Final report

Improving beef production through management of plant toxins

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Abstract

The economic impacts of plant toxins on the Australian cattle industry are significant in terms of animal deaths, production losses and control measures ranging from several million dollars up to $50 million annually for Pimelea poisoning. This project sought to deliver a microbial inoculum together with an effective toxin adsorbent to enable cattle to better deal with the Pimelea toxin.

Microbiome analysis of rumen fluid collected from cattle with and without apparent resistance to Pimelea did not reveal any significant differences.

A series of 15 *in vitro* fermenter trials were conducted utilising rumen fluids collected from cattle, sheep, goats and kangaroos, with 157 bacterial isolates obtained. However, none of the bacterial isolates tested were capable of degrading simplexin as determined by LC-MS/MS.

An experimental inoculum was developed based on goat and sheep fermentations but was unfortunately unsuccessful in preventing Pimelea poisoning in a three-month Pimelea feeding trial with Droughtmaster steers. However, steers consuming bentonite adsorbent showed an increased resistance to toxin impacts, particularly in terms of the haematological parameters for the non-regenerative anaemia seen in severe Pimelea poisonings.

Biopolymer composites in *in vitro* fermentations and in fistulated steers demonstrated tailorable degradation rates depending on composition and degree of porosity and are good candidates for intraruminal sustained release of bioactives, including toxins to foster toxin-degrading microbe populations.

Executive summary

Background

The economic impact of plant toxins on the Australian cattle industry has been variously quantified in terms of animal deaths, production losses and control measures ranging from several million dollars annually for such individual plants as Lantana and Georgina gidgee, to $50 million for Pimelea poisoning. Less readily quantified, but equally important, are the detrimental impacts of significant poisoning events (such as Pimelea) on animal welfare and as well as the emotional wellbeing of producers who are managing poisoned livestock.

The intent of this project was the development of management strategies to achieve improved welfare, production, and profitability of the cattle industry with the main target audience being cattle producers across the inland grazing areas of Australia. Toxic Pimelea plants contain the toxin simplexin and are native species endemic to almost one half of Australia's pastoral lands. The distinctive syndrome of Pimelea Poisoning is unique to Australia with simplexin causing potent activation of Protein Kinase C with resultant pulmonary venule constriction leading to characteristic oedema, anaemia and heart failure. Occurrence of poisoning can be difficult to predict but is generally associated with winter rainfall and minimal pasture competition.

Objectives

This project sought to capitalise on natural rumen response of animals with prior toxin exposure by isolating microbes capable of degrading toxins (for use as preventative probiotics), to investigate biopolymers to foster toxin-degrading microbe populations, and to provide an effective toxin adsorbent to enable cattle to better deal with this toxin.

Despite best efforts, microbes able to degrade simplexin were not isolated during this study and an experimental inoculum administered to steers did not reduce Pimelea impacts. The use of toxin adsorbents demonstrated no effect on any variable measured. The biopolymer research has also demonstrated potential to act as slow-release delivery devices within the rumen, with wide and varied possible applications.

Methodology

1. Rumen microbial populations were determined by 16S rRNA gene amplicon sequencing of 112 field collected samples from cattle, sheep, goats and kangaroos exposed to Pimelea, and from cattle reported by producers to be resistant/non-resistant to Pimelea poisoning.
2. Collected rumen fluid samples were utilised as starter for 15 anaerobic laboratory fermentations fed daily with Pimelea to enrich for potential simplexin degrading microbes with simplexin concentrations monitored by LC-MS/MS analysis.
3. Degradation kinetics of different biopolymers impregnated with Pimelea particles/extract were studied in laboratory fermentations for up to 63 days. To determine degradation potential *in vivo*, solid and porous PHA biopolymers were studied during a four-month trial in fistulated steers.
4. Laboratory studies were undertaken with a range of adsorbents to study their capacity to remove simplexin from rumen fluid as monitored by LC-MS/MS analysis.
5. A Pimelea feeding pen trial (30 steers) was conducted with six treatment groups to determine effects of selected adsorbents and experimental inoculum treatments relative to positive

(Pimelea-fed) and negative (no Pimelea) groups and impacts on the rumen microbiome, blood and other parameters.

Results/key findings

Gastrointestinal microbial populations were found to vary between the animal species sampled (cattle, sheep, goats and kangaroos), but there were only small differences between cattle regardless of origin or previous experience with Pimelea. A series of 15 *in vitro* fermenter trials were conducted based on rumen fluids collected from diverse animal species, regions, and reported Pimelea susceptibility, with 57 bacterial isolates obtained. However, none of the bacterial isolates tested were capable of degrading simplexin as determined by LC-MS/MS.

Extruded biopolymer composites demonstrated a high degree of stability under both rumen-like fermenter conditions and within the rumen of fistulated steers, with surface erosion degradation providing options to control potential bioactive release rates. For example, porous 3D printed PHA biopolymer pieces degraded more than twice as fast as the solid biopolymers in the *in vivo* study.

In vitro studies indicated that adsorbents such as activated biochar and bentonite showed some potential as simplexin adsorbents, with activation of biochar by heating to 1,000°C required to enhance biochar potential. Pen trials however, demonstrated that steers fed Pimelea and a mitigating treatment as a constant part of their daily diet showed no difference in resistance to poisoning compared to a control group fed a similar Pimelea dose. The rumen microbes in trial animals consuming Pimelea were not significantly different to the animals consuming a hay diet and the rumen microbiome appeared to be very resilient, continuing to function despite the deteriorating health condition of the animals.

Benefits to industry

Overall outcomes from this project suggest that the administration of a mitigating treatment such as bentonite, an inoculum or activated charcoal as part of a loose lick will not prevent the onset of Pimelea poisoning. A small number of producers with Pimelea affected pastures provided bentonite in loose lick supplements to cattle during the past year at a rate about double used in our pen trial and reported very encouraging results with noticeable improvements in animal health and production. The proposed rate of ~100–200 g per head is within range approved by the European Food Safety Authority (EFSA) who assessed bentonites as being safe for all animal species, the consumers and the environment when used at a maximum level of 20,000mg/kg or 20g/kg complete feed.

Future research and recommendations

Further research is required to understand the fate of simplexin. Neither simplexin nor its presumed metabolites were detectable in weekly blood samples, yet characteristic oedema only became evident in some animals up to two weeks after cessation of Pimelea intake, which suggests an unidentified reservoir of toxin within the animal. Preliminary computer-based molecular modelling studies are being conducted to enable a better understanding of the purported interactions between simplexin and Protein Kinase C (PKC). The activation of this PKC enzyme is key to the causative pulmonary venule constriction and modelling the dynamics of the interaction of simplexin with PKC may yet lead to a novel solution to Pimelea poisoning which continues to plague producers across our inland grazing regions.

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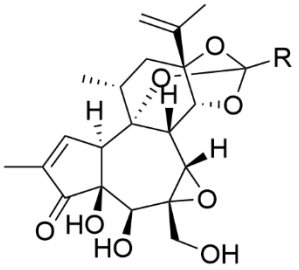
1. Background

1.1 Strategies to enable toxin breakdown in the rumen before uptake into the bloodstream

Devising strategies to deal with diverse plant toxins is not easy as the chemical action and target organ varies considerably, and the best line of action is prevention rather than remedial treatment. In pasture systems it is difficult to prevent consumption of poisonous plants, other than by total removal from the pasture which is generally not possible. However, plant consumption does not necessarily equal uptake of the toxin, and the approach of the proposed research is to devise strategies to enable toxin breakdown within the rumen before absorption into the animal's circulatory systems.

Our approach in this project encompassed three key strategies that were applied to Pimelea as a high economic impact plant in the first instance, but with the potential to expand the devised methodologies to other plant toxins in the future. This project sought to capitalise on the variable response in cattle observed with ingestion of plant toxins by isolating microbes capable of degrading toxins (for use as preventative probiotics), and to investigate toxin adsorbents and/or biopolymers to foster toxin-degrading microbe populations. This three-pronged approach was to devise strategies to enable toxin breakdown in the rumen before absorption into the bloodstream. Initially this research was applied to the Pimelea toxin, simplexin (Figure 1.1), which causes frequently fatal poisoning in cattle grazing inland pastures of Australia. Pimelea plant species are native plants widespread across arid inland grazing regions and have been previously reported to contain the toxin simplexin with lesser amounts of the related toxin huratoxin (Figure 1.1) (Chow et al. 2010).

Figure 1.1. Chemical structure of the toxic orthoester simplexin (1) and the related daphnane orthoester huratoxin (2) with differing alkyl groups (R).



simplexin (**1**) R = -$(CH_2)_8CH_3$
huratoxin (**2**) R = -$(CH{=}CH)_2(CH_2)_8CH_3$ -(*E*,*E*)

1.1.1 Microbial inoculum for toxin degradation in the rumen

"Experienced" cattle with long-term exposure to particular poisonous plants are frequently noted to have improved resistance to the toxin when compared to "naïve" stock. This improved "resistance" in experienced livestock can generally be attributed to increased ruminal degradation of toxin by appropriate degrading bacteria in the rumen. Our previous research demonstrated in a four month feeding trial that cattle exposed to prolonged low doses of Pimelea developed the ability to deal with the toxin and overcome toxic effects (Fletcher et al. 2014). It was considered that microbial adaptation had occurred during this prolonged low dose exposure, and this project therefore sought to isolate these detoxifying bacteria to enable the production of an inoculum with the capacity to

degrade the Pimelea toxin simplexin as a preventative probiotic, akin to the inoculum produced by DAF for Leucaena.

1.1.2 Biopolymers for slow release

The controlled delivery of low dose Pimelea toxin would be an ideal mechanism in bolstering beneficial rumen bacterial populations without adverse toxin effects, and the second component of our proposal is the design and development of rumen boluses or similar constructs containing Pimelea toxin material within a biopolymer matrix. Polyhydroxyalkanoate biopolymers are water insoluble, stable under rumen pH and degrade via a surface erosion mechanism, making them ideal candidates as novel delivery systems to provide timely low-dose toxin release and encourage beneficial microbes to propagate.

Despite the numerous controlled release technologies available, the options for the design of a ruminal device to cattle which can deliver such an agent are limited. Most existing systems are basically designed to release the drug by a single rate-controlling physical or chemical phenomenon such as diffusion-dissolution through a polymer matrix or membrane, chemical degradation leading to erosion, controlled corrosion, or swelling/osmotic pressure. Because of the poor aqueous solubility of the Pimelea toxin, designs based on the partition-diffusion of drug through a rate-controlling membrane are not likely to yield therapeutic release rates using commonly employed polymeric materials of reasonable thickness. Likewise, the use of swelling/osmotic pressure as a driver for release is less likely to be successful. Designs based on the erosion of chemically degradable polymers are therefore the most effective strategy, and, in practice, it is necessary to restrict the degradation to the surface of the device, which makes the use of biopolymers such as polyhydroxyalkanoates, which are water insoluble and degrades via a surface erosion mechanism, very attractive. In this project, four types of biopolymer were selected for their biodegradability as well as their compatibility for medical applications (Asghari et al. 2017; Barouti et al. 2017; Laycock et al. 2017; Davoodi et al. 2018; Elmowafy et al. 2019; Harting et al. 2019; Rodríguez-Contreras et al. 2019) or veterinary applications (Rathbone and McDowell 2013; Bilhalva et al. 2018).

- Polyhydroxyalkanoate (PHA): which is a polyester produced by numerous microorganisms, through bacterial fermentation of carbon feedstocks. It is typically biodegradable in any environment where bacteria are present.
- Polycaprolactone (PCL): which is a biodegradable polyester produced by ring opening polymerisation of ε-caprolactone using a catalyst.
- Polylactic acid (PLA): which is a biodegradable (compostable) thermoplastic derived from renewable resources (fermented plant starch).
- Starch: natural polymer derived from plants, which is composed of two macromolecules: amylose and amylopectin. To generate thermoplastic starch polymers, the complex semicrystalline structure needs to be destroyed to produce an amorphous material. This process called gelatinisation, is achieved by heating the starch in the presence of water and/or some other plasticiser.

1.1.3 Enteroadsorbents for toxin adsorption in the rumen

The use of clay adsorbent has been routinely employed for foods and feeds containing mycotoxins such as aflatoxin (Bryden 2012), and these and other adsorbents such as activated charcoal has also shown some promise to treat plant poisoning of sheep and cattle (McKenzie 1991b). Activated charcoal is well known as an effective adsorbent of many organic toxins with particular application in

acute medical interventions for drug overdose and poison ingestion (Cooney 1980). Activated charcoal can similarly be utilised to treat livestock and forms for example the basis of therapy for Lantana poisoning in livestock (McSweeney and Pass 1982). Bentonite has also been reported to be an effective and considerably cheaper alternative therapy for Lantana poisoning in sheep (McKenzie 1991a). The mechanism of organic toxin adsorption by bentonite was perhaps less clear and it was suggested that bentonite as a swelling clay could absorb water and expand within the rumen and potentially sequester the toxin within its ionic structure (McKenzie 1991a). Bentonite (Dadswell et al. 1994) and kaolin (Cantello 1969) clay treatments have also been suggested for Pimelea treatment but their therapeutic value has never been scientifically tested. Biochar is carbonized biomass like charcoal and although less "activated" (pyrolysed) than activated charcoal has found use in acute treatment of animals for many centuries (Schmidt et al. 2019). A review of impacts of biochar in animal feed by Schmidt et al. (2019) found that positive effects on parameters including toxin adsorption were generally reported, although it was noted that a considerable number of studies provided statistically non-significant results, though tendencies were mostly positive.

In the past decade, there has been some anecdotal reported use of biochar and bentonite in reducing impacts of Pimelea poisoning, although no scientific studies have been reported to date. As a third prong to this research, this project investigated the Pimelea toxin adsorption capacity of biochar and bentonite *in vitro* and tested efficacy *in vivo*. Before the commencement of this project, the project team presented at a series of AgForce producer forums to discuss impacts of Pimelea and prospects for further research, including well attended forums at Begonia (May 2016), St George (March 2017), and St George (August 2017). A small number of producers reported some but varied success in preventing/treating Pimelea Poisoning using lick blocks containing bentonite (Olsson's Bentobite Block, 33% bentonite) and biochar (Olsson's Biochar Agricultural charcoal Stockchar block, 35% biochar).

Additional producer Pimelea Research Update forums were held at Begonia, Roma and St George in October 2018, and producers again reported some success with these blocks or with bentonite or biochar as a component of loose lick mixes. The adsorbent products most employed by these Pimelea-affected producers appear to be RCRA Biochar and Sibelco Trufeed Sodium Bentonite, with both products produced in western Queensland. RCRA Biochar is a certified organic livestock grade biochar produced from Gidgee biomass by Renewable Carbon Resources Australia and distributed by Bos Rural supplies. Additionally, RCRA Biochar is incorporated in production of licks/blocks by Olssens, Nutralick (Mitchell) and Top Ration (Roma) among others. Trufeed Sodium Bentonite is produced by Sibelco Australia at the Gurulmundi Bentonite Mine near Miles and is a highly swelling natural clay, noted for its benefits of high water absorption, cation exchange ability and organic adsorption properties.

2. Project objectives

Original objectives for this project were as stated below in Section 2.1 and Section 2.2, with the success in meeting these objectives described in Section 2.3.

2.1 Outputs

2.1.1 Microbial inoculum

Development of new natural microbial inoculums that influence rumen function and enable management of harmful plant compounds (specifically Pimelea toxins in the first instance).

2.1.2 Enterosorbents

Development of natural or synthetic adsorbents that bind plant toxins and/or provide timely controlled release to enable manipulation of rumen function to improve management of harmful plant toxins.

2.2 Outcomes

2.2.1 Better information for industry

Better information available to the cattle industry to manage the impact of plant toxins on reproduction and productivity.

2.2.2 Management strategies

Development of management strategies through extension, pasture management or treatments to reduce impacts of these plant toxins and increase productivity.

2.3 Success in meeting project objectives and outcomes

2.3.1 Microbial inoculum

The project was not able to produce an inoculum that was effective in preventing impacts of Pimelea, as demonstrated in a three-month Pimelea feeding trial with 30 Droughtmaster steers. A series of 15 *in vitro* fermenter trials were conducted based on rumen fluids collected from diverse species, regions, and reported susceptibility of animals, with 157 bacterial isolates obtained. However, none of the bacterial isolates tested were capable of degrading simplexin.

2.3.2 Enterosorbents

Biopolymer-Pimelea composites were successfully trialled in fermenter studies with release rates able to be manipulated by selection of biopolymer composition and inclusion of porogen. A range of enterosorbents were successfully tested for their capacity to bind simplexin *in vitro*, with three selected adsorbents also tested in the Pimelea feeding trial. Co-administered bentonite provided a degree of protection to steers consuming Pimelea. It has been postulated that bentonite administered at a higher level could offer higher protection, but this is yet to be tested.

2.3.3 Better information for industry

A series of regional producer days, info sessions and presentations have been held throughout this project (with support from AgForce, FutureBeef and MLA) with the aim to keep producers informed and involved, see Section 9.1**.** It is expected that a summary of project outcomes will be made available to producers through these same outlets.

2.3.4 Management strategies

Overall outcomes from this project suggest that the administration of bentonite as part of a loose lick may aid in the prevention of onset of Pimelea poisoning. The proposed rate of ~100–200 g per head is within range approved by EFSA who assessed bentonites as being safe for all animal species,

the consumers and the environment when used at a maximum level of 20,000 mg/kg or 20g/kg complete feed (EFSA Panel on Additives Products or Substances used in Animal Feed et al. 2017).

Other management strategies as previously published (and available on the FutureBeef website) remain unchanged (Fletcher et al. 2009).

3. Methodology

3.1 Introduction and overview of methodology

This project was undertaken as a series of separate but interrelated components, as outlined below.

3.1.1 Development of microbial probiotic

Field collected Pimelea plant and rumen fluid from livestock exposed to Pimelea in pasture (both affected and non-affected animals) was utilised as source material for toxin (simplexin) isolation and as a source for potential detoxifying bacteria, respectively. Rumen fluid obtained from these field animals were utilised in a rumen microbiome field survey and as starter for a series of 15 *in vitro* fermenter studies. These fermenter studies were all fed daily with Pimelea plant material for up to 63 days to select for toxin-degrading microbes. Bacteria isolates obtained from these fermenter trials were identified and tested for their ability to degrade simplexin *in vitro*. Isolated simplexin was utilised as a substrate for degradation assays and as a quantitation standard for LC-MS/MS analysis. Rumen inoculums for use in the cattle feeding trial outlined below were harvested from two of these fermentation trials.

3.1.2 Biopolymer composites for toxin slow-release within the rumen

Selected biopolymer composites (PHA, PCL, PLA and blends thereof) were extruded with incorporation of either milled Pimelea plant material or ethanolic Pimelea extracts. Porous PHA biocomposites were also prepared by incorporation of water-soluble porogens, other components readily assimilated by microbes, and sodium bicarbonate (foaming agent). Each biopolymer was characterised before and after bacterial degradation in *in vitro* fermenter trials to determine both the rate of biopolymer degradation and also the simplexin release/uptake (as determined by LC-MS/MS). The degradation of selected biopolymers (without toxin inclusion) was also assessed *in vivo* over a 4-month period in fistulated calves.

3.1.3 Enterosorbent studies

A range of adsorbents (including Biochar, activated Biochar and bentonite) were characterised and tested for their capacity to adsorb simplexin under rumen-like conditions *in vitro* with monitoring of toxin uptake by adsorbents carried out by LC-MS/MS analysis. Selected adsorbents were then further studied for their efficacy in the *in vivo* feeding trial outlined below.

3.1.4 Demonstration of treatment efficacy in cattle feeding trials

A Pimelea feeding trials was undertaken with six cattle treatment groups comprising T1, Pimelea only (positive control); T2, Pimelea + Biochar; T3, Pimelea + activated Biochar; T4, Pimelea + inoculum; T5, no treatment (negative control) and T6, Pimelea + bentonite. Thirty cattle with no previous exposure to Pimelea (naïve cattle) were selected and consumed Pimelea over a three-

month period in individual pens with careful monitoring of animal health to determine the efficacy of each treatment in mitigating adverse impacts of Pimelea.

3.2 Animal ethics approvals

Appropriate animal ethics approvals were obtained for all studies conducted in this project. Copies of relevant approvals are included in Appendix (Section 9.5), and include approvals for collection of rumen fluid from fistulated cattle at Gatton campus (SAFS/296/17); collection of rumen fluid from cattle, sheep and goats in the field (SA 2016/11/586), extended and further modified to include collection of lymph nodes, blood and other tissues from animals euthanased in the field by DAF officers (SA 2019/11/722); biopolymer trials in fistulated steers at Gatton Campus (SA 2020/03/737 and DAF/QAAFI/125/20); Pimelea feeding trial conducted in individual pens at QASP (QAFFI/QASP/337/20/DAF).

3.3 Pimelea plant collections and processing

Pimelea plant material has been collected, freeze dried and milled as recorded in the project P.PSH.0900 (Fletcher and Ouwerkerk 2018). For each collection a voucher specimen has been lodged with the Queensland Herbarium and species identification confirmed with AQ number recorded.

Further bulk sources of *P. trichostachya* were identified and additional plant material (100kg for fermenter trials and 50kg for feeding trial) collected with details as recorded in Table 3.1 **.**

Table 3.1. Summary of additional Pimelea plant samples collected and some of the metadata recorded.

Collection date	Property number[A]	Local Government Area	Property ID (PIC)	Site #[B]	Geographic location	Altitude	Plant Species	Herbarium Voucher Number[C]	Amount collected (FW kg)	Freeze dried and milled	Predominant pasture	Habitat	Soil type
16/9/18	8	Balonne	QEBL0048	2	27° 58' 48" S 148° 11' 9" E	NR[D]	*Pimelea trichostachya*	AQ522769	100	NA	Oats cultivation	Yellow jacket, poplar box (cleared)	Red loam with sedimentary surfaces
14/09/20 13/10/20	-	Balonne	QGBR0175	-	-27.570230 147.686327	NR[D]	*Pimelea trichostachya*	AQ952584	50	Yes	Pasture	Pasture	-

[A] Collections from Properties 1-30 were recorded in P.PSH.0900 (Fletcher and Ouwerkerk 2018).

[B] Multiple collection sites on same property

[C] Queensland Herbarium

[D] NR = not recorded

3.4 Isolation of simplexin and huratoxin from Pimelea plant material

3.4.1 Purified simplexin and huratoxin

Simplexin was isolated from dried and milled roots of *Pimelea elongata* (Qld Herbarium ID AQ522483) by repeated chromatography in an adaptation of methods described previously (Hayes et al. 2010) with final purification by preparative High Performance Liquid Chromatography (HPLC). The identity of the purified compound was confirmed by Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis and comparison with literature mass spectra (Chow et al. 2010).

Additional simplexin and huratoxin were similarly isolated from *Pimelea trichostachya* seeds (Qld Herbarium ID AQ522769, 40.77 g). Isolated simplexin (25.5 mg, 0.06% yield) was 95% pure and confirmed to be simplexin by NMR and LC-MS/MS. Huratoxin (1.51mg) was also isolated.

Extraction from *P. trichostachya* seeds (Qld Herbarium ID AQ522769, 58.65 g) was repeated giving simplexin (23.7 mg, 0.04% yield), and huratoxin (6.7 mg, 0.01% yield). The structure of huratoxin was confirmed and shown to be 80% pure by nuclear magnetic resonance spectroscopy (NMR) and LC-MS/MS. Extraction from *P. trichostachya* seeds (Qld Herbarium ID AQ522769, 121.54 g) was again repeated giving simplexin (126.89 mg, 0.1% yield), 95% pure.

3.4.2 Pimelea plant extract

A crude Pimelea plant extract was prepared from green aerial portion of *Pimelea elongata* (AQ522483). Freeze-dried and milled plant material (40g) was extracted with ethanol as previously described (Fletcher and Ouwerkerk 2018) to provide a 250 mL solution in 90% ethanol which was utilised in the preparation of media for simplexin degradation studies (Section 3.7.4).

3.4.3 Semi-pure simplexin for media preparation

Semi-pure simplexin was prepared from 114g of *P. trichostachya* seeds (Qld Herbarium ID AQ522769) by extraction followed by repeated chromatography as described above. Without further purification, this gave the semi-pure extract (230.6mg). Repetition of this procedure yielded semi-pure extract (332.0 mg). Based on previous extractions, the total amount of simplexin in these semi-pure extracts was estimated to be 60 mg. This semi-pure material was used in the preparation of media for bacterial isolate degradation studies (Section 3.8.4).

3.5 Analysis of Pimelea toxins by LC-MS/MS

Simplexin was analysed in various samples throughout this project including fermenter fluid, plant material, biopolymer samples and animal tissues. Preparation and clean-up of samples varied depending on the samples, but UHPL-Q-Orbitrap-MS parameters were based on a common method.

3.5.1 LC-MS/MS analysis method and parameters

Extracted samples were analysed on a Dionex Ultimate 3000 UPLC coupled to a Thermo Q Exactive high resolution accurate mass (HRAM) spectrometer (Thermo Fisher Scientific, Scoresby, Australia) as previously described (Fletcher and Ouwerkerk 2018). The analytical column was a Waters UPLC BEH RP18 1.7um 2.1 x 100mm (Milford, MA, USA). The mobile phase consisted of (A) ammonium formate (10 mM) in water with 0.1% formic acid and (B) 98% v/v methanol/water with ammonium formate (10 mM) and 0.1% formic acid. The column was eluted at 0.3 mL/min with mobile phase B

held at 5% for 0.3 min followed by linear gradients of B from 5-80% (0.3–0.5 min), 80-93% (0.5-0.8 min), 93-99% (0.8–1.3 min), held at 99% (1.3–5.8 min) before 99-5% over 12 sec, where it was held until stopped at 7 min. The column temperature was maintained at 35˚C. Instrument control and data acquisition were conducted using Xcalibur software (version 3.0.63), and data analysis was conducted using Tracefinder (version 4.1). All software was from Thermo Fisher Scientific, Waltham, MA, USA. The Thermo Q Exactive mass spectrometer was equipped with an electrospray ionisation (ESI) interface and was operated in positive ionisation mode, using the following settings: electrospray voltage 3.50 kV, sheath gas: 45 arbitrary units, auxiliary gas: 10 arbitrary units, capillary temperature 250 ˚C.

Full-scan MS was operated in positive polarity, with a resolving power of 70,000 (at m/z 200) and a scan range of m/z 80–1,200. The automatic gain control was set to a target value of 3 $x10^6$. The maximum time of accumulating ions per scan event was 200 ms. Parallel reaction monitoring (PRM) was also operated in positive polarity, with a resolving power of 70,000 (at m/z 200) and automatic gain control was set to a target value of 2 $x10^5$. The maximum time of accumulating ions per scan event was 100 ms and an isolation window of 4.0 m/z was utilised. Normalised collision energy was 35%. The inclusion list corresponded to simplexin ($C_{30}H_{44}O_8$, $M+H^+$) 533.31089.

Mass calibration was carried out using ThermoTune software (Version 2.8 SP1 Build 2806).

3.5.2 Quantitation of simplexin using external standard

For generating extracted ion chromatograms, a relative mass extraction window size of ±5 ppm was applied during data analysis. The most dominant daughter ion was chosen as the transition for quantitation (533.3109 -> 253.1225) and the next dominant daughter ion was selected as the transition (533.3109 - > 267.1382) used for verification.

Simplexin was isolated by repeated chromatography in an adaptation of methods described previously (Hayes et al. 2010) as described in Section 3.4.1. Isolated simplexin (>95% pure) was used as an external standard in LC-MS/MS analysis with standard solutions prepared in methanol (5 - 3,000 µg/L, i.e. ppb). The linearity of each standard curve was shown by plotting the peak area versus simplexin concentration. The unknown concentrations were determined from the standard curve weighted least-squares regression analysis.

3.5.3 Quantitation of huratoxin using external standard

The simplexin-related toxin, huratoxin (as well as additional simplexin) was isolated from *Pimelea trichostachya* seeds (AQ522769) as described in Section 3.4.1.

The LC-MS/MS method inclusion list was then expanded to include huratoxin ($C_{34}H_{48}O_8$, $M+H^+$) 585.3416 and the most dominant daughter ion was chosen as the transition for quantitation (585.3416 -> 253.1225) and the next dominant daughter ion was selected as the transition (585.3416 - > 267.1382) used for verification. The unknown concentrations of huratoxin in extracts were estimated using the simplexin standard curve weighted least-squares regression analysis.

3.5.4 Quantitation of simplexin with added internal standard

MS detection was performed as described in Section 3.5.1 with some modification of MS parameters. HESI probe in positive ionisation mode was optimised for simplexin with spray voltage 3.5kV, sheath gas flow 45 arbitrary units, auxiliary gas flow 10 arbitrary units, capillary voltage 5V, capillary temperature 250°C and tube lens 50 V.

Full-scan and parallel reaction monitoring (PRM) was employed with a resolution of 70,000 (FWHM at m/z 200) and a full MS mass range of 400–800 m/z, automatic gain control target of 3e6 and maximum injection time of 200 ms. MS/MS parameters were set to resolution 70,000 (FWHM at m/z 200) with automatic gain control target of 2e5, maximum injection time of 100 ms, isolation width of 40 m/z, isolation off-set of 20 m/z, normalised collision energy of 35 eV for simplexin and loop count set to 1. Inclusion list of simplexin used protonated simplexin ($(M+H)^+$, m/z 533.3108). The major fragment ions of m/z 533.3109 - > 253.1220 and m/z 533.3109 - > 267.1381 were used for quantification and verification respectively. Phorbol 12-myristate 13-acetate (PMA, internal standard, Sigma Aldrich) was monitored at m/z 617.4080 - > 311.1642 with normalised collision energy set to 15 eV.

Isolated simplexin (>95% pure) prepared in methanol was used for the calibration curve (10 – 2000 ng/mL) with PMA (50 ng/mL) used as the internal standard for quantitative analysis using the response ratio.

3.6 Rumen Microbiome: Field Survey

3.6.1 Source rumen material

A total of 110 rumen/forestomach pellet samples had previously been obtained from 82 cattle, 11 goat, nine sheep and eight kangaroos. These included rumen samples collected from 61 cattle, 11 goats (unknown, feral breeds and Boer), nine sheep (Merino, Dohne and Dorper) and forestomach samples from eight kangaroos (Eastern Grey, Red and Wallaroo) in the MLA Donor Company project P.PSH0900 (Fletcher and Ouwerkerk 2018), 17 cattle rumen samples collected in the Agri-Science Queensland Innovation Opportunity project AS10583 (Ouwerkerk and Milson 2017) and four rumen samples from heifers within the Pimelea feeding trial conducted in 2008 (Fletcher et al. 2014). Details of the samples and source properties 1- 17 are contained in Appendix Section 9.6

3.6.2 Extraction of gDNA and sequencing

The gDNA from each of 1.0 mL frozen rumen pellet samples were extracted using the RBB+C method (Yu and Forster 2005) The quantity and quality of the extracted gDNA was determined prior to sequencing using the Invitrogen Qubit® (Thermo Fisher Scientific, USA) with the dsDNA BR assay kit (Thermo Fisher Scientific) as per the manufacturer's instructions. The extracted gDNA quality was confirmed by 1% agarose gel electrophoresis in TBE buffer with 1Kb DNA ladder and the DNA was visualised using GelRed® stain. The gDNA were diluted to within the range of 10 - 50 ng/µL in a final volume of 50 µL.

The samples were tested for PCR inhibition by using extracted gDNA as template in the universal 16S rRNA gene PCR assay with the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (3'-AAGGAGGTGWTCCARCC-5') (Lane 1991) to PCR amplify 16S rRNA genes. Each PCR reaction contained 5X Phire buffer (4 µL); 10 µM dNTP mix (0.4 µL); 27F primer 12.5 pmol/µL (0.8 µL); 1525R pmol/µL (0.8 µL); ultrapure H_2O (12.6 µL); template DNA (1 µL) and Phire Hot Start II DNA polymerase (0.4 µL) (Thermo Fisher Scientific). PCRs were carried out in a C1000 Thermal Cycler PCR machine (Bio-Rad Laboratories Pty., USA) with the hot lid set to 105 °C. The amplification conditions were denaturation at 98°C for 30 sec, followed by 35 cycles of 98°C for 5 sec, 60 °C for 5 sec, and 72°C for 25 sec; followed by final extension step 72°C for 3 min and then held at 12°C until manually stopped. The quality and quantity of the 16S rRNA gene amplicons were determined by 1% agarose

gel electrophoresis in Tris Acetate EDTA (TAE) buffer at 85 V for 45 min with a GeneRuler1Kb DNA ladder and the DNA was visualised using GelRed® stain (Biotium, USA).

The gDNA were sent to an external sequencing provider, the Australian Genomic Research Facility (AGRF) for microbial diversity profile sequencing using 16S rRNA gene barcoded amplicons generated of the 16S rRNA gene V3-V4 region using the forward primer 341F (5'-CCTAYGGGRBGCASCAG-3') and reverse primer 806R (5'- GGACTACNNGGGTATCTAAT-3') with overhang adapters and sequenced using the Illumina MiSeq platform to obtain 300 bp paired end reads. The sequencing was undertaken in two submissions, the first submission contained 17 samples from two properties and the second submission contained 93 samples from 14 properties plus a negative control and one sample from the first submission.

The sequence data received from AGRF consisted of .fastq files of forward and reverse paired end reads for each sample. These raw sequence files were archived on (1) a restricted access Queensland Cyber Infrastructure Foundation (QCIF) data collection (Rumen Ecology Q0259); and (2) a restricted access archive within the Queensland Government, Department of Environmental Science, High Performance Computing (HPC) facility (Apollo); and (3) the DAF \\lands\data server.

3.6.3 Rumen microbiome field survey: Sequence data analysis and statistics

The analysis of microbiome sequence data obtained for rumen field survey samples, was undertaken for samples collected from project P.PSH.0900 (Fletcher and Ouwerkerk 2018). The 17 samples collected from two properties (Property 6 and 7) within DAF Agri-Science Queensland Innovation Opportunity grant AS10583 were excluded from further analysis due to insufficient quality of the reverse sequence reads. To facilitate sample description and statistical analysis, a metadata mapping file was created, incorporating information relating to each sample, for example: Property from which the sample was collected and region (Station clusters, including the Animal Research Station, Augathella, Blackall, Bollon St George, Miles, Roma, and St George); type or species of animal (Animal Breed, including Kangaroo, Small ungulates, *Bos taurus*, *Bos indicus* cross, *Bos taurus* and *Bos indicus*); Sex (male, female); Lactation; Body Condition Score i.e. scale from 1 to 5, with 1 being very low Body Condition and 5 being very good Body Condition; extent of clinical signs of Pimelea Poisoning i.e. Severe, Moderate, Mild or None (Clinical signs); and the overall presence or absence of clinical signs of Pimelea poisoning (Clinical signs, yes/no); use of any dietary supplements, for example licks or protein meal (Dietary supplements): and grain supplementation (High Grain).

The quality of the raw sequence data was initially checked using FASTQC software https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ then quality filtered, trimmed of barcodes and primers and size filtered to retain sequences with a minimum length of 200nt using Trimmomatic version 0.36 (Bolger et al. 2014). These sequences were then analysed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software pipeline package Version 2018.6 (Caporaso et al. 2010; Bokulich et al. 2018). The forward and reverse sequence reads were formatted for import into QIIME2 and using the DADA2 software for modelling and correcting Illumina-sequenced amplicon errors (Callahan et al. 2016), the input sequences were further quality filtered, the forward and reverse reads merged, unique sequences (sequence variants) grouped, and chimeras removed. A Feature table containing the counts (frequencies) of each unique sequence in each sample in the dataset (Feature), a representative sequences file and a FeatureData file which maps Feature identifiers in the Feature table to the sequences they represent, was then created. A multiple sequence alignment using the Multiple Alignment using Fast Fourier Transform software (Katoh et al. 2002) and a phylogenetic tree was created to relate Features to one another and assign

phylogenetic groups to the Feature table. Taxonomy was then assigned using a pre-trained Naïve Bayes classifier trained on the Greengenes database (2015 update 13_8, 99% sequence similarity) (DeSantis et al. 2006; McDonald et al. 2012). Following the release of new software and a more comprehensive database for assigning taxonomy to 16S rRNA reads, the analysis presented in Milestone Report 2, was revised and updated using the QIIME2 software pipeline package version 2021.4 (Caporaso et al. 2010; Caporaso et al. 2012) and taxonomy assigned using a pre-trained Naïve Bayes classifier trained on the SILVA database December 2019 update, version 138 (Yilmaz et al. 2014), which has adopted the revised taxonomy detailed in the Genome Taxonomy Database (GTDB) (Parks et al. 2020).

The Feature table was filtered to remove samples containing relatively low numbers of sequences (< 14,820), samples with unusually high numbers of sequences (> 100,000 sequences) and sequencing control samples. A total of 87 samples were retained for further analysis. Throughout the analysis the original Feature table was filtered to retain only samples of interest, for example, to remove the effect of species variation on microbial populations, the Feature table was filtered to retain cattle samples only.

For each Feature table, alpha diversity measures (microbial diversity within a sample) and beta diversity measures (differences in diversity between samples) were calculated using QIIME2 software. Alpha diversity analysis was determined on the basis of four measures: (1) counts of observed species (Observed Species); (2) Phylogenetic Diversity (PD); (3) Shannon entropy of counts (Shannon); and (4) Pielou's Evenness; a measure of community evenness (Evenness). The four alpha diversity measures were analysed in Genstat Release 21.1 (27 January 2022; VSN International Ltd., 2022) using a general linear model (GLM, Regression analysis) and accumulated analysis of variance (ANOVA). Predicted means and standard errors (s.e.) were calculated. Metadata categories tested included animal breed, and then when breed effects were removed by examining cattle only, additional metadata categories including station clusters, sex, dietary supplementation, clinical signs (yes/no) and the interaction of clinical signs and breed were examined. Additional tests undertaken with Genstat, include the Fisher's least significant difference test to identify differences occurring between groups within each metadata category.

For determination of the differences in the microbial communities occurring between samples (Beta diversity), the respective metadata files, as well as the table, representative sequence (rep set), and unrooted phylogenetic tree (.tre) files generated using QIIME2, were imported into the R packages, Phyloseq (version 1.30.0; https://joey711.github.io/phyloseq/index.html) (McMurdie and Holmes 2013); and MixOmics (version 6.10.6; http://mixomics.org/methods/pls-da/)(Rohart et al. 2017). Statistical exploration and microbial community analysis used a multivariate projection-based approach with repeated measures. For the identification of indicator species and determination of microbial signatures, a sparse Partial Least Squares Discriminant Analysis (SPLSDA) was undertaken. This method was conducted using the MixOmics R package.

Briefly, an unsupervised analysis with Principal Component Analysis (PCA) (Jolliffe 2002) was conducted using the Feature table data generated using QIIME 2, transformed using the centred log ratio (CLR). To determine the most discriminative Features or OTUs (Features being referred to as OTUs within the MixOmics package), that best characterised factors of interest, a supervised analysis and selection of discriminative OTUs was undertaken with a multivariate analysis SPLSDA on three components (Shen and Huang 2008; Lê Cao et al. 2011). Contribution plots showing the most discriminative OTUs were generated based on the coefficient derived from the component analysis. This indicated the importance of the respective OTUs in determining the microbial signature, with the sign indicating the positive or negative correlations between the OTUs, relative to the

proportions of the others. Due to the high numbers of OTUs within the microbial signatures, results were presented as tables of the top 10 most important OTUs with respective assigned taxonomy, for each of the three components of the sPLSDA.

Core microbial communities were determined following taxonomic classification of Features identified using QIIME2. Features which were present in 100% of samples according to the metadata category of interest (e.g., animal species), were designated as "core" microbial communities. For comparison of the numbers of core and overall microbial communities, the on-line tool Venny was used (Oliveros 2007-2015). This method was used to generate Venn diagrams and lists of microbial populations which were designated as either shared or unique, according to the metadata category of interest.

3.7 Screening for detoxifying bacteria in field collected material

3.7.1 Plant nutritional analysis

A dried, milled sample of each Pimelea species was selected (*Pimelea trichostachya* AQ522479, *P. simplex* subsp. *continua* AQ522485 and *P. elongata* AQ522480)(Fletcher and Ouwerkerk 2018) and submitted for nutritional analysis by both DAF Biosecurity Sciences Laboratory (Coopers Plains, Qld) and SGS Australia (Cairns, Qld) utilising their respective standard laboratory methods. Buffel grass was collected from a single location near Blackall in western Queensland transported to Dutton Park where it was oven dried at 55 °C for 48 h, milled (3mm screen) on a Christy and Norris Ltd. Mill (Suffolk, UK), and similarly submitted for nutritional analysis.

3.7.2 Rumen fluid collections

Cryopreserved rumen fluid samples were used in 16 separate *in vitro* fermentations to enrich for bacterial populations able to utilise Pimelea and its toxin simplexin or investigate microbial degradation of different biopolymer compositions. Rumen fluid samples collected from fistulated steers #1990 and #1998 at the Queensland Animal Science Precinct at UQ Gatton (Animal Ethics Approval SAFS/296/17), cryopreserved and stored frozen at -20 °C were used either individually to inoculate Fermentation 1 or together Fermentations 8, 9, 12, and 14. Rumen fluid samples collected in the project P.PSH.0900 (Fletcher and Ouwerkerk 2018) under Animal Ethics Approvals SA 2016/11/586 and SA 2019/11/722, cryopreserved and stored frozen at -20 °C were thawed and used to inoculate Fermentations 2, 3, 5, 7, 10, and 11. Fermentation 4 was inoculated with rumen fluid collected in 2008 from Pimelea feeding trial Calf 4 (Heifer tag number #694) (Fletcher et al. 2014), cryopreserved and stored frozen -20 °C. Collection details and volumes of rumen fluid samples used to start each fermentation are listed in Table 3.2.

Table 3.2. Cryopreserved samples (rumen/fermentation fluid) used for *in vitro* fermentations 1-16.

Fermentation Number	Animal number	Sample date	Sample origin	Sex/Age	Breed	Volume (mL)
FERM#1	1990	27/04/18	UQ, Gatton[A]	♂/6 yr	Brahman X	100
FERM#2	096 120 MZ120	24/08/17	Property 1[B]	♀/2+ yr ♀/12 yr ♂/2 yr	Shorthorn X	50 50 50
FERM#3	5192 5143 5393	24/08/18	Property 3[B]	♂/18 mth ♂/18 mth ♂/2 yr	Droughtmaster Droughtmaster Shorthorn	50 50 50
FERM#4	694	26/08/08	Animal Research Institute[C]	♀/9 mth	*Bos taurus*	250
FERM#5	n/a[D]	29/09/17	Property 13[B]	2 ♂/6 yr 1 ♀/2yr	Feral goat	Mix 200
FERM#6	1990 1998	15/03/19 15/03/19	UQ, Gatton[A] UQ, Gatton[A]	♂/6 yr ♂/6 yr	Brahman X Brahman X	100 100
FERM#7	n/a	26/09/17	Property 8[B]	♂/5 yr ♂/5 yr ♂/5 yr	Merino Merino Merino	Mix 200
FERM#8	1990 1998	15/03/19 15/03/19	UQ, Gatton[A] UQ, Gatton[A]	♂/6 yr ♂/6 yr	Brahman X Brahman X	100 100
FERM#9	1990 1998	15/03/19 15/03/19	UQ, Gatton[A] UQ, Gatton[A]	♂/6 yr ♂/6 yr	Brahman X Brahman X	100 100
FERM#10	n/a	28/09/2017	Property 11[B]	♀ ♂	Red Kangaroo Red Kangaroo	200
FERM#11	n/a	27/09/19	Property 10[B]	2 ♀/4 to 5 2 ♀/4	Dorper sheep Dohne sheep	200 200
FERM#12	1990 1998	15/03/19 15/03/19	UQ, Gatton[A] UQ, Gatton[A]	♂/6 yr ♂/6 yr	Brahman X Brahman X	100 100
FERM#13	Day10 Day30		FERM#11		n/a	100 100
FERM#14	1990 1998	15/03/19 15/03/19	UQ, Gatton[A] UQ, Gatton[A]	♂/6 yr ♂/6 yr	Brahman X Brahman X	100 100
FERM#15	Day30 Day43		FERM#5 FERM#13	n/a	n/a	100 100
FERM#16	blank	n/a	n/a	n/a	Sterile RF/Gly	100

[A]Animal Ethics Approval SAFS/296/17; [B]Fletcher and Ouwerkerk (2018); [C]Fletcher et al. (2014); [D]n/a – Not applicable

3.7.3 *In vitro* anaerobic Fermentations 1 to 16

Anaerobic fermentations were conducted in a Labfors 3 benchtop fermentation system (Infors HT, Switzerland) using a fermentation volume of three litres. The fermenter vessel was maintained at 39 °C, continuously bubbled with a mixture of CO_2:H_2 (95:5 v/v) at 1.2 L/min to ensure anaerobic conditions and maintained at a pH 6.7. Total fermentation time per experiment was 11 days for Fermentation 1, 20 days for Fermentation 2 and 30 days for Fermentations 3 and 4 (Table 3.3).

Fermentations were commenced using 3 L of a fermenter starter medium (Appendix Section 9.7.1) including cryopreserved samples of rumen fluid or fermentation fluid as shown in Table 3.2. For Fermentations 1 to 4, at the commencement of the fermentation 20 g of dried, milled (3 mm screen) *Pimelea trichostachya* AQ522479 (NTL1714) collected in Maranoa during project P.PSH.0900 (Fletcher and Ouwerkerk 2018), and 20 g of dried, milled (3 mm screen) Buffel Grass (*Astrebla sp*.) pasture hay, was added as substrate for the fermentation. For Fermentation 4, at commencement 40 g of dried, milled (3 mm screen) *Pimelea trichostachya* AQ522479 (lab id = NTL1714) was added as substrate for the fermentation.

Table 3.3. Duration and start feed composition for *in vitro* Fermentations 1 to 16.

Fermentation Number	Duration (Days)	*Pimelea trichostachya* AQ522479 (g)	Buffel grass (g)
FERM#1	11	20	20
FERM#2	20	20	20
FERM#3	30	20	20
FERM#4	30	40	-
FERM#5	30	40	-
FERM#6	30	20	20
FERM#7	30	40	-
FERM#8	30	20	20
FERM#9	63	20	20
FERM#10	30	40	-
FERM#11	30	40	-
FERM#12	30	20	20
FERM#13	43	40	-
FERM#14	30	-	40
FERM#15	56	40	-
FERM#16	10	40	-

During each of the fermentations, half of the fermenter liquid volume (i.e. 1.5 L) was removed into 3 x 500 mL Wheaton bottles on a daily basis and replaced with 1.5 L of fermenter salts solution (Appendix Section 9.7.2), a balanced anaerobic salt solution containing minimal nutrients. From the second day of fermentation onwards, Fermentations 1 to 3, 6, 8, 9 and 12 had 5.0 g of Pimelea and 5.0 g of Buffel grass added daily, whilst Fermentations 4, 5, 7, 10, 11, 13, 15 and 16 had 10 g of Pimelea added daily. Fermentation 14 had 10 g of Buffel grass added daily from the second day of fermentation onwards. In Fermentations 3 to 5, from Day 20 to Day 30 the fermenter fluid removed was filtered through nylon stocking into plastic bottles and frozen at -20 °C for use in the development of a selective media for isolating bacteria (Section3.8.1). Fermenter fluid sampled from selected days of seven fermentations were also used for serial dilutions leading to bacterial isolations (Section 3.8.2).

Fermenter fluid aliquots for analysis

For each fermentation sample aliquots were taken from the second 500 mL of removed fermentation fluid and the types and numbers of samples taken on various days throughout each of the four fermentations are listed in Table 3.4. Each day a drop of fermentation fluid was placed on a slide and the bacterial diversity present visually examined daily using 400X magnification on an Eclipse 80i microscope (Nikon Instruments Inc., USA) and a representative field of view photo taken to allow the progression of community shifts occurring during the fermentation to be captured.

The fermentation fluid (FF) aliquots (1.0 mL) were placed into 1.5mL microcentrifuge tubes, centrifuged at 16,100 xg for 10 min, the resulting supernatant removed, and the remaining cell pellet stored frozen at -20 °C for future gDNA extraction and microbiome analysis. The 20 mL and 2.0 mL FF aliquots were placed into 50 mL or 15 mL falcon tubes respectively and frozen at -20°C until transfer to the Natural Toxin Laboratories for LC-MS/MS measurement of simplexin concentration.

Table 3.4. Details of samples of fermenter fluid taken for analysis during each *in vitro* fermentation from Ferm#1 to Ferm#16.

	Fermentation days	gDNA (1.0 mL)	LC-MS/MS (2.0 mL)	LC-MS/MS (20 mL)	Simplexin degradation assay
FERM#1	1	4	5	5	
11 Days	2 to 11	4 daily	5	5	
	5, 11	-	-	-	9 x 2.0 mL per time point (0 h, 48 h, 168 h)
FERM#2	1	8	39	-	
20 Days	2 to 20	4 daily	3	-	
	5, 10, 15, 20	-	-	-	9 x 2.0 mL per time point (0 h, 48 h, 168 h)
FERM#3 to #8, 10 to 12, #14	1	8	39	-	
	2 to 30	4 daily	3	-	
30 Days	10, 15, 20, 24, 30	-	-	-	18 x 1.0 mL time point (0 h, 48 h, 72 h, 168 h)
FERM#9	1	8	39	-	
63 Days	2 to 63	4 daily	3	-	
	10, 15, 20, 24, 30, 35, 40, 45, 50, 55, 60,63	-	-	-	18 x 1.0 mL/time point (0 h, 48 h, 72 h, 168 h)
FERM#13	1	8	39	-	
43 Days	2 to 63	4 daily	3	-	
	10, 15, 20, 24, 30, 35, 40, 43	-	-	-	18 x 1.0 mL per time point (0 h, 48 h, 72 h, 168 h)
FERM#15	1	8	39	-	
56 Days	2 to 56	4 daily	3	-	
	10, 15, 20, 24, 30, 35, 40, 45, 50, 56	-	-	-	18 x 1.0 mL per time point (0 h, 48 h, 72 h, 168 h)
FERM#16	1	8	39	-	
10 Days	2 to 10	4 daily	3	-	

3.7.4 Fermentation fluid simplexin degradation assay

A simplexin degradation assay was developed to determine if microbial degradation of the toxin was occurring in the fermentation (Appendix Section 9.7.12). This assay underwent a number of modifications with each successive fermentation trial. In Fermentation 1, nine Hungate tubes (3 x 3) containing 10 mL of fermentation fluid (FF) from Day 5 and from Day 11 (Table 3.4) were set up and allocated (in triplicate) to three treatment groups –

1. Fermentation Fluid + Simplexin (FFS) where 200 µL of an ethanol extract of Pimelea plant material containing simplexin at 1,465 ng/mL was added to each tube;
2. Fermentation Fluid (FF) + 200 µL of Fermenter Salts solution was added to each tube;
3. Fermentation Fluid + Ethanol (FFE) where 200 µL of 90% ethanol was added to each tube.

In Fermentation 2 the same nine Hungate tubes (FFS/FF/FFE) were set up and an extra three Hungate tubes containing 10 mL of fermentation fluid (Day 5, 10, 15 and 20) were immediately

autoclaved at 105 °C for 45 min, allowed to cool and 200 µL of Fermenter Salts solution was added to each tube (Killed Fermentation Fluid (KFF).

In Fermentations 3 and 4 the original nine Hungate tubes (FFS/FF/FFE) containing 10 mL of fermentation fluid (Day 10, 15, 24 and 30) were set up as above and a complete second set of nine tubes were set up and immediately autoclaved at 121 °C for 30 min, allowed to cool then allocated (in triplicate) to three treatment groups

1. Killed FF + Simplexin (KFFS) where 200 µL of an ethanol extract of Pimelea plant material containing simplexin at 1,465 ng/mL was added to each tube;
2. Killed FF (KFF) – 200 µL of Fermenter Salts solution was added to each tube; and
3. Killed FF + Ethanol (KFFE) where 200 µL of 90% ethanol was added to each tube.

For Fermentations 1 and 2, 2.0 mL aliquots were removed at time zero and placed into 15 mL Falcon tubes. The Hungate tubes were then incubated at 39 °C with rocking and further 2.0 mL samples taken at 48 h and 168 h. For Fermentations 3 and 4, 1.0 mL aliquots were removed at time zero, with further 1.0 mL aliquots taken at 48 h, 72 h and 168 h. All samples were stored frozen at -20 °C until extraction for LC-MS/MS measurement of simplexin concentration by LC-MS/MS

For Fermentations 5, 7, 10 to 13 and 15, a set of Hungate tubes (FFS/FF/FFE) containing 10 mL of fermentation fluid collected every five days from Day 10 onwards across the fermentations (Table 3.4) were allocated (in triplicate) to three treatment groups

1. FF + Simplexin (FFS) where 200 µL of an ethanol extract of Pimelea plant material containing simplexin at 1,465 ng/mL was added to each tube;
2. FF (FF) where 200 µL of Fermenter Salts solution was added to each tube; and
3. FF + Ethanol (FFE) where 200 µL of 90% ethanol was added to each tube.

On Day 20 Fermentation 5, Day 10 Fermentation 7, Day 10 Fermentation 10 and Days 10 and 24 of Fermentation 13, a second complete set of nine tubes of FF were immediately autoclaved at 121 °C for 30 min, allowed to cool then allocated (in triplicate) to three treatment groups

1. Killed FF + Simplexin (KFFS) where 200 µL of an ethanol extract of Pimelea plant material containing simplexin at 1,465 ng/mL was added to each tube;
2. Killed FF (KFF) where 200 µL of Fermenter Salts solution was added to each tube; and
3. Killed FF + Ethanol (KFFE) where 200 µL of 90% ethanol was added to each tube.

For each degradation assay, 1.0 mL aliquots were removed at time zero, with further 1.0 mL aliquots taken at 48 h, 72 h and 168 h (Table 3.4). All samples were stored frozen at -20 °C until extraction for LC-MS/MS measurement of simplexin concentration by the Coopers Plains Natural Toxins Lab.

3.7.5 *In vitro* dry matter digestion (DMD) assay

Within a number of *in vitro* fermentations, a series of nylon bags were suspended within the fermenter system and removed at various time points for both dry matter digestion of Pimelea or Buffel Grass and biopolymer degradation (Table 3.5) To determine the ability of the microorganisms to digest the dried, milled Pimelea, an *in vitro* dry matter (DM) digestion assay was used (Appendix Section 9.8). Briefly, milled Pimelea plant material (3 mm screen) was dried at 55 °C for 48 h–72 h, as were Nylon bags with a pore size of 45 µm measuring 98 mm x 38 mm sewn with polyester thread (Allied Filter Fabrics, Berkeley Vale NSW) each with unique number written in permanent marker. The nylon bags and a length of fishing line were weighed, and their combined weights recorded before 1.0 g of Pimelea, or biopolymer piece, was added and the opening secured with fishing line. The filled nylon bags were then weighed, and the weights recorded. These bags were incubated in

the fermentation and taken out (in duplicate) as detailed in Table 3.5. Upon removal from the fermentation, the nylon bags were washed, dried at 55 °C for 48 h – 72 h and re-weighed to determine the reduction in weight of the Pimelea. Once weighed the bags were sent to the Coopers Plains Natural Toxin Lab for simplexin measurement.

Table 3.5. Nylon bags containing either biopolymer pieces or plant material taken for analysis during each *in vitro* fermentation.

	Day of removal	Number of nylon bags removed for analysis	
		Dry matter	Biopolymer
FERM#1	1	2[A]	
11 Days	3, 5, 7, 9, 11	10[A] (2/day)	
	11		12 (3 replicate bags; 4 biopolymers – PHA, PCL, PLA, Starch)
FERM#2	1	2[A]	
20 Days	3, 5, 7, 15, 20	10[A] (2/day)	
	20		12 bags (2 replicate bags; 3 biopolymers – PHA, PCL, PLA with either5% or 40% Pimelea inclusion)
FERM#3	1	4[B]	
30 Days	30	4[B]	
	10, 30		12 bags/day (2 replicate bags; 3 biopolymers PHA, PCL, PLA with either 0% or 20% Pimelea inclusion)
FERM#4	1	6[C]	
30 Days			
	15, 30	12[C] (6/day)	
	10, 20, 30		21 bags (7 bags/day with 2 biopolymer pieces/bag; 3 biopolymers PHA, PCL, PLA – with either 0% Pimelea or 30% Pimelea inclusion and one bag of PHA biopolymer with 20% Pimelea inclusion)
FERM#6	10, 20, 30	-	26 bags (8 bags/day with 2 biopolymer pieces/bag; 2 biopolymers PHA, PCL; PHA, PHA with 30% *Pimelea* inclusion or *Pimelea* ethanol extract; PCL with 30% *Pimelea* inclusion; PHA containing 50% PCL; PHA containing 35%PCL with 30% *Pimelea* inclusion; an additional 2 bags of PHA and PHA with 30% *Pimelea* inclusion were left till the end for method development of biofilm on the surface of the biopolymer DNA extraction)
30 Days			
FERM#8	10, 20, 30	-	24 bags (8 bags/day; 1 biopolymer; 2 biopolymer pieces/bag; all biopolymers had either 0% Pimelea or 30% Pimelea)
30 Days			
FERM#9	20, 41, 63	-	26 bags (8 bags/day with 2 biopolymer pieces/bag; PHA; PHA + 30% *Pimelea* inclusion; PHA + 50% Sugar; PHA + 35% Sugar + 30% *Pimelea*; PHA + 50% Starch, PHA + 35% Starch + 30% *Pimelea*; PHA + 5% $NaHCO_3$, PHA + 3.5% $NaHCO_3$ + 30% *Pimelea*; remaining 2 bags PCL and PCL with 30% Pimelea inclusion were removed on Day 30)
63 Days			
FERM#10	23, 30	-	Bags added into Fermenter on Day 3 - 12 bags (6 bags/day with 2 biopolymer pieces/bag; PHA; PHA + 30% *Pimelea*; PHA + 50% Sugar; PHA + 35% Sugar + 30% *Pimelea*)
30 Days			
FERM#12	10, 20, 30		24 bags with 2 biopolymer pieces/bag (4 bags/day; PHA; PHA + 30% Pimelea; PHA + 30% cryo-milled Pimelea; PHA + Pimelea ethanol extract; PHA + 35% icing sugar + 30% Pimelea; PHA + 35% icing sugar + 30% cryo-milled Pimelea; PHA + 50% icing sugar + Pimelea Ethanol extract; 35 % PHA + 35 % starch + 30% cryo-milled Pimelea)
30 Days			
FERM#14	2, 5, 10, 20, 30		20 bags with 2 biopolymer pieces/bag (4 bags/day; PHA + 50% icing sugar + 30% cryo-milled Pimelea; PHA + 50% starch + 30% cryo-milled Pimelea; PHA + 30% cryo-milled Pimelea; PHA)
30 Days			

[A]bags contained milled Pimelea plant

[B]bags contained milled Buffel Grass or were empty

[C]bags contained Pimelea or Buffel Grass or were empty

3.7.6 Biodegradation of biopolymers

To test the biodegradation rate of samples of different biodegradable biopolymers by rumen bacteria, small pieces of each biopolymer were placed in nylon bags, incubated in the fermenters and removed for analysis, as detailed in Table 3.5. The nylon bags containing the biopolymers were washed several times with reverse osmosis (RO) water prior to opening and retrieving the biopolymer ribbon. The biopolymer ribbons rinsed with RO before being dried at 45 °C for a minimum of 24 h prior to being analysed. Biopolymer pieces were returned to the Chemical Engineering research group at UQ and were physically characterised as detailed in Section 3.9.10.

3.7.7 Cryopreservation for bacterial isolations

On the final day of Fermentations 2 through to 13 and 15, four 50 mL aliquots of fermentation fluid were mixed with 50 mL cryopreserving medium RF/Gly (Appendix Section 9.7.4) and frozen at -25 °C for future fermentations. Three 4.0 mL aliquots of fermentation fluid were mixed with 4.0 mL cryopreserving medium RF/Gly and frozen at -25 °C for use in bacterial isolations.

3.7.8 Fermentation microbiome methodology

Fermenter fluid was sampled from the majority of *in vitro* fermentations of Pimelea plant material, conducted during the course of this project (Table 3.4). Throughout the fermentations, at each sample collection time point, four 1.0 mL samples of fermenter fluid were taken into 1.5 mL microcentrifuge tubes, centrifuged at 16,100 x g for 10 min and the resulting supernatant discarded. The remaining cell pellet was stored frozen at -20°C for future gDNA extraction and microbiome analysis. Although fermenter fluid was collected from all fermentations, only selected fermentations were chosen for microbiome analysis, including Fermentation numbers. 4, 5, 6, 7, 11, 13 and 15.

If cryopreserved fermenter fluid was used to start the fermentation system, either replicate 1 mL subsamples of the cryopreserved fermenter fluid were collected prior to inoculation of the fermenter, or the microbial populations of this starter material was estimated by in silico addition of respective fermenter fluid microbiome samples. For example, Fermentation 13 was inoculated with fermenter fluid obtained from Fermentation 11 on days 10 and 30; and Fermentation 15 was inoculated with fermenter fluid obtained from Fermentation 5 on day 30 and Fermentation 13 on day 43. In the instance of Fermentation 4, there was no subsample taken of the cryopreserved rumen fluid used to start the fermentation, therefore the microbiome sequence data for the original bovine rumen fluid sample (*Bos taurus* cow, Ear tag number 694), generated in the Illumina MiSeq sequencing project for the Field Survey samples (Section 3.6.1) was used to indicate the microbial populations inoculated into Fermentation 4.

DNA extraction and PCR amplification

DNA was extracted from microbial cell pellets obtained from 1mL subsamples of fermenter fluid and cryopreserved rumen fluid samples. As detailed in methods Section 3.6.2 above, the gDNA from each of 1.0 mL frozen microbial pellet samples were extracted using the RBB+C method (Yu and Forster 2005). The extracted gDNA quality was confirmed by 1% agarose gel electrophoresis in TBE buffer with 1Kb DNA ladder and the DNA was visualised using GelRed® stain. Quantitation of extracted DNA was undertaken using a Spectrophotometer (Nanodrop 8000, Thermo Scientific).

In contrast to the methodology used for the rumen fluid samples (Section 3.6.2), the first round of PCR amplification was conducted in our laboratory, with the primers used to amplify the V3 to V4 region of the 16S rRNA gene also containing an Illumina sequence tag (tagged PCR primers, 16S-

341F-tagged 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTAYGGGRBGCASCAG '3; and 16S-806R-tagged 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGACTACNNGGGTATCTAAT '3). PCR reactions were prepared with a total volume of 50 µL and used the Platinum SuperFi II DNA Polymerase enzyme (Thermofisher Scientific). The PCR protocol involved an initial denaturation of 98 °C for 1 min, then 25 cycles of 98 °C for 10 s, an annealing step of 60 °C for 10 s, extension step of 72 °C for 30 s and a final extension step of 72 °C for 5 min. All PCR reactions were performed using a SimpliAmp Thermo Cycler (Applied Biosystems). The inclusion of a sequence tag enabled the external sequence provider (AGRF) to undertake the second round of PCR to multiplex the samples and proceed with sequencing within a single lane of the Illumina MiSeq sequencing platform.

Sequence data analysis and statistics

The sequence data received from AGRF, consisting of 300 bp paired end reads, were initially quality filtered, paired and size trimmed (retaining reads >200 bp in length) using Trimmomatic version 0.36 (Bolger et al. 2014). Paired reads were then imported into the High Performance Computer (HPC) and the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software pipeline package version 2021.4 (Caporaso et al. 2010; Caporaso et al. 2012) and the DADA2 software (Callahan et al. 2016) used to model and correct any remaining Illumina sequencing errors. In this way the reads were further quality filtered, the forward and reverse reads merged, unique sequences (sequence variants) grouped, and chimeras removed. A Feature table containing the counts (frequencies) of each unique sequence in each sample in the dataset (Feature), a representative sequences file and a FeatureData file which maps Feature identifiers in the Feature table to the sequences they represent, was then created. A multiple sequence alignment was done in the 'Multiple Alignment using Fast Fourier Transform' software (Katoh and Standley 2013) and a phylogenetic tree was created to relate Features to one another and assign phylogenetic groups to the Feature table.

For the 16S rRNA gene reads, taxonomy was assigned using a pre-trained Naïve Bayes classifier trained on the SILVA database December 2019 update, version 138 (Yilmaz et al. 2014), which has adopted the revised taxonomy detailed in the Genome Taxonomy Database (GTDB) (Parks et al. 2020). Taxonomic analysis of negative control samples included in the Illumina MiSeq sequencing project, identified some contamination of the dataset with non-specific amplification and detection of chloroplast DNA and a single feature classified as "d__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae; g__Burkholderia-Caballeronia-Paraburkholderia". These two features were removed from the overall Feature table and from the file of representative sequences corresponding to this Feature table. The Feature table was also filtered to retain only those features represented by at least 5 sequence reads, and the negative control samples removed from the overall dataset. In addition, the samples obtained for the negative control Fermentation 16, containing no rumen fluid or fermenter fluid starter microbial populations), were partitioned from the overall Feature table and analysed separately due to the extreme differences in the microbial populations arising within this fermentation. The Feature table, taxonomy and Metadata files using for the QIIME2 analysis were also reformatted to enable data transformation for PCA and differential abundance analysis, using the MixOmics R package (details in Section 3.6.3).

For each Feature table, alpha diversity measures (microbial diversity within a sample) and beta diversity measures (differences in diversity between samples) were calculated using QIIME2 software. Alpha diversity analysis was determined on the basis of four measures: (1) counts of observed species (Observed Species); (2) Phylogenetic Diversity (PD); (3) Shannon entropy of counts (Shannon); and (4) Pielou's Evenness; a measure of community evenness (Evenness). Metadata categories such as time (days of fermentation) and fermentation starter source material, were used as variables to determine whether there were any significant effects on alpha diversity ($P < 0.05$), using an ANOVA with Fisher's LSD testing using Genstat Release 21.1 (27 January 2022; VSN International Ltd., 2022). The effects of fermentation time (days) on microbial diversity were

determined using an accumulated Analysis of Variance (ANOVA) and modelled using a General Linear Model (GLM), with an exponential curve fitted.

The filtered representative sequence file was also used to create a custom database which was used to determine whether the 40 bacteria isolated purified from the *in vitro* fermentations and employed in the simplexin degradation assays were representative of the microbial populations present in the Fermentations. The similarity (homology) of each of the 16S rRNA gene sequences of each of the bacterial isolation to the representative sequence file was determined using the blast+ suite (blastn version 2.9.0 (Camacho et al. 2009)).

3.8 Bacterial isolations and simplexin degradation screening

3.8.1 Selective culture media preparation

Initial Pimelea Media selective medium

Many anaerobic microbial growth mediums contain a rumen fluid base, which is rumen fluid that has been clarified through centrifugation, to provide essential cofactors, vitamins and other nutrients that the anaerobic bacteria require for growth. Initially when developing the selective Pimelea Medium, clarified fermentation fluid was used in place of rumen fluid base. In brief, from Days 20 through to 30 in Fermentations 3 and 4, the fermentation fluid (1.5 L) removed from the fermenter was filtered through a plastic funnel lined with nylon stocking into 2 L plastic bottles and stored frozen at -20°C. To prepare the Pimelea fermentation fluid base (PFFB), bottles from the same fermentation were thawed and the fluid centrifuged at 18,566 *xg* for 30 min at 4 °C using the FA14-250Y lite rotor in a Sorvall Lynx 6000 high speed centrifuge (Thermo Fisher Scientific, Waltham USA). The resulting supernatant was removed, pooled and 33 mL or 66 mL volumes aliquoted into glass bottles and stored frozen at -20 °C until used in media preparation. The composition of the Pimelea Media (PM) initially used for bacterial isolations is outlined in Table 3.6.

Table 3.6. Ingredients required for selective media Pimelea Medium (PM) to make 100 mL

Ingredient	/100 mL
Peptone	0.1 g
Yeast extract	0.1 g
$NaHCO_3$	0.5 g
Salts A solution[A]	16.5 mL
Salts B solution[A]	16.5 mL
Resazurin solution[A]	0.1 mL
Pimelea fermentation fluid base	33 mL
Pimelea plant ethanol extract[B]	10 mL
RO H_2O[C]	33 mL
After boiling add:	
VFA solution[A]	1.0 mL
Cysteine-HCl	0.02 g

[A] Composition of Salts A, Salts B, Resazurin and VFA solutions are contained in Appendix Section 9.7
[B] Ethanol extract of 40g milled *P. elongata* in 250 mL 90% ethanol (52,941 ng simplexin/mL) (Fletcher and Ouwerkerk 2018)
[C] RO H_2O = reverse osmosis water

The processes involved in the preparation of PM broths and agar plates follows that described for anaerobic media (Appendix Section 9.7). PM broth was aliquoted into Hungate tubes as 5 mL or 9 mL volumes and into serum bottles as 100mL containing either 1.5 g agar for agar plates or 0.9 g agar for the soft agar overlays. The PM broth tubes, and agar bottles were autoclaved at 105°C for 45 min and then stored in a cupboard at room temperature until use.

The PM agar was melted by heating to 105°C for 10 min before the molten agar was taken into the anaerobic chamber (Coy Laboratory Products Inc., USA). The molten agar was allowed to cool to approx. 60°C before the agar plates were poured and allowed to set under anaerobic conditions.

Modifications of the PM selective media

After transfer from plates to broths it was noted that the bacterial isolates exhibited poor growth in PM broths. A modified selective media (RF^+modPM) was developed, replacing the Pimelea fermentation fluid base with rumen fluid (RF) base and adding small amounts of cellobiose and glucose along with a new ethanol extract of *Pimelea* plant material as detailed in Section 3.4.2 The new Pimelea plant ethanol extract was prepared using *P. trichostachya* (AQ522769) plant material following the method detailed in Section 3.4.2 with the modification of a sonication probe (Qsonica QL125 with 25Hz CL-18 fine probe) used at 75% amplification for 25 min in an ice bath instead of a sonication bath. A sample of the resulting Pimelea plant extract Batch# JG012019 was sent to the Natural Toxins Lab for measurement of simplexin concentration.

A second modification to the selective media was made replacing the Pimelea plant ethanol extract with a semi-pure extract of simplexin prepared from Pimelea seeds as detailed in Section 3.4.3 and reconstituted in 90% ethanol. The semi-pure simplexin extract was added to the media as detailed in Appendix Section 9.7.11 to give a simplexin concentration of either 3,000 ng/mL (RF^+modPM3000) or 6,000 ng/mL (RF^+modPM6000) in the media. The bacterial isolations undertaken from Fermentation 13 (days 30 and 43) used RF^+modPM3000 selective culture media for the serial dilutions, isolation broths and agar plates.

A final modification to the selective media was made for use in the simplexin degradation trials which was the RF^+mod broth media prepared without the addition of the semi-pure simplexin extract following the preparation protocol described for anaerobic media (Appendix Section 9.7.3). The semi-pure simplexin extract was added to individual Hungate tubes at a rate of 0.01mL per 10 mL (equivalent to approximately 6000 ng/mL simplexin) or 0.005mL per 10mL (equivalent to approximately 3000 ng/mL simplexin) just prior to inoculation with the bacterial isolate.

3.8.2 Bacterial isolations

Bacterial isolations were undertaken from fermentation fluid collected on the final day of a fermentation. A 10 mL volume of fresh fermenter fluid was taken into the Coy anaerobic chamber (Coy Laboratory Products USA) and a serial dilution prepared by the addition of 1.0mL of fermenter to a Hungate tube containing of 9 mL of selective culture media (PM; RF^+ModPM or RF^+mod3000) and mixed well (dilution 10^{-1}). Using a 1.0mL syringe and a 25G needle, 1.0mL of the 10^{-1} dilution was transferred to a fresh Hungate tube containing 9mL of selective culture media and mixed well (10^{-2} dilution). This process was repeated using fresh syringes and needles for each dilution of the series down to a maximum dilution of 10^{-8}.

Four dilutions were selected (e.g., 10^{-5} to 10^{-8}) and an aliquot was plated out on PM agar plates by either as a –

1. Spread plate, with a 100 µL of the dilution pipetted onto the surface of the 1.5% PM agar plate and spread around on each plate using a sterile plastic spreader, or
2. Soft agar overlay, with 1.0 mL of the dilution pipetted into 3.0mL of molten 0.9% PM overlay agar in a 15mL falcon tube and mixed by gentle inversion prior to being poured onto the surface of a PM agar plate and allowed to set.

The two dilution sets of spread and soft agar plates were split between two BBL® GasPak® 100 vented lid anaerobic jars, taken out of the anaerobic chamber and incubated at 39°C for 24 hours. After 24-hour incubation the anaerobic jars were taken into the anaerobic chamber, opened and the agar plates visually examined for the presence of bacterial colonies. The locations of visible colonies were marked on the bottom of the petri dish and the number of visible colonies for each dilution counted and recorded.

From the spread plates, well-spaced colonies were selected with the colony growth characteristics recorded (e.g. colour (white, cream, yellow, brownish), size (tiny, small, large), opacity (opaque, translucent), profile (domed, flat, egg-like) and surface (mucoid, glossy, matt)) recorded prior to the colony being picked. A sterile plastic loop was used to pick the chosen colony and transfer it into a Hungate tube containing 5.0mL of selective broth (PM; RF^{+}ModPM or RF^{+}mod3000).

From the soft agar overlays, well-spaced colonies were selected, and their growth characteristics recorded before a 200 µL tip was used to take a plug of the soft agar containing the colony from the soft agar overlay plates. This agar plug was then ejected into a Hungate tube containing 5.0 mL of selective broth (PM; RF^{+}ModPM or RF^{+}mod3000).

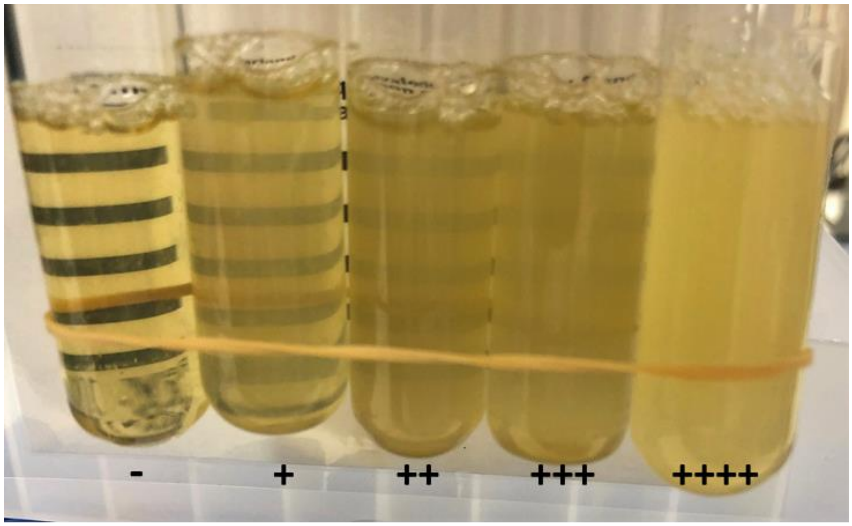
Both sets of agar plates were then returned to their anaerobic jars and, along with the broths containing the selected colonies, taken out of the anaerobic chamber and incubated at 39°C. After a further 24 h of incubation the agar plates were re-examined for further colony growth with any new colonies marked and counted. New colonies were selected for isolation as detailed above. The broth cultures were examined for visible growth and any unusual growth characteristics recorded (e.g. clumping, visible pellet at the bottom of the tube, stringy growth). A 1.0mL syringe with 25G needle was used to anaerobically remove a drop of the culture which was placed on a glass microscope slide and examined microscopically at 400x magnification on a Nikon Eclipse 80i microscope. The culture's bacterial cell morphologies (rod, cocci, spiral, spindle, motile, spores, singles, chains) were recorded and an image of a field of view taken.

A second purification round of the bacterial isolates was undertaken in the anaerobic chamber by 16 streaking a loop full of the isolate broth culture onto a selective media (PM; RF^{+}ModPM or RF^{+}mod3000) agar plate. The agar plates were placed into anaerobic jars, taken out of the Coy anaerobic chamber and incubated at 39°C and visually checked for colony growth daily until visible colonies were seen. A single, well-spaced colony was described and then picked off the agar plate, using a sterile plastic loop, into a Hungate tube containing 5.0mL of selective broth. The second-round broths were incubated for 24 h at 39°C and then examined as described above and this process was repeated until the bacterial isolate was considered pure. Once considered pure, the isolate was assigned a DAF culture collection identifier (DP number) and prepared for entry into the DAF culture collection.

For entry into the culture collection, a 0.1mL and 0.2mL aliquot of broth culture were transferred anaerobically into a 5 mL and 9 mL RF^{+}modPM broth respectively and incubated at 39 °C for 24 h. The broth culture was examined microscopically for growth. If a good cell density (good growth) was seen, an image of a field of view was recorded and three 4.0 mL aliquots of the broth were cryo-preserved by mixing with 4.0 mL of RF/Glycerol medium in a 15 mL serum bottle and freezing, one bottle at -20 °C and two bottles at -80°C. From the remaining broth a 1.0 mL aliquot was transferred to a 1.5 mL microcentrifuge tube and the cells pelleted by centrifugation at 16,100 x*g* at room temperature for 10 min and the resulting supernatant was removed and discarded. The remaining cell pellet was stored at -20°C until used for isolate species identification (Section 3.8.3)

To standardise the assessment of the growth of isolates in broth cultures, a method loosely based on the McFarland turbidity standards methodology was developed. The method uses the level of turbidity to standardise culture density by comparing different bacterial cultures, holding them next to each other in front of a 'Wickerham card' (a white card with printed black lines). Cultures of a similar turbidity to the standard will blur the black lines to approximately the same extent. In our modification to this method, bacterial cultures in Hungate tubes were held next to a Hungate tube of sterile media in front of a Wickerham card and a rating of -, +, ++, +++ or ++++ was assigned for cell density based on visibility of the printed black lines through the culture Figure 3.1

Figure 3.1. Broth cultures of bacterial isolates in front of a Wickerham card showing different levels of cell growth (turbidity) from no growth (-) to very good growth (++++).



