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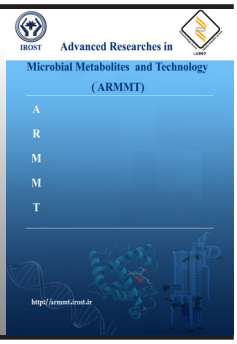

A study of rumen microbial community of Baluchi lambs fed a high concentrate diet containing conventional ingredients

Seyed Hadi Ebrahimi[1*], Reza Valizadeh[1], Vahideh Heidarian Miri[1] and Peter H. Janssen[2]

[1] **Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran**

[2] **AgResearch Limited, Grasslands Research Centre, Palmerston North 4442, New Zealand**

Article Info**

Received: 10 Febuary 2017
Received in revised form: 13 April 2017
Accepted: 5 May 2017

Keywords:
Baluchi Lamb
Rumen Microbes
High Concentrate Diet
Pyrosequencing

Abstract

Baluchi sheep is the dominant fat-tail breed in Iran. We studied the microbial communities in the rumen of four Baluchi lambs fed a high concentrate conventional diet. Using DNA extracted from the rumen samples, we found the partial ribosomal rRNA of bacterial and archaeal were amplified by polymerase chain reaction (PCR). The amplicons were sequenced using 454 Titanium pyrosequencing and the data analyzed using the QIIME software package. The results indicated that Prevotella, a member of thephylum Bacteroidetes, dominated and its relative abundance accounted for 70.7 ± 2.68% of the bacteria. Firmicutes was the second most abundant phylum, and Selenomonas and unclassified Veillonellaceae and Lachnospiraceae were present at relative abundances of 2.4 ± 0.62, 2.1 ± 0.21 and 1.9 ± 0.58%, respectively. Entodiniumwas the most abundant genus of protozoa, comprising 61.6 ± 4.52% of the protozoal community, followed by Polyplastron and Isotricha with relative abundances of 18.2 ± 2.35 and 9.7 ± 5.62%, respectively. More than half of the archaeal community (53.3 ± 1.87%) was composed of members of the *Methanobrevibacter gottschalkiiclade*. The second and third most dominant archaea were members of the order Methanomassiliicoccales (28.3 ± 5.23%) and *Methanobrevibacter wolinii* and relatives (8.5 ± 4.26%). Based on this, rumen microbes of Baluchi lambs fed a total-mixed rations diet were similar to rumen microbes of ruminants fed similar diets in the other geographic regions around the world.

1. Introduction

The rumen, the modified forestomach of ruminant animals, is an anaerobic ecosystem in which multiple different microorganisms form a symbiotic relationship with their host (Hungate and Hobson, 1988). The animal provides a suitable environment for the activity of the microbes, and during feeding it delivers complex plant material into the rumen. The microorganisms break down polymeric components of the feed and ferment the smaller components released to produce products that the animal can use, like volatile fatty acids. The microorganisms can also break down

*Corresponding author. Tel: + 985138805744
Email address: shebrahimi@um.ac.ir

** This article has first been published online as 1(2017) 12-21

DOI: 10.22104/ARMMT.2018.2459.1008

toxins that might otherwise adversely affect the animal. The multiplicity and diversity of ruminal bacteria, protozoa, anaerobic fungi and methanogens has been previously reported (Callaway et al., 2010) and factors affecting microbial community composition have been also studied (Liu et al., 2016).

Manipulation of the rumen microbial ecosystem is a strategy to enhance nutrient utilization, aiming to increase desirable processes, pathways and microbial populations (Castro-Carrera et al,. 2014). Processes that might be manipulated include improved fiber digestion or minimizing the number of microorganisms that produce deleterious substances or are involved in processes that do not benefit the animal, such as proteolysis, methanogenesis, etc. (Heidarian Miri et al., 2015). Success or failure of such strategies depends upon the relative abundance of specific target organisms. Transferable strategies for altering populations of microorganisms rely on the similarity of the communities in different ruminants, but if specific ruminant populations have unique and different rumen microorganisms, manipulation techniques developed for other breeds might not be applicable.

