TITLE

The effect of diet of the donor cows on *in vitro* measurements of methane production from wheat and corn incubated in various forage to grain ratios.

RUNNING TITLE

Comparison of *in vitro* methane production from wheat and corn grains

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ABSTRACT

BACKGROUND Supplementation of ruminant diets with wheat and corn grains influences ruminal fermentation. *In vitro* fermentation is a methodology that can be used to screen feeds for their potential to produce enteric methane. However, there is evidence that the diet of the donor cows could impact the results of *in vitro* analysis. This research investigated the *in vitro* fermentation of wheat and corn grain when incubated in ruminal fluid from cows fed different grain types and different forage to grain ratios.

RESULTS The type of grain fed to the donor cows, as well as forage to grain ratio affected the outcome of fermentation of wheat and corn grain. Differences in methane production (MP) between grains were only observed when incubated with ruminal fluid adapted to each specific grain type. Increasing proportions of wheat but not of corn decreased *in vitro* MP in a linear manner compared to MP produced from forage only.

CONCLUSIONS Wheat grain has greater *in vitro* antimethanogenic effect than corn. However, to detect the different fermentations between wheat and corn, grains should be incubated in ruminal fluid from cows adapted to that specific grain type.

Keywords: *In vitro*, methane, ruminal fluid, wheat, corn, ruminant

INTRODUCTION

High producing animals (dairy and beef cattle) require high energy diets and this is achieved by feeding grains, such as wheat and corn. Previous studies have demonstrated that feeding wheat or corn grain to dairy cows has different effects on ruminal fermentation.¹⁻³ One of the differences reported between feeding wheat or corn grain to dairy cows is associated with enteric methane production (MP).²

An option for screening feeds for their methane mitigating effect is the *in vitro* fermentation technique, which involves incubating a test feed in a fermentation vessel containing ruminal fluid and a buffering solution.⁴⁻⁶ However, some aspects within the methodology can affect the results and hence the conclusions drawn. A previously published review made a series of recommendations to standardize the *in vitro* methodology.⁶ One recommendation was that *in vitro* incubations should use ruminal fluid from donor animals fed a diet similar to the substrates that are screened *in vitro* in order to have an inoculum containing microbial populations adapted to the substrate in question.⁶ For example, it has been reported that starch-rich substrates ferment more rapidly *in vitro* when incubated in ruminal fluid from donor cows fed a starch rich diet when compared with ruminal fluid from donor cows fed a forage based diet.⁷⁻⁹ Several recent studies have screened a large number of substrates for MP using ruminal fluid

from donor animals fed a common diet for all substrates.¹⁰⁻¹² Thus, when screening two or more grains that induce different ruminal conditions *in vivo*,^{1,2} it is currently unclear if these grains should be incubated in ruminal fluid from cows fed the same diet or if each grain should be incubated with ruminal fluid from donor cows adapted to that specific grain type.

It has also been recommended that the *in vitro* incubation should use a range of substrates that reflect the diet offered within a given production system.⁶ *In vitro* studies that have tested the methane mitigating capacity of different feeds have used a single substrate in the incubation although the diets fed to the donor cows contain a mixture of ingredients.¹²⁻¹⁴ For example, studies have measured *in vitro* MP with a substrate composed of 100% corn.¹⁰ However, cows rarely eat a diet composed of a single ingredient, with dairy cow diets commonly including up to 50% corn or wheat grain, and the rest of the diet composed of a fibre source.¹⁵ Moreover, it has been shown that diets containing approximately 50% wheat result in substantially less methane than diets containing approximately 50% corn.² Thus, it is unclear if MP obtained *in vitro* using pure substrates has relevance to the *in vivo* MP. Furthermore, even though it has been recommended that the substrate used *in vitro* should reflect diets fed *in vivo*, there appears to be little evidence available as to how different forage to grain ratios of wheat and corn grain influence *in vitro* MP of wheat and corn grain.

The research studied the *in vitro* fermentation of wheat and corn grain when incubated in ruminal fluid from donor cows fed different grain types and in different forage to grain ratios. We hypothesized that 1) the *in vitro* fermentation of grains would

produce more methane when incubated with ruminal fluid from cows adapted to each specific grain type (homologous ruminal fluid) compared with incubated in ruminal fluid not adapted to that specific grain type (heterologous ruminal fluid) and 2) that when incubated with ruminal fluid from cows adapted to each specific grain type (homologous ruminal fluid) MP would be negatively related to the proportion of grain (i.e. wheat or corn) in the substrate incubated.

