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Prepared by: Dr Carl K. Davis
Department of Agriculture and Fisheries

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Leucaena Rumen Inoculum – composition and activity along the supply chain

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Abstract

For the past 20 years a fermentor-grown inoculum containing *Synergistes jonesii* has been produced and supplied to the beef industry as an oral rumen drench to protect cattle from Leucaena toxicity. The aim of the current study was to define the bacterial composition of the inoculum, determine if there had been major changes in inoculum composition over the last 20 years and to determine the survivability of *S. jonesii* along the supply chain. 16S rRNA gene amplicon sequencing showed that the inoculum contains an estimated 300-400 bacterial species (taxa) and these are predominated by commensal, rumen bacteria of the Bacteriodes and Firmicutes phyla. Significant, albeit, small changes in the overall bacterial composition were seen in batches produced between 1999 and 2012. However, it was determined that there was one strain of *S. jonesii* present in the inoculum and no changes in the levels of this strain of *S. jonesii* in the inoculum over the 20 years of production. Viable cell counts of *S. jonesii* in the inoculum showed that thawing followed by storage at 4 °C for 30 hours decreased viability from 1.1 $x10^5$ to 9.4 $x10^4$ colony forming units (CFU's) which represents 0.05 ± 34 % log_{10}fold change. These results were consistent with experiments performed using pure cultures of *S. jonesii* which showed a slightly higher 0.21 ± 18 % log_{10}fold decrease in viability. Additionally, supply chain conditions tested had no effect on the ability of pure cultures to resume DHP degradation. These findings suggest that reductions in *S. jonesii* viability due to conditions along the supply chain are unlikely to impact on the overall ability of the inoculum to establish and maintain its activity *in vivo*.

Executive Summary

The Leucaena inoculum produced by the Queensland Department of Agriculture and Fisheries (DAF) has been provided to livestock producers since 1995 as a probiotic drench for cattle. The inoculum is used to decrease symptomatic toxicity associated with Leucaena-fed cattle and to increase live-weight gains associated with this high nutrient fodder. Every batch of the fermentor grown inoculum undergoes stringent quality controls, including its ability to degrade the anti-nutrient components of Leucaena (mimosine and DHP compounds), and enumeration of *Synergistes jonesii* (an organism that degrades DHP compounds). However, little is known about the inoculums overall bacterial content or how the conditions throughout the supply chain may affect the viability and activity of the inoculum. A recent study has reported levels of DHP compounds excreted in the urine of inoculated cattle grazing Leucaena. This has raised speculation about the efficacy of the inoculum, and has resulted in apprehension amongst livestock producers.

To address these concerns, the current project aimed to determine the bacterial composition of the Leucaena inoculum, both in regard to overall composition and the number of *S. jonesii* strains present, and whether the inoculum has changed over time.

In addition, the project aimed to characterise, evaluate and sequence the genomes of all known *S. jonesii* reference strains, since some reports have suggested that *S. jonesii* strains have different abilities to degrade the toxic component of Leucaena. Culture-based methods were developed to isolate *S. jonesii* strain(s) from the fermentor and to compare it to the reference strains. Molecular screening methods were also developed to investigate the number of strains present in the inoculum, and elucidate whether they are different from the known strains or have changed during the production period of the inoculum.

Finally, this project determined the impact of the transport system on the viability and activity of *S. jonesii*. This was performed using simulated conditions to replicate the transport chain, such as changes in temperature and storage conditions, and exposure to oxygen prior to inoculation.

The bacterial composition of the fermentor inoculum contains approximately 300-400 identifiable bacterial species (taxa) and these are predominated by commensal, rumen bacteria of the Bacteriodes and Firmicutes phyla. These bacteria contribute important functions in rumen ecosystems. The 10 most abundant bacteria in the inoculum, which represented nearly half of all bacteria in each inoculum batch, are found at similar proportions in nearly all batches. The fermentor inoculum appears to have undergone

relatively little change in regards to the most abundant taxa or the relative abundance of phylum-level taxa during its time in production.

One strain of *S. jonesii* is present in the fermentor and the 16S rRNA gene sequence of this strain has not changed during the course of the inoculum's production. The fermentor strain is a different strain based on 16S rRNA gene sequence (a gene used to classify bacteria) to all other reference cultures, (which are all identical). Levels of *S. jonesii*, determined by molecular quantification, were not significantly different between batches studied. *S. jonesii* maintained levels in excess of 1 million cells/mL in these batches, which translates to approximately 100, 000 CFU/mL.

There is little or no true genomic variation between the genomes of the *S. jonesii* reference strains. All reference strains of *S. jonesii* can completely degrade 2,3- and 3,4-DHP, although small differences exist in the overall rate of DHP-degradation which may be explained by biological variability associated with *in vitro* culturing rather than true phenotypic variation.

Cold chain treatments performed on a pure culture of *S. jonesii* showed viable cells of *S. jonesii* decreased by approximately 60 % when thawed and refrozen from the cryoprotectant. When this treatment was additionally exposed to oxygen for 30 minutes no further effect on viable cell numbers was seen. Interestingly, refrigeration of the inoculum following thawing led only to a 37 % decrease in viable cell numbers (lower than the freeze-thaw). Even though the percentage decease in viable cell numbers may appear large in these treatments, the inoculum contains over 100,000 viable *S. jonesii* cells/ mL of inoculum. Furthermore, none of treatments had any effect on the overall ability to degrade DHP. Experiments using the fermentor inoculum as opposed to the pure culture gave very similar results in the viable cell numbers following the same treatments as the pure culture.

Industry benefits/implications of the work

- The Leucaena inoculum contains a well-defined bacterial community containing Bacteroides and Firmicutes phyla, which are commensal rumen microorganisms implicated in a wide array of beneficial functions.
- The inoculum undergoes strict quality assessment prior to supply to guarantee that every batch contains *S. jonesii* at high levels and is able to completely degrade mimosine and DHP compounds.
- The total cell count of *S. jonesii* as determined by DNA-based assays, has changed little during its production with a steady population of greater than one million cells/mL.

- Sensitive molecular-based assays based on Reverse transcriptase quantitative PCR (RT-q-PCR) have been developed that increase the detection level of *S. jonesii* in rumen fluid.
- Methods to isolate *S. jonesii* from complex environmental samples have been developed that allow further research into other strains of *S. jonesii* present in other locations and host ecosystems. There is one strain of *S. jonesii* in the inoculum which is present throughout the entire production process. There has been no change, based on 16S rRNA gene classification, of *S. jonesii* since the earliest archived samples taken in 1999 .
- Best practice for maintaining the viability and DHP-degrading activity of the Leucaena inoculum has been proposed.

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1 Background

Leucaena (*Leucaena leucocephala*) is a leguminous shrub that is widely cultivated in tropical and subtropical regions worldwide for use as high nutrient fodder for ruminant livestock. However, its use is limited by the presence of the toxic amino acid mimosine, which occurs at various concentrations in the Leucaena plant, and its breakdown products 3,4- and 2,3-DHP. In Australia, the toxicity of Leucaena limited its usefulness as a fodder crop until the early 1980s with productivity from Leucaena-based fodder being reduced by DHP-induced depressions in intake. A solution to Leucaena toxicity in livestock animals was discovered when populations of goats in Hawaii were observed eating Leucaena without effect and studies showed they were able to degrade 3,4-DHP within the rumen (Jones, 1981). Subsequent studies showed that transferring a mixed bacterial culture derived from these goats to Australian cattle conferred protection from the clinical effects of Leucaena toxicity (Jones and Megarrity, 1986).

The organism responsible for DHP degradation in the rumen has been isolated, characterised and named *Synergistes jonesii* (Allison et al., 1992). *S. jonesii* converts 3,4-DHP to the slightly less toxic isomer 2,3-DHP, then degrades this isomer to non-toxic products (Dominguez-Bello et al., 1998). *S. jonesii* can establish self-maintaining populations in the rumen of cattle, sheep and goats feeding on Leucaena, that have been inoculated with rumen fluid of animals in which it is already present, and rapidly spreads to uninoculated animals in the same herd (Quirk et al., 1988, Quirk et al., 1990).

In Queensland, an inoculum consisting of a mixed bacterial culture containing *S. jonesii* has been produced by the Department of Agriculture and Fisheries (DAF) in an *in vitro* fermentation system since 1995 (Klieve et al., 2002). This mixed culture is distributed to cattle producers who administer it to their herds as a probiotic rumen drench . Recently, the ability of *S. jonesii* to persist and continue to effectively detoxify DHP in the rumens of inoculated cattle has been questioned on the basis of the presence of elevated concentrations of 2,3-DHP in the urine of some inoculated cattle (Dalzell et al., 2012, Graham et al., 2013). This has led to the establishment of the current project which aims to investigate the survivability of *S. jonesii* in the production and transport chain of the inoculum, and to assess archival samples of the inoculum to determine to what extent the composition of the inoculum may have changed over the period during which it has been produced by DAF.

In this report we present findings on:

1. The bacterial composition of the fermentor-grown inoculum in batches produced from 1999 to 2008, and the number of *S. jonesii* strains present in the Leucaena inoculum.

2. Isolation and characterisation of the *S. jonesii* strains in the fermentor and genetic comparisons with the reference strains.

3. The survival, persistence and activity of *S. jonesii* in pure culture and in the fermentor-grown inoculum subjected to various treatments to simulate conditions occurring along the inoculum supply chain.

2 Project objectives

Determine the bacterial composition of the *in vitro* inoculum, including the number of strains of *S. jonesii* present, and how this has changed over time and in comparison with ruminal contents.

Determine the survivability, persistence and activity of *S. jonesii* strains throughout the inoculum supply chain.

3 Methodology

3.1 Composition of the fermentor-grown inoculum

In order to determine the bacterial composition of the fermentor-grown inoculum, 16S rRNA gene 454 amplicon pyrosequencing was performed on archival fermentor DNA from fermentor runs conducted between 1999 and 2012. The samples were chosen to allow us to determine whether there has been a change in bacterial composition due to changes in the starter culture (passage effect), and the type of fermentor system used during production.

3.1.1 Sample collection and experimental design

The 16S rRNA gene dataset consisted of the following samples in three independent replicates:

- Eight time points taken during a single fermentation (Run 79, 27/6/2012) - days 2, 3, 5, 7, 9, 11, 13 and 15.

- Sixteen fermentor batches produced between 1999 and 2012 sampled mostly on day 15 are summarised in Table 1. To determine whether the starter culture (which originated from a cannulated Brahman cross steer) had an effect on bacterial composition, batches were chosen that originated from different starter cultures which had been passaged between two and six times.

Table 1 Details of the samples used for 16S rRNA gene 454 amplicon pyrosequencing. Fermentor run number, fermentor day sampled, year of production, starter passage number, starter date and run origin used to start the fermentation, amount of Leucaena leaf fed daily, apparatus, fermentation volume and the length of fermentation.

Batch/ Run #	Day sampled	Production year	Starter passage no.	Starter date harvest (Run #)	Leucaena (g/day)	Apparatus type	Fermentation Volume	Duration (days)
15	9	1999	2	Unknown	10	3 L chemostat/ Eyela	1.5 L	9
23	15	2002	3	13/7/1999 (15)	10	3 L chemostat/ Eyela	1.5 L	15
30	12	2004	4	5/5/2002 (23)	10	3 L chemostat/ Eyela	1.5 L	15
34	15	2005	5	28/5/2004 (30)	10	3 L chemostat/ Eyela	1.5 L	15
35	15	2005	5	28/5/2004 (30)	10	3 L chemostat/ Eyela	1.5 L	15
36	15	2005	5	28/5/2004 (30)	10	3 L chemostat/ Eyela	1.5 L	15
37	15	2005	5	28/5/2004 (30)	10	3 L chemostat/ Eyela	1.5 L	15
39	15	2006	5	28/5/2004 (30)	10	3 L chemostat/ Eyela	1.5 L	15
45	15	2007	5	28/5/2004 (30)	15	7 L chemostat/ Infors	3.0 L	15
49	15	2008	5	28/5/2004 (30)	15	7 L chemostat/ Infors	3.0 L	15
51	15	2008	6	10/7/2008 (49)	15	7 L chemostat/ Infors	3.0 L	15
58	15	2009	6	10/7/2008 (49)	15	7 L chemostat/ Infors	3.0 L	15
59	15	2009	6	10/7/2008 (49)	15	7 L chemostat/ Infors	3.0 L	15
67	15	2010	6	10/7/2008 (49)	30	7 L chemostat/ Infors	3.0 L	15
72	15	2012	6	10/7/2008 (49)	30	7 L chemostat/ Infors	3.0 L	15
79	15	2012	6	10/7/2008 (49)	30	7 L chemostat/ Infors	3.0 L	15

DNA was extracted from 1 mL aliquots of fermentor liquor collected into 1.5 mL microcentrifuge tubes. Biomass was pelleted by centrifugation at 16,000 x g for 10 minutes, the supernatant was removed before the pellets were stored frozen at -20 °C prior to DNA extraction. Archival samples of the *in vitro* inoculum consisted of pellets that had been processed as above and stored at -20 °C for up to 14 years. Samples for RNA extraction were collected from fermentation runs conducted in 2013 and consisted of 500 µL fermentor liquor collected into 1.5 mL centrifuge tubes and immediately snap frozen in liquid nitrogen to preserve RNA integrity and copy numbers. These samples were thawed and pelleted by centrifugation at 16,000 x g at 4° C for 10 minutes prior to RNA extraction.

Prior to October 2005, the mixed bacterial culture containing *S. jonesii* that is used to inoculate cattle was produced as described by Klieve et al. (2002). After October 2005, the inoculum was produced in the same way, except that fermentation was scaled up and conducted in a 3 L volume using a 7 L chemostat fermentor (Infors HT, Bottmingen, Switzerland). Daily 1.5 L of media was replaced and 15 g of chopped Leucaena leaf added and the amount of chopped Leucaena leaf was increased to 30 g from 2009. Under current production conditions the fluid removed for the first nine days of the fermentation run is discarded. From days 10 to 30, 250 mL aliquots of the fluid removed are mixed with equal volumes of sterile anaerobic rumen fluid medium, which contains 50 % glycerol as a cryopreservative. This harvested fluid is immediately frozen and stored at -20° C prior to distribution to cattle producers. A schematic diagram illustrating the production of the *in vitro* Leucaena inoculum culture in the fermentor is provided in Fig. S 1 (Appendix 9.1).

3.1.2 Amplicon pyrosequencing and analysis

DNA was extracted from rumen fluid and fermentor fluid samples according to the method of Yu and Forster (2005). For DNA purification the total nucleic acids were processed with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and eluted in AE buffer, then made up to 500 µL in nuclease free water.

Barcoded amplicons of the V3-V4 region of the 16S rRNA gene were generated using high fidelity DNA polymerase and barcoded primers listed in **Table S 1** (Appendix 9.1). Amplicon pyrosequencing was conducted on the pooled amplicons using the Roche 454 GS FLX Titanium platform (Macrogen, South Korea). The resulting sequences were processed using the UPARSE pipeline. In summary, raw reads were quality filtered with a maximum expected error of 1.0 using USEARCH. Barcodes were removed using python script fastq_strip_barcode_relabel2.py and globally trimmed to a fixed length of 300 bp. Sequences were de-replicated, sorted by abundance and singletons removed. Sequences

were clustered into OTU's (minimum identity 97 %) and chimeras removed using the cluster_otus command. Additional reference-based chimera removal was performed with UCHIME (Edgar et al., 2011) against the Gold database (version microbiomeutil-r20110519). Chimeric sequences were verified by BLAST searches against NCBI cultured isolates and Decipher (Wright et al., 2012). Reads were mapped back to OTU's using the usearch_global command and an OTU table generated using the 'uc2otutab.py' script. Taxonomic assignments were made using the UTAX algorithm at a confidence level of 70 %. Remaining OTU's, were assigned Greengenes (release 13_8) taxonomy using the 'assign_taxonomy.py' script in QIIME (Caporaso et al., 2010). Representative OTU sequences were aligned to the Silva NR SSU reference database (release 119) (Pruesse et al., 2007) in MOTHUR using the 'align_seqs.py' script (Schloss et al., 2009). A phylogenetic tree in Newick format was produced using the make_phylogeny command in QIIME employing the FASTTREE program (Price et al., 2010).

The OTU table, sample metadata, Newick phylogenetic tree and taxonomy assignments were imported into the Phyloseq package (McMurdie and Holmes, 2013) in R (https://www.r-project.org; R version 3.2.2). Alpha diversity and richness measurements were computed with Chao estimator (Chao, 1984), Observed and ACE estimator (Kocherginskaya et al., 2001) using the 'plot_richness' function in Phyloseq.

To calculate phylum level changes within fermentor samples, a subset of the data was made using prune_taxa command in Phyloseq. Replicate sample OTU counts were summated, zero taxonomic counts removed and transformed to relative abundance. The 100 most abundant OTU's were calculated based on the highest relative abundance values for OTU's in the entire dataset. Data was plotted using the package ggplot2 (Wickham and Wickham, 2009). Similarly, the relative abundance of taxa at time points during a single fermentor run was calculated by creating a subset of data, removing zero taxonomic counts and replicate sample OTU counts summated and transformed to relative abundance. The 10 most abundant OTU's were calculated based on the highest relative abundances in the entire dataset.

Phylogenetic trees with measures of abundance were prepared using the plot_tree function in Phyloseq. Abundances were standardised to median sequencing depth of the dataset. Replicates were merged and summated, and data plotted based on putative taxonomic classification. In order to perform weighted ordinations, abundance tables were normalised using variance stabilisation transformation implemented in the R package Deseq2 (Love et al., 2014). Ordinations and confidence ellipses were calculated in R using the Vegan package (Dixon, 2003) and plotted using ggplot2. Subsets of relevant data were initially

produced using the prune_samples and prune_taxa functions in Phyloseq. Analyses of similarities (ANOSIM) were performed in R using the vegan package. Unifrac dissimilarity matrices were calculated using the GUniFrac package (Lozupone and Knight, 2005).

3.2 Characterisation of *S. jonesii* strains present within the Leucaena inoculum

S. jonesii in the inoculum was characterised using several approaches. The reference 'strains' of S. jonesii obtained from Dr Milton Allison and originally isolated from a single goat in Hawaii in 1989 (strains 78-1, 100-6, 113-4 and 147-1) were compared using growth curves and DHP degradation assays. The genomes of the reference strains were sequenced, functionally annotated and compared to attempt to identify genes involved in DHP degradation. In order to determine the number of strains present in the inoculum and whether the strains had changed over the years of production, *S. jonesii*-specific assays based on Denaturing Gradient Gel Electrophoresis were developed. Another assay based on Reverse Transcriptase quantitative PCR (RT-qPCR) was also developed to enumerate *S. jonesii* in the fermentor as well in the rumen. Finally, genome sequence information was used to develop a specific growth medium to isolate *S. jonesii* from the Leucaena inoculum.

3.2.1 Bacterial strains and growth conditions

S. jonesii (ATCC 49833) was obtained from the American Type Culture Collection (ATCC). Other *S. jonesii* strains 78-1, 100-6, 113-4 and 147-1 were obtained from Dr Milton Allison at Iowa State University. Anaerobic culturing was performed using the techniques of Hungate (Hungate, 1966) and Bryant (Bryant, 1972). Cultures were grown in Hungate tubes under strictly anaerobic conditions in media (10 mL) containing rumen fluid and various carbon/nitrogen sources under a headspace of CO_2/H_2 (95:5) at 39 °C. Where noted, media contained 2,3- and/or 3,4- dihydroxypyridine (DHP) at 0.25 g/L.

