*Irish Journal of Agricultural and Food Research* **53:** 21–36, 2014

# The effect of water-soluble carbohydrate concentration and type on *in vitro* rumen methane output of perennial ryegrass determined using a 24-hour batch-culture gas production technique

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**The objective of this study was to examine the effects of water-soluble carbohydrate (WSC) concentration and type on the *in vitro* rumen methane ($CH_4$) output of perennial ryegrass (PR) using a 24-hour batch-culture gas production technique. Dried and milled PR was incubated either alone (PR-O) or with added sucrose (PR-S), inulin (PR-I), or sucrose plus inulin (PR-S+I; sucrose:inulin ratio of 1:4) in sealed glass bottles [0.5 g total substrate dry matter (DM) per bottle] at 39 °C for 24 hours with buffered rumen fluid. The WSC types were added (except for PR-O) so that the WSC concentration in each fermentation bottle at the start of the incubation was either 180 (i.e., PR-O), 225, 270, 315, or 360 g/kg of total substrate DM incubated. There were linear decreases in $CH_4$ output per gram of DM disappeared ($CH_4$/*iv*DMD) and per mmol of total volatile fatty acid output ($CH_4$/tVFA) with increasing WSC concentration in the incubated substrate. The WSC type had no effect on *in vitro* rumen $CH_4$ output. It is concluded that since $CH_4$/*iv*DMD and $CH_4$/tVFA were reduced by increasing the concentration of WSC incubated with PR, it would be worthwhile to undertake *in vivo* experiments to examine these effects on *in vivo* $CH_4$ emissions per unit of animal product.**

*Keywords*: *in vitro* rumen; methane; perennial ryegrass; water-soluble carbohydrate

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## Introduction

The increase in the atmospheric concentration of methane ($CH_4$) over the last two centuries has contributed significantly to the concurrent increase in total radiative forcing from greenhouse gases (GHG; Wuebbles and Hayhoe 2002). As a result of international legislation, many countries are obligated to significantly reduce their total GHG emissions, including $CH_4$ emissions from agriculture. In addition to representing a loss of energy from animals' diets, the formation of $CH_4$ during enteric fermentation in ruminant livestock is a significant source of this GHG. For example, approximately 0.30 of total GHG emissions in Ireland are attributed to agriculture (Environmental Protection Agency 2011), and about half of this is enteric $CH_4$ from ruminants (Duffy *et al.* 2011).

Approximately 0.9 of total agricultural land in Ireland is grassland, and intensively grazed grass is the lowest-cost feed available for ruminant production systems (Finneran *et al.* 2012). Within these systems, perennial ryegrass (PR) is the dominant grass species sown, and accounts for 0.95 of grass seed used when reseeding approximately 0.02 of total grassland area annually (Grogan and Gilliland 2011). Thus, any $CH_4$ mitigation strategies must be applicable within the PR-dominated animal production systems that prevail in Ireland and in numerous other countries with temperate climates.

Cultivars of PR that exhibit high concentrations of water-soluble carbohydrate (WSC) can contain a lower concentration of neutral detergent fibre (NDF), and be of greater dry matter (DM) digestibility, compared to PR cultivars of intermediate WSC concentration (Lee *et al.* 2001; Miller *et al.* 2001; Radojevic *et al.* 1994). Such an increase in digestibility may result in a decrease in *in vitro* rumen $CH_4$ production per unit of feed DM digested (Janssen 2010).

Lovett *et al.* (2004) found no difference in *in vitro* rumen $CH_4$ output between what were designated as high and standard WSC PR cultivars. This finding can be explained by the small-scale difference in WSC concentration between these cultivars (i.e., 169 and 158 g WSC/kg DM for the high and standard WSC PR cultivars, respectively). Lee *et al.* (2003) found that increasing the supply of WSC (i.e., sucrose plus inulin) with PR resulted in a linear decrease in the proportion of acetic acid and a linear increase in the proportion of propionic acid in the total volatile fatty acids (VFA) produced, when analysed using the rumen simulation technique (Rusitec). Furthermore, Lee *et al.* (2002) found lower acetic and greater propionic acid proportions in the total VFA in the rumen fluid (RF) of steers fed a high WSC (243 g WSC/kg DM) PR cultivar *versus* a control cultivar of lower WSC concentration (161 g WSC/kg DM). Although $CH_4$ output was not measured in the two latter studies, such a change in the profile of rumen VFA production can decrease the amount of $H_2$, and thus $CH_4$, produced per unit of feed fermented in the rumen (Mills *et al.* 2001; Janssen 2010). Therefore, it was hypothesised that increasing the concentration of WSC incubated with PR would reduce *in vitro* rumen $CH_4$ output.

In Ireland, the majority (~0.70) of the WSC fraction of PR consists of fructan with the remainder being sugars (mainly the disaccharide sucrose and monosaccharides glucose and fructose) (McGrath 1988). However, the proportion of fructan in the WSC fraction of PR can vary considerably throughout the year (e.g., Radojevic *et al.* 1994).

Experiment 1 of Heldt *et al.* (1999) reported a lower proportion of acetic acid

in the total rumen VFA of beef steers fed hay supplemented with a disaccharide (sucrose) compared to those supplemented with monosaccharides (glucose and fructose), and also a lower rumen fluid acetic acid proportion for beef steers fed hay supplemented with sugar (sucrose, glucose and fructose) than with the storage polysaccharide starch. Although $CH_4$ output was not measured in the latter study, such changes in the proportions of the main VFA produced could affect $CH_4$ output through effects on the availability of $H_2$ for methanogenesis. Similar to the role of starch in other forages, fructans act as storage polysaccharides in PR (see review by Chalmers *et al.* 2005). Thus, it was hypothesised that the type of WSC incubated with PR might affect *in vitro* rumen $CH_4$ output due to changes in the *in vitro* rumen VFA profile.

