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Fungal secondary metabolites from *Monascus* spp. reduce rumen methane production in vitro and in vivo[1]

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ABSTRACT: Decreasing methanogenesis without affecting fermentation and digestion of feeds in the rumen can reduce the environmental impact of ruminant production and have a beneficial effect on feed conversion efficiency. In this work, metabolites produced by *Monascus* spp. molds were assayed for their antimethanogenic activity in vitro and in vivo. The capacity of 7 strains of *Monascus* to produce secondary metabolites was assessed in solid media. Monitored metabolites included the statins monacolin K, pravastatin, and mevastatin, and the mycotoxin citrinin. Ethanolic extracts from 5 different solid media from 2 selected strains were tested in vitro. Fermentation was not negatively affected by any treatment, but one extract decreased methane production ($P < 0.05$). This extract was further assayed in 3 consecutive batch incubations where a marked decrease in methane was observed in the third batch ($P < 0.05$). In contrast, methane produced in flasks with pure monacolin K was not different from controls ($P > 0.05$). Rice on which the selected *Monascus* sp. was grown also decreased methane production when used as substrate for in vitro incubations ($P < 0.05$). The effect of *Monascus*-fermented rice on methane production was then assayed in vivo. Six wethers were adapted to a diet containing rice grain and hay (1:1 ratio). Rice was then replaced by fermented rice and given to animals for nearly 2 wk. Animals were monitored for a further 2 wk after the treatment. Daily methane emissions decreased ($P < 0.05$) by 30% after 2 to 3 d into the treatment and remained low throughout the administration period. This change was associated with reduced ruminal acetate to propionate ratio and decreased numbers of methanogens as detected by quantitative PCR ($P < 0.05$). In contrast, no changes in the methanogenic community were observed by denaturing gradient gel electrophoresis (DGGE). Total bacteria numbers increased ($P < 0.05$) with changes in the DGGE profile community, whereas protozoa were not affected by the treatment. Methane emissions and the acetate to propionate ratio remained numerically less in the 2 wk posttreatment as compared with measures before treatment. Metabolites produced by *Monascus* appear to have an inhibitory effect on methanogens and decreased methanogenesis in vitro and in short-term in vivo without any apparent negative effect on rumen fermentation. This strategy deserves to be further explored and could be an abatement option under certain feeding situations.

Key words: enteric methane, fungal metabolite, inhibitor, rumen methanogenic *archaea*

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INTRODUCTION

A major criticism of modern ruminant production is its emission of greenhouse gases and its impact on natural resources (Steinfeld et al., 2006). About one-half of the equivalent CO_2 required for producing a kilogram of milk or beef meat comes from enteric methane.

The reduction of enteric methane is being explored through different angles at the farm, animal, and

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[3]Use of *Monascus* metabolites as feed additives is covered by patent FR1052213

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microbial level (Martin et al., 2010). The use of feed additives and supplements as modulators of rumen methanogenesis is a common area of study. There is also a demand by consumers, or imposed by the legislation, for the development of additives issued from natural sources (Jouany and Morgavi, 2007). Plant secondary metabolites fit well into this classification and are being intensively studied (reviewed in Hart et al., 2008; Patra, 2010; Benchaar and Greathead, 2011). In contrast, there are fewer studies on other natural alternatives like feed enzymes, probiotics, and the use of molecules produced by bacteria and fungi (Lee et al., 2002; Chung et al., 2011; Grainger and Beauchemin, 2011). *Monascus* spp. are molds used in food production and medicine and are known to produce a number of biologically active substances, among them 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (**HMGCR**) inhibitors (Shi and Pan, 2011) that have been described to inhibit the growth of rumen methanogens in vitro (Miller and Wolin, 2001). However, not all secondary metabolites produced by *Monascus* have a beneficial effect, as some strains can also produce mycotoxins, particularly citrinin under some conditions (Liu et al., 2005). We hypothesized that secondary metabolites of *Monascus* could modulate rumen fermentation and methane production. We monitored the production of secondary metabolites of field isolates and type strains under various culture conditions and conducted a series of in vitro and in vivo experiments that, taken together, showed an inhibitory effect of secondary metabolites of *Monascus* on methanogenesis.

MATERIALS AND METHODS

Procedures with animals were conducted in accordance with the guidelines for animal research of the French Ministry of Agriculture and applicable European guidelines and regulations for experimentation with animals (www2.vet-lyon.fr/ens/expa/acc_regl.html; verified January 24, 2013).

Monascus Strains and Culture

Seven strains of *Monascus* sp. were tested. Three isolates, unidentified at the species level were obtained from corn silage in our laboratory (Strains 1 to 3); a *Monascus* sp. and a *Monascus ruber* strain were a gift from INSA Toulouse (Strains 4 and 5); and the last 2 strains of *Monascus purpureus* 1603 isolated from Chinese anka and *Monascus ruber* 62748 isolated from corn silage, Strains 6 and 7, respectively, were purchased through the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The media used to grow the strains were 2 rice-based solid media and 2 liquid media. Rice media was prepared by soaking rice overnight with tap water on a 1:1 ratio. The next day, free water was drained, 100 g of wet rice were transferred to 250-mL Erlenmeyer flasks and autoclaved (120°C, 20 min). The sterilized wet rice was used as is (i.e., without any additional component), or enriched by aseptically adding 1 g of ammonium sulfate, reported to increase monacolin K production (Su et al., 2003). Liquid medium favoring monacolin K production was prepared according to Su et al. (2003). The medium contained per liter 60 g glucose, 25 g peptone, 5 g corn-steep liquor, and 5 g ammonium chloride. Liquid medium favoring citrinin production was based on a YES medium according to Blanc et al. (1995). Fifty milliliters of liquid media were dispensed in culture flasks and autoclaved (120°C, 20 min).

Fungal inocula were prepared by culturing the strains in potato dextrose agar medium, except for number 6 that was maintained in Yeast protein Soluble starch (YpSs, M190 medium of DSMZ) agar medium. After 5 d of incubation at 25°C, spores were collected by washing the surface of cultures with 0.05% Tween 80 and enumerated in a Malassez hemocytometer. Each flask was inoculated with 3.5×10^5 spores, incubated at 30°C for 2 d and then at 25°C for 2 wk. Cultures were manually agitated every 24 h for the first 3 d of incubation. Each medium-strain combination was incubated in duplicate. At the end of the incubation, 100 mL of 67% ethanol were added to the solid media, stirred in a rotary agitator for 90 min, and filtered through filter paper (0.45 µm, Sartorius, Aubagne, France). Aliquots (20 µL) of filtrates were then injected into the HPLC system for analysis and the remaining volumes were stored at 4°C until use in in vitro assays. Several ethanolic extracts from cultures were prepared as above and used in the different in vitro experiments described below.

