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Impact of Rumen Fluid Storage on In Vitro Feed Fermentation Characteristics

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Abstract: Storing rumen fluid (RF) has the potential to standardize subsequent in vitro feed fermentation studies. The first phase of this experiment aimed to evaluate the effect of two RF storage methods on gas composition and dry matter disappearance (DMD) in wheat grain and lucerne hay under in vitro fermentation. The storage methods were as follows: (1) snap-freezing RF using liquid nitrogen and then storing it at −80 °C (−80 °C); and (2) mixing RF with 5% dimethyl sulfoxide (DMSO), subsequently freezing it at −20 °C (D−20 °C), and comparing it to fresh RF on days 1, 14, and 30 post collection. The objective of the second phase was to quantify the impact of preserving the RF for 180 days at D−20 °C on the in vitro fermentation parameters. The methane composition was lower ($p < 0.001$) in both the preserved RFs than in the fresh RF. There was no difference ($p < 0.05$) in DMD values between days 14 and 30. The average cumulative gas production and DMD from the RF stored at D−20 °C was higher than that from the RF stored at −80 °C. Moreover, there was no difference between day 30 and day 180 in the total gas production and lag time when fermenting with RF preserved at D−20 °C. Therefore, storing RF at D−20 °C is preferable to storing it at −80 °C when access to fresh RF is limited.

Keywords: preservation; gas production; methane; dry matter disappearance; volatile fatty acids

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1. Introduction

In vitro fermentation procedures are used as a source of information for assessing the fermentability and nutritional value of potential feeds and feed additives [1,2] and require the collection of rumen fluid (RF). RF storage may help to reduce collection times, minimize the cost of handling cannulated animals, reduce the ethical concerns associated with cannulation, and avoid the impact of seasonal factors on RF quality [3–5]. Moreover, storing RF has the potential to standardize subsequent in vitro feed fermentation studies by reducing the need for frequent access to fistulated animals and decreasing variations in RF quality [6,7].

Most studies evaluating preserved RF have done so only over a short time (48 h to 7 days) [8,9], or without cryoprotectants [10]. Cryoprotectants such as sucrose, glycerol, and 5% dimethyl sulfoxide (DMSO) are able to protect the cell membranes of micro-organisms during freezing by reducing cell dehydration and intracellular ice crystal formation [11,12]. DMSO is a more effective cryoprotectant than some others, such as glycerol, due to its rapid diffusion through cell membranes and its ability to reduce the formation of intracellular ice crystals [5,13]. However, there is no published information on the storage of RF using DMSO as a preservative for more than 30 days.

In a previous study, Tunkala, et al. [14] evaluated a series of storage techniques and observed that RF preserved by mixing with 5% DMSO (*v*/*v*) and then freezing at −20 °C (D−20 °C) and RF preserved by snap-freezing using liquid nitrogen and storing at −80 °C (−80 °C) showed the best performance when compared with RF stored according to other

methods. However, these methods were not evaluated for gas composition and dry matter disappearance (DMD). Therefore, the aims of this experiment were twofold. Firstly, to evaluate the effects of these RF storage methods on gas composition and DMD for 14 days and 30 days. Secondly, to evaluate the impact of using RF mixed with 5% DMSO and then frozen at −20 °C (D−20 °C) for an extended period of 180 days on various in vitro feed fermentation parameters.

2. Materials and Methods

All procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [15]. Approval to conduct the experiment was obtained from the Agricultural Research and Extension Animal Ethics Committee of the Department of Jobs, Precincts and Regions.

2.1. Experiment Design

Wheat grain and lucerne hay were used as the substrates to be fermented, representing concentrates and forage feeds. The D−20 °C and −80 °C RF storage methods were selected as they were the two best-performing methods in our previous experiment [14] in terms of in vitro fermentation characteristics, such as gas production, protozoa counting, fatty acids, and ammonia-N concentration. Therefore, two substrates (wheat grain and lucerne hay), three RF types (fresh RF, RF preserved at D−20 °C, and RF preserved at −80 °C), and three storage periods (1 day, 14 days, and 30 days) were computed in a 2 × 3 × 3 factorial design in the first phase of this study. Fresh RF was used for incubation on day 1 as a control, and preserved RF was used in other periods. The above substrates were fermented again on the 180th day post collection using RF preserved at D−20 °C in the second phase. The D−20 °C storage method was selected for the second phase because it performed better than the −80 °C storage method.

2.2. Chemical Composition

The samples of wheat grain and lucerne hay were analyzed using near-infrared spectroscopy for dry matter, dry matter digestibility, fat, neutral detergent fiber (NDF), acid detergent fiber (ADF), non-fiber carbohydrate (NFC), and ash in a commercial laboratory (FeedTest Laboratory, Agrifood Technology, Werribee, Victoria). Crude protein was estimated according to the Kjeldahl method (method 954.01) [16].

