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Forage-fungal associations and effects on methanogenesis

Report to MAF on SLMACC Project C10X0829

June 2010

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Forage-fungal associations and effects on methanogenesis

Contract no. C10X0829 Report to MAF/FRST

Gerald Cosgrove, Stefan Muetzel and Bob Skipp

June 2010

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EXECUTIVE SUMMARY

Reducing enteric methane emissions from pasture-fed animals is difficult. Some evidence suggested that pasture-related fungi may reduce methane production. This study was conducted to test the antimethanogenic potential of saprophytic and endophytic fungi.

An *in vitro* procedure was used to screen candidate fungi in a 24 hour batch-culture fermentation assay, with provision for promising candidates to be evaluated further in a longer-term continuous-culture assay, ultimately confirmed by measuring methane emissions in sheep using a calorimeter.

For perennial ryegrass-endophyte and tall fescue endophyte associations it was hypothesised that alkaloids produced by the endophyte may be implicated in suppressing enteric methane production, and candidates for screening were selected on the basis of their production of various alkaloid compounds. For saprophytic fungi, candidates were selected on the basis of their involvement with spoilage of silage, and production of various classes of secondary compounds. Eleven perennial ryegrass, 7 tall fescue and 1 meadow fescue-endophyte associations and 10 saprophytic fungi consisting of different strains of 4 species, were evaluated in batch-culture assays.

Samples of grass-endophyte associations were obtained from existing field plots growing in Canterbury and Northland in autumn when concentrations of alkaloids are normally at their highest, freeze dried and ground in preparation for fermentation assay. Starter cultures of saprophytic fungi were obtained from culture collections held in New Zealand or The Netherlands, grown in culture medium and the mycelia mat and supernatant liquid for each candidate culture separated and assayed independently.

No grass-endophyte candidates suppressed methane production in batch-culture sufficiently to warrant the conduct of a continuous-culture assay. The supernatant fraction of the 3 strains of the fungus *Mortierella wolfii* was the most promising saprophytic fungus in terms of its ability to suppress methane production without compromising the beneficial aspects of forage fermentation such as the production of short chain fatty acids. This candidate was identified in the final stages of this project, after broadening the selection of candidates for screening. At this point there was insufficient time for continuous-culture screening. This candidate should be investigated further by growing fresh cultures under contrasting conditions to establish some variation in putative bioactive compounds for further batch-culture and then continuous-culture screening. If suppression is sustained, then it should be evaluated in an animal-based study and fractionation and extraction steps undertaken to identify the bioactive compounds.

INTRODUCTION

The predominance of pasture in the diet of ruminants in NZ presents some specific challenges for mitigation of methane generation, but also opportunities for some unique solutions. Plant and plant-related attributes that offer mitigation potential are highly relevant for NZ where the forage-dominant diets characteristically result in high emissions of methane per kg of dry matter consumed and per unit of animal product.

There is preliminary evidence that suppression of enteric methanogenesis by fungi may be possible. This evidence comes from both laboratory and animal-based studies. In a study using an *in vitro* continuous fermenter, a novel strain of the endophytic fungus (*Neotyphodium coenophialum*) in tall fescue (*Festuca arundinacea*) suppressed methanogenesis under a particular set of fermenter conditions (Vibart *et al*., 2007). Similarly the presence of the spoilage fungus *Penicillium roquefortii* in silage reduced methane production in a dose-dependent manner (Mauruschat, 1996). While the effect in this study was probably due to the suppression of fermentation rather than the presence of a specific methane inhibition agent, mouldy silage fed to cattle also reduced methane production to about half of the output normally produced by cattle (Clark and Krause, 2007). Methane emissions from sheep grazing pastures composed of kikuyu (*Pennisetum clandestinum*) were on occasions considerably lower than predicted (Ulyatt *et al*. 2002a, b). In this study, it was expected that emissions would be higher than from ryegrass, on the basis of the higher fibre content of this sub-tropical grass (with a C4 pathway of photosynthesis, and therefore a greater concentration of cell wall carbohydrates). The fact that they were lower was attributed by the author to the possibility of mycotoxins associated with kikuyu, although it was not clear if this was a direct effect on the animal (e.g. kikuyu poisoning, Martinovich et al., 1972) or on rumen microbes and methanogenesis. Other studies have demonstrated that endophytic fungi may have inhibitory effects on other fungi (Christensen *et al*., 1996), and they have been shown to affect soil biota in a way that slows the decomposition of soil organic matter (Antunes *et al*. 2008; Omacini *et al*. 2004; Ryan *et al*. in press). Collectively, this evidence warrants investigation to validate the findings described above, and to establish whether endophytic or saprophytic fungi may have the capacity to selectively inhibit rumen methanogenic bacteria and so methanogenesis. This should be followed if necessary by further study to establish the mechanism of action, as first steps towards exploiting a possible opportunity.

There is a considerable base of knowledge of the biology of both endophytic and saprophytic fungi. This has developed because of the economic significance of pasture-related fungi in New Zealand. Endophyte has a big influence on plant metabolism and the metabolism of grazing ruminants. Saprophytes are ubiquitous in pasture and are seasonally important in influencing ruminant metabolism. The naturally occurring species of endophytic fungi in tall fescue and in ryegrass are both amenable to selection, and such selection can substantially alter the profile of the secondary metabolites produced by the fungus. For both species of grass, particularly ryegrass, there are clearly established precedents for selecting endophytic fungal strains for beneficial purposes (Easton *et al*. 2001; Easton and Tapper, 2005). For example, there are novel non-toxic strains that retain the ability to produce alkaloids responsible for conferring resistance to insect feeding (predominantly peramine, lolines), yet do not produce the alkaloids responsible for animal toxicosis (predominantly lolitrem B,

ergovaline). Furthermore, there is a clear pathway for the ensuing benefits from development of novel strains of endophyte to be delivered. This pathway encompasses expertise in fungal manipulation, introduction of novel strains into the appropriate grass species (including examining the strain x cultivar interactions which are important in determining the production of secondary metabolites) and *in vitro* and *in vivo* testing, and ultimately seed production, sales and marketing. The main advantage of delivery via a fungal endophyte is the low cost and simplicity associated with the continual dietary supply of a mitigating agent with pasture. An alternative method, particularly if saprophytic fungi are shown to be antimethanogenic, could be the synthesis of analogue bioactive compounds and delivery by other means e.g. via drinking water or intra-ruminal controlled release technology, such as already used for delivery of therapeutic compounds to animals.