Growth and DHP degradation experiments of *S. jonesii* were performed on the panel of five *S. jonesii* strains. All experiments were inoculated from actively growing cultures. Optical density (OD_{600}) was monitored in 96-well Nunclon DS plates (Thermo Fisher Scientific, Waltham, USA) using a Fluostar OPTIMA microplate reader (BMG labtech GmbH, Ortenberg, Germany). All reported results are the averages from triplicate cultures and triplicate readings.

3.2.2 Quantification of 2,3- and 3,4-DHP

Preliminary analysis for the detection of DHP was performed using a ferric chloride colorimetric assay described by Allison et al. (1990). Briefly, 100 µL of samples was mixed with 500 µL of ferric chloride reagent (2X) and the presence/absence of 2,3- or 3,4-DHP determined by visual inspection.

Quantification of DHP was performed using HPLC and ultra-violet/visible detection (280 nm) described by Tangendjaja and Wills (1983). Briefly, culture media (200 µL) was filtered through a 0.22 µm syringe-filter. Samples (10 µL) were separated on an Aqua C18 column, (Particle size 5µm, Pore size 125A, 250 x 4.6mm; Phenomenex) using 0.1 % phosphoric acid/ 1 % methanol as mobile phase at a flow rate of 1 mL/min. Quantification of 2,3- and 3,4–DHP was determined from comparison to standard curves.

3.2.3 Synergistes jonesii specific DGGE

Primers for a nested PCR approach to produce *S. jonesii* specific amplicons for DGGE PCR were developed and tested *in silico* and the primer sequences are listed in **Table S 1** (Appendix 9.1). Extracted fermentor DNA samples were amplified in an initial 25 µL volume PCR reaction containing: 10 ng DNA template, Phire Green Buffer (ThermoFisher, Scoresby Victoria, Australia), 0.2 mM dNTP's, 400 nM primers SJ062F and SJ1009R, and 0.1 U Phire Hot Start DNA Polymerase (ThermoFisher). Reactions were performed in a BioRad C1000 thermocycler (Biorad Laboratories, Richmond, California) using an initial denaturation at 98 °C for 30 seconds, followed by a three-step amplification profile consisting of 35 cycles of 95 °C for 5 seconds, 60 °C for 5 seconds and 72 °C for 15 seconds, with a final extension 72 °C for 3 minutes. Excess primers were removed in a reaction (10 µL) containing PCR product (1 µL), ExoI buffer (NEB, Ipswich, MA, USA), and exonuclease I (NEB). A 1 µL aliquot of this reaction was used as a template for the nested PCR using identical conditions as the initial PCR with primers SJ193F and SJ492R-GC. Products were separated on 6 % polyacrylamide gels with a linear 40 to 60 % denaturing agent gradient (100 % denaturing agent was defined as 7 M urea and 40 % deionized formamide) and electrophoresed for 18 hours at 100 volts following the method of Muyzer et al., (1993). Bands were visualised by silver staining (Kocherginskaya et al., 2005). For sequence characterisation, PCR's were purified using QIAquick PCR purification kit (Qiagen) and purified amplicons were Sanger sequenced by AGRF (Brisbane, Australia) using the primers SJ193F and SJ492.

3.2.4 Development of qPCR and RT-qPCR assay for *S. jonesii*

For RNA purification the total nucleic acids were processed with the RNeasy kit (Qiagen) following the manufacturer's instructions to remove contaminating DNA and residual protein. RNA was eluted in 50 µL nuclease free water and quantified using the RiboGreen RNA quantification kit (Life Technologies, Carlsbad, California, USA).

A real time quantitative PCR (qPCR) assay was designed to allow accurate quantification of the cell numbers and level of metabolic activity of *S. jonesii* in the fermentor. A pair of PCR primers, Syn061F and Syn172R within the 16S rRNA gene V1 and V2 variable regions, an internal oligonucleotide probe SynProbe2 were designed using Primer 3 (Untergasser et al*.*, 2012) and tested *in silico*. The primer and probe sequences are listed in **Table S 1**, Appendix 9.1. The same primers and probe were also used in a reverse-transcription quantitative PCR (RT-qPCR) assay to quantify the copy number of 16S rRNA transcripts of *S. jonesii*.

Bacterial cell number standards were prepared as described by Ouwerkerk et al*.,* (2002). *In vitro* transcribed RNA standards for *S. jonesii* 16S rRNA were prepared using the strategy described by Fey et al*.,* (2004). Briefly, a 603 bp fragment of the 16S rRNA gene of *S. jonesii* was amplified by PCR using a set of primers that incorporated the T7 promoter sequence (**Table S 1**, Appendix 9.1). The resulting T7-tagged amplicons were transcribed *in vitro* using T7 RNA polymerase. This produced transcripts that could be accurately quantified fluorometrically and used to make standard series with precisely calculated numbers of transcript copies.

PCR reactions (25 µL) contained 10 µL 2.5× Real Master Mix Probe reaction buffer (5 prime, Hilden, Germany), 200 nM of each primer, 100 nM of oligonucleotide probe and 5 µL DNA template (10 ng/µL). PCR was performed in a Rotor-Gene model RG-6000 (Corbett Life Science) using an initial denaturation at 95 °C for 60 seconds, followed by a two-step amplification profile consisting of 45 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds.

3.2.5 Genome sequencing, assembly, annotation and analysis

High molecular weight genomic DNA from pure cultures of *S. jonesii* strains were prepared with enzymatic, detergent and chaotrophic lysis followed by phenol-chloroform extraction. Cultures (50 mL) of *S. jonesii* were grown to mid-log phase (OD_{600} ~ 0.35), centrifuged at 10000 x g for 20 minutes at 4 °C and washed with PBS. Cell pellets were resuspended in 50 mM Tris, 10 mM EDTA (400 µL, pH 8.0) then 50 µL of 100 mg/mL lysozyme (Sigma) and

10 µL RNaseA (Qiagen) added and the mix incubated in a shaker for 2 hours at 37 °C. A 50 µL volume of Proteinase K (Qiagen) and 25 µL of 10 % SDS were added, mixed gently, and incubated with shaking at 50 °C for 30 minutes. Buffer AL (375 µL, Qiagen) was added and incubated for 10 minutes at room temperature. Tris buffered phenol/chloroform/isoamyl alcohol was added (900 µL, 25:24:1, pH 8.0, Sigma) and thoroughly mixed by inversion before centrifuging at 13,200 x g for 15 minutes. The supernatant was removed into a new tube and the extraction repeated with additional phenol/chloroform/isoamyl alcohol (900 µL). The resulting supernatant was washed twice with chloroform/isoamyl alcohol (900 µL). The supernatant was precipitated by adding a 0.7 volume of isopropanol and 0.1 volume of 3M sodium acetate (pH 5.6). DNA was spooled with a glass pipette, washed twice with 70 % ethanol, air-dried for 5 minutes and resuspended in 0.1 X TE buffer (Qiagen).

Complete genome sequences of *S. jonesii* strains 100-6, 113-4, and 147-1 were generated by PacBio sequencing (Millennium Science, Mulgrave, Victoria) using either 10 kb (P4C2 chemistry, 2 flow cells per genome) or 20 kb insert libraries (BluePippin kit, P6-C4 chemistry, 1 flow cell per genome). Genome sequences were assembled using SMRT portal software (v2.3.0) using default settings. The genome sequence of the type strain 78-1 (ATCC 49833) was kindly provided by Jonathan Eisen of the University of California, Davis.

Annotation of genome sequences were performed using Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008) and Prokka (Seemann, 2014). Genomes of the three strains 100-6, 113-4, 147-1 were visualised in the software package, Geneious v7.1.1 (Kearse et al., 2012), and whole genome alignments performed with Mauve (Darling et al., 2010).

3.2.6 Isolation of *S. jonesii* from Leucaena inoculum

A selective isolation media was developed using biological data relating to growth requirement and antimicrobial resistance obtained from the genomes of closely related bacteria. *S. jonesii*, *Pyramidobacter piscolins* and *Cloacibacillus evryensis* were obtained from this project and IMG database and annotated with RAST and Prokka. Genome-scale metabolic reconstructions were drafted with Model-SEED (Overbeek et al., 2005) and curated with Metacyc (Caspi et al., 2015), literature and in-house biochemical data. Antimicrobial resistance traits were identified from functional gene annotations and active subsystems within SEED. Blast searches of putative genes were performed against the Conserved Domain Database (Marchler-Bauer et al., 2015).

Several putative antimicrobial-resistance and metabolic traits were identified from genome-scale reconstructions and published literature sources and validated empirically using pure cultures of *S. jonesii* strains. Targeted isolations from the Leucaena inoculum were performed in the developed anaerobic medium SS-4 containing protein hydrolysates and amino acids to select for indicative traits, and various antimicrobials to suppress the growth of non-target organisms (media composition listed in Appendix 9.2). A sample of Leucaena inoculum fermentor fluid was obtained on day 30 of the fermentation run and was diluted with anaerobic diluent and spread directly onto solidified SS-4 media containing both 2,3- and 3,4-DHP (0.25 g/L). Plates were incubated in anaerobic metal canisters at 39 °C for seven days. Colonies were re-streaked onto new SS-4 plates and single colonies inoculated into 10 mL of DHP-containing broth media. Cultures were verified for purity microscopically and identified by direct sequencing of 16S rRNA genes using universal primers 27F and 1492R (Lane, 1991).

3.2.7 Phylogenetic analysis

Representative 16S rRNA gene sequences from the Synergistetes phylum were obtained from the SILVA database v119 (Pruesse et al., 2007). The 16S rRNA gene sequences from bacteria isolated in this study were aligned with SINA (v1.2.11) (Pruesse et al., 2012). Alignment files were imported in Geneious software v7.1.1 (Kearse et al., 2012). Distance trees were constructed using the neighbour joining algorithm and TN93 substitution model. Robustness of the tree was verified by calculating bootstrap values (1000 replicates) (Holmes, 2003).

3.3 Assessing the effect of cold storage on viability and DHP-degrading ability of *S. jonesii*

To assess the effect of supply chain from the production of the Leucaena inoculum through to its receipt by the farmer, two sets of experiments were performed; one using a pure culture of *S. jonesii* str. 78-1 and other using fermentor-growth inoculum (Run 11 , Day 9, 9/12/2014).

3.3.1 Experimental design

The following treatments were used to replicate various conditions along the supply chain and to determine their effect on viability and DHP-degrading ability (a schematic of the supply chain is shown in **Fig. 1**).

(1) Control (no treatment, for pure culture only)

(2) Cryoprotectant: 2 mL culture mixed with 2 mL crytoprotectant in 10 mL serum vial

(3) Freeze/thaw: as with (2), stored at -20 °C for 24 hours and thawed at room temperature to room temperature and left for 30 minutes

(4) Exposure to oxygen: as with (3) then opened to air and left standing at room temperature for 30 minutes

(5) Freeze/thaw/refrigeration: as with (3) then refrigerated at 4 to 6 °C for 30 hours.

Three independent replicate experiments were performed and triplicate CFU counts done for each treatment.

3.3.2 Cell viability assays and DHP-degrading ability

Following each of the various treatments, CFU counts were used to determine viability of *S. jonesii* in pure cultures and in the Leucaena inoculum. Cultures were first serially diluted in anaerobic diluent to 5000 cells/mL (based on the control culture) for pure cultures and a fixed 1/1000 dilution was used for the Leucaena inoculum. These dilutions were calculated to achieve no more than 250 CFU's in accordance with reference (Sutton, 2011) CFU counting theory and practice. Diluted treatment cultures were spread onto selective plates prepared according to the method described in Appendix 9.2 Plates were incubated in an anaerobic metal canister at 39 °C and after seven days each plate was scanned using the BioRad GelDoc system (BioRad, Gladesville, New South Wales, Australia) and colonies quantified using OpenCFU software (Geissmann, 2013).

DHP-degrading activity was determined in the case of pure cultures experiments. Three colonies from each treatment were inoculated into 5 mL of media containing 2,3- and 3,4-DHP and grown for 10 days at 39 °C. Samples for DHP analysis were taken after 0, 5 and 10 days. The cultures were also assessed for 2,3- and 3,4-DHP degradation by HPLC (Section 3.2.2).

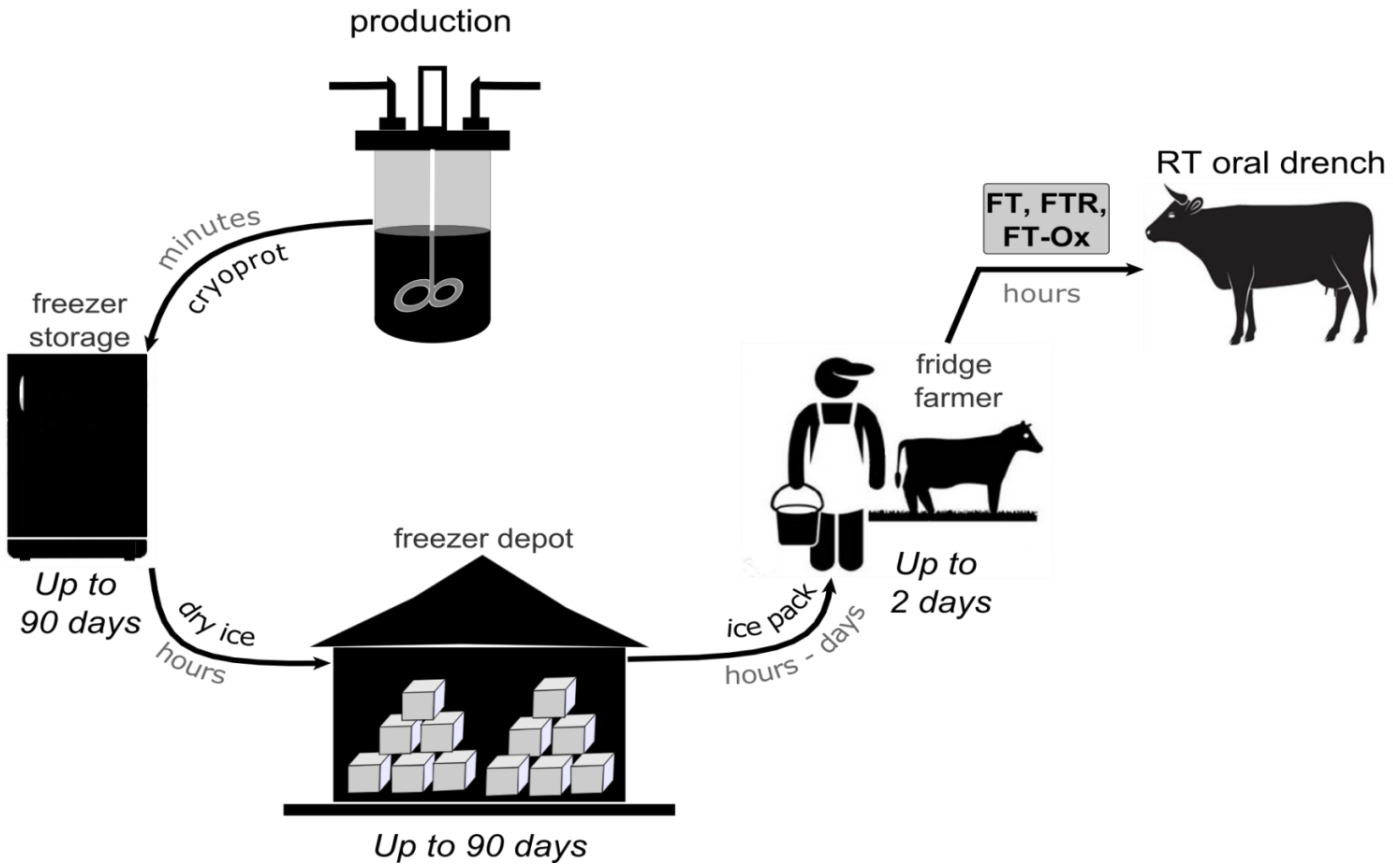

Fig. 1: Schematic diagram of the supply chain between the laboratory *in vitro* production of the mixed bacterial inoculum (production) and the oral drenching of cattle on farm (RT oral drench).The image also reports how the inoculum is refrigerated (ice pack or dry ice) and stored (freezer storage or fridge at the farm) while reaching the end users and the approximate travelling time from one storage to another and then to the farm (hours, minutes, days); various treatments to the inoculum simulating conditions that occur, or are likely to occur, along the supply chain are represented by (CP) Crytoprotectant, (FT) Freeze/thawing, (FTR) Freeze/thawing/refrigeration and (FT-Ox) Freeze/thawing and exposure to oxygen.

4 Results

4.1 Bacterial composition of the fermentor-grown inoculum

4.1.1 Composition during a production run

Twenty-three archival fermentor samples (from 1999 to 2012) were studied together with seven rumen samples (three inoculated with the fermentor-grown inoculum and four feeding Mitchell grass) and Leucaena leaf as a negative control. One fermentor run (Batch 79) was studied from day 2 to day 15.

A total of 1,793,865 raw sequence reads (776.7 Mbp) were generated by amplicon pyrosequencing of the V3-V4 region of the 16S rRNA gene. The percent of reads passing various Phred score filters across the length of reads are shown in **Fig. S 2** (Appendix 9.1). This information was used to determine appropriate settings for quality filtering prior to OTU clustering. After pre-processing, 1,425,733 sequences (85.1 %) were used for cluster-based Operational Taxonomic Unit (OTU) reference picking (minimum identity of 97 %) (**Table 2**). A total of 2,946 OTU's were identified. A total of 1,406,462 reads were mapped back to these OTU's

Table 2 Details of samples, 16S rRNA gene amplicon sequences passing quality filtering and the total number of OTUs assigned for fermentor fluid, rumen fluid and Leucaena leaf samples.

Sample	**No. samples**	**Mapped reads**	**Mean**	**Min**	**Max**	**SD**	**OTU's**
Fermentor	23	731,076	31,786	2,533	21,873	4,772.86	838
Rumen	7	668,695	95,527	18,345	63,692	12,952.17	2,866
Leucaena leaf	2	6,691	3,345	510	1,910	565.32	372
Total	**32**	**1,406,462**	**43,952**				**2,946**

Species richness and diversity in the fermentor-grown inoculum were calculated with three diversity measures; observed (the actual number of different taxa in a sample), Chao 1 (the predicted number of taxa in a sample extrapolated from the number of rare taxa) (Chao, 1984) and ACE (an abundance-based estimator which accounts for rare species and singletons) (Kocherginskaya et al., 2001). Diversity increased in the fermentation from day two of the fermentor run (approx. 200 identifiable OTU's; Chao richness estimator) and plateaued at days 11 -15 (approx. 400 OTU's) (**Fig. 2**).

