# final report

Project code: B.CCH.1024

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Date published: May 2012

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

# Variability in methanogenic potential of the pasture legume Biserrula pelecinus

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

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**Abstract**
A cultivar of *Biserrula pelecinus* (biserrula) has been demonstrated to have a ten-fold lower methanogenic potential in the rumen than lucerne (*Medicago sativa*) when grown in the glasshouse and tested in batch culture *in vitro*. However, it also reduced overall microbial fermentation. A core collection of biserrula accessions has recently been established and the main aim of this study was to investigate this core collection for candidates that may have low methanogenic potential without affecting ruminal fermentation more generally. The initial observations of antimethanogenic bioactivity in biserrula were based on plants grown in a glasshouse, under controlled conditions. The second aim of this project was to investigate the antimethanogenic potential of biserrula in more detail by screening samples of biserrula grown in-field under different environmental conditions, and at various stages of growth *in vitro*, to determine the degree of variation in its methanogenic potential. We also examined the level of inclusion of biserrula needed within a mixed substrate to reduce methane without reducing overall fermentation as well as the persistency of the effects. All samples taken during this experiment were screened using an *in vitro* batch culture system based on rumen fluid collected from sheep. The parameters that were measured as indicators of the bioactive effects of biserrula were: methane, total gas, VFA and ammonia production and the acetate to propionate ratio. The most bioactive accession was also examined at different levels of inclusion in a mixed substrate for fermentation in batch culture and then extended to a continuous RUSITEC culture systems to better mimic the ruminal environment and test the persistency of the bioactive effects. The most bioactive accession was also used to examine crude extracts of biserrula using different solvents to help identify the fractions that contained the bioactivity. There was significant variation in methanogenic potential amongst the cultivars within the core collection, but they all reduced both methane and overall microbial fermentation. However, biserrula reduced methane production when it was used as part of a mixed diet and affected methane and most of the fermentation parameters in a favorable way when it was included as 50% of the diet. We have preliminary evidence that these results occur in continuous culture and persist for over a week. There was a definite effect of eco-geographical factors and the age of plant on bioactivity and testing the plant extracts suggested that there is more than one compound responsible for the bioactivity we observed in the plant. Our results will be used to determine which cultivars should be evaluated *in vivo* and identify how these cultivars are to be integrated into farming systems of the future.

**Executive summary**
Ruminant production in Australia relies on extensive, forage-based feeding systems, with various conventional legumes such as subclover and red clover (Nichols et al., 2007). Recently, the potential to broaden the feed base with novel species has encouraged the inclusion of some other legumes. *Biserrula pelecinus L.* (biserrula) is an annual legume that originates from Mediterranean countries and has been considered as a pasture legume for the wheat belt of southern Australia (Loi *et al*. 1997). Recently, we demonstrated that one cultivar had a ten-fold lower methanogenic potential in the rumen than lucerne (*Medicago sativa*) when grown in the glasshouse and tested in batch culture *in vitro*. However, it also reduced overall microbial fermentation. In a preliminary study of some common pasture forages in Australia we found that biserrula, when fermented by rumen microbes, produced nearly ten times less methane than the other legumes tested, such as *Trifolium subterraneum* L. and *Medicago sativa* L. Biserrula also produced some other favourable fermentation profiles, such as reduced acetate to propionate ratios. A core collection of biserrula accessions has recently been established and the main objective of this study was to investigate this collection for candidates that inhibited, or reduced significantly, methane production without affecting overall fermentation. We tested the bioactivity of plants under field rather than glasshouse conditions and examined whether phenology affected bioactivity and, in particular, whether we could reduce the amount of methane produced without affecting overall fermentation simply by including biserrula as part of a mixed substrate rather than the sole source of substrate. We also attempted to obtain preliminary data about the compounds that may be involved in the bioactivity associated with biserrula.

Our approach was to build on our preliminary results about the beneficial bioactive properties of *B. pelecinus* and investigate its potential to be integrated better into grazing systems and developed into a CFI methodology. We used both batch and continuous culture (RUSITEC) *in vitro* fermentation systems to screen and identify the most promising cultivars, dose and persistency effects and to test crude aqueous and solvent extracts of biserrula to help identify the compounds responsible for the bioactivity. The batch system was used for large-scale screening purposes and to measure the variability of the anti-methanogenic bioactivity of biserrula as a function of where it was planted and its phenology. The RUSITEC system was used to confirm the bioactive effects in a continuous culture system that more closely mimics the rumen and to measure persistency. Samples were also taken during the RUSITEC experiment that will be analysed using molecular techniques to study the effects on microbial ecology and gain some insight into the mechanism behind the bioactivity. A range of crude extracts was obtained from biserrula using various organic solvents and water, which were tested for antimethanogenic effects *in vitro*. The most potent extract was then fractionated further using solvent gradients and these fractions were tested to help narrow down more specifically the fractions that contained the compounds responsible for the antimethanogenic effects. We propagated biserrula in the field at several sites to establish whether the results we obtained using plant material from the glasshouse were replicated when plants were propagated in the field under a less controlled environment. We also collected plant samples at different phenological stages at each field site to establish whether stage of growth influences the antimethanogenic properties of the plant.

We found that there was significant variation in the antimethanogenic properties amongst the cultivars within the core collection, but they all reduced both methane and overall microbial fermentation. To establish whether we could make use of the antimethanogenic potential of biserrula in a practical sense, we tested whether biserrula reduced methane production when it was used as part of a mixed substrate for the *in vitro* fermentation. We found that including biserrula at 50% of the substrate fermented in batch and continuous culture *in vitro* significantly

reduced methane without affecting overall fermentation. In addition, the bioactivity varies according to eco-geographical factors (site) and is affected by the age of the plants at some sites.

The extracts we tested provided evidence that the bioactivity is linked to secondary compounds, which is perhaps not surprising. However, the results did indicate that there was bioactivity presented in different solvent and water extracts (not just in one fraction) and there is likely to be more than one compound responsible for the bioactivity. The bioactivity in the water extract was interesting because it reduced methane without inhibiting overall fermentation. Further purification, isolation and analysis of fractions are needed to identify more specifically the PSC in biserrula responsible for the antimethanogenic bioactivity. Purifying these compounds will make it possible to examine the mechanisms behind the bioactivity we have observed in biserrula.

The results from this project have enabled us to determine which cultivars should be evaluated in more detail at the plant chemistry level and *in vivo* and have also assisted in developing a strategy to start integrating biserrula into future grazing systems that maintain or improve productivity but reduce emissions.

## General background

Ruminant production in Australia relies on extensive, forage-based feeding systems, with various conventional legumes such as subclover and red clover (Nichols et al., 2007). Recently, the potential to broaden the feed base with novel species has encouraged the inclusion of some other legumes. *Biserrula pelecinus L.* (biserrula) is an annual legume that originates from Mediterranean countries and has been considered as a pasture legume for the wheat belt of southern Australia (Loi *et al*. 1997). It is an annual species that germinates in autumn and produces a large number of pods. It has been grown successfully in areas receiving 390 mm average annual rainfall in southern NSW, and 450 mm in northern NSW.

A preliminary study of some common pasture forages in Australia revealed that biserrula, when fermented by the rumen microbes, produced nearly ten times less methane than the other legumes tested, such as *Trifolium subterraneum* L. and *Medicago sativa* L. (Banik et al. submitted). Biserrula also produced some other favourable fermentation profiles, such as reduced acetate to propionate ratios. However, it also inhibited overall gas production but had sustained volatile fatty acid production by the rumen microbes. These observations imply a selective effect of the plant on rumen microbes that may be exploited for development of environmentally-friendly grazing methodologies.

Evaluation of the agromorphological data is often used by plant breeders to select for better cultivars. Intra-specific variation in morphological traits is well documented in forage legumes (Black and Wilkinson, 1963; Dear et al., 1993). These differences are often associated with geographic origin as a set of environmental factors or as genetic factors that influence long-term selection for certain characteristics. While various plant morphology traits are used for plant selection and breeding, the effects of plant morphology in the animals and their gut fermentation have not been studied. The Australian Trifolium Genetic Resource Centre (ATGRC) has a germplasm collection of 279 accessions of biserrula. These collections of native populations and their related rhizobia provide great genetic resource for further breeding and selection within the species. A core collection of biserrula has recently been developed (Ghamkhar *et al.* 2010), providing a structured sample that maximizes the variation with minimum repetitiveness and potentially allows finding more diverse candidates for screening fermentation profiles (i.e. with less detrimental effects on microbial fermentation).

