



Candidate metabolites for methane mitigation in the forage legume *biserrula*

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Accepted: 18 May 2018 / Published online: 30 May 2018
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Abstract

The forage legume species *biserrula* (*Biserrula pelecinus*) is among a few forage species with low enteric methane emission when fermented by rumen microbes and it is unclear whether metabolites in *biserrula* play a role in this. The hypothesis of this study was that specific metabolites in *biserrula* are candidate biomarkers for and associated with low methanogenesis. We characterized the metabolomic profiles of 30 accessions of *biserrula* or its core collection using nuclear magnetic resonance and liquid chromatography mass spectrometry. These profiles were then analyzed for association with methanogenesis potential in the rumen. Metabolomic profile was predictive of methanogenesis. Forty-seven putative metabolites were identified by liquid chromatography mass spectrometry that were highly expressed ($P < 0.05$) in the associations with low methanogenic potential. Fragmentation analysis of a subset of these metabolites suggested saponins, with one feature tentatively identified as an ursolic or oleanolic terpene glucoside, consistent with the nuclear magnetic resonance data. Accessions with Eritrean geographic origin were metabolomic outliers. Here, we show for the first time that some metabolites contribute to the methane mitigation effects of *biserrula*. If in vivo results confirm this, the environmental impact of this study would be the availability of biomolecules for livestock vaccination to mitigate methane emission resulting in an economic impact of lower cost of production in countries with a price on environmental emissions.

Keywords *Biserrula pelecinus* (*biserrula*) · Enteric methane mitigation · Geographic origin · Legume · Metabolomics · Saponin

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13593-018-0510-x>) contains supplementary material, which is available to authorized users.

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1 Introduction

CH₄ is a common byproduct of anaerobic microbial fermentation of forage in the rumen (Fig. 1a). Ruminants are vital to food production in most countries of the world, and in Australia and New Zealand, their products are important contributors to economic and social wellbeing. Enteric CH₄ emission occurs as a result of microbial fermentation of feed components (Johnson and Johnson 1995). CH₄ produced by domesticated ruminants represents a loss of 2–15% of the gross energy intake (Holter and Young 1992), thus being an important inefficiency in ruminant production systems (Moss et al. 2000). To abate CH₄ emission and increase the efficiency of digestion in ruminants, the focus is to divert some of the energy lost as CH₄ into animal products (Mitsumori and Sun 2008). Enteric fermentation can vary widely depending on factors such as type of the animal, feed intake, the type and quality of feed, environment, adding dietary fat, feed additives, rumen microbiota, and, indirectly, body weight of the animal through feed intake (Moss et al. 2000).

There are urgent needs to understand the various factors affecting variability in enteric CH₄ production to decrease

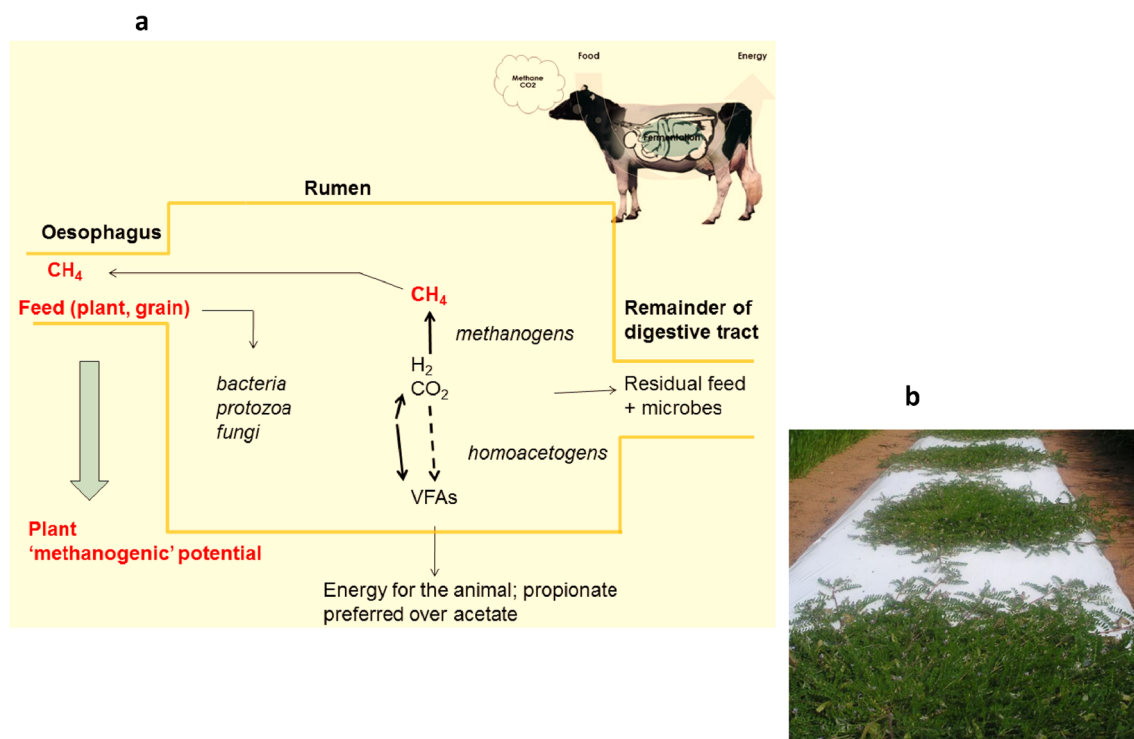


Fig. 1 **a** Schematic presentation of methanogenesis in rumen. The type of feed can affect the extent of methanogenesis. The type of rumen flora also affects the final outcome of methanogenesis/homoacetogenesis. **b** Spaced biserrula plants in the field