3.8.3 Bacterial isolate species identification

The gDNA from each of 1.0 mL frozen bacterial isolate pellets were extracted using the RBB+C method (Yu and Forster 2005). Genomic DNA was extracted from the cell pellets of pure cultures of the isolates using method detailed in Section 3.6.2 and used as template in a universal 16S rRNA gene PCR assay with the primers 27F and 1525R. The resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) as per the manufacturer's instructions. The purified PCR products were Sanger sequenced at the Australian Genome Research Facility (AGRF) using the following primers 27F, 530F, 787R and 1525R. The returned sequences were quality checked and assembled using Sequencher® (version 5.4.1) sequence analysis software into near full-length 16S rRNA gene sequences. The Basic Local Alignment Search Tool (BLAST) nucleotide BLASTn was used to compare the bacterial isolate 16S rRNA gene sequence to the National Center for Biotechnology Information (NCBI) sequence database and obtain species identification.

The phylogeny of forty selected bacterial isolates was determined by creating a .fasta format file of the near full-length 16S rRNA gene sequences and trimming all the sequences to the same length (1318 nt). These sequences were then tested using a Model Test in Mega7 (Kumar et al. 2016) and the model with the highest BIC score was determined to be the Kimura 2-parameter model (Kimura 1980), with a discrete gamma distribution (5 sites). A tree with the highest log likelihood (- 14403.24) was then generated with a replication of 100 bootstraps. The tree was rooted with the 16S rRNA gene sequence of strain DP168 and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates), shown next to the branches (Felsenstein 1985), with values < 80 % not shown in the final phylogenetic tree.

3.8.4 Bacterial isolate simplexin degradation screening assays

A method for determining the ability of isolated bacteria to degrade simplexin each trial was developed and in total six experiments (Degradation Trials) were undertaken with the methodology refined based on results obtained in the previous trial. Across the trials, modifications were made to the culture media formulations, the source of simplexin, incubation conditions, sample collection and the method used to measure the culture's cell density. The bacterial isolates used in the degradation trials also varied, with new bacteria isolated from different fermentations, and the range of bacterial isolates screened are summarised in Table 3.7.

Table 3.7. Details of bacterial isolates screened in simplexin degradation trials 1 to 6

DP#	Species ID (16S rRNA sequence match)	Isolation Ferm#	Screening Trial #
DP25	*Streptococcus macedonicus*	4	1, 3
DP27	*Pseudobutyrivibrio ruminis*	4	1, 2
DP28	*Prevotella ruminicola*	4	3
DP31	*Enterocloster clostridioformis*	4	1, 3
DP40	*Butyrivibrio hungatei*	4	1, 3
DP47	Lachnospiraceae bacterium CA60	4	1, 2, 5, 5S, 6
DP51	Lachnospiraceae bacterium CA60	4	3,
DP77	*Streptococcus henryi*	5	1, 3
DP78	*Succinivibrio dextrinosolvens*	5	1, 3
DP81	*Agathobacter ruminis*	5	1, 3
DP88	*Escherichia coli*	5	1, 3
DP89	*Pseudobutyrivibrio xylanivorans*	5	1, 3
DP90	*Eubacterium sp.*	5	1, 3
DP93	*Selenomonas ruminantium subsp. lactilytica*	3	1, 3
DP94	*Streptococcus lutetiensis*	3	1, 2
DP95	*Kandleria vitulina*	3	1, 3
DP97	*Butyrivibrio fibrisolvens*	3	1, 2,
DP98	*Enterocloster clostridioformis*	3	1, 2
DP100	*Streptococcus henryi*	3	1, 3
DP101	*Enterocloster clostridioformis*	7	3
DP162	*Streptococcus sp.*	10	3
DP163	*Streptococcus gallolyticus*	10	3
DP167	*Mannheimia succiniciproducens*	10	3
DP168	*Fusobacterium varium*	10	3
DP172	*Streptococcus equinus*	10	3
DP179	*Eubacterium sp*.	13	4, 5
DP181	*Megasphaera elsdenii*	13	4, 5
DP186	*Butyrivibrio sp*.	13	4, 5
DP188	*Pseudobutyrivibrio xylanivorans*	13	4, 5, 6
DP189	*Ruminococcus flavefaciens*	13	4
DP190	*Prevotella ruminicola*	13	4, 5, 6
DP191	*Pseudobutyrivibrio xylanivorans*	13	4, 5, 6
DP198	*Pseudobutyrivibrio xylanivorans*	13	4, 5
DP210	*Treponema sp*.	13	4, 5, 6
DP211	*Lachnospira pectinoschiza*	13	4, 5, 6
DP218	*Butyrivibrio crossotus*	13	4, 5

Trial 1 – Bacterial isolate simplexin degradation screening

For Trial 1, a single batch of PM broths (Table 3.6) were prepared for the experiment and ten extra 2.5 mL samples from the same batch of PM broth were supplied to Dr Natasha Hungerford for use in determining recovery efficiency, through spiking with known amounts of simplexin. Frozen stock cultures of each of the 17 microbial isolates (Table 3.7) were revived by thawing, transferring a 0.1 mL aliquot into 5 mL PM broth and incubating at 39 °C for 48 h to obtain a dense culture with cell density determined by $OD_{600\ nm}$ measurement using an Eppendorf BioPhotometer and Wickerman card. A 0.2 mL volume of well-grown broth cultures were used to inoculate duplicate fresh 5 mL PM broths and duplicate negative controls were inoculated with 0.2 mL of sterile PM broth. The negative controls were sampled at time 0 (T-0 h) prior to all the Hungate tubes being incubated at 39 °C for 168 h (1 week) with full rocking. After one week (T-168 h) of incubation, the negative controls and inoculated cultures were all sampled and similarly processed as detailed below. From the remaining culture broths an aliquot was examined microscopically at 400x magnification on a Nikon Eclipse 80i microscope for cell density, cell condition and an image of a representative field of view recorded utilising Nikon NIS Elements basic research imaging software.

For each time point, a 2.5 mL aliquot was removed anaerobically from the Hungate tube and transferred to a 5 mL Eppendorf tubes. A 0.1 mL sub-sample was removed and the $OD_{600\ nm}$ measured as before. The remaining sample was centrifuged at 10,000 x *g* (Eppendorf 5810R Centrifuge) for 10 min at room temperature, the supernatant transferred to a fresh 5 mL Eppendorf tube and frozen at -20°C. The pellet was washed by adding 2.5 mL of dilution solution and briefly vortexing before centrifuging again at 10,000 x *g* (Eppendorf 5810R Centrifuge) for 10 min at room temperature. The wash supernatant was transferred into fresh 5 mL Eppendorf tubes, labelled appropriately and frozen at -20°C, the pellets were then also stored at -20 °C and transferred to the Natural Toxins Laboratory for analysis of simplexin levels.

Trial 1 – Bacterial isolate simplexin degradation screening simplexin LC-MS/MS analysis

Culture supernatants, wash solution supernatants and pellets from Trial 1 were extracted using a solid phase (SPE) procedure. The culture supernatants (2 x 2.5 mL), wash supernatants (2 x 2.5 mL) and pellets (2x) were subjected to SPE to reduce the matrix and background noise for LC-MS/MS measurement of simplexin concentration. Nine Pimelea media matrix samples (already containing simplexin) were also extracted, with three utilised for pre-extraction simplexin spikes in which simplexin standard is added prior to sample extraction. Three matrix samples were utilised for post-extraction simplexin spikes with simplexin standard added after sample extraction. A further three samples were analysed as unchanged matrix.

Methanol (2.5 mL) was added to all frozen isolate culture supernatants (2.5 mL), wash solutions supernatants (2.5 mL) and pellets, except the pre-extraction spike tubes, to which 100 µL of 10 ppm simplexin standard (in methanol) was added, followed by methanol (2.4 mL). Defrosted samples were vortexed (20 s), ultrasonicated (5 min) and shaken for 1 h, then centrifuged (3,214 x g, 10 min). The supernatant was poured into labelled 50 mL tubes. To the residual plug, methanol (3.0 mL) was added and the samples were vortexed (20 s), ultrasonicated (5 min), shaken for 1 h, and centrifuged (3,214 x g, 10 min). The supernatant was poured into the same corresponding labelled 50 mL tubes. Again, to the residual plug, methanol (3.0 mL) was added and the samples were vortexed (20 s), ultrasonicated (5 min), shaken for 1 h, and centrifuged (3,214 x g, 10 min). The supernatant was poured into the same corresponding labelled 50 mL tubes, and the plug was then discarded.

For supernatant samples, to each 50 mL tube RO water (6.0 mL) was added, and for pellet samples RO water (8.5 mL) was added, to give 50 % methanol/water mixtures. These were vortexed (20 s) and shaken (10 min) prior to loading onto the Solid Phase Extraction (SPE) sorbent cartridges. Oasis HLB SPE cartridges (200 mg) (Waters, USA) were preconditioned with methanol (5 mL) and water (5

mL) using a Varian Vac Elute SPS 24 manifold connected to house vacuum to maintain flow rates of 1 mL/min (for loading and elution) or 5 mL/min (for preconditioning and washing). The entire contents of the 50 mL tubes were loaded onto the HLB SPE cartridges. Each tube was washed consecutively with 2 x 1.5 mL of 50 % methanol/water and added after the initial load. Each cartridge was washed with 5 % methanol/water then 60 % methanol/water. Residual solvent was removed by increasing the vacuum and then simplexin elution was achieved with the addition of 100 % methanol (10.0 mL) and collected into 15 mL tubes. Final volumes were adjusted to 10 mL with methanol, if needed. To the post-extraction simplexin spike tubes was added simplexin standard (100 µL of 10 ppm solution in methanol). Methanol (100 µL) was added to all other tubes. Three tubes of methanol (10 mL) were spiked with simplexin standard (100 µL of 10 ppm solution in methanol) to create solvent spikes. All tubes were shaken to mix (10 min) then 1.0 mL was transferred via 0.2 µm membrane GHP Acrodisc syringe filter (Pall USA) into vials for LC-MS/MS analysis (using external standards as described in Section 3.5). Statistical analyses were performed using GraphPad Prism 8.0.1 using 2-way ANOVA multiple comparisons.

Trial 2 – Bacterial isolate simplexin degradation screening

The $OD_{600\ nm}$ results obtained for isolates in Trial 1 indicated poor growth of isolates in the PM broth and LCMS/MS analysis of the PM broth and PFFB revealed very low levels of simplexin present (12.5 ng/mL and 5.6 ng/mL respectively), so the decision was made to use RF^{+}modPM broths and only screen five isolates (Table 3.7) selected from Trial 1. Frozen stock cultures of the five isolates were thawed and a 0.1 mL aliquot transferred anaerobically to Hungate tubes of 5 mL RF^{+}modPM broth containing approx. 1,821 ng/mL of *Pimelea* plant ethanol extract (prepared similar to Section 3.4.2, Batch #JG012019). The Hungate tubes were secured on a rocking platform and incubated at 39 °C for 24 h, checked visually and their $OD_{600\ nm}$ measured. For Trial 2, 10 mL RF^{+}modPM broths were prepared in Hungate tubes with a simplexin concentration of approximately 5,463 ng/mL by adding 3X the volume of *Pimelea* plant extract (RF^{+}modPM3X). Five negative control 10 mL RF^{+}modPM3X broths were set up and 0.2 mL from a sterile 5 mL RF^{+}modPM broth was added to each negative Hungate tube. For each isolate a 0.2 mL aliquot from the 5 mL RF^{+}modPM broth culture was used to inoculate a 10 mL RF^{+}modPM3X broths in duplicate. All Hungate tubes were incubated at 39 °C with full rocking. Samples were taken anaerobically through the butyl rubber stopper using a syringe and 25 G needle at two extra time points (24 h and 48 h) for $OD_{600\ nm}$ and simplexin measurement. The pellet washing step was removed. The frozen samples were transferred to the Natural Toxins Laboratory for analysis of simplexin levels.

Trial 2 – Bacterial isolate simplexin degradation screening simplexin LC-MS/MS analysis

Trial 2 culture supernatants and pellets were extracted using a solid phase (SPE) procedure. For the culture supernatants to ensure a consistent volume, the samples were defrosted, vortexed and 1.5 mL was transferred to a new 5 mL tube. The culture supernatants (2 x 1.5 mL) and pellets (2x) for each time point (24 h, 48 h and 168 h) and negative control supernatants (5 x 1.5 mL) and negative control pellets (5x) for each time point (0 h, 24 h, 48 h and 168 h) were subjected to SPE to reduce the matrix and background noise for LC-MS/MS measurement of simplexin concentration. Nine RF^{+}modPM broth samples (RF media spiked with the *Pimelea* plant extract (already containing simplexin)) were also extracted as matrix samples, with three utilised for pre-extraction simplexin spikes in which simplexin standard is added prior to sample extraction. Three matrix samples were utilised for post-extraction simplexin spikes with simplexin standard added after sample extraction. A further three samples were analysed as unchanged matrix.

The same SPE procedure was used as for the bacterial isolates from Degradation Trial 1, except that after extraction with methanol (2.5 mL, then 2 x 3 mL), for culture supernatant samples, to each 50 mL tube was added RO water (7.0 mL), and for pellet samples was added RO water (8.5 mL), to give 50 % methanol/water mixtures which were vortexed (20 s) and shaken (10 min) prior to loading onto

the Solid Phase Extraction (SPE) sorbent cartridges. The same protocol was followed as for bacterial isolates from Degradation Trial 1 for LC-MS/MS analysis of simplexin and huratoxin.

Trial 3 – Bacterial isolate simplexin degradation screening

Analysis of the simplexin concentrations in the media from Trial 2 identified that the media's actual simplexin concentration was considerably lower than the calculated concentration. The extraction of a semi-pure simplexin extract replaced the Pimelea plant extract JG012019. The semi-pure simplexin extract was added to the media to give a calculated final concentration of simplexin in the media of 6,000 ng/mL (RF^+modPM6000). A panel of 20 isolates were selected (Table 3.7) and for each microbial isolate the following was done:

1. The isolate was revived from a frozen microbial stock culture by thawing and inoculating 0.1 mL into a 5mL broth of RF^+ModPM3000 and incubating for 24 to 48 h as required to obtain a dense culture.
2. For the trial, an actively growing overnight culture was used with the $OD_{600\ nm}$ of the inoculum culture recorded.
3. A time T0h sample was taken from each Hungate tube of 5 mL RF^+modPM6000 using a syringe and needle to remove 2.5 mL through the butyl rubber stopper and transfer into a 5 mL Eppendorf tube. From this a 2.0 mL aliquot was pipetted into a 15 mL falcon tube and frozen at -20 °C as the representative T 0h sample for each of the isolates. The remaining 0.5 mL broth of the aliquot was used to obtain $OD_{600\ nm}$ readings for T 0h.
4. The remaining 2.5 mL in the Hungate were inoculated with 0.1 mL of the fresh culture with each isolate set up in triplicate.
5. Triplicate negative controls were set up by removing 2.5 mL into a 5mL Eppendorf tube and pipetting 2.0 mL into a 15 mL falcon tube and freezing at -20 °C as the negative control T 0h samples. To mimic the culture inoculations, 0.1 mL of sterile RF^+modPM3000 broth was added to the remaining 2.5 mL RF^+modPM6000 broth and all Hungate tubes were incubated with full rocking at 39 °C for one week (168 h).
7. After 168 h of incubation the Hungate tubes of all triplicate cultures and negative controls had their cell density recorded utilising both the $OD_{600\ nm}$ measurement and Wickerham card method and a photo of each tube taken alongside a tube of un-inoculated medium.
8. A drop of culture was removed, placed on a glass microscope slide and examined at 400x magnification on a Nikon Eclipse 80i microscope for cell density and to determine the condition of cells with an image taken of a representative field of view.
9. The Hungate tubes were frozen at -20 °C until transportation to the Natural Toxins Laboratory at Coopers Plains for simplexin analysis.

Trial 3 – Bacterial isolate simplexin degradation screening simplexin LC-MS/MS analysis

Culture samples from Trial 3 above were extracted using a solid phase (SPE) procedure. Each culture and negative controls were subjected to SPE to reduce the matrix and background noise for LC-MS/MS measurement of simplexin concentration.

Samples in Hungate tubes were defrosted, vortexed and transferred to a new plastic tube. MeOH (2 x 2.5 mL) was added into the Hungate tubes and gently vortexed without touching the rubber stopper. The contents were transferred to the respective tubes. The entire sample was vortexed (20 s), ultrasonicated (5 min) and shaken (1 h) then centrifuged (3,214 x g, 10 min). The supernatant was poured into labelled 50 mL tubes. To the residual plug, methanol (3.0 mL) was added and the samples were vortexed (20 s), ultrasonicated (5 min), shaken for 1 h, and centrifuged (3,214 x g, 10 min). The supernatant was poured into the same corresponding labelled 50 mL tubes. Again, to the residual plug, methanol (3.0 mL) was added and the samples were vortexed (20 s), ultrasonicated (5 min), shaken for 1 h, and centrifuged (3,214 x g, 10 min). The supernatant was poured into the same corresponding labelled 50 mL tubes, and the plug was then discarded.

To each 50 mL tube RO water was added to give 50 % methanol/water mixtures. These were vortexed (20 s) and shaken (10 min) prior to loading onto the Solid Phase Extraction (SPE) cartridges. The same SPE protocol was followed as for bacterial isolates from Trial 1 (above). A subsample was diluted to give a final volume of 1 mL and internal standard PMA (50 µL, 1 µg/mL) was added ready for LC-MS/MS analysis of simplexin and huratoxin using the internal standard method (Section 3.5.4).

MS detection was performed and operated on similar mode as previously described in Section 3.5.1 with MS parameters, HESI probe and inclusion list tuned for simplexin and PMA. HESI probe in positive ionisation mode was optimised for simplexin; spray voltage 3.5 kV, sheath gas flow 45 arbitrary units, auxiliary gas flow 10 arbitrary units, capillary voltage 5 V, capillary temperature 250 °C and tube lens 50 V.

MS was operated in PRM mode. MS parameters were shown as follows: Full MS parameters were set with resolution setting of 70,000 (RFWHM at m/z 200), Full MS mass range of 400 – 800 m/z, automatic gain control target of 3e6 and maximum injection time of 200 ms. MS/MS parameters were set to resolution 70,000 (RFWHM at m/z 200) with automatic gain control target of 2e5, maximum injection time of 100 ms, isolation width of 40 m/z, isolation off-set of 20 m/z, normalised collision energy of 35 eV for simplexin and loop count set to 1. Inclusion list of simplexin was used based on fragmentation of protonated simplexin ($(M+H)^+$, m/z 533.3108) utilising two major fragment ions of m/z 533.3109 > 253.1220 as transition of quantification and m/z 533.3109 > 267.1381 as transition of verification respectively. PMA (internal standard) was monitored at m/z 617.4080 > 311.1642 with normalised collision energy set to 15 eV.

Isolated simplexin (>95% pure) prepared in methanol was used for the calibration curve (10 – 2000 ng/mL) with PMA (50 ng/mL) used as internal standard for quantitative analysis.

Simplexin binding in Hungate tubes

After the analysis of the simplexin concentration in samples from Trial 3 still being considerably lower than the expected calculated concentration in the negative controls, an experiment designed to determine where possible losses of simplexin were occurring throughout the process of media preparation, Hungate tube set-up, incubation, rocking and sampling of Hungate tubes.

Media preparation – A 100 mL volume batch of RF^+modPM6000 broth was prepared and during preparation samples were taken at each of these stages:

- ‘Prep 1’ – 3 x 1.0 mL aliquots of the semi-pure extract were pipetted into 15 mL falcon tubes and frozen at -20 °C.
- ‘Prep 2’ - after addition of the semi-pure simplexin extract and boiling of the media, but before the media is placed on ice 3 x 2.0 mL aliquots were pipetted into 15 mL falcon tubes and frozen at -20 °C.
- ‘Prep 3’ - after the media had been cooled on flake ice, the VFAs and cysteine had been added, 3 x 2.0 mL aliquots were pipetted into 15 mL falcon tubes and frozen at -20 °C.
- ‘Prep 4’ - when aliquoting the 5.0 mL volumes into Hungate tubes, aliquot 2 mL into three Hungate tubes which were autoclaved at 105 °C for 45 min with the rest of the media. Once cooled to room temperature the Hungate tubes were frozen at -20 °C.

Incubation rocking – three sets of Hungate tubes containing 5 mL of RF^+modPM6000 broth were incubated in different ways – 1. Stationary (tubes upright in rack on shelf); 2. Full rocking (media moving the full length of tube hitting the inside of butyl rubber stopper); and 3. Controlled rocking (angled rocking with media not touching the butyl rubber stopper) and each sample was taken using

a 2 mL sterile syringe with a 25 G needle (the tubes were held on an angle without allowing the broth to touch the stoppers) as follows –

- Time 0 h:
  - Stationary T0h -2 mL removed from each of the three 5 mL Hungate tubes into 15 mL falcon tubes and frozen at -20 °C.
  - Rocking T0h: 2 mL taken from each of the three 5 mL Hungate tubes into 15 mL falcon tubes and frozen at -20 °C.
  - Controlled Rocking T0h: 2 mL taken from each of the three 5 mL Hungate tubes into 15 mL falcon tubes and frozen at -20 °C.
- Time 168 h:
  - Stationary T168h - 2 mL taken from each of the three 5 mL Hungate tubes and frozen at -20 °C.
  - Rocking T168h - 2 mL taken from each of the three 5 mL Hungate tubes and frozen at -20 °C.
  - Controlled Rocking T168h - 2 mL taken from each of the three 5 mL Hungate tubes and frozen at -20 °C.
- Remaining:
  - Stationary T168h remainder - each of the three 5 mL Hungate tubes with the remainder of liquid in the Hungate tube frozen at -20 °C.
  - Rocking T168h remainder - each of the three 5 mL Hungate tubes with the remainder of liquid in the Hungate tube frozen at -20 °C.
  - Controlled Rocking T168h remainder - each of the three 5 mL Hungate tubes with the remainder of liquid in the Hungate tube frozen at -20 °C.

All samples were stored frozen at -20 °C until transportation to the Natural Toxins Laboratory at Coopers Plains for simplexin analysis.

Simplexin binding in Hungate tubes LC-MS/MS analysis

Samples were extracted using a solid phase (SPE) procedure and LC-MS/MS analysis as described for Bacterial Isolate Trial 3 (above).

Trial 4 – Bacterial isolate simplexin degradation screening

The results from the simplexin binding in Hungate tube experiment indicated that simplexin appeared to bind to the butyl rubber stopper used to seal the Hungate tubes, so for Trial 4 the incubation conditions were modified. The Hungate tubes were incubated at 39 °C with controlled rocking (media not contacting the butyl rubber stopper). For sampling, the Hungate tubes were taken into the Coy Anaerobic chamber and the lids fitted with a butyl rubber stopper completely removed to allow samples to be taken anaerobically using a P 1000 pipette with a 1 mL filtered tip. A panel of 11 isolates (Table 3.7) were selected for screening in Trial 4 and for each of the 11 microbial isolates the following was undertaken:

1. Each isolate was revived from frozen microbial stock culture by thawing and inoculation of 0.1 mL into a 5 mL of RF^{+}modPM3000 broth followed by incubation at 39 °C with controlled rocking for 24 to 48 h with daily checks until grown to a dense culture.
2. The revived cultures were inoculated (0.1 mL) into a fresh Hungate tube of RF^{+}modPM3000 broth and incubated for 24 h at 39 °C for use in Trial 4 with each culture's Wickerham card score recorded for culture growth. Hungate tubes of 5 mL RF^{+}modPM6000 broth were taken into the Coy Anaerobic chamber where the lid with butyl rubber stopper were removed and a 2.5 mL

aliquot was transferred into a 5 mL Eppendorf tube. From this volume of culture, a 2.0 mL aliquot was pipetted into a 15 mL falcon tube and frozen at -20 °C (T 0h for each isolate). Triplicate cultures of each isolate were then set up by adding 0.1 mL of fresh isolate culture into the remaining 2.5 mL of RF$^+$modPM6000 broth.

3. Negative controls were set up in triplicate with a negative T 0h sample taken as described above. To mimic the culture inoculations, 0.1 mL of sterile RF$^+$modPM3000 broth was added to the remaining 2.5 mL of RF$^+$modPM6000 broth in the Hungate tubes.
4. All Hungate tubes were secured onto an angled rack on the rocking platform and controlled rocked visually checking the media didn't touch the butyl rubber stopper when rocking and incubated at 39 °C for one week (T 168h).
5. After T 168h all triplicate cultures and negative control Hungate tubes were removed from the incubator and:
    a. Observed for density with the Wickerham card score for culture cell density recorded of each Hungate tube and a photo taken beside a Hungate tube of sterile RF$^+$modPM6000 broth.
    b. Taken into the Coy Anaerobic chamber, where the lids with butyl rubber stopper were removed and a drop of broth removed, placed on a microscope slide for examination at 400x magnification on a Nikon Eclipse 80i microscope to assess the cell density and overall health or condition of cells with an image of a representative field of view taken.
    c. The Hungate tubes then stored frozen at -20 °C until transportation to the Coopers Plains Natural Toxin Laboratory for simplexin analysis.

Trial 4 – Bacterial isolate simplexin degradation screening with simplexin LC-MS/MS analysis

Samples were extracted using a solid phase (SPE) procedure and LC-MS/MS analysis as described for Bacterial Isolate Trial 3 (above).

Trial 5 – Bacterial isolate simplexin degradation screening

Trial 5 was very similar to Trial 4 with 11 isolates (Table 3.7) that included 10 of the 11 isolates from Trial 4 along with DP47 and for each of the 11 isolates the following was undertaken -

1. Each isolate was revived from frozen stock cultures into two different growth media (5 mL RF$^+$modPM3000 broth or 5 mL of a standard RF$^+$ media broth) by inoculating each Hungate tube of broth with 0.1 mL of thawed stock culture and incubating at 39 °C with controlled rocking for 24 to 48 h. The culture broths were visually assessed, via Wickerham card, for culture density and the media resulting in the best growth of the isolate was used to grow the inoculum culture for Trial 5.
2. The inoculum culture Hungate tubes were taken into the Coy Anaerobic chamber and for each isolate, 0.1 mL of the revived isolate was transferred via 1 mL syringe with 25G needle into media in triplicate as follows -
    a. Set 1 - Hungate tubes of 5 mL RF$^+$mod broth and then the lid and butyl rubber stopper were removed from the Hungate tubes and a 50 μL aliquot of semi-pure simplexin extract (600,000 ng/mL JG-256-91-2) added;
    b. Set 2 - Hungate tubes of 5 mL RF$^+$modPM6000 broth.
3. For both media types, negative controls in triplicate were set up and pseudo inoculated by adding 0.1 mL of sterile media from selected media used to grow isolates. The negative controls were treated in parallel with the broth cultures of respective isolates.
4. Both sets of Hungate tubes were incubated at 39°C with controlled rocking for 1 week (168 h).

5. At 48 h, observations of growth habit were recorded and then each set of isolate cultures were gently vortexed, without allowing the liquid to touch the lid, and a Wickerham card score was recorded.
6. After 168 h incubation, all triplicate culture and negative control Hungate tubes were frozen at - 20 °C until transferred to the Coopers Plains Natural Toxin Laboratory for simplexin analysis.

A supplementary trial (5s) was run to investigate the unexpected very high simplexin concentrations in the RF$^+$mod broths spiked with 50 μL of semi-pure simplexin extract compared to the RF$^+$modPM6000 media. The protocol from Trial 5 was followed for a single isolate DP47.

Trial 5 – Bacterial isolate simplexin degradation screening with simplexin LC-MS/MS analysis

Samples were extracted using a solid phase (SPE) procedure and LC-MS/MS analysis as described for Bacterial Isolate Trial 3 (above).

Trial 6 – Bacterial isolate simplexin degradation screening

A subset of six isolates from the panel used in Trial 5 (Table 3.7), were used in Trial 6 which was modified to include simplexin in the media at calculated concentrations of either 6,000 or 12,000 ng/mL and for each of the six isolate es the following was undertaken -

1. Each isolate was revived from freezer microbial stock culture in 5 mL RF$^+$mod broth which was spiked with 50 μL of semi-pure simplexin (600,000 ng/mL JG-256-91-2) by inoculating with 0.1 mL of thawed stock culture and incubating at 39 °C with controlled rocking for 24 to 48 h. The culture broths were visually assessed, via Wickerham card, for culture density. The revived isolates were passaged in the spiked RF$^+$mod broth media for two weeks to establish strong growth.
2. The Hungate tubes of 5 mL RF$^+$mod broths were taken into the Coy anaerobic chamber and the lid with butyl rubber stopper removed to allow the broth to have 50 μL of semi-pure simplexin extract added via filtered pipette tip (600,000 ng/mL JG-256-91-2). Sets of three Hungate tubes/isolate were then each inoculated with 0.1 mL of isolate culture added via 1 mL syringe with 25G needle into the Hungate tube.
3. A second set of Hungate tubes of 10 mL RF$^+$mod broth media with 200 μL of semi-pure simplexin extract (600,000 ng/mL JG-256-91-2) added as described previously were inoculated with 0.2 mL of isolate culture via 1 mL syringe with 25G needle into the Hungate tube in duplicate.
4. For both sets, negative controls (5 mL and 10 mL) broths were set up and either 50 μL or 200 μL of sterile spiked RF$^+$mod broth media was added to mimic inoculation.
5. The first set of Hungate tubes (5 mL) were incubated with controlled rocking at 39°C for 1 week (168 h) whilst the second set of Hungate tubes (10 mL) were incubated upright and static as the volume was too great a volume to ensure that the liquid didn't touch the butyl rubber stopper when controlled rocked.
6. After 48 h incubation, each set of Hungate tubes were gently vortexed without allowing media to touch the lid of the tube and a Wickerham card score was recorded.
7. After 168 h incubation, both sets of Hungate tubes were frozen at -20 °C until transported to the Coopers Plains Natural Toxin Laboratory for simplexin analysis.

Trial 6 – Bacterial isolate simplexin degradation screening simplexin LC-MS/MS analysis

Culture samples from Trial 6 above were extracted using a solid phase (SPE) procedure. The samples (3 x 5 mL and 2 x 10 mL) for each culture and negative controls (3 x 5 mL and 2 x 10 mL) incubated for 168 h were subjected to SPE to reduce the matrix and background noise for LC-MS/MS measurement of simplexin concentration.

The 5 mL samples in Hungate tubes were defrosted, vortexed and transferred to a new plastic tube. MeOH (2 x 2.5 mL) was added into the Hungate tubes and gently vortexed without touching the rubber stopper. The contents were transferred to the respective tubes. The entire sample was vortexed (20 s), ultrasonicated (5 min) and shaken (1 h) then centrifuged (3,214 x g, 10 min). The supernatant was poured into labelled 50 mL tubes. To the residual plug, methanol (3.0 mL) was added and the samples were vortexed (20 s), ultrasonicated (5 min), shaken for 1 h, and centrifuged (3,214 x g, 10 min). The supernatant was poured into the same corresponding labelled 50 mL tubes. Again, to the residual plug, methanol (3.0 mL) was added and the samples were vortexed (20 s), ultrasonicated (5 min), shaken for 1 h, and centrifuged (3,214 x g, 10 min). The supernatant was poured into the same corresponding labelled 50 mL tubes, and the plug was then discarded.

To each 50 mL tube RO water (6.0 mL) was added to give 50 % methanol/water mixtures. These were vortexed (20 s) and shaken (10 min) prior to loading onto the Solid Phase Extraction (SPE) cartridges. The same SPE protocol was followed as for bacterial isolates from Trial 1.

A subsample (200 µL) were diluted 1 in 5 to give a final volume of 1 mL and internal standard PMA (50 µL, 1 µg/mL) was added ready for LC-MS/MS analysis of simplexin and huratoxin using the internal standard method as per Isolates Trials 3 (above).

The 10 mL samples in Hungate tubes were defrosted, vortexed and transferred to a new plastic tube. MeOH (2.5 mL) was added into the Hungate tubes and gently vortexed without touching the rubber stopper. The contents were transferred to the respective tubes. A further wash of the Hungate tube with MeOH (1.5 mL) was completed and added to the respective tubes. The entire sample was vortexed (20 s), ultrasonicated (5 min) and shaken (1 h) then centrifuged (3,214 x g, 10 min). The supernatant was poured into labelled 50 mL tubes. To the residual plug, methanol (3.0 mL) was added and the samples were vortexed (20 s), ultrasonicated (5 min), shaken for 1 h, and centrifuged (3,214 x g, 10 min). The supernatant was poured into the same corresponding labelled 50 mL tubes. Again, to the residual plug, methanol (3.0 mL) was added and the samples were vortexed (20 s), ultrasonicated (5 min), shaken for 1 h, and centrifuged (3,214 x g, 10 min). The supernatant was poured into the same corresponding labelled 50 mL tubes, and the plug was then discarded.

Each 50 mL tube RO water (6.0 mL) was already 50 % methanol/water mixtures. These were vortexed (20 s) and shaken (10 min) prior to loading onto the Solid Phase Extraction (SPE) cartridges. The same SPE protocol was followed as for bacterial isolates from Trial 1 above. A subsample (100 µL) were diluted 1 in 10 to give a final volume of 1 mL and internal standard PMA (50 µL, 1 µg/mL) was added ready for LC-MS/MS analysis of simplexin and huratoxin using the internal standard method as per Isolates Trials 3 above.

3.9 Novel biopolymer manufacturing, characterisation and release of simplexin under simulated rumen conditions *in vitro*

3.9.1 Biopolymer materials

Four different biopolymers were selected for their properties and capacity to degrade in biological environments:

1. Polyhydroxyalkanoate (PHA): Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) biopolymer with 1 mol% 3-(hydroxyvalerate) (HV) content was purchased from TianAn Biopolymer (Ningbo, China) in powder form with no additives, under the trade name of ENMAT Y1000 (Batch: EDW2189 and B2173).

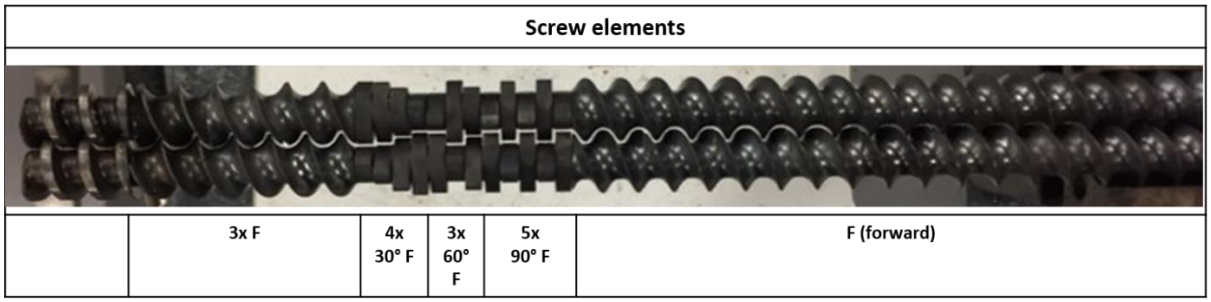
2. Polycaprolactone (PCL): polycaprolactone pellets were purchased from Sigma Aldrich, with an average molecular weight of 80,000 g/mol (Lot # 20170315).
3. Polylactic acid (PLA): polylactic acid pellets under the trade name of Ingeo 2500HP (batch number: BG 1428BIII) were purchased from NatureWorks; this grade is a high viscosity product designed for extrusion applications.
4. Starch: grade Hylon VII was obtained from Ingredion this grade is a high amylose food starch, in a powder form.

Pimelea trichostachya plant material incorporated in biopolymer composites was dried and milled (3 mm screen size) AQ522479 collected from Property 21 in Maranoa during project P.PSH.0900 (Fletcher and Ouwerkerk 2018) or AQ522769 collected from Property 8 in Balonne area (Table 3.1). Porous PHA samples were also prepared by extrusion with sugar, starch and sodium bicarbonate. Icing sugar (Black and Gold) and sodium bicarbonate (Black and Gold) were purchased from the supermarket and used as received.

3.9.2 Biopolymer extrusion

Prior to extrusion, all biopolymers and Pimelea plant material were dried separately in a vacuum oven at 65 °C with a gauge pressure of −80 kPa for 24 h. A co-rotating twin screw extruder (EuroLab 16 XL, Thermo Fisher Scientific Inc, Waltham, USA) with a diameter of 16 mm and a length-to-diameter ratio of 40:1 was used for this work. The screw profile is shown in Figure 3.2**,** and was kept consistent for PHA, PCL and PLA biopolymers.

Figure 3.2. Screw profile utilised for PHA, PCL and PLA biopolymers.



The extrusion die was set as a ribbon shape with a width of 10 mm and a thickness of 2 mm. The extruder temperature profiles and screw speeds for each biopolymer are presented in Table 3.8**.** As each biopolymer has a different melting point, different temperature profiles were designed for optimum processing.

Table 3.8. Extruder temperature profile and screw speeds used for PHA, PCL and PLA biopolymers.

Polymer	Screw speed (rpm)	Zone Temperature									
		Die	8	7	6	5	4	3	2	1	Feed
PHA	300	160°C	160°C	165°C	180°C	180°C	180°C	180°C	180°C	180°C	180°C
PCL[A]	20	70°C	80°C	85°C	90°C	90°C	90°C	90°C	90°C	90°C	90°C

PLA	20	190°C	210°C	210°C	210°C	210°C	210°C	210°C	210°C	180°C	180°C	

[A]A water bath was used to collect the PCL ribbon due to the sticky nature of material coming out from the die.

3.9.3 Starch extrusion

As starch requires water to plasticise, it was necessary to use a different approach to process this material. It was extruded with the aim to produce a final 40% moisture content ratio. The extruder screw profile is presented in Figure 3.3. The screw profile comprises a mixing zone (zones 3 and 4) which is positioned just after the water inlet, which allows the starch to be well mixed with water in order to plasticise. The extruder temperature profiles and screw speeds for starch are presented in Table 3.9.

Figure 3.3. Screw profile utilised to process starch.

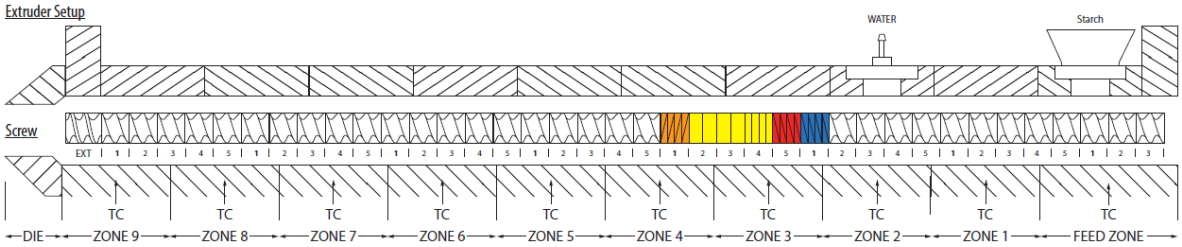


Table 3.9. Extruder temperature profile and screw speeds utilised for starch.

Polymer	Screw speed (rpm)	Zone Temperature											
		Die	10	9	8	7	6	5	4	3	2	1	Feed
Starch	250-300	90°C	90°C	90°C	90°C	90°C	90°C	90°C	100°C	100°C	90°C	80°C	70°C

3.9.4 Biopolymer processing

Processing of the biopolymers alone was achieved by feeding the dried biopolymer directly into the extruder, except for the starch sample which was co-fed with water, as described above.

3.9.5 Biopolymer/Pimelea composite processing

The biopolymer composite materials were prepared using a two-step process, consisting of dry mixing followed by melt compounding. In the case of PHA, the milled Pimelea plant material was combined with the PHA powder using a Homemaker double-blade kitchen stick mixer. A batch of 150 g total combined mass was ground for 2 min at 200 rpm to fully mix the two materials. In the case of polymers in pellet form (PCL and PLA), the milled dried plant material was combined with the biopolymer pellets in a bucket and lightly mixed by manual stirring until a visually homogenous mixture was obtained. Table 3.10 summarises the different formulations that were processed. The pre-mixed formulations were then fed to the extruder. The optimised extrusion parameters were determined based on a preliminary study.

Table 3.10. Biopolymer composite formulation, where the biopolymer was PHA, PCL, or PLA.

Biocomposite formulation	Biopolymer (wt%)	*Pimelea* (wt%)
Biopolymer + 5% Pimelea	95	5
Biopolymer + 10% Pimelea	90	10
Biopolymer + 20% Pimelea	80	20
Biopolymer + 30% Pimelea	70	30
Biopolymer + 40% Pimelea	60	40

3.9.6 Processing of composites of blends of PHA and PCL biopolymers with Pimelea

PHA and PCL were selected to be blended together at different weight ratios due to their differing biodegradability and water permeability. PCL pellets were cryo-milled in order to reduce them into powder form, prior to being mixed with PHA powder and milled Pimelea plant material in varying compositions as shown in Table 3.11. The composite materials were then prepared using a two-step process, consisting of dry mixing followed by melt compounding.

Table 3.11. Composite blends of PHA and PCL at different weight ratios with different loadings of Pimelea.

Biocomposite blend	PHA (wt%)	PCL (wt%)	Pimelea (wt%)
PHA + 25% PCL	75	25	0
PHA + 20% PCL + 20% Pimelea	60	20	20
PHA + 17.5% PCL + 30% Pimelea	52.5	17.5	30
PHA + 50% PCL	50	50	0
PHA + 40% PCL + 20% Pimelea	40	40	20
PHA + 35% PCL + 30% Pimelea	35	35	30
PHA + 75% PCL	25	75	0
PHA + 60% PCL + 20% Pimelea	20	60	20
PHA + 52.5% PCL + 30% Pimelea	17.5	52.5	30

3.9.7 Preparation of composites of biopolymer with ethanol extracts of Pimelea

The previously prepared ethanol extract of Pimelea plant material (AQ522479) (Section 3.4.2) was mixed with PHA powder followed by evaporation of the ethanol from the mixture using a rotary evaporator until the mixture was dry. The theoretical simplexin concentration of 280 µg/g was determined based on concentration of simplexin in the ethanol extract (56 µg/mL). Extrusion was performed as described in Section 3.9.2 and the actual simplexin concentration determined post extrusion as 217 µg/g by the methods described in Section 3.9.12.

3.9.8 Preparation of porous PHA Pimelea composites

Three approaches were taken in order to produce porous PHA. The first approach was to add a component (porogen) into the extrusion mixture that is water soluble, such as sugar or salt; after processing, that component was then leached out with water to form pores within the polymer. The second approach was to use a component that can easily be assimilated by microorganisms once in the fermenter/rumen environment to create a large pore network. This would facilitate the internal access for microorganisms to digest PHA that is present in the bulk of the biopolymer. The third

approach was to use a foaming agent, sodium bicarbonate ($NaHCO_3$) (SB), as it is one of the most common and green foaming agents available, producing CO_2 *in-situ* during extrusion. For these samples, the Pimelea sample used was AQ522769, which has a higher content of simplexin.

Use of the porogen: icing sugar

Prior to extrusion, different ratios of icing sugar and PHA were combined using a Homemaker double-blade kitchen stick mixer. Batches of total weight 100 g were ground for 1 min at 200 rpm to fully mix the two materials. Milled Pimelea was then added at a ratio of 30 wt%, and again mixed using a stick mixer, as described above.

The samples were extruded into a ribbon profile, using the same screw profile as described in Figure 3.2. Table 3.12 lists the four different formulations that were successfully extruded.

Table 3.12. Composite blends of PHA and sugar at different weight ratios with different loadings of Pimelea.

Biocomposite blend	PHA (wt%)	Sugar (wt%)	Pimelea (wt%)
PHA + 50% Sugar	50	50	0
PHA + 35% Sugar + 30% Pimelea	35	35	30
PHA + 80% Sugar	20	80	0
PHA + 56% Sugar + 30% Pimelea	14	56	30

Use of a component easily assimilated by microorganisms: starch

Here the same process as used for the porogen icing sugar was used to blend the PHA, starch and Pimelea prior to extrusion. The samples were extruded into a ribbon profile, using the same screw profile as described in Figure 3.2. Table 3.13 lists the four different formulations that were extruded successfully.

Table 3.13. Composite blends of PHA and starch at different weight ratios with different loadings of Pimelea.

Biocomposite blend	PHA (wt%)	Starch (wt%)	Pimelea (wt%)
PHA + 10% Starch	90	10	0
PHA + 7% Starch + 30% Pimelea	63	7	30
PHA + 20% Starch	80	20	0
PHA + 14% Starch + 30% Pimelea	56	14	30
PHA + 50% Starch	50	50	0
PHA + 35% Starch + 30% Pimelea	35	35	30

Use of a foaming agent during extrusion: Sodium bicarbonate

Sodium bicarbonate (SB) was used as a foaming agent and was added at different weight ratios in order to optimise the porosity of the final biopolymer composite. Prior to extrusion all materials were mixed together following the same procedure as described above.

The extruder was set with a string die instead of the ribbon die, as it was not possible to extrude these materials with a ribbon die. All the other extrusion parameters were kept the same as for normal PHA extrusion (Figure 3.2). A total of six formulations were processed as described in Table 3.14.

Table 3.14. PHA extrusion with foaming agent sodium bicarbonate (SB) at different weight ratios with different loadings of Pimelea

Biocomposite blend	PHA (wt%)	Sodium bicarbonate (wt%)	Pimelea (wt%)
PHA + 2%SB	98	2	0
PHA + 1.4%SB + 30% Pimelea	68.6	1.4	30
PHA + 5%SB	95	5	0
PHA + 3.5%SB + 30% Pimelea	66.5	3.5	30
PHA + 10%SB	90	10	0
PHA + 7%SB + 30% Pimelea	56	7	30

3.9.9 *In vitro* biopolymer degradation in Fermentations 1 to 4, 6, 8, 9, 10, 12 and 14

Biopolymers and biopolymer composites were included in Fermentation 1 (11 days), Fermentation 2 (20 days), Fermentations 3, 4, 6, 8, 10, 12 and 14 (all 30 days), and Fermentation 9 (63 days) as described in Section 3.7.3

A series of nylon bags were suspended within the fermenter system and removed at various time points for assessment of biopolymer degradation (Table 3.5). Biopolymers were placed in nylon bags with a pore size of 45 µm measuring 98 mm x 38 mm and sewn with polyester thread (Allied Filter Fabrics, Berkeley Vale NSW). These bags were incubated in the fermenters and taken out (in duplicate) as detailed in Table 3.5. In order to optimise the samples within each fermenter run, it was decided to sew the nylon bags in the middle in order to be able to place two biopolymer ribbons per bag; this protocol was used from Fermentation 4 onwards.

3.9.10 Biopolymer characterisation

To test the bio-degradation rate of samples of different biodegradable biopolymers by rumen bacteria, small pieces of each biopolymer were placed in nylon bags, incubated in the anaerobic *in vitro* fermentations as described in Section 3.7.3.

Percentage weight loss

Before fermenter exposure, the samples were dried in an oven at 45 °C overnight, placed in a desiccator and allowed to cool to room temperature then weighed, marked with unique identification codes and placed in nylon bags as described in Section 3.7.3. At each time-point, two samples in nylon bags exposed specimens were removed from the fermenter, washed with RO water and wiped before drying in an oven at 45 °C overnight, placed in a desiccator and allowed to cool to room temperature and then weighed. The percentage dry weight loss of the recovered samples was determined following the equation below:

$$Dry\ weight\ loss\ (\%) = \frac{Initial\ dry\ weight - Dried\ weight\ after\ fermenter\ exposure}{Initial\ dry\ weight} \times 100$$

Scanning electron microscopy (SEM)

A representative section of the biopolymer composite (before and after bacterial degradation) was cut using scissors or pliers. This central piece avoiding the ribbon edges was mounted on a stud covered with a sticky carbon stab. Samples were coated with ~11 nm of Iridium and imaged using a Hitachi SU3500 scanning electron microscope. Image acquisition was done on the Iridium-coated

samples at 5 kV accelerating voltage, with a spot size of 40 and approximately 10 mm working distance.

Differential scanning calorimetry (DSC)

A differential scanning calorimeter Q2000 (TA Instruments) under a constant nitrogen flow of 50 mL/min was used to determine the thermal properties of the biopolymer composites. Samples of 5–10 mg were placed in a sealed aluminium pan and analysed using standard DSC heating and cooling scans.

1. For PHA, each sample was heated from 25 °C to 185 °C at 10 °C/min and kept isothermal for 0.1 min, and then cooled to −70 °C at 10 °C/min, heated back to 190 °C.
2. For PCL, each sample was heated from 25 °C to 85 °C at 10 °C/min and then cooled to −70 °C at 5 °C/min, heated back to 85 °C at 10 °C/min.
3. For PLA, each sample was heated from 25 °C to 280 °C at 10 °C/min and then cooled to 0 °C at 5 °C/min, finally heated back to 280 °C at 10 °C/min.
4. For starch, each sample was heated from 25 °C to 90 °C at 10 °C/min and then cooled to 0 °C at 5 °C/min, and heated back to 90 °C at 10 °C/min
5. For PHA/PCL blends, each sample was heated from 25 °C to 280 °C at 10 °C/min and then cooled to 0 °C at 5 °C/min.

The melting temperature, T_m, and enthalpy of fusion, ΔH_m, were determined from the second heating cycle. The crystallisation temperature, T_c, was determined from the first cooling cycle.

Colour measurement (LAB)

The colour analysis of all samples was performed using Adobe Photoshop software. The samples were scanned using a Konika Minolat Bizhub C454e document scanner with a white background. The colour of the scanned images was determined by using the eyedropper tool with a 31 by 31 average across the sample according to the CIE 1976 L*, a*, b* colour space. L* (from 0 to +100) represents lightness: an increase in L* indicates a lighter colour. a* and b* (from −300 to +300) represent colour components from green to red and from blue to yellow respectively. The mean values were obtained from the analysis of five areas on each of three replicate samples.

3.9.11 Additional characterisation of biopolymers in Fermentation 14

This study was undertaken as an independent study and has been published as Yuan et al. (2022).

Gel Permeation chromatography (GPC)

Gel permeation chromatography (GPC) was utilised to quantify the molecular weight of PHA in all the cryo-ground biopolymers. In this case, 4.9-23.6 mg of each of the biocomposites were added to ~2 mL HPLC grade chloroform in capped glass tubes. The mixture was heated in a dry heating block at 80 °C for 30 min or until fully dissolved, and then the suspension was filtered before analysis.

An Agilent 1260 Infinity instrument (G7116A Multicolumn Thermostat, G7110B Isocratic Pump, G7128A Vialsampler, G7800A Multi-Detector GPC/SEC System) was used with a column set consisting of a guard column (PLgel MIXED (5 µm, 7.5 × 50 mm)) followed by two columns of PLgel 105Å, (5 µm, 7.5 x 300 mm) and PLgel 104Å, (5 µm, 7.5 x 300 mm). The columns were maintained at 30 °C. The refractive index (RI) signal was used for data processing. Narrowly distributed polystyrene standards were used for calibration

X-ray computed tomography (CT) (μ -CT)

The non-destructive and microscopic μ-CT analysis was conducted to investigate the changes in the interior pore network of the biopolymer samples after exposure to the *in vitro* fermentation environment. The two-dimensional (2D) scanning images were firstly acquired with a Skyscan 1272 (Skyscan, Bruker, Belgium) for a single ribbon piece of each type of biopolymer at every time point (Day 0, 1, 5, 9, 19 and 29), where Day 0 is the commencement day of the trial. The following parameters were used: X-ray source of 50 KV voltage and 200 uA current, pixel size of 7.5 μm, exposure time of 275 ms, rotation step of 0.25°, no filter, 2×2 binning, and averaging of 2. NRecon Reconstruction Software was then used for reconstructing all the obtained images with the same threshold range (0-0.13). As the ribbon pieces were oversized and scanned by 3-4 smaller parts, the images were combined, too. Several steps were applied to the obtained reconstructed images in CTan software (Skyscan), prior to the three-dimensional (3D) analysis of the pore structure. An automatic algorithm (Ridler-Calvard), which can best represent pores as black pixels and solid material as white pixels, was chosen to binarize the reconstructed images to avoid bias resulting from manual decided threshold. Then, possible artifacts were removed using the functions integrated in the software, including 'despeckle' and 'sweep'. At last, the volume of interest (VOI) was defined using the shrink-rap algorithm by stretching the boundary over holes lager that 60 pixels (60*7.5 μm= 450 μm). The percentage of closed pores (definition: a connected assemblage of space (black) voxels that is fully surrounded on all sides in 3D by solid (white) voxels) and the percentage of open pores (definition: any space located within a solid object or between solid objects, which has any connection in 3D to the space outside the object or objects.) within the VOI were calculated by 3D analysis. In order to further estimate the pore diameter distribution, the pore structure was turned into object while the solid material turned into voids by changing the black pixels into white and white pixels into black in the binarized images. These processed images were saved and 3D analysis was performed to obtain the structure thickness as an indication of the pore diameter within our biopolymers. The term of structure thickness was defined by Hildebrand and Rüegsegger (1997)as the diameter of the largest sphere which fulfills two conditions: the sphere encloses the point (but the point is not necessarily the centre of the sphere), the sphere is entirely bounded within the solid surfaces.

Toxin release performance analysis

After the μ-CT scanning, the majority of the ribbon pieces including the Day 0 samples of all the biopolymers were split and cryo-ground for 3×10 min in liquid nitrogen using a freezer mill (6875 Freezer/Mill®, Spex, Metuchen, NJ, USA). The cryo-ground particles were dried again at 45 °C for 24 h (-80 kPa) in a vacuum oven, restored to room temperature in a desiccator and stored in a freezer (-20 °C). The concentration of simplexin in the cryo-ground biocomposites were determined by using the established LC-MS/MS methodology described below in Section 3.9.12. Given the known concentration of simplexin in these biocomposite samples, the cumulative release of simplexin was calculated using the following equation:

$$\text{cumulative release of simplexin } (\mu g) = M_0 \cdot C_0 - M_i \cdot C_i$$

where M_0 is the dry weight of the ribbon piece before exposure to the fermentation system (g); C_0 is the initial concentration of simplexin within the sample (μg/g); M_i is the dry weight of the sample after exposure to the fermentation system for *i* days (g); C_i is the concentration of simplexin within the sample piece after exposure to the fermentation system for *i* days (μg/g).

3.9.12 LC-MS/MS analytical method for determination of simplexin concentration in biopolymer composites

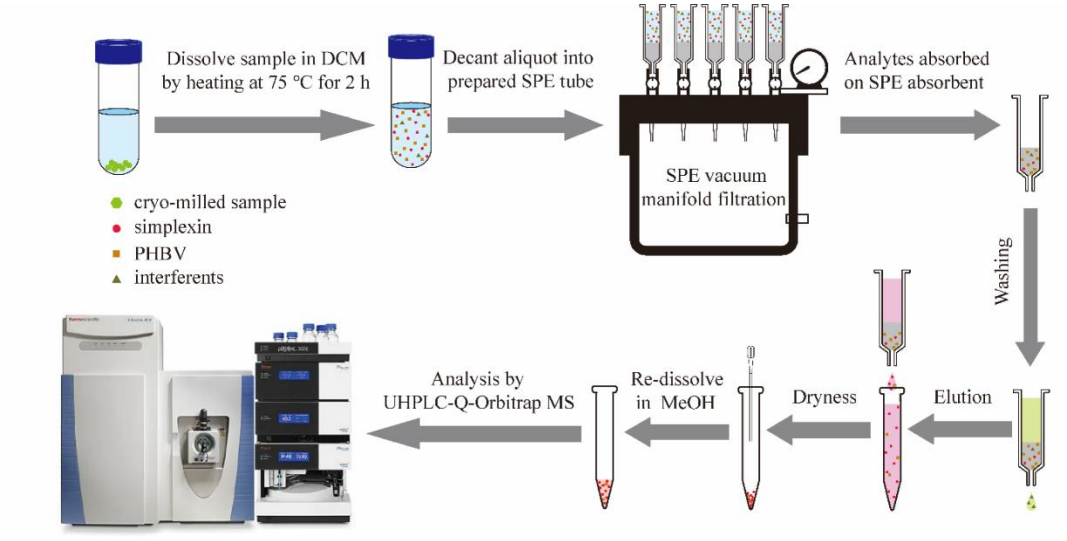
Sample selection and processing

Composites of biopolymer PHA + 30% Pimelea and of PHA with crude ethanolic extracts of Pimelea (5 mL of a crude ethanolic Pimelea extract with 1 g PHA powder and 10 mL of a crude ethanolic extract with 1 g PHA powder) were used for method development (optimisation and validation) in the determination of simplexin concentration in a biopolymer matrix. Samples were also collected after being exposed in a fermenter simulating the rumen environment for 9, 10 and 29 days and the residual simplexin content analysed. Due to the non-availability of Pimelea plant material without simplexin, pure PHA extruded under the same conditions as the biopolymer composites was used as a substitute for a blank matrix. All samples were cryogenically ground for 3×10 min in liquid nitrogen using a freezer mill (6875 Freezer/Mill®, Spex, Metuchen, NJ, USA) to reduce distributional heterogeneity before analysis.

Biopolymer extraction

A portion (50 mg biopolymer PHA + 30% Pimelea, 35 mg for Pimelea ethanol extract/PHA) of the cryo-milled sample was accurately weighed (± 0.01 mg) into a 50 mL glass tube with PTFE-lined screwcap, and 35 mL dichloromethane (DCM) was added. The tube was then capped, heated at 75 °C in a dry block heater (Ratek Instruments, Boronia, Victoria, Australia) for 2 h, and vigorously shaken by hand every 30 min until the polymer was completely dissolved. A solution of milled extruded PHA (1 mg/mL) was prepared in the same manner, as a substitute for blank matrix. A 5 mL aliquot of the resulting mixture from PHA + 30% Pimelea, or a 1 mL aliquot from Pimelea ethanol extract /PHA, was then transferred into 15 mL polypropylene tubes prior to sample clean-up by solid-phase extraction (SPE). Mega BE-SI SPE cartridges (1 g, Agilent Technologies, Santa Clara, CA, USA) paired with 10 mL syringe barrels were set up on a Vac Elut SPS-24 vacuum manifold system (Varian, Harbor City, CA, USA). After preconditioning the SPE cartridges with DCM (10 mL), each sample and its sequential rinsing with 2 mL and 1 mL DCM were loaded. Before elution of simplexin, the sorbent was washed with 10 mL n-hexane containing 5% (v/v) DCM to remove interfering non-target compounds. The final eluent of 10 mL DCM with 5% (v/v) methanol under gravity, was collected in another 15 mL polypropylene tube, and evaporated to dryness under a gentle nitrogen stream at 40 °C. The residue was reconstituted in 1 mL methanol, vortexed for 1 min and filtered through a polypropylene membrane syringe filter (13 mm, 0.2 µm, Pall Corporations, Ann Arbor, MI, USA) prior to being injected into the UHPLC Q-Orbitrap MS system for simplexin analysis. A schematic of the workflow is shown in Figure 3.4.

Figure 3.4. Schematic workflow for biocomposite sample extraction for simplexin concentration measurement.



LC-MS/MS analysis of biopolymer extracts

Quantification of simplexin by UHPLC-Q-Orbitrap-MS analysis (Section 3.5) was achieved from the external calibration curve obtained from the standards in pure MeOH.

Considering the existence of a matrix effect in LC-MS/MS analysis of complex matrices, the concentration of simplexin within the biopolymer composites was determined by external calibration of matrix-matched standards. The stock solution of simplexin at 10 µg/mL was prepared in methanol using previously purified simplexin. A nine-level standard solution set, ranging from 10 ng/mL to 1,000 ng/mL, were made by dilution of the stock solution with methanol. The matrix residue extracted from 5 mL PHA dissolution through SPE was used to make matrix-matched calibration standard solutions for PHA + 30% Pimelea and matrix residue extracted from 1 mL PHA dissolution for Pimelea ethanol extract /PHA sample. A further stock solution of simplexin at 1 µg/mL was prepared in dichloromethane for spiking purposes. All solutions were stored at -18 °C and restored to room temperature before use.

3.9.13 Bolus manufacture

Several small-scale boluses were 3D printed using a Flashforge 3D printer. This printer was set with PCL filament. The PCL was placed in a stainless-steel reservoir and heated at 120 °C through a heated cartridge unit. When the polymer reached a molten phase, air flow was used to extrude solid fibres through a needle. A continuous printing process resulted in fabrication of properly connected fibres forming a construction with 0−90° or 0-45° angle between consecutive layers with a fibre inter-distance of 1 mm.

3.10 *In vivo* rumen microbial degradation of biopolymer composites

3.10.1 Animal Ethics Approval

An animal ethics application '*Use of fistulated Holstein Friesian steers to determine rumen degradation rates of biopolymer boluses'* was developed and submitted through the DAF Animal Ethics Committee (AEC) in March 2020 and was approved as AEC reference number SA 2020/03/737,

and this was subsequently ratified by the UQ AEC and a certificate issued DAF/QAAFI/125/20 (Appendix Section 9.5.4). The trial design was a repeated-measures randomised-block design and was assessed by a DAF Biometrician as an acceptable design with three animals used to determine between animal variations, and two replicates sets of biopolymers in each animal allowing measurement of within animal variation.

3.10.2 Preparation of biopolymer pieces for *in vivo* trial

The biopolymer (PHA) used in the *in vivo* rumen degradation trial was a poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV, with 1 mol% HV, from TianAn in China). Two set of PHA samples were prepared for the animal trial. The first set of samples was prepared to assess the degradation rate of pure PHA under *in vivo* conditions, to compare with the *in vitro* testing done to date. For this purpose, a solid rod-like sample of PHA was extruded using a large circular die (to give solid cylinders of dimensions ~ 10 mm (D) x 50 mm (L)) (

Figure 3.5). A second set of samples was prepared to assess the effective of macro-scale porosity on the biodegradation rate of PHA under *in vivo* conditions. These open porous samples were 3D printed using a Flashforge Inventor 3D printer die (to give lattice-like cylinders of dimensions ~ 13 mm (D) x 50 mm (L)), with the intent being to have a large available surface area of PHA for biodegradation (Figure 3.6).

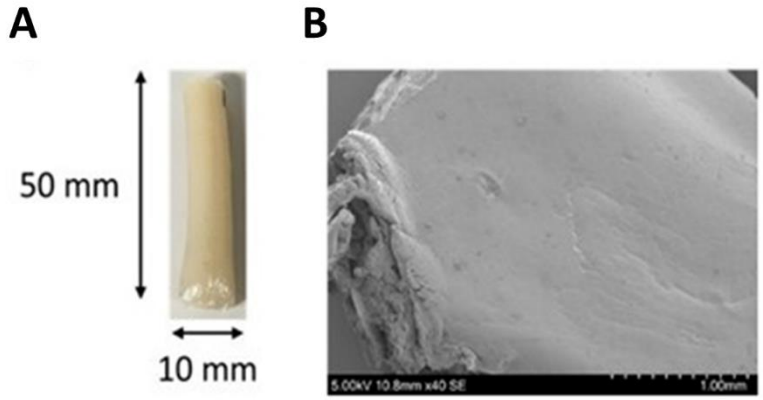
Extrusion of solid PHA test pieces:

Prior to extrusion, the PHA biopolymer was dried separately in a vacuum oven at 65 °C with a gauge pressure of −80 kPa for 24 h. A PolyLab OS (Haake RheoDrive 7, Thermo Scientific) co-rotating twin screw extruder with a diameter of 16 mm and a length-to-diameter ratio of 40:1 was used for this work. The screw profile was the same as presented in Figure 3.2.

The extruder was equipped with a melt pump OS (HAAKE, Thermo Scientific) set at 135 °C and running at 60 rpm. A circular die of 15 mm, which was orientated vertically to enable smooth and constant output, was used to extrude cylindrical PHA (

Figure 3.5). The extruded PHA was cooled down with a fan at the exit of the extruder in order to keep a consistent diameter.

Figure 3.5. (A) solid PHA biopolymer piece with approximate size and (B) SEM of extruded surface of solid PHA piece.



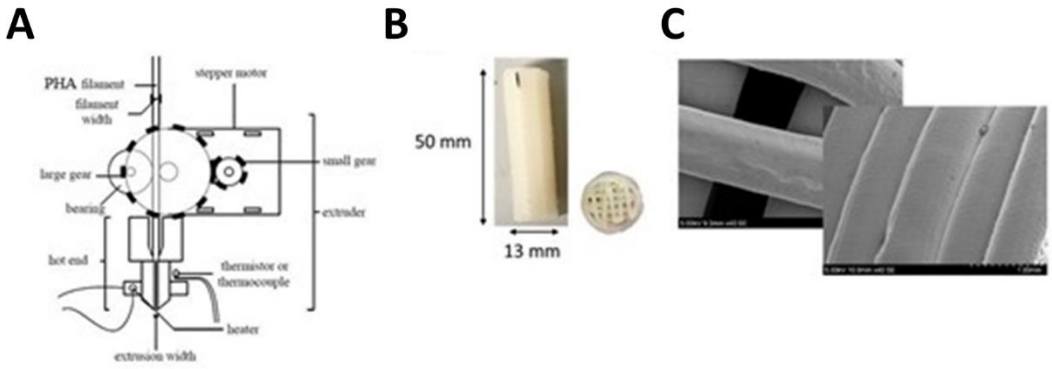
3D printing of PHA test pieces

Prior to 3D printing the PHA was dried as described in the previous section.

To enable 3D printing of PHA, it was first necessary to extrude PHA as a consistent filament of about 1.6 to 1.7 mm diameter. This was achieved by using the same screw profile as described above, with the extruder running at 100 rpm**.** The extruder was set up with the melt pump equipped with a strand die on this occasion. The melt pump was set at 160 °C to produce a homogeneous filament. A PHA filament with a diameter varying between 1.65 and 1.8 mm was successfully extruded and used for 3D printing.

Cylinders of porous PHA were 3D printed using a Flashforge Inventor 3D printer (Zhejiang Flashforge3D technology, China), using the fused filament fabrication method. The printer was equipped with a 0.8 mm diameter nozzle. The printer was loaded with PHA extruded filament. The filament was fed into the 3D printer with gears set at the desired speed in the heated nozzle (Figure 3.6A). The filament cooled and solidified instantaneously on leaving the nozzle. A continuous 3D printing process resulted in fabrication of properly connected fibres forming a construction with either 0−90° or 0-45° angle between consecutive layers (see Figure 3.6B for the pattern of angles of filament within a layer) and with a fibre inter-distance of 1 mm.

Figure 3.6. (A) Flashforge Inventor 3D printer extruder schematic showing PHA filament, gears for consistent filament feed in, filament melting and extrusion printing of 3D PHA porous structure, (B) 3D printed PHA piece with size indicated and (C) SEM of 3D printed porous PHA biopolymer piece.



3.10.3 Allocation of biopolymer pieces to animal treatment set bags

The experimental design for the *in vivo* biodegradation testing using the two sets of biopolymer samples (i.e. solid cylinder or 3D printed porous cylinder) in three Holstein Friesian steers is summarised in Table 3.15. The experiment used duplicate samples of each of the two polymer forms, with four time points of removal for each polymer form, in each steer.

The biopolymer pieces (both solid and porous) were dried and weighed before being randomised by weight and then split into either a high or low weight cluster, for the intra-animal set so that each animal received one high and one low weight piece. The four time points were randomised within the low and high sets and the animals randomised within time and replicate so that each animal received a random weight.

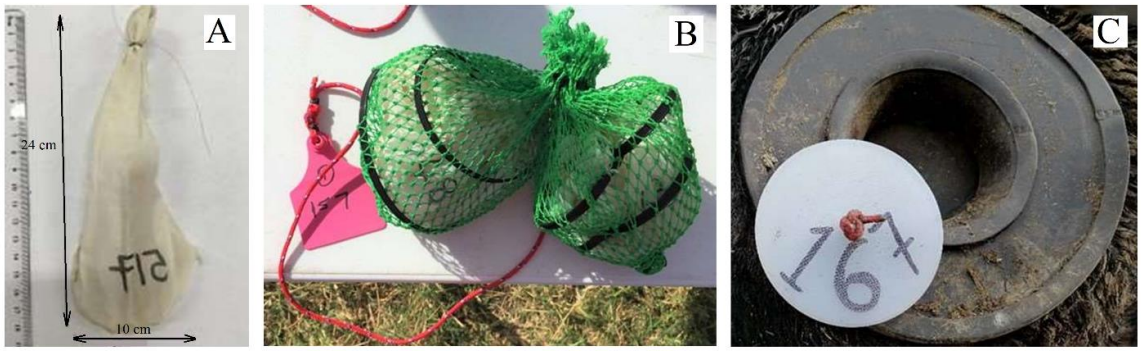
Table 3.15. Summary of experimental design for *in vivo* testing of biopolymer degradation.

Sample type	Sample #	Nylon Bag #	Dry weight Biopolymer (g)	Sample type	Sample #	Nylon Bag #	Dry weight Biopolymer (g)	Intra animal Set #	Time Point	Animal Number	Animal Eartag
Solid biopolymer	32	648	5.153	Porous Biopolymer	20	128	5.281	1	1	1	157
Solid biopolymer	31	863	5.967	Porous Biopolymer	11	5	5.733	2	1	1	157
Solid biopolymer	28	758	5.751	Porous Biopolymer	10	25	5.696	1	2	1	157
Solid biopolymer	25	647	6.361	Porous Biopolymer	17	710	5.948	2	2	1	157
Solid biopolymer	2	14	5.107	Porous Biopolymer	23	143	5.162	1	3	1	157
Solid biopolymer	19	548	6.123	Porous Biopolymer	16	730	5.841	2	3	1	157
Solid biopolymer	5	588	5.28	Porous Biopolymer	15	154	5.451	1	4	1	157
Solid biopolymer	4	596	6.583	Porous Biopolymer	6	112	6.506	2	4	1	157
Solid biopolymer	26	635	5.232	Porous Biopolymer	21	583	5.301	1	1	2	167
Solid biopolymer	29	586	5.993	Porous Biopolymer	7	220	5.771	2	1	2	167
Solid biopolymer	27	434	5.837	Porous Biopolymer	4	111	5.708	1	2	2	167
Solid biopolymer	15	620	6.438	Porous Biopolymer	3	91	6.017	2	2	2	167
Solid biopolymer	9	30	4.858	Porous Biopolymer	24	150	5.16	1	3	2	167
Solid biopolymer	24	605	6.096	Porous Biopolymer	22	964	5.801	2	3	2	167
Solid biopolymer	30	572	5.441	Porous Biopolymer	8	196	5.495	1	4	2	167
Solid biopolymer	11	562	6.474	Porous Biopolymer	9	96	6.251	2	4	2	167
Solid biopolymer	17	80	5.259	Porous Biopolymer	12	53	5.365	1	1	3	168
Solid biopolymer	16	549	5.931	Porous Biopolymer	18	74	5.725	2	1	3	168
Solid biopolymer	12	735	5.746	Porous Biopolymer	5	536	5.636	1	2	3	168
Solid biopolymer	20	808	6.272	Porous Biopolymer	19	90	5.931	2	2	3	168
Solid biopolymer	18	640	5.113	Porous Biopolymer	13	69	5.272	1	3	3	168
Solid biopolymer	7	848	6.266	Porous Biopolymer	1	22	5.893	2	3	3	168
Solid biopolymer	10	556	5.692	Porous Biopolymer	14	866	5.593	1	4	3	168
Solid biopolymer	6	265	6.754	Porous Biopolymer	2	10	6.773	2	4	3	168

Each biopolymer piece was placed into a numbered polyester bag (Allied Filter Fabrics; monofilament polyester, 24 X 10 cm outer dimension, pore size 45 µm) which, following folding, were secured with nylon fishing line and rubber bands (Figure 3.7A). Nylon mesh bags were then secured with weights (large nuts secured to the bottom of the bag with a zip tie) to keep the bags below the rumen raft and zip tie spacer rings used to keep bags more open Figure 3.7B. Once the set of solid and porous PHA pieces was added into the bags as per the allocation in Table 3.15, they were secured closed with a further zip tie, and then the paired intra-animal sets were attached to a nylon rope labelled with an ear tag (Figure 3.7B). The bags were pushed through the cannula and pushed down to sit below the rumen raft, while the eartag was kept outside the rumen cannula with the rope threaded down the side of the bung. A modification was made to the setup four days into the trial with the eartag replaced with a solid disc of high-density polyethylene (cut from a chopping board) to prevent the rope pulling through the bung (Figure 3.7C).

Before the bags were placed into the rumen via the cannula, four 1.0 mL samples of rumen fluid were taken into 1.5 mL microcentrifuge tubes and centrifuged at 16,100 x g for 10 min. The resulting supernatant was removed, and the remaining cell pellet placed on ice for transport back to the laboratory and then stored frozen at -20 °C for future gDNA extraction and microbiome analysis.

Figure 3.7. (A) Numbered polyester bag folded and secured with fishing line prior to application of a rubber band; (B) Two sets of biopolymer pieces within set bags containing weights and showing zip tie spacer rings, nylon rope and initial ear tag used to secure through the rumen canula, and (C) Solid disc securing bags through the rumen canula.



3.10.4 Biopolymer animal trial

The animal trial was conducted at the UQ/DAF Dairy facility at the UQ Gatton Campus with the three fistulated steers in the trial housed in a paddock with a fourth fistulated steer. The steers were maintained on a diet of sorghum silage (whole plant/grain on) and occasionally barley hay at the start of the trial on 12/5/2020 but were transferred to a Rhodes grass hay diet and had access to a urea lick block. The animals were checked daily for the first week to ensure there were no impacts on the animal's health and well-being.

The animals were visually sighted daily and physically checked weekly to ensure the bags were still tethered to the outside of the rumen canula and there were no health issues.

3.10.5 Biopolymer removal and sample collection

The two nylon mesh bags containing the sets of biopolymer pieces were removed from the rumen of each animal via the rumen cannula on days 30, 62, 92 and 122 and opened to identify and remove

the numbered bags of biopolymer pieces allocated for removal at that time point. Once the individual bags had been removed, the nylon mesh bags were closed with a new zip tie, re-attached to the tether rope with a new zip tie and replaced back through the rumen cannula and pushed down to below the rumen raft. The individual bags were rinsed in reverse osmosis (RO) water in plastic buckets to remove excess rumen fluid and plant debris from the bag. The individual bags from each animal were placed into zip-lock bags labelled with the animal number and placed on ice for transportation back to the laboratory. At each time point, four 1.0 mL samples of rumen fluid were taken into 1.5 mL microcentrifuge tubes, centrifuged at 16,100 x *g* for 10 min, the resulting supernatant discarded, and the remaining cell pellet placed on ice for transport back to the laboratory. The cell pellets were stored frozen at -20 °C for future gDNA extraction and microbiome analysis.

Once at the lab the nylon bags were individually rinsed again in RO water in a 2 L beaker with gentle massaging and the rubber band removed to allow access to the folds for rinsing. The beaker RO water was changed several times and once the water was relatively clean, the bags were patted dry with paper towel and the nylon fishing line removed. The bag was inverted over a beaker and the biopolymer pieces removed into the beaker. If the biopolymer piece was completely intact, it was washed with RO water and then placed into a pre-weighed, numbered petri dish labelled with the animal number, biopolymer piece number and date and placed into a vacuum oven (-80 kPa) at 45 °C for 24 h. The petri dish was removed from the oven and placed into a desiccator containing fresh silica and allowed to cool to room temperature. Once at room temperature, the biopolymer pieces were weighed and placed into individual small ziplock bags labelled with the animal number, biopolymer number and date of removal from the rumen. The biopolymer pieces were stored frozen at -20 °C until transportation to UQ for analysis by the UQ Biopolymer group. If the biopolymers were present as fragments within the bag it was inverted, and the contents rinsed into a beaker with RO water and the biopolymer pieces allowed to settle to the bottom before the majority of the water was decanted off. Fresh RO water was added and the process of settling, and decanting was repeated several times before a disposable pipette with the tip cut off was used to transfer the biopolymer fragments to a petri dish containing a round Whatman grade 1 filter paper (Cytvia, UK). A dissection microscope and tweezers were used to transfer the fragments of biopolymer to a fresh pre-weighed petri dish labelled with the animal number, biopolymer piece number and date of removal from the rumen. The samples were then treated as described above.

3.10.6 Analysis of biopolymers after removal from rumen

Percentage weight loss

As described in Sections 3.2.3 and 3.2.5, the dried samples were weighed before and after animal rumen exposure, with the percentage dry weight loss of the recovered samples was determined following the equation below:

$$Dry\ weight\ loss\ (\%) = \frac{Initial\ dry\ weight - Dried\ weight\ after\ animal\ exposure}{Initial\ dry\ weight}$$

The weight loss results were analysed using Genstat v19 (VSN International, UK) by fitting a simple linear regression with groups with the percent biopolymer weight loss as the response variable and time of extraction as the explanatory variable, with biopolymer type as the group. Curvature of the fit was also tested by fitting a quadratic response however the quadratic coefficient was not significantly different to 0 (p=0.352), showing no indication of curvature. The porous biopolymer only recorded results at two time points and so the linear response should only be taken as an indication of a possible linear result. Both animal number and weight group were also tested within each biopolymer type and found to be non-significant and so not tested further.