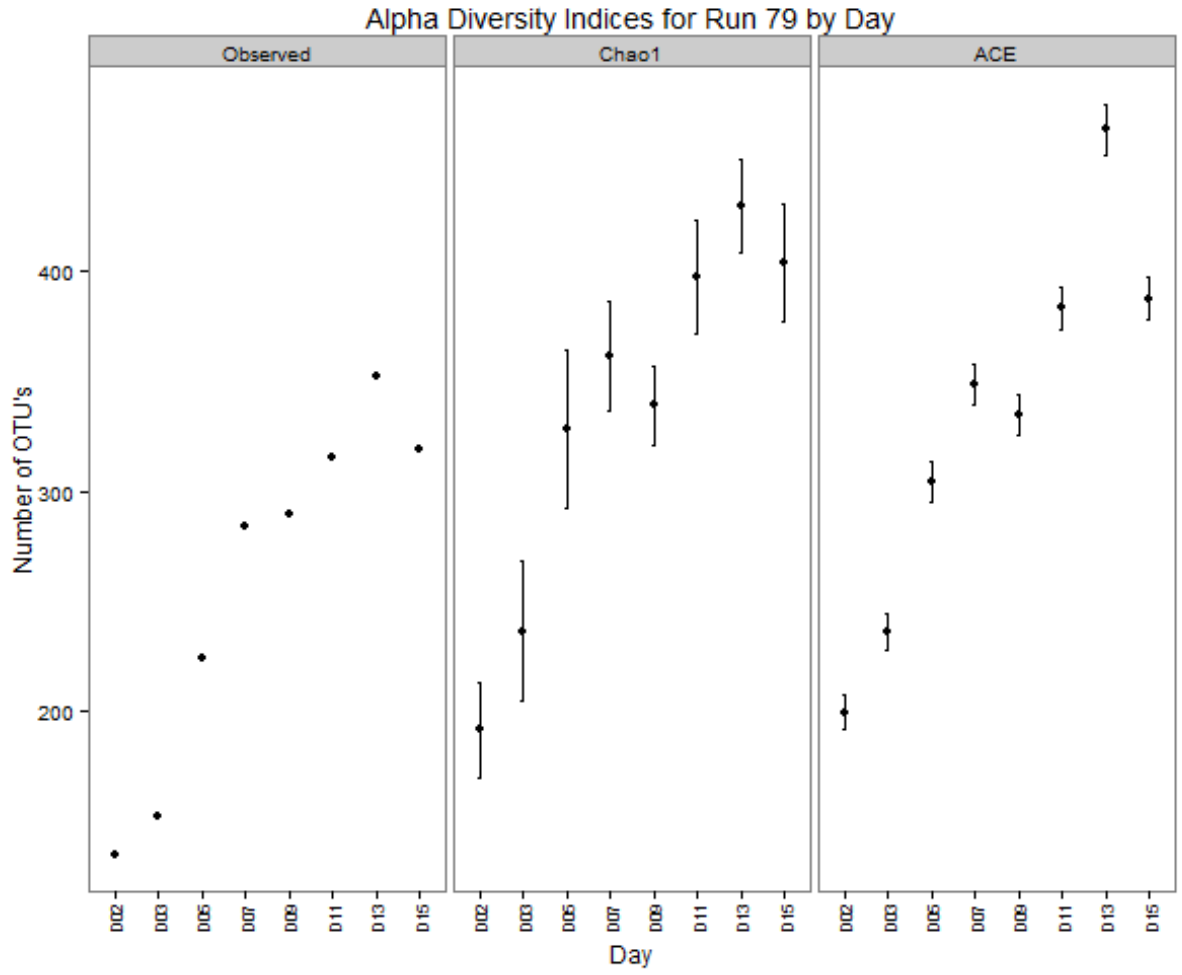

Fig. 2: Alpha diversity (richness) of samples from the Leucaena Inoculum Fermentor Run 79, days 2 to 15 using three diversity measures (Observed, Chao 1 and ACE).

The relative abundance of bacterial phyla during the first 15 days of a typical fermentor production run is shown in **Fig. 3**. Eight bacterial phyla were present in the fermentor-grown inoculum. Firmicutes and Bacteroidetes phyla represented approximately 85 to 90 % of bacterial sequences in the fermentor at any time. Firmicutes predominated the fermentor during the first three days of production, after which, Bacteroidetes rapidly increased until

day nine. Firmicutes and Bacteriodetes were present at similar relative abundances from days 9-15. Proteobacteria declined from day two and stabilised during days 3 to 15 of the fermentor run, while Synergistetes and Tenericutes increased and stabilised.

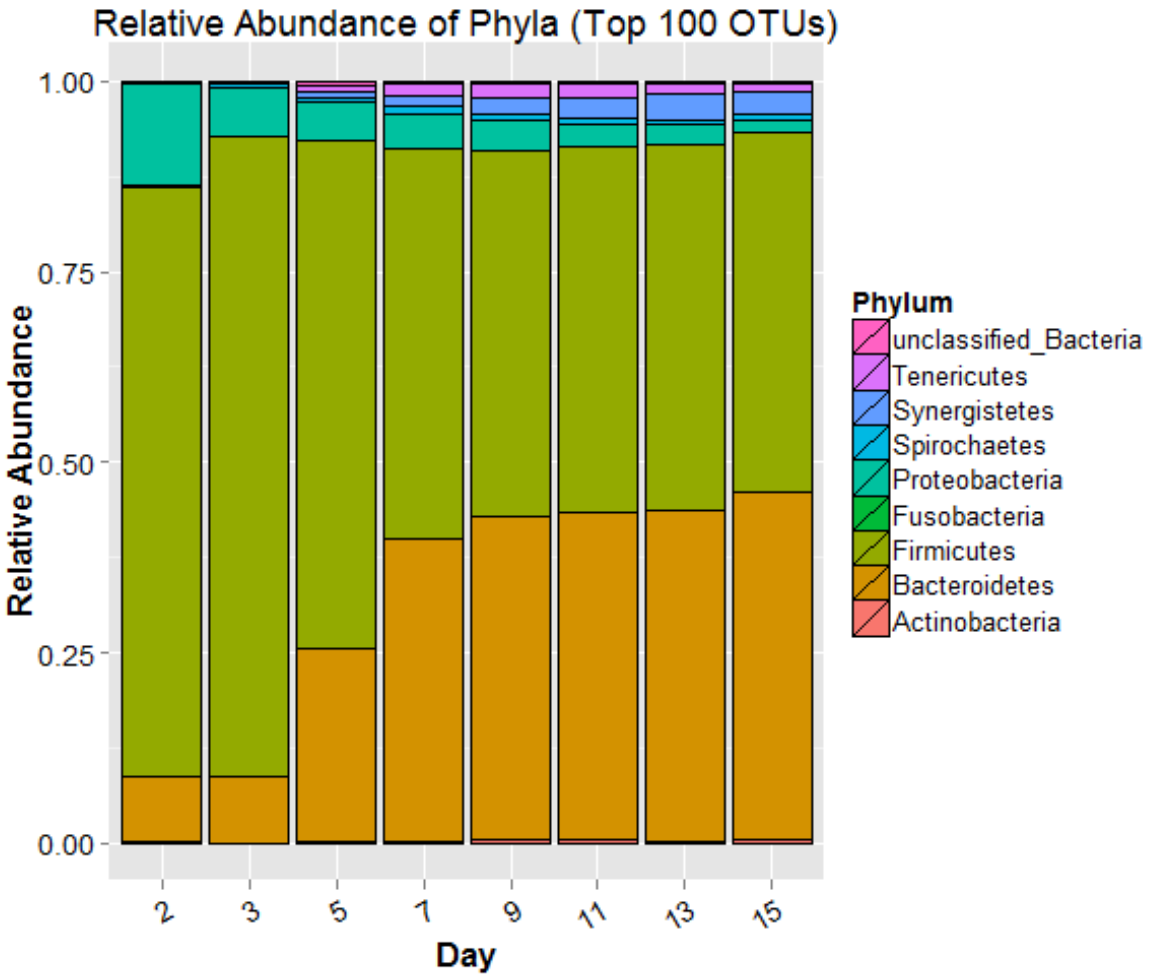

Fig. 3: Relative Abundance of major bacterial phyla from days 2 to 15 of a fermentor run (Run 79), mean of triplicate samples. The 100 most abundant OTUs represent 97.4 % of sequences

A large proportion of the fermentor-grown inoculum is represented at the species level by 10 OTUs (**Fig. 4**). Days two and three of production were dominated (approximately 90 % of sequences) by five OTUs, Selenomonadales (Order), Bacteroidetes (Phylum), *Anaerovibrio* sp., *Schwartzia* sp. and *Basfia* sp. On days five and seven, five other OTUs, *Prevotella sp*., Prevotellaceae (Family), Lachnospiraceae (Family), Bacteroidetes (Phylum) and another Lachnospiraceae (Family) started to increase in relative abundance until day 11 when there were only minor changes until day 15. During the course of fementation all other bacteria increased from representing approximately 15 % of all sequences on day two to over 50 % of all sequences on day 15. *Anaerobvibro* sp., *Basfia* sp., and *Swartzia* sp. were initially the

most abundance bacteria gradually decreased becoming very minor populations of the inoculum by day 15.

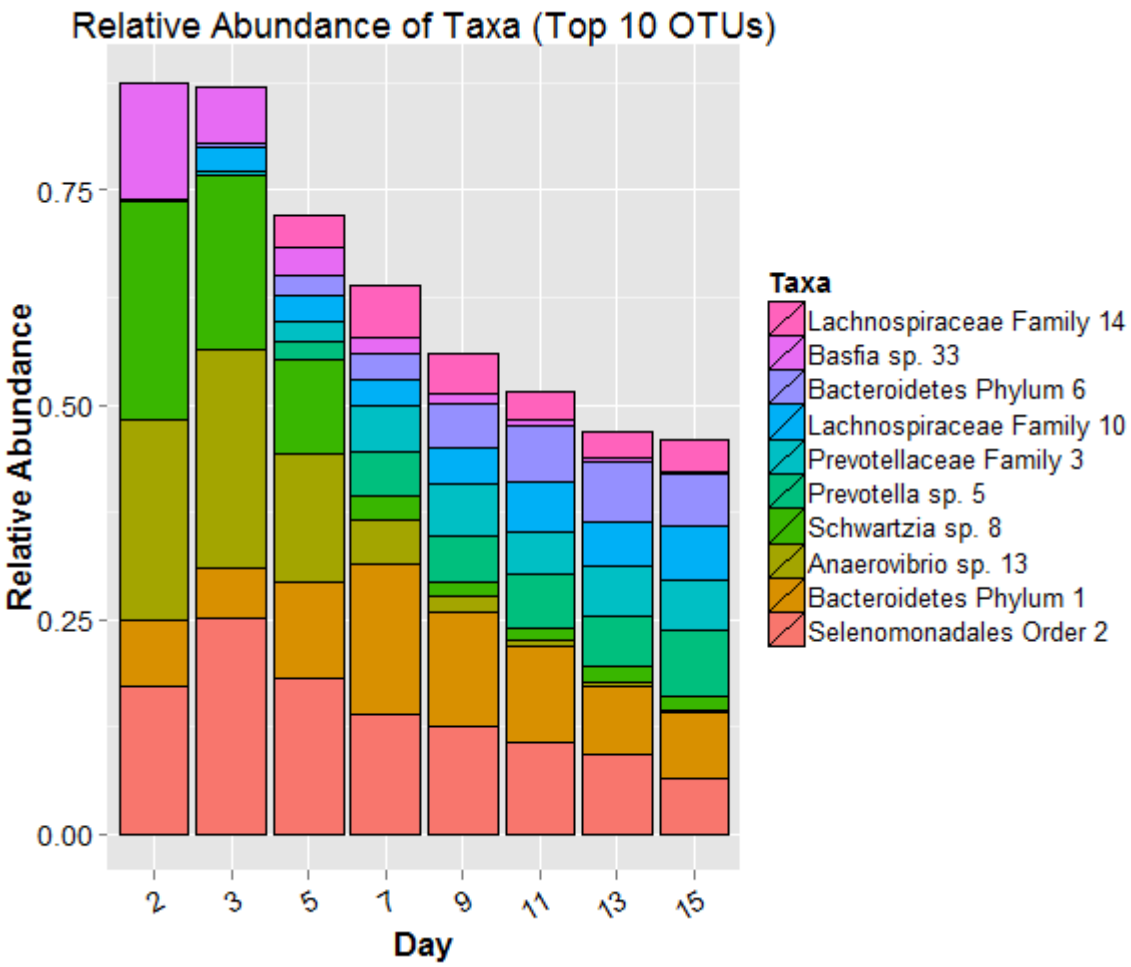

Fig. 4: Relative Abundance of the 10 most abundant OTU's across fermentation run 79 days 2 to 15 and their putative classification (Legend notation is highest level of assigned classification followed by the OTU identifier).

Beta diversity (diversity between samples) of the Leucaena inoculum fermentation, analysed using NMDS ordinations, showed clustering according to the length of time of the fermentation (**Fig. 5**). Unweighted Unifrac distances which account for the presence or absence of OTU's showed distinct clustering of day five through to day 15 of the fermentor run **Fig. 5**B. Replicates from the same biological sample also clustered together. Weighted Unifrac distances which account for OTU abundances showed clustering at day 13 and 15.

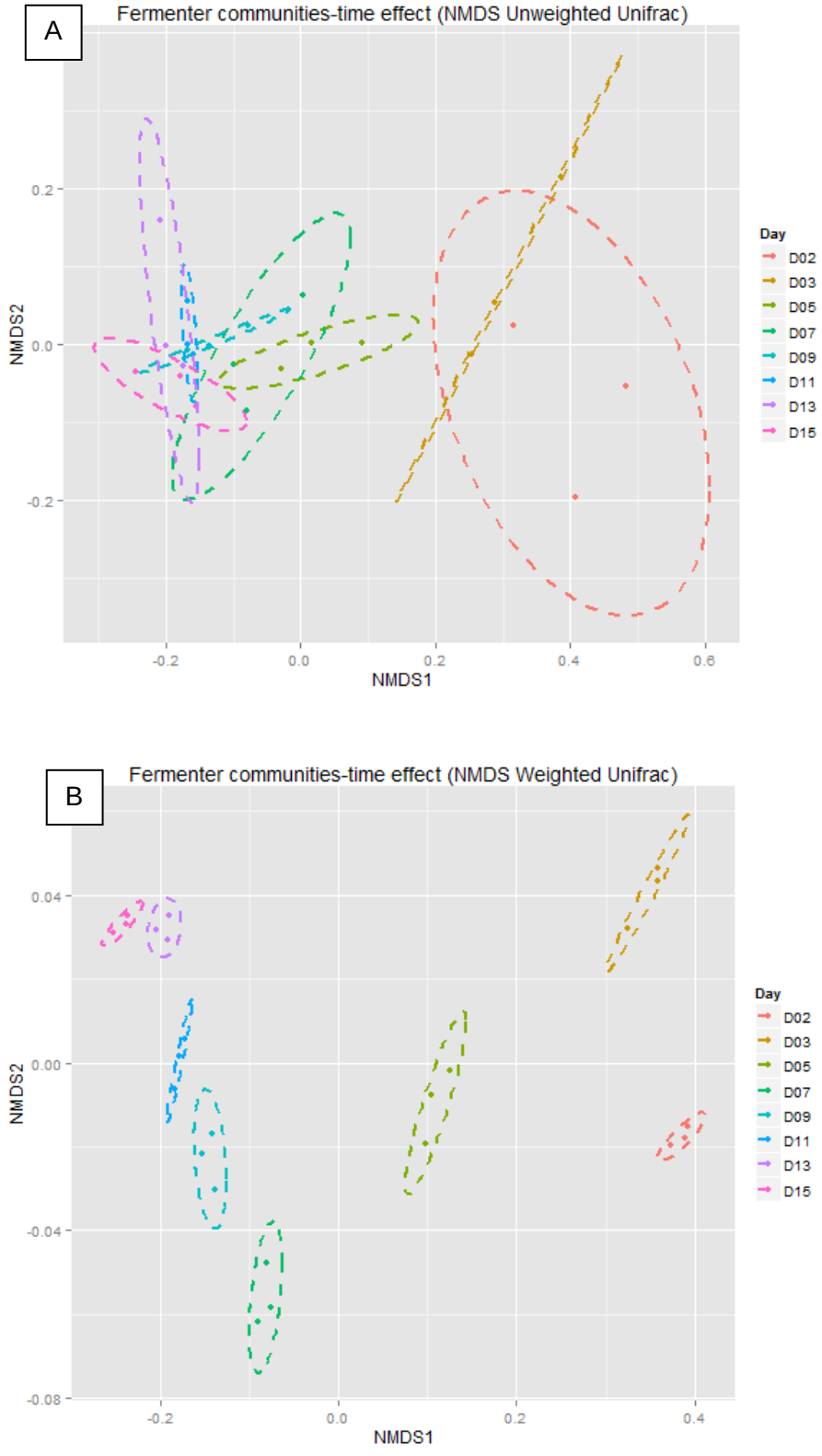

Fig. 5: Beta diversity measures for elucidating the relationships of fermentor microbiota across different days of a single fermentor run (Run 79, days 2 to 15) non-metric multidimensional scaling (NMDS) ordination plots using weighed (A) and unweighted (B) Unifrac distances. Ellipses represent a 95 % confidence level.

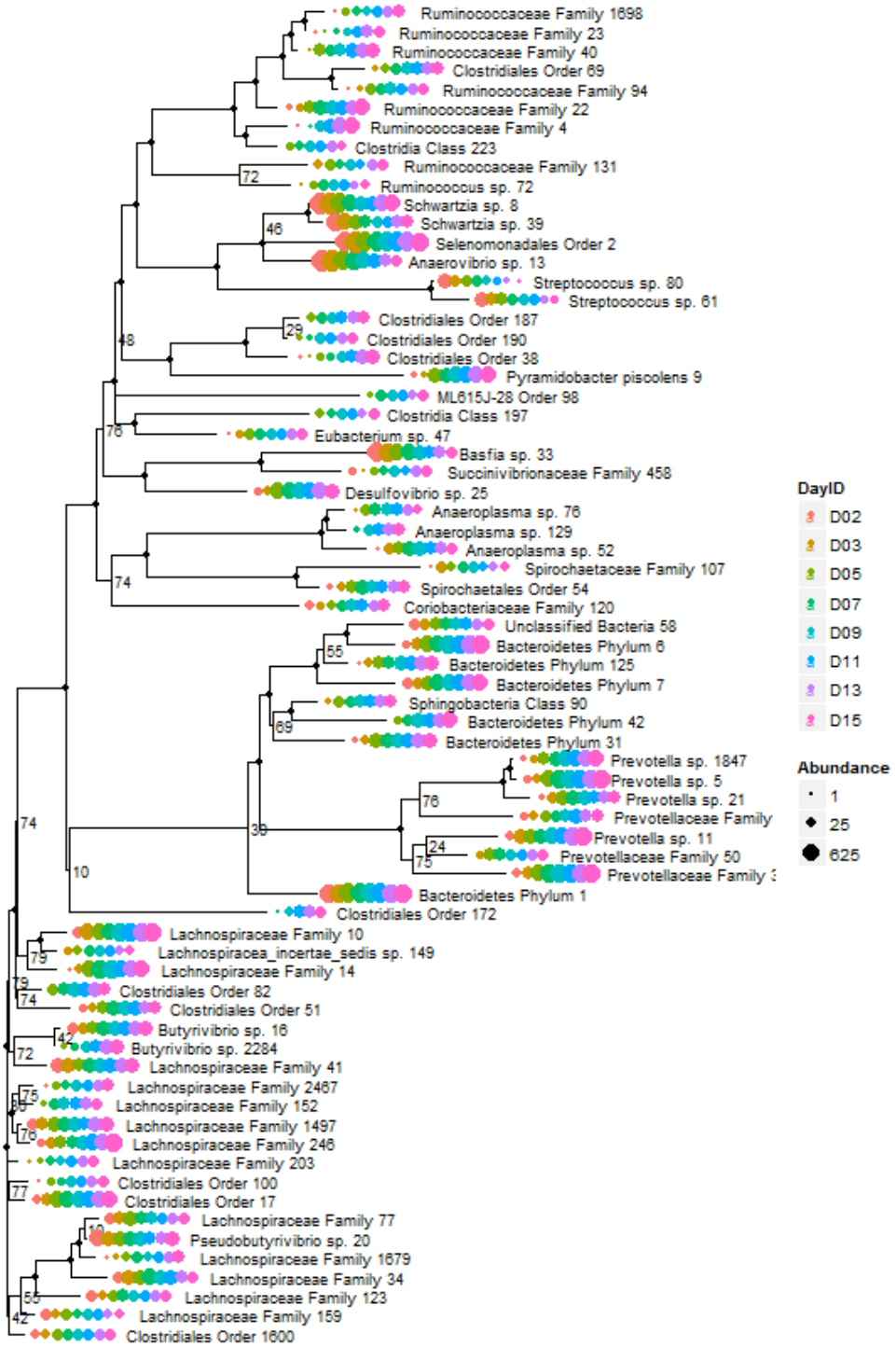

Fig. 6: Phylogenetic tree of the top 70 most abundant taxa of fermentor Run 79 showing the standardised abundance of taxa from day 2 to 15 of fermentor run 79. Tip labels represent taxonomic classification followed by the OTU ID. Bootstrap values greater than 80 are shown as solid black dot on branches. Other values are as indicated.

