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Technical note: Comparison of automated ribosomal intergenic spacer analysis and denaturing gradient gel electrophoresis to assess bacterial diversity in the rumen of sheep[1]

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ABSTRACT: The aim of this study was to compare automated ribosomal intergenic spacer analysis (ARISA) and denaturing gradient gel electrophoresis (DGGE) techniques to assess bacterial diversity in the rumen of sheep. Sheep were fed 2 diets with 70% of either alfalfa hay or grass hay, and the solid (SOL) and liquid (LIQ) phases of the rumen were sampled immediately before feeding (0 h) and at 4 and 8 h postfeeding. Both techniques detected similar differences between forages, with alfalfa hay promoting greater ($P < 0.05$) bacterial diversity than grass hay. In contrast, whereas ARISA analysis showed a decrease ($P < 0.05$) of bacterial diversity in SOL at 4 h postfeeding compared with 0 and 8 h samplings, no variations ($P > 0.05$) over the postfeeding period were detected by DGGE. The ARISA technique showed lower ($P < 0.05$) bacterial diversity in SOL than in LIQ samples at 4 h postfeeding, but no differences ($P > 0.05$) in bacterial diversity between both rumen phases were detected by DGGE. Under the conditions of this study, the DGGE was not sensitive enough to detect some changes in ruminal bacterial communities, and therefore ARISA was considered more accurate for assessing bacterial diversity of ruminal samples. The results highlight the influence of the fingerprinting technique used to draw conclusions on factors affecting ruminal bacterial diversity.

Key words: automated ribosomal intergenic spacer analysis, denaturing gradient gel electrophoresis, forage, postfeeding time, ruminal bacteria, sheep

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INTRODUCTION

In the last few years several molecular fingerprinting techniques have been used to assess the diversity of ruminal microbial communities, the denaturing gradient gel electrophoresis (**DGGE**) probably being the most widely used. The DGGE theoretically separate the 16S rRNA gene based on the difference in the nucleotides with distinct melting properties, and its efficiency to analyze changes in ruminal bacterial communities produced by feeding different diets, defaunation, and other factors has been shown (Kocherginskaya et al., 2001; Huws et al., 2010; Mosoni et al., 2011). The automated ribosomal intergenic spacer analysis (**ARISA**) is based on the analysis of intergenic 16S-23S internally transcribed spacer sequences within the ribosomal DNA (rDNA) operon, and has also been used in the last five years to investigate ruminal bacterial diversity (Ramos et al., 2009; Weimer et al., 2010; Welkie et al., 2010). The ARISA technique has been shown to be more sensitive than DGGE in detecting changes in bacterial diversity in soils (Cherif et al., 2008), but to our knowledge there has not been a direct comparison of the efficacy of both techniques in the examination of factors influencing bacterial communities in the rumen. We hypothesized that the fingerprinting technique used to assess bacterial diversity of ruminal samples may influence the results, thus helping to explain some inconsistent results in the literature. In this study, we aimed for the first time to compare the results obtained by using ARISA and DGGE to assess how several factors

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(diet, rumen phase, and postfeeding time) affect bacterial diversity in the rumen of sheep.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