The microbes in a sample collected from a rumen can be surveyed using marker genes for the different taxa present. The 16S rRNA gene is widely used to identify bacteria and archaea present in the rumen, and the 18S rRNA gene is used to identify protozoa (Kittelmann et al., 2013). DNA is extracted from rumen contents, the marker genes are amplified using targeted primers, and the resultant amplicons sequenced, for example, using 454 pyrosequencing technology. The sequences are compared to databases of marker genes that are annotated with taxonomic identities, allowing each sequence read to be assigned to a taxon. Multiple samples can be analyzed in a single experiment by including a unique DNA barcode in the amplification primers used for each sample, so that the resultant sequence reads can be attributed to the sample the sequences were amplified from. By generating hundreds or thousands of sequences for each sample, a picture can be built up of the microbial composition in the rumen of the animal from which the sample came.

The Baluchi sheep is a dominant fat-tail breed in Iran and is well adapted to warm and dry environmental conditions. The Baluchi sheep has the physiological ability to deal with drought and thirst (Gholizadeh and Ghafouri-Kesbi, 2015). It is also an important part of livestock farming in southwest Pakistan and southern Afghanistan, and fat-tailed breeds have been raised in this area of southwest Asia for at least 5000 years (Ryder, 1983). The impact of this breed on the carpet and meat industries in the areas where it is farmed is large (Valizadeh, 2010), which make it attractive to study to attempt to understand and potentially improve production and production efficiency. The aim of this study was to investigate the rumen microbial community in the Baluchi breed.

2. Materials and methods

Animals, feed and management

Rumen samples used in the present study were collected from four growing Baluchi lambs (9 months old and 35 ± 1.4 kg body weight) maintained at the livestock research center, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran in 2015. Experimental procedures used in this study were approved by the Principles of Research Ethics, Ministry of Science, Research and Technology, Iran. The sheep were fed twice a day with a total-mixed rations diet containing 70 % chopped alfalfa hay and 30 % concentrate, by dry weight at 7:00 am and 4:00 pm. Animals were maintained in individual pens while on the diets (ad libitum feeding with about 10 % refusals) with free

access to water at all times for more than two months. The composition of the diet is given in Table 1. Rumen contents samples were collected before the morning feeding using a stomach tube, immediately transferred to the laboratory where the pH was measured. As described in our previous work (Ebrahimi et al., 2011), 10 and 3 ml aliquots of the rumen samples were used for estimation of ammonia nitrogen and volatile fatty acids (VFA), respectively. Approximately 300 ml of rumen contents were frozen and freeze-dried for DNA extraction. To determine diet composition, AOAC methods were applied to determine diet composition including method 930.15 and 928.08 for dry matter and crude protein, respectively, (AOAC 2006). Acid and neutral detergent fibers were estimated by the Van Soest et al. (1991) method.

Table 1. Composition of the diet

Ingredient	% of dry matter
Alfalfa hay	30
Barley	36.2
Wheat bran	24
Cotton seed meal	7
Soybean meal	7
Sunflower meal	7
Rice bran	6
Bagasse (enriched with molasses)	6
Salt	1
Sugar beet molasses	4
Calcium carbonate	0.8
Mineral and vitamin supplement[1]	0.5
Sodium bentonite	0.5
Chemical composition, % DM	
CP	16.85 ± 0.09
ADF	18.4 ± 0.06
NDF	32.14 ± 0.9

[1]Contained: Fe, 40 mg/kg Mn, 60 mg/kg Zn, 20 mg/kg Cu, 1 mg/kg I, 0.2 mg/kg Se, 0.2 mg/kg Co, 2,200 IU of vitamin A/kg, 275 IU of vitamin D/kg, and 50 IU of vitamin E/kg.