A forage-based mitigation technology, while elusive to date, could provide a simple, cost-effective and enduring solution for a significant proportion of ruminant livestock in New Zealand. This proof-of-concept project uses existing and developing expertise in plant-fungi-animal interactions and applies it to assessing the potential for plant-related fungi to reduce methane production, using initially lab-based screening assays followed by animal-based validation.

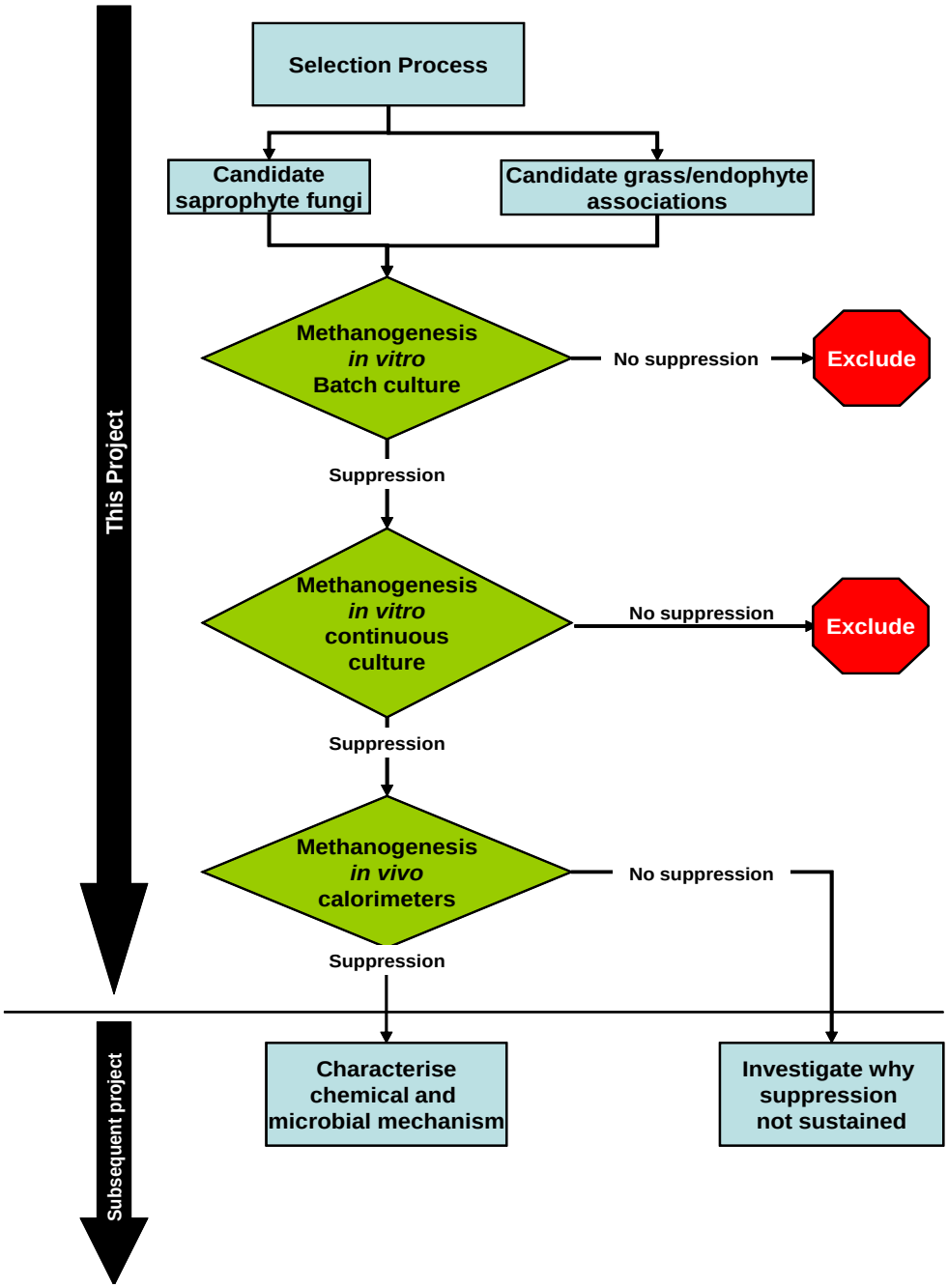
OBJECTIVES

The objective of the project was to screen contrasting classes of fungi to determine their potential for reducing enteric methane production. For each class of fungi the hypothesis was that the presence of the fungus would suppress methane production and a likely mode of action was the effect of endophyte alkaloids and in the case of saprophytic fungi various secondary metabolites, on methanogenesis.

MATERIALS AND METHODS

This study was conducted at AgResearch Grasslands, Palmerston North during 2009-10. The study was designed to use a newly-developed *in vitro* (laboratory) simulated rumen fermentation system, and an *in vivo* (animal-based) system for measuring total methane production by animals in fully enclosed calorimeters. The study was planned for the screening to be conducted in a sequential manner (Figure 1), and for any sample evidence of suppression of methane production was required in the short-term (24 hour) batch-culture laboratory assay, before it would advancing to a longer term (12-14 day) continuous-culture laboratory assay, and ultimately to an animal-based test to validate laboratory results. In the case that no candidate fungus warranted continuous-culture or animal-based testing, provision was made to broaden the batch-culture screening.

Figure 1. Diagram of planned sequential screening process of candidate endophytic and saprophytic fungi.



Initially, six candidate saprophytic and 12 candidate grass-endophyte associations were selected for screening. At the completion of the batch-screening for this set, the list of candidates was broadened and by the end of the study, 10 saprophytic (two fractions for each fungus) and 21 grass-endophyte associations (this included 2 associations that were repeated because of initially promising results, which were not confirmed at the second screening) had been screened.

Because of delays with commissioning laboratory assay equipment necessary for the screening to be conducted, the batch-culture screening for the endophytic fungi was conducted using a manual system, whereas for the saprophytic fungi the automated system

was used. This one-year project was extended by six months from December 31 to June 30, as a consequence of these delays.