MATERIALS AND METHODS

Substrates, animals and ruminal fluid

This study was conducted in two *in vitro* experiments. The substrates for these *in vitro* studies were ground corn, ground wheat or ground alfalfa hay (Table 1). Substrates were analyzed by Dairy One Laboratories (Ithaca, NY, USA) for dry matter (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), ash and starch by chemical analytical methods according to their published analytical procedures.¹⁶

///Insert Table 1 around here///

All experiments involving sampling of ruminal fluid from rumen cannulated cows were approved by the Animal Ethics Committee of the Department of Economic Development Jobs Transport and Resources – Victoria. Cows were cared for according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.¹⁷

Twelve lactating Holstein-Friesian dairy cows fitted with ruminal cannulae (www.rumencannula.com) were used to source rumen fluid. Donor animals were offered a mixed diet containing forage (11.0 kg DM d⁻¹ of chopped alfalfa hay (*Medicago sativa* L) and either crushed corn (*Zea mays* L, n = 6 cows, rumen fluid labelled 'C') or crushed wheat (*Triticum aestivum* L, n = 6 cows, rumen fluid labelled 'W'), both at 10.0 kg DM d⁻¹. For both experiments, fresh ruminal fluid was collected 2 h after feeding, using a 100 mL syringe and copper pipe inserted through the fistula, and ruminal fluid was collected from multiple sites within the rumen. The ruminal fluid was kept in 2 L glass bottles that had been pre-warmed to 39°C and then transported to the laboratory in an incubator set at 39°C.¹⁸ The ruminal fluid was filtered through four layers of cloth to remove particles. During the filtering process, the fluid was under constant flow of carbon dioxide. A 0.5 mL representative sub-sample of ruminal fluid was transferred to a 5 mL plastic vial and then diluted with 4.5 mL of a solution containing formalin (400 mL L⁻¹), saline (9 mL L⁻¹) and methylene blue (4 mL L⁻¹). These samples were used to count ciliate protozoa using a counting chamber (Mod-Fuchs-Rosenthal; Hawksley & Sons Ltd, Lancing, UK) and a microscope (Leitz Laborlux S; Leica Microsystems Pty Ltd, North Ryde, NSW, Australia).

Experiment Designs

Experiment 1 tested the effect of donor animal diet (grain type) on fermentation of two grain types *in vitro* in a 2 × 2 factorial design (donor diet × grain type). Experiment 1 was conducted in four *in vitro* runs. Runs 1 and 2 both used ruminal fluid from the same group of six cows comprising three cows on wheat and three on corn,

and runs 3 and 4 both used ruminal fluid from another group of six cows, three on wheat and three on corn. In each run, grains were incubated separately in the ruminal fluid of each individual cow replicated in three fermentation bottles.

In Experiment 1, in each run, each type of ruminal fluid (W or C) was obtained from three cows. Four treatments were tested: C-C, a corn grain substrate ($1000 \text{ mg g}^{-1} \text{ DM}$) incubated in 'C' ruminal fluid; C-W, a corn grain substrate ($1000 \text{ mg g}^{-1} \text{ DM}$) incubated in 'W' ruminal fluid; W-W, a wheat grain substrate ($1000 \text{ mg g}^{-1} \text{ DM}$) incubated in 'W' ruminal fluid; and W-C, a wheat grain substrate ($1000 \text{ mg g}^{-1} \text{ DM}$) incubated in 'C' ruminal fluid. At the time of collection of ruminal fluid, the cows had been consuming their allocated diets for two weeks. Based on previous research, this period was considered of adequate duration to ensure that the ruminal microbiota were adapted to the specific diets.¹⁹

In this experiment, substrates were incubated separately in the ruminal fluid of each individual cow replicated in three fermentation bottles. Per run, a total of 36 fermentation bottles (2 types of grains $\times$ 2 types of ruminal fluid $\times$ 3 cows $\times$ 3 bottles) were used for substrates and 6 fermentation bottles were used for "blanks." i.e. bottles containing ruminal fluid without substrate.

Experiment 2 tested different forage to grain ratios as substrate in a 2×5 factorial design (donor diet $\times$ forage to grain ratio of substrate). Experiment 2 was conducted in two *in vitro* runs where each treatment was incubated in four incubation bottles within each run. Based on the results of experiment 1, in experiment 2, each

grain type was incubated only in the corresponding ruminal fluid adapted to that specific grain type in experiment 2.

In Experiment 2, five treatments that contained different proportions of grain (0, 250, 500, 750 and 1000 mg g⁻¹ DM) were tested. Alfalfa hay was used as forage and was substituted with grain as the grain proportions increased, resulting in a total of ten treatments (2 grains and 5 proportions of grain). Ruminal fluid was collected from three cows with 'C' rumen fluid and three with 'W' ruminal fluid and fluid was mixed between the cows of the same rumen fluid type. Substrate was incubated in a homologous rumen fluid type. The *in vitro* experiment was repeated in two runs using ruminal fluid from the same six cows. Each run involved four fermentation bottles for each grain at each grain proportion meaning that per run a total of 40 fermentation bottles were used for treatments (2 types of grains × 5 grain proportions × 4 bottles) and 8 fermentation bottles were used for "blanks