Some recent results on antimethanogenic plants have implied that it is possible to include more potent candidates as a part of a mixed diet to reduce methane while avoiding detrimental effect on rumen fermentation (Li et al. 2010, 2011). The basis of plant bioactivity is often related to the presence of plant secondary compounds (PSC). These compounds are synthesised in response to environmental stressors, and hence variability in plant bioactivity is reported between plants grown at different eco-geographical locations (Durmic *et al*. 2010). Chemical profiles of the plants also vary with age, which in turn may affect bioactivity as the plant gets older.

Finally, developing CFI methodology will require that the plant bioactivity is maintained in the field and expressed in the gut when consumed by animals. Results to date have shown that plants identified as antimethanogenic *in vitro* produce similar fermentation profiles and maintain bioactivity in the field (Durmic *et al*. 2010) and when fed to the animal (Li, pers. comm.).

The overall aim of this project was to examine the bioactivity of biserrula in more detail including the fermentative characteristics, chemical profiles, variability and the optimal dose for reducing methane production without disrupting overall rumen fermentation.

## Section 1. Screening for diversity in morphological traits and rumen fermentation profiles within the core collection of *Biserrula pelecinus*

*Abstract*

Thirty accessions from the core collection of *Biserrula pelecinus* L. were examined for variability in morphology and *in vitro* rumen fermentation, including methane production by rumen microbes. Accessions were grown in a glasshouse for 11 weeks, during which time morphological traits were recorded. Plant material was then harvested and tested in the *in vitro* batch culture for fermentability and methane production. Amongst the accessions tested, there were some marked differences in plant morphology, including stem pigmentation, leaf mid-rib pigmentation, leaflet size and growth habit**.** All accessions had reduced microbial gas methane and ammonia concentrations ($P<0.05$), but not volatile fatty acid production when compared to the control (subclover). There was also significant variation in all fermentability parameters tested amongst accessions ($P<0.05$) and methane production varied from 0.63 mL/g DM (accession 138972) to 8.41 mL/g DM (accession 2004ERI1PEL). There was a significant association between morphological traits and methane production ($P<0.05$), however methane production did not correlate to the other parameters of rumen fermentation measured. In conclusion, there was genetic variability within the core collection of *B. pelecinus* in morphological and fermentability traits. During microbial fermentation of plant material, all accessions caused a significant reduction in methane but also in overall fermentation.

*Introduction*

*In vitro* fermentation of plant material in rumen fluid offers a screening tool that may predict methane output from microbial fermentation plant material in the rumen. However, this methodology is costly, requires specialized equipment and access to surgically-altered animals. In a recent study (Banik, 2010), it was found that some plant morphological traits are also associated with their effect on rumen fermentation. Establishing the links between morphology markers and the effect in the animal would assist plant breeders to make better choices in plant selection for methane mitigation from the livestock.

Overall, the objective of the current study was to i) search for candidate(s) within a core collection of biserrula that can significantly reduce methane without affecting overall microbial fermentation and ii) to find a simple tool for genetic breeding by linking morphological characteristics to the plant effect in the animal.

The specific aims of the current study were to investigate the variation in morphological traits and microbial fermentation of the accessions in the core collection of *B. pelecinus* and examine any association between morphological and fermentability traits within these samples.

*Materials and methods*

*Experimental outline*

Thirty accessions from the core collection of *biserrula* were grown in pots in a glasshouse. Fifteen accessions were grown in a single plot and the other fifteen were replicated in two pots (partial replication). Plants were examined for morphological variability and then harvested at 11 weeks of growing, freeze-dried and analysed for *in vitro* fermentability by rumen microbes using a batch culture.

*Plant material*

A core collection, containing 30 accessions of *B. pelecinus* has recently been developed (Ghamkhar *et. al.,* pers. comm.). Briefly, a total of 279 accessions of this plant that were originally collected from sites in Eritrea, France, Greece, Israel, Italy, Morocco and in the Canary Islands of Spain (Table 1) and conserved in the Australian Trifolium Genetic Resources Centre (ATGRC, Perth, Western Australia) were examined. Thirty accessions were selected based on 18 agro-morphological traits and 22 eco-geographical specifications to develop a subset representing a maximum level of diversity in nature with the lowest level of repetitiveness. This core collection provides a structured sample that maximizes the variation with minimum repetitiveness (Brown, 1995) and potentially allows finding more diverse candidates.

All accessions were obtained from the Germplasm Centre of Department of Agriculture and Food Western Australia (DAFWA) trough collaborative project between UWA and DAFWA. Seeds were scarified prior to sowing and, if required, a petri dish germination procedure was used to ensure plant germination. For each accession, ten seeds/germinated seeds were transferred to individual pots (22 cm diameter) and placed in controlled temperature room, where the temperature will be maintained at 22°C in the day and 10°C at night. After seven days, the pots were transferred to the UWA Phytotron, where they were be kept at 20°C in day/ 15°C in night, and relative humidity of 60%. Specific clustered rhizobia were used for legume nodulation when cotyledons emerged and plants were given adequate moisture throughout the growing stage.

*Morphological traits*

Eight morphological traits, including, time to flower (i.e. 'early', 43-60 days; 'medium', 61-77 days and 'late' > 77 days), colour of flower (violet or white), podding (presence or absence), colour of the pod (violet or green), size of leaflet (small, medium and large), pigmentation of midrib (unpigmented green, pigmented purple and mixed), pigmentation of stem (unpigmented green, pigmented purple and mixed) and growth habit of plant (prostrate or semi-prostrate) were taken at week 11 from date of sowing.

In vitro *fermentability testing*

After scoring morphological traits at 11 weeks, plant material was collected for *in vitro* fermentability testing. The edible parts of the plants (leaf and stem less than 10 cm long) was harvested from individual plants, the material was freeze-dried and ground to pass through a 1.0 mm screen. Material was stored at room temperature in sealed containers until analysis.

*In vitro* testing was done as per protocol of Durmic et al. (2010). Briefly, one day before the experiment, 0.1 g of plant material was prepared in bellco tubes and transferred to an anaerobic chamber (Coy anaerobic chamber; 80% N2 : 10% CO2 : 10% H2) to expel oxygen from the tubes. On the experimental day, rumen fluid was collected 2 h after feeding from two fistulated sheep fed a general maintenance diet. Rumen fluid was strained, pooled and buffered to pH 7.2 using McDougall's buffer. Ten mL of this mix was dispensed into prepared bellco tubes inside the chamber, where the tubes will be sealed with rubber stoppers, crimped and then incubated for 24 h at 39°C, with constant shaking at 50 rpm. At the end of the incubation period, gas pressure was measured using a pressure transducer and 5 mL of headspace gas was transferred to an exetainer tube for subsequent methane analysis by gas chromatography. One mL of the liquid phase was aliquoted for VFA and $NH_3$ analysis.

*Statistical analysis*

A Chi-square ($\chi^2$) test was used along with a contingency table, to get the significance values of different morphological traits. One-way analyses of variance (ANOVA) for all morphological and fermentability traits and correlation coefficients among the traits was calculated using GENSTAT version 12.1 (VSN International Ltd.). Student's t-test in JMP® software (Sall et al., 2005) was used for multiple pair-wise comparisons of the fermentation parameters and significance was accepted at $P < 0.05$.

## Results

*Variability in morphological traits*

Selected morphological traits varied among the different accessions of *B. pelecinus* (Table 1).

