Effects of the addition of non-fibre carbohydrates with different rumen degradation rates in dairy cow high-forage diets using the Rumen Simulation Technique

Hanlon, M.E.; Simoni, M.; Moorby, J.M.; Righi, F.; Tsiplakou, E.; Kantas, D.; Foskolos, A.

Published in:
Animal

DOI:
10.1016/j.animal.2023.100732

Publication date:
2023

Citation for published version (APA):
Hanlon, M. E., Simoni, M., Moorby, J. M., Righi, F., Tsiplakou, E., Kantas, D., & Foskolos, A. (2023). Effects of the addition of non-fibre carbohydrates with different rumen degradation rates in dairy cow high-forage diets using the Rumen Simulation Technique. *Animal*, *17*(4), [100732]. https://doi.org/10.1016/j.animal.2023.100732

Animal 17 (2023) 100732

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Effects of the addition of non-fibre carbohydrates with different rumen degradation rates in dairy cow high-forage diets using the Rumen Simulation Technique

M.E. Hanlon [a,d], M. Simoni [b], J.M. Moorby [c], F. Righi [b], E. Tsiplakou [d], D. Kantas [a], A. Foskolos [a,*]

[a] Department of Animal Sciences, University of Thessaly, GR-41500 Larisa, Greece
[b] Department of Veterinary Science, University of Parma, IT-43126 Parma, Italy
[c] Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, SY23 3EE Aberystwyth, United Kingdom
[d] Department of Animal Science, Agricultural University of Athens, GR-11855 Athens, Greece

ARTICLE INFO

Article history:
Received 21 April 2022
Revised 1 February 2023
Accepted 3 February 2023
Available online 11 February 2023

Keywords:
Digestibility
Efficiency of nitrogen utilisation
Microbial flow
Microbial protein synthesis
Rumen fermentation

ABSTRACT

Nutrient synchronisation of protein and carbohydrates is a promising practice to improve ruminal nutrient utilisation. However, dietary sources supplying these nutrients can vary in ruminal nutrient availability due to differing degradation rates, therefore potentially affecting utilisation of nitrogen (**N**). The effects of the addition of non-fibre carbohydrates (**NFCs**) with different rumen degradation rates in high-forage diets on ruminal fermentation, efficiency and microbial flow were investigated *in vitro* using the Rumen Simulation Technique (**RUSITEC**). Four diets were tested: control with 100% ryegrass silage (**GRS**) and substitution of 20% on a DM basis of ryegrass silage with corn grain (**CORN**), processed corn (**OZ**) or sucrose (**SUC**). The four diets were assigned to 16 vessels in two sets of RUSITEC apparatuses in a randomised block design over a 17 d experimental trial; 10 d consisted of adaptation and 7 d for sample collection. Rumen fluid was collected from four rumen-cannulated dry Holstein-Friesian dairy cows and was treated without mixing. Then, rumen fluid from each cow was used to inoculate four vessels, and diet treatments were randomly allocated to each one. This was repeated for all cows resulting in 16 vessels. The inclusion of SUC in ryegrass silage diets improved DM and organic matter digestibility. The only diet to significantly lower ammonia-N concentration compared with GRS was SUC. The outflows of non-ammonia-N, microbial-N, and efficiency of microbial protein synthesis were not affected by diet type. However, the efficiency of nitrogen utilisation was improved by SUC compared with GRS. This indicates that the inclusion of an energy source with a high rumen degradation rate in high-forage diets improves rumen fermentation, digestibility, and N utilisation. Specifically, this effect was observed for the more readily available energy source, SUC, compared with the more slowly degradable NFC sources, CORN and OZ.

Implications

The nutrient synchronisation of carbohydrates and proteins in dietary rations to improve utilisation and reduce loss of nitrogen has been explored considerably; however, the influence of ruminal degradation rates of these sources has not. Grass silage-based diets, supply large amounts of readily available protein, the partial replacement with sucrose as an energy source that has a similar degradation rate of energy, resulted in improved efficiency of nitrogen utilisation in the rumen. This can be applied to ruminant diet formulation in grass silage or pasture-based diets to reduce nitrogen loss and improve nutrient synchronisation in the rumen.

Introduction

The environmental impact of dairy farming affecting atmospheric, soil, and water quality through nitrogen (**N**) emissions is of growing concern (Oenema et al., 2009). Multiple nutritional mitigation strategies have been proposed to reduce N excretion (Calsamiglia et al., 2010; Dijkstra et al., 2011), focusing on enhanced N use efficiency. Synchronisation of protein and carbohydrate availability in the rumen has been shown to improve N and microbial protein synthesis (Chumpawadee et al., 2005;

* Corresponding author.
E-mail address: afoskolos@uth.gr (A. Foskolos).

https://doi.org/10.1016/j.animal.2023.100732
1751-7311/

Higgs et al., 2013), which in turn reduces N loss and enhances productivity.

An *in vitro* study by Lee et al. (2003) reported improvements of microbial-N, efficiency of microbial protein synthesis (**EMPS**), and reduced ammonia-N when increasing energy availability in a grass silage-based diet with sucrose infusion. In contrast, when comparing the partial replacement of corn starch with sucrose *in vitro*, no effects on N utilisation occurred (Vallimont et al., 2004). Additionally, an *in vivo* study reported no effect on ruminal ammonia-N concentration when comparing corn grain and sucrose in a total mixed ration (Penner and Oba, 2009). It needs to be noted that different quantities and sources of proteins and carbohydrates were investigated. Feed sources differ in degradation rates and fractions of nutrients, therefore affecting energy and protein availability in the rumen (Hall and Huntington, 2008). This suggests that rate and extent of degradation of feeds should be of higher consideration than solely the synchronisation of protein and carbohydrate sources in a ration.

