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Effectiveness of *S. jonesii* inoculum for cattle grazing leucaena

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Abstract

The thrust of the work was to investigate the effectiveness of the *S. jonesii* inoculum produced in anaerobic fermenters by QPIF for distribution to graziers utilizing leucaena grass pastures. A second objective was to produce a quantitative real time PCR assay that specifically identifies and enumerates *S. jonesii*.

When using recommended and modified synthetic media for degradation studies, frozen QPIF inoculum and frozen rumen fluid collected from one property did not degrade the toxins 3,4-DHP and 2,3-DHP which are by-products of the breakdown of mimosine. However, fresh rumen fluid collected from another property did successfully degrade the toxins in the same modified media. Also fresh fermenter fluid which is the basis of the QPIF inoculum degraded both toxins when used in an undiluted form to evaluate metabolism of added toxin.

A quantitative real time PCR assay was developed for detection of *S. jonesii* in the rumen but the target gene is identical between all isolated strains of *S. jonesii* which precludes the differentiation of individual strains by this approach.

These experiments demonstrate that the ability of *S. jonesii* to degrade the toxin (DHP) *in vitro* is not always predictable and the efficiency of DHP degradation is likely to be regulated by the environment in which *S. jonesii* is growing. Thus the efficacy of the inoculum once established in animals cannot be inferred from these experiments. Isolation and evaluation of the DHP degrading bacteria (presumably *S. jonesii* strains) in Australian cattle would aid in the evaluation of the effectiveness of these naturalised microorganisms. Furthermore, the development of methods for the production and distribution of pure strains of *S. jonesii* to graziers may add to the quality control for the distribution of the inoculum.

Executive Summary

Following a number of reports, dating back to 2003, of mimosine and DHP toxicity from graziers using leucaena grass pastures, there has been concern that subclinical toxicity may be reducing response of cattle to this highly productive feeding system. This was confirmed by a survey conducted by The University of Queensland of 44 herds grazing leucaena. Urine tests showed the presence of mimosine break-down products, the toxins 3,4-DHP or 2,3-DHP, or both, in 52% of herds tested.

Of special concern, was the discovery that many herds previously inoculated with *Synergistes jonesii* (the leucaena bug), and considered protected, had high levels of DHP in urine. One possible explanation was that the current *in vitro* source of rumen inoculum containing *S. jonesii* is not working effectively to fully degrade 3,4-DHP and 2,3-DHP produced during leucaena digestion in cattle. The reasons for this may relate to management factors affecting the spread and retention of *S. jonesii* within a herd or the loss of strains of *S. jonesii* from the current inoculum cultures which are produced in an anaerobic fermenter in the laboratories of QPIF. Another issue of concern was the lack of a test for directly detecting the presence and abundance of the *S. jonesii* strains in inoculum or samples of rumen fluid from cattle grazing leucaena. This limits our ability to determine whether presence of toxins in urine is due to absence, low numbers of bacterium or sub-optimal function of bacteria with DHP-degrading capacity.

This study was designed to assess the effectiveness of the QPIF *in vitro* rumen inoculum for degrading both 3,4-DHP and 2,3-DHP and to develop a real time PCR test able to determine the presence, and enumerate strains of *S. jonesii* in rumen fluid.

Different sources of inoculum, including rumen samples obtained from properties thought to be protected from toxicity and pure cultures of the rumen bacteria *S. jonesii*, displayed varying ability to degrade the toxins 3,4-DHP and 2,3-DHP *in vitro*, despite all strains being phylogenetically identical. Fresh and frozen samples from 2 batches of QPIF *in vitro* fermenter material lacked ability to completely degrade both isomers of DHP under the *in vitro* media conditions employed. However subsequent experiments conducted by QPIF on the degradation of mimosine, and the two isomers of DHP, using pure fresh undiluted inoculum, demonstrated that the inoculum was capable of metabolising all these compounds but the results (extent of degradation) were variable especially for 3,4-DHP.

Frozen rumen fluid collected from cattle at CSIRO Lansdown did not degrade DHP, while fresh rumen fluid from commercial cattle at Banana completely degraded 3,4-DHP without the accumulation of 2,3-DHP when media containing high concentrations of clarified rumen fluid was used. Pure cultures of 4 different strains of *S. jonesii* obtained from the American Type Culture Collection and the University of Iowa, USA had varying ability to degrade 3,4-DHP and 2,3-DHP. These results highlight that the rate and extent of degradation of 3,4- and 2,3-DHP by closely related strains of *S. jonesii* is variable and also likely to be influenced by conditions used for the studies.

A quantitative real-time PCR (qPCR) assay that targets the 16S rRNA gene and is specific for the bacterium *S. jonesii* has been developed. The assay is able to detect the presence of all available strains of *S. jonesii* which were originally isolated from the rumen of a goat in Hawaii. The 16S rDNA gene of these strains were sequenced and found to be 99-100% identical and therefore cannot be differentiated using this technology. The qPCR assay was able to detect *S. jonesii* in rumen fluid and does not appear to cross react with sequences of other species of the 'Synergistes' division that were also present in the rumen.

Use of the PCR marker to establish the presence of the bacterium *in vitro* did not necessarily correlate with degradation of the toxin. It was found that *S. jonesii* could be positively identified by

the PCR, for both pure strains and rumen inocula, some of which degraded DHP, some partially degraded DHP while some fully degraded DHP.

Extensive and numerous attempts to enrich and isolate strains of *S. jonesii* from the QPIF inoculum, using various media and antibiotics, were unsuccessful due primarily to the low number of *S. jonesii* relative to other bacteria in the inoculum. More research is required to identify modified media that can successfully enrich strains of *S. jonesii* prior to isolation and purification.