The objective of this study was to examine the effects of WSC concentration (180, 225, 270, 315, and 360 g WSC/kg total substrate DM incubated) and type (added sucrose only, inulin only, or inulin plus sucrose; inulin was the only commercially available fructan) on *in vitro* rumen $CH_4$ output and other *in vitro* rumen fermentation characteristics of PR using a 24-hour batch-culture gas production technique.

## Materials and Methods

All animal procedures used in this study were conducted under experimental license from the Irish Department of Health and Children in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005.

### *Experimental treatments*

The treatments examined in this study comprised a factorial arrangement of "WSC types" and "WSC concentrations". The WSC types examined were PR plus sucrose (PR-S), PR plus inulin (PR-I), or PR plus sucrose plus inulin (PR-S+I). Inulin was used as a source of fructan. The WSC concentrations examined were 180 (PR-O; the basal WSC concentration of the PR samples used where no WSC type was added), 225, 270, 315, or 360 g/kg of total substrate DM incubated.

### *Perennial ryegrass samples*

Perennial ryegrass [*Lolium perenne* (L.), cv. Greengold] was grown in quadruplicate field plots at Grange, Dunsany, Co. Meath, Ireland (53°30'N, 6°40'W; 83 m above sea level). The PR herbage from each plot was harvested to a 50-mm stubble height using a Haldrup plot harvester (Haldrup, Lögstör, Denmark) on 15 November 2011 after 55 days of regrowth. Once collected, the PR samples were stored at –18 °C and subsequently thawed at 4 °C for 24 hours, after which each sample was separately comminuted (Muller MKT 204 Special Food Processor, Saarbrücken, Germany) and thoroughly mixed**.** Representative sub-samples were then taken from each sample, oven-dried at 40 °C for 48 hours, and milled through a sieve with 1-mm apertures prior to chemical composition analyses and *in vitro* rumen incubation. The mean (and standard deviation) NDF, acid detergent fibre (ADF), crude protein (CP) and ash concentrations of the PR [which contained a WSC concentration of 180 (standard deviation 0.9) g/kg DM and had an organic matter (OM) digestibility (OMD) value of 816 (4.8) g/kg] are presented in Table 1. The calculated chemical compositions of the total substrate incubated at each of the remaining WSC concentrations (i.e., 225, 270, 315, and 360 g WSC/kg of total substrate DM incubated) are also presented in Table 1.

**Table 1. The mean (and standard deviation) neutral detergent fibre (NDF), acid detergent fibre (ADF), crude protein (CP), and ash concentrations [g/kg of dry matter (DM)] of the perennial ryegrass substrate only [i.e., a water-soluble carbohydrate (WSC) concentration of 180 g/kg of DM], and the calculated values for total incubated substrate (i.e., perennial ryegrass substrate plus added WSC) at WSC concentrations of 225, 270, 315, or 360 g/kg of total substrate DM incubated**

| | NDF | ADF | CP | Ash |
|---|---|---|---|---|
| WSC concentration | | | | |
| 180 | 470 (2.5) | 242 (1.7) | 217 (6.8) | 111 (0.6) |
| 225 | 443 | 228 | 205 | 105 |
| 270 | 417 | 215 | 193 | 98 |
| 315 | 390 | 201 | 180 | 92 |
| 360 | 364 | 187 | 168 | 86 |

*Rumen microbial inoculum*

Four rumen-fistulated steers [682 (standard deviation: 50.2) kg mean live weight] were individually offered a grass silage plus concentrate diet (60:40 grass silage:concentrate on a DM basis) *ad libitum* for several weeks prior to RF collection, and switched to a restricted allowance (0.9 of *ad libitum* intake) three days prior to RF collection. The composition of the concentrate was 830 g rolled barley, 100 g soyabean meal, 50 g molasses and 20 g mineral plus vitamin (premix) per kilogram of fresh weight. Each animal had continuous access to fresh drinking water. Representative solid-phase rumen digesta samples were obtained from different parts of the rumen of each animal one hour prior to feeding and squeezed through a sieve with 1-mm apertures. The filtrate (i.e., RF) was then pooled across animals on an equal volume basis and under a constant stream of carbon dioxide ($CO_2$). This RF was then strained through four layers of cheesecloth.

In vitro *rumen incubation*

The *in vitro* rumen batch-culture gas production technique of Navarro-Villa *et al.* (2011) and Purcell *et al.* (2011) was employed in this study. The study was undertaken in a single *in vitro* rumen incubation, where replication (×4) was provided by the PR field plots. Half of a gram (0.5 g) of PR DM only (PR-O) was added to *in vitro* rumen fermentation bottles. This PR-O contained a WSC concentration of 180 g/kg total substrate DM. For the remaining WSC concentrations either PR-S (sucrose = 99.5%; Sigma-Aldrich Co., St. Louis, MO, USA), PR-I (inulin from dahlia tubers; Sigma-Aldrich Co.) or PR-S+I in a sucrose:inulin ratio of 1:4 as per Uden (2006) and Lee *et al.* (2003) were added. These WSC types were added so that the total WSC concentration (i.e., the basal WSC concentration of the PR of 180 g/kg plus the added WSC types) in the fermentation bottles at the start of the incubation was either 225, 270, 315, or 360 g/kg total substrate DM incubated. The appropriate amount of PR DM and WSC were added to each bottle so that there was a total of 0.5 g of total substrate DM of the appropriate WSC concentration and type in each bottle. This allowed the evaluation of the effects of WSC concentration of PR *per se* on *in vitro* rumen $CH_4$ output. In the case of PR-O, there were three groups of four replicates, one for each of the PR-S, PR-I, and PR-S+I WSC type treatments. Thus, 60 fermentation bottles [i.e., 5 (WSC concentrations) × 3 (WSC types) × 4 (replicates)] were incubated (excluding blank fermentation bottles). Buffered mineral solution (i.e., artificial saliva) was prepared according to McDougall (1948). The RF was then

