 'home pen' for each animal. The pens were orientated with a slope 3˚ West to East (from front of pen to back), along the row of pens. Each pen provided 50 $m^2$/head pen space, and was fitted with a reticulated water trough of 150 cm length. All animals were checked for health daily.

## 3.3 Feeding and dietary treatments

Three days after induction, the steers commenced their adaptation to the AACo Grower total mixed ration (TMR) through a 17-day, 5-ration transition (Table 1). The steers were maintained on the Grower TMR for 132 days (until d150 post-induction), then were adapted to the AACo Finisher ration over 3 days. They were fed the finisher TMR until the day of feedlot dispatch (120 days total). All diets in the feeding program contained canola oil, at the same inclusion rates across the two treatment diets. Total oil inclusion was stepped up with the preliminary transition program, and *Asparagopsis* oil was included for the Asp-Oil Treatment from d 7 (Table 1). The final *Asparagopsis* oil inclusion rate was achieved in the Grower TMR and maintained through the Finisher TMR (delivering a formulated 25 mg bromoform ($CHBr_3$)/kg feed dry matter (DM)).

The dietary oil was a mix of test oil – a solvent-extracted canola oil base, supplied by Sea Forest Ltd, Triabunna, Tasmania – and diluent oil, also a solvent-extracted canola oil (supplied by Purkiss Rural, Armidale, NSW). The test oil in the Asp-Oil treatment was a stock solution of *Asparagopsis* extract in the canola oil carrier, supplying 3.47 g $CHBr_3$, 73 mg iodine and 24.4 g bromide per kg canola oil in the stock solution. This was diluted 0.6:1.0 with the diluent oil, to achieve a formulated $CHBr_3$ content of 1.30 g/kg dietary oil in the test oil. The Control test oil was a mix in the same ratios of the unmodified stock oil base and the diluent oil. The dietary oils were mixed on d 0 and d 180. The dietary oils were stored outside in pallet tanks covered by a reflective insulation jacket, and pumped into the feed mixer. The oil in the pallet tanks was mixed by recirculation for 15 minutes (sufficient for the entire volume of the tank to circulate at least once) once every week. The Asp-Oil was sampled from the pallet tanks on 3 occasions and analysed for $CHBr_3$ content using a headspace gas extraction protocol (Analytical Services Tasmania, New Town, Tasmania). $CHBr_3$ content was consistent over the duration of the experiment (1.26 ± 0.04 g $CHBr_3$/kg dietary oil, Appendix 2).

*Table 1. Experimental feeding program, and program of adaptation of Asparagopsis-supplemented treatment group to final experimental bromoform ($CHBr_3$) inclusion*

| Diet | Feeding period | Formulated $CHBr_3$ inclusion rate in Asp-Oil treatment (g $CHBr_3$/kg DM) |
|---|---|---|
| Starter ration (no oil) | 4 days (d 0 – 4) | 0 |
| AC1 ration (control oil only) | 3 days (d 5 – 7) | 0 |
| AC1 ration (treatment oils[1]) | 4 days (d 7 – 10) | 6.8 |
| AC2 ration (treatment oils[1]) | 4 days (d 11 – 14) | 13.3 |
| AC3 ration (treatment oils[1]) | 3 days (d 14 – 17) | 19.3 |
| Grower ration (treatment oils[1]) | 132 days (d 18 – 150) | 25.2 |
| Finisher ration[2] (treatment oils[1]) | 138 days (d 151 – 289) | 25.0 |

[1]Treatment oils were: Control – canola oil; Asp: canola oil mixed with *Asparagopsis* extract to achieve a $CHBr_3$ content of 1.30 g/kg canola oil; [2]Grower and finisher diets were titrated at 50% of each diet on days 151 - 153.

*Table 2. Formulated ingredient composition and analysed nutrient composition of Grower and Finisher diets fed to F1 Wagyu steers.*

| | Diet (days fed) | |
|---|---|---|
| Item | Grower (18 - 150) | Finisher (151 - 275) |
| Ingredient, % as fed | | |
| Tempered Barley | 69.4 | 77.9 |
| Cereal hay | 7.8 | 3.5 |
| Barley straw | 7.9 | 3.5 |
| Whole cottonseed | 8.0 | 8.0 |
| Liquid supplement | 5.5 | 5.5 |
| Canola oil blend | 1.6 | 1.6 |
| Analysed nutrient composition (DM-basis) | | |
| Dry Matter (%) | 81.7 | 81.5 |
| Organic Matter, % DM | 93.9 | 94.4 |
| Ash, % DM | 6.1 | 5.7 |
| Crude Protein, % DM | 12.2 | 11.6 |
| Fat, % DM | 5.8 | 5.6 |
| NDF, % DM | 31.1 | 26.8 |
| ADF, % DM | 15.2 | 12.5 |
| Starch, % DM | 42.0 | 44.9 |
| ME, MJ/kg DM | 13.2 | 13.2 |
| Ca, % DM | 0.9 | 1.0 |
| P, % DM | 0.3 | 0.3 |
| K, % DM | 0.8 | 0.7 |
| Mg, % DM | 0.2 | 0.2 |
| Zn, ppm | 117 | 125 |
| Monensin (formulated), ppm | 22.5 | 22.8 |

To achieve sufficient batch volume for homogenous mixing, the TMRs were mixed in a single batch once every 2 days (alternate days for Control and Asp-Oil diets) for a period of 20 minutes, in an NDE vertical mixer wagon with 1.0 kg scale intervals (NDECo, Sioux Falls, SD), and fed out daily, alternating daily between treatments at either 0715 or 0815 h. This protocol meant that for each diet, a freshly mixed ration was fed every second day, and on the alternate days, a 23-hour-old ration was fed out, before mixing of the other treatment ration. The entire mixer was emptied between mixing of treatment rations. No flush was implemented. A pre-experimental mixer test, using the grower ration, was conducted to determine the mixing time required to achieve a coefficient of variation of < 5% for ether extract content over 10 grab samples collected across the entire feed-out of the mixer. The mixer scale was assessed twice weekly using

a weighbridge as a reference weight and recalibrating if the difference between the net weight and scale display exceeded the sensitivity of the weighbridge (20 kg).

A representative mixing batch sample, bulked from a grab sample collected from each pen within a treatment (n = 4), was collected for each TMR batch mixed, frozen immediately, and then bulked weekly within a diet. Dry matter content of was determined on a single ~150 g sub-sample of the weekly bulked sample by oven drying at 65 ºC, until there was no change in weight. For mixed, diets, a further ~150g subsample was bulked weekly and analysed for content of crude protein (AOAC Method 2001.11), acid detergent fibre (ADF, AAFCO Method 008.08), neutral detergent fibre (NDF, NFTA Method 2.2.2.5), organic matter (ISO 5984:2002(E)), ether extract (AFIA Method 1.14R), starch (AOAC Method 996.11), water soluble carbohydrates (AFIA Method 1.11A), digestible dry and organic matter (AFIA Method 1.7R) (NSW DPI Laboratory Services - Wagga Wagga Chemistry Services Laboratory, Wagga Wagga NSW; Table 1). Metabolizable energy (ME) content was calculated according to the equations for grains and concentrates of AFIA Method 2.2R (MAFF, 1990).

Each pen contained one custom-designed and built automatic feeder (capacity 140 kg Grower ration), fitted with load cells (with scale intervals of 0.01 kg) and a RFID-sensor to identify individual steers and record the time, weight of feed removed and duration of every feeding bout individually (Bindon 2001). The autofeeder scales were verified and then calibrated to a 0.00 kg empty weight and 50.0 kg calibration weight, monthly. Feed was available to the steers *ad libitum*, but daily feed addition to the feeders was managed so that feed remaining in the autofeeder each morning was minimal. Feed remaining from the previous day was not removed before daily feed addition.

## 3.4 Measurement of cattle performance and methane emissions

Liveweight was measured (without withholding feed) on d -3, d18 (at the commencement of the grower ration and allocation to experimental pens), d98 (grower ration), d150 (at the end of the grower ration), and d199 (finisher ration) on a Gallagher cattle scale calibrated with 600 kg of certified weights before each weigh-day. A final shrunk-liveweight was measured immediately after stunning (d276, before ex-sanguination and evisceration) at the processing plant, and a 5 % shrink value applied to calculate final liveweight on d275.

Methane was measured in individual steers using 4 out of a pool of 5 Greenfeed Emissions Monitoring (GEM) units (C-Lock, Cedar Rapids, SD). The 4 GEM units were rotated around pens, so that each pen was fitted with a GEM on a 3-weeks-on, 3-weeks-off rotation for the duration of the experiment. The GEM units were rotated across all pens to account for any effect of GEM unit. A millmix-based commercial pellet (Pryde's Easiride, 11.6 MJ DE, 12.0 % CP) was used as a lure to attract the steers to visit the GEM. To increase novelty and attractiveness of this lure, from d150 onwards, the pellets were sprayed with an aniseed flavour (Fluidarom 1957; Norel Animal Nutrition, Madrid, Spain). The GEM units were set to allow 6 drops of pellets per visit, 35 s apart, with 4 intervals of 4 h between allowable visits, and a maximum of 5 visits per day.

## 3.5 Slaughter and carcase measurements

All steers were transported to slaughter on the same day (d275) in two trucks, penned such that each truck contained 2 pens of each treatment, and each deck contained one treatment only, alternated between trucks so that each treatment had 2 pens on a top deck and 2 pens on a bottom deck. The abattoir was 450 km from the research feedlot. The steers were held overnight and slaughtered in pen groups on d276, such that the kill order alternated between 2 pens of Control, followed by 2 pens of Asp-Oil steers.

Immediately post slaughter and dressing, hot standard carcass weight (HSCW, kg) was determined according to AUS-MEAT carcass standards (AUS-MEAT Limited 2005). Post-chilling (at 2 °C), the carcases were electrically stimulated with an immobiliser and hide puller. The carcases were then evaluated between the 12/13$^{th}$ rib by a single expert Meat Standards Australia (MSA) grader (Meat Standards Australia 2007) for hump height (mm); fat colour; meat colour; MSA marbling score; rib fat depth (mm); ossification score; ultimate pH (pHu); eye muscle area (EMA, $cm^2$). Hump heights were measured using a 5 mm graduated metal ruler (Meat Standards Australia 2007). Fat colours were scored against the AUS-MEAT fat colour reference standards against the intermuscular fat positioned laterally to the rib eye muscle using a 0 (white) to 9 (deep yellow) scale (AUS-MEAT Limited 2005). Meat colour was scored using the AUS-MEAT colour reference standards on the bloomed rib eye muscle (*longissimus thoracis et lumborum*) using a 1 (light pink) to 7 (deep purple) scale (AUS-MEAT Limited 2005). MSA marbling score was evaluated in chilled carcases against MSA reference standards at the quartering site and was estimated by scoring the amount and distribution of intramuscular fat deposited between individual fibres, on a scale ranging from 100 to 1100 (Romans and Ziegler 1985; AUS-MEAT Limited 2005; Meat Standards Australia 2007). Rib fat depth was measured using a graduated metal ruler, at the quartering site positioned between the 12th and 13th rib (AUS-MEAT Limited 2005). Ossification score was determined a scale between 100 and 590 in accordance with the guidelines described by the United States Department of Agriculture (Romans and Ziegler 1985). Ultimate pH and loin temperature were measured in the *l. thoracis et lumborum* at the time of carcase grading. Carcass temperature and pH were measured using an MSA approved temperature and pH probes (TPS MC-80 or TPS WP-80M pH Meter, TPS Pty Ltd., Springwood, Brisbane, Qld, 4127, Australia). Eye muscle area was determined for each carcase by measuring the *l. thoracis et lumborum* at the quartering site using a standardised grid (AUS-MEAT Limited 2005).

150 g of chuck meat, kidney, liver and fat were collected after boning and subsampled into 50 g aquilots for analysis of residues of $CHBr_3$, Bromide ($Br^-$), and Iodide ($I^-$). All samples were stored at -20 °C for 24 h then shipped frozen for analysis. Bromide and iodide residues were analysed by ICP-MS (Symbio Laboratories, Eight Mile Plains, Queensland), and $CHBr_3$ residues were measured using a headspace gas extraction protocol (Analytical Services Tasmania, New Town, Tasmania).

## 3.6 Trained taste panel assessment of eating quality

Two cuts from each carcase (cube roll, *Longissimus dorsi*, *Spinalis dorsi* and *Longissimus thoracis;* and rosbiff, *Gluteus medius*) were assessed using conventional descriptive profiling techniques with a panel of experienced trained assessors rating 27 traits that covered a wide spectrum of aroma, flavour, texture and mouthfeel properties. Samples were prepared for tasting according to a strict sample preparation and cooking methodology and were immediately presented in a randomised presentation design to panellists in a controlled environment via purpose built sensory booths. Panel scores (0-100) for each of the 27 defined sensory attributes were collected for each sample on tablets using the software Redjade. Refer to Appendix 5 for a full trained sensory sensory evaluation report including detailed methodology. Cuts were also taken for Meat Standards Australia (untrained sensory panel data) with results available in May, 2024 via MLA Project L.EQT.2306.

## 3.7 Data management

Four steers (1 steer from Block 1 Asp-Oil treatment, 2 steers from Block 1 Control treatment, and 1 steer from Block 2 Asp-Oil treatment) were removed from the trial within the first 4 days of commencing in the

autofeeder pens, due to failure to adapt to the autofeeders and thus low, or fluctuating intakes causing acidosis. These steers were not replaced and all of their data were excluded from the analysed dataset.

Records of each feeding bout from the autofeeder in each pen (N = 469,581) were downloaded through a FileZilla® file transfer protocol (FTP) platform. Each file consisted of eartag (the individual steer electronic identity number, EID), entry time, entry weight, leave time, leave weight, session time, and session weight. The entry and leave times were the times the animal entered and left the auto-feeder, while the entry and leave weights were the initial and final weights of the feed in the autofeeder after the entry and leave times are recorded. The session time was the time an individual animal spent for every bout (duration) (leave time – entry time), while the session time was the feed intake for every bout (entry weight - leave weight). The individual intake and feeding rates were quantified within the R statistics environment (R Core Team 2020). The 258 files of feed intake were grouped into one folder per pen. The files were merged into one file with a new column added to represent day 18 to day 275 (03-05-2022 – 15-01-2023). Visit frequency (VF) of all individuals (*eartag*) within a pen was tabulated to exclude EID numbers with low VF (<1000, i.e. test tags) using the *dplyr* package (Wickham *et al.* 2022).

A small number of systematic errors in the individual feeding bout data resulted in loss or corruption of all data from an individual autofeeder for periods of several hours to several days; where this occurred, data was excluded for the entirety of any affected 24-hour period. 1.89 % of day-total feed intakes for individual steers were excluded or missing from the final analysed dataset as a result. Feeding rate per feeding bout was calculated by dividing feed intake (g) and feeding duration (s). Feed intake, duration, and daily feeding rates were then summarised using the dplyr package and aggregate function of base R.