The dry organic matter digestibility (DOMD) and metabolizable energy (ME) were determined using the formulae from SCA [17] and AFFIA [18], respectively.

$$\text{DOMD} = 0.95\text{DMD}\% - 0.9$$

where DMD represents dry matter digestibility.

$$\text{ME} = 0.858 + (0.138 \times \text{DOMD}\%) + (0.272 \times \text{Fat}\%)$$

where DOMD represents dry organic matter digestibility and fat % based on the chemical composition analysis of substrates.

2.3. Rumen Fluid Collection

The RF was collected, transported, pooled, and filtered according to the procedure described by Gonzalez-Rivas, et al. [19]. Four cannulated mid-lactation Holstein Friesian dairy cows at Agriculture Victoria (Ellinbank, Victoria) served as the donors in equal proportions, and a total of four liters of RF was collected twice in the morning before feeding, as recommended by Belanche, et al. [20]. The cows grazed a perennial ryegrass (*Lollium perenne* L.) pasture, and a wheat and barley grain mix (6 kg DM per day per cow) was supplied in the milking parlor. A handful of solid rumen content was placed in each two-liter bottle to maintain the microbial profile [9]. The bottles were capped with one-way

gas lids and transported to the laboratory in a pre-warmed incubator (Premium blanket warmer, Thermoline Scientific, Wetherill Park, NSW, Australia) at 39 °C.

The RF was mixed and filtered through two layers of cheesecloth to separate the liquid RF and the residual solids. The liquid portion was sub-divided into 12 pre-warmed 0.5 L bottles. Two of these 0.5 L bottles were used as fresh RF and incubated in two consecutive runs on the same day they were collected. Another six 0.5 L bottles of RF were stored at −20 °C after mixing with 5% DMSO (CSA Scientific, 500 mL, Chemsupply, Gillman, Australia) for the D−20 °C treatment. The remaining four 0.5 L bottles of RF were snap-frozen using liquid nitrogen and stored at −80 °C, to be used for the −80 °C treatment. Four bottles of RF stored at D−20 °C and another four stored at −80 °C were used on days 14 and 30. The last two bottles of RF were stored frozen at D−20 °C and were used on 180th day post collection. The RF manipulations were performed under continuous flushing of carbon dioxide.

2.4. Incubation

The substrates were ground to pass through a 1 mm sieve, and 1 g was placed in 250 mL ANKOM bottles with 75 mL of buffer solution dispensed to the bottles using a liquid dispenser. The Kansas buffer solution with a 6.8 pH value was used [21,22]. The RF was thawed in three steps. First, it was placed in a 4 °C refrigerator for 30 min, then it was placed in a cold water bath (around 15 °C) for 30 min, and finally the water bath was turned up to 39 °C to warm the water gradually and thaw the RF for one additional hour.

Twenty-five ml of RF was added to the substrate and buffer mixture. The bottles were capped with pressure sensor modules of the ANKOM system, and carbon dioxide was flushed into the bottles before they were placed in water baths (20-L Analogue Waterbath, WB20; Ratek Instruments Pty Ltd., Boronia, VIC, Australia) pre-warmed to 39 °C. Four blank bottles were also incubated with buffer and RF as a control. The incubation was carried out in two runs for 24 h with a total of eight replicates per treatment. The incubations using RF preserved at −80 °C and D−20 °C were conducted simultaneously using the same sources of RF.

The gas pressure measured by the ANKOM gas production system was converted to moles of gas produced using the 'ideal' gas law and Avogadro's law [22].

$$n = P(V/RT)$$

where n = quantity of gas in moles, P = pressure in psi (1 psi = 6.8948 kPa), V = gas volume (mL), R = gas constant (8.1345 L kPa/K·mol), and T = temperature in Kelvin. This was then converted to volume (mL) of gas produced using Avogadro's law, where 1 mol of gas holds 25.6 L at 39 °C (312 Kelvin), with pressure converted to kilopascals; therefore, gas measured in moles was converted to gas volume in milliliters as follows:

$$\text{Gas volume (mL)} = n \times 22.4\ \text{L} \times 1000$$

2.5. Variables Measured

Gas is accumulated in the headspace and vented through the valve of each ANKOM gas production module. Gas production data were collected every five minutes through an automated pressure sensor connected to the computer via a wireless connection. The lag time and gas production rate were computed using GenStat with the Gompertz model:

$$y = A + C^{\exp\{-\exp[-B(X - M)]\}}$$

in which A is the y-intercept, B is the rate of gas production (mL/h), C is the maximum gas produced (maximum gas mL/g DM), X is the total time (h) of incubation, and M is the time (h) at which the maximum rate of gas production is reached.

