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Article

Evaluation of nutritional methods to reactivate preserved ruminal inoculum assessed through *in vitro* fermentation kinetics and forage digestibility

María G. Domínguez-Ordóñez [a]

Luis A. Miranda-Romero [a]

Pedro A. Martínez-Hernández [a]

Maximino Huerta-Bravo [a]

Ezequias Castillo-Lopez [b]*

[a] Universidad Autónoma Chapingo. Departamento de Zootecnia, 56220. Texcoco, Estado de México, México.

[b] Universidad Nacional Autónoma de México. Facultad de Estudios Superiores Cuautitlán, Medicina Veterinaria y Zootecnia. Cuautitlán, Estado de México, México.

*Corresponding author: ezequias@huskers.unl.edu

Abstract:

This study evaluated the effect of the media and pre-incubation time for reactivating preserved ruminal inoculum, assessed through *in vitro* fermentation kinetics and dry matter digestibility (IVDMD). In the first experiment, treatments were 1) CONTROL, fresh ruminal fluid, 2) LOW24, inoculum reactivated by 24 h pre-incubation in a basal culture solution, 3) MODE24, inoculum reactivated by 24 h pre-incubation in basal culture solution, yeast extract and peptone from casein; and 4) HIGH24, inoculum reactivated by 24 h pre-incubation in basal culture solution, yeast extract, peptone from casein and carbohydrates. In the second experiment, treatments evaluated were 1) CONTROL, fresh ruminal fluid, and 2) HIGH12, similar to HIGH24 but inoculum was pre-incubated for 12 h. Each experiment included three replicates. Maximum gas volume (*Vm)*, lag phase (*L*), gas production rate (*S*) and IVDMD were measured using four fermentation substrates. Main effects of inoculum and fermentation substrate, and interactions, were analyzed. Compared to CONTROL, *Vm*, *L* and *S* were negatively affected ($P<0.01$) by preservation of inoculum. However, HIGH24 displayed an

improvement (*P*<0.01) in fermentation kinetics and IVDMD compared to MODE24 or LOW24. In the second experiment, HIGH12 displayed lower (*P*<0.01) IVDMD at 72 h compared to CONTROL. Alfalfa and orchardgrass had higher (*P*<0.01) *Vm* and IVDMD compared to cocuite and guinea grass. Overall, reactivation of preserved ruminal inoculum by pre-incubation for 24 h in a medium containing yeast extract, peptone from casein and carbohydrates performed better compared to reactivation by pre-incubation for 12 h; however, fermentation kinetics and IVDMD were still depressed compared to fresh ruminal fluid.

Key words: Forage digestibility, Fermentation, Preservation, Ruminal inoculum.

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Introduction

In vitro techniques are commonly used to evaluate fermentation and digestibility of feed ingredients utilized in ruminant rations[1,2,3]. However, the need for fistulated animals for ruminal fluid collection is an important limitation of these techniques[4,5,6]. Thus, preservation of ruminal fluid might overcome this limitation as it allows the use of inoculum without having to keep donor animals[7,8,9]. This is conducted by using glycerol to minimizes microbial cell damage[10,11,12] and maintain the microbial community[13,14].

The appropriate reactivation of preserved inoculum before being used remains largely unknown. Lyophilized ruminal fluid underestimates *in vitro* fermentation and dry matter digestibility compared to fresh ruminal fluid when reconstituted in McDougall's buffer[8]. The depression in fermentation parameters[15] presumably due to cell damage[8,9] or microbial death[9] may explain this underestimation. In addition, limitations in the availability of nutrients such as nitrogen[16,17] and carbohydrates[18,19,20] may influence reactivation, growth and activity of ruminal microbes. Thus, it has been recently acknowledged that the reactivation of preserved bacteria is a critical step in obtaining active microorganisms, and that the reactivation conditions should be optimized[21,22,23].

Because of the limited information on strategies to improve the reactivation of ruminal inoculum, research to find a cost-effective and practical approach is warranted. Thus, the objectives of this study were to evaluate the effects of the culture medium used and the incubation time needed for proper reactivation of lyophilized ruminal inoculum. Response variables assessed were based on *in vitro* fermentation kinetics and dry matter digestibility of alfalfa, orchardgrass, cocuite and guinea grass. The hypothesis was that there will be no difference in *in vitro* forage digestibility and fermentation kinetics between preserved and fresh ruminal inoculum.