Daily intake of TMR ration from autofeeders was calculated by summing the weight of all individual feeding bouts (*k*) of an individual steer (*i*) in a calendar day (*j*).

$$Ration\ intake_{ij}, kg\ DM = \sum_{j=1}^{j} DM\ weight\ bout_{ij}$$

Total DM intake for an individual steer was calculated as the sum of TMR DM intake from autofeeders plus the sum of DM intake from the GEM units for the same calendar day, and this value was used for subsequent calculation of Gain:Feed and $CH_4$ yield. To account for missing days of total DM intake data for individual steers Gain:Feed was calculated for each period (*l*) using the average total DM intake for the period, weighted by period length (*P*, days), and liveweight change over the period.

$$Gain{:}Feed_{il} = \frac{LW_l - LW_{l-1}}{\sum_{i=1}^{i} Daily\ total\ DM\ intake, kg_{ij}/n_{ij} \times Period\ duration, days_l}$$

Intake for a period (*l*) weighted for liveweight was calculated using the same calculation of period intake divided by the mean liveweight for the period

$$Intake, g/kg\ liveweight_{il} = \frac{\sum_{i=1}^{i} Daily\ total\ DM\ intake, kg_j/n_j \times P_l}{(LW_l - LW_{l-1})/2}$$

Visitation to the GEM units was highly variable among steers (Table 3). Methane production (g $CH_4$/day) was predicted for each visit (*m*) to the GEM unit by an individual steer exceeding 2 minutes duration, using the proprietary algorithms of the manufacturer. The predicted $CH_4$ production measured by GEM Unit 31 included some values that were unexpectedly high for the period d 180 – 214, and so all data from this unit were excluded from the analysis of methane emissions. For analysis of $CH_4$ production, these individual visit predictions of $CH_4$ production were considered as individual observations within a replicate pen. For analysis of $CH_4$ yield, individual visit predictions of $CH_4$ production were summarised as mean estimate of

daily $CH_4$ production for each steer (*i*) visiting the GEM on a day (*j*). Methane yield ($Y_m$, g $CH_4$/kg DM intake) was then calculated by dividing this calculated mean $CH_4$ production for a day by the total daily feed intake for each steer for the same day.

$$Ym_{ij} = \frac{\frac{1}{n_{ml}}\sum_{m=1} methane\ production_{ij}}{Total\ intake, DM_{ij}}$$

Methane intensity (g $CH_4$/kg liveweight gain) was calculated by the mean $CH_4$ production for a period (*l*) weighted for the number of days in a period (*p*), divided by the liveweight gain for the period:

$$Methane\ intensity, g/kg\ liveweight_{il} = \frac{\frac{1}{n_{ml}}\sum_{m=1} methane\ production_{il}\ \times p_l}{LW_l - LW_{l-1}}$$

Because $CH_4$ intensity on a HSCW-basis will also include emissions from the period between birth and feedlot entry (which were not measured as part of this project), effects of *Asparagopsis*-supplementation during the grain-feeding period on this trait are reported as difference from control only.

## 3.8 Statistical analysis

All data was analysed in R (R Core Team 2020), and in all analyses pen was considered the experimental unit. Several models of variance-covariance were tested for each response, and the model of best fit chosen by the lowest Akaike Information Criterion value. Data from single observations (i.e. liveweights, average daily gain, gain:feed, $CH_4$ intensity, carcass measurements) were analysed in a linear mixed model (*lme4*, Bates *et al.* (2015)) with Treatment and Block as fixed effects and Pen within Block as a random effect, and an unstructured model of variance-covariance. Repeated measures of intake from the autofeeders were analysed in a mixed effects linear model (*lme4*, Bates *et al.* (2015)) with Treatment, Block and Day as fixed effects, and steers nested within Pens as random effects and a first-order autocorrelation structure of Steers within Pens as a random effect. Methane production and yield estimates by the GEM units from individual steers were considered as observations within a pen, and analysed in a mixed effects linear model with Treatment, Block and GEM unit as fixed effects, Day as a random effect and a first-order autocorrelation structure of Steers within Pens as a random effect. The interaction of Day and Treatment on methane yield was tested as a fixed effect in this model, but was not significant, and not able to converge for methane production, and so was discarded from the final model. Since liveweight varied between Treatments at the start of the experimental period (d 18), d 18 liveweight was tested for effect on intercepts and slopes of methane emissions, but was not significant, and so was discarded from the final model.

Data from single observations (i.e. sensory attribute scores) were analysed using a linear mixed model ANOVA (*lme4*) with Treatment, Cut, Marbling and Block as fixed effects and Pens and Animals nested within Pen as random effects.

*Table 3. Number of observations of methane ($CH_4$) production from individual F1 Wagyu steer visits to the Greenfeed Emissions (GEM) units (total visit observations for a period, used in the estimate of $CH_4$ production), unique steers recorded during a period, median number of observations (visits) per steer per period, and total number of daily mean observations of $CH_4$ production (steers observed at the GEM units at least once within a day in the period, used in the estimate of $CH_4$ yield and intensity).*

| Period | Control | Asp-Oil |
|---|---|---|
| d 0 – 98[1] | | |
| Total visit observations, n | 985 | 853 |
| Unique steers recorded, n | 32 | 36 |
| Median number of observations/steer.period (min, max) , n | 21.0 (1, 39) | 11.5 (1, 107) |
| Total daily mean observations, n | 399 | 390 |
| d 99 – 150[1] | | |
| Total visit observations, n | 241 | 330 |
| Unique steers recorded, n | 20 | 19 |
| Median number of observations/steer.period (min, max) , n | 7.0 (1, 39) | 11.0 (1, 80) |
| Total daily mean observations, n | 155 | 158 |
| d 151 – 199[2] | | |
| Total visit observations, n | 148 | 202 |
| Unique steers recorded, n | 23 | 22 |
| Median number of observations/steer.period (min, max) , n | 5.0 (1, 35) | 7.5 (1, 43) |
| Total daily mean observations, n | 101 | 93 |
| d 200 – 275[2,3] | | |
| Total visit observations, n | 269 | 303 |
| Unique steers recorded, n | 31 | 23 |
| Median number of observations/steer.period (min, max) , n | 8.0 (1, 49) | 13.0 (1, 39) |
| Total daily mean observations, n | 177 | 163 |

# 4. Results

## 4.1 Feedlot Performance

There was a consistent effect of Asp-Oil on cumulative intake of the TMR (and hence total DM intake) in all periods of the feeding period. Associated with this depression in DM intake was a reduction in cumulative average daily gain in periods d18 – 98, 18 – 150, and 18 – 275 ($P$ <0.05) such that there was no effect on cumulative conversion of gain to feed (G:F) in any period (Table 4). Feed intake and liveweight gain were reduced in the Asp-Oil treatment group during the adaptation period (Appendix 2), so that although the treatment groups were balanced for liveweight on allocation to treatments at d -3, liveweight on commencement of the experimental period (d18) differed between groups (Table 4). Total dry matter intake when expressed on a g DM/kg LW basis was 7.74 % lower overall when fed Asp-Oil. Despite a consistently lower ration intake, there was no statistically significant effect of Asp-Oil on eating time or eating rate (Table 5).

## 4.2 Methane emissions

Over the 275-day feeding period, Control F1 Wagyu steers produced 138.6 ± 6.50 g $CH_4$/day, or 38.12 kg of $CH_4$ for the 275-d feeding period, and 156.5 ± 14.16 g $CH_4$/kg liveweight (Table 6). For the Grower period, Control $CH_4$ emissions were 137.1 ± 6.14 g/day and 14.6 ± 0.986 g/kg DMI, and for the Finisher period, 154.0 ± 5.57 g/day and 14.6 ± 0.75 g/kg DMI. Supplementation with Asp-Oil reduced $CH_4$ production by 28.0 % from Control levels overall ($P$ = 0.004, Table 6). When considered against the reduced feed intake and liveweight gain of Asp-Oil treated steers, $CH_4$ yield overall was reduced by 22.0 %, and there was no effect of Asp-Oil supplementation on $CH_4$ intensity/kg liveweight gain. There was no interaction of treatment with time ($P$ = 0.193, Appendix 3). On a HSCW basis, $CH_4$ carcase intensity did not differ between the two groups (Asp-Oil – Control = -27.2 g $CH_4$ /kg HSCW, $P$ = 0.256). During all measurement periods, $CH_4$ production was significantly affected by GEM unit ($P$ < 0.001).

## 4.3 Carcase grading

Consistent with the differences in growth rate and feedlot data presented from the feedlot the control carcases trended heavier than the treatment carcases ($P$ = 0.094, Table 7). There were no significant differences for any of the of the other traits apart from pH for which there was a trend for Asp-Oil animals to have a slightly higher $pH_u$ (Table 7).

## 4.4 Carcase residues

$CHBr_3$ residues were below detectable limits in all carcases at the liver, kidney, fat and flank (Table 8). Bromide concentrations were below detectable limits in all samples of meat (Table 9). Bromide residues accumulated in Asp-Oil kidneys significantly more than in control kidneys ($P$ = 0.022, Table 9). Iodide concentrations were below detectable limits in most samples of meat, liver and fat, and did not differ from control in kidney (Table 10).

## 4.5 Trained taste panel eating quality

By conventional sensory descriptive profiling a trained taste panel were able to clearly distinguish between samples that differed in many factors that are known to influence eating quality of beef (cut, marble score,

Appendix 4). However, no significant difference were detected between the treatment and control animals for any of the 27 sensory properties scored (Table 11).

*Table 4. Least-squares means and SE of liveweight (LW), cumulative average daily gain, cumulative dry matter intake and cumulative gain:feed of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM, or a control canola oil.*

| | Control | Asp-Oil | SE | *P* |
|---|---|---|---|---|
| Liveweight, kg | | | | |
| d -3 | 449.6 | 447.9 | 1.47 | 0.417 |
| d 18 | 485.2 | 477.7 | 2.63 | 0.048 |
| d 98[1] | 571.1 | 547.7 | 4.29 | < 0.001 |
| d 150[1] | 625.4 | 599.6 | 5.52 | 0.001 |
| d 199[2] | 658.5 | 639.3 | 6.02 | 0.030 |
| d 275[2,3] | 732.2 | 701.5 | 6.83 | 0.004 |
| Average daily gain, kg/hd.d | | | | |
| d 18 – 98[1] | 1.06 | 0.87 | 0.041 | 0.002 |
| d 18 – 150[1] | 1.05 | 0.92 | 0.337 | 0.006 |
| d 18 – 199[1,2] | 0.96 | 0.89 | 0.280 | 0.099 |
| d 18 – 275[1,2] | 0.96 | 0.87 | 0.025 | 0.007 |
| Ration intake, kg DM/hd.d | | | | |
| d 18 – 98[1] | 9.90 | 8.82 | 0.293 | 0.009 |
| d 18 – 150[1] | 9.67 | 8.75 | 0.227 | 0.004 |
| d 18 – 199[1,2] | 9.30 | 8.49 | 0.169 | 0.001 |
| d 18 – 275[1,2] | 9.11 | 8.35 | 0.232 | 0.021 |
| Total intake[4], kg DM/hd.d | | | | |
| d 18 – 98[1] | 10.03 | 8.94 | 0.343 | 0.025 |
| d 18 – 150[1] | 9.79 | 8.85 | 0.270 | 0.015 |
| d 18 – 199[1,2] | 9.41 | 8.59 | 0.171 | 0.001 |
| d 18 – 275[1,2] | 9.21 | 8.48 | 0.248 | 0.037 |
| Total intake[4], g DM/kg LW | | | | |
| d 18 – 98[1] | 18.7 | 16.9 | 0.30 | < 0.001 |
| d 18 – 150[1] | 17.4 | 16.1 | 0.25 | < 0.001 |
| d 18 – 199[1,2] | 16.2 | 14.9 | 0.24 | < 0.001 |
| d 18 – 275[1,2] | 15.5 | 14.3 | 0.22 | < 0.001 |
| Gain:feed[5], kg/kg DM | | | | |
| d 18 – 98[1] | 0.106 | 0.099 | 0.0036 | 0.200 |
| d 18 – 150[1] | 0.108 | 0.105 | 0.0026 | 0.424 |
| d 18 – 199[1,2] | 0.103 | 0.107 | 0.0023 | 0.200 |
| d 18 – 275[1,2] | 0.105 | 0.106 | 0.0019 | 0.681 |

[1]Grower diet, d18 – 150; [2]Finisher diet, d151 – 275; [3]Shrunk liveweight measured after stunning and before exsanguination on d 276 was adjusted by 5 % to calculate liveweight on d 275; [4]Total intake, including pellets from Greenfeed Emissions Monitoring units and basal ration; [5]Mean daily total intake for period and mean liveweight for period.

*Table 5. Least-squares means (± s.e.) of feeding behaviour of F1 Wagyu steers fed tempered-barley based Grower (d18 – 150) and Finisher (d151 – 275) diets supplemented with either Asparagopsis extract in canola oil at 25 mg bromoform/kg feed dry matter (Asp-Oil), or a control canola oil.*

| | Control | Asp-Oil | SE | *P* |
|---|---|---|---|---|
| Eating rate, g as-fed/s | | | | |
| d 18 – 98[1] | 3.53 | 3.38 | 2.273 | 0.694 |
| d 18 – 150[1] | 3.60 | 3.50 | 0.213 | 0.720 |
| d 18 – 199[2] | 3.79 | 3.66 | 0.189 | 0.649 |
| d 18 – 275[2] | 4.08 | 3.92 | 0.175 | 0.510 |
| Eating time, min/day | | | | |
| d 18 – 98[1] | 64.4 | 59.6 | 3.66 | 0.349 |
| d 18 – 150[1] | 64.5 | 59.6 | 3.65 | 0.342 |
| d 18 – 199[1,2] | 61.2 | 57.0 | 3.06 | 0.332 |
| d 18 – 275[1,2] | 56.5 | 52.9 | 3.05 | 0.418 |

[1]Grower diet, d 18 – 150; [2]Finisher diet, d 151 – 275.