the uncertainty in GHG emission inventories and to identify viable GHG reduction strategies (Sejian et al. 2011). Improving pasture quality is viewed as a means of reducing CH_4 emissions because animal productivity may be enhanced (i.e., lowering CH_4 emissions per unit of animal product) with less dietary energy lost as CH_4 due to reduced fiber content. In New Zealand, Friesian and Jersey dairy cows grazing sulla (*Hedysarum coronarium*), a condensed tannins-containing legume, emitted less CH_4 per unit of dry matter than cows grazing perennial ryegrass pasture (Woodward et al. 2002). Further, the antimicrobial properties of essential oils have been shown through in vitro and in vivo studies to inhibit a number of bacteria and yeasts and to control fermentation gases, livestock waste odors, and human pathogenic bacteria (Helander et al. 1998; Lee et al. 2002; Wallace et al. 2002). Experiments conducted in vitro (Ulyatt et al. 2002) and in vivo (Bayaru et al. 2001) show that addition of organic acids to the diet reduces CH_4 production.

In a recent study, a broad range of dietary pasture and forage species and their effect on CH_4 production in ruminants was reported (Banik et al. 2013). The most promising among these species was biserrula (*Biserrula pelecinus*), an important annual legume for the wheat belt of southern Australia (Fig. 1b).

Recently, a core collection of 30 accessions of biserrula has been developed from the genetic resource of 279 accessions in the Australian Trifolium Genetic Resource Centre (Perth,

Western Australia), held by the Department of Agriculture and Food Western Australia (DAFWA) (Ghamkhar et al. 2012). Preliminary LCMS analysis of a subset (12) of the biserrula accessions has previously been reported by Ghamkhar et al. (2016). Here, we report the use of metabolomics techniques to investigate the effect of all accessions of the core collection of biserrula on CH_4 production when supplied to a rumen fluid suspension in vitro. In the past, some phytochemicals have been suggested to have a direct toxic effect on methanogens (e.g., condensed tannins) or protozoa (e.g., saponins) (Patra and Saxena 2009) and the heterogeneous group of compounds known as essential oils also show correlation with methanogenesis (Martin et al. 2010). Recent studies suggested plant secondary compounds as a cue in methanogenic potential of biserrula (Swinney et al. 2015; Banik et al. 2016). Identification of metabolites in this study will provide the same, similar, or different candidate phytochemical targets for development of novel classes of methanogen inhibitors. These measurements and correlations should assist plant breeders to make better choices in plant selection, in particular targeting CH_4 mitigation from livestock.

The aims of the current study were to:

1. Compare the metabolomics profiles of the accessions of the biserrula core collection and their association with CH_4 emission using an in vitro fermentation technique (IVFT); and

- Identify candidate metabolites that can reduce methane production in the rumen and suggest future environmentally and phytochemically targeted plant breeding options in biserrula.

2 Materials and methods

2.1 Plant material

The core collection of biserrula (*Biserrula pelecinus* L.), made up of 30 accessions conserved in the ATGRC, and two controls, red clover (*Trifolium pratense* L. cv. Redquin) and subterranean clover (*Trifolium subterraneum* L. cv. Woogenellup) (Table 1) were sampled and screened for their in vitro CH₄ output analysis as described by (Banik et al. 2013). Briefly, 47 plants of 30 accessions of the biserrula core collection with half of them replicated, and two control species, subterranean and red clover, were grown in pots in a glasshouse, and plant material was collected for in vitro analysis of methanogenic potential at 77 days post-sowing. The herbage consisting of leaf and stem 3 cm above ground level when elevated were harvested from individual plants, and the material was freeze-dried in a bench-top freeze dryer (BenchTop, VirTis/SP Scientific, Warminster, PA) and ground in a grinding mill (Glen Creston, Stanmore, UK) to pass through a 1.0-mm screen. Material was stored at room temperature in sealed containers until analysis by Banik et al. (2013).

The same ground plant material was used for metabolomics analysis except that accession numbers 11, 21, 29, and 37, the latter two being replications of the same accession were missing and not available for this analysis (Table 1). However, 27 accessions of biserrula from different geographic origins and a diverse range of CH₄ output as well as the two clover controls were still available for metabolomics analysis as shown in Table 1.

2.2 In vitro fermentation technique

Data from Banik et al. (2013) were sorted and processed for use as the base information for the 27 accessions of biserrula and the control species (Table 1). Briefly, plants were harvested at 10 weeks. The edible parts of the plants was freeze-dried in a bench-top freeze dryer (BenchTop, VirTis/SP Scientific, Warminster, PA) and ground in a grinding mill (Glen Creston, Stanmore, UK). Fermentability was examined in an in vitro batch fermentation system commonly used to examine plant fermentability (Bodas et al. 2008). One day before the testing, 0.1 g of plant material was weighed and transferred to an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) maintained at 39 °C and supplied with 80% N₂:

10% CO₂: 10% H₂ to expel oxygen from the tubes. Before the commencement of fermentation, rumen fluid was collected from two fistulated sheep fed a general maintenance diet consisting of lupins and oaten chaff 2 h after feeding.