Fig. 6 shows a phylogenetic tree of the top 70 OTU's (for clarity of presentation) and their standardised abundance throughout the course of a typical fermentor run (Run 79). Two OTUs, Selenomonadales Order (OTU 2) and Bacteroidetes Phylum (OTU 1), were present in the fermentor at high abundance throughout the run and a number of other OTU's also maintained their abundance at lower abundance levels across the fermentor run. Many OTU's increased over the course of the fermentor run from an initial low abundance, like the Ruminoccaceae Family OTU's 23, 40 and 1698. Other OTU's were initially present at high abundance and decreased across the fermentation, *Anaerovibrio* sp. (OTU 13) and *Basfia* sp. (OTU 33).

The most abundant Synergistetes phylum bacteria in the fermentor were *Pyramidobacter* sp. (OTU 9) followed by *Synergistes jonesii* (OTU 60) and three other unclassified bacteria, OTU 145, OTU 419 and OTU 438 (**Fig. 7**). Each of these was found throughout the course of production, while other bacteria were not consistently observed in multiple days of the fermentor run.

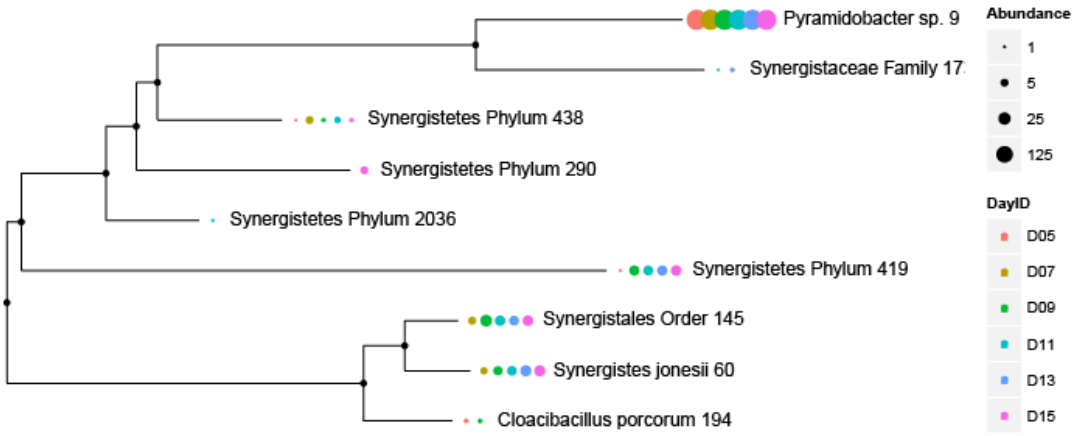

Fig. 7: Phylogenetic tree of the Synergistetes phylum with quantitative measures of abundance on different days (DayID).

4.1.2 Composition between different batches

Species richness, determined using Chao1 and ACE richness estimators, of various batches of the Leucaena inoculum from 1999 to 2012 sampled mostly on day 15 of the fermentor run, had between 350 and 450 OTU's (**Fig. 8**). Also shown is the actual observed number of species which doesn't take into account further species that could be found with further sequencing.

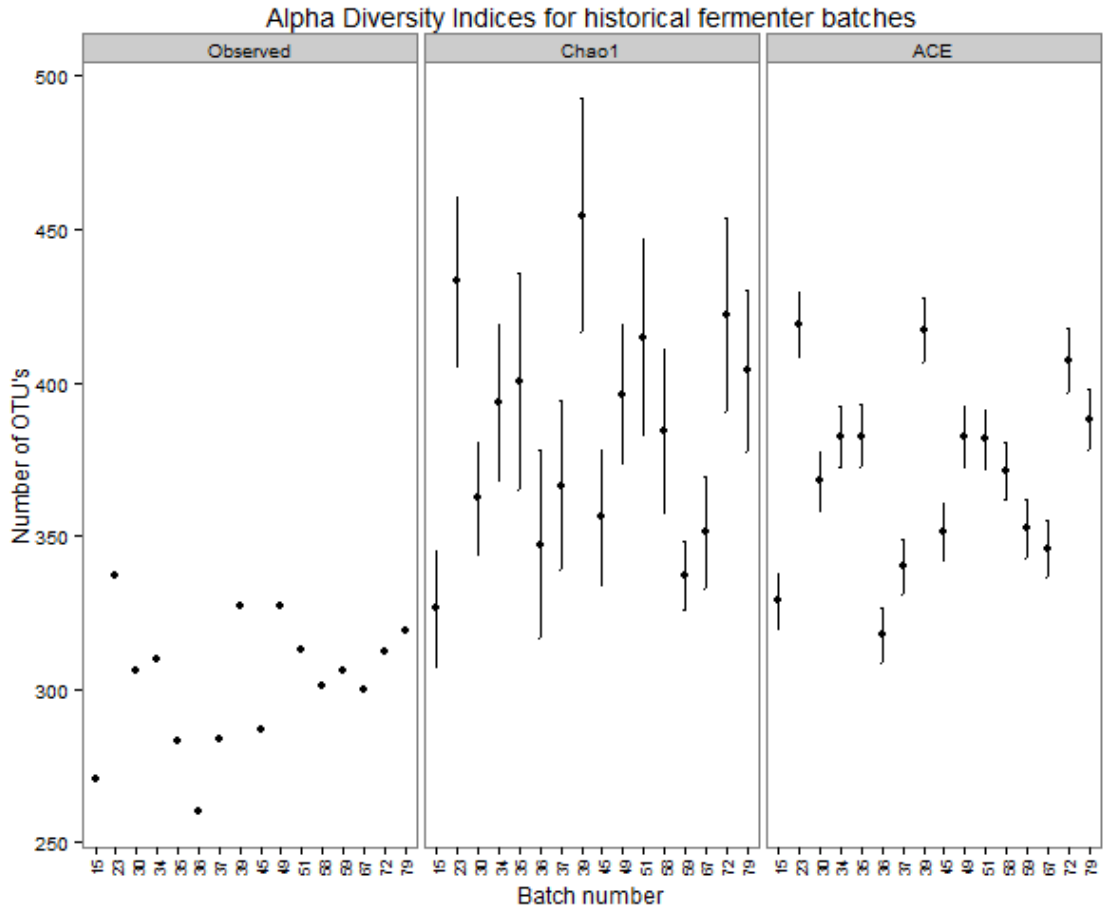

Fig. 8: Alpha diversity (richness) of samples from the Leucaena inoculum using three diversity measures (Observed, Chao 1 and ACE) of different batches of the fermentor inoculum produced between1999-2012

To assess phylum-level changes in the fermentor inoculum between fermentor runs, a subset of the 100 most abundant OTU's was extracted representing 95.4 % of all sequences in the entire fermentor batch dataset (**Fig. 9**). The 100 most abundant OTU's contained seven phyla and all fermentations were dominated by Bacteriodetes and Firmicutes phyla with smaller proportions of Proteobacteria and Synergistetes. Tenericutes, Fusobacteria and Spirochaete phyla represented only a very small proportion of fermentor bacteria.

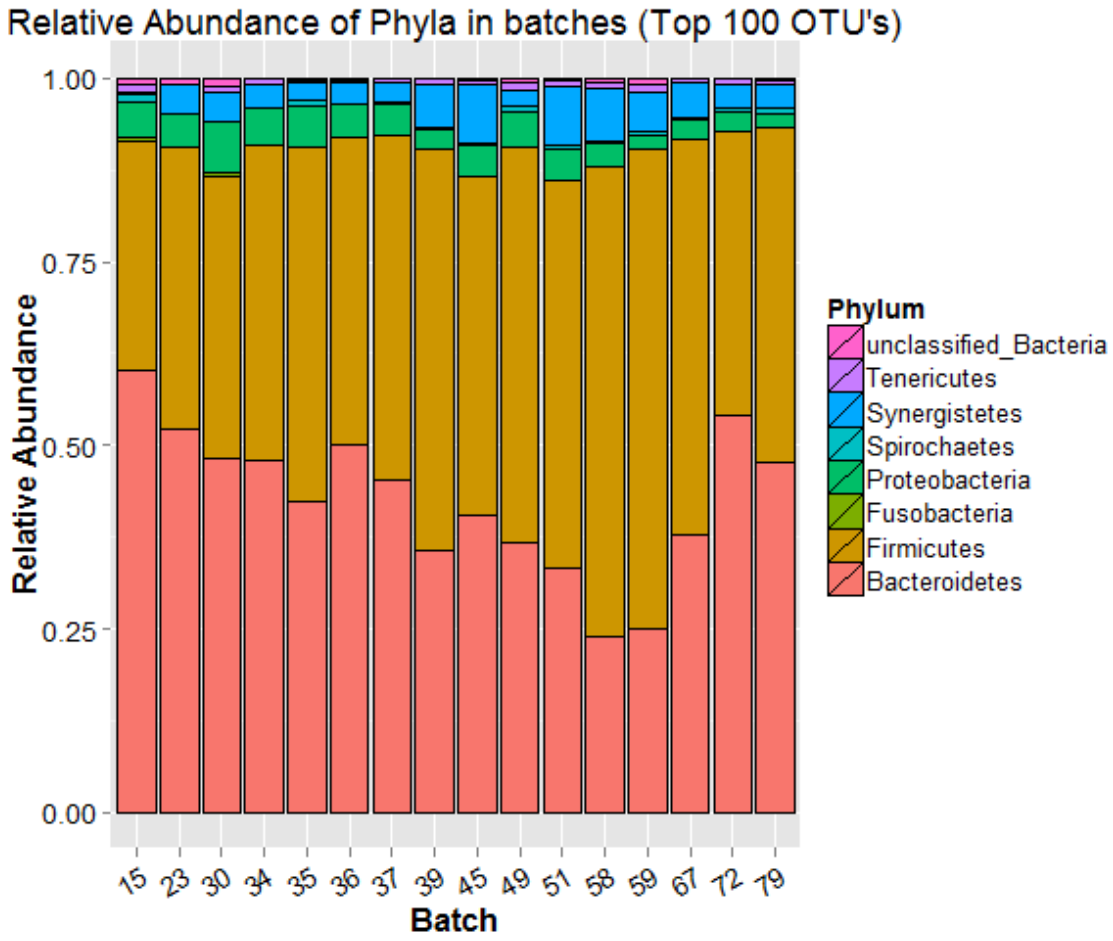

Fig. 9: Relative abundance of Phylum level taxa of the 100 most abundant OTU's across different fermentor runs sampled at day 15 (or 9 for batch 15, 12 for batch 30).

The 10 most abundant OTU's, which represented 63.4 % of sequences in the entire fermentor batch dataset, were consistently found across most fermentor batches, with the exception being batch 15 (taken on day nine) and batch 30 (taken on day 12) (**Fig. 10**). Four fermentor runs (batches 34 to 37) had high abundances of an unclassified Bacteriodes phylum (OTU 1) and an unclassified Selemonodales order (OTU 2). There were also clear absences or decreases of Prevotellaceae family (OTU 3) and Ruminococcaceae family (OTU 4). These fermentor runs were 1.5 L fermentations produced in a 3 L base-heated fermentation vessel. However, when production moved to the 3 L fermentation run, in an Infors 7 L water-jacketed fermentor system at batch 45, the abundance of the unclassified Bacteroidetes phylum bacteria (OTU1) and the abundance of all 10 OTUs decreased, representing approximately 50 to 55 % of sequences. The 16S rRNA gene sequences corresponding to *S. jonesii* at day 15 of the fermentor batches represented a maximum of 1.07 % (mean 0.28 % ± 0.04, n =42) of sequences in any sample.

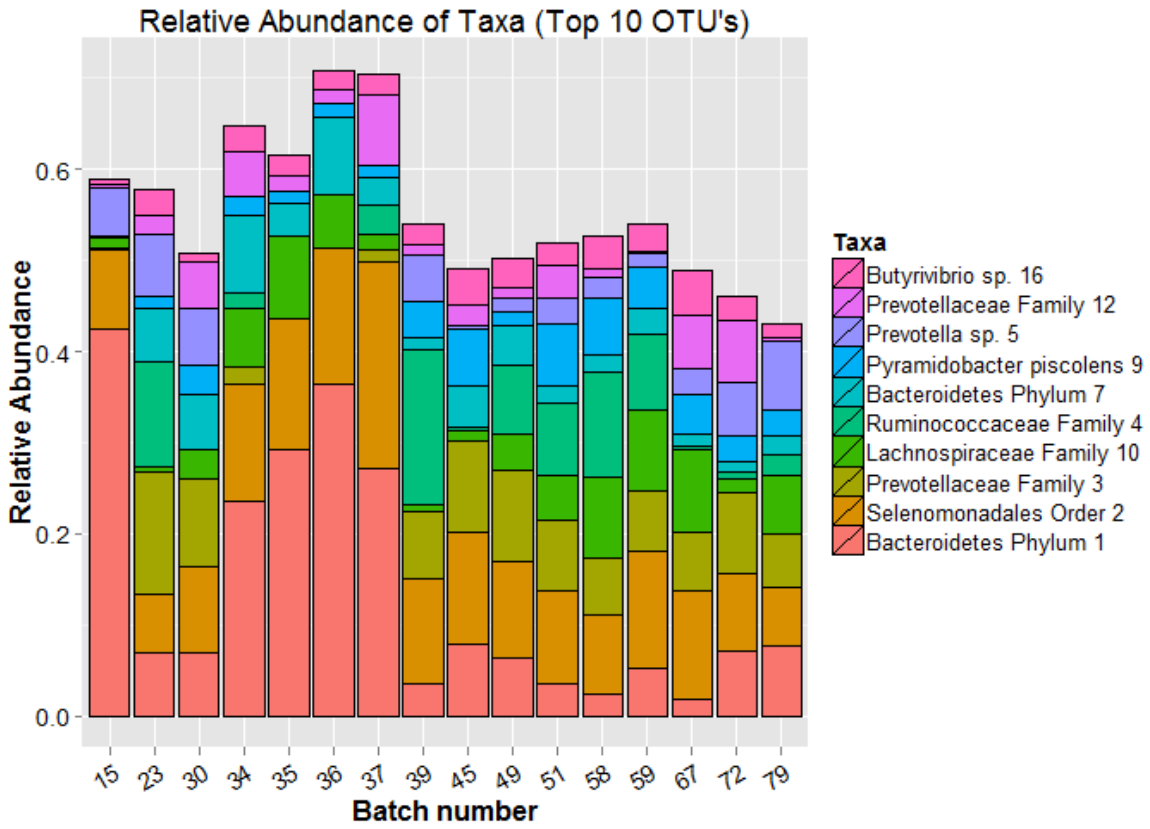

Fig. 10: Relative abundance of the 10 most abundant OTU's present in day 15 of different fermentation runs (batches) from 1999 to 2012 classified at species level (97 % sequence similarity).

Results of NMDS ordinations of the unweighted Unifrac distances indicated that bacterial communities clustered in respect to different starter cultures as shown by 95 % confidence ellipses (**Fig. 11**A). All bacterial communities from different starter cultures showed some overlap with passage 5 indicating the presence of common bacterial communities. Two main clusters from starter cultures 5 and 6 (n = 7 and n = 6 respectively), had the most overlap indicating similar bacterial communities present in the fermentation runs. Weighted Unifrac also showed overlap between fermentations using starters from passage 5 and 6 indicating phylogenetic similarity between the abundance of bacterial communities (**Fig. 11**B). Analysis of similarity (ANOSIM) between passage 5 and 6 with the unweighted and weighted datasets gave R statistics of 0. 3876 and 0.3403 (P = 0.001), respectively, which indicated small albeit significant differences due to passage. Similarly, the effect of the type of fermentor system gave R statistics of 0.645 and 0.308 (P = 0.001), respectively, indicating small but significance changes in bacterial composition due to change in apparatus.

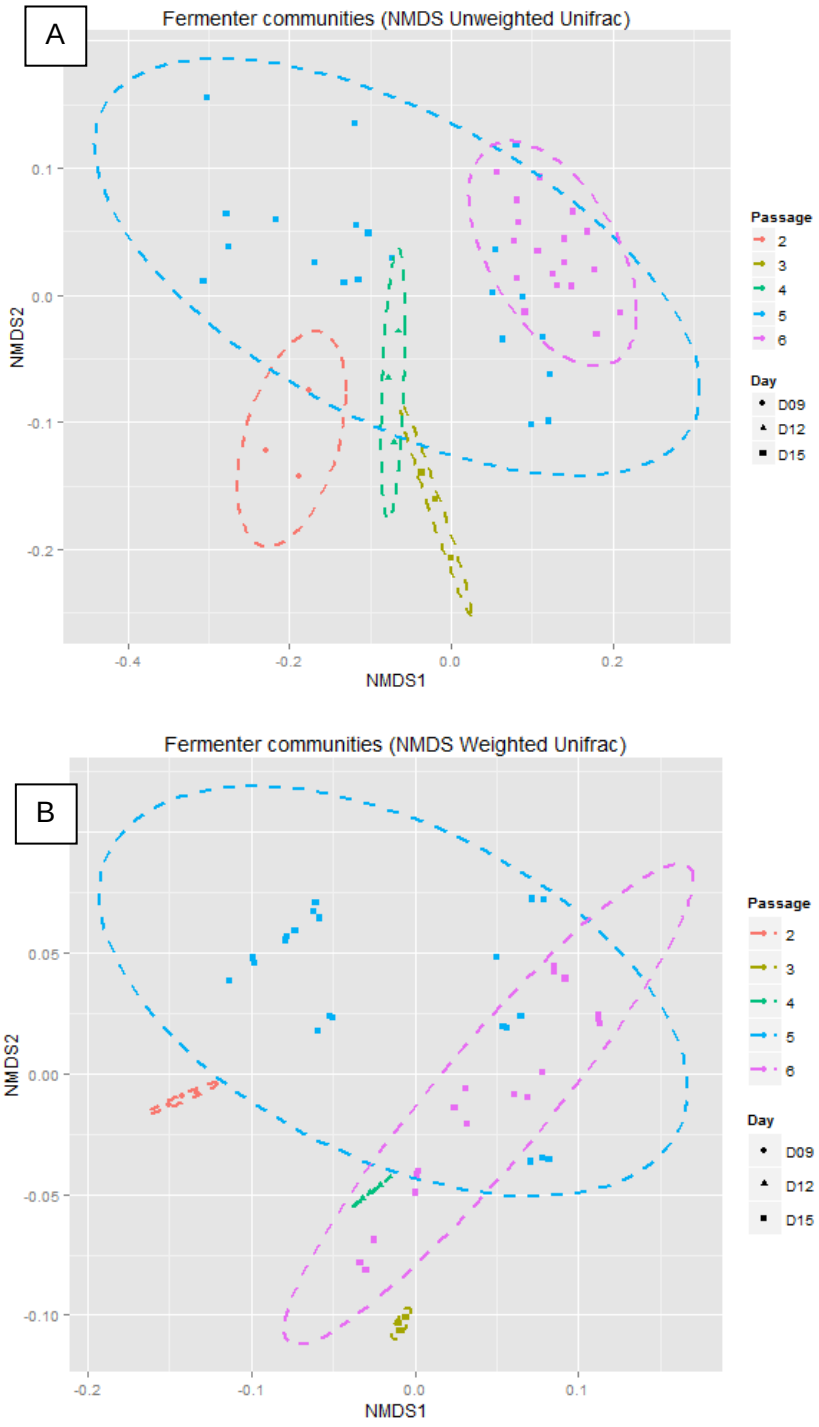

Fig. 11: Multidimensional reduction analysis for elucidating the relationships of fermentor microbiota across different batches from starter cultures obtained from previous fermentation batches used to investigate the effect of passage on bacterial community structure. A. Unweighted Unifrac distance matrix. B. Weighted Unifrac distance matrix.