*Table 6. Least-squares means (± s.e.) of methane ($CH_4$) production, yield and intensity of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with either Asparagopsis extract in canola oil at 25 mg bromoform/kg feed dry matter (DM), or a control canola oil.*

| | Control | Asp-Oil | *P* |
|---|---|---|---|
| Methane production, g $CH_4$/day | | | |
| d 18 – 98[1] | 137.1 ± 6.14 | 102.7 ± 6.26 | 0.037 |
| d 18 – 150[1] | 140.4 ± 3.7 | 106.3 ± 5.44 | 0.023 |
| d 18 – 199[2] | 131.0 ± 3.43 | 101.8 ± 3.31 | 0.057 |
| d 18 – 275[2] | 138.6 ± 6.50 | 99.8 ± 6.57 | < 0.001 |
| Methane yield, g $CH_4$/kg total DM intake | | | |
| d 18 – 98[1] | 14.6 ± 0.986 | 11.0 ± 1.00 | 0.164 |
| d 18 – 150[1] | 15.0 ± 0.74 | 12.0 ± 0.68 | 0.059 |
| d 18 – 199[2] | 14.5 ± 0.70 | 11.6 ± 0.66 | 0.067 |
| d 18 – 275[2] | 15.0 ± 0.60 | 11.7 ± 0.62 | 0.032 |
| Methane intensity, g $CH_4$/kg liveweight gain | | | |
| d 18 – 98[1] | 132.1 ± 11.47 | 118.8 ± 10.84 | 0.464 |
| d 18 – 150[2] | 134.7 ± 13.36 | 127.9 ± 11.72 | 0.728 |
| d 18 – 199[2] | 136.4 ±20.99 | 144.1 ± 30.65 | 0.855 |
| d 18 – 276[2, 3] | 153.6 ± 23.59 | 147.3 ± 12.48 | 0.925 |

[1]Grower diet, d 18 – 150; [2]Finisher diet, d 151 – 275; [3]Shrunk liveweight measured after stunning and before exsanguination on d 276; [4]Hot Standard Carcase Weight.

*Table 7 Least-squares means of post-slaughter carcase grading of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with a control canola oil (Control) or Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (Asp-Oil).*

| Trait | Control | Asp-Oil | SE | *P* |
|---|---|---|---|---|
| Hot standard carcase weight, kg | 405.9 | 390.8 | 6.27 | 0.094 |
| Carcass yield, % | 58.3 | 58.6 | 0.33 | 0.505 |
| Dentition | 4.5 | 4.9 | 0.27 | 0.111 |
| MSA Marble Score | 514.9 | 521.6 | 25.31 | 0.792 |
| Marble Score | 2.8 | 2.9 | 0.24 | 0.695 |
| EMA, $cm^2$ | 97.7 | 97.2 | 2.75 | 0.853 |
| Meat Colour | 3.6 | 3.8 | 0.23 | 0.486 |
| Fat Colour | 0.3 | 0.2 | 0.15 | 0.429 |
| Ossification Cold | 166.5 | 165.1 | 4.39 | 0.751 |
| $pH_u$ | 5.5 | 5.6 | 0.02 | 0.051 |
| Hump height, cold, mm | 106.4 | 108.0 | 3.62 | 0.652 |

*Table 8 Bromoform ($CHBr_3$) concentration[1] (mg/kg) of kidney, liver, fat and meat samples from carcases from F1 Wagyu steers (N = 76) fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM, or a control canola oil.*

| | | Sample site | | | |
|---|---|---|---|---|---|
| Treatment | Animal | Kidney | Liver | Fat | Chuck |
| Control | | | | | |
| | 1 | ND | ND | ND | ND |
| | 6 | ND | ND | ND | ND |
| | 11 | ND | ND | ND | ND |
| | 16 | ND | ND | ND | ND |
| | 38 | ND | ND | ND | ND |
| | 43 | ND | ND | ND | ND |
| | 48 | ND | ND | ND | ND |
| | 53 | ND | ND | ND | ND |
| Asp-Oil | | | | | |
| | 19 | ND | ND | ND | ND |
| | 24 | ND | ND | ND | ND |
| | 29 | ND | ND | ND | ND |
| | 34 | ND | ND | ND | ND |
| | 58 | ND | ND | ND | ND |
| | 63 | ND | ND | ND | ND |
| | 67 | ND | ND | ND | ND |
| | 72 | ND | ND | ND | ND |

[1]Bromoform tolerable daily intake for humans is 17.9 µg/kg BW (WHO 2004)

*Table 9 Bromide (Br-) concentration1 (mg/kg) of kidney, liver, fat and meat samples from carcases from F1 Wagyu steers (N = 76) fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at*

| | | Sample site | | | |
|---|---|---|---|---|---|
| Treatment | Carcase number | Kidney | Liver | Fat | Chuck |
| Control | | | | | |
| | 1 | 14 | 6 | ND | ND |
| | 6 | 15 | ND | 5 | ND |
| | 11 | 14 | ND | ND | ND |
| | 16 | 20 | 5 | 5 | ND |
| | 38 | 16 | 6 | 9 | ND |
| | 43 | 17 | 5 | 7 | ND |
| | 48 | 18 | 5 | ND | ND |
| | 53 | 15 | ND | ND | ND |
| | Mean ± SE | 16.1 ± 0.83 | - | - | - |
| Asp-Oil | | | | | |
| | 19 | 23 | 8 | 9.2 | ND |
| | 24 | 18 | 7 | 6 | ND |
| | 29 | 20 | 7 | 6 | ND |
| | 34 | 19 | 7 | 6 | ND |
| | 58 | 25 | 6 | ND | ND |
| | 63 | 23 | 8 | ND | ND |
| | 67 | 19 | 7 | ND | ND |
| | 72 | 23 | 7 | ND | ND |
| | Mean ± SE | 21.3 ± 0.83 | - | - | - |
| | *P* | 0.022 | | | |

[1]Bromide acceptable daily intake for humans is 1 mg/kg BW; with ranges for 1-3-year-old (13kg) to average adults (70kg) (FAO Panel of Experts on Pesticide Residues in Food 1999).

*Table 10 Iodide ($I^-$) concentration[1] (mg/kg) of kidney, liver, fat and meat samples from carcases from F1 Wagyu steers (N = 76) fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (Asp-Oil), or a control canola oil.*

| | | Sample site | | | |
|---|---|---|---|---|---|
| Treatment | Animal | Kidney | Liver | Fat | Chuck |
| Control | | | | | |
| | 1 | 0.2 | 0.2 | 0.2 | ND |
| | 6 | 0.2 | ND | ND | ND |
| | 11 | 0.2 | 0.1 | ND | ND |
| | 16 | 0.2 | 0.1 | ND | ND |
| | 38 | 0.2 | 0.1 | ND | ND |
| | 43 | 0.2 | 0.1 | ND | 0.4 |
| | 48 | 0.1 | ND | ND | ND |
| | 53 | 0.2 | 0.1 | ND | 0.1 |
| | Mean ± SE | 0.19 ± 0.017 | - | - | - |
| Asp-Oil | | | | | |
| | 19 | 0.2 | ND | ND | ND |
| | 24 | 0.1 | ND | ND | ND |
| | 29 | 0.2 | ND | ND | ND |
| | 34 | 0.1 | ND | ND | ND |
| | 58 | 0.1 | ND | 0.1 | ND |
| | 63 | 0.2 | 0.1 | 0.1 | 0.1 |
| | 67 | 0.1 | 0.5 | ND | ND |
| | 72 | 0.2 | ND | ND | ND |
| | Mean ± SE | 0.15 ± 0.017 | - | - | - |
| | *P* | 0.227 | | | |

[1] Iodine recommended upper limit for humans (mg/d)(sustained): 1-3 years (0.2); 4-8 years (0.3); 9-13 years (0.6); 14-18 years (0.9); 19+ years (1.1, (Trumbo et al. 2001)).

*Table 11 Least-squares means of test panel results of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM, or a control canola oil.*

| Taste panel attribute | Control | Asp-Oil | SE | *P* |
|---|---|---|---|---|
| Aroma | | | | |
| Aroma intensity | 55.6 | 54.4 | 1.09 | 0.287 |
| Roasted | 47.2 | 47.5 | 1.30 | 0.782 |
| Caramelised | 37.0 | 38.2 | 2.06 | 0.594 |
| Buttery - fatty | 36.7 | 36.7 | 1.40 | 0.982 |
| Gamey | 23.2 | 19.4 | 2.27 | 0.193 |
| Barnyard | 16.3 | 13.3 | 1.19 | 0.087 |
| Vegetative – corn | 18.9 | 19.2 | 1.28 | 0.801 |
| Texture | | | | |
| Tenderness | 58.1 | 60.1 | 5.50 | 0.734 |
| Juiciness | 54.0 | 52.5 | 2.28 | 0.570 |
| Dissolving | 40.4 | 41.6 | 4.99 | 0.826 |
| Fattiness | 33.2 | 35.1 | 2.30 | 0.481 |
| Chewiness | 48.4 | 47.4 | 5.11 | 0.860 |
| Fibrous | 47.3 | 44.4 | 3.71 | 0.481 |
| Flavour | | | | |
| Flavour intensity | 60.8 | 59.9 | 0.96 | 0.331 |
| Sweetness | 32.1 | 33.7 | 1.39 | 0.259 |
| Sourness | 21.1 | 21.0 | 1.21 | 0.938 |
| Saltiness | 25.8 | 27.2 | 1.26 | 0.253 |
| Umami | 42.6 | 43.7 | 1.41 | 0.452 |
| Roasted | 53.1 | 53.3 | 1.25 | 0.850 |
| Gamey | 13.9 | 12.6 | 1.40 | 0.420 |
| White meat | 28.1 | 26.8 | 1.84 | 0.533 |
| Buttery-fatty | 35.5 | 38.1 | 2.24 | 0.338 |
| Metallic-mineral | 25.9 | 25.0 | 1.23 | 0.433 |
| Other attributes | | | | |
| Lingering flavour | 48.2 | 48.7 | 1.11 | 0.631 |
| Metallic | 32.3 | 33.3 | 1.41 | 0.470 |
| Umami -roasted | 39.8 | 40.4 | 1.33 | 0.628 |
| Fatty mouth coating | 35.9 | 39.1 | 1.75 | 0.160 |

# 5. Discussion

## Methane emissions of long-fed Wagyu cattle

The Control group of F1 steers in this project provide the first estimates of baseline emissions in a Wagyu finishing system in Australia. Methane yield did not differ between grower and finisher rations, but $CH_4$ production increased modestly (by ~13 %) from Grower to Finisher periods.

## Reduction in methane emissions with Asp-Oil supplementation

The reduction in $CH_4$ production achieved with Asp-Oil supplementation at 25 mg $CHBr_3$/kg DM over the whole feeding period was 28 %. This was substantially less than predicted for this $CHBr_3$ inclusion rate from the result of a dose-response trial testing the same Asp-Oil in a short-fed finisher ration (85 % grain, DM-basis) in respiration chambers (P.PSH.1351, Cowley *et al.* (2023)), which found reductions of 64.1 – 96.3% reduction in $CH_4$ production with 17 – 34 mg $CHBr_3$/kg DM inclusion (*cf.* 86.3 g/day Control $CH_4$ production). The $CH_4$ abatement was also less than has previously been achieved with FD whole *Asparagopsis:* for example, 70 % reduction in $CH_4$ production in a 72 % grain (DM-basis) finishing diet providing 35 mg $CHBr_3$/kg DM (Roque *et al.* 2021). Methane yield was reduced by 20 – 22 %, suggesting that the reduction in $CH_4$ production was only partially due to a reduction in DM intake in the *Asparagopsis*-supplemented group. However, as the grain inclusion in the present experiment was ~67 % DM, an interaction of $CH_4$ abatement with diet should be considered. Such an interaction has previously been demonstrated with FD *Asparagopsis*: methane mitigation was reduced from 70 % to 52 % with 35 mg $CHBr_3$/kg DM when grain content decreased from 72 to 37 % (Roque *et al.* 2021).

This experiment reports, to our knowledge, the longest period for which an *Asparagopsis* product has been fed to cattle, to date, and is an opportunity to consider whether rumen adaptation to $CH_4$ inhibition occurs over the long-term, particularly in rumens which are only partially inhibited. There was no interaction of Asp-Oil treatment with time on $CH_4$ yield over the whole feeding period, suggesting that there was no adaption of rumen methanogenesis during the experiment. At 17 mg $CHBr_3$ inclusion/kg DM, Cowley et al. (2023) found steady increase in $CH_4$ production and yield from day 49 of feeding onwards, suggesting adaptation of the rumen to $CHBr_3$. No such adaptation was evident in that research when Asp-Oil-derived $CHBr_3$ was included at 34 mg/kg DM over a 77-day feeding period, although very high (85 – 90 %) $CH_4$ inhibition was achieved at 34 mg $CHBr_3$/kg DM. When feeding freeze-dried *Asparagopsis* at 35.1 mg $CHBr_3$/kg DM for 147 days, Roque *et al.* (2021) reported a persistent reduction in $CH_4$ yield, ~ 50 % (measured with GEM units).

There was a strong effect of GEM unit on $CH_4$ emissions prediction; the rotational deployment of GEM units in the experimental design allowed for this to be accounted for in the statistical analysis. The lower than expected $CH_4$ inhibition observed in the present experiment may be partially due to the use of in-field GEM units, instead of gold-standard respiration chambers, since $CH_4$ measurement equipment (chamber v greenfeed v sulfur hexaflouride-6) has previously been suggested as a cause of study to study variability in $CH_4$ mitigation from feeding 3-NOP (Yu *et al.* 2021). However, GEM units have recorded levels of $CH_4$ production below 40 g/day (much lower than levels observed in the current experiment), in experiments with beef steers supplemented with FD *Asparagopsis* (Roque *et al.* 2021) or 3-NOP (Alemu *et al.* 2021).

## Effect on cattle feedlot performance

Feed intake of the TMR was affected by *Asparagopsis* oil inclusion throughout the experiment, including during the adaptation period, and this resulted in a lower growth rate for all periods. The lower intake in *Asparagopsis*-supplemented steers was not explained by their lower body weight (intake/kg liveweight), and there was no improvement in feed efficiency with *Asparagopsis* supplementation.

Ration intakes in the *Asparagopsis*-supplemented steers reduced upon first introduction of Asp-Oil, in the adaptation period, and were maintained below those of the Control steers for the entirety of the experiment. Slow adaptation of animals to *Asparagopsis* products is recommended as best practice to reduce initial feed intake depression. Best practice management of the transition to feedlot diets normally also includes incremental increases in total fat inclusion, and so a single Asp-Oil stock inclusion in dietary oil was used, and incremental increase in $CHBr_3$ inclusion was achieved with incremental increase in oil inclusion. The $CHBr_3$ inclusion increments during the adaptation period in the present research were at each step between the incremental adaptation inclusion rates of the Low and Medium Asp-Oil treatments from P.PSH.1351 (Cowley *et al.* 2023), although in the present experiment, time at each increment was 4 days, cf. 7 days in P.PSH.1351.