For the in vivo trial, wet rice was prepared in the same way as above, but the incubation was done in 1.2-L plastic jars (SACO2, Eke, Belgium) that were inoculated with a piece of a *Monascus* culture grown on agar (1/8 of a 100 mm Petri dish) and incubated at 30°C for 3 wk with periodic manual mixing. At the end of the 3-wk incubation, cultured rice was taken out of jars, spread in trays, and partially dried at 45°C for 2 d to eliminate excess humidity. Contents of all jars were thoroughly mixed to produce a homogenous lot and stored at –20°C in plastic bags until use. The amount needed to feed experimental animals for 1 d was thawed at room temperature overnight before use.

Metabolite Analysis

Separation and quantification of *Monascus* metabolites were performed at room temperature by HPLC, using UV detection for monacolin K,

mevastatin, and pravastatin; and fluorometry for citrinin. The HPLC system consisted of a P1000XR pump (SpectraSYSTEM, San Jose, CA) and an automatic sampler (SpectraPhysics, Santa Clara, CA). Separation was performed on a C18 Gravity Nucleodur reversed-phase column: 150 × 4.6 mm, 5 µm (Macherey-Nagel, Hoerdt, France) using a gradient solvent (Solvent A: 0.5% acetic acid, and Solvent B: 0.5% acetic acid/acetonitrile) at a flow rate of 1 mL/min. The gradient was started at 40% of B, which increased to 75% in 15 min; maintained at 75% for 4 min, and then returned to the initial condition in 1 min. The detection was set at 238 nm in UV and at 336 nm excitation and 440 nm emission in fluorescence mode. The retention times were 3, 8, 19, and 20 min for pravastatin, citrinin, mevastatin, and monacolin K, respectively. Standards were obtained from Sigma (St. Louis, MO). Working solutions at adequate concentrations were prepared by dissolving in ethanol, except for pravastatin, which was dissolved in methanol. Aliquots were stored at –20°C until use.

In Vitro

Rumen fluid was obtained from 3 rumen-cannulated Texel wethers fed a hay diet twice daily, at 0800 and 1600 h. Wethers had unrestricted access to water and a mineral salt block supplement. Whole rumen contents were collected before the morning feeding and strained through a polyester monofilament fabric (250-µm mesh aperture) to remove solids. Equal volumes of rumen fluid from each animal were pooled and then mixed in a 1:5 ratio with an anaerobic buffer solution (Weller and Pilgrim, 1974) kept at 39°C under oxygen-free carbon dioxide gas. This rumen fluid–buffer mixture was used immediately after preparation for fermentation experiments.

A total of 5 mL of the rumen fluid–buffer mixture were transferred to Hungate tubes containing 100 ± 2 mg alfalfa hay (470 g NDF, 156 g CP per kg DM) followed by the addition of 100 µL of *Monascus* extracts. Control tubes contained 100 µL of extract from unfermented rice prepared as per *Monascus* extracts. Extracts were adjusted so as to have a final concentration of monacolin K in the fermentation media of 5 and 20 µg/mL. Pure monacolin K and citrinin were also used as treatments. The tubes were capped, keeping the anaerobic conditions in the head space and incubated horizontally at 39°C in a shaking water bath for 18 or 24 h. At the end of the incubation period gas production was measured with the aid of a pressure transducer and samples were collected for analysis of constituents by gas chromatography. For VFA, 0.8 mL fermentation liquid were transferred to a microcentrifuge tube containing 0.5 mL of a 0.5 N HCl solution containing 2% (wt/vol) metaphosphoric acid and 0.4% (wt/vol) crotonic acid, kept at 4°C for 2 h and centrifuged (16,500 × *g*, 10 min, 4°C). Supernatants were stored at –20°C until analysis. All treatments were assayed in triplicate and the experiment was repeated twice (3 × 2).

In a second series of experiments, the effect of *Monascus* extracts was tested using a consecutive batch culture technique (Theodorou et al., 1987). The first culture was prepared as described above and incubated for 48 h in triplicate. Two milliliters of each replicate were mixed and 1.5 mL was used to inoculate 3 Hungate tubes containing alfalfa hay substrate, extracts, to have a final concentration of monacolin in the fermentation media of 20 µg/mL, and 3.5 mL of anaerobic buffer and incubated for another 48 h. Including the first incubation, a total of 3 serial cultures were performed.

In addition to extracts, *Monascus*-fermented rice was also used as a substrate for in vitro incubations. Mixed rumen fluid was prepared as above and 10 mL were immediately inoculated into 120-mL vials containing 30 mL of an anaerobic buffer solution kept at 39°C, and 500 mg of ground (1-mm sieve) *Monascus*-fermented rice (868 g starch, 10 g NDF, 94 g CP per kg DM– values before fermentation from INRA Tables 2007; Beaumont et al., 2007) alone (or combined at a 1:1 ratio (250 mg each) with alfalfa hay (470 g NDF, 156 g CP per kg DM). *Monascus*-fermented rice was replaced by unfermented rice in controls. Vials were incubated anaerobically at 39°C for up to 24 h. At the end of the incubation period gas production was measured with the aid of a pressure transducer and samples were collected for analysis of constituents by gas chromatography. Samples for VFA were prepared as above. All treatments were assayed in triplicate and the experiment was repeated twice. The consecutive batch culture technique was also used on the *Monascus*-fermented rice using 3 consecutive series transferred every 48 h. For the second and third series, at the end of each 48-h incubation, 15 mL of culture were transferred into vials containing 250 mg fermented or unfermented (control) substrate and 15 mL of anaerobic buffer.

In Vivo

The experiment was conducted at the animal experimental facilities of the INRA's Herbivores Research Unit (Saint-Genès Champanelle, France). Six adult Texel wethers fitted with rumen cannulae were used in this study. Animals were housed in individual pens and had unrestricted access to water and a mineral salt block supplement. They were weighed regularly during the study; at the start and end of the experiment, average BW was 63.7 ± 3.7 kg and 63.3 ± 4.5 kg, respectively. Wethers were restricted fed on a DM basis 600 g of rice and 600 g of a poor quality prairie hay

(NDF 730 g/kg DM and ADF 399 g/kg DM) twice a day at 0800 and 1600 h during 10 wk. Wet rice was prepared daily by adding 40% (wt/vol) of warm water and letting it soak overnight. In wk 7 and 8, wet rice was replaced by an equal amount of *Monascus*-fermented rice (60% DM). Methane production measurements were done as before (Martin et al., 2008; Morgavi et al., 2008) on wk 3 and 4 and daily from wk 7 to 10 (Fig. 1). Rumen content samples for fermentation and microbiological parameters were taken through the cannulae before the morning feeding at the end of each methane measurement week (5 times). The pH was measured immediately after collection. A portion of the sample (~150 g) was strained through a polyester monofilament fabric (250-µm mesh aperture) and samples of the liquid filtrate were taken and stored as described for in vitro experiments for VFA analysis. For protozoa counts, strained rumen fluid was sampled, stored, and analyzed as described below. Another portion of the whole rumen sample (~50 g) was homogenized for three 1-min cycles with 1-min intervals on ice using a Polytron grinding mill (Kinematica GmbH, Steinhofhalde, Switzerland). Approximately 0.5 g were transferred to 2-mL Eppendorf tubes and stored at –80°C for molecular biology analyses.