***In vitro* fermentations**

All substrate samples (wheat, corn or mixtures of these grains with alfalfa hay) for incubation were dried for 24 h at 60°C, then ground in a cyclone mill (Smartgrind® Stainless Steel Coffee Grinder; Black and Decker, Baltimore, MD, USA) and passed through a 1 mm sieve. The dried substrate was placed into 310 mL Ankom incubation bottles and 75 mL of Kansas State buffer solution at a pH of 6.8²⁰ and 25 mL of ruminal fluid was added, leaving 210 mL of head space volume. An Ankom GP (Ankom GP; Ankom Technology, Macedon, NY, USA) module was placed on top of each bottle and

the bottles were placed in a water bath maintained at 39°C. The fermentation bottle's head space was flushed with carbon dioxide prior to commencing incubation. For gas collection, the module's venting tube was connected to an empty gas collection bag through a 22 cm long extension tube with an internal diameter of 4 mm (Extension Line Type Heidelberger; Braun, Bella Vista, NSW, Australia) and a three way stopcock (Model MGT110000; Vitalmed, Kingsgrove, NSW, Australia).²¹ The gas collection bags had a capacity of 400 mL and were manufactured in house using a 3-layered gas-impervious foil (Code 1 MAC-15UM Nylon/9UM Foil/80UM LLDPE; Wests Packaging Services P/L, Carrum Downs, Vic, Australia). A small quantity of plumber's silicon (Selleys Roof and Gutter Speed Seal Silicone; Selleys, Padstow, NSW, Australia) was placed on two corners of each gas collection bag to act as septa, enabling needle access to each gas collection bag.²¹

Measurements

In both experiments, measurements included total microbial gas production (GP), MP, degraded dry matter (DDM), ammonia and volatile fatty acid (VFA) concentration in the incubation medium, and post fermentation pH after 24 hours of *in vitro* incubation. Measurement of GP were conducted with an Ankom Technology *in vitro* cumulative GP system fitted with pressure transducers and gas sample collection ports. *In vitro* incubations were conducted over 24 h. The data were logged to a computer spreadsheet by radio frequency transmission with 5 min intervals. Additionally, each time the pressure in the bottle reached 6.89 kPa above atmospheric pressure, an internal valve opened for 1 s to release the gas produced. This avoided the

fermentation gas produced in the head space of the fermentation bottle from diffusing back into the ruminal fluid.²² This vented gas was collected into the gas impermeable bags described above. Blanks were used to correct for gas produced from residual feed particles remaining in the ruminal fluid. Ruminal fluid was measured for pH (Ecoscan pH +5; Eutech Instruments, Vernon Hills, IL, USA) prior to and immediately after incubation.

At the completion of the incubation period, DDM was assayed following a previously published method,²³ with the modification that the remaining residue was filtered through a filter with 114 microns mesh openings (54T DP Screen Mesh, GJS Machinery, NSW, Australia) rather than centrifuged.²⁴ The final residue was then dried at 60°C for 48 h and weighed to establish its mass difference with the initial sample. Ammonia concentration in the ruminal fluid was determined with an ion selective electrode (SM703; Metrhom AG, Herisau, Switzerland).²⁵ At the conclusion of the *in vitro* fermentations, a 50 mL sample of the fermentation fluid was collected, chilled on ice and centrifuged at 4200 *g* for 10 min at 4°C. A 1 mL aliquot of the supernatant was collected and mixed with 9 mL of a dilute acid made up of formic acid (10 mL L⁻¹), orthophosphoric acid (10 mL L⁻¹) and an internal standard of 4-methylvaleric acid (184 µL L⁻¹). This aliquot was then stored frozen at -20°C until analysed for VFA concentrations, by capillary gas chromatography using an Agilent 7890B gas chromatograph (Agilent, CA, USA) with an auto-sampler and auto-injector.¹⁸ All results were calculated as ppm and converted to mmol L⁻¹ for subsequent statistical analyses.

After the incubation period, a gas-tight glass syringe with a volume of 50 mL (SGE International Pty Ltd, Ringwood, Vic, Australia) with attached needle was used to collect a gas sample from the head space of each incubation bottle and another sample from the gas bag attached to each module, with 20 mL gas samples transferred into separate Exetainers[®] (12 mL soda glass vial; Labco ltd., Buckinghamshire, UK). Methane proportion in the samples of head space gas and in the gas collected in the bags was determined by gas chromatography as described previously.²⁶

Calculations

Cumulative pressure measured by the Ankom GP modules over 24 h was used to estimate total GP using the ideal gas law. Methane production measured over the 24-h period was calculated through the equation:²²

$$MP = (HPM \times HV) + (BPM \times BV) \quad (1)$$

Where HPM is the methane proportion in the head space gas, HV is the known head space volume (210 mL), BPM is the methane proportion in gas voided into the collection bag and BV is volume (mL) of gas collected in the collection bag (also called GP) which is the equivalent of the total vented gas volume as measured by the Ankom GP system.²¹