Selection of candidate fungi

Two classes of fungi, endophytic and saprophytic fungi, were chosen for this project. For both classes there was some prior indication that they could have an effect on methane production in the rumen, although this evidence was limited and unsubstantiated.

Endophytic fungi:

Endophytic fungi grow in mutualistic association with host grasses. For NZ, the primary relevant fungi are the ryegrass endophyte (*Neotyphodium lolii*) and tall fescue endophyte (*Neotyphodium coenophialum*). Within each species of endophyte, there are numerous strains. Prior observation of an antimethanogenic effect related to one strain of tall fescue endophyte. For the current study ryegrass-endophyte associations were included as well because it is more common in NZ and to test whether any anti-methanogenic effect is restricted to a species of grass, and to a species and strain of endophyte, or more general across different genuses.

Prior observation with tall fescue was strain-specific, but several different strains were included in the selection.

Saprophytic fungi:

There are numerous strains of saprophytic (spoilage) fungi that could potentially be tested. Based on prior observations, the selection was confined to fungi that have been observed in spoilage of silage and hay, and those commonly seen in association with pasture. Consultation with Dr Margaret di Menna led to the choice of 4 species of fungi most commonly associated with silage spoilage for testing in the *in vitro* assay (Table 1). Although *Aspergillus fumigatus* had also been considered as a possible candidate it was not used because of its potential to cause mycotic infections (aspergillosis) and allergic reactions in humans and other animals. *Mortierella wolfii* is not known as a human pathogen but can cause mycosis in sheep and cattle (Munday *et al*., 2010). All the fungi tested are known to produce secondary metabolites with a range of biological activities.

Table 1. The species and strains of saprophytic fungi associated with spoilage of silage and selected for screening, their source and some characteristic production of secondary metabolites that may influence enteric methane production.

Fungus	Strain	Code	Source	Characteristics of fungus
Mortierella wolfii	68/707w2	C	di Menna[1]	Produces a protein nephrotoxin, grows at 37°C and causes mycosis in cattle (abortion and pneumonia; encephalitis when injected into brain of mice).
	69/3891	A	di Menna	
	74/2649	B	di Menna	

Penicillium roquefortii	A2	F	di Menna	Regarded as a complex of 3 species (*P. roquefortii*, *P. paneum* and *P. carneum* – the first 2 being associated with mouldy silage *P. carneum* mainly with bread).
	G2	D	di Menna	Produces extensive range of mycotoxins including: - Roquefortine (several) Mycophenolic acid Eremofortin C PR toxin Penitrem A
	I2	E	di Menna	
Monascus ruber	15220	G	ICMP[2]	A red yeast that produces Citrinin (active against G+ bacteria) and Monacolin K = lovastatin (statins may affect methanogenesis, but also growth of anaerobic fungi).
	503.70	H	CBS[3]	
	554.76	J	CBS	
Geotrichum candidum	240.62	I	CBS	May produce clavine alkaloids (ergot). A secondary mycosis in people with TB.

[1] Dr Margaret di Menna, AgResearch, Hamilton

[2] International Collection of Microorganisms from Plants, Landcare Research, New Zealand

[3] Centraalbureau voor Schimmelcultures, The Netherlands

Collection of samples

Endophytic fungi:

Established plots of ryegrasses at AgResearch Lincoln, and tall fescues at AgResearch KeriKeri, each with a range of strains of endophyte were sampled in early autumn 2009. At each site the samples were taken from each replicate plot in the trial and composited into a single sample for the purposes of providing 200-300 g dry weight for bioassay. Samples were frozen as soon after collection as possible and then transferred to Palmerston North for freeze drying and grinding. At Lincoln, the plots consisted of a single experimental line of ryegrass (GA66) infected with each of a range of 30 commercial and experimental strains of endophytes, and an endophyte-free control. The characteristics of the grass-endophyte association, and particularly the concentrations of secondary metabolites produced by the fungus, are influenced by both the host grass and the endophyte strain. Using a single line of ryegrass has the advantage of allowing a more systematic evaluation of variation among endophyte strains. However, a potential disadvantage is this may restrict the extent of

variation in the concentrations of alkaloids produced, and a combination may express strain-specific attributes differently from that same strain in another cultivar.

The 11 candidates for screening (Table 1) were chosen according to two criteria; i) to represent the genetic diversity in endophyte strains, using a dendrogram of genetic separation between strains (prepared by Dr M. Faville, AgResearch) and ii) to achieve a set such that there were as many combinations as possible of expected presence and absence of the three primary alkaloids lolitrem B, ergovaline and peramine (4 of 7 combinations of the presence of a single, a pair and all three alkaloids were possible). Nil endophyte (no alkaloids) and wildtype (all three alkaloids) provided negative and positive controls.

Table 2. The expected concentrations of alkaloids in perennial ryegrass-endophyte, tall fescue-endophyte and meadow fescue-endophyte associations selected for screening.

Species	Cultivar (or Line)	Endophyte strain	Code	Alkaloid (mg/kg DM)		
				Lolitrem	Ergovaline	Peramine
P. ryegrass	GA66	Nil	4/20	ND	ND	ND
		Wildtype	21	High	Med	Med
		AR1	14	ND[1]	ND	Med
		AR6	15	ND	High	High
		AR37	16	ND	ND	ND
		AR47	17	ND	ND	ND
		AR50	1	ND	ND	High
		AR68	5/18	ND	Trace	Med
		AR73	19	ND	Low	High
		AR96	2	High	ND	ND
		AR100	3	ND	High	Low
Tall fescue	Jesup	Nil	6	ND	ND	ND
		Wildtype	7	ND	High	Low
		AR525	8	ND	Trace	Low
		AR542	9	ND	ND	Low
		AR584	10	ND	ND	Low
		AR593	11	ND	ND	Low
	Resolute II	AR542	12	ND	ND	Low

Meadow fescue	Selection	Wildtype	13	ND	ND	Low

[1] Not detectable

At Kerikeri the plots consisted of a combination of cultivars of tall fescue with commercial and experimental strains of endophyte (Table 2). The 3 combinations of primary interest, Jesup tall fescue with no endophyte, wildtype endophyte, and the commercial strain Max Q (AR 542), were comparable to ryegrass in being a single cultivar of grass in association with each of 2 different strains of endophyte and an endophyte-free control, and replicated those used in the study reported by Vibart *et al*., (2007). Other combinations are shown in Table 2.