Unlike other research (Nyløy *et al.* 2023) observing reduced intake of a TMR mixed with FD *Asparagopsis taxiformis*, the present experiment did not observe a statistically significant reduction in as-fed eating rate or duration in *Asparagopsis*-supplemented steers, although numerically, both were consistently lower than Control steers. The present experiment measured eating rate using calculated rate of disappearance of feed during a steer's attendance at the feeder, whereas (Nyløy *et al.* 2023) calculated eating rate by total daily intake as a function of time spent eating, measured with a pressure-sensor nose band technology.

Reduced feed intake in cattle supplemented with *Asparagopsis* has been observed in numerous experiments in beef and dairy cattle. Inherent aversiveness (taste) and negative post-ingestive feedback mechanisms may explain reduced intake where *Asparagopsis*, especially in a FD state, has been homogenised with a TMR. However, reduced intake of basal diet has also been observed where *Asparagopsis* has been offered as a separate supplement. Furthermore, the use of Asp-Oil in the present experiment removes many potential confounding factors associated with feeding whole seaweed biomass, as the Asp-Oil consists only of the lipid-soluble compounds from the seaweed, including $CHBr_3$, iodide and bromide. Some intake suppression has also been demonstrated when beef cattle $CH_4$ production is suppressed by other ameliorants, such as 3-NOP, either initially, or when very high inclusion rates are used, although this is not sustained in the long term (Yu *et al.* 2021). Together, this may support hypotheses that reduced feed intake is a result of methane suppression in general, rather than specific characteristics of *Asparagopsis*, or Asp-Oil.

For example, although respired $H_2$ gas varies inversely to respired $CH_4$ from ruminants supplemented with a $CH_4$ inhibitor, the $H_2$ gas production is less than the stoichiometric prediction leading to the hypothesis that hydrogen is instead directed to other sinks (Martinez-Fernandez *et al.* 2016). Alternatively, $H_2$ may accumulate in the rumen fluid, increasing $H_2$ partial pressure and potentially stimulating intake suppression, although, since most research has examined hydrogen production by measuring respired $H_2$, this has rarely yet been demonstrated. However, in the present experiment, $CH_4$ abatement was relatively low, approximately 22 % of Control yield; it is not known whether under such conditions accumulation of $H_2$ would be sufficient to inhibit intake; and $H_2$ -driven suppression of intake is currently a hypothesis only. The reduction of intake observed in *Asparagopsis*-supplemented steers during the adaptation period may be driven by a different mechanism (e.g. novelty or taste) than the sustained suppression. The long duration of this experiment and use of a TMR to supply *Asparagopsis* provided the best opportunity for animals to overcome any initial aversion caused by novelty or taste in the absence of underlying negative post-

ingestive feedback. Future research could explore preference tests with dose titration of Asp-Oil to determine if the product affects palatability of diets.

Further research on the drivers of intake suppression with $CH_4$-inhibition, and methods to overcome this, is imperative. The business case for adoption of $CH_4$ inhibitors is strongly affected by their effect on animal performance and feed cost. Should $H^+$ accumulation prove to be the link between $CH_4$ suppression and reduced intake, research on alternative $H^+$ sinks may yield recommendations on feeding of $CH_4$-ameliorants in combination with promoters of $H^+$ incorporation into alternative sinks. Efforts on this front are already progressing. Phloroglucinol fed together with *Asparagopsis* reduced by ~74 % the respired $H_2$ emissions produced in methane-inhibited goats (Romero *et al.* 2022), and proves at least conceptually that supplementing *Asparagopsis* with $H^+$ sinks may reduce $H_2$ accumulation. In another example of increasing $H^+$ sinks, feeding the propionate precursor fumarate with another $CH_4$ inhibitor (3-NOP) enhanced $CH_4$ mitigation above feeding 3-NOP alone, and feeding fumarate, alone or in combination with 3-NOP, increased propionate production *in vitro* (Liu *et al.* 2022).

## Residues

The lack of accumulation of $CHBr_3$ in the carcase is consistent with previous studies demonstrating that $CHBr_3$ acts in the rumen and on its microbiota, and is not bioavailable to the host ruminant (Glasson *et al.* 2022; Cowley *et al.* 2023). Although Asp-Oil can be high in $Br^-$ content (Cowley *et al.* 2023), there was no deposition of $Br^-$ in the meat, and $Br^-$ levels in kidney was at safe levels. Iodine was concentrated in kidney also, but levels did not differ from Control.

## Carcase grading and trained sensory panel evaluation

Despite consistently lower liveweight gains, and a 30.7 kg difference in liveweight at the end of the experiment, a statistical trend ($P$ = 0.09) for decreased HSCW, and no difference in carcase grading traits at slaughter was reported. The treatment difference in HSCW of 15.1 kg is a 3.7 % difference in total HSCW, which was below the statistical power of this analysis. However, if this numerical difference were to hold when larger-scale performance experiments are undertaken, it would be a commercially significant impact. Ossification, hump height and marble score were all very similar between the two groups, and are important predictors of eating quality. Consistent with these results, there was no effect of Asp-Oil supplementation on trained sensory panel attributes.

Effects of *Asparagopsis* supplementation on marbling have not previously been reported. Despite the lower intake and liveweight gain of Asp-Oil steers during the feedlot period, this did not carry over into any effect on marbling. Reduction in $CH_4$ production as a result of *Asparagopsis* supplementation has frequently been associated with a reduction in acetate: propionate production in the rumen (since acetate is a $H_2$ producer, and its production is likely inhibited by higher rumen $H_2$ concentrations). Despite intramuscular fat (IMF) synthesis being reliant on acetate as a substrate for *de novo* synthesis in adipose tissue, glucose (derived from propionate) is thought to be the limiting factor for lipogenesis (Pethick *et al.* 2004).

Results from a linear mixed model ANOVA found that there were significant differences between different cuts and marbling levels for many of the 27 sensory attributes rated. This result was expected and provides evidence of the panellist's performance during sensory evaluation. However, there were no differences observed in scoring for any of the sensory attributes between samples from animals fed the control and the *Asparagopsis* supplemented diet. These results demonstrate that *Asparagopsis* supplementation has no impact on the sensory properties of these two cuts (cube roll, *Longissimus dorsi*, *Spinalis dorsi* and *Longissimus thoracis*; and rosbiff, *Gluteus medius*).

Results of Meat Standards Australia (untrained sensory panel data) will be available in May, 2024 via MLA Project L.EQT.2306.

# 6. Conclusion

## 6.1 Key findings

- Feeding an *Asparagopsis* extract in canola oil (Asp-Oil) at 25 mg/kg DM to F1 Wagyu steers for 258 days (including grower and finisher diets) reduced methane production and yield by 28 and 22%, respectively from Control.
- Asp-Oil reduced feed intake consistently without improvement in feed efficiency.
- This resulted in persistently reduced liveweight gain, liveweight and a trend of a lower HSCW in Asp-Oil supplemented steers.
- The decline in performance, resulted in no advantage for cattle fed Asp-Oil for methane intensity (g/kg LW gain) over control animals.
- Other carcase traits, and trained sensory panel attributes (including flavour and texture traits) were not affected by Asp-Oil supplementation.
- Consistent with other research, there was no bromoform detectible in meat or offal, nor concerning residues of iodine or bromide.
- Canola oil stabilised bromoform over the duration of use in this study with no volatilisation evident in vegetable oil tanks at the feedlot.

## 6.2 Benefits to industry

The research project has been one of the first commercialisation trials of *Asparagopsis* products conducted under Australian feedlot industry-relevant conditions, and is the largest-scale, and longest feeding experiments conducted to-date on *Asparagopsis* products. As such, it provides essential data to support the business case for adoption of Asp-Oil into long-fed feedlot programs, in terms of cattle productivity and $CH_4$ abatement. It has highlighted that further research is required to understand the reasons for feed intake depression caused by $CH_4$ inhibitors, and management strategies which could overcome this, so that supplementary $CH_4$ inhibitors can be widely adopted with confidence.

# 7. Future research and recommendations

Further research is required to refine feeding protocols for $CH_4$ inhibiting supplements to overcome intake depression. This may not be unique to *Asparagopsis*, and should consider not only dose titration and adaptation protocols, but potentially, co-feeding of hydrogen sinks or products that promote hydrogen utilisation in the rumen together with $CH_4$ inhibitors. Results of Meat Standards Australia (untrained sensory panel data) will be available in May, 2024 via MLA Project L.EQT.2306.

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# 9. Appendix 1. Adaptation period performance

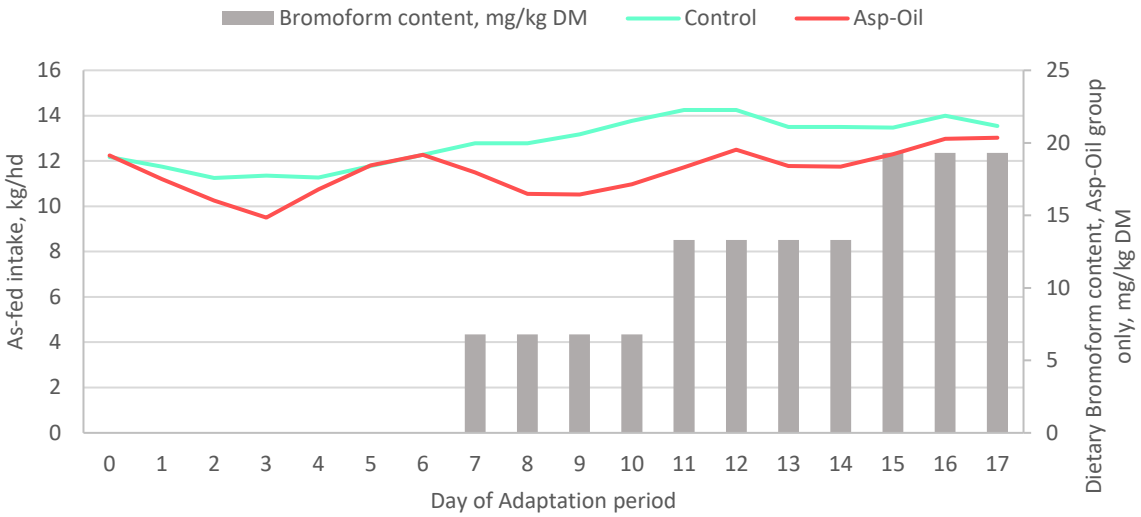


*Figure A1. As-fed intake (kg/head.day) of Control (Green) and Asparagopsis-oil-supplemented F1 Wagyu steers during the adaptation period (d 0 – 18), when Asp-Oil group diets were gradually increased in content of grain, and either Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (grey bars, Asp-Oil only), or a control canola oil.*

*Table A1. Observed means ± SE of liveweight, average daily gain, dry matter intake and gain:feed of F1 Wagyu steers during the adaptation period (d -3 – 18), when Asp-Oil group diets were gradually increased in content of grain, and either Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (Asp-Oil only), or a control canola oil.*

| | Control | Asp-Oil | SE |
|---|---|---|---|
| Liveweight, kg | | | |
| d -3 | 449.6 | 447.9 | 1.47 |
| d 18[1] | 485.2 | 477.7 | 2.63 |
| Average daily gain, kg/hd.d | 1.97 | 1.66 | NA |
| Ration intake, kg DM/hd.d | 9.83 | 9.24 | NA |
| Total intake[5], kg DM/hd.d | 9.83 | 9.24 | NA |
| Gain:feed[5], kg/kg DM | 0.200 | 0.180 | NA |

# 10. Appendix 2. Analysed bromoform content of test Asp-Oil mixture

| | $CHBr_3$ content (g/kg) |
|---|---|
| Day 100 | 1200 |
| Day 180 | 1320 |
| Day 250 | 1260 |

# 11. Appendix 3. Interval measures of feedlot performance and methane emissions

There was a modest, but consistent effect of period on feed DM intake, such that intake reduced in both groups with increasing days on feed, but there was no interaction of treatment with days on feed (Figure A2). Average daily gain (ADG) slowed for both groups after 151 days on feed (Figure A3), but there was a difference between treatment groups, such that there was a steeper decline between the d 99 – 150 and d 151 – 199 periods for Control cattle than for Asp-Oil cattle, but between the d 151 – 199 and d 200 - 275 periods, decline in ADG was steeper for Asp-Oil cattle than control cattle. This is also reflected in G:F for these last two periods (Figure A4).

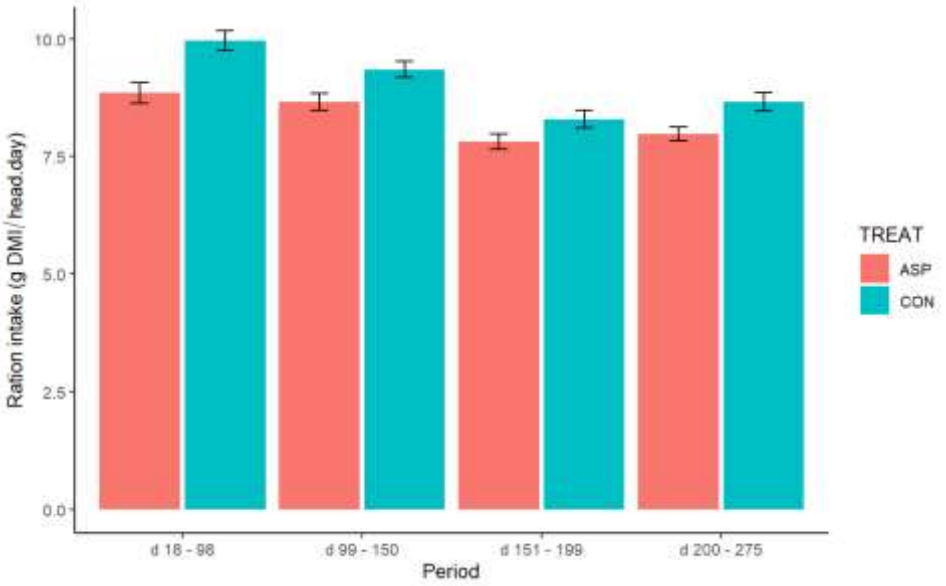


*Figure A2: Observed means ± standard error of TMR intake (dry matter basis), of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (ASP), or a control canola oil (CON).*