Measurements

Volatile fatty acids were analyzed by gas chromatography (CP 9002 Chrompack, Middelburg, Germany) using a wall-coated open-tubular fused silica column (0.25 mm i.d. × 25 m) coated with CP-wax 58 (FFAP)-CB (Morgavi et al., 2003). Fermentation gases were analyzed by gas-chromatography (Micro GC 3000A, Agilent Technologies, Les Ulis, France). Individual gas molar concentration was calibrated using a certified standard (relative accuracy of 2%, ALPHAGAZ No. 073562.00).

Methane production in vivo was determined using the sulfur hexafluoride (**SF_6**) tracer technique (Johnson et al., 1994) as described by Martin et al. (2008). A calibrated permeation tube containing ultrapure SF_6 was introduced into the rumen of each sheep through the rumen cannulae 2 wk before the initiation of measures. The expected useful life, which is the length of time when SF6 release is constant (i.e., zero-order release of each permeation tubes), was calculated based on the diffusion rates of SF6 (1115 ± 149 ng/min, mean ± SD). As a precaution, only tubes that had a calculated useful life that was at least 8 wk longer than the expected end of the experiment were used.

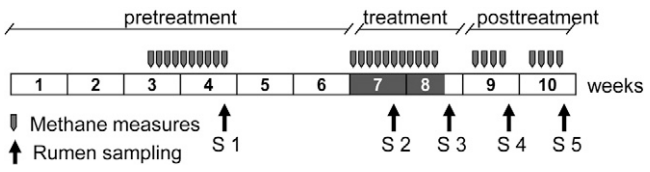

Figure 1. Diagram showing methane measurement days and rumen content sampling (S1 to S5) on wethers (n = 6) before (pretreatment), during (treatment), and after (posttreatment) feeding with *Monascus*-fermented rice. Rumen samples were taken before feeding.

The protozoal, bacterial, and archaeal communities present in rumen contents were characterized by different methods. For protozoa, strained rumen fluid samples were mixed with methylgreen-formalin solution in a 1:1 ratio and stored at room temperature in the dark until used for protozoal counting. Samples were diluted in methylgreen-formalin solution if necessary and enumeration was done using a Neubauer counting chamber. Bacteria and archaea were evaluated by quantitative (**q**) PCR and PCR-denaturing gradient gel electrophoresis (**DGGE**). Total genomic DNA was extracted using the method described by Yu and Morrison (2004) and quantified by spectrophotometry using a NanoQuant Plate on an Infinity spectrophotometer (TECAN, Männedorf; Switzerland).

The qPCR for methanogens was done using primers targeting the methyl coenzyme-M reductase (*mcrA*) gene of the methanogenesis pathway as described by Denman et al. (2007) and total bacteria were quantified using primers targeting the *rrs* gene (Edwards et al., 2007). Assays were run in triplicate using the SYBR Premix Ex Taq kit (Lonza, Levallois-Perret, France) on a StepOnePlus system (Applied Biosystems, Foster City, CA). Negative controls without DNA template were run with every assay to assess the overall specificity. The PCR amplification efficiency was checked as described before (Mosoni et al., 2011) using standard curves, 10^8 to 10^3 copies prepared from *mcr*A or *rrs* DNA fragments amplified from genomic DNA of *Methanobrevibacter smithii* DSM861 and a mixture of 11 rumen bacterial species, respectively. The slope and efficiency for *mcr*A and *rrs* primers were: –3.74 and 85% and –3.58 and 90%, respectively, with R^2 being greater than 0.99 in both cases. The PCR efficiency with DNA samples was confirmed to be similar to that obtained with the standard curves. For each rumen content sample, the results were expressed as the mean of 3 replicates in *rrs* or *mcrA* copies per gram of rumen content. In addition the relative abundance of methanogens relative to the abundance of total bacteria considered as the reference gene was calculated using the comparative C_T method ($2^{-\Delta C_T}$; Denman and McSweeney, 2006; Schmittgen and Livak, 2008).

For PCR-DGGE, the *mcrA* gene was also targeted using primers 5′-GGTGGTGTMGGATTCACACARTAYGC-WACAGC-3′ and 5′-TTCATTGCRTAGTTWGGR-TAGTT-3′, forward and reverse, respectively (Luton et al., 2002). The use of *mcrA* as a target in DGGE analysis

has been used before (Galand et al., 2002; Wilms et al., 2007). The forward primer had a 40 bp GC clamp added at its 5′ end (Muyzer et al., 1993). The PCR mixture (50 μL) contained 1 × PCR buffer (Qiagen GmbH, Hilden, Germany), 4.5 m*M* $MgCl_2$, 0.25 μ*M* of each primer, 200 μ*M* of each dNTP, 2.5 U HotStar *Taq* polymerase (Qiagen) and 50 ng extracted DNA. The PCR conditions were those used by Luton et al. (2002), with a 5-cycle slow ramp protocol to allow the extension of mismatched primers, except for the initial denaturation and final extension steps. The initial hotstart denaturation was done for 15 min at 95°C and the final extension step at 72°C was done for 30 min to eliminate artifactual double bands (Janse et al., 2004). For bacteria, the V3 variable region of the *rrs* gene was targeted with primers 341f-(5′– CCTACGGGAGGCAGCAG–3′) and 534r (5′–ATTACCGCGGCTGCTGG– 3′; Muyzer et al., 1993) as described by Sadet et al. (2007).

The DGGE was performed as described by Sadet et al. (2007). Briefly, the gel contained 8% (wt/vol) polyacrylamide in 0.5 × Tris-acetate-EDTA buffer (**TAE**) with a denaturant gradient of 20 to 55% [100% denaturant is 7 M urea and 40% (vol/vol) formamide]. Electrophoresis was done for 5 h at 200 V at 60°C. Gels were silver stained using a commercial kit (Bio-Rad Laboratories, Hercules, CA) and images were acquired using an optical density calibrated scanner (ImageScanner, GE Healthcare, Piscataway, NJ) at a spatial resolution of 400 dpi. Images were analyzed using GelCompar II version 4.0 package (Applied Maths, Kortrijk, Belgium). GelCompar II was used to normalize and compare all the DGGE patterns using hierarchical clustering to join similar patterns into groups (Fromin et al., 2002). To this end, all the images of DGGE gels were matched using the internal control sample and the bands were quantified after a local background subtraction. A tolerance in the band position of 1% was applied. The similarity among patterns was calculated with the Pearson product-moment correlation coefficient, recommended for the analysis of this type of profiles (Savelkoul et al., 1999), and the clustering was done using the unweighted pair-group method with arithmetic averages (**UPGMA**). The lowest percentage of similarity between standards on the gel was 77.8 and 90.4 for bacteria and methanogens, respectively.