Scanning electron microscopy (SEM) analysis

For all SEM analyses, a representative section of the central part of the ribbon was cut from the dried biopolymer or biocomposite samples using a saw and pliers. This central piece, which avoided the ribbon edges, was mounted on a stud covered with a sticky carbon stab. Samples were sputter coated with ~11 nm of platinum and imaged using a Hitachi SU3500 scanning electron microscope. Image acquisition was done on the Iridium-coated samples at 5 kV accelerating voltage, with a spot size of 40 and approximately 10 mm working distance.

Differential scanning calorimetry (DSC) analysis

The thermal properties of selected biopolymers retrieved from *in vivo* animal trial as well as the Day 0 samples were analysed using the same DSC instrument and 5-cycle heating-cooling protocol described in Section 3.9.10 whereby a differential scanning calorimeter Q2000 (TA Instruments) operating under a constant nitrogen flow of 50 mL/min was used to determine the thermal properties of the biopolymer composites. Samples of 5–10 mg were placed in a sealed aluminium pan and analysed using standard DSC heating and cooling scans, whereby each sample was heated from 25 °C to 185 °C at 10 °C/min and kept isothermal for 0.1 min, and then cooled to −70 °C at 10 °C/min, before being heated back to 190 °C at 10 °C/min.

To best represent different degrees of biodegradation, a series of the solid extruded biopolymers were analysed (See Section 4.2, Fig. 4.12). The 0-day samples and those that still had a solid cylinder shape after exposure to the *in vivo* rumen environment were cut in half from the middle, and both the interior area and the surface area were sampled for analysis. Otherwise, the small particles left from the extensively degraded final solid extruded biopolymers and the 3D-printed porous biopolymers were analysed in duplicate. The melting temperature, T_m, and enthalpy of fusion, ΔH_m, were determined from the first heating cycle. The crystallisation temperature, T_c, was determined from the first cooling cycle.

Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) was utilized to quantify the molecular weight of all the biopolymer samples. Same as DSC analysis, both interior and surface area were analysed for the solid extruded biopolymers with an integrated shape (as shown in the first two rows in Figure 4.58). Other samples were analysed in duplicates. Samples were weighed into HPLC grade chloroform to obtain a concentration of 2.5 mg PHA/mL mixture in capped glass tubes. The mixture was then heated in a dry heating block at 80 °C for 30 min or until fully dissolved. An Agilent 1260 Infinity instrument (G7116A Multicolumn Thermostat, G7110B Isocratic Pump, G7128A Vialsampler, G7800A Multi-Detector GPC/SEC System) was used with a column set consisting of a guard column (PLgel MIXED (5 µm, 7.5 × 50 mm)) followed by two columns of PLgel 105Å, (5 µm, 7.5 x 300 mm) and PLgel 104Å, (5 µm, 7.5 x 300 mm). The columns were maintained at 30 °C. The refractive index (RI) signal was used for data processing. Narrowly distributed polystyrene standards were used for calibration.

X-ray computed tomography (CT) (μ -CT)

Selected biopolymer samples including both the 0-Day extruded and 3D-printed biopolymers and a 3D-printed porous biopolymer retrieved after 62 days, were scanned by µ-CT. The two-dimensional (2D) transaxial images were acquired from a quick scan of the middle position of the samples with a Skyscan 1272 (Skyscan, Bruker, Belgium). The following parameters were used: X-ray source of 50 KV voltage and 200 uA current, pixel size of 9.0 µm, exposure time of 100 ms, 360° rotation on, rotation step of 0.30°, no filter, 4×4 binning, and frame averaging of 2. NRecon Reconstruction Software was then used for reconstructing all the obtained images with the threshold range of 46-255.

3.11 *In vitro* Enterosorbent studies

3.11.1 Adsorbent characterisation

Adsorbents tested

Oral intestinal adsorbents (enterosorbents) are orally administered materials which pass through the gut where they bind (adsorb) various substances. Enterosorbents include activated carbons or charcoals, inorganic minerals, polymeric and silicon-containing resins, and have found application in both human and animal therapies. Although frequently referred to interchangeably as absorbents or adsorbents, "adsorbents" or enterosorbents is the correct term. In this study adsorbents of different product type (Table 3.16) were sourced for simplexin adsorbent studies in clarified rumen fluid.

These adsorbent products were provided in differing particle sizes and a number were pre-processed and/or milled before use (Table 3.17). PAC 1000 (activated carbon from coconut) was a powder with particle size less than 0.18 mm (180 microns or US Mesh 80) and a median diameter between 15 to 30 micron. The Supelco activated charcoal was provided in powdered form with an average particle size of 80 microns. The PAC 1000 and activated charcoal were used without further modification. All biochars and activated products were milled using a Culatto Type MFC CZ13 hammer mill fitted with a 0.5 mm screen to provide powders of a similar particle size. Elitox, Solid HT Clay and the bentonites produced a fine dispersion in solution and were not milled prior to use. Biochar (Gidgee) was also activated as described below.

Table 3.16. Product type and supplier details for the adsorbents used in *in vitro* simplexin adsorbent studies.

Adsorbent product name	Product type	Supplier/source, location
Biochar (Gidgee)	Ligno-cellulosic biochar from 'Gidgee' (*Acacia cambagei*)	Renewable Carbon Resources Australia, Charleville Qld
GAC coconut	Granular activated carbon from coconut	Clarence Water filters, Yamba New South Wales
Acticarb PC 1000 (PAC 1000)	Powdered activated carbon from coconut	Activated Carbon Technologies, Brisbane Queensland
Sugarcane waste biochar temperature activated	Sugarcane waste	Sugarcane tops sourced from Rockypoint, activated at USQ
Sugarcane waste biochar steam activated	Sugarcane waste	Sugarcane tops sourced from Rockypoint, activated at USQ
Activated charcoal (Supelco)	Powdered activated carbon	Sigma Aldrich / Merck Australia
Sodium bentonite	Sodium dominate montmorillonite	Trufeed®, Sibelco Australia
Calcium bentonite	Calcium dominate montmorillonite	Bentonite Resources Pty Ltd, Ebenezer, Queensland
Bentonite (product #285234)	Montmorillonite	Sigma Aldrich / Merck Australia
Solid HT Clay	Organically modified lucentite with choline chloride and hexadecylpyridinium functional surface groups	The University of Queensland
Elitox	Mixture of hydrated sodium calcium aluminium silicates (HSCAS), *Carica papaya* extract, chitosan and xylanase.	Feedworks, Australia

Table 3.17. Processing of adsorbents before use in adsorbent trials.

Adsorbent	Modification completed
Biochar (Gidgee)	Milled to <0.5mm
Temperature treated Biochar (Gidgee)	Biochar (Gidgee) heated to 1,000 °C and milled to <0.5 mm
Steam activated Biochar (Gidgee)	Biochar (Gidgee) heated to 1,000 °C with steam activation and milled to <0.5 mm
Nitrogen purged Biochar (Gidgee)	Biochar (Gidgee) heated to 1,000 °C under nitrogen and milled to <0.5 mm
GAC coconut	Milled to <0.5 mm
PAC 1000	Used without modification
Sugarcane waste biochar temperature activated	milled to <0.5 mm
Sugarcane waste biochar steam activatedactivated	milled to <0.5 mm
Activated charcoal (Supelco)	Used without modification
Sodium bentonite	Used without modification
Calcium bentonite	Used without modification
Bentonite (product #285234)	Used without modification
Solid HT Clay	Used without modification
Elitox	Used without modification

Biochar (gidgee) modifications

The Gidgee Biochar that is widely used by producers was characterised and used (after milling, Table 3.17) in most of the *in vitro* adsorbent studies (Table 3.18).This Biochar (Gidgee) was also modified (activated) to improve its binding properties by Dr Les Bowtell at University of Southern Queensland (USQ), Toowoomba by three different methods as described below.

Briefly for temperature activation, Biochar (Gidgee) (~150 g) was placed into a graphite crucible, which was transferred into a kiln (Rio-Grande, PMC kiln) and heated to 1,000 °C at a rate of 600 °C/h. The temperature was held at 1,000 °C for 1 h, and the sample then allowed to cool to room temperature before being removed from the kiln. The cooled sample was transferred into a plastic container and stored sealed at room temperature until needed.

For steam activation: distilled water (~2 mL) was injected onto the Biochar after the heating period at 1,000 °C, and this water injection was repeated three times over 3 min. For the nitrogen purged Gidgee Biochar, the crucible, Biochar and kiln were continuously flushed with nitrogen gas throughout the entire activation process.

These activated products and the original Biochar (Gidgee) were hammer milled as described above before use in adsorbent assays. Characterisation studies were performed on the tested adsorbents to assist with understanding differences in simplexin uptake performance:

Net surface charge analysis

The net surface charge of adsorbents was measured by assessing the effect of adsorbents on the pH of 1 M solutions of KCl (pH KCl) compared with the effect of the same adsorbents on the pH of water

(pH(H_2O)), which provides an assessment of the net charge of the colloidal system. Adsorbent (1 g) was added to a 50 mL falcon tube containing either 20 mL of 1M KCl or 20 mL of H_2O, and the tubes agitated using a magnetic stirrer bar at speed setting 3, for 1 h. The numerical difference in the pH measured in 1M KCl and H_2O (pH KCl – pH H_2O) is referred to as the delta pH. When this difference is negative, the colloid has a net negative charge (cation exchange capacity), and when positive, it has a net positive charge (anion exchange capacity) (Enders et al. 2012).

Zero-point charge analysis

The zero-point charge pH (pH(Zpc)) of the adsorbents was measured using the pH drift method. Solutions (50 mL) of 0.01 M NaCl solution were adjusted to a selected range of initial pH (pHi) values (ranging between pH 2 – 10) by addition of HCl (0.1 M) or NaOH (0.1 M). Adsorbent material (200 ± 10 mg) was then added, and the sample stirred using a magnetic stirrer bar at speed setting 3, for 48 h. After this, the final pH (pH_f) was measured. The zero point charge pH(zpc) value is the point where the curve of ΔpH (pH_f–pH_i) versus pH_i crosses the x axis (Gupta and Nayak 2012).

3.11.2 Preliminary enterosorbent studies in rumen fluid media

Adsorbent trials in rumen fluid media

Rumen fluid media (RF Media) was prepared by standard protocol (Appendix Section 9.7.3). Adsorbents (biochar, bentobite, elitox) in amounts of 10, 30, 50 and 70 mg were placed in 5 mL Eppendorf tubes (conducted in triplicate), and aliquots of RF media (2.5 mL) were added together with simplexin (100 µL of 50 µg/mL solution in ethanol) and the resulting suspension shaken for 1 h using a Paton Scientific Reciprocating Shaker RP1812. After shaking, the adsorbent in the tubes were allowed to settle for 10 min and an aliquot removed from the supernatant for analysis. Aliquots of supernatant (0.5 mL) were diluted with ethanol (0.5 mL) and then filtered through GHP Acrodisc 0.2 µm membrane syringe filters (Pall) in preparation for LC-MS/MS analysis as described in Section 3.5.

3.11.3 Adsorbent *in vitro* testing methodology in clarified rumen fluid

Preliminary adsorbent trials were conducted in prepared rumen fluid media as described in Section 3.11.2. However further trials in different media batches proved unreliable largely due to the poor solubility of simplexin in such media and salting out effects (results not shown). We then conducted a further series of adsorbent trials conducted in clarified rumen fluid with different adsorbents, duration and adsorbent mass as shown in Table 3.18.

Clarified rumen fluid

Rumen fluid was obtained from a rumen-fistulated Brahman cross steer (#1990) that had not been exposed to Pimelea, located at the Queensland Animal Science Precinct (QASP) at UQ Gatton (UQ Animal Ethics Approval SAFS/296/17) and frozen at -20 °C until needed. Clarified rumen fluid was prepared via centrifugation following standard protocol as described in Appendix Section 9.7.5 and stored frozen at -20 °C until required.

Clarified rumen fluid in vitro adsorbent testing method

In each experiment, complete sets of 15 mL falcon tubes (one tube for each replicate for each adsorbent for each time point) were set up with designated amounts of adsorbent added to each tube (Table 3.18) and 9.9 mL clarified rumen fluid (Section 9.7) added to each tube. Studies were carried out in triplicate unless stated elsewhere. Three no adsorbent blanks for each timepoint were also included in each set, and similarly treated with clarified rumen fluid. A 100 µL aliquot of simplexin solution in methanol was added to provide the simplexin concentration shown in Table

3.18. The tubes were then vortexed for 20 s and placed on an orbital shaker in a temperature-controlled incubator at 39 °C.

After the designated incubation time, the samples were taken from the incubator and centrifuged at 845 x*g* for 1 min to separate the adsorbent from the liquid. A pipette was then inserted 2 cm below the liquid surface and 1 mL of the supernatant was removed for SPE clean-up followed by LC-MS/MS analysis as described in Section 3.5.

Table 3.18. Adsorbents tested, simplexin concentration and duration of adsorbent trials 1 to 6 in clarified rumen fluid (RF).

RF Trial	Adsorbents tested	Duration (h)	Simplexin concentration (ng/mL)	Adsorbent mass (mg)
RF AdsorbTrial #1	Biochar (Gidgee) Na bentonite	6	100	120
RF AdsorbTrial #2	Biochar (Gidgee) Na bentonite	42	200	120
RF AdsorbTrial #3	Biochar (Gidgee) Na bentonite Elitox HT Clay	4	100	60
RF AdsorbTrial #4	Biochar (Gidgee) Na bentonite Ca Bentonite Bentonite (Sigma) Elitox HT Clay Activated charcoal (Supelco)	4	100	120
RF AdsorbTrial #5	Biochar (Gidgee) Na bentonite Bentonite (Sigma) Activated charcoal (Supelco)	24	100	120
RF AdsorbTrial #6	Na bentonite Elitox Biochar (Gidgee) Biochar (Gidgee) temperature treated Biochar (Gidgee) steam activated Biochar (Gidgee) nitrogen purged PAC 1000 GAC activated coconut Sugarcane waste steam activated Sugarcane waste temperature activated Activated charcoal (Supelco)	1	100	5, 10, 50, 120, 240

Adsorbent assay SPE extraction process

Methanol (3 mL) was added to each supernatant sample (1 mL), and the extract vortexed (30 s) and shaken for 1 h and centrifuged at 3,220 x*g* for 10 min. The extract was decanted, and the pellet extracted twice more. The pooled extracts were then diluted with MilliQ water to give a 50% methanol/water mixture which was vortexed for 20 s prior to loading onto the SPE cartridge. The

SPE cartridges (Oasis® HLB 6CC 200MG, Waters Corporation) were preconditioned with methanol (5 mL) and water (5 mL). Then, the sample was loaded onto the cartridge. The cartridge was washed with 5% methanol (10 mL), 60% methanol (10 mL), and finally the analytes were eluted with 100% methanol (10 mL). Methanol eluents were filtered (0.2 µm) ready for LC-MS/MS analysis as described in Section 3.5.

Statistical analysis of adsorbent tests

Statistical tests were performed in Microsoft Excel. The results are presented as mean ± standard deviation. Comparisons between simplexin binding of adsorbents were completed using the *t*-test function in Microsoft Excel. Statistically significant difference was set at $p < 0.05$.

3.12 Pimelea feeding trial in individual pens

3.12.1 Animal Ethics

Animal ethics approval was obtained for this feeding trial through the UQ Production and Companion Animal Ethics Committee (QAFFI/QASP/337/20/DAF) see Section 9.5.

3.12.2 Animal husbandry and management

Cattle used in this trial belonged to DAF and were sourced from Spyglass commercial herd steers agisted at 'Berrigurra' [Emerald Agricultural College (QATC) 'Berrigurra', Blackwater QLD 4717]. Forty Droughtmaster steers (born *ca*. June 2020) were selected by DAF staff based on weight (~ 200 kg) and temperament, and then trucked to QASP arriving 28 January 2021. After a period of habituation and induction at QASP in feedlot pens, steers were assessed by QASP staff for temperament, general health check, behaviour and willingness to consume molasses and weighed. From this assessment, 30 animals were selected for the trial and assigned to one of five treatment groups (A)-(E) stratified by weight and assigned to individual pens. Steers at the commencement of the trial weighed between 237 kg and 272 kg.

During the trial, steers were housed in the QASP individual pens facility (Figure 3.8) to enable monitoring of plant intake and health assessment. This facility has 32 outdoor individual pens (3 m x 10 m each) (Figure 3.9), 30 of which were used for this trial. The facility has a partial roof over the central work area and animal pens. Feed bins are concrete bunk type troughs which line the central work area for ease of dispensing feed and collection of refusals. Pens also have an auto water bowl with individual water intake meters and SureFoot® mat in each pen. The facility has a dedicated cattle crush with weigh scales (Figure 3.9 (B)) and multi drafting and gate penning off options with a laneway system which is used for the secure movement of animals and also for restriction of animals for faecal collections as described below.

Animals were fed medium quality chaffed Rhodes grass (*Chloris gayana*) hay (9% protein) as a maintenance diet throughout the trial. Before feeding, diluted molasses (300 g) in 1 litre of water was added to this hay followed by calculated aliquot of milled Pimelea material plus additives (depending on treatment group), and thoroughly mixed by hand.

Figure 3.8. Project steers in individual pen facility.

Figure 3.9. (A) Project steers housed in individual 10 x 3 pens with pen number, steer number and treatment group indicated on each pen. (B) Steers were moved to central crush for weighing, blood collection and detailed condition assessments.

A **B**

3.12.3 Treatment design

The trial was undertaken with six groups of five animals + 10 spare = 40 animals. The six treatment groups were as described below:

T1: Pim = Pimelea only (positive control)
T2: Pim + Bchar = Pimelea + non-activated Biochar
T3: Pim + aBchar = Pimelea + heat activated Biochar
T4: Pim + Inoc = Pimelea + inoculum
T5: No Pim control = no treatment (negative control)
T6: Pim + Bent = Pimelea + bentonite

One treatment group (T5) was a no treatment (no Pimelea) control, all other treatment groups included milled Pimelea at a dose rate to deliver 5 µg simplexin /kg bodyweight (bw) per day (bw/day). T1 was the Pimelea only positive control, while T4 had the same daily Pimelea dose but included the addition of an experimental inoculum which was administered fortnightly to the steers in this treatment group. T2, T3 and T6 had the same daily Pimelea dose and included the addition of adsorbents (non-activated Biochar, activated Biochar and bentonite respectively) with all adsorbents fed at 0.3 g/kg bw/day.

After assessment of the 40 animals for temperament, general health check, behaviour and willingness to consume molasses 30 animals were selected for the trial and stratified by weight in a block design across the 6 treatment groups. The experimental design was a 6x5 randomised block design (Figure 3.10) giving 19 degrees of freedom for the residual term. This design was approved by David Mayer (Principal Biometrician, Animal Science, Agri-Science Queensland DAF and ensured adequate statistical power without being excessive.

Figure 3.10. Layout of steers (#number) and Treatment Group (TG: T1-T6) in QASP individual Pens 1-32. The experimental design was a 6x5 randomised block design stratified by weight across the 6 treatment groups. Blocks are shown with solid green lines.

Pen No	#Steer/TG	Central work area	#Steer/TG	Pen No
32	#207 / T6		#377 / T2	1
31	#338 / T1		#352 / T5	2
30	#280 / T4		#381 / T3	3
29	#176 / T3		#186 / T5	4
28	#375 / T4		#157 / T6	5
~~27~~	*spare pen*		*spare pen*	~~6~~
	Laneway		Crush Laneway	
26	#082 / T2		#105 / T1	7
25	#002 / T3		#037 / T5	8
24	#198 / T2		#079 / T4	9
23	#051 / T6		#147 / T1	10
22	#267 / T2		#145 / T5	11
21	#191 / T3		#226 / T1	12
20	#326 / T4		#119 / T6	13
19	#401 / T2		#102 / T1	14
18	#163 / T5		#286 / T6	15
17	#086 / T4		#359 / T3	16

3.12.4 Pimelea plant material

Flowering *P. trichostachya* plants were collected at a single location on a collaborator property (QGBR0175) 50 km north of Bollon in October 2020. A separate pressed sample was submitted as a voucher specimen to the Queensland Herbarium with the species identification confirmed as *P. trichostachya* (AQ952584). The collected Pimelea plant material was air-dried at DAF Charleville and then transported to Brisbane. The combined aerial portion of these plants (leaves, stems and flowers) was separated, and hammer milled using a Christy and Norris 8000RPM 8” Laboratory Mill fitted with a 1 mm screen. The 1 mm screen was chosen to ensure no seeds remained intact after milling (to meet AEC requirements). The milled plant material (~30 kg) was thoroughly mixed to ensure consistency of milled material and then stored frozen (-20 °C) in two L air-tight containers (approx. 750 g per container) until required. Milled Pimelea plant material was added to the daily feed of animals in the Pimelea treatment groups at a rate calculated to deliver 5 µg simplexin/kg bw per day (based on weekly bodyweight measures).

3.12.5 Toxin analysis of Pimelea plant material

Milled *P. trichostachya* (AQ952584) was weighed accurately (0.1 g) from 3 different storage bottles, with 3 replicates from each. Methanol (20 mL) was added to each sample, which were then vortexed (20 s), sonicated (5 min), shaken (20 min) and centrifuged (3,214 x*g*, 10 min). The supernatant was poured into a new tube and to the residue was added further methanol (20 mL) with the sample again vortexed, sonicated, shaken and centrifuged in the same way. The supernatant was combined with the previous supernatant for each sample. To the residue was added methanol (10 mL) and the sample again vortexed, sonicated, shaken and centrifuged in the same way. The supernatant was combined with the previous supernatant (50 mL total). The combined supernatants were shaken and a sample of each (1 mL) was filtered (0.2 µm) into a MS vial ready for LC-MS/MS analysis. Another sample of each was diluted (1 in 10) with methanol and filtered (0.2 µm) into a MS vial. A further dilution with methanol (1 in 2) gave a final dilution (1 in 20) which was also filtered (0.1 µm) ready for LC-MS/MS analysis.

Concentrations of simplexin in replicate plant extracts were determined from parallel reaction monitoring (PRM) with protonated simplexin ($[M+H]^+$, 533.3109) selected as the precursor ion (mass tolerance window of 5 ppm) as described previously (Section 3.5). The area of the dominant product ions 253.1225 and 267.1382 were used for quantification and confirmation, respectively.

3.12.6 Adsorbent materials (source and preparation)

Three adsorbents were tested for their efficacy in binding simplexin in this animal trial.

Non-activated Biochar

Commercially available Biochar (Renewable Carbon Resources Australia, Charleville Qld) was purchased from Bos Rural Supplies, Kandanga, Qld and milled using a Christy and Norris 8000RPM 8” Laboratory Mill fitted with a 2 mm screen to provide a uniform product for administration to each steer in treatment group 2. In this report this material is referred to as “non-activated Biochar” to clearly distinguish this material from the activated Biochar referred to below.

Activated Biochar

Activated Biochar was prepared from commercially available Biochar (non-activated, as purchased above) and was transported to P7 Lab at Southern Queensland University, Toowoomba campus, and activated using a Woodrow kiln HF Mini (Woodrow Kilns, Picton NSW). Crucibles containing 500 g of Biochar were heated four at a time in the kiln (Figure 3.11). The kiln temperature was set at 1,000 °C with a one hour holding time at 1,000 °C. The heating procedure (400 °C/h) required 3 hours to reach 1,000 °C, one hour holding time and then left for one hour to cool down before taking samples from the kiln. Activated Biochar was then milled using a Christy and Norris 8000RPM 8” Laboratory Mill fitted with a 2 mm screen to provide a uniform product for administration to each steer in treatment group 3.

Figure 3.11. Crucibles containing biochar activated in the Woodrow Kiln.

Bentonite

Sodium bentonite (Trufeed®, Sibelco Australia) was purchased from Buckhams General Produce, North MacLean, Qld) and was milled using a Christy and Norris 8000RPM 8" Laboratory Mill fitted with a 2 mm screen to provide a uniform product for administration to each steer in treatment group 6.

3.12.7 Rumen inoculum preparation

The rumen inoculums for use in the animal trial were harvested from final four days of the 56-day Fermentation 15 which was inoculated using two 100 mL volumes of cryopreserved fermentation fluid from Day 30 of Fermentation 5 and Day 43 of Fermentation 13 (Section 3.7.3). The harvested fermentation fluid was cryopreserved following the method of Klieve *et al.* (2002), in brief a 250 mL volume of fermentation fluid was removed and mixed with an equal volume of anerobic rumen fluid glycerol medium in 500 mL Wheaton bottles and mixed well before freezing at -30 °C. The bottles of inoculum were stored frozen at -30 °C until transportation to Gatton on wet ice and allowed to completely thaw prior to use.

3.12.8 Health checks

I-auditor software app (https://safetyculture.com/iauditor/) was used to generate daily health checks on all animals including BAR (bright, alert and responsive), feed refusals, health concerns, oedema, demeanour, faecal consistency, body condition/score, plus photos where relevant. Feed intake of each animal was calculated daily on the basis of measured feed refusals.

As the trial progressed, dehydration score, heart rates, rectal temperatures and thickness of dewlap/brisket fold were measured on occasion in the crush as part of the weekly weighing and blood sampling regime.

3.12.9 Blood and other tissue collections

For ease of handling and sample collection logistics, steers were allocated to Groups 1 (Block 2 and Block 5; 12 animals) and Group 2 (Blocks 1, 3 and 4; 18 animals) for sampling on Wednesday and Thursday each week.

Steers were individually taken to the crush and weighed weekly, with this bodyweight being used to calculate the amount of Pimelea and adsorbent to be fed for the following week. Blood samples were collected: 3 x 10 mL jugular blood samples into lithium heparin anticoagulant [1x 10 mL for clinical chemistry profile; 2 x 10 mL for simplexin assay] and 1 x 5 mL jugular blood into EDTA anticoagulant. Lithium heparin and EDTA tubes were submitted on day of collection to UQ

Veterinary Laboratory Services for both biochemical and haematology profiles respectively. Lithium heparin replicate tubes for simplexin analysis were combined in a single 50 mL plastic tube and stored frozen (-20 °C) until required for analysis.

Adhoc faecal samples were collected from the rectum by a licenced veterinarian to exclude presence of worms etc. as a cause of diarrhea. Earwax samples were collected using cotton buds for assessment of both cortisol and simplexin levels.

At the conclusion of the trial, tail hair samples have been collected from the tail switch of each animal for future analysis.

3.12.10 Rumen fluid sampling of steers

All steers had samples of rumen fluid collected every 14 days as outlined in the trial schedule. Samples were collected by use of a stomach tube as outlined in the Standard Operating Procedure #3 Use of stomach tube for the collection of rumen fluid in cattle (Appendix Section 9.9). Briefly, the animals were taken from their pens to the crush and restrained using the head bale. A plastic polypipe gag was placed into their mouth and positioned across the tongue and a length of clear braided PVC hose inserted through the gag, down the oesophagus into the reticulum/rumen. A hand pump was used to create a vacuum drawing rumen fluid into a side arm flask. Approximately 200 mL of rumen fluid was collected per animal. The collected rumen fluid was filtered through stocking material into a labelled plastic container and the presence of saliva or blood in in the collected rumen fluid was noted. The collected sample was briefly placed on ice for transport to the sample processing room where the following samples were taken -

- Four 1.0 mL samples of rumen fluid were placed in 1.5 mL microcentrifuge tubes and centrifuged at 11,000 x*g* for 10 min. The resulting supernatant removed and discarded with the resulting pellet frozen on dry ice. The frozen samples were kept on dry ice until the return to the MEORG laboratory then transferred to a -20 °C freezer for storage until analysis.
- Two 1.0 mL samples of rumen fluid were transferred to 1.5 mL cryotubes, flash frozen in liquid nitrogen. The samples were kept in dry ice until the return to the MEG laboratory where upon they were transferred to a -80 °C freezer for storage.
- A 50 mL volume of rumen fluid was transferred into a 70 mL sample container, placed on ice and transported to the Natural Toxin Laboratories for storage at -20 °C.

Following collection of subsamples, the remaining rumen fluid was used for determination of pH using a portable pH meter (pH Testr® 30, Oakton Instruments). Rumen fluid samples collected from Group 2 animals on the 6/5/2021 were cryopreserved by mixing with an equal volume of rumen fluid glycerol and freezing on dry ice.

3.12.11 Inoculum administration

Directly after samples were taken from the Treatment 4 steers every 14 days each animal received a 200 mL volume of the inoculum administered orally using a multi-volume drench gun (Bainbridge, Luscombe Qld). From each bottle of inoculum used, two aliquots of 2.0 mL were taken, centrifuged at 11,000 x*g* for 10 min with the resulting supernatant removed and discarded. The resulting pellet was frozen on dry ice. Samples were kept on dry ice until the return to the MEORG laboratory and stored at -20 °C freezer until analysis.

3.12.12 Faecal collection

Every 14 days (in alternate weeks to rumen fluid collection), 24 h faecal samples were obtained from individual pens. Before collection pens were hosed clean and the multi drafting and gate penning off options of the individual pens then used to restrict steers to the front portion of each pen for 24 h. After the 24 h period of restriction, the concrete floor of each pen was then scraped, and a composite faecal sample obtained for each animal. After thorough mixing a sub-sample (500 mL) of each faecal sample was frozen and stored -20 °C before transfer to individual trays for freeze drying using a CSK Climatek Freeze Dryer.

3.12.13 Necropsy

Four steers were euthanased and necropsied due to animal welfare issues. Before necropsy animals were killed humanely by Lethabarb® injection and then transported to the UQ Veterinary Science for post-mortem examination. Samples of liver, kidney, heart, skeletal muscle, renal fat, lymph nodes, and earwax were frozen for simplexin assay, and a complete range of tissues collected into 10% buffered neutral formalin for histopathology. Tail hair samples were also retained for possible future DNA comparisons.

3.12.14 Post-trial animal destination

After cessation of Pimelea feeding, all steers were fed Rhodes grass hay and molasses in the individual pens until any remaining symptoms of Pimelea poisoning had resolved (particularly diarrhea and oedema). On assessment of individual status, steers were progressively removed from the individual pens to the larger feedlot pens and then into pasture. All 26 remaining steers were returned to DAF and transported to Brian Pastures Research Station on 29th June 2021. All animals remained healthy with no unresolved effects. The remaining three steers from treatment group 4 (inoculum) were held at Brian Pastures for 12 months after completion of the trial as required under APVMA PER7250 Small scale trial permit (as noted in Animal Ethics application) and then sold.

3.13 Pen trial sample analysis

3.13.1 Simplexin analysis of faecal samples

The faecal samples were spread across stainless steel trays and freeze-dried (CSK Climatek, Darra, QLD, Australia) to avoid any potential thermal degradation of simplexin. The freeze-dried samples were then milled to a powder using either a knifemill (Knifetec 1095, FOSS, Hillerød, Denmark) or a laboratory blender (8010s, Waring, Conair Corporation, Stamford, U.S.A).

The freeze dried and milled faeces, ~1 g was weighed into a 50mL falcon tube. Then, 20 mL of acetonitrile (HPLC grade, Merck, Germany) was added and the samples were vortexed for 20s, followed by 5 min in the ultrasound bath (ELMA, Transsonic digital), shaken for 1 hour (reciprocating platform RP1812, Paton Scientific, Adelaide, SA, Australia) and finally centrifuged for 10 min at 3,220 x*g* (Eppendorf Centrifuge 5810, Eppendorf AG, Hamburg, Germany). The supernatant was decanted into a clean 50 mL falcon tube, and the extraction repeated twice with 10 mL acetonitrile. The pooled acetonitrile extracts were dried under a gentle nitrogen flow at room temperature, and the sample reconstituted in 5 mL methanol (HPLC grade, Merck, Germany) which was filtered through a 0.2 µm pore size syringe filter (PTFE Acrodisc® 13mm, Pall Corporation) into a LCMS vial for analysis as previously described (Section 3.5).

3.13.2 Analysis of selected rumen fluid samples

Microbiome analysis

The genomic DNA from selected rumen fluid samples (1.0 mL) across the animal trial were extracted as detailed in Section 3.6.2 and used as template in PCR assays to produce 16S rRNA gene V3/V4 region tagged amplicons as described in Section 3.7.8. The PCR amplicons were submitted to an external sequence provider (AGRF), to undertake the second round of PCR to multiplex the samples and proceed with sequencing within a single lane of the Illumina MiSeq sequencing platform.

To facilitate sample description and statistical analysis, a metadata mapping file was created, incorporating information relating to each sample, for example: treatment group (described in Section 3.12.3 including, Pimelea plant material (Pim); Pimelea plant material + Biochar (Pim + Bchar); Pimelea plant material + activated Biochar (Pim + aBchar); Pimelea plant material + mixed microbial inoculum (Pim + Inoc); no Pimelea plant material, control group (no Pim Control); Pimelea plant material + bentonite (Pim + Bent); rumen fluid sample collection time point (four time points, 17-18th February [1]; 7-8th April [2]; 5-6th May ([3]; 26th May [4]); inclusion of Pimelea in the diet (with or without Pimelea); and use of adsorbents (Adsorbent; Pimelea Only; no Pimelea).

Once the 300bp paired ends reads were downloaded from the sequencing provider, the sequence reads sequence reads de-multiplexed, quality filtered, paired and size trimmed (>200 bp in length remaining) and imported into the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software pipeline package, either version 2019.10 or 2021.4 (Caporaso et al. 2010; Caporaso et al. 2012) where and the DADA2 software (Callahan et al. 2016) was used to model and correct any remaining Illumina sequencing errors (as described in Section 3.6.3). Using the protocols described in Section 3.6.3, a Feature table containing the counts (frequencies) of each unique sequence in each sample in the dataset (Feature or sequence variant, similar to the Operational Taxonomic Unit determined by previous versions of QIIME), a representative sequences file (rep set) and a FeatureData file which maps Feature identifiers in the Feature table to the sequences they represent, was created.

For the 16S rRNA representative sequences (rep set), taxonomy was assigned using a pre-trained Naïve Bayes classifier trained on the SILVA database December 2019 update, version 138 (Yilmaz et al., 2014). Taxonomic analysis of negative control samples included in the Illumina MiSeq sequencing project, identified contamination of the dataset, with non-specific amplification of two features classified as "d__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae; g__*Burkholderia-Caballeronia-Paraburkholderia*" and “d__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae; g__*Ralsonita*”. These two features were removed from the overall Feature table and from the file of representative sequences corresponding to this Feature table.

The taxonomy of specific samples was depicted using taxonomic bar plots generated using QIIME 2, with samples ordered on the x-axis on the basis of specific metadata categories of interest (e.g., rumen fluid sample collection time). Alpha diversity analysis (microbial diversity within a sample) was determined on the basis of four measures: (1) counts of observed species (Observed Species); (2) Faith phylogenetic diversity (Faith-PD); (3) Shannon entropy of counts (Shannon); and (4) Peilou evenness. The four alpha diversity measures were analysed in Genstat Release 21.1 (27 January 2022; VSN International Ltd., 2022) using an accumulated ANOVA and regression model. Predicted means and standard error differences (s.e.d.) were calculated and tested using a Fisher’s LSD test.

For determination of the differences in the microbial communities occurring between samples (Beta diversity), the respective metadata files, as well as the table, representative sequence (rep set), and

unrooted phylogenetic tree (.tre) files generated using QIIME2, were imported into the R packages, Phyloseq (version 1.30.0; https://joey711.github.io/phyloseq/index.html)(McMurdie and Holmes 2013) and MixOmics (version 6.10.6;http://mixomics.org/methods/pls-da/)(Rohart et al. 2017). Statistical exploration and microbial community analysis used a multivariate projection-based approach with repeated measures. An unsupervised analysis with Principal Component Analysis (PCA) (Jolliffe 2002) was conducted using the Feature table data generated using QIIME 2, transformed using the centred log ratio (CLR). For the identification of indicator species and determination of microbial signatures, a sparse Partial Least Squares Discriminant Analysis (SPLSDA) was undertaken. Contribution plots showing the most discriminative OTUs were generated based on the coefficient derived from the component analysis. Due to the high numbers of OTUs within the microbial signatures, results were presented as tables of the top 10 most important OTUs with respective assigned taxonomy, for each of the three components of the sPLSDA.

Simplexin analysis in rumen fluid

Rumen fluid samples were collected from selected affected trial steers (#51, #82, #86 and # 375) during week 9 (21-22/4/21) and frozen until analysed. The bulk rumen fluid samples were thawed and shaken (reciprocating platform RP1812, Paton Scientific, Adelaide, SA, Australia) to mix the contents. For each sample in triplicate, rumen fluid (10 mL) was transferred to a 50 mL centrifuge tube, methanol (10 mL) was added and the mixture vortexed (Select Vortexer, Select BioProducts, NJ, USA, 10 sec). Dichloromethane (DCM, 20 mL) was added and the mixture vortexed (10 sec) again. The samples were shaken (1 h) and centrifuged at 3220 x*g* (20 min) to separate the layers. The aqueous layer was removed, and the DCM layer was filtered (Whatman qualitative filter paper grade #1, Merck Millipore, USA) into a separatory funnel. The DCM layer was washed once with 1% sodium chloride solution (5 mL) and once with MeOH/NaCl solution (1:1, 5 mL). The organic layer was dried with anhydrous sodium sulfate (Na_2SO_4) and concentrated under reduced pressure. The resulting residue was reconstituted in hexane (10 mL) and partitioned with acetonitrile (4 mL) and MeOH (2 mL). The acetonitrile/MeOH layer was collected, and the hexane layer was again extracted with acetonitrile (2 x 4 mL). The combined acetonitrile layers were dried under nitrogen flow at room temperature. The residue was redissolved in MeOH (1 or 2 mL) and then filtered through a syringe filter (0.2 µm PTFE, Acrodisc®, Pall Corporation) into vials with simplexin content determined by UHPL-Q-Orbitrap-MS analysis as previously described (Section 3.5).

3.13.3 Simplexin analysis of blood samples

Initial blood analysis method.

Frozen blood samples were freeze-dried separately using a CSK Climatek Freeze Dryer before analysis. Freeze-dried blood (0.5 g) was shaken with dichloromethane/methanol (3:1, 15 mL) for 30 min and centrifuged at 3,900 x*g* for 10 min; the supernatant was decanted, and the residue was re-extracted by the same process. The combined decanted supernatant was washed once with brine (6 mL) and then with methanol/brine (1:1, 6 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was partitioned between hexane (4 mL) and acetonitrile (4 mL). The hexane layer was extracted with further acetonitrile (2 × 2 mL), and the combined acetonitrile extract was evaporated. The evaporated residue was taken up in methanol (0.5 mL) and filtered (0.2 µm PTFE), and the simplexin content was determined by UHPL-Q-Orbitrap-MS analysis as previously described (Section 3.5).

***Improved method to remove phospholipid interference*.**

Frozen blood samples were freeze-dried separately using a CSK Climatek Freeze Dryer before analysis. Freeze-dried blood (0.5 g) was shaken with acetonitrile (15 mL) for 30 min and centrifuged

at 3,900 x*g* for 10 min; the supernatant was decanted, and the residue was re-extracted by the same process. The combined decanted supernatant was evaporated under nitrogen and the residue was dissolved in dichloromethane (15 mL). Sample was washed once with brine (6 mL) and then with methanol/brine (1:1, 6 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was partitioned between hexane (4 mL) and acetonitrile (4 mL). The hexane layer was extracted with further acetonitrile (2 × 2 mL), and the combined acetonitrile extract was evaporated. The evaporated residue was taken up in methanol (0.5 mL) and filtered (0.2 μm PTFE), and the simplexin content was determined by UHPL-Q-Orbitrap-MS analysis as previously described (Section 3.5).

3.13.4 Statistical analysis of feeding trial data

Data has been analysed by Dr David Mayer (DAF Principal Scientist-Biometry) using GenStat (21.1. VSNI). The time-series nature of the data was taken into account by an analysis of variance of repeated measures (Rowell and Walters 1976) via the AREPMEASURES procedure of GenStat (2021).This forms a split-plot analysis of variance (split for time). The Greenhouse-Geisser epsilon estimates the degree of temporal autocorrelation and adjusts the probability levels for this. All observations were analysed by repeated-measures ANOVA (split-plot across the times), with pre-treatment (week 0) values as covariate. Adjusted means were standardised for the blocks, covariate, group and missing-values. The standard error of the mean (se) was plotted for the interaction means of time and treatment. The 5% least significant difference (LSD) were used to compare the means between treatment and time. ANOVA (adjusted for covariate) was conducted at each time point with Fisher's Protected Least Significant Differences (LSD) of means (5% level) to determine which means were significantly different from each other ($p < 0.05$).

4. Results

4.1 Rumen microbiome: Field Survey

4.1.1 Comparison of rumen microbiome across animal species and field exposure

A total of 112 samples (110 from Properties 1-16 and Property 31 Appendix Section 9.6, one repeat and one negative control) were 16S rRNA gene amplicon sequenced. Of these 112 samples, 25 were excluded from the analysis, including the negative control, 17 cattle samples from two properties that had insufficient reverse sequence quality, five samples (four cattle, one goat) from three properties which had extremely low sequence numbers and two cattle samples from two properties which returned extremely high sequence numbers. For the remaining 87 samples retained for microbial community (microbiome) analysis, the extent of bacterial and archaeal population diversity within each sample (alpha diversity) was calculated using four alpha diversity measures (Faith Phylogenetic Diversity, Shannon, Observed species and Pielou evenness; Table 4.1). Statistical analysis of each of these diversity measures showed that the bacterial and archaeal diversity was most significantly affected by the species of animal from which the sample was collected. All measures of phylogenetic diversity indicated that forestomach samples collected from kangaroos had significantly lower microbial diversity than rumen fluid samples collected from cattle ($P < 0.001$).

The taxonomic composition of the microbial communities was determined for all samples collected. A total of 14,327 Features were identified across 87 samples, representing 1,190,974 sequences and a representative sequence for each Feature compared to a database of taxonomically defined microbial groups SILVA database, version 138, December 2019 update (Yilmaz et al., 2014).

Taxonomy was assigned on the basis of 99% homology and if the Features did not match any of the reference sequences in the database, then they were classified as either unassigned or classified to the highest taxonomic level possible, for example, Kingdom Bacteria. This showed that the microbial populations identified in the rumen fluid of sheep, cattle and goats and the forestomach material from Kangaroos, varied according to the species of animal. When core microbial communities, defined as the microbial genera found in 100% of samples from each animal species, were compared, distinct, species-specific differences were observed (

Figure 4.1 and Table 4.2). This showed how the ruminant species shared several key bacterial populations classified within the orders Bacteroidales, Oscillospirales and Lachnospirales, whereas Kangaroos had species-specific bacteria, classified within the class Gammaproteobacteria and order Pasteurellales.

The variation in the bacterial and archaeal populations (beta-diversity) of all the samples collected were compared using Principal Components analysis (PCA,

Figure 4.2), which transforms the data using a centred log ratio to maximise the visualisation of sample variation. This analysis further indicated that although the rumen microbial populations of cattle, sheep and goats showed some variation in microbial communities, when compared to the microbial populations present in forestomach samples from kangaroos, there was also some overlap occurring in microbial population composition. This supported the findings indicated by the Venn analysis, which showed that although variation occurred, there was also commonalities occurring between the microbial populations of each of the herbivore gut systems examined. Microbial communities that contributed to the species-specific differences seen, were identified by sPLSDA (results presented in Appendix 9.10, Figure 9.1, Table 9.12). Bacterial taxonomic groups contributing to the differences seen included those found to be more predominant in sheep (classified in the families Anaerovoracaceae and Acholeplasmataceae), goats (classified in the families Prevotellaceae and Rikenellaceae), and kangaroos (also classified in the families Prevotellaceae and Rikenellaceae, as well as Absconditabacteriales and Defluviitaleaceae).

The majority of the gut samples used for microbiome analysis were obtained from cattle (n = 57) therefore, to remove the effect of animal species, the dataset was subdivided to retain only cattle-derived samples. The effects of additional experimental parameters on the diversity of rumen bacterial and archaeal populations of cattle, were then determined (Table 4.3), including the presence or absence of clinical signs of Pimelea poisoning (Clinical signs, yes/no) and the extent of any Pimelea poisoning (Clinical signs). This analysis indicated that although differences in microbial diversity were observed, the effects were often slight and were not shown by all four of the microbial diversity indexes determined. For example, there was no significant statistical differences ($P > 0.05$) for the Observed features and Faith PD diversity indexes for any the experimental parameters examined. The two scaled measures of microbial diversity (Shannon and Pielou evenness) both indicated that the geographical location from which cattle were rumen sampled had a significant effect on the diversity of rumen bacterial and archaeal populations ($P < 0.001$). Cattle sampled from the St George region had the highest rumen microbial diversity for these indexes, whereas cattle sampled from the neighbouring Bollon and Roma had the lowest microbial diversity.

Determination of the highly abundant bacterial and archaeal populations associated with rumen fluid collected from cattle (Figure 4.3), showed that there were few differences occurring between cattle showing clinical signs of Pimelea poisoning, and cattle not showing any clinical signs of Pimelea poisoning. While some differences appeared to occur in response to the geographical location from which cattle were sampled, all rumen samples were dominated by bacteria classified within the orders Bacteroidales, Lachnospirales, Christensellales and Oscillospirales.

When the overall, highly abundant, and core rumen microbial communities of cattle were examined on the basis of the presence or absence of clinical signs of Pimelea poisoning (Figure 4.4**,** Table 4.4), the majority of bacterial and archaeal genera identified were found to be the same (82% of highly abundant bacterial and archaeal genera shared; 68.8% of core bacteria and archaea shared). Of these shared core bacteria, the orders Bacteroidales, Lachnospirales, Christensellales and Oscillospirales also dominated. The three core bacterial populations found only in the cattle not

showing any clinical signs of Pimelea poisoning were also classified within these orders (Bacteroidales, Lachnospirales, and Oscillospirales) and were classified only to the taxonomic levels of Bacteroidales family F082, Oscillospiraceae UCG-005, and Lachnospiraceae NK3A20 group. Only two core bacterial populations were associated with cattle showing clinical signs of Pimelea poisoning, also classified within the dominant bacterial orders found in all cattle (Oscillospirales and Bacteroidales), however these two populations were classified to genus level (*Eubacterium coprostanoligenes* group and *Prevotella* UCG-001).

When the microbial communities of cattle rumen fluid samples were examined on a between-sample basis (beta-diversity), cattle showing signs of Pimelea poisoning, showing slightly greater variation in rumen microbial communities present, as indicated by the wider ellipse for this treatment group in the PCA (Figure 4.5). Differential abundance analysis indicated the bacterial populations contributing to these differences (Figure 4.6, Table 4.5) however the bacteria which differed according to the presence or absence of clinical signs of Pimelea poisoning, where often taxonomically related, for example belonging to the Prevotellaceae, Lachnospiraceae, and Rikenellaceae families. Bacterial populations classified within the order Oscillospirales however, did appear to be found more frequently in cattle showing clinical signs of Pimelea poisoning, including the genera *Ruminococcus* and *Papillibacter*. Some bacterial populations classified in the Class Clostridia, were also found to differentially abundant in cattle showing clinical signs of Pimelea poisoning, including those classified in the genera *Anaerovorax* and *Anaerofustis.*

Table 4.1. Effect of the type of animal (animal breed) on the microbial diversity of rumen or forestomach samples, described by four measures of within-sample (alpha) diversity.

	Observed features		Faith PD		Shannon entropy		Pielou evenness	
Animal Breed[A]	**Mean[B]**	**s.e.[C]**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**
Kangaroo	178.3[a]	75.07	19.99[a]	3.696	6.177[a]	0.255	0.817[a]	0.011
Small Ungulates	484.5[b]	41.61	44.56[b]	2.049	7.822[b]	0.142	0.881[b]	0.006
Bos taurus, Bos indicus cross	527.3[b]	34.65	45.19[b]	1.706	8.334[b]	0.118	0.929[b]	0.005
Bos Taurus	501.1[b]	39.20	42.86[b]	1.930	8.149[b]	0.133	0.917[b]	0.006
Bos indicus	465.4[b]	78.59	42.88[b]	3.869	8.059[b]	0.267	0.915[b]	0.012

[A] For all four diversity measures, breeds were found to be significantly different (P. < 0.001) using a General Linear model (GLM), Accumulated ANOVA. Breed groupings: Kangaroos (Wallaroo, Eastern grey, and Red kangaroos); Small Ungulates (Dorper, Dohne and Merino sheep); *Bos taurus, Bos indicus* cross (Brahman cross, Brangus, Droughtmaster, Droughtmaster cross, Santa Charalais Cross and Simbra cattle); *Bos taurus* (Angus, *Bos taurus* cross, Hereford cross, Murray Grey, Red Angus, Shorthorn and Shorthorn cross cattle); *Bos indicus* includes Brahman cattle. [B]Adjusted means with letters indicating results of Fisher's least significant difference testing. [C]Standard error of the mean (s.e.).

Figure 4.1. Venn diagram of highly abundant bacterial genera identified as core communities (present in 100% of samples) in rumen samples collected from cattle, sheep and goats, and forestomach samples from kangaroos.

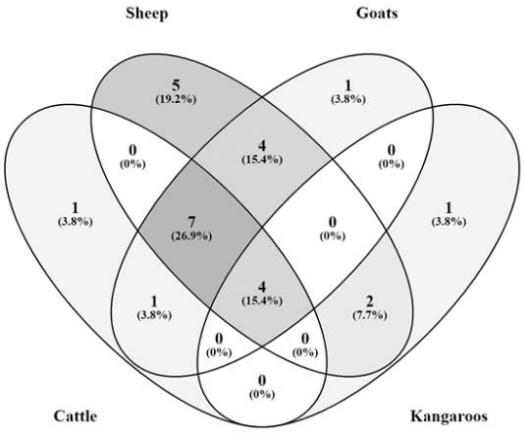


Table 4.2. Table of core microbial communities in rumen samples from cattle, sheep and goats, and forestomach samples from kangaroos (highly abundant, core bacterial and archaeal genera present in 100% of samples from each respective sample group). This table corresponds to the Venn diagram presented in Fig. 4.1. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g), or to the lowest level of classification possible.

4 common elements in "Cattle", "Sheep", "Goats" and "Kangaroos"
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__*Prevotella*
p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae;g__Christensenellaceae_R-7_group
p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__NK4A214_group
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae
1 element included exclusively in "Cattle":
p__Firmicutes;c__Clostridia
5 elements included exclusively in "Sheep"
p__Firmicutes;c__Clostridia;o__Clostridia_UCG-014;f__Clostridia_UCG-014;g__Clostridia_UCG-014
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidales_RF16_group;g__Bacteroidales_RF16_group
d__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__*Methanobrevibacter*
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales
p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae;g__*Succinivibrio*
1 element included exclusively in "Goats"
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnospiraceae_NK3A20_group
1 element included exclusively in "Kangaroos":
p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae
1 common element in "Cattle" and "Goats"
p__Firmicutes;c__Clostridia;o__Oscillospirales;f__[Eubacterium]_coprostanoligenes_group; *Eubacterium coprostanoligenes* group
4 common elements in "Sheep" and "Goats"
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__F082

p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidales_BS11_gut_group;g__Bacteroidales_BS11_gut_group
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__*Butyrivibrio*
p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__Selenomonadaceae;g__*Selenomonas*
2 common elements in "Sheep" and "Kangaroos"
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__*Eubacterium ruminantium* group
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__*Oribacterium*
7 common elements in "Cattle", "Sheep" and "Goats"
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Rikenellaceae_RC9_gut_group
p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__*Ruminococcus*
p__Firmicutes;c__Clostridia;o__Clostridia;f__Hungateiclostridiaceae;g__*Saccharofermentans*
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnospiraceae_XPB1014_group
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__*Pseudobutyrivibrio*
p__Firmicutes;c__Negativicutes;o__Acidaminococcales;f__Acidaminococcaceae;g__*Succiniclasticum*
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotellaceae_UCG-001

Figure 4.2. Differences in variation occurring between the bacterial populations associated with rumen and forestomach samples collected from cattle (o), goats (Δ), kangaroos (+) and sheep (×), determined by PCA. Results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).

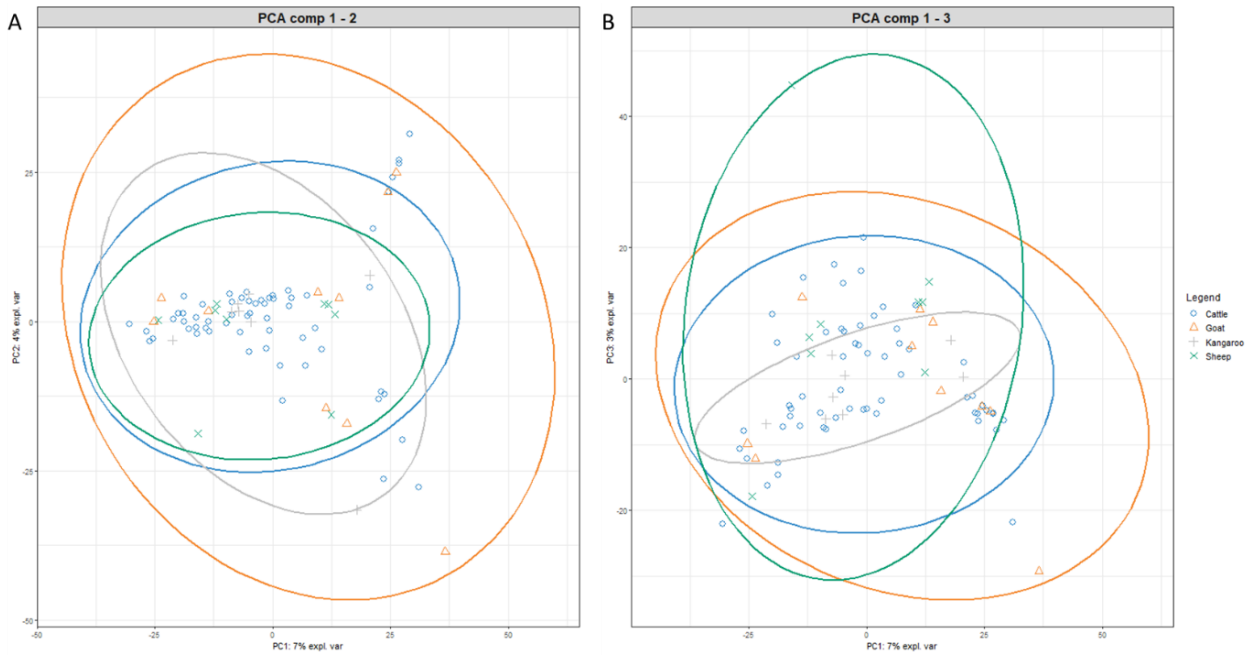


Table 4.3. Microbial diversity of rumen fluid samples collected from cattle, described by four measures of within-sample (alpha) diversity. Experimental parameters examined include: Station clusters (locations from which cattle were sampled); sex of the cattle; whether dietary supplementation was being provided or not; the extent of any clinical signs of Pimelea toxicity; the presence or absence of clinical signs of Pimelea toxicity; the interaction of Clinical signs(yes/no) and the breed of cattle from which rumen fluid was collected.

Experimental parameters[A]	Observed Features		Faith PD		Shannon entropy		Pielou evenness	
	Mean[B]	s.e.[C]	Mean[B]	s.e.[C]	Mean[B]	s.e.[C]	Mean[B]	s.e.[C]
Station clusters								
Animal Research Institute (Brisbane)	223.6[a]	110.4	30.17[a]	5.564	6.918[a]	0.347	0.860[a]	0.012
Augathella	409.8[abc]	103.4	39.72[abc]	5.213	8.051[bc]	0.325	0.925[bc]	0.011
Blackall	727.4[cd]	131.1	51.78[bcd]	6.609	8.845[bc]	0.412	0.930[bc]	0.014
Bollon St George	319.0[ab]	85.20	35.97[ab]	4.295	7.582[ab]	0.268	0.905[b]	0.009
Miles	728.1[cd]	108.8	54.04[cd]	5.486	8.971[c]	0.342	0.946[c]	0.012
Roma	429.6[bc]	76.90	40.07[abc]	3.879	7.822[b]	0.242	0.903[b]	0.008
St George	909.9[d]	149.3	63.24[d]	7.527	9.890[d]	0.469	0.985[d]	0.016
Sex								
female	536.5	35.38	45.22	1.784	8.397	0.111	0.929	0.004
male	441.6	69.93	40.41	3.526	7.726	0.220	0.895	0.007
Dietary supplementation								
no	291.2	100.7	34.73	5.079	7.446	0.317	0.899	0.011
yes	650.5	66.94	49.74	3.375	8.696	0.211	0.933	0.007
Clinical signs								
mild	470.0	43.31	41.87	2.184	8.073	0.136	0.918	0.005
moderate to severe	475.3	57.60	43.77	2.905	8.222	0.181	0.926	0.006
none	549.7	34.35	45.25	1.732	8.292	0.108	0.918	0.004
Clinical signs (yes/no)								
No	540.8	33.77	44.92	1.685	8.269	0.106	0.918	0.004
Yes	479.6	32.61	42.80	1.627	8.132	0.103	0.920	0.003
Clinical signs(yes/no).Breed								
yes.Brahman	440.9	84.36	41.68	4.208	8.055	0.266	0.922[ab]	0.009
yes.Taurus	478.0	51.37	42.13	2.562	8.117	0.162	0.921[ab]	0.005
no.Taurus Indicus cross	500.6	41.74	43.45	2.082	8.168	0.132	0.919[ab]	0.004
yes.Taurus Indicus cross	524.6	46.87	44.84	2.338	8.373	0.148	0.931[b]	0.005
no.Taurus	577.6	66.62	45.16	3.323	8.321	0.210	0.913[a]	0.007
no.Brahman	507.3	92.06	44.21	4.592	8.233	0.290	0.918[ab]	0.010

[A] All listed parameters were found to have no statistical differences using a General Linear model (GLM), Accumulated ANOVA ($P > 0.05$) for both the Observed features and Faith PD diversity indexes; For the Pielou evenness diversity measure, significant differences were seen for the parameters of Station clusters ($P < 0.001$), Sex ($P < 0.05$), and Breed.Clinical signs (yes/no) ($P < 0.05$) only; For the Shannon diversity measure, significant differences were seen for the Station clusters parameters ($P < 0.05$) only. [B]Adjusted means with letters indicating results of Fisher's least significant difference testing. Results shown only when differences were observed. [C]Standard error of the mean (s.e.).

Figure 4.3. Stacked bar graph of highly abundant bacterial and archaeal families identified in rumen fluid samples from cattle showing no clinical signs of Pimelea poisoning (No clinical signs) and cattle showing clinical signs of Pimelea poisoning (Clinical signs). The geographical regions from which cattle were sampled (Station Clusters) are also indicated, with samples from cattle employed in the animal trial undertaken at the Animal Research Institute, Yeerongpilly (ARI) included. The top 25 most highly abundant bacterial and archaeal populations are listed.

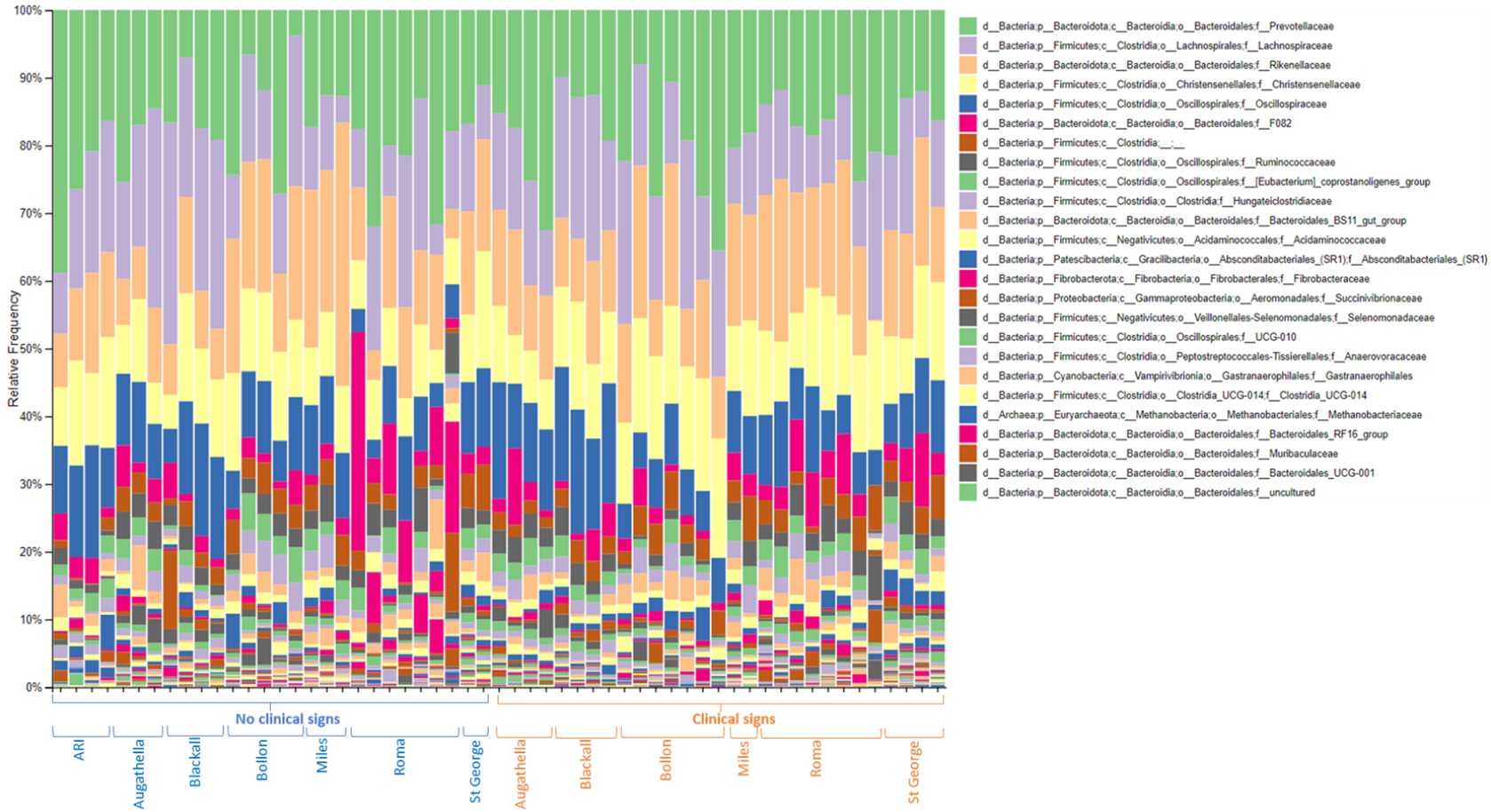


Figure 4.4. Venn diagrams of indicating the numbers of either shared or unique highly abundant bacteria and archaea present in (A) the rumen of cattle showing no Clinical signs of Pimelea poisoning and cattle showing clinical signs of Pimelea poisoning; and (B) communities of core bacteria and archaea (present in 100% of animals in each group) present in the rumen of cattle showing no Clinical signs of Pimelea poisoning and cattle showing clinical signs of Pimelea poisoning.

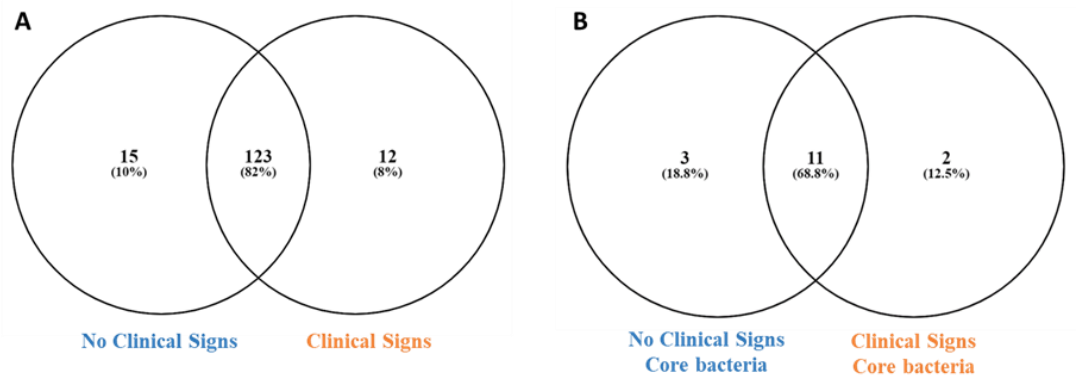


Table 4.4. Table of highly abundant, core microbial communities (bacteria) found to be either shared or unique to the rumen of cattle showing no clinical signs of Pimelea poisoning (no clinical signs) and cattle showing clinical signs of Pimelea poisoning (Clinical signs). This table corresponds to the Venn diagram presented in Figure 4.4B. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g), or to the highest level of classification possible.

11 common elements in "No clinical signs" and "Clinical signs"
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__*Prevotella*
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Rikenellaceae_RC9_gut_group
p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae;g__Christensenellaceae_R-7_group
p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__NK4A214_group
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae
p__Firmicutes;c__Clostridia
p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__*Ruminococcus*
p__Firmicutes;c__Clostridia;o__Clostridia;f__Hungateiclostridiaceae;g__*Saccharofermentans*
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnospiraceae_XPB1014_group
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__*Pseudobutyrivibrio*
p__Firmicutes;c__Negativicutes;o__Acidaminococcales;f__Acidaminococcaceae;g__*Succiniclasticum*
3 elements included exclusively in "No clinical signs"
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__F082
p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__UCG-005
p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnospiraceae_NK3A20_group
2 elements included exclusively in "Clinical signs"
p__Firmicutes;c__Clostridia;o__Oscillospirales;f__[Eubacterium]_coprostanoligenes_group;g__*Eubacterium coprostanoligenes* group
p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__*Prevotella* UCG-001

Figure 4.5. Differences in variation occurring between the bacterial populations in rumen samples collected from cattle with clinical signs of Pimelea poisoning (Yes, Δ) and cattle without any clinical signs of Pimelea poisoning (No, o), determined by PCA. Results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).

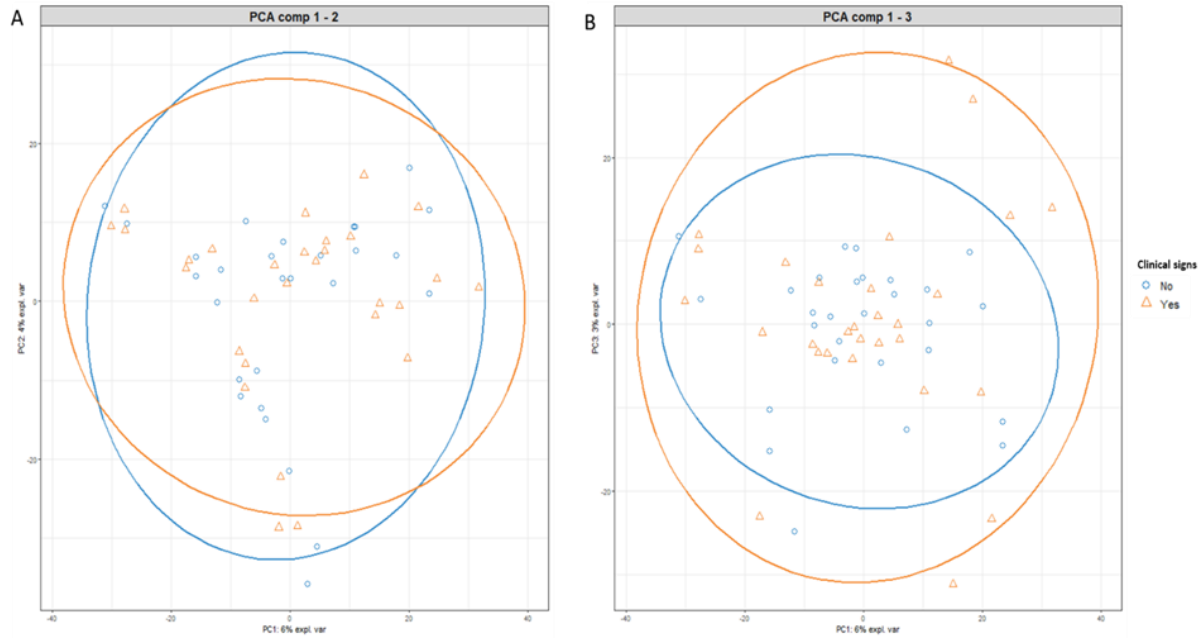


Figure 4.6. Differences in variation occurring between the bacterial populations in rumen samples collected from cattle with clinical signs of Pimelea poisoning (Yes, Δ) and cattle without any clinical signs of Pimelea poisoning (No, o), determined by sPLSDA. Results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1-2); and (B) Components 1 vs 3 (sPLSDA comp 1-3).

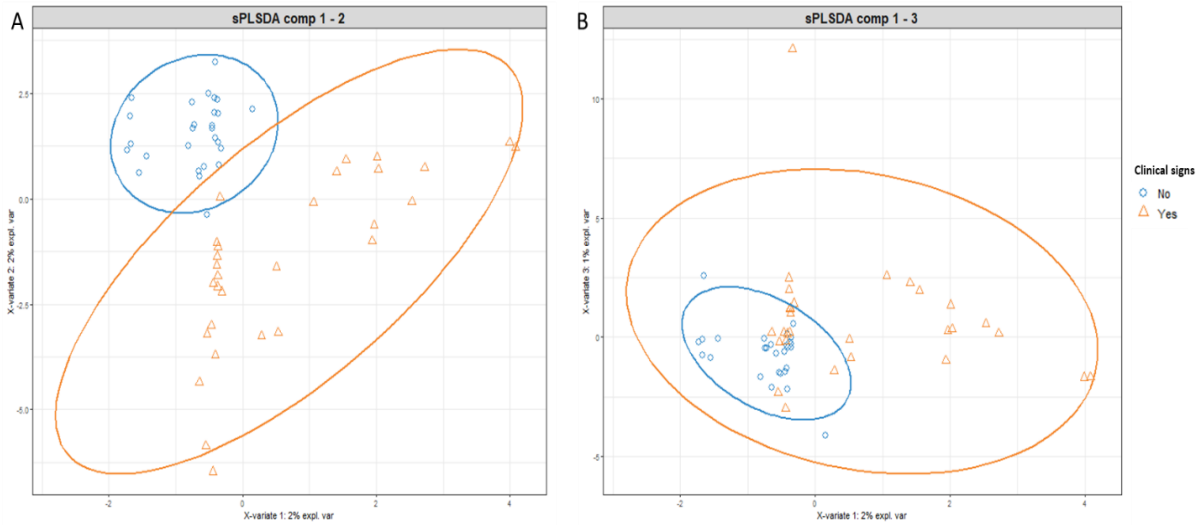


Table 4.5. Bacteria contributing to the differences in variation occurring between the rumen bacterial populations of cattle with or without clinical signs of Pimelea poisoning, determined by sPLSDA. The top 10 bacteria are listed (where available), and the respective positive or negative correlation value (Importance) given. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g). Where bacterial taxons with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset that could not be classified beyond the lowest taxonomic level listed.

Contribution to sPLSDA component 1 - Bacterial taxon	**Importance[A]**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_UCG-003	0.790
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae	0.343
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__*Papillibacter*	0.331
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.108
p__Firmicutes; c__Clostridia; o__Peptococcales; f__Peptococcaceae	-0.370
Contribution to sPLSDA component 2 - Bacterial taxon	**Importance**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.300
p__Firmicutes; c__Clostridia; o__Clostridia; f__Hungateiclostridiaceae; g__*Saccharofermentans*	0.277
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.240
p__Firmicutes; c__Clostridia; o__Clostridia; f__Hungateiclostridiaceae; g__*Saccharofermentans*	0.208
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.200
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	-0.210
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__*Ruminococcus*	-0.216
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Lachnospiraceae_NK3A20_group	-0.218
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__*Ruminococcus*	-0.236
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.311
Contribution to sPLSDA component 3 - Bacterial taxon	**Importance**
p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales	0.474
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.374
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.332
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales	0.255
p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Anaerovoracaceae; g__*Anaerovorax*	0.232
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.185
p__Firmicutes; c__Clostridia; o__Eubacteriales; f__Anaerofustaceae; g__*Anaerofustis*	-0.186
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.236
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	-0.262
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	-0.277

[A]Correlation values (Contribution) TRUE for Clinical signs, Yes (orange numbers); TRUE for Clinical signs, No (blue numbers).

4.1.2 Summary discussion – field survey

The microbiome of rumen contents collected from cattle and other animals grazing in areas with Pimelea present have been examined. Differences ($P < 0.001$) in the rumen microbial populations were found to occur between the animal species sampled (cattle, sheep, goats and kangaroos). The small differences in the rumen microbiome seen to occur for cattle from different stations, could be attributed to differences in the pasture and forage diets provided at each location, or herd effects. The microbial populations were otherwise relatively similar to each other, and to those previously identified in cattle grazing a forage diet (Henderson et al. 2015). The cattle sampled, were identified by producers as either unaffected by Pimelea consumption or showing clinical signs of Pimelea poisoning. Analysis of rumen fluid collected from these groups of cattle showed there was no significant differences in rumen microbial community structure between cattle unaffected by Pimelea and those exhibiting clinical signs of Pimelea poisoning. The bacteria identified in each respective group (presence or absence of clinical signs of poisoning) were very similar, and no bacteria known to be uncommon to the rumen microbial community were identified.

4.2 Screening for detoxifying bacteria

4.2.1 Plant nutritional analysis

Nutritional testing of the collected Pimelea and Buffel grass species collected from producer properties in western Queensland was undertaken by the DAF Biosecurity Sciences Laboratory (Coopers Plains, Qld) and at SGS Australia (Cairns, Qld) to determine the level of inclusion in the *in* vitro fermentations (Table 4.6). Two species of Pimelea, *P. simplex* and *P. trichostachya* had slightly higher levels of crude protein than *P. elongata*. All three species of Pimelea had similar levels of acid digested fibre (ADF) around 30% compared to Buffel grass which had 40.8%.

Table 4.6. Nutritional analysis of three species of Pimelea and a Buffel grass. All results are reported on a dry matter basis.

Nutritional Test	***Pimelea simplex* subsp. *continua* (AQ522485)**		***Pimelea elongata* (AQ522480)**		***Pimelea trichostachya* (AQ522479)**		**Buffel grass (BGB2018)**	
	BSL[A]	SGS[B]	BSL	SGS	BSL	SGS	BSL	SGS
% Ash	6.7	ND[C]	5.7	ND	4.9	ND	9.0	ND
% Nitrogen	1.63	1.5	1.31	1.2	1.63	1.5	0.84	0.8
% Protein (N% x 6.25)	10.17	9.4	8.16	7.5	10.17	9.4	5.27	5.0
% Crude fibre	21.6	ND	24.5	ND	24.2	ND	32.1	ND
% Acid detergent fibre	30.0	ND	30.7	ND	28.4	ND	40.8	ND
% Phosphorous	0.19	0.18	0.15	0.15	0.10	0.10	0.15	0.15
% Calcium	ND	0.42	ND	0.52	ND	0.53	ND	0.23
% Potassium	ND	1.8	ND	1.5	ND	1.2	ND	2.1
% Sulphur	ND	0.18	ND	0.14	ND	0.14	ND	0.09

[A]BSL = DAF Biosecurity Sciences Laboratory, Coopers Plains, Qld; [B]SGS = SGS Australia Laboratory, Cairns, Qld; [C]ND – not determined

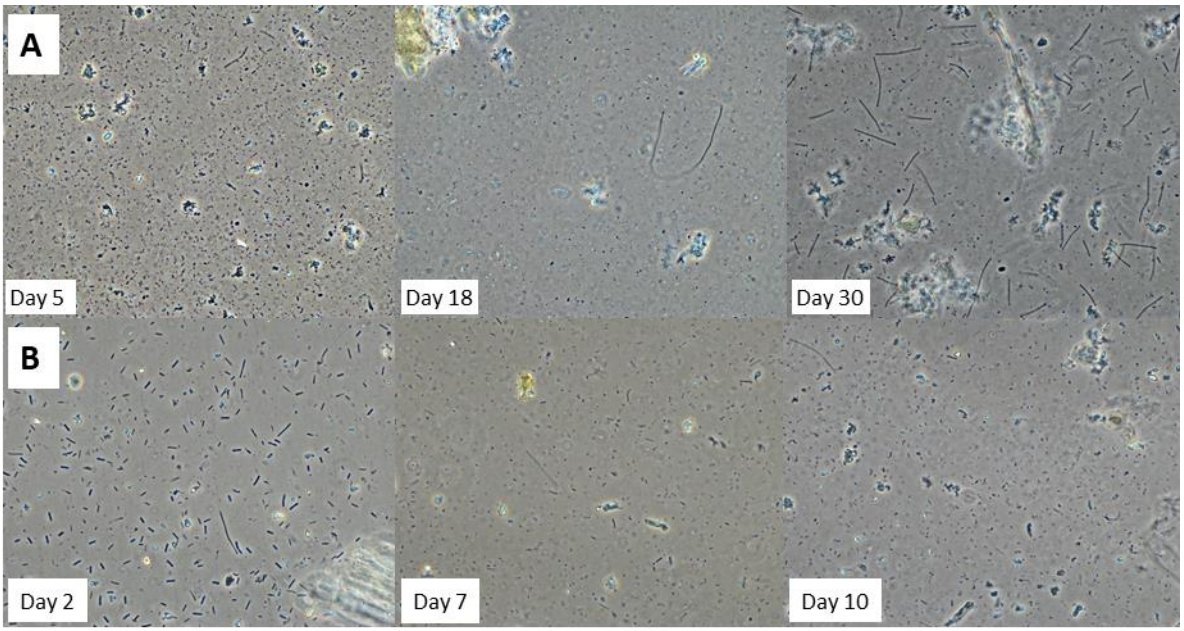
4.2.2 *In vitro* anaerobic fermentations

Sixteen *in vitro* fermentations ranging in length from 11 days to 63 days were undertaken to enrich microbial communities capable of digesting Pimelea plant material and potentially the compound simplexin contained within. For Fermentations 1 to 3, 6, 8, 9 and 12 a 50:50 mix of Pimelea and

Buffel grass hay was added to the fermenter daily. Fermentations 4, 5, 7, 10,11, 13, 15 and 16 had only Pimelea plant material added as the feed source for the microbial populations. Fermentation 14 was conducted to determine the release profiles of biopolymer containing Pimelea plant material so only Buffel grass hay was added daily to this fermentation. The decision to only add Pimelea plant material as the feed source for the microbial populations was made as it was expected that feeding only Pimelea would push the selection and growth of microbial populations able to digest Pimelea and provide increased levels of simplexin available as a potential feed substrate for bacteria which might be able to degrade it.