Saprophytic fungi:

Cultures of candidate saprophytic fungi were obtained from culture collections, held in New Zealand (Dr Margaret di Menna; AgResearch; International Collection of Microorganisms from Plants, Landcare Research) or overseas (Centraalbureau voor Schimmelcultures, The Netherlands).

Preparation for screening

Endophytic fungi:

Samples of grass-endophyte combinations were stored frozen, then freeze-dried, and ground to pass a 1 mm sieve.

Saprophytic fungi:

Cultures received from culture collections were transferred to potato dextrose agar (PDA) and sub-cultured on this medium until required for culture in the liquid medium used to prepare extracts for testing. The liquid medium used for all fungi was Czapek Dox Yeast Extract Liquid Medium (CDYE; Sumarah *et al*., 2005). Agar plugs taken from the edge of colonies growing on PDA were macerated in sterile water and used to initiate seed cultures grown in 50 ml CDYE in 250 ml flasks at 25°C for 3 days. Macerated fungal mycelium from the seed cultures was used to inoculate stationary production cultures in 200ml quantities of the medium in 1l flasks at 25°C for 16 days. The mat of fungal mycelium was then removed from the liquid culture medium by straining through nylon cloth and the combined fungal material from two culture flasks macerated in 250ml sterile water to produce the 'mycelial mat' fraction (M). The remaining liquid medium fraction (L) was freed from any remaining fragments of fungal material by centrifugation. The two fractions were stored frozen until required for testing.

Bioassays

The project used batch and continuous culture fermenters that provide controlled conditions to simulate the rumen environment and microbial processes (Cone *et al*. 1996), with some minor modifications. This allowed for a sequential screening procedure, where early phase batch tests eliminate ineffective fungi and only those fungi demonstrating potential for inhibition proceed to longer-term continuous-culture testing. Provision was made to conduct the most rigorous test of efficacy, using sheep in open-circuit calorimeters (e.g. Pinares-Patino et al., 2007; 2008). Reliable estimates of methane emissions can be obtained over comparatively short durations of 2-days. This short-term duration makes it feasible to test potential inhibitory compounds and diets, and this short duration is particularly valuable

where experimental diets may be available only in restricted quantity which necessarily limits the duration of animal feeding assays. No continuous-culture or animal-based assays were conducted in the duration of this project.

Batch culture fermentation

Endophytic fungi:

In vitro incubation

Samples of 600mg of substrate were weighed into 120 ml serum bottles. The bottles were pre-warmed for 2 hours before the incubation started. Artificial saliva (McDougall 1948) was prepared the day before incubation and heated to 39°C in a water bath. One hour before incubation the buffer was flushed with CO_2 and 20 min before incubation started, 100mg/L of sodium sulphide (Na_2S) was added to the buffer to completely reduce the solution. Rumen fluid was collected from rumen-cannulated grazing cows in the morning at 08:00 manually into a pre-warmed thermos bottle. The rumen fluid was then brought to the lab, filtered through one layer of cheese cloth and mixed at a ratio of 1:4 with reduced artificial saliva. The mixture was kept at 39°C, stirred and continuously flushed with CO_2. A total of 60 ml of the mixture was dispensed into each serum bottles containing the substrate under a stream of CO_2 and the bottle was closed with a butyl rubber stopper and placed in an incubator at 39°C.

Gas analysis

After 2, 6, 12, and 24 hours of incubation the gas volume was determined by releasing the gas into a calibrated syringe and a 1 ml aliquot was taken for determination of methane concentration of the gas. 200 µl of the gas sample was injected manually into a gas chromatograph (GC-2010, Shimadzu corporation, Kyoto, Japan) fitted with a flame ionisation detector (FID). Gases were separated in a HP-Plot Molsieve column (length 35 m, ID 0.53 mm, Agilent Technologies Inc, Santa Clara, California, USA) and methane was detected with an FID detector (250 °C). The GC was run under isocratic conditions at 85 °C with N_2 as a carrier gas.

Sampling

At the end of the incubation a sample was collected from each bottle to determine the concentrations of short chain fatty acids (SCFA) and ammonia. For SCFA and ammonia analysis, an aliquot of 2 ml rumen fluid in duplicate was centrifuged at 21,000g for 10min at room temperature. 900 µl of the supernatant was transferred into a fresh vial containing 100 µl of internal standard (19.87 mM ethyl-butyric acid, 20% (v/v) ortho-phosphoric acid). The samples were then frozen overnight, and centrifuged (21,000g, 10min at room temperature) the next day. 800 µl of the supernatant was transferred into a crimp cap vial and samples for SCFA were analysed in a gas chromatograph (HP 6890) equipped with a capillary column (ZB FFAP &HK-G009-22) (Attwood, Klieve et al. 1998). Part of the final supernatant was used for a colorimetric ammonia assay (Weatherburn 1967).

Saprophytic fungi:

Samples of saprophytes were added on top of 600 mg hay, also used as substrate for a positive control. Either the macerated mat of the fungus or the incubation medium the fungus was grown in was added to the incubation. This allowed independent testing for the effects of the fungus itself and the metabolites of the fungus that were released into the medium. A known inhibitor of methane, Bromoethane sulphonate (BES) was also added to the hay substrate and included in each incubation run as a positive control, at a final concentration of 30 µmoles/l. The hay with no BES added or no saprophyte sample added served as a negative control.

Incubation, sampling and sample analysis was done as described above for grass-endophyte samples, but an automated analysis system was used to determine gas volume and composition. Each incubation bottle was attached to a pressure sensor (40PC015G1A, Honeywell International Inc., Morristown, New Jersey, USA), and gas volume was determined from pressure. Pressure was recorded every minute for each sensor. A calibration curve for every sensor was used to convert pressure into gas volume. When the threshold pressure of 90 mbar was reached a solenoid valve was opened and the gas sample was injected into the GC. Gas analysis was done using the same conditions as described above.

Other chemical analyses

Subsamples of the freeze-dried herbage supplied for fermentation bioassay were also analysed for the concentrations of the main known alkaloids produced by the endophytic fungi, according to standard methods described for lolitrem B (Spiering *et al*., 2005), ergovaline (Spiering *et al*., 2002), peramine (Spiering et al., 2002), lolines (Kennedy and Bush 1983; Yates *et al*., 1989) and epoxyjanthitrems (Tapper and Lane, 2004).