The post-fermentation pH value of the RF was measured using a pH meter (Oakton® Acorn™ series pH 6 m, Sigma-Aldrich, North Ryde, Australia). The gas sample collection

and analysis followed the laboratory procedure previously described by Alvarez Hess, et al. [23]. Briefly, a gas sample was collected manually through the vent valve adapter of the module using a needle and an air-tight glass syringe (SGE International Pty Ltd., Ringwood, VIC, Australia). The gas samples were transferred into pre-vacuumed exetainers® screw capped with septa (12 mL soda glass vial, Labco Ltd., Buckinghamshire, UK). The exetainers® were pre-vacuumed with a pressure of $<5 \times 10^{-3}$ torr using the vial evacuation manifold. The samples' methane, carbon dioxide, and nitrous oxide proportions were determined using a gas chromatograph (7890A Agilent, Santa Clara, CA, USA) equipped with an autosampler and flame ionization detector.

A filter bag with a pore size of 25 µm (F57 ANKOM bag, ANKOM Technology, Macedon, NY, USA) was used to quantify the DMD of the incubated feeds according to the method described by Prates, de Oliveira, de la Fuente Oliver, Abecia, and Fondevila [8]. Heat-sealed F57 filter bags were incubated holding 1 g of substrate in a 250 mL ANKOM bottle with 100 mL of 3:1 ratio buffer media to RF solution. The filter bags were washed slightly using cold water in a plastic water diffuser (tattoo squeeze) after 24 h of incubation, and the residue was dried in a 60 °C incubator for 48 h. The samples were weighed for the remaining dry matter and computed from the initial weight using the following equation [14,24].

$$\% \text{ DMD} = ((W_1 + W_2) - W_3) \times 100$$

where W_1 is the weight (g) of the empty filter bag, W_2 is the weight (g) of the substrate, and W_3 is the weight (g) of the final oven-dried bag with the feed residue.

The partition factor (PF) was calculated as DMD (mg) divided by the ml of gas produced in vitro after 24 h of incubation [25], quantifying in vitro fermentation efficiency.

In addition to the above variables, volatile fatty acids (VFA) and ammonia-N concentrations were estimated after the 180th day of storage in the second phase of this study. The VFAs were determined using a gas chromatograph fitted with a flame ionization detector [26] and using methyl valerate as the internal standard. The ammonia concentration was quantified using the colorimetric technique with a multiscan colorimetric plate reader (Thermo Multiskan Spectrum, Thermo Fisher Scientific, Waltham, MA, USA), according to the process described by Weatherburn [27].

2.6. Data Analysis

The data on gas production, composition, lag time, gas production rate, DMD, pH, ammonia-N, and VFA were analyzed via unbalanced ANOVA using the GenStat V18 statistical package:

$$Y_{ijk} = \mu + S_i + P_j + D_k + (SPD)_{ijk} + e_{ijk}$$

where Y is the continuous dependent variable, µ is the overall mean of the population, S_i is the fixed effect of the substrate (i = wheat, lucerne hay), P_j is the fixed effect of the RF storage method (j = fresh RF, D−20 °C, and −80 °C), Dk is the fixed effect of the duration of storage (k = 1 day, 14 days, 30 days, or 180 days), $(SPD)_{ijk}$ is the interaction effect between the independent variables, and e is the residual error.

Each ANKOM module was used as a unit of analysis to test the variation and compare the mean differences for *p*-values less than 5%. The treatment structure was substrate × storage methods × days of storage, with the ANKOM systems (n = 2) as a blocking factor. Interactions between fixed effects were analyzed for each variable using the Linear Mixed Model of GenStat [28].

3. Results

The chemical compositions of the wheat grain and lucerne hay used as substrates in this experiment are presented in Table 1. The crude protein, NDF, ADF, fat, and ash content of the lucerne hay was greater than that of the wheat grain, while the wheat grain possessed higher NFC, ME, dry matter digestibility, and organic matter digestibility compared with the lucerne hay.

Table 1. Chemical composition of wheat grain and lucerne hay used as substrates.

Parameter	Lucerne Hay	Wheat Grain
Dry Matter	95.2	90.3
Moisture	4.8	9.7
Crude Protein	22.5	13.5
ADF	24.6	4.9
NDF	39.9	11.1
Fat	4.1	1.5
Ash	9.9	<1.0
NFC	23.7	75.9
Dry matter digestibility	65.1	94.3
Organic matter digestibility [1]	62.0	92.7
ME [1]	9.60	14.2

[1] calculated values; ADF: acid detergent fiber; NDF: neutral detergent fiber; NFC: non-fiber carbohydrate; ME: metabolizable energy. The dry matter and moisture were measured as a percentage of the sample. The crude protein, ADF, NDF, fat, ash, NFC, dry matter digestibility, and organic matter digestibility were measured as a percentage of dry matter. The ME was measured as megajoules of energy per kilogram of dry matter (MJ/kg DM).