Material and methods

Experiments were carried out at Universidad Autónoma Chapingo. Animals used in the experiments were managed according to the guidelines and University regulations.

Fermentation substrates and chemical analysis

Four forage species commonly used for grazing ruminants in Mexico were utilized as fermentation substrates (Table 1): alfalfa (*Medicago sativa* L*)* cv San Miguel, orchardgrass (*Dactylis glomerata* L) cv Potomac, cocuite (*Gliricidia sepium* (Jacq.) Kunth ex Walp.) and guinea grass (*Panicum maximum* Jacq.*)* cv Tanzania. Alfalfa, guinea grass and orchardgrass were cut at 7 cm above ground level; only leaves of cocuite were collected by hand from branches of several trees. Enough sample material was collected to obtain at least 1 kg of sample (DM basis) for each forage species. Collected forage samples were dried in a forced air oven at 60 ºC for 96 h; they were ground to pass through a 1 mm screen (Wiley Mill, Arthur H. Thomas Co., Philadelphia, PA) and analyzed for crude protein, ash, ether extract[24] (methods # 976.06; # 942.05; # 920.39, respectively). Acid detergent fiber (ADF)[25], neutral detergent fiber (NDF) were assayed without heat stable amylase and expressed inclusive of residual ash[25], and soluble sugars[26].

Table 1: Analyzed chemical composition (g/kg DM) of the four forage species used as fermentation substrates for *in vitro* fermentation kinetics and *in vitro* dry matter digestibility experiments

	Chemical composition (g/kg DM)					
Forage species	**Crude protein**	**Ash**	**Ether extract**	**ADF**	**NDF[A]**	**Sugars**
Alfalfa	206	119	11	350	442	41
Orchardgrass	197	163	26	400	540	35
Cocuite	183	85	24	367	465	47
Guinea grass	65	123	5	564	779	29

ADF= acid detergent fiber; NDF= neutral detergent fiber.

[A] neutral detergent fiber was assayed without heat stable amylase and was expressed inclusive of residual ash.

Ruminal fluid collection, preservation and reactivation

In vitro procedures reported for each experiment included three replicates[27,28]. Similar to previous studies[29], fresh ruminal fluid was collected for each *in vitro* run from three ruminally fistulated adult Creole rams with an average body weight of 53.0 kg. Donor

rams were fed a diet containing 80 % forage and 20 % concentrate. Feed was offered at 0900 and 1500 h every day; *ad libitum* intake was allowed. In addition, clean fresh water was available *ad libitum*. Rams were fitted with a ruminal cannula to collect ruminal fluid by suction[30]. The collected ruminal fluid was filtered through four layers of cheesecloth and equal volumes of ruminal fluid from each donor were combined in order to obtain a representative sample and to prevent animal-to-animal variations[31,32,33].

Five percent (v/v) glycerol (Sigma-Aldrich, St. Louis, MO) was incorporated to serve as a cryoprotectant of ruminal inoculum[12,13]. Aliquots were placed in 10-mL sterile glass containers. Containers were hermetically sealed and frozen at -70 ºC for 3 d. Lyophilization was conducted as previously described[8] (Labconco Lyph Lock, model 195) under vacuum (-0.133 mBar), and inoculum was stored until later use. The reactivation of the preserved inoculum was conducted by reconstituting lyophilized samples in a cysteine solution to a volume equal to that of the original strained ruminal fluid. This solution contained 2.5 g of L-cysteine, 2.5 g of sodium sulfite and 0.1 mL of resazurin (1%) dissolved in 15 mL of sodium hydroxide (2N), distilled water was added to make a total volume of 100 mL (Table 2), which served as a buffer and created a reduced environment in the media, simulating the reduced conditions of the rumen. Reconstituted inoculum was incubated at room temperature for 10 min to allow rehydration, the reconstituted inoculum was transferred to 100 mL of culture medium, then it was pre-incubated at 39 °C for 24 or 12 h. The culture medium used and the pre-incubation time varied depending on the experiment, as described below.