Proportion of methane (PM; mL L⁻¹) in the fermentation gas was calculated as:

$$PM = MP \times 1000/GP \quad (2)$$

Methane produced per gram of DDM (MPgDDM, MP g⁻¹ DDM) was defined as:

$$\text{MPgDDM} = \text{MP} \times 1000/\text{DDM} \quad (3)$$

Statistical Analysis

The statistical analysis was performed with the Genstat statistical package (64 bit Release 19.1; VSN International, Hemel Hempstead, UK). The data were analyzed by Anova. Experiment 1 had a 2×2 factorial treatment structure of ruminal fluid type (ruminal fluid from cows fed the wheat or corn based diets) by substrate (ground wheat and ground corn), specified in GenStat as Diet * Substrate. The blocking structure was nested within set, a factor with 2 levels distinguishing between the first pair and second pair of runs that used different sets of 6 cows. The blocking structure was specified in GenStat as Set/((Run * Cow)/Substrate)/Bottle. The analysis was conducted according to the following model:

$$Y_{rcb} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + S_s + R_r + C_c + RC_{rc} + C\beta_{cj} + RC\beta_{rcj} + \varepsilon_{rcb} \quad (4)$$

Where Y_{rcb} = dependent variable for run r , cow c , and fermentation bottle b , μ = overall mean, with effects α_i = ruminal fluid diet i , β_j = substrate j , $\alpha\beta_{ij}$ = interaction between ruminal fluid diet and substrate, S_s = set s , R_r = run r , C_c = cow c , RC_{rc} = interaction of run r and cow c , RC^2_{rcj} = run by cow by substrate, and ε_{rcb} = residual error that comprised individual substrate sample, bottle measurement error effects. Under this model, cow was the experimental unit for ruminal fluid diet treatment, and the cow by substrate treatment interaction was the unit for substrate comparisons.

Data from experiment 2 were analyzed by Anova with a 2×5 factorial treatment structure of rumen fluid type (corresponding to grain type fed to the donor cows) by proportion of grain, and nested blocking structure of run split for treatment split for fermentation bottle, using the following model:

$$Y_{rb} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + R_r + R\alpha\beta_{rij} + \varepsilon_{rbi}$$

(5)

Where Y_{rb} = dependent variable for fermentation bottle b in run r , μ = overall mean, α_i = grain type i , β_j = grain proportion j , $\alpha\beta_{ij}$ = interaction between grain type and proportion, R_r = run r , $R\alpha\beta_{rij}$ = treatment by run effect, and ε_{rbi} = residual error, comprising individual substrate sample, bottle and measurement error effects. The residual error was allowed to have a separate variance for the two grain types, i . Under this model, treatment by run was the unit for treatments. Linear and quadratic orthogonal polynomial contrasts for grain proportion were calculated separately for each grain type and tested by t-test, using the means and variance covariance matrix.

RESULTS

Experiment 1

Methane production in the fermented gas was not different between substrates (Table 2). However, treatments that used ruminal fluid from cows fed corn had greater ($P = 0.043$) MP than treatments that used ruminal fluid from cows fed wheat. In addition, C-C had 32% greater MP than W-W. Similar results were obtained for PM. Methane produced per gram of DDM was greater ($P = 0.022$) in treatments that used corn as substrate than in treatments that used wheat as substrate, and greater ($P = 0.031$)

in treatments that used ruminal fluid from cows fed corn than in treatments that used ruminal fluid from cows fed wheat. Gas production was less ($P = 0.002$) in treatments that used corn as substrate than in treatments that used wheat as substrate. Degraded dry matter was less ($P < 0.001$) in treatments that used corn for substrate than in treatments that used wheat for substrate. Ammonia concentration was greater ($P = 0.006$) in the incubation medium of treatments that contained corn as substrate than in treatments that contained wheat as substrate. Total VFA was lower ($P < 0.001$) in the post-incubation medium of treatments that used corn as substrate than in treatments that used wheat as substrate. There was an interaction effect between ruminal fluid and grain type on A:P ($P = 0.018$) as C-C was different from C-W, but there was no difference between W-W and W-C. Post fermentation pH of the incubation medium was higher ($P < 0.001$) in treatments that used corn as substrate than in treatments that used wheat as substrate. Variance between cows fed the same diet was significantly greater than zero for MP ($P = 0.001$), PM ($P = 0.001$), MPgDDM ($P = 0.010$), VFA ($P < 0.001$), A:P ($P = 0.023$) and post-fermentation pH ($P = 0.003$).

///Insert table 2 around here///

The pre-fermentation pH in ruminal fluid from cows fed diets that contained corn or wheat was not different (Table 3). Protozoa count was greater ($P = 0.020$) in cows fed diets that contained corn than in cows fed diets that contained wheat.