The effects of using RF preserved at −80 °C across storage periods (14 days and 30 days) on the fermentation parameters are presented in Table 2 and Figure 1. The gas production, methane concentration, and DMD of the feeds fermented using the fresh RF were larger than those of the feeds fermented using RF preserved at −80 °C ($p < 0.001$). There was no difference between gas production values on days 14 and 30 for −80 °C. The lag time of the feeds fermented using the fresh RF was lower ($p < 0.001$) than that of the feeds fermented using RF preserved at −80 °C. The gas production rate was decreased, and the pH was increased when RF preserved at −80 °C was used ($p < 0.001$). There was no difference between the fermentation parameters on days 14 and 30 for DMD and methane for either feed. The partition factor of the wheat grain fermented using RF preserved at −80 °C did not differ on days 14 and 30.

Table 2. The effects of feedstuffs (wheat grain vs. lucerne hay) and rumen fluid storage days (14 days and 30 days) using RF snap-frozen in liquid nitrogen and preserved at −80 °C on fermentation parameters [1].

Parameter	Wheat Grain			Lucerne Hay			SED [2]	S	D	S × D
	1	14	30	1	14	30				
Gas production, mL/g	132.9	78.2	71.3	71.6	51.7	44.8	6.85	<0.001	<0.001	0.065
Lag time, h	0.75	5.26	5.20	0.45	1.37	1.39	0.195	<0.001	<0.001	<0.001
Gas prod. rate, mL/h	5.39	3.26	2.97	3.01	2.12	1.83	0.293	<0.001	<0.001	0.018
pH	5.48	5.61	5.67	6.11	6.23	6.28	0.009	<0.001	<0.001	0.052
DMD, %	58.6	53.9	53.1	56.0	49.1	48.5	1.63	<0.001	<0.001	0.51
Methane, % gas	8.79	3.62	3.03	6.33	3.79	2.75	1.980	0.42	0.003	0.56
CO_2, % gas	91.2	96.4	97.0	93.7	96.2	97.3	2.03	0.32	0.007	0.77
Partition factor, mg/mL	4.80	7.22	7.47	8.04	9.56	11.3	0.660	<0.001	<0.001	0.54

[1] All measures taken after 24 h of fermentation and pooled across day of treatment. [2] Standard error of the difference for substrate (S) × days of storage (D).

The volume and rate of the gas production, amount of methane, and DMD of the incubations using the RF preserved at D−20 °C were lower ($p < 0.001$) compared with the incubations using the fresh RF (Table 3). In contrast, compared with the incubations using the preserved RF, the incubations using the fresh RF had lower ($p < 0.001$) lag time, pH, carbon dioxide, and PF for both feeds. The gas production of the wheat grain fermented using the RF preserved at D−20 °C for 30 days was 106.3 mL/g and was reduced ($p < 0.001$) by 8.5% after using the same RF preserved for 180 days (Table 3 and Figure 2). There was no difference between the gas production from the lucerne hay fermented using the RF preserved at D−20 °C for 14 days, 30 days, and 180 days.

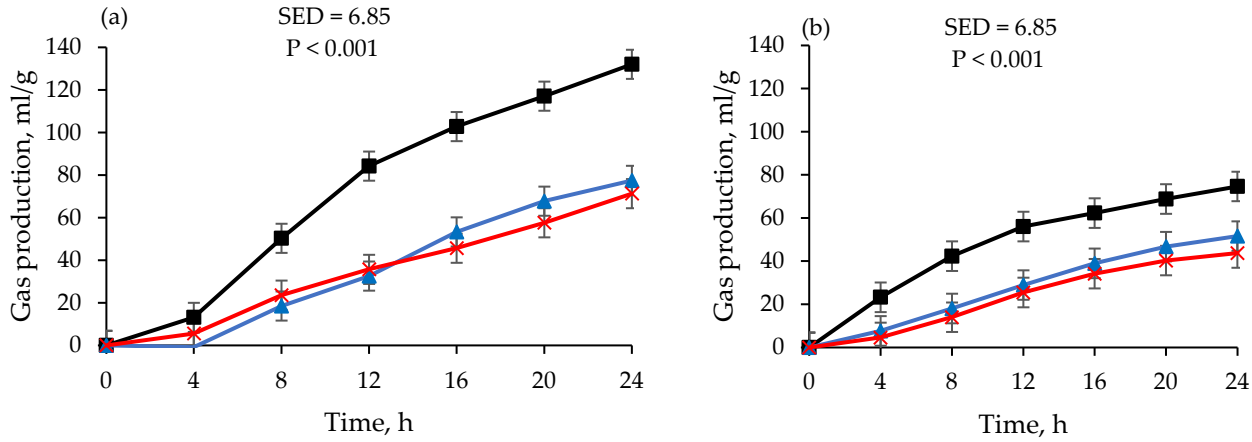

Figure 1. Gas production curves for 24 h incubation of feedstuffs ((**a**) wheat grain and (**b**) lucerne hay) at 39 °C using fresh rumen fluid on day 1 (—■—) and rumen fluid snap-frozen using liquid nitrogen and stored at −80 °C (−80 °C) for 14 days (—▲—) and 30 days (—×—) at varying incubation times. Data at day 1 are for fresh RF. Data are least-square means of gas production after converting gas pressure into gas volume using Avogadro's law.