Statistical Analysis

In vitro data were statistically analyzed by 1-way ANOVA using the General Linear procedures (SAS Inst. Inc., Cary, NC) and fermentation vials as experimental units. Differences among means were tested against a unique control using the Dunnet adjustment option in the first in vitro experiment. In the other experiments containing more than 1 control treatment, the Tukey adjustment option was used. In vivo data were analyzed in repeated measures using the MIXED procedure of SAS. The model included the fixed effect of sampling period and the random effect of wether. Best fitting covariance structure was compound symmetry for all variables studied. Significance was declared at the $P < 0.05$ probability level, and trends were discussed at $P < 0.10$ probability level.

RESULTS

Production of Metabolites by Different Monascus Strains

All strains grew well on the media used to monitor metabolite production. The concentration in different media of the 4 metabolites monitored is shown in Table 1. In liquid media the only metabolite detected was monacolin K, but only for some strains and at less concentrations than that found in solid media (data not shown). Monacolin K was also the only metabolite produced by all strains although the differences in concentration were large, ranging from 7 up to 570 mg/kg. Addition of a nitrogen source to increase production (Su et al., 2003) was effective in Strains 1, 2, and 3 only. Strains 4 and 5 did not produce or were low producers of metabolites. Strain 6 was the largest producer of monacolin K and mevastatin but also of citrinin and was not further considered because the concentration of this toxic metabolite was considered too high. Maximum acceptable levels of citrinin proposed in the EU for feeds and foods is 100 ng/g whereas in Japan the legal limit has been set at 200 ng/g (Shi and Pan, 2011). Strain 7 cultured on rice, which had the greatest monacolin K to citrinin ratio, and Strain 1, which had a lower monacolin K to citrinin ratio were chosen for in vitro studies with rumen fluid.

In Vitro Experiments

Monascus extracts, particularly those from Strain 7, reduced the production of methane in 18-h incubations without negatively affecting fermentations as assessed by total gas and VFA production (Table 2). When used at the concentration of 20 μg of monacolin equivalent (*Monascus* 7 high), the production of gas and VFA were even stimulated. Pure secondary metabolites gave contrasting results. Citrinin had a marked negative effect on methane production that was reduced to trace amounts but also reduced fermentation. In contrast, monacolin K did not affect either gas or methane production. γ-Aminobutyric acid, another common secondary metabolite with biological activity produced by *Monascus* did not negatively affect fermentations.

Table 1. Metabolite production of various *Monascus* strains grown on different culture media

Metabolite	Medium[1]	Strain 1	2	3	4	5	6	7
		mg/kg						
Monacolin K	rice	260	265	239	7	8	570	240
	N-enriched rice	322	349	297	ND[2]	1	331	14
Citrinin	rice	0.15	0.16	0.12	ND	0.41	18.50	0.06
	N-enriched rice	0.17	0.20	0.15	ND	0.19	0.56	ND
Pravastatin	rice	151	139	84	ND	ND	56	ND
	N-enriched rice	39	32	43	ND	ND	ND	ND
Mevastatin	rice	ND	1	1	ND	ND	78	1
	N-enriched rice	ND	1	1	ND	ND	ND	ND

[1]Media composition described in *Materials and Methods* section. Data not shown for liquid media.

[2]ND, not detected.

Monascus extracts and citrinin increased the proportion of propionate and butyrate and reduced that of acetate.

As the original extract preparations were all consumed in the in vitro tests replications, new extracts from *Monascus* Strains 1 and 7 cultured in different media as described in the Materials and Methods section were prepared. The effect of the different extracts obtained on gas production and methane production were tested as before, and whereas most extracts stimulated gas production, the effect on methane was disappointing as none of them had a reducing effect (data not shown). *Monascus* cultures were regenerated and in vitro batch experiments repeated, but similar results were obtained.

In a second series of experiments, we used the consecutive batch culture technique (Theodorou et al., 1987). Only extracts from *Monascus* Strain 7 were used in this sequence of experiments. In the first batch, no major differences were observed between *Monascus* extract and control incubations (Table 3). In the second and third batch there was a shift in VFA proportions, with a decrease in acetate and an increase in propionate. Methane production decreased by 86% in the third batch with a smaller reduction, about 10%, in the amount of gas and VFA produced, although changes in VFA were not significant. The recovery of hydrogen estimated from the amount of acetate, propionate, butyrate, and methane formed (Demeyer, 1991) was 100% in controls, but only 60% in *Monascus* extracts. The effect of Monacolin K was intermediate with methane production reduced by 35% in the third batch ($P = 0.041$).

In vitro experiments were also performed with *Monascus*-fermented rice without extraction. The rice employed as the solid media for culturing *Monascus* Strain 7 was dried and used as substrate in 72-h batch incubations and in consecutive batch technique experiments. In batch incubations, fermented rice was used as the sole substrate or combined with hay. Results at the end of the 72-h incubation are shown in Table 4. Gas production kinetics throughout the incubation for rice alone are shown in Fig. 2A. *Monascus*-fermented rice was more rapidly degraded at early incubation times, but gas production was the same between treatments at the end of the incubation time. *Monascus*-fermented rice substrate, when used alone, increased VFA production ($P = 0.040$). Fermented rice also tended to increase molar proportions of propionate

Table 2. Effect of *Monascus* spp. metabolites on rumen fermentation characteristics of 18-h in vitro incubations

Treatment[1]	Gas[2]	Methane[2]	VFA Total	Acetate (Ac)	Propionate (Pr)	Butyrate	Iso-acids	Minor[3]	Ac:Pr ratio
	mL	µmol	mmol/L	mol per 100 mol					
Control	5.8	61.2	96.1	71.2	22.1	4.8	1.0	0.9	3.2
Monascus 1 low	5.9	64.4	98.5	70.6	22.6	5.0	0.9	0.9	3.1
Monascus 1 high	5.5	45.1	93.5	63.6*	28.1*	6.2*	0.9	1.2[4]	2.3*
Monascus 7 low	5.7	28.4*	102.1	63.2*	29.3*	5.9*	0.6*	1.0	2.2*
Monascus 7 high	7.4*	33.6[4]	111.3*	54.8*	35.3*	8.3*	0.4*	1.2	1.6*
Monacolin low	6.1	60.3	99.0	70.8	22.2	4.9	1.0	1.0	3.2
Monacolin high	6.4	67.4	100.6	72.2	21.2	4.6	1.0	0.9	3.4
Citrinin low	4.7*	ND*[5]	82.0*	63.7*	28.0*	5.9*	1.2*	1.2	2.3*
Citrinin high	3.1*	2.4*	64.4*	63.6*	28.4*	5.8[4]	1.4*	0.9	2.2*
SEM	0.19	6.59	2.99	0.76	0.70	0.27	0.04	0.10	0.08
P-value[6]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

*Means values ($n = 6$) within a column differ from control ($P < 0.05$).