added to the buffered mineral solution at a ratio of 1:4 (RF: buffered mineral solution) and maintained at 39 °C and under a constant stream of $CO_2$ at all times to ensure anaerobic conditions. When the pH of the buffered RF stabilized at 6.85, 50 mL was dispensed into each fermentation bottle, thereby resulting in 10 mg of total substrate DM per mL of buffered RF and an effective fermentation bottle headspace volume of ~110 mL. The former value is between those of Newbold *et al.* (2005; 8 mg DM/mL) and Bodas *et al.* (2008; 11 mg DM/mL), where accumulated gas pressures were also measured after 24 hours of *in vitro* rumen incubation. However, the effective headspace volume in this study is greater than the 70 mL of Bodas *et al.* (2008) and Newbold *et al.* (2005). Blank fermentation bottles containing 50 mL of buffered RF only were also incubated to correct total gas production (TGP), $CH_4$ output, and *in vitro* DM disappearance (*iv*DMD) for any direct contribution from the buffered RF only. The gas headspace in each inoculated fermentation bottle was thoroughly flushed with $CO_2$, and the bottles were immediately sealed with butyl rubber stoppers, crimped with aluminium caps, and incubated *in vitro* at 39 °C for 24 hours. Based on the assumption of Mould *et al*. (2005) that 25 mg of N is required to degrade 1.0 g of carbohydrate, it was calculated that the amount of N in the strained RF and incubated forage supplied sufficient N and that N was not a limiting factor for microbial growth during the fermentation. After the 24 hour *in vitro* rumen incubation, the pressure in the gas headspace of each fermentation bottle was measured using a pressure transducer (Tracker 220, Gems Sensors and Controls, Basingstoke, UK), and the volume of gas produced in each bottle was determined using the equation of Mauricio *et al.* (1999):

$$TGP\,(mL) = \left(\frac{bottle\ headspace\ volume\ [mL]}{atmospheric\ pressure\ [hPa]}\right) \times bottle\ headspace\ pressure\ (hPa)$$

The mL of TGP were then converted to mmol using the ideal gas law, which allowed the calculation of the volume of 1 mmol of gas at 39 °C under the atmospheric pressure at the time of gas sampling:

$$Volume\ (V) = \frac{no.\ moles\ (n) \times the\ ideal\ gas\ constant\ (R) \times temperature\ (T;\ 39\ °C)}{pressure\ (P;\ atmospheric\ pressure)}$$

Following measurement of the fermentation bottle headspace pressures, a 0.8 mL gas sample was extracted from each fermentation bottle using a gas-tight syringe (1 mL capacity) and a hypodermic needle (25 gauge), and transferred to pre-evacuated chromatography vials (2 mL capacity) with silicon-teflon septums (8 mm; Sun-Sri, Rockwood, TN, USA; supplied by Labquip Ireland Ltd.).

The $CH_4$ concentration was determined using an automated gas chromatograph (Shimadzu GC-17A) with a flame ionisation detector and equipped with a Chrompack column (2.4 m length × 5.0 mm external diameter × 3.4 mm internal diameter; glass column). Temperatures were 150 °C in the column, 150 °C in the injector, and 180 °C in the detector. The carrier gas was N and the gas samples were injected on-column. Triplicates of gas standards of known $CH_4$ proportion (0.05, 0.10 and 0.15; Air Products, Dublin, Ireland) were used to determine $CH_4$ output. The total mL of $CH_4$ output in each bottle was then calculated using the equation outlined in Lopez *et al.* (2007):

$$CH_4(mL) = \frac{\left(TGP\,[mL] + bottle\ volume\,[160\ mL] - buffered\ RF\ volume\,[50\ mL]\right) \times \%\ CH_4}{100}$$

The mL of $CH_4$ were converted to mmol using the ideal gas law as described for TGP. As a result of the extensive flushing of both the buffered RF and the *in vitro* rumen fermentation bottle headspace with $CO_2$ until the bottles were sealed, and considering the low solubility of $CH_4$ in $H_2O$ (Windholz *et al.* 1976) and thus in the fermentation medium, it was considered that the gas sample collected from the bottle headspace at the end of the 24 hour *in vitro* incubation was representative of the $CH_4$ produced during the fermentation. Following gas sampling, the remaining accumulated gas pressure inside each of the fermentation bottles was released, and the bottles were stored at 4 °C to halt fermentation. A 0.8 mL sample of the post-incubation fermentation medium was then obtained from each bottle, aliquoted into 2 mL Eppendorf tubes containing 20 $\mu$L of a 9M $H_2SO_4$ solution, and stored at –18 °C for subsequent VFA, ammonia ($NH_3$), and lactic acid analyses. The contents of each fermentation bottle were filtered through sintered Pyrex glass crucibles (porosity number 1), after which the crucibles containing the fermentation residue were oven-dried at 98 °C for 48 hours and weighed. The *iv*DMD (g/g) was then calculated using the equation:

$$ivDMD\,(g/g) = \frac{\left(crucible\ \ [g]\ +\ DM\ incubated\ \ [g]\right) - dried\ \ crucible\ \ and\ \ residue\ \ (g)}{DM\ incubated\ \ (g)}$$