RESULTS

Endophyte alkaloid concentrations

The grass-endophyte alkaloid concentrations (Table 3) showed the expected structure of presence in high, medium or low concentrations, or absence (not detectable) relating to whether the strain was wildtype, a selected strain or did not contain endophyte. However, concentrations particularly in the fescue cultivars were generally lower than expected. Trace concentrations of ergovaline and peramine in the nil-endophyte Jesup tall fescue probably indicate a low level of contamination in those plots from tall fescue containing the wildtype strain. The analysis also included lolines and epoxy-janthitrems. While the presence or absence of these alkaloids was not taken into account in the selection of candidates for testing, they were included in this chemical analysis because the epoxy-janthitrems have shown tremorgenic effects similar to lolitrem B in animal studies, and lolines have shown antifeeding and toxicity in insect-based studies that is broader than shown by peramine.

Table 3. Concentrations of the major classes of alkaloids lolitrem B, ergovaline (Ev), peramine (Per), loline and epoxy-janthitrems (Epox) in samples of perennial ryegrass-endophyte, tall fescue-endophyte and meadow fescue endophyte associations.

			Alkaloid (mg/kg DM)				
Species	Cultivar (or Line)	Endophyte strain	Lolitrem	Ev	Per	Loline	Epox
P. ryegrass	GA66	Nil	ND	ND	ND	ND	ND
		WT	1.7	0.4	22.7	ND	ND
		AR1	ND	ND	21.3	ND	ND
		AR6	ND	1.1	14.2	ND	ND
		AR37	ND	ND	ND	ND	21.7
		AR47	ND	ND	ND	ND	ND
		AR50	ND	<0.1	24.3	ND	ND
		AR68	ND	0.2	9.7	ND	ND
		AR73	ND	0.6	7.7	ND	ND
		AR96	1.3	<0.1	ND	ND	ND
		AR100	ND	1.1	9.1	ND	ND
Tall fescue	Jesup	Nil	ND	0.1	0.4	ND	0.2
		Wildtype	ND	0.2	3.9	1188	ND
		AR542	ND	ND	2.5	217	ND
		AR525	ND	ND	5.0	687	0.1
		AR584	ND	ND	4.0	344	ND
		AR593	ND	ND	5.0	419	0.1
	Resolute II	AR542	NA[2]	NA	NA	NA	NA
Meadow fescue	Selection	Wildtype	ND	ND	0.5	2106	ND

[1] None detected
[2] Insufficient sample for analysis

Endophyte fermentation products

Total gas production was significantly different for both perennial ryegrass and tall fescue infected with various endophytes (Table 4). Methane production (ml/g) also showed

significant differences. However the proportion of methane did no differ between the various endophyte strains. Largely similar results were obtained for the tall fescue (Table 4). Significant differences were observed for gas and methane production but here also the proportion of methane was significantly different (P = 0.017). The differences nevertheless are relatively small (less than 10%) and were observed only during early stages of fermentation at 6 hours and 12 hours, while no differences in the proportion of methane were observed at 24 hours of incubation (P = 0.364).

Table 4. Total gas production (GP), methane gas production and proportion from perennial ryegrass or tall fescue infected with different strains of endophyte, after 24 hour *in vitro* incubation.

Grass	Endophyte strain	GP ml/g	CH_4 ml/g	CH_4 %
P. ryegrass	Nil	129.5	24.8	18.5
	AR1	121.1	24.3	19.7
	AR100	128.8	27.3	20.8
	AR37	127.9	25.7	19.8
	AR47	127.1	25.4	19.7
	AR50	126.0	24.8	20.1
	AR6	122.1	23.2	17.9
	AR68	125.3	24.7	19.4
	AR73	136.1	25.9	18.3
	AR96	126.2	25.2	20.4
	Wildtype	130.8	24.5	19.1
	LSD	5.60	0.60	1.30
	P value	<.001	<.001	0.913
Tall fescue	Nil	99.1	19.6	19.9
	AR525	102.4	19.8	20.4
	AR542	120.7	24.4	19.9
	AR584	116.0	23.1	19.5
	AR593	125.7	23.8	18.0
	Wildtype	113.0	21.2	18.4
	LSD	3.10	0.60	1.15
	P value	<.001	<.001	0.017

Substrate	SED	3.13	1.47	1.14
	P value	<.001	0.004	0.178

Between the two substrates (perennial ryegrass and tall fescue) significant differences were observed in total gas production and methane production (Table 4) with gas production and methane production higher from ryegrass than from tall fescue. The proportion of methane however was similar between the two grasses. However, the higher gas production did not result in a higher SCFA concentration for perennial ryegrass (Table 5) and also the proportion of individual SCFA was similar between the two grasses. Only NH_4 concentrations differed between the substrates with ryegrass yielding less NH_4 than tall fescue.

Table 5. The concentrations of ammonia (NH_4), total short chain fatty acids (SCFA), and the proportions of the predominant short chain fatty acids acetate, propionate and butyrate, and the total proportion of all other minor SCFAs.

		NH_4 mM	SCFA mM	Acetate %	Propionate %	Butyrate %	Minor %
P. ryegrass	Nil	9.0	38.6	61.8	22.8	10.3	5.1
	AR1	7.9	35.7	63.8	24.2	7.8	4.2
	AR100	8.1	44.6	61.5	21.4	12.1	5.1
	AR37	9.1	38.7	62.7	23.7	9.0	4.6
	AR47	12.6	37.6	63.3	23.6	8.3	4.8
	AR50	7.4	42.8	61.5	21.4	12.2	4.8
	AR6	9.0	37.5	62.7	23.8	8.9	4.6
	AR68	8.4	39.7	60.3	24.1	10.9	4.8
	AR73	10.0	37.2	62.0	24.3	9.0	4.7
	AR96	7.9	43.1	61.9	21.3	12.0	4.8
	Wildtype	8.0	35.1	62.3	24.6	8.5	4.5
	LSD	2.61	4.55	3.31	1.94	2.60	1.19
	P value	0.103	0.013	0.110	<.001	0.149	0.018
Tall fescue	Nil	13.4	37.0	63.4	22.6	9.4	4.7
	AR525	14.9	37.8	64.3	23.5	7.8	4.4
	AR542	21.7	47.4	63.3	22.5	8.7	5.5
	AR584	20.1	42.5	62.9	23.2	8.5	5.4
	AR593	18.2	51.5	64.0	22.4	8.8	4.7