All animal procedures were conducted in accordance with the Spanish Animal Care Regulations and were approved by the León University Institutional Animal Care and Use Committee. Four rumen-fistulated Merino sheep (58.3 ± 3.27 kg BW) were housed in individual pens and had continuous access to fresh water over the trial. The 2 experimental diets had forage:concentrate ratio (DM basis) of 70:30 with alfalfa hay (**AL**) or grass hay (**GR**) as forage (**FOR**). The AL contained 2.67, 46.6, and 33.1% of N, NDF, and ADF, respectively, whereas the GR contained 1.46, 56.9, and 28.6% of N, NDF, and ADF, respectively. The AL-diet had greater N and ADF (2.69, 26.9% of DM, respectively), but lower NDF (42.6%) content than GR-diet (1.94, 23.8 and 49.9% of DM, respectively). Animals were fed twice daily (0800 and 2000 h) at a daily rate of 52 g of DM/kg $BW^{0.75}$. Statistical design was a crossover design with 2 experimental periods of 24 d each, the first 21 d for dietary adaptation. On d 22 and 24 of each period, ruminal content from each sheep was sampled immediately before (0 h) and at 4 and 8 h after the morning feeding. A mix of liquid (**LIQ**) and solid (**SOL**) samples from the dorsal, central, and ventral regions of the rumen were collected through the ruminal cannula to form one composited sample (about 500 g) for each sheep at each sampling time. Ruminal content was strained through 4 layers of cheesecloth, and about 100 g of SOL and 20 mL of LIQ samples were placed in independent sterile containers and stored frozen at –80°C until DNA extraction. Within each experimental period and sampling time, the 2 samples taken on the 2 sampling days from each sheep were composited before DNA analysis, and therefore there were 3 SOL and 3 LIQ samples per sheep in each period.

DNA Extraction, PCR-ARISA, and PCR-DGGE Analysis of Bacterial Communities

Liquid samples were defrosted and centrifuged (4 mL, 20,000 × *g*, 5 min, 4°C), and the pellets were used for DNA extraction. Freeze-dried SOL samples (120 mg) and pellets from LIQ samples were treated with a MiniBeadbeater (3 min; Biospec Products, Bartlesville, OK) for mechanical disruption of microorganisms before DNA extraction. Total DNA was extracted in duplicate following the procedure described by Yu and Morrison (2004), with the exception that an additional step involving the treatment of samples with cetyltrimethylammonium bromide was included to remove PCR inhibitors. The QIAamp DNA Stool Mini Kit columns (QIAgen, Valencia, CA) were used to purify the DNA.

For ARISA, DNA was amplified using universal bacterial primers 16S-1392F and 23S-125R (Danovaro et al., 2006). Primer 23S-125R was fluorescently labelled with the phosphoramidite dye 6-FAM. Thermocycling was conducted in a 2720 Thermal Cycler (Applied Biosystem, Foster City, CA) as described by Ramos et al. (2009) and the PCR products were analyzed for quality by electrophoresis in 1.5% agarose gels. The DNA concentrations were determined spectrophotometrically in a Nanodrop ND-1000 (Nano-Drop Technologies, Wilmington, DE), and about 5 ng of amplicons were mixed with an internal size standard (GS 1200 LIZ; Applied Biosystems, Foster City, CA) in deionized formamide, then denatured at 94°C for 2 min, and immediately chilled on ice as described by Ramos et al. (2009). Automated detection of fragments was performed using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) with 36-cm by 50-µm capillary and POP-7 polymer (Applied Biosystems). Peaks were identified by comparison with the internal size standard using the GeneMaker Software v1.80 (SoftGenetics, State College, PA) using a threshold of 100 fluorescence units.

Fragments of the 16S rDNA suitable for DGGE analysis were obtained by using the primers GM5clamp and 907R, which amplified a 550-bp fragment of the 16S rRNA gene (region hypervariable v3) and the PCR conditions described by Muyzer et al. (1993). The PCR amplifications were performed with an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Warrington, UK) in 25 µL volumes as described by Muyzer et al. (1993). Amplification products were analyzed first by electrophoresis in 1.5% (wt/vol) agarose gels, and then by ethidium bromide staining. Ten µL of amplicons were charged onto 6% polyacrylamide gels with a 20–60% denaturing gradient (100% denaturant defined as 7 mol/L urea, and 40% (vol/vol) formamide; Muyzer et al., 1993). Electrophoresis was performed in a Bio-Rad DCode system (Bio-Rad UK Ltd, Hemel Hempstead, UK) at a constant voltage of 100 V and 60°C of temperature for 18 h. Gels were stained with SYBR Gold and photographed with UV transillumination, and DGGE bands were detected using Quantity One software (Biorad, El Prat de Llobregat, Spain). All samples from 1 sheep were loaded in a single gel to facilitate the analysis of experimental treatments.