[1]Ethanolic extracts from rice cultures of *Monascus* Strains 1 and 7 adjusted to an equivalent concentration of monacolin K of 5 (low) or 20 µg/mL (high). Pure monacolin and citrinin treatments were used at a concentration of 5 (low) or 20 µg/mL (high). Control contained the same amount of ethanol than treatments.

[2]Total gas and methane produced in 5 mL fermentation volume containing 100 mg alfalfa hay substrate at the end of the incubation period.

[3]Valerate.

[4]Means values ($n = 6$) within a column differ from control ($P < 0.10$).

[5]ND: not detected, less than 100 mg/m^3.

[6]Main effect of treatment.

Table 3. Effect of *Monascus* Strain 7 metabolites on rumen fermentation characteristics of 48-h consecutive batch cultures

Treatment[1]	Gas[2]	Methane[2]	VFA						
			Total	Acetate (Ac)	Propionate (Pr)	Butyrate	Iso-acids	Minor[3]	Ac:Pr ratio
	mL	µmol	mmol/L	mol per 100 mol					
First batch									
Control	10.5	155.9	98.3	72.2	17.3	6.7	2.4	1.5	4.2
Monascus 7 extract	10.0	162.0	106.5	71.9	18.0	7.1[4]	1.6*	1.4[4]	4.0
Monacolin K	10.3	162.5	96.3	72.6	17.3	6.4	2.3	1.5	4.2
SEM	0.54	10.70	3.0	0.24	0.33	0.11	0.12	0.02	0.08
P-value[5]	0.79	0.85	0.11	0.23	0.30	0.012[4]	0.011	0.056	0.28
Second batch									
Control	7.2	57.8	86.3	68.1	22.2	7.2	1.6	0.9	3.1
Monascus 7 extract	5.3	24.6	77.8	62.1*	30.0*	6.4	0.7*	0.8	2.1*
Monacolin K	6.2	16.2	76.5	64.3*	25.7*	7.7	1.4	0.9	2.5*
SEM	0.59	21.12	6.52	0.66	0.56	0.25	0.08	0.09	0.08
P-value[5]	0.15	0.40	0.55	0.002	<0.001	0.027[4]	<0.001	0.77	<0.001
Third batch									
Control	8.7	90.2	101.1	64.2	25.4	6.8	1.6	1.9	2.5
Monascus 7 extract	6.7*	12.8*	90.6	56.0*	35.4*	6.0*	0.5*	2.1	1.6*
Monacolin K	7.6*	59.1*	95.1	60.2*	30.0*	6.9	1.0*	1.9	2.0*
SEM	0.18	4.79	3.99	0.53	0.45	0.12	0.02	0.09	0.05
P-value[5]	0.001	<0.001	0.25	<0.001	<0.001	0.003	<0.001	0.41	<0.001

*Means values ($n = 3$) within a column and for each batch differ from control ($P < 0.05$). Significant main effect of treatment with no differences between control and *Monascus* metabolites are not indicated by * symbol.

Significant main effect of treatment with no differences between control and *Monascus* metabolites are not indicated by * symbol.

[1]Ethanolic extracts from rice culture of *Monascus* Strain 7 adjusted to an equivalent concentration of monacolin K of 20 µg/mL. Pure monacolin was used at a concentration of 20 µg/mL. Control contained the same amount of ethanol than treatments.

[2]Total gas and methane produced in 5 mL fermentation volume containing 100 mg alfalfa hay substrate at the end of the incubation period.

[3]Valerate and caproate.

[4]Means values ($n = 3$) within a column and for each batch followed by differ from control ($P < 0.10$).

[5]Main effect of treatment.

($P = 0.074$) and decrease that of acetate and the acetate to propionate ratio ($P = 0.088$). Methane production, although not significantly, was numerically less with fermented rice as substrate, particularly when used alone (Table 4 and Fig. 2B). Amount of methane per milliliter of gas produced was less than control at 8 h, and was numerically reduced after 72-h incubation times (Fig. 2B inset). In consecutive incubations, *Monascus*-fermented rice differed from the control in the molar proportion of VFA, with increases in propionate and butyrate and decreases in acetate (Table 5). In the first 24-h batch, the amount of gas and VFA produced was greater than control. Methane production did not differ from control in the first batch, but decreased in the third batch by up to 60% of control ($P = 0.006$). At the same time, an increase ($P = 0.029$)

Table 4. Effect of *Monascus* Strain 7 with fermented rice used as substrate on rumen fermentation characteristics of 72-h in vitro incubations

Substrate	DMD	Methane[1]		Gas[1]	VFA					
					Total	Acetate (Ac)	Propionate (Pr)	Butyrate	Iso-acids	Ac:Pr ratio
	%	µmol	µmol/100 mg DMD	mL	mmol/L	mol per 100 mol				
Rice	63.7	852.2	267.7	103.6	74.8	50.6	27.1	20.6	0.6	1.9
Fermented rice	66.2	754.4	229.1	104.8	78.4*	48.8	30.0[2]	19.1	0.7	1.6[2]
Rice–hay (1:1)	64.7	824.8	256.6	98.0	74.9	59.6	25.1	11.0	2.2	2.4
Fermented rice–hay (1:1)	65.8	796.8	242.8	93.2	74.2	57.5[2]	25.8	12.4	2.2	2.2
SEM	1.31	32.92	12.62	1.91	0.86	0.59	0.79	0.93	0.10	0.07
P-value[3]	0.55	0.22	0.19	0.001[4]	0.009	<0.001[4]	0.002[4]	<0.001[4]	<0.001[4]	<0.001[4]

*Means values ($n = 6$) within a column differ from corresponding, nonfermented substrate control ($P < 0.05$).

[1]Total gas and methane produced in 40-mL fermentation vials containing 500 mg substrate at the end of the incubation period.

[2]Means values ($n = 6$) within a column differ from corresponding, nonfermented substrate control ($P < 0.10$).

[4]Significant main effect of treatment with no differences between non-fermented control substrate and fermented substrate are not indicated by * symbol.

[3]Main effect of treatment.

in the amount of dihydrogen accumulation, which was not observed in the other in vitro experiments (data not shown), was detected.