We conclude that the current *in vitro* QPIF inoculum and rumen fluid collected from protected cattle grazing leucaena were able to degrade (varying rates and extent of degradation) the leucaena toxins *in vitro* when the *in vitro* assay is conducted using fresh 100% inoculum conditions. The inconsistent degradation of 2,3- and 3,4-DHP by pure strains of *S. jonesii* in prepared media in conjunction with the variable results of the experiments using QPIF inoculum suggest that the standardised *in vitro* methods used in these studies may influence the detoxifying ability of *S. jonesii* and require further work so that they can be routinely used. These experiments demonstrate that the ability of *S. jonesii* to degrade the toxin (DHP) *in vitro* is not always predictable and the efficiency of DHP degradation is likely to be regulated by the environment in which *S. jonesii* is growing. Thus the efficacy of the inoculum once established in animals cannot be inferred from these experiments.

We recommend that industry continue using the QPIF inoculum as presently produced as it appears effective at least in the short term. A related project *(B.NBP.0494)* will provide much needed information on the retention of degrading ability post-inoculation. However, further work is required to develop methods to isolate specific strains of *S. jonesii* and to compare them with culture strains for effectiveness and longevity. In the longer term, new methods for the production and distribution of pure cultures of effective *S. jonesii* strains could benefit the delivery of the organism to industry users.

Contents

Page

1 Background 6

2 Project Objectives 7

3 Methodology 7

3.1 *In vitro* testing of *S. jonesii* inoculum 7
3.2 Development of real time PCR assay 8
3.3 Enrichment and isolation of *S. jonesii* strains from inoculum of *S. jonesii* supplied to industry by QPIF 8

4 Results and Discussion 12

4.1 *In vitro* testing of *S. jonesii* inoculum to determine effectiveness of DHP degradation 12
4.2 Development of real time PCR assay 12
4.3 Enrichment and isolation of *S. jonesii* strains from inoculum of *S. jonesii* supplied to industry by QPIF 14

5 Success in Achieving Objectives 15

6 Impact on Meat and Livestock Industry – now & in five years time 15

7 Conclusions and Recommendations 16

8 Bibliography 17

9 Appendices 18

9.1 Appendix 1 - Report prepared by Rumen Ecology Unit QPIF 18

1 Background

At the break of the 2002/2003 drought, deaths (acute mimosine toxicity) and clinical symptoms of 3-hydroxy-4(1H)-pyridone (3,4-DHP) toxicity in cattle grazing leucaena were reported in herds previously thought protected by the *S. jonesii* 'bug'. With support from MLA (NBP.340), a UQ survey of leucaena growers in 2004 revealed that of 44 herds (385 animals) tested, 52% showed subclinical DHP toxicity (presence of high concentrations of 3,4-DHP and/or 2,3-DHP in the urine of cattle showing no visible symptoms). Leucaena toxicity is known to reduce appetite and can suppress liveweight gain by 30-50% (Quirk *et al.*, 1988; Prachett *et al*., 1991).

While some graziers may have used inappropriate rumen inoculation methods and/or were unaware of the need for on-going 'bug' management in their herds, of particular concern, was the discovery that many herds previously inoculated with the 'bug', and considered protected, were suffering from toxicity. Reports from several graziers have highlighted the continuing problem that many are having with management of leucaena toxicity even though they had followed recommended inoculation procedures.

The finding of high levels of 2,3-DHP in some herds was unexpected and contrasted previous research findings that reported that 2,3-DHP was a transitory breakdown product (Ford *et al*., 1984) of 3,4-DHP degradation by rumen bacteria (Allison *et al*., 1992; Rincon *et al*., 1998 & 2000). A number of species of rumen bacteria, in addition to strains of *S. jonesii*, have been identified that can degrade 2,3-DHP but not 3,4-DHP (Hammond *et al*., 1989). The reason why *S. jonesii* was not efficiently degrading 2,3-DHP in cattle in the 2004 UQ study remains unclear.

A possible explanation is the loss of a strain that efficiently degrades 2,3-DHP from the current *in vitro* inoculum. When the *S. jonesii* culture was first released, it included two strains selected from a collection of strains held by Dr Raymond Jones in Townsville but now discarded. Both were capable of degrading mimosine and both isomers of DHP, however, one strain was noticeably superior at degrading 2,3-DHP (Raymond Jones, personal communication). Therefore, the unexpected 2,3-DHP toxicity observed in the 2004 UQ study may be due to the temporary or permanent loss of 2,3-DHP degrading capacity of *in vitro* cultures.

Interestingly, there are several herds in Queensland that have retained long-term protection against toxicity without the need for re-inoculation. One such example is the CSIRO Lansdown herd which has now been sold. This may indicate that the original *in vivo* source of *S. jonesii* has retained long-term effectiveness to a greater extent than the current *in vitro* source.

We therefore hypothesise several possible reasons for the occurrence of subclinical leucaena-induced toxicity being observed in cattle herds previously thought to be protected. Our hypotheses are: (a) there has been a possible loss of a strain, or change of the original strains of *S. jonesii*, in the current *in vitro* inoculum; (b) there has been loss of a strain/s or change in detoxifying ability of *S. jonesii* while animals are 'off' leucaena and being fed alternative diets; and/or (c) there has been failure of the inoculation process which involves oral drenching with recently thawed, anaerobic *in vitro* culture of *S. jonesii.*

This project explores the first hypothesis by studying the effectiveness of the current *in vitro* source of rumen inoculum containing *S. jonesii*. The latter hypotheses (b and c) are being studied in a separate project investigating factors affecting the presence and dynamics (introduction, impact and retention) of *S. jonesii* in cattle herds grazing leucaena (MLA Project B.NBP.0494).