4.2 Characterisation of *S. jonesii* in the fermentor-grown inoculum

4.2.1 Growth and DHP degradation by five 'strains' of *S. jonesii*

Pure cultures of representative strains of *S. jonesii* (ATCC 49833, 78-1, 100-6, 113-4 and 147-1) completely degraded both 2,3-DHP and 3,4-DHP to completion (**Fig. 12**). Most strains showed bi-phasic degradation; degrading 3,4-DHP before 2,3-DHP resulting in an increase in the 2,3 isomer as 3,4-DHP decreased. Small differences were seen in the cell densities of cultures measured as maximum overall OD_{600}, which ranged from approx. 0.6 to 0.8, when grown under optimal growth conditions.

Cell morphology was consistent between all strains which displayed slightly elongated cocci, forming pairs and sometimes chains. Arginine and histidine added to the media improved overall growth yields. Cells required a source of protein for growth. Growth yields dramatically increased when mixed proteins sources (peptone, tryptone, phytone, and yeast extract) were supplied into growth media as opposed to any single protein source alone. Rumen fluid was essential for good growth when reviving cultures from stationary phase or cryopreservation. Rumen fluid did not affect the growth yield or rate of continuously sub-cultured, actively growing cells. Suboptimal growth conditions such as those of cultures grown on published media greatly reduced growth rates and yields and and highly pleomorphic cells displaying irregular sizes and shapes which sometimes appeared elongated or filamentous. Greatly diminished or temporary loss of DHP-degrading activity was seen under suboptimal growth conditions. Cultures grown without DHP in media for undefined periods of time appeared to lose DHP-degrading activity, however, activity returned with further extended culturing. DHP degradation could occur at any time from early log-phase to extended stationary phase, but frequently occurred in early log phase when cultures were continuously grown under optimal conditions.

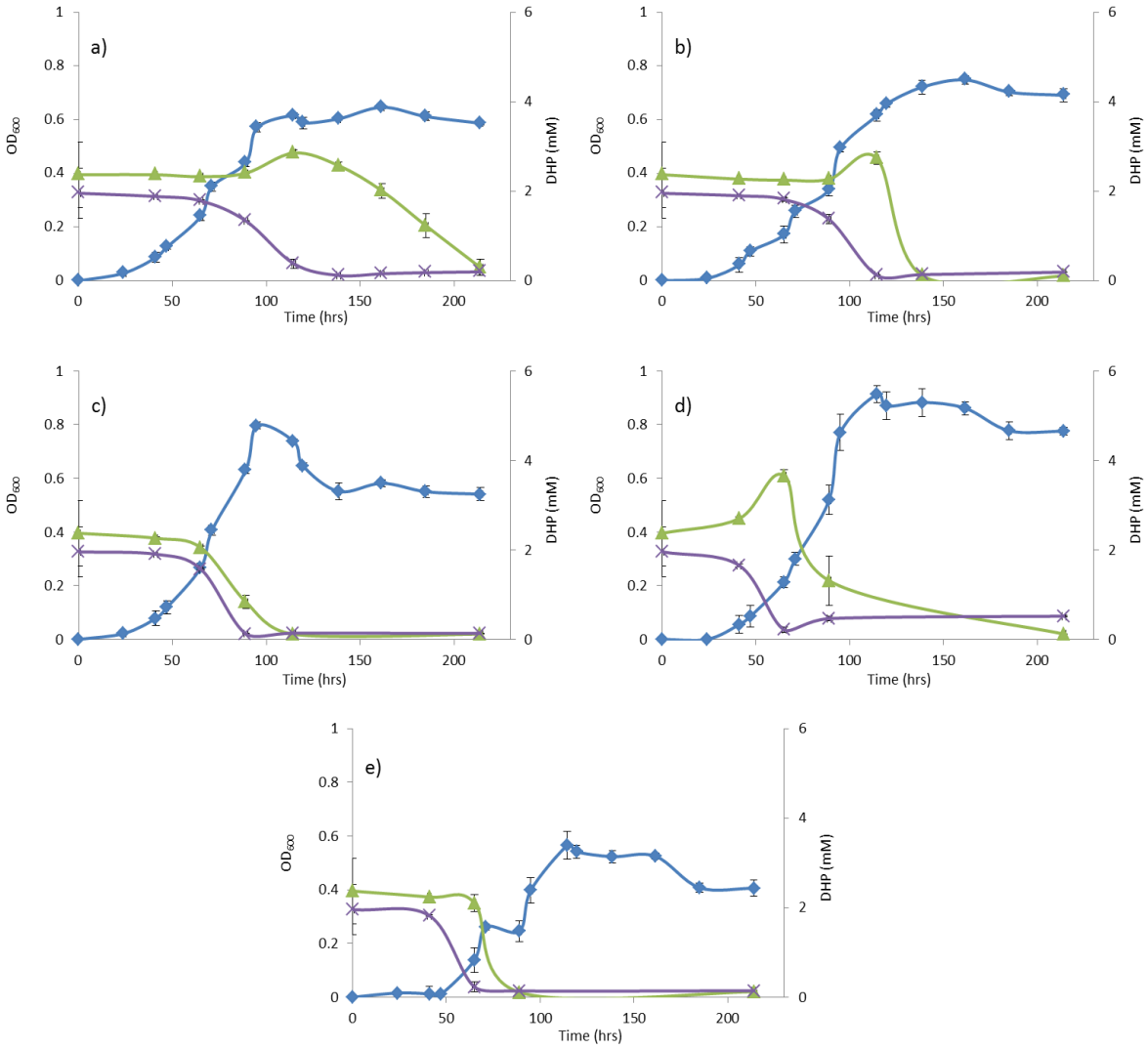

Fig. 12: Growth and DHP degradation profiles for *S. jonesii* reference strains. Growth at OD_{600} (blue diamonds), concentration of 2,3-DHP (green triangles) and 3,4-DHP (purple crosses). *S. jonesii* strains studied were (a) ATCC 49833, (b) 78-1, (c) 100-6, (d) 113-4 and (e) 147-1 as indicated on the top left of each graph. Error bars represent SD of triplicates.

4.2.2 Quantification of *S. jonesii* in the fermentor inoculum

A real-time RT-q PCR assay was developed to improve the sensitivity of our current qPCR assay for the quantification of *S. jonesii* within the fermentor inoculum and rumen fluid. The assay is approximately 1000 times more sensitive than the previous DNA-based assay. RT-qPCR targeting 16S rRNA correlated well with the results of the qPCR targeting the 16S rRNA gene (DNA) (Pearson correlation coefficient p=0.94 for log transformed data). Within a single fermentation run the numbers of *S. jonesii* increased rapidly for the first week of the fermentation and stabilised after approximately eight days. The number of *S. jonesii* cells as determined by the qPCR for different production batches from 1999 through to 2013 is shown in **Fig. 13**. With the exception of a very early batch produced in 1999 when the

fermentation was only run for nine days, the numbers of *S. jonesii* cells present in the *in vitro* fermentation system by the end of an inoculum production run have consistently been greater than 1 x 10^6 cells per mL (**Fig. 14**).

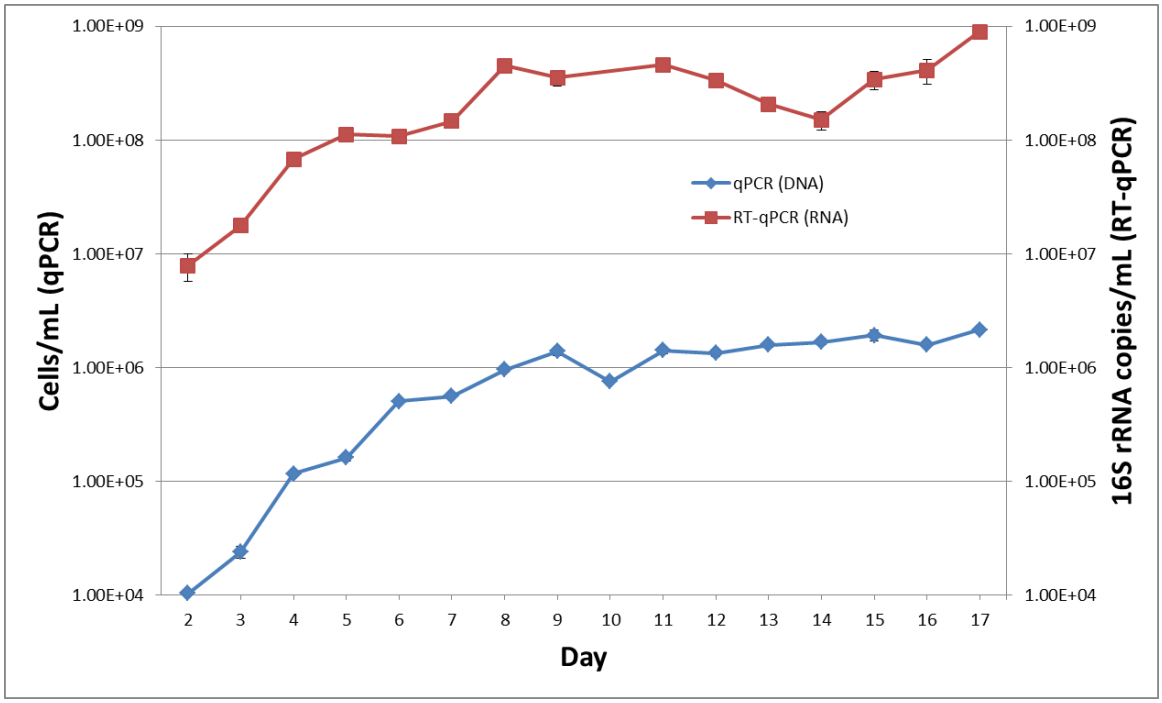

Fig. 13: Cell numbers and ribosomal RNA copy numbers of *S. jonesii* sampled daily in a fermentation run used to produce the Leucaena inoculum.

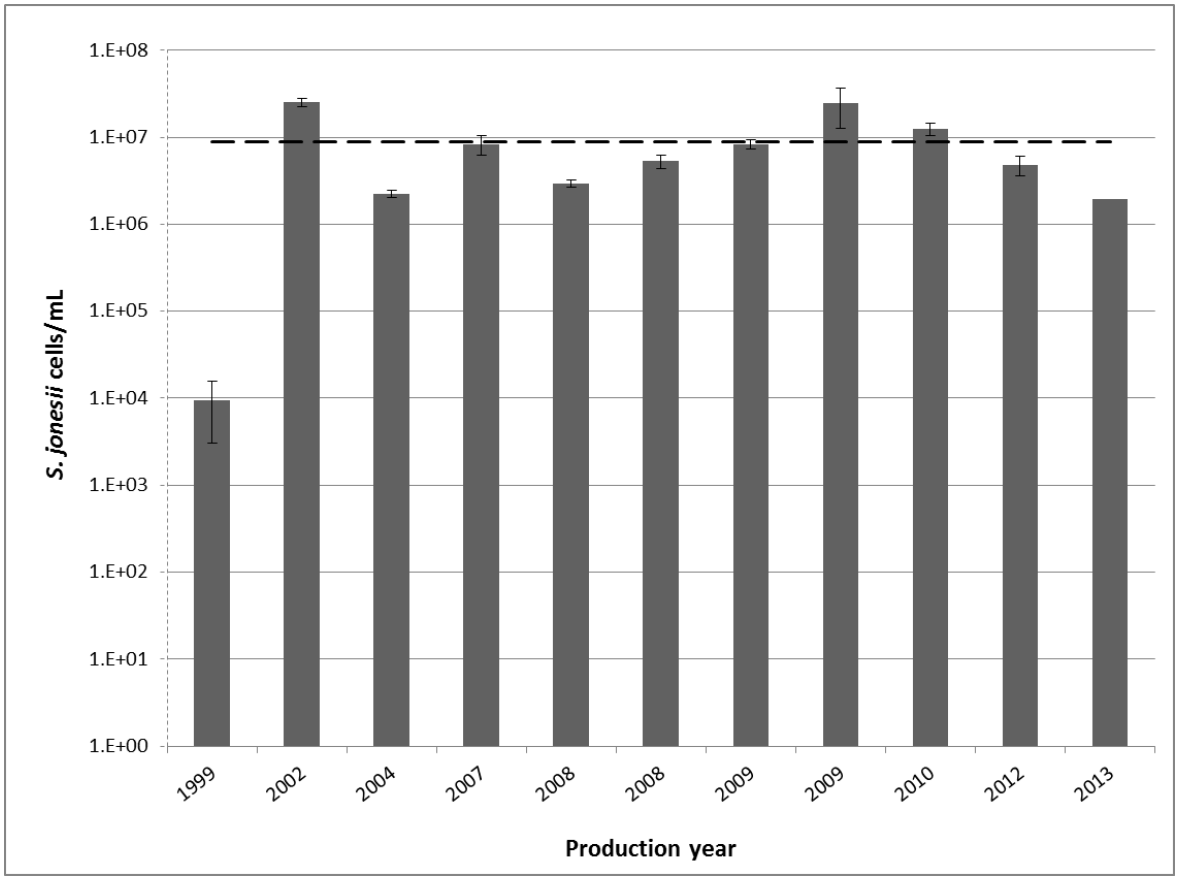

Fig. 14: *S. jonesii* cell numbers, determined by qPCR, present on the last day of selected fermentations used to produce individual batches of inoculum between 1999 and 2013. Error bars represent standard error of triplicate samples. Dashed line represents average cell/mL.

4.2.3 *S. jonesii* strains present in fermentor-grown inoculum

Historical fermentor samples analysed using a *S. jonesii*-specific DGGE showed one specific band which did not correspond to the band from *S. jonesii* 78-1. Sequencing of the band confirmed it as *S. jonesii*. The presence of this single band indicated that only one strain of *S. jonesii* has been present in the fermentor over its 20-year course of production. The DGGE profiles of various batches of the Leucaena inoculum, spanning a nine year period from 1999 - 2008, are shown in Gel A (**Fig. 15**A). No changes were seen following an upgrade of the production apparatus and increase in fermentation volume to 3 L in 2005 or when the fermentor was inoculated from different starter cultures. Also, no changes were seen during a single fermentor run (**Fig. 15**B). Interestingly, the fermentor DGGE amplicon (which is approx. 300 bp) differs by one base pair (nucleotide 306 G -> A, of the *E.coli* reference position) from that of the *S. jonesii* reference stains which are 100 % identical across this region.

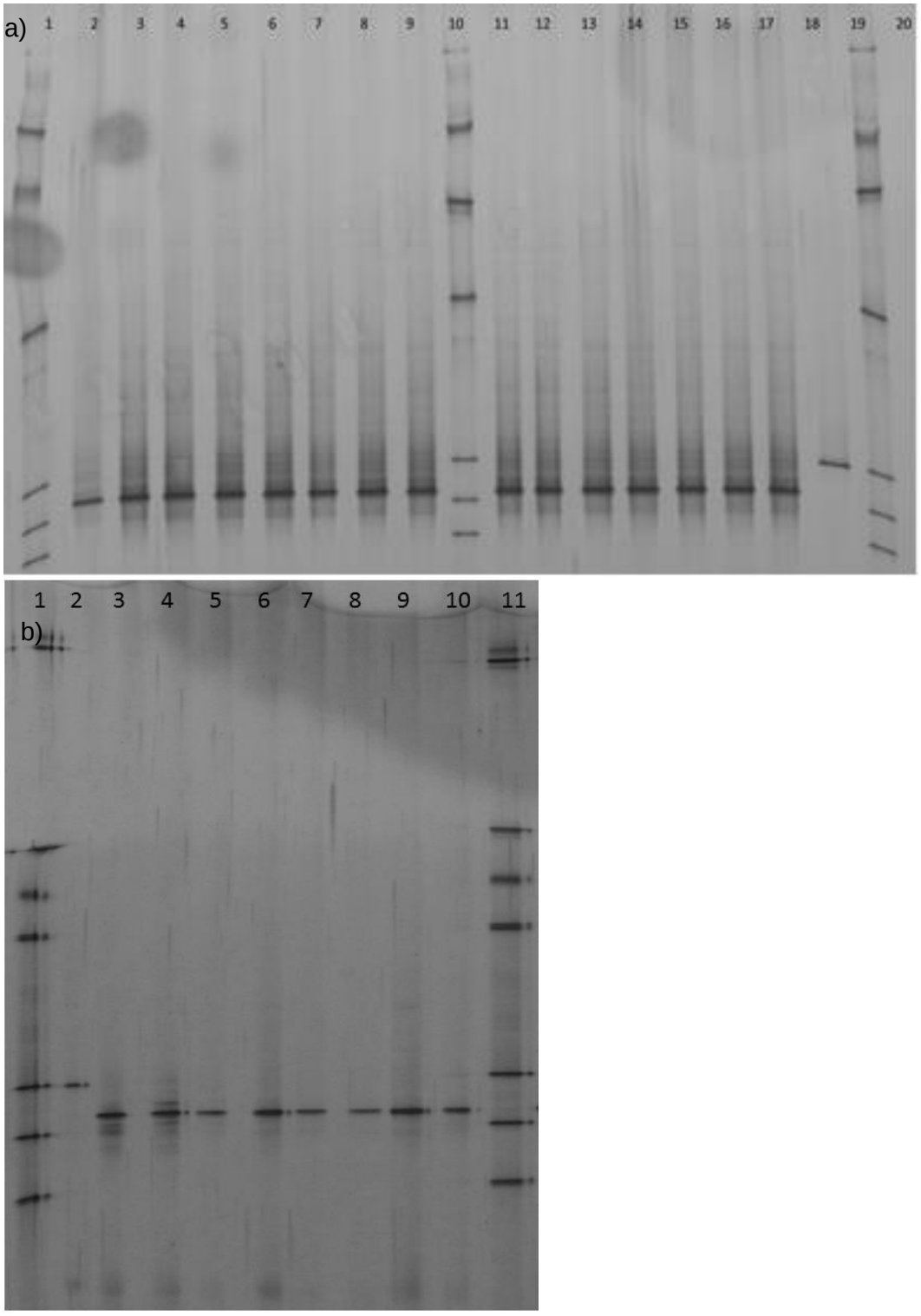

Fig. 15: Taxon-specific DGGE gels comparing the *S. jonesii* 16S rRNA sequences from archival fermentor production runs (A) and across a fermentor run (B). **A**: Day 15 fermentor fluid from fermentor run #15 (1999) through to fermentor run #51 (2008) (Lanes 2-9, 11-17), *S. jonesii* 78-1 (Lane 18), DNA ladder (Lanes 1, 10, 19), no template control (NTC) (Lane 20); **B**: *S. jonesii* 78-1 (Lane 2), Fermentor Run days 2, 3, 5, 7, 9, 11, 13, 15 (Lanes 3-10).