In Vivo Trial

Methane emissions in adult wethers decreased when rice was replaced by *Monascus*-fermented rice ($P < 0.001$; Table 6). The abatement was up to 30% compared with measures before the treatment and, as shown in Fig. 3, the effect was relatively quick to take place and was clearly observed on the third day of feeding. Figure 3 boxplot also shows that values 1.5 times lower and higher of the interquartile range were often attributed to the same individuals. Wethers were on restricted intake during the 10-wk-long experiment, and results are expressed as grams of methane per day, as no refusals were recorded. Body weight was measured 5 times at regular intervals and was stable over the whole experiment ($P > 0.1$; 63.5 ± 3.92 kg, mean ± SD). Rumen concentrations of VFA varied throughout the trial. During the treatment (S2 and S3) they were less than at the beginning of the trial (S1), but did not differ from the values obtained at the end of the trial (S5). Concentrations of VFA posttreatment were greater than in the previous periods (Table 6). Compared with the pretreatment period, the molar proportion of acetate to total VFA decreased ($P < 0.001$) and that of propionate (S1 vs. S2; $P = 0.077$) and butyrate increased numerically during treatment. The acetate to propionate ratio decreased as well.

The treatment stimulated slightly but in a significant manner the concentration of bacteria in the rumen (*rrs* copies/g DM rumen content) and decreased the concentration of methanogens (*mcr*A copies/g DM rumen contents; $P = 0.006$ and 0.012, respectively). The proportion of methanogens relative to bacteria decreased threefold during treatment as compared with values before treatment ($P < 0.001$; Table 6). In contrast, no changes were observed in the number and proportion of main protozoal groups. Protozoa populations were composed of 97.1% small entodiniomorphs, 1.6% large entodiniomorphs (>100 µm), and the rest composed by Holotrichs *Dasytricha ruminantium* and *Isotricha* spp., 1.23 and 0.12%, respectively.

The profiles of the bacterial community from individual wethers obtained by PCR-DGGE and analyzed by clustering of the whole densitometric curves showed that samples obtained in Sampling Periods 2 and 3 formed 3 small clusters at the first, second, and third nodes, whereas samples taken before (S1) and after the treatment (S4 and S5) grouped together and exhibited a greater percentage of similarity between them (Fig. 4). Wether Number 6 had an unusual, highly different profile in S2, with only 26% similarity with the rest of the samples (data not shown). As it was not possible to exclude a technical problem with this sample, Wether 6 was not included in the analysis. In contrast, methanogens (*mcr*A gene) profiles were not affected by the treatment (data not shown).

DISCUSSION

Secondary metabolites from *Monascus* reduced rumen methane production in vitro and in vivo without any apparent negative effect on fermentation variables. However, not all the strains tested were effective for decreasing methanogenesis. In addition, the culture conditions also seem important for the production of the active molecules. Screening of strains is important, as we show that among the secondary compounds monitored, citrinin had a marked negative effect on fermentations. Strains producing this mycotoxin have also to be avoided for feed safety reasons (Shi and Pan, 2011) and for reducing toxicity incidents in ruminants (Griffiths and Done, 1991). Culture conditions favoring

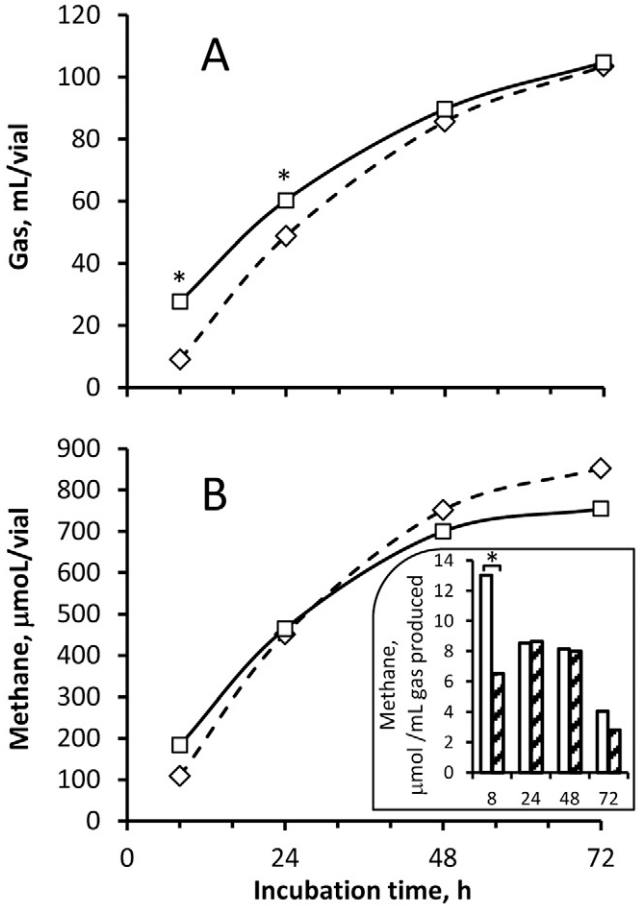

Figure 2. Production of (A) gas and (B) methane by mixed rumen microbes in the presence of rice (diamonds) or *Monascus* Strain 7 fermented rice (squares) used as substrate. Inset shows methane production in µmol per mL of gas produced at each incubation time. Values are means ($n = 6$); SEM at 8, 24, 48, and 72 h, respectively; 1.42, 2.71, 2.56, and 1.91 for A; 22.03, 32.19, 41.49, and 32.92 for B; and 1.19, 0.68, 0.77, and 1.13 for inset. *Significant ($P < 0.05$) between treatments at equal incubation time.

Table 5. Effect of *Monascus* Strain 7 with fermented rice used as substrate on rumen fermentation characteristics of 24-h consecutive batch cultures

Substrate	Gas[1]	Methane[1]	H_2[1]	VFA				Minor[2]	Ac:Pr ratio
				Total	Acetate (Ac)	Propionate (Pr)	Butyrate		
	mL	μmol		mmol/L	mol per 100 mol				
First batch									
Rice	54.5	616.6	2.7	49.3	57.0	25.7	15.3	1.2	2.2
Fermented rice	63.6	640.6	8.3	53.2	51.9	30.7	15.3	1.5	1.7
SEM	1.61	33.99	1.84	1.41	0.87	0.61	0.44	0.06	0.08
P value[3]	0.003	0.63	0.06	0.08	0.002	<0.001	0.97	0.014	<0.001
Second batch									
Rice	41.2	377.4	2.0	49.7	52.6	27.9	17.9	1.2	1.9
Fermented rice	43.7	342.1	9.8	51.3	46.9	31.5	19.9	1.4	1.5
SEM	0.91	22.04	2.88	0.96	0.89	0.86	0.28	0.09	0.08
P value[3]	0.08	0.28	0.09	0.26	0.001	0.015	<0.001	0.08	0.005
Third batch									
Rice	29.4	208.5	3.7	35.6	46.2	32.2	20.1	0.9	1.4
Fermented rice	29.7	83.2	46.6	32.4	39.7	37.2	22.3	1.2	1.1
SEM	0.84	25.64	12.8	1.56	1.32	1.60	0.35	0.14	0.09
P-value[3]	0.93	0.028	0.029	0.12	0.004	0.049	0.001	0.20	0.018

[1]Total production in 40-mL fermentation vials containing 500 mg substrate at the end of the incubation period.