Table 3. The effects of feedstuffs (wheat grain vs. lucerne hay) and rumen fluid storage conditions (fresh rumen fluid vs. rumen fluid mixed with 5% DMSO and stored at −20 °C) across storage days 1, 14, 30, and 180 on fermentation parameters [1].

Parameter	Wheat Grain				Lucerne				SED [2]	S	D	S × D
	1	14	30	180	1	14	30	180				
Gas production, mL/g	132.9	88.3	106.3	97.3	71.6	60.3	54.9	57.5	6.94	<0.001	<0.001	0.015
Lag time, h	0.73	2.04	3.69	3.90	0.45	0.52	0.31	0.80	0.699	<0.001	<0.001	<0.001
Gas prod. rate, mL/h	5.38	3.69	4.43	4.10	3.00	2.53	2.08	2.41	0.269	<0.001	<0.001	0.023
pH	5.48	5.61	5.59	5.58	6.11	6.22	6.25	6.28	0.092	<0.001	<0.001	0.64
DMD, %	58.6	57.3	55.1	50.4	56.0	52.0	52.0	48.8	1.53	<0.001	<0.001	0.58
Methane, % gas	8.79	3.72	3.13	0.66	6.33	1.83	2.62	0.42	0.337	<0.001	<0.001	0.002
Carbon dioxide, % gas	91.2	96.3	96.9	99.3	93.7	98.2	97.4	99.6	0.34	<0.001	<0.001	0.002
Partition factor, mg/mL	4.80	6.85	5.20	5.14	8.04	8.87	10.5	8.48	0.581	<0.001	<0.001	0.001
Ammonia-N, mL/g	0.29	0.26	0.25	0.22	0.95	0.69	0.31	0.29	0.016	<0.001	<0.001	0.003

[1] All measures taken after 24 h of fermentation and pooled across day of treatment. [2] Standard error of the difference for substrate (S) × days of storage (D).

The wheat grain lag time (h) was longer ($p < 0.001$) when the RF preserved at D−20 °C was used than when the fresh RF was used; however, no difference was observed between successive runs of lucerne hay (days 14, 30, and 180) fermented using the same RF (it ranged from 0.31 h to 0.80 h). The lucerne hay lag time was lower ($p < 0.001$) than that of the wheat grain in all the fermentation periods. Conversely, the gas production rate (mL/h) was greater ($p < 0.001$) when the fresh RF was used for the incubation of both feeds than when the RF preserved at D−20 °C was used. Moreover, the lucerne hay gas production rate showed a linear reduction as the RF storage period increased, and was lower ($p < 0.001$) than that of the wheat grain for all storage times.

The DMD values were the same for the wheat grain fermented using the fresh RF and the RF preserved at D−20 °C for 14 days, and for the lucerne hay at days 14 and 30. The DMD declined ($p < 0.001$) by 8.5% for the wheat grain and 6.2% for the lucerne hay when the RF stored at D−20 °C for 180 days was used instead of that stored for 30 days. The methane percentage of the wheat grain (8.79%) and the lucerne hay (6.33%) fermented using the fresh RF was larger ($p < 0.001$) than that fermented using the RFs preserved at D-20 °C, while there was no difference in methane production between days 14 and 30 for

either feed. The percentage of methane in the total gas production was below 1% for both feeds fermented using the RF preserved at D−20 °C for 180 days.