///Insert table 3 around here///

Experiment 2

There was a negative linear relationship ($P < 0.001$) between wheat proportion and MP; however, there was no relationship between corn proportion and MP (Table 4). Methane production was less ($P < 0.001$) in treatments that used wheat as substrate than in treatments that used corn as substrate. Similar results were observed for PM. When the grain in the substrate was $250 \text{ mg g}^{-1} \text{ DM}$, the MP from the treatments that contained wheat was 61% of the MP from the substrate that contained corn, with this comparison decreasing to 47%, 43% and 42% for grain proportions of 500, 750 and $1000 \text{ mg g}^{-1} \text{ DM}$ respectively. There was a negative linear relationship ($P < 0.001$) between the MPgDDM and the proportion of wheat, and a negative linear relationship ($P < 0.001$) between MPgDDM and the proportion of corn. Incubations that used wheat as substrate had smaller ($P < 0.001$) MPgDDM than incubations that used corn as substrate. There was a negative relationship ($P < 0.001$) between proportions of wheat and GP, and there was a negative relationship ($P < 0.001$) between the proportion of corn and GP. Mean GP from incubations that used wheat as substrate was less ($P < 0.001$) than that of incubations that used corn as substrate. There was a positive relationship ($P < 0.001$) between wheat and corn proportions and DDM, for wheat, this relationship was linear ($P < 0.001$) while for corn, this relationship was quadratic ($P = 0.021$). Ammonia concentration in the incubation medium was not affected by the proportion of grain or grain type. Total VFA concentration increased linearly ($P < 0.001$) with the proportion of either wheat or corn and was greater ($P < 0.001$) in treatments that contained wheat as substrate than in treatments that contained corn as substrate. Acetate to propionate ratio was negatively linearly related ($P < 0.05$) to the

proportions of wheat or corn in the substrate. The incubations that used wheat substrate and media derived from ruminal fluid from cows fed wheat resulted in lower ($P = 0.019$) A:P, than those that used corn substrate and media derived from ruminal fluid from cows fed corn. There was a negative relationship ($P < 0.001$) between proportions of wheat and post fermentation pH, and there was also a negative relationship ($P < 0.001$) between the proportions of corn and post fermentation pH. Incubations that used corn as substrate and medium, had greater ($P < 0.001$) post-fermentation pH than incubations that used wheat.

///Insert table 4 around here///

Pre-incubation pH in ruminal fluid from cows fed corn was 6.2 and it contained a protozoa population of 5.52 (Log10 count mL⁻¹ ruminal fluid) while ruminal fluid from cows fed wheat had a pre-incubation pH of 5.8 and contained a protozoa population of 5.32 (Log10 count mL⁻¹ ruminal fluid) (data not shown).

DISCUSSION

Experiment 1

The greater MP of grains incubated in ruminal fluid from cows fed diets that contained corn grain than wheat grain, despite no difference between substrates, indicates that the ruminal fluid used had greater effect on *in vitro* MP than the substrate incubated. Thus, we reject our first hypothesis that the *in vitro* fermentation of grains would produce more methane when each grain was incubated with ruminal fluid from cows adapted to each specific grain type. The microbial population in the rumen has been reported to adapt to the starch source fed thereby facilitating the *in vitro*

degradation of the substrate and influencing its fermentation parameters.⁹ While previous studies have found that more gas and methane were produced when substrates were incubated in ruminal fluid from cows adapted to the substrate, this did not occur in our study. It is well known that the rumen inoculum is one of key factors that influence *in vitro* fermentations.⁶ However, most previous studies that investigated this issue compared rumen inocula from donor cows fed forage (low starch) vs concentrate (high starch) diets.^{7,9,27,28} Indeed, we could find no previous studies that compared impacts on MP when ruminal fluid inoculum was obtained from donor cows fed two different grain types. This is an important novel feature of our experiment. In this research we found that when donor cows were fed a diet similar to the substrate incubated *in vitro*, the source of ruminal fluid had a substantial effect on methane production. Furthermore, if we had used only one source of ruminal fluid from this experiment measuring wheat grain and corn grain fermentation, the results would have been quite different from the results obtained when using two different sources of ruminal fluid. Therefore, the novel finding of this manuscript is that we have compared the effect of two similar concentrate diets and have found that this also affected MP. In fact, we have made the important novel finding that the source of the donor fluid had a greater effect on MP than did the substrate used in the incubation. This is an indication that the original recommendation that the diet fed to donor animals should be of similar composition to the substrate incubated *in vitro*,⁶ is in some cases not enough to elucidate the different methane productions between two different feeds.

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In our study there was no interaction between type of ruminal fluid used and grain type in the substrate. Furthermore, the 32% greater MP of corn grain incubated in ruminal fluid adapted to that specific grain type, compared with the incubation of wheat in ruminal fluid adapted to that specific grain type, was similar to that observed in *in vivo* experiments, where dairy cows fed corn produced between 30% and 33% more methane than cows fed wheat at the same rate.² This finding shows that the ruminal fluid used affected the *in vitro* fermentation in terms of representing the diet effect, but would have the same effect on any type of grain. However, these results support the need for using ruminal fluid adapted to each specific grain type when analysing MP from wheat and corn *in vitro*, as incubating these grains in the same ruminal fluid would not mimic the differences reported *in vivo*. When screening multiple grains for methane mitigating effects, one approach would be to use ruminal fluid from donor animals fed a mixture of the feeds to be evaluated. However, this approach was beyond the scope of this research and further research would be required to elucidate the effects on methane production of ruminal fluid from donor animals fed a mixture of the feeds evaluated. That the variance in MP, PM and MPgDDM from incubations using ruminal fluid from cows fed the same diet was greater than zero further supports previous statements that ruminal fluid from more than one cow should be used in *in vitro* incubations,⁶ as there can be significant variation in the ruminal fluid of individual cows fed the same diet.