In the UK, dairy cattle diets are traditionally grass silage and/or forage based (Miller et al., 2001, Moorby et al. 2016), with perennial ryegrass (*Lolium perenne*) typically being the main forage. Grass silages are a readily available protein source with high amounts of soluble N (Givens and Rulquin, 2004), and at the same time have a high NDF content (Ahvenjärvi et al., 2006) that is a slowly available energy source, thus creating asynchrony in the availability of N and energy for microbial growth in the rumen in addition to a potential imbalance in the amounts of each (Hall and Huntington, 2008). Therefore, for optimal utilisation of the rapidly available N by microbes for microbial protein synthesis, energy sources with similar rumen degradation rates should essentially be supplied (Hall and Huntington, 2008). A major energy substrate for rumen microorganisms is non-fibre carbohydrate (**NFC**), which contributes to an increase in protein N uptake, mainly as ammonia for microbial protein synthesis when supplied in higher amounts (Schwab, 2005). A common NFC source supplied in dairy cattle diets is corn (*Zea mays*) grain, although its rumen starch degradation rate can be considered relatively low (15%/h; Van Amburgh et al., 2015). However, proprietary methods of processing corn starch consisting of heat and pressure resulted in higher degradation rates. For example, the product of Matrix Nutrition LLC (Phoenix, Arizona), OZ 45, had a 45%/h starch degradation rate (Holt and Garner, 2017). Lascano et al. (2016) reported a 35.6% higher 7-h starch degradability for OZ 45 compared with unprocessed corn, therefore, it is more rapidly supplied energy in the rumen. Sugars, such as sucrose or fructose, are another common NFC source, which are rapidly fermented providing energy at rates higher than starch and fibre (Lee et al., 2003), with degradation rates of 40–60%/h (Van Amburgh et al., 2015). Therefore, the objective of this study was to examine the effects of the addition of NFC with different rumen degradation rates on ruminal fermentation, efficiency, and microbial flow in high-forage diets with the Rumen Simulation Technique (**RUSITEC**). We hypothesised that partially supplementing NFC sources with higher degradation rates in the diets would improve ruminal N utilisation.

Material and methods

Experimental diets and design

Two identical sets of RUSITEC apparatus (Czerkawski and Breckenridge, 1997) were used at the same time to provide a total of 16 fermentation vessels. The four diets investigated consisted of (1) GRS: a control forage comprising 100% ryegrass silage, (2) CORN: 20% of the forage DM replaced by corn grain, (3) OZ: 20% of the forage DM replaced by OZ 45, and (4) SUC: 20% of the forage DM replaced by sucrose. The main objective of the study was to evaluate nutrient synchronisation in the rumen; therefore, the control of solely ryegrass silage was used to provide maximum soluble N without the interference of other nutrients from additional feed ingredients. The ingredient OZ 45 was obtained from Matrix Nutrition LLC, Phoenix, Arizona and described as finely ground particle size prepared from unprocessed whole corn and was processed using proprietary heat and pressure treatments that sheared the prolamine protein structure, eliminating the crystalline and hydrophobic properties of the unprocessed corn (Holt and Garner, 2017). The sucrose used was obtained from the local supermarket as granulated sugar.

Ryegrass silage was freeze-dried and then ground to pass a 2-mm sieve, corn grain was ground to pass through a 1.5 mm sieve (Wiley hammer mill - John Wiley & Sons Ltd, Chichester, UK), and OZ 45 and sucrose were used as procured. The chemical composition of forage and NFC sources are reported in Table 1. The chemical composition of the diets were mathematically calculated based on the chemical composition of the ingredients and the offered amount of each ingredient, and this is presented in Table 2.

Animal procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the Aberystwyth University Animal Welfare and Ethical Review Body. Rumen fluid was obtained prior to morning feeding from four rumen-cannulated Holstein-Frisian cows (878 ± 67 kg of BW) that were being fed 100% grass silage diets. Rumen contents were strained through a double layer of muslin and transferred to the laboratory under anaerobic conditions at 39 °C in Dewar flasks, keeping the rumen fluid from each cow separate. The experiment consisted of a single incubation period using 16 experimental units (vessels). Each experimental treatment diet had four replicates that were randomly allocated to the vessels and each was inoculated with the rumen fluid from one of the four cows, with each cow used on four vessels. Vessels had an effective volume of 800 mL and were maintained at 39 °C under permanent vertical agitation. As described by Ramos-Morales et al. (2018) with some modifications, on day 1, the vessels were inoculated with the 265 mL of rumen fluid strained through a double layer of muslin, artificial saliva (McDougall, 1948) and demineralised water, mixed together using a 1:1:1 ratio (vol/vol). Artificial saliva was continuously infused into each vessel throughout the experimental period at a rate of 640 mL/d (dilution rate of 3.33%/h) using a multi-channel peristaltic pump (Watson–Marlow 200 series, Cornwall, UK). For day one only, one nylon bag containing squeezed rumen solids (20 g) (10 × 10 cm, pore size 50 μm; R1020 ANKOM Forage Bag-Stockport, UK) was placed in each vessel for 24 hrs to provide solid-associated bacteria and was replaced with a bag containing the experimental diet. In addition, on day 1, another bag containing the experimental diet was also placed in each vessel for 48 hrs. On subsequent days, there were consistently two feed bags in each vessel. The bag that was removed after 48 hrs was squeezed and rinsed with 30 mL of artificial saliva. The rinsing liquid was returned to the vessel along with a new bag of feed (20 g DM). The pH and volume of effluent and gas produced from each vessel were recorded daily.

Experimental procedure and sample collection

The experimental period consisted of 17 days, with the first 10 days for adaptation and the last 7 days for sample and data collection. Days 11, 12, 13 and 14 were allocated to the measurement of digestibility, gas production and the outflow of rumen fermentation characteristics. Nylon bags containing the diet were collected following incubation and rinsed with cold water for 20 min then stored at −20 °C and freeze-dried to determine residual DM content. The samples were then pooled per vessel to further determine

Table 1
Chemical composition of forage and non-fibre carbohydrate sources in dairy cattle experimental diets used in the Rumen Simulation Technique.

Item	Grass silage	Corn	OZ 45[1]	Sucrose
DM, %	30.0	90.0	94.0	100
CP, % of DM	20.4	6.85	8.57	–
Soluble N, % CP[2]	74.4	–	–	–
WSC, % of DM[2]	10.1	1.5	4.5	100
Starch, % of DM	0.56	49.4	64.5	–
NDF, % of DM	43.9	27.8	11.5	–

[1] OZ 45 = Processed corn grain using proprietary heat and pressure treatments (Matrix Nutrition LLC, Phoenix, AZ, USA).
[2] N = nitrogen; WSCs = water-soluble carbohydrates.