The dried residue in each crucible was then analysed for its NDF concentration, after which NDF disappeared (NDFD; g/g) for each fermentation bottle was calculated using the equation:

$$aNDFd\,(g\ g^{-1}) = \frac{NDF\ incubated\,(g) - NDF\ in\ the\ fermentation\ residue\,(\text{g; } post\text{-}incubation)}{NDF\ incubated\,(g)}$$

*Herbage chemical composition and* in vitro *rumen VFA, ammonia, and lactic acid analyses*

*In vitro* OMD of the dried, milled PR samples was determined using the Tilley and Terry (1963) technique, where the final residue was isolated by filtration (Whatman GF/A 55 mm, pore size 1.6 $\mu$m, Whatman International, Maidstone, UK) rather than centrifugation. Both ADF (expressed exclusive of residual ash) and NDF (assayed with a heat stable amylase and sodium sulphite, and expressed exclusive of residual ash) were analysed using filter bag techniques (ANKOM 2006a,b). Ash concentration was determined by complete combustion in a muffle furnace at 550 °C for 5 hours. The CP (calculated as N × 6.25) concentration was determined using a Leco FP 528 N analyser based on method 990-03 of the AOAC (1990). The WSC concentration was determined using the anthrone method (Thomas 1977).

The VFA [acetic, propionic, butyric (n-butyric + iso-butyric) and valeric (n-valeric + iso-valeric) acids] concentrations of the post-incubation fermentation medium of each bottle were determined using gas chromatography (as for $CH_4$) as described by Ranfft (1973). The total lactic acid concentration was determined using the ACE Alera Clinical Chemical Analyser (Alfa Wassermann, NJ, USA) and the UV-method test kit (Roche/R-Biopharm, Darmstadt, Germany), with

D-lactic acid and L-lactic acid determined using the enzymes D-lactate dehydrogenase and L-lactate dehydrogenase, respectively. Concentrations of $NH_3$ were determined using the ACE Alera Clinical Chemical Analyser and the Thermo Electron Infinity $NH_3$ liquid stable reagent kinetic method (Thermo Fisher Scientific Inc., Middletown, VA, USA).

*Statistical analysis*

Data for WSC concentration were subjected to analysis of variance using a model that accounted for WSC concentration (180, 225, 270, 315, and 360 g WSC/kg total substrate DM incubated). Each of the four independent PR field replicates was incubated with each treatment combination [including each of the three PR-O (i.e., 180 g WSC/kg of total substrate DM incubated) treatment combinations], where the experimental unit was therefore the *in vitro* rumen fermentation bottle. This meant that the PR-O treatment was incubated in a total of 12 fermentation bottles. Linear and quadratic effects of WSC concentration were tested for all variables. Data for WSC type were analysed as a 3 (WSC type) × 4 (WSC concentration: 225, 270, 315, and 360 g WSC/kg total substrate DM incubated) factorial arrangement of treatments. The model included WSC type, WSC concentration and WSC type × WSC concentration. Only the WSC-type and WSC type × WSC concentration results are stated and discussed from the latter model. Differences among the individual WSC types, WSC concentrations, or WSC type × WSC concentration combinations were tested using Tukey-adjusted comparisons where these factors were significant in the models. All data were analysed using GenStat ($16^{th}$ edition).

## Results

In vitro *rumen methane output*

*Water-soluble carbohydrate concentration*. There were no effects ($P>0.05$) of WSC concentration on the mmol of $CH_4$ output per gram of total substrate DM incubated (DMi) ($CH_4$/DMi), per gram of OM incubated ($CH_4$/OMi), per gram of *iv*DMD ($CH_4$/*iv*DMD), per mmol of TGP ($CH_4$/TGP), or per mmol of tVFA ($CH_4$/tVFA). There were linear decreases ($P<0.05$) in $CH_4$/*iv*DMD and $CH_4$/tVFA with increasing WSC concentration of the incubated substrate (Table 2). There were no quadratic effects of WSC concentration on any of the aforementioned $CH_4$ output variables ($P>0.05$; Table 2).

*Water-soluble carbohydrate type*. The WSC type had no effect ($P>0.05$) on $CH_4$/DMi, $CH_4$/OMi, $CH_4$/*iv*DMD, $CH_4$/TGP, or $CH_4$/tVFA. There was a tendency ($P<0.1$) towards significance for $CH_4$/*iv*DMD, but there were no differences between the WSC types when contrasted using Tukey-adjusted comparisons (Table 3). There were no WSC concentration × WSC type interactions for any *in vitro* rumen $CH_4$ output variables ($P>0.05$; Table 3).

*Other* in vitro *rumen fermentation variables*

*Water-soluble carbohydrate concentration*. There were linear increases in TGP/DMi ($P<0.01$), tVFA/DMi ($P<0.05$) and *iv*DMD ($P<0.001$), and linear decreases in grams of NDF disappeared per gram of DM disappeared (NDFD/*iv*DMD; $P<0.001$), pH ($P<0.001$) and mmol of ammonia per gram of total substrate DM incubated ($NH_3$/DMi; $P<0.001$), as the WSC concentration of incubated substrate increased (Table 4). There was no effect ($P>0.05$) of WSC concentration on TGP/OMi [mean values across WSC concentrations of 7.98 (s.e. 0.187) mmol/g], tVFA/