Table 2. Qualitative data of the morphological traits of different accessions from the core collection of *Biserrula pelecinus*

| Acession | Country of origin | Time to flower | Flower color | Presence of pod | Pod color | Leaflet size | Color of midrib | Color of stem | Growth habit |
|---|---|---|---|---|---|---|---|---|---|
| 2006MAR29PEL | Morocco | Medium | Violet | Present | Violet | Medium | Unpigmented | Unpigmented | Prostrate |
| 139058 | Greece | Late | N/A | Absent | N/A | Large | Unpigmented | Unpigmented | Prostrate |
| 2004ERI37PEL | Eritrea | Early | White | Present | Green | Small | Unpigmented | Pigmented | Semi-prostrate |
| 2004ERI56PEL | Eritrea | Early | White | Present | Green | Medium | Unpigmented | Mixed | Semi-prostrate |
| 2005GRC77PEL | Greece | Late | N/A | Absent | N/A | Large | Unpigmented | Unpigmented | Prostrate |
| 2004ERI1PEL | Eritrea | Early | White | Present | Green | Medium | Pigmented | Mixed | Semi-prostrate |
| 2004ESP19PEL | Spain | Early | White | Present | Green | Large | Unpigmented | Mixed | Semi-prostrate |
| 2004ESP39PEL | Spain | Late | N/A | Absent | N/A | Large | Pigmented | Pigmented | Prostrate |
| 2004ESP64PEL | Spain | Late | N/A | Absent | N/A | Medium | Pigmented | Pigmented | Prostrate |
| 143415 | Spain | Medium | Violet | Absent | N/A | Large | Unpigmented | Mixed | Prostrate |
| 143464 | Italy | Late | N/A | Absent | N/A | Large | Unpigmented | Mixed | Prostrate |
| 143467 | Italy | Medium | Violet | Absent | N/A | Medium | Unpigmented | Unpigmented | Prostrate |
| 143469 | Italy | Early | Violet | Absent | N/A | Large | Unpigmented | Mixed | Prostrate |
| 143474 | Italy | Late | N/A | Absent | N/A | Small | Unpigmented | Unpigmented | Prostrate |
| 95ITA7/16PEL (MAURO) | Italy | Late | N/A | Absent | N/A | Medium | Unpigmented | Unpigmented | Prostrate |
| 143267a | Italy | Late | N/A | Absent | N/A | Medium | Unpigmented | Unpigmented | Prostrate |
| 143267b | Italy | Late | N/A | Absent | N/A | Large | Unpigmented | Unpigmented | Prostrate |
| 139049.2 | Greece | Medium | Violet | Absent | N/A | Medium | Mixed | Mixed | Prostrate |
| 139362 | Morocco | Medium | Violet | Present | Violet | Medium | Unpigmented | Unpigmented | Prostrate |
| 139363 | Morocco | Medium | White | Present | Green | Medium | Unpigmented | Unpigmented | Prostrate |
| 138972 | Morocco | Late | N/A | Absent | N/A | Large | Unpigmented | Unpigmented | Prostrate |
| 139026 | Greece | Late | N/A | Absent | N/A | Medium | Mixed | Unpigmented | Prostrate |
| 93FRA4PEL | France | Late | N/A | Absent | N/A | Large | Unpigmented | Mixed | Semi-prostrate |
| 93ITA45PEL | Italy | Late | N/A | Absent | N/A | Small | Pigmented | Mixed | Prostrate |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GEH71PEL | Greece | Late | N/A | Absent | N/A | Large | Unpigmented | Unpigmented | Prostrate |
| GEH77PEL | Greece | Late | N/A | Absent | N/A | Large | Unpigmented | Unpigmented | Prostrate |
| CASBAH | Morocco | Late | N/A | Absent | N/A | Large | Unpigmented | Unpigmented | Prostrate |
| 2006ISR20PEL | Israel | Late | N/A | Absent | N/A | Medium | Mixed | Unpigmented | Prostrate |
| 2006MAR22PEL | Morocco | Late | N/A | Absent | N/A | Large | Unpigmented | Unpigmented | Semi-prostrate |
| 2004ERI38PEL | Eritrea | Early | White | Present | Green | Medium | Unpigmented | Pigmented | Semi-prostrate |

In vitro *fermentability*

When compared to the control legumes (subterranean clover and red clover), all fermentation parameters measured, including gas pressure, methane production, acetate to propionate ratio and ammonia concentrations (except for one accession) were significantly lower ($P < 0.001$) in all biserrula samples (Table 2 and Figure 1). This inhibition in methane was as high as 98%, but reduction in gas did not exceed 50% in any of the biserrula samples, while reduction in VFA was not significant and not more than 10%.

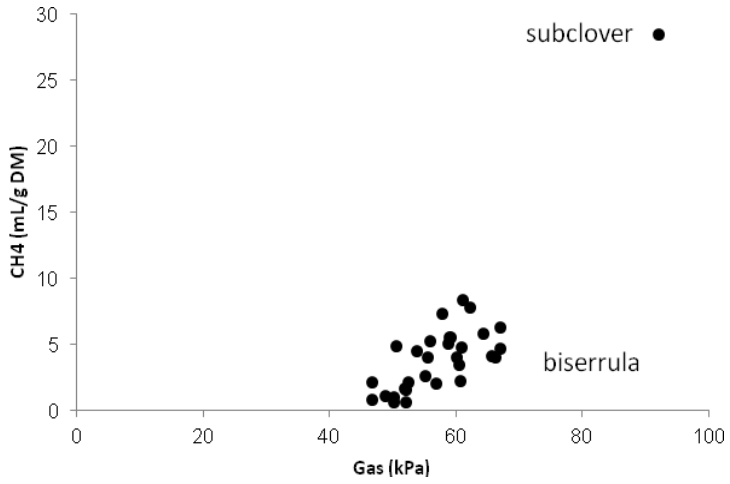



Figure 1. Distribution of samples according to gas and methane production in all samples

All *in vitro* fermentation parameters varied between different accessions of biserrula ($P < 0.001$). Methane production was the lowest in accession 138972 (0.63 mL/g DM) and the highest in accession 2004ERI1PEL (8.41 mL/g DM). Gas production and VFA were lowest in 143267b (47 kPa and 66 mmol/L), while the lowest acetate to propionate ratio and $NH_3$ were in 139058 (1.31 and 139 mg/L). All accessions except for one produced acetate to propionate ratios below 2.0, while all except for GEH71PEL had $NH_3$ below 400 mg/L.

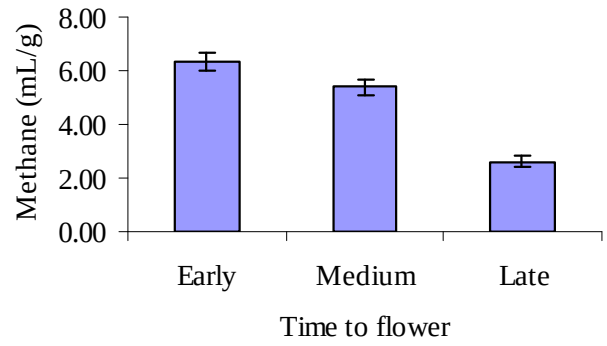
Table 2: *In vitro* fermentation parameters of the different accessions from the core collection of *Biserrula pelecinus* and the two legume controls

| Accession | Country of origin | Gas (kPa) | | Methane (mL/g DM) | | VFA (mmol/L) | | A : P | | NH3 (mg/L) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Controls | | | | | | | | | | | |
| subterranean clover | - | 92 | b | 28.48 | b | 73 | abcd | 3.01 | a | 440 | a |
| red clover | - | 118 | a | 36.18 | a | 42 | e | 3.09 | a | 152 | jk |
| Biserrula | | | | | | | | | | | |
| 2004ERI37PEL | Eritrea | 56 | hij | 3.99 | hi | 69 | bcd | 1.45 | de | 175 | ijk |
| 2004ERI56PEL | Eritrea | 62 | def | 7.80 | cd | 72 | abcd | 1.59 | cde | 154 | jk |
| 2004ERI1PEL | Eritrea | 61 | efg | 8.41 | c | 69 | bcd | 1.55 | de | 283 | defg |
| 2004ERI38PEL | Eritrea | 64 | cde | 5.84 | ef | 75 | abcd | 1.50 | de | 301 | cdefg |
| 93FRA4PEL | France | 52 | ijkl | 1.57 | klmno | 67 | bcd | 1.38 | de | 317 | bcdef |
| 139058 | Greece | 67 | cd | 4.70 | fgh | 78 | abc | 1.31 | e | 139 | k |
| 2005GRC77PEL | Greece | 57 | ghi | 2.04 | klm | 73 | abcd | 1.35 | de | 142 | k |
| 139049.2 | Greece | 66 | cd | 4.13 | hi | 73 | abcd | 2.41 | b | 206 | hijk |
| 139026 | Greece | 59 | fgh | 5.08 | fgh | 72 | abcd | 1.46 | de | 356 | abcd |
| GEH71PEL | Greece | 51 | jklm | 4.89 | fgh | 70 | bcd | 1.59 | cde | 402 | ab |
| GEH77PEL | Greece | 52 | jkl | 1.64 | klmn | 74 | abcd | 1.45 | de | 348 | bc |
| 2006ISR20PEL | Israel | 66 | cd | 4.00 | hi | 69 | bcd | 1.34 | e | 300 | cdef |
| 143464 | Italy | 60 | efg | 4.02 | hi | 73 | abcd | 1.43 | de | 204 | hijk |
| 143467 | Italy | 56 | hij | 5.24 | fg | 74 | abcd | 1.54 | de | 330 | bcd |
| 143469 | Italy | 55 | hij | 2.65 | jk | 74 | abcd | 1.37 | de | 199 | hijk |
| 143474 | Italy | 47 | m | 2.16 | k | 69 | cd | 1.52 | de | 315 | cde |
| 95ITA7/16PEL (MAURO) | Italy | 61 | efg | 3.44 | ij | 77 | ab | 1.39 | e | 266 | efgh |
| 143267a | Italy | 50 | klm | 1.00 | lmno | 69 | bcd | 1.39 | de | 231 | fghij |
| 143267b | Italy | 47 | m | 0.77 | mno | 66 | cd | 1.45 | de | 231 | fghij |
| 93ITA45PEL | Italy | 53 | ijkl | 2.15 | kl | 75 | abcd | 1.43 | de | 337 | bcde |
| 2006MAR29PEL | Morocco | 67 | c | 6.31 | e | 71 | bcd | 1.51 | de | 231 | ghi |
| 139362 | Morocco | 59 | fgh | 5.54 | ef | 67 | d | 1.52 | de | 316 | cde |
| 139363 | Morocco | 59 | fgh | 5.54 | ef | 70 | bcd | 1.51 | de | 339 | bcd |
| 138972 | Morocco | 50 | klm | 0.63 | no | 72 | abcd | 1.42 | de | 314 | cdef |
| CASBAH | Morocco | 61 | efg | 2.22 | k | 81 | a | 1.35 | e | 304 | cdef |
| 2006MAR22PEL | Morocco | 52 | jkl | 0.66 | o | 66 | d | 1.36 | e | 336 | bcd |
| 2004ESP19PEL | Spain | 58 | fgh | 7.36 | d | 70 | bcd | 1.79 | cd | 190 | ijk |
| 2004ESP39PEL | Spain | 49 | lm | 1.07 | lmno | 70 | bcd | 1.43 | de | 298 | cdef |
| 2004ESP64PEL | Spain | 54 | ijk | 4.50 | gh | 69 | cd | 1.97 | c | 340 | bcd |
| 143415 | Spain | 61 | efg | 4.74 | fgh | 75 | abcd | 1.43 | de | 206 | hijk |
| S. E. M. | | 1.79 | | 0.52 | | 5.85 | | 0.15 | | 10.69 | |