Table 2
Ingredient and nutrient composition of dairy cattle experimental diets in the Rumen Simulation Technique.

Item	GRS[1]	CORN[1]	OZ[1]	SUC[1]
Ingredient, % DM				
Ryegrass silage	100	80	80	80
Corn	–	20	–	–
OZ 45	–	–	20	–
Sugar	–	–	–	20
Nutrient composition, % DM				
DM, %	89	90	90	92
CP	20.4	17.7	18.1	16.3
Soluble N, % CP[1]	74.4	62.8	69.9	71.7
WSC[1]	10.1	8.32	8.92	28.0
Starch	0.56	10.4	13.4	0.40
NDF	43.9	40.7	37.4	35.1
NFC:CP[1]	1:1.91	1:0.95	1:0.81	1:0.57

[1] GRS = 100% grass silage; CORN = 20% replacement of grass silage with corn grain; OZ = 20% replacement of grass silage with OZ 45 (processed corn grain using proprietary heat and pressure treatments; Matrix Nutrition LLC, Phoenix, AZ, USA); SUC = 20% replacement of grass silage with sucrose; N = nitrogen; WSCs = water-soluble carbohydrates; NFCs = non-fibre carbohydrates.

nutrient digestibility. Fermentation gases were collected in hermetic bags to measure total gas, methane and carbon dioxide production. Total effluent volume was measured by weight difference of the flask, and 10 mL of a water solution saturated with $HgCl_2$ (diluted 1:5) was added to stop the fermentation processes. A 1.5 mL sample was taken from the effluent every 24 hrs for volatile fatty acid (**VFA**) determination and was diluted with 0.5 ml of a preservative solution, consisting of orthophosphoric acid (50% v/v) and valerenic acid (5% w/v) as internal standards. In addition, to describe diurnal changes of the fermentation patterns, on days 15, 16, and 17, fermenter fluid was sampled from each vessel by aspiration at 0, 2, and 8 hrs after inserting new bags of feed for VFA determination with the same process described for days 11–14.

To determine microbial protein synthesis, ammonium sulphite ($^{15}NH_4SO_4$; 98% enrichment) was used as a microbial marker (Broderick and Merchen, 1992). As described by García-González et al. (2010) with modifications, each vessel was dosed with 3.15 mg of ^{15}N on day 12, and from that point until the end of the experiment, ^{15}N was added to the artificial saliva at a rate of 3.7 mg/L. On days 16 and 17, at 24 hrs, the nylon bag residuals were mixed with their associated effluent, homogenised for 1 minute with a stomacher and pooled per vessel to create a sample of total digesta. Total digesta was subsampled in three parts: (1) a 1 mL sample was taken to determine ammonia-N concentration and acidified with 0.1 mL 2 M HCl; (2) 100 g were freeze-dried for N determination; and (3) 200 g was used to isolate associated bacteria. Liquid-associated bacteria were isolated by differential centrifugation at 1 000*g* for 10 min to separate feed particles, and the supernatant was centrifuged at 20 000*g* for 20 min to isolate bacterial cells. Bacterial pellets were rinsed twice with saline solution and recentrifuged at 20 000*g* for 20 min. The final pellet was recovered with distilled water to prevent contamination of bacteria with ash. Bacterial cells were then freeze-dried and further analysed for DM, organic matter (**OM**), N, and ^{15}N.

Sample analysis

Feed offered and residuals were analysed for DM, OM, total N, NDF, starch and water-soluble carbohydrate (**WSC**). The analysis of DM was conducted by heating in an oven at 103 °C for 24 hrs and OM content by heating in a muffle furnace at 550 °C for 6 hrs. Total N concentration was determined by combustion analysis using a Leco FP 428 nitrogen analyzer (Leco Corporation, St. Joseph, MI). As described by Van Soest et al., (1991), neutral detergent fibre was determined with heat-stable amylase and expressed inclusive of residual ash using the Fibertec equipment (Tecator Ltd., Thornbury, Bristol, Somerset, UK). The WSC concentrations were determined spectrophotometrically using an auto-analyzer (SEAL Analytical Ltd., Southampton, UK) as described by Thomas (1977). Starch concentration was determined as the difference between the total carbohydrate concentration (measured as glucose) and WSC concentration (measured as glucose). The total carbohydrate concentration was determined after boiling in water and subsequent hydrolysis with amyloglucosidase (Rhizopus mould 20 100 units/g solid; Sigma-Aldrich Co. Ltd.). Briefly, 0.4 g of the sample was refluxed in 80 mL of deionised water for 30 min, 20 mL of cold acetate buffer (comprising 40% vol/vol 1 M sodium acetate and 60% vol/vol 1 M acetic acid) was then added, and the mixture was allowed to cool for 20 min. This was then shaken for 1 h following the addition of 10 mL of amyloglucosidase solution (1 g in 100 mL of deionised water). This solution was then made up to 250 mL, filtered, and a portion of the filtrate was analysed for WSC concentration. A coefficient of 0.9 was used as a factor for converting measured glucose release into the original starch concentration Thomas (1977).

Nutrient digestibility was calculated using the following equation: digestibility_x, % = $((\text{input}_x - \text{residuals}_x)/\text{input}_x) \times 100$, where x = DM, OM, N, NDF, starch, and WSC. Total gas volume of the bags were measured by the weight difference between the empty bag and bag containing gas. Then, a sample was taken using a syringe (50 mL) and analysed for methane and carbon dioxide concentration using an ADC 5000 Series Gas Analyser (Analytical Development Co. Ltd, St Albans, UK). The fermenter fluid samples and effluent samples were analysed for VFA concentration using gas chromatography as described by Zhu et al. (1996). The samples collected for ammonia-N concentration were determined enzymatically using glutamate dehydrogenase on a discrete analyzer (FP-901M Chemistry Analyzer, Labsystems Oy, Helsinki, Finland; test kit No. 66–50, Sigma-Aldrich Co. Ltd., Poole, Dorset, UK; Lee et al., 2003). Freeze-dried digesta and microbial pellet were analysed for total N content and ^{15}N to estimate microbial-N enrichment using an isotope ratio mass spectrometer (IRMS; ANCA/SL 20/20, PDZ Europe Ltd., Crewe, Cheshire, UK). The microbial-N contribution to the effluent was estimated based on the relationship between the ^{15}N enrichment in the total digesta and in the total bacterial-N pellet (Calsamiglia et al., 1996). Then, N outflows were calculated as follows: (1) non-ammonia-N (**NAN**) = total digesta N – ammonia-N, and (2) non-ammonia non-microbial-N (**NANM-N**) = NAN – microbial-N. Moreover, EMPS was calculated as microbial-N (mg) per mg of digested organic matter, and the efficiency of nitrogen utilisation (**ENU**) was calculated as microbial-N (mg) per mg of N intake.