**Table 2. Effect of water-soluble carbohydrate (WSC) concentration [180, 225, 270, 315, or 360 g/kg of total substrate dry matter (DM) incubated] on *in vitro* rumen methane ($CH_4$) output from perennial ryegrass after 24 hours of batch-culture incubation**

| | $CH_4$/DMi | $CH_4$/OMi | $CH_4$/*iv*DMD | $CH_4$/TGP | $CH_4$/tVFA |
|---|---|---|---|---|---|
| WSC concentration | | | | | |
| 180 | 1.09 | 1.23 | 1.42 | 0.154 | 0.214 |
| 225 | 1.07 | 1.19 | 1.37 | 0.150 | 0.206 |
| 270 | 1.09 | 1.19 | 1.37 | 0.149 | 0.202 |
| 315 | 1.07 | 1.16 | 1.32 | 0.147 | 0.196 |
| 360 | 1.02 | 1.09 | 1.22 | 0.139 | 0.181 |
| s.e. | 0.046 | 0.050 | 0.060 | 0.0056 | 0.0094 |
| Significance | | | | | |
| WSC concentration | | | | | |
| Linear | | | * | | * |
| Quadratic | | | | | |

$CH_4$/DMi = mmol of $CH_4$ output per gram of total substrate DM incubated; $CH_4$/OMi = mmol of $CH_4$ output per gram of total substrate organic matter incubated.
$CH_4$/*iv*DMD = mmol of $CH_4$ output per gram of DM disappeared.
$CH_4$/TGP = mmol of $CH_4$ output per mmol of total gas produced.
$CH_4$/tVFA = mmol of $CH_4$ output per mmol of total volatile fatty acids.

OMi or NDFD. There were no effects (P>0.05) of WSC concentration on the mmol of total lactic acid (D-lactic acid + L-lactic acid) per gram of DMi [tLAC/DMi; mean value across WSC concentrations of 0.032 (s.e. 0.0052) mmol/g], mmol of total lactic acid per gram of OMi [tLAC/OMi; 0.034 (0.0050) mmol/g], or the proportion of D-lactic acid in the total lactic acid [854 (10.4) mmol/mol of total lactic acid]. There were no quadratic effects of WSC concentration on any of the aforementioned *in vitro* rumen fermentation variables (P>0.05).

*Water-soluble carbohydrate type*. The WSC type had no effect on any of the aforementioned *in vitro* rumen fermentation

**Table 3. Effect of water-soluble carbohydrate (WSC) type [perennial ryegrass plus sucrose (PR-S), inulin (PR-I), or sucrose plus inulin (PR-S+I)] on *in vitro* rumen methane ($CH_4$) output from perennial ryegrass after 24 hours of batch-culture incubation**

| | $CH_4$/DMi | $CH_4$/OMi | $CH_4$/*iv*DMD | $CH_4$/TGP | $CH_4$/tVFA |
|---|---|---|---|---|---|
| WSC type | | | | | |
| PR-S | 1.10 | 1.20 | 1.38[a] | 0.151 | 0.202 |
| PR-I | 0.99 | 1.08 | 1.22[a] | 0.139 | 0.184 |
| PR-S+I | 1.09 | 1.18 | 1.35[a] | 0.148 | 0.202 |
| s.e. | 0.042 | 0.045 | 0.054 | 0.0052 | 0.0081 |
| Significance | | | † | | |
| WSC concentration × WSC type | | | | | |
| s.e. | 0.083 | 0.091 | 0.107 | 0.0104 | 0.0163 |
| Significance | | | | | |

[a]Means within a column with common superscripts do not differ (P>0.05).
† = P<0.1
$CH_4$/DMi = mmol of $CH_4$ output per gram of total substrate DM incubated.
$CH_4$/OMi = mmol of $CH_4$ output per gram of total substrate organic matter incubated.
$CH_4$/*iv*DMD = mmol of $CH_4$ output per gram of DM disappeared.
$CH_4$/TGP = mmol of $CH_4$ output per mmol of total gas produced.
$CH_4$/tVFA = mmol of $CH_4$ output per mmol of total volatile fatty acids.

**Table 4. Effect of water-soluble carbohydrate (WSC) concentration [180, 225, 270, 315, or 360 g/kg of total substrate dry matter (DM) incubated] on other *in vitro* rumen fermentation characteristics of perennial ryegrass after 24 hours of batch-culture incubation**

| | TGP/DMi | tVFA/DMi | tVFA/OMi | *iv*DMD | NDFD | NDFD/*iv*DMD | pH | $NH_3$/DMi | $NH_3$/OMi |
|---|---|---|---|---|---|---|---|---|---|
| WSC concentration | | | | | | | | | |
| 180 | 7.10 | 5.24 | 5.89 | 0.771 | 0.763 | 0.466 | 6.42 | 0.726 | 0.817 |
| 225 | 7.13 | 5.20 | 5.78 | 0.784 | 0.762 | 0.433 | 6.41 | 0.715 | 0.794 |
| 270 | 7.30 | 5.39 | 5.91 | 0.799 | 0.767 | 0.403 | 6.39 | 0.630 | 0.691 |
| 315 | 7.26 | 5.45 | 5.91 | 0.811 | 0.762 | 0.371 | 6.39 | 0.547 | 0.593 |
| 360 | 7.59 | 5.63 | 6.04 | 0.835 | 0.760 | 0.336 | 6.38 | 0.525 | 0.564 |
| s.e. | 0.10 | 0.111 | 0.123 | 0.0089 | 0.0085 | 0.0043 | 0.010 | 0.0299 | 0.0333 |
| Significance | | | | | | | | | |
| WSC concentration | * | † | | *** | | *** | * | *** | *** |
| Linear | ** | * | | *** | | *** | *** | *** | *** |
| Quadratic | | | | | | | | | |

† = $P<0.1$.
TGP/DMi = mmol of total gas produced per gram of total substrate DM incubated.
tVFA/DMi = mmol of total volatile fatty acids per gram of total substrate DM incubated.
tVFA/OMi = mmol of total volatile fatty acids per gram of total substrate organic matter (OM) incubated.
*iv*DMD = grams of DM disappeared per gram of DM incubated.
NDFD = grams of neutral detergent fibre (NDF) disappeared per gram of total substrate NDF incubated.
NDFD/*iv*DMD = grams of NDF disappeared per gram of DM disappeared.
$NH_3$/DMi = mmol of ammonia per gram of total substrate DM incubated.
$NH_3$/OMi = mmol of ammonia per gram of total substrate OM incubated.

variables (P>0.05; Table 5). There were no WSC concentration × WSC type interactions for any *in vitro* rumen fermentation variables (P>0.05), except *iv*DMD (P<0.05; Table 5).