	Wildtype	23.3	48.3	64.8	22.1	7.8	5.2
	LSD	4.11	4.69	2.37	1.47	0.99	0.46
	P value	0.008	0.001	0.570	0.437	0.075	0.010
Substrate	SED	1.07	3.21	1.22	1.37	1.44	0.44
	P value	0.010	0.271	0.514	0.883	0.387	0.865

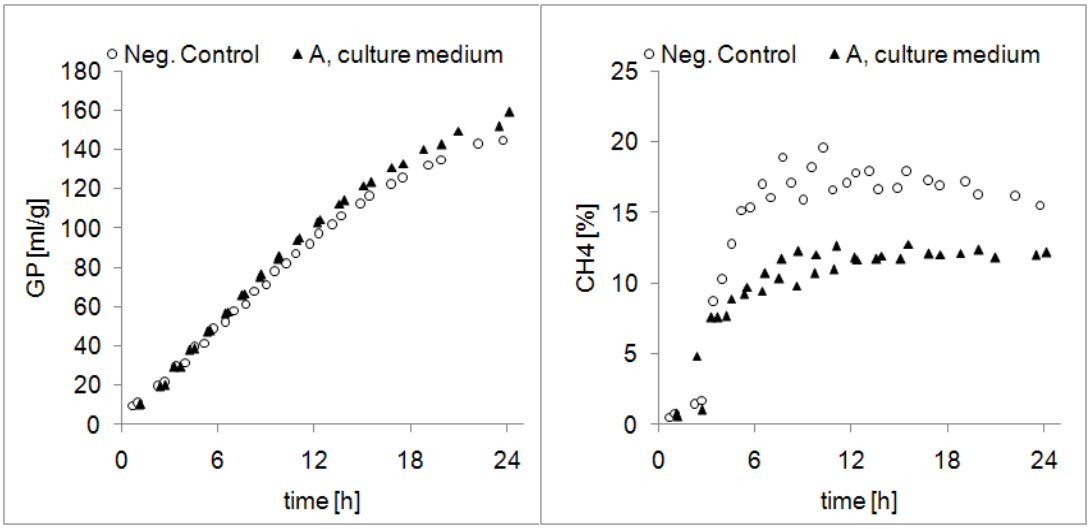
For the ryegrass samples there were no differences among endophyte strains in NH_4 concentration but there were differences in the total SCFA production. Within the SCFA only propionate and the sum of the minor fatty acids differed among endophyte strains, while the proportions of acetate and butyrate were similar among the strains. In contrast, for the tall fescue incubation, there were no differences in either the total SCFA concentration, or the proportions of individual SCFA were observed, but some of the samples showed a significantly increased NH_4 concentration after 24 hours of *in vitro* incubation.

Saprophyte fermentation products

Sapropytes were incubated in the automated incubation system and the samples were added on top of the hay substrate. Either the macerated mat of the fungus or the residual culture growth medium was added to the batch cultures to test for effects of the fungus itself or metabolites released into the medium. In addition a positive and a negative control were used. The negative control was the hay on its own while the positive control consisted of the hay with BES added to a concentration of 30 µmoles/l.

The automated system continuously measures gas and methane production. An example of the data is given in Figure 2 for Culture A (culture medium) where no effect on total gas production was observed but the proportion of methane was significantly decreased over the course of the experiment. The evaluation of gas data was done at 9 and 24 hours of incubation reflecting the time of maximum fermentation activity and the end point of the incubation.

Figure 2. Example of data generated by the automated batch culture system.



The saprophytes had on average a much more pronounced effect on the fermentation than the endophyte infected grasses. Significant differences among the fungal cultures and fractions were observed for every parameter except for the proportion of butyrate. The proportions of methane differed at 9 and 24 hours of incubation, which was the main parameter of the screening. Differences however were more pronounced at early stages of fermentation (9h) than at the end of the incubation.

Between the fractions, incubations with the fungal mat tended to increase gas production, the proportion of methane and the total SCFA concentrations. However no differences in the composition of the SCFA were observed.

Table 6. Total gas production (GP) and methane gas production as a proportion of total gas production after 9 hours and 12 hours of fermentation of fungal fractions and strains of candidate fungi.

Fungus			Total gas production ml/g		Methane production % of GP	
Species	Strain	Fraction	GP_9	GP_24	CH_4_9	CH_4_24
Control			81	154	14.8	16.6
Mortierella wolfii	69/3891	fungal mat	76	141	14.6	15.9
Mortierella wolfii	74/2649	fungal mat	110	186	10.2	12.2
Mortierella wolfii	68/707w2	fungal mat	98	173	14.8	16.9
Penicillium roquefortii	G2	fungal mat	77	150	13.0	13.9
Penicillium roquefortii	I2	fungal mat	95	173	15.6	17.8
Penicillium roquefortii	A2	fungal mat	100	176	15.4	17.9
Monascus ruber	15220	fungal mat	87	162	13.7	16.3
Monascus ruber	503.7	fungal mat	110	197	9.1	12.4
Geotrichum candidum	240.62	fungal mat	111	206	8.8	12.3
Monascus ruber	554.76	fungal mat	107	194	8.4	11.9
Mortierella wolfii	69/3891	supernatant	82	155	10.0	11.6
Mortierella wolfii	74/2649	supernatant	103	181	14.6	16.6
Mortierella wolfii	68/707w	supernata	105	186	8.8	10.6

	2	nt				
Penicillium roquefortii	G2	supernatant	76	143	11.1	13.4
Penicillium roquefortii	I2	supernatant	87	161	17.3	19.2
Penicillium roquefortii	A2	supernatant	98	174	17.9	19.2
Monascus ruber	15220	supernatant	112	192	15.8	17.6
Monascus ruber	503.7	supernatant	76	158	11.8	14.0
Geotrichum candidum	240.62	supernatant	74	157	12.2	14.3
Monascus ruber	554.76	supernatant	73	152	11.8	13.9
	fungal mat mean		97	176	12.4	14.8
	supernatant mean		89	166	13.1	15.1
Fungus	LSD		19.0	1.9	1.60	3.39
	P value		<.001	0.004	<.001	<.001
Fraction	LSD		7.9	12.1	1.20	1.03
	P value		0.041	0.057	<.001	<.001
Fun.x Frac.	P value		<.001	0.004	0.620	0.065

Table 7. Total short chain fatty acids (SCFA) and acetate, propionate and butyrate as a proportion of total SCFA after 24 hours fermentation of fungal fractions and strains of candidate fungi.