Statistical analysis

All statistical analyses were conducted using JMP (version 13; SAS Institute Inc., Cary, NC). All variables were checked for normality based on their distribution, and no transformation was required to achieve normal distribution. Results of VFA concentration, ammonia-N, nutrient digestion, and flows were analysed using a mixed-effects model. The model accounted for the effects of diets (fixed effect) and the animal used to obtain rumen fluid (random effect). Differences between means of diets were tested using the Tukey option, and significance was declared at $P < 0.05$ with tendency declared at $P > 0.05$ to $P < 0.10$.

Results

Digestibility

As shown in Table 3, the digestibility of DM, OM, WSC and starch were all significantly affected by diet type. Partial replacement with sucrose (SUC) as an NFC source in the diet increased DM ($P = 0.01$) and OM ($P = 0.03$) digestibility compared with GRS, by 17 and 13%, respectively. The N digestibility had a tendency ($P = 0.07$) to be greater with the partial replacement with SUC in the diet. The NDF digestibility was unaffected by the experimental diets. The partial replacement with OZ increased starch digestibility compared with CORN by 49% ($P = 0.02$). The digestibility of WSC was high for all diets, averaging 96%. However, SUC resulted in the highest digestibility, with a 2% increase compared with OZ ($P = 0.02$).

Rumen fermentation and gas production

Rumen fermentation profile of the effluent is shown in Table 4. The total effluent volume, total VFA and individual VFA production were not affected by diet, with the exception for branched-chained volatile fatty acids (**BCVFAs**) which was greater in OZ compared with GRS and CORN (2.1 and 1.7 mol/100 mol, respectively, $P = 0.01$).

The diurnal rumen fermentation characteristics of the vessels are reported in Table 5. Prior to morning feeding, at hour 0, SUC had 17.3 mM higher total VFA concentration in the vessel fluid ($P = 0.02$) compared with GRS. At two-hrs postfeeding, the CORN vessel fluid showed a 24.6 mM higher total VFA concentration compared with OZ and an 8.8 mol/100 mol higher molar proportion of acetate compared with SUC, whereas the molar proportion of propionate was greater in the SUC diet ($P < 0.01$) compared with all other diets for both 2- and 8-hrs postfeeding. At two-hrs postfeeding, the molar proportion of BCVFA for SUC and OZ averaged 9.4 mol/100 mol, 3.3 mol/100 mol greater than the 6.1 mol/100 mol average molar proportion of GRS and CORN ($P < 0.001$). At eight-hrs postfeeding, the molar proportion of BCVFA for SUC and OZ averaged 9.6 mol/100 mol, significantly higher compared with the 7.2 mol/100 mol average for GRS and CORN ($P < 0.001$). At 8-hrs postfeeding, SUC had a 22.5 mol/100 mol higher total VFA concentration compared with GRS ($P = 0.02$). Gas production is reported in Table 6. Total gas production, carbon dioxide and methane concentration and yield were all unaffected by experimental diet treatments.

Microbial efficiency and nitrogen flow

The microbial efficiency and N flows are presented in Table 7. Total nitrogen input was significantly different among all diets ($P < 0.001$), GRS had the highest input followed by OZ, CORN and SUC. Therefore, the N outflows were expressed as a percentage of N input from the diets and also as the mg/d measured in the effluent fluid in Table 7. For the N outflows as a percentage of N input, the ammonia-N concentration was significantly higher in GRS and CORN compared with SUC ($P < 0.01$). The N and microbial-N were unaffected by diet type, while there was a tendency for the NAN to be great in OZ and SUC ($P = 0.09$). The NANM-N was significantly higher in OZ compared with GRS and CORN ($P = 0.01$). When expressing the N outflows as mg/d measured in the effluent fluid, ammonia-N was significantly lower for SUC compared to all other diets, while CORN and OZ were not significantly different from one another but lower than GRS ($P < 0.001$). Additionally, the N, NAN, and microbial-N were not affected by diet type, however, the NANM-N was significantly higher for OZ compared with CORN ($P = 0.03$). The EMPS was not affected by diet type, while the ENU was positively affected by SUC, resulting in a 0.11 mg/mg higher efficiency compared with GRS ($P = 0.05$).

Discussion

Digestibility

It was expected that the partial replacement of grass silage with highly digestible energy sources, namely corn grain, OZ 45 and sucrose, would have resulted in increased DM and OM digestibility. However, this occurred only for SUC, while CORN and OZ had only numerical differences compared to GRS. Based on previous literature, WSC has a higher rumen degradation rate compared with starch from corn grain or OZ 45 (Higgs et al., 2015, Lascano et al., 2016), and this was demonstrated by our results for SUC with the current rate of inclusion (20% on a DM basis). However, the inclusion rate was not enough to provoke overall changes in the case of CORN and OZ. Moreover, starch digestibility was unexpectedly lower for CORN compared with all other diets (30.5 vs 59.2% for CORN vs GRS, respectively). A potential reason for this is that the starch content of the corn grain used in the current study was relatively low and outside of normal ranges. Higgs et al., (2015) reported a starch content of 72.1 ± 1.49% DM for corn grain and a NDF content of 11.4 ± 1.30% DM. However, the corn grain used in the current study had 49.4 and 27.8% DM of starch and

Table 3
Effects of 20% replacement of grass silage (GRS) with corn grain (CORN), OZ 45[1] (OZ) or sucrose (SUC) in dairy cattle experimental diets on digestibility in the Rumen Simulation Technique.