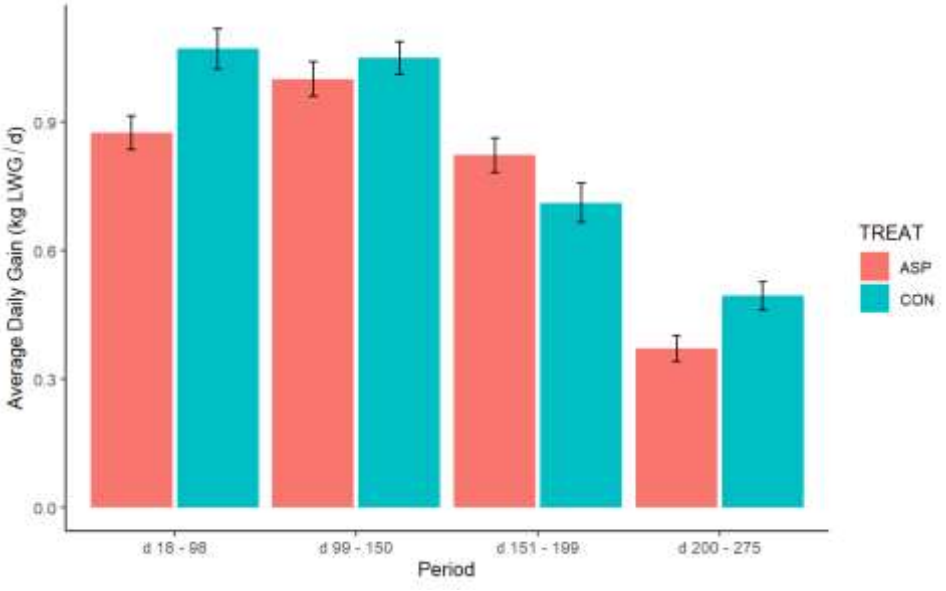

*Figure A3: Observed means ± standard error of average daily gain, of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (ASP), or a control canola oil (CON). Weight on d 275 measured as shrunk weight, after stunning and before exsanguination, all other weights are unshrunk.*

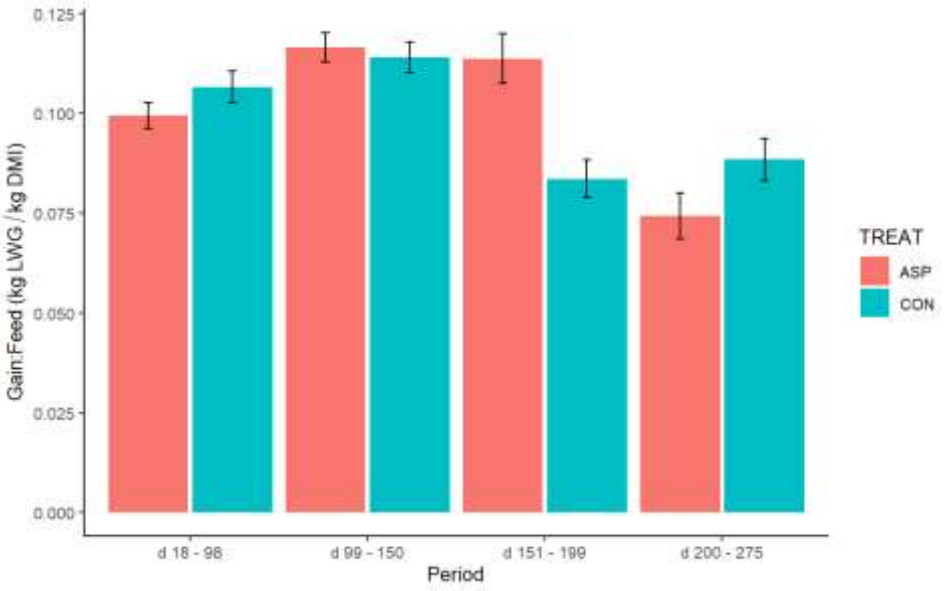


*Figure A4: Observed means ± standard error of Gain:Feed, of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (ASP), or a control canola oil (CON). Intake (dry-matter basis) includes basal TMR and intake of pellets from Greenfeed units. Weight on d 275 measured as shrunk weight, after stunning and before exsanguination, all other weights are unshrunk.*

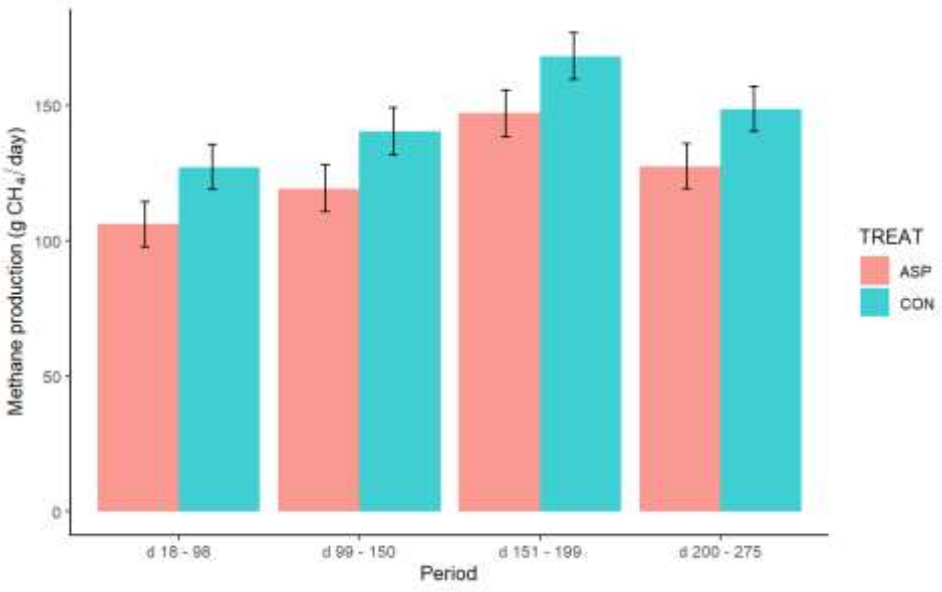


*Figure A5: Least squared means ± standard error of methane production of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (ASP), or a control canola oil (CON).*

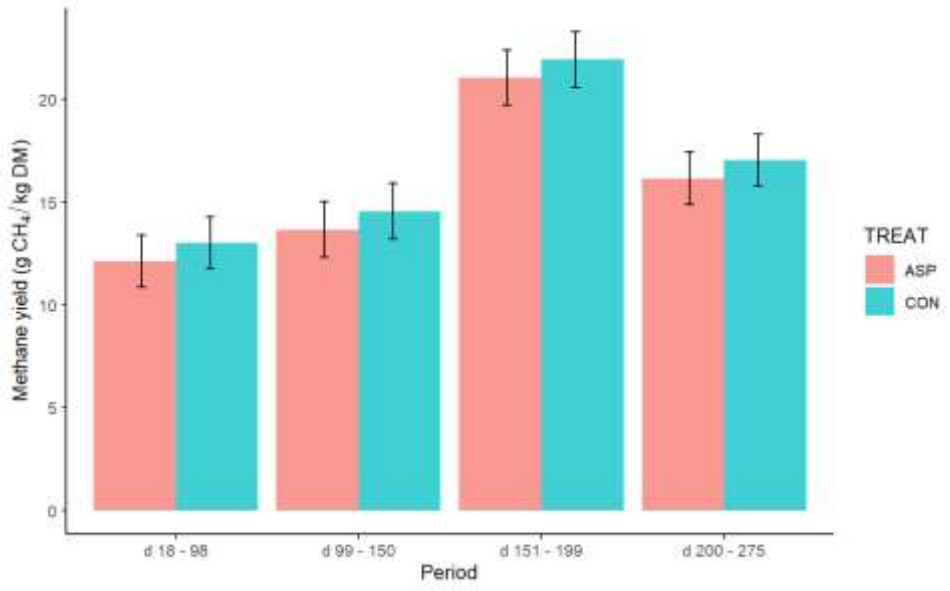


*Figure A6: Least squared means ± standard error of methane yield of F1 Wagyu steers fed tempered-barley based Grower (d 18 – 150) and Finisher (d 151 – 275) diets supplemented with Asparagopsis extract in Canola oil at 25 mg bromoform/kg feed DM (ASP), or a control canola oil (CON).*

# 12. Appendix 4. Empirical demonstration of taste panel experiment using factors expected to influence eating quality as positive controls

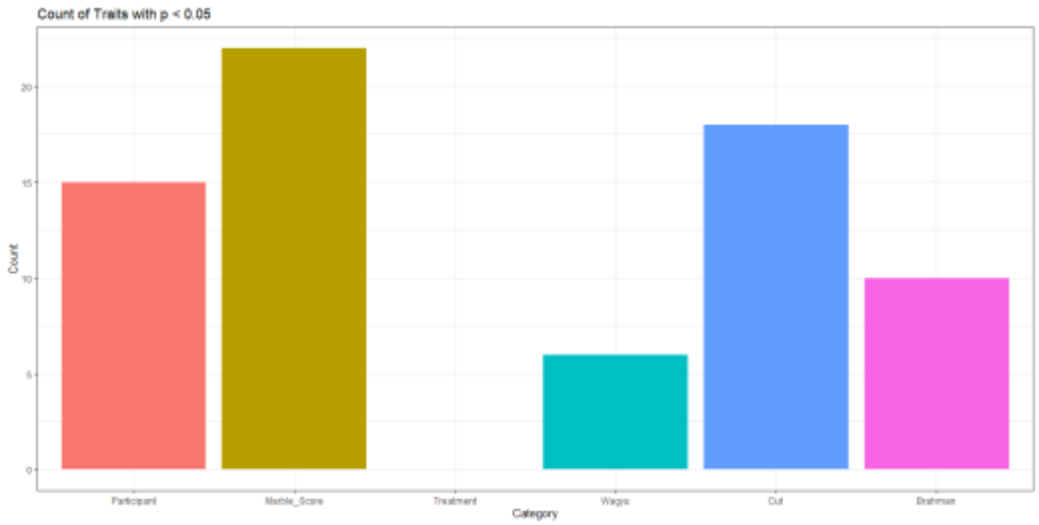


Figure A7. Number of taste panel attributes significantly associated with participant, marble score, wagyu content, brahman content, cut and treatment.

# 13. Appendix 5. Report on trained sensory panel analysis from the University of Queensland

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The trained sensory panel of the Food Quality team at the University of Queensland.

## Statistical support

Allan Lisle

# Executive Summary

Environmental sustainability in agriculture, specifically beef cattle, has become increasingly important for producers and consumers. Developing a safe and cost-effective method to decrease cattle emissions could increase sustainability and create an advantage over competitors, however, the high quality of the wagyu beef which is prized for its unique flavour and texture must be retained. In the current study, reduction of emission of wagyu cattle was investigated by feeding cattle red Asparagopsis for 300 days. The sensory properties of the beef were evaluated by an experienced trained sensory panel who had previously been involved in development of the Westholme Wagyu Flavour Wheel (2019, AACo Westholme Wagyu project).

A total of 152 different samples of beef were collected for this study including two cuts (rump rostbiff and cube roll), two diets (control and Asparagopsis treated diet) and a range of different marbling levels (10–70). The beef samples were prepared according to a strict sample preparation methodology and presented to sensory panellists immediately after cooking. Conventional sensory descriptive profiling was the method applied whereby a trained panel of 11 experienced tasters evaluated samples for 27 discrete attributes covering the aroma, texture, flavour and after taste of the beef samples. The Westholme Wagyu Flavour Wheel and sensory lexicon were used as tools to inform the sensory properties selected during panel training.

Results from a linear mixed model ANOVA found that there were significant differences between cuts and marbling levels for many of the 27 sensory attributes rated. This result was expected and provides evidence of the panellist's performance. The model also found there were no differences observed between samples from the different diets (control and Asparagopsis) for any of the 27 sensory attributes scored.

The results from this study demonstrate that feeding cattle a diet of red Asparagopsis for 300 days has no impact on any of the sensory properties of rump (rostbiff) and cube roll cuts of beef.

# Background

AACo is pursuing safe and cost-effective methane emission reduction through inclusion of Asparagopsis in feedlot rations (see AACo project summary for more details). AACo have designed a collaborative study with ANU, Sea Forest and UQ for MLA funding to conduct an animal trial comparing Wagyu beef fed a control and an Asparagopsis diet.

Given her experience with the development of the Westholme Flavour Wheel (2020) Dr Heather Smyth's Food Quality team at QAAFI, the University of Queensland, were engaged to evaluate the sensory properties of two cuts of beef from animals coming from the diet trial. The deliverables and milestones of the project are detailed in Table 1.

*Table 1 Deliverables and Milestones*

| Milestone / Deliverable | Date |
|---|---|
| Signing contract | TBA (late 2022) |
| Sensory profiling | ~18 sessions (10 formal sessions)<br>Feb-March 2023 |
| Top line results | 24th March |
| Final Report | May 2023 |

# Project Objectives

The aim of the sensory work is to ensure there is no impact on meat product quality of animals on a treatment versus control diet. The existing lexicon for Wagyu from AACo's Westholme flavour wheel will be utilised for profiling samples from treatments.

Scope and outline of work:

- Selection of samples includes two cuts (rump rostbiff and cube roll), variation in marbling and the numbers of animals per treatment (80).
- Application of existing presentation formats and method of assessment. Each of the 80 (x 2 cuts) samples will be assessed by a panel of 10 trained panellists. Conduct 8 training and 10 formal sessions (16 samples per session).
- Development of detailed product sensory profiles (product descriptions) as required for appearance, aroma, flavour, texture, mouth feel and aftertaste (relying on existing lexicon)
- Data collection, rigorous analysis, and provision of a detailed written report.

A panel of 10 experienced tasters will be engaged for the study. Conventional descriptive profiling involves a training phase for lexicon development and attribute definition, followed by formal assessments in a purpose-built sensory laboratory (more details below). Data will be collected using RedJade sensory software and analysed statistically using XLSTAT.