The greater MP in treatments that used ruminal fluid from cows fed a diet that contained corn compared with that of treatments that used ruminal fluid from cows fed a diet that contained wheat, is possibly explained by the greater numbers of protozoa in

the ruminal fluid from cows fed the diet that contained corn compared to the number of protozoa in the ruminal fluid from cows fed the diet that contained wheat. It has been reported that some methanogenic archaea attach to the exterior surface of protozoa and within the ciliates of the protozoa in an endosymbiotic relationship.^{29,30} Approximately 10-20% of the methanogenic archaea in the rumen are attached to protozoa and these contribute approximately 25 to 37% of MP in the rumen.³⁰⁻³² Therefore, the reduction of protozoa in the rumen can decrease MP by 13 to 50%.³³ Similarly, it has been observed that dairy cows fed wheat at a rate of 753 g kg⁻¹ DM had a lower ruminal protozoa population than when the cows were fed corn at 673 g kg⁻¹ DM.¹

In experiment 1, the greater MPgDDM in incubations that used corn as substrate than in treatments that used wheat as substrate is at least partly explained by the lower *in vitro* DDM of corn substrate compared with wheat substrate. The calculation of MPgDDM also involves DDM and in experiment 1 there was no difference between substrates in terms of their MP or their PM. The different DDM between corn grain and wheat grain is related to the structure of the grains, as the starch granules in corn grain, unlike those in wheat grain, are almost completely embedded within a protein matrix, which infers greater protection from enzymatic hydrolysis, thus reducing rumen degradability.^{34,35} The greater DDM observed in the incubation of wheat grain is possibly also associated with the greater GP than that of corn grain. However, as the PM in the corn grain was slightly higher than that of wheat grain, when using PM and GP to calculate MP, this resulted in a lack of effect of grain type on MP.

The main novel finding from this experiment was that it was only when corn and wheat grain were incubated in homologous ruminal fluid, that relative differences in MP were similar to those observed *in vivo*. This finding suggests that when two or more feed ingredients that have different effects on ruminal ecosystems are to be incubated *in vitro*, they should be incubated in ruminal fluid adapted to that specific feed type to better mimic *in vivo* conditions. However, we suggest that further research is required to analyse the effect of various substrates.

Experiment 2

We reject our second hypothesis, that MP would be negatively related to the proportion of grain (i.e. wheat or corn) in the substrate incubated, as MP was negatively related to the proportion of wheat grain in the substrate, but not to the proportion of corn in the substrate. That MP of the treatment that contained wheat at 500 mg g⁻¹ DM was 47% of the treatment that contained the same proportion of corn was similar to previous *in vivo* studies.^{2,3} In a previous *in vivo* study cows were fed diets containing either corn or wheat at 440 g kg⁻¹ dry matter intake, and cows fed wheat had a MP which was 50% of the MP of cows fed corn.³ However, it should be considered that *in vitro* experiments are used to screen substrates for their potential methane mitigation effect, and in that context, all rates used in experiment 2 detected the methane mitigating potential of wheat grain when compared to corn grain. It has been suggested that ruminal fluid for *in vitro* studies should come from donor animals fed a diet similar to the feed to be tested.⁶ This may not always be practical or desirable for the health of the donor animals. For example, cows are rarely fed a diet containing 100% grain, and such a diet might be

expected to result in acidosis or death.³⁶ Nevertheless, we suggest that when screening feed ingredients for their methane mitigating potential, the *in vitro* incubation of a pure substrate, for example a substrate made up 100% of a single ingredient, could nevertheless be carried out in order to determine the methane mitigating potential of that substrate. However, the extent of methane mitigation as determined *in vitro* should not be expected to apply to *in vivo* situations where animals are fed a combination of ingredients.