Statistical Analyses

Analysis of ARISA peaks and DGGE band profiles was performed with the MVSP v3.12d software (Kovach Computing Service, Anglesey, UK). The Shannon-Wiener index (H') was used to evaluate the diversity of bacterial

Table 1. Number of peaks**,** Shannon index, and similarity of bacterial communities in solid (SOL) and liquid (LIQ) phases of the rumen from sheep fed diets with 70% of alfalfa hay (AL) or grass hay (GR) as forage (FOR) assessed by ARISA and DGGE[1]

Fingerprinting technique	Rumen phase	Item	Diet	Time after feeding (h)			SEM	*P*-value		
				0	4	8		FOR	Time	FOR × Time
ARISA	SOL	Number of peaks	AL	49.2^{b}	32.8^{a}	48.5^{b}	3.28	0.01	< 0.001	0.35
			GR	44.2^{b}	22.1^{a}	42.3^{b}				
		Shannon index	AL	3.82^{b}	3.50^{a}	3.75^{b}	0.073	< 0.001	< 0.001	0.12
			GR	3.61^{b}	2.88^{a}	3.71^{b}				
		Similarity AL-GR, %	–	51.2^{b}	40.7^{a}	54.9^{b}	3.49	–	0.04	–
	LIQ	Number of peaks	AL	52.0^{b}	46.3^{ab}	44.0^{a}	2.57	0.02	0.03	0.62
			GR	46.0^{b}	38.5^{a}	41.3^{ab}				
		Shannon index	AL	3.95^{b}	3.82^{ab}	3.77^{a}	0.061	0.03	0.04	0.67
			GR	3.83^{b}	3.65^{a}	3.70^{ab}				
		Similarity AL-GR, %-		46.2	49.0	45.2	5.07	–	0.86	–
SOL-LIQ		Similarity SOL-LIQ, %	AL	48.3	47.2	49.7	4.00	0.005	0.11	0.25
			GR	35.4	30.4	46.5				
DGGE	SOL	Number of peaks	AL	12.8	12.5	11.0	1.05	0.03	0.46	0.84
			GR	9.5	10.4	9.5				
		Shannon index	AL	2.53	2.51	2.39	0.105	0.02	0.52	0.90
			GR	2.21	2.30	2.15				
		Similarity AL-GR, %	-	49.9	52.0	55.2	8.21	–	0.49	–
	LIQ	Number of peaks	AL	14.3	13.8	13.5	1.27	0.01	0.45	0.20
			GR	9.3	11.3	13.0				
		Shannon index	AL	2.61	2.54	2.57	0.100	0.008	0.27	0.14
			GR	2.19	2.38	2.50				
		Similarity AL-GR, %	–	50.7	55.4	55.6	3.44	–	0.55	–
SOL-LIQ		Similarity SOL-LIQ, %	AL	49.3	49.1	51.4	3.82	0.004	0.37	0.24
			GR	35.0	46.3	36.8				

[a,b]Within a row, means without a common superscripts differ ($P < 0.05$). Superscripts are shown only when a significant ($P < 0.05$) *P* value for time effect was detected.

[1]A percent similarity index was calculated from the peak/band profile patterns in the electropherograms/gels of samples from the same sheep, considering only the presence/absence of the different peaks/bands.

communities, and was calculated for the presence/absence of ARISA-peaks and DGGE-bands data as follows:

$$H' = -\sum_{i-1}^{S} pi \ln pi$$

where S is the total number of peaks/bands, *pi* is the proportion of the *i*th peak/band in the sample, and ln is the natural logarithm. Similarities between the peaks/bands profiles of the experimental samples were analyzed using the Pearson correlation and displayed graphically as a dendrogram. The clustering algorithm used to calculate the dendrograms was an unweighted pair-group method with arithmetic averages (UPGMA) in the MVSP v3.12d software.