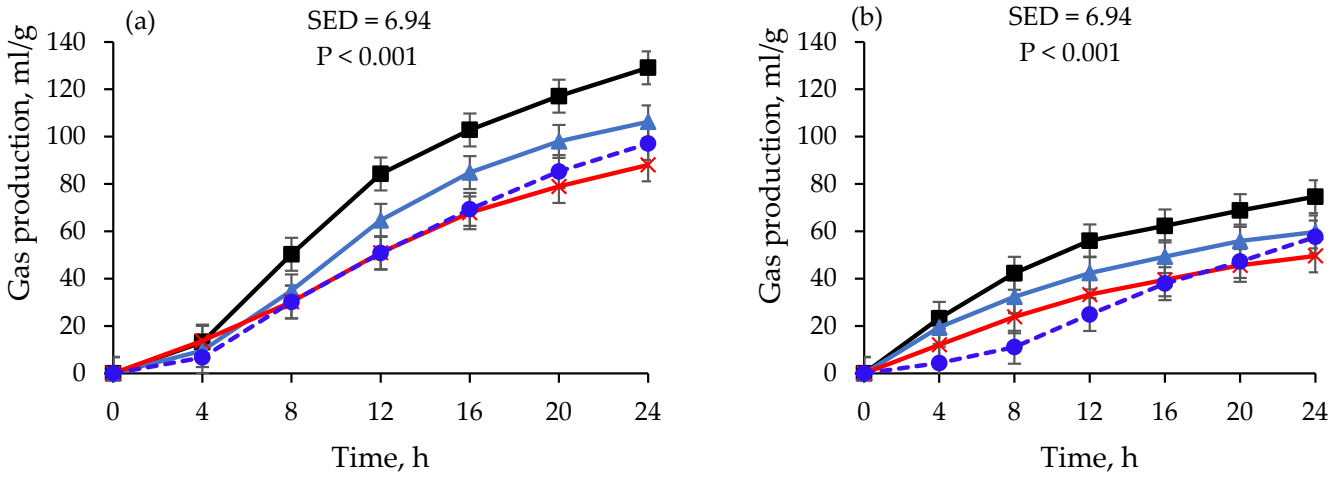

Figure 2. Gas production curves for 24 h incubation of feedstuffs ((**a**) wheat grain and (**b**) lucerne hay) at 39 °C using fresh rumen fluid on day 1 (—■—) and rumen fluid treated with DMSO and kept at D−20 °C for 14 days (—▲—), 30 days (—×—), and 180 days (- -●- -) at varying incubation times. Data at day 1 are for fresh rumen fluid. Data are least-square means of gas production after converting gas pressure into gas volume using Avogadro's law.

The partition factor was the same between days 30 and 180 for the wheat grain, and between days 14 and 180 for the lucerne hay fermented using the RF preserved at D−20 °C. The ammonia-N values fell ($p < 0.001$) for the lucerne hay over the preservation days, but was stable for the wheat grain fermented using the RF stored at D−20 °C. The ammonia-N concentration of the lucerne hay (0.26 mL/g) was larger ($p < 0.001$) than that of the wheat grain (0.22 mL/g) fermented using the RF preserved for 180 days at D−20 °C. There was significant interaction ($p < 0.05$) between the feeds and the duration of RF storage at D−20 °C, except for the pH and DMD parameters.

The total VFA values of the wheat grain and lucerne hay incubated using the RF preserved at D−20 °C for 180 days were 86.8 mM/L and 79.3 mM/L, respectively (Figure 3). The acetic acid produced from the wheat grain (37.3 mM/L) was greater ($p < 0.001$) than that of produced from the lucerne hay (31.8 mM/L), and the propionic acid produced from the wheat grain (24.4 mM/L) was lower ($p < 0.001$) than that produced from the lucerne hay (36.9 mM/L). The isobutyric, butyric, isovaleric, and valeric acid contents (mM/L) from the feeds fermented using the RF preserved for 180 days at D−20 °C were 2.98, 3.63, 1.55, and 2.00 for the lucerne hay and 11.8, 6.62, 3.54, and 3.10 for the wheat grain, respectively.

Comparing −80 °C and D−20 °C (Tables 2 and 3), the incubations using the RF stored at D−20 °C had greater ($p < 0.001$) gas volume and rate of production than those of the RF stored at −80 °C for both feeds over the days of storage, except for the lucerne hay on day 30. The lag time for the feeds fermented using the RF preserved at D−20 °C was lower than that of the feeds fermented using the RF stored at −80 °C. There was no difference between D−20 °C and −80 °C in terms of concentration of methane and carbon dioxide on days 14 and 30. However, the DMD of both feeds was higher ($p < 0.001$) for the RF stored at D−20 °C than for the RF stored at −80 °C, and the PF of both of the feeds fermented at D−20 °C was also lower ($p < 0.05$) than that of the feeds fermented at −80 °C.

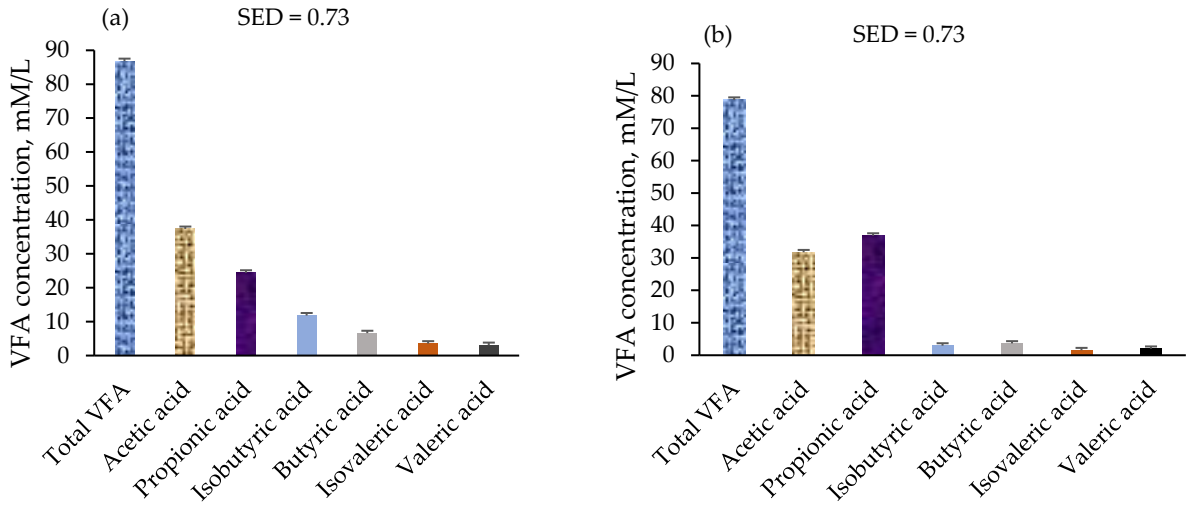

Figure 3. The amount of volatile fatty acids (VFAs) produced from (**a**) wheat grain and (**b**) lucerne hay after 24 h fermentation using rumen fluid, either fresh or treated with DMSO and kept at D−20 °C for 180 days. Data are least-square means of different VFAs in the substrate.