In our *in vitro* incubations, we used a weak buffer to manifest differences among treatments on pH to reflect the *in vivo* effects of differences between grains and forages.¹⁸ Mean MP and mean post fermentation pH were lower in treatments that incubated wheat substrate than in treatments that incubated corn substrate. Which was consistent with previous reports that have reported a negative relationship between MP and pH *in vitro*³⁷ and *in vivo*.^{2,38,39} It has been reported that low pH is associated with low MP, as the fermentation acids produced in low ruminal fluid pH are considered to be toxic to methanogenic microorganisms.^{35,37} Similar results have been reported for previous *in vivo* studies, where MP was decreased by up to 85% when ruminal fluid pH declined to 5.7³⁸ and where cows fed 10 kg DM wheat day⁻¹ had a greater duration and area of ruminal pH below 6 and also lower methane yield (MP kg⁻¹ dry matter intake) than cows fed the same amount of corn.² This finding is likely caused by the structural differences between the grains as previously discussed, which leads to a faster fermentation of wheat compared to corn and to faster formation of lactate which decreases ruminal fluid pH.⁴⁰⁻⁴²

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The negative relationship between proportion of wheat and MP could also be attributed to the ruminal fluid pH. The treatment that contained wheat grain at a proportion of 500 mg g⁻¹ DM produced 26% less MP than the control treatment and the treatment that contained wheat grain at 1000 mg g⁻¹ DM produced 40% less MP than the control treatment. An increased wheat content in the diet leads to greater starch degradation, which decreases ruminal fluid pH, favours propionate production and inhibits methanogenic function.^{34,43} These results are in accordance with the results of previous *in vivo* research that fed different forage to grain ratios to dairy cows,³⁹ as there was a negative relationship between grain content in the treatments and post-fermentation pH. Similar results have been observed in previous studies where increased proportions of grain in the diet of ruminants decreased methane when the proportion of grain was above 300 mg g⁻¹ DM.⁴¹ However, in our research, this effect did not occur for corn, as there was a negative relationship between proportion of corn and post-fermentation pH and A:P, and a positive relationship between proportion of corn and DDM but no relationship between proportion of corn and MP. A possible explanation for this could be the small decrease in PM with increased proportions of corn grain in the substrate, but a marked increase in GP. As the magnitudes of both PM and GP influence the magnitude of MP, this results in a lack of effect of increasing proportions of corn grain on MP.

CONCLUSIONS

An important novel finding from this research is that using a single source of ruminal fluid to screen two different feed grains (wheat and corn) may provide a

misleading indication as to the methane mitigation potential of these grains. Indeed, our results indicate that to mimic *in vivo* conditions, *in vitro* incubations that compare or screen different feeds for their methane mitigation potential should be performed using ruminal fluid adapted to each specific feed type. Further research is necessary to test this across a wider range of substrates. In addition, *in vitro* MP was reduced when wheat was included in a grain/alfalfa hay substrate, and the magnitude of the reduction was related to the proportion of wheat in the substrate. Inclusion of corn in a grain/alfalfa hay substrate did not reduce MP. These findings suggest that the feeding of wheat to ruminants may be an especially effective strategy to reduce their enteric methane emissions.

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TABLES

Table 1. Dry matter content (g kg ⁻¹ fresh weight) and chemical compositions (g kg ⁻¹ DM) of hay and cereal grains fed to the donor cows and used for <i>in vitro</i> experiments						
Grain	Dry Matter	Crude Protein	ADF	NDF	Ash	Total starch
Alfalfa	902	185	389	476	105	12
Corn	973	103	31	82	12	685

Wheat	975	144	25	136	17	614
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Table 2. Comparison of *in vitro* fermentation parameters obtained when ground corn and ground wheat were incubated in either homologous or non-homologous ruminal fluids in experiment 1

Treatments	MP ¹	PM ²	MPgDDM ³	GP ⁴	DDM ⁵	NH ₃ ⁶	VFA ⁷	A:P ⁸	pH
C-C ⁹	64	197	97	322	659	86	112	3.7 ^b	6.3
C-W ¹⁰	46	147	67	315	705	86	117	2.2 ^a	6.3
W-C ¹¹	64	190	83	334	780	74	125	2.6 ^a	6.1
W-W ¹²	44	134	58	329	795	79	126	2.2 ^a	6.2
SED w/i ¹³									
RF ¹⁴	2.7	9.0	6.2	4.6	28.6	3.8	2.1	0.26	0.03
SED b/w ¹⁵ RF	8.1	24	12	6.7	33.4	4.6	5.5	0.43	0.06
Substrate									
Corn	55	172	82	319	682	86	114	3.0	6.3
Wheat	54	162	70	332	788	77	125	2.4	6.1
SED	1.9	6.3	4.4	3.3	20.2	2.7	1.5	0.18	0.02
RF									
Corn	64	194	90	328	720	80	118	3.2	6.2
Wheat	45	140	62	322	750	83	122	2.2	6.2
SED	7.8	23.0	10.9	5.8	26.6	3.7	5.3	0.39	0.06
<i>P</i> values									
Substrate	0.567	0.157	0.022	0.002	<0.001	0.006	<0.001	0.011	<0.001
RF	0.043	0.045	0.031	0.333	0.292	0.480	0.549	0.040	0.821
Substrate × RF	0.540	0.611	0.512	0.825	0.461	0.262	0.126	0.018	0.270