Significance: within the same column, values not sharing the same superscript significantly differ ($P < 0.001$)

*Correlations between the measured traits*

There was some association between the fermentability parameters and selected morphology characters in biserrula core (Figure 2). Methane production was highly correlated ($P < 0.001$) with time to flower and growth habit, with the lower methane content observed in late flowering and prostrate accessions. There was no strong correlation between methane and gas pressure ($R^2 = 0.43$) or methane and VFA concentration ($R^2 = 0.0039$) (Figure 3). There was also no grouping of samples according to country of origin (Figure 4).



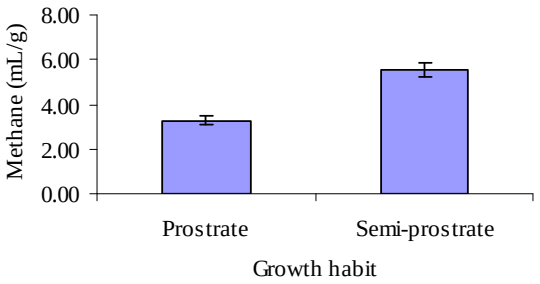




Figure 2. Methane production by different plant samples of biserrula according to flowering time or growth habit

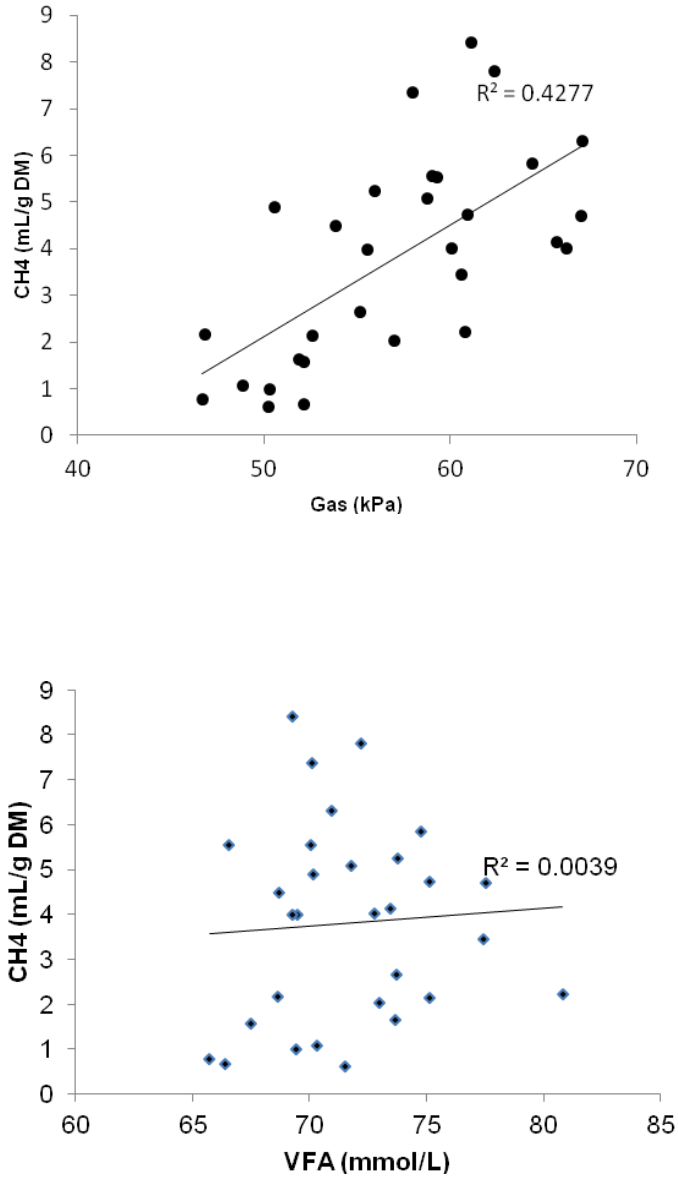



Figure 3. Correlation between gas or VFA concentration and methane in biserrula samples

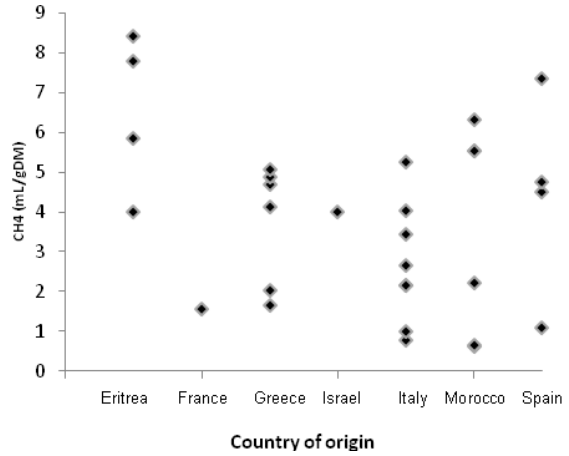



Figure 4. Methane production amongst accessions of biserrula grouped by the country of origin

*Discussion*

Our results confirmed previous observations that forage legume *Biserrula pelecinus* has significant inhibitory effects on selected parameters of rumen fermentation (Banik et al., submitted). All accessions produced at least three times less methane than subclover. There were some accessions, for example 138972 and 2006MAR22PEL that nearly inhibited methane production completely, (i.e. producing less than 1 mL of methane per gram of substrate supplied). However, in all accessions there was a significant reduction in overall gas production, but to a lesser extent than the reduction in methane. Furthermore, there was no inhibition in VFA production and consequently, there was no correlation between gas and methane production. There were accessions that caused 30% reduction in gas but nearly 80% reduction in methane (eg. 2006MAR29PEL). In our previous studies we found that plants like these may maintain their bioactivity and not inhibit overall fermentation if included at a lower dose, mixed with another fermentable substrate (Li et al. 2010). It is therefore possible that inclusion of some of these biserrula accessions as part of mixed diet would result in inhibition of methane without affecting normal rumen parameters. It should be noted that gas production is often used as main indicator of microbial function, however the results from this study confirmed our previous observations that there may be dissociation between fermentation parameters (Durmic et al. 2010). Gas production therefore may not be a good parameter to be used as a sole measure of microbial function, because the functions and activities of the ruminal microorganisms are complex.

The study also confirmed our previous observations (Banik et al., submitted) that morphological traits of the plant do not necessarily align with bioactivity in the rumen, as only two parameters were associated with methane production. These parameters may prove useful in future breeding programmes, but these results need to be confirmed in-field and over larger number of samples.