Item	GRS	CORN	OZ	SUC	SEM	*P*-value
Digestibility, %						
DM	34.5[b]	40.0[a,b]	43.0[a,b]	51.4[a]	2.60	0.01
OM[2]	52.9[b]	58.9[a,b]	59.9[a,b]	66.1[a]	2.20	0.03
Nitrogen	36.6	38.6	35.3	43.4	1.69	0.07
NDF	11.0	16.7	17.1	14.7	3.84	0.74
Starch	59.2[a,b]	30 .5[b]	79.2[a]	54.9[a,b]	8.42	0.02
WSC[2]	96.1[a,b]	96.7[a,b]	93.0[b]	98.5[a]	0.50	0.02

[a–b]Within a row, means without a common superscript differ ($P < 0.05$).
[1] OZ 45 = Processed corn grain using proprietary heat and pressure treatments (Matrix Nutrition LLC, Phoenix, AZ, USA).
[2] OM = organic matter; WSCs = water-soluble carbohydrates.

Table 4
Effects of 20% replacement of grass silage (GRS) with corn grain (CORN), OZ 45[1] (OZ) or sucrose (SUC) in dairy cattle experimental diets on the ammonia-N[2] and VFA[2] concentration of the effluent after 24hr of incubation in the Rumen Simulation Technique.

Item	GRS	CORN	OZ	SUC	SEM	*P*-value
Effluent volume, L	1.21	1.21	1.20	1.21	0.01	0.86
Total VFA, mM	46.9	58.2	59.3	64.2	6.86	0.30
VFA (mol/100 mol)						
Acetate	42.4	42.1	39.7	40.7	2.14	0.50
Propionate	23.6	24.4	23.7	24.9	2.27	0.70
Butyrate	15.9	15.1	17.2	15.8	2.34	0.51
Valeric	10.9	10.7	10.1	10.4	0.82	0.80
BCVFA[2]	7.18^{b}	7.56^{b}	9.28^{a}	8.15^{a,b}	0.53	0.01

$^{a-b}$Within a row, means without a common superscript differ ($P < 0.05$).
[1] OZ 45 = Processed corn grain using proprietary heat and pressure treatments (Matrix Nutrition LLC, Phoenix, AZ, USA).
[2] N = nitrogen; VFA = volatile fatty acid; BCVFAs = branched-chain volatile fatty acids.

Table 5
Effects of 20% replacement of grass silage (GRS) with corn grain (CORN), OZ 45[1] (OZ) or sucrose (SUC) in dairy cattle experimental diets and sampling time on vessel rumen fermentation characteristics in the Rumen Simulation Technique.

Item	GRS	CORN	OZ	SUC	SEM	*P*-value
Vessel volume, L	1.13	1.13	1.11	1.12	0.02	0.91
0 h relative to feeding						
Total VFA,[2] mM	45.5^{b}	53.9^{a,b}	55.0^{a,b}	62.8^{a}	4.51	0.02
VFA profile, mol/100 mol						
Acetate	46.4	46.0	43.7	40.1	1.89	0.15
Propionate	23.1	24.7	22.9	27.6	1.91	0.36
Butyrate	15.2	13.6	15.6	12.6	1.62	0.39
Valeric	8.31	8.64	8.26	9.22	0.64	0.75
BCVFA[2]	6.99	6.92	9.62	10.4	0.87	0.45
2 h relative to feeding						
Total VFA, mM/d	59.5^{a,b}	79.2^{a}	54.6^{b}	71.2^{a,b}	4.50	0.02
VFA profile, mol/100 mol						
Acetate	43.9^{a,b}	47.3^{a}	43.5^{a,b}	38.5^{b}	1.25	<0.01
Propionate	22.4^{b}	24.0^{b}	24.4^{b}	30.3^{a}	0.91	<0.01
Butyrate	15.3	14.5	13.8	12.0	1.01	0.27
Valeric	9.19	8.43	8.67	8.85	0.72	0.86
BCVFA	6.50^{b}	5.78^{b}	9.62^{a}	9.33^{a}	0.55	<0.001
8 h relative to feeding						
Total VFA, mM	47.9^{b}	63.0^{a,b}	64.0^{a,b}	70.4^{a}	4.55	0.02
VFA profile, mol/100 mol						
Acetate	44.5	43.5	42.3	38.0	1.84	0.17
Propionate	24.9^{b}	25.0^{b}	22.8^{b}	31.0^{a}	1.13	<0.01
Butyrate	15.5	13.0	15.0	11.8	3.57	0.39
Valeric	9.70	8.16	8.31	9.41	0.51	0.05
BCVFA	7.42^{b}	7.07^{b}	9.44^{a}	9.89^{a}	0.60	<0.001

$^{a-b}$Within a row, means without a common superscript differ ($P < 0.05$).
[1] OZ 45 = Processed corn grain using proprietary heat and pressure treatments (Matrix Nutrition LLC, Phoenix, AZ, USA).
[2] VFA = volatile fatty acid; BCVFAs = branched-chain volatile fatty acids.

Table 6
Effects of 20% replacement of grass silage (GRS) with corn grain (CORN), OZ 45[1] (OZ) or sucrose (SUC) in dairy cattle experimental diets on gas production in the Rumen Simulation Technique.

Item	GRS	CORN	OZ	SUC	SEM	*P*-value
Total gas, L/d	1.9	2.4	2.2	2.1	0.2	0.60
Methane, %	5.2	6.8	5.6	6.3	0.5	0.30
Methane, mmol/d	0.04	0.07	0.05	0.06	0.30	0.43
Carbon dioxide, %	42.0	48.4	47.8	43.7	2.55	0.32
Carbon dioxide, mmol/d	0.35	0.53	0.48	0.42	0.04	0.46

[1] OZ 45 = Processed corn grain using proprietary heat and pressure treatments (Matrix Nutrition LLC, Phoenix, AZ, USA).

NDF, respectively. Even though we purchased corn grain from a regular feed-meal of the area, but in a small quantity (2 kg), the chemical composition was not within normal ranges; thus, the results of the CORN diet should be considered with conscious.