Another objective of the project is to develop a real-time PCR test able to distinguish the presence and enumerate strains of *S. jonesii* in rumen fluid. This is needed as there is also currently no test for directly detecting the presence and abundance of the *S. jonesii* strains in

inoculum or samples of rumen fluid from cattle grazing leucaena. This limits our ability to determine if there is a loss of 'the leucaena bug' or loss of DHP-degrading capacity.

2 Project Objectives

At completion of the project in July 2009, the project will have:

1. Established the *in vitro* effectiveness of the current source of *Synergistes jonesii*, including both the QPIF in vitro inoculum and rumen fluid from the CSIRO Lansdown herd, for degrading mimosine, 3,4-DHP and 2,3-DHP.
2. Developed real-time PCR tests able to distinguish the presence and enumerate strains of *Synergistes jonesii* in rumen fluid.

3 Methodology

3.1 *In vitro* testing of *S. jonesii* inoculum

Several sources of inoculum were obtained and tested for effectiveness in degrading the two isomers of the dihydroxypyridine toxin (2,3-DHP and 3,4-DHP) in several media**.** Degradation of both isomers of DHP in *in vitro* cultures was initially estimated by the colorimetric ferric chloride test (Megarrity, 1978) and confirmed using HPLC (Tangendjaja and Wills, 1980). The experiments involved inoculum from QPIF fermenter culture, rumen fluid from cattle from two properties, and pure strain inocula obtained from the USA. Some experiments were replicated in both the CSIRO and UQ laboratories, and run for approximately 1 month to ensure any degradation was detected. Mimosine was not included in the degradation studies because plant enzymes and a diverse suite of rumen bacteria convert mimosine to 3,4-DHP in the rumen.

The sources of inoculum tested were:

(a) Frozen *in vitro* inoculum obtained from QPIF laboratories at Yeerongpilly in November 2007 representative of the frozen stock distributed to producers. Subsequently, fresh *in vitro* inoculum obtained from QPIF in May 2008 was also tested.
(b) Frozen rumen fluid collected from 8 cattle at the CSIRO Lansdown Research Station herd that was inoculated 24 years ago with 2 original strains of *S.jonesii* introduced to Australia by Dr Raymond Jones.
(c) Fresh rumen fluid collected from 6 cattle from a Banana property demonstrated to be protected from leucaena toxicity (UQ unpublished data).
(d) Four individual strains of *S. jonesii* obtained from Dr Milt Allison at the University of Iowa (strains 78-1, 147-1, 100-6 and 113-4) and the type strain 78-1 obtained from the American Type Culture Collection.

The culturing techniques and medium used were similar to those described by Allison *et al.* (1992) which were used to isolate the original culture of *S. jonesii*. Some modifications of the media were also used. Media used included**:**

1. Modified basal medium 10 (modified BM10). The BM10 medium was made according to the original pre-reduced medium of Caldwell and Bryant (1966), with clarified and depleted rumen fluid added at a rate of either 15% or 30%. A high level of peptone (2%) and trace elements were also added. The pH of both media was adjusted to 6.8 – 7.0.

2. A buffered rumen fluid based media (BRF) was prepared consisting of clarified depleted rumen fluid (93%), sodium bicarbonate (7%), peptone (0.4%), trace elements, and a freshly prepared reducing solution after boiling.

3.2 Development of real time PCR assay

Primers designed for the detection of *S. jonesii* were targeted against signature 16S rRNA gene sequences found to be unique to this microorganism by analysis using ARB database and software. Analysis of the 16S rRNA gene sequence from all isolates of *S. jonesii* (strains 78-1, 23-35, 100-6, 113-4, 147-1) obtained from international culture collections demonstrated 99-100% similarity of the gene and thus the primers detected all these strains. The 16S rDNA forward primers (SynJ998F) ACTACTGTTACTTGAGAGAGATCGA and reverse primer CGCTCGTTGCGGGACTTG (SynJ1091R) were analysed using Vector NTI (Invitrogen) for primer melting temperatures (Tm) and possible intra- and intermolecular secondary structure formation. qPCR assays were performed on a ABI PRISM ® 7900HT Sequence Detection System (Applied Biosystems). Assays were set up using ABI SYBR green PCR master mix (Applied Biosystems). Optimisation of assay conditions was performed for annealing temperature, primer, DNA template and $MgCl_2$ concentrations. An optimal concentration of 200 nM primer, 2 mM $MgCl_2$ and 1-100 ng/µl DNA was used for each assay. The following cycle conditions were used: one cycle of 50°C for 2 mins, one cycle of 95°C for 10 mins for enzyme activation, 40 cycles of 95°C for 15 seconds and 60°C for 1 min for primer annealing and product extension. Amplification specificity was performed using dissociation curve analysis of PCR end-products using the following conditions; increased temperature from 60 to 95°C at 2°C/min. PCR products were also cloned and sequenced to verify amplification of the microorganism of interest and verified as *S. jonesii* in origin by checking against the CSIRO and public databases of Synergistes division 16S rDNA sequences.

A standard curve for the absolute quantification of *S. jonesii* was performed using gDNA extracted from a known number of *S. jonesii* cells. Amplification efficiency for the qPCR assay was calculated over a six-log dilution series (10^7-10^1 cells).