Cow ¹⁶	0.001	0.001	0.010	0.109	0.313	0.346	<0.001	0.023	0.003
¹ methane production (mL) ² proportion of methane (mL L ⁻¹) ³ methane produced per unit of degraded dry matter (mL g ⁻¹ DDM) ⁴ gas production (GP; mL) ⁵ degraded dry matter degraded (g kg ⁻¹ DM) ⁶ ammonia (mg L ⁻¹) ⁷ total volatile fatty acids (mmol L ⁻¹) ⁸ acetate to propionate ratio ⁹ corn grain substrate in ruminal fluid from cows fed alfalfa hay and corn grain ¹⁰ corn grain substrate in ruminal fluid from cows fed alfalfa hay and wheat grain ¹¹ wheat grain substrate in ruminal fluid from cows fed alfalfa hay and corn grain ¹² wheat grain substrate in ruminal fluid from cows fed alfalfa hay and wheat grain ¹³ Within ¹⁴ Ruminal fluid ¹⁵ Between ¹⁶ Variance components of cows fed the same diet									

Table 3. Total pre-fermentation protozoa count (Log10 count mL⁻¹) and pre-fermentation pH in ruminal fluid of six cows fed a diet containing wheat and six cows fed the diet containing corn used as ruminal fluid donors for experiment 1

Cow	Run	Diet	Ruminal fluid pH	Protozoa
1	1 and 2	Corn	6.08	5.69
2	1 and 2	Corn	5.83	5.00
3	1 and 2	Corn	6.33	5.54
4	3 and 4	Corn	6.34	5.56
5	3 and 4	Corn	6.30	5.45
6	3 and 4	Corn	6.66	5.02
7	1 and 2	Wheat	6.26	4.98
8	1 and 2	Wheat	6.21	5.00
9	1 and 2	Wheat	5.80	4.98
10	3 and 4	Wheat	6.10	5.00
11	3 and 4	Wheat	6.35	5.02
12	3 and 4	Wheat	6.13	4.89
Mean Corn			6.25	5.38
Mean Wheat			6.14	4.98
SED			0.14	0.12
<i>P</i> value			0.424	0.020

Table 4. Comparison of *in vitro* fermentation parameters resulting from the incubation of different proportions of corn and wheat (C and W) in homologous grain type ruminal fluid in experiment 2

RF ¹ -Grain proportion (mg g ⁻¹)	MP ²	PM ³	MPgDDM ⁴	GP ⁵	DDM ⁶	NH ₃ ⁷	VFA ⁸	A:P ⁹	pH
C-C 0	64	227	181	282	353	68	80	3.9	6.73
C-C 250	70	235	145	299	495	56	86	3.2	6.57
C-C 500	74	234	127	318	573	62	94	3.3	6.39
C-C 750	78	236	115	330	581	69	99	2.9	6.23
C-C 1000	69	209	92	332	579	71	100	2.5	6.13
W-W 0	49	170	133	285	375	58	90	3.1	6.43
W-W 250	43	147	96	291	451	62	97	2.8	6.22
W-W 500	35	120	66	301	549	64	101	2.9	6.04
W-W 750	34	110	51	310	673	53	110	2.2	5.87
W-W 1000	29	92	40	315	746	56	115	1.8	5.78
SED corn	5.7	17.3	14	2.9	4.7	22.4	3.4	0.5	0.03
SED wheat	1.9	5.9	14	2.9	4.7	22.4	2.5	0.5	0.03
<i>P</i> (wheat linear)	<0.001	<0.001	<0.001	<0.001	<0.001	0.832	<0.001	0.017	<0.001
<i>P</i> (wheat quadratic)	0.266	0.134	0.105	0.788	0.882	0.823	0.873	0.463	0.010

<i>P</i> (corn linear)	0.220	0.394	<0.001	<0.001	0.001	0.746	<0.001	0.016	<0.001
<i>P</i> (corn quadratic)	0.109	0.212	0.407	0.001	0.021	0.642	0.230	0.860	0.118
Substrate Means									
Corn	71.2	228	132.3	312.1	51.6	65.2	91.9	3.1	6.4
Wheat	38.1	128	77.1	300.6	55.9	58.5	102.7	2.6	6.1
SED	1.9	5.8	13.7	2.9	4.7	22.4	1.3	0.5	0.03
<i>P</i> (Substrate)	<0.001	<0.001	<0.001	<0.001	0.072	0.518	<0.001	0.019	<0.001
<i>P</i> (Proportion)	0.002	<0.001	<0.001	<0.001	<0.001	0.998	<0.001	0.020	<0.001
<i>P</i> (Substrate $\times$ Proportion)	<0.001	0.004	0.886	0.001	0.057	0.943	0.480	0.953	0.655
¹ Ruminal fluid ² methane production (mL) ³ proportion of methane (mL L ⁻¹) ⁴ methane produced per unit of degraded dry matter (mL g ⁻¹ DDM) ⁵ gas production (GP; mL) ⁶ degraded dry matter degraded (g kg ⁻¹ DM) ⁷ ammonia (mg L ⁻¹) ⁸ total volatile fatty acids (mmol L ⁻¹) ⁹ acetate to propionate ratio

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