# Materials and Methods

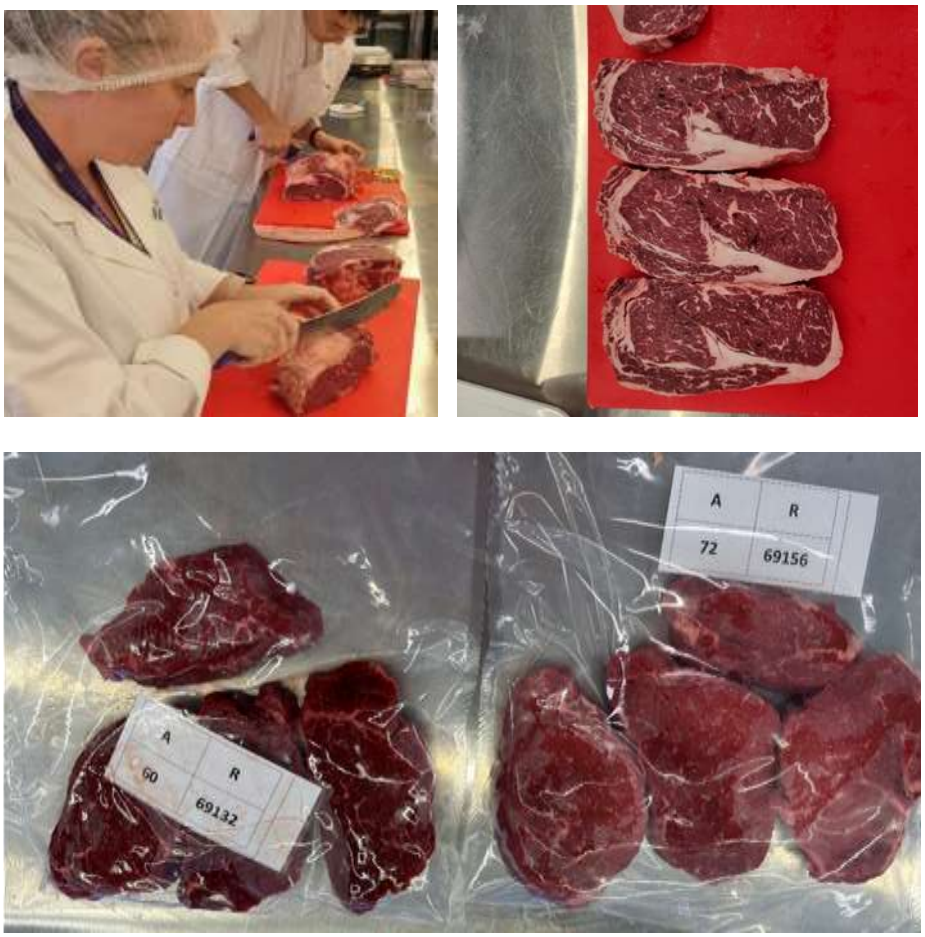
## Samples

Beef samples that were evaluated during formal sensory assessments were provided by the client (Figure 1) in the week prior to the sensory training sessions commencing and are detailed in Table 2. On arrival samples were shrink-wrapped as blocks of meat and stored chilled (2°C).

*Table 2 Samples assessed during the sensory study*

| | Treatment Diet | Control Diet | Sample requirement |
|---|---|---|---|
| **Rump (Rostbiff)** | 40 animals | 40 animals | 2 steaks per animal |
| **Cube Roll** | 40 animals | 40 animals | 2 steaks per animal |
| **Total Samples** | **80** | **80** | |

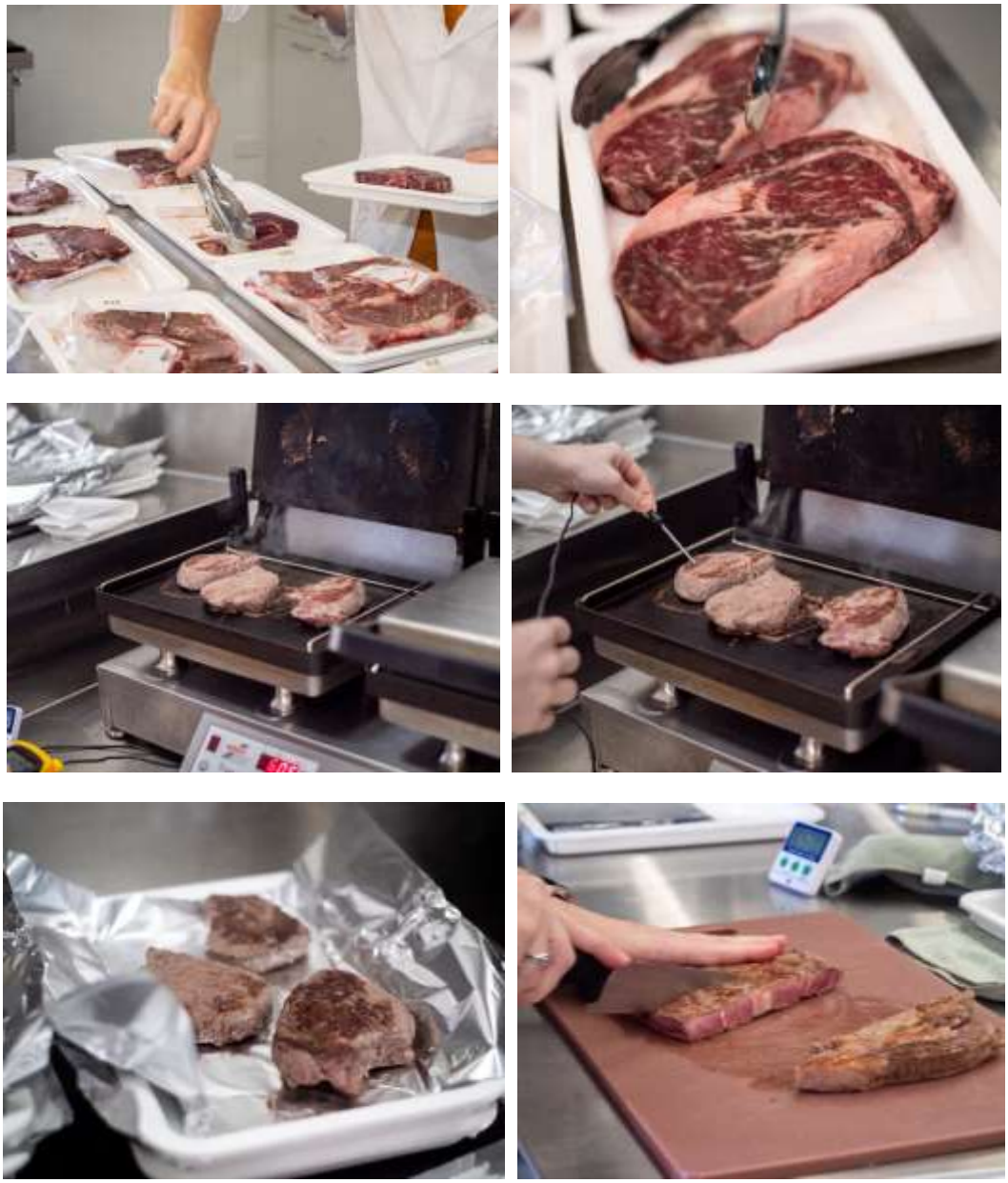
*Figure 1 Photographs of the samples received for sensory evaluation after being cut into steaks and packed in vacuum bags prior to sensory evaluation.*




## Sample Preparation

Within two weeks after arrival, the required samples were removed from 2°C and immediately opened, rinsed and patted dry before cutting into 25 mm thick steaks perpendicular to the grain (while still cold). A ruler and scoring with a paring knife ensured accuracy in sample steak thickness. These steaks were then denuded of excess fat and a block of maximum length by 60-80 mm wide was prepared. Due to the size of the blocks of meat, approximately 2-4 bags of 2-3 steaks each were prepared ensuring sufficient samples for training and formal evaluation (one bag comprised sufficient sample to serve one session of a panel evaluation - 10-12 panellists). The steaks after being cut as well as the vacuum sealed bags of various steaks are shown in Figure 1. Given the smaller size of the rostbiff rump, fewer bags of sample were obtained after cutting (~2 or in some cases 3).

*Figure 2 Photographs of cooking procedure of wagyu steaks for sensory evaluation.*



All steaks were vacuum sealed and stored frozen at -19°C until required. Bags of sample were transferred to 2°C the night before tasting and were removed to room temperature at least an hour before cooking to allow a precooking temperature of >10°C.

Figure 2 shows the steps involved in cooking the prepared steaks for sensory evaluation. A clamshell Silex Grill was preheated to 160°C and programmed to run for 3 minutes. A set of sacrificial steaks and fat were used to oil and commence the cooking cycle to stabilise temperature variation.

Steaks were placed on the grill with the lid on and cooked at 160°C. An internal on-grill temperature of 50 - 55°C was desired to produce a medium doneness steak. Some steaks took longer to reach this internal temperature (depending on size and amount of fat), an initial time of 1 minute was allowed per side and then 30 second increments after that for the cube rolls. Generally, the rump (rostbiff) steaks only required 45 seconds per side. A thermocouple was used to check steak internal temperature.

Once the desired internal temperature was achieved, the steaks were removed and placed in deep plastic pans, covered with aluminium foil, and set to rest on a cutting board (covered with towels) for a minimum of 4 minutes, resulting in an internal temperature of approximately 60°C.

*Figure 3 Photographs of cutting protocol for cooked, rested samples for sensory evaluation.*

After resting, the steaks were removed one at a time and transferred to the cutting board where the crust of the steak was removed from the four sides producing a block of maximum length, approximately 50 mm wide and 25 mm thick. A ruler was used as a guide to allow for accuracy. Figure 2 depicts the steps involved in preparing the cooked, rested samples for panel evaluation.

Cooked steaks were sliced parallel to the fibres to produce 50x10x25 mm strips and then divided in two to produce two 25x10x25 mm bite sized pieces for each sampler.

Any slices that contained large amounts of fat and gristle were discarded, a constant doneness was attempted to be maintained between all samples.

Two slices were placed on small aluminium tart trays and wrapped with a labelled aluminium foil and served immediately to the sensory panel.

For initial training trials the prepared samples were immediately placed in a preheated baker's oven at 50°C, and then transferred to another preheated baker's oven set at 70°C 8 minutes prior to serving. This re-heat step was not implemented during formal evaluation as the samples were typically overcooked by this method.

## Trained Sensory Panel

Ten trained sensory panellists (10 female and 1 male) participated in the study, aged between 30 and 69 years (with an average age of 48). These panellists were sourced from a pool of experienced trained assessors who had previously been screened for sensory acuity.

## Sensory evaluation

Conventional quantitative sensory descriptive analysis method was used to characterise the sensory properties. The study consisted of fifteen sessions including 4 training sessions, 1 practice formal assessments and 10 formal assessments. The sessions lasted up to 2 hours over a period of 4 weeks (February 21st 2023 – March 16th 2023). A total of 152 beef samples were evaluated during the formal sessions with no repetitions (each sample was evaluated a single time). During the training sessions (Figure 4), the wagyu beef flavour wheel was used and the attributes of the wagyu beef study completed in 2019 was also used as a reference.

The training involved familiarising the panellists with the samples; developing an assessment protocol, developing a concise vocabulary to describe the sensory properties of the samples; defining the sensory attributes; developing corresponding sensory reference standards and developing scales and anchors for rating the attributes. Due to the large number of animals, most but not all samples were introduced to the panellists at least once during training. Practice sessions were held at the end of the training phase that mimicked a formal evaluation session, wherein panellists' discrimination performance was assessed.

The samples were randomly selected from each treatment group daily with a roughly equal distribution of rump (rostbiff) and cube roll. 15-16 samples were presented any single day. In each formal evaluation session, panellists were asked to first go through the definitions of the attributes and re-assess the sensory reference standards, before assessing samples.

The method developed for assessment of each sample is detailed as follows:

- Lift lid and assess aroma
- Take a piece of beef to assess texture
- First bite with molars perpendicular to the cooked edge of the piece.
- Take another piece of beef to assess flavour and aftertaste
- Repeat as necessary
- Cleanse between samples with green apple, water crackers, and filtered/sparkling water.

*Figure 4 Photographs of the training sessions using the wagyu beef flavour wheel.*

The sensory properties rated included 6 aroma, 6 texture, 9 flavour and 3 aftertaste attributes. An *'other aroma'* and *'other flavour'* attribute were also included for panellists to rate and describe if any other sensory property was perceived during tasting. The sensory attributes, together with their definitions and composition of the sensory reference standards are detailed in Table 3. All attributes were rated on unstructured line scales (0-100), anchored from 'none' to 'high'. Within each 2-hour session, a maximum of 16 samples were presented with forced 1-5 minute breaks between samples with samples presented on-demand. Data were collected electronically using the software Redjade (Redjade Software Solutions, LLC, Tragon Corporation, California, USA, 2019).

All training sessions were conducted in a meeting room equipped with an electronic white board. Practice and formal evaluation sessions were held in the purpose-build sensory evaluation laboratory of QAAFI located at the Elkhorn building (UQ Long Pocket Campus, Indooroopilly).

The sensory evaluation area consists of 14 isolated sensory booths, tablets, temperature control (22°C) and under day-light equivalent lighting.

*Table 3 Sensory attributes and definitions used in the sensory descriptive study of wagyu beef on various diets.*

| Attribute | Marketing term | Definition | Reference Standard |
|---|---|---|---|
| **Aroma** | | (none to high) | |
| *aroma intensity* | **aroma intensity** | The overall aroma intensity of the sample. | nil |
| *roasted* | **roasted** | The rich aroma of roasted or pan-fried beef, rich beef stock, vegemite, savoury richness. | 2 cm2 piece of pan-crusted beef. |
| *caramelised* | **caramelised** | A sweet note, sweet potato-like, caramelised meat or fat. | ½ $cm^3$ of sweet potato which had been oven roasted until soft wrapped in foil PLUS 2 cm2 piece of pan-crusted beef. (Fresh daily) |
| *buttery/fatty* | **rich butter** | Aroma of rich butter, a fatty note. | Cooked beef fat ¼ tsp. (Fresh daily) |
| *gamey* | **game meat** | A game-meat aroma, liver, animal hormonal, porky. | Kangaroo fillet slice 2 cm2 (Woolworths, cooked med-rare on dry pan) and tiny piece of cooked beef liver. (Fresh every second day) |
| *barnyard* | **white pepper** | Aroma of white pepper, barnyard, pooey. | white pepper (McKenzie's Ground White Pepper). (Fresh each week) |
| *vegetative / corn* | **herbaceous** | Aroma of steamed green vegetables, sweet corn, herbaceous. | Fresh sweet corn kernels steamed (3 kernels) (Fresh every second day) |
| *other aroma* | | Any other aroma | nil |
| **Texture** | | | |
| *tenderness* | **tenderness** | The tenderness of the sample on the first few bites. | nil |
| *juiciness* | **juiciness** | The juiciness perceived during the first few bites. | nil |
| *dissolving* | **dissolving** | The dissolving, disintegrating nature of the sample in the first 3-5 bites. | nil |
| *fattiness* | **buttery texture** | The fattiness of the sample felt in-mouth. | nil |
| *chewiness* | **bite** | The amount of chewing required before swallowing. | nil |
| *fibrous* | **fibrous** | Presence and size of meat fibres in-mouth upon chewing. | nil |
| **Flavour** | | | |
| *flavour intensity* | **flavour intensity** | The overall flavour intensity of the sample. | nil |
| *sweetness* | **sweetness** | Sweet taste | Sweet (sucrose 10 g/l) |
| *sourness* | **tart** | Sour taste, tart, tangy. | Sour (citric acid 0.3 g/l) |
| *umami* | **umami** | Umami, vegemite, savoury richness, beef stock. | Umami (monosodium glutamate 0.6 g/l) |
| *roasted* | **roasted** | Flavours associated with roast beef | "as above for aroma" |
| *gamey* | **game meat** | A game-meat flavour, liver, animal hormonal, porky. | "as above for aroma" |

| Attribute | Marketing term | Definition | Reference Standard |
|---|---|---|---|
| *white meat* | **white meat** | The delicate flavour of poached chicken. | "as above for aroma" |
| *buttery / fatty* | **buttery** | Flavour of butter, fatty taste. | "as above for aroma" |
| *other flavour* | | Any other flavour | |
| **Aftertaste** | | | |
| *lingering flavour* | **lingering flavour** | A lingering flavour sensation in the mouth persists after swallowing. | nil |
| *metallic* | **mineral** | A metallic taste in the mouth after swallowing | nil |
| *umami/roasted* | **roasted** | An umami or roasted taste in the mouth after swallowing. | nil |
| *fatty mouth coating* | **buttery mouth coating** | A fatty oiliness coating the oral surfaces that remains after swallowing. | nil |

## Data Analysis

Data was exported from Redjade into Microsoft Excel for analysis. XLSTAT (version 5) was used to analyse the sensory data and R (R Core Team, 2022) was used to model effects with pen considered the experimental unit.