After straining, rumen fluid was pooled, transferred into the anaerobic chamber, and buffered to pH 7.2. Then 10 mL of this mix was dispensed into prepared tubes. Inside the chamber, the tubes were sealed and incubated for 24 h at 39 °C, with constant shaking at 50 rpm. Tubes were then placed in a water bath at 39 °C. After measuring gas pressure, 5 mL of headspace gas was used for subsequent methane analysis by gas chromatography (GC). Methane concentration in the gas sample was determined using a Micro-GC with a PoraPLOT Q 0.25 mm × 10 m column (Varian/Agilent Technologies, Australia Pty Ltd., Mulgrave, Vic.), at 50 °C, a thermal conductivity detector, and Galaxie software (Varian, Australia). The methanogenic potential of the plant was expressed as total methane produced (mL/g DM incubated, (Patra et al. 2006)).

2.3 Mass spectrometry

LCMS was based on the methodology described by the authors (Ghamkhar et al. 2016), but in this experiment, a much larger sample of 30 accessions was examined with the following steps:

2.3.1 Extraction

In brief, ground, freeze-dried samples (100 mg) were extracted in 1 mL 80% methanol:water, by sonication for 10 min. After centrifugation, 200 µL of supernatant from each sample was placed into an HPLC vial for LCMS analysis. This LCMS sample was stored at 4 °C overnight prior to analysis. The extraction process was repeated on the pellet for a second time. Combined extracts were then reduced in volume to less than 300 µL by evaporation under N₂ gas whilst heating at 30 °C. Samples were frozen at −80 °C and then freeze-dried using a Christ Freeze Drying System (ice condenser, −55 °C; vacuum: 0.030 mbar) (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Freeze-dried samples were stored at −80 °C over desiccant. Duplicates were prepared for three samples to test method reproducibility.

2.3.2 LCMS sample analysis

LCMS data was acquired in negative ionization mode at high resolution. Negative Fourier transform mode lenses were tuned to *m/z* of 739.20 by infusion of sample number 3 when teed with mobile phase at initial chromatographic conditions.

HPLC separation was achieved using a 150 mm × 2.1 mm ThermoFisher 1.9 µm C18 HPLC column (Thermo Fisher Scientific, Australia) fitted to an Agilent series 1290 high-performance liquid chromatograph (binary pump, cooled

Table 1 Core collection of biserrula and the two control species, their origin, and in vitro methane output. Methane output was derived from IVFT over short incubations by Banik et al. (2013). High-end methane groups are high within biserrula germplasm not at interspecific level

Accession ID	Accession number	Country of origin	Mean methane output (mL g ⁻¹ DM)	Methane group
Red clover (cv. Redquin)	47	N/A	36.18	Very high
Subterranean clover (cv. Woogenellup)	46	N/A	28.38	Very high
2004ERI1PEL	43	Eritrea	7.96	High
2004ERI56PEL	4	Eritrea	7.71	High
2004ESP19PEL	7, 31	Canary Islands	7.36	High
2006MAR29PEL	1, 35	Morocco	6.31	High
2004ERI38PEL	30	Eritrea	5.75	High
139362	19, 40	Morocco	5.55	High
139363	20, 41	Morocco	5.54	High
143467	12, 38	Italy	5.24	High
139026	22	Greek Islands	4.99	Low
GEH71PEL	25	Greek Islands	4.80	Low
143415	10	Spain	4.65	Low
139058	2	Greek Islands	4.60	Low
2004ESP64PEL	9, 44	Spain	4.50	Low
139049	18, 45	Greek Islands	4.13	Low
2006ISR20PEL	28, 42	Israel	4.00	Low
143464	11	Italy	3.93	Low
2004ERI37PEL	3	Eritrea	3.91	Low
cv. MAURO	15, 39	Italy	3.44	Low
143469	13	Italy	2.57	Low
cv. CASBAH	27, 33	Morocco	2.22	Low
143474	14, 32	Italy	2.16	Low
93ITA45PEL	24	Italy	2.06	Low
2005GRC77PEL	5	Greek Islands	1.95	Low
GEH77PEL	26, 36	Greek Islands	1.64	Low
93FRA4PEL	23	France	1.49	Low
2004ESP39PEL	8, 34	Canary Islands	1.07	Low
143267a	16	Italy	0.91	Low
143267b	17	Italy	0.68	Low
2006MAR22PEL	29, 37	Morocco	0.66	Low
138972	21	Morocco	0.54	Low

auto-sampler maintained at 4 °C, column heater maintained at 30 °C and photodiode array detector) utilizing a gradient elution, (98 to 0%A over 20 min followed by a 5 minute wash with 100% B before returning to the initial gradient conditions for 4 minutes; A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile, at 0.3 ml min⁻¹). The HPLC was connected to a ThermoFisher LTQ Velos orbitrap mass spectrometer (Thermo Fisher Scientific, USA), operating with a heated electrospray ionization probe operating in a negative FT mode over 100–2000 *m/z*. The heated capillary was maintained at 300 °C with a source heater temperature of 350 °C and the sheath, auxiliary, and sweep gases at 37, 13, and 7 units respectively. The source voltage was 3.3 kV.