*Volatile fatty acids*

*Water-soluble carbohydrate concentration*. There were linear decreases (P<0.001) in the non-glucogenic VFA ratio (NGGR), the acetic acid to propionic acid ratio (A:P), and the acetic acid proportion, and linear increases (P<0.001) in the proportions of propionic and butyric acids, as the WSC concentration in the incubated substrate increased. There was no effect of WSC concentration on the proportion of valeric acid (P>0.05; Table 6). There were no quadratic effects of WSC concentration on any of the aforementioned *in vitro* rumen VFA variables (P>0.05; Table 6).

*Water-soluble carbohydrate type*. The WSC type had no effect (P>0.05) on the NGGR, or the proportions of acetic, butyric, or valeric acids in the tVFA (Table 7). However, PR-I had a greater (P<0.05) A:P ratio, and a lower proportion of propionic acid in the total VFA concentration, than PR-S (Table 7).

There were no WSC concentration × WSC type interactions for any *in vitro* rumen VFA proportion variables (P>0.05; Table 4), except for the proportion of propionic acid in the tVFA concentration (P<0.05; Table 7).

## Discussion

The polysaccharide inulin and the disaccharide sucrose were added as the WSC types in this study to provide sources of fructan and sugars, respectively, for the *in vitro* rumen fermentation. The ratio of sucrose to inulin (1:4) added to the PR substrate is similar to the ratio of fructan to sugars typically found in PR. For example, Lattanzi *et al.* (2012) found a mean fructan proportion of 0.82 in the total WSC fraction of PR leaves, with the remainder consisting of sugars (i.e., sucrose, fructose, and glucose). Similarly, Lee *et al.* (2003) examined PR for which 0.80 of the WSC fraction was fructan and 0.20 consisted of monosaccharides and disaccharides.

The mean basal WSC concentration of the grass herbage examined in this study (i.e., 180 g/kg total substrate DM) is similar to that of the diets examined in the study of Ellis *et al.* (2011; range of mean WSC concentration values across diets of 179 g/kg of DM). In addition, the 360 g WSC/kg total substrate DM treatment examined in this study was generally similar to the "basal × 1.75" treatment of Lee *et al.* (2003; corresponding value of approximately 350 g WSC/kg of total substrate DM incubated). However, the basal WSC concentration of the PR herbage (180 g/kg of DM) used in this study was lower than that of Lee *et al.* (2003; basal concentration of 250 g/kg of DM).

The overall range of mean *in vitro* rumen TGP/DMi (178 to 189 mL/g of DM incubated) and TGP/OMi (201 to 204 mL/g of OM incubated) values across WSC types and concentrations in this study were generally similar to those reported for PR by Purcell *et al.* (2012b; 135 to 180 mL/g DM incubated), Navarro-Villa *et al.* (2011; 161 to 178 mL/g of DM incubated), and Lovett *et al.* (2004; 206 to 237 mL/g of OM incubated), all of whom measured accumulated gas pressure after 24 hours of *in vitro* rumen incubation.

Overall, the effects of WSC concentration × WSC type on *iv*DMD and the proportion of propionic acid in the tVFA generally did not contradict the overall main effect of WSC concentration

**Table 5. Effect of water-soluble carbohydrate (WSC) type [perennial ryegrass plus sucrose (PR-S), inulin (PR-I), or sucrose plus inulin (PR-S+I)] on other *in vitro* rumen fermentation characteristics of perennial ryegrass after 24 hours of batch-culture incubation**

| | TGP/DMi | tVFA/DMi | tVFA/OMi | *iv*DMD | NDFD | NDFD/*iv*DMD | pH | $NH_3$/DMi | $NH_3$/OMi |
|---|---|---|---|---|---|---|---|---|---|
| WSC type | | | | | | | | | |
| PR-S | 7.32 | 5.49 | 5.98 | 0.798 | 0.753 | 0.385 | 6.39 | 0.617 | 0.674 |
| PR-I | 7.28 | 5.40 | 5.89 | 0.816 | 0.769 | 0.386 | 6.39 | 0.597 | 0.656 |
| PR-S+I | 7.35 | 5.37 | 5.85 | 0.808 | 0.766 | 0.387 | 6.39 | 0.595 | 0.652 |
| s.e. | 0.077 | 0.062 | 0.067 | 0.0064 | 0.0083 | 0.0031 | 0.008 | 0.0179 | 0.0196 |
| Significance | | | | | | | | | |
| WSC concentration × WSC type | | | | | | | | | |
| s.e. | 0.153 | 0.124 | 0.134 | 0.0128 | 0.0166 | 0.0063 | 0.016 | 0.0357 | 0.039 |
| Significance | | | | * | | | | | |

TGP/DMi = mmol of total gas produced per gram of total substrate DM incubated.
tVFA/DMi = mmol of total volatile fatty acids per gram of total substrate DM incubated.
tVFA/OMi = mmol of total volatile fatty acids per gram of total substrate organic matter (OM) incubated.
*iv*DMD = grams of DM disappeared per gram of DM incubated.
NDFD = grams of neutral detergent fibre (NDF) disappeared per gram of total substrate NDF incubated.
NDFD/*iv*DMD = grams of NDF disappeared per gram of DM disappeared.
$NH_3$/DMi = mmol of ammonia per gram of total substrate DM incubated.
$NH_3$/OMi = mmol of ammonia per gram of total substrate OM incubated.