The plant material in this experiment was grown under controlled conditions (in the glasshouse) and sampled at only one phenological stage. Further studies are required to examine if the bioactive effects are maintained when the plant is grown in the field and over different phenological stages. Finally, these findings also imply that there might be a possible selective effect against methanogen microbes. This needs to be investigated further by identifying and

purifying active secondary compounds from biserrula and testing these against pure and mixed cultures of rumen microbes.

In conclusion, *Biserrula pelecinus* was capable of significantly reducing methane from rumen fermentation (*in vitro*) and this bioactive property was present in all accessions across the core collection. However, the plant affected some aspects of rumen fermentation and needs to be investigated further as part of a mixed diet. There were limited links between plant morphology and plant fermentability, implying that more complex factors may be involved in the bioactive properties of this plant.

## Section 2. Is the bioactive effect of biserrula replicated in-field and is there site variation?

*Introduction*

Plant fermentability and antimethanogenic potential may be related to the location where the plant was grown (Durmic *et al*. 2010). Previous reports on biserrula (Banik et al. submitted; Section 1) used plant material that was grown under controlled conditions in a glasshouse. It is not known if the plant maintains its antimethanogenic potential when grown in the field and if this property is affected by eco-geographical factors. The aim of the current study was to examine this variability on a limited number of field samples.

*Materials and methods*

*Plant material*

Material was collected from three research farms in WA – DAFWA Esperance, DAFWA/UWA Shenton Park and UWA Ridgefield. Farms were selected based on their geographical location and some differences in climatic factors. DAFWA Esperance is positioned in the far south of WA on the coast; UWA Ridgefiled is south/east and inland with lower rainfall rain; Shenton Park is in Perth (central) and was irrigated during the plant growth.

Plant material was collected at 8 weeks post sowing, then processed and analysed in the same way as described in Section 1.

*Results*

There was no significant difference in gas and VFA production between samples of biserrula grown at three different sites (Table 1). However, samples collected from Esperance and Ridgefield produced significantly ($P<0.05$) less methane, ammonia and had lower acetate to propionate ratios than plants grown under irrigation at Shenton Park. Methane production in French serradella also varied significantly ($P<0.05$) between samples from Esperance and Ridgefield. All biserrula samples produced less gas, methane and acetate to propionate ratios when compared to control legume French serradella grown at respective sites ($P<0.05$). The Ridgefield samples of biserrula also produced lower VFA concentrations than French serradella samples taken from the same site ($P<0.05$).

Table 1. Fermentation profiles of biserrula and French serradella collected at different sites and at 8 weeks from sowing

| Treatment | Gas (kPa) | CH4 (mL/g DM) | VFA (mmol/L) | A:P | NH3 (mg/L) |
|---|---|---|---|---|---|
| Biserrula | | | | | |
| Esperance | 58 b | 6.1 d | 67.0 b | 1.30 c | 151 c |
| Ridgefield | 52 b | 3.1 d | 69.1 b | 1.48 c | 224 bc |
| Shenton Park | 65 b | 12.1 c | 79.4 ab | 2.00 b | 328 a |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SEM | 2.3 | | 1.2 | | 2.7 | | 0.10 | | 26 | |
| French serredella | | | | | | | | | | |
| Esperance | 85 | a | 37.5 | a | 70.3 | ab | 2.63 | a | 173 | bc |
| Ridgefield | 98 | a | 20.1 | b | 93.3 | a | 2.41 | ab | 290 | ab |
| SEM | 8.8 | | 4.2 | | 9.5 | | 0.08 | | 30 | |

Significance: within the same column, values not sharing the same superscript differ significantly ($P < 0.001$)

*Discussion*

Our results demonstrate that methane production from fermentation of biserrula is consistently lower to the control legume when grown at different sites in WA. The effect was accompanied with significant reduction in gas and VFA concentrations, but also some favourable effects, such as reduced acetate to propionate ratios. One of the samples grown at Ridgefield produced half the methane than the samples from Esperance and four times less than the samples from Shenton Park, but with comparable gas and VFA concentrations to these two samples. It also produced half of the methane of the biserrula of a similar age, but grown in a glasshouse (i.e. 11 mL/g DM; Banik et al., submitted). It is possible that the poor soil and lack of water during growth at this Ridgefield, that the plants were more stressed than at other sites and produced more plant secondary compounds as a result of that stress, which in turn reduced fermentability and methane production *in vitro*. In support of this theory is the fact that the control legume, French serradella, which normally has a relatively high methane production (i.e. 41 mL/gDM when grown in the glasshouse; Banik et al., submitted) produced less methane when grown at Ridgefield (i.e. 20.1 mL/g DM). Conversely, plants at Shenton Park that were grown under more favourable conditions, i.e. were watered during dry periods, had less pronounced effects on methane and overall fermentation.

The bioactive effect of biserrula may be affected by the site/conditions where (and how) plant is grown. It appears that the most pronounced effect on methane was when plants were grown at under relatively poor soli and moisture conditions. Further investigation into the effect of climatic and eco-geographical influence on bioactivity in biserrula is required before developing an appropriate CFI methodology.

## Section 3. Is the bioactive effect of biserrula maintained at all stages of plant growth and plant age?

*Introduction*

Plant fermentability and antimethanogenic potential may be related to the age of the plant (Durmic et al. 2010). Previous reports on biserrula used plant material that was collected at vegetative and reproductive stage, but no later than 11 weeks from sowing (Banik et al. submitted and Section 1). The aim of this experiment was to examine the effect of plant age on the antimethanogenic potential of biserrula.

*Materials and methods*

*Plant material*

Material was collected from two research farms in WA –DAFWA/UWA Shenton Park and UWA Ridgefield (described above). Plant material from Ridgefield was collected from multiple plants at 8, 9 and 11 weeks post sowing and material was bulked for analysis. Material from Shenton Park was collected from three separate replicates and was not bulked. Material from both sites was processed and analysed according to the description in Section 1.

*Results*

Amongst samples of biserrula collected at different ages from Ridgefield, there was no significant difference in fermentation profiles except for ammonia in week 11, while in French serradella there were some differences between the collection times (Table 1). Nevertheless, biserrula consistently produced significantly ($P<0.05$) lower amounts methane, gas and acetate to propionate ratios compared to the control, while VFA concentrations were reduced only in week 8 and 11 and $NH_3$ in week 8 only.

Table 1. Fermentation profiles of biserrula and French serradella collected at 8, 9 and 11 weeks at Ridgefield, WA

| Treatment | Gas (kPa) | | $CH_4$ (mL/g DM) | | VFA (mmol/L) | | A:P | | $NH_3$ (mg/L) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Biserrula | | | | | | | | | | |
| 8 weeks | 52 | cd | 3.1 | b | 69.1 | b | 1.48 | b | 224 | b |
| 9 weeks | 59 | cd | 3.5 | b | 66.7 | b | 1.47 | b | 215 | b |
| 11 weeks | 47 | d | 2.8 | b | 68.9 | b | 1.88 | b | 167 | d |
| SEM | 1.9 | | 0.2 | | 1.7 | | 0.11 | | 9.0 | |
| French serredella | | | | | | | | | | |
| 8 weeks | 98 | a | 20.1 | a | 93.3 | a | 2.41 | a | 290 | a |
| 9 weeks | 82 | ab | 18.9 | a | 70.8 | ab | 2.59 | a | 200 | bc |
| 11 weeks | 74 | bc | 16.4 | a | 68.4 | b | 2.70 | a | 179 | cd |

| | | | | | |
|---|---|---|---|---|---|
| SEM | 6.6 | 1.0 | 6.5 | 0.06 | 18.3 |

Significance: within the same column, values not sharing the same superscript differ significantly (P < 0.05)

In samples collected from Shenton Park, there was a significant variation in fermentation parameters from biserrula collected at different ages, but also between plant replicates of the same age (P<0.05; Table 2). In general, all three replicates had the highest antimethanogenic bioactivity at 8 weeks, with methane ranging from 7.7 mL/g DM to 15.7 mL/g DM. Methane production increased in at 16 weeks (16.9 – 18.4 mL/g DM) and peaked at 28 weeks (19.2 - 23 mL/g DM). A similar trend was observed with acetate to propionate ratios. There was significant variation between some of the replicate plants, in particular at week 8. For example, at 8 weeks, replicate 1 produced only half the amount of methane of replicate 2 and had significantly lower gas, VFA concentrations, acetate to propionate ratio and ammonia to the other two replicates. However, these differences diminished over time and methane was not different between the replicates at 16 and 28 weeks.