MS2 and MS3 analyses were carried out on selected samples using a data-dependent approach with accurate mass in MS1 and MS2 set at resolution of 15,000 for MS2 and MS3. Dynamic exclusion was engaged with a repeat duration of 30 s and an exclusion duration of 180 s.

2.3.3 LCMS data analysis

Initial data processing was carried using Expressionist Refiner MS (Genedata, Basel, Switzerland) to align MS data, carry out noise reduction, and for peak picking. Cluster analysis was undertaken to group isotope peaks and singletons removed. The cluster volume was analyzed statistically in Expressionist

Analyst (Genedata, Basel, Switzerland). Samples were classified into groups according to methanogenic potential and by country of origin (Table 1). Principal components analysis (PCA) and hierarchical cluster analysis (HCA)—Manhattan—were used to explore the relationship between the accessions. For both methods valid values were set to a minimum of 20%. To investigate specific features that were elevated in the low methanogenic potential group compared to the high group, a *t* test was used with 30% valid values per group with bootstrapping (10 repeats). *P* values were adjusted using Bonferonni's correction ($P=0.05$ equivalent to $P=7.7e-6$). In order to identify specific metabolomic features that could be contributing to the low methanogenic potential of the biserrula accessions, the LCMS data was re-examined. The clover samples were omitted from analysis so that only the high and low biserrula accessions were considered. These were then analyzed by a *t* test. The features that were highly statistically significant and that had an effect size of ten or greater (elevated in the low CH₄ accessions) were selected as the most likely candidate features with CH₄ lowering potential.

2.4 NMR sample preparation

Freeze-dried sample extracts were re-dissolved for NMR analysis in 600 μ L of DMSO-*d*₆ by vortexing and sonicating. Samples were centrifuged for 10 min at 14,000 rpm and 20 °C. A total of 550 μ L was transferred to NMR tubes for analysis.

2.4.1 NMR analysis

NMR data was acquired on a Bruker 700-MHz instrument equipped with a cryoprobe and an autosampler. The noesypr1d pulse sequence was used with a spectral width of 18.6 ppm, 256 scans (8 dummy scans), and a total acquisition time of 1.26 s. The data were processed in Topspin 3.2 (Bruker Biospin, Germany) with a line broadening of 0.3 Hz. Spectra were referenced to DMSO (2.5 ppm). 2D NMR data was acquired for samples 4 (high) and 14 (low). The pulse sequences used were socygpqf, hsqcedetgpsisp 2.2, and hmbcplpndqf.

2.4.2 NMR data analysis

Proton spectra were imported into MatLab (R2015b, Mathworks) using a modified Prometab script, as previously described (Rochfort et al. 2015). Spectra were baseline corrected and normalized (total spectral area set to one) in PLSToolbox (Ver 8.0.2, Eigenvector Research). The spectra were analyzed by PCA with mean centering pre-processing. This analysis confirmed that the replicate samples (for 17, 36, and 46) clustered closely to each other and revealed the spectrum of sample 2 to be an outlier due to poor spectral quality. The spectra from

replicates and sample 2 were removed and the residual solvent (2.48–2.55 ppm) was deleted. This data was used in all subsequent PCA analyses. Samples were classified into groups according to methanogenic potential (Table 1).

3 Results and discussion

Initially, the “high” and “low” categories in methanogenic potential assigned in Table 1 involved the drawing of a somewhat arbitrary line through a continuous distribution. However, analysis of the LCMS data by unsupervised modeling techniques including PCA and HCA revealed clear trends in the data. In the PCA scores plot (data not shown), each accession was classified as having low or high (within biserrula's already low range of methanogenesis), and very high (in two clovers) methanogenic potential based on the in vitro CH₄ output (Banik et al. 2013). In general, the accessions cluster based on methanogenic potential with low and high groups separated on PC1 and the very high group separated on PC2. The clear outlier here was sample 3, which did not appear to cluster according to CH₄ group. Generally, the HCA showed that the clustering of the accessions by their methanogenic potential was in line with their origin (Fig. 2).

Initial analysis of NMR data with all accessions and control clover species using PCA revealed a similar pattern to the LCMS results with low and high accessions separating on PC1 and very high accessions separating on PC2 with the expected graduation across the two groups (not shown here). Accession 3 still stayed with its contrasting CH₄ output group.

Results obtained from both LCMS and NMR PCA suggest that high and low CH₄ output accessions are, to a large extent, two chemically distinct groups within the core collection of biserrula. Further, hierarchical cluster analysis indicates that within the high CH₄ output group of accessions, the metabolic profile of accessions from Eritrea is clearly distinct from accessions of the northern Mediterranean region. This supports the previous suggestion (Ghamkhar et al. 2012) that the Eritrean accessions are a differentiated group of accessions within *B. pelecinus* germplasm and distinctly different from the other accessions of biserrula, in agro-morphological traits, eco-geographical distribution, and genomic profile. However, this study suggests that they still share some metabolites with some accessions from Morocco and Canary Islands, regions geographically isolated from the northern Mediterranean region.

It can be seen that the outlier in PCA (accession 3) clusters with its respective eco-geographical origins after the HCA (Fig. 2), while the latter analysis identified 6484 features in metabolomics data. It separated the control species more clearly from the biserrula accessions (Fig. 2). It also separated the accessions with higher CH₄ output from the ones with lower CH₄ output, although there were three exceptions.