[2]Valerate and caproate.

[3]Main effect of treatment.

citrinin production in *Monascus* are known and should be used to screen out any citrinin-producing strain before testing them on animals. It should be noted that *Monascus* spp. produce many secondary metabolites (Shi and Pan, 2011) but only 4 were monitored as they were commercially available to us and, at this stage, we cannot affirm that these metabolites were responsible of the antimethanogenic activity.

There is a large body of literature describing the effect of natural compounds, particularly plant-

Table 6. In vivo rumen fermentation characteristics and abundance of bacteria, archaea methanogens, and protozoa in the rumen of wethers ($n = 6$) before, during, and after feeding with *Monascus*-fermented rice

	Pretreatment	Treatment		Posttreatment		SEM	*P*-value[2]
	S1[1]	S2	S3	S4	S5		
Fermentation products							
Methane, g/kg DMI	35.2^{a}	24.9^{b}	25.8^{b}	33.5^{a}	25.8^{ab}	2.75	<0.001
Total VFA, mmol/L	73.4^{b}	61.8^{c}	75.3^{b}	94.4^{a}	89.6^{a}	3.84	<0.001
Acetate (A),[3] %	70.8^{a}	60.0^{b}	63.8^{bc}	66.2^{ac}	67.5^{ac}	1.25	<0.001
Propionate (P), %	12.5	18.0	16.4	15.6	15.7	1.41	0.06
Butyrate, %	13.1	16.3	15.7	13.7	12.8	1.13	0.013[4]
Iso-acids, %	2.6^{ab}	3.0^{a}	1.9^{b}	3.0^{a}	2.5^{ab}	0.28	0.39
A:P	5.8^{a}	3.5^{b}	4.0^{b}	4.6^{ab}	4.3^{ab}	0.38	0.002
Microbial numbers							
Bacteria,[5] $\log_{10}$ rrs copies/g DM rumen content	11.1^{b}	11.3^{ab}	11.4^{a}	11.2^{ab}	11.2^{ab}	0.04	0.006
Archaea,[5] $\log_{10}$ mcrA copies/g DM rumen content	9.9^{a}	9.5^{b}	9.6^{ab}	9.6^{ab}	9.7^{ab}	0.07	0.012
% archaea[6]	6.2^{a}	1.7^{b}	1.9^{b}	2.9^{b}	3.3^{ab}	0.79	<0.001
Protozoa,[7] $\log_{10}$ cells/mL rumen fluid	5.9	5.8	6.0	5.8	5.8	0.08	0.68

[a–c]Within a row, means without a common superscript letter differ ($P < 0.05$).

[1]Sampling time, see Fig. 1.

[2]Main effect of treatment.

[3]Mol/100 mol.

[4]Significant main effect of treatment with no differences between sampling time means compared by Tukey's test.

[5]Estimated by quantitative PCR.

[6]Relative quantification assay using methanogens' *mcrA* gene and total bacteria *rrs* gene for normalization.

[7]Estimated by microscopy. Protozoa were composed of 97.1% small entodiniomorphs, 1.6% large entodinimorphs (> 100 μm), 1.23% *Dasytricha ruminantium*, and 0.12% *Isotricha* spp.

derived products, as antimethanogenic agents on rumen fermentations in vitro (Calsamiglia et al., 2007; Benchaar and Greathead, 2011). However, the assessment in animals of compounds selected in vitro is still scarce. Animal trials performed using some of these in vitro promising compounds failed to show a practical methane mitigation effect under the conditions studied (e.g., Holtshausen et al., 2009; Klevenhusen et al., 2011b; van Zijderveld et al., 2011). In the work presented here, the traditional in vitro batch system was marginal or ineffective at detecting an antimethanogenic activity (except for the extracts used in 1 experiment; discussed below). In vitro effects were clearly observed using a consecutive batch culture approach. Whether this method is representative of the rumen function could be a matter of debate, as the composition of the microbiota at the end of the incubation period is certainly not the same than the initial inoculum. However, in this work the method was useful, as it gave a better indication of the antimethanogenic potential of different strains and solid state fermentation conditions (H. Boudra and D. Morgavi, unpublished data) than traditional batch fermentations. In contrast to other products reported in the literature, reduction in methane by metabolites of *Monascus* was more apparent in our in vivo than in our in vitro studies. In the proof of concept, sheep trial, an important 30% enteric methane reduction effect was observed. An observation emanating from the trial was that methane production values placed outside the whiskers in the box plot graph might suggest different animal susceptibility to the treatment and perhaps also differences in the capacity to reestablish the methanogenesis function after the disturbance to the microbial community produced by the treatment, akin to that described after antibiotic treatment (Dethlefsen and Relman, 2011). It should be mentioned that there was a confounding effect of time in the experimental design used that could also have an effect on methane emissions. This effect, if any, should be minor under the controlled conditions of the assay but certainly need to be considered in future trials. We were limited in the amount of *Monascus*-fermented rice available and that was the reason of the relatively short duration of the animal experiment. We are working on improving the solid state fermentation process and on the use of other feed substrates to conduct other research on this subject.

A hypothesis was that monacolin K was the active molecule. This compound has been cited as one of the most biologically active in red mold rice (Shi and Pan, 2011). Monacolin K and other polyketide compounds belonging to the statin group are HMGCR inhibitors and have cholesterol-lowering effect in mammals. The HMGCR inhibitors, such as lovastatin and mevastatin, have been shown to suppress the growth of rumen methanogens in pure culture (Miller and Wolin, 2001). In mixed rumen cultures, lovastatin was not effective in short-term batch incubations (Busquet et al., 2005), but reduced methane production by ~40% in a semicontinuous rumen fermentor (Rusitec; Soliva et al., 2011). The incubation and adaptation of the microbiota to the treatment were different between these 2 reports but also the doses used; 5 mg/L in the study of Busquet et al. (2005) and 150 mg/L in that of Soliva et al. (2011). Lovastatin was not effective in sheep in the only in vivo trial that we are aware of (Klevenhusen et al., 2011a). Monacolin K was not previously assayed in vitro or in vivo on the rumen and, based on our results, it is not possible to confirm or refute the hypothesis that monacolin K was responsible for the effect on fermentation and methane. Extracts used in in vitro experiments were all adjusted to have equal concentration of monacolin K, yet their activity was not the same. In addition, monitoring monacolin K during solid state fermentations is not clearly correlated to the antimethanogenic activity (H. Boudra and D. Morgavi, unpublished data). We also used LC-MS to detect monacolin K and analogs produced by *Monascus* spp., but until now it is difficult to correlate metabolic profiles with biological activities (H. Boudra and D. Morgavi, unpublished data). The lack of an effect reported with pure lovastatin (Klevenhusen et al., 2011a) might suggest that the activity of secondary metabolites produced by *Monascus* spp. is due to a mix of compounds and not only HMGCR inhibitors. In contrast, *Monascus*-fermented rice reduced the number of methanogens in wethers, whereas bacterial populations were increased. In addition, the results obtained with the consecutive batch method suggest a microbiostatic and specific