Table 2. Fermentation profiles of biserrula collected at 8, 9 and 11 weeks at Shenton Park, WA. Numbers in brackets refer to filed replicate.

| Treatment | Gas (kPa) | | CH4 (mL/gDM) | | VFA (mmol/L) | | A:P | | NH3 (mg/L) | |
|---|---|---|---|---|---|---|---|---|---|---|
| 8 weeks SP (1) | 50 | d | 7.7 | d | 61 | c | 1.40 | e | 187 | f |
| 8 weeks SP (2) | 75 | a | 15.7 | bc | 90 | a | 2.35 | d | 410 | a |
| 8 weeks SP (3) | 68 | c | 12.8 | c | 87 | ab | 2.26 | d | 387 | b |
| SEM | 3.8 | | 1.6 | | 4.7 | | 0.15 | | 36 | |
| 16 weeks SP (1) | 70 | c | 16.9 | bc | 85 | ab | 2.65 | c | 322 | de |
| 16 weeks SP (2) | 73 | bc | 18.4 | b | 82 | b | 2.79 | c | 320 | e |
| 16 weeks SP (3) | 68 | c | 18.4 | b | 80 | b | 2.63 | c | 358 | c |
| SEM | 1.0 | | 0.4 | | 1.6 | | 0.03 | | 6 | |
| 28 weeks SP (1) | 74 | ab | 23.0 | a | 81 | b | 3.47 | a | 336 | d |
| 28 weeks SP (2) | 75 | ab | 19.2 | ab | 81 | b | 3.22 | b | 327 | de |
| 28 weeks SP (3) | nt | | nt | | nt | | nt | | nt | |
| SEM | 1.4 | | 0.9 | | 1.0 | | 0.10 | | 3 | |

Significance: within the same column, values not sharing the same superscript differ significantly (P < 0.05)

*Discussion*

The aim of our experiment was to examine the effect of plant age on *in vitro* fermentation profiles, in particular methanogenic potential amongst samples of biserrula. The plants at Ridgefield consistently produced significantly less methane and gas, while maintaining VFA production comparable to the control throughout all sampling times, but the effect was not

related to plant age. Fermentation profiles of biserrula samples collected from Shenton Park were however affected by the age of the plant, with younger plants in general being more 'bioactive', i.e. producing less methane and more propionate than the older ones. An important observation is that there were also significant differences between replicates of the plant. It is not clear why there was not a clear effect of age on methane production in plants collected at Ridgefield, but it was noted earlier (Section 2) that at 8 weeks plants sampled from Ridgefield produced half the methane of the biserrula of a similar age but grown in a glasshouse (i.e. 11 mL/g DM; Banik et al., submitted), and two to four times less methane compared to those from other sites (see Section 2). At 11 weeks however, the amount was more comparable to that reported earlier (Banik et al., submitted) when grown in the glasshouse (i.e. 2.8 mL/g DM at Ridgefield vs 4 mL/g DM in the glasshouse). It is possible that the factors such as plant age are only important until the plant reaches a certain plateau of 'bioactivity' and once this plateau is reached, no further improvement in bioactivity occurs.

Our results indicate that bioactivity in biserrula may be affected by plant age (site related), varies between individual plants and is affected by eco-geographical factors.

## Section 4. Bioactivity of biserrula when included as part of a mixed substrate

*Introduction*

The results from the experiment reported in Section 1 did not identify any cultivars that, when used as the sole substrate *in vitro*, reduced methane without affecting fermentation. In the field, a practical application of biserrula may be to use it as of a mixed diet rather than the sole component of the diet. It has been demonstrated that E. glabra, which inhibits overall fermentation when used as the sole substrate, is effective at reducing methane without reducing overall fermentation when included at 15% of the substrate *in vitro* (Li et al. 2010) and of the diet *in vivo* (Li pers. Comm; BCCH1012 final report). The aim of this experiment was to investigate whether there was an optimal dose of biserrula that reduced methane without affecting rumen fermentation.

*Methods*

*Plant material*

A total of approx. 1700 g of DM of biserrula was collected from Esperance and Shenton Park when the plants were 8 weeks old. Material was bulked, mixed and processed. A portion of this material (100 g) was then ground to pass through a 1 mm screen and used in the current study, while the remaining portion was set aside for a RUSITEC experiment (Section 5) and for extracting and testing plant secondary compounds (Section 6).

*In vitro* batch fermentation testing was conducted according to the description in Section 1, except that it was done in 100 mL serum vials containing 50 mL buffered rumen fluid and 500 mg substrate. Treatments were as follow: Control – 500 mg oaten chaff; 25 % B.p – 125 mg biserrula + 325 mg oaten chaff; 50 % B.p. – 250 mg biserrula + 250 mg oaten chaff; 75 % B.p. – 325 mg biserrula + 125 mg oaten chaff; and 100 % B. p. – 500 mg biserrula.

*Results*

When biserrula was included at different levels in combination with oaten chaff, there were significant differences in fermentation profiles (Table 1). When compared to the control (100% OC), methane was significantly ($P<0.05$) reduced when biserrula was included at >50% of the diet, while gas, VFA concentration and acetate to propionate ratio were reduced with inclusion at >75 %, and ammonia levels seem to increase with the increase of biserrula inclusion. All fermentation parameters were highly correlated to the level of biserrula, with the highest correlations occurring with methane and ammonia ($R^2$ = 0.96) and the weakest with VFA concentration ($R^2$ = 0.70).

Table 1. Fermentation parameters in treatments with a different level of inclusion of biserrula (B. p.) and oaten chaff (OC)

| Treatment | Gas (Kpa) | | $CH_4$ (mL/g DM) | | VFA (mmol/L) | | A:P | | $NH_3$ (mg/L) | |
|---|---|---|---|---|---|---|---|---|---|---|
| 100 % OC | 105 | a | 29.3 | a | 74 | a | 3.43 | b | 99 | d |
| 25 % B.p + 75 % OC | 105 | a | 23.9 | a | 71 | a | 3.46 | b | 119 | c |
| 50 % B.p + 50 % OC | 101 | a | 15.0 | b | 73 | a | 3.36 | b | 122 | c |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 75 % B.p + 25 % OC | 70 | b | 10.6 | b | 64 | b | 2.64 | c | 148 | b |
| 100 % B.p | 66 | b | 8.2 | b | 66 | b | 2.64 | c | 163 | a |
| SEM | 2.6 | | 2.1 | | 1.4 | | 0.08 | | 3.9 | |
| $R^2$ (level x parameter) | 0.83 | | 0.96 | | 0.70 | | 0.79 | | 0.96 | |

Significance: within the same column, values not sharing the same superscript differ significantly ($P < 0.05$)

*Discussion*

Our results indicate that biserrula acts in a dose-dependant manner, with bioactive effects increasing with the level of inclusion of biserrula. If the in vitro results reflect what occurs in the field then biserrula could be included as 50% of the diet and have the potential to reduce methane (50% reduction) without having a detrimental effect on other rumen fermentation parameters. This effect needs to be confirmed over a period of time using a continuous culture system (RUSITEC) and *in vivo*. It should be noted that the control substrate used in this study was oaten chaff as it does not contain any PSC and it is a common supplementary fodder in WA. However, the effects we have reported here need to be confirmed with other mixtures, for example, combining biserrula with other pasture species that may offer complementary effects in terms of agronomy and nutritive values.

## Section 5. Does the bioactive effect of biserrula persist over time under continuous culture system (RUSITEC)?

*Please note: this experiment was not scheduled to be done within the project. It is an additional investigation that we decided to conduct, given the results in the Section 4 and the fact that there were additional resources (plant material from the original sampling) to do so. We had access to plant material and a student through our collaborative projects, which meant we had the labour and some additional operating expenses linked to the student that made it possible to conduct the additional experiment. The RUSITEC experiment commenced on 13th April 2012 and is scheduled to finish on 3rd May 2012. All analyses should be completed in 2 months. We have presented the experimental design and some preliminary results in this section.*

*Introduction*

In the previous sections, we have demonstrated that biserrula his highly antimethanogenic. We have also demonstrated that mixing biserrula with oaten chaff can reduce the inhibitory effect on overall fermentation and, at 50 % inclusion in the substrate, biserrula still reduces methane significantly. However, all of these results have come from experiments done in a batch *in vitro* fermentation system. While this system is convenient for large-scale screening and preliminary testing, it has limitations. In this system, the amount of substrate, buffer and rumen fluid is limited and not renewed. The fermentation is measured at one time-point and because the end-products of fermentation are not removed they accumulate in the system. This is very different to what occurs in the animal, where there is a constant influx of feed, water and saliva, and removal of gasses and end products of fermentation. However, animal experimentation is laborious, costly and above has animal welfare implications. An open, continuous *in vitro* system called an 'artificial rumen' or RUSITEC is designed to mimic rumen fermentation, with constant influx of saliva, removal of end-products and daily feeding. It also allows regular monitoring and daily collection of samples over a prolonged period of time (i.e. 3 weeks), which enables an assessment of the persistency of the effects and the adaptation of the ruminal microbes to the substrate. The system also allows taking some additional measurements, such as dry matter disappearance and digestibility.