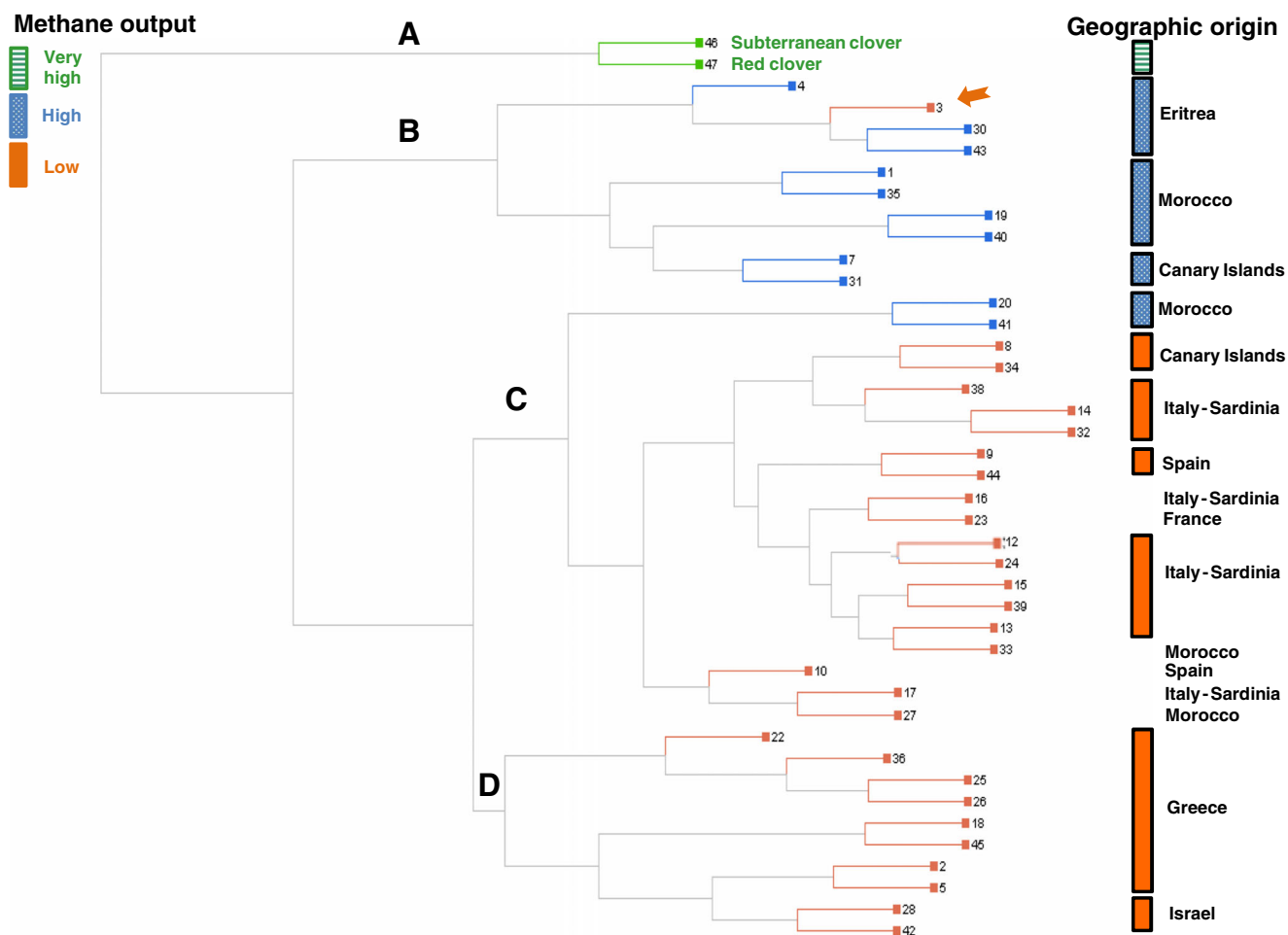


Fig. 2 Hierarchical clustering of liquid chromatography mass spectrometry (LCMS) data in the accessions of biserrula and the two clover control species. Accessions are clustered based on their color-

coded methane output (arrow indicating accession 3). Countries of origin of the accessions are projected on the clusters

Accession number 20 (same accession as 41) with a high CH₄ output was clustered with the lower CH₄ output cluster. Also, as in the PCA, accession 3 was grouped with the high emitters but it was clustered with its geographical group (Fig. 2). In general, this analysis, although on metabolites, indicates a pattern from high CH₄ output to low CH₄ output accessions. When the geographical site of the accessions is added to the equation, Eritrea is identified as the hotspot of accessions with high CH₄ output. A change in CH₄ output, from high to low, was observed in the collection sites from Africa to Europe via Morocco and the Canary Islands. The dendrogram was sub divided into four separate clusters (Fig. 2). Clovers were clearly separate from all biserrula accessions (cluster “A” in Fig. 2). All accessions from Eritrea clustered, firstly, together and then, distinctly with some Moroccan and Canary Islands accessions. The rest of the accessions could be geographically categorized to two regions: the western Mediterranean region or cluster C (Morocco, Canary Islands, Spain, France, and

Italy), and the eastern Mediterranean region or cluster D (Greece and Israel) (Fig. 2).