The *S. jonesii*-specific DGGE PCR assay was used on a range of samples and the *S. jonesii* identities of the DGGE band amplicons were confirmed by sequencing. Rumen fluid

obtained from University of New England (Armidale) steers (n = 2) possessed a *S. jonesii* strain that differed by two base pairs to the reference strain *S. jonesii* 78-1, two sheep from Gatton were the same as the fermentor strain whilst the DGGE band from a third sheep differed by one base pair (nucleotide 273 C -> T). Cattle (n = 3) inoculated with the Leucaena inoculum and feeding on Leucaena (Brian Pastures, Gayndah) showed a single band identical to that of *S. jonesii* strain present in the Leucaena inoculum (**Fig. 16**).

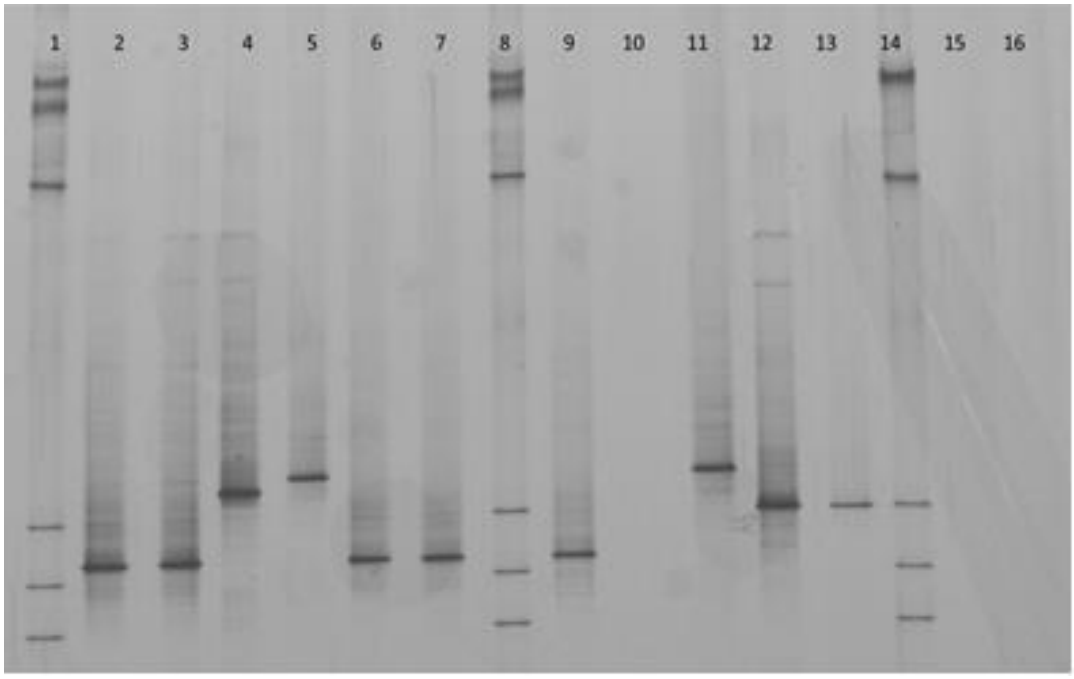

Fig. 16: *S. jonesii*-specific DGGE gels showing the banding pattern from various ruminants: Sheep, University of Queensland, Gatton (Lanes 2, 3, 4), Steer, University of New England, Armidale (Lane 5), Leucaena-fed cattle, Brian Pastures Research Station, Gayndah (Lanes 6, 7, 9), Steer, University of New England, Armidale (Lane 11), *S. jonesii* 78-1 (Lanes 12, 13), DNA ladder (Lanes 1, 8, 14), NTC (Lanes 15-16).

4.2.4 Genome comparison between *S. jonesii* strains 100-6, 113-4 and 147-1

The genomes of strains 100-6, 113-4 and 147-1 isolated by Allison (1992) have been sequenced to allow inter-strain variation to be characterised. Genomes sizes varied between the three strains (**Table 3**). A large episome ranging in size from 165 - 221 Kbp, possibly originating from a bacteriophage, was also identified . Comparative analysis of these genomes indicates that the major variations between the strains are due to transient elements such as phage sequences, mobile genetic elements and duplications and deletions of some of these elements (**Table S 2**, Appendix 9.1). In total, 13 single nucleotide polymorphisms (SNP's) were observed across the three genomes. However these may be due to sequencing error (calculated to be 26 nucleotides per genome based on PacBio sequencing accuracy of 99.999 %). Nonetheless, all three strains appear to have almost no true genomic variation. Alignments of the genomes of representative cultures 100-6, 113-4 and 147-1 are shown in **Fig. S 3** (Appendix 9.1).

Table 3 Genome and putative episome sizes found in *S. jonesii* reference strains

Strain	Genome Size (bp)	Putative episome Size (bp)	Relative Genome Size (bp)
100-6	2,705,803	221,850	2,483,953
113-4	2,705,788	221,850	2,483,938
147-1	2,637,532	165,828	2,471,704

4.2.5 Isolation of *S. jonesii* and other Synergistetes Phylum bacteria from the fermentor-grown inoculum

Plating of a rumen-derived fermentor inoculum directly onto selective medium SS-4, under anaerobic conditions, led to the exclusive isolation of novel strains representing three Synergistetes taxa. These included a new strain of *Synergistes jonesii*, termed strain YE330, a *Cloacibacillus* sp. and a potential new species of the *Pyramidobacter* genus. Altering the antimicrobial content in media SS-4 and the dilution of the inoculum before plating allowed for the isolation of *S. jonesii* colonies on single growth plate without contaminating species. *S. jonesii* str. YE330 had a partial 16S rRNA gene sequence identity of 99.7 % to that of *S. jonesii* str. 78-1 (four base pairs were different over the 1,389 bp that were sequenced). This new isolate represents the first strain of *S. jonesii* since the initial isolation over 20 years

ago. Sequence comparison between this isolate and the one amplified from the fermentor and analysed by DGGE were identical.

Other bacteria from the Synergistetes phylum isolated from the fermentor inoculum include a new strain of *Cloacibacillus porcorum* (99.4 % similarity, approx. 1,400 bp). This species included the bacterium Synergistetes str. MFA1 (99.4 % similarity), which has been studied for its ability to degrade another plant toxin, monofluoroacetate (Davis et al., 2012). The third Synergistetes phylum bacterium isolated from the inoculum showed 16S rRNA gene similarity to the *Pyramidobacter* genus (97.2 % similarity, approx. 1,400 bp). This represents the first bacterium within the *Pyramidobacter* genus to be isolated from a ruminant-derived ecosystem. The phylogenetic tree including the three organisms reported above is presented in **Fig. 17**.

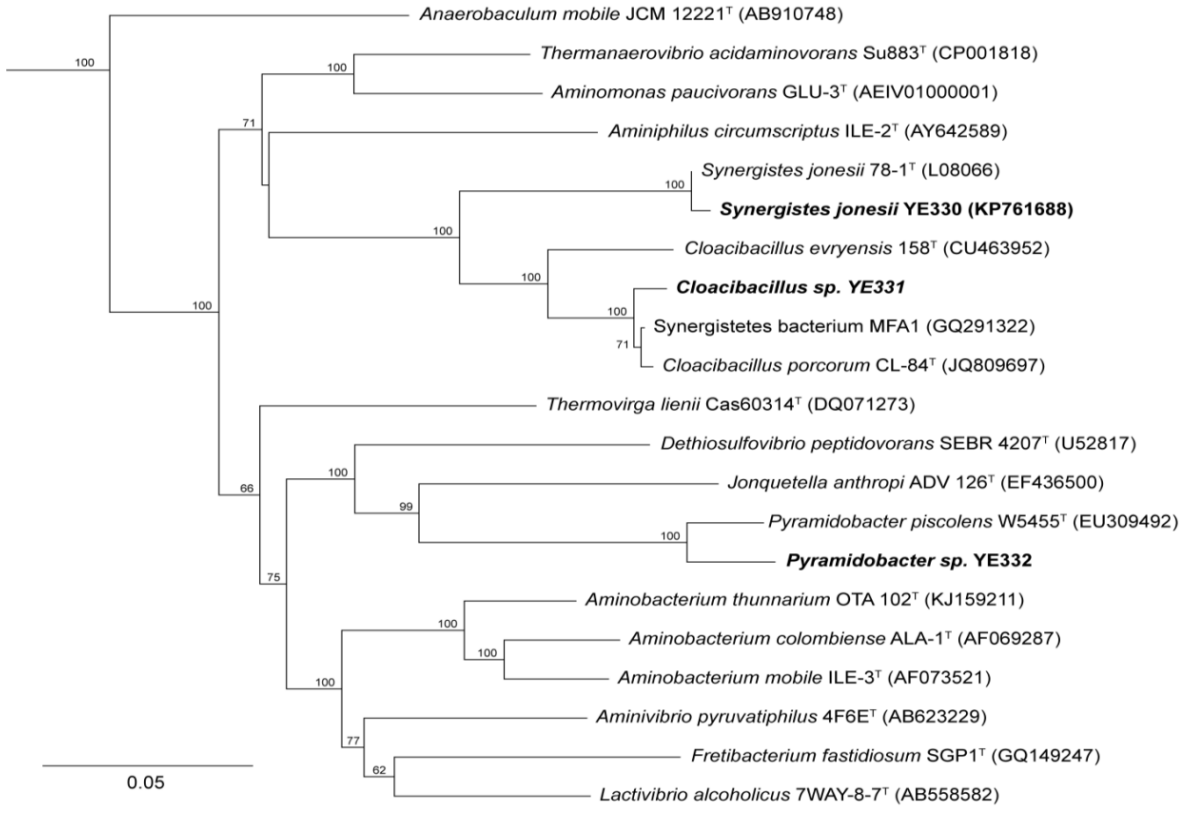

Fig. 17: Neighbour-joining phylogenetic tree of the Synergistetes phylum based on partial 16S rRNA gene sequences of representative strains. Cultures isolated in this study highlighted in bold. The tree is rooted to *Aquifix pyrophilus* (M83548). Three phylum members isolated from the fermentor-grown inoculum are shown in bold. Numbers on branches of the tree represent percentage bootstrap values of 1000 replicates. Only values greater than 50 % are shown. Bar, 5 nt substitutions per 100 nt.

4.2.6 Effect of cold storage on viability and DHP-degrading ability of *S. jonesii*

The cold storage experiments were conducted in order to simulate how particular events along the supply chain, from lab to farm and finally drenching, are likely to impact on the survivability, viability and activity of *S. jonesii* in the inoculum. A pure culture of strain 78-1 was used in the inoculum to allow a preliminary understanding of the bacterium's response. The results on the viability and DHP-degrading ability of pure cultures of *S. jonesii* in response to the various treatments are summarised in Fig. 18.

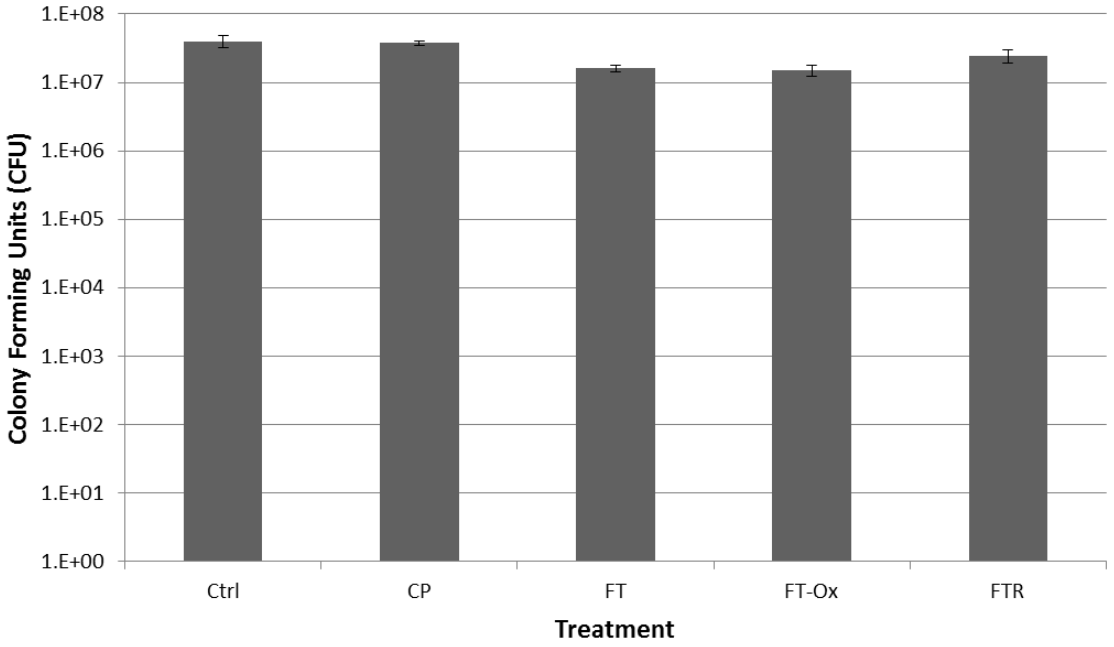

Fig. 18: Survivability of *S. jonesii* measured as Colony Forming Units (CFU) following various Cold chain experimental treatments: ctrl - control pure culture; CP – addition of cryoprotectant; FT - addition of cryoprotectant and Freeze/Thaw cycle; FT-Ox - addition of cryoprotectant, Freeze/Thaw cycle and exposure to Oxygen and FTR - addition of cryoprotectant and Freeze/Thaw/Refrigerate cycle. Error bars represent SD of triplicate measures.

All cultures remained viable after each treatment. Very small differences in viability were seen between the control (fresh culture) and when it was mixed with the cryoprotectant (0.03 ±9 % $\log_{10}$fold change). A single freeze-thaw cycle resulted in a viable cell count drop 0.4 ± 12 % $\log_{10}$fold change of compared to the control. Additional exposure to oxygen for 30 minutes resulted in a slightly larger decrease in viable cell counts (0.42 ± 18 % $\log_{10}$fold change). Interestingly, refrigeration for 30 hours following thawing resulted in a smaller decrease in the viability of the *S. jonesii* (0.21 ± 18 % $\log_{10}$fold change) compared to a single freeze/thaw event. Although there were decreases in viability following some of these

treatments, all were able to resume complete DHP-degradation within 10 days of inoculation. Subsequently, the cold chain treatment was applied to the Leucaena inoculum, a mixed bacterial population containing *S. jonesii,* and CFU's measured. The results on the viability of *S. jonesii* within the Leucaena inoculum in response to the various treatments are summarised in **Fig. 19**.

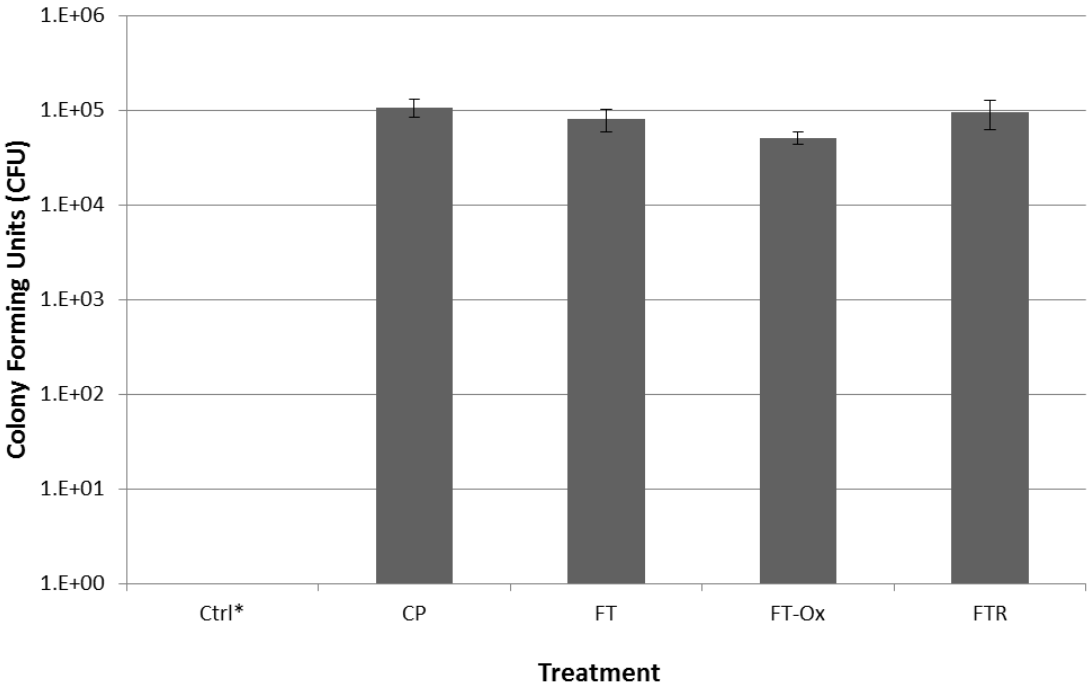

Fig. 19: Cold chain experiment on inoculum – S. jonesii Colony Forming Units (CFU) on Synergistes-Selective (SS-1) Media measured for the cryoprotectant (CP), one Freeze/Thaw cycle (FT), one Freeze/Thaw and exposure to Oxygen (FT-Ox) and Freeze/Thaw/ followed by refrigerate (FTR). Error bars represent SD of triplicate independent measures in three separate experiments. (*Control condition not applicable since inoculum is mixed immediately with cryoprotectant after harvest). CP used for baseline measurements.

The viability of *S. jonesii* had a small decrease compared to the reference condition (CP) for one freeze-thaw (0.12 ± 27 % $\log_{10}$fold change). A single freeze thaw followed by exposure to oxygen deceased viability (0.32 ± 14 % $\log_{10}$fold change) compared to CP. Thawing followed by refrigeration for 30 hours showed a smaller decrease in the viability of the *S. jonesii* (0.05 ± 34 % $\log_{10}$fold change) compared to a single freeze/thaw with no refrigeration. These results are consistent with the relative decreases viability observed for pure cultures (**Fig. 18**).

5 Discussion

5.1 Bacterial composition of the fermentor grown inoculum

Objective 1 of the project determined the bacterial composition of the *in vitro* inoculum, including the number of strains of *S. jonesii* present, and how this has changed over time and in comparison with ruminal contents.

5.1.1 The fermentor reaches microbial stability within nine days

The current study evaluated the bacterial composition of the fermentor-grown inoculum during the production process as well as for different batches spanning more than 13 years. The fermentor microbiota diversifies over the first week of production to create a homogenous, rich and stable anaerobe-dominated bacterial community by the first day of inoculum harvest on day 10 of the production process. The bacterial community consisted of an estimated 400 species (OTU's). In comparison, a rumen bacterial community typically includes 2,000 – 3,000 bacterial species (Jami and Mizrahi, 2012). The inoculum community is dominated by unclassified bacteria from the phyla Bacteroidetes and Firmicutes, genus *Prevotella* and family Lachnospiraceae. All of these bacterial taxa are common and important members of the normal rumen microbiota (Hobson and Stewart, 1997).

Interestingly, the most dominant species in the fermentor was an unclassified Bacteroidetes bacterium which had only 85 % 16S rRNA gene similarity to any cultured bacteria. It also appears to be an important member of the rumen as it is commonly observed in 16S rumen microbial studies (Kong et al., 2010). The Bacteroidetes phylum are the most abundant and found in ruminants on many diets and are responsible for breaking down complex polysaccharides and plant cell wall components to short chain fatty acids. If these bacteria can be isolated from the fermentor it represents an opportunity to investigate the metabolic capabilities of this diverse and abundant group of rumen microorganisms.