Fungus			SCFA			
Species	Strain	Fraction	Total mM	Acetate %	Propionate %	Butyrate %
Control			39.3	66.0	22.8	8.3
Mortierella wolfii	69/3891	fungal mat	36.4	61.0	27.6	7.8
Mortierella wolfii	74/2649	fungal mat	46.6	58.8	30.3	7.9
Mortierella wolfii	68/707w2	fungal mat	41.3	68.7	20.6	7.8
Penicillium roquefortii	G2	fungal mat	33.7	58.6	29.4	8.4
Penicillium roquefortii	I2	fungal mat	43.1	67.9	20.0	8.6

Penicillium roquefortii	A2	fungal mat	44.0	67.5	20.2	8.9
Monascus ruber	15220	fungal mat	40.4	68.7	20.7	7.9
Monascus ruber	503.7	fungal mat	45.3	61.1	26.3	9.9
Geotrichum candidum	240.62	fungal mat	45.1	61.7	25.9	9.8
Monascus ruber	554.76	fungal mat	45.2	60.7	26.9	9.8
Mortierella wolfii	69/3891	supernatant	38.5	61.0	28.5	7.3
Mortierella wolfii	74/2649	supernatant	41.6	68.6	20.7	7.8
Mortierella wolfii	68/707w2	supernatant	44.5	57.4	30.6	7.7
Penicillium roquefortii	G2	supernatant	32.4	56.4	31.5	8.5
Penicillium roquefortii	I2	supernatant	39.7	66.5	20.6	9.2
Penicillium roquefortii	A2	supernatant	41.1	67.3	20.0	9.0
Monascus ruber	15220	supernatant	45.0	67.1	20.7	8.8
Monascus ruber	503.7	supernatant	34.9	64.3	23.8	9.0
Geotrichum candidum	240.62	supernatant	33.0	64.3	23.8	8.8
Monascus ruber	554.76	supernatant	32.4	63.6	24.5	9.0
	fungal mat mean		42.1	63.5	24.8	8.7
	supernatant mean		38.3	63.6	24.5	8.5
Fungus	LSD		3.31	2.86	0.65	0.58
	P value		<.001	<.001	<.001	0.979
Fraction	LSD		2.15	2.10	1.82	0.41
	P value		<.001	0.286	0.080	0.100
Fun.x Frac.	P value		<.001	<.001	<.001	<.001

In order to identify possible candidates for further examination, the samples were ranked according the to their effects on methane inhibition in early and late stages of fermentation as well as their effect on SCFA production and the proportion of propionate. The ranking of the samples is given in Table 8. For each the sample the ranking was calculated from the inhibition of methane relative to the positive control plus the change in SCFA concentration relative to negative control plus the change in the proportion of propionate relative to the negative control, in percent. This integrated the effects of a fungus on methane suppression with the effects on overall fermentation, so as to separate out candidates (rank low) where

suppression of methane was related to suppression of fermentation (undesirable) rather than an effect of specific methane inhibition.

Table 8. Ranking of the saprophyte fungi according to effects on methane and short chain fatty acids produced during *in vitro* fermentation.

Fungus	Strain	Fraction	Ranking
Mortierella wolfii	68/707w2	supernatant	12.0
Mortierella wolfii	74/2649	supernatant	10.8
Mortierella wolfii	69/3891	supernatant	9.1
Monascus ruber	554.76	supernatant	8.3
Geotrichum candidum	240.62	supernatant	7.4
Monascus ruber	503.7	supernatant	7.2
Penicillium roquefortii	G2	supernatant	6.1
Penicillium roquefortii	G2	fungal mat	4.3
Mortierella wolfii	74/2649	fungal mat	2.9
Monascus ruber	15220	supernatant	2.3
Mortierella wolfii	69/3891	fungal mat	2.3
Penicillium roquefortii	A2	supernatant	2.0
Penicillium roquefortii	I2	supernatant	1.7
Monascus ruber	15220	supernatant	1.5
Monascus ruber	15220	fungal mat	1.1
Monascus ruber	15220	fungal mat	0.8
Monascus ruber	503.7	fungal mat	-0.2
Monascus ruber	554.76	fungal mat	-0.5
Penicillium roquefortii	I2	fungal mat	-0.5
Mortierella wolfii	68/707w2	fungal mat	-0.6
Penicillium roquefortii	A2	fungal mat	-0.8
Geotrichum candidum	240.62	fungal mat	-1.3

DISCUSSION

The differences in total gas production, SCFA and NH_4 concentrations may reflect differences in the composition and degradability of the grass samples, rather than an effect of the endophyte. However, the limited effects on the proportion on methane indicate that there is little scope of manipulating enteric methane emissions by different strains of endophyte fungi or their metabolites. Perennial ryegrass tends to have a better nutritional value than tall fescue as was shown by the higher total gas production. However, the differences between species in SCFA concentration were not significant. Also, the utilisation of nitrogen from ryegrass seemed to be better than that from tall fescue since less NH_4 accumulated in vitro.

The candidate grass-endophyte associations provided the structured set of presence and absence of alkaloids required to test whether any possessed antimethanogenic properties. There was no suppression observed for any of the combinations, suggesting that alkaloids are not antimethanogenic. The samples were collected in March, the time of year when the concentrations of alkaloids would normally be at their seasonal peak (Watson *et al*., 1999; Fletcher, 1999) and were cut as close to the ground as possible so as to include grass tiller bases where the concentrations of lolitrems and ergovaline tends to be highest (Keogh *et al*., 1996; Lane *et al*., 1997). The concentrations in ryegrass were within the expected range, whereas for both tall fescue and meadow fescue, the concentrations of the main alkaloids ergovaline and loline were low. This was possibly a consequence of the dry summer conditions at that site prior to sampling. A more rigorous screening test may be to separate tillers into leaf lamina and stem and pseudostem, and conduct the assay on the basal herbage containing the higher concentrations. There may be other explanations for a nil result. There may be other, possibly unknown compounds that interfere with an anti-methanogenic effect, or its detection.