**Table 6. Effect of water-soluble carbohydrate (WSC) concentration [180, 225, 270, 315, or 360 g/kg total substrate dry matter (DM) incubated] on the proportions of volatile fatty acids (VFA) after 24 hours of batch-culture incubation of perennial ryegrass**

| | NGGR | A:P | Acetic | Propionic | Butyric | Valeric |
|---|---|---|---|---|---|---|
| WSC concentration | | | | | | |
| 180 | 3.46 | 2.59 | 622 | 240 | 104 | 33.7 |
| 225 | 3.40 | 2.53 | 619 | 244 | 106 | 30.9 |
| 270 | 3.34 | 2.47 | 611 | 248 | 108 | 34.9 |
| 315 | 3.23 | 2.42 | 606 | 251 | 109 | 34.3 |
| 360 | 3.28 | 2.39 | 607 | 254 | 113 | 28.9 |
| s.e. | 0.043 | 0.024 | 3.32 | 1.5 | 1.0 | 2.0 |
| Significance | | | | | | |
| WSC concentration | ** | *** | ** | *** | *** | |
| Linear | *** | *** | *** | *** | *** | |
| Quadratic | | | | | | |

NGGR = non-glucogenic to glucogenic VFA ratio [(acetic acid + (butyric acid × 2)]/propionic acid).
A:P = acetic acid to propionic acid ratio.
Acetic = acetic acid proportion (mmol/mol of total VFA).
Propionic = propionic acid proportion (mmol/mol of total VFA).
Butyric = butyric acid proportion (mmol/mol of total VFA).
Valeric = valeric acid proportion (mmol/mol of total VFA).

**Table 7. Effect of water-soluble carbohydrate (WSC) type [perennial ryegrass plus sucrose (PR-S), inulin (PR-I), or sucrose plus inulin (PR-S+I)] on the proportions of volatile fatty acids (VFA) after 24 hours of batch-culture incubation of perennial ryegrass**

| | NGGR | A:P | Acetic | Propionic | Butyric | Valeric |
|---|---|---|---|---|---|---|
| WSC type | | | | | | |
| PR-S | 3.30 | 2.42$^{b}$ | 608 | 252$^{a}$ | 110 | 32.4 |
| PR-I | 3.38 | 2.49$^{a}$ | 613 | 246$^{b}$ | 109 | 31.5 |
| PR-S+I | 3.27 | 2.45$^{ab}$ | 611 | 250$^{ab}$ | 108 | 32.9 |
| s.e. | 0.039 | 0.021 | 3.3 | 1.1 | 0.9 | 1.93 |
| Significance | | * | | ** | | |
| WSC concentration × WSC type | | | | | | |
| s.e. | 0.077 | 0.041 | 6.5 | 2.2 | 1.8 | 3.87 |
| Significance | | | | * | | |

$^{a-b}$Means within a column with common superscripts do not differ (P>0.05).
NGGR = non-glucogenic to glucogenic VFA ratio [(acetic acid + (butyric acid × 2)]/propionic acid).
A:P = acetic acid to propionic acid ratio.
Acetic = acetic acid proportion (mmol/mol of total VFA).
Propionic = propionic acid proportion (mmol/mol of total VFA).
Butyric = butyric acid proportion (mmol/mol of total VFA).
Valeric = valeric acid proportion (mmol/mol of total VFA).

(Table 8). Therefore, the overall effects of WSC concentration on both variables are discussed.

*Water-soluble carbohydrate concentration*

Overall, increasing the WSC concentration in the substrate at the start of the incubation [where the quantity of total substrate DM incubated was the same (i.e., 0.5 g) for all treatments] resulted in an expected subsequent increase in the extent of the *in vitro* rumen fermentation. The latter was evidenced by the linear increases in *in vitro* TGP/DMi, total

**Table 8. Significant two-way interactions between water-soluble carbohydrate (WSC) concentration [225, 270, 315, or 360 g/kg total substrate dry matter (DM) incubated] and type [sucrose (PR-S), inulin (PR-I), and sucrose plus inulin (PR-S+I)] on *in vitro* rumen DM disappearance (*iv*DMD) and the proportion of propionic acid in the total volatile fatty acids (mmol/mol) after 24 hours of batch-culture incubation of perennial ryegrass**

| | *iv*DMD | Propionic acid |
|---|---|---|
| PR-S | | |
| 225 | 0.794$^{abc}$ | 245$^{bc}$ |
| 270 | 0.766$^{c}$ | 246$^{bc}$ |
| 315 | 0.798$^{abc}$ | 251$^{abc}$ |
| 360 | 0.837$^{ab}$ | 258$^{a}$ |
| PR-I | | |
| 225 | 0.773$^{bc}$ | 242$^{c}$ |
| 270 | 0.820$^{abc}$ | 245$^{bc}$ |
| 315 | 0.842$^{a}$ | 252$^{abc}$ |
| 360 | 0.828$^{abc}$ | 246$^{bc}$ |
| PR-S+I | | |
| 225 | 0.784$^{abc}$ | 246$^{bc}$ |
| 270 | 0.812$^{abc}$ | 253$^{ab}$ |
| 315 | 0.792$^{abc}$ | 249$^{abc}$ |
| 360 | 0.845$^{a}$ | 258$^{a}$ |
| s.e. | 0.0128 | 2.2 |
| Significance | * | * |

$^{a-c}$Means within a column with common superscripts do not differ (P>0.05).