2.1. Assessment of the microbial community composition

2.1.1. DNA extraction, PCR amplification and 454 pyrosequencing

DNA extraction was performed from 30 mg of freeze-dried, homogenised rumen contents using the PCQI method (Rius et al., 2012) in the rumen microbiology laboratory, AgResearch, Grasslands Research Centre, Palmerston North, New Zealand.

Table 2. Group-specific primers used to amplify bacterial and archaeal 16S rRNA genes and ciliate 18S rRNA genes, and their arrangement with adapters, barcodes, and linkers. Sequences of adapters A and B are given in the text. The barcodes are described by Fiereret al. (2008).

Microbes	Primers name	Primer Seq (5'-3') [Adaptor-[Barcode]-Linker-Specific primer	Primers Location	Amplicon (bp)	Tm (°C)
Bacteria	Ba9F	Adapter B-AC-GAGTTTGATCMTGGCTCAG	16S RNA (9-27)	525	52
	Ba515Rmod1	Adapter A-barcode-GT-CCGCGGCKGCTGGCAC	(515-530)		
Archaea	Ar915aF	Adapter A-barcode-GT-AGGAATTGGCGGGGGAGCAC	16S RNA (896-915)	492	59
	Ar1386R	Adapter B-CA-GCGGTGTGTGCAAGGAGC	(1386-1403)		
Protozoa	RP841F	Adapter B-AA-GACTAGGGATTGGARTGG	18S RNA (824-841)	511	54
	Reg1302R	Adapter A-barcode-TC-TCAATTGCAAAGATCTATCCC	(1302-1322)		

Adapter A: 5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3'

Adapter B: 5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG-3'

The extraction protocol combined mechanical (bead-beating) and chemical (phenol-chloroform) protocols in the Kittelmann *et al.* (2013) method.

16S and 18S rRNA gene regions of microbial rumen community were each amplified seprataely, in triplicate as in Rius *et al.* (2012) with primers shown in Table 2. One μg DNA of each microbial group was gel electrophresed on agarose gel (1%). Bands were visualized and excised under blue light transillumination and amplicons were gel purified with the QIA quick Gel Extraction Kit (Qiagen). Using 454 GS FLX Titaniumchemistry at Eurofins MWG Operon (Ebersberg, Germany), microbial amplicons were then sequenced.

2.1.2. Phylogenetic analysis of pyrosequencing reads

The data processes and analysis of 454 Pyrosequencing were conducted using QIIME software (Caporaso *et al.*, 2010). Operational taxonomic units (OTUs) were determined based on the 97% similarity threshold for bacteria (Edgar, 2010), 99% for archaea and 100% for protozoa (usually 97% similarity). BLAST analysis (Altschul *et al.*, 1990) of bacterial (McDonald *et al.*, 2012), archaeal (Seedorf *et al.*, 2014), and protozoal (Kittelmann and Janssen, 2011) rRNA genes was also carried out. Data, including the total number of reads assigned to each taxon, were summarized for each sample by phylum at phylum, class, order, family and genus levels for bacteria and using a mixed taxonomic scheme for archaea (Seedorf *et al.*, 2014), and at the genus level for protozoa.

3. Results and discussion

Table 3 shows rumen fermentation characteristics of Baluchi lambs which were *ad libitum* fed a diet composed of 70 % concentrates. The average pH value of rumen fluid was 6.08 ± 0.01 and the concentration of total VFA was estimated at 120.12 ± 0.58 mM.

Table 3. Volatile fatty acids, ammonia nitrogen and ruminal pH of Baluchi lambs fed a high concentrate diet.