Data on the number of peaks/bands and Shannon index were analyzed independently for each fingerprinting technique and rumen phase as a mixed model with repeated measures using the PROC MIXED of SAS (SAS Inst. Inc., Cary, NC). The statistical model used included FOR, period, time, and FOR × time as fixed effects, and sheep as a random effect. When a significant effect of FOR × time interaction ($P < 0.05$) was detected, differences among means were tested using Tukey's multiple comparison test. Effects were declared significant at $P < 0.05$.

RESULTS AND DISCUSSION

Because the main objective of the present study was to assess the ability of ARISA and DGGE to detect similar changes in ruminal bacterial communities, the effects of FOR and sampling time on bacterial diversity were analyzed independently for each technique, and the results are shown in Table 1. For the analysis, it was considered that the peak/band profile in ARISA electropherograms/DGGE gels reflects the predominant bacterial species or populations present in the samples. However, it should be noted that in both techniques, each peak/band can represent multiple species, and that individual operational taxonomic units within a peak may be lost if they are at low abundance whereas the peak/band could still be de-

tected if the other operational taxonomic units remained. The number of ARISA-peaks for the different treatments was similar to that previously reported in sheep (Ramos et al., 2009) and cows (Weimer et al., 2010; Welkie et al., 2010), and comparable numbers of DGGE-bands have also been shown in sheep (Belanche et al., 2012) and steers (Kocherginskaya et al., 2001) using the same primers utilized in our study. Both techniques detected that SOL and LIQ samples from AL-fed sheep had higher ($P < 0.001$ to 0.03) peaks/bands and Shannon index values than samples from GR-fed sheep. The influence of FOR type on bacterial diversity has been previously shown in cattle (Huws et al., 2010; Kong et al., 2010), and may indicate an adaptation of ruminal bacteria in response to the physicochemical composition of each FOR.

Although it is well known that ruminal microbial communities vary with time after feeding in response to changes in the rumen conditions produced by feed intake and fermentation, no previous studies have analyzed the evolution of bacterial diversity in sheep rumen over the feeding cycle. With ARISA, sampling time affected ($P < 0.01$ to 0.04) the number of peaks and Shannon index in both SOL and LIQ samples. Bacterial diversity in SOL decreased ($P < 0.05$) at 4 h postfeeding and recovered before feeding values at 8 h postfeeding for both diets, and similar results were observed in LIQ samples for GR diet. In contrast, no variations ($P = 0.27$ to 0.52) with time in number of bands or Shannon index were detected with DGGE. In agreement with this, Li et al. (2009) found no differences among samples from cows collected at different times relative to feeding using DGGE, but Welkie et al. (2010) observed changes over the feeding cycle in bacterial communities in the LIQ phase of the rumen from cows by using ARISA. An explanation may be that 16S rRNA DGGE relies on the amplification of a short fragment (about 500 bp) of the conserved gene coding for the 16S rRNA, whereas ARISA does contain the intergenic sequence flanked by the 16S and 23S rRNA genes, which may range between 100 and 1,500 bp (Fisher and Triplett, 1999), is highly variable, and generally encodes tRNAs. Moreover, ARISA uses capillary electrophoresis to separate the amplicons, which provides a much higher resolution than can be achieved in the gels used for DGGE. As stated before, there are no previous studies comparing DGGE and ARISA techniques in ruminal samples, but our results seem to confirm that ARISA technique is particularly well suited to detecting subtle changes in ruminal bacterial communities (Welkie et al., 2010).