Rumen fermentation and gas production

The rumen fermentation characteristics for the effluent after 24 hrs of incubation were within the normal range observed in RUSITEC studies (Belanche et al., 2013; Ramos-Morales et al., 2018) and not affected by diet type, except for the molar proportion of BCVFA. Indeed, OZ resulted in a higher concentration of BCVFA compared with GRS and CORN. In addition, at 2- and 8-hrs postfeeding in the vessel fluid, the molar proportion of BCVFA was higher for OZ and SUC compared to CORN and GRS. Branch-chain volatile fatty acids are a mixture of isobutyrate, isovalerate and 2-methylbutyrate, which are derived from protein degradation in the rumen (Andries et al., 1987), suggesting that

Table 7
Effects of 20% replacement of grass silage (GRS) with corn grain (CORN), OZ 45[1] (OZ) or sucrose (SUC) in dairy cattle experimental diets on nitrogen input and outflow and microbial protein synthesis in the Rumen Simulation Technique.

Item	GRS	CORN	OZ	SUC	SEM	*P*-value
Total nitrogen input, mg/d	653[a]	567[c]	576[b]	523[d]	0.38	<0.0001
Outflows						
NH_3-N[2], mg/d	114.3[a]	96.3[b]	88.2[b]	63.4[c]	3.77	<0.0001
NH_3-N[2], % N input	17.5[a]	16.98[a]	15.3[a,b]	12.6[b]	0.66	<0.01
Nitrogen, mg/d	350	333	355	327	18.6	0.60
Nitrogen, % N input	53.7	58.7	61.7	60.0	2.91	0.31
NAN[3], mg/d	236	237	267	263	15.4	0.40
NAN, % N input	36.2	41.7	46.4	47.4	2.98	0.09
NANM-N[4], mg/d	73.9[a,b]	63.1[b]	92.3[a]	73.9[a,b]	7.20	0.03
NANM-N, % N input	11.3[b]	11.1[b]	16.0[a]	14.1[a,b]	1.20	0.01
Microbial-N, mg/d	162	173	175	191	11.7	0.55
Microbial-N, % N input	24.9	30.6	30.3	33.3	2.44	0.14
EMPS[5], mg/mg	16.8	15.2	15.7	15.6	1.24	0.87
ENU[6], mg/mg	0.25[b]	0.31[a,b]	0.30[a,b]	0.36[a]	0.02	0.05
Microbial-N: NAN	0.69	0.73	0.66	0.71	0.02	0.06

[a–b]Within a row, means without a common superscript differ ($P < 0.05$).
[1] OZ 45 = Processed corn grain using proprietary heat and pressure treatments (Matrix Nutrition LLC, Phoenix, AZ, USA).
[2] NH_3-N = ammonia–nitrogen.
[3] NAN = non-ammonia-nitrogen; NAN = nitrogen outflow-ammonia-nitrogen.
[4] NANM-N = non-ammonia non-microbial nitrogen; NANM-N = non-ammonia-nitrogen outflow-microbial-N.
[5] Efficiency of microbial protein synthesis (EMPS) = Microbial-N/digested organic matter, (mg/mg).
[6] Efficiency of nitrogen utilisation (ENU) = Microbial-N/N Intake (mg/mg).

protein degradation was higher for SUC and OZ. This result is not in line with the study of Penner and Oba (2009), where sucrose addition did not affect BCVFA concentration *in vivo*, and the study of Lascano et al. (2016) where OZ 45 addition decreased the molar proportion of BCVFA *in vitro* 4-hrs postfeeding. Furthermore, this is not supported by the digestibility results of the current study where N digestibility was not affected by diet type. However, the observation was constant both at the 24 h effluent and the diurnal pattern of the fermentation vessel at 2- and 8-hrs postfeeding.

For individual VFA production, acetate and propionate were affected by diet type. The diet CORN increased the molar proportion of acetate compared with SUC 2-hrs postfeeding, while that of propionate was greater with SUC compared with all other diets at 2- and 8-hrs postfeeding. It is expected that when the proportion of propionate increases, acetate in turn will be reduced. A previous *in vitro* study demonstrated that the increased inclusion of WSC content through a gradual increase of sucrose in fresh perennial ryegrass reduced acetate concentration while increasing propionate (Lee et al., 2003). This suggests that there was an increase in sugar and starch fermentation, shifting from fibre digestion of the grass silage due to the 20% of DM removal and replacement with an NFC source. This was expected due to more readily available energy carbohydrate sources, specifically WSC, potentially increasing N and energy utilisation.

For both the effluent and vessel fluid, the molar proportion of butyrate was not affected by diet type. The inclusion of dietary sugar in a ration is expected to increase the butyrate concentration in the rumen (Oba, 2011); however, this was not observed in the current study. Similar to the current study, increasing the total dietary sugar content up to 10% of DM (Broderick and Radloff, 2004) or replacing dietary corn starch with sucrose (Broderick et al., 2008) did not alter ruminal butyrate concentration *in vivo*. In contrast, a previous *in vitro* study by Vallimont et al. (2004) observed increased butyrate concentrations with high-sugar diets. Findings of greater total VFA concentration specifically butyrate are inconsistent among studies and can also vary by sugar type fed and starch source replaced (Oba, 2011).

All gas production was unaffected by diet type. Similarly, an *in vitro* study by Hatew et al. (2015) found that when comparing starch sources of fast or slow degradation, gas production and methane percentage of total gas were unaffected by source, but significantly affected by the level of inclusion which were lower in greater amounts of dietary starch inclusion. Therefore, a difference may have not been seen between diets due to similar inclusion rates of NFC sources and low quantities.