3.3 Enrichment and isolation of *S. jonesii* strains from inoculum of *S. jonesii* supplied to industry by QPIF

The aim of this experiment was to enrich for DHP degrading bacteria in the inoculum of *S. jonesii* supplied to industry by QPIF, using the techniques of Allison *et al.* (1990 & 1992). This strategy should aid in the isolation of DHP degrading bacteria if the diversity and abundance of background bacteria can be restricted and the DHP degraders elevated. Hence, choosing media for culturing is an important step. The anaerobic techniques of Hungate (1969) as modified by Bryant (1972) were applied in the preparation of media for enrichment. A range of nutrient media was trialled to enrich bacteria that could degrade DHP. Basal medium was adjusted by changing the quantity and type of amino acid source (protein digests such as yeast extract, trypticase and casein), the amount and type of substrates (mimosine, 2,3-DHP and/or 3,4-DHP) and type of buffer systems (PIPES or Na_2CO_3) (Table 1). Two of those media (DHP7 and DHP8) were similar to media 2,3A and 3,4A reported in Allison *et al.* studies (1990 & 1992) which were used to successfully isolate *S. jonesii*. In addition, a basal medium similar to that in Table 1 was prepared except that high amounts of protein digests or amino acids were added with clarified 30% rumen fluid and a Na_2CO_3 buffer. Protein digests or amino acids were tryptone (Allison *et al*., 1990), histidine, arginine (McSweeney *et al.*, 1993), phenylalanine (Rincon *et al*., 1998), soybean (plant protein), yeast extract, peptone and leucaena leaves (Table 2). Degradation of DHP was estimated by the colorimetric ferric chloride test described above.

Antibiotic sensitivity An antibiotic sensitivity experiment was also performed to enrich the DHP degrading bacteria in cultures and to test the antibiotic sensitivity of *S. jonesii.* It was proposed

that certain antibiotics would kill or inhibit growth of some background bacteria but favour *S. jonesii* with the expectation that *S. jonesii* in the QPIF inoculum would increase relative to the other bacteria for ease of isolation. Allison *et al*. (1992) mentioned that *S. jonesii* was resistant to some antibiotics which were used in this experiment. Five antibiotics including vancomycin, streptomycin, colistin, and chloramphenicol were added into 22 tubes of the medium DHP4 as shown in Table 3.

Enrichment process Frozen and fresh *in vitro* inoculum obtained from QPIF laboratories at Yeerongpilly in March 2009 and frozen *S. jonesii* (strain 78-1) was thawed and inoculated (1-2%) into the different media described above. Cultures were transferred after 3 to 6 days when the colorimetric test showed that DHP had been degraded. After several transfers, the serial dilution to extinction principle was applied to verify relative proportions of *S. jonesii* compared to all other microorganisms in the enriched culture using the real time PCR primers for *S. jonesii* as described above in conjunction with universal bacterial primers to determine relative abundance of *S. jonesii* to total bacteria in the culture.

Table 1. Components of different media used to enrich DHP degrading bacteria

Media Components	DHP1	DHP2	DHP3	DHP4	DHP5	DHP6	DHP7	DHP8
							Allison	Allison
							et al	*et al*
Reference		TRF					1990	1990
Undefined								
Clarified rumen fluid (ml)	300	900	300	300	300	300	300	300
Carbon&Nitrogen								
Yeast extract (g)	0.02	0.02	0.02	5	0.04	5		
Casitone (g)				5		5		
Casamino acids (g)	0.1	0.1	0.1		0.2			
Leucaena blend (g)	7500	7500						
Mimosine			0.396					
3,4-DHP (final 2.5mM) (g)	0.2775	0.2775	0.2775	0.2775	0.2775		0.2775	
2,3-DHP (final 2.5mM) (g)						0.2775		0.2775
Buffer&Macro Minerals								
PIPES (100mM stock) (ml)	100	100	100		100			
NaHCO3 (g)	6	6	6		6			
Na2CO3 (g)				4		4	4	4
Mineral solution 2 (ml)				37.5		37.5	37.5	37.5
Mineral solution 3 (ml)				37.5		37.5	37.5	37.5
Mineral PA2 (ml)	100		100	0	100			
Additionals								
Trace elements (ml)	1		1	1	1	1	1	1
10X VFA mix 1 (ml)	100		100		100			
Haemin (ml)	1		1	1	1	1	1	1
L-Cysteine HCl-H_2O (g)	1		1	1	1	1	1	1
Resazurin (ml)	1	1	1	1	1	1	1	1
Vitamin solution (ml)	1	1	1	1	1	1	1	1
pH (before autoclave)	6.7	6.7	6.7	6.5	6.7	6.5	6.5	6.5
Volume (ml)	1000	1000	1000	1000	1000	1000	1000	1000

Table 2. Amino acids or protein digests added to basal media

Media	Amino acids or protein digests (g per 1000ml)
P1. Tryptone	Tryptone 5
P2. Soy bean	Soy bean 3
P3. Yeast extract	Yeast extract 5
P4. Peptone	Peptone 5
P5. Casamino acid	Casamino acid 5
P6. Arginine	Arginine 5
P7. Histidine	Histidine 5
P8. Phenylalanine	Phenylalanine 5
P9. Tryptone + Histidine + Arginine	Tryptone (5g) + Histidine (5g) + Arginine (5g)
P10. Basal media	None
P11. DHP4	Yeast extract 0.5 + Casitone 0.5

Table 3. Type and quantity of antibiotics added into media DHP4

DHP4+antibiotics	Streptomycin (μg/ml)	Vancomycin (μg/ml)	Colistin (μg/ml)	Chloramphenicol (μg/ml)
A1	25			
A2		8		
A3			20	
A4				15
A5	25			15
A6		8	20	
A7	25	8	20	15
A0 (control)				

4 Results and Discussion

4.1 *In vitro* testing of *S. jonesii* inoculum to determine effectiveness of DHP degradation

There was no degradation of either isomer of DHP in cultures inoculated with frozen rumen fluid from Lansdown Research Station, nor frozen inoculum from the QPIF fermenter. The rumen inocula from Banana cattle rapidly converted 1-2 mM of 3,4-DHP to 2,3-DHP within 72 h but 2,3-DHP was not metabolised further. Furthermore, cultures from Banana cattle grown on media with 2,3-DHP did not degrade this isomer. However, inocula from two of these cattle completely degraded 3,4-DHP without 2,3-DHP accumulating when grown in medium which contained higher concentrations of clarified sterile rumen fluid (BRF media). Molecular PCR techniques identified the presence of *S. jonesii* in enriched samples of QPIF inocula, Lansdown rumen fluid and Banana rumen fluid. They also confirmed that all the imported strains were *S. jonesii*. This indicated that positive molecular identification occurred for strains that did not degrade DHP, partially degraded DHP or fully degraded DHP when grown on synthetic media.