The aim of the current study was to confirm that the effects observed with biserrula described in Section 5 occur in a continuous culture system (RUSITEC) and persist.

*Methods*

*Plant material*

Plant material was the same as described in Section 4, with the exception that it was ground to pass through a 4 mm screen instead of 1 mm screen to prevent plant material passing through the nylon bag.

*Experimental design*

The amount of biserrula plant material that was available for testing was limited so we examined only two levels of inclusion, 50 % and 100 %, and in a sequential manner, rather than in parallel (Figure 1). Briefly, six fermentors were set up and allowed to acclimatize for 7 days (day 1-7) on a control substrate (oaten chaff). Following this, biserrula was introduced in three fermentors

('biserrula fermentors') at a level of 50 % combined with oaten chaff, while the remaining three fermentors ('control fermentors') continued receiving oaten chaff only and these treatments were maintained for 7 days (Day 8-15). After 7 days, biserrula was then included as a sole substrate (100 %) in the three 'biserrula' fermentors for another 7 days (day 16-21) to examine the effect of this dose on fermentation parameters.

*Setting up and running the RUSITEC, sampling and analyses*

Rumen fluid (approx. 1200 mL in total) was collected from three donor sheep maintained on autumn pasture (Shenton Park) and supplemented with 100 g lupins/head/day. Rumen fluid was pooled and strained to separate the liquid (900 mL) and solid (270 g) portions. Each fermentor was filled with 150 mL of liquid portion of the strained rumen fluid and 250 mL of prewarmed McDougal's buffer. The solid portion was aliquoted into six nylon bags (45 g in each) and one bag each was placed alongside a nylon bag containing 15 g of oaten chaff in respective perforated feed containers. These were then immersed in respective fermentors and immediately flushed with nitrogen. Fermentors were then attached to a supply of buffer and to outflow tubing for the collection of gas (in silver bags) and liquid overflow (in scott bottles kept in a portable freezer at 0°C to prevent secondary fermentation in the overflow). On day one, pH was measured hourly and buffer flow was adjusted to maintain pH at approx 6.7 in each fermentor. The next day, bags containing solids were replaced with a bags containing 15 g of oaten chaff. Following this, bags containing oaten chaff were replaced with feresh plant material each day. During this period, pH, gas and overflow liquid were monitored daily. Samples were collected prior to the introduction of treatments and analysed for pH, VFA, acetate to propionate ratio, microbial profiling (3 hours after feeding), gas, methane, outflow volume and dry matter digestibility (DMD) (total over 24 hours).

From day 8 -14, three fermentors were allocated as 'biserrula fermentors' and in these, bags of oaten chaff were replaced with a bag containing 7.5 g biserrula and 7.5 g of oaten chaff daily ('50 % BP'). From day 15 – 21, these three fermentors were supplied daily with 15 g biserrula only ('100% BP'). The remaining three fermentors received 15 g of oaten chaff throughout ('Control'). In these two periods, samples were also collected and analysed as described above.

Figure 1. Distribution of daily treatments day 1-21

| Fermentor | Day 1-7 | Day 8-14 | Day 15-21 |
|---|---|---|---|
| 1,3,5 (Control) | 15 g oaten chaff | 15 g oaten chaff (Control) | 15 g oaten chaff (Control) |
| 2,4,6 (Biserrula) | 15 g oaten chaff | 7.5 g biserrula+ 7.5 g oaten chaff (BP 50%) | 15 g biserrula (BP 100%) |

*Results*

At the time of preparation of this report, only preliminary results were available for day 9-11 and are presented in Table 1. On day 9 (first day when the full treatment, i.e. BP 50% was fermented for at least 24 h) there were no significant differences in the parameters measured. On day 10, some trends were observed in total gas, total methane and methane per unit of dry

matter (DM) digested. Some of these differences became significant (P<0.05) on day 11. Dry matter disappearance was also significantly higher in BP 50% than in the control.

Table 1. Preliminary results from testing two levels of biserrula and oaten chaff in a RUSITEC. Control – 15 g oaten chaff supplied daily; BP 50 % - 7.5 g biserrula + 7.5 g oaten chaff, supplied daily. DMD – dry matter digestibility (dry matter disappeared/dry matter) supplied). DM – dry matter.

| Treatment | DMD | | Total gas produced (mL/24 h) | Total $CH_4$ produced (mL/24 h) | $CH_4$ yield (mL/g DM digested) | |
|---|---|---|---|---|---|---|
| Day 9* | | | | | | |
| Control | 0.34 | | 5140 | 122 | 21.5 | |
| BP 50% | 0.29 | | 4555 | 134 | 37.3 | |
| SEM | 0.04 | | 393 | 16 | 7.7 | |
| Day 10 | | | | | | |
| Control | 0.44 | | 4703 | 81.7 | 12.3 | |
| BP 50% | 0.45 | | 4351 | 70.3 | 10.1 | |
| SEM | 0.01 | | 316 | 24 | 3.6 | |
| Day 11 | | | | | | |
| Control | 0.19 | b | 4660 | 74.2 | 26.5 | b |
| BP 50% | 0.34 | a | 4138 | 48.8 | 9.8 | a |
| SEM | 0.04 | | 257 | 9 | 4.1 | |

* first day when both bags in the fermentor contained the biserrula treatment

Significance: within the same column and same sampling day, values not sharing the same superscript differ significantly (P < 0.05)

*Discussion*

Some trends in the bioactive effect of biserrula on rumen fermentation started to emerge on day 11. Methane appeared to be affected and when expressed per unit of dry matter digested, it was nearly three times lower in the biserrula treatment compared to the control. A complete analysis of all fermentation parameters will be done at the completion of the experiment. We will have a much clearer idea of the persistency of the effects on methane production and whether there are any longer-term adverse affects on fermentation when this analysis is done.

## Section 6 – Linking the antimethanogenic bioactivity of biserrula to plant secondary compounds

*Introduction*

The mechanism behind low methanogenic potential in biserrula is unknown, but in our previous experiments, we have confirmed that the bioactive effect of biserrula is complex. It is not simply linked to plant morphology and may have some selective effects against rumen methanogens because it affected methane production without affecting VFA production (in batch culture). Low methanogenic potential is often linked to the presence of PSC in plants (Patra and Saxena, 2010). A wide range of PSC, including saponins, phenolics, alkaloids (Pistelli 2002) and proanthocyanidins (Aerts *et al*. 1999) are found in *Astragalus*. Inhibition of ruminal cellulose fermentation by extracts of other species of this genera have been reported (Weimer 1993), but in biserrula, the type, amounts, distribution of PSC or their effects on rumen fermentation are unknown. It has been reported that during flowering and at seed-set, biserrula can be unpalatable and cause photosensitivity in sheep, which is likely to be linked to the presence of PSC (Hackney et al., 2007). Recently, it has been suggested that plants containing PSCs may be potential candidates to reduce ruminal methanogenesis (Bodas *et al*., 2008) and it is therefore possible that selected PSCs might be responsible for the bioactivity we have observed in biserrula. The aim of the current study was to examine the effects of crude and more purified extracts of biserrula on rumen fermentation *in vitro*.

*Methods*

*Experimental design*

Crude extracts were obtained using five different solvents and tested in an *in vitro* batch fermentation system. Following this, water and organic fractions were separated and tested in a dose dependant manner.