Among the low methanogenic accessions, Greek accessions have a similar metabolic profile with the Israeli accessions as they form a cluster together. Using agromorphological characteristics and molecular diversity profiles, Ghamkhar et al. (2012) concluded that accessions from the western and eastern Mediterranean regions can be distinguished as two separate categories. In this study, the two regions are distinct in their metabolomics profile as well, although Eritrean accessions are, again, an exception. However, within cluster B, the East-West separation is also true.

The discovery of 47 candidate metabolites (Table 2) that may affect CH₄ output in the rumen is a starting point for further investigation into these candidate metabolites as markers for this trait in other pasture species. It also provides an opportunity to discover candidate genes for controlling this trait in a reverse genetics approach, from metabolites to the

Table 2 Clusters elevated in accessions of the biserrula core collection with low compared to high methanogenic potential

Cluster	Effect size	Adjusted <i>p</i>	Mass	<i>m/z</i> [M-H] ⁻	RT (min)	Possible molecular formula
1	10.0	0.00051	858.4961	428.2408	8.35	C ₂₂ H ₃₆ O ₈
2	19.9	0.00003	650.4024	649.3951	8.41	C ₃₆ H ₅₇ O ₁₀
3	19.4	0.00005	652.4175	651.4102	8.42	
4	15.1	0.00279	680.4483	679.4410	9.17	C ₃₈ H ₆₃ O ₁₀
5	20.0	0.00006	684.4436	683.4363	8.72	
6	14.7	0.00059	690.4115	689.4042	7.17	
7	47.5	0.00249	696.4440	695.4368	7.44	C ₃₈ H ₆₃ O ₁₁
8	12.9	0.00001	696.4441	695.4368	7.55	C ₃₈ H ₆₃ O ₁₁
9	402.7	0.00020	696.4443	695.4370	7.73	C ₃₈ H ₆₃ O ₁₁
10	151.8	0.00007	698.4234	697.4161	7.22	C ₃₇ H ₆₁ O ₁₂
11	43.0	0.00026	700.4383	699.4310	7.17	C ₃₇ H ₆₃ O ₁₂
12	34.4	0.00031	700.4393	699.4320	7.40	C ₃₇ H ₆₃ O ₁₂
13	14.1	0.00169	706.4286	705.4213	12.33	C ₃₉ H ₆₁ O ₁₁
14	10.5	0.00054	722.4604	721.4531	9.29	C ₄₀ H ₆₅ O ₁₁
15	13.3	0.00153	724.4376	723.4303	9.17	C ₃₉ H ₆₃ O ₁₂
16	8.8	0.00252	728.3089	727.3017	3.84	C ₃₁ H ₅₁ O ₁₉
17	138.6	0.00026	740.4328	739.4255	7.44	C ₃₉ H ₆₃ O ₁₃
18	17.2	0.00003	740.4332	739.4259	7.55	C ₃₉ H ₆₃ O ₁₃
19	106.6	0.00005	740.4332	739.4260	7.73	C ₃₉ H ₆₃ O ₁₃
20	12.7	0.00015	752.4703	751.4630	8.46	C ₄₁ H ₆₇ O ₁₂
21	32.7	0.00298	768.4028	767.3955	7.17	C ₅₀ H ₅₅ O ₇ /C ₃₂ H ₆₃ O ₂₀
22	11.0	0.00014	782.4810	781.4737	7.98	C ₄₂ H ₆₉ O ₁₃
23	11.0	0.00008	782.4811	781.4738	8.63	C ₄₂ H ₆₉ O ₁₃
24	25.3	0.00212	794.4828	793.4755	10.33	C ₄₃ H ₆₉ O ₁₃
25	16.5	0.00004	796.4541	795.4468	10.28	C ₄₉ H ₆₃ O ₉
26	19.6	0.00054	798.4752	797.4679	8.25	C ₄₂ H ₆₉ O ₁₄
27	21.3	0.00115	816.4862	815.4790	6.27	C ₄₂ H ₇₁ O ₁₅
28	33.0	0.00155	830.4803	829.4731	8.03	
29	11.3	0.00020	830.5010	829.4937	8.11	
30	11.2	0.00114	838.5149	837.5076	9.41	
31	10.0	0.00015	846.4966	845.4893	7.81	C ₄₃ H ₇₃ O ₁₆
32	29.3	0.00169	862.4916	861.4843	6.27	C ₄₃ H ₇₃ O ₁₇
33	29.4	0.00003	862.4913	861.4840	6.44	
34	28.2	0.00001	884.4711	883.4638	8.08	C ₅₂ H ₆₇ O ₁₂
35	72.0	0.00022	902.4854	901.4781	6.71	C ₄₅ H ₇₃ O ₁₈
36	342.2	0.00032	902.4862	901.4790	6.51	
37	12.0	0.00065	922.4543	921.4471	10.50	C ₄₇ H ₆₉ O ₁₈
38	9.2	0.00064	926.5208	925.5135	9.96	C ₅₅ H ₇₃ O ₁₂ /C ₄₈ H ₇₇ O ₁₇
39	11.7	0.00023	926.4872	925.4799	9.42	
40	13.7	0.00028	972.4871	971.4798	7.96	C ₅₅ H ₇₁ O ₁₅
41	98.2	0.00069	1014.6110	1013.6040	7.54	C ₅₃ H ₈₉ O ₁₈
42	18.3	0.00000	1046.3781	1045.3710	5.40	C ₅₄ H ₆₁ O ₂₁
43	15.9	0.00019	1096.6862	1095.6790	7.78	C ₆₆ H ₉₅ O ₁₃
44	53.3	0.00004	1098.7047	1097.6970	7.84	C ₆₆ H ₉₇ O ₁₃
45	52.1	0.00148	1098.7025	1097.6953	8.03	C ₆₇ H ₉₉ O ₁₄
46	43.1	0.00016	1128.7149	1127.7080	7.82	C ₆₆ H ₉₇ O ₁₃
47	71.1	0.00046	1308.8670	1307.8600	7.16	C ₇₂ H ₁₂₃ O ₂₀