For all sensory attribute scores, minimum, maximum, mean, standard deviation and coefficient of variation were calculated. Principal component analyses (PCA) were performed on the mean scores for all samples to visually observe sample grouping and differentiation. Sample data were also plotted on a cobweb plot to aid in interpretation of differences observed.

Data from single observations (i.e. sensory attribute scores) were analysed using a linear mixed model ANOVA (lme4) with Treatment, Cut, Marbling and Block as fixed effects and Pens and Animals nested within Pen as random effects.

## Results and Discussion

A summary of the sensory data collected is provided in Table 4 including the minimum and maximum scores for each attribute used across the 152 samples as well as the mean, standard deviation and standard error.

*Table 4 Summary of the descriptive analysis scores (n= 76 animals x 2 cuts x 1 replicate tasting by 11 sensory panellists).*

| Variable | Minimum | Maximum | Mean | Std. deviation | Std. Error of mean |
|---|---|---|---|---|---|
| *aroma intensity AR* | 2 | 100 | 55 | 22 | 0.53 |
| *roasted AR* | 0 | 100 | 48 | 26 | 0.63 |
| *caramelised AR* | 0 | 100 | 38 | 30 | 0.73 |
| *buttery / fatty AR* | 0 | 98 | 37 | 28 | 0.68 |
| *gamey AR* | 0 | 100 | 21 | 26 | 0.64 |
| *barnyard AR* | 0 | 100 | 15 | 21 | 0.51 |
| *vegetative / corn AR* | 0 | 99 | 19 | 25 | 0.62 |
| *tenderness TX* | 0 | 100 | 59 | 27 | 0.65 |
| *juiciness TX* | 0 | 100 | 53 | 24 | 0.59 |
| *dissolving TX* | 0 | 100 | 41 | 29 | 0.72 |
| *fattiness TX* | 0 | 100 | 34 | 30 | 0.74 |
| *chewiness TX* | 0 | 100 | 48 | 28 | 0.70 |
| *fibrous TX* | 0 | 100 | 46 | 29 | 0.72 |
| *flavour intensity FL* | 5 | 100 | 60 | 19 | 0.48 |
| *sweetness FL* | 0 | 100 | 33 | 28 | 0.69 |
| *sourness FL* | 0 | 100 | 21 | 25 | 0.60 |
| *saltiness FL* | 0 | 95 | 27 | 25 | 0.61 |
| *umami FL* | 0 | 100 | 43 | 28 | 0.69 |
| *roasted FL* | 0 | 100 | 53 | 25 | 0.62 |
| *gamey FL* | 0 | 100 | 13 | 21 | 0.51 |
| *white meat FL* | 0 | 100 | 27 | 26 | 0.64 |
| *buttery / fatty FL* | 0 | 100 | 37 | 31 | 0.76 |
| *metallic/mineral FL* | 0 | 97 | 26 | 26 | 0.63 |
| *lingering flavour AT* | 0 | 99 | 48 | 22 | 0.54 |
| *metallic AT* | 0 | 100 | 33 | 29 | 0.71 |
| *umami/roasted AT* | 0 | 100 | 40 | 26 | 0.65 |
| *fatty mouth coating AT* | 0 | 100 | 38 | 30 | 0.73 |

The results from the mixed model analysis are given in **Error! Reference source not found.**. As expected, marbling and cut had a significant effect on sensory scoring for a number of attributes. However, treatment (diet) had no effect on sensory scores indicating the diet had no impact on sensory quality.

*Table 5. F-ratios and significance for effects in mixed model*

| Sensory attribute | Marbling | Block | **Treatment (Diet)** | **Cut** | Treatment x Cut |
|---|---|---|---|---|---|
| *aroma intensity AR* | 2.97 | 2.03 | 0.70 | **13.68*** ** | 1.40 |
| *roasted AR* | 0.49 | **3.55*** | 0.13 | 0.14 | 0.23 |
| *caramelised AR* | 0.34 | 1.61 | 0.44 | 0.14 | 0.08 |
| *buttery / fatty AR* | 0.0009 | 1.17 | 0.003 | 0.014 | 3.80 |
| *gamey AR* | **4.90*** | 1.51 | 3.88 | **49.96*** ** | 0.016 |
| *barnyard AR* | **7.31**** | 0.62 | 5.87 | 3.21 | 3.78 |
| *vegetative / corn AR* | 0.046 | 2.59 | 0.13 | **6.72*** | 2.47 |
| *tenderness TX* | **9.85**** | 0.14 | 0.29 | 1.59 | 1.57 |
| *juiciness TX* | **6.56*** | 0.62 | 0.27 | **25.43*** ** | 3.85 |
| *dissolving TX* | **11.52**** | 0.43 | 0.26 | **9.67**** | 1.28 |
| *fattiness TX* | **19.14*** ** | 0.64 | 1.37 | **88.21*** ** | 0.11 |
| *chewiness TX* | **8.15**** | 0.58 | 0.18 | 1.38 | 0.025 |
| *fibrous TX* | 3.07 | 0.56 | 1.04 | 1.61 | 0.0051 |
| *flavour intensity FL* | **22.38*** ** | 0.66 | 0.091 | 0.19 | 0.0044 |
| *sweetness FL* | **14.25*** ** | 2.09 | 1.18 | **46.26*** ** | 0.16 |
| *sourness FL* | **5.36*** | 0.81 | 0.053 | **105.67*** ** | 0.50 |
| *saltiness FL* | **7.09**** | 0.63 | 2.08 | **10.07**** | 0.23 |
| *umami FL* | **26.59*** ** | 2.52 | 1.29 | **18.48*** ** | 0.26 |
| *roasted FL* | **7.06*** ** | 2.12 | 0.40 | **17.58*** ** | 2.02 |
| *gamey FL* | 0.003 | 1.62 | 0.49 | **95.81*** ** | 0.35 |
| *white meat FL* | **14.93*** ** | 0.26 | 1.21 | 0.11 | 1.2 |
| *buttery / fatty FL* | **25.19*** ** | 0.72 | 2.01 | **89.91*** ** | 0.000 |
| *metallic/mineral FL* | **5.45*** | 1.64 | 0.11 | **155.16*** ** | 0.90 |
| *lingering flavour AT* | **9.28**** | 0.74 | 0.89 | **7.37**** | 0.67 |
| *metallic AT* | **8.03**** | 1.51 | 1.21 | **142.64*** ** | 1.92 |
| *umami/roasted AT* | **16.50*** ** | 0.69 | 0.75 | **12.04*** ** | 0.000 |
| *fatty mouth coating AT* | **19.12*** ** | 0.46 | 3.78 | **50.13*** ** | 0.0017 |

Significant F-ratios are indicated in bold and by * ($p < 0.05$), ** ($p < 0.01$) and ***($p<0.001$).

## Results of sensory evaluation of beef from the diet study - overview

The mean scores of 27 sensory attributes used for profiling the 76 animals from the diet study (average scores across the two cuts) were evaluated by principal component analysis (PCA). In the PCA bi-plot (PC1 v PC2, Figure 5) 51% of the variation in the data explained by the first two PC's. Animals from the control diet are shown in orange font, while animals from the treatment diet are shown in blue font (Figure 5). There was no significant difference in sensory scoring between samples from animals according to diet regime.

*Figure 5 PCA bi-plot of sensory profile data from 76 animals (average of 2 cuts) divided into 2 diet regimes (PC1 vs PC2 51%) (n = 1 animal x 2 cuts x 11 panellists).*

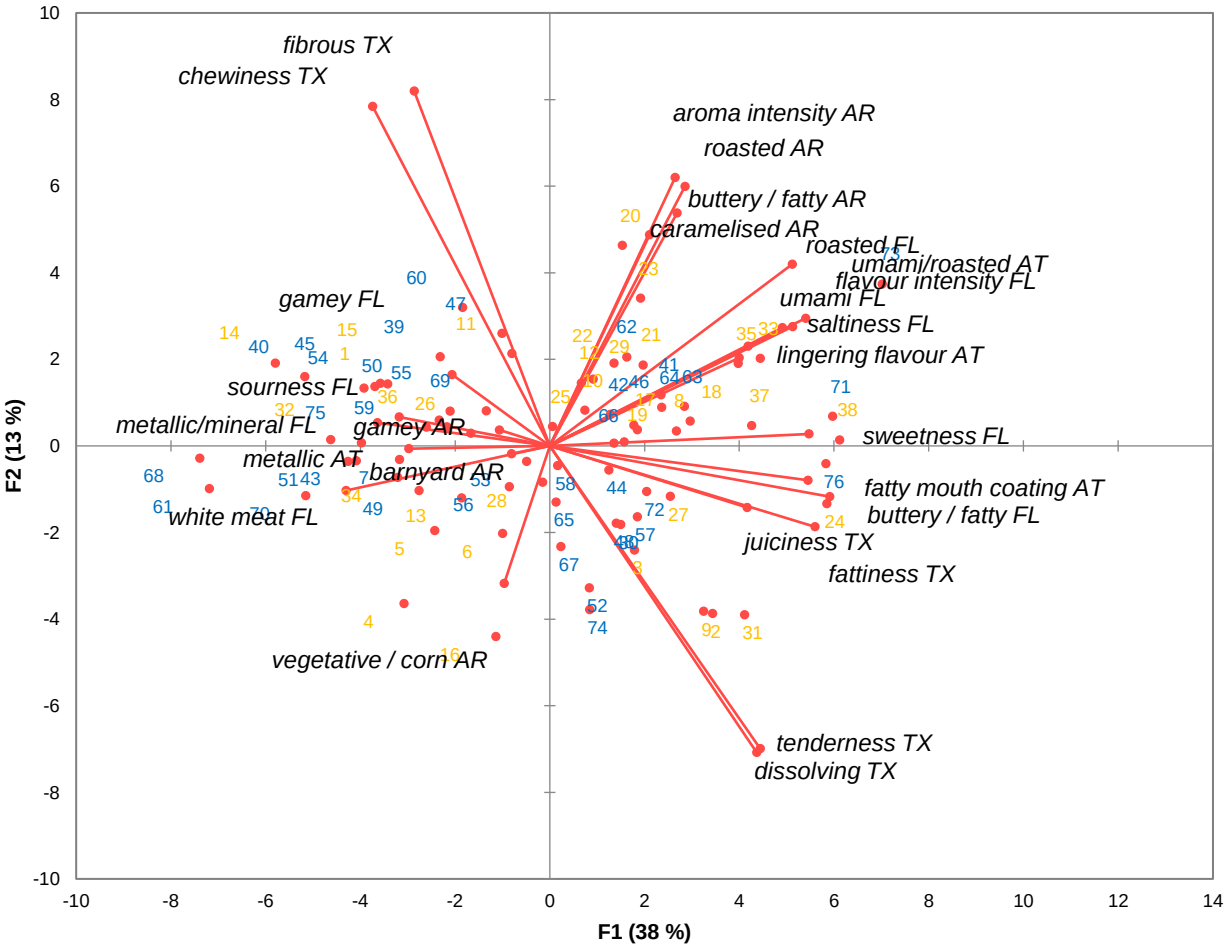



Figure 6 shows a PCA bi-plot of the average scores for samples from the two diets and two cuts. While significant differences were observed between cuts (left to right of the plot in Figure 6), there were no significant difference observed between samples of the same cut but from different diet regime.

*Figure 6 PCA bi-plot of average sensory profile data obtained from 2 cuts and 2 diets (PC1 vs PC2 83%) (n = 38 animals x 1 tasting by 11 sensory panellists)*

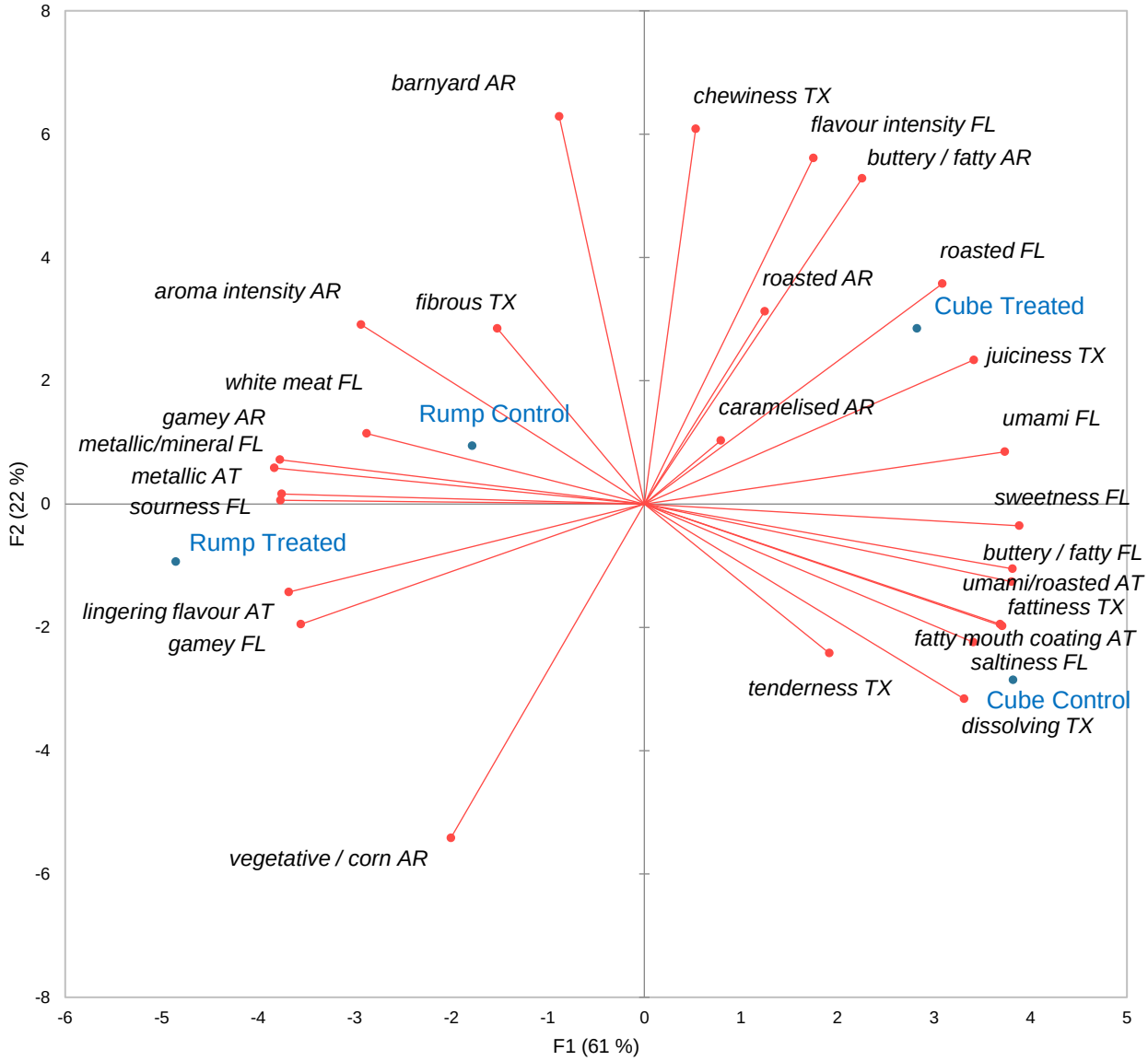



Figure 7 and Figure 8 provide cobweb plots comparing average sensory profiles by diet and cut, respectively. As shown in the mixed model (Table 5. ) 18 of the 27 sensory attributes differed significantly in score between samples according to cut, while none of the attributes differed significantly according to diet.