The one previous study that showed suppression of methane production by an endophytic fungus was based on tall fescue containing the selected strain AR542, but no effect was shown for the same cultivar containing wildtype endophyte (Vibart *et al*., 2007). If the hypothesis that alkaloids are the bioactive agent is correct, it would be expected that the wildtype would show at least the same effect. This also tends to suggest that endophyte alkaloids are not involved. It is hard to explain how a strain selected to eliminate the production of ergovaline would create an effect not seen with the wildtype strain that does produce ergovaline, unless there is some other unknown difference between strains. However, a further test using herbage with higher concentrations, or perhaps even extracted alkaloids where a dose-response relationship could be tested, would be required before ruling out any prospect with endophytes.

The saprophytic fungi showed much greater effects on reducing methane production. Some of the samples reduced methane at the equivalent of 30 µM BES. Generally the effects were much more pronounced in the culture supernatant than in the fungal mats, suggesting that the active component is a metabolite of the fungus. Our initial screening results also indicated that *Mortierella wolfii* is the most promising candidate, since the supernatant fraction of each of the three strains of this species were grouped at the top of the ranking list. However, when samples were analysed again several months later the effect was decreased markedly suggesting that the bioactive component(s) is not stable when stored at -20 ^{o}C.

When new preparations of two strains (*Penicillium roquefortii* G2 and *Mortierella wolfii* 69/3891) were prepared they did not grow as well as the first preparations and when tested *in vitro* it was shown that the activity of G2 was greatly reduced in that preparation.

Two candidate species of saprophytic fungi showed some evidence of properties that suppress methane production *in vitro*. This appears to be a specific inhibition, in that methane was reduced as a proportion of total gas production, rather than as a consequence of overall suppression of fermentation. For the saprophytic fungi chosen for this study, there were no analyses undertaken comparable with those available for the endophytes. The profile of secondary metabolites produced by some of the candidate fungi is well known because of their importance in industrial fermentations for production of commercially important preparations such as pharmaceuticals (see Table 2). The anecdotal information suggesting strong links between ingestion of mouldy silage and suppression of methane production by ruminants gave no indication of the specific spoilage fungus involved, let alone any specific secondary bioactive metabolite. The suppression of methane production which followed addition of crude extracts from the mycelium and culture filtrate of two of the four candidate species in the batch test is very encouraging. To obtain a positive result in a bioassay of unknown material will always depend on a bioactive constituent being present in the extract at a concentration that will elicit a measurable effect, and without any other constituents of the sample masking the effect. Activity can be greatly affected by fungal strain, growth medium, culture conditions and whether special methods are required to preserve activity of labile components after harvest. Once any degree of activity has been detected, the processes of bioassay-guided fractionation and purification are required to establish the range of bioactive compounds and to establish their chemical identity and essential properties (e.g. specificity, mode of activity). The importance of persevering with bioassay based fractionation and purification before eliminating potential candidates is highlighted by results with *Monascus ruber.* This red yeast fungus is known to produced monicolin K (lovastatin) which is reported to suppress growth of methanogenic Archaea but not other rumen bacteria (Refer to in comment in Table 2), yet extracts of none of the three strains suppressed methane production. At this stage it is unclear whether the strains used will produce lovastatin at a detectable concentration, or at all, either under the conditions we used or under one of many other possible combinations of growth medium, incubation conditions or extraction method. The batch fermentation technique provides a valuable tool to investigate the potential of saprophytic fungi to ameliorate excessive methane production by ruminants. It is therefore recommended that more work is done with *Mortierella wolfii* including some more batch culture tests, a continuous culture test and eventually some extracts to identify the components.

While the results of this screening have not shown conclusive evidence that fungi can suppress methane production, it should be remembered that the nature of the study makes it possible to draw that conclusion only for the candidates selected for screening. It is not possible to conclude that suppression by this means is not possible under any circumstances. There is a wide diversity of fungi and even for the two classes chosen here there is considerable diversity among strains of the same species. Candidates were selected to encompass the diversity between and within species as much as possible, given the constraints on the number that could be screened. The number of species of fungi was narrow, and confined to those where there was prior indication of a possible link between the

presence of fungi and suppression of enteric methane production during the digestion of fresh or ensiled pasture. It is possible there may be species or strains of fungi with antimethanogenic properties outside of those selected for screening. Furthermore, there is year-to-year and seasonal variation in the concentrations of endophyte alkaloids, so results must be interpreted in the context of the particular concentrations of alkaloids and other secondary metabolites in the year in which these samples were collected. Similarly, for the saprophytic fungi, the culture conditions can influence the characteristics of the fungal mycelia mat, and this could influence potential antimethanogenic properties.

CONCLUSIONS

For the species and strains of endophytic fungi tested none demonstrated potential to significantly reduce methane production in batch-culture assay. On this basis it is unlikely that any suppression would be demonstrated under the more challenging conditions of continuous-culture fermentation and animal-based testing, where even promising short term suppression may be lost as the rumen microbial population adjusts. However, one species of saprophyte, *Mortierella wolfii* demonstrated the properties to suppress methane production without suppressing the beneficial production of short chain fatty acids during fermentation. Simple separation of the mycelia mat from the liquid supernatant and assaying these fractions independently suggests that the bioactive constituent may be a metabolite of the fungus. This should be investigated further, firstly in continuous-culture to verify that the effect can be sustained, and then undertaking fractionation steps to identify the bioactive constituent and tests to determine if the effect can be enhanced in a dose-dependent manner by, for example, concentrating the active compound.

RECOMMENDATIONS

One species of saprophytic fungi showed evidence of properties that suppress methane production *in vitro*. This was in the supernatant fraction, indicating that the bioactive constituent was a metabolite of the fungus released into the culture medium. This appears to be a specific inhibition, in that methane was reduced as a proportion of total gas production, rather than as a consequence of overall suppression of fermentation. Evidence of this nature that commonly occurring fungi can suppress methanogenesis, is very limited, suggesting that further investigation of these strains is warranted. This would include conducting further batch-culture evaluation of material grown under contrasting culture conditions and prepared for assay by different methods to determine if the apparent effect can be enhanced, as a prerequisite to attempts to fractionate and isolate bioactive components.

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