4. Discussion

The cumulative gas production after 24 h was lower when using the preserved RF than when using the fresh RF. Previous researchers have reported similar results for gas production with various feeds fermented in fresh and preserved RF [14,29,30]. The differences in microbial concentration caused by mechanical damage to cell structures from cold shock could be the reason for the reduced gas production when using preserved RF [29,31]. Nevertheless, despite the effect of the cold shock on the micro-organisms, the RF stored under both storage conditions utilized in the present experiment still possessed substantial fermentative capacity. Moreover, there was no difference between the gas production values of either feed after using the RF preserved at D−20 °C for 30 days and 180 days. The niche of the microbes most affected during the storage could have been occupied by the less affected groups, resulting in stable fermentation [8] and implying the possibility of using preserved RF for up to 180 days.

Lag time (h) is the time prior to gas production, or the initial delay before the release of gas begins [32,33]. The micro-organisms re-establish the required inputs, multiply, and grow during the lag time to enable the fermentation of the substrate [34]. The lag time was lower when the feeds were fermented using the fresh RF, and it was stable between day 30 and day 180 for the wheat grain, while for the lucerne hay fermented at D−20 °C, it was stable between day 1 and day 180. This stability demonstrated the potential of D−20 °C RF storage to maintain microbial density and diversity. Moreover, the lag time of the lucerne hay was shorter compared with that of the wheat grain. The excessive availability of soluble starch in the wheat grain can lower the pH, resulting in reduced microbial activity and degradation in the initial phase [35]. Additionally, when concentrates such as wheat grain are fermented, the microbial population needs to be altered towards osmophilic and sugar-loving microbes such as protozoa before fermentation commences [9]. Thus, this result confirms that lag time is affected by the differences in substrate quality [36,37].

Methane and carbon dioxide compositions are used as a source of information for the examination of potential feeds and feed additives that can reduce greenhouse gas emissions from livestock [1,2]. However, reports on the impact of RF storage on methane and carbon dioxide production from in vitro experiments are limited. The proportion of methane gas decreased by over 50% in both feeds following the use of the preserved RF and reached below 1% when using the RF preserved for 180 days. This shows that methanogens are highly susceptible to the process of cold storage and thawing, and this can be attributed to a reduction in the protozoa population caused by freezing [10,14] as protozoal concentration and methane emissions are linearly correlated [38]. A study by Belanche, Palma-Hidalgo,

Nejjam, Serrano, Jimenez, Martin-Garcia, and Yanez-Ruiz [9] also demonstrated declining methanogens in frozen RF. The reduction in methanogens resulted in a lower methane concentration and an increased percentage of carbon dioxide in gas samples collected after using preserved RF. Therefore, preserved RF muse be used with caution in methane studies.

The interactions between RF storage methods, feeds, and duration of storage could be attributed to differences in microbial concentration between the fresh and preserved RFs and differences in chemical composition between the feeds. Therefore, it was not surprising that the duration of storage was inversely related to the volume of gas production and methane concentration. Confirming the interaction, the carbon dioxide percentages and PF values increased with the increasing duration of RF storage for both feeds.

The use of 5% DMSO as an additive for RF storage limits the effect of cell dehydration during freezing and prevents intracellular ice crystal formation [13]. Abdel-Aziz, et al. [39] and Denek, Can, and Avci [5] demonstrated that DMSO was a more effective cryoprotectant than glycerol because of its rapid diffusion into cells, whereby it protects the cell membranes of micro-organisms and allows a superior protozoa recovery rate. Moreover, the RF mixed with 5% DMSO and frozen at −20 °C exhibited higher fermentation parameters than the RF preserved at −80 °C (with and without DMSO) and at D−20 °C [14], suggesting that the effect of DMSO depends on the storage temperature.

There was no difference between the storage methods in terms of the concentration of methane and carbon dioxide on days 14 and 30. However, the incubations using the RF stored at D−20 °C had greater gas volume, gas production rate, and DMD values than the incubations using the RF stored at −80 °C for both feeds over all the days of storage, and hence, reduced PF values. The lag time was also lower for the feeds fermented using the RF preserved at D−20 °C as the reactivation and proliferation of micro-organisms required less time than in the case of the feeds fermented using the RF stored at −80 °C. These findings support those of a previous study [14], which suggested D−20 °C as a modest option for RF storage.