Microbial efficiency and nitrogen flow

The effect of partial replacement of grass silage with sucrose as an NFC source on ruminal ammonia-N utilisation was positive, reducing the outflow of ammonia-N concentration compared with the GRS diet when expressed as a percentage of N input. The reduction of rumen ammonia-N concentration suggests a greater microbial utilisation of dietary N supplied in the rumen for microbial growth or protein synthesis (Oba, 2011). Previous studies evaluating the inclusion of NFC sources and increase in WSC content in fresh perennial ryegrass and ryegrass silage diets using the RUSITEC system also reported a reduction of ammonia-N concentration in the ruminal outflow effluent (Lee et al., 2003; Jaurena et al., 2005). However, although a reduction in the outflow of ammonia-N might be expected in the NFC diet treatments compared with the control grass silage-only diet, only sucrose significantly lowered levels. Similar to our findings, when more readily available energy was increased in a diet through the replacement of dietary starch with lactose or sucrose *in vivo*, ruminal ammonia-N concentration was reduced (Chibisa et al., 2015). This could be a result of the more rapidly available energy supplied with sucrose to be utilised by microbes compared with CORN and OZ 45. However, when the ammonia-N concentration was expressed as mg/d, the diets were all significantly different. This could be a result of a difference in N input (mg/d) for each diet.

In the current study, diets were not formulated to be isonitrogenous due to the main focus being on the inclusion of NFC sources and the respective degradation rates, therefore, the N inputs were significantly different for each diet treatment. Although the microbial-N was not different between diet treatments, ranging from 24.9 to 33.3% of total N input or 162 to 191 mg/d, the ENU of the SUC diet was significantly higher compared with GRS, demonstrating greater microbial-N per unit of N intake. Therefore, as an NFC source, sucrose allowed for greater N utilisation and microbial protein efficiency when included in ryegrass silage diets, while corn and OZ 45 did not to that extent. This suggests that their

associated ruminal degradation rates were not sufficient enough in supplying energy at the most optimal rate for maximum N utilisation. Although CORN and OZ did not significantly affect ammonia-N concentration per N input or ENU, both diets had numerically lower ammonia-N concentrations and a higher ENU compared to GRS. As expected, due to the high inclusion of ryegrass silage, which contains a large portion of CP in the soluble form (74.4% in the current study), the ammonia-N concentration of the outflow for SUC was still considered high compared with similar *in vitro* studies (Bach et al., 2005). This may suggest that N utilisation could have been improved further with the additional inclusion of sucrose in the diet.

Conclusion

The results of this study demonstrated that compared to a control grass silage-only diet, partial replacement of the feed DM with sucrose led to increases in DM and OM digestibility without significantly altering rumen fermentation and increased ENU. However, the replacement of silage DM with corn and OZ 45 as NFC sources did not enhance N utilisation to the extent of sucrose. In ryegrass silage-based diets with readily available N, the NFC source sucrose provides energy at a more similar rate due to its more rapid degradation rate compared with corn and processed corn (OZ 45), increasing ruminal N utilisation. Collectively, the results of this study suggest that partial replacement with sucrose is likely to be a beneficial NFC source in pasture-based systems with the potential to improve N efficiency and reduce N loss.

Ethics approval

Not applicable.

Data and model availability statement

None of the data were deposited in an official repository. The data/models that support the study findings are available to reviewers.

Author ORCIDs

Hanlon M.E.: https://orcid.org/0000-0003-3791-4220.
Simoni M.: https://orcid.org/0000-0003-3920-9744.
Moorby J.M.: https://orcid.org/0000-0002-4449-8432.
Righi F.: https://orcid.org/0000-0001-9274-4143.
Tsiplakou E.: https://orcid.org/0000-0002-2544-8966.
Kantas D.: https://orcid.org/0000-0003-2451-309X.
Foskolos A.: https://orcid.org/0000-0002-7339-1140.

Author contributions

Hanlon M.E.: Data Analysis, Investigation, Methodology, Review and Editing, Writing; **Simoni M.:** Data Analysis, Investigation, Methodology; **Moorby J.M.:** Conceptualisation, Funding Acquisition, Investigation, Project Administration, Review and Editing; **Righi F.:** Conceptualisation, Review and Editing, Supervision; **Tsiplakou E.:** Conceptualisation, Review and Editing, Supervision; **Kantas D.:** Conceptualisation, Funding Acquisition, Project Administration, Review and Editing, Supervision; **Foskolos A.:** Conceptualisation, Data Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Review and Editing, Supervision, Writing; All authors critically reviewed the draft and contributed to the final version of the manuscript.

Declaration of interest

None.

Acknowledgements

The authors are grateful to Mark Holt (Matrix Nutrition, USA) for providing OZ 45.

Financial support statement

Funding for this analysis was provided as part of the EU Horizon 2020 Marie Sklodowska-Curie Research and Innovation Staff Exchanges project CowficieNcy (grant number: 777974).