*Obtaining and testing crude extracts*

The biserrula accession 2006MAR22PEL (1) was selected for this study as the most potent amongst the accessions we have tested. Plant material that was used for the study in Section 1 was also used to obtain crude extracts for this study. Five different solvents – water (100% v/v water), methanol (80% v/v methanol), ethanol (70% v/v ethanol), acetone (100% acetone) and methanol/chloroform (50% : 50% methanol : chloroform) were used and a modified version of the methods for obtaining crude extracts of plant materials (Billo et al., 2005; Geyid et al., 2005; Mothana and Lindequist, 2005; Neto et al., 2005; Sweeney et al., 2001) were used in this experiment. Briefly, plant material (0.5 g) was extracted with 5 mL of solvent by macerating for 3 hours in a shaking incubator (100 rpm at 22°C). This was followed by centrifugation for 12 minutes at 2320 rpm and collecting the supernatant in pre-weighed bellco tubes. The second step of extraction was done with the remaining pellet and 2 mL of solvent. The two supernatant portions were combined. Following this, a speedvac was used to evaporate the liquid phase to get the dry crude extract.

Prior to testing, the dry crude extract was redissolved with 500 µL of 70% ethanol. Plant extracts were then tested in a modified *in vitro* fermentation system for testing plant extracts (Durmic *et al*. 2008). Briefly, 100 µL of this reconstituted extract was added to a bellco tube

containing 100 mg ground oaten chaff and 10 mL of buffered rumen fluid. Each 100 µL of this reconstituted extract therefore corresponded to the amount extracted from 100 mg of plant material, which was the amount of plant material included previously (Section 1) in the *in vitro* batch assay. A control consisted of ground oaten chaff + 100 µL ethanol. The treatments were incubated and analysed for methane only as described previously (Section 1).

*Separating and testing aqueous and organic extracts*

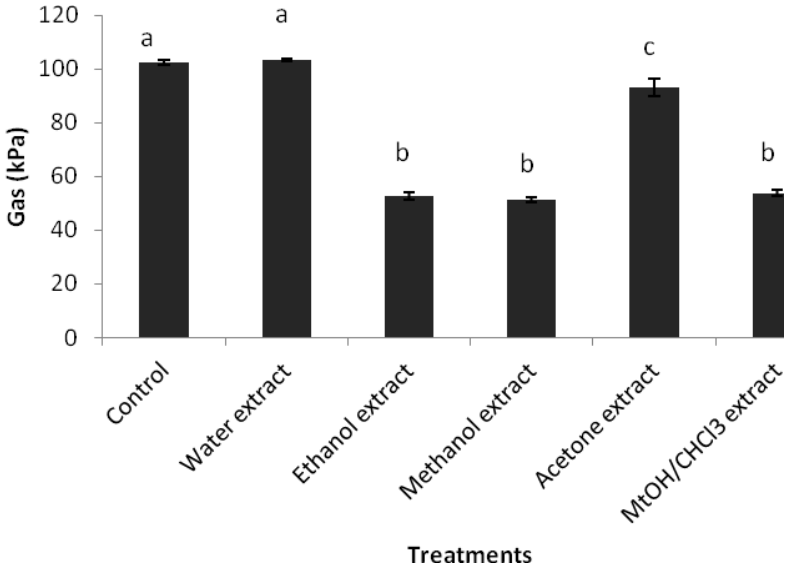
Methanol/chloroform was selected as the most effective solvent and the procedure described above was used to obtain the organic fraction, with the exception that water was also used to obtain an aqueous fraction. Briefly, different amounts of plant material (0, 100, 200 and 400 mg) were extracted with 10 mL of methanol by macerating for 45 minutes in a sonicated water bath at 40° C. This was followed by centrifugation for 5 minutes at 3000 rpm and collecting 7 mL of supernatant in glass tubes. This supernatant was then extracted by adding distilled water and chloroform to obtain the aqueous fraction, or methanol and chloroform, to obtain the organic layer followed by centrifugation for 3 minutes at 1000 rpm. Layers were collected separately and the solvent was evaporated in a methane reaction chamber. Dry crude extract were reconstituted and tested *in vitro* as described above.

## *Results*

*Crude extracts*

*In vitro* gas production from fermentation of oaten chaff was not affected by the addition of ethanol or water extracts of biserrula, but it was significantly ($P<0.05$) affected by the addition of the other redissolved crude extracts. Methane production was significantly reduced with all extracts when compared to the control (Figure 1). The methane production amongst treatments varied, with the water extract treatment producing the most, i.e. 14 mL/g DM and methanol/chloroform extract treatment the least, i.e. 4.47 mL/g DM amongst the extract treatments, and these two treatments were significantly different.

a




b

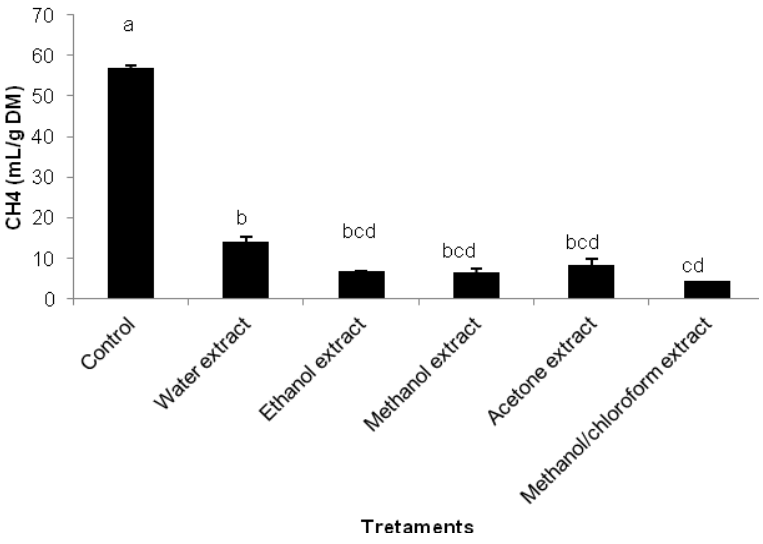



Figure 1. Gas (a) and methane (b) production (mean±SEM) from the fermentation of oaten chaff (control) or oaten chaff in the presence of different extracts from biserrula. Control – 0.1 g of oaten chaff + 100 µL ethanol. Treatments – 0.1 g oaten chaff + 100 µL crude extract reconstituted in ethanol.

*Aqueous vs organic fraction*

*In vitro* methane production was reduced by the addition of reconstituted aqueous or organic fractions (values ranging from 4 to 7 mL/g DM) when compared to the control ($P<0.05$) (not

shown; 57 mL/g DM) (Figure 2). However, there was no difference between aqueous and organic fraction or with respect to the amount of plant material used for extraction.


Figure 2. Methane production (mean±SEM) from the fermentation of control (oaten chaff only, not shown) in the presence of aqueous and organic fractions extracted from three different quantities of biserrula plant material. Control – 0.1 g of oaten chaff + 100 µL ethanol. Treatments – 0.1 g oaten chaff + 100 µL fraction reconstituted in ethanol.

*Discussion*

The extracts we tested have provided evidence that the bioactivity in biserrula is linked to secondary compounds, which is perhaps not surprising. However, the results did indicate that there was bioactivity present in different solvent and water extracts (not just in one fraction) and there is likely to be more than one compound responsible for the bioactivity. The bioactivity in the water extract in particular was interesting because it reduced methane without inhibiting overall fermentation. Further purification, isolation and analysis of fractions are needed to identify more specifically the PSC in biserrula responsible for the antimethanogenic bioactivity.

This study has provided the basis for choosing the extracts to focus on first for identifying the PSCs of interest and for unravelling the mechanisms behind their action. The compound(s) responsible for the bioactivity of the aqueous phase/extract is of particular interest because it seemed to show some specificity towards the rumen methanogens rather than a general inhibition of the ruminal microbes.

## Section 7. Publications arising from BCCH 1024

*Biserrula pelecinus* L. - genetic diversity in a promising pasture legume for the future. K. Ghamkhar, et al. (2011), submitted to Crop and Pasture Science

## Section 8. Collaboration with other projects within RELRP

This project was linked directly with projects BCCH1012, 1067, 1004, 1009, 1010 and 1014 in the sense that the methods and the objectives of screening plants for their potential to reduce methane and modify rumen fermentation were closely aligned. It was also linked to BCCH 1031 (Ridgefield demonstration site), which was one of the sites where biserrula was planted to test whether the bioactivity we observed from plants grown under controlled conditions in a glasshouse was present in plants grown under field conditions. We have interacted with the RELRP team as a whole at all technical meetings and workshops held since the inception of the programme.

## Section 9. Media coverage

The media coverage for this project has been entirely linked to the more general media coverage of the BCCH1012 and BCCH1031 demonstration site projects. The interest in biserrula stemmed from the studies and screening that was being undertaken in BCCH1012, which received considerable press coverage, and the biserrula that was planted at the demonstration site was a key interest point for the second field day that was held there in October 2011.