The DMD of the fresh RF was greater than that of the preserved RF, and it further reduced with the increasing storage period. This was possible because a higher amount of dry matter could be consumed by active and concentrated micro-organisms in the fresh RF, and the longer RF storage period reduced the microbial population [14]. This finding is consistent with the study of [10], in which it was demonstrated that fresh RF resulted in higher DMD, followed by RF preserved at −20 °C for rapeseed meal and grass nuts. Prates, de Oliveira, de la Fuente Oliver, Abecia, and Fondevila [8] also reported that the DMD of fresh RF was greater than that of RF preserved using liquid nitrogen. However, there was no difference in the DMD values on days 14 and 30 between the different storage methods.

The PF values increased for the preserved RF compared with those of the fresh RF as a response to the declining gas production; however, it was stable for the lucerne hay incubated at D−20 °C on days 14, 30, and 180. The PF of the wheat grain did not differ between fermentations on days 30 and 180 of RF storage at D−20 °C. Lower PF indicates a lower dry matter requirement for gas production [25,40]. Therefore, the efficiency of the fresh RF is higher in terms of dry matter conversion and gas production. The stable PF value of the feeds fermented at D−20 °C validates the possibility of using D−20 °C for in vitro feed fermentation as a storage method.

The freezing of RF can reduce ammonia-N by minimizing protein metabolism [9], which explains the ammonia-N reduction when the RF preserved at 180 days was compared with the fresh RF. The decline in ammonia-N concentration with increased storage duration is consistent with a prior study by Tunkala, DiGiacomo, Hess, Dunshea, and Leury [14]. However, there was no difference between the ammonia-N values of the lucerne hay incubated using the RF preserved for 180 days (0.29 mL/g) compared with that incubated for 30 days (0.32 mL/g) at D−20 °C, indicating the potential of D−20 °C for 180 days as an RF storage method.

The total VFA declined for the feeds fermented using the RF preserved for 180 days at D−20 °C, and the reduction reached 20–25% for both feeds compared with the RF

stored for 14 days and 30 days in the study by Tunkala, DiGiacomo, Hess, Dunshea, and Leury [14]. However, the VFA values from the feeds fermented using the RF stored at D−20 °C for 180 days indicates the survival of a sufficient quantity of beneficial micro-organisms required for feed fermentation.

The differences between the wheat grain and lucerne hay in in vitro fermentation parameters were expected. The wheat grain promoted higher fermentation in terms of gas production, DMD, and the proportion of methane gas in both the fresh and preserved RFs as the NFC content and the organic and dry matter digestibility of the wheat grain were greater than those of the lucerne hay. NFC and feed digestibility are positively correlated with the volume of cumulative gas [41,42] and methane production [43,44]. The lucerne hay produced a greater carbon dioxide percentage and PF, and a lower lag time. The lower digestibility and the higher ADF and NDF contents of the lucerne hay may also account for the increased PF in the lucerne hay. PF is inversely proportional to cumulative gas production, as energy is consumed to produce gas [3,40]. Therefore, the differences between the fermented feeds may be attributed to variations in their chemical composition.

In summary, the effect of the storage methods was consistent in magnitude for each substrate in terms of the variables used, excepting the insignificant differences between both methods in terms of gas composition values. All the quantified parameters of the substrates incubated using the preserved RF were lower than those of the substrates incubated using the fresh RF. However, D−20 °C produced fewer PF differences in both feeds, and the gas volume, rate of gas production, and DMD of the substrates fermented at D−20 °C were higher than those of the substrates fermented at −80 °C. Moreover, for the feeds fermented using the RF preserved at D−20 °C, there was no difference between day 30 and day 180 in total gas production for either feed, in PF for the wheat, or in ammonia-N for the lucerne hay. The lag times of the feeds fermented using the RF stored at D−20 °C were also lower than those of the feeds fermented using the RF stored at −80 °C. Therefore, D−20 °C showed relatively better performance than −80 °C and has the potential to be used as an RF storage method for up to 180 days. Future work may focus on whether extended fermentation beyond 24 h with preserved RF may yield fermentation characteristics quantitatively similar to those of fresh RF fermented for 24 h.

5. Conclusions

The general trend of in vitro fermentation parameters was that the fresh RF had greater gas production, DMD, and PF values, followed by the RF stored at D−20 °C and the RF stored at −80 °C. Therefore, storing RF at D−20 °C is preferable to storing it at −80 °C. Moreover, D−20 °C can preserve RF for up to 180 days for the in vitro screening of feeds when access to fresh RF is limited. Nevertheless, additional RF storage studies are required to minimize the existing differences between fresh and preserved RF in terms of in vitro feed fermentation characteristics. Further research on the microbial quality and enzymatic activities of preserved RF could also help us to understand the basic chemical and biological changes that occur during and after the storage process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9040392/s1.

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Data Availability Statement: The data presented in this study are available and have been uploaded as "Supplementary Files".

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