The microbial communities were diverse in all fermentations and followed similar patterns in the visual changes seen microscopically. For example, in Fermentation 4 the first Pimelea only fermentation, the microbial community progressively changed with the number of motile bacteria, large rods and large cocci in pairs dropping away and long chains of cocci appearing around Day 18 (Figure 4.7). The number of long chains of cocci continued to increase, however by Day 25 motile bacteria were again seen along with the large cocci in pairs and they remained through to Day 30.

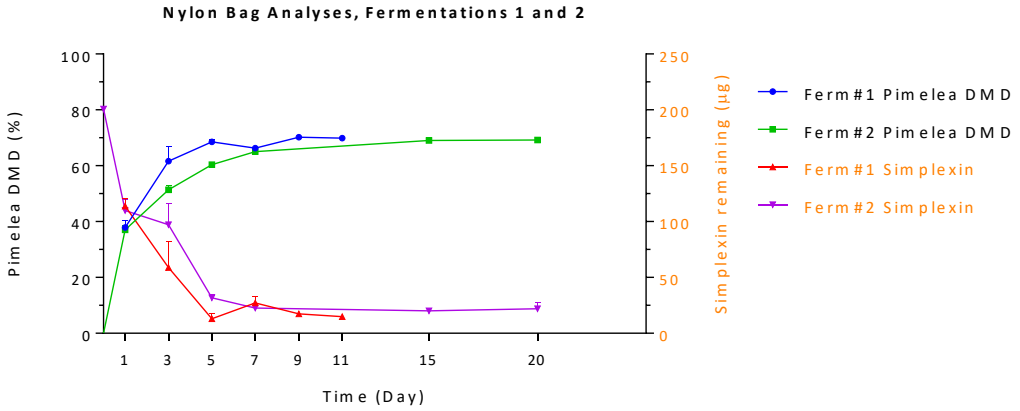
Figure 4.7. Microbial diversity changes in fermentation fluid collected from A. Ferm#4 on Day 5, Day 18 and Day 30 and B. Ferm#16 on Day 2, Day 7 ad Day 10 viewed under 400X magnification.



4.2.3 *In vitro* dry matter digestion (DMD) assay

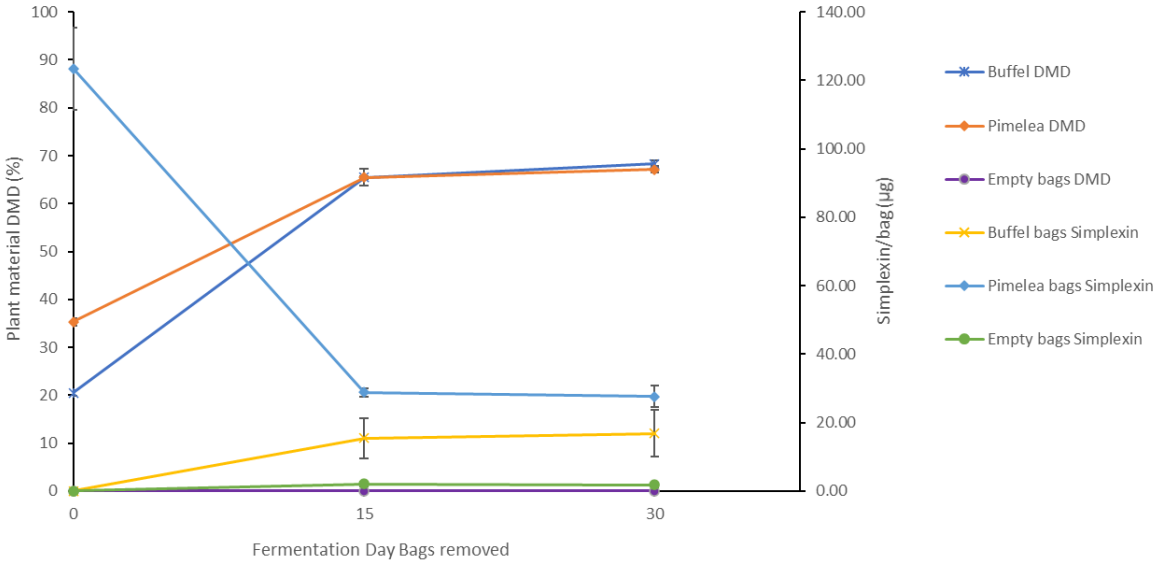
The digestibility of the milled Pimelea was determined in Fermentations 1 and 2 by incubating known weights of Pimelea, contained in nylon bags, within the fermenter for various lengths of time. Figure 4.8 shows the disappearance of the Pimelea plant material over the length of the fermentation with an average of 69.6% of the Pimelea being digested by Day 9 of the fermentation and no further digestion occurring. The Day 0 bags were dried and weighed in the same manner as the other bags but were not treated in any other way until extraction for simplexin measurement. The Day 1 nylon bags were not placed in the fermenter but processed exactly as the bags coming out of the fermenter were to determine the percentage of plant material that would be immediately soluble and lost and resulted in a drop in weight of 40 % for the Pimelea. The levels of simplexin present dropped, mirroring the disappearance of the plant material (Figure 4.8) with the non-digestible Pimelea plant material still containing simplexin at levels between 49 µg/g to 79 µg/g.

Figure 4.8. Percentage of Pimelea plant material utilised (dry matter digestion, DMD) and amount of simplexin (µg) in remaining plant material within duplicate nylon bags after incubation within *in vitro* Fermentations 1 and 2.



To determine if simplexin bound to other plant materials or the nylon bag itself, sets of duplicate nylon bags which were either empty, containing Buffel grass or containing Pimelea were incubated for different lengths of time within Fermentation 4. The set of six Day 0 bags (empty, Pimelea, Buffel grass) were not placed in the fermenter but processed exactly as all the bags coming out of the fermenter were to be processed to determine the percentage of plant material that would be immediately soluble. The Pimelea and Buffel grass in the Day 0 nylon bags dropped in weight by 35.4 % and 20.5% respectively. The percentages of the plant material utilised within the duplicate bags, along with the amount of simplexin detected within the bags, are shown in Figure 4.9. After the empty bags were processed, simplexin was detected at low levels (approx. 2 µg) after 15 days and 30 days of incubation whilst in the bags containing Buffel grass 15.44 µg simplexin was detected after 15 days incubation and 16.82 µg after 30 days incubation. After 15 days incubation 28.81 µg simplexin was measured in the bags containing Pimelea and 27.6 µg measured after 30 days incubation.

Figure 4.9. Percentage of either Pimelea or Buffel plant material utilised and amount of simplexin (µg) measured within duplicate nylon bags including empty nylon bags after different lengths of incubation within *in vitro* Fermentation 4.



4.2.4 Fermentation daily sample simplexin analysis

Daily samples taken from all fermentations were analysed for simplexin and they show a similar simplexin concentration pattern (Figure 4.10). The recoveries were estimated from the ratio of the pre-extraction spike samples to the post-extraction spike samples (85-93 %). The matrix factor was estimated from the ratio of the post-extraction spike samples to the solvent spike samples (0.98 – 1.03) corresponding to matrix effects of -1.9% to 1.3%. An average accuracy of 76 % was estimated by comparison of observed pre-extraction spike concentration to spiked concentration.

Overall, in the first three fermentations, started with cattle rumen fluid and fed a 50:50 mix of Pimelea and Buffel grass hay, the simplexin present in the fermenter levelled out from Day 5 onwards. Anova 1-way analysis using Tukey's multiple comparisons test (using GraphPad Prism) showed no statistical difference between the averages of days 5-11 in Fermentation 1, between the averages of days 5-20 in Fermentation 2, and between the days 5-30 in Fermentation 3. Fermenter fluid is removed and Pimelea plant material added on a daily basis throughout each of the fermentation runs and this is reflected in the daily simplexin analysis results of the removed fermenter fluid (Figure 4.10). This level represents the net level after any degradation. The situation is complicated by the diet provided with Fermentations 1 to 3 receiving half the amount of Pimelea plant material compared to the other fermentations which were provided with 100 % Pimelea plant material. Other factors such as the presence of biofilms in the system meant that simplexin may not be evenly distributed through the fermenter system. Another Pimelea toxin, Huratoxin, was analysed for from Fermentation 4 onwards and the concentrations present in the daily samples followed a similar pattern to simplexin concentration (Figure 4.11).

Figure 4.10. Concentration of simplexin (ng/mL) measured in representative samples selected from daily aliquots taken from selected i*n vitro* Fermentations, with the legend indicating source of starter inoculum for each. Fermentations 1 to 3 were supplied with a 50:50 mix of Pimelea and Buffel grass hay, all other fermentations received only Pimelea plant material.

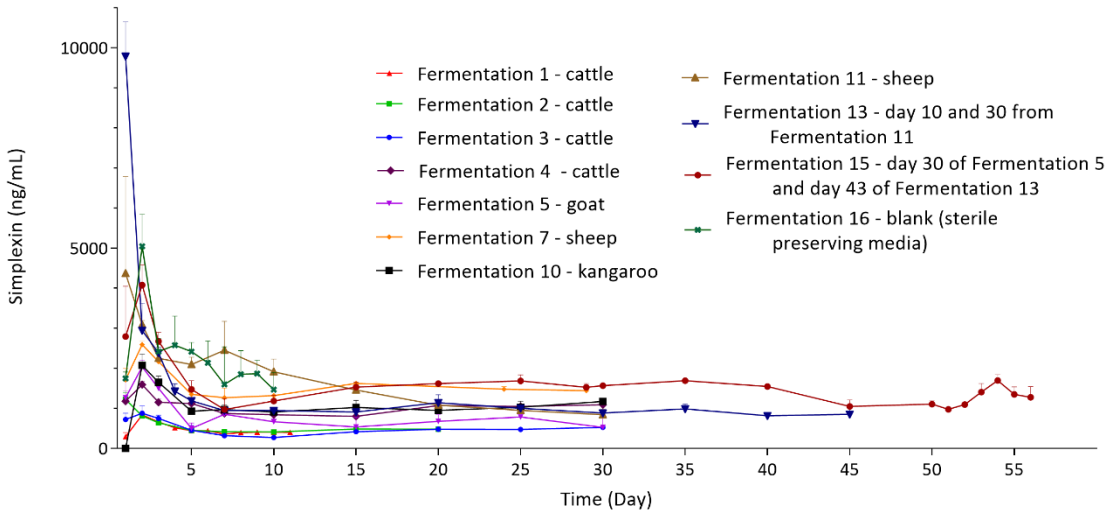
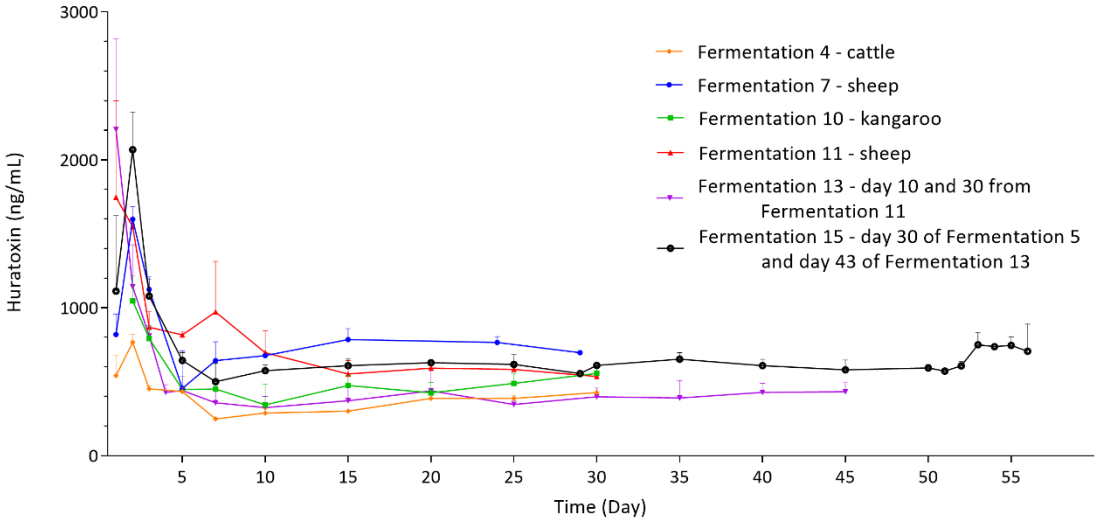


Figure 4.11. Concentration of huratoxin (ng/mL) measured in representative samples selected from daily aliquots taken from selected i*n vitro* Fermentations, with the legend indicating source of starter inoculum for each.



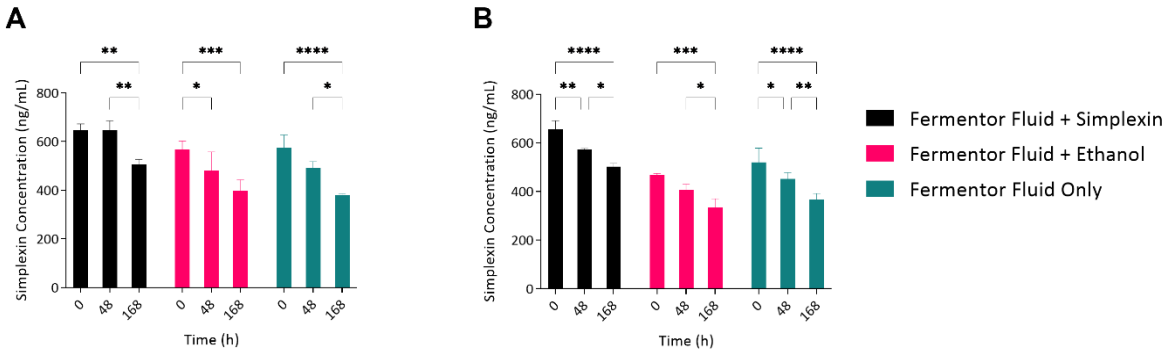
Overall, analysis of simplexin in daily fermentation fluid samples in all fermentation trials showed significant loss of simplexin between day 1 and day 5 of the fermentation. Based on the initial simplexin loss up to day 5, goat-based, sheep-based and goat & sheep-based fermentations showed higher simplexin decrease compared to cattle-based and kangaroo-based fermentations. Simplexin levels remained unchanged from day 5 until the end of the fermentation. Statistical analysis of the degradation curves of Fermentations 5, 7, 10, 11 and 13 showed that Fermentation 11 was significantly different ($P < 0.001$) to the other four fermentations and that the daily simplexin levels of Fermentations 5, and 13 (goat, Dorper sheep) plateaued at a significantly lower level ($P < 0.001$) than in Fermentations 7 and 10 (Merino sheep, Red Kangaroo).

4.2.5 Fermentation fluid simplexin degradation assays

A degradation bioassay was developed to determine if microbial degradation of simplexin was occurring in the fermentation (Appendix Section 9.7.12). This assay underwent a number of modifications with each successive fermentation trial. In assessing the accuracy of this LC-MS/MS analysis, an average recovery of 88 % was observed, matrix factors ranged from 0.94-1.15, with matrix effects between -5.6 to 14.69 %. Average accuracy was estimated to be 86 %.

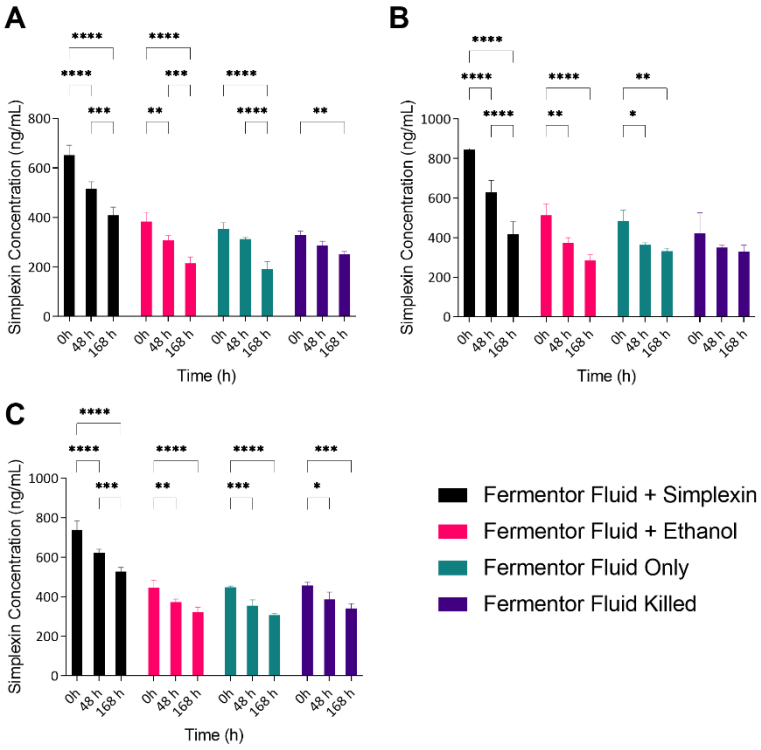
Fermentation 1 carried out over 11 days with starter rumen fluid from fistulated cattle at Gatton (Table 3.2 and Table 3.3) unexpectedly showed significant simplexin decreases in assays with fermenter fluid collected at Day 5 and Day 11, with P values varying between time points.(Figure 4.12, P values shown).

Figure 4.12. Simplexin degradation assays with fermenter fluid from (A) Day 5, (B) Day 11 of Fermentation 1. Significant differences within treatments were determined using two-way ANOVA with Tukey's multiple comparisons test, giving adjusted P values, ** *P* < 0.0001, *** *P* = 0.0001–0.001, ** *P* = 0.001–0.01, * *P* = 0.01–0.05, ns *P* ≥ 0.05 (not shown).**



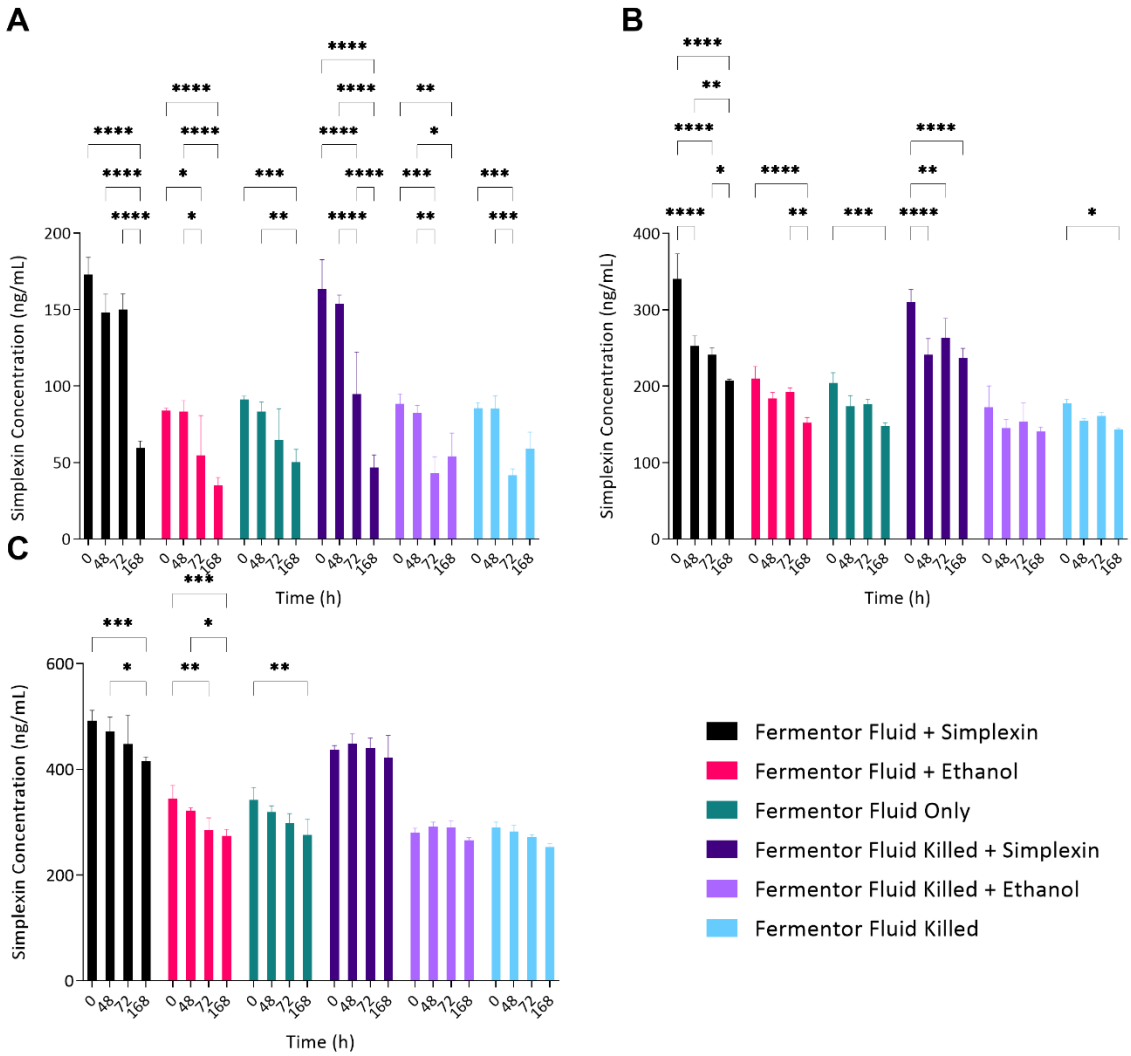
Fermentation 2, carried out over 20 days with starter rumen fluid from cattle on Property 1 (Table 3.2 and Table 3.3), showed significant simplexin decreases in assays with fermenter fluid collected at Day 10, Day 15 and Day 20 (Figure 4.13, P values shown). There was also apparent simplexin decreases/degradation with killed (autoclaved) fermenter fluid for Day 10 (P = 0.0035 for 0 h vs 168 h) and Day 20 (P = 0.0002 for 0 h vs 168 h), but not significant for Day 15 (P > 0.05), and it was considered that the microbes could be absorbing rather than degrading the toxin.

Figure 4.13. Simplexin degradation assays with fermenter fluid from (A) Day 10, (B) Day 15 and (C) Day 20 of Fermentation 2. Significant differences within treatments were determined using two-way ANOVA with Tukey's multiple comparisons test, giving adjusted P values, ** *P* < 0.0001, *** *P* = 0.0001–0.001, ** *P* = 0.001–0.01, * *P* = 0.01–0.05, ns *P* ≥ 0.05 (not shown).**



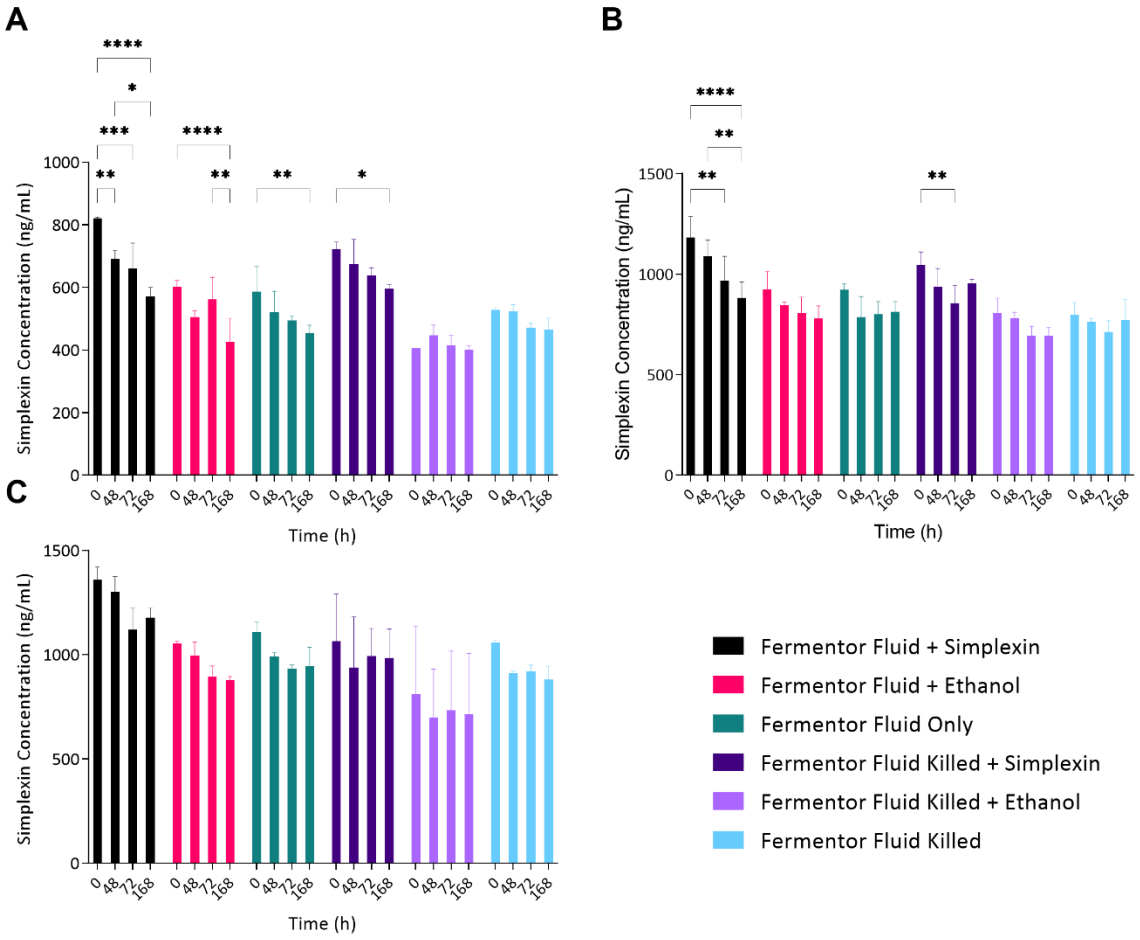
Fermentation 3, carried out over 30 days with starter rumen fluid from cattle on Property 3 (Table 3.2 and Table 3.3), showed substantial simplexin decreases in assays with fermenter fluid collected at Day 10 ($P < 0.0001$ for 0 h vs 168 h) and Day 20 ($P \leq 0.0001$ for 0 h vs 168 h), but lesser decreases with fermenter fluid collected at Day 30 (Figure 4.14, P values shown). Notably, the decreases in simplexin levels in Day 10 killed (autoclaved) samples were also significant ($P < 0.0001$ for 0 h vs 168 h), whereas for Day 20 and Day 30 killed (autoclaved) samples there were mostly no significant differences ($P > 0.05$).

Figure 4.14. Simplexin degradation assays with fermenter fluid from (A) Day 10, (B) Day 20 and (C) Day 30 of Fermentation 3. Significant differences within treatments were determined using two-way ANOVA with Tukey's multiple comparisons test, giving adjusted P values, ** *P* < 0.0001, *** *P* = 0.0001–0.001, ** *P* = 0.001–0.01, * *P* = 0.01–0.05, ns *P* ≥ 0.05 (not shown).**



Fermentation 4, carried out over 30 days with starter rumen fluid from a nine-month *Bos taurus* heifer that had been fed Pimelea for three months (Table 3.2 and Table 3.3), showed similar simplexin degradation in assays to previous fermentations with better degradation in fermenter fluid collected at Day 10 and 20, ($P < 0.0001$ for 0 h vs 168 h for live fermenter fluid with added simplexin), compared with fermenter fluid collected at Day 30 (all $P > 0.05$, Figure 4.15).

Figure 4.15. Simplexin degradation assays with fermenter fluid from (A) Day 10, (B) Day 20 and (C) Day 30 of Fermentation 4. Significant differences within treatments were determined using two-way ANOVA with Tukey's multiple comparisons test, giving adjusted P values, ** *P* < 0.0001, *** *P* = 0.0001–0.001, ** *P* = 0.001–0.01, * *P* = 0.01–0.05, ns *P* ≥ 0.05 (not shown).**



Fermentation 5, carried out over 30 days with starter rumen fluid from feral goats collected from property 13 (Table 3.2 and Table 3.3), showed substantial simplexin decreases (P < 0.0001 for 0 h vs 168 h) in assays with fermenter fluid with added simplexin collected at Day 10, Day 15 and Day 20 but minimal decreases with fermenter fluid collected at Day 30 (P = 0.002 for 0 vs 168 h for samples with added simplexin (

Figure 4.16). Day 20 killed (autoclaved) samples with added simplexin (+ simplexin) however also showed decreased simplexin levels (P = 0.0021 for 0 h vs 168 h) again suggestive of an absorption phenomenon.

Fermentation 7, carried out over 30 days with starter rumen fluid from Merino sheep collected from property 8 (Table 3.2 and Table 3.3), showed substantial simplexin decreases in assays with fermenter fluid collected at Day 10 and Day 30 (P < 0.0001 for 0 h vs 168 h for samples with added simplexin) but minimal degradation with fermenter fluid collected at Day 15 or Day 20 (Figure 4.17, P values shown). Day 10 killed (autoclaved) fermentation fluid + simplexin samples also showed significant differences (P = 0.0029 for 0 h vs 168 h) suggesting that the observed decreases were not due to the actions of live microorganisms.

Figure 4.16. Simplexin degradation assays with fermenter fluid from (A) Day 10, (B) Day 15, (C) Day 20 and (D) Day 30 of Fermentation 5. Significant differences within treatments were determined using two-way ANOVA with Tukey's multiple comparisons test, giving adjusted P values, ** *P* < 0.0001, *** *P* = 0.0001–0.001, ** *P* = 0.001–0.01, * *P* = 0.01–0.05, ns *P* ≥ 0.05 (not shown).**

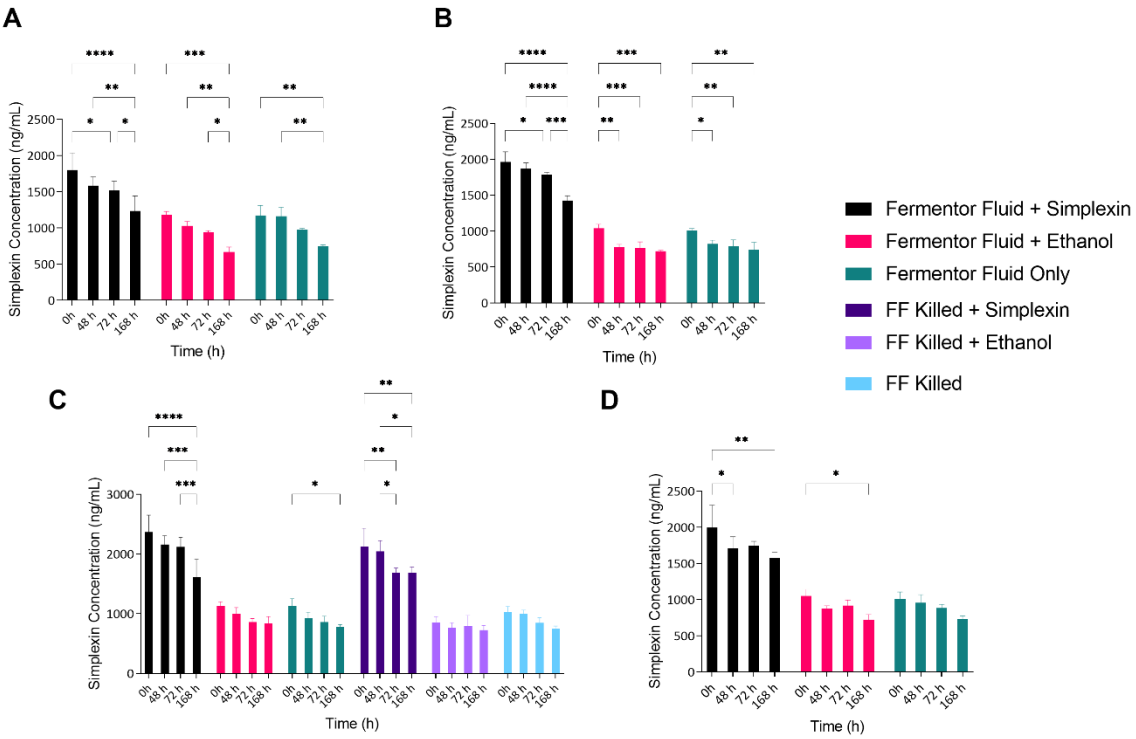
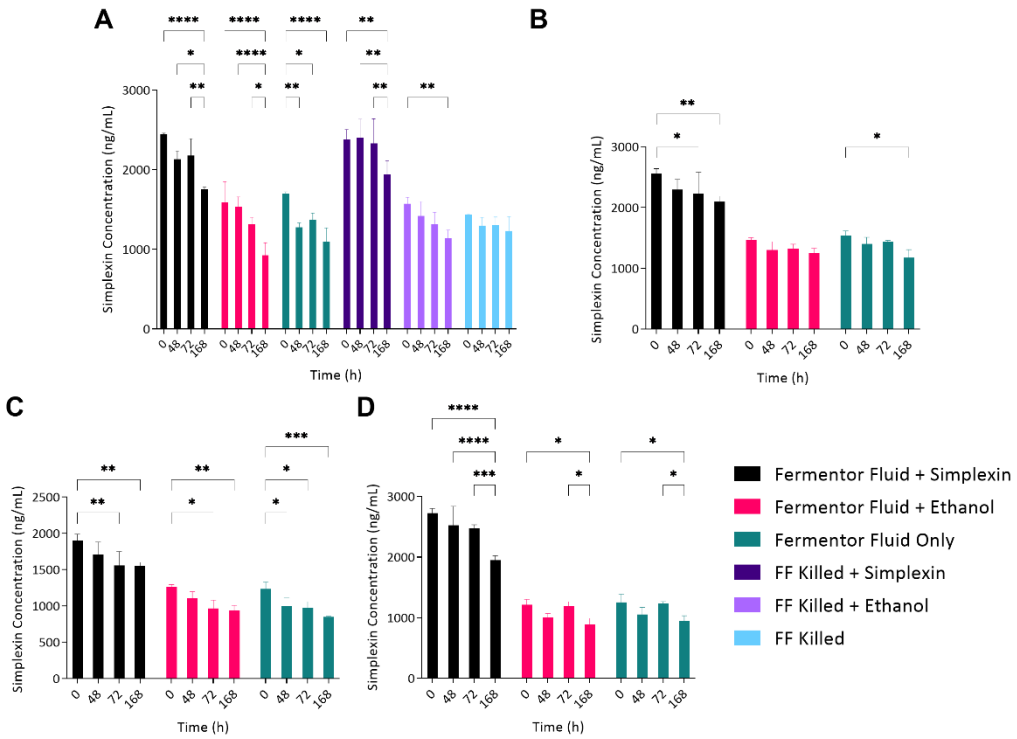
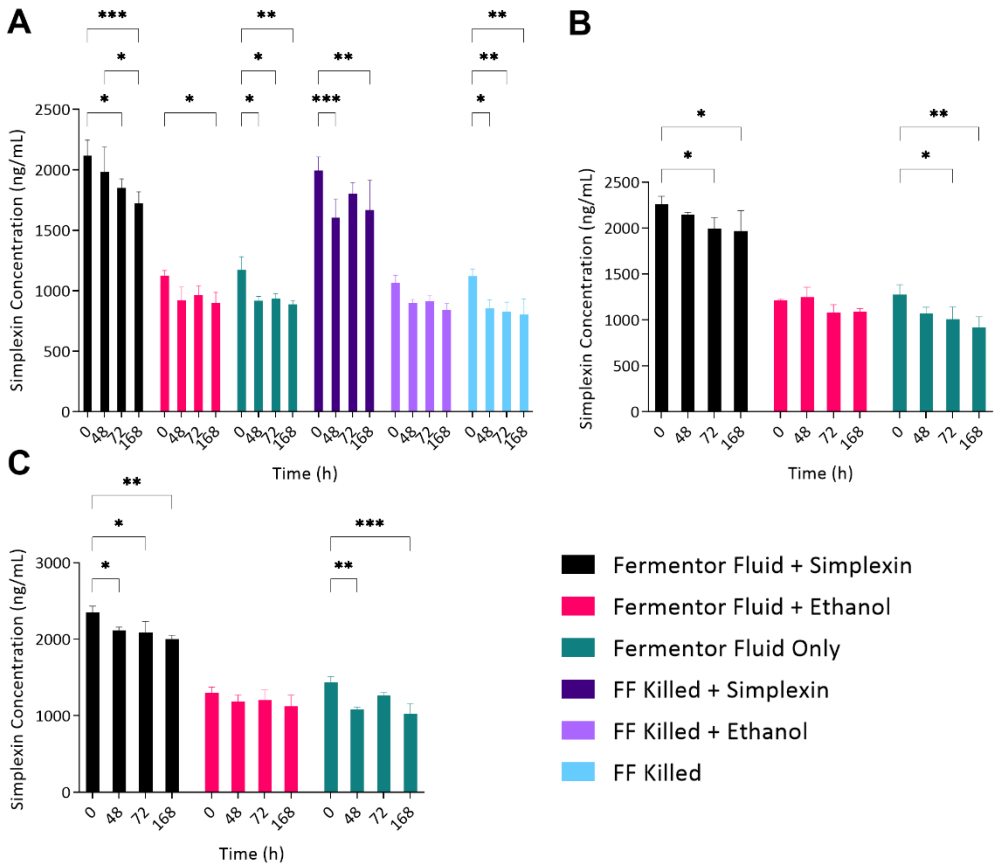


Figure 4.17. Simplexin degradation assays with fermenter fluid from (A) Day 10, (B) Day 15, (C) Day 20 and (D) Day 30 of Fermentation 7. Significant differences within treatments were determined using two-way ANOVA with Tukey's multiple comparisons test, giving adjusted P values, ** *P* < 0.0001, *** *P* = 0.0001–0.001, ** *P* = 0.001–0.01, * *P* = 0.01–0.05, ns *P* ≥ 0.05 (not shown).**



Fermentation 10, carried out over 30 days with starter rumen fluid from red kangaroos collected from property 11 (Table 3.2 and Table 3.3), showed similar simplexin decreases in assays with fermenter fluid collected at Day 10, Day 20 and Day 30. Day 10 live Fermenter Fluid versus killed (autoclaved) treatments suggestive decreases due to absorption (Figure 4.18, P values shown).

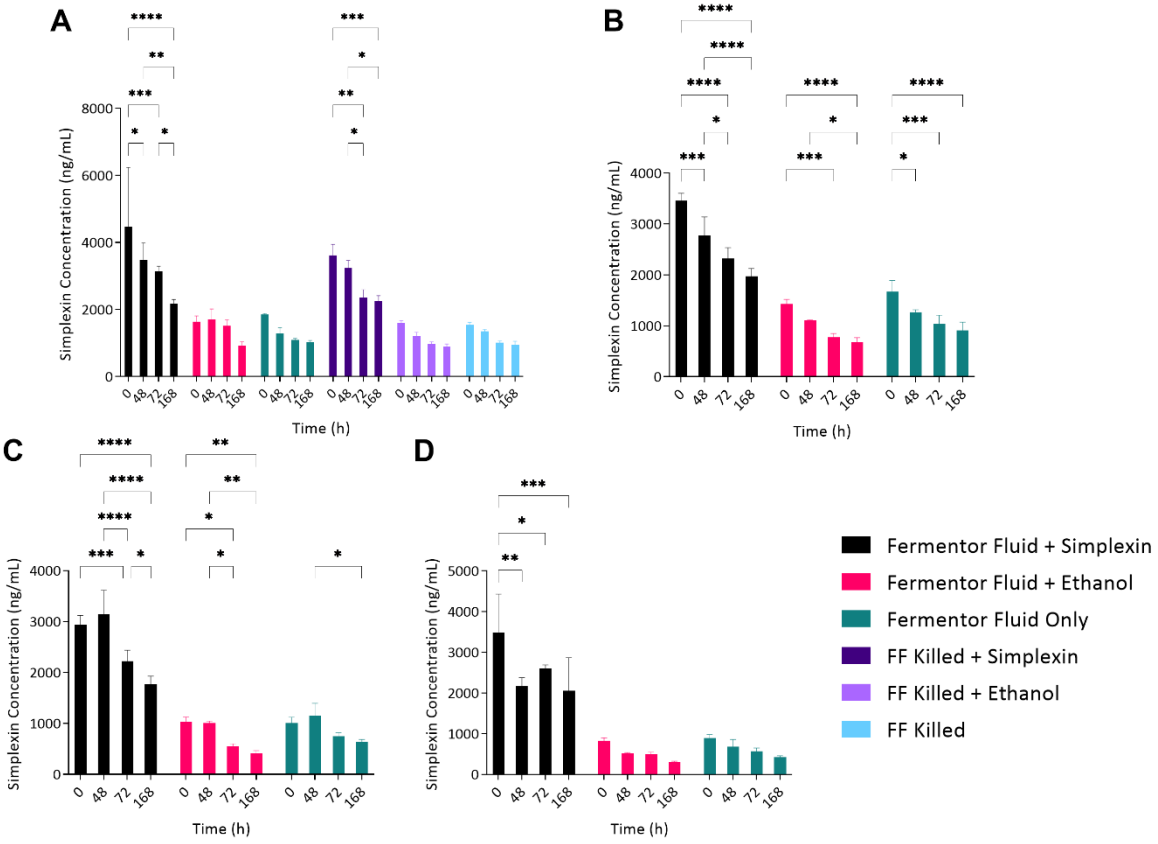
Figure 4.18. Simplexin degradation assays with fermenter fluid from (A) Day 10, (B) Day 20, (C) Day 30 of Fermentation 10. Significant differences within treatments were determined using two-way ANOVA with Tukey's multiple comparisons test, giving adjusted P values, ** *P* < 0.0001, *** *P* = 0.0001–0.001, ** *P* = 0.001–0.01, * *P* = 0.01–0.05, ns *P* ≥ 0.05 (not shown).**



Fermentation 11, carried out over 30 days with starter rumen fluid from Dorper and Dohne sheep collected from property 10 (Table 3.2 and Table 3.3), showed substantial simplexin decreases in assays with fermenter fluid collected at Day 10 in fermentation fluid + simplexin ($P < 0.0001$ for 0 h vs 168 h) in comparison to the killed fermentation fluid + simplexin ($P = 0.0008$ for 0 h vs 168 h), with Day 15, Day 20 and Day 30 showing lesser patterns of simplexin decreases (

Figure 4.19).

Figure 4.19. Simplexin degradation assays with fermenter fluid from (A) Day 10, (B) Day 15, (C) Day 20 and (D) Day 30 of Fermentation 11. Significant differences within treatments were determined using two-way ANOVA with Tukey's multiple comparisons test, giving adjusted P values, ** *P* < 0.0001, *** *P* = 0.0001–0.001, ** *P* = 0.001–0.01, * *P* = 0.01–0.05, ns *P* ≥ 0.05 (not shown).**

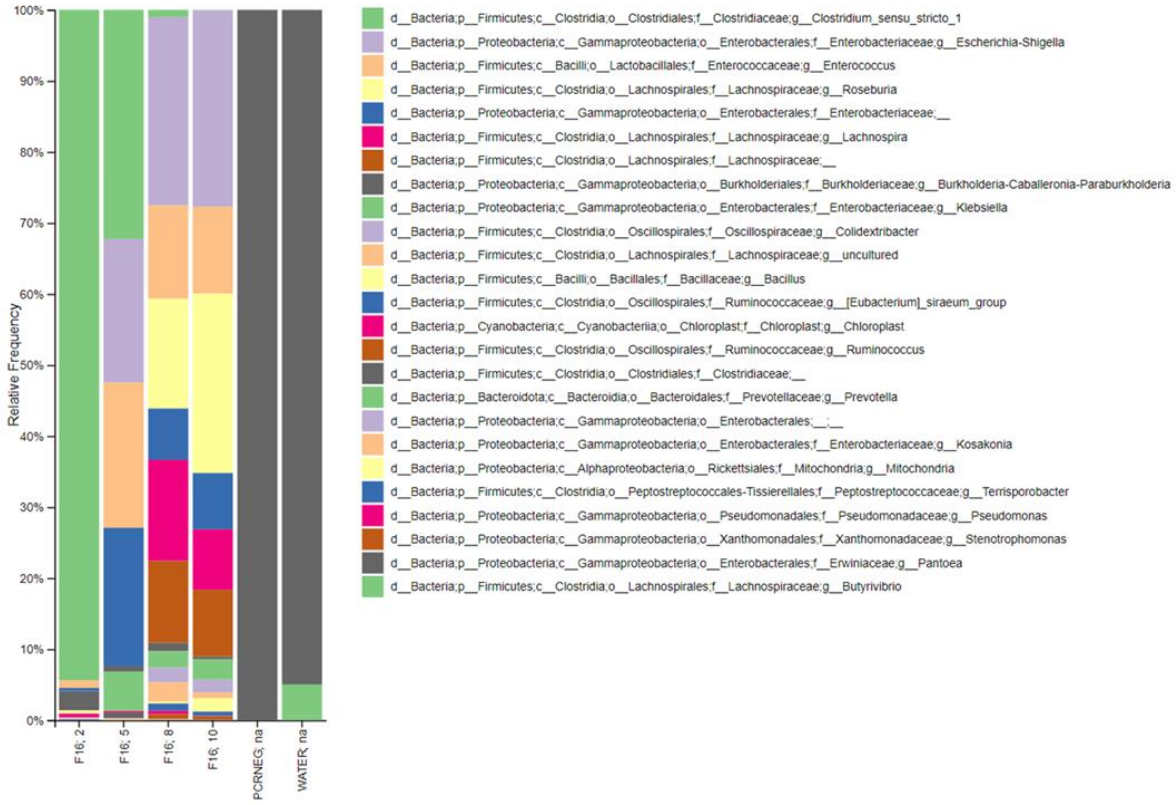


4.2.6 Microbiome of *in vitro* Fermentations

Initially the *in vitro* fermentations of Pimelea plant material were commenced with a mixed microbial inoculum from cattle rumen samples and later fermentations were conducted with either pooled goat rumen samples or pooled sheep rumen samples. The final fermentations (Fermentations 13 and 15) were inoculated on the first day of fermentation with fermenter fluid harvested from previous fermentations (Table 3.2 and Table 3.3). In addition, a 10-day fermentation (Fermentation 16), was undertaken in late October to determine the levels of simplexin in a Pimelea fermentation, with minimal bacterial activity. The fermentation was set up exactly as all other Pimelea fermentations but instead of a bacterial inoculum, a 'blank inoculum' was added, consisting of a 100 mL volume of sterile cryopreserving media. Although the media and blank inoculum were sterile, the milled Pimelea plant material was not, therefore the microbial populations observed in this fermentation may have arisen from microbes present on the plant material introduced into the fermentation apparatus on a daily basis and from common laboratory microbial contaminants (e.g., airborne, spore-forming or skin-associated microbes) that were able to multiply within the anaerobic conditions provided by the fermentation apparatus. The dominant microbial populations identified in this fermentation included the bacterial genera *Clostridium*, *Escherichia-Shigella*, *Enterococcus* and *Roseburia* (Figure 4.20). Due to the major differences in the way this fermentation was conducted, the samples from this fermentation and the negative control samples (water and PCR blanks) were removed from the overall Feature table and from subsequent analyses. In addition, the

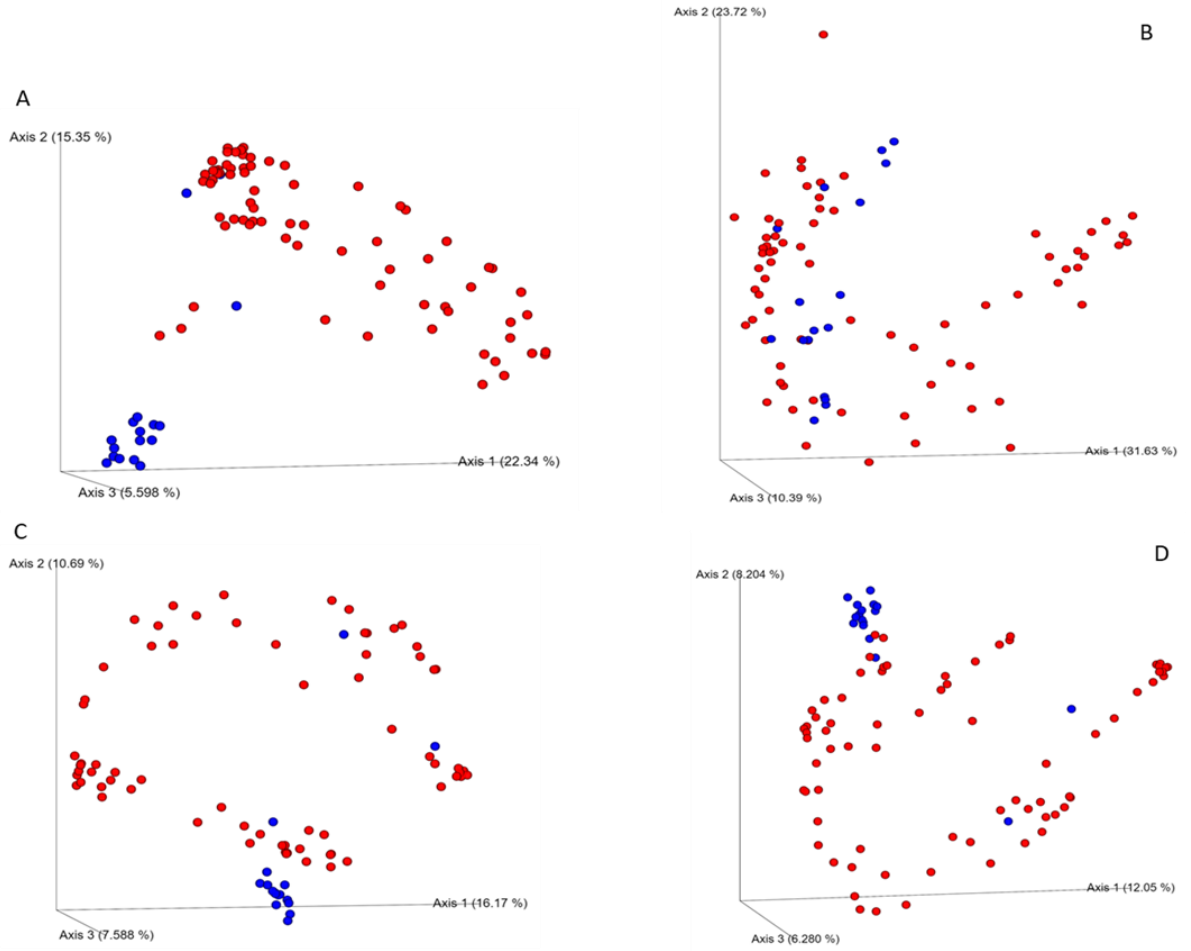
major microbial contaminant identified within the water and PCR negative samples (genus Burkholderia-Caballeronia-Paraburkholderia) was removed from all subsequent analysis.

Figure 4.20. Stacked bar plot of highly abundant microbial genera identified in samples collected from days 2, 5, 8 and 10 of Fermentation 16 (F16) and two Negative control samples (PCRNEG and Water). Highly abundant genera designated as those with a frequency greater than the median frequency of 31 sequences per Feature.



Rumen fluid samples collected from a range of ruminant species (cattle, sheep and goats), which were either known or suspected to be grazing pastures containing Pimelea, were used to inoculate Fermentation numbers 4, 5, 6, 7 and 11. These rumen fluid samples differed in microbial community structure when compared to the microbial populations which were cultivated in the fermenter apparatus (Figure 4.21). The most highly abundant taxonomic groups identified in each of the fermentations after 10 days of fermentation with Pimelea plant material were similar, containing microbial populations which preferred the physical, anaerobic conditions of the fermentation system and could be maintained on the relatively high protein, ground Pimelea feedstock. Dominant bacterial genera identified in fermenter fluid samples collected throughout each of the fermentations and analysed, included *Prevotella, Butyrivibrio, Rikenellaceae* (RC9 gut group), *Streptococcus* and *Succiniclasticum*.

Figure 4.21. Differences in total microbial populations present in fermenter and microbial populations used to start the *in vitro* fermentation system, with Fermenter samples (●) and the microbial populations of either rumen fluid or mixtures of fermenter fluid (●), coloured accordingly. Results of PCoA on the basis of distance matrices were generated using four measures of microbial between-sample (beta) diversity: (A) unweighted Unifrac; (B) Weighted Unifrac; (C) Bray-Curtis; and (D) Jaccard.



Each of the fermentations showed a distinct and significant change in microbial diversity ($P < 0.001$) and population structure over time as shown by taxonomic analysis (Figure 4.22) and statistical analysis of alpha (within-sample) diversity measures (Table 4.7). The most variation in microbial community structure occurred within the first 10 days of fermentation, with microbial diversity occurring within each fermentation increasing exponentially (Figure 4.23 and Figure 4.24). Each of the four microbial diversity measures determined, indicated that the diversity plateaued in the later stages of the fermentation, indicating that the microbial populations had stabilised to the nutritional and environmental conditions provided by the fermentation apparatus. Interestingly a single fermentation (fermentation 7), which was inoculated with rumen fluid from sheep, was shown to contain small ciliate protozoa, which were observed by microscopy of fermenter fluid collected on the 9th, 10th and 11th day of fermentation. The presence of protozoa in Fermentation 7, may have attributed to the relatively low microbial diversity observed on the 10th and 15th day of this fermentation, as protozoa consume bacteria as a food source.

The microbial population used to inoculate, or start the fermentation also had a significant effect ($P < 0.001$) on the diversity of the microbial populations of the fermentation (Table 4.7). This result was supported by the PCA analysis, where the effects of time of fermentation were minimised by only

including fermenter samples collected once the microbial diversity had stabilised (≥ 10 days of fermentation) (Figure 4.25). Distinct differences in the microbial populations of each fermentation were observed. The microbial populations of Fermentations 11 and 13 were most similar, as the fermenter fluid generated during Fermentation 11, was used to inoculate Fermentation 13. Otherwise, the microbial populations of each fermentation were determined by the respective rumen fluid sample used to establish the microbial populations. As fermentation 15 was inoculated with fermenter fluid from fermentations 5 and 13, this fermentation developed it's own distinct microbial population.

Differential abundance analysis results explained the differences occurring between each fermentation (Figure 4.26 and Table 4.8). Interestingly fermentation fluid from fermentation 7, appeared contained the highest proportion of differentially abundant bacterial populations, including the bacterial genus *Muribaculaceae* and several genera classified in the order Christensenellales. Other bacterial populations that differed in abundance include those classified in the genus *Succiniclasticum* with different bacterial strains belonging to this genus identified in fermentations 4, 6, and F11, which were inoculated with rumen fluid from cattle from an animal trial (ARI), cattle from Gatton (UQ), and fermenter fluid from a previous fermentation inoculated with sheep rumen fluid, respectively. Other differentially abundant bacteria were classified in genera commonly observed in rumen fluid and contributing to the breakdown of plant fibre, such as bacteria classified in the families Lachnspiraceae, Rikenellaceae and Christensenellaceae.

Figure 4.22. Stacked bar plot of highly abundant microbial classes identified in Fermenter fluid (FF) samples collected from Fermentation numbers 4, 5, 6, 7, 11, 13 and 15 on consecutive time-points (e.g. days 2, 3, 4, 5, 10, 15, 20, 25 and 30), with the microbial source material (cryopreserved rumen fluid sources including Bos Taurus cows, feral Goats, Brahman cross cattle, Merino sheep, Dorper and Dohne sheep; and fermenter fluid [FF]). Highly abundant Features were designated as those with a frequency greater than the median frequency of 78 sequences per Feature.

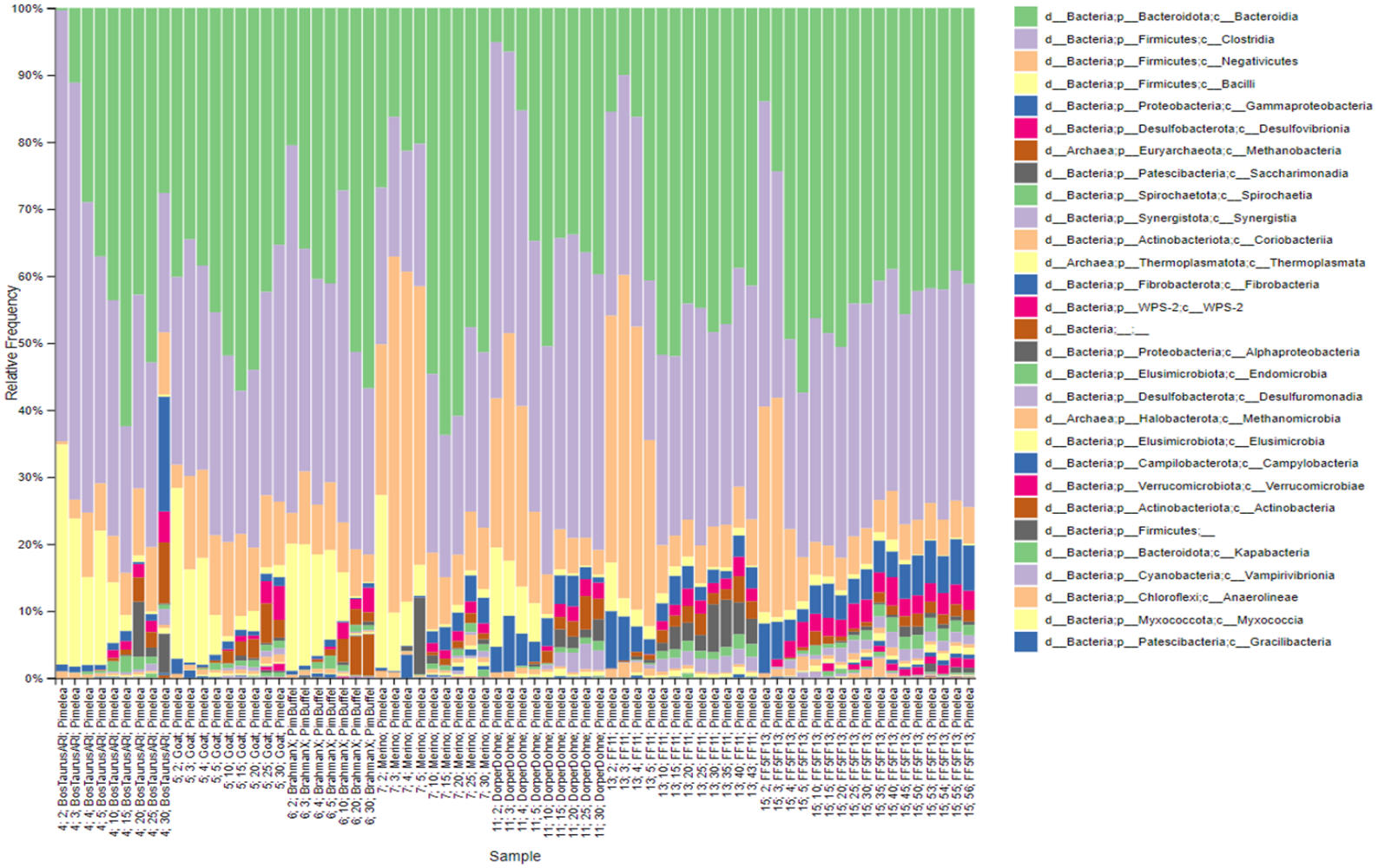


Table 4.7. *In vitro* fermentations of Pimelea plant material, statistical analysis of four measures of within-sample (alpha) diversity, results of an Accumulated analysis of Variance, including the effect of time of fermentation (Day); Fermentation starter source and the interaction occurring between Day and Fermentation starter source material. Results listed include the degrees of freedom (d.f.); sum of squares (s.s.), mean square (m.s.), variance ratio (v.r.), and the probability value corresponding to a variance ratio (F probability = P)

Change	d.f	s.s.	m.s.	v.r.	P
Observed Features					
Day[A]	2	1,241,045	620,522	272.7	<.001
Fermentation starter source[B]	6	625,350	104,225	45.81	<.001
Day.Fermentation starter source	6	70,968	11,828	5.200	<.001
Faith PD					
Day	2	6406.8	3203.4	503.0	<.001
Fermentation starter source	6	1019.2	169.9	26.70	<.001
Day.Fermentation starter source	6	240.8	40.10	6.300	<.001
Shannon entropy					
Day	2	32.90	16.45	242.6	<.001
Fermentation starter source	6	11.70	1.949	28.75	<.001
Day.Fermentation starter source	6	1.582	0.264	3.890	0.003
Pielou evenness					
Day	2	0.123	0.061	91.67	<.001
Fermentation starter source	6	0.049	0.008	12.27	<.001
Day.Fermentation starter source	6	0.021	0.004	5.270	<.001

[A] Day includes all days of each fermentation for which fermentation fluid (FF) samples were collected for microbiome sequencing, including days 2,3,4,5,10,15,20,25,30 (for Fermentation numbers 4,5,6,7 and 11) and further days of fermentation (days 35, 40, 43 of Fermentation number 13; and days 35, 40, 45, 50, 53, 54,55, 56 of Fermentation 15)

[B] Fermentation starter source includes the groupings: Cattle rumen fluid; Sheep rumen fluid; Goat rumen fluid; Sheep FF; Sheep and Goat FF.

Figure 4.23. Changes in microbial diversity over time (days) for fermentations listed according to the source of microbial starter, including rumen fluid from cattle (Brahman cross, *Bos taurus*); goats and sheep (Dorper dohne and Merino); and fermenter fluid harvested from the final days of a fermentation started with sheep rumen fluid (Sheep FF) and fermenter fluid harvested from the final days of two fermentations, started with either sheep or goat fermenter fluid (Sheep and Goat FF). Microbial (alpha) diversity measures include (A) Shannon entropy; and (B) Pielou evenness.

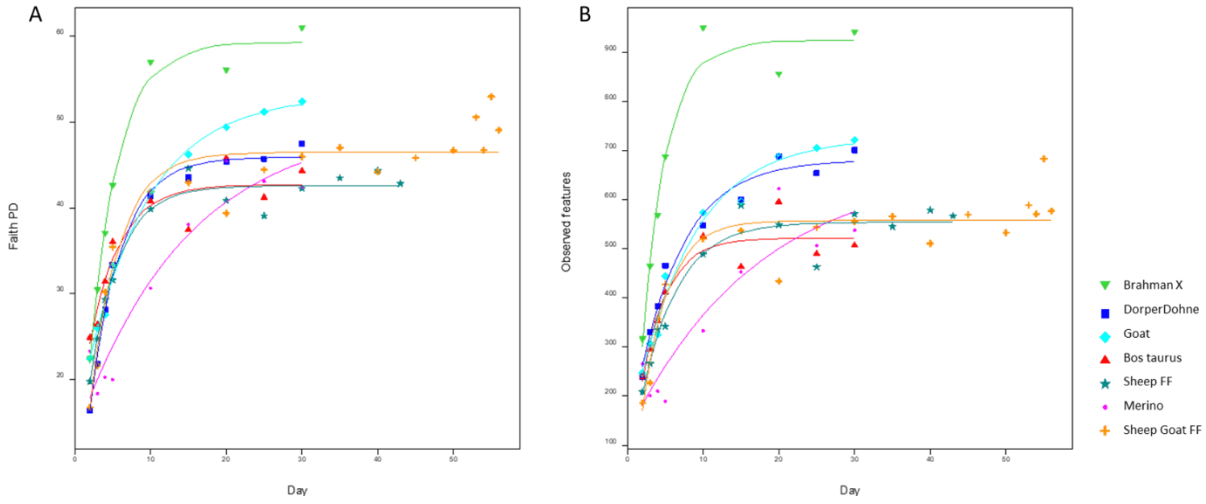


Figure 4.24. Changes in microbial diversity over time (days) for fermentations listed according to the source of microbial starter, including rumen fluid from cattle (Brahman cross, *Bos taurus*); goats and sheep (Dorper dohne and Merino); and fermenter fluid harvested from the final days of a fermentation started with sheep rumen fluid (Sheep FF) and fermenter fluid harvested from the final days of two fermentations, started with either sheep or goat fermenter fluid (Sheep and Goat FF). Microbial (alpha) diversity measures include (A); and (B) Pielou evenness.

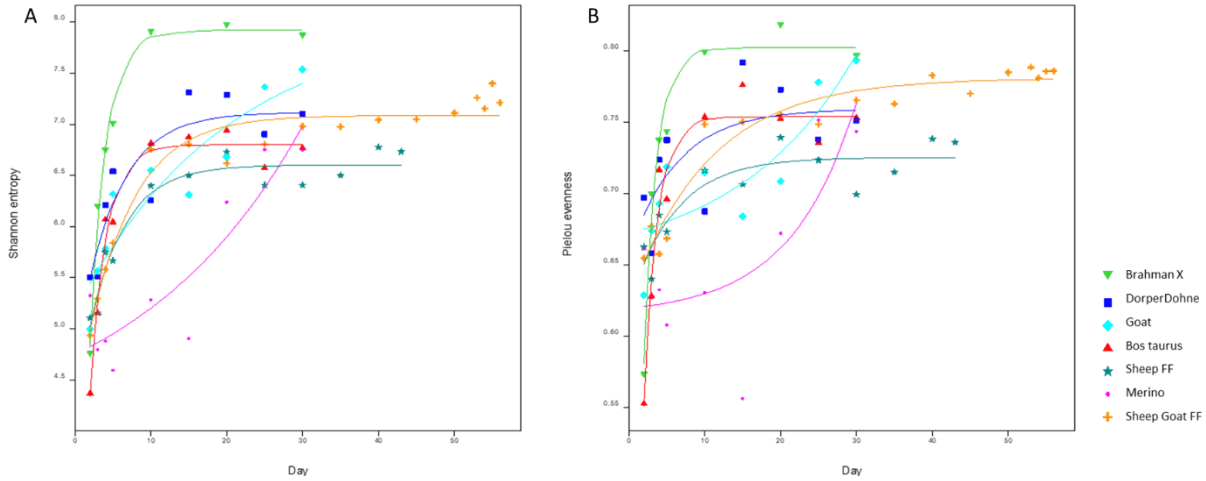


Figure 4.25. Variation in the microbial populations from the 10th day of fermentation for seven *in vitro* fermentations (F4, F5, F6, F7, F11, F13 and F15), with the microbial starter material indicated (either cryopreserved rumen fluid [RF] or Fermenter fluid from previous fermentations [FF]). PCA plot with bacterial and archaeal populations, with each point representing a sample of fermenter fluid collected on days 10, 15, 20, 25 and 30 for F4 to F11; and additional days for F13 and F15, coloured according to the fermentation number. PCA results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).

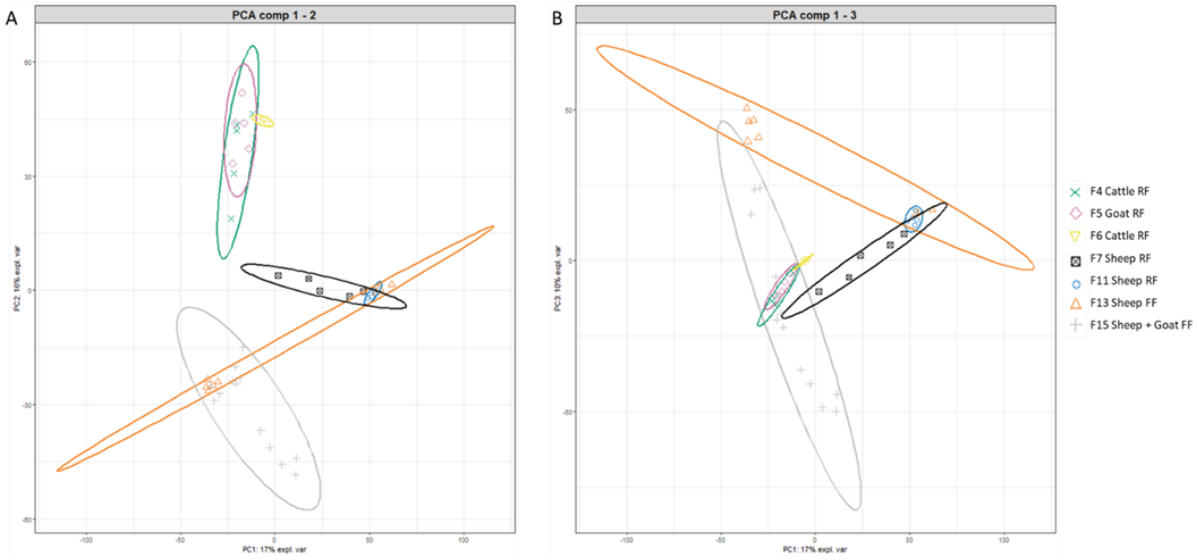


Figure 4.26. Variation in the microbial populations from the 10th day of fermentation for seven *in vitro* fermentations (F4, F5, F6, F7, F11, F13 and F15), with the microbial starter material indicated (either cryopreserved rumen fluid [RF] or Fermenter fluid from previous fermentations [FF]). PCA plot with bacterial and archaeal populations, with each point representing a sample of fermenter fluid collected on days 10, 15, 20, 25 and 30 for F4 to F11; and additional days for F13 and F15, coloured according to the fermentation number. PCA results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).

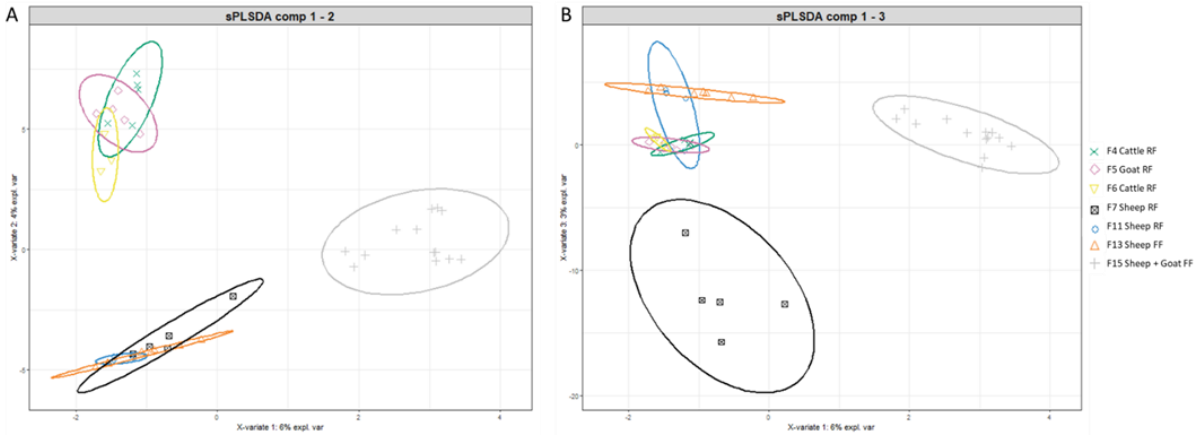


Table 4.8. Table of bacteria and archaea contributing to the differences in variation occurring between the bacterial populations associated the microbial populations from the 10th day of fermentation, for seven *in vitro* fermentations (F4, F5, F6, F7, F11, F13 and F15), determined by sPLSDA presented in Fig. 4.25. The top 10 microbial populations are listed and the respective positive or negative correlation value (Importance) given. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); genus (g) and species (s). Where microbial taxons with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset, that could not be classified beyond the lowest taxonomic level listed.

Contribution to sPLSDA component 1	**Importance[A]**
p__Firmicutes; c__Negativicutes; o__Acidaminococcales; f__Acidaminococcaceae; g__*Succiniclasticum*	-0.698
p__Firmicutes; c__Negativicutes; o__Veillonellales-Selenomonadales; f__Selenomonadaceae; g__*Selenomonas*	0.536
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	-0.317
p__Firmicutes; c__Clostridia; o__Peptococcales; f__Peptococcaceae	0.277
p__Firmicutes; c__Negativicutes; o__Acidaminococcales; f__Acidaminococcaceae; g__*Succiniclasticum*	-0.221
Contribution to sPLSDA component 2	**Importance**
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__*Eubacterium ruminantium* group; s__*Eubacterium ruminantium*	0.296
p__Proteobacteria; c__Gammaproteobacteria; o__Pasteurellales; f__Pasteurellaceae; g__*Basfia*	0.281
p__Firmicutes; c__Negativicutes; o__Acidaminococcales; f__Acidaminococcaceae; g__*Succiniclasticum*	0.272
p__Firmicutes; c__Negativicutes; o__Acidaminococcales; f__Acidaminococcaceae; g__*Succiniclasticum*	0.264
p__Spirochaetota; c__Spirochaetia; o__Spirochaetales; f__Spirochaetaceae; g__*Treponema*	0.254
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.248
p__Spirochaetota; c__Spirochaetia; o__Spirochaetales; f__Spirochaetaceae; g__*Treponema*	0.239
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__UCG-005	0.237
p__Proteobacteria; c__Gammaproteobacteria; o__Pasteurellales; f__Pasteurellaceae; g__*Basfia*; s__*Basfia succiniciproducens*	0.227
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*; s__*Prevotella ruminicola*	0.207
Contribution to sPLSDA component 3	**Importance**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__*Muribaculaceae*	-0.315
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__*Muribaculaceae*	-0.314
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	-0.296
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	-0.240
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	-0.234
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	-0.223
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	-0.214

p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	-0.214
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	-0.204
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Enterobacteriaceae; g__*Escherichia-Shigella*	-0.190

[A] Respective positive or negative correlation values (Importance) are coloured according to the Fermentation number which contributed to the difference observed (TRUE by sPLSDA). Fermentation numbers include, F4 (green text); F5 (purple text); F7 (black text); F11 (blue text); F15 (grey text), with colours corresponding to the colour scheme used in the corresponding sPLSDA plot.

4.2.7 Summary discussion – fermenter studies screening for detoxifying bacteria

To enrich for bacteria which could potentially degrade simplexin, a series of *in vitro* fermentations were conducted using the bacterial populations from rumen fluid samples collected during the property survey. The microbial populations adapted over time to the feedstock and physical conditions provided by the *in vitro* fermentation apparatus, with most changes in microbial community structure occurring within the first 10 days of fermentation. The microbial community structure was then found to stabilise in the later days of fermentation. The microbial populations cultivated in each of the Pimelea fermentations differed in composition, according to the microbial populations used to initially inoculate the fermenter apparatus. The final fermentations, which were inoculated with fermenter fluid harvested from prior fermentations, continued to successfully encourage the growth of organisms present in the original fermentation. Statistical analysis of simplexin concentrations in fermenter fluid samples collected throughout each fermentation, using LC-MS/MS, indicated that there were several fermentations with significantly lower levels of simplexin present occurring with time. The final fermentation (Fermentation 15) was inoculated with fermenter fluid harvested from prior fermentations (Fermentation 5 and Fermentation 13) for which analysis of the daily simplexin concentrations indicated the greatest potential to contain microbial populations most likely to degrade Pimelea plant material and simplexin. The fermenter fluid harvested on the final days of this Fermentation 15 was therefore cryopreserved for use in the Pimelea feeding trial and was supplied to cattle as a mixed microbial drench (inoculum), within the three-month animal pen trial.

4.3 Bacterial isolations and simplexin degradation screening

4.3.1 Bacterial isolations

Bacterial isolations using selective media containing simplexin were undertaken using fermentation fluid collected seven different Fermentations 3, 4, 5, 7, 9, 10 and 13. Serial dilutions of fresh fermentation fluid were set up in Hungate tubes of broth and aliquots from selected dilutions between 10^{-3} to 10^{-7} were spread onto an agar plate (spread) or mixed with molten agar then poured onto an agar plate (soft agar overlay). Following 24 h of incubation at 39 °C, the colonies present on each of the spread and soft agar overlay plates for each dilution were counted to determine the colony forming units (CFU) obtained from each isolation method and are detailed for the spread plates in Table 4.9 and for the soft agar overlay method in Table 4.10.

Table 4.9. Counts of CFU on spread plates of dilutions 10^{-3} to 10^{-7} dilutions of fermentation fluids from anaerobic fermentations, started with different source inoculums, fed milled Pimelea plant material after 24 h incubation at 39 °C.

Ferm #	Original Source	Fermentation Day Sampled	Spread plates (colonies/dilution plate)					Estimated bacteria/mL
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
3	Shorthorn steer	30	TMTC[A]	137	11	2	ND[B]	1.49×10^{6}
4	Dairy steer	30	TMTC	TMTC	56	5	ND	5.3×10^{6}
5	Feral Goat	30	TMTC	TMTC	TMTC	28	ND	2.8×10^{7}
7	Merino Sheep	13	TMTC	TMTC	TMTC	97	ND	9.7×10^{7}
9	Brahman X	63	TMTC	51	26	4	ND	2.4×10^{6}
10	Red Kangaroo	30	TMTC	TMTC	101	40	ND	2.51×10^{7}
13	Dorper sheep	30	TMTC	TMTC	97	11	0	1.04×10^{7}
13	Dorper sheep	43	ND	TMTC	73	42	3	2.6×10^{7}

[A]TMTC – too many to count; [B]ND – not done

Table 4.10. Counts of CFU on soft agar overlay plates of dilutions 10^{-3} to 10^{-7} dilutions of fermentation fluids from anaerobic fermentations, started with different source inoculums, fed milled Pimelea plant material after 24 h incubation at 39 °C.