Items	Mean ± SE
Total VFA (mM)	120.12 ± 0.58
Molar proportion (%)	
Acetate	68.14 ± 0.12
Propionate	29.31 ± 0.09
Butyrate	2.55 ± 0.01
Ammonia N (mg/ dL)	26.52 ± 0.04
pH	6.08 ± 0.01

As part of the Global Rumen Census project, we characterized the overall ruminal microbial composition by using pyrosequencing data generated from16SrRNA and 18S rRNA gene amplicons. The sequences were deposited in the GenBank as BioSamples SAMN03293962 to SAMN03293965. In total, 40189 bacterial 16S rRNA gene sequence reads were generated with an average of 10047 reads per sample. We detected 105 bacterial groups, of which thirty-four groups (Table 4) comprised 98.82 % of all bacterial sequences. The remaining 1.38 % (grouped and labeled as "Others") had a relative abundance of smaller than 0.1 % each. Twenty of these 34 dominant groups were classified to the genus level, and contained a total relative abundance of 82.86 % of all bacterial reads, while 14 groups not resolved to the genus level had a combined relative abundance of 15.76 %. Reads originating from members of the genus Prevotella (in the phylum Bacteroidetes) dominated the dataset, and on average its relative abundance accounted for 70.71 ± 2.68 % of total bacteria. The second most abundant (6.68 ± 0.09 %) bacteria were assigned to an unclassified group of Bacteroidales (also belonging to the phylum Bacteroidetes).

The second most abundant bacterial phylum was Firmicutes, and Selenomonas, unclassified Veillonellaceae and unclassified Lachnospiraceae (all members of that phylum) had relative abundances of 2.43 ± 0.62, 2.14 ± 0.21 and 1.88 ± 0.58%, respectively. The relative abundance of sequences from the genus Fibrobacter was 1.83 ± 0.38 %, and so Fibrobacteres was the third most abundant phylum. *Prevotella* spp. are strictly anaerobic gram negative bacteria that degrade and use starch and plant cell wall polysaccharides such as xylans and pectins.

Table 4. Bacterial community composition in the rumens of Baluchi lambs fed a high concentrate diet.

Phylum	Class	Order	Family	Genus	Percent ± SE
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	70.71 ± 2.68
Bacteroidetes	Bacteroidia	Bacteroidales	unclassified	unclassified	6.68 ± 0.09
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Selenomonas	2.43 ± 0.62
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified	2.14 ± 0.21
Firmicutes	Clostridia	Clostridiales	unclassified	unclassified	1.88 ± 0.58
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	unclassified	1.85 ± 0.25
Fibrobacteres	Fibrobacteres	Fibrobacterales	Fibrobacteraceae	Fibrobacter	1.83 ± 0.38
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter	1.65 ± 0.31
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	1.08 ± 0.14
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified	0.94 ± 0.22
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.91 ± 0.18
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	unclassified	0.81 ± 0.23
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	0.56 ± 0.16
Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	TG5	0.50 ± 0.44
Tenericutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Bulleidia	0.44 ± 0.22
Tenericutes	Erysipelotrichi	Erysipelotrichales	vadinHA31	RFN20	0.42 ± 0.26
Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio	0.40 ± 0.18
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified	0.39 ± 0.26
Tenericutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Sharpea	0.36 ± 0.20
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	0.30 ± 0.07
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospira	0.28 ± 0.11
Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma	0.26± 0.12
Firmicutes	Clostridia	Clostridiales	Incertaesedis	unclassified	0.20 ± 0.04
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	0.19 ± 0.12
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema	0.19 ± 0.08
Lentisphaerae	Lentisphaerae	Victivallales	Victivallaceae	unclassified	0.18 ± 0.04
Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	unclassified	0.17 ± 0.04
Cyanobacteria	4C0d-2	YS2	unclassified	unclassified	0.15 ± 0.04
Proteobacteria	Gammaproteobacteria	Aeromonadales	unclassified	unclassified	0.13 ± 0.06
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Pseudobutyrivibrio	0.12 ± 0.02
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Shuttleworthia	0.12 ± 0.05
Proteobacteria	Alphaproteobacteria	unclassified	unclassified	unclassified	0.12 ± 0.06
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231	0.10 ± 0.03
TM7	TM7-3	CW040	F16	unclassified	0.10 ± 0.04
Others					1.38 ± 0.01