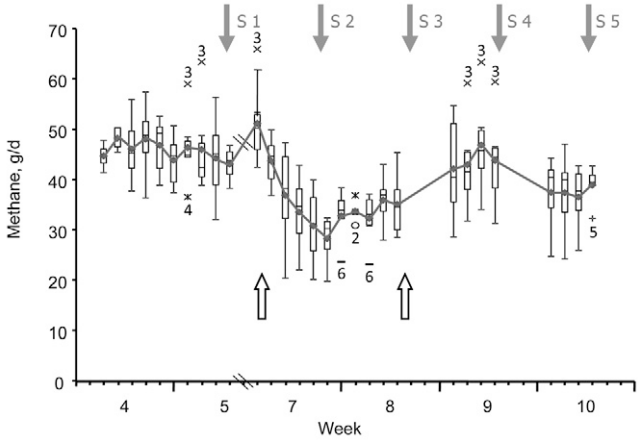

Figure 3. Daily methane emissions of wethers (n = 6) before, during, and after feeding with *Monascus*-fermented rice. Each box gives the mean values (diamonds), the median (line across the box), the upper and lower quartiles, and the maximum and minimum value (bars) within 1.5 interquartile range of the higher and lower quartile, respectively. Outlier symbols are followed by wether number. Unfilled arrows indicate the start and end of the treatment and gray arrows indicate rumen content sampling (S1 to S5).

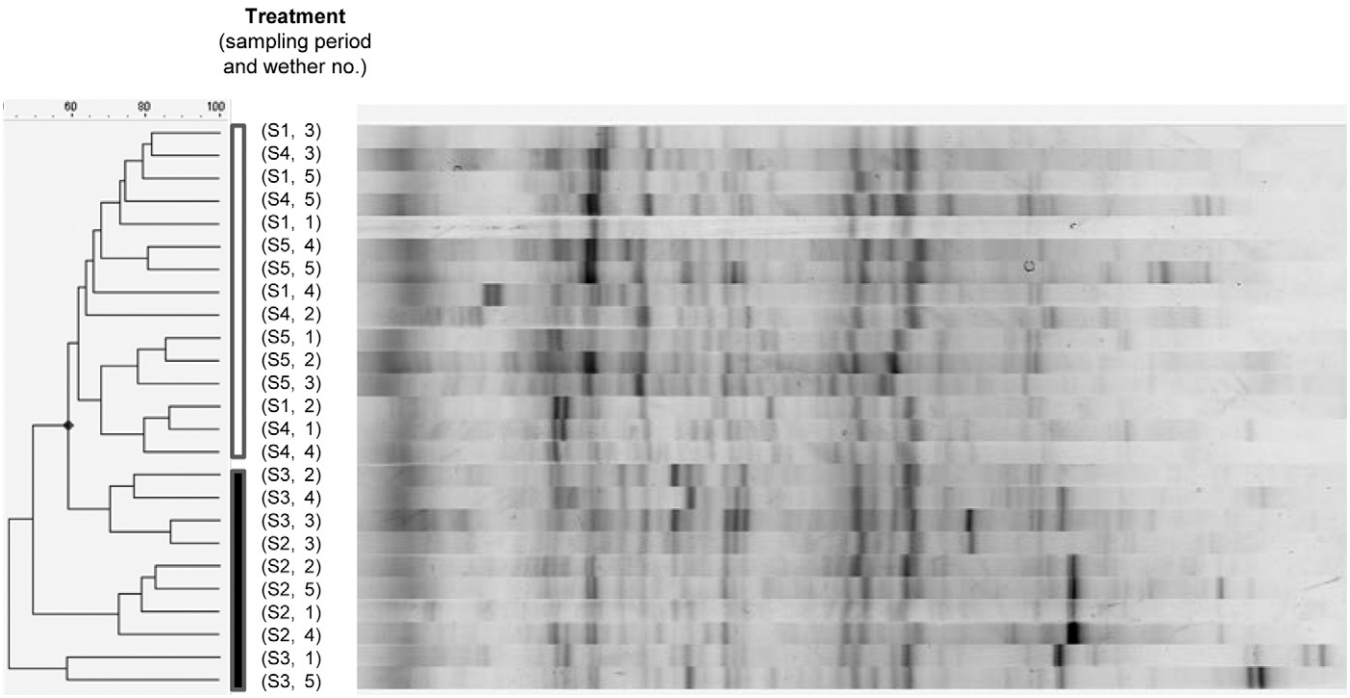

Figure 4. Denaturing gradient gel electrophoresis profiles and unweighted pair-group method using arithmetic averages dendrogram generated from rumen bacteria (*rrs* gene). Rumen content samples from wethers (n = 5) taken before (open square, S1), during (filled square, S2 and S3), and after (open square, S4 and S5) feeding with *Monascus*-fermented rice.

effect against methanogens. Taken together, these effects could be ascribed to the inhibition of HMGCR enzymes involved in the mevalonate pathway, which is present in archaea methanogens but absent in most prokaryotes (Friesen and Rodwell, 2004). The mechanism of action of *Monascus* metabolites, however, remains to be validated. It is worth noting that there was a significant effect on numbers and structure (evidenced with PCR-DGGE) of the rumen bacterial community in treated wethers, indicating that reduction in methane emissions are not exclusively due to changes in methane producers, but also to other members of the rumen microbiota (Morgavi et al., 2010).

This work showed that commonly used in vitro batch cultures were not adequate to detect an antimethanogenic activity that was observed in vivo. This result challenges the principle that if it does not work in vitro, it is not worth testing in vivo. We have recourse to a consecutive batch technique that was capable of detecting the antimethanogenic activity in vitro. Although consecutive batch cultures might be less representative of the rumen ecosystem than other in vitro techniques, it could be useful for improving the chances of finding promising candidates using a battery of relatively inexpensive in vitro methods.

Monascus metabolites stimulated in vitro fermentation, suggesting that they do not negatively affect digestibility and feed utilization, an essential aspect for a potential adoption of the technology. The effect on digestibility needs to be confirmed in vivo though. Future works for establishing the economic viability and sustainability of the technology should be directed to the improvement of the solid fermentation process, the evaluation of an optimal dose, and the long-term efficacy.

Conclusions

Secondary fungal metabolites from *Monascus* spp. reduced enteric methane emissions in sheep by 30% in a short-term trial. Reduction of methane was accompanied, both in vitro and in vivo, by a shift in VFA pathways, decreasing the acetate to propionate ratio. The main microbial modifications observed were a reduction in methanogens numbers, suggesting a specific and toxic effect on this microbial group and a concomitant increase in bacterial numbers, with marked changes in the structure of the bacterial community.

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