Ferm #	Original Source	Fermentation Day Sampled	Overlay plates (colonies/ dilution plate)					Estimated bacteria/mL
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
3	Shorthorn steer	30	TMTC[A]	TMTC	45	1	ND	2.75×10^{6}
4	Dairy steer	30	TMTC	TMTC	38	1	ND	2.4×10^{6}
5	Feral Goat	30	TMTC	TMTC	TMTC	TMTC	ND	
7	Merino Sheep	13	TMTC	TMTC	TMTC	77	ND	7.7×10^{7}
9	Brahman X	63	TMTC	TMTC	120	30	ND	3.0×10^{7}
10	Red Kangaroo	30	TMTC	TMTC	TMTC	90	ND	9.0×10^{7}
13	Dorper sheep	30	TMTC	TMTC	TMTC	72	8	7.2×10^{7}
13	Dorper sheep	43	ND	TMTC	TMTC	70.5	3	5.0×10^{7}

[A]TMTC – too many to count; [B]ND – not done

A range of different colony types were selected from the spread and soft agar overlay plates and transferred into selective media broths. Once grown each isolate was passaged through several cycles taking an aliquot of the broth culture and 16 streaking on agar plates, transferring well-spaced colonies into broths, growing and checking microscopically for purity until the isolate was considered to be a pure culture.

Bacterial isolate species identification

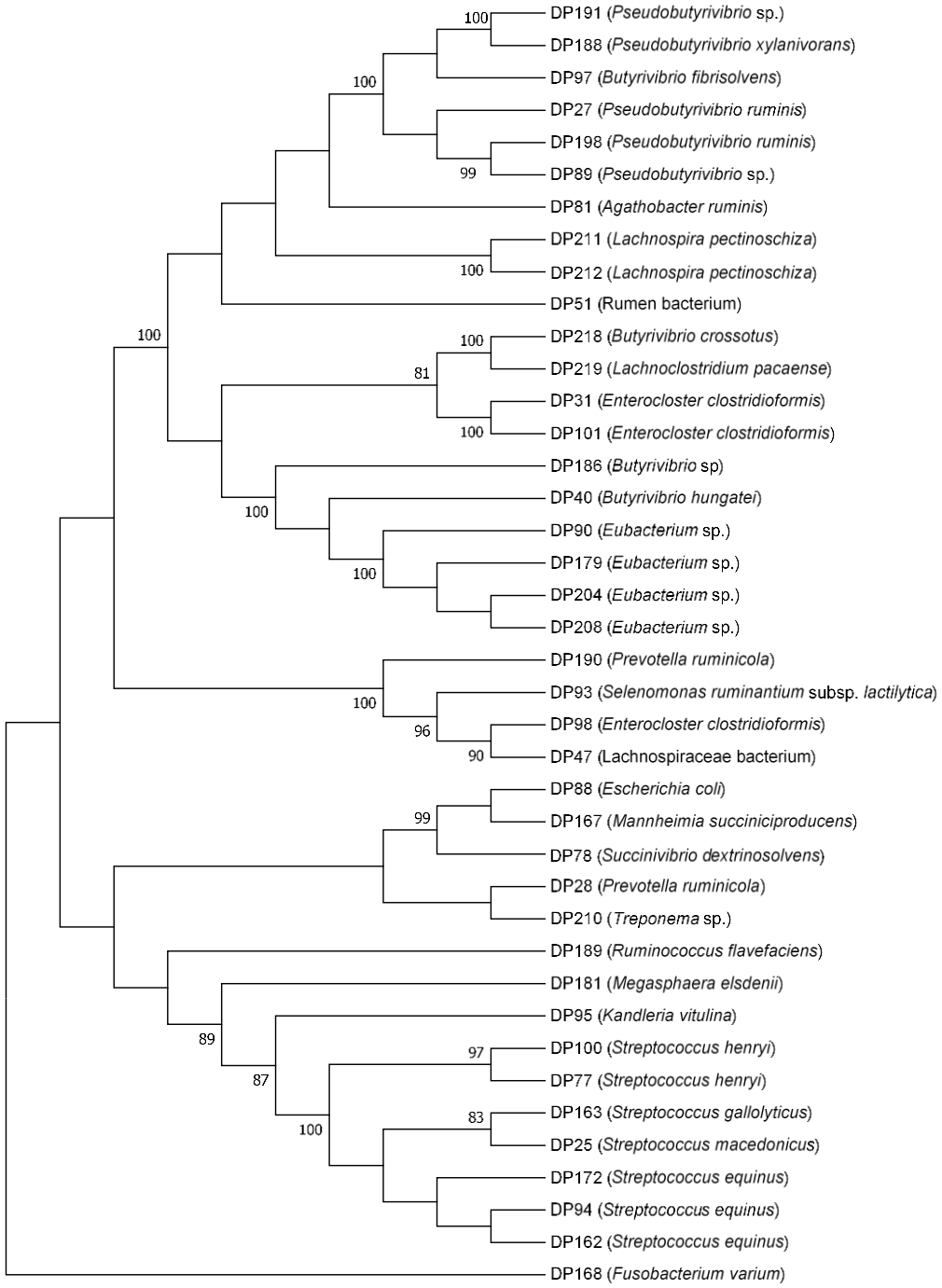
In total 157 bacterial isolates were obtained from seven different fermentations as pure cultures using selective media, assigned a culture collection identifier (DP number) and placed in the DAF culture collection. The 157 isolate cultures were grown and cryo-preserved in triplicate and are stored in three separate freezers located at the EcoSciences Precinct. The species identities of the isolates were determined by BLASTn of near full-length 16S rRNA gene sequences to 16S rRNA sequences in the NCBI GenBank database (Table 4.11). A total of 27 different species were isolated across 18 different genera. The phylogeny of forty of the bacterial isolates using approximately 1318 bp of 16S rRNA sequence was determined using the Kimura 2-parameter model and a 100 bootstrap tree generated which was rooted with bacterial isolate DP168 (Figure 4.27). Whilst these bacteria were isolated on a selective media containing the toxin simplexin, it was a crude ethanol extract of Pimelea plant material containing other plant compounds. To assess the ability of the isolated bacteria to degrade simplexin, a degradation assay was developed and is reported in Section 4.3.2.

Table 4.11. Bacterial isolates species ID based on near full-length 16S rRNA gene sequence BLASTn match (NCBI database), isolation source fermentation and number of isolates.

Nearest Relative (% Homology to near full length 16S rRNA gene seq)[A]	**Fermentation # (# of isolates)**
Agathobacter ruminis strain JK623	5 (1)
Butyrivibrio crossotus strain DSM 2876	13 (1)
Butyrivibrio fibrisolvens strain YE44	3 (1); 9 (1)
Butyrivibrio hungatei strain MB2003	13 (6)
Butyrivibrio hungatei strain Su6	4 (6)
Butyrivibrio sp. strain CG9	13 (1)
Enterocloster clostridioformis strain FDAARGOS_739	3 (2); 4 (6); 7 (7); 9 (4)
Escherichia coli strain PK5086	5(1)
Eubacterium sp. strain TW2	5 (3); 13 (9)
Fusobacterium varium strain NCTC10560	10 (2)
Kandleria vitulina strain JCM 1143	3 (7); 7 (1)
Lachnospira pectinoschiza isolate M46	13 (1)
Lachnospiraceae bacterium CA60	4 (2)
Mannheimia succiniciproducens strain MBEL55E	10 (1)
Megasphaera elsdenii strain 14-14	13 (5)
Prevotella ruminicola strain BP1-40	4 (1)
Prevotella ruminicola strain CA6	13 (7)
Pseudobutyrivibrio ruminis isolate L4	4 (12)
Pseudobutyrivibrio xylanivorans strain MA3014	5 (1); 13 (12)
Ruminococcus flavefaciens strain LP-C14-Adx	13 (1)
Selenomonas ruminantium	5 (1)
Selenomonas ruminantium strain S2	9 (2)
Selenomonas ruminantium subsp. *lactilytica* strain TAM6421	7 (2); 9 (1)
Streptococcus equinus strain CNU 77-78	4 (1)
Streptococcus equinus strain JB1	10 (5)
Streptococcus gallolyticus strain FDAARGOS_755	10 (2)
Streptococcus henryi strain OZK31	3 (8); 7(1); 5 (12)
Streptococcus lutetiensis strain DTK428	3 (8)
Streptococcus macedonicus strain W64	4 (2)
Streptococcus sp. strain CNUG3	10 (5)
Succinivibrio dextrinosolvens strain CA76	5 (1)
Succinivibrio dextrinosolvens strain CA81	9 (3)
Treponema sp. strain JC4	13 (1)

[A]highest 16S rRNA gene sequence BLASTn match

Figure 4.27. Phylogenetic tree based on near full length 16S rRNA gene sequences of the forty bacterial strains isolated from rumen-derived *in vitro* fermentations of Pimelea plant material and later used in simplexin degradation assays. The tree with the highest log likelihood (-14403.24) is shown (topology only and scale bar not shown due to high degree of diversity occurring between bacterial species). This tree was generated using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura *et al*., 1980), with a discrete gamma distribution (5 sites). There were a total of 1318 nt positions in the final dataset and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein, 1985), with values < 80% not shown. The tree is rooted with the 16S rRNA gene sequence of strain DP168 also isolated from an *in vitro* fermentation of Pimelea plant material.



4.3.2 Bacterial isolate simplexin degradation screening

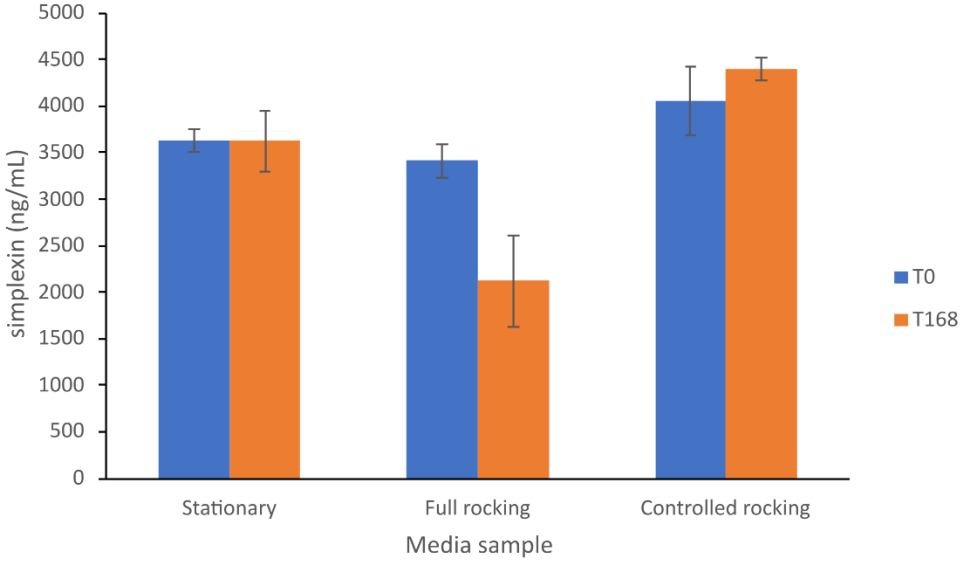
Trials 1–3

The first three bacterial isolate simplexin degradation trials were conducted and reported in Milestone Reports. However, these results are not included in this report as subsequent experiments showed the simplexin binding to the butyl rubber stopper of the Hungate tubes used in testing experiments, therefore these results were determined to be invalid.

Simplexin binding in Hungate tube experiment

An experiment was designed to investigate the losses of simplexin at different stages of media preparation and during the incubation with rocking. The simplexin level dropped slightly during the preparation of RF$^+$modPM6000 broth media making process, however it was not significant. Sterile RF$^+$modPM6000 media in Hungate tubes was incubated in three different treatments – Stationary with the tubes upright in a rack; Controlled rocking with tubes angled to ensure the media did not touch the butyl rubber stopper; and Full rocking. Analysis of simplexin in the T 0h and T 168h samples showed the level of simplexin present in the broth from the Full rocking Hungate tubes had significantly decreased ($P < 0.05$) whilst the Stationary and Controlled rocking samples had not (Figure 4.28). Analysis of the butyl rubber stopper for simplexin in the rocking treatment Hungate tubes showed approximately 16% of the total simplexin measured was bound to the it after one week of rocking at 39 °C.

Figure 4.28. Levels of simplexin measured in sterile RF$^+$modPM6000 broth measured initially (T 0h) and following incubation at 39°C for one week (T 168h) either upright (Stationary), secured to a rocking platform with the liquid hitting the butyl rubber stopper (Full rocking) or rocking on an angle that prevented the liquid touching the butyl rubber stopper (Controlled rocking).



Trial 4

The growth of the 11 isolates in RF$^+$modPM6000 broths, assessed as cell density after one week of rocking on an angle (T 168h), ranged from low levels of growth (+) through to good growth (+++) (Table 4.12).

However due to a technical error the simplexin levels in Trial 4 showed an apparent increase after 168h of incubation (data not shown) with the majority of the cultures with simplexin levels higher than the negative. This trial was then repeated as Trial 5.

Table 4.12. Trial 4 isolates, their identity based on highest 16S rRNA sequence match and growth assessment Wickerham card score at T 168h

Isolate ID	Nearest relative (16S rRNA gene species ID)	Culture density [A] T 168h
DP179	*Eubacterium* sp.	++
DP181	*Megasphaera elsdenii*	++
DP186	*Butyrivibrio* sp.	++
DP188	*Pseudobutyrivibrio xylanivorans*	++
DP189	*Ruminococcus flavefaciens*	+
DP190	*Prevotella ruminicola*	+
DP191	*Pseudobutyrivibrio xylanivorans*	+++
DP198	*Pseudobutyrivibrio xylanivorans*	+++
DP210	*Treponema* sp.	++
DP211	*Lachnospira pectinoschiza*	++
DP218	*Butyrivibrio crossotus*	+
Negative	-	-

[A]Wickerham card score – no growth through to +++ good growth

Trial 5

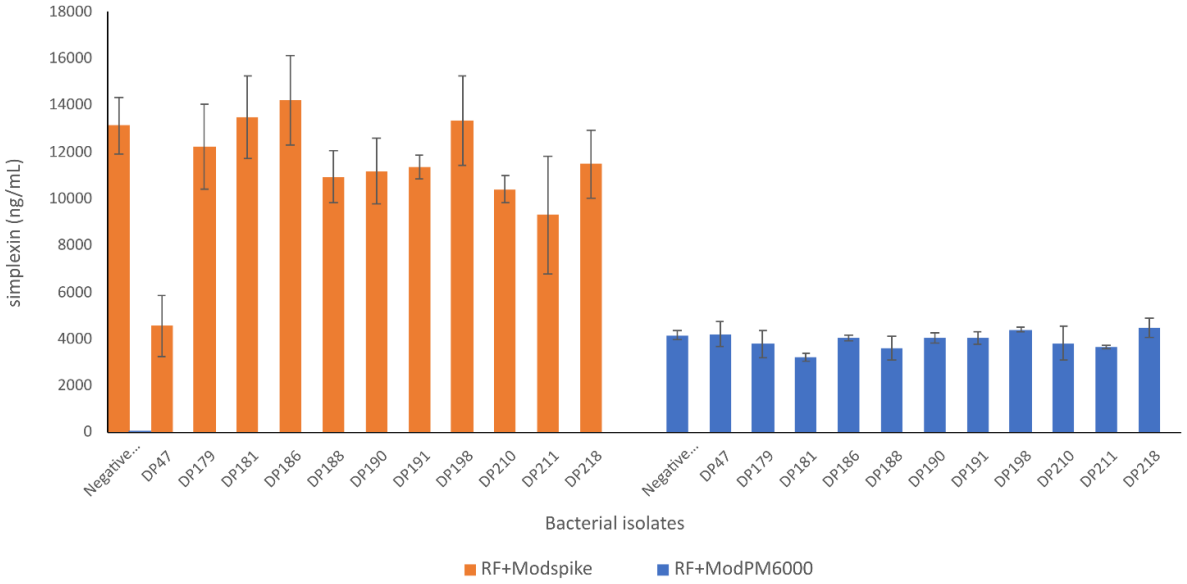
The growth of the 11 isolates from Trial 4 plus an additional isolate DP47 in RF$^+$modPM6000 and RF$^+$mod spiked broths, assessed for cell density after 48 hours of controlled rocking on an angle (T 48h), ranged from low levels of growth (+) through to good growth (+++) (Table 4.13). The concentration of simplexin was measured after one week's growth (T 168h) with controlled rocking for each isolate in both broths (Figure 4.29). DP47 appeared to exhibit significantly decreased ($P < 0.05$) simplexin degradation potential and a further supplementary degradation assay (Trial 5s) was undertaken to substantiate this.

Table 4.13. Trial 5 isolates, their identity based on highest 16S rRNA sequence match and culture density (Wickerham card score) in pre-trial media and in Trial media after 48 h incubation at 39°C with controlled rocking

Isolate ID	Nearest relative (16S rRNA gene species ID)	Pre-trial RF$^+$ (24 h)	Pre-trial RF$^+$mod (24 h)	RF$^+$mod Spiked T 48h	RF$^+$mod PM6000 T 48h
DP179	*Eubacterium* sp.	+++	+++	+++	+++
DP181	*Megasphaera elsdenii*	++++	++++	+++	+++
DP186	*Butyrivibrio* sp.	++++	+++	+++	+++
DP188	*Pseudobutyrivibrio xylanivorans*	++++	+++	+++	+++
DP189	*Ruminococcus flavefaciens*	+++	++	ND[A]	ND
DP190	*Prevotella ruminicola*	++++	+++	++	+++
DP191	*Pseudobutyrivibrio xylanivorans*	++++	+++	+++	+++
DP198	*Pseudobutyrivibrio xylanivorans*	++++	+++	+++	+++
DP210	*Treponema* sp.	+++	+++	++	+++
DP211	*Lachnospira pectinoschiza*	+++	+++	++	+++
DP218	*Butyrivibrio crossotus*	+	+	+	++
DP47	Lachnospiraceae bacterium	-	+[B]	++	++
Negative		-	-	-	-

[A]ND – not done, [B]DP47 inoculum grown in RF$^+$modPM6000

Figure 4.29. Trial 5 levels of simplexin in bacterial cultures measured at the initial time of inoculation (T 0h) and following incubation at 39 °C for one week (T 168h), when grown in Hungate tubes of either spiked RF+mod broth or RF+modPM6000 broth. Hungate tubes were secured to a rocking platform rocking on an angle that prevented the liquid culture touching the butyl rubber stopper (Controlled rocking).

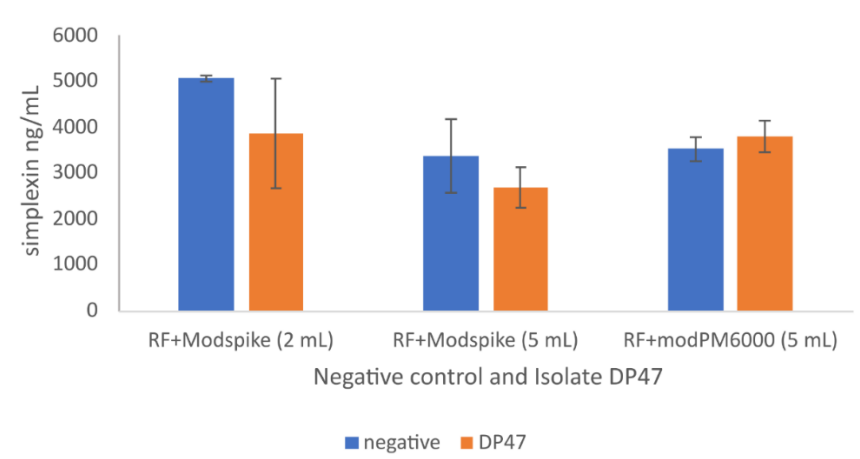


In Trial 5s, a single isolate, DP47 (Lachnospiraceae bacterium), was grown in 2 mL RF+mod spiked broth, 5 mL RF+mod spiked broth and RF+modPM6000 with a Wickerham card scores for cell density recorded after 48 h and 168 h incubation at 39 °C with controlled rocking (Table 4.14). The concentration of simplexin was measured after one week's growth (T 168h) with controlled rocking of all broth cultures and controls. The simplexin levels present in the DP47 cultures were not significantly different ($P > 0.05$) compared to the negative controls Figure 4.30.

Table 4.14. Trial 5s isolate (DP47), growth assessment (Wickerham card score) in Trial media after 48 h incubation at 39 °C with Controlled rocking.

Isolate ID	Nearest relative (16S rRNA gene species ID)	Culture Density RF^+mod spiked (2 mL)		Culture Density RF^+mod spiked (5 mL)		Culture Density RF^+modPM6000 (5 mL)	
		48 h	168 h	48 h	168 h	48 h	168 h
DP47	Lachnospiraceae bacterium)	+	+	+	+	++	+++
Negative		-	-	-	-	-	-

Figure 4.30. Levels of simplexin measured in negative Hungate tubes of sterile RF$^+$modPM6000 and 2mL or 5 mL of RF$^+$mod spiked broths and the corresponding cultures of DP47, following incubation at 39°C for one week (T 168h) with Controlled rocking.



Trial 6

The six isolates in Trial 6, were grown in RF+mod spiked broth (x1 and x2 simplexin concentration) with Wickerham card scores for cell density recorded after 28 h incubation at 39 °C with Controlled rocking (Table 4.15). The concentration of simplexin was measured after one week's incubation (T 168h) with either Static or Controlled rocking Figure 4.31. The simplexin levels present in the six isolate cultures either incubated static or with controlled rocking were not significantly different ($P > 0.05$) to the levels present in their corresponding negative control.

Table 4.15. Trial 6 isolates, their identity based on highest 16S rRNA sequence match and growth assessment (Wickerham card score) in pre-trial media and in Trial media after 28 h incubation at 39 °C with Controlled rocking.

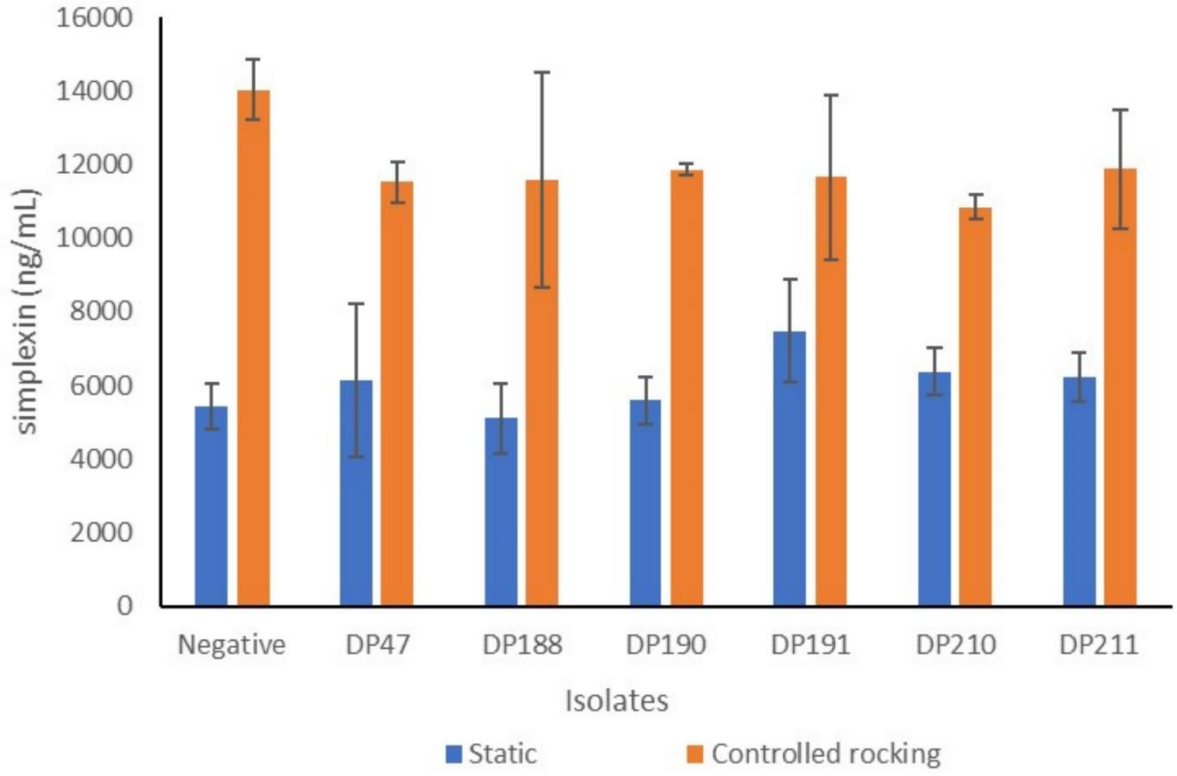
Isolate ID	16S rRNA gene species ID	Pre-trial RF$^+$mod spiked x1[A]	RF$^+$mod spikedx1 (5 mL) T 28h[B]	RF$^+$mod spikedx2[C] (10 mL) T 28h
DP188	*Pseudobutyrivibrio xylanivorans*	++	+++	+
DP190	*Prevotella ruminicola*	+	+	+
DP191	*Pseudobutyrivibrio xylanivorans*	+++	+++	+++
DP210	*Treponema* sp.	++	+	+
DP211	*Lachnospira pectinoschiza*	+++	+++	+
DP47	Lachnospiraceae bacterium	+	+	+
Negative		-	-	-

[A]RF$^+$mod spikex1 equivalent simplexin concentration of 6,000 ng/mL

[B]Data recorded at 28 h post inoculation due to snap 3-day COVID lockdown commencing

[C] RF$^+$mod spikex2 equivalent simplexin concentration of 12,000 ng/mL

Figure 4.31. Levels of simplexin measured in RF$^+$mod spike x1 (6,000 ng/mL simplexin) and spiked RF$^+$mod spike x2 (12,000 ng/mL) broth cultures and negatives (sterile media) following incubation at 39 °C for one week (T 168h) either upright (Static, spike x1) or secured to a rocking platform rocking on an angle that prevented the liquid touching the butyl rubber stopper (Controlled rocking, spike x2).



Isolate abundance in fermentation microbiomes

The microbiomes of selected *in vitro* fermentations have been sequenced and analysed (**Section 4.2.6**) To determine if the bacteria, isolated using the selective media, were present as detectable populations within the *in vitro* fermentations, the V3-V4 variable region of the 16S rRNA sequences from 40 isolates were selected and trimmed. Initially, the trimmed sequences were compared to the sequences of Feature representatives in 89 samples in the Fermentation microbiome datasets from seven fermentations consisting of the inoculum sample (rumen fluid or Day 30 fermentation fluid) used to start the fermentation and selected days across the fermentation. Table 4.16 details the percentage homology match to a representative Feature, the number of samples it was in and relative abundance across the microbiome data set.

Table 4.16. Relative abundance of bacterial isolates, as indicated by the proportion of homologous (>97% homology) sequence reads present within the overall microbiome dataset of fermenter fluid samples and rumen fluid starters.

Isolate ID	Nearest Relative (% Homology to near full length 16S rRNA gene seq)[A]	Feature representative seq homology[B] (%)	Number of sequences[C]	Number of samples detected in[D]	Relative abundance in total dataset[E] (%)
DP94 ★	*Streptococcus equinus* strain CNU 30	100	238,577	68	3.04
DP162 ★	*Streptococcus equinus* strain CNU 30	100	238,577	68	3.04
DP172 ★	*Streptococcus equinus* strain CNU 30	100	238,577	68	3.04
DP190	*Prevotella ruminicola* strain CA6	100	147,864	48	1.88
DP181	*Megasphaera elsdenii* strain 14-14	99.77	118,474	36	1.51
DP27	*Pseudobutyrivibrio ruminis* strain L4	100	112,203	87	1.43
DP97	*Butyrivibrio fibrisolvens*	100	112,203	87	1.43
DP188	*Pseudobutyrivibrio xylanivorans* strain MA3014	100	112,203	87	1.43
DP191	*Pseudobutyrivibrio sp*. strain CA18	100	112,203	87	1.43
DP28	*Prevotella ruminicola* strain BP1-40	100	71,782	50	0.91
DP90	*Eubacterium sp*. strain TW2	100	62,337	56	0.79
DP179	*Eubacterium sp*. strain TW2	100	62,337	56	0.79
DP204	*Eubacterium sp*. strain TW2	100	62,337	56	0.79
DP208	*Eubacterium sp*. strain TW2	100	62,337	56	0.79
DP211	*Lachnospira pectinoschiza* isolate M46	100	37,688	46	0.48
DP212	*Lachnospira pectinoschiza* isolate M46	100	37,688	46	0.48
DP40	*Butyrivibrio hungatei*	100	34,440	10	0.44
DP89	*Pseudobutyrivibrio sp.* strain CA18	100	18,381	63	0.23
DP198	*Pseudobutyrivibrio sp.* strain CA18	100	18,381	63	0.23
DP167	*Mannheimia succiniciproducens* MBEL55E	99.77	16,228	30	0.21
DP189	*Ruminococcus flavefaciens* strain LP-C14-Adx	100	16,070	40	0.20
DP218	*Butyrivibrio crossotus* strain DSM 2876	100	14,070	43	0.18
DP219	*Lachnoclostridium pacaense* strain Marseille-P3100	100	14,070	43	0.18
DP31	*Enterocloster clostridioformis* strain FDAARGOS_739	100	12,506	60	0.16
DP98	*Enterocloster clostridioformis* strain FDAARGOS_739	100	12,506	60	0.16
DP101	*Enterocloster clostridioformis* strain FDAARGOS_739	100	12,506	60	0.16
DP88	*Escherichia coli* strain PK5086	100	11,454	41	0.15

DP77	*Streptococcus henryi* strain OZK31	100	7,598	41	0.10
DP100	*Streptococcus henryi* strain 126	100	7,598	41	0.10
DP78	*Succinivibrio dextrinosolvens* strain ASCUSBF53	100	6,578	46	0.08
DP186	*Butyrivibrio sp*. strain CG9	100	5,328	58	0.07
DP95	*Kandleria vitulina* strain JCM 1143	100	3,802	39	0.05
DP210	*Treponema sp*. strain JC4	100	2,824	19	0.04
DP81	*Agathobacter ruminis* strain JK623	100	1,721	28	0.02
DP25	*Streptococcus macedonicus* strain W64	100	1,103	7	0.01
DP163	*Streptococcus gallolyticus* strain FDAARGOS_755	100	1,103	7	0.01
DP93	S*elenomonas ruminantium* subsp. *lactilytica* strain JCM 7528	100	44	3	<0.01
DP47	Lachnospiraceae bacterium CA60	100	26	2	<0.01
DP51	Rumen bacterium strain NK4C38	100	26	2	<0.01
DP168	*Fusobacterium varium* strain NCTC10560	97.54	15	2	<0.01

★ Bacterial isolates representative of the most highly abundant Feature identified within the microbiome sequence dataset (of 89 fermenter fluid and rumen fluid starter samples).

[A]Closest strain-level identity assigned following BLASTn of near full-length 16S rRNA gene sequences for each isolate against the NCBI nr nt database (accessed July- August 2021).

[B]% homology of the 16S rRNA gene sequence for each isolate, to the representative sequences for each Feature identified in the 16S rRNA gene amplicon sequence dataset (Features identified from a total of 7,855,927 sequence reads).

[C]Number of sequence reads corresponding to each Feature (quality filtered and merged F and R amplicon sequence reads).

[D]Number of daily Fermenter samples and starter rumen fluid samples that each isolate's 16S rRNA gene sequence was detected in (from a total of 89 samples).

[E]Relative abundance as a % of the total number of sequence reads in the total dataset (7,855,927 quality filtered and merged R and R amplicon sequence reads).

4.3.3 Summary results – bacterial isolations

Selective mediums were developed to isolate individual bacterial species, potentially capable of degrading simplexin, from the mixed populations present in the *in vitro* fermentations. The original selective media contained fermentation fluid as a base and Pimelea plant material as the source of simplexin. Due to poor growth of the bacteria the media was modified to use rumen fluid as a base and the Pimelea plant material replaced with an ethanol extract of Pimelea plant material which contained simplexin along with a range of other ethanol soluble plant compounds. A third selective isolation media contained a semi-pure extract of simplexin prepared by HPLC.

Initial isolation experiments using fermenter fluid from two cattle-derived fermentations as source material, and Pimelea plant material to provide simplexin, resulted in 56 bacterial isolates identified as 11 different species. These 11 species represent bacteria commonly found in the rumen and involved in the degradation of plant structural carbohydrates, proteins and starch. Source material was also obtained from four different fermentations started with either rumen fluid from goats, sheep, cattle or kangaroo forestomach, and bacterial isolation experiments undertaken using an ethanol extract of Pimelea plant material. In this way, a total of 157 bacterial isolates were obtained and identified as 21 different species. Many of these were species that are well known in the rumen as carbohydrate fermenters producing volatile fatty acids such as succinate, acetate, formate and lactate. In addition, source material was obtained from a sheep rumen fluid fermentation and isolations undertaken using the selective media containing semi-pure extract simplexin extract. This resulted in 44 bacterial isolates, identified as 10 species. As well as many species similar to those already isolated, one isolate was identified as *Treponema sp*., a spiral-shaped bacterial species found in the rumen which is largely uncharacterised. However, a previous published study genome sequenced *Treponema sp*., identifying an assortment of novel enzymes important for plant fibre breakdown, including cellulases, endohemicellulases, and debranching enzymes as well as a suite of carbohydrate esterases (Rosewarne et al. 2012).

A simplexin degradation assay was developed and used to assess a representative bacterial culture of each of the 27 isolated species. The results of the initial assays looked promising with a number of bacterial isolates having lower simplexin concentrations after growth, than the negative control of media only. However, inconsistencies in the simplexin concentrations within the negative controls prompted further experiments to investigate the losses of simplexin at different stages of media preparation and during the incubation with rocking. The results determined that simplexin was binding to the butyl rubber stopper of the Hungate tubes used to maintain the anaerobic conditions when growing the bacterial isolates, when full rocking of the tube was undertaken. Further experiments indicated that none of the bacterial isolates tested, were capable of degrading simplexin.

4.4 Novel Biopolymers tested for capacity to hold and release simplexin under simulated rumen conditions *in vitro*

4.4.1 Selected biopolymers

A total of 27 biopolymer composites were tested to evaluate their degradation performance in the *in-vitro* simulated rumen environment. Four types of biopolymer were selected for their biodegradability as well as their compatibility for medical applications (Asghari et al. 2017; Barouti et al. 2017; Laycock et al. 2017; Davoodi et al. 2018; Elmowafy et al. 2019; Harting et al. 2019;

Rodríguez-Contreras et al. 2019) or veterinary applications (Rathbone and McDowell 2013; Bilhalva et al. 2018).

The four polymers chosen were:

1. Polyhydroxyalkanoate (PHA): which is a polyester produced by numerous microorganisms, through bacterial fermentation of carbon feedstocks. It is typically biodegradable in any environment where bacteria are present.
2. Polycaprolactone (PCL): which is a biodegradable polyester produced by ring opening polymerisation of ε-caprolactone using a catalyst.
3. Polylactic acid (PLA): which is a biodegradable (compostable) thermoplastic derived from renewable resources (fermented plant starch).
4. Starch: starch is a natural polymer derived from plants. Structurally, starch is composed of two macromolecules: amylose and amylopectin. To generate thermoplastic starch polymers, the complex semicrystalline structure needs to be destroyed to produce an amorphous material. This process called gelatinisation, is achieved by heating the starch in the presence of water and/or some other plasticiser.

4.4.2 *In vitro* biopolymer degradation in Fermentations 1 to 4, 6, 8, 9, 10 and 12

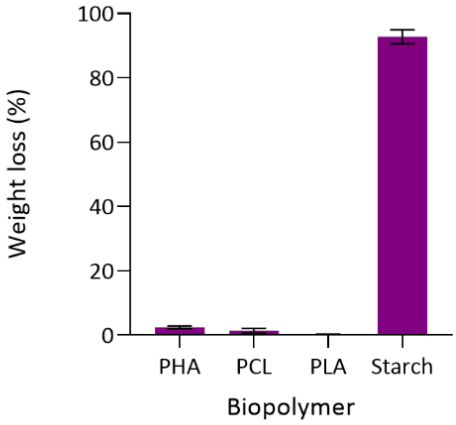
Initial screening (Fermentation 1)

The initial fermenter (Fermentation 1) was run for 11 days, in order to do a first screening of the polymer performances of selected four biopolymers (PHA, PCL, PLA and Starch) *in vitro*. The initial image of the different polymer ribbons is presented in Figure 4.32**.**

Figure 4.32. Extruded ribbon of the four different biopolymers prior to fermentation.

Biopolymers placed in the fermenter for 11 days did show some weight loss, which is associated with microorganisms present in the fermenter that are using the biopolymers as a source of energy. The starch sample was almost completely degraded after 11 days (Figure 4.33), being reduced to small flakes, rendering it very hard to retrieve from the nylon bag at the end of the trial. All three other biopolymers were significantly less degraded than starch ($P < 0.0001$), with less than 2% weight loss, compared to the percentage weight loss of starch which was 92%.

Figure 4.33. Percentage weight loss of the four different polymers after 11 days in Fermentation 1.



The surfaces of the samples were also analysed using SEM, with the images of the samples before and after 11 days in the fermentation being presented in Appendix Section 9.11. Prior to fermentation exposure, all the samples presented a smooth surface. After 11 days in the fermenter, the PHA samples had a large number of holes on the surface of the ribbon, which is characteristic of biodegradation, while PCL had fewer holes, and PLA appeared to be as smooth as at the start of the fermentation. The SEM results are in agreement with the percentage weight loss observed for the different samples.

The DSC results did not show any variation between the initial sample and the sample that spent 11 days in the fermenter, suggesting that the degradation was mainly occurring on the surface of the polymer, in line with expectation.

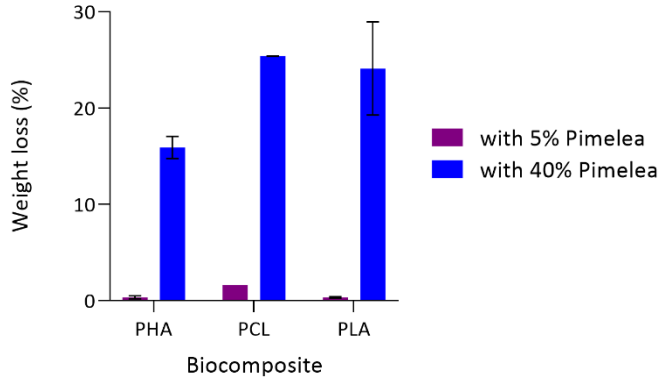
From this preliminary trial, it was concluded that the biopolymers could be a source of energy for the microbial populations present in the fermenter. In addition, the microbial populations maintained high levels of diversity, indicating that the presence of the biopolymers in the fermenter did not have a negative impact on the microbial populations. From this trial it was decided to exclude the biopolymer starch from further investigation as it degraded too rapidly; only PHA, PCL and PLA were investigated further with different Pimelea loadings. This work is presented in the next section.

Performance of biopolymer composites loaded with 5 wt% and 40 wt% Pimelea (Fermentation 2)

A 20-day fermentation trial with biopolymer loaded with 5 wt% or 40 wt% of milled Pimelea was undertaken in order to establish the effect of the Pimelea loading on both the biopolymer degradation rate and the fermenter microbial populations.

Discoloration of the biopolymer containing 40 wt% Pimelea was observed following fermenter exposure, with a slight whitening of the material being due to the formation of small surface holes and associated light scattering. This correlates with the percentage weight loss of the biopolymer samples, where samples with the 40 wt% Pimelea showed over 15% weight loss, while the samples with 5 wt% Pimelea presented less than 2% weight loss (Figure 4.34). This is linked to the presence of Pimelea, which may be easier for microorganisms to use as a source of energy compared to the biopolymers but also which allows pathways for ingress of the bacteria and associated enzymes into the bulk of the polymer, accelerating degradation. Statistically, the weight loss was significantly higher for samples with 40 wt % Pimelea than 5 wt% Pimelea ($P < 0.0001$ for PCL and PLA and $P=0.0008$ for PHA).

Figure 4.34. Percentage weight loss of the biopolymer composites after 20 days in Fermentation 2.



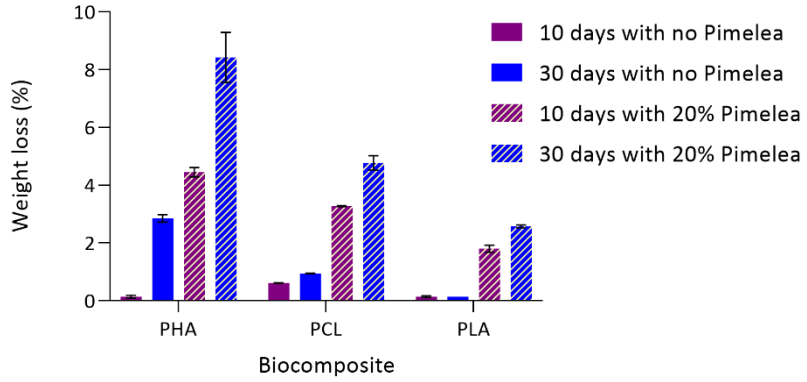
The surfaces of the samples were analysed using SEM, with the images of the samples before and after exposure being presented in Appendix Section 9.11. Overall, the initial samples with only 5 wt% Pimelea loading showed very smooth surfaces initially, which is in contrast with the samples loaded with 40 wt% Pimelea, where the surface was rougher and some Pimelea fragments were directly exposed on the surface of biopolymer matrix. This rough surface may be enabling the microbial population to more easily attach and access both Pimelea and biopolymer.

After 20 days in the fermenter SEM images indicated that the biopolymer surface was similar to the initial stage. Only PCL + 40 wt% Pimelea showed some holes on the surface. From this trial it was decided to investigate the performance of biopolymer composites with a Pimelea loading of 20% over 20 days in a fermenter.

Performance of biopolymer composites loaded with 20 wt% milled Pimelea in vitro (Fermentation 3)

A 30-day fermenter trial including biopolymer loaded with 20 wt% of Pimelea was completed. Biopolymer samples were retrieved after 10 and 30 days in Fermentation 3 to establish the biopolymer degradation rate (Figure 4.35). All the samples loaded with 20 wt% Pimelea lost significantly more weight ($P < 0.0001$ at 30 days) than the neat biopolymer. This again suggests that Pimelea is more easily digested by the fermenter microbial population and/or enables better access to the interior of the biopolymer composite for the bacteria and associated enzymes, promoting biodegradation. From this study, it was observed that the different biopolymer composites had different degradation rates, with the PHA + 20 wt% Pimelea sample showing the largest mass loss of 9%, followed by PCL + 20 wt% Pimelea, with a mass loss of 5%, then finally PLA + 20 wt% Pimelea, which showed the slowest degradation rate, with a mass loss of 3% (Figure 4.35).

Figure 4.35. Percentage weight loss of the biopolymer composites over time in Fermentation 3.



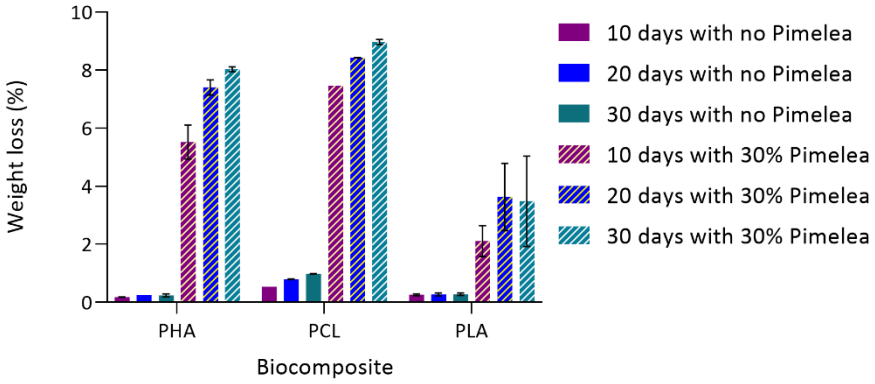
From the SEM imaging of the surfaces of the samples (Appendix Section 9.11), it was observed that both PHA samples had more visible holes on the surface following exposure to the fermenter, which are good signs of degradation. The presence of microorganisms was also observed, which is confirmation of bacterial attachment and activity. By contrast, the PCL samples and PLA samples had fewer holes detected on the surface, which is consistent with them being less degraded. In the case of PLA + 20 wt% Pimelea, it was clearly observed that the Pimelea fragments that are accessible on the surface of the biopolymer were being degraded, while the PLA surface remained smooth.

Performance of biopolymer composites loaded with 30 wt% milled Pimelea (Fermentation 4)

A 30-day fermenter trial with biopolymer composites loaded with 30 wt% of Pimelea was completed. This Fermentation 4 was fed daily with Pimelea only instead of a mix of Buffel grass and Pimelea. This feed change seemed to have an effect on the biopolymer degradation, as presented below.

For this fermentation, biopolymer samples were retrieved every 10 days to establish the biopolymer degradation rate. The percentage weight loss over time of the different formulations is presented in Figure 4.36. Both biopolymer composites (PCL + 30 wt% Pimelea and PHA + 30 wt% Pimelea*)* showed a steady weight loss over time after 20 days. Neat biopolymers showed a significantly slower degradation rate compared to biopolymers loaded with Pimelea (after 30 days, $P < 0.0001$ for PCL and PHA, $P = 0.0002$ for PLA). The biopolymer PLA composites, with Pimelea, were significantly slower to degrade compared to biopolymer composites containing PHA and PCL ($P < 0.0001$ at 30 days). Without Pimelea there was no significant difference between the three biopolymers at each time point ($P > 0.05$).

Figure 4.36. Percentage weight loss of the biopolymer composites over time in Fermentation 4.



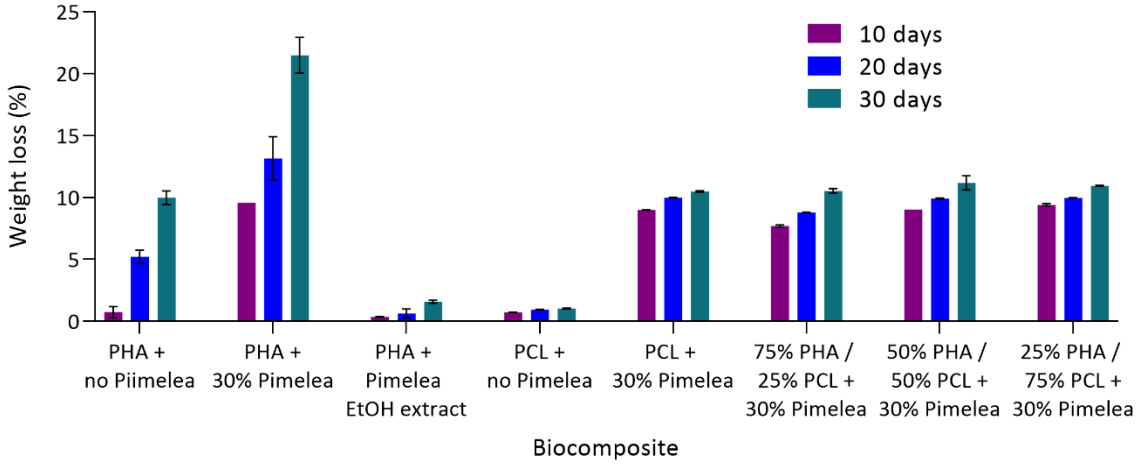
Interestingly, for this trial, at the 30-day mark, almost all samples showed the presence of microorganisms attached in pockets and surfaces with holes, as revealed by the SEM analysis (Appendix Section 9.11). This had not been observed before and could be linked to the different feeding regime of this fermenter, which was only fed Pimelea instead of a mix of Buffel grass / Pimelea as used for previous fermentations.

Here again the SEM images correlate well with the percentage weight loss, where PLA samples did not present a large degradation, while PHA showed the presence of holes and good degradation of Pimelea on the surface of the biopolymer.

Degradation performance of biopolymer loaded with 30 wt% milled Pimelea vs biopolymer loaded with Pimelea ethanol extract in vitro (Fermentation 6)

For this fermentation, a biopolymer PHA loaded with Pimelea ethanol extract was evaluated, the aim being to compare the degradation behaviour of a biopolymer composite loaded with a higher concentration of simplexin and without fibres in the final material. A blend of PHA with PCL was also tested in order to assess how this altered the degradation rate of the overall biopolymer. This trial ran for 30 days, and samples were retrieved every 10 days in order to evaluate the degradation rate of each biocomposite formulation. The percentage weight loss over time of the different formulations is presented in Figure 4.37.

Figure 4.37. Percentage weight loss of the biopolymer composites over time in Fermentation 6.

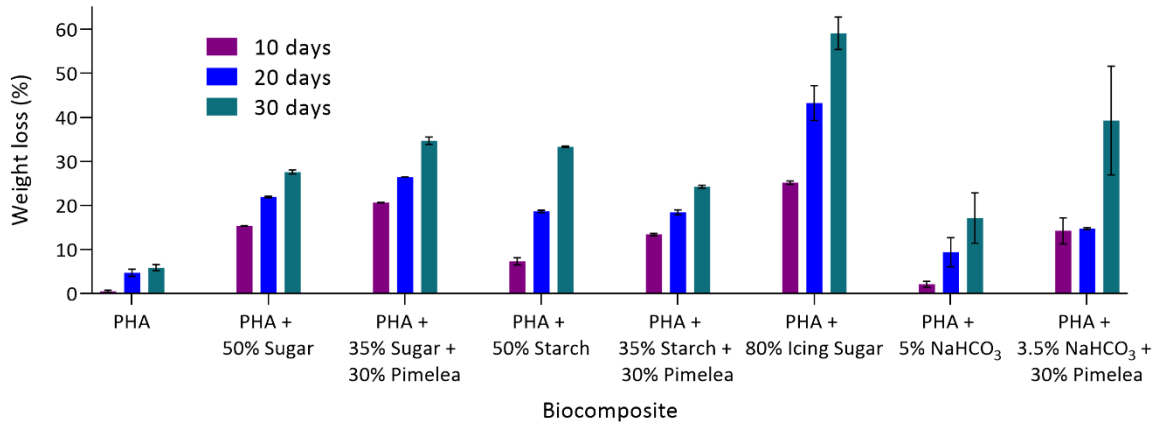


The biopolymer composite of PHA loaded with 30 wt% Pimelea was the fastest to degrade, reaching about 23% weight loss over 30 days in the fermenter (Figure 4.37). Interestingly, the mix of PHA/Pimelea ethanol extract had a different degradation profile than PHA alone. The presence of Pimelea extract rather than 30 % Pimelea plant material seemed to significantly slow down the degradation process, as observed by both the weight loss ($P < 0.0001$ at each time point) and microscopy analysis (Appendix Section 9.11), with little evidence of surface pitting in this case. It is noted that this particular material had a glossy, dark green surface and was more rigid overall. It is possible that a combination of factors are playing a role in this low mass loss outcome: the components in the extract may be crosslinking or otherwise being modified during extraction to form a more rigid, water impermeable skin, acting as a barrier to water and enzyme penetration; and the relative hydrophobicity and/or smoother surface may slow the formation of a biofilm and/or affect the initial adsorption of the hydrolases to the surface. It was however observed that this extract-based biocomposite exhibited a more consistent and sustained release when compared with the biopolymers incorporating milled material (Figure 4.37). Further work is needed to fully understand and characterise the release from these extract-based formulations. The degradation rate for the combination of PHA/PCL (4:1, 1:1, 1:4) with 30 wt% milled Pimelea was similar to that of PCL with 30 wt% milled Pimelea over the first 30 days ($P > 0.05$). It was again observed that when the Pimelea plant material was accessible it was easily assimilated by microorganisms present.

Performance of porous biopolymer with 30% Pimelea in vitro (Fermentation 8)

In order to further enhance the biocomposite degradation rate, porous biocomposites were produced, with the expectation that the presence of holes and interconnections should allow for a faster degradation rate given that there is an increased exposed surface area and potentially microorganisms will be able to access further the bulk of the biopolymer via the interconnected pathways (Raeisdasteh Hokmabad et al. 2017; Jin et al. 2019). In order to do so, soluble porogens such as sugar (Hou et al. 2003; Wei and Ma 2006; Tan et al. 2011; Song et al. 2016) and starch were introduced into the polymer matrix. It is noted that many other porogens can be used, such as salt. However, sugar and starch were selected based on their food grade quality as well as their ability to dissolve in water and be easily digested by rumen bacteria. Following extrusion, extruded samples were immersed in water for 1 day in order to dissolve out some of the sugar. The samples were then dried prior to use in the fermenter. Another alternative was to use a foaming agent such as sodium bicarbonate (SB), which is also a food grade ingredient. The sodium bicarbonate produces CO_2 in the extruder, thus acting as a foaming agent. However, it was only possible to extrude the PHA foamed with sodium bicarbonate in a string profile not in a ribbon stage, as testing showed it was not possible to get the material to extrude and hold in a large ribbon shape. The different samples were tested *in vitro*, in a fermenter for 30 days, with samples being retrieved every 10 days (Figure 4.38).

Figure 4.38. Percentage weight loss of non-porous and porous biopolymer composites over time in Fermentation 8.



The PHA sample loaded with 80% sugar showed a very fast degradation rate compared to PHA ($P < 0.0001$ at 10, 20 and 30 days), with over 40% mass loss after 20 days. The combinations of PHA + 35% Sugar or starch with 30 wt% milled Pimelea composites presented a gradual degradation rate (Figure 4.38). The porous PHA with 3.5% sodium bicarbonate + 30 wt% milled Pimelea showed a high percentage loss in the samples after 30 days in the fermenter, which might be due to the fact that the samples were very brittle and were difficult to retrieve from the nylon bag, so this value might be over-estimated.

Porous biopolymer samples prepared using sugar presented numerous holes that were well interconnected after 30 days in the fermenter as seen in SEM images (Appendix Section 9.11). This was also observed for the sample incorporating starch. However, when the PHA + starch sample was loaded with Pimelea, the degradation rate seemed to slow down although not significantly ($P > 0.05$), (Figure 4.38). Finally, the foamed samples using sodium bicarbonate presented some pores in the cross section, but not so many were detected on the surface (Appendix Section 9.11); the biopolymer was degraded but not as drastically as the other samples. This study demonstrated that by producing a more porous biocomposite the degradation rate could be accelerated.

From those biodegradation studies, that showed some very fast degradation rates *in vitro*, the simplexin release for some selected samples were measured following the method developed using UHPLC-Q-Orbitrap-MS analysis as described in Section 3.9.12 and Section 3.5, as it is crucial to evaluate how the rate of toxin release is related to the degree of mass loss of the biocomposite. This LC-MS/MS analysis is presented below. In addition, given that the biocomposite will need to remain in the cattle rumen for a longer period of time than 30 days, a further fermentation study (Fermentation 9) over two months was performed, using the most promising formulations.

LC-MS/MS analysis of Simplexin release in vitro from Fermentations 6 and 8

A reliable and robust method for the quantification of simplexin present in biopolymer composites containing both Pimelea plant material and Pimelea extract was developed and validated utilising solid phase extraction (SPE) combined with ultra-high-performance liquid chromatography quadrupole orbitrap mass spectrometry (UHPLC-Q-Orbitrap MS/MS). This method demonstrated good recoveries of simplexin (> 93%) and was used to undertake preliminary analysis of simplexin release in selected biocomposites recovered from Fermentations 6 and 8.

These selected biocomposite samples from Fermentations 6 and 8 were used to determine the simplexin levels in samples before and after exposure to microbial degradation over periods of time

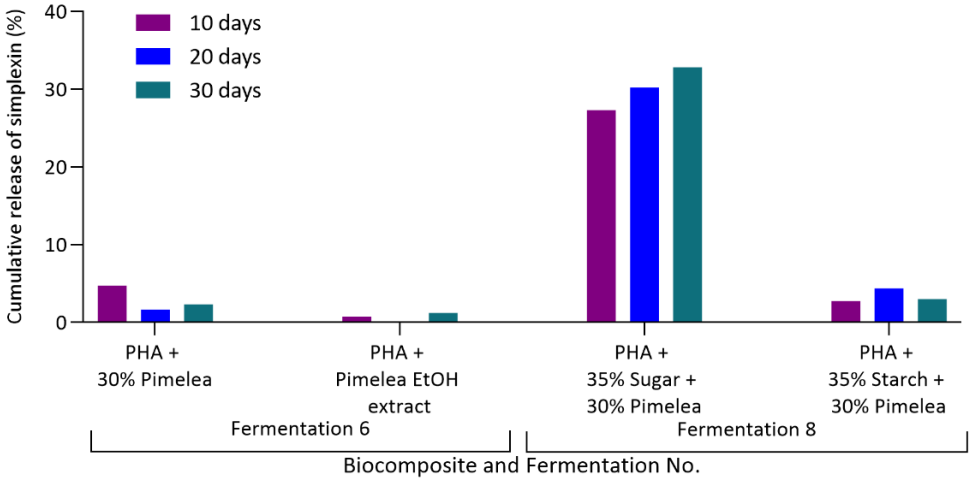
within *in vitro* fermentations. Given the known concentration of simplexin in these biocomposite samples, the cumulative release of simplexin was calculated using the following equation:

$$\text{cumulative release of simplexin } (\mu g) = M_0 \cdot C_0 - M_i \cdot C_i$$

where M_0 is the mass of the matrix before placement into the fermentation system; C_0 is the initial concentration of simplexin within the matrix (µg/g); M_i is the mass of the matrix after exposure to the fermentation system for *i* days (g); and C_i is the concentration of simplexin within the matrix after exposure to the fermentation system for *i* days (µg/g). The percentage cumulative loss was then calculated as a percentage of the starting mass of simplexin in the biopolymer.

The percentage cumulative released amounts of simplexin from the biocomposites PHA + 30 wt% milled Pimelea, PHA + Pimelea ethanol extract, PHA + 35 wt% Sugar + 30 wt% milled Pimelea and PHA + 35 wt% Starch + 30 wt% Pimelea, after being placed within an *in vitro* fermentation system for 10, 20, and 30 days, are shown in Figure 4.39. The results shown represent preliminary results and are analyses of single samples at each time point with non-homogeneity in the extruded biopolymer causing issues in some instances, particularly where samples were analysed retrospectively.

Figure 4.39. Percentage cumulative release of simplexin over time for the different biocomposite formulations in *in vitro* Fermentation 6 (PHA + 30% Pimelea, PHA + Pimelea extract) and Fermentation 8 (PHA + 35% Sugar + 30% Pimelea, PHA + 35% starch + 30 % Pimelea).



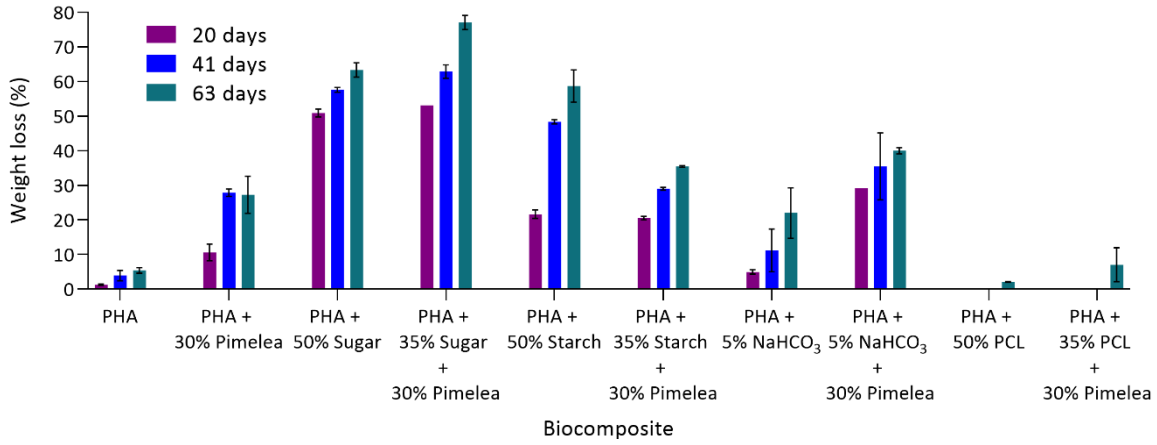
For PHA + 30 wt% Pimelea, the calculated results appeared to present only a small release of simplexin from the biopolymer (less than 5% simplexin release). The simplexin concentration in the PHA + Pimelea ethanol extract biopolymer was determined as 217 µg /g biocomposite post extrusion. The simplexin release from this PHA + Pimelea ethanol extract biopolymer in Fermentation 6 was also small (Figure 4.39), and was consistent with the sustained slow degradation rate of this biopolymer composite as seen in both percentage weight loss (Figure 4.37) and SEM studies (Appendix Section 9.11). Compared with these two types of non-porous PHA biocomposites, sugar and starch were incorporated into PHA matrix to allow for the manufacturing of a porous biocomposite matrix, enabling internal access for microorganisms, enzymes and water. The intent was to demonstrate that it was possible to significantly accelerate the release of simplexin as well as the biodegradation rate of PHA + Pimelea. A promising release of simplexin was achieved from the PHA + 35 wt% Sugar + 30 wt% milled Pimelea, with 27% of simplexin released after 10 days and 32 % after 30 days and these results parallel the percentage mass loss seen in Figure 4.38. However, for PHA + 35 wt% Starch + 30 wt% Pimelea, the released amounts of simplexin were less than expected (< 5%), which might be due to a slower degradation rate of this biocomposite system compounded

by the heterogeneity of the extruded matrix. An initial analysis of biocomposite reproducibility and methods to improve matrix uniformity such as cryo-milling of Pimelea plant material has subsequently been conducted and is reported in Section 4.4.4**.**

Performance of porous biocomposite loaded with 30 wt% milled Pimelea in vitro over 63 days (Fermentation 9)

A 63-day fermentation trial was performed to evaluate the biocomposite degradation rate over a longer period of time, as the final biocomposite bolus will need to remain in the cattle rumen for a longer period of time than 30 days. Details of the fermentation are contained in Section 3.9, and Figure 4.40 summarises the degradation pattern of the samples over the 63 day trial measured as percentage mass loss for both porous and non-porous biocomposites.

Figure 4.40. Percentage weight loss of biocomposites over a 63-day trial (Fermentation 9).



After 63 days, the PHA + 35 wt% Sugar + 30 wt% milled Pimelea biocomposites were almost completely degraded and were hard to remove from the nylon bags as they were falling apart. It appears that combination of PHA + 35 wt% Sugar with 30 wt% Pimelea would be a good candidate for fast degradation (toxin release) as it lost 77% of its initial weight after 63 days. This conclusion is also valid for PHA + 35 wt% Starch with 30 wt% Pimelea biocomposite, which also presented around 35% weight loss after 63 days. The porous PHA with 5 wt% $NaHCO_3$ (SB) + 30 wt% Pimelea biocomposite showed a slower degradation rate compared to the samples with sugar or starch, although not significant ($P > 0.05$) until 63 days compared to the sugar biocomposite ($P < 0.0001$).

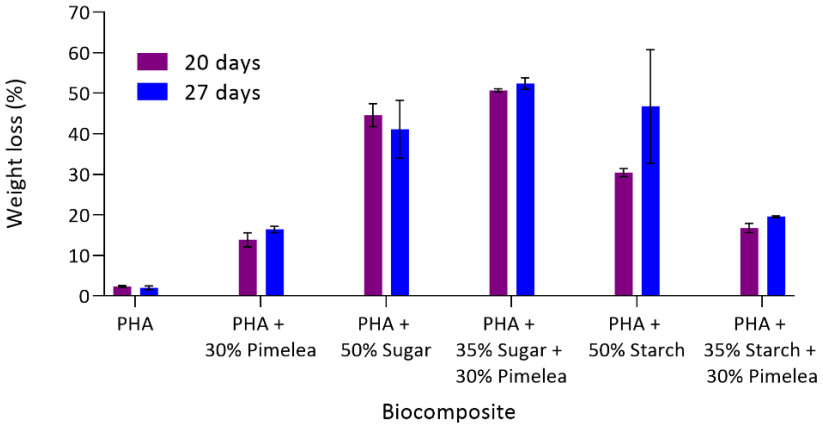
The PHA sample loaded with 35 wt% Sugar and 30 wt% milled Pimelea showed very significant evidence of degradation under SEM analysis, with interconnection of pores (Appendix Section 9.11) that increased in size over time. At the end of the trial, it was difficult to remove the sample from the bag without breaking it. This was not observed for the PHA + 35 wt% Starch + 30 wt% milled Pimelea. It was again observed that when Pimelea particles are accessible, they are easily assimilated by microorganisms present in the fermentation. The combination of PHA + 50% Sugar or starch with 30% Pimelea seemed to be the best candidates for a fast-degrading biopolymer.

Performance of porous biocomposites loaded with 30 wt% milled Pimelea in vitro over 30 days (Fermentation 10)

The opportunity was available to assess performance of the most promising biocomposite formulations in fermentations containing microorganisms from gut environments other than the cattle rumen. Fermentation 10 investigated the biocomposite behaviour in a fermentation started using forestomach contents obtained from a red kangaroo and fed only Pimelea plant material with fermentation details contained in Section 3.9. The biopolymers were introduced into the fermenter

on Day 3, instead of Day 1, and samples were retrieved after 20 and 27 days in the fermentation. The degradation pattern of the samples over the 27-day trial measured as percentage mass loss for both porous and non-porous biocomposites are presented in Figure 4.41**.**

Figure 4.41. Percentage weight loss of biocomposite over 27 days in Fermentation 10.

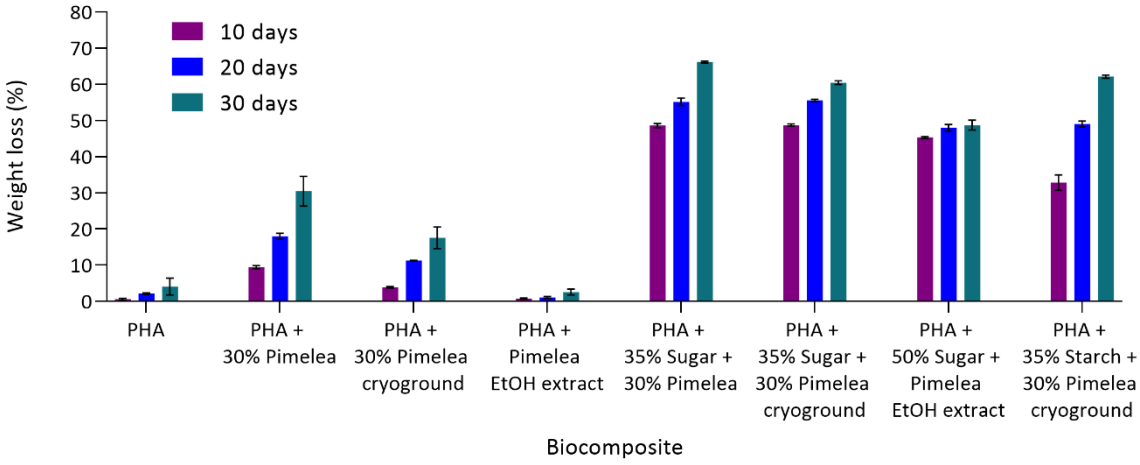


In the kangaroo forestomach based Fermentation 10, samples loaded with Sugar and Pimelea (PHA + 35 wt% Sugar + 30 wt% milled Pimelea) presented the largest amount of degradation, with approximately 50% weight loss after 27 days, which was similar to the weight loss observed at 20 days in the previous cattle rumen based Fermentation 9 (Figure 4.40). In the case of the samples loaded with Starch and Pimelea (PHA + 35 wt% Starch + 30 wt% Pimelea), they showed similar degradation rates (not significantly different, $P > 0.05$) to the PHA + 30 wt% Pimelea, and this was also observed at 20 days in Fermentation 9. This was also confirmed by the SEM imaging, which showed little degradation on the surface, as presented in Appendix Section 9.11. However, by Day 27 the PHA + 50 wt% Starch had degraded by approximately 45% in Fermentation 10 which was higher than seen at 20 days in Fermentation 9 where it had lost only approximately 20% of its mass. This increase in degradation could be due to a number of factors, such as the bacterial community being of kangaroo forestomach origin and hence potentially containing more starch/PHA degraders, or this particular material combination showing non-linear degradation performance that is only evident over the longer time period. Samples containing sugar presented large pores after 27 days of exposure in the fermenter, hence increasing the surface area of contact between the microorganisms and the biocomposite.

Performance of porous biocomposites loaded with 30 wt% of Pimelea either milled or cryo-milled Pimelea in vitro over 30 days (Fermentation 12)

In order to further extend the understanding of the biocomposite degradation rate, PHA biocomposites were produced with smaller size Pimelea particles, with the expectation that smaller particles would be easier to disperse in the polymer matrix than larger particles. This would allow for a better dispersion of Pimelea within the PHA matrix, and possibly increase the number of nucleation sites which would increase the overall PHA crystallinity. To do so Pimelea leaves were cryo-milled into a powder with particles size smaller than 150 µm. Pimelea ethanol extract was also investigated, as this form allows for a more concentrated Simplexin content. The performances of porous and non-porous PHA with three different types of Pimelea forms were assessed *in-vitro* for 30 days, with samples being retrieved every 10 days (Figure 4.42).

Figure 4.42. Percentage weight loss of biocomposite over 30 days in Fermentation 12.



The PHA sample loaded with 35% Starch and 30% Pimelea cryo-milled showed a fast linear degradation rate, with over 60% mass loss after 30 days. While samples containing sugar presented a burst release within the 10 first day which is associated with the loss in sugar which has been assimilated first. After 10 days it is slower to degrade compared to PHA with 35% Starch + 30% Pimelea. PHA with 35% Sugar and 30% Pimelea presented a weight loss around 65% after 30 days, while the sample with 35% Sugar and 30% cryo-milled Pimelea presented a lower weight loss, which reached 60% after 30 days. PHA samples loaded with 30% Pimelea presented a linear weight loss reaching 30% after 30 days, while the same samples loaded with 30% cryo-milled Pimelea presented also a linear weight loss but only reaching 17% after 30 days. As expected, the smaller size Pimelea particles are significantly slowing down the degradation rate ($P < 0.0001$ for PHA + 30% Pimelea vs PHA + 30% Pimelea cryoground at 30 days) of the overall biopolymer, which might be linked to an increase crystallinity as smaller particles can provide nucleation sites for more perfect crystals and would hence reduce the degradation rate of the overall biopolymer composite.

Samples containing Pimelea ethanol extract were significantly slower ($P < 0.0001$ from 20 days) to degrade compared to the same samples containing milled Pimelea plant material or cryo-milled Pimelea plant material. This was also confirmed by the SEM imaging, which showed little degradation on the surface, as presented in Appendix (Section 9.11).

It was again observed that when Pimelea plant particles are accessible, they are easily assimilated by microorganisms present in the fermentation. The combination of PHA + 35% Sugar or starch with 30% Pimelea seemed to be the best candidates for a fast degrading biopolymer.

4.4.3 Biodegradation of biopolymers *in vitro* (Fermentation 14)

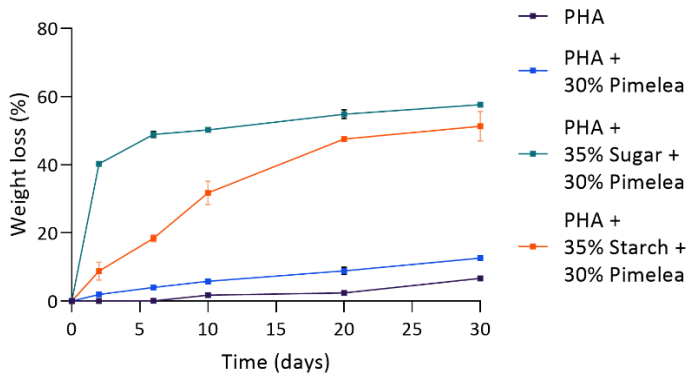
This study was undertaken as an independent study and has been published as Yuan et al. (2022).

Quantification of degradation by weight loss

Dry weight loss has been considered as an index for biodegradation of biopolymers. The percentage weight loss of the four biopolymers incubated in Fermentation 14 was plotted against different time points (Day 0, 1, 5, 9, 19 and 29) in Figure 4.43, where Day 0 is the commencement day of the trial. All biopolymers showed a linear weight loss trend, which is consistent with results obtained from previous fermentations, indicating a dominant surface erosion mechanism. Compared to neat PHA, the mass loss rate of PHA+30% Pimelea was higher due the incorporation of plant material. The

addition of the porogen, starch, accelerated the biodegradation rate even further. The sugar-porogen biopolymer had the greatest rate of weight loss, with an acute increase of weight loss from 0 to 40.3% being observed after only 24 h exposure to the fermentation environment. This is mostly due to leaching out of the theoretically 35 wt% of icing sugar in this biocomposite, which is apparently more rapidly removed/used by bacteria than the starch. After Day 5, this sugar-porogen biocomposite also showed a flattened trend of mass loss, in parallel with PHA+30% Pimelea biocomposite.

Figure 4.43. Percentage weight loss of biopolymer incubated in Fermentation 14



Micro-pore structure revealed by µ-CT

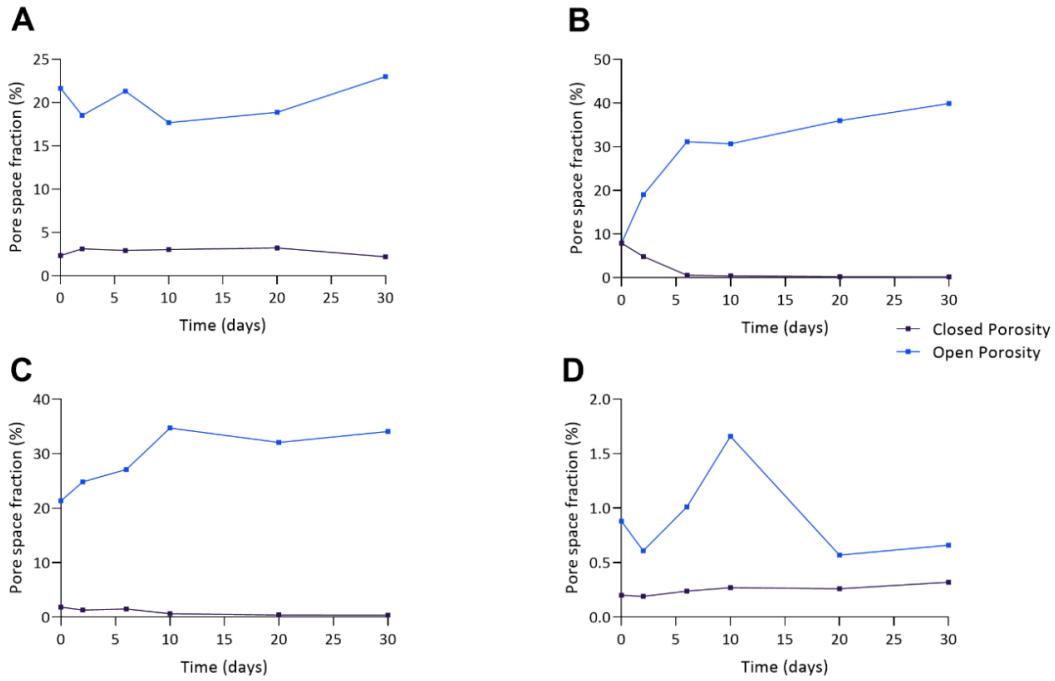
A direct visual illustration of the reconstructed 2D cross-sectional images for each type of biopolymer at different time point was presented in Appendix Section 9.12**.** No significant changes were observed for PHA+30%Pimelea and neat PHA. For both of these two biopolymers, huge voids with diameters larger than 500 μ m were observed in the middle of the matrix along the whole ribbon piece, as a result of air bubbles entrained in the matrix during the extrusion process. For sugar-porogen biopolymer, the interior pore network of the Day 0 sample looked very similar to PHA+30%Pimelea. After 24 h incubation in the fermenter, the Day 1 ribbon showed a much darker colour, suggesting a significant drop in the biocomposite's density, which is in agreement with the 40.3% weight loss and the remaining integrated shape. Based on the homogeneously distributed small voids that appeared in the Day 1 sample image, the incorporated sugar has leached out of the system. However, unlike these three biopolymers, the starch-porogen biopolymer showed a very different pore development. The density of this biocomposite didn't decrease as whole, as for sugar-porogen biopolymer, but layer by layer from the surface, indicating a moving front of starch removal and diffusion. The void size in the starch-porogen biopolymer was also much smaller, possibly due to the different extrusion approach used to manufacture this biocomposite.

Changes in open porosity and closed porosity

The computed open porosity and closed porosity for all the four biopolymers after 3D analysis are shown in Figure 4.44. There were no obvious changes in the open pore space fraction and closed pore space fraction in the PHA+30%Pimelea biopolymer samples at different time points, with a relative standard deviation (RSD) of 10.4% and 15.3%. A limited biodegradation degree was also confirmed for the neat PHA samples by the constant and small porosity compared with the plant material incorporated biopolymers. A sharp increase in open porosity from 7.95% to 31.1% of the sugar-porogen biopolymer was observed during the initial 5-day exposure to the fermenter. A consistent increase in open porosity from 21.4% to 34.7% was also observed for the starch-porogen biopolymer during the first 9-day incubation. For these two porogen incorporated biocomposites,

more and more closed pores became accessible to the environment over time resulting a decrease in the closed porosity.

Figure 4.44. The changes in open porosity and closed porosity of biopolymers incubated in Fermentation 14 for (A) PHA with 30% Pimelea, (B) PHA with 30% Pimelea and 35% sugar, (C) PHA with 30% Pimelea and 35% starch and (D) PHA.