VFA (/DMi) and *iv*DMD with increased WSC concentration of incubated substrate. This outcome, which most likely reflects the extensive degradation and microbial utilisation of the WSC added, is in agreement with Umucalilar *et al.* (2010), who found a linear increase in *in vitro* rumen TGP (mL) with increasing addition of inulin to a forage plus concentrate feed.

When the WSC concentration of the incubated substrate was increased, there was a change in the direction of the *in vitro* rumen fermentation (i.e., the relative proportions of the main VFA produced), as indicated by the overall linear decrease in NGGR. These findings are in general agreement with Lee *et al.* (2002) who found steers grazing a PR cultivar containing a greater WSC concentration (243 *versus* 161 g WSC/kg of DM) to have a lower acetic acid (620 *versus* 650 mmol/mol) and a greater propionic acid (230 *versus* 200 mmol/mol) molar proportion in total *in vivo* rumen VFA compared to a control PR cultivar. The less methanogenic VFA profile with increasing WSC concentration is also in accord with the *in vitro* findings of Lee *et al.* (2003), who found a linear decrease in the proportion of acetic acid (570 to 430 mmol/mol) and a linear increase in propionic acid (260 to 350 mmol/mol) in the total VFA of PR due to comparable extents of WSC addition as was undertaken in the current experiment.

The aforementioned less methanogenic VFA profile with increasing pre-incubation WSC concentration of substrate most likely resulted in a decrease in the total amount (i.e., mmol) of $H_2$ produced per unit of total substrate digested during the incubation (Mills *et al.* 2001; Janssen 2010). Conversely, the aforementioned increase in the extent of the *in vitro* rumen fermentation with increased WSC concentration would be expected to result in an increase in the total amount of $H_2$ produced. Rumen methanogenic Archaea use $H_2$ to reduce metabolic $CO_2$ to $CH_4$ (Hungate 1967; Janssen and Kirs 2008). It is thus likely that the lack of an effect of WSC concentration on $CH_4$/DMi in the current experiment was due to the increased extent of the fermentation being counteracted by the less methanogenic direction of fermentation with increasing WSC concentration of incubated substrate. This outcome is in accord with the similar finding of Purcell *et al.* (2012a). When expressed relative to *iv*DMD ($CH_4$/*iv*DMD), the less methanogenic direction of fermentation contributed to the observed linear decrease in $CH_4$ output with increasing WSC concentration of incubated substrate.

In contrast to the lack of effect of WSC concentration on NDFD in the current study, Lee *et al.* (2003) found a decrease in *in vitro* NDF digestion (g/kg of DM incubated) of PR with increasing WSC availability due to a concurrent decrease in pH from 6.4 to 6.0. This apparent discrepancy in the findings for *in vitro* NDF digestibility between this study and Lee *et al.* (2003) most likely reflects the generally greater pH values in the current study for all WSC concentrations (range of mean pH values of 6.38 to 6.42) compared to those of Lee *et al.* (2003; pH range of 6.0 to 6.4). These relatively greater pH values in this study, which reflect the strongly buffered medium in the *in vitro* rumen fermentation bottles, were most likely not sufficiently low (i.e., < 6.2) to negatively impact on the rate of NDF disappearance (Grant and Mertens 1992; Grant and Weidner 1992), as occurred in the study of Lee *et al.* (2003).

Overall, the range of mean NDFD values across WSC concentrations in this study (0.760 to 0.767 g NDF disappeared/g NDF incubated) were within the range of mean *in sacco* rumen NDF degradability results of 0.61 to 0.80 for three PR cultivars reported by Sun *et al.* (2010). These similarly high NDFD values across the WSC concentrations in this study indicate that the NDF in the incubated substrate was extensively degraded during the *in vitro* rumen fermentation for all WSC concentrations. The linear decrease in the pH of the *in vitro* fermentation medium with increasing WSC concentration of the incubated substrate is in accord with the lower rumen fluid pH in steers fed a high WSC *versus* a control PR cultivar reported by Lee *et al.* (2002), and reflected the simultaneous linear increase in tVFA (/DMi and /OMi) concentration in this study. However, the overall range of mean pH values across WSC concentrations was small.

*Water-soluble carbohydrate type*

Overall, WSC type had a small-scale effect on both the extent and the direction of the *in vitro* rumen fermentation, as evidenced by the lack of effects on all *in vitro* rumen fermentation characteristics, and the lack of or small-scale effects on *in vitro* rumen VFA proportion variables. Considering this, the lack of effects of WSC type on all $CH_4$ output variables was in line with expectation.

## Conclusions

Increasing the WSC concentration of PR using either sucrose, inulin, or sucrose plus inulin decreased $CH_4$/*iv*DMD and $CH_4$/tVFA. Overall, doubling the WSC concentration in the total substrate from 180 to 360 g/kg of DM resulted in decreases of 0.14 and 0.15, respectively, for these variables. This most likely reflected the less methanogenic direction of the fermentation with increasing WSC concentration, resulting in a decrease in $H_2$ production per unit of total substrate fermented *in vitro*. *In vitro* rumen $CH_4$ output was not affected by the WSC type incubated with PR, which was in general agreement with the findings for other *in vitro* rumen fermentation variables. The results obtained in this study suggest that it would be worthwhile to undertake *in vivo* animal nutrition experiments to examine the effects of elevating the WSC concentration of PR on $CH_4$ emissions per unit of animal product.

**Acknowledgements**

Funding for this study was provided under the National Development Plan through the Research Stimulus Fund administered by the Department of Agriculture, Food & the Marine (RSF no. 07 517). The authors thank Belynda Weldon and Gabriel Costello for assistance with sample collection and processing, and Grange Laboratories staff for assistance with chemical analyses.

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*Received 8 November 2012*