transcriptome and eventually the genome by investigating enzymes that control the biochemical pathways where these markers are most active/present. Ursolic acid, a candidate marker of interest in this study, is a pentacyclic triterpene saponin and a phytosterol. It is a biochemical component with cytotoxic activity and also used as an emulsifier in pharmaceuticals, cosmetics, and foods, similar to pomolic acid found in the wax coats of apples (He and Liu 2007). Both ursolic acid and its isomer, oleanolic terpene, identified as the two main candidate markers for CH₄ output mitigation in rumen, originate from the oleanyl cation in biosynthesis.

The loadings plot for PC1 (Fig. 3a) reveal some clear metabolic signatures that are responsible for the discrimination on PC1 and so between the low and high groups. The resonances between 0 and 2.5 ppm are typical of terpenes with methyl singlets resonating between 0.7 and 1.2 ppm. Methylene and methine resonances between 0.2 and 2.4 are separated and well resolved, typical of protons on more complex cyclic structures such as diterpenes and triterpenes. These terpene resonances are elevated in the spectra from the low CH₄ accessions. The majority of the carbohydrate residues are resonating between 2.8 and 4.2 ppm with anomeric methines resonating between 3.8 and 5.5 ppm. This is a crowded region of the spectrum and there are resonances contributing to both the positive and negative loadings. The olefinic methines between 5.2 and 5.5 ppm seem to be contributing to the separation on

the positive loadings. The aromatic methine resonances are elevated in the high accessions although, again, this is a crowded spectral region and there are some resonances contributing to the positive loadings as well.

Oleanolic acid and ursolic acid and their derivatives have anti-inflammatory and antioxidant effects as well (Pollier and Goossens 2012). In plants, specifically, they exist in the epicuticular waxes as a first defense barrier against pathogens (Heinzen et al. 1996) and may function as defense compounds against pathogens or as allelopathic agents (Szakiel et al. 2005). This suggests a potentially crucial role for these components as key biomolecular components to be added to future vaccines against high methanogenesis in the rumen, possibly through their antibacterial (Pollier and Goossens 2012) or anti-proliferative (Neto 2007) activity. However, Banik et al. (2016) suggested that rumen microflora can change or adapt over time in response to biserrula substrate; therefore, the short-term bioassay data has to be used with some caution. Ultimately, in vivo and in-field testing is required to confirm these properties.

3.1 Data mining for specific metabolites for CH₄ output

A total of 47 features were identified (Table 2) that were significantly elevated in the low group ($P < 0.05$ and 10 fold or