Toxin (simplexin) release performances

The cumulative release of simplexin in the Pimelea biocomposites over time is presented in Figure 4.45. Within the 29-day incubation, only PHA+30%Pimelea biocomposite in Fermentation 14 showed a limited cumulative release of simplexin of 8.7% after 19 days and 10.5% after 29 days. Although the biodegradation of these biocomposites was strongly evidenced by the increasing weight loss over time and the microscopic μ-CT analysis, simplexin release from the matrix is not occurring at the rate expected, with an increasing concentration of simplexin being observed in all the three biocomposites over time of exposure. As shown in Figure 4.46, the estimated concentration of simplexin obtained by dividing the initial amount of simplexin embedded in the biocomposites at Day 0 by the dry weight of biocomposites at Day 1, 5, 9, 19 and 29, hypothesising no simplexin was released with the mass loss, closely matched the actual concentration of simplexin determined by the extraction and LC-MS/MS analysis. The addition of porogens indeed increased open porosity and the surface area, but the accelerated biodegradation of the biocomposites didn't contribute to the release of toxin.

Indeed, it may be considered that simplexin with its aliphatic chain may be preferentially adsorbed by the hydrophobic PHA surface rather than diffusing into the hydrophilic aqueous fermentation medium. As such the increased porosity afforded by inclusion of sugar/starch may have served to expose further PHA surface area for adsorption.

In previous fermentation trials additional Pimelea plant material was added daily to the fermentation media and our preliminary calculations suggested simplexin uptake by biopolymers rather than release. This phenomenon needs to be explored more rigorously with individual biopolymers but does suggest the potential for biopolymers to act as simplexin-sponges within rumen systems.

Figure 4.45. The cumulative release of simplexin in Biocomposites incubated in Fermentation 14.

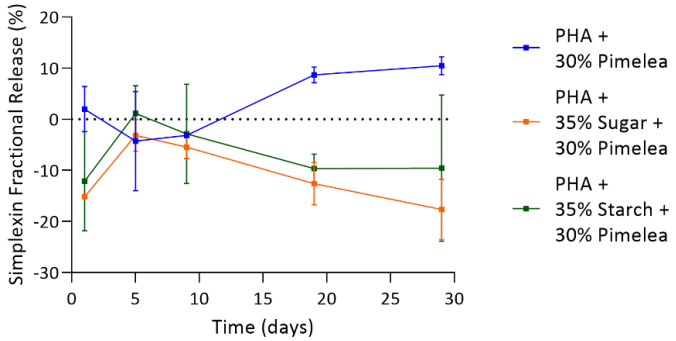
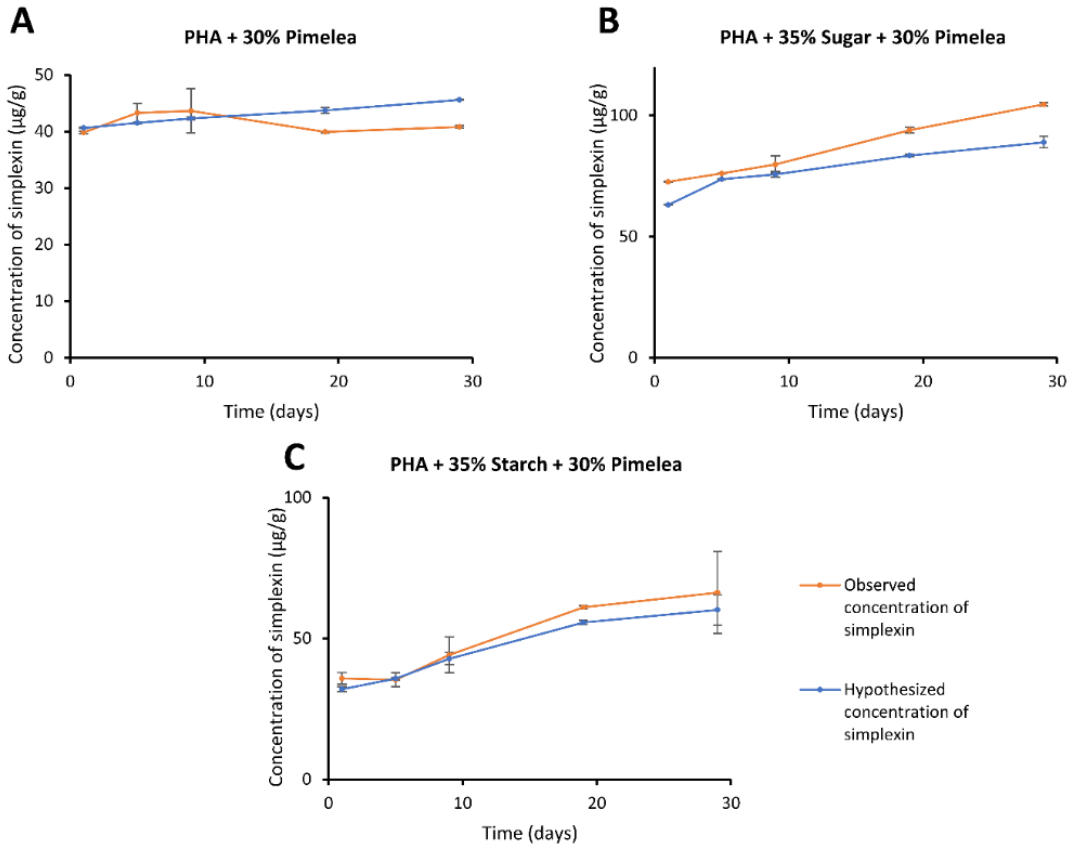


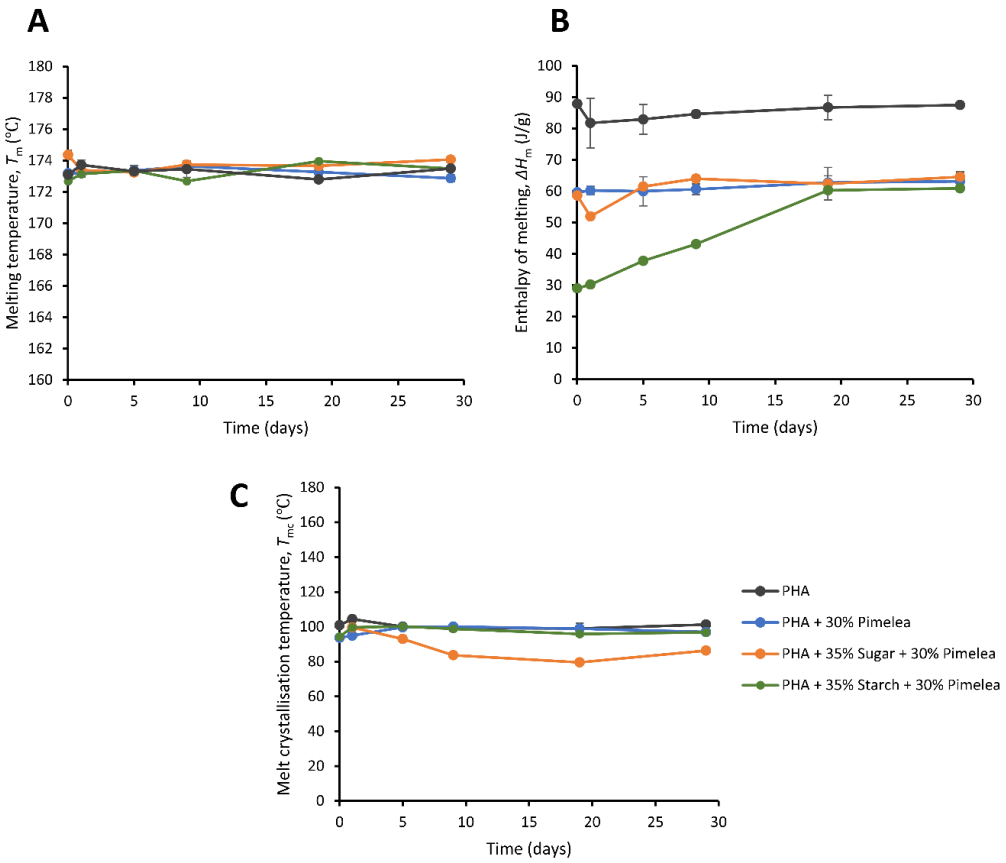
Figure 4.46. The changes in the concentration of simplexin within the biocomposites over time during Fermentation 14 for (A) PHA with 30% Pimelea, (B) PHA with 30% Pimelea and 35% sugar, (C) PHA with 30% Pimelea and 35% starch.



Thermal properties

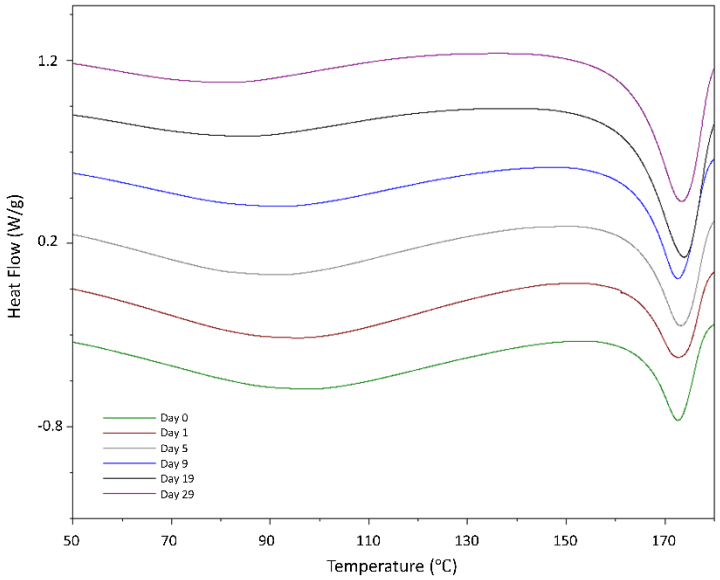
Figure 4.47 shows the changes in the melting temperature (T_m), enthalpy of melting (ΔH_m) and the melt crystallisation temperature (T_{mc}) of the different biopolymers during the incubation in Fermentation 14.

Figure 4.47. The changes in thermal properties of the biopolymers incubated in Fermentation 14 for (A) PHA with 30% Pimelea, (B) PHA with 30% Pimelea and 35% sugar, (C) PHA with 30% Pimelea and 35% starch.



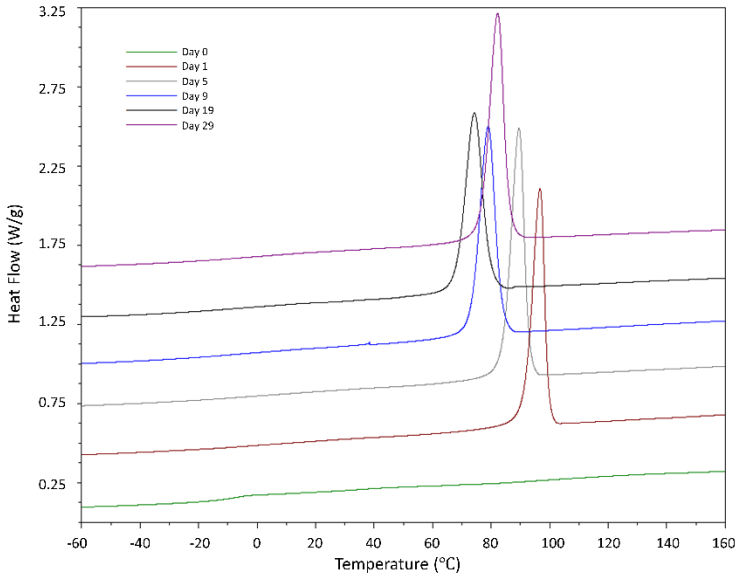
The melting temperature values ranging from 172 to 174 °C, and remained consistent for all the biopolymers overtime, with an RSD of 0.16% for PHA+30%Pimelea, 0.24% for sugar-porogen biopolymer, 0.27% for starch-porogen biopolymer and 0.24% for neat PHA. The thermal stability during the first melting ramp confirmed, again, that the PHA in all the biopolymers degraded via a surface erosion mechanism. Compared with neat PHA, the three biocomposites showed a lower ΔH_m value as a result of the incorporated fillers (plant material and porogens). The sugar-porogen biopolymer again showed a similar thermal behaviour with PHA+30%Pimelea after Day 1 when the sugar had been removed from the matrix. On the other hand, the starch-porogen biopolymer has a much lower ΔH_m value during the initial 9-day incubation, which can be attributed to the starch integrated in the matrix. As shown in Figure 4.48, there was an obvious broad shoulder peak before the melting peak of PHA in the Day 1-5 samples of starch-porogen biopolymer, indicating the existence of starch. It seemed that after 9-day incubation, the content of starch has dropped to a low level and the melting behaviours of the starch-porogen biopolymer matched with the other two biocomposites.

Figure 4.48. DSC thermograms of Biocomposite (PHA + 35% Starch + 30% Pimelea) during the first heating scan.



No significant changes were observed in the crystallisation behaviour after quench cooling among the biopolymers over time. As shown in Figure 4.47, the T_{mc} values neat PHA (100.8 °C in average) was a little higher than PHA+30%Pimelea and the starch-porogen biopolymer (97.4 and 97.6 °C in average), and kept stable with RSDs of 2.2 %, 2.6% and 2.3% for PHA, PHA+30%Pimelea and the starch-porogen biopolymer respectively. It is noteworthy that the sugar-porogen biocomposite showed about 10 °C lower T_{mc} values than the other two Biocomposites. The melted sugar in the Day 0 samples of the sugar-porogen biocomposite also brought about failure of crystallisation as shown in Figure 4.49.

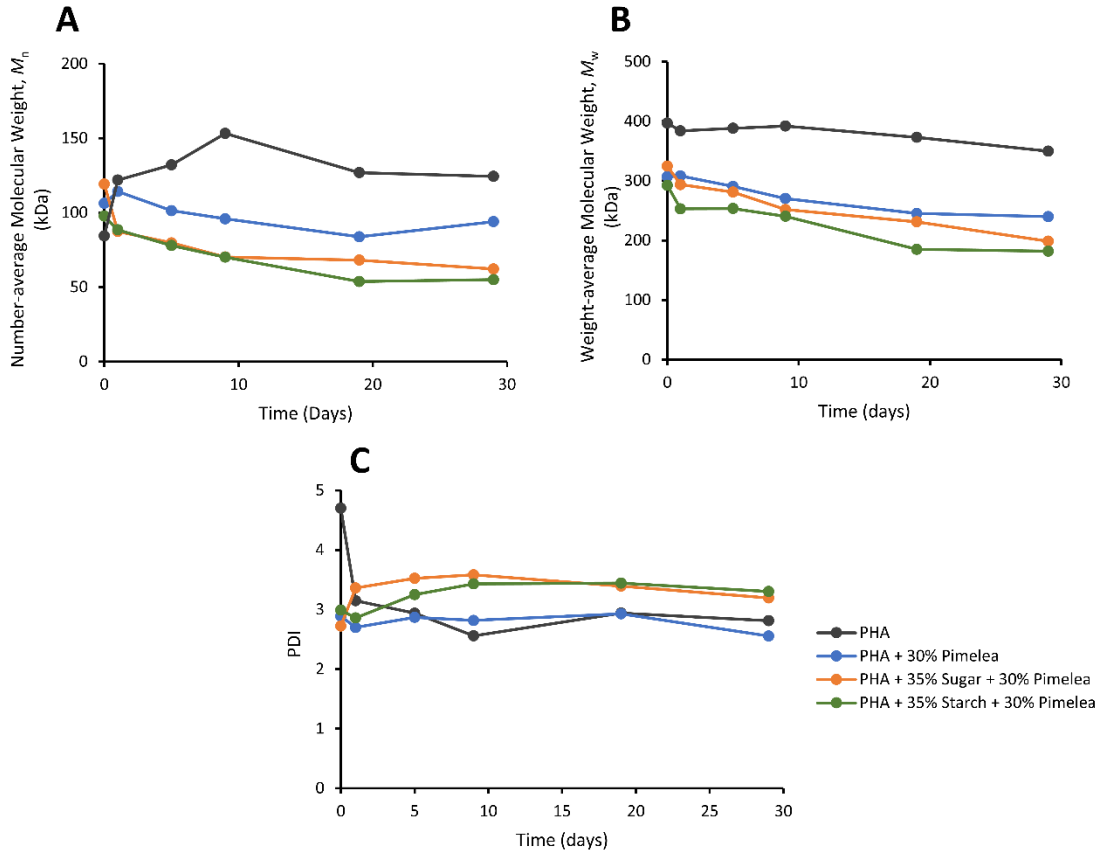
Figure 4.49. DSC thermograms of Biocomposite (PHA + 35% Sugar + 30% Pimelea) during the first cooling scan.



Quantification of biodegradation by molecular weight analysis

The number-average molecular weight ($\overline{M_n}$), weight-average molecular weight ($\overline{M_w}$) and polydispersity (PDI) of the PHA in all the biopolymers during the incubation in Fermentation 14 were shown in Figure 4.50, respectively.

Figure 4.50. The changes in the average molecular weight profile of PHA in biopolymers incubated in Fermentation 14 for (A) PHA with 30% Pimelea, (B) PHA with 30% Pimelea and 35% sugar, (C) PHA with 30% Pimelea and 35% starch.



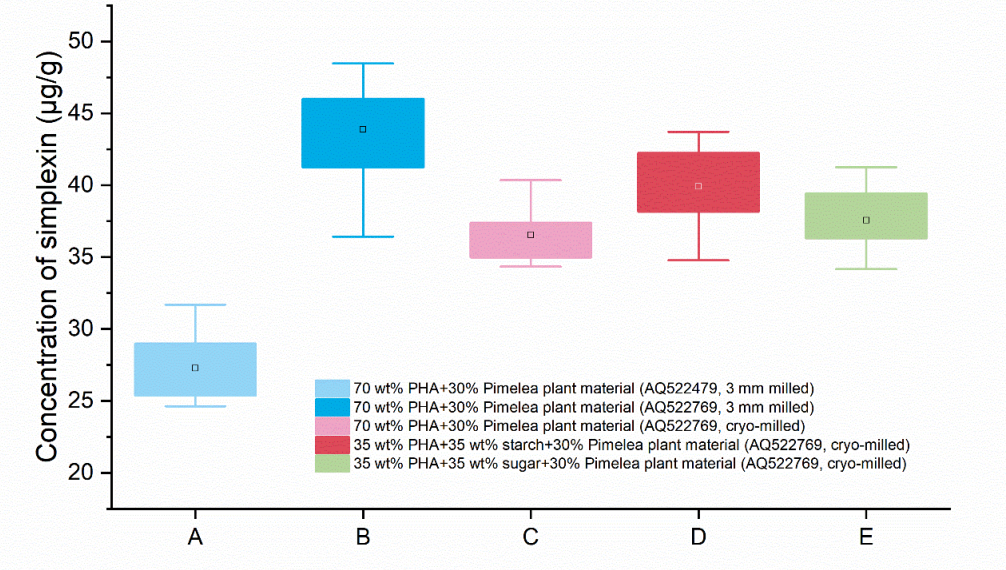
A constant decrease in $\overline{M_w}$ can be observed for all the biopolymers over the whole incubation time. However, the variations were not significant, as PHA undertook a surface erosion mechanism and the molecular weights tended to remain relatively unchanged until bulk depolymerization starts to take place. As $\overline{M_n}$ is more sensitive than $\overline{M_w}$ to the changes in the proportion of low molecular weight polymer chains, it is noteworthy that PHA+30%Pimelea and the neat PHA both showed a slight increase during the initial time period. After 24 h exposure to the fermenter, the $\overline{M_n}$ of PHA in the Day 1 samples of PHA+30%Pimelea increased from 106.3 to 114.2 kDa and started decreasing ever since. For the neat PHA, the $\overline{M_n}$ increased from 84.5 to 153.2 kDa during the 9-day incubation and then started decreasing. This phenomenon has been seen in other biodegradation experiments of PHA in soil environments, as the lower molecular weight polymer chains are more likely to be at an amorphous state and thus more readily migrated and degraded.

4.4.4 Biocomposite reproducibility studies with LC-MS/MS analysis

Given the importance of controlling the reproducibility of the initial biocomposite product, particularly after milling or grinding followed by significant thermal exposure and mechanical processing through an extruder, analyses were conducted of the variability of simplexin loads in

extruded samples using the protocols described in Section 3.9.12 with results shown in Figure 4.51. The biocomposite samples assayed included three based on PHA alone with 30 wt% Pimelea (from two different milled Pimelea sources) as well as from one that was cryo-milled (into much finer particle sizes) and two based on the 30 wt% cryo-milled Pimelea with 35 wt% PHA and either starch or sugar as porogen. For each biocomposite, five different samples were randomly taken from along the extruded ribbon and each was analysed in triplicate, with the exception of the biocomposite prepared from AQ522479 Pimelea for which only four different samples could be taken.

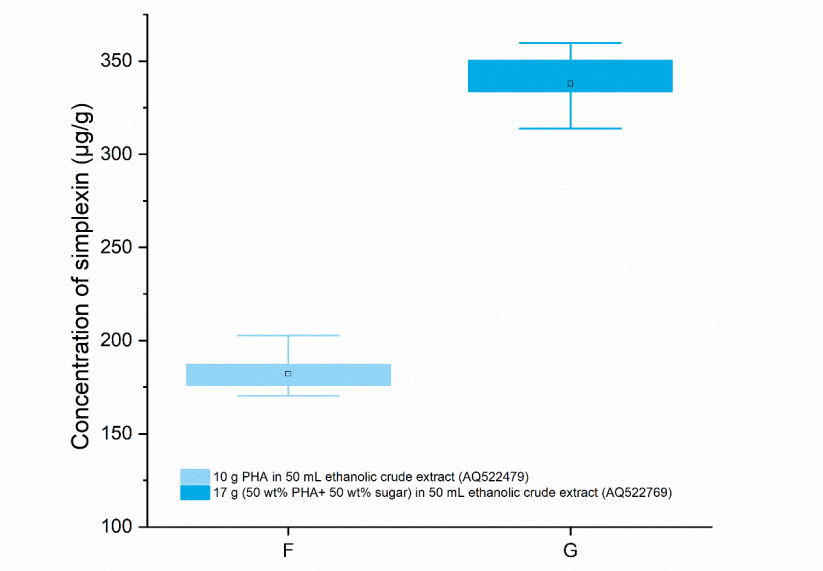
Figure 4.51. Reproducibility of simplexin loading in extruded biocomposites shown as boxplots with the median marked as a square within the interquartile range (solid colour rectangle).



It is evident that there was less simplexin present in the AQ522479 Pimelea sample than for AQ522769, which is an indication that it is important to quantify this simplexin content in plant samples in order to have consistent loading in composites. In addition, based on ANOVA analysis, there was found to be a significant difference in simplexin loading across the samples prepared using the same Pimelea plant material (AQ522769) at the same loading (Figure 4.51), depending on sample preparation method and/or formulation ($P << 0.05$). Milling has resulted in higher simplexin loadings than cryo-milling, which is likely due to less mechanical work required for the former process. The differences between the composites based on cryo–milled samples were also significant at the 95% confidence level, indicating that formulation does play a role in final loading and this will need to be monitored for each future batch to assess the factors that play into this difference.

The same procedure was repeated for the composites prepared using ethanol extract (Figure 4.52) showing that there was a significantly higher loading in the final composites using this approach. The relative amounts of the simplexin loading in the two samples again reflected the fact that there was less simplexin in the AQ522479 Pimelea sample.

Figure 4.52. Simplexin loading in extruded biocomposites based on ethanol extracts of Pimelea AQ522479 and AQ522769 shown as boxplots with the median marked as a square within the interquartile range (solid colour rectangle).



On a relative error basis, where relative error in this case refers to the 95% confidence interval divided by the mean (expressed as a percent) (Table 4.17), the variability is smaller for the samples made from extracts than it is for the cryo-milled samples, which in turn have smaller relative error than the milled sample. Thus, these latter processes will give us more confidence in the reproducibility of the biocomposite product.

Table 4.17. Relative error of simplexin loading in selected biocomposites (based on confidence interval relative to mean, expressed as a %).

Biocomposite	Relative error (%)
70 wt% PHA+30% Pimelea plant material (AQ522479, 3 mm milled)	4.7
70 wt% PHA+30% Pimelea plant material (AQ522769, 3 mm milled)	3.8
70 wt% PHA+30% Pimelea plant material (AQ522769, cryo-milled)	2.4
35 wt% PHA+35 wt% starch+30% Pimelea plant material (AQ522769, cryo-milled)	3.7
35 wt% PHA+35 wt% sugar+30% Pimelea plant material (AQ522769, cryo-milled)	2.4
10 g PHA in 50 mL ethanolic extract (AQ522479)	2.6
17 g (50 wt% PHA + 50 wt% sugar) in 50 mL ethanolic extract (AQ522769)	1.7

A full journal paper detailing the validation of this extraction and determination of simplexin in these plant-polymer biocomposites has been published (Yuan et al. 2021). This study also demonstrated a limited mass transfer of simplexin (< 3.5%) from the PHA/sugar/cryo-milled Pimelea biocomposites into a sterilised rumen fluid environment after a 10-day incubation.

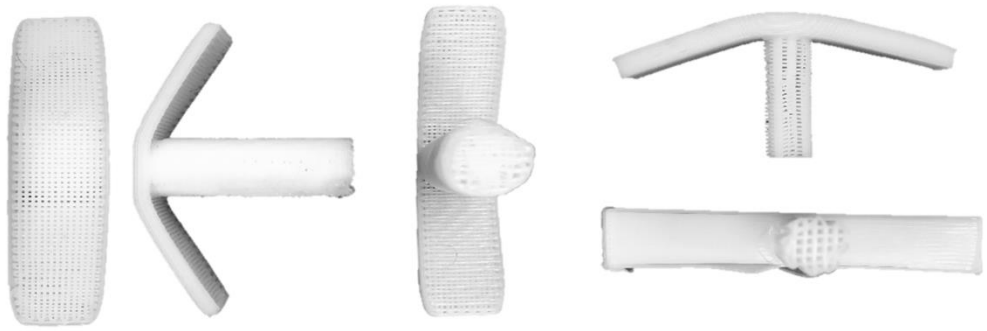
4.4.5 Bolus manufacture investigations

Biopolymer bolus prototypes

A bolus could be manufactured by either 3D printing, injection moulding or extrusion. At this stage, several-model small scale boluses (3 cm x 5 cm) have been successfully 3D printed using a Flashforge 3D printer (Figure 4.53). This printer was set with PCL filament. The aim was to first optimise the

design of the 3D printed bolus, before moving to another 3D printer that will allow the use of PHA, and eventually PHA loaded with Pimelea.

Figure 4.53. Different views of 3D printed PCL model small scale boluses (3 cm x 5 cm).



The advantage of using 3D printing is that the final bolus can be constructed with some pores, allowing for a better diffusion of the rumen fluid through the bolus, hence enhancing the biodegradation of the bolus. PCL being flexible, it was possible to achieve the construction of bendable wings. This would not be achievable with PHA only, but the bolus could be manufactured such that the main core is comprised of PHA + Pimelea while the wings could be made of PCL or PCL + *Pimelea*.

Regarding the injection moulding, it will first be necessary to design the final bolus to be able to manufacture the mould accordingly.

Literature survey on potential bolus design

To be effective an intraruminal bolus needs to remain in the rumen of the animal for long periods of time, and to achieve this, two approaches have been developed. The first approach is the commonly employed involving the incorporation of components into the bolus that provide an overall device density greater than 2 g/mL. This ensures that the device remains at the bottom of the reticulo-rumen and will not be regurgitated. The second approach is a bolus that can expand in the reticulo-rumen cavity after passing through the oesophagus. This is typically achieved by incorporating polymeric wings that are constrained by a water-soluble tape or adhesive during administration of the bolus into the animal. The tape or the adhesive dissolves and the wings expand preventing regurgitation. This can also be accomplished by using polymeric sheets that are rolled as a cylinder and then can unwrap in the rumen. The effective diameter of the wings has to be larger than the opening of the oesophagus to avoid regurgitation (Cardinal 2000).

Intraruminal boluses are usually administered via the oral route into the rumen of animals. Depending on the design, upon depletion of drug, the device erodes away or if it is composed of non-degradable polymeric or metallic shell which will remain with the animal throughout its lifetime.

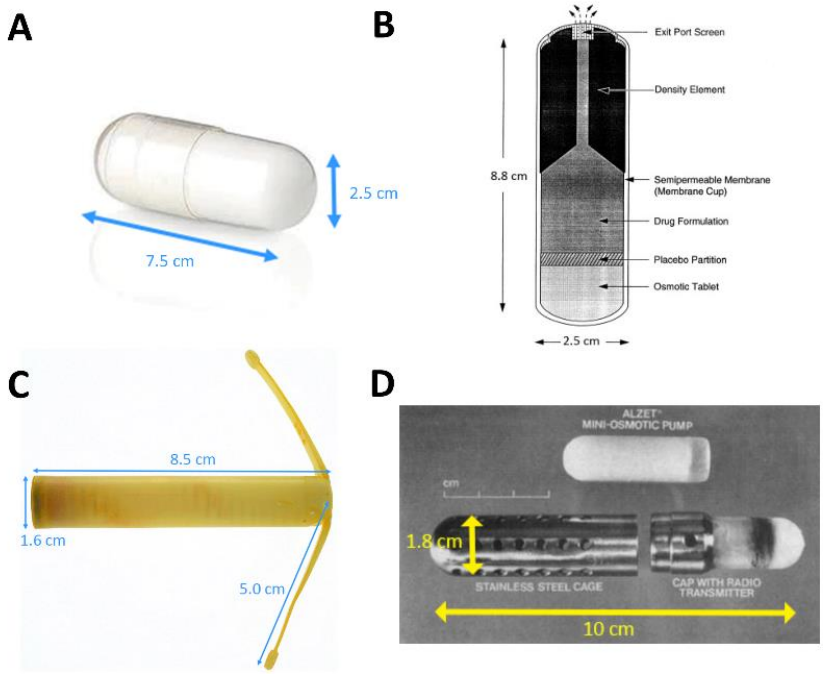
In the case of erodible systems, Riner et al. (1982) investigated the effects of the density on the retention and location of boluses in cattle (Table 4.18). They demonstrated that minimum density of 1.6 g/cm^3 was required to prevent regurgitation from the ruminoreticulum and a minimum of 2.0 g/cm^3 for retention in the reticulum. For electronic rumen boluses, a density above 3 3 gm/cm^3 has been recommended(Fallon 2001).

Table 4.18. Effect of density on retention in the rumen (Riner et al. 1982)

Density (g/cm^3)	Number of doses retained		
	Reticulum	Rumen	Missing
1.2	3	54	123
1.4	37	65	48
1.6	130	32	19
1.6	85	90	5
1.8	133	47	0
2.0	178	2	0
2.0	153	27	0
2.2	180	0	0
2.4	180	0	0

A survey from the literature on bolus dimension found that bolus diameters can vary between 1.5 cm to 2.5 cm, and between 8 cm to 10 cm in length as presented in Figure 4.54. The only bolus with wings is produced Argenta Manufacturing Limited, NZ and its wings were ca. 5 cm each in length.

Figure 4.54.Different bolus designs and dimensions, as reported in the literature including (A) Gel capsule size 7, 24 mL (The Capsule Guy 2022), (B) Ivomec® SR Bolus (Cardinal 2000), (C) Argenta Manufacturing Limited, NZ (Carlsson et al. 2012) and (D) ALZET 2ML4 mini-osmotic pump (ALZA Corp., Palo Alto, CA 94304) (Pope et al. 1985).



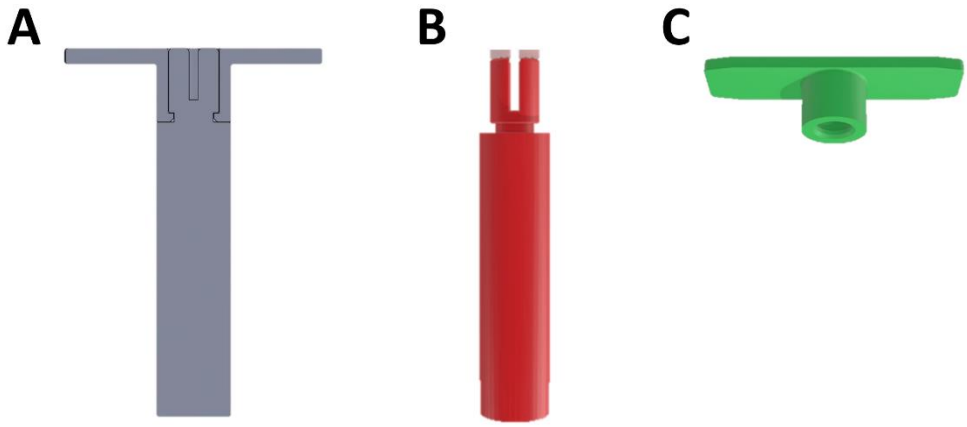
The different type of bolus presented in Figure 4.54, are orally introduced into the animal, and typically administered by using a “gun” as presented in Figure 4.55.

Figure 4.55. Balling gun from Bio-Vet.

Bolus requirements

From the literature findings (Table 4.18) a biopolymer bolus with density lower than 2 $g.cm^{-3}$ will be regurgitated and to avoid this issue wings will need to be added to the bolus. PHA loaded with 30% Pimelea has a density lower than 1.2 $g.cm^{-3}$, and as such it will be necessary to add bendable wings to avoid regurgitation of the device. The aim is to design the bolus such that the body of the bolus will be like a cylinder made of PHA loaded with Pimelea, and the wings will perhaps be made of PCL which is more flexible than PHA. Figure 4.56 depicts an initial first design proposal where the wings could be easily clipped on the cylinder. This design is a prototype for possible production of bolus via injection moulding. For initial feasibility testing, it is envisaged that the body and the wing would first be 3D printed to allow for further optimisation of the design.

Figure 4.56. Proposed initial prototype biopolymer bolus design (A) Cross-section, (B) Bolus body in PHA plus Pimelea, and (C) Bolus wings in flexible biopolymer such as PCL.



4.4.6 Summary discussion - biopolymer composites for intraruminal slow release

In initial fermenter studies, pure biopolymers were shown to degrade by surface erosion in the order: starch >> polyhydroxyalkanoate (PHA) > polycaprolactone (PCL) > polylactic acid (PLA), with only PHA, PCL and PLA considered for further study (Section 4.4.2). Biopolymer composites were prepared with both Pimelea plant particles and ethanol extracts, and their degradation kinetics determined in fermenter studies of up to 63 days. Biopolymers incorporating Pimelea plant particles degraded faster than those based on plant extracts. Observed surface degradation (SEM and DSC) suggested that Pimelea plant particles are more easily digested by the fermenter microbial population and/or enable better access to the interior of the biopolymer composite for the bacteria and associated enzymes, promoting biodegradation. The degradation rate for the biopolymers PHA, PCL and PLA incorporating Pimelea particles followed the same order as the pure biopolymers.

Interestingly degradation rates of combinations of PHA/PCL (4:1, 1:1, 1:4) with milled Pimelea were similar to that of PCL with milled Pimelea. The inclusions of porogens (starch and sugar) were shown to accelerate biopolymer degradation rates in *vitro*, further demonstrating the capacity to tailor biopolymer composition to achieve a range of release rates (Section 4.4.3). LC-MS/MS methodology was also developed and validated to analyse the Pimelea toxin simplexin within the biopolymers demonstrating both the consistency of composition and also toxin release rates (Yuan et al. 2021) (Section 4.4.4).

Overall, these extruded biopolymer composites demonstrated a high degree of stability under rumen-like fermenter conditions, with surface erosion degradation providing options to control potential bioactive release rates. This potential was further demonstrated in a 120-day study within the rumen of fistulated cattle (Section 4.5), wherein porous 3D printed PHA biopolymer pieces degraded more than twice as fast as the solid biopolymers.

4.5 *In vivo* rumen microbial degradation of biopolymer composites

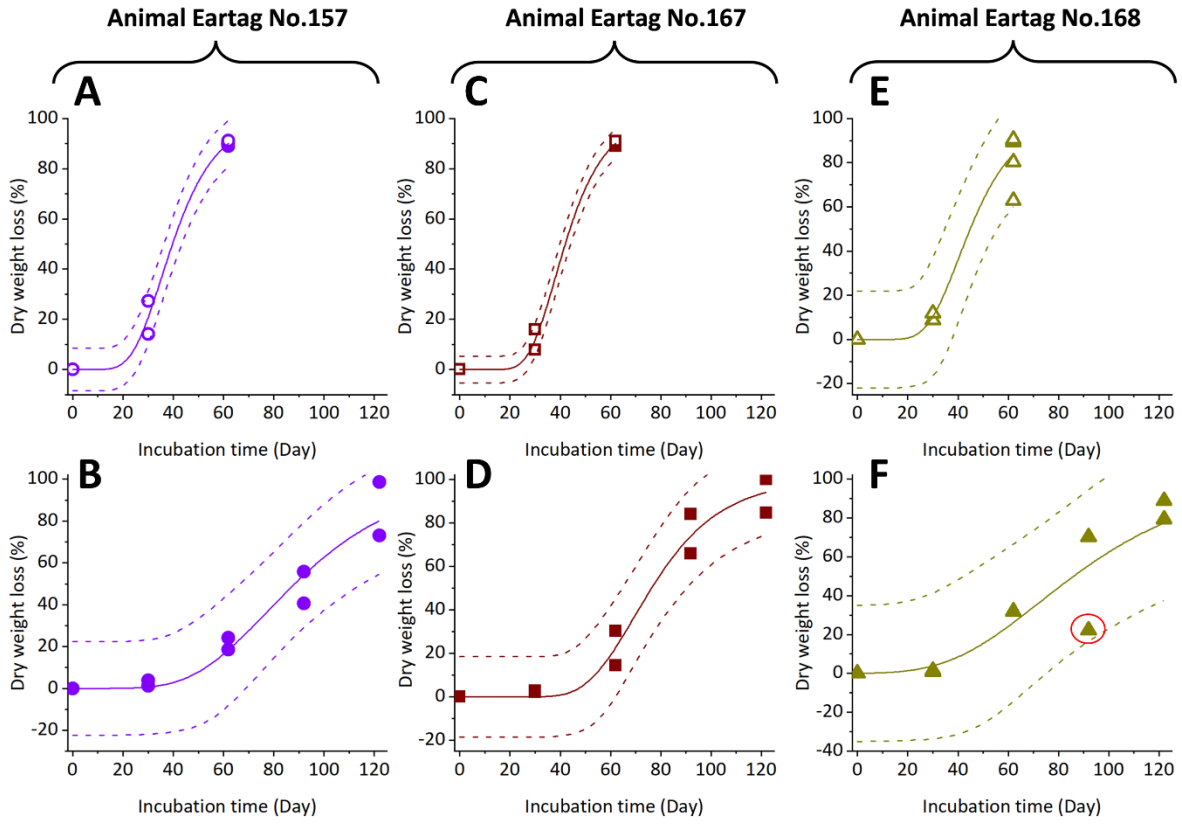
The animal trial utilising three fistulated Holstein Friesian steers to determine the rumen degradation rates of biopolymer boluses ran for 122 days with the biopolymer pieces being removed on Days 30, 62, 92 and 122. Each animal was allocated two sets of biopolymer pieces to investigate intra-animal variations. The two types of biopolymers pieces, porous and solid, were sorted by weight and split into low/high weight for the intra-animal sets so that each animal received one of each. The four sample times were randomised within the low/high sets and the animals randomised within time and replicate so that each animal received a random weight. The first set of biopolymer pieces were removed after 30 days, where the solid biopolymer pieces had weight loss between 0.85% to 2.8%, while the porous biopolymer pieces had weight loss between 7.8% to 27.3% (Figure 4.57. When bags were removed after 62 days in the rumen, the porous biopolymer pieces were largely degraded, so the decision was made to remove all the remaining porous biopolymer pieces. The percentage weight loss of the removed porous pieces ranged from 62.9% to 91.6% with an average of 87.8% loss while the solid biopolymer pieces had lost between 14.4% to 32.1% of the original weight with an average loss of 25.2%. By day 92, the solid pieces had lost between 22.3% to 84.0% and by day 122 the last set of solid biopolymer pieces had lost between 73.1% to 99.9% of the original weight Figure 4.57.

4.5.1 Analysis of biopolymers after removal from rumen

Percentage weight loss

The degradation of the two types of biopolymers over time was analysed using a simple linear regression with groups, with days in rumen as the explanatory variable, percent weight loss as the response variate and biopolymer type as the group. Analysis showed that the biopolymers degraded at significantly different rates ($P < 0.001$) with the porous biopolymer degrading 2.46 times more rapidly than the solid biopolymer (Figure 4.57). However, the porous biopolymer only had assessments for two time points (days 30 and 62) so results should be interpreted with care.

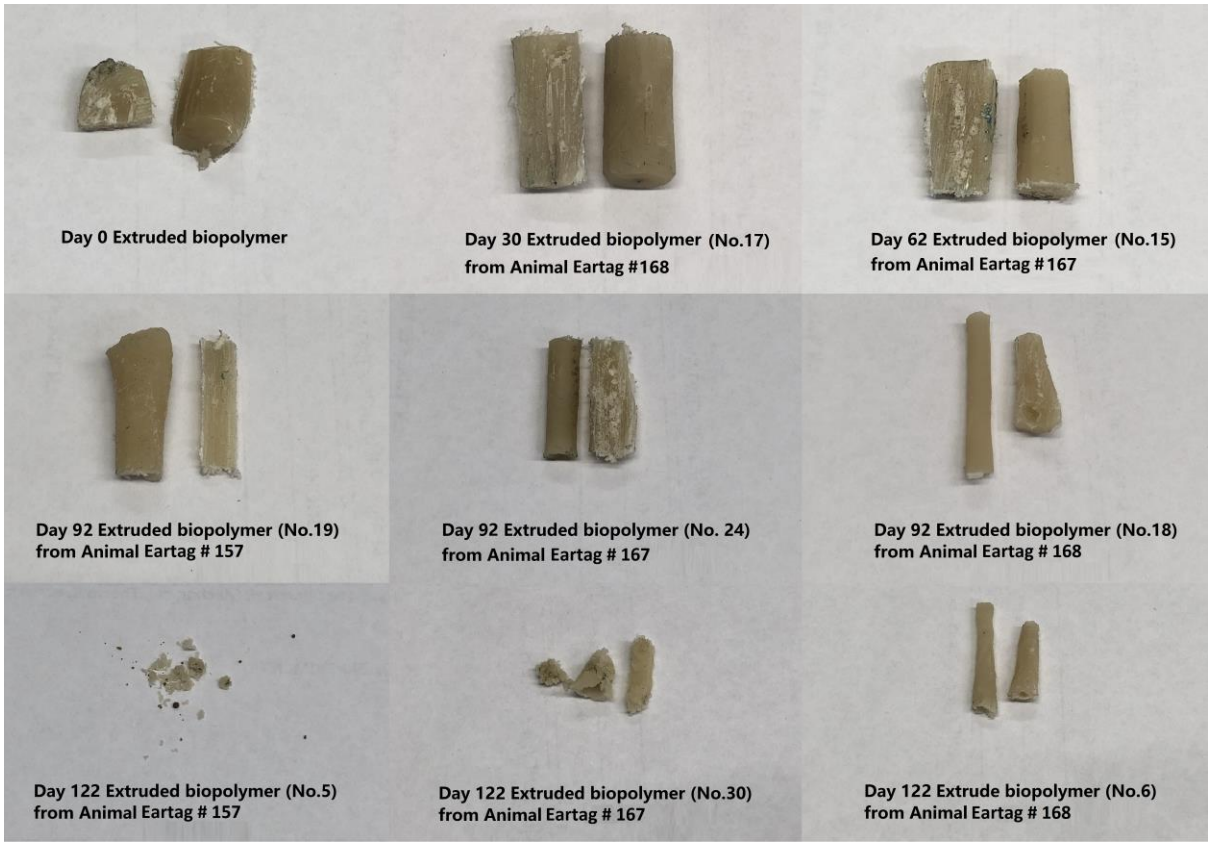
Figure 4.57. Dry weight losses (wt.%) of (A) the 3D printed scaffold PHBV samples and (B) the extruded solid cylinder samples incubated in the steer with Eartag #157, (C) the scaffolds and (D) solid samples incubated in the steer #167, and (E) the scaffolds and (F) solid samples incubated in the steer #168, versus different lengths of time (data points are shown as symbols, with the outlier data point red circled, the fits with models as solid lines, and the 95% confidence prediction bands as dashed lines).



Differential Scanning Calorimetry (DSC)

To best represent different degree of biodegradation, a series of the solid extruded biopolymers were analysed (Figure 4.58, as described in Section 3.10.6).

Figure 4.58. Illustration of the selected extruded biopolymers with varying degrees of biodegradation analysed via DSC



The melting temperature (T_m) and enthalpy of fusion (ΔH_m) of the solid extruded biopolymers and the porous 3D-printed biopolymers after different exposure time to the *in vivo* rumen environment are shown in Table 4.19.

Table 4.19. Summary of the non-isothermal melting behaviours of selected solid cylinder biopolymer samples and the 3D printed scaffold samples before and after biodegradation *in sacco*.

Incubation time (day)	Extruded solid cylinder biopolymers				Scaffold biopolymers	
	T_m (℃)		ΔH_m (J/g)			
	Interior areas	Surface areas	Interior areas	Surface areas	T_m (℃)	ΔH_m (J/g)
0	175	176	83.3	88.1	176	91.1
30	176	174	101	93.7	174	86.4
62	175	175	88.4	88.0	173	96.2
92	175	175	92.1	88.8	-	-
122	175		89.9		-	-

No significant changes can be observed in the thermal properties of the biopolymers, regardless of their different structure, different extent of biodegradation and the different rumen environment of different animals. Over the 120-day *in vivo* rumen fermentation, the average T_m value of the solid extruded biopolymers was 175 ℃ with a relative standard deviation (RSD) of 0.60%, and the average ΔH_m value as 90.1 J/g with a RSD of 4.3%. Similarly, over the 62-day *in vivo* rumen fermentation, the average T_m value of the porous 3D-printed biopolymers was 173 ℃ with a relative standard deviation (RSD) of 0.77%, and the average ΔH_m value as 91.5 J/g with a RSD of 6.2%. The consistency

in the melting behaviour of PHA confirmed that in a real rumen environment, surface erosion is still the dominant degradation pattern.

Gel permeation chromatography (GPC)

The GPC analysis revealed the different molecular weight composition of the solid extruded biopolymer and the porous 3D-printed biopolymer, as shown in Table 4.20.

Table 4.20. Summary of the molecular weight distribution profiles of selected solid cylinder biopolymer samples and the 3D printed scaffold samples before and after biodegradation *in sacco*.

Incubation time (day)	Extruded solid cylinder biopolymers						3D printed scaffold biopolymers		
	$\overline{M_w}$ (kDa)		$\overline{M_n}$ (kDa)		Đ		$\overline{M_w}$ (kDa)	$\overline{M_n}$ (kDa)	Đ
	Interior areas	Surface areas	Interior areas	Surface areas	Interior areas	Surface areas			
0	243	98.4	73.7	40.9	3.3	2.4	359	146	2.5
30	311	263	90.0	98.0	3.5	2.7	289	121	2.4
62	222	210	86.8	76.5	2.6	2.7	-	-	-
92	180	195	74.0	83.9	2.4	2.5	-	-	-
122	189		70.0		2.7		-	-	-

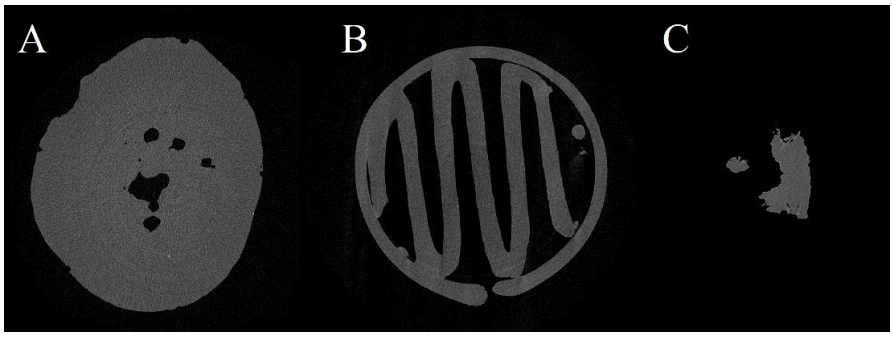
Comparing the 0-day samples of the solid extruded biopolymer and the porous 3D-printed biopolymer, the latter obviously showed a much higher average molecular weight, indicating the presence of longer chains of PHA. Although the two types of biopolymers were manufactured from the same PHA powder, the extrusion process might have caused greater degradation of the polymer than the 3D printing process. There was a noticeable difference between the average molecular weight in the surface area as opposed to the interior region in the 0-day samples of the extruded biopolymers. The interior part of biopolymer had both a much higher number-average molecular weight ($\overline{M_n}$) and weight-average molecular weight ($\overline{M_w}$) values compared to the surface of the biopolymer. The reason for this phenomenon is due to the high temperature the surface area has been exposed to during the extrusion process, which brought about more degradation of PHA. After 30 days of *in vivo* fermentation, both the $\overline{M_n}$ and $\overline{M_w}$ values of the solid biopolymer have increased, especially in the surface area where $\overline{M_n}$ increased from 40.9 to 98.0 kDa and $\overline{M_w}$ from 98.4 to 262 kDa. This is understandable as the low molecular weight chains located in the exterior layer were more readily degraded and, with direct contact to the rumen microbes, they can be enzymatically hydrolysed more rapidly. The big difference in the molecular weight profile of surface area and interior area also no longer existed from this time point. By the end of the animal trial, some of the solid biopolymers were close to disappearing and had broken down into small particles, Figure 4.58. However, the molecular weight of these samples with high extent of biodegradation still didn't show a significant change (Table 4.20). Likewise, the distribution of molecular weight of the 3D-printed biopolymers exhibited neglectable difference after 30-day *in vivo* fermentation (Table 4.20). These results have certainly solidified the well-known degradation mechanism of PHA, which is surface erosion rather than bulk erosion.

X-ray computed tomography (CT) (μ -CT)

The reconstructed μ -CT scanning images also showed an obvious surface erosion mechanism as seen in the cross-section of the 3D-printed biopolymer after 60-days of *in vivo* fermentation (Figure 4.59). No pore development was observable from inside, with biodegradation being all from the

surface. The huge pores in the middle of the cross-section of the solid biopolymer as shown in Figure 4.59 were due to the extrusion process.

Figure 4.59. Reconstructed μ -CT scanning images of the cross section of (A) 0-day solid extruded biopolymer, (B) 0-day 3D-printed biopolymer and (C) 60-day samples of 3D-printed biopolymer



SEM analyses of selected retrieved biopolymer samples

The surfaces of representative solid and porous samples were analysed using SEM, with the images of the samples before and after rumen exposure presented in Appendix Section 9.13. Prior to fermentation exposure, all the samples presented a smooth surface. After 30 days of *in vivo* fermentation, both solid and porous PHA samples had large numbers of holes/hemispherical divots on the surface, indicative of bacterial enzymatic degradation. In addition, the porous samples showed similar surface degradation and porosity, even on the inside, which is due to the large mesh size allowing for the rumen fluid to penetrate the sample. The SEM results were in agreement with the percentage weight loss observed for the different samples.

4.5.2 Summary discussion – future applications of biopolymer for bioactive delivery

This 120-day study within the rumen of fistulated steers has further demonstrated the stability and gradual surface erosion of extruded biopolymers by surface erosion degradation providing options to control potential bioactive release rates. Porous 3D printed PHA biopolymer pieces degraded more than twice as fast as the solid biopolymers. These results serve to highlight the potential application of such biopolymers as slow-release delivery devices for drugs and other bioactives.

4.6 *In vitro* enterosorbent studies

4.6.1 Characterisation of adsorbents

Net surface charge analysis

The results of the net surface charge analysis are summarised in Table 4.21. It should be noted that the purpose of the 1 M KCl solution is to infer the presence of exchangeable aluminium and provide an assessment of the net charge of the colloidal system, in that the salt solution displaces H^+ and Al^{3+} ions from the surface of the adsorbent. In the process, the aluminium that is displaced by K^+ on the exchange interface consumes OH^- ions and the number of H^+ increases in solution, lowering the pH. Based on these results, the Biochar (Gidgee) had a negative surface charge but the modified Gidgee biochars showed a positive surface charge. The pH(KCl) was lower for the non-activated Biochar (Gidgee), the Supelco activated charcoal and the GAC coconut activated carbon. This result indicates that these adsorbents released more protons in the 1 M KCl solution compared to in distilled water or alternately that the 1M KCl solution has suppressed the “suspension effect” or “junction potential

effect", which results in the exchange of excess K^+ with H^+ on the surface of the adsorbent (Kome et al. 2018).

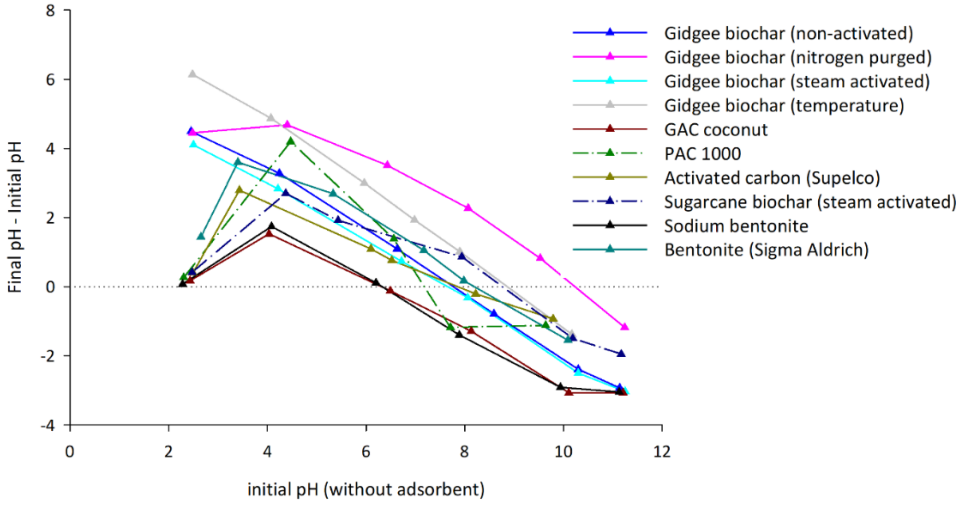
Table 4.21. Surface charge of adsorbents. Results presented as mean of 2 measurements for pH(H_2O) and pH(KCl)

Material	pH(H_2O)	pH(KCl)	ΔpH	Implied surface charge
Biochar (Gidgee) (milled < 0.5 mm)	9.8	8.55	-1.25	Negative
Biochar (Gidgee) (steam activated, milled < 0.5 mm)	11.38	12.97	1.59	Positive
Biochar (Gidgee) (nitrogen purged, milled < 0.5 mm)	11.53	12.34	0.81	Positive
Steam activated sugarcane waste (milled <0.5 mm)	9.21	9.77	0.56	Positive
PAC 1000 (activated carbon)	8.85	9.72	0.87	Positive
Activated charcoal (Supelco)	8.74	7.45	-1.29	Negative
GAC (granular coconut activated carbon) milled to <0.5 mm	8.48	7.97	-0.51	Negative

Zero-point charge analysis

The results for the zero-point charge analysis are given in Figure 4.60. The pH(zpc) was determined to be 6.3 for the GAC coconut biochar, 7.8 for the Biochar (Gidgee) and 10.2 for the nitrogen purged Biochar (Gidgee), 8.8 for the Steam activated sugarcane waste and 7.2 for the PAC 1000. When the solution pH was below these values, the adsorbent's surface would be positively charged. The larger the difference between the solution pH and the pH(zpc), the greater the density of positive ions on the surface of the adsorbent, which would in turn allow for less adsorption of positively charged compounds and more adsorption of negatively charged compounds. Likewise, when the solution pH is increased above the pH(zpc), a negative charge is dominant on the surface of adsorbent and there would be greater cation adsorption. When the solution pH is at the pH(zpc) the charge is balanced.

Figure 4.60. Zero-point charge analysis of a range of carbon and non-carbon-based adsorbents.



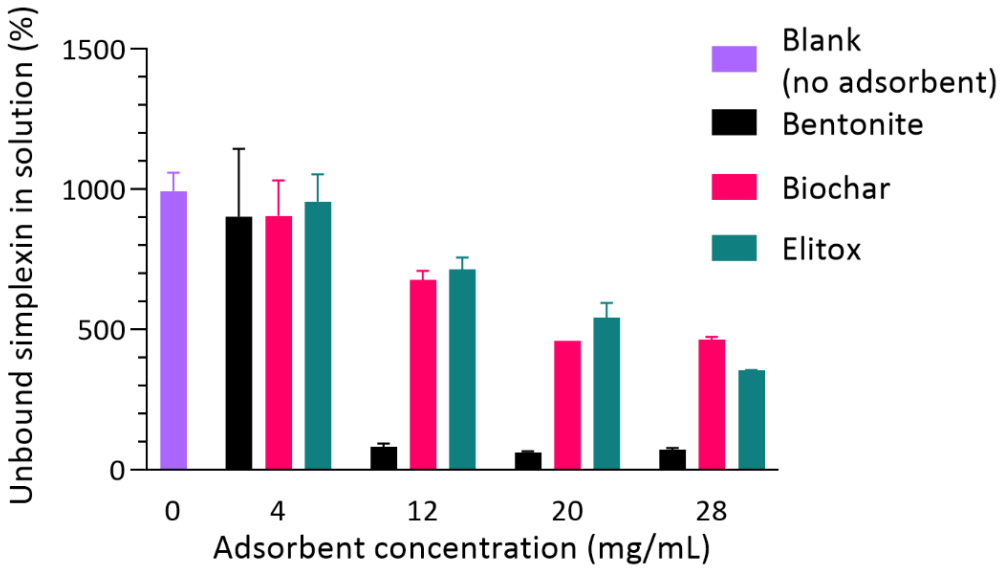
4.6.2 Preliminary enterosorbent studies in rumen fluid media

Several studies have been undertaken to simulate toxin adsorption *in vitro* under pseudo-rumen conditions, particularly with mycotoxins such as aflatoxin (Diaz et al. 2002; Spotti et al. 2005; Moschini et al. 2008; Gallo and Masoero 2010). The most simplistic of these simulation studies utilised a simple aqueous system with changing pH to mimic passage through rumen, stomach and small intestine. In this study, similar adsorption trials were initially attempted with simplexin in aqueous PBS buffer, but this proved problematic due to poor solubility of simplexin in such simple buffers.

Adsorbent studies in rumen fluid media

Adsorbent trials were conducted in rumen fluid media initially and appeared to demonstrate that bentonite had superior adsorbent simplexin efficacy as compared to either Biochar or Elitox (Figure 4.61). No significant difference ($n > 0.05$) was observed between the blank (no adsorbent) and samples containing 10 mg of the respective adsorbent material in 2.5 mL of rumen fluid media (4 mg/mL) (Figure 4.61). However, it was noted that the simplexin added to the rumen fluid media should have produced a concentration of 2,000 ng/mL, yet only 1,000 ng/mL was measured by LC-MS/MS indicating again a problem with solubility. Notwithstanding this issue, samples with 12 mg/mL or more of adsorbent were significantly different to the blank ($P < 0.05$). For bentonite, simplexin levels were greatly reduced ($P < 0.0001$) in all samples containing 12 mg/mL or more, with more than 90% of the simplexin being removed from the rumen fluid media at each of the higher bentonite adsorbent loads. For Biochar and Elitox, a gradual decline in simplexin in the media was observed with increasing amounts of adsorbent material, but this removal efficacy remained significantly less than that of bentonite. Given the poor solubility of simplexin in this rumen fluid media (~50%), it was considered that the observed removal of simplexin from the media (particularly by bentonite) could be due to precipitation/coagulation rather than adsorption. Rumen fluid media is comprised of 33% rumen fluid with the remainder being aqueous salts solutions (Section 9.7.3).

Figure 4.61. Comparison of simplexin removal efficacy of different masses of adsorbents in rumen fluid media, potentially due to simplexin precipitation/coagulation rather than adsorption



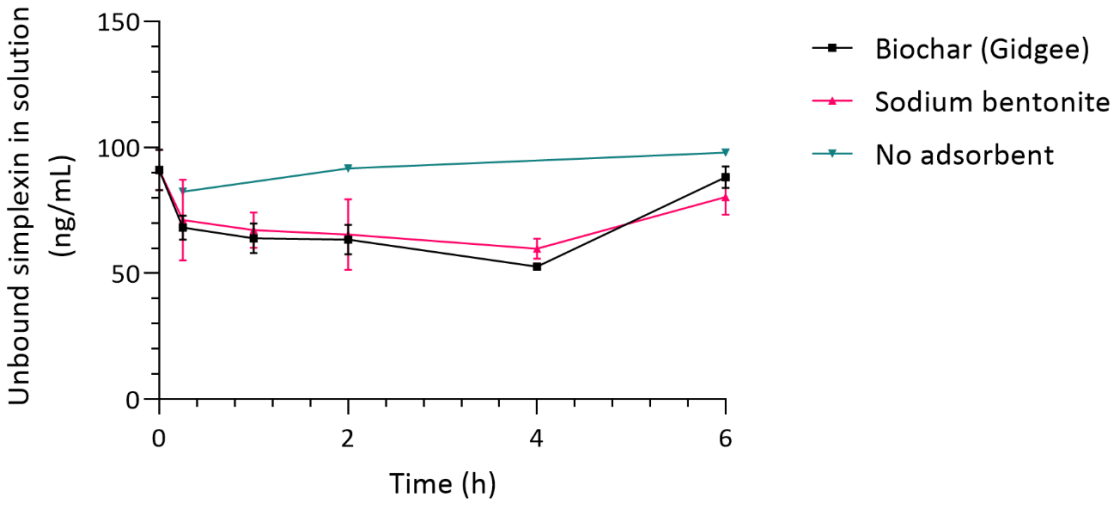
Subsequent adsorbent studies in this project were carried out in clarified rumen fluid (Section 9.7.5) with simplexin concentrations of only 100 ng/mL to minimise this ambiguity due to poor simplexin solubility. Estimates of concentrations of simplexin possible within the rumen based on previous feeding trials would suggest that expected simplexin levels are much less than 100 ng/mL.

4.6.3 Adsorbent *in vitro* testing methodology in clarified rumen fluid

In this study, a number of adsorbents have been tested for their capacity to adsorb simplexin in clarified rumen fluid in six adsorbent trials, as outlined in Table 3.18, over varying incubation time periods.

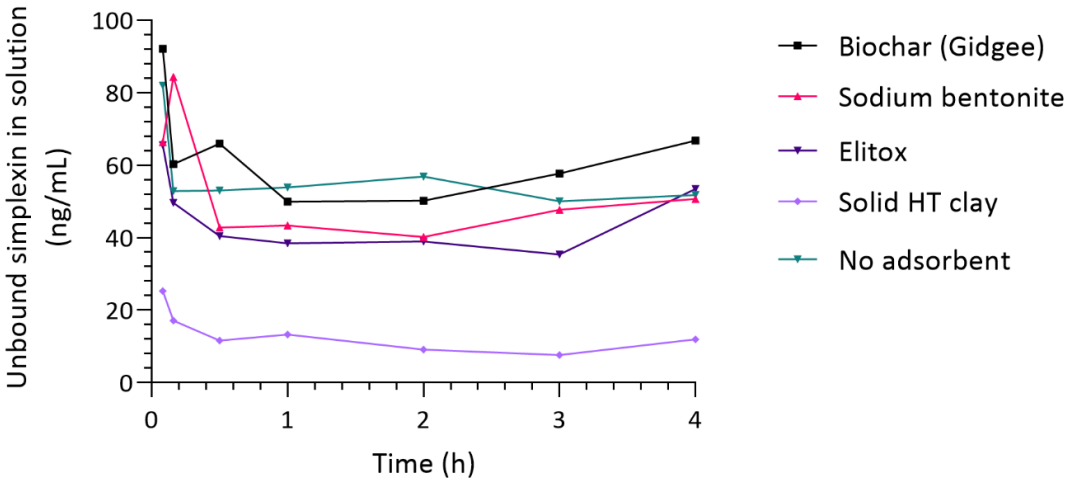
The initial clarified rumen fluid adsorbent trial (RF AdsorbTrial #1) is shown in (Figure 4.62). This study showed a ~30% reduction in simplexin concentration in the samples with Biochar adsorbent (12 mg/mL) after 4 h and a similar reduction in the samples with sodium bentonite (12 mg/mL) with no significant change in the no adsorbent control samples. The difference between the two adsorbents in this trial was not statistically significant ($P > 0.05$at the 95% confidence level). The second trial, conducted over a 42 h timeframe (RF AdsorbTrial #2), however showed a reduction in simplexin in this extended timeframe, even for the no adsorbent blank (results not shown), suggesting that this assay system was less reliable over extended incubation times as fermentation may have occurred under the non-anaerobic conditions of these trials. Similarly, a 24 h trial conducted as RF AdsorbTrial #5 is also not reported.

Figure 4.62. Comparison of the adsorption differences between sodium bentonite (12 mg/mL) and Biochar (Gidgee) (12 mg/mL) with 100 ng/mL simplexin in clarified rumen fluid over 6 h incubation time (RF AdsorbTrial #1). Each timepoint sample taken from different tubes.



In RF AdsorbTrial#3 Figure 4.63, four adsorbents (6 mg/mL) were tested in a 4 h assay in clarified rumen fluid (non-replicated). The Solid HT clay exhibited the greatest surface hydrophobicity and was the best at adsorbing simplexin, though its efficacy in animals is unknown and the cost to implement makes it uneconomical. HT clay is lucentite, a phyllosilicate clay ($Na_{0.33}Mg_{2.67}Li_{0.33}Si_4O_{10}(OH)_2.(H_2O)_n$) that has magnesium as the main structure with lithium as the substitute. The sodium has been exchanged with a mixture of choline chloride (75%) and hexadecylpyridinium chloride (25%). The HT clay material is a small particle size <10 µm.

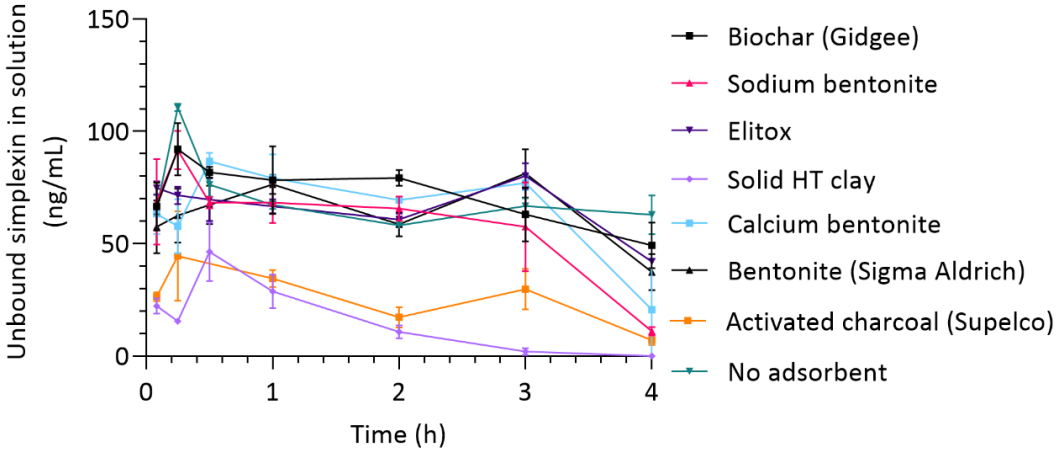
Figure 4.63. Comparison of the adsorption differences between Solid HT Clay and more common adsorbents (6 mg/mL) with simplexin (100 ng/mL) in clarified rumen fluid over 4 h incubation time (RF AdsorbTrial #3). Successive timepoint samples taken from the same tubes for each adsorbent.



In RF AdsorbTrial#4 (

Figure 4.64), a more extensive array of adsorbents was tested at 12 mg/mL. Activated charcoal (Supelco) performed the best in the group (comparable to Solid HT Clay ($P > 0.05$) adsorbing 70% in 1 h and reaching a maximum of 90% after 4 h. For the other materials, after 3 h there was no statistically significant difference compared with the no-adsorbent sample (spiked rumen fluid) for the sodium bentonite ($P > 0.05$), calcium bentonite ($P > 0.05$), Sigma Aldrich bentonite ($P > 0.05$), Elitox adsorbent ($P > 0.05$), and the Gidgee biochar (non-activated) ($P > 0.05$) which all showed non-significant reduction in simplexin levels after 3 h incubation. However, at the 4 h time point the simplexin concentration had decreased significantly for the samples containing sodium bentonite, calcium bentonite or bentonite (Sigma Aldrich) ($P < 0.05$). Results are presented as mean with standard deviation error bars. In RF AdsorbTrial#4 it should be note that successive timepoint samples for each adsorbent were taken from the same tube, so that the relative amount of adsorbent per mL increased after each sampling. This may have contributed to the increased adsorption seen at 4 h.

Figure 4.64. Comparison of the adsorption differences between a range of adsorbents (12 mg/mL) with simplexin (100 ng/mL) in clarified rumen fluid over 3 h incubation time (RF AdsorbTrial #4). Successive timepoint samples were taken from the same tubes.



The relative efficiency of removal of simplexin from suspension by a range of adsorbents at different adsorbent loadings (5 - 240 mg) in 10 mL clarified rumen fluid in a 1 h incubation at 39 °C is provided in

Figure 4.65 (RF AdsorbTrial #6). With a starting simplexin concentration of 100 ng/mL, the two adsorbents that performed the best at removing simplexin were GAC coconut activated carbon and Supelco activated charcoal, with the results showing <5 ng/mL was detected with adsorbent levels of 12 mg/mL and 24 mg/mL (

Figure 4.65). For the other materials, almost 100% of the simplexin remained suspended in solution at low concentrations of adsorbent (0.5 and 1 mg/mL) (

Figure 4.65). As the amount of adsorbent increased, less simplexin remained in the solution because it was bound to the adsorbent. The sodium bentonite, Elitox and Biochar (Gidgee) (<0.5 mm milled) were the least effective of the adsorbents tested (

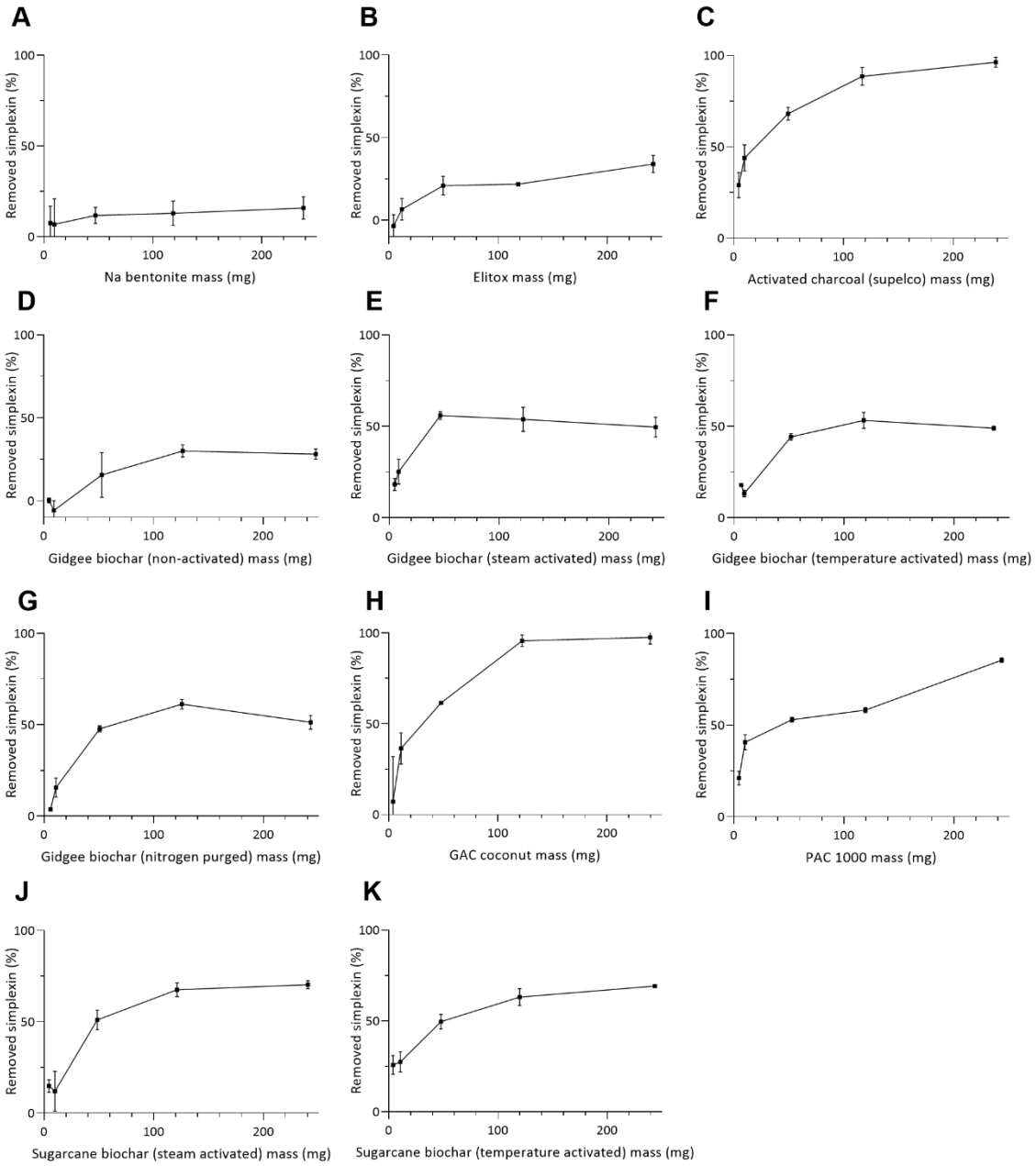
Figure 4.65), with <20% reduction in the concentration of simplexin at sorbent loadings of 12 mg/mL or more. The activated Biochar (Gidgee) (steam, nitrogen purged, temperature) all performed equally and showed a statistically significant improvement over the original Biochar (Gidgee) material. Overall, the reduction in the concentration of simplexin in this 1 h trial was generally less than that seen in the longer 4 h trial (

Figure 4.64), and this difference may reflect the length of time required for some adsorbent to reach equilibrium in this rumen fluid matrix.

Based on a two tailed test hypothesis test comparison at a significance level of 0.05 (with heterogeneous variation), the Supelco activated charcoal performed significantly better at simplexin uptake at adsorbent amounts of 50 mg or more compared with PAC 1000. The Supelco activated charcoal was also significantly better at simplexin uptake compared to Gidgee Biochars (crude and modified) at all adsorbent amounts, and to sugarcane biochars at adsorbent amounts above 5 mg, but borderline not significant at 5 mg ($P = 0.053$). Compared to GAC coconut activated carbon (milled to <0.5 mm) the difference was not significant ($P > 0.05$).

The activated Gidgee Biochars (heat, steam and nitrogen purged) were significantly better at simplexin uptake at adsorbent amounts above 5 mg compared to the unactivated Biochar (gidgee) (milled <0.5 mm). The GAC coconut performed equally well as the Supelco activated charcoal. The simplexin uptake of GAC coconut compared to activated Gidgee Biochars (heat, steam and nitrogen purged) was not significantly different at levels less than 5 mg/mL but significantly different at 12 mg/mL and 24 mg/mL mass levels.

Figure 4.65. Comparison of the simplexin removal efficiency between adsorbent doses with 100 ng/mL simplexin in clarified rumen fluid (10 mL) over 1 h incubation time (RF AdsorbTrial #6).



4.6.4 Summary discussion - selection of adsorbent treatments for the feeding trial

Of the adsorbents tested in the laboratory, only a small number are economically available on the scale required for administration to livestock on a regular basis. Activated charcoal (Supelco) for example was highly effective at removing simplexin from clarified rumen fluid (Section 4.6.3). However, at a cost of $624 per kilogram (Sigma Aldrich), this highly refined product is not economical when compared with Biochar (gidgee) at $40 per 20L (Bos Rural, Kandanga).

All three forms of activated Biochar demonstrated better adsorbent efficacy in the laboratory trials than the original commercial Biochar (Gidgee) material (Section 4.6.3). To demonstrate whether this increased efficacy translated to improved toxin adsorption with the rumen, both Biochar (Gidgee)

and heat-activated Biochar were selected as treatment groups for the feeding trial. Bentonite had demonstrated some efficacy as an adsorbent *in vitro* (particularly in the 4 h trial

Figure 4.64) and was included as a third adsorbent in the feeding trial based on anecdotal use of bentonite in the field (and its readily available to producers in western Queensland).

4.7 Animal trial monitoring and analysis results

The aim of this feeding trial was to investigate the effect of adsorbents and an inoculum on mitigating effects of Pimelea poisoning in cattle. The six treatment groups as described in Section 3.12.3 were: Pim (Pimelea positive control, T1), Pim + Bchar (Pimelea plus Biochar – non-activated, T2), Pim + aBchar (Pimelea plus heat-activated Biochar, T3), Pim + Inoc (Pimelea plus inoculum, T4), no Pim Control (negative control, T5), Pim + Bent (Pimelea plus Bentonite, T6).

4.7.1 Toxin analysis of Pimelea plant material

Simplexin LC-MS/MS analysis results were highly reproducible both across and within the 3 bottles (3 replicates each) analysed, demonstrating a consistent simplexin concentration of 103 µg/g throughout the milled *P. trichostachya* (AQ952584) plant material used in the cattle feeding trial.