The four individual strains of *S. jonesii* obtained from Dr Milt Allison at the University of Iowa (strains 78-1, 147-1, 100-6 and 113-4) were able to degrade either one or both 3,4-DHP and 2,3-DHP at various rates and capabilities in the modified BM10 media.

The type strain 78-1 which was imported from the American Type Culture Collection initially did not degrade either isomer of DHP even though it was grown on DHP medium and passaged multiple times over a 2 month period at CSIRO. However, it eventually regained the ability to completely degrade 3,4-DHP and 2,3-DHP but did not always degrade 2,3-DHP when provided as the sole DHP source. The identity and purity of this strain as *S. jonesii* has been verified through DNA sequence analysis.

The irreversible loss of capability to degrade DHP has been reported previously (Dominguez-Bello *et al*., 1997). The *S. jonesii* strains imported from Dr Milt Allison all degraded 3,4-DHP and 2,3-DHP except strain 78-1 which only degraded 3,4-DHP.

Appendix 1
The results of experiments conducted by QPIF on the degradation of mimosine, 3,4-DHP and 2,3-DHP by fresh fermenter liquor is attached. This liquor is frozen and is used by the cattle industry as a source of *S. jonesii* inoculum (QPIF inoculum). These studies demonstrate that fresh QPIF inoculum was capable of degrading mimosine, 3,4-DHP and 2,3-DHP but the degradation of 3,4-DHP did not occur consistently in all trials.

4.2 Development of real time PCR assay

Analysis of sequences generated from the qPCR assay using the *S. jonesii* primers (Primer 998F – 1091R) and the genomic DNA from a rumen digesta sample showed 100% identity to this microorganism of interest and the melt curves for the amplicons from rumen samples and pure cultures of *S. jonesii* were identical demonstrating the specificity of the PCR primers (Figure 1). The standard curve for detection of *S. jonesii* was linear over the range tested (10^7-10^1 cells) (Figure 2). Furthermore, the PCR primers did not cross react with a rumen bacterial isolate from the ‘Synergistes’ division which was a different species to the target bacterium but is the closest isolated relative to *S. jonesii* that does not degrade toxins in leucaena. This further demonstrated the specificity of the primers to *S. jonesii.* The 16S rDNA sequence was identical for all four strains of *S. jonesii* which are available in culture collections.

Figure 1a. Dissociation curves for PCR products generated from genomic DNA isolated from a pure culture of *S. jonesii* (Primer 998F – 1091R).

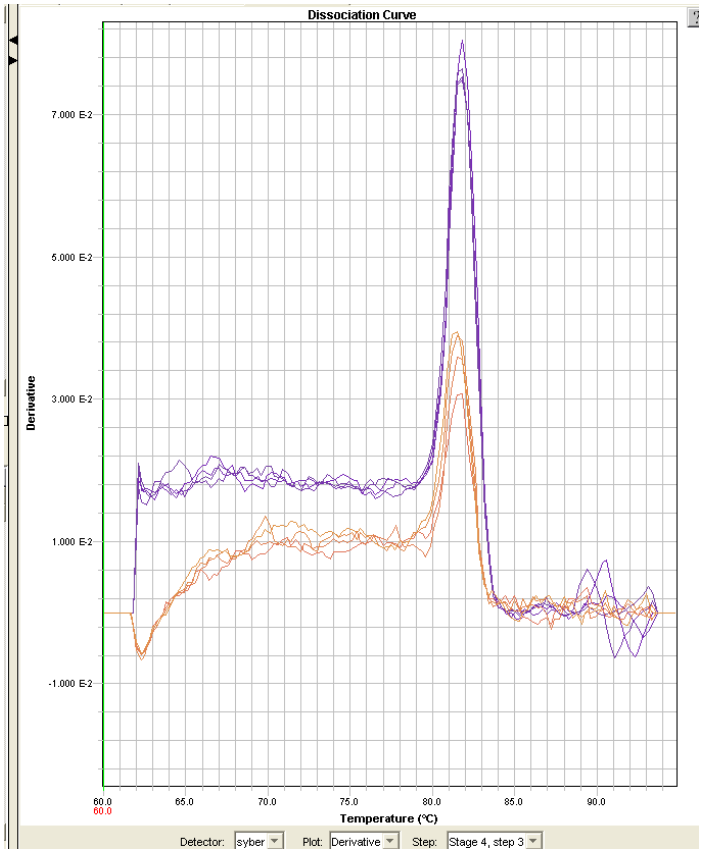

Figure 1b. Dissociation curves for PCR products generated from genomic DNA isolated from a pure culture of *S. jonesii* (pink) and a mixed rumen microbial population (brown).