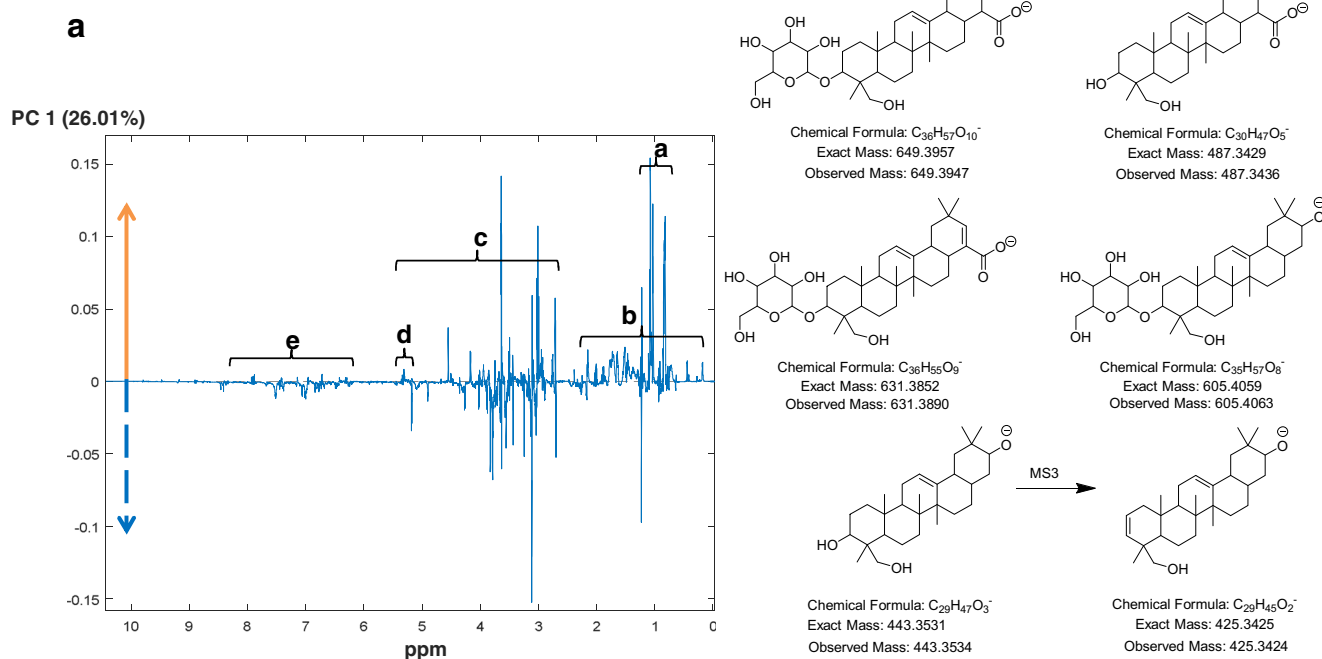


Fig. 3 a Principal component analysis loadings plot of NMR data for biserrula. The resonances on the positive side of the Y axis are elevated in the accessions with low methanogenic potential (upward arrow) and on the negative side of the Y axis in accessions with high methanogenic potential (downward arrow). Several structural

features can be identified that may play a role in diversity of the methanogenic potential of the accessions: a–e. b MSn analysis of m/z 649.3957 where the parent ion (m/z 649.3957) generates 5 fragments in MS2 and the MS2 base peak (m/z 443.3534) generates m/z 425.3424 in MS3

greater change in cluster volume). These 47 features correspond to metabolites of relatively high molecular weight, between 650 and 1310 amu. Accurate mass measurement was used to predict the most probable molecular formulae. However, even with a mass error of 3–5 ppm, allowed there are over 30 possible formulae for many of the ions. For this reason, and considering both the NMR and MS² data, formulae were restricted to CHO composition. This allowed putative molecular formulae to be proposed.

Joblin (1999) stated that the management of H₂ production in the rumen is the most important factor to be considered when developing strategies to control ruminant CH₄ emissions. It should therefore be possible to reduce CH₄ production by inhibiting H₂-liberating reactions or by promoting alternative H₂-using reactions or routes for disposing of H₂ during fermentation. An achievement in reducing CH₄ production in livestock farms would be the development of a vaccine containing an antigen derived from methanogenic bacteria (Gworgwor et al. 2006) and an immunogenic preparation which reduces the activity of rumen protozoa (Baker et al. 2004). Such vaccines have the potential to provide a cost-effective treatment to reduce CH₄ emission and enhance animal production. The candidate metabolites are also potential precursors for future vaccine development if their inhibiting properties on methanogenic bacteria are proved.

MSⁿ data was acquired in a data independent mode in separate analyses. Useful MSⁿ data was acquired on only a few of the 47 potential biomarkers. The best data was obtained for *m/z* 649.3957. The parent ion (*m/z* 649.3957) generates five main fragments in MS² (*m/z* 631.3890, 605.4063, 487.3436, 443.3534, and 425.3424). In addition, the MS² base peak (*m/z* 443.3534) generates *m/z* 425.3424 in MS³. Accurate mass measurements on the fragment ions greatly facilitated the assignment of the molecular formulae of this molecule and allowed determination of the molecular formulae of the fragments. The mass and fragmentation pattern are consistent with an ursolic or oleanolic terpene glycoside. The fragmentation of this molecule follows the same general trend reported by Xia et al. (2015). In particular, the observation of the loss of CO₂ to form the ion at *m/z* 605.4063 confirmed that the glycosylation cannot occur at this position and also confirms the presence of the carboxylic acid moiety in the molecule. The regiochemistry of the molecule cannot be defined by the MS data, hence the structure may be an ursolic or oleanolic terpene. The NMR data is consistent with this interpretation in that there are resonances in the regions appropriate for both the carboxylic acids and terpene glycosides (Fig. 3b). Indeed, the NMR data suggests there are many such molecules in the accessions. However, the NMR data is complex and no single molecule could be positively identified.

Although the remaining biomarkers remain unidentified, it seems very likely that they are also terpene glycosides. Further work will need to be done with both targeted MSⁿ approaches

and isolation of metabolites for NMR structure determination to confirm this. Several of the proposed molecular formulae do not have a match in databases (Scifinder, FoodDB, etc.) suggesting that it is very likely that some of these metabolites will be novel biomarkers for methane mitigation.

4 Conclusion

In conclusion, metabolomic and methanogenic profiles of the biserrula core collection are very congruent. We suggest that metabolites discovered in this study and most likely the ursolic or oleanolic terpene glycosides affect the methanogenic potential in the rumen and the novel ones can be potentially used for vaccine manufacturing in the future. Further mass spectrometry analysis of samples with CH₄ output measurements in the field will be needed to confirm the relevance and true effects of the discovered metabolic markers. The results obtained from all datasets suggest that the Eritrean accessions likely belong to a different taxon within the species *B. pelecinus* with potentially different agronomic traits. Finally, crossing the low and high methanogenic accessions of biserrula investigated in this study will lead to materials and populations for gene discovery of CH₄ output through transcriptomics and reverse genomics tools and techniques in the near future.

Acknowledgements The authors would like to thank Vilnis Ezemiaks of the Department of Economic Development, Victoria, for MS lab experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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