Isolated simplexin (>95% pure) was used as an external standard in LC-MS/MS analysis with standard solutions at 8 levels prepared in methanol (5-2,000 ng/mL) with squared weighted correlation coefficients (R^2) of 0.9954. Method validation was conducted by comparing the results for calibration using external simplexin standards to a standard addition method for determining simplexin concentrations for each of the 3 bottles (3 replicates each), with standards added at 4 levels. For standard additions, squared linear correlation coefficients (R^2) were in the range of 0.986–0.998. Percentage recoveries of standard additions to plant extracts at four levels for each of simplexin were calculated, with values for spiked samples calculated by subtraction of the endogenous, no-spike value. Recoveries for spiked samples were calculated by using the expected value and averaged 97-108% with standard deviations of 2.6% at the highest spike level (320 ng/mL), and 18% at the lowest spike level (40 ng/mL).

The measured concentration of 103 µg/g in milled plant material was used for calculations of the Pimelea dose based on steer weight. For example, the calculated Pimelea intake for a 200 kg steer is 50 mg Pimelea/kg bw/day to deliver the desired dose of 5 µg simplexin /kg bw/day.

4.7.2 Feeding

The 40 Droughtmaster steers were fed *ad lib* hay for 2 weeks in the QASP feedlot pens (10 animals per pen), during which time the animals were assessed for temperament, general health, behaviour and willingness to consume molasses. Thirty animals were selected, stratified by weight across treatments and transferred to the individual pens as shown in Figure 3.10.

For the 2 weeks pretrial period, animals in individual pens consumed molasses mixed with water (diluted molasses) as a supplement (in trays) in addition to hay 2.25% bw.

In week 1 of the trial, the daily Pimelea dose was added to the diluted molasses in the trays to provide 5 µg/kg bw/day of the toxin simplexin (initial dose introduced incrementally over first 4 days of the trial - Day 1 25%, Day 2 50%, Day 3 75%, Day 4 100%) together with added adsorbents for T2, T3 and T6 treatment groups. However, by the end of Week 1, it was noted that some steers were showing a reluctance to consume the molasses with the added adsorbents.

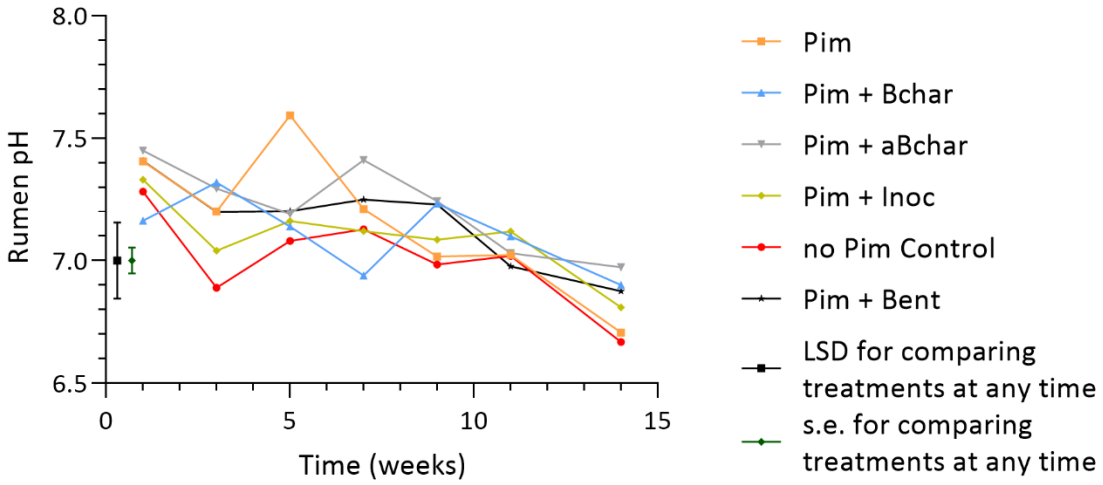
To overcome this rejection issue, the diluted molasses was thereafter added to the top of the hay in the bunker with Pimelea and adsorbents then sprinkled across the diluted molasses and thoroughly mixed by hand into the hay. The no Pim Control (Treatment Group 5) had the same diluted molasses mixed into the hay in a similar manner. To ensure that all animals consumed their treatments, the hay intake was restricted to *ad lib* feeding, with a minimum hay amount of 1.7% bw (maintenance) and increasing amounts only offered to animals that had consumed all of the previous day's ration. This enables the calculation of DMI/bw ratios to compare relative intake between treatment groups.

4.7.3 Analysis of collected rumen fluid

pH of collected rumen fluid

The pH of rumen fluid was relatively constant across all treatment groups throughout the trial (to week 11, Figure 4.66). Slight drop in pH in the post-trial recovery period may have been influenced by change in diet during this period as animals transitioned to feedlot pens and pasture (no molasses). There did not seem to be an overall effect of treatments on the rumen pH with the pH of the rumen fluid ranging from 6.61 to 8.44 in individual animals. The rumen fluid samples where the pH measurement was above 8.0 the presence of saliva was generally noted.

Figure 4.66. Rumen pH across treatment groups measured during the Pimelea feeding trial. LSD = Least significant difference of means (5% level); s.e. = standard error of means.



Simplexin concentration in collected rumen fluid

Measured simplexin levels in selected rumen fluids collected during week 9 were in the range 0.5 - 4 ng/mL which is an order of magnitude lower than the administered daily dose of 7.5 µg simplexin /kg bw/day (commenced week 9). On the day of rumen fluid collection, none of the animals were showing physical signs of oedema. Interestingly, the two animals (steers #86 and #51) displaying a faecal consistency score of 3 (diarrhea) had the lower levels of rumen simplexin detected (0.5 ng/mL and 0.7 ng/mL, respectively). Steer#86 (Pim + Inoc treatment group) was taken off the Pimelea treatment on the day of rumen fluid collection. Steer#51 belonged to the Pimelea plus bentonite (Pim + Bent) treatment group. Of the other two animals, Steer#82 (Pim + Bchar treatment group) with measured rumen fluid simplexin of 3.6 ng/mL, had a better faecal consistency score of 2, whilst Steer#375 (Pim + Inoc treatment group) with 3.6 ng/mL had faecal consistency score of 1 on

collection day. Steer#82 displayed the higher feed refusal score on collection day (1.5–3kg remaining). Further rumen fluid analysis is needed, but likely the low simplexin level analysed in the filtered rumen fluid reflects the adherence of simplexin to undigested plant material.

Microbiome

Microbial DNA was extracted from rumen fluid collected from cattle at four time-points throughout the Pimelea feeding trial, over a 10-week period, and used to generate 16S rRNA gene tagged PCR amplicons, for a total of 136 samples including negative controls. Following sequencing using the Illumina MiSeq platform and bioinformatic analysis of results, bacterial and archaeal populations of each rumen fluid sample was determined. Across the dataset of 112 rumen fluid samples, 12,166 features were identified representing 3,639,897 sequences. This large dataset provided good sequence coverage and therefore enabled determination of microbial populations contained in rumen fluid samples.

Analysis of the sequence data indicated that the microbial populations of cattle in each of the six treatment groups, which were all maintained on the same base diet of medium quality chaffed hay (9% protein), had similar rumen microbial populations, irrespective of the experimental treatment being applied. Some changes in bacterial community profile, as indicated by taxonomic analysis occurred over time (Figure 4.67). For all rumen fluid samples analysed, bacterial populations were dominated by bacteria classified within the orders Bacteroidales, Oscillospirales, Lachnospirales and Christensenellales. The most highly abundant methanogenic archaea present were classified within the order Methanobacteriales.

Microbial diversity occurring within the rumen fluid samples (alpha diversity) was determined using four diversity measures (Table 4.22). Statistical analysis (GLM, accumulated ANOVA) indicated that there was a significant effect ($P < 0.001$) of time (Sample collection No.) on rumen microbial diversity. The first two rumen fluid sample collections had similar microbial diversity, when measured by the Observed features, Faith PD and Shannon entropy indexes. By the third and fourth rumen fluid sample collections, the extent of within-sample microbial diversity slightly decreased from that observed at the first and second collections, as indicated by two of the four diversity measures (Observed features and Faith PD). The effect of treatment group however did not show a consistent effect on within-sample microbial diversity, with only a single diversity measure (Pielou evenness), showing any significant differences ($P < 0.05$), with the Pimelea with activated Biochar treatment group having the lowest microbial diversity, and Pimelea with Bentonite treatment group having the highest microbial diversity.

When the variation in microbial populations occurring between rumen fluid samples was further examined on the basis of experimental treatment group, with all four sample collection time-points included, there appeared to be very little difference in rumen microbial populations present in all animals. This was indicated by the large overlap in the ellipses in the PCA (Figure 4.68) for each respective experimental treatment group. When the data was further transformed in the sPLSDA and the microbial populations contributing to the differences identified (Figure 4.69; Table 4.23), the greatest variation was found to occur between the Pimelea plant material control group (Pim), the no Pimelea plant material negative control group (no Pim Control), and the Pimelea + Bentonite treatment (Pim + Bent) groups. The remaining treatment groups, clustering together in the sPLSDA plot, indicating that less variation was occurring in the microbial populations of these treatment groups (Pim + Bchar; Pim + aBchar; and Pim + Inoc). The microbial populations contributing to the differences seen between experimental treatment groups included genera classified within the order Bacteroidales (Rikenellaceae RC9 gut group and *Prevotella*), being more predominant in the

Pimelea plant material control group. Different bacterial populations classified as *Prevotella* however, were also differentially abundant in both the no Pimelea control group and the Pimelea + bentonite treatment group. The no Pimelea control group animals however did have some additional bacterial populations contributing to the differences seen, specifically bacteria classified in the family Saccharimonadaceae, and the two genera, *Quinella* and *Anaerovorax.*

When the effect of time was addressed by analysing the data from each rumen fluid sample collection time-point individually, different effects on microbial variations were observed at each time-point (Figure 4.70 to Figure 4.77; and corresponding Table 9.13 to Table 9.16 in Appendix Section 9.10). For the first sample collection time-point, PCA indicated that there was only a low level of variation in rumen microbial populations occurring between animals in each experimental treatment group (Figure 4.70). Following further data transformation in the sPLSDA (Figure 4.71), the Pimelea + Biochar, Pimelea only, and the Pimelea + Inoculum treatment groups were observed to contribute most to the variation in microbial populations, with several microbial populations less abundant in these treatment groups, compared to the other experimental treatment groups and the control group, as indicated by the negative correlation values obtained (Appendix Table 9.13). The Pimelea plant material + Biochar treatment group had less bacteria classified within the families Saccharimonadaceae, Spirochaetaceae and Prevotellaceae; the Pimelea only control treatment group had less bacteria classified in the order Bacteroidales, and genus *Butyrivibrio*; and the Pimelea + inoculum treatment group had less bacteria classified in the families Christensenellaceae, Prevotellaceae and Rikenellaceae. Interestingly, the Pimelea + inoculum treatment group also had less archaeal populations classified within the genus *Methanobrevibacter*, which are known to produce methane.

At the second sample collection time-point, the animals within the Pimelea only treatment group appeared to have more variation in rumen microbial populations (PCA, Figure 4.72). Following further data transformation in the sPLSDA (Figure 4.73) the no Pimelea control group had the most variation in microbial populations, with the Pimelea + activated Biochar and Biochar, also showing some variation in microbial populations. The no Pimelea control group had higher populations of bacteria classified within the Class Clostridia and genus *Clostridia* (UCG-014), and family Christensenellaceae; the Pimelea + Biochar treatment group had higher levels of the genera *Prevotella* and *Quinella*, and the family Ruminococcaceae; and the Pimelea + activated Biochar treatment group had higher levels of the order Oscillospirales, and the genera *Anaeroplasma* and *Succiniclasticum.* In addition, in the first sPLSDA component, a single bacterial population classified within the family Lachnospiraceae was found to be much lower (negative correlation value) in the Pim + Bentonite treatment group (Appendix Table 9.14)

At the third sample collection time-point, in contrast to the previous two time-points, the animals within the no Pimelea control group showed the most variation in rumen microbial populations, with the Pimelea only and Pimelea + Biochar treatment groups, also showing variation in respective rumen microbial populations (PCA, Figure 4.74). When the data was transformed in the sPLSDA, the Pimelea + inoculum treatment group also showed variation in rumen microbial populations, when compared to the other experimental treatment and control groups (sPLSDA, Figure 4.75). Specific microbial populations contributing to this variation included, bacteria classified within the families Clostridia (UCG-014) and Prevotellaceae, which were less in the Pimelea only treatment group (negative correlation values); the genera *Prevotella, Clostridia* (UCG-014) and *Anaeroplasma*, which were present in higher numbers in the no Pimelea control group; and the genera *Eubacterium coprostanoligenes*, and *Prevotella,* which were present in higher proportions in the Pimelea + Inoculum treatment group (Appendix Table 9.15).

At the fourth sample collection time-point, there was still a lack of variation occurring between the rumen microbial populations present in cattle within each of the six experimental groups (PCA, Figure 4.76). At this final sample collection time-point, transformation of the data indicated that the experimental groups showing the most variation in microbial populations were the Pimelea + inoculum, Pimelea + activated Biochar and Pimelea + bentonite treatment groups (sPLSDA, Figure 4.77). The rumen bacterial populations contributing to this variation included those classified within the order Oscillospirales and families Saccharimonadaceae and Clostridia (UCG-014), which were less predominant in the Pimelea + inoculum treatment group (negative correlation values); the genera *Eubacterium coprostanoligenes,* and *Prevotella* and *Gastranaerophilales,* which were more predominant in the Pimelea + Bentonite treatment group; and the genera *Prevotella, Eubacterium ruminantium,* and *Treponema,* which were present in higher proportions in the Pimelea + activated Biochar treatment group (Appendix Table 9.16).

While several microbial populations were consistently associated with the six treatment groups at all sample collection time-points, for example, bacterial populations classified within the genus *Prevotella* contributed to the between-sample variation observed at most sample collection time points, there were no time or treatment-specific bacterial populations identified. The majority of bacterial populations identified throughout the duration of the experiment, were those known to contribute to the breakdown of fibrous plant material, including populations classified within the bacterial orders, Bacteroidales, Oscillospirales, Lachnospirales and Christensenellales (Seshadri et al. 2018; Wallace et al. 2019). This lack of variation in rumen microbial populations can possibly be attributed to the experimental design, whereby cattle in every control and treatment groups, were maintained on the same basal diet, and diet has previously been shown to significantly influence rumen microbial community composition (Henderson et al. 2015; Newbold and Ramos-Morales 2020). The physical proportion of milled Pimelea plant material supplied to the cattle was low (added to the daily feed of animals in the Pimelea treatment groups at a rate calculated to deliver 5 µg simplexin/kg bw per day, based on weekly bodyweight measures) and did not appear to change the overall nutritional composition of the diet. As animals in the Pimelea treatment groups increasingly showed clinical signs of Pimelea poisoning, the rumen microbial populations did not appear to reflect these changes. This suggests that the rumen microbial populations were very resilient, with dominant bacterial populations being maintained, despite decreases in feed intake, changes in blood parameters and body condition score and the development of oedema (treatment group effects described in Section 4.7.4 and Section 4.7.5).

Figure 4.67. Rumen microbial populations present in cattle in each Pimelea feeding trial treatment group over time (10 weeks). Bacterial and archaeal populations for each animal presented at order level taxonomy, represented in each column of the stacked bar chart. The top 20 most highly abundant orders are listed, and samples are ordered according to the sample collection time (collections 1 to 4) and the respective treatment group, including, Pimelea only (Pim); Pimelea + Biochar (Pim + Biochar); Pimelea + activated Biochar (Pim + aBiochar); Pimelea + mixed microbial inoculum (Pim + Inoc); No Pimelea control group (No Pim Control); Pimelea + bentonite (Pim + Bent).

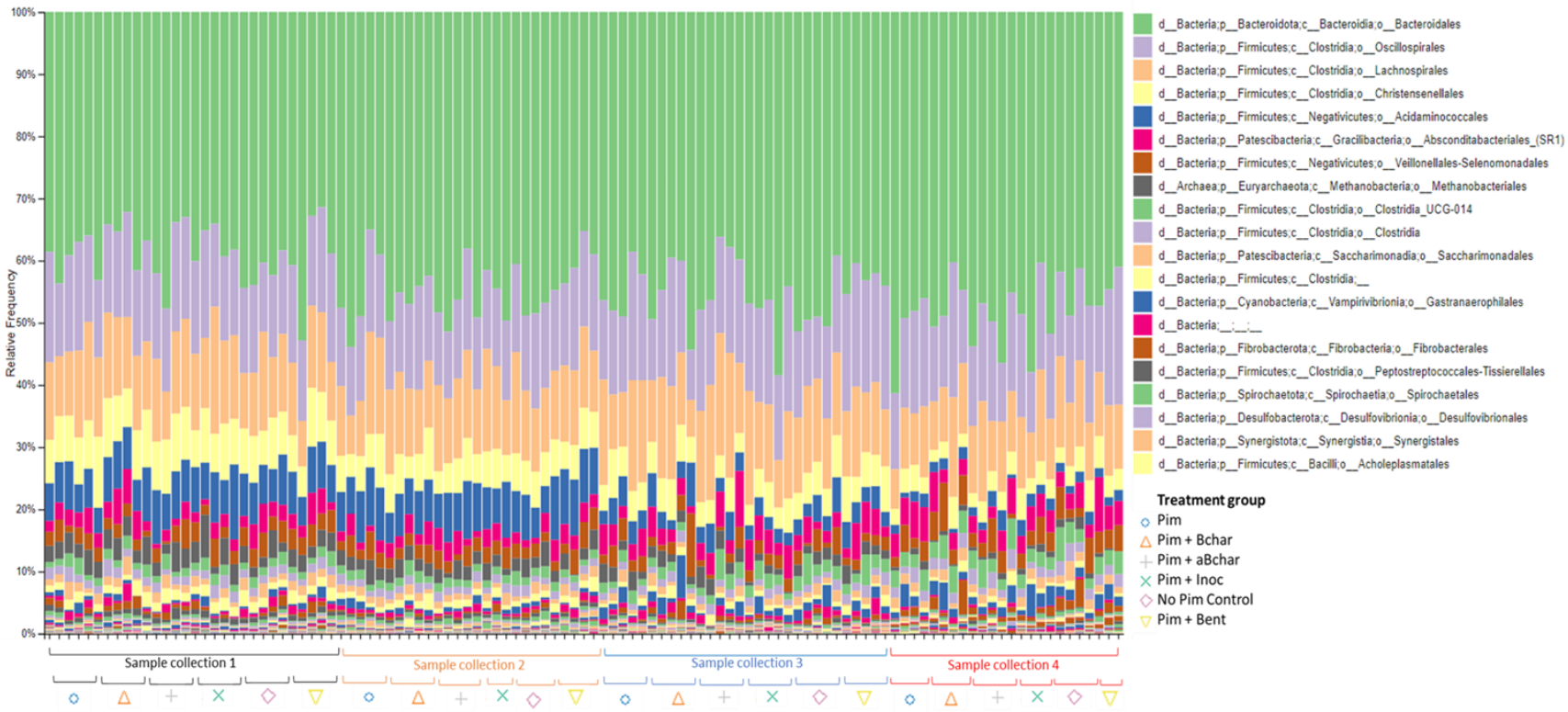


Table 4.22. Pimelea feeding trial, microbial diversity of rumen fluid samples collected from cattle, described by four measures of within-sample (alpha) diversity. Experimental parameters examined include: Sample collections in February (1), April (2), and two collections in May (3,4); and Experimental treatment groups at all sample collection times. The 6 treatment groups included, Pimelea plant material (Pim); Pimelea plant material + Biochar (Pim + Bchar); Pimelea plant material + activated Biochar (Pim + aBchar); Pimelea plant material + mixed microbial inoculum (Pim + Inoc); no Pimelea plant material, control group (no Pim Control); Pimelea plant material + bentonite (Pim + Bent).

Experimental parameters[A]	Observed features		Faith PD		Shannon entropy		Pielou evenness	
	Mean[B]	s.e.[C]	Mean[B]	s.e.[C]	Mean[B]	s.e.[C]	Mean[B]	s.e.[C]
Sample collection No.								
1	936.6[c]	30.02	63.52[c]	1.272	8.916[b]	0.075	0.905	0.004
2	924.2[c]	31.68	63.09[c]	1.343	8.854[b]	0.079	0.900	0.004
3	648.1[b]	30.02	52.98[b]	1.272	8.314[a]	0.075	0.896	0.004
4	556.6[a]	33.62	48.43[a]	1.425	8.187[a]	0.084	0.905	0.005
Treatment group								
Pim	824.1	37.70	59.36	1.598	8.710[b]	0.094	0.907[bc]	0.005
Pim + Bchar	779.7	37.70	57.96	1.598	8.557[ab]	0.094	0.897[ab]	0.005
Pim + aBchar	728.2	36.77	55.12	1.559	8.383[a]	0.092	0.889[a]	0.005
Pim + Inoc	755.4	41.10	56.73	1.745	8.582[ab]	0.103	0.903[abc]	0.006
no Pim Control	763.5	37.74	56.96	1.600	8.522[ab]	0.094	0.897[ab]	0.005
Pim + Bent	790.0	38.78	57.75	1.644	8.749[b]	0.097	0.917[c]	0.005

[A]All experimental parameters were tested using a GLM, Accumulated ANOVA. For the Sample collection No. three measures, Observed Features, Faith PD and Shannon entropy, were found to have significant differences ($P < 0.001$). For the Treatment group, only the Pielou evenness measure indicated significant differences ($P < 0.05$).

[B]Adjusted means with letters indicating results Fisher's least significant difference testing, results shown only when differences were observed.

[C]Standard error of the mean (s.e.).

Figure 4.68. Variation in the rumen microbial populations of cattle throughout the duration of the Pimelea feeding trial (10 weeks of rumen fluid sampling). PCA plot with bacterial and archaeal populations of rumen fluid samples from each animal at four sample collection time-points, coloured according to the experimental treatment group, and results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).

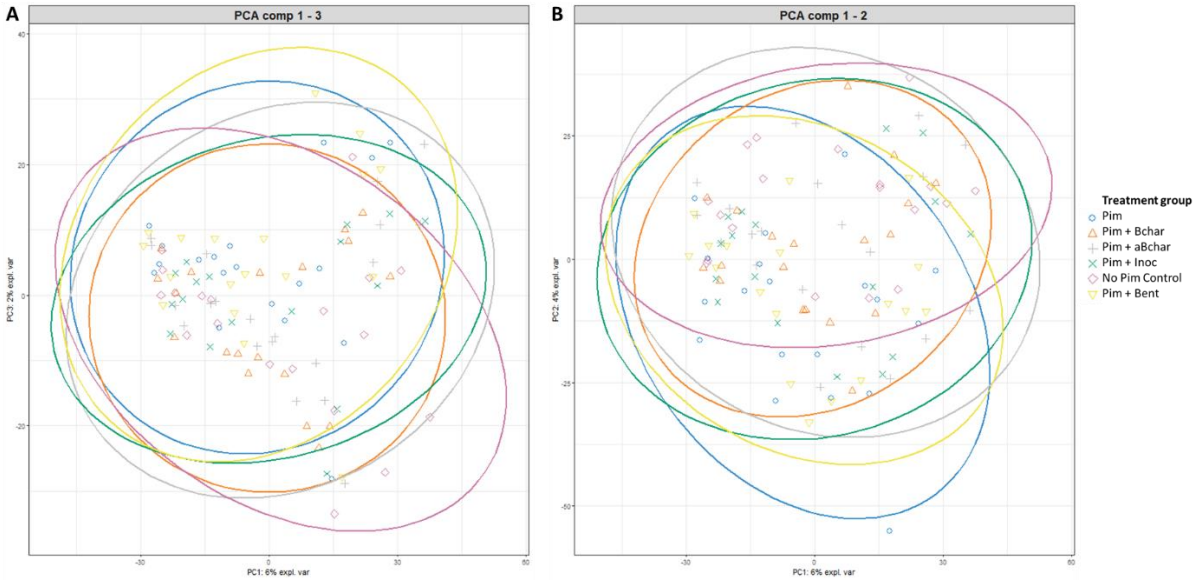


Figure 4.69. Variation in the microbial populations of cattle throughout the duration of the Pimelea feeding trial (within 10 weeks of rumen fluid sampling), results from sPLSDA, with bacterial and archaeal populations of rumen fluid samples from each animal at four sample collection time-points, coloured according to the experimental treatment group, and results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1–2); and (B) Components 1 vs 3 (sPLSDA comp 1–3).

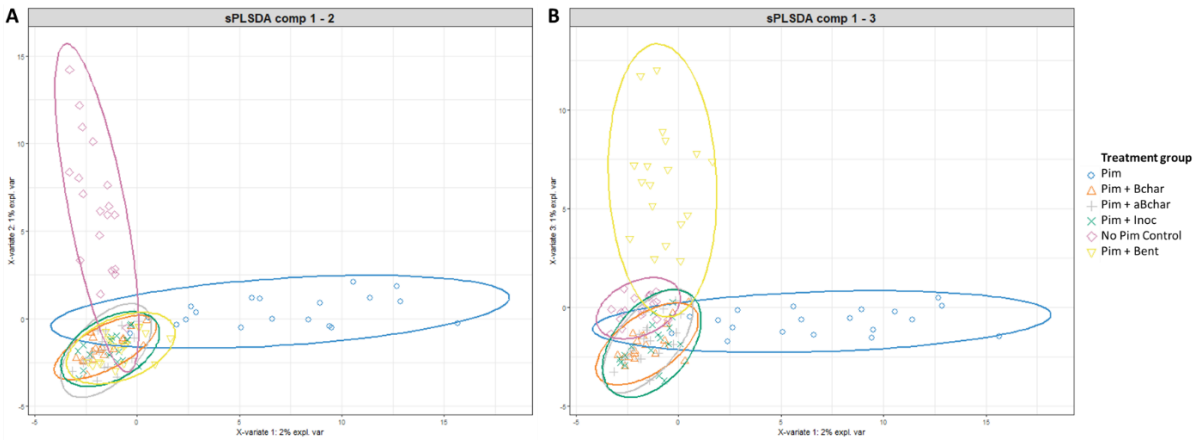


Table 4.23. Table of bacteria and archaea contributing to the differences in variation occurring between the rumen bacterial populations associated with cattle from each experimental treatment group, at all sample collection times, determined by sPLSDA presented in Figure 4.70 . The top 10 microbial populations are listed and the respective positive or negative correlation value (Importance) given. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g). Where microbial taxons with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset, that could not be classified beyond the lowest taxonomic level listed.

Contribution to sPLSDA component 1	**Importance[A]**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.366
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.269
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.248
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.209
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.206
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.179
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.171
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.168
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.157
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.156
Contribution to sPLSDA component 2	**Importance[B]**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.379
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.327
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Lachnospiraceae_NK3A20_group	0.243
p__Patescibacteria; c__Saccharimonadia; o__Saccharimonadales; f__Saccharimonadaceae; g__*Candidatus_Saccharimonas*	0.233
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	0.223
p__Firmicutes; c__Negativicutes; o__Veillonellales-Selenomonadales; f__Selenomonadaceae; g__*Quinella*	0.178
p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Anaerovoracaceae; g__*Anaerovorax*	0.168
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.157
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.157
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.154
Contribution to sPLSDA component 3	**Importance[C]**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.245
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__*Acetitomaculum*	0.238
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	0.227
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-011; g__UCG-011	0.220
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.209
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	0.204

p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.198
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.192
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_UCG-001	0.182
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_UCG-003	0.178

[A] Importance, all listed genera contributed from the Pimelea only treatment group (blue text; TRUE by sPLSDA);
[B]Importance, all listed genera contributed from the no Pimelea Control treatment group (purple text; TRUE by sPLSDA);
[C]Importance, all listed genera contributed from the Pimelea + Bentonite treatment group (yellow text, TRUE by sPLSDA).

Figure 4.70. Sample collection 1: Variation in the rumen microbial populations of cattle, at time point 1. PCA plot with each dot-point representing the bacterial and archaeal populations present in rumen fluid collected from a single animal, coloured according to the experimental treatment group. PCA results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).

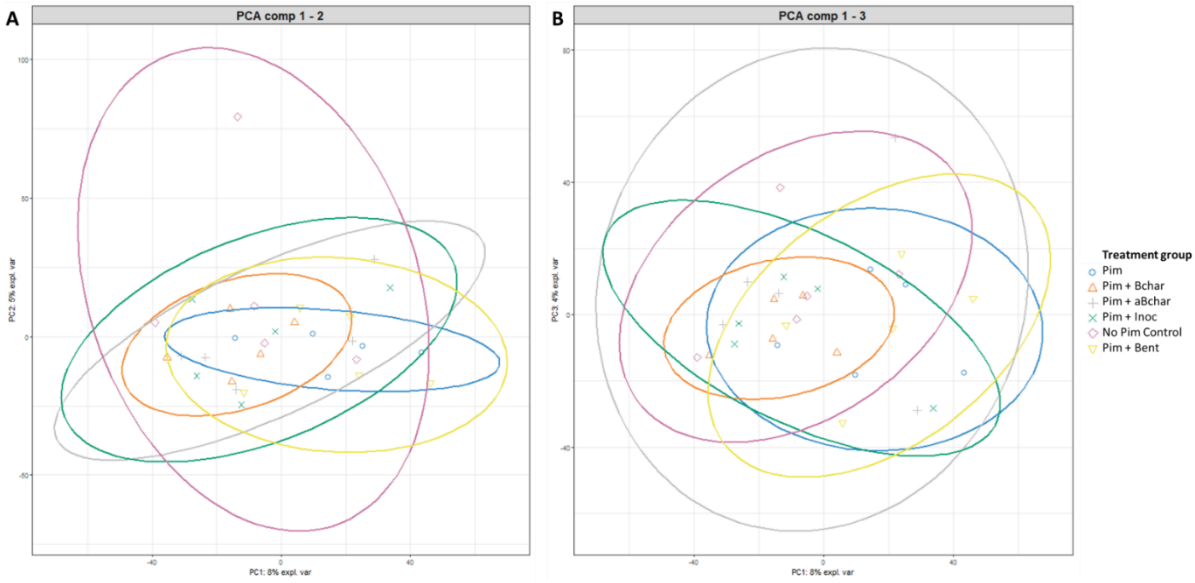


Figure 4.71. Sample collection 1: Variation in the rumen microbial populations of cattle at time-point 1 of the Pimelea feeding trial, results from sPLSDA, with rumen fluid samples from each animal coloured according to the experimental treatment group and results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1–2); and (B) Components 1 vs 3 (sPLSDA comp 1–3).

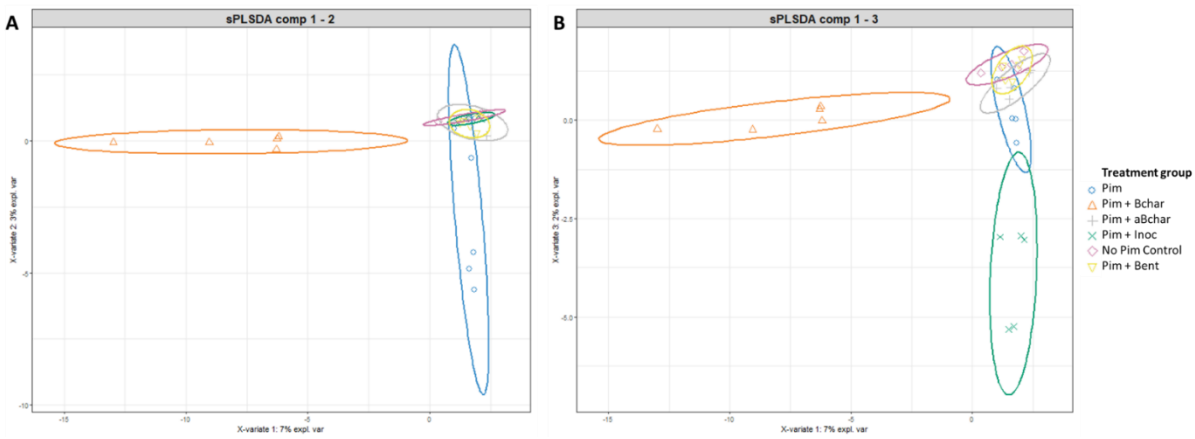


Figure 4.72. Sample collection 2: Variation in the rumen microbial populations of cattle, at time-point 2. PCA plot with each dot-point representing the bacterial and archaeal populations present in rumen fluid collected from a single animal, coloured according to the experimental treatment group. PCA results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1–2); and (B) Components 1 vs 3 (PCA comp 1–3).

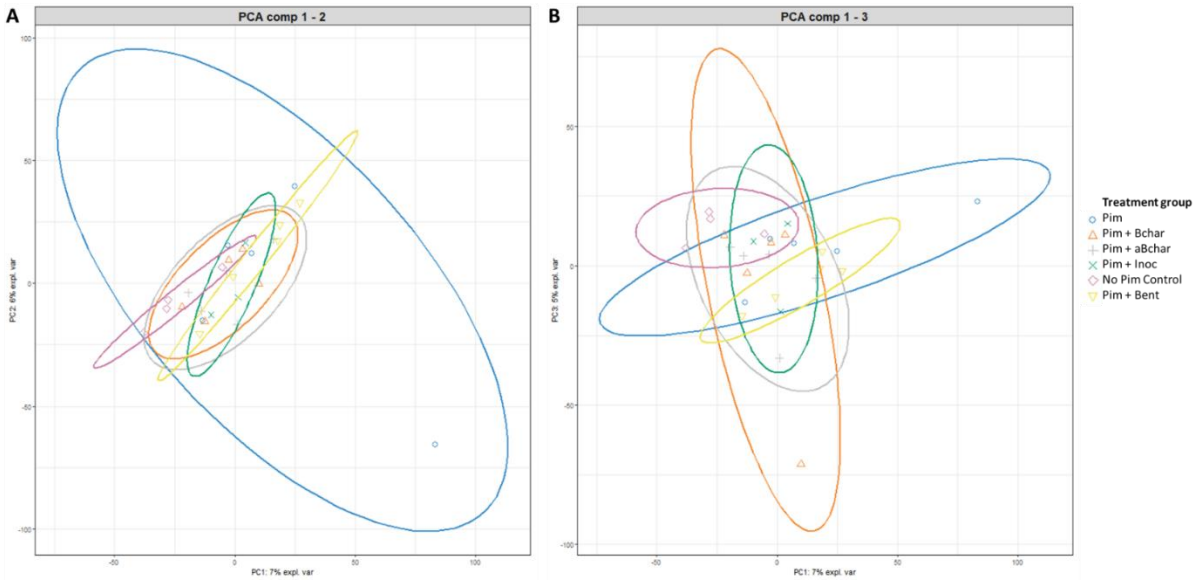


Figure 4.73. Sample collection 2: Variation in the rumen microbial populations of cattle, at time-point 2 of the Pimelea feeding trial, results from sPLSDA, with rumen fluid samples from each animal at coloured according to the experimental treatment group and results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1–2); and (B) Components 1 vs 3 (sPLSDA comp 1–3).

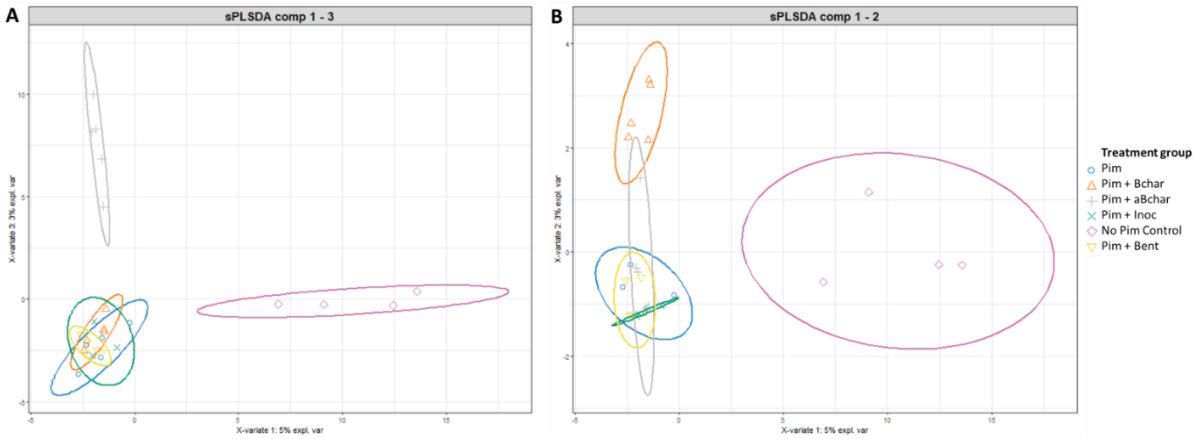


Figure 4.74. Sample collection 3: Variation in the microbial populations of cattle, at time-point 3. PCA plot with each dot-point representing the bacterial and archaeal populations present in rumen fluid collected from a single animal, coloured according to the experimental treatment group. PCA results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1–3).

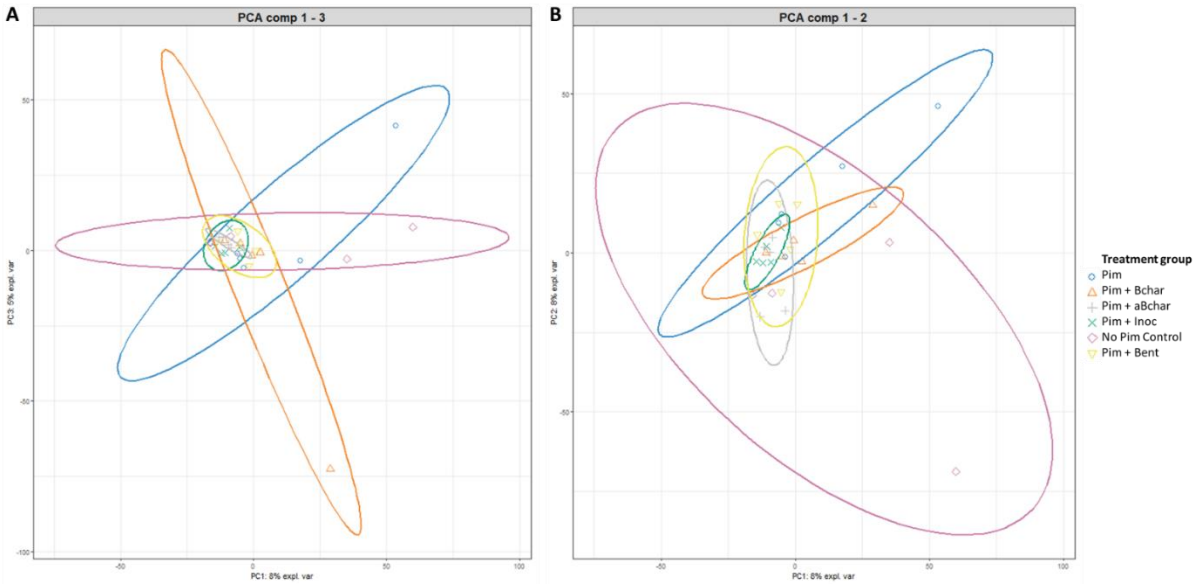


Figure 4.75. Sample collection 3: Variation in the rumen microbial populations of cattle, at time-point 3 of the Pimelea feeding trial, results from sPLSDA, with rumen fluid samples from each animal at the third sample collection time-point coloured according to the experimental treatment group and results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1-2); and (B) Components 1 vs 3 (sPLSDA comp 1–3).

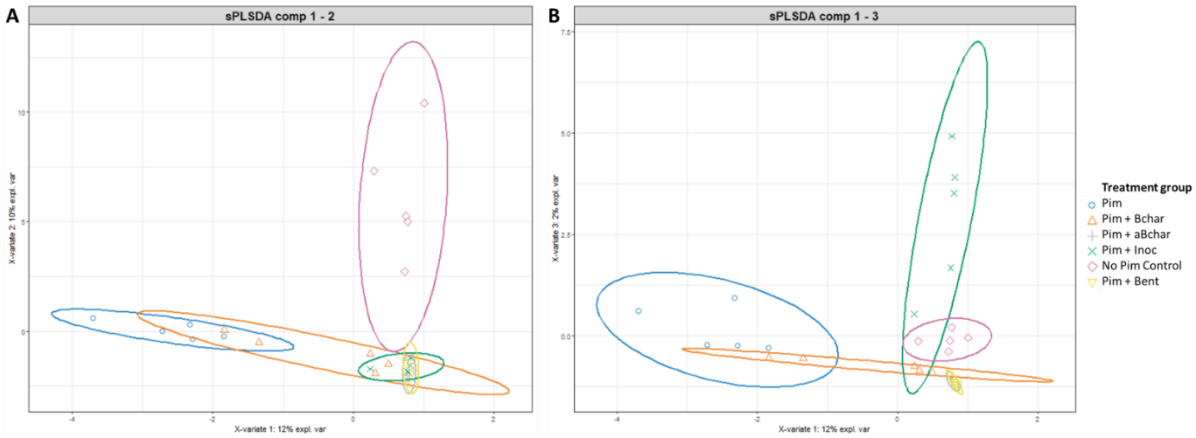


Figure 4.76. Sample collection 4: Variation in the microbial populations of cattle, at the fourth sample collection time point. PCA plot with each dot-point representing the bacterial and archaeal populations present in rumen fluid collected from a single animal, coloured according to the experimental treatment group. PCA results shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1–2); and (B) Components 1 vs 3 (PCA comp 1–3).

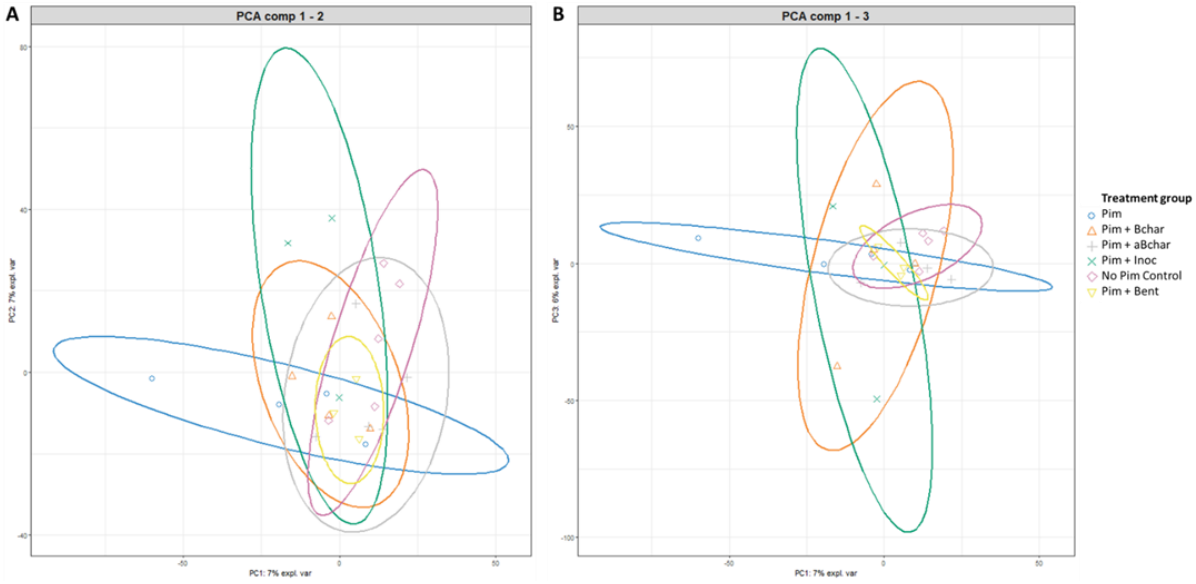
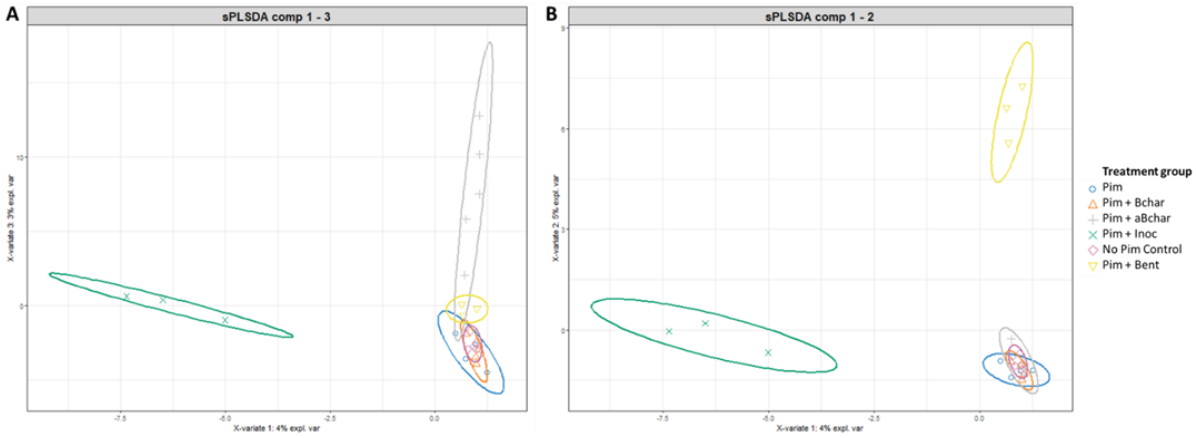


Figure 4.77. Sample collection 4: Variation in the rumen microbial populations of cattle, at time-point 4 of the Pimelea feeding trial, results from sPLSDA, with rumen fluid samples from each animal at time-point 4 coloured according to the experimental treatment group and results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1–2); and (B) Components 1 vs 3 (sPLSDA comp 1-3).



4.7.4 Clinical and haematological analysis

Blood samples were collected weekly and analysed by UQ Veterinary Laboratory Services for both haematology and biochemistry. Genstat analysis provided average values for each treatment group across time, with Estimated standard errors (s.e.) and Least Significant Difference (LSD) values for each parameter. Repeated measures ANOVA across time, with pre-treatment measures as a covariate, showed the treatment by time interaction was significant ($P < 0.05$, except for total protein $P = 0.103$ and lymphocytes $P = 0.079$) for the analyses discussed below (see

Figure 4.78, Figure 4.79 and

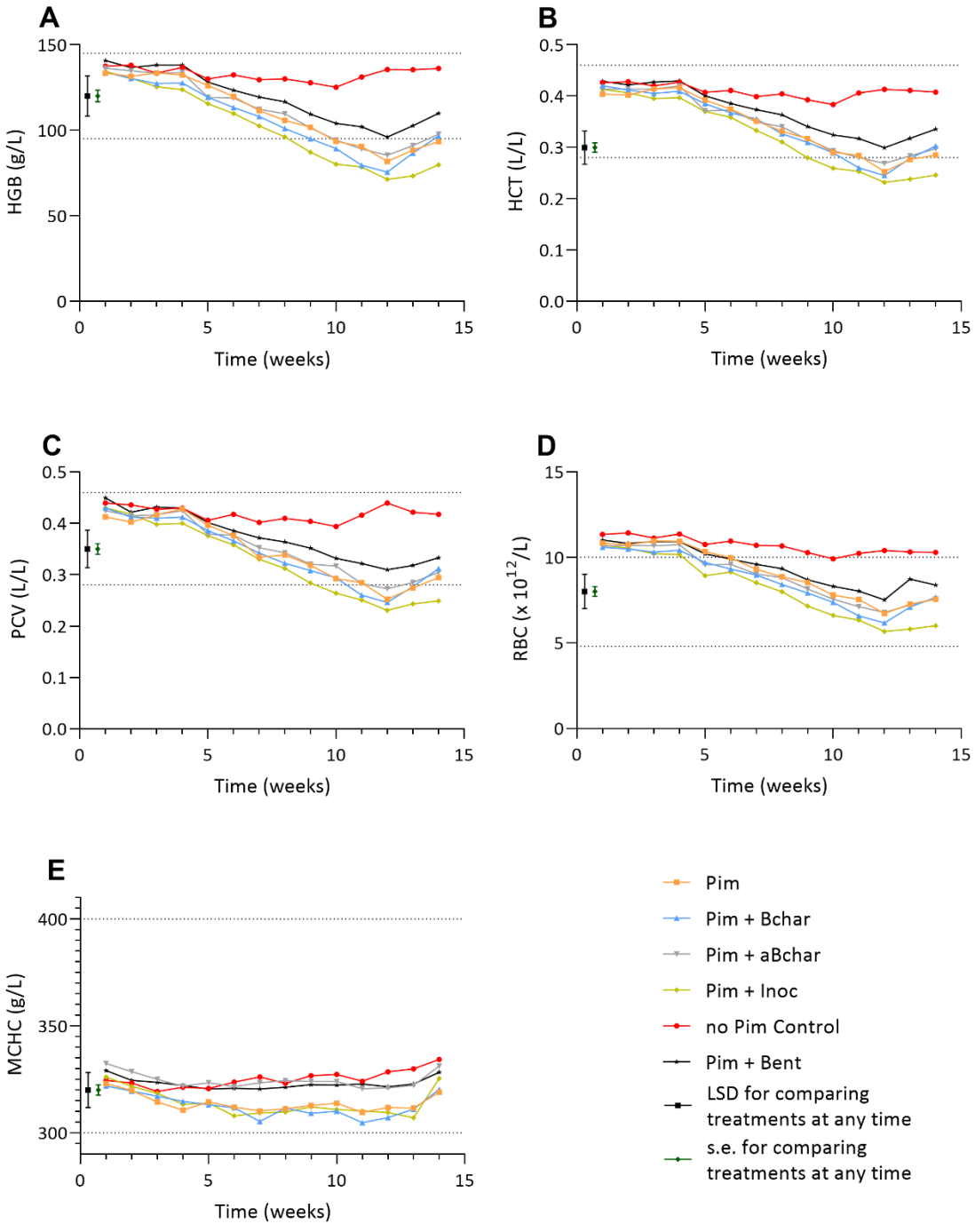
Figure 4.80), indicating differing treatment patterns over time. The treatment by time interaction means (adjusted for block, covariate, group and missing values) showed differences between the Pimelea-fed treatments compared to the negative control (no Pim Control), indicating the importance of time as well as treatment on the effects of Pimelea poisoning.

Haematological values (haemoglobin concentration, haematocrit, packed cell volume, erythrocyte, platelet, total and differential leucocyte counts and erythrocyte indices) were measured. For Pimelea dosed animals there was a general trend in decreasing haemoglobin (HGB), haematocrit (HCT), packed cell volume (PCV), red blood cells (RBC) and mean corpuscular haemoglobin concentration (MCHC) significant over time ($P < 0.05$) compared to the negative control (

Figure 4.78).

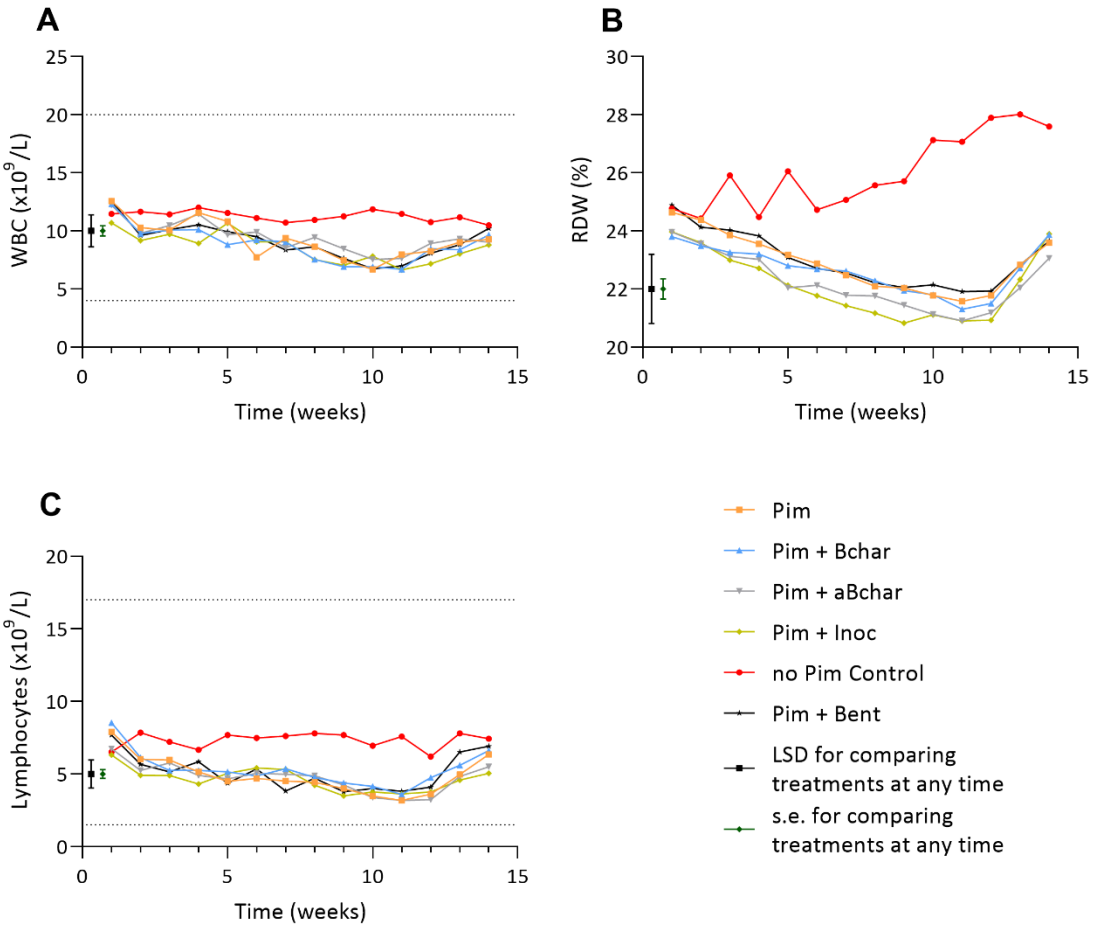
These parameters remained relatively unchanged in the negative control (no Pim Control) group as shown by the treatment versus time plots. In all five of these parameters, there was no difference between the Pimelea-fed treatments ($P > 0.05$). There were however no clear impacts of any of the five Pimelea treatments on eosinophils, fibrinogen, MCV, monocytes, platelets, RET% and RET (Abs).

Figure 4.78. Changes in (A) haemoglobin (HGB), (B) haematocrit (HCT), (C) packed cell volume (PCV), (D) red blood cells (RBC) and (E) mean corpuscular haemoglobin concentration (MCHC) across treatment groups measured during the Pimelea feeding trial (dotted lines show references intervals). LSD = Least significant difference of means (5% level); s.e. = standard error of means.



White blood cells (WBC), lymphocytes and red cell distribution width (RDW) decreased over time (significant ($P < 0.05$) for WBC in weeks 10–12, RDW in weeks 7–15 and lymphocytes in weeks 3,6, and 8-12), in all Pimelea treatment groups (Figure 4.79) compared to the negative control (no Pim Control), with no discernible trends between Pimelea treatments groups.

Figure 4.79. Changes in (A) white blood cells (WBC), (B) red cell distribution width (RDW) and (C) lymphocytes across treatment groups measured during the Pimelea feeding trial (dotted lines show references intervals). LSD = Least significant difference of means (5% level); s.e. = standard error of means.

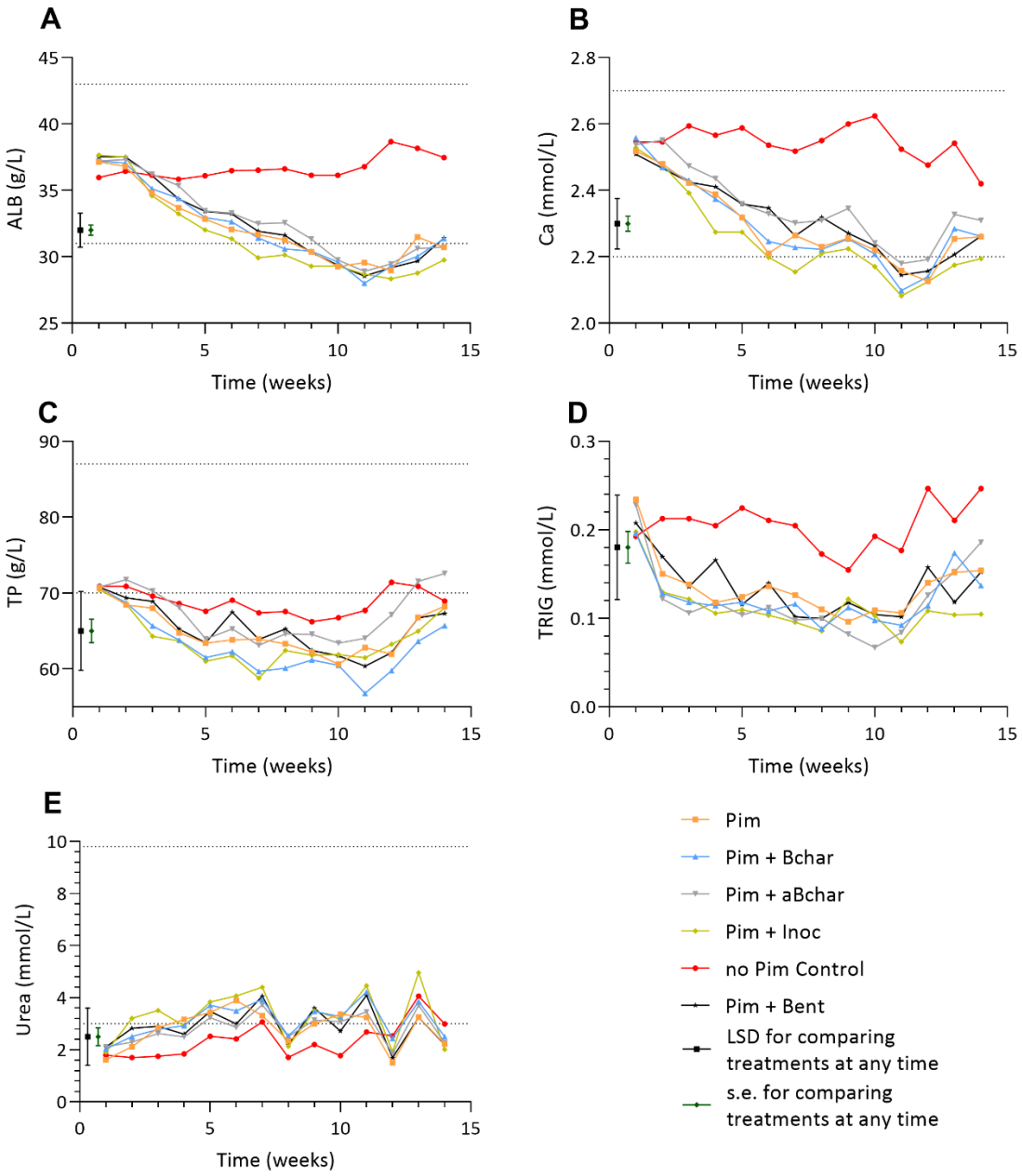


Chemical concentrations and enzyme activities (aspartate aminotransferase, creatine kinase, glutamate dehydrogenase, γ-glutamyl transferase, total protein, albumin, bile acids, triglycerides, non-esterified fatty acids, glucose, β-hydroxybutyrate, phosphate, bicarbonate, calcium, magnesium, sodium, potassium, chloride, total bilirubin, urea and creatinine) were measured in plasma. Lipaemia, icterus and haemolysis were assessed and graded from 1 (mild) to 4 (marked). N=normal. Downward trends over time were noted particularly for albumin ($P < 0.05$), Calcium (Ca) ($P < 0.05$) and triglycerides ($P < 0.05$) and for total protein (not significant, $P > 0.05$), in the bloods of the Pimelea treatment groups when compared to the no Pimelea control group (

Figure 4.80).

Urea on the other hand showed an apparent upward trend (not significant, $P > 0.05$) in the Pimelea treatment groups compared to the negative control. No consistent significant changes ($P > 0.05$) were noted in other biochemical parameters during the trial, in either treatment or control groups

Figure 4.80. Changes in (A) Albumen (ALB), (B) Ca, (C) Total Protein (TP), (D) Triglycerides (TRIG), and (E) Urea across treatment groups measured during the Pimelea feeding trial. LSD = Least significant difference of means (5% level); s.e. = standard error of means.

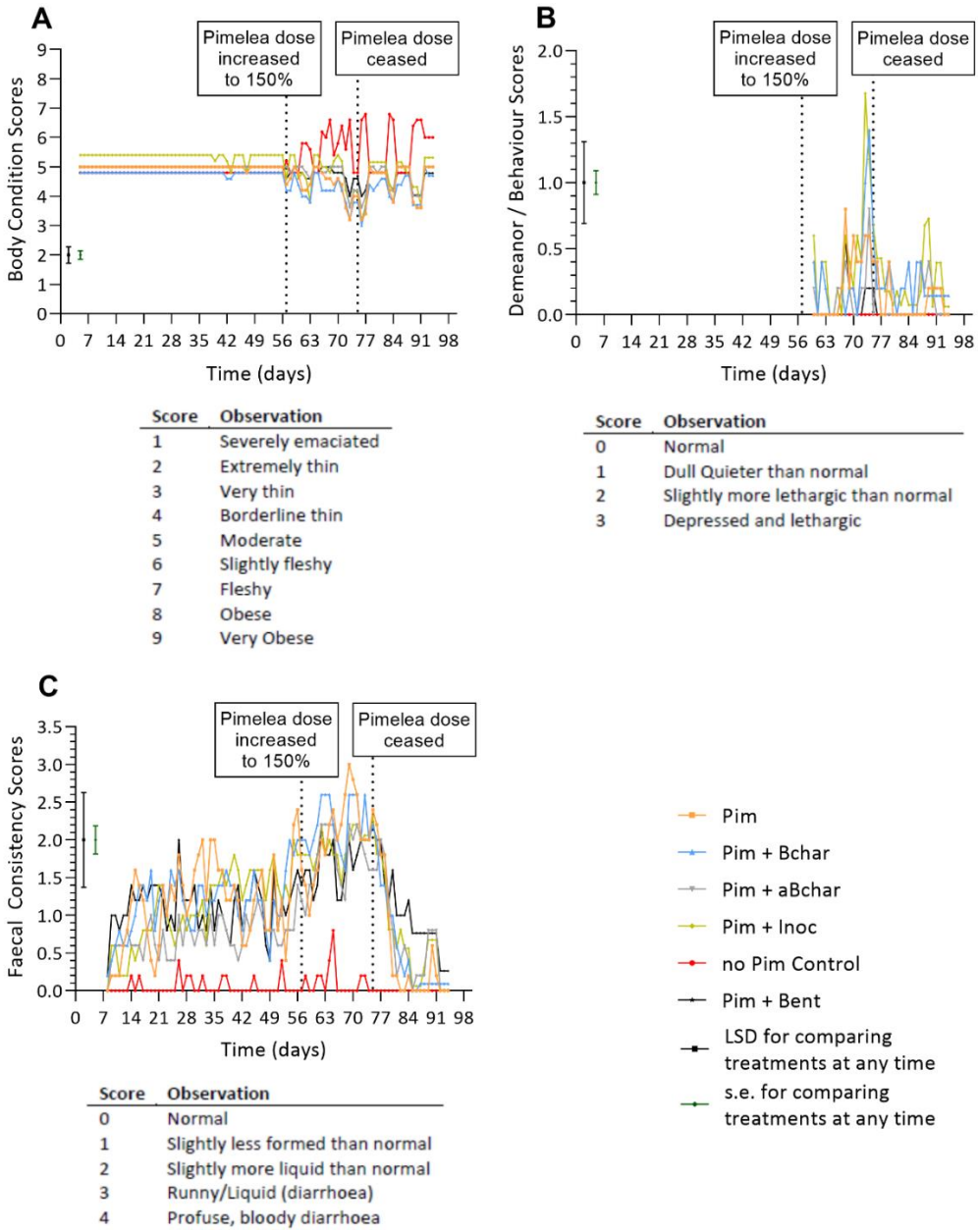


4.7.5 iAuditor reports

iAuditor reports were recorded on a daily basis throughout the trial and were extracted back into excel for statistical analysis by Genstat. All-zero dates were excluded from the statistical analyses and the graphs show means adjusted for missing values. Scores from animals that were removed from the treatment for periods on animal ethics grounds remained in the statistical analyses keeping them indicative of the range of responses from those treatments. The LSD bar on each graph of Genstat output shows where the treatments significantly diverged.

Negative control animals remained healthy throughout the trial with no diarrhea, oedema, demeanour changes or loss of condition noted. After the Pimelea dose was increased (week 9), the negative control animals body condition and demeanour scores were consistently better than the Pimelea treatment groups (Figure 4.81). Throughout the trial, the negative control treatment animals had largely normal faecal consistency whilst those receiving Pimelea doses demonstrated diarrhea, particularly after the dose was increased, with it gradually returning to normal after the dose was ceased. Body condition losses and behaviour changes were first observed at week 8 (Figure 4.81).

Figure 4.81. Genstat treatment versus time output based on iAuditor Scores given to animals in each treatment for (A) Body Condition, (B) Demeanour/Behaviour, (C) Faecal consistency showing the meaning of each score applied for each observation. All-zero dates were excluded from the analyses. LSD = Least significant difference of means (5% level); s.e. = standard error of means.



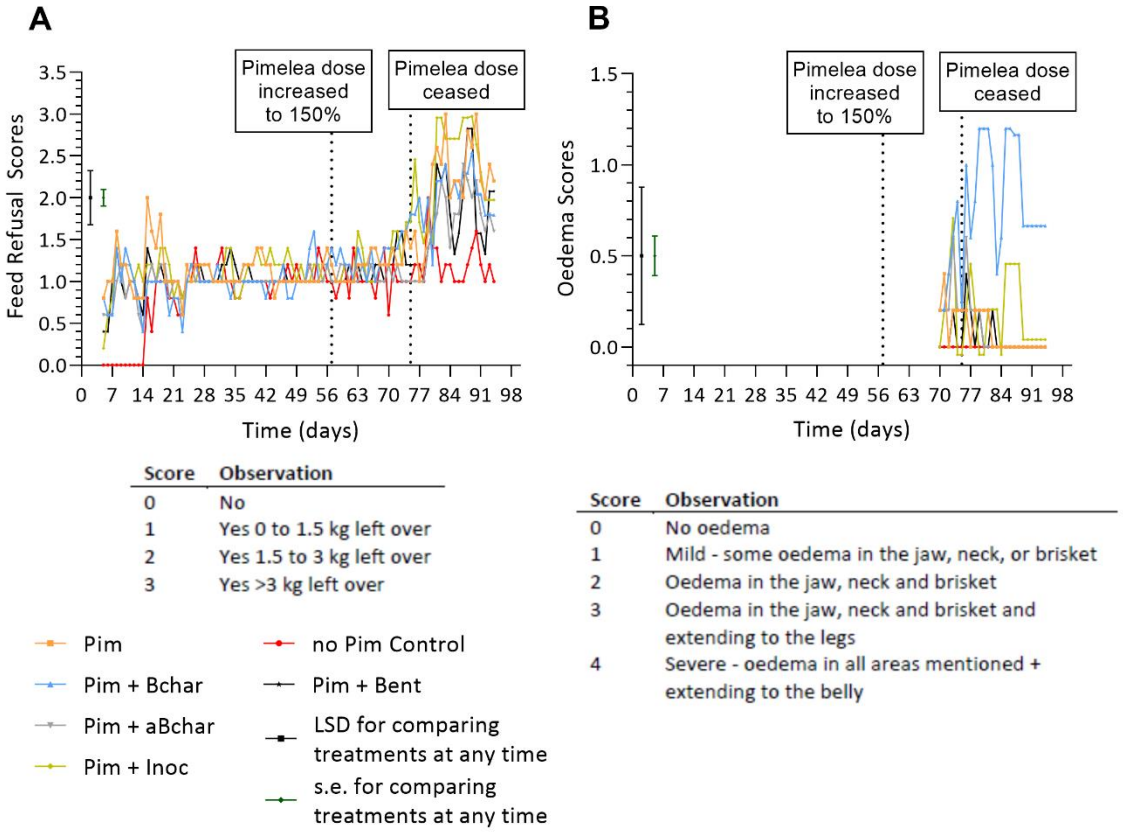
Score	Observation
1	Severely emaciated
2	Extremely thin
3	Very thin
4	Borderline thin
5	Moderate
6	Slightly fleshy
7	Fleshy
8	Obese
9	Very Obese

Score	Observation
0	Normal
1	Dull Quieter than normal
2	Slightly more lethargic than normal
3	Depressed and lethargic

Score	Observation
0	Normal
1	Slightly less formed than normal
2	Slightly more liquid than normal
3	Runny/Liquid (diarrhoea)
4	Profuse, bloody diarrhoea

Oedema was first noted at week 10 of the trial (after the Pimelea dose increased in week 9) and was particularly pronounced for the Pim+Bchar group (Figure 4.82), with some animals developing oedema up to 2 weeks after cessation of Pimelea intake. The treatment by time interaction was significant for body condition and feed refusals (both $P < 0.001$) and faecal consistency ($P < 0.003$).

Figure 4.82. Genstat treatment versus time output based on iAuditor Scores given to animals in each treatment for (A) Feed Refusals and (B) Oedema showing the meaning of each score applied for each observation. All-zero dates were excluded from the analyses. LSD = Least significant difference of means (5% level); s.e. = standard error of means.



4.7.6 Animal health and welfare

Three animals (#338 T1 Pim positive control; #280 T4 Pim + Inoc; #086 T4 Pim + Inoc) were withdrawn from treatment for periods of up to 7 days when this diarrhea became extreme. #280 (Pim + Inoc) coincidentally experienced an unfortunate collision with a laneway rail and broke 4 front teeth on his lower jaw. This steer was examined by UQ Vets and received pain relief (meloxicam-20 6.25mL, SC batch 200828 exp 02/22). On examination the affected teeth were deemed to be baby teeth, and the animal continued in the trial with no apparent impact on feed intake.

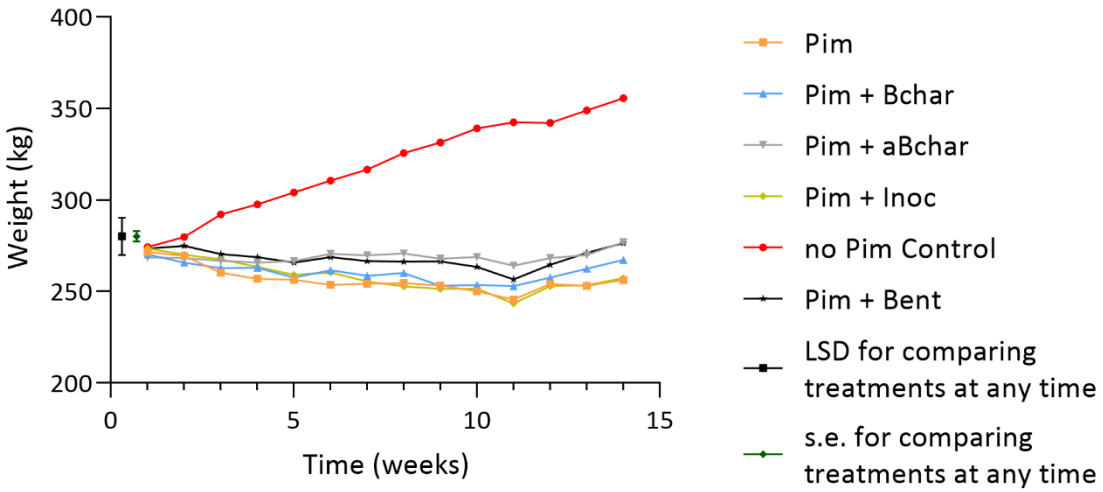
No sign of fluid accumulation or oedema was seen in any of the treatment groups after 8 weeks of Pimelea inclusion of 5 µg simplexin /kg bw/day. At week 9, the daily dosing was increased to 7.5 µg simplexin /kg bw/day (50% increase). After a further 2 weeks of feeding, mild sign of oedema was first noted with mild swelling around the face of # 082. In the following week, further evidence of oedema was noted together with reduced feed intake and increased heart rates in the crush. All Pimelea dietary treatments were ceased on 6th May (11 weeks). Thereafter steers were fed with the

same diluted molasses as before added. The Pimelea treatment animals gradually increased hay intake, and all animals were administered 8 kg hay per day after cessation of Pimelea intake.

4.7.7 Weight gain and feed intake

Negative control animals (no Pim Control) consistently gained weight throughout the trial. Analysis of variance (adjusted for covariate) indicated the treatment by time interaction was significant ($P < 0.001$) for steer weights and feed intake. All Pimelea treatment groups had significantly ($P < 0.05$) reduced feed intake and decreased weight gains over time, consistent with a general dislike of the Pimelea diets. Pim + aBchar and Pim + Bent treatment groups tended to maintain weight across the trial better than did treatment groups Pim (positive control), Pim + Bchar or Pim + Inoc (Figure 4.83). Animals were fed adlib, so it is not surprising that the negative control group (no Pim Control) had a higher daily intake and weight gain. The difference in bodyweight between the positive control (Pim) group and the negative control (no Pim Control) group was ca. 100 kg bodyweight at the end of the trial.

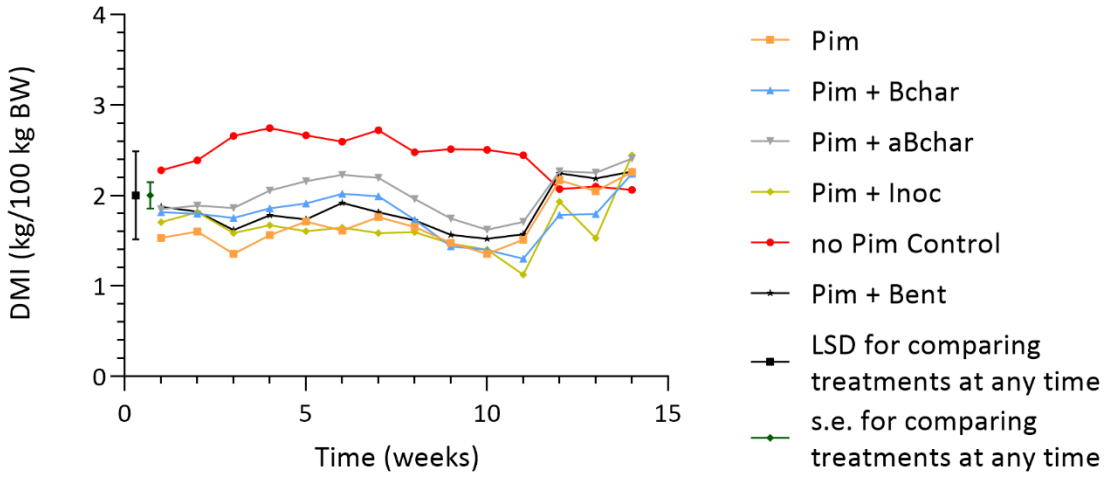
Figure 4.83. Changes in steer body weight across treatment groups measured weekly during the Pimelea feeding trial. LSD = Least significant difference of means (5% level); s.e. = standard error of means.



The feed intake plot DMI/100 kg bodyweight (

Figure 4.84) demonstrates the reduced feed intake that occurred when the Pimelea intake was increased in week 9, and subsequent increased feed intake that occurred across most treatment groups when the Pimelea intake was ceased at week 11.

Figure 4.84. Changes in DMI/100 kg bodyweight across treatment groups measured weekly during the Pimelea feeding trial. LSD = Least significant difference of means (5% level); s.e. = standard error of means.



4.7.8 Simplexin analysis of blood samples

Lithium heparin bloods for toxin analysis have been freeze dried using a CSK Climatek Freeze Dryer. The LC-MS/MS analysis method previously reported by Fletcher et al. (2014) was adapted to suit current LC-MS/MS instrumentation. Analysis of blank blood spiked with simplexin showed acceptable recoveries (65-70%) from blank blood spiked at 0.055 and 1.10 mg/kg (Table 4.24). At lower level spikes 0.010 mg/kg, interference was noted from phospholipids, and an improved method developed with the initial extraction step carried out with acetonitrile rather than with dichloromethane/methanol (3:1) (Section 3.13.3).

Table 4.24. Average simplexin levels in spiked dried blood determined by LC-MS/MS using external standards method.

Sample		0.010 mg/kg simplexin spiked in dried blood[A]	0.055 mg/kg simplexin spiked in dried blood	1.10 mg/kg simplexin spiked in dried blood
Pre-extraction spike	Measured (mg/kg)	0.005 ± 0.0005	0.033 ± 0.003	0.75 ± 0.07
Post-extraction spike	Measured (mg/kg)	0.008 ± 0.0004	0.046 ± 0.001	1.15 ± 0.11
Solvent spike	Measured (mg/kg)	0.010 ± 0.0002	0.059 ± 0.005	1.10 ± 0.05
Recovery (%)		57	70.5	65.4
Matrix factor		0.8	0.8	1.04
Matrix effect (%)		-17	-21.5	4.2
Accuracy (%)		47	59.4	68.2

[A]As measured with the improved acetonitrile extraction method.

The interference by phospholipids initially presented false positive results (0.01 -0.02 mg/kg) in the analysis of a selected number of dried blood samples from steers within the feeding trial. Analysis of

these blood samples with the improved acetonitrile extraction method (Section 3.13.3) confirmed that these results were largely false positives.