5.1.2 Bacterial composition changes between batches

The bacterial composition of the inoculum revealed significant, albeit minor, changes due to repeated passage of the starter culture. Archival samples of the inoculum showed that the numbers of *S. jonesii* in the mixed culture have remained stable over time. This is confirmed by statistical analysis of the 16S rRNA gene amplicon sequence data generated from archival samples, which showed that there was no significant correlation between *S. jonesii* sequence numbers and the number of times the *in vitro* inoculum had been passaged. While the *S. jonesii* population has remained stable over time, a gradual shift in the overall

composition of the bacterial population in the inoculum is apparent from the earliest archived batch produced in 1999 towards a more stable composition which has changed very little between 2006 and the present. The system used to produce the inoculum has been continuously refined over the 20 year period in which it has been in operation. It is not surprising that a complex biological ecosystem such as the Leucaena inoculum which contains approximately 400 species of bacteria has had minor changes in composition over time. There are multiple factors such as the length of the fermentation run and the amount of Leucaena leaf fed into the system per day that may have affected the relative sizes of the various bacterial populations present in the mixed culture. The most pronounced changes in the composition of the drench occurred between batches produced before and after October 2006 (Fig. 3). These changes can be attributed to a change in the *in vitro* fermentation system used from a 1.5 L fermentation carried out in a 2 L chemostat with a heated base (Mini Jar Fermentor M-100, Tokyo Rikakikai Co. Ltd) to a 3 L fermentation carried out in a 7 L computer controlled chemostat with a waterjacket heating system and increased gas flow rate (Infors HT, Bottmingen, Switzerland) (Fig. S 1, Appendix 9.1), which facilitates production of larger quantities of the inoculum. Furthermore, the size of the inoculum used to 'start' the fermentor remained constant, whereas the volume increased by 33 % hence permitting fast-growing organisms to reach larger numbers initially. However, as shown during the time course of fermentation, bacterial composition soon reaches equilibrium.

5.1.3 *S. jonesii* strain evaluation in the fermentor

One strain of *S. jonesii* is present in the Leucaena inoculum and no phylogenetic changes in this strain were seen in the fermentor, either during the production process, between batches inoculated from different starter cultures or any of the batches we analysed from 1999 to the most recent. These findings were supported by two independent methods; one using DGGE and the other using 16S rRNA gene amplicon pyrosequencing. These results correlate well with the fact that there has been no change in the fermentor's ability to degrade DHP during this course of production. Interestingly, our results of *S. jonesii*-specific DGGE showed that Leucaena-fed cattle inoculated with the fermentor-grown inoculum (Brian Pastures cattle, Gayndah) have the same *S. jonesii* 16S rRNA sequence to that of the fermentor, affirming that the inoculum does indeed establish in animals feeding on Leucaena.

5.1.4 Differences between degradation of DHP by strains of *S. jonesii* and relation to genomics

Previous studies have suggested that variation exists within the strains of *S. jonesii* in relation to the ability to degrade DHP compounds (Jones, 1994, DominguezBello et al., 1997, Padmanabha et al., 2014). Recently, concerns have been raised that *S. jonesii* 'strains' differ in their ability to degrade DHP and this may account for the presence of 2,3-DHP in the urine of some cattle, observed *in vivo* (Graham et al., 2013). This was also speculated as the perceived cause of reduction in live-weight gains in cattle inoculated with the Leucaena inoculum. Work conducted during this project focused on improving the *in vitro* culturing conditions for *S. jonesii.* Past culturing efforts using published methods showed high variability in terms of growth rate, biomass yield and DHP-degrading ability. We obtained four representative cultures of *S. jonesii* from a culture collection held by Milton Alison, who originally isolated the bacterium from a goat in Hawaii (Allison et al., 1992). We also obtained an additional culture from the ATCC (ATCC 49833, originally called 78-1, deposited by Milton Alison). Our results indicate that all five representative cultures have the ability to degrade both DHP compounds to completion. However, this only occurred under optimal conditions and prolonged continuous subculturing with the target compounds. Interestingly, two of the cultures display a bi-phasic degradation pattern, that is, 3,4-DHP degrades before 2,3-DHP (thus an increase in the 2,3-DHP isomer as 3,4-DHP decreases). Other cultures degrade both compounds in tandem. However, 3,4-DHP was always degraded more rapidly than 2,3-DHP, which may explain why 2,3-DHP is most readily seen the urine of cattle feeding Leucaena. Although small differences exist in the rate DHP degrades in representative cultures, further continuous culturing of these cultures is likely to lead to very similar or identical DHP degrading profiles.

The ability of *S. jonesii* to increase its rate of DHP degradation when cultured under optimal and continuous exposure to DHP compounds is also supported by our study of two identical cultures, strain 78-1 and ATCC 49833, which were obtained from different culture collections. These two representative cultures were most likely cultivated using different conditions prior to being cultured in our lab. It would almost appear certain that the media recommended by ATCC for culturing ATCC 49833 did not include DHP in their medium. And indeed culturing using this media gives suboptimal biomass. Interestingly, initial culturing of ATCC 49833 resulted in no DHP degradation, while strain 78-1, from the Milt Alison collection, which had an unknown history of culture conditions but likely contained DHP in the medium, was able to slowly degrade both DHP compounds. Continuous subculturing of both of these cultures with the target compounds increased their rate of degradation. This is

likely to continue to improve with further culturing. These results indicate that DHP is required to be present at a relatively constant rate and under optimal *in vitro* conditions for efficient degradation to occur. Taken together, sporadic or intermittent exposure to the mimosine toxin in the rumen could lead to less efficient degradation of DHP and elevated levels of toxicity. Therefore, it is recommended that cattle drenched with the Leucaena inoculum have constant access to Leucaena rather than intermittent feeding regimes that are reported in the some production systems.

Temporary and permanent loss of the DHP degrading ability of *S. jonesii* has been reported elsewhere (Dominguez-Bello et al., 1998, Rincon et al., 1998). The temporary loss of DHP degrading ability resulting from a lack of access to DHP compounds may be the perceived cause of residual 2,3-DHP observed in the urine of some inoculated cattle (Graham et al., 2013), and is likely to have implications on the management practices adopted by livestock producers. The trait of degrading DHP compounds appears to be more complex than expected and possibly bi-phasic or tandem depending on compound concentration and the extent of time of exposure to DHP compounds. Erratic and sudden exposure to high percentages of dietary Leucaena by cattle that have been rumen drenched with the Leucaena inoculum could possibly lead to the presence of measurable levels of DHP in the urine. Further research is needed to establish the optimal levels of Leucaena and the exact feeding regime for optimal live weight gains (LWG) in cattle. Studies also need to be undertaken to establish what levels of DHP can be present in the urine before they are to be considered an adverse indicator of live weight gain or suggestive of symptomatic toxicity. Nonetheless we have shown that the processes governing DHP-degradation are highly regulated and dependant on the substrate being present at a steady rate, otherwise DHP-degrading ability of *S. jonesii* can be compromised.

The genomes of *S. jonesii* sequenced in this study showed little genetic variation between the representative cultures. Some variation did exist however, in particular around mobile insertion elements and phage related sequences. This included some duplications and truncations for certain phage–related genes but no new genetic sequences were seen in one genome and not the others. Phage sequences and other mobile elements are common in many bacterial species and are usually promiscuous in nature (Kenzaka et al., 2010). Continuous subculturing of the *S. jonseii* representative cultures is likely to evoke further movement of these types of genetic sequences. Bacteria also have defensive immune systems known as the CRISPR/Cas system that adapts to cleave invading DNA preventing infection of the cell by bacteriophage (Barrangou et al., 2007). Therefore, it is not surprising that the foreign DNA is found in different locations of the genomes of the *S. jonesii* strains. The apparent similarity in the genomes also suggests that all the representative cultures of

S. jonesii are not in fact different strains by the true definition, but could be considered clonal cultures. Indeed, all these cultures originated from the same goat. Indications of minimal genetic differences were also supported by the culture-based studies in this project that showed little differences in biomass, morphology and DHP-degrading ability between the strains.

5.1.5 Isolation of *S. jonesii* from the fermentor

Efforts in our lab to isolate *S. jonesii* from the fermentor using published methods were unsuccessful. We therefore developed a selective medium using metabolic and antimicrobial resistance traits unique to the Synergistetes phylum which were obtained by searching the genome data, literature and in-house biochemical data. Development of a Synergistetes-specific medium (SS-4) in the current study led to the isolation of a new strain of *S. jonesii* YE330, which is the first time this species has been re-isolated in over 20 years. The strain is different based on 16S rRNA gene sequence to the four representative cultures which are all identical to the original strain 78-1. The new isolated strain was identical to sequences we obtained from the fermentor and from cattle inoculated with the Leucaena inoculum. The isolation technique reported has the potential to be applied to other environmental samples, possibly leading to isolation of other *S. jonesii* strains. Padmanabha et. al., (2014) reported genetic diversity of *S. jonesii* from different animals from geographically diverse locations (Padmanabha et al., 2014). Some of these animals are reported to have superior detoxification properties. This potentially allows an opportunity to study whether these strains of *S. jonesii* have greater abilities to degrade DHP.

The selective media used to isolate *S. jonesii* in this study was also modified slightly allowing the isolation of two other Synergistetes bacteria, *Cloacibacillus sp*. YE331 and *Pyramidobacter sp*. YE332. Numerous 16S rRNA gene studies have shown that both these bacterial genera are ubiquitous in gastrointestinal ecosystems, especially the rumen (Godon et al., 2005, Davis, 2011). *Pyramidobacter sp*. has never been isolated from any animals other than humans prior to this study (Downes et al., 2009) and the strain isolated in this study was the ninth most dominant OTU in the Leucaena inoculum, and was also present in detectable abundance in cattle inoculated with the Leucaena inoculum grazing Leucaena. The Synergistetes phylum appears to be metabolising proteins and amino acids which suggest that they may contribute to important environmental functions such as nitrogen recycling. The isolation of the *Pyramidobacter sp*. into culture may allow further investigations into its role in the rumen. The *Cloacibacillus sp*. isolated in this study is also interesting because it is closely related to *Synergistetes* bacterium MFA1 (99.4 % 16S rRNA gene sequence similarity), which has the ability to detoxify another plant toxin fluoroacetate

(Davis et al., 2012). The study of such bacteria is important to increaseour understanding of the rumen microbial ecosystems and their potential roles in animal health and nutrition.

S. jonesii comprised a relatively small fraction of the total bacterial population in the Leucaena inoculum. 16S rRNA gene sequences corresponding to *S. jonesii* comprised a maximum of 0.55 % of the total sequences for any sample. No study has yet determined the number of *S. jonesii* which would typically occur in the rumen. However, given that *S. jonesii* is rarely detected in the rumen by current molecular techniques, a theoretical detection limit in the order of 10^3 to 10^5 cells/mL is proposed. Assuming the rumen contains an average of 10^{11} bacterial cells/mL (Hobson and Stewart, 1997), *S. jonesii* represents an extremely small proportion of approximately one in 10^6 to 10^8 cells. Comparatively, the fermentor-produced inoculum contains on average one *S. jonesii* cell in 500 , which is at least three to five log-folds higher than in the rumen. The reason why such a high proportion of *S. jonesii* occurs in an *in vitro* fed system compared to the rumen is may be associated with the fermentor having less bacterial diversity compared with the rumen; there is less competition for available nutrients and co-factors. It is also possible that provision of a uniform nutrient substrate and the continuous exposure to a consistent amount of DHP on a daily basis allows the increased populations of *S. jonesii.* This may have implications for producers in that cattle are possibly more likely to build stable populations of *S. jonesii* with steady continuous feeding of Leucaena as opposed to sporadic or on-off feeding.

5.1.6 Development of sensitive RT-q-PCR for detection of *S. jonesii*

PCR assays targeting both the 16S rRNA gene (DNA) and 16S ribosomal subunit (RNA) were successfully developed in this project. The specificity of the PCR primers and probe were tested *in silico* by comparison of their sequences to publically available sequence databases. These comparisons indicated that the assays were highly specific. The specificity of the assay was also verified by testing against a panel of common culturable rumen bacteria as described previously (Klieve et al., 2007). Using standard criteria for assessing qPCR assays it was determined that our DNA-based assay allows effective quantification of *S. jonesii* cells between 10^5 and 10^{10} cells/mL, with reproducible positive detection of cell numbers in the range of 10^4 cells/mL. This detection limit is comparable to the limit reported by Graham et al., (2013) for their nested PCR assay which allows qualitative (i.e. positive or negative) detection of *S. jonesii* in rumen fluid.

A RNA-based (RT-qPCR) assay was developed in order to improve upon the sensitivity of the DNA-based assay. A RT-qPCR assay targeting ribosomal RNA (rRNA) has two distinct advantages over an assay targeting the corresponding DNA copies of the rRNA genes. The

first advantage is that rRNA transcripts typically occur in much higher copy numbers than their corresponding DNA templates in the cells of actively metabolising organisms, which makes them easier to detect. The second advantage is that the copy number of rRNA transcripts is directly related to the physiological status of the cell. Ribosomes are an essential component of the cellular machinery required for gene expression, so rRNA is produced by the cell as the genes it requires for growth are expressed. This means that an assay that allows RNA copy numbers to be measured also provides a measure of the level of metabolic activity of the organism.

Assessment of our *S. jonesii* 16S rRNA RT-qPCR assay showed that it can reliably quantify 16S rRNA transcripts ranging from 1×10^4 to 1×10^{11} copies/mL. In order to relate this range of transcript copy numbers to actively growing *S. jonesii* cells, daily samples collected from a 17 day Leucaena inoculum production fermentation run were analysed using both the DNA-based qPCR, and the rRNA-based RT-qPCR assays. The results show very good correlation between the number of *S. jonesii* cells and the copy number of 16S rRNA (Pearson correlation coefficient $\rho = 0.94$ for log transformed data). This result indicates that the RT-qPCR targeting the 16S rRNA of *S. jonesii* accurately reflects cell numbers of the bacterium in actively growing cultures. In addition, the 16S rRNA copy number as determined by the RT-qPCR assay was consistently two to three orders of magnitude higher than the cell numbers determined by DNA-based qPCR. This result indicates that the RT-qPCR assay offers a 250 – 1,000 fold increase in sensitivity over the DNA-based qPCR assay, and the nested PCR assay of Graham et al., (2013). This suggests that this assay could be a useful tool for accurately quantifying the numbers of actively growing *S. jonesii* cells in the rumen of inoculated animals.

5.2 Fate and dynamics of *S. jonesii* along the supply chain

The Leucaena inoculum is supplied to farmers as a cyroprotected product, which occurs at the point of manufacture before being stored frozen at -20° C.The frozen inoculum is then transported to producers in plastic eskies with cold packs. This project evaluated the effect of various conditions along the supply chain which may adversely affect the viability and DHP-degrading ability of *S. jonesii* in the inoculum.

Studies on pure cultures of *S. jonesii* and the Leucaena inoculum showed that the cryoprotectant alone, without any freeze-thaw cycle, had no effect on the viability of *S. jonesii* in the inoculum or its ability to degrade 3,4 or 2,3-DHP. This is not surprising since the cryoprotectant is based on media which is used to produce the inoculum. Glycerol, which is the principle cryoprotectant, is not toxic to microorganisms and serves to penetrate

bacterial cells preventing the formation of ice crystals that can cause membrane rupture during freezing and storage (Izawa et al., 2004). After a single freeze thaw cycle we found small decreases in the viability of *S. jonesii* in the pure cultures and inoculum, in the order of 0.12 log fold decrease , this reduction was far less than expected. For instance, the gut bacteria *Heliocobacter pylori* shows nearly a one log reduction in bacterial colony counts in response to cryopreservation and almost two log reduction with other well established methods of cryopreservation (Oskouei et al., 2010). Some species of bacteria enter a physiological dormant state or viable, but not culturable state under environmental stress (Oliver, 2005). This could be why the CFU counts in the inoculum were nearly one log lower than indicated by our qPCR assay. Nonetheless, small losses in viability due to freeze thawing of the inoculum lead us to recommend that the number of freeze thaw cycles be minimised during throughout the supply chain.

Interestingly, following one freeze thaw cycle, storage for 24 hours at 4 to 8 °C improved the viability of both *S. jonesii* pure cultures and in the inoculum. The reason for this increased viability is not clear.It is possible that refrigeration allows *S. jonesii* to slowly transition back to a non-dormant state improving its viability under *in vitro* culture conditions. According to these results, refrigeration of the Leucaena inoculum for 24 to 48 hours is unlikely to be detrimental to the viability of the inoculum. Since the greatest reduction was seen during freezing and thawing, it is recommended that the inoculum not be refrozen if it is received already thawed.

Exposing the pure cultures and the inoculum to oxygen for 30 minutes after being thawed appeared to have little impact on viability of *S. jonesii.* This is reassuring since exposure to oxygen is inevitable when drenching cattle under farm conditions. *S. jonesii* is an obligate anaerobe (Allison et al., 1992), meaning that it can only grow without the presence of oxygen. But it also appears to be able to tolerate oxygen for some time. Further experiments are needed to determine the exact level of aerotolerance of *S. jonesii*. Our culture based studies indicate that *S. jonesii* viability and DHP-degradation are unaffected by short term (30 minutes) exposure to oxygen, suggesting that *S. jonesii* is partially aerotolerant. However, being an obligate anaerobe we recommend that the inoculum not be exposed to oxygen until the point of drenching cattle and exposure to oxygen should then be kept to minimum.

The current practice of dispatching the frozen Leucaena inoculum in an esky with cold packs which maintains temperatures below 4 °C for 34 hours and results in the producer receiving a thawed product does not detrimentally affect the viability of *S. jonesii*. Our recommendations for Leucaena inoculum best practice is for it to be used immediately on

arrival or refrigerate without exposure to oxygen for a further 24 hours prior to drenching animals that have been grazing Leucaena.

6 Conclusions/recommendations

6.1 Project insights and implications for industry

The production of the Leucaena inoculum by *in vitro* fermentation for over 20 years has not resulted in fundamental changes to its composition and the population size of *S. jonesii* has remained consistent in fermentor produced inoculum. The Leucaena inoculum contains a mix of micro-organisms that can effectively break down DHP and detoxify Leucaena.

There has been only one strain of *S. jonesii* present in the fermentor inoculum since production started. The same strain has been found in steers from Brian Pastures that were inoculated with the inoculum and feeding on Leucaena.

Continuous exposure of *S. jonesii* to 2,3 and 3,4-DHP improves its ability to degrade these compounds. It is our recommendation that cattle rumen drenched with the Leucaena inoculum have constant access to Leucaena.

The current practice of dispatching the frozen Leucaena inoculum in eskies with cold packs which have been tested and shown to take a minimum of 15 hours to reach an average internal temperature of 0 °C and a minimum of 34 hours to reach an average internal temperature of 4 °C resulting in producers receiving a thawed product does not detrimentally affect the viability of *S. jonesii*. Our recommendations for Leucaena inoculum best management practice is to either use the thawed Leucaena inoculum immediately on arrival or refrigerate without exposure to oxygen for a further 24 hours prior to drenching animals that have been grazing Leucaena.

New tools (e.g. quantitative PCR assay based on RNA) have become available that allow for more sensitive detection of *S. jonesii* in environmental samples. This assay has potential for monitoring the levels of *S. jonesii* in the rumen of inoculated animals grazing Leucaena.

6.2 Adoption activities.

An article outlining the project key findings and recommendations for the most effective use of the Leucaena inoculum will be circulated to producers via the FutureBeef website, the Leucaena Network and at Producer Demonstration Site events.