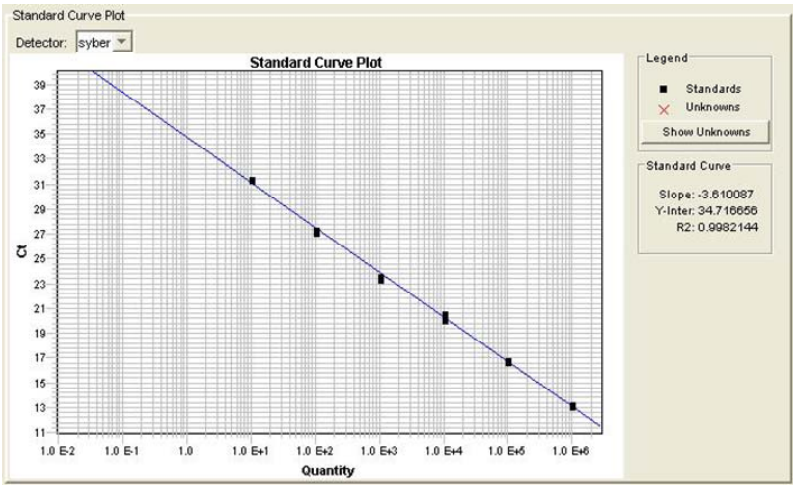

Figure 2. Standard curve plot of log10 DNA template dilutions (represented as number of *S. jonesii* cells) verses Ct value.

In conclusion, the PCR primers designed to target *S. jonesii* are specific for this species of bacterium and detect all current isolated strains of *S. jonesii.* The close identity of these isolates at the level of the 16S rRNA gene precluded the design of primers to differentiate these strains and thus presumably other uncultured strains of *S. jonesii*.

4.3 Enrichment and isolation of *S. jonesii* strains from inoculum of *S. jonesii* supplied to industry by QPIF

Enrichment of basal media. Eight types of media were initially inoculated in an attempt to enrich DHP-degrading microorganisms. Except for media DHP4&6 (Table 1), the other media were generally nutrient-poor containing only substrates (mimosine and/or DHP) and either none or very low amounts of protein digests (yeast extract) as nutrients for bacterial growth. Three media including DHP1, DHP3 and DHP8 showed signs of degradation (up to 10%) after 3 days but no further degradation was detected 60 days later. Cultures of those media were further transferred into new tubes. Meanwhile, in 5 other media, over 60 days, colorimetric assays could not detect any change in concentration of mimosine, 3,4-DHP and/or 2,3-DHP. Despite the similarity of media DHP7 and DHP8 with media 3,4A and 2,3A (Allison *et al*., 1990; Allison *et al*., 1992), these media failed to enrich the DHP degrading bacteria.

The PCR test indicated that in media DHP1&3, *S. jonesii* was present at a concentration of one in several thousand background bacteria. In other words, to isolate *S. jonesii* bacterium from these cultures would require at least several thousand colonies to be picked. That means *S. jonesii* was present in QPIF inoculum, but at quite a low proportion. The lower proportion of *S. jonesii* in the other enriched cultures indicated that efforts to significantly enrich DHP degrading bacteria using poor nutrient media were not successful and in fact promoted the background bacteria.

Enrichment with media containing high levels of protein digests or amino acids. Except media P10, (basal media), all other media used in this experiment contained high amounts of protein digests or amino acids which could be alternative energy sources to 3,4-DHP for bacterial growth. No degradation could be detected in cultures inoculated with QPIF inoculum over 60 days. Pure cultures of *S. jonesii* in media containing tryptone, yeast extract, peptone (P1, P2, P4, P9 and P11) completely degraded DHP within 4 days. Pure cultures of media P5 (casamino acid) and P8 (phenylalanine) degraded DHP partly and the colorimetric assay turned blue (indicating more 2,3-DHP than 3,4-DHP in the cultures). Results from pure cultures P5, P6, P7, P8 and P10 suggested that *S. jonesii* did not degrade DHP when histidine, arginine or phenylalanine were the sole source of amino acids.

Real-time PCR results suggested that *S. jonesii* was present in the cultures started with QPIF inoculum at a ratio of about 1:6000 compared to other bacteria and this ratio improved to 1:5000 in the P4 medium. In other words, to isolate one *S. jonesii* bacterium, it would be necessary to pick 5000 colonies on an agar growth plate. In conclusion, the results of DHP degradation and growth from pure cultures suggested that the *S. jonesii* was able to grow well on many substances present in protein digests. However, increased nutrient levels in media at times promoted growth and relative proportion of other fast growing bacteria in the mixed cultures. Media P4 (peptone) and P11 (DHP4) seemed to both support DHP degradation in pure cultures and elevate the number of *S. jonesii* in the mixed cultures.

Antibiotic enrichment. Pure strain 78-1 was used to confirm the resistance of *S. jonesii* to vancomycin, streptomycin and colistin. However, pure cultures containing chloramphenicol did not grow or degrade DHP which was different from the Allison *et al.* (1992) study which reported the resistance of *S. jonesii* to chloramphenicol. All antibiotic enrichment cultures with QPIF inoculum did not degrade DHP. Real-time PCR results indicated *S. jonesii* was highest in the cultures A4, A5 and A7 where chloramphenicol was present even though DHP did not degrade.

This probably indicated that chloramphenicol inhibited all the bacteria in the QPIF inoculum including *S. jonesii* and thus DHP degradation would not occur. Thus abundance of *S. jonesii* was highest in the QPIF inoculum and enrichment in the presence of antibiotics did not increase *S. jonesii* relative to the background bacteria. In this experiment, the QPIF inoculum contained one *S. jonesii* to about 6000 other bacteria.

Although vancomycin, colistin and streptomycin did not enrich for *S. jonesii* in the mixed culture QPIF inoculum these antibiotics were used in agar isolation plates to reduce background bacteria once the mixed cultures were diluted and applied to the agar medium. However a pure isolate was not obtained after picking several hundred colonies from agar plates spread with an inoculum from an *S. jonesii* enrichment culture.