Few studies have compared bacterial diversity in SOL and LIQ phases of the rumen, and results are inconsistent. A paired Student's *t* test was performed for each technique to compare peaks/bands number and Shannon index in both ruminal fractions. With ARISA, LIQ samples taken at 4 h postfeeding had greater ($P < 0.05$) bacterial diversity than those from SOL for both diets (results not shown). In contrast, no differences ($P = 0.37$ to 0.91) between the 2 rumen phases were detected by DGGE at any sampling time, which agrees with the lack of differences reported by Belanche et al. (2012), also using DGGE in sheep. In accordance with our ARISA results at 4 h postfeeding, Cunha et al. (2011) reported greater bacterial diversity in LIQ than in SOL samples in grazing goats by using cloning and sequencing 16S rRNA. Our results indicate that differences in bacterial diversity between SOL and LIQ phases of the rumen within the same animal may be affected by sampling time, but also by the fingerprinting technique used for the analysis, and these factors should be considered when comparing results from different studies.

With both fingerprinting techniques, the similarity between bacterial communities in SOL and LIQ samples was greater ($P = 0.005$ and 0.004 for ARISA and DGGE, respectively) in sheep fed AL than in those fed GR, showing a clear influence of FOR; in contrast, similarity was unaffected ($P > 0.05$) by sampling time. Similarity values between SOL and LIQ ranged from 30.4 to 51.4%, and were similar to the 51% of operational taxonomic units shared between the 2 ruminal phases reported by Cunha et al. (2011) in grazing goats, but much lower similarities (from 8 to 21% of operational taxonomic units) were observed by Kong et al. (2010) in the rumen of cows fed alfalfa or triticale. On the whole, these results seem to indicate that changes in bacterial diversity observed in cattle cannot be directly extrapolated to sheep or goats.

Figure 1 shows the dendrograms of the ARISA and the DGGE profiles of SOL and LIQ ruminal digesta from sheep fed the 2 experimental diets. DGGE dendrograms showed a clear discrimination between samples from SOL digesta and those from ruminal fluid in sheep 1, 3, and 4, for which SOL and LIQ samples formed 2 large clusters; however, for sheep 2 one sample from ruminal fluid (AL0) was placed in the cluster formed by 5 samples from SOL digesta. Within each major cluster, no clear pattern of subclustering was observed, with the exception of sheep 4, for which samples from the same FOR grouped together. Similar results were found by Mosoni et al. (2011), who observed that samples from SOL and LIQ phases of the rumen from wethers separated into 2 distinct clusters in DGGE dendrograms, but no further subclustering according to other factors was observed. In contrast, no clear pattern of clustering according to ruminal phase, FOR type, or sampling time was observed in ARISA-dendrograms for any sheep, with the exception of sheep 2, for which 5 samples of ruminal fluid grouped together. The ARISA clustering pattern was more complex,