*Figure 7 Cobweb plot showing differences between beef samples from each **diet** (n=38 animals x 2 cuts x 11 panellists).*

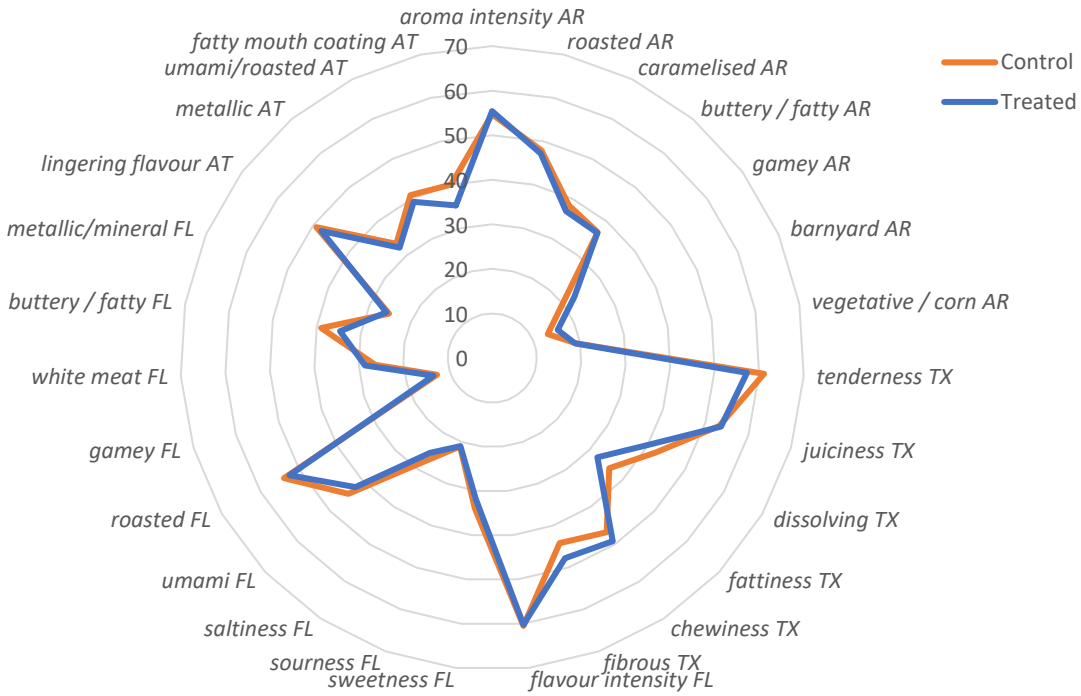
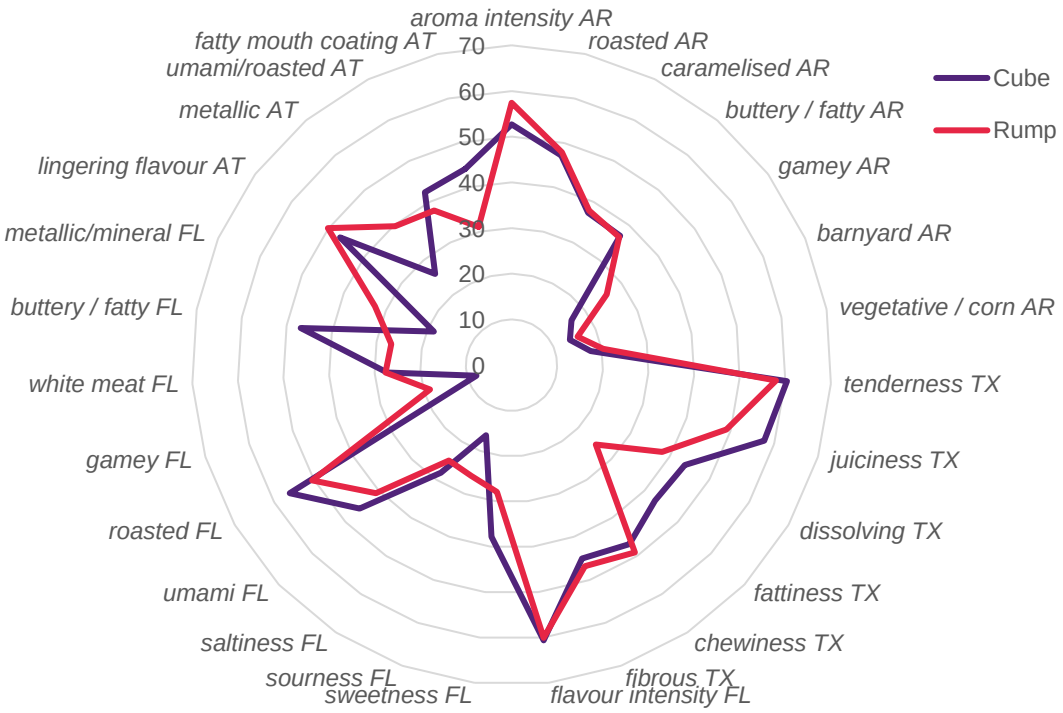



*Figure 8 Cobweb plot showing differences between different **cuts** of beef (n = 38 animals x 2 cuts x 11 panellists).*



# Appendix A-1 Block design for formal evaluation

Formals 1

| Red Jade numbers | Sensory code | Sample number | Sample type | Marbling |
|---|---|---|---|---|
| 1 | 171 | 56 | CR | |
| 2 | 374 | 73 | R | 30 |
| 3 | 258 | 60 | CR | 30 |
| 4 | 847 | 23 | R | 20 |
| 5 | 272 | 37 | R | |
| 6 | 201 | 35 | CR | 30 |
| 7 | 238 | 24 | R | |
| 8 | 643 | 19 | CR | 20 |
| 9 | 771 | 62 | R | 20 |
| 10 | 810 | 55 | CR | 30 |
| 11 | 971 | 12 | R | |
| 12 | 405 | 57 | CR | 20 |
| 13 | 320 | 54 | CR | 20 |
| 14 | 010 | 40 | R | 10 |
| 15 | 583 | 28 | CR | 40 |

Formals 2

| Red Jade numbers | Sensory code | Sample number | Sample type | Marbling |
|---|---|---|---|---|
| 16 | 038 | 5 | CR | 20 |
| 17 | 211 | 36 | R | |
| 18 | 257 | 58 | CR | 20 |
| 19 | 432 | 5 | R | 20 |
| 20 | 555 | 31 | R | |
| 21 | 333 | 75 | CR | 30 |
| 22 | 162 | 35 | R | 30 |
| 23 | 631 | 31 | CR | |
| 24 | 711 | 6 | CR | 30 |
| 25 | 823 | 58 | R | |
| 26 | 901 | 50 | R | 10 |
| 27 | 492 | 48 | CR | 70 |
| 28 | 905 | 73 | R | 30 |
| 29 | 52 | 53 | R | |
| 30 | 853 | 74 | CR | 30 |

Formals 3

| Red Jade numbers | Sensory code | Sample number | Sample type | Marbling |
|---|---|---|---|---|
| 31 | 391 | 66 | R | 40 |
| 32 | 081 | 47 | R | |
| 33 | 183 | 22 | R | |
| 34 | 592 | 53 | CR | 20 |
| 35 | 982 | 55 | R | 30 |
| 36 | 290 | 37 | CR | |
| 37 | 483 | 1 | CR | 10 |
| 38 | 685 | 29 | R | 30 |
| 39 | 733 | 66 | CR | 40 |
| 40 | 953 | 8 | CR | |
| 41 | 666 | 17 | R | 40 |
| 42 | 500 | 57 | R | 20 |
| 43 | 412 | 17 | CR | 40 |
| 44 | 368 | 1 | R | 10 |
| 45 | 812 | 49 | CR | 20 |

Formals 4

| Red Jade numbers | Sensory code | Sample number | Sample type | Marbling |
|---|---|---|---|---|
| 46 | 154 | 20 | R | |
| 47 | 131 | 64 | CR | 20 |
| 48 | 910 | 32 | R | |
| 49 | 743 | 19 | R | 20 |
| 50 | 709 | 36 | CR | |
| 51 | 347 | 3 | CR | 30 |
| 52 | 677 | 47 | R | 20 |
| 53 | 600 | 46 | R | |
| 54 | 870 | 70 | CR | 10 |
| 55 | 096 | 50 | CR | |
| 56 | 423 | 52 | R | 20 |
| 57 | 368 | 39 | CR | 20 |
| 58 | 819 | 11 | R | |
| 59 | 585 | 45 | CR | |
| 60 | 691 | 41 | CR | 20 |

Formals 5

| Red Jade numbers | Sensory code | Sample number | Sample type | Marbling |
|---|---|---|---|---|
| 61 | 420 | 61 | CR | 20 |
| 62 | 899 | 59 | R | 20 |
| 63 | 705 | 15 | CR | 20 |
| 64 | 424 | 72 | R | 30 |
| 65 | 621 | 7 | CR | |
| 66 | 506 | 60 | R | 30 |
| 67 | 966 | 29 | CR | 30 |
| 68 | 377 | 27 | CR | 40 |
| 69 | 813 | 2 | CR | 30 |
| 70 | 599 | 28 | R | 40 |
| 71 | 860 | 27 | R | 40 |
| 72 | 739 | 15 | R | 20 |
| 73 | 126 | 52 | CR | 20 |
| 74 | 114 | 51 | R | 30 |
| 75 | 680 | 30 | R | |

Formals 6

| Red Jade numbers | Sensory code | Sample number | Sample type | Marbling |
|---|---|---|---|---|
| 76 | 362 | 847 | CR | 40 |
| 77 | 853 | 10 | R | |
| 78 | 925 | 9 | CR | |
| 79 | 746 | 201 | R | 30 |
| 80 | 712 | 69 | R | 20 |
| 81 | 447 | 42 | CR | 70 |
| 82 | 179 | 65 | CR | 20 |
| 83 | 650 | 72 | CR | 30 |
| 84 | 261 | 68 | CR | 10 |
| 85 | 965 | 56 | R | 30 |
| 86 | 603 | 64 | R | 20 |
| 87 | 873 | 48 | R | 70 |
| 88 | 168 | 7 | R | |
| 89 | 292 | 30 | CR | |
| 90 | 402 | 10 | CR | |

| Formals 7 | | | | | Formals 8 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Red Jade numbers | Sensory code | Sample number | Sample type | Marbling | Red Jade numbers | Sensory code | Sample number | Sample type | Marbling |
| 91 | 824 | 63 | R | 20 | 106 | | 4 | R | |
| 92 | 234 | 51 | CR | 30 | 107 | | 69 | CR | |
| 93 | 435 | 76 | R | 40 | 108 | | 43 | R | |
| 94 | 809 | 46 | CR | 30 | 109 | | 61 | R | |
| 95 | 136 | 9 | R | | 110 | | 76 | CR | |
| 96 | 539 | 25 | CR | 20 | 111 | | 74 | R | |
| 97 | 400 | 11 | CR | | 112 | | 33 | R | |
| 98 | 047 | 16 | R | | 113 | | 16 | CR | |
| 99 | 263 | 63 | CR | 20 | 114 | | 18 | CR | |
| 100 | 086 | 11 | R | | 115 | | 40 | CR | |
| 101 | 185 | 2 | R | 30 | 116 | | 62 | CR | |
| 102 | 637 | 38 | CR | | 117 | | 54 | R | |
| 103 | 485 | 8 | R | | 118 | | 43 | CR | |
| 104 | 609 | 59 | CR | 30 | 119 | | 13 | R | |
| 105 | 668 | 75 | R | 30 | 120 | | 14 | R | |

| Formals 9 | | | | | Formals 10 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Red Jade numbers | Sensory code | Sample number | Sample type | Marbling | Red Jade numbers | Sensory code | Sample number | Sample type | Marbling |
| 121 | 840 | 26 | CR | 30 | 137 | 611 | 49 | R | 30 |
| 122 | 590 | 34 | R | 20 | 138 | 801 | 68 | R | 20 |
| 123 | 327 | 32 | CR | | 139 | 511 | 71 | CR | 20 |
| 124 | 492 | 38 | R | | 140 | 047 | 67 | R | |
| 125 | 813 | 26 | R | 30 | 141 | 630 | 45 | R | 30 |
| 126 | 596 | 14 | CR | 20 | 142 | 250 | 44 | R | 20 |
| 127 | 304 | 21 | R | | 143 | 305 | 34 | CR | |
| 128 | 402 | 33 | CR | | 144 | 732 | 44 | CR | |
| 129 | 700 | 71 | R | 30 | 145 | 777 | 18 | R | 30 |
| 130 | 540 | 65 | R | 20 | 146 | 150 | 3 | R | 20 |
| 131 | 404 | 12 | CR | | 147 | 350 | 25 | R | |
| 132 | 087 | 67 | CR | | 148 | 417 | 23 | CR | 40 |
| 133 | 938 | 70 | R | 10 | 149 | 936 | 20 | CR | 10 |
| 134 | 740 | 39 | R | 20 | 150 | 747 | 42 | R | 20 |
| 135 | 358 | 21 | CR | | 151 | 614 | 24 | CR | |
| 136 | 336 | 22 | CR | | 152 | 923 | 13 | CR | |