In conclusion, the presence of complex amino acids in the medium is important to sustain the growth of *S. jonesii* relative to background bacteria, and pure cultures of *S. jonesii* rapidly degraded DHP in this medium. However we were unable to demonstrate significant degradation of DHP in this medium with the QPIF inoculum even though *S. jonesii* was present and appeared to be growing in significant numbers. This suggests that current *in vitro* enrichment culture techniques for evaluating efficacy of DHP degradation of mixed rumen cultures are inconsistent and need to be interpreted with caution.

5 Success in Achieving Objectives

The project was partially successful in achieving its objectives.

Objective 1.
In our work, the assays of effectiveness of the QPIF inoculum involving use of modified basal media and buffered rumen fluid, as published by the early workers with *S. jonesii*, failed to demonstrate functional effectiveness of the QPIF inoculum even though pure strains of *S. jonesii* effectively degraded DHP in the same media. Subsequent work that attempted to enrich *S. jonesii* on a range of modified media was not successful. More research is required.

However, in parallel work, the effectiveness of the QPIF inoculum in degrading mimosine, 3,4-DHP and 2,3-DHP was demonstrated by QPIF assays of undiluted unfrozen bacterial culture fresh from the fermenter. Whist the results of their assay were variable especially in relation to the degradation of 3,4-DHP, it could be concluded that that *S. jonesii* was present and generally active in the cultures. Subsequent use of this inoculum on properties appears to have proved successful in protecting cattle herds from leucaena toxicity (*B.NBP.0494)*.

Objective 2.
A quantitative real-time PCR was developed to distinguish the presence and enumerate *S. jonesii* in bacterial cultures of pure strains, rumen fluid and *in vitro* produced QPIF inoculum. However, presence and number of *S. jonesii* did not necessarily relate to capacity to degrade the toxins 3,4-DHP and 2,3-DHP.

6 Impact on Meat and Livestock Industry – now & in five years time

The results of this project, together with the results and recommendations of a linked project (B.NBP.0494), will strongly and positively impact the northern Australian grazing industry. Leucaena toxicity, caused by high levels of the toxic amino acid mimosine in young growth of leucaena, is generally subclinical in nature and affects approximately half of all cattle herds

grazing leucaena pastures (NBP.340). Most graziers are unaware of the existence of the problem. Previous work has shown that subclinical leucaena toxicity can reduce appetite and thus productivity by up to 30-50% (Quirk *et al*., 1988). Since there are currently >500 leucaena growers with approximately 150,000 ha of leucaena, and half of these graziers are experiencing subclinical toxicity, effective treatment of the toxicity could increase average live weight of cattle by 50 kg/head/year, giving a gross financial valued at approximately $5.4 million annually.

Impact will be immediate as the significance of leucaena toxicity is publicised to graziers and regular monitoring using the University of Queensland developed urine test kit is employed.

7 Conclusions and Recommendations

1. These studies demonstrated that a standardised medium for testing the ability of a rumen inoculum to degrade DHP has not been defined. Currently the most consistent degradation of DHP occurs under conditions where DHP (or leucaena) is added to an undiluted fresh rumen or *in vitro* fermenter inocula.

2. The viability and effectiveness of *S. jonesii* in QPIF inoculum which has been frozen, stored and thawed has not been established in this work but should be investigated as a quality control measure in the production and delivery chain for the inoculum.

3. Isolation of further strains of *S. jonesii* is essential to determine whether there is variation in DHP degrading ability among members of this species. New isolation techniques need to be developed since published procedures appear inefficient or ineffective. Molecular methods to distinguish *S. jonesii* strains will be more complex than the currently developed real-time PCR.

4. It is recommended that graziers continue to use the QPIF inoculum produced in the anaerobic fermenter, at least in the short term. In the longer-term, methods need to be developed for the production and distribution of inoculum containing the most effective pure strain(s).

8 Bibliography

Allison MJ, Hammond AC, Jones RJ (1990) Detection of rumen bacteria that degrade toxic dihydroxypyridine compounds produced from mimosine. *Applied and Environmental Microbiology* **56**, 590-594

Allison MJ, Mayberry WR, McSweeney CS, Stahl DA (1992) *Synergistes jonesii*, gen. nov., sp. nov.: A rumen bacterium that degrades toxic pyridinediols. *Systematic and Applied Microbiology* **15**, 522-529.

Bryant, MP (1972) Commentary on the Hungate technique for culture of anaerobic bacteria. *American Journal of Clinical Nutrition* **25**, 1324–1328.

Caldwell DR, Bryant MP (1966) Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. *Applied Microbiology* **14**, 794-801.

Dominguez-Bello MG, Lovera M, Rincon MT (1997) Characteristics of dihydroxypyridine-degrading activity in the rumen bacterium *Synergistes jonesii*. *FEMS Microbiology Ecology* **23**, 361-365.

Ford CW, Megarrity RG, Meehan GV (1984) 2,3-DHP, a novel mimosine metabolite. *Leucaena Research Reports* **5**, 2.

Hammond AC, Allison MJ, Williams MJ, Prine GM, Bates DB (1989) Prevention of leucaena toxicosis of cattle in Florida by ruminal inoculation with 3-hydroxy-4-(1H)-pyridone-degrading bacteria. *American Journal of Veterinary Research* **50**, 2176-2180.

Hungate, RE (1969) A roll tube method for cultivation of strict anaerobes. In *'Methods in Microbiology*' Vol. 3 (eds Norris IR, Ribbons EW). pp. 117–132. (Academic Press: New York)

McSweeney CS, Allison MJ, Mackie RI (1993) Amino acid utilization by the ruminal bacterium *Synergistes jonesii* strain 78-1. *Archives of Microbiology* **159**, 131-135.

Megarrity, RG (1978) An automated colorimetric method for mimosine in Leucaena leaves. *Journal of the Science of Food and Agriculture* **29**, 182-186.