They are not cellulolytic, but do degrade proteins (Hungate and Hobson, 1988). *P. bryantii, P. ruminicola, P. albensis*, and *P. brevis* species are four known ruminal species. When grown in the laboratory, production of polysaccharidases was influenced by the growth substrates and each ruminal Prevotella responded differently (Matsui et al., 2000). This versatility may account for the abundance of this genus in the rumen of the Baluchi lambs studied here. A

similar dominance of *Prevotella* spp. has been found in the rumen of adult cows fed 70 % concentrate (Jami *et al.*, 2013) and in heifers fed a diet with 100 % concentrate (Petri *et al.*, 2012). Stevenson and Weimer (2007) also found that the genus Prevotella accounted for 60 % of total bacteria when cows consumed a diet containing 63 % concentrate and that uncultured species of the genus predominated while the relative abundances of known strains were maximally about 2 %. Because Lin *et al.* (2015) reported that Prevotella made up 58.3 % of total ruminal bacteria in the rumens of buffalo maintained on a high proportion of concentrate in the ration, it may be concluded that diet is the main factor for the dominance of *Prevotella* spp. in the rumen of ruminants, rather than the location and animal species. Thus, it seems that the bacterial community of the Bauchi lambs reflects the proportion of high concentrate in the diet. Considering that the pH of the rumen samples was not in the acidotic range (i.e., < 5.5), that the animals showed no signs of acidosis, and noting that there was only a very low population of *Streptococcus* spp. (< 0.1 %), it can be concluded that there was sufficient time for adaptation to the high concentrate diet under the feeding regime used in this study.

We obtained 5861 good-quality reads for protozoa, with an average of 1465 reads per sample. Twelve protozoal genera were identified (Table 5). Entodinium was the most abundant genus, comprising 61.62 ± 4.52 % of the protozoal community, followed by Polyplastron and Isotricha with relative abundances of 18.19 ± 2.35 and 9.69 ± 5.62, respectively. Three other dominant genera having more than 0.1 % relative abundance were Ophryoscolex (4.84 ± 2.93), the Eremoplastron-Diploplastron group (3.63 ± 1.52) and Dasytricha (1.32 ± 1.13). Henderson *et al.* (2015) in a recent global study of rumen and foregut samples from ruminants and other foregut fermenters identified 12 genus-equivalent protozoal groups: Anoplodinium-Diplodinium, Enoploplastron, Entodinium, Epidinium, Eremoplastron-Diploplastron, Eudiplodinium, Metadinium, Ophryoscolex, Ostracodinium, Polyplastron, Dasytricha and Isotricha.

Table 5. Protozoal community composition in the rumens of Baluchi lambs fed a high concentrate diet.

Genus	Percent ± SE
Entodinium	61.62 ± 4.51
Polyplastron	18.19 ± 2.35
Isotricha	9.69 ± 5.62
Ophryoscolex	4.84 ± 2.93
Eremoplastron-Diploplastron	3.63 ± 1.52
Dasytricha	1.32 ± 1.13
Enoploplastron	0.31 ± 0.31
Epidinium	0.21 ± 0.09
Anoplodinium-Diplodinium	0.09 ± 0.04
Ostracodinium	0.03 ± 0.03
Eudiplodinium	0.03 ± 0.02
Metadinium	0.03 ± 0.02