References

Ahvenjärvi, S., Joki-Tokola, E., Vanhatalo, A., Jaakkola, S., Huhtanen, P., 2006. Effects of replacing grass silage with barley silage in dairy cow diets. Journal of Dairy Science 89, 1678–1687.
Andries, J.I., Buysse, F.X., De Brabander, D.L., Cottyn, B.G., 1987. Isoacids in ruminant nutrition: Their role in ruminal and intermediary metabolism and possible influences on performances—A review. Animal Feed Science and Technology 18, 169–180.
Bach, A., Calsamiglia, S., Stern, M.D., 2005. Nitrogen metabolism in the rumen. Journal of Dairy Science 88, E9–E21.
Belanche, A., Lee, M.R.F., Moorby, J.M., Newbold, C.J., 2013. Comparison of ryegrass and red clover on the fermentation pattern, microbial community and efficiency of diet utilisation in the rumen simulation technique (Rusitec). Animal Production Science 53, 1052–1064.
Broderick, G.A., Luchini, N.D., Reynal, S.M., Varga, G.A., Ishler, V.A., 2008. Effect on production of replacing dietary starch with sucrose in lactating dairy cows. Journal of Dairy Science 9, 4801–4810.
Broderick, G., Merchen, N., 1992. Markers for quantifying microbial protein synthesis in the rumen. Journal of Dairy Science 75, 2618–2632.
Broderick, G., Radloff, W., 2004. Effect of molasses supplementation on the production of lactating dairy cows fed diets based on alfalfa and corn silage. Journal of Dairy Science 87, 2997–3009.
Calsamiglia, S., Stern, M.D., Firkins, J.L., 1996. Comparison of nitrogen-15 and purines as microbial markers in continuous culture. Journal of Animal Science 74, 1375–1381.
Calsamiglia, S., Ferret, A., Reynolds, C.K., Kristensen, N.B., Van Vuuren, A.M., 2010. Strategies for optimizing nitrogen use by ruminants. Animal 4, 1184–1196.
Chibisa, G.E., Gorka, P., Penner, G.B., Berthiaume, R., Mutsvangwa, T., 2015. Effects of partial replacement of dietary starch from barley or corn with lactose on ruminal function, short-chain fatty acid absorption, nitrogen utilization, and production performance of dairy cows. Journal of Dairy Science 98, 2627–2640.
Chumpawadee, S., Sommart, K., Vongpralub, T., Pattarajinda, V., 2005. Effects of synchronizing the rate of dietary energy and nitrogen release on ruminal fermentation, microbial protein synthesis, blood urea nitrogen and nutrient digestibility in beef cattle. Asian-Australasian Journal of Animal Science 19, 181–188.
Czerkawski, J.W., Breckenridge, G., 1997. Design and development of a long-term rumen simulation technique (Rusitec). British Journal of Nutrition 38, 371–384.
Dijkstra, J., Oenema, O., Bannink, A., 2011. Dietary strategies to reducing N excretion from cattle: implications for methane emissions. Current Opinion in Environmental Sustainability 3, 414–422.
García-González, R., González, J.S., López, S., 2010. Decrease of ruminal methane production in Rusitec fermenters through the addition of plant material from rhubarb (*Rheum* spp.) and alder buckthorn (*Frangula alnus*). Journal of Dairy Science 93, 3755–3763.
Givens, D.I., Rulquin, H., 2004. Utilisation by ruminants of nitrogen compounds in silage-based diets. Animal Feed Science and Technology 114, 1–18.
Hall, M., Huntington, G., 2008. Nutrient synchrony: Sound in theory, elusive in practice. Journal of Animal Science 86 (suppl_14), E287–E292.
Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta, S.C., Bannink, A., Hendriks, W.H., Dijkstra, J., 2015. Relationship between in vitro and in vivo methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle. Animal Feed Science and Technology 202, 20–31.
Higgs, R.J., Sheahan, A.J., Mandok, K., Van Amburgh, M.E., Roche, J.R., 2013. The effect of starch-, fiber-, or sugar-based supplements on nitrogen utilization in grazing dairy cows. Journal of Dairy Science 96, 3857–3866.
Higgs, R.J., Chase, L.E., Ross, D.A., Van Amburgh, M.E., 2015. Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. Journal of Dairy Science 98, 6340–6360.
Holt, M.D., Garner, M.R., 2017. United States Patent Application No., 15/269,747. Patent and Trademark Office, Washington, DC, USA.
Jaurena, G., Moorby, J.M., Davies, D.R., 2005. Efficiency of microbial protein synthesis on red clover and ryegrass silages supplemented with barley by

rumen simulation technique (RUSITEC). Animal Feed Science and Technology 118, 79–91.
Lascano, G.J., Alende, M., Koch, L.E., Jenkins, T.C., 2016. Changes in fermentation and biohydrogenation intermediates in continuous cultures fed low and high levels of fat with increasing rates of starch degradability. Journal of Dairy Science 99, 6334–6341.
Lee, M., Merry, R., Davies, D., Moorby, J., Humphreys, M., Theodorou, M., MacRae, J., Scollan, N., 2003. Effect of increasing availability of water-soluble carbohydrates on *in vitro* rumen fermentation. Animal Feed Science and Technology 104, 59–70.
McDougall, E.I., 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal 43, 99–109.
Miller, L.A., Moorby, J.M., Davies, D.R., Humphreys, M.O., Scollan, N.D., MacRae, J.C., Theodorou, M.K., 2001. Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.): Milk production from late-lactation dairy cows. Grass and Forage Science 56, 383–394.
Moorby, J.M., Ellis, N.M., Davies, D.R., 2016. Assessment of dietary ratios of red clover and corn silages on milk production and milk quality in dairy cows. Journal of Dairy Science 99, 7982–7992.
Oba, M., 2011. Review: Effects of feeding sugars on productivity of lactating dairy cows. Canadian Journal of Animal Science 91, 37–46.
Oenema, O., Witzke, H.P., Klimont, Z., Lesschen, J.P., Velthof, G.L., 2009. Integrated assessment of promising measures to decrease nitrogen losses from agriculture in EU-27. Agriculture, Ecosystems & Environment 133, 280–288.
Penner, G.B., Oba, M., 2009. Increasing dietary sugar concentration may improve dry matter intake, ruminal fermentation, and productivity of dairy cows in the postpartum phase of the transition period. Journal of Dairy Science 92, 3341–3353.
Ramos-Morales, E., Rossi, G., Cattin, M., Jones, E., Braganca, Newbold, C.J., 2018. The effect of an isoflavonid-rich liquorice extract on fermentation, methanogenesis and the microbiome in the rumen simulation technique. FEMS Microbiology Ecology 94, fiy009.
Schwab, C.G., 2005. Nitrogen requirements of cattle. In: Hristov, A., Pfeffer, E. (Eds.), Nitrogen and phosphorus nutrition of cattle. CABI Publishing, Wallingford, UK, pp. 13–71.
Thomas, A.T., 1977. An automated procedure for the determination of soluble carbohydrates in herbage. Journal of the Science of Food and Agriculture 28, 639–642.
Vallimont, J.E., Bargo, F., Cassidy, T.W., Luchini, N.D., Broderick, G.A., Varga, G.A., 2004. Effects of replacing dietary starch with sucrose on ruminal fermentation and nitrogen metabolism in continuous culture. Journal of Dairy Science 87, 4221–4229.
Van Amburgh, M.E., Collao-Saenz, E.A., Higgs, R.J., Ross, D.A., Recktenwald, E.B., Raffrenato, E., Foskolos, A., 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. Journal of Dairy Science 98, 6361–6380.
Van Soest, P., Robertson, J., Lewis, B., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.
Zhu, W.Y., Theodorou, M.K., Longland, A.C., Nielsen, B.B., Dijkstra, J., 1996. Trinci. A. P.J. Growth and survival of anaerobic fungi in batch and continuous-flow cultures. Anaerobe 2, 29–37.