Key findings and recommendations from the project will be provided to Queensland DAF extension staff to enable them to better inform producers on the use of the Leucaena inoculum for animals grazing Leucaena.

The recommended best management practice guidelines will be updated from the recommendations from the project.

6.3 Future research and development

Given the importance of the Leucaena inoculum in protecting cattle from mimosine toxicity and ensuring efficiency of forage utilisation it is imperative to determine that this will still be the case with cattle being fed the new Redlands cultivar. The release of Redlands poses a new range of questions in regards to the survival of *S. jonesii* and the efficacy of the current inoculum in relation to the new cultivar. Things that we do not know in relation to the Redlands cultivar are:-

- Will the inoculum be protective against DHP toxicity in cattle grazing Redlands?
- How will the 'anti-psyllid' properties impact on the survival of *S. jonesii,* its ability to degrade DHP, and the overall bacterial diversity within the fermentor produced inoculum which may in turn affect the population size of *S. jonesii* present in the inoculum?
- What is the impact on other species in the rumen, particularly other inoculant species (which help to efficiently use Leucaena) and the ability of fibrolytic species to efficiently digest plant fibre? In other words, what is the impact on overall feed utilisation?

In addition, we need to increase our understanding of the mechanisms of DHP degradation by *S. jonesii* and identify the functional genes involved. This may lead to the development of molecular assays targeting the genes involved in DHP degradation. This assay could allow screening for DHP degradation potential in other bacteria which could be used to increase the effectiveness of the Leucaena inoculum.

7 Key messages

- The Leucaena inoculum has not changed in 20 years of production and the numbers of *S. jonesii* in the Leucaena inoculum have remained consistent over this time.
- Producers should either use the Leucaena inoculum immediately upon receipt or store at refrigerator temperature (3 to 5 °C) for up to 24 hours to minimise the impact on populations of *S. jonesii*.
- Producers should mix the Leucaena inoculum well before opening the bottle, keep it cool on ice or with ice bricks prior to rumen drenching and minimise the introduction of oxygen into the inoculum.
- Cattle should be already grazing Leucaena before being drenched with the Leucaena inoculum.
- Producers should ensure cattle have continual access to some Leucaena as this will maximise the effective detoxification by the Leucaena inoculum.
- The Leucaena inoculum should NOT be refrozen if it has thawed.
- Cattle should not be taken off Leucaena and then put back on as this may result in decreased effectiveness of DHP degradation by *S. jonesii* and increased levels of DHP may become present in the urine.

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9 Appendix

9.1 Extra Tables and Figures

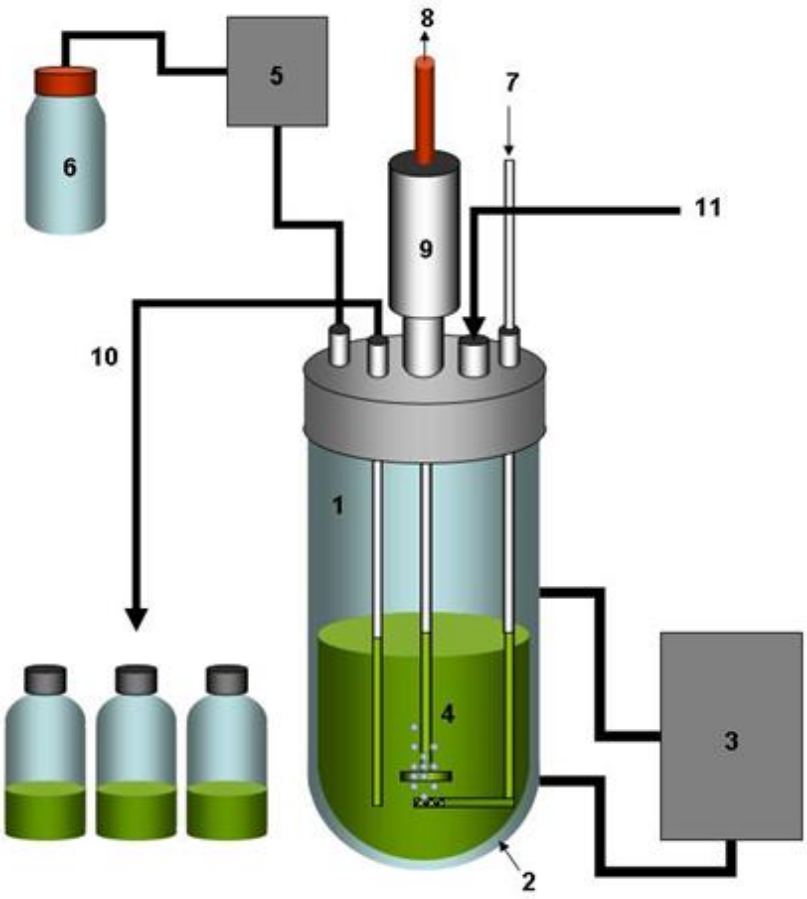

Fig. S 1 Schematic representation of the *in vitro* fermentation system used to produce the Leucaena rumen inoculum. The system consists of 7 L sealed glass fermentation vessel (1) with an outer water-filled jacket (2) which is plumbed to a temperature control unit (3). During operation the vessel contains 3 L of liquid medium which is constantly agitated with a stirrer (4). Temperature is maintained at 39 °C by a computer controller (5) which receives input from temperature and pH sensors immersed in the fermentation medium. The pH of the fermentation medium is maintained between 6.6 and 6.8 by computer controlled addition of 5 M NaOH solution (6). Anaerobic conditions are maintained by constantly sparging the medium with a CO_2:H_2 (95:5) gas mixture (7). Exhaust gases (8) are vented through a condenser (9) which prevents excessive loss of water vapour from the system. Each day 1.5 L of fluid is removed through a siphon (10). The culture is maintained by adding 1.5 L fresh liquid medium and 30 g macerated Leucaena leaf (11) through an aperture in the lid of the fermentor, which is then stoppered to prevent entry of oxygen into the system.

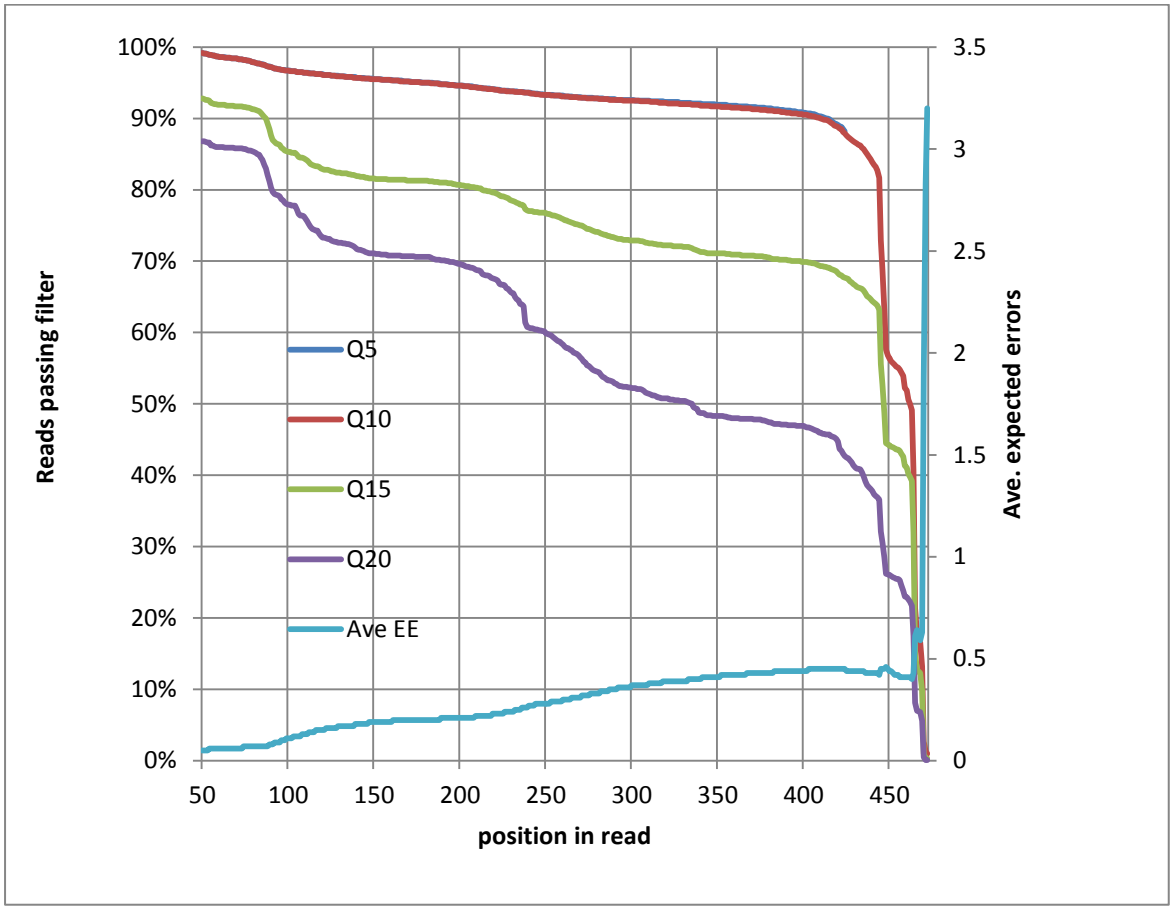

Fig. S 2 Percent of reads passing filter vs position in read at different Phred scores.

Table S 1 Details of PCR primers and probes used in this study.

Name	Description	Sequence (5' to 3')	Reference
341F	Universal bacterial 16S forward primer tagged with a 10 bp barcode sequence and 454 sequencing adapters. Used to produce amplicons for 454 sequencing	Fusion A-Barcode-CCTACGGGAGGCAGCAG	Watanabe et al., (2001)
787RSYN	Universal bacterial 16S reverse primer tagged with 454 sequencing adapters. Modified to include a variable base (bold) to allow annealing to published 16S rRNA gene sequences from the phylum Synergistetes. Used to produce amplicons for 454 sequencing.	Fusion B–TACC**R**GGGTATCTAAT	Baker et al., (2003)
SJ62F	Forward primer specific to V1 region of *S. jonesii* 16S rRNA gene	TCGAACGGGGATCATGTAGAA	this study
SJ176R	Reverse primer specific to V2 region of *S. jonesii* 16S rRNA gene for qPCR	ACCTCTCGGCTTATGGGGTA	this study
SJ193F	Forward nested primer specific to *S. jonesii* 16S rRNA gene	TAAAAGGAGCGATCCGGTAACA	this study
SJ492R (GC)	Reverse DGGE primer [b] specific to *S. jonesii* 16S rRNA gene	GCCGTGGCTTTCTCTTGC-(GC-clamp)	this study
SJ589R	*S. jonesii* specific reverse primer for production of T7 tagged amplicons for *in vitro* transcription	CAGTTTCAACTGTGATGCAAA	this study
SJ1009R	Reverse primer specific to *S. jonesii* 16S rRNA gene	GCTAAGGTCCTCTCGATCTCTCTC	this study
SJprobe87	Fluorescent oligonucleotide probe specific to V1 region of *S. jonesii* 16S rRNA gene for qPCR	FAM-CTTAGACATGATTTTAGTGGCGGACGGGT-BHQ1	this study
T727F	T7 promoter tagged [a] universal 16S forward primer for production of T7 tagged amplicons for *in vitro* transcription	<u>TAATACGACTCACTATAGGG</u>GAGTTTGATCCTGGCTCAG	Weisburg et al. (1991)
27F	Universal bacterial 16S rRNA gene forward primer	AGAGTTTGATCCTGGCTCAG	Lane (1991)
1492R	Universal bacterial 16S rRNA gene reverse primer	GGTTACCTTGTTACGACTT	Lane (1991)

[a] Sequences corresponding to the T7 promoter are underlined;
[b] GC-clamp = CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

Table S 2 Differences between genomes of *S. jonesii* strains 100-6, 113-4, and 147-1

Position (Bp)	Strain	Gene	Description/Comment
186 386	147-1	Periplasmic [Fe] hydrogenase large subunit (EC 1.12.7.2) CDS	T insertion 147-1
290 202-290 457	147-1	Aldehyde dehydrogenase B (EC 1.2.1.22) CDS	Truncated gene - CRISPR elements upstream
394 694	147-1	Transcriptional regulator, AraC family CDS	SNP G->A
399 427	147-1	Iron-sulfur flavoprotein CDS	SNP G->T
399 621	100-6	Iron-sulfur flavoprotein CDS	SNP A->G
460 971-464 656	113-4	putative type IIS restriction/modification enzyme CDS	partial gene duplication
542 058	147-1	YheO-like PAS domain CDS	A insertion
589 001	147-1	Large Subunit Ribosomal RNA	SNP A->G
779 762-779 773	147-1	Transcriptional regulator, GntR family CDS	deletion
964 964	147-1	Periplasmic [Fe] hydrogenase (EC 1.12.7.2) CDS	SNP T-> C
1 024 760-1 024 997	147-1	tRNA-Ile-GAT; tRNA-Ala-TGC insertion	duplication
1 241 760-1 255 913	147-1	metabolic gene cluster deletion; flanked by mobile element	deletion; flanked by mobile element
1 263 050	147-1	RAP transporter solute receptor, unknown substrate 5 CDS	SNP G->C
1 274 575	147-1	Activator of (R)-2-hydroxyglutaryl-CoA dehydratase CDS	SNP T-> C
1 447 777-1 448 617	147-1	LysR family transcriptional regulator STM2281 CDS	insertion of Mobile element protein CDS
1 571 211	147-1	Exoenzymes regulatory protein AepA precursor CDS	SNP G->A
1 693 562	147-1	Preprotein translocase subunit YajC (TC 3.A.5.1.1) CDS	SNP T-> G
1 811 534	100-6	GTP-sensing transcriptional pleiotropic repressor codY CDS	SNP C-> T
1 812 116	113-4	GTP-sensing transcriptional pleiotropic repressor codY CDS	SNP A->G
1 900 050-1 900 891	147-1	Transcriptional regulator CDS; Permeases (DMT) superfamily CDS	Insertion Mobile element protein CDS
1 918 065-1 918 905	147-1	Hypothetical protein 374 CDS; LuxR family CDS	Insertion Mobile element protein CDS
1 926 659	147-1	Homoserine dehydrogenase (EC 1.1.1.3) CDS	SNP T-> C
2 074 482	147-1	2-oxoglutarate oxidoreductase, alpha subunit (EC 1.2.7.3) CDS	SNP C-> T
2 110 240-2 111 080	147-1	Zinc metalloprotease (EC 3.4.24.-) CDS	Insertion Mobile element protein CDS
2 441 330-2 611 497	147-1	metabolic genes/plasmid, mobile element	possible assembly artifact

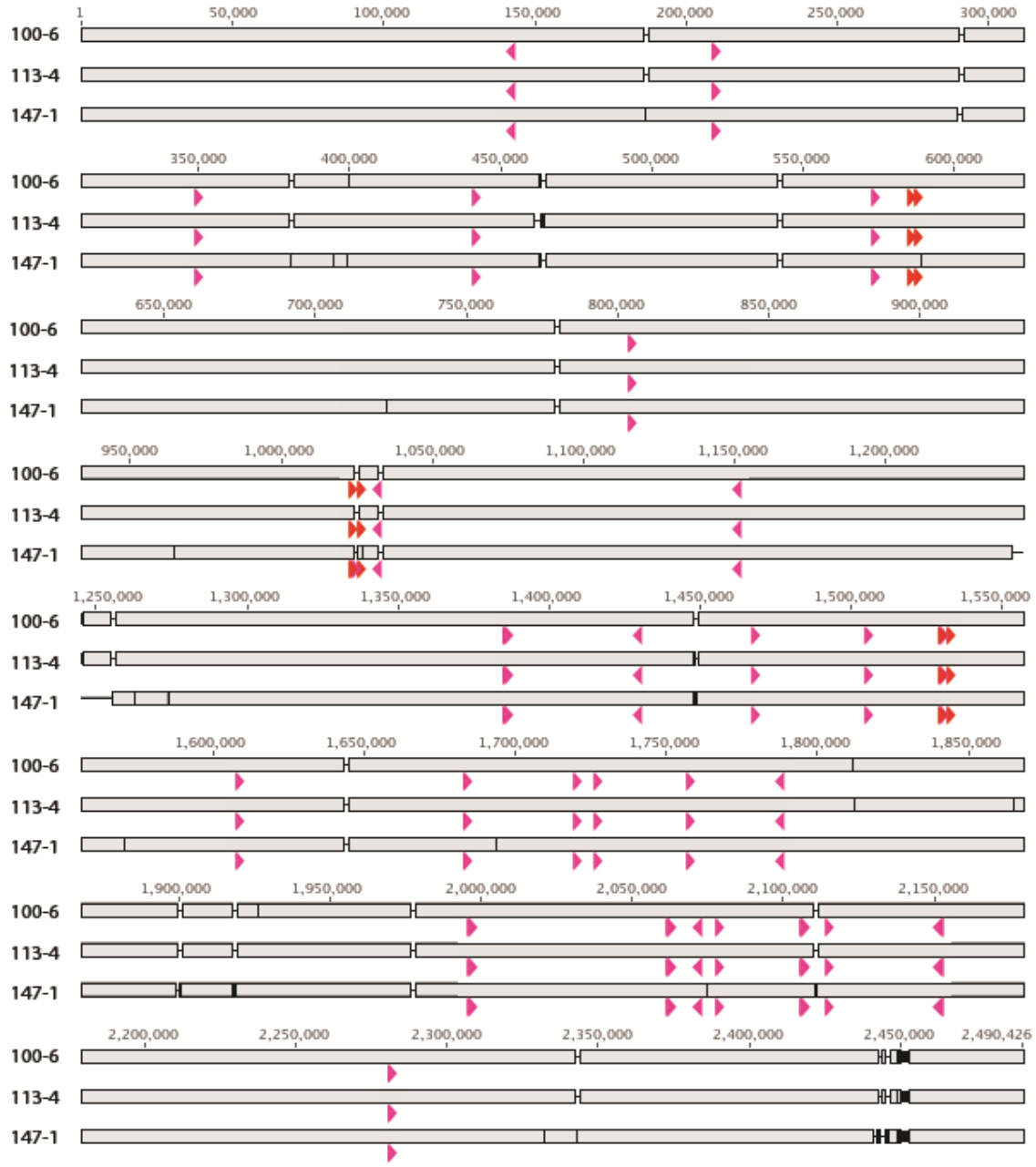

Fig. S 3 Genome alignments of the *S. jonesii* strains 100-6, 113-4, and 147-1. Ribosomal RNA genes (red arrows) and tRNA genes (pink arrows) are identical in all three strains. Black vertical lines and gaps represent regions that differ from the consensus sequence in the respective genomes either as a single nucleotide or as multiple nucleotides.

9.2 Synergistetes-Selective media (SS-4) composition

Routine culturing of *S. jonesii* was performed in a basal media containing (per L) mineral solution 2 and 3 (37.5 mL) (McSweeney et al., 2005), clarified rumen fluid (200 mL), mixed protein hydrolysate (5 g), trace elements solution (1 mL) (McSweeney et al., 2005), haemin (1 mL), arginine hydrochloride (2.11 g), histidine (1.55 g), cysteine (0.5 g), sodium carbonate (4 g) and resazarin as redox indicator (1 mg). Final pH for media was 6.7-6.9. Selective-Synergistetes media (SS-4) was emended with one or more of the following antimicrobials: vancomycin, neomycin, colisten, rifampicin, and erythromycin at minimal inhibited concentrations