Only five of these genera had relative abundances of greater than 1 % as found in the rumen samples of the present study. Over wide ranges of diets, species, breeds and geographical locations, the genus Entodinium (the smallest protozoa in the rumen) was reported as a predominant group of ruminal protozoa (Henderson *et al.*, 2015; Gürelli *et al.*, 2016). It has been reported that diets containing high levels of concentrate favor *Entodinium* spp. (Abrar *et al.*, 2016). In this study, in which the sheep were fed a diet containing 70 % concentrate, the high abundance of Entodinium which has the capability of engulfing particulate materials (highly fermentable carbohydrates), could have helped the host by preventing different bacterial fermentations that cause lactic acid

acidosis by pH reduction (Mackie *et al.*, 1978). However, Singh and Kundu (2011) reported the dominance of entodiniomoph protozoa in the rumens of sheep fed tree and grass leaves at the ratio of 75 to 25, so the explanation of their abundance may be more complicated than that. Polyplastron, a large cellulolytic protozoan with greater endoglucanase and xylanase activity than genus Entodinium (Newbold *et al.*, 2015), and the second most predominant group of the protozoa present in the rumen of the Baluchi lambs were also reported in a similar study by Lin *et al.*(2015), followed by the holotrich genus Isotricha. Polyplastron spp. were also the closest protozoal group associated with concentrate intake in a global study (Henderson *et al.*, 2015).

Table 6. Archaeal community structure in the rumen of Baluchi lambs fed a high concentrate diet.

Group	Percent ± SE
Methanobrevibacter gottschalkii clade	53.32 ± 1.87
Methanomassiliicoccales	28.29 ± 5.23
Methanobrevibacter wolinii and relatives	8.49 ± 4.26
Methanobrevibacter ruminantium clade	6.23 ± 1.53
Methanosphaera spp.	3.65 ± 0.74
Methanimicrococcus spp.	0.02 ± 0.02

The number of good quality reads obtained for rumen archaea was 4433, and on average there were 1108 reads per sample. Six archaeal groups were detected in the rumens of the sheep (Table 6). More than half of the archaeal community (53.32 ± 1.87 %) was composed of members of the *Methanobrevibactergottschalkii* clade. The second and third most dominant groups were Methanomassilii coccales (28.29 ± 5.23 %) and *Methanobrevibacter wolinii* and its relatives (8.49 ± 4.26 %). Two other dominant archaeal groups with relative abundances greater than 0.1 % were the *Methanobrevibacter ruminantium* clade and *Methanosphaera* spp. The abundance of members of the *Methanobrevibactergottschalkii* clade of rumen methanogens observed in the present study is similar to that reported elsewhere (Henderson *et al.*, 2015; Lin *et al.*, 2015; Huang *et al.*, 2016). These methanogens use hydrogen plus carbon dioxide for their growth. Their major competitors would appear to be the physiologically similar members of the *Methanobrevibacter ruminantium* clade, but these were rare in the samples studied here. This pattern fits well with the global pattern of *M. gottschalkii* being more strongly favored in concentrate-rich diets and *M. ruminantium* being favored in forage-rich diets (Henderson *et al.*, 2015). Members of the order Methanomassilii coccales are implicated in methane emissions in the rumen, possibly from methylamines (Poulsen *et al,*. 2013) and were found as major methanogens in the rumen of Australian sheep and small ruminants of Tibetan Plateau (Huang *et al.*, 2016).

3. Conclusion

In the present study, lambs of the indigenous Baluchi sheep were fed a ration commonly used in the nutrition of ruminants, and the microbial communities in their rumen studied using 454 pyrosequencing. The dominant microorganisms observed in the rumen of these lambs were similar to those previously reported from different species and breeds fed similar diets. This indicated that when an indigenous breed consumed conventional feeds, the effect of the diet determined the rumen microbial community structure. Knowledge of rumen microbial community function from other studies may therefore be applicable to these sheep. However, it remains to be determined how the rumen microbial community of such animals changes when they are grazing on natural pastures under dry and warm conditions, and if there are indications that this may be specialized to the diet or even the host.

Conflict of interest

The authors confirm that there is no conflict of interest.

Acknowledgements

This work was supported by Ferdowsi University of Mashhad, Mashhad, Iran. The New Zealand Government (as part of its support for the Global Research Alliance on Agricultural Greenhouse Gases) supported the molecular analysis in the current study. We thank Faith Cox and Gemma Henderson for their assistance with this project.

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