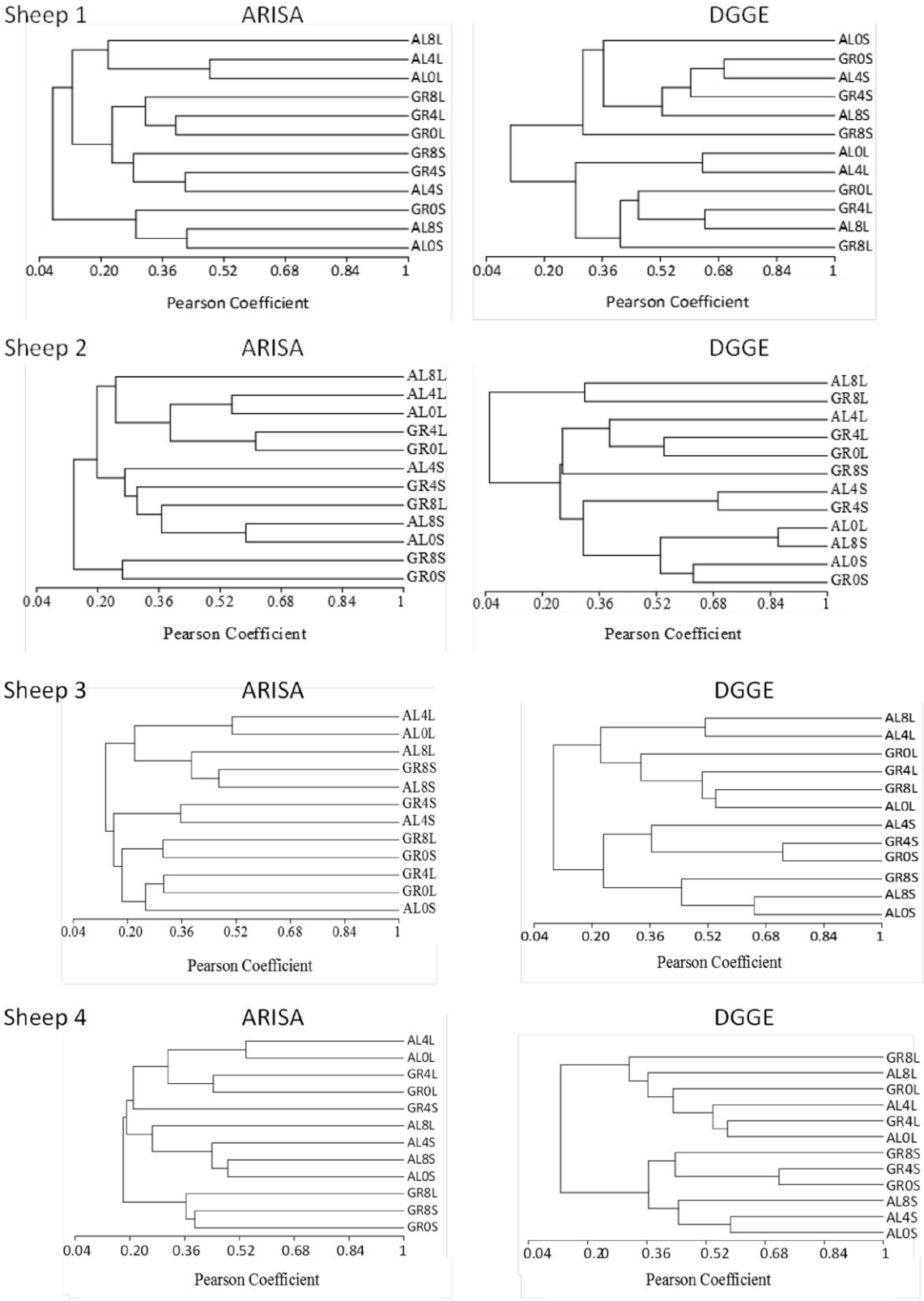

Figure 1.Dendrograms of ARISA and DGGE profiles of solid (SOL) and liquid (LIQ) ruminal digesta from sheep fed diets with 70% of either alfalfa hay (AL) or grass hay (GR). Samples were taken at 0, 4, and 8 h postfeeding. Each dendrogram correspond to samples taken from an individual sheep (sheep 1 to sheep 4).

which was probably related to the greater sensitivity of this technique compared with that of DGGE.

In conclusion, our results with both fingerprinting techniques indicate that ruminal bacterial diversity in SOL and LIQ fractions of the rumen were influenced by the FOR fed to sheep. However, ARISA detected changes in bacterial communities over the feeding cycle and differences between SOL and LIQ samples that were not

revealed by DGGE, resulting in a different interpretation of the information obtained from the same samples. Higher resolution in ARISA would be expected, especially for communities sharing close relative genera and species, whereas DGGE may be suitable to understand community changes at higher taxonomic ranks. Overall, our results show that ARISA is more accurate than DGGE for assessing bacterial diversity of ruminal samples; moreover, ARISA was found to be easier, less time consuming, and more appropriate for the rapid analysis of a great number of samples. However, it should be noted that neither ARISA nor DGGE provides any taxonomic or phylogenetic information on community members, and additional analysis will be required to identify specific bacteria.

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