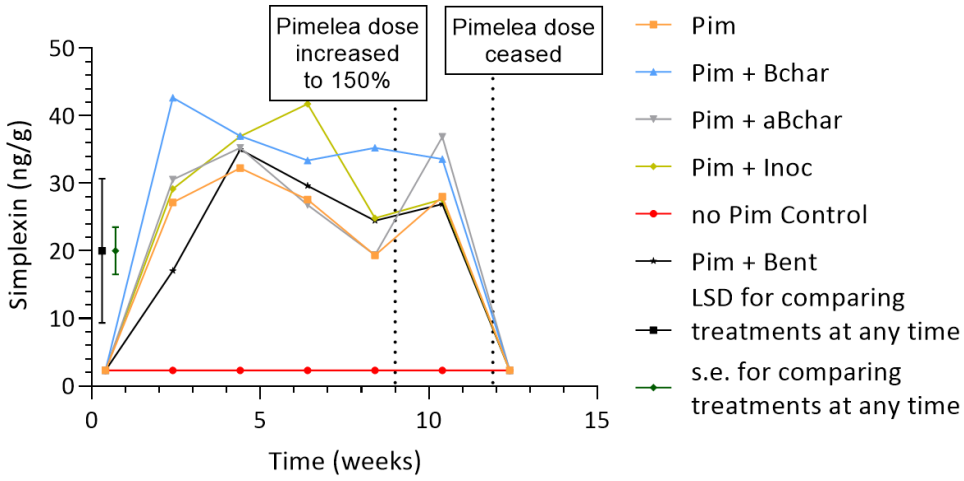
An LC-MS/MS analytical method was developed which successfully eliminated the phospholipid interference and was subsequently utilised for simplexin analysis of freeze-dried blood samples. The method limit of detection (LOD) was determined to be 3 ng/g simplexin in dried blood and limit of quantification (LOQ) of 9 ng/g simplexin in dried blood. The LOD was significantly lower than developed previously (Fletcher et al. 2014).

Week 10 steer blood samples were analysed for simplexin as steers in that week were fed the higher Pimelea plant dose of 7.5 µg simplexin/kg bw/day which started in week 9. Week 10 was the final week that steers in all treatment groups ate most of their feed. However, simplexin concentration was below the limit of detection (LOD) for all treatment groups, despite animals in Pimelea containing treatments displaying physical signs of poisoning. Targeted MS analyses to search for possible metabolites (e.g. hydroxylated, sulfate or glucuronide derivatives of simplexin) were unsuccessful.

4.7.9 Simplexin analysis of faecal samples

Faecal samples collected fortnightly throughout the trial have been freeze-dried and analysis conducted on three replicate samples by LC-MS/MS. Limit of detection was determined to be 4.6 ng/g and LOQ 10 ng/g. For samples in which no simplexin was detected (including pre and post-trial samples and no Pimelea control samples), LOD/2 was substituted for statistical analysis purposes. Any simplexin detected between LOD and LOQ were assigned LOQ/2. Each of the 3 replicate samples were averaged before statistical analysis by Genstat. A statistically significant difference ($P < 0.05$) was observed between the no Pimelea control treatment and the remainder of the treatments (Figure 4.85). A trending pattern over time (not significant, $P > 0.05$) indicated slightly higher levels of simplexin in the Pim + Bchar treatment samples compared to other Pim containing samples (except Pim + Inoc).

Figure 4.85. Changes in simplexin detected in faeces across treatment groups measured fortnightly during the Pimelea feeding trial. LSD = Least significant difference of means (5% level); s.e. = standard error of means.



4.7.10 Animal euthanasia and necropsy

Death as an end point was not the objective of this trial, with all animals expected to recover after cessation of Pimelea plant material intake. Four animals however experienced severe symptoms some related to Pimelea and in two cases coincidental physical causes. Feed intake was severely reduced over 3–4 days in all 4 animals, and these animals were euthanased on veterinary advice due to animal welfare considerations as detailed in Section 3.12.13, with necropsy details provided in Appendix Section 9.14. Steer #082 (T2 Biochar treatment group (Pim +Bchar)) and steer #086 (T4 inoculum treatment group (Pim + Inoc)) experienced marked Pimelea poisoning symptoms and were both euthanased in week 13 of the trial. Two further animals experienced unrelated oesophageal injuries (Steer #375, T4 inoculum treatment group (Pim + Inoc)) and abscess (Steer #051, T6 bentonite treatment group (Pim + Bent)) and were euthanased in week 11 and week 13 respectively. Notably, the significant fluid accumulation in both abdomen and chest cavities seen at necropsy in Steer#082 and Steer #086 was not present in Steer#051 which had received the bentonite treatment (Appendix Section 9.14).

4.7.11 Summary discussion - Treatment efficacy in cattle feeding trials

Efficacy of adsorbent treatments

Pimelea toxicity was not prevented by any of the mitigation treatments. All mitigation treatments were similar to the positive control group fed Pimelea for overall effects of pimelea toxicity. The lack of efficacy for the mitigation treatments was particularly evident in body weight, dry matter intake and the haematology parameters haemoglobin (HGB), haematocrit (HCT), packed cell volume (PCV), red blood cells (RBC) and mean corpuscular haemoglobin concentration (MCHC). Decreases in these erythrocyte indices are considered indicative of the non-regenerative anaemia seen in Pimelea poisoning (Kelly 1975). These parameters are more reliably measured compared to the extent of oedema which is somewhat subjective and dependent on the individual person scoring the animal.

Simplexin was detected in faecal samples of cattle fed Pimelea diets (all treatment groups except the negative control group) and represented only a small portion of simplexin present in the diet (<10%). Some variability was observed between (and within) treatment groups but none of the adsorbents seemed to greatly enhance the amount of simplexin in collected faeces, and it is plausible that this represents at least in part undigested milled Pimelea plant material. The addition of bentonite to the diet appears to have acted to reduce toxin impacts on blood parameters and suggests that this adsorbent is able to bind the toxin and prevent or delay toxin uptake from the gastric system. The absence of increased toxin in the faeces however suggests that this binding is insufficient to survive passage through the entire gastric system.

Simplexin was not detectable in blood samples from any steers (collected weekly throughout the trial), even in those exhibiting marked signs of Pimelea poisoning. Simplexin is known to bind strongly to Protein Kinase C (PKC) with the binding suggested to be irreversible (Pegg et al. 1994) with the toxin not able to be washed out (Mason 1976) and re-enter the bloodstream. This may explain the inability to extract and detect simplexin in animal tissues. Alternately simplexin may be metabolised either in the gastric tract or within circulatory system to an analogue which is not detected by our methodology. It has been reported that the daphnane orthoester yuanhuapine is metabolised through hydroxylation, methylation, glucuronidation and cysteine conjugation during the phase I and phase II biotransformation pathways with the suggestion that the initial step was P450 hydroxylation of one or more methyl groups (Chen et al. 2015). Our attempts to detect the corresponding simplexin metabolites in blood and faecal samples was however not successful.

Efficacy of amicrobial rumen inoculum treatment

The fortnightly administration of the experimental mixed microbial rumen inoculum did not convey any advantage to steers fed Pimelea within their daily diet when compared to a control group fed a similar Pimelea dose. The rumen microbiomes of steers in each of the six treatment groups had similar microbial populations, irrespective of the experimental treatment being applied. As the trial progressed there were some changes in the rumen bacterial communities in all animals. While several microbial populations were consistently associated with the six treatment groups at all sample collection time-points only a few, for example, bacteria classified within the genus *Prevotella* contributed to the between-sample variation. There were no time or treatment-specific bacterial populations consistently identified. Many of the bacterial populations identified throughout the duration of the animal trial were those known to contribute to the breakdown of fibrous plant material, including populations classified within the bacterial orders, Bacteroidales, Oscillospirales, Lachnospirales and Christensenellales (Seshadri et al. 2018; Wallace et al. 2019).

The lack of variation in rumen microbial populations between treatment groups can possibly be attributed to the experimental design, whereby cattle in every control and treatment groups, were maintained on the same basal diet of a medium quality, chaffed Rhodes grass hay (9% protein) at a minimum of 1.7% of bw, and diet has previously been shown to significantly influence rumen microbial community composition (Henderson et al. 2015; Newbold and Ramos-Morales 2020). The physical proportion of milled Pimelea plant material (10% protein), supplied to the cattle was low (added to the daily feed of animals in the Pimelea treatment groups at a rate calculated to deliver 5 µg simplexin/kg bw per day, based on weekly bodyweight measures). As an example, for in the diet of a 250kg animal 12.5 g of Pimelea plant material was supplied with 4.25 kg of Rhodes grass hay. This low proportion of Pimelea in the diet did not appear to change the overall nutritional composition of the diet and the inclusion of the enterosorbents in the diet of those treatment group animals also did not have a significant effect on the rumen microbial populations. As the animals in the Pimelea treatment groups increasingly showed clinical signs of Pimelea poisoning, the rumen microbial populations did not appear to reflect these changes. This suggests that the rumen microbial populations were very resilient, with the dominant bacterial populations being maintained, despite decreases in feed intake, changes in blood parameters and body condition score and the development of oedema. This maintenance of the rumen microbial populations indicates that it is likely that the rumen populations will be able to respond to new diets during the recovery of animals from the effects of Pimelea poisoning.

5. Conclusion

5.1 Key findings

This project has provided key insights into the understanding of Pimelea poisoning.

1. Our rumen microbiome studies have largely refuted the hypothesis that the rumen population of “experienced” cattle was adapted to deal with the Pimelea toxin. Differences in the gastrointestinal microbial populations were found to occur between differing species sampled (cattle, sheep, goats and kangaroos) in Pimelea infested regions, but there were only small differences in the rumen microbiome between cattle regardless of origin or previous experience with Pimelea. A series of 15 *in vitro* fermenter trials were conducted based on rumen fluids collected from diverse animal species, regions, and reported Pimelea

susceptibility, with 157 bacterial isolates obtained. However, none of the bacterial isolates tested were capable of degrading simplexin as determined by LC-MS/MS.

2. Our studies further demonstrated that a relatively small amount Pimelea in the diet of cattle can cause poisoning with simplexin. When Pimelea plant material was provided as the sole feedstock to rumen enrichment fermentations, microbes isolated from these enrichments using selective mediums containing simplexin, were unable to break down the simplexin molecule. Whilst this study was unsuccessful in isolating toxin degrading rumen microbes, there may be microbes capable of breaking down simplexin in the environment, which would not be otherwise found in the rumen e.g. soil fungi, which may have a constant higher level of environmental exposure to Pimelea plant material and simplexin.

3. Extruded biopolymer composites demonstrated a high degree of stability under both *in vitro* rumen-like fermenter conditions and *in vivo* within the rumen of fistulated steers, with surface erosion degradation providing options to control potential bioactive release rates. For example, porous 3D printed PHA biopolymer pieces degraded more than twice as fast as the solid biopolymers in the *in vivo* study. *In vitro* fermenter studies with Pimelea plant material and extracts showed that such biopolymers are good candidates for intraruminal sustained release of bioactives, including toxins to foster toxin-degrading microbial populations.

4. *In vitro* studies in clarified rumen fluid demonstrated that adsorbents such as activated biochar and bentonite showed potential as adsorbents for the toxin simplexin, with the precautionary note that activation of biochar by heating to 1,000°C is required to enhance biochar potential. Pen trials further demonstrated that steers fed Pimelea as a constant part of their daily diet and were also consuming 0.3 g bentonite/kg bw/day showed more resistance to poisoning than a control group fed a similar Pimelea dose, whereas an experimental inoculum administered to steers did not reduce Pimelea impacts.

5. An unexpected observation in the pen trial was that some feeding trial animals developed characteristic signs of Pimelea poisoning (particularly oedema) up to 2 weeks after complete cessation of Pimelea consumption. Such information on the delayed onset of symptoms is useful to producers who detect signs of Pimelea poisoning after moving animals to new or different pastures where Pimelea is not evident. In such cases the poisoning may well have occurred in the previous pasture. This situation is also relevant to producers purchasing animals that appear healthy and then later develop signs of Pimelea poisoning.

6. Feeding trial animals which developed signs of Pimelea poisoning throughout the pen trial possessed rumen microbial communities which were very similar to those present in animals maintained on the hay only diet. In this way the rumen microbes of the Pimelea-fed steers appeared to be very resilient, continuing to function despite the deteriorating health condition of the animals. This information has implications for producers managing the recovery of animals following Pimelea poisoning, as the rumen may retain function and should respond to feeding strategies.

5.2 Benefits to industry

All mitigation treatments in this study were insufficient to prevent the onset of Pimelea poisoning. A small number of producers with Pimelea affected pastures provided bentonite in loose lick supplements to cattle during the past year at a rate about double used in our pen trial and reported

very encouraging results with noticeable improvements in animal health and production. The improvements in animal health and production is however an anecdotal finding that lacks evidence. The proposed rate of ~100–200 g per head is within range approved by the European Food Safety Authority (EFSA) who assessed bentonites as being safe for all animal species, the consumers and the environment when used at a maximum level of 20,000 mg/kg or 20 g/kg complete feed.

A series of industry presentations have formed a key component of this project, from MLA BeefUp forums, Biosecurity Field days, NABRC meetings, Future Beef updates, AgForce Producers meetings and updates (see Section 9.1.1). Producers whilst awaiting a "silver bullet" solution, have taken on board the potential application of adsorbents and the theory that prevention of toxin uptake is better than a cure. Anecdotal feedback has indicated some successful producer outcomes which require further follow up (and validation).

The use of bentonite as an enterosorbent represents a management strategy that can be readily applied in the field at minimal cost but is ineffective within the conditions of these reported studies in preventing pimelea toxicity in cattle. During this project, a number of DAF field staff and Livestock Agents of Nutrien Ag Solutions (Roma) have related reports from several producers in the Roma-St George region who are administering a 1:1 mix of bentonite/loose lick with "great results", with some producers buying the bentonite by the "truck load" direct from the mine at Miles. These producers report the bentonite/loose lick mix is readily consumed by cattle (~200g bentonite/animal per day), which then show no obvious Pimelea impacts even though Pimelea was evident in pasture. By comparison our feeding trial steers were consuming an approximate daily dose of 75g bentonite per 250kg steer. An individual producer in the Blackall-Jerricho area reported he had developed a regime of administering a loose lick containing Biochar at ~0.5kg charcoal per head per week all year (~70g/animal per day) with reports of general health improvement in stock, and the perception that the animals perform better against Pimelea with the added Biochar than without Biochar. His conclusion was that Biochar was effective as a preventative against Pimelea, but only up to a point. The adsorbent can only adsorb so much toxin, and even with this rate of Biochar ingested Pimelea can still have an effect on animals if they consume enough Pimelea.

Whilst these project studies have focused on Pimelea poisoning, the wider use of enterosorbents with other plant toxins should also be considered as an economically viable treatment. It is noted for example that activated charcoal is considered a highly effective but expensive antidote for Lantana poisoning in livestock, whilst bentonite presents effective and considerably cheaper alternative therapy for such poisoning, particularly in sheep.

6. Future research and recommendations

Pimelea poisoning remains an on-going challenge for producers in affected regions. Whilst this research has not uncovered the long-hoped for silver bullet, it has provided valuable insights into the disease and the use of enterosorbents as a preventative measure. Further research is recommended as follows:

- Faecal results suggest bentonite is not adsorbing simplexin and carrying the toxin through the gastric tract. It is hypothesised that the interaction of bentonite with simplexin may result in degradation of the toxin as has been seen in soil studies (Loh 2022). Further work is required to investigate this mechanism and identify the potential degradation products.

- **Use of biopolymers for release of toxin and/or agvet chemicals within the gastric tract.** Preliminary studies in fistulated animals have highlighted the potential of biopolymers for the slow-release delivery of active agents within the rumen.
- **Metabolic fate of simplexin.** Further research is also required to better understand the fate of simplexin. In the current study neither simplexin nor its presumed metabolites were detectable in weekly blood samples, yet characteristic oedema only became evident in some animals up to 2 weeks after cessation of Pimelea intake, which suggests an unidentified reservoir of toxin (or an active metabolite) within the animal.
- **Molecular modelling of interaction of simplexin with Protein Kinase C.** Preliminary computer-based molecular modelling studies have also demonstrated potential to better understand the purported interaction between simplexin and Protein Kinase C (PKC). This activation of PKC by simplexin is key to the causative pulmonary venule constriction observed in Pimelea-affected cattle and modelling the interaction may yet lead to a novel solution to Pimelea poisoning which continues to plague producers across our inland grazing regions. A research area which may have potential, once the binding interactions are understood, is the emerging field of peptide-nanoparticle conjugates that can employ specific small binding-motifs of proteins (peptides) on nanoparticles to capture targets ranging from enzyme inhibitors to bacterial toxins.

Adoption of our project research outcomes regarding the efficacy of bentonite as a preventative would be best achieved through producer demonstration sites, which could also be used to substantiate the effects in animals with and without bentonite intake in grazing pastures with Pimelea present.

7. Acknowledgements

This project would not be possible without the willing co-operation seen between both UQ and DAF project participants and field staff. We would like to acknowledge the input into the project of DAF Microbial Ecology Group (formerly RENU) staff under the leadership of Diane Ouwerkerk including Dr Ros Gilbert, Jenny Gravel, Anita Maguire, Cathy Minchin, Dr Ben Schofield and UQ masters student Dukho Chung. Dr Natasha Hungerford, Dr Simon Loh, Russell Gordon and Jiali Zhang (QAAFI) are also gratefully acknowledged for their contributions including the LC-MS/MS analysis of various materials.

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biopolymer pieces in animals and statistical analysis of biopolymer weight loss from the fistulated animal trial.

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The three-month feeding trial was conducted at the UQ Gatton Queensland Animal Science Precinct with invaluable assistance provided by Milou Dekker and her team particularly Scott Cullen, Stacey Groves, Mitchell Harris and Samantha Armour, who all went above and beyond under sometimes trying circumstances. UQ Vets provided on-going care particularly Dr Sarah Rodwell and Dr Ben Wood.

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9. Appendix

9.1 Presentations

9.1.1 Oral presentations

Oral - 1: Fletcher, M.T. "Mitigating the effects of the toxin simplexin in Pimelea poisoning of cattle." *2nd RACI Queensland Annual Chemistry Symposium*, Brisbane, Australia, 27 November 2017 **(Plenary presentation).**

Oral - 2: Ouwerkerk, D.; Fletcher, M.; Laycock, B.; Gilbert, R.; Hungerford, N.; Gauthier, E.; Dixon, R.; Silva, L.; Klieve, A. "Improving beef production through management of plant toxins – strategies for the Pimelea problem" *Beef Australia 2018*, Rockhampton, May 2018.

Oral - 3: Dixon, R. **"**Update for the WQRBRC on some recent projects with QAAFI, UQ and partnerships, and some future proposed directions for comment." *Western Queensland Beef Research Council*, May 2018.

Oral - 4: Fletcher, M. "Novel approach to control Pimelea spp. toxicity." *Australian Veterinary Association South-East Queensland Branch Seminar*, Brisbane, 12th July 2018

Oral - 5: Fletcher, M. "Novel approaches to managing Pimelea toxicity" *Beefup Forum*, Augathella, 10th October 2018.

Oral - 6: Laycock, B. "Polyhydroxyalkanoates in the Circular Economy". *International Symposium on Biopolymer*, Beijing China, 21-24 October2018 (*300+ conference attendees*)

Oral - 7: Fletcher, M. Ouwerkerk, D. "Pimelea Research Update Session". Selling Ring, Roma Cattle Yards, 30th October 2018 (*40 producers in attendance*)

Oral - 8: Fletcher, M. Ouwerkerk, D. "Pimelea Research Update Session". Begonia Hall and Sports Club, 31st October 2018 (*40 producers in attendance*)

Oral - 9: Fletcher, M. Ouwerkerk, D. "Pimelea Research Update Session". St George, 31st October 2018 (*60 producers in attendance*)

Oral - 10: Chung, D.; Ouwerkerk, D.; Phan, M.-D.; Gilbert, R.A. "Rumen microbiome analysis of cattle affected by the toxic plant, *Pimelea*." Australian Society for Microbiology Qld Branch - *Microbiology in Moreton Bay*, Sandstone Point, 10th November 2018.

Oral - 11: Fletcher, M. "Novel approaches to prevent Pimelea toxicity in cattle" *Veterinary Laboratory Queensland Management System (VLQMS) Seminar Series*, Biosecurity Queensland, Brisbane 10th December 2018.

Oral - 12: Fletcher, M. "Ruminant Nutrition and Managing impacts of poisonous plants for improved production" University of Queensland/Yangzhou University Workshop, Brisbane 12th December 2018.

Oral - 13: Fletcher, M., Ouwerkerk, D. "The Pimelea story – application of novel approaches to control toxicity in cattle." *Australian Veterinary Association Conference*, Gold Coast QLD Australia, 23-24 March 2019. (Invited speaker)

Oral - 14: Gauthier, E.; Ouwerkerk, D.; Laycock, B.; Fletcher, M. "Biopolymer composites for slow release to manage Pimelea poisoning in cattle." *7th International Conference on Biobased and Biodegradable Polymers*, Stockholm, Sweden, 17-19th June 2019.

Oral - 15: Fletcher, M., Managing impacts of poisonous plants for improved production. *Northern Beef Research Update Conference,* Brisbane, Australia, 20-21 August 2019.

Oral - 16: Loh, Z. H.; Hungerford, N. L.; Ouwerkerk, D.; Klieve, A.V.; Fletcher, M.T. "Mitigating the effects of the toxin simplexin in pimelea poisoning of cattle by development of a microbial probiotic." *4th Queensland Annual Chemistry Symposium*, Brisbane, QLD, Australia, 29 November 2019.

Oral - 17: Yuan, Y.; Gauthier, E.; Hungerford, N.L.; Ouwerkerk, D.; Fletcher, M.T.; Laycock, B. "Modelling the controlled release of toxins in a rumen environment." *4th Queensland Annual Chemistry Symposium*, Brisbane, QLD, Australia, 29 November 2019.

Oral - 18: Gordon, R.J.; Hungerford, N.L.; Laycock, B.; Ouwerkerk, D.; Fletcher, M.T. Are toxic *Pimelea* secondary compounds absorbed via the intestinal lymph? *4th Queensland Annual Chemistry Symposium*, Brisbane, QLD, Australia, 29 November 2019.

Oral - 19: Loh, Z.H. Probiotics to combat plant toxins. *QAAFI Three Minute Thesis (3MT)*. University of Queensland June 2020. https://vimeo.com/424495265 (student presentation)

Oral - 20: Gordon, R.J. Shielding cattle from death in the paddock. *QAAFI Three Minute Thesis (3MT)*. University of Queensland June 2020. https://vimeo.com/431167617 (student presentation)

Oral - 21: Yuan, Y. Food poisoning in cattle – Not a Joke. *School of Chemical Engineering Three Minute Thesis (3MT)*. University of Queensland June 2020. https://vimeo.com/431335753 (student presentation) (Runner-up award)

Oral - 22: Loh, Z.H., Hungerford, N.L., Ouwerkerk, D., Klieve, A.V., Fletcher, M.T. "Identification of volatile plant compounds in *P. trichostachya* plants responsible for livestock aversion." *3rd Queensland Annual Chemistry Symposium*, Queensland University of Technology, November 2020. (student presentation)

Oral - 23: Fletcher, M.T. *Pimelea Management Update*, Roma Beefup Forum, Roma Cultural Centre 26th March 2021

Oral - 24: Fletcher, M.T. Managing the impacts of Pimelea poisoning on cattle. *Precision Beef - Improving breeding, feed efficiency, production and beef flavours.* Beef 2021, Rockhampton 5th May 2021.

Oral - 25: Marie Vitelli, *Pimelea Research Update*, Animal Welfare and Biosecurity Workshop, Tambo Wednesday 21 July 2021. - https://www.eventbrite.com/e/158853287459

Oral - 26: Marie Vitelli, *Pimelea Research Update,* Animal Welfare and Biosecurity Workshop, Toompine Friday 23 July 2021 - https://www.eventbrite.com/e/158856248315

Oral - 27: Fletcher, M. Ouwerkerk, D. "Pimelea Research Info Day". Eldwick Station, Jundah, 15th August 2022 (*50 producers in attendance*)

Oral - 28: Fletcher, M. "Pimelea Update and Producer Info Afternoon". Begonia Sports Club, Begonia, 15th August 2022 (*120 producers in attendance*)

9.1.2 Poster presentations

Poster - 1: Gauthier, E.; Ouwerkerk, D.; Laycock, B.; Fletcher, M.; "Slow release biopolymer composites for management of Pimelea poisoning in cattle" *5th Animal Science Poster Olympics*, University of Queensland, St Lucia, 30th November 2018**. (Winner: *Australian Society for Animal Production Poster Prize).***

Poster - 2: Gordon, R.J.; Hungerford, N.L.; Fletcher, M.T; "In vitro study of the effectiveness of commercial sequestering agents for the adsorption of the Pimelea toxin, simplexin" *5th Animal Science Poster Olympics*, University of Queensland, St Lucia, 30th November 2018**.** (**Runner-up: *Australian Society for Animal Production Poster Prize).***

Poster - 3: Loh, Z.H.; Hungerford, N.L.; Ouwerkerk, D.; Gilbert, R.A.; Klieve, A.V.; Fletcher, M.T. "Towards a Microbial Probiotic: LC-MS/MS Analysis of Simulated Rumen Degradation of the *Pimelea* Toxin Simplexin" *Queensland Mass Spectrometry Symposium*, Queensland University of Technology, Brisbane 6-7th December 2018.

Poster - 4: Gordon R.J.; Hungerford, N.L.; Laycock, B.; Ouwerkerk, D.; Fletcher, M.T. "Adsorbents for the sesquestration of the *Pimelea* toxin, simplexin." *Northern Beef Research Update Conference,* Brisbane 20-21 August 2019.

Poster - 5: Gauthier, E.; Yuan, C.Y.; Ouwerkerk, D.; Fletcher, M.; Laycock, B. "Biopolymer composites for slow release of toxins to manage *Pimelea* poisoning in cattle. *Recent Advances in Animal Nutrition*, University of New England, Armidale 23-25 October 2019

Poster - 6: Gordon, R.; Hungerford, N.L.; Laycock, B.; Ouwerkerk, D.; Fletcher, M.T. "Adsorbents for the sequestration of the *Pimelea* toxin, simplexin. *Third International Tropical Agriculture Conference*, Brisbane, Australia, 11–13 November 2019.

Poster - 7: Gauthier, E.; Yuan, C.Y.; Ouwerkerk, D.; Laycock, B.; Fletcher, M. "Biopolymer composites for slow release to manage *Pimelea* poisoning in cattle." *Third International Tropical Agriculture Conference*, Brisbane, Australia, 11–13 November 2019.

Poster - 8: Yuan, Y.; Gauthier, E.; Hungerford, N.L.; Ouwerkerk, D.; Fletcher, M.T.; Laycock, B. "Modelling the controlled release of toxins in a rumen environment." *Third International Tropical Agriculture Conference*, Brisbane, Australia, 11–13 November 2019.

Poster - 9: Loh, Z.H.; Hungerford, N.L.; Ouwerkerk, D.; Gilbert, R.A.; Gravel, J.; Minchin, C.M.; Maguire, A.J.; Yong, K.; Klieve, A.; Fletcher, M. (2019). "Mitigating the effects of the toxin simplexin in pimelea poisoning of cattle by development of a microbial probiotic." *Third International Tropical Agriculture Conference*, Brisbane, Australia, 11–13 November 2019

Poster - 10: Loh, Z.H., Hungerford, N.L., Ouwerkerk, D., Klieve, A.V., Fletcher, M.T. Analysis of the *Pimelea* toxin simplexin for the development of a cattle microbial probiotic, *1st International Electronic Conference on Toxins*, January 2021.

Poster - 11: Yuan, Y., Gauthier, E., Ouwerkerk, D., Laycock, B., Fletcher, M.T. Revealing the micro- and macroscopic mechanisms of biodegradable toxin-loaded biocomposites exposed to *in vitro* rumen environments. *1st International Electronic Conference on Toxins*, January 2021.

Poster - 12: Loh, Z.H., Hungerford, N.L., Ouwerkerk, D., Klieve, A.V., Fletcher, M.T. UPLC-MS/MS analysis of the *Pimelea* toxin simplexin and its potential degradation products. *33rd Australian Association of Animal Sciences Conference*, 1-4 February 2021 (eposter student presentation).

Poster - 13: Ouwerkerk, D., Gilbert, R.A., Gravel, J.L., Minchin, C.M., Hungerford, N.L., Loh, Z.H., Fletcher, M.T. Enriching for rumen bacteria to degrade the *Pimelea* plant toxin simplexin, in an anaerobic *in vitro* fermenter. *33rd Australian Association of Animal Sciences Conference*, 1-4 February 2021 (eposter).

Poster - 14: Yuan, Y., Gauthier, E., Hungerford, N.L., Ouwerkerk, D. Laycock, B. Fletcher M.T. Combating *Pimelea* poisoning with biodegradable biocomposite-based boluses: an investigation into the slow release of toxins in the rumen environment. *33rd Australian Association of Animal Sciences Conference*, 1-4 February 2021 (ePoster student presentation).

Poster - 15: Loh, Z.H.; Hungerford, N.L.; Ouwerkerk, D.; Klieve, A.V.; Fletcher, M.T. Analysis of the Pimelea toxin simplexin and identification of its potential degradation products using UPLC-MS/MS. *6th Animal Science Poster Olympics*, University of Queensland 19th February 2021. (**runner-up award for the "Innovation for the red meat industry award" supported by MLA**).

Poster - 16: Yuan, Y.; Gauthier, E.; Hungerford, N.L.; Ouwerkerk, D.; Fletcher, M.T.; Laycock, B. Combating Pimelea Poisoning using biodegradable biocomposite-based boluses: A n investigation into the slow release mechanism of toxins in a rumen environment. *6th Animal Science Poster Olympics*, University of Queensland 19th February 2021. (**winner *General Animal Science Research award* $1,000 prize supported by the Centre for Animal Science, QAAFI the University of Queensland. *Open category*)**

Poster - 17: Ouwerkerk, D.; Gauthier, E.; Yuan, Y.; Costa, D.F.A.; Laycock, B.; Fletcher, M.T. *In vivo* rumen microbial degradation of polyhydroxyalkanoate biopolymers. *34th Australian Association of Animal Sciences Conference*, Cairns, 5-7 July 2022 (ePoster student presentation)

Poster - 18: Loh, Z.H.; Hungerford, N.L.; Ouwerkerk, D.; Klieve, A.V.; Fletcher, M.T. Effects of adsorbents and probiotics in mitigating simplexin poisoning effects in cattle fed Pimelea. *34th Australian Association of Animal Sciences Conference*, Cairns, 5-7 July 2022 (ePoster student presentation).

9.2 Manuscript publications

Publication - 1: Fletcher, M., Ouwerkerk, D. The *Pimelea* story – application of novel approaches to control toxicity in cattle. In 'Toxicology – Threats, treatment and other toxic topics.' *Conference Proceedings Australian Veterinary Association Queensland Division*, **2019**, 119-121 (*Invited manuscript*).

Publication - 2: Gauthier, E., Ouwerkerk, D., Laycock, B., Fletcher, M. Biopolymer composites for slow release to manage *Pimelea* poisoning in cattle. *Proceedings* 2019 *36 (1)*, 97. https://doi.org/10.3390/proceedings2019036097 (*Published conference abstract*).

Publication - 3: Gordon, R., Hungerford, N.L., Laycock, B., Ouwerkerk, D., Fletcher, M.T. Adsorbents for the sequestration of the *Pimelea* toxin, simplexin. *Proceedings* 2019 *36 (1)*, 90. https://doi.org/10.3390/proceedings2019036090 (*Published conference abstract*).

Publication - 4: Yuan, Y., Gauthier, E., Hungerford, N.L., Ouwerkerk, D., Fletcher, M.T., Laycock, B. Modelling the controlled release of toxins in a rumen environment. *Proceedings* 2019 *36* (1) 89. https://doi.org/10.3390/proceedings2019036089 (*Published conference abstract*).

Publication - 5: Loh, Z.H., Ouwerkerk, D., Klieve, A.V., Hungerford, N.L., Fletcher, M.T. Toxin degradation by rumen microorganisms: A review. *Toxins*, 2020 12 (10), 664. https://doi.org/10.3390/toxins12100664

Publication - 6: Gordon, R.J.; Hungerford, N.L.; Laycock, B.; Fletcher, M.T. A review of *Pimelea* poisoning of livestock. *Toxicon,* 2020, 186, 46-57. https://doi.org/10.1016/j.toxicon.2020.07.023

Publication - 7: Yuan, Yue, Hungerford, Natasha L., Gauthier, Emilie, Ouwerkerk, Diane, Yong, Ken W. L., Fletcher, Mary T., and Laycock, Bronwyn (2021). Extraction and determination of the Pimelea toxin simplexin in complex plant-polymer biocomposites using ultrahigh-performance liquid chromatography coupled with quadrupole Orbitrap mass spectrometry. *Analytical and Bioanalytical Chemistry 413* (20) 5121-5133. doi: 10.1007/s00216-021-03475-5

Publication - 8: Yuan, Y, Gauthier, E, Ouwerkerk, D, Fletcher, MT, Laycock, B (2022). Unravelling biodegradation and toxin release kinetics of poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-based biocomposites in a simulated rumen environment: a preliminary study. *Sustainable Materials and Technologies* e00498. https://doi.org/10.1016/j.susmat.2022.e00498

9.3 Research promotion

Display - 1: Beef 2021 (Rockhampton): QAAFI stand display: Handout "Improving beef production through management of plant toxins"

Display - 2: Beef 2021 (Rockhampton): Biopolymers at MLA stand: "Cattle Rumen biopolymers" – physical display.

9.4 Project media coverage

Media - 1: Beef Central webpage, 30 November 2017: MLA's Plan to pitch preventative probiotic against pimelea. https://www.beefcentral.com/production/mla-research-targets-preventative-probiotic-in-fight-against-pimelea/

Media - 2: Queensland Country Life, 30 November 2017: *Pimelea research to continue*

Media - 3: Beef Central, 5 February 2018 *Pimelea cattle poisoning research enters next stage*" https://www.beefcentral.com/production/pimelea-cattle-poisoning-research-enters-next-stage/

Media - 4: Feedback Magazine, February 2018: *Putting a stop to poisoning*

Media - 5: QAAFI Annual Report, April 2018: New project targets pimelea poisoning

Media - 6: Central Station, August 22 2018: *The Problematic Pimelea plant* https://www.centralstation.net.au/the-problematic-pimelea-plant/

Media - 7: Beef Central, 9 August 2018: *Pimelea warning to livestock producers ahead of spring.* https://www.beefcentral.com/news/pimelea-warning-to-livestock-producers-ahead-of-spring/

Media - 8: Beef Central, 3 September 2018: *Producers urged to be on alert for Pimelea + research project update.* https://www.beefcentral.com/production/grazing-land-management/producers-urged-to-be-on-alert-for-pimelea-research-project-update/

Media - 9: Western Star (Roma), 18 September 2018: Researchers hunt for pimelea breakthrough

Media - 10: Queensland Country Life 27 September 2018: *Beware of pimelea risk*

Media - 11: Beef Central, 2 October 2018: *Producers to learn more about Pimelea cattle poisoning research.* https://www.beefcentral.com/production/producers-to-learn-more-about-pimelea-cattle-poisoning-research/

Media - 12: Western Star (Roma) 26 Oct 2018: Pimelea studies to be presented to region

Media - 13: Queensland Country Life Pimelea research update tours southern Qld

Media - 14: "Conditions ripe for *Pimelea*" Queensland Country Life, July 11, 2019

Media - 15: "Researchers deliver latest *Pimelea* news at AgForce animal health days" @Sally Cripps QCL July 9 2019. https://www.queenslandcountrylife.com.au/story/6264878/no-simple-answer-in-pimelea-simplexin-puzzle/

Media - 16: ABC Longreach Radio interview Mary Fletcher (10 July 2019), in relation to Industry Animal Health Forum presentation in Longreach

Media - 17: AgForce Pimelea Update April 2020. AgForce has in past years invited us to present our research at regional Pimelea Information sessions for producers. Such Information days were not able to be held during 2020 due to Covid restrictions. Instead a Pimelea Research Update April 2020 was prepared and circulated to producers. This *Pimelea Research Update* was included as a link in the *Southern Inland Queensland* and *South West Queensland* sections of AgForce QLD's weekly Action e-newsletter as a PDF attachment (Pimelea Update-April 2020).

Media - 18: QAAFI Innovation in Agriculture May 2020. A similar update article was included in the QAAFI Innovation In Agriculture May 2020, which was circulated Monday June 1 2020. This update article also appears on the QAAFI website "Combating Pimelea Toxicity".

Media - 19: QAAFI Seminar at Beef 2021: https://qaafi.uq.edu.au/Beef-2021

Media - 20: Pimelea presentation at Roma Beefup: https://www.mla.com.au/globalassets/mla-corporate/news-and-events/documents/events/roma-beefup-flyer.pdf

Media - 21: FutureBeef facebook. https://www.facebook.com/futurebeef/posts/pimelea-/4251138991609694/

Media - 22: Beeftalk August 2021. Leanne Hardwick, "Be on the lookout for Pimelea", FutureBeef CQBEEF section of the *Queensland Country Life* 26th August 2021.

Media - 23: Winter rain sees toxic plant spread quickly across the west. ABC Western Queensland. August 11 2022 (Promotion for Pimelea Info Day at Eldwick Station) https://www.abc.net.au/westqld/programs/north-west-and-western-queensland-rural-report/pimelea-concerns-in-western-queensland/101322256

9.5 Animal ethics approval and amendments

All animal ethics approvals associated with this project are provided as a single separate file

9.5.1 Rumen Fluid collection (Gatton) SAFS/296/17

9.5.2 Isolation and investigation of simplexin toxin-metabolising microbiota in cattle poisoned by pimelea SA 2016/11/586

9.5.3 Improving beef production through management of plant toxins SA 2019/11/722

This approval was obtained for the collection of rumen fluid and other samples from field animals

(a) Initial approval
(b) Amendment for field tissues of Pimelea affected animals

9.5.4 Use of fistulated Holstein Friesian steers to determine rumen degradation rates of biopolymer boluses.

This fistulated animal trial was approved by both a) DAF AEC (SA 2020/03/737) and b) UQ AEC (DAF/QAAFI/125/20)

a) DAF AEC (SA 2020/03/737)
b) UQ AEC (DAF/QAAFI/125/20)

9.5.5 Mitigating effect of the Pimelea toxin simplexin (Pen trial)

Animal ethics for the Pimelea feeding trial was approved in September 2020 by UQ AEC (QAFFI/QASP/337/20/DAF).

AEC Amendments to change the trial design from 5 treatments x 6 animals per treatment to 6 treatments x 5 animals, and to include further staff subsequently approved. A further amendment in relation to collecting tail hair and earwax was also approved.

9.6 Microbiome sample details

Table 9.1. Summary of collaborator producer properties visited, animal samples collected in P.PSH.0900 and AS10583 projects and submitted for gDNA analysis (modified from P.PSH.0900 final report *(Fletcher and Ouwerkerk 2018)*), together with additional Pimelea feeding trial samples (Property 32).

Collection date	Property number	Local Government Area	Property ID (PIC)	Site #[A]	Geographic location	Altitude	Number of animals gDNA extracted				Predominant pasture	Habitat	Soil type
							Cattle	Goats	Sheep	Roos			
24/8/17	1	Maranoa	QHMZ0023		27° 15' 4" S 148° 52' 13" E	240m	6	0	0	0	Buffel grass	Pine, box, Brigalow, Mulga, River flood out	Sandy loam
23/8/17	2	Balonne	QHBL0107		28° 27' 40" S 147° 59' 13" E	178m	1	3	0	0	Buffel grass	Some Mulga	Sandy
23/8/17	3	Balonne	QEBL0400		28° 24' 56" S 148° 00' 9" E	180m	6	0	0	0	Buffel grass	Buffel, Mulga	Sandy loam
24/8/17	4	Maranoa	QCBN1478		26° 49' 9" S 148° 53' 24" E	266m	6	0	0	0	Buffel grass	River flood out - Box, Cyprus Pine	Light Black soil
25/9/17	5	Maranoa	QCWR0206		27° 09' 24" S 148° 40' 5" E	256m	6	0	0	0	Buffel grass	Box, Ironbark, Mulga	Red
16/2/17	6	Blackall	QABA0059		24° 03' 52" S 145° 54' 44" E	394m	12	0	0	0	Spinifex, Buffel, Button and Wandery	Bloodwood, Ironwood, Wattle, Gumtree	Desert upland, Sandy flat
18/12/16	7	Maranoa	QKWR0091		27° 10’ 19” S 148° 25’ 58” E	246m	5	0	0	0	Buffel grass	Variable Mulga, hard ridges, Silver leaf Ironbark, Poplar Box	Sandy loam
26/9/17	8	Balonne	QEBL0048		27° 59' 10" S 148° 10' 57" E	209m	0	0	3	0	Buffel with pulled box pine pasture	Yellow Jacket, Poplar Box country	Red sandy loam
26/9/17	9	Balonne	QHBL0342		28° 07' 5" S 147° 59' 54" E	200m	3	3	0	0	Buffel grass - very low coverage	Box country	Red sandy loam
27/9/17	10	Balonne	QBBL0296		28° 00' 53" S 147° 38' 26" E	197m	7	2	6	3	Buffel grass	Semi open range, Poplar Box, Mulga	Red sandy

												loam (bit of black)
28/9/17	11	Paroo	QCPA0266	A	27° 44' 53" S 146° 38' 3" E	204m	0	0	0	4	Native grasses	Mulga	Red sandy loam
				B	27° 46' 10" S 146° 40' 48" E	204m	0	0	0	1	Native grasses	Mulga	Red sandy loam
29/9/17	13	Balonne	QBBL0202		27° 55' 59" S 147° 47' 42" E	204m	6	3	0	0	Buffel grass	Box and Sandalwood	Red sandy loam (bit of black)
30/9/17	14	Balonne	QJBL0530		26° 43' 60" S 150° 09' 50" E	294m	5	0	0	0	Speargrass, Buffel and Rhodes grass	Open Box country	Sandy loam
6/11/17	15	Murweh	QCMW0343		25° 52’ 3” S 146° 31’ 2” E	356m	7	0	0	0	Buffel and Blue grass	Bottle tree / Mulga tree country Downs country, red loam ridges	Red loam to Dark Chocolat e soil
1/11/17	16	Barcaldine	QCBT0064		23° 54’ 18” S 145° 48’ 51” E	348m	8	0	0	0	Spinifex	Ironbark country (broadleaf)	Red sandy
26/8/08	31[B]	Yeerongpilly	Animal Research Institute		NR	NR	4	0	0	0	Rhodes grass/Lucerne	Pimelea pen trial (Fletcher et al. 2014)	

[A]Multiple collection sites on same property

[B]Plant collections only were obtained from Properties 17-30 as recorded previously in P.PSH.0900 (Fletcher and Ouwerkerk 2018).

9.7 Preparation of anaerobic media (DAF Rumen Ecology SOP)

For all media:

1. Add dry components to a 2 L conical flask. Add reverse osmosis water (RO H_2O) to the flask and dissolve dry components by swirling. Add salt solutions A and B, and resazurin. Ensure that the volume of the media is above 1 L by adding excess RO H_2O (~1100 mL final volume works well).
2. Place flask on a fire-retardant mat on a Bunsen stand and set up a Bunsen burner under the flask. Light the Bunsen burner and adjust air intake until it is burning with a blue flame.
3. Boil under constant flow of 95% CO_2/5% H_2 until the volume has reduced to 1 L. The colour of the solution will normally change from blue to a purple or even bright pink as the pH of the media changes during boiling.
4. Cool the media on a bed of fresh ice (with as little liquid water as possible) until it is comfortable to hold (35-50 °C). Continue to gas the media with a constant flow of 95% CO_2/5% H_2 while it is cooling. Do not let the media get cold – this will create a vacuum in the bottle following autoclaving.
5. While the media is cooling, begin gassing Wheaton bottles with 95% CO_2/5% H_2.
6. Add Volatile Fatty Acid (VFA) solution (use a filtered pipette or tip and avoid drips – VFA solution is very pungent) and Cysteine-HCl (use a weigh boat) to the media.
7. Mix well before dispensing into Wheaton bottles (500 mL/bottle), or other bottle volumes as required, under a constant flow of CO_2/H_2 to maintain anaerobic conditions. Ensure the Wheaton bottle lids have a rubber septum in place, and the lid is tight.
8. Place an autoclave tape label on the bottle with the type of media, date made, and initials written on the tape.
9. Autoclave at 105 °C for 45 minutes.

9.7.1 Preparation of fermenter starter medium

Table 9.2. Ingredients for fermenter starter medium required to make 1 L of media

Component	/1,000 mL
Peptone	0.5 g
Yeast extract	0.5 g
Sodium hydrogen carbonate ($NaHCO_3$)	5.0 g
Glucose	0.5 g
Cellobiose	0.5 g
RO H_2O	505 mL+
Salt solution A	165 mL
Salt solution B	165 mL
Rumen fluid base	165 mL
Resazurin	1.0 mL
After boiling add:	
VFA solution	10 mL
Cysteine-HCl	0.22 g

[A]RO H_2O = reverse osmosis water

Preparation Notes:
Mix well before dispensing 500 mL per Wheaton Bottle and autoclave at 105 °C for 45 minutes.

9.7.2 Preparation of fermenter salt solution

Table 9.3. Ingredients for Fermenter Salts Solution required to make 1 L of media

Component	/1,000 mL
Peptone	0.1 g
Yeast extract	0.1 g
Sodium hydrogen carbonate ($NaHCO_3$)	5.0 g
RO H_2O[A]	670 mL +
Salt solution A	165 mL
Salt solution B	165 mL
0.1% w/v Resazurin	1.0 mL
After boiling, add:	
VFA solution	10 mL
Cysteine-HCl	0.22 g

[A]RO H_2O = reverse osmosis water

Preparation Notes:
Mix well before dispensing 500 mL per Wheaton Bottle and autoclave at 105 °C for 45 minutes.

9.7.3 Preparation of rumen fluid (RF^+) medium

Table 9.4. Ingredients for RF^+ required to make 1 L of media

Component	/1,000 mL
Peptone	0.1 g
Yeast extract	0.1 g
Sodium hydrogen carbonate ($NaHCO_3$)	5.0 g
Glucose	2.0 g
Cellobiose	2.0 g
RO H_2O[A]	330 mL+
Salt solution A	165 mL
Salt solution B	165 mL
Rumen fluid base	330 mL
Resazurin	1.0 mL
Add after boiling	
VFA solution	10 mL
Cysteine-HCl	0.2 g

[A]RO H_2O = reverse osmosis water

9.7.4 Preparation of rumen fluid/glycerol (RF/Gly) medium

Table 9.5. Ingredients for RF/Gly medium required to make 1 L of media

Component	/1,000 mL
Peptone	0.1 g
Yeast Extract	0.1 g
Sodium hydrogen carbonate ($NaHCO_3$)	5.0 g
Glucose	2.0 g
Cellobiose	2.0 g
Distilled H_2O[A]	330 mL+
Salt solution A	165 mL
Salt solution B	165 mL
Rumen fluid base	330 mL
Resazurin	1.0 mL
Add after boiling	
VFA solution	10 mL
Cysteine-HCl	0.22 g

[A]RO H_2O = reverse osmosis water

Preparation Notes:

1. Mix well before dispensing.
2. Gassed glycerol: dispense the appropriate volume of glycerol and bubble CO_2 gas through it for at least 30 minutes before adding the medium.
3. Dispense 50 mL medium into a serum bottle containing 50 mL of gassed glycerol. This makes 100 mL of medium and an equal volume (100 mL) of fermenter fluid is added.
4. Dispense 125 mL of medium into Wheaton bottle containing 125 mL of gassed glycerol (250 mL of RF/Gly medium).
5. Normally used to store bacteria after harvesting in a fermenter run (250 mL of fermenter fluid is added).

9.7.5 Preparation of rumen fluid base

Rumen fluid was collected from pasture grazing fistulated Brahman x steers located at the Queensland Animal Science Precinct at UQ Gatton (Animal Ethics Approval SA 2014/06/473) and filtered through nylon stocking into 2.5 L plastic bottles and frozen at -20 °C until needed. Clarification via centrifugation is required to ensure the fluid is free of plant material and microbial contaminants. Using the Thermo Lynx 6000 high speed centrifuge (Thermo Fisher Scientific) and the FA14-250Y lite rotor in the Rumen fluid program (found in settings/ runs) 11,000 rpm (18,566 xg) for 30 min at 4°C with a slow deceleration to prevent pellet dislodging.

Preparation Notes

1. Thaw frozen 2.5 L bottles of rumen fluid (in bucket of warm/hot water OR in fume cupboard overnight in a bunded tray) before preparation.
2. Pre-chill rotor and centrifuge.
3. Weigh out the rumen fluid evenly into Nalgene PPCO 250 mL centrifuge bottles #3141-0250 - (max. weight of fluid, bottle and lid 290 g - approx. 250 mL).
4. Gently pour off the clarified fluid without disturbing the pellet.
5. Take a drop of the clarified fluid and look at it under the microscope to ensure there is not any possible contaminants remaining in the processed fluid such as: bacteria (ghosts or alive) and large fragments of plant material.

6. If there are residual contaminants at this point, re-centrifuge the fluid.
   Dispense the pooled, clarified rumen fluid using the Brand Dispensette (Wertheim, DE) as 33 mL, 66 mL or 165 mL aliquots into glass screwcap bottles and store frozen at -20°C.

9.7.6 Preparation of volatile fatty acid (VFA) solution

Table 9.6. Ingredients for volatile fatty acid solution required to make 1 L of solution

Component	/1,000 mL
Acetic Acid	170 mL
Propionic Acid	60 mL
Butyric Acid	40 mL
Isobutyric Acid	10 mL
n-Valeric Acid	10 mL
Isovaleric Acid	10 mL
D-L-α Methyl butyric Acid	10 mL

Preparation Notes:

1. Add above contents to a 2L beaker
2. Adjust pH to 7.5 using NaOH pellets, (use liquid 5 M NaOH at the end). This is done on a stirrer and must be done in the fume hood. As the addition of NaOH produces heat, this must be slowly achieved and will take a whole day to complete. Do not rush adjusting the final pH at the end as the solution can rapidly overshoot the desired pH and then the solution will need to be discarded.
3. Add NaOH pellets in 20 g lots and RO H_2O can be added to approximately the 500-600 mL mark.
4. Cover the beaker with foil to prevent evaporation.
5. Once pH reaches 7.5 make up volume to 1,000 mL with ROH_2O.
6. Store at 4 °C.

9.7.7 Preparation of salts A solutions

Table 9.7. Ingredients for salts A required to make 1 L of solution

Components	/1,000 mL
KH_2PO_4	3.0 g
NaCl	6.0 g
$(NH_4)_2SO_4$	3.0 g
$CaCl_2$	0.3 g

1. Add the above chemicals to a 2L beaker and add RO H_2O to 1 L and mix thoroughly until dissolved. This may require use of the magnetic stirrer.
2. Prepare a label including the preparation date and store at 4°C.

9.7.8 Preparation of salts B solutions

Table 9.8. Ingredients for salts B required to make 1 L of solution

Components	/1,000 mL
K_2HPO_4	3.0 g

1. Add the K_2HPO_4 to a 2L beaker and add RO H_2O to 1 L and mix thoroughly until dissolved. This may require use of the magnetic stirrer.
2. Prepare a label including the preparation date and store at 4°C.

9.7.9 Preparation of resazurin solutions

Table 9.9. Ingredients for resazurin solution required to make 100 mL of solution

Components	/100 mL
Resazurin	0.1 g

1. Add 100 mL of RO H_2O to the Resazurin and mix thoroughly until dissolved.
2. Prepare a label including the preparation date and store at 4°C.

9.7.10 Preparation of RF$^+$modPM

Table 9.10. Ingredients for RF$^+$modPM selective media required to make 100 mL

Ingredients	/100 mL
Peptone	0.1 g
Yeast Extract	0.1 g
$NaHCO_3$	0.5 g
Glucose	0.1 g
Cellobiose	0.1 g
RO H_2O[c]	33 mL+
Salt Solution A[A]	16.5 mL
Salt Solution B[A]	16.5 mL
Rumen Fluid	33 mL
Resazurin[A]	0.1 mL
Pimelea plant extract (168,618 ng simplexin/mL)[B]	3.24 mL
Add after boiling	
VFA Solution	1.0 mL
Cysteine-HCl	0.02 g

9.7.11 Preparation of RF$^+$modPM3000 and RF+modPM6000 medium

Table 9.11. Ingredients for RF$^+$modPM3000 and RF$^+$modPM6000 selective media required to make 100 mL

Ingredient	/100 mL RF$^+$modPM3000	/100 mL RF$^+$modPM6000
Peptone	0.1 g	0.1 g
Yeast Extract	0.1 g	0.1 g
$NaHCO_3$	0.5 g	0.5 g
Glucose	0.1 g	0.1 g
Cellbiose	0.1 g	0.1 g
RO H_2O[c]	33 mL+	33 mL+
Salt Solution A	16.5 mL	16.5 mL
Salt Solution B	16.5 mL	16.5 mL
Rumen fluid base	33 mL	33 mL
Resazurin solution	0.1 mL	0.1 mL
Semi-pure simplexin extract (600,000 ng/mL)	0.5 mL	1.0 mL
RO H_2O	33 mL	33 mL
Add after boiling		
VFA Solution	1.0 mL	1.0 mL
Cysteine-HCl	0.02 g	0.02 g

9.7.12 Procedure for simplexin degradation assay

1. Set up the required number of 10 mL Hungate tubes in rack and flush with CO_2/H_2 gas mix.
2. Remove sufficient fermenter fluid into a container and add 10 mL of fermenter fluid to each tube and kill one set by autoclaving and allow to cool. To the tubes add
    a. 200 µL of simplexin plant extract to three tubes of fresh fermenter fluid (FFS) and three tubes of autoclaved fermenter fluid (KFFS)
    b. 200 µL of ethanol to three tubes of fresh fermenter fluid (FFE) and three tubes of autoclaved fermenter fluid (KFFE).
    c. 200 µL of salts solution to three tubes of fresh fermenter fluid (FF) and three tubes of autoclaved fermenter fluid (KFF)
3. Immediately take 2.0 mL sample from each tube using a 2 mL syringe and 21G needle gassing the syringe with the CO_2/H_2 gas mix (Time 0) and place into a 15 mL falcon tube and freeze at -20 °C.
4. Place all Hungate tubes onto rocker in the 39 °C incubator.
5. After 48 h remove tubes from incubator and take 2.0 mL sample using a 2 mL syringe and 21G needle gassing the syringe with the CO_2/H_2 gas mix into a labelled 15 mL falcon tube and freeze at - 20 °C. Return Hungate tubes to the incubator.
6. After 72 h take 2.0 mL samples from each tube as before (Step 5.) and return the Hungate tubes to the incubator.
7. After 168 h (7 days) remove tubes from incubator and take 2.0 mL sample using a 2 mL syringe and 21G needle gassing the syringe with the CO_2/H_2 gas mix into a labelled 15 mL falcon tube and freeze at - 20 °C. Measure the pH of the culture remaining in each tube.

9.8 Procedure for Pimelea dry matter digestion (DMD) assay

This protocol is for the measurement of digestion of Pimelea dry matter within a fermenter system.

1. Check the nylon bags carefully for wear, tears and holes. Replace any that are not sound and intact.
2. Dry the bags required plus some spares (in a wire mesh container) and the milled *Pimelea trichostachya* (spread about 1-2 cm thick in a metal tray with an alfoil lid) at 55 °C for approximately 48-72 h.
3. While these are drying, ensure that the silica in the desiccator is dry. If necessary, refresh the silica by heating the silica in a metal tray at 140 °C for at least 2 h.
4. While wearing gloves, weigh each bag with length of nylon fishing line on a three-place balance, record the weight, and add 1.0 g of milled *Pimelea trichostachya* to the bag.
5. Fold the top of the bag lengthwise in half three times and then fold the top over once to seal the bag. Wrap the fishing line around the top of the bag several times tie and repeat until the bag is tightly sealed and tied securely.
6. Weigh the bag plus *Pimelea trichostachya* plus fishing line and record the weight.
7. Place two of the bags plus *Pimelea trichostachya* into the desiccator and leave until all bags are sent to Coopers Plains.
8. Take two bags for Day 1, (set aside until time available to wash), and wash the bags and contents in 500 mL distilled water measured with a cylinder, wash bags for 1minute by moving the contents up and down with your fingers. Repeat the wash in two changes of water. Take 2 x 2.0 mL samples of wash water from each wash into 15 mL falcon tubes and freeze at -20 °C.
9. Dry the bags in a wire basket at 55 °C for 48-72 h, and then store the dried bags in the desiccator at ambient temperature until all the bags are dried.
10. Attach the bags going into the fermenter to a pole with elastic bands and leave in the fermenter for the desired time.
11. Wear gloves while removing or handling bags. Following removal of the bag from the fermenter liquid, place on paper towel, remove any plant material from the outside of the bag, wash the bag and contents in at 500 mL distilled water measured with a cylinder, wash bag for 1 minute by moving the contents up and down with your fingers. Repeat the wash in two changes of water. Take 2 x 2.0 mL samples of wash water from each wash into 15 mL falcon tubes and freeze at -20 °C
12. Dry the bag in a wire basket at 55 °C for 48-72 h, and store the dried bag in the desiccator at ambient temperature until all the bags are dried.
13. Wearing gloves, weigh all the dried bags plus *Pimelea trichostachya* plus fishing line.
14. Keep all bags to send for simplexin level testing by the Natural Toxin Laboratory.

9.9 Standard Operating Procedure: Use of stomach tube for the collection of rumen fluid in cattle

Policy: This procedure may only be performed by, or under the supervision of operators skilled in the technique identified in active Animal Ethics Approvals to collect rumen fluid.

Precautions: Restraint of the animal's head is required for the procedure. It is preferable to have the animal in a crush with its head restrained in the head bail. Regurgitation and aspiration of rumen fluid into the lungs may occur if the animal is forced to elevate its head. The presence of

horns often requires modification of the procedure to ensure operator safety. Stomach tubes should be checked after use to ensure all surfaces are smooth.

Equipment: Stomach tube (approx.1.5 – 2 m of 16 mm diameter reinforced tubing with holes burnt through one end and end of tubing bevelled);Polypipe gag (approx. 80 cm length of 25 mm diameter black pipe with bevelled ends)Plastic side arm flask (2 L volume) with rubber bung that fits stomach tubing and tubing that fits side arm and long enough to go to hand pump; Hand pump (e.g. Wanderer Large Dual Action Hand Pump); Plastic Buckets (2 or 3 set up to rinse gag, stomach tubing and 2 L flask between animals)

Procedure:

1. Check the hand pump is connected to deflate (pull a vacuum) and connect the tubing from the pump to the side arm of the 2 L plastic flask. Place the rubber bung into the top opening of the flask and insert the non-bevelled end of the stomach tubing through the hole. Place a hand around the holes in the end of the stomach tube and pump a couple of times to ensure you can feel a vacuum being pulled.
2. Run your hand along the length of the stomach tube and the ends feeling for any burrs or other sharp edges.
3. The size and disposition of the animal will determine if an assistant is required to restrain the animal and open its mouth to place the gag across the tongue. The stomach tube is inserted through the gag and down the back of the throat, down the oesophagus into the rumen.
4. Once the tube is gently inserted, working as a team one person commences slow hand pumping to create a vacuum whilst the other person moves the stomach tubing slightly back and forth in the rumen. Fluid should come up the tube and into the 2 L flask. Do not collect more than 1.5 L into the flask.
5. Withdraw the tubing with the animal's head as low as possible to minimise the risk of accidental aspiration of rumen content. Once the tube is removed, remove the gag and restraint of the animal's head.
6. Between animal's rinse gag, stomach tubing (inside and out) and 2 L side arm flask in sequential buckets of water if a tap is not available.

9.10 Additional Results of Microbiome Analysis

Figure 9.1. Variation occurring between the bacterial populations associated with rumen and forestomach samples collected from cattle (o), goats (Δ), kangaroos (+) and sheep (×), determined by sPLSDA. Results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1-2); and (B) Components 1 vs 3 (sPLSDA comp 1-3).

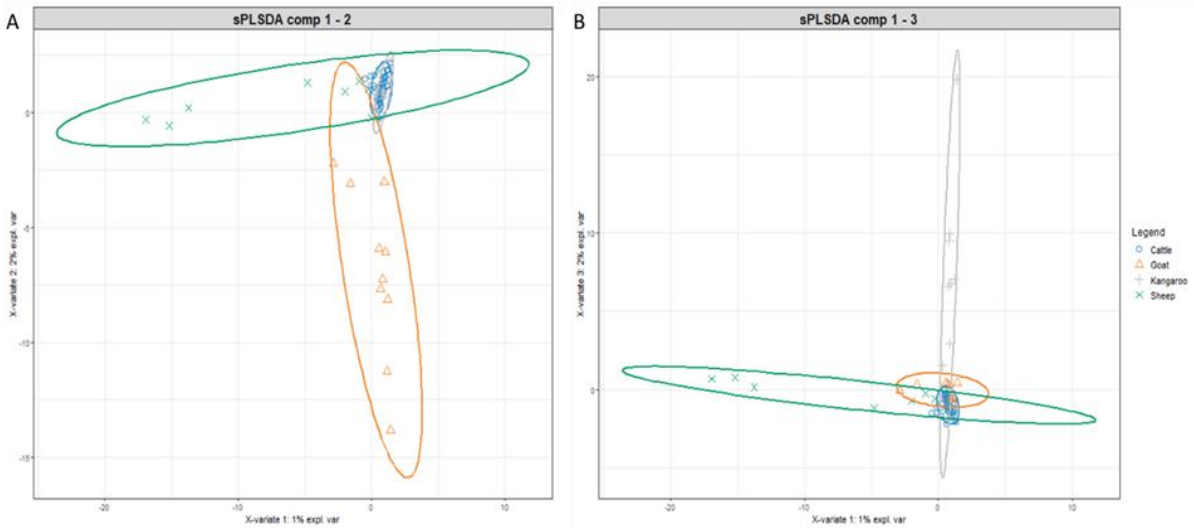

Table 9.12. Table of bacteria contributing to the differences in variation occurring between the bacterial populations associated with rumen and forestomach samples from cattle, sheep, goats and kangaroos, determined by sPLSDA (Figure 9.1). The top 10 bacteria are listed, and the respective positive or negative correlation value (Importance) given. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g). Where bacterial taxons with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset that could not be classified beyond the lowest taxonomic level listed.

Contribution to sPLSDA component 1 - Bacterial taxon[A]	**Importance**
p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Anaerovoracaceae	-0.440
p__Firmicutes; c__Bacilli; o__Acholeplasmatales; f__Acholeplasmataceae; g__*Anaeroplasma*	-0.337
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__*Lachnospiraceae*_XPB1014_group	-0.330
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__*Rikenellaceae*_RC9_gut_group	-0.319
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.314
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__U29-B03	-0.299
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__[*Eubacterium*]_*ruminantium*_group; s__bacterium_YSB2008	-0.255
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__UCG-005	-0.250
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__UCG-002	-0.230
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__*Clostridium*_sensu_stricto_1	-0.185
Contribution to sPLSDA component 2 - Bacterial taxon[B]	**Importance**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae	-0.263

p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__*Rikenellaceae*_RC9_gut_group	-0.228
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.227
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__*Rikenellaceae*_RC9_gut_group	-0.220
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	-0.217
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__*Lachnospiraceae*_ND3007_group	-0.216
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.213
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__NK4A214_group	-0.210
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Bacteroidales_RF16_group; g__*Bacteroidales*_RF16_group	-0.206
p__Firmicutes; c__Clostridia; o__Clostridia_UCG-014; f__Clostridia_UCG-014; g__*Clostridia*_UCG-014	-0.205
Contribution to sPLSDA component 2 - Bacterial taxon[C]	**Importance**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.272
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__*Rikenellaceae*_RC9_gut_group	0.232
p__Firmicutes; c__Clostridia; o__Oscillospirales	0.226
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.222
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__*Rikenellaceae*_RC9_gut_group	0.221
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__*Rikenellaceae*_RC9_gut_group	0.219
p__Patescibacteria; c__Gracilibacteria; o__Absconditabacteriales_(SR1); f__Absconditabacteriales_(SR1); g__*Absconditabacteriales*_(SR1)	0.219
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	0.217
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Defluviitaleaceae; g__*Defluviitaleaceae*_UCG-011	0.217
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__*Christensenellaceae*_R-7_group	0.215

[A]All listed genera contributed from sheep samples (TRUE by sPLSDA; importance coloured green); [B] All listed genera contributed from goat samples (TRUE by sPLSDA; importance coloured orange); [C]All listed genera contributed from kangaroo samples (TRUE by sPLSDA, importance coloured grey).

Table 9.13. Sample collection 1: Bacteria and archaea contributing to the differences in variation occurring between the rumen microbial populations associated with cattle from each experimental treatment group, at time-point 1, determined by sPLSDA presented in Figure 4.71. The top 10 microbial populations are listed and the respective positive or negative correlation value (Importance) given. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g). Where microbial taxons with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset, that could not be classified beyond the lowest taxonomic level listed.

Contribution to sPLSDA component 1	**Importance[A]**
p__Patescibacteria; c__Saccharimonadia; o__Saccharimonadales; f__Saccharimonadaceae; g__*Candidatus_Saccharimonas*	-0.298
p__Spirochaetota; c__Spirochaetia; o__Spirochaetales; f__Spirochaetaceae; g__*Treponema*	-0.294
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_Ga6A1_group	-0.293
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae	-0.281
p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales	-0.280
p__Bacteroidota; c__Bacteroidia	-0.259
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__*Ruminococcus*	0.241
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella;* s__Prevotella_bryantii	-0.150
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.148
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	-0.141
Contribution to sPLSDA component 2	**Importance**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	-0.680
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	-0.634
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__*Butyrivibrio*	-0.347
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__*Butyrivibrio*	0.087
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.046
p__Spirochaetota; c__Spirochaetia; o__Spirochaetales; f__Spirochaetaceae; g__M2PT2-76_termite_group	-0.033
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Lachnospiraceae_UCG-008	-0.031
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.030
p__Fibrobacterota; c__Fibrobacteria; o__Fibrobacterales; f__Fibrobacteraceae; g__*Fibrobacter*	-0.024
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	-0.023
Contribution to sPLSDA component 3	**Importance**
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	-0.483
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.440
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	-0.415
p__Euryarchaeota; c__Methanobacteria; o__Methanobacteriales; f__Methanobacteriaceae; g__*Methanobrevibacter*	-0.407
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__[Eubacterium]_coprostanoligenes_group; g__*Eubacterium coprostanoligenes*_group	-0.357
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.266
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_UCG-003	-0.132

p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__*Eubacterium ruminantium*_group	-0.120
p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Succinivibrionaceae; g__*Succinivibrio*; s__*Succinivibrio_dextrinosolvens*	-0.057
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	-0.048

[A] Respective positive or negative correlation value (Importance) coloured according to the experimental treatment group which contributed to the difference observed (TRUE by sPLSDA). Treatment groups include Pimelea + Biochar (orange text); Pimelea only (blue text); no Pimelea control group (purple text); Pimelea + mixed microbial inoculum (green text).

Table 9.14. Sample collection 2: Bacteria and archaea contributing to the differences in variation occurring between the rumen microbial populations associated with cattle from each experimental treatment group, at time point 2, determined by sPLSDA presented in Figure 4.73. The top 10 microbial populations are listed and the respective positive or negative correlation value (Importance) given. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g). Where microbial taxons with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset, that could not be classified beyond the lowest taxonomic level listed.

Contribution to sPLSDA component 1	**Importance[A]**
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	-0.307
p__Firmicutes; c__Clostridia; o__Clostridia_UCG-014; f__Clostridia_UCG-014; g__*Clostridia*_UCG-014	0.299
p__Firmicutes; c__Clostridia	0.296
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	0.292
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	0.292
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.267
p__Firmicutes; c__Negativicutes; o__Acidaminococcales; f__Acidaminococcaceae; g__*Succiniclasticum*	-0.242
p__Firmicutes; c__Negativicutes; o__Acidaminococcales; f__Acidaminococcaceae; g__*Succiniclasticum*	-0.230
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.205
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__NK4A214_group	-0.162
Contribution to sPLSDA component 2	**Importance***
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.750
p__Firmicutes; c__Negativicutes; o__Veillonellales-Selenomonadales; f__Selenomonadaceae; g__*Quinella*	0.478
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae	0.387
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__*Eubacterium coprostanoligenes*_group; g__*Eubacterium coprostanoligenes*_group	0.235
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.062
Contribution to sPLSDA component 3	**Importance***
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	0.227
p__Firmicutes; c__Bacilli; o__Acholeplasmatales; f__Acholeplasmataceae; g__*Anaeroplasma*	0.211
p__Firmicutes; c__Negativicutes; o__Acidaminococcales; f__Acidaminococcaceae; g__*Succiniclasticum*	0.206
p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Anaerovoracaceae; g__Family_XIII_AD3011_group	0.203

p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__UCG-005	0.193
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Lachnospiraceae_XPB1014_group	0.185
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.185
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__*Ruminococcus*	0.184
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	0.177
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	0.177

[A] Respective positive or negative correlation value (Importance) coloured according to the experimental treatment group which contributed to the difference observed (TRUE by sPLSDA). Treatment groups include Pimelea only (blue text); Pimelea + bentonite (yellow text); no Pimelea control group (purple text); Pimelea + mixed microbial inoculum (green text); Pimelea + Biochar (orange text); and Pimelea + Biochar (grey text).

Table 9.15. Sample collection 3: Bacteria and archaea contributing to the differences in variation occurring between the microbial populations associated with cattle from each experimental treatment group, at time-point 3, determined by sPLSDA, presented in Figure 4.75. The top 10 microbial populations are listed and the respective positive or negative correlation value (Importance) given. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g). Where microbial taxons with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset, that could not be classified beyond the lowest taxonomic level listed.

Contribution to sPLSDA component 1	**Importance[A]**
p__Firmicutes; c__Clostridia; o__Clostridia_UCG-014; f__Clostridia_UCG-014; g__*Clostridia*_UCG-014	-0.930
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.290
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae	-0.212
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.070
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	-0.022
Contribution to sPLSDA component 2	**Importance[B]**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.647
p__Firmicutes; c__Clostridia; o__Clostridia_UCG-014; f__Clostridia_UCG-014; g__*Clostridia*_UCG-014	0.370
p__Firmicutes; c__Bacilli; o__Acholeplasmatales; f__Acholeplasmataceae; g__*Anaeroplasma*	0.272
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.255
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__*Muribaculaceae*	0.244
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.206
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_UCG-001	0.139
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__*Ruminococcus*; s__*Ruminococcus_flavefaciens*	0.133
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	0.131
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__*Syntrophococcus*	0.114
Contribution to sPLSDA component 3	**Importance[C]**

p__Firmicutes; c__Clostridia; o__Oscillospirales; f__*Eubacterium coprostanoligenes*_group; g__*Eubacterium coprostanoligenes*_group	0.647
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.635
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.379
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.151
p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales	0.058
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae	0.037
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.034
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__F082; g__F082	0.032
p__Firmicutes; c__Negativicutes; o__Acidaminococcales; f__Acidaminococcaceae; g__*Succiniclasticum*	0.031
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__*Ruminococcus*	0.031

[A]Respective positive or negative correlation value (Importance) coloured according to the experimental treatment group which contributed to the difference observed (TRUE by sPLSDA). For component 1, the treatment group contributing was the Pimelea only group (blue text). [B] For component 2, the treatment group contributing was the no Pimelea control group (purple text). [C] For component 3, the treatment group contributing was the Pimelea + mixed microbial inoculum (green text).

Table 9.16. Sample collection 4: Bacteria and archaea contributing to the differences in variation occurring between the rumen microbial populations associated with cattle from each experimental treatment group, at time-point 4, determined by sPLSDA, presented in Figure 4.77. The top 10 microbial populations are listed and the respective positive or negative correlation value (Importance) given. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g). Where microbial taxons with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset, that could not be classified beyond the lowest taxonomic level listed.

Contribution to sPLSDA component 1	**Importance[A]**
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	-0.743
p__Patescibacteria; c__Saccharimonadia; o__Saccharimonadales; f__Saccharimonadaceae; g__*Candidatus_Saccharimonas*	-0.244
p__Firmicutes; c__Clostridia; o__Clostridia_UCG-014; f__Clostridia_UCG-014; g__*Clostridia*_UCG-014	-0.244
p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group	-0.237
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.235
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010	-0.234
p__Proteobacteria; c__Alphaproteobacteria; o__Paracaedibacterales; f__Paracaedibacteraceae	-0.234
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_UCG-001	-0.229
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	-0.146
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_NK3B31_group	-0.113
Contribution to sPLSDA component 2	**Importance[B]**
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__*Eubacterium coprostanoligenes*_group; g__*Eubacterium coprostanoligenes*_group	0.399
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales	0.388

p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.383
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.376
p__Cyanobacteria; c__Vampirivibrionia; o__Gastranaerophilales; f__Gastranaerophilales; g__*Gastranaerophilales*	0.373
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae	0.277
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.238
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenellaceae_RC9_gut_group	0.201
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.180
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.175
Contribution to sPLSDA component 3	**Importance[C]**
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	0.234
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__[Eubacterium]_ruminantium_group; s__Lachnospiraceae_bacterium	0.225
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella*	-0.184
p__Spirochaetota; c__Spirochaetia; o__Spirochaetales; f__Spirochaetaceae; g__*Treponema*	0.171
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_UCG-004	0.166
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__p-251-o5; g__p-251-o5	0.162
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Ruminococcaceae	0.162
p__Cyanobacteria; c__Vampirivibrionia; o__Gastranaerophilales; f__Gastranaerophilales; g__Gastranaerophilales	0.159
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.159
p__Cyanobacteria; c__Vampirivibrionia; o__Gastranaerophilales; f__Gastranaerophilales; g__Gastranaerophilales	0.158

[A]Respective positive or negative correlation value (Importance) coloured according to the experimental treatment group which contributed to the difference observed (TRUE by sPLSDA). For component 1, the treatment group contributing was the Pimelea + mixed microbial inoculum group (green text). [B] For component 2, the treatment group contributing was the Pimelea + Bentonite group (yellow text). [C] For component 3, the treatment group contributing was the Pimelea + activated Biochar group (grey text) and the Pimelea + Biochar group (orange text).

9.11 SEM images of biopolymers from *in vitro* Fermentations

SEM images provided as a separate file.

9.12 Micro-pore structure revealed by µ-CT of biopolymers from *in vitro* fermentation experiments

µ-CT images provided as a separate file

9.13 SEM images of biopolymers from *in vivo* trial in fistulated steers

SEM images provided as a separate file

9.14 Details of animal euthanasia and necropsy

Four steers were euthanased during the Pimelea feeding trial on veterinary advice due to animal welfare considerations. Steer #082 (T2) and steer #086 (T4) experienced marked Pimelea poisoning. Two further animals experienced unrelated oesophageal injuries (Steer#375, T4) and abscess (Steer#051, T6) as detailed below. An Adverse Incident Report was submitted to, and accepted by, the UQ PCA AEC in relation to these animals and a description of the circumstances of each of these four animals is included below.

Steer #375, Pen 28 (T4 – Pim + Inoc (Pimelea plus inoculum) treatment group)

On Friday 7th May, steer #375 was first noted to show mild oedema which significantly increased during the day. Temperature 40.4 °C, Heart rate 156; Respiratory rate 40. This steer had reduced feed intake in preceding days with no feed intake on Thursday 6th May. On veterinary advice this animal was euthanased on the afternoon of 7th May (week 11), and at autopsy had significant fluid accumulation in both abdomen and chest cavities. On post-mortem there was a small (0.5-1cm diameter) transmural perforation to the cervical oesophagus with associated inflammation and necrosis of the surrounding tissue, extending caudally along the adventitia. Histologic examination showed that inflammatory changes in the oesophagus were acute (1 - 2 days duration), suggesting the perforation occurred within the previous days, not weeks. Liver histology showed moderate, chronic sinusoidal dilation (peliosis hepatis) and acute, multifocal hepatocellular necrosis and suppurative hepatitis. It was considered likely that the observed oesophageal damage had occurred during rumen tubing on Wednesday 5th May, and that this and ensuing necrosis and inflammatory responses had exacerbated the effects of Pimelea on this animal. Full PM report available.

Steer #082, Pen 26 (T2 – Pim + Bchar (Pimelea plus non-activated Biochar) treatment group)

The mild oedema first noted as face swelling in Steer #082 on 28th April, continued to develop despite cessation of Pimelea intake on 6th May. Feed intake was reduced and on veterinary advice this animal was euthanased on 18th May (week 13). At necropsy, this animal had significant fluid accumulation in both abdomen and chest cavities. The right heart was dilated two-fold normal; the free wall was distended and flaccid. Full PM report available.

Steer #086, Pen 17 (T4 – Pim + Inoc (Pimelea plus inoculum) treatment group)

Despite having no oedema at cessation of Pimelea intake, Steer #086 developed significant oedema on 17th May 11 days after cessation of Pimelea intake and due to reduced feed intake was euthanased on veterinary advice and necropsied on 20th May (week 13). At necropsy, this animal had significant fluid accumulation in both abdomen and chest cavities. The right heart was dilated two-fold normal; the free wall was distended and flaccid. Full PM report available.

Steer #051, Pen 23 (T6 – Pim + Bent (Pimelea plus Bentonite) treatment group)

Steer #051 experienced little apparent impact from Pimelea but was observed to experience diarrhea that was not resolved on cessation of Pimelea and was noted to have significantly reduced feed intake on 19th May. On veterinary inspection a large abscess was detected on the oesophagus by ultrasound, which the veterinarian attempted to drain and when this was unsuccessful surgically operate on (following day). Despite best efforts, the oesophagus was still restricted compromising this animal's feed intake, and this animal was euthanased and necropsied on 21st May (week 13). At necropsy, this animal had no significant fluid accumulation in either abdomen or chest cavities. The right heart was only slightly dilated; the free wall was distended and firm. Examination of the

abscess showed that it was within the oesophageal muscle and external to the oesophagus, and this lesion appeared unrelated to the Pimelea treatment. Full PM report available.