Pratchett D, Jones RJ, Syrch FX (1991) Use of DHP-degrading rumen bacteria to overcome toxicity in cattle grazing irrigated leucaena pasture. *Tropical Grasslands* **25**, 268-274.

Quirk MF, Bushell JJ, Jones RJ, Megarrity RG, Bulter KL (1988) Live-weight gains on leucaena and native grass pastures after dosing cattle with rumen bacteria capable of degrading DHP, a ruminal metabolite from leucaena. *Journal of Agricultural Science, Cambridge* **111**, 165-170.

Rincon MT, Allison MJ, Michelangeli F, De Sanctis Y, Dominguez-Bello MG (1998) Anaerobic degradation of mimosine-derived hydroxypyridines by cell free extracts of the rumen bacterium *Synergistes jonesii*. *FEMS Microbiology Ecology* **27**, 127-132.

Rincon MT, Dominguez-Bello MG, Lovera M, Romero MR (2000) Degradation of toxic pyridine diols derived from mimosine by rumen bacteria: 1. Microbiological aspects. *Revista Cientifica, Facultad de Ciencias Veterinarias, Universidad del Zulia* **10**, 222-232.

Tangendjaja B, Wills RBH (1980) Analysis of mimosine and 3-hydroxy-4(1H)-pyridone by high-performance liquid chromatography. *Journal of Chromatography* **202**, 317-318.

9 Appendices

9.1 Appendix 1 - Report prepared by Rumen Ecology Unit QPIF

Report prepared by Rumen Ecology Unit QDPI&F

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We have recently (completed 08/07/2008) undertaken three more analyses of the efficacy of the fermentor produced inoculum and found some variability in response but overall are still satisfied that this product is efficacious after 13 years of production.

Experiment 1:

- Control tubes (no additions) were virtually unchanged throughout the 72h cycle of the experiment. A low background level of 3,4 DHP was maintained throughout.
- Added mimosine was rapidly converted to 3,4 DHP (complete conversion in 6 h).
- In this experiment further conversion of 3,4 DHP was not apparent although it did appear that a small amount of 2,3 DHP had been produced.
- It should be noted that the fluid used in this experiment was taken at the end of the fermentation period and that technical difficulties had arisen a couple of days earlier that may have impacted on some microbial populations.
- Added 2,3 DHP was virtually completely degraded within 72h of addition.

Experiment 2:

- Both experiment 2 and 3 were based on fluid from the same fermentor run. The fluid used in Experiment 2 was taken at the earliest point in the run that we thought was reasonable, on day 4, which is the day prior to the day that harvesting normally begins.
- Control tubes (no additions) were virtually unchanged throughout the 72h cycle of the experiment. A low background level of 3,4 DHP was maintained throughout.
- Added mimosine was rapidly converted to 3,4 DHP (almost complete conversion in 5 h).
- In this experiment further conversion of 3,4 DHP was not apparent although a small amount of 2,3 DHP had been produced by the end of the experiment at 72h. In hindsight (see experiment 3 below), this experiment was probably concluded too early.
- Added 2,3 DHP was completely degraded within 72h of addition.

Experiment 3:

- Experiment 3 used fermentor fluid from the same run as Experiment 2 except that the fluid was taken from later in the run, on day 7.
- Control tubes (no additions) were largely unchanged throughout the 96h cycle of the experiment. A low background level of 3,4 DHP was maintained throughout. However, toward the end of the experiment this residual 3,4 DHP did appear to reduce slightly and a small amount of what appeared to be 2,3 DHP was present.
- The addition of mimosine resulted in a scenario that was very similar to that observed in 2006. Added mimosine was converted completely to 3,4 DHP within 5h. 3,4 DHP was completely degraded and converted to 2,3 DHP over the next 72h. The 2,3 DHP peak formed as 3,4 DHP disappeared and was itself completely degraded within 120h (not shown).
- Added 2,3 DHP was completely degraded within 72h of addition.

As experiment 3 used fluid that was three days further into the fermentation than in experiment 2, it could be expected that the population density of those bacteria with 3,4 DHP degrading ability was too low in experiment 2 to effect degradation within the time scale used. Day 4 was a very minimal time to expect a slow growing bacterium to reach high density in the fermentor, and at 72h there were signs that 2,3 DHP had been starting to be produced. Obviously, over the

following three days this population had increased to the level where it could easily degrade 3,4 DHP.

General points from the most recent re-evaluation.

- Mimosine was always rapidly converted to 3,4 DHP (within hours).
- Added 2,3 DHP was always degraded completely and the rate of degradation appeared similar in each experiment. It is possible that this enzymic activity is constitutively expressed (whether or not one or more strains of S. jonesii are involved).
- Results for the degradation of 3,4 DHP are more variable and may be dependent upon bacterial density and/or DHP concentration. This enzymic activity may have to be induced.
- In control fermentations, a low background level of 3,4 DHP was maintained throughout. As with the previous dot point this may suggest that there is a threshold concentration, below which 3,4 DHP is not degraded. This is in agreement with the classical literature (Jones and Megarrity, 1986) which showed that in a goat that had been inoculated it took 14 days to reduce DHP in the urine to a very low level but that a low level of DHP was still detectably excreted by the end of the experiment, at 58 days (refer to Fig. 3 in the paper).
- The current fermenter based inoculum is capable of degrading mimosine and both isomers of DHP to completion.

From the experiment 3 fermentation we have harvested a large number of “starter” cultures that will be stored at -80oC and will be used to initiate each new fermentation run.

As an on-going improvement to our quality assurance program, in addition to the PCR assay to detect the presence of S. jonesii, we will use HPLC to analyse the degradation of mimosine, 3,4 DHP and 2,3 DHP in each batch of fermenter fluid. The costs of this additional QA will be re-couped through increasing the price of the inoculum to producers (which is supplied at a cost-recovery rate).