Table 2: Ingredient composition of the three media utilized for the reactivation of lyophilized ruminal inoculum

Ingredient of medium used for reactivation	**Inoculum type**		
	LOW24	**MODE24**	**HIGH24**
	----------	Amount per 100 mL	--------
	--		
Distilled water, mL	50	50	50
Ruminal fluid[A], mL	29	29	29
Sodium carbonate solution (8%), mL	5	5	5
Mineral solution I[B], mL	7	7	7
Mineral solution II[C], mL	7	7	7
Cysteine solution[D], mL	2	2	2
Resazurin solution 1%, mL	0.10	0.10	0.10
Yeast extract, g	---	0.50	0.50
Peptone from casein, g	---	0.50	0.50
Ground forage[E], g	0.25	0.25	0.25
Glucose, g	---	---	0.30
Cellobiose, g	---	---	0.30
Starch, g	---	---	0.25

[A]Strained through 4 layers of cheesecloth, 2 times centrifuged at 13,416 ×g and sterilized at 15 psi for 15 min.

[B]Containing 6.0 g of potassium hydrogen phosphate per liter of distilled water[44].

[C]Containing 6.0 g of monobasic potassium phosphate; 6.0 g ammonium sulfate; 12 g sodium chloride; 2.45 g magnesium sulfate monohydrate; and 1.6 g calcium chloride monohydrate per liter of distilled water[44].

[D]2.5 g of L-cysteine dissolved in 15 mL of sodium hydroxide (2N), 2.5 g of sodium sulfide and 0.1 mL of rezasurin (1%) volume was brought to 100 mL; the solution was heated and it was sterilized using an autoclave.

[E]Ground guinea grass.

Ruminal inocula evaluated

Experiment 1. Four types of ruminal inoculum were evaluated for measurements of fermentation kinetics and IVDMD. A fresh ruminal fluid (Control) was compared to lyophilized inocula reactivated by pre-incubation for 24 h in 1 of 3 culture media (Table 2). Specifically, treatments were 1) CONTROL, fresh ruminal fluid; 2) LOW24, inoculum reactivated in a medium containing 100 mL of a basal culture solution (composed of 50 % distilled water, 29 % clarified ruminal fluid, 14 % mineral solutions I and II, 5 % sodium carbonate, 2 % cysteine solution) and 0.1 % resazurin; 3) MODE24, inoculum reactivated in a medium containing 100 mL of the basal culture solution, 0.1 % resazurin, 0.5 g of yeast extract (Sigma-Aldrich, St. Louis, MO) and 0.5 g of peptone from casein (Bioxon Becton Dickinson, Mexico); and 4) HIGH24, inoculum reactivated in a medium containing 100 mL of a basal culture solution, 0.1 % resazurin, 0.5 g of yeast extract (Sigma-Aldrich, St. Louis, MO), 0.5 g of peptone from casein (Bioxon Becton Dickinson, Mexico), 0.3 g of glucose, 0.3 g of cellobiose and 0.25 g of starch. Media also included 0.25 g of ground forage on a DM basis (Table 2).

Experiment 2. In the second experiment, two types of ruminal fluid were compared: 1) CONTROL, fresh ruminal fluid, and 2) HIGH12, inoculum reactivated using a medium previously described for HIGH24. However, in this experiment, preserved ruminal inoculum was reactivated by pre-incubation for only 12 h as an attempt to find a more practical and faster approach for the reactivation process.

Fermentation kinetics and IVDMD

In each experiment, fresh and reactivated ruminal inocula were combined with a diluting agent containing the reduced mineral solutions I and II and the cysteine solution[34] at a ratio of 1:9 (v/v, ruminal fluid: diluting agent, Table 2). CO_2 was flushed while adding the diluting agent to the ruminal fluid, which was maintained at 39 ºC.

Afterwards, the fermentation kinetics and forage IVDMD were determined by combining 90 mL of diluted ruminal inoculum with 0.5 g of fermentation substrate using 125-mL

glass bottles. The determination of the parameters of fermentation kinetics was based on the procedure utilized for the measurement of gas[35,36]. More specifically, gas pressure (kg/cm^2) was recorded at 1, 2, 4, 6, 10, 14, 18, 24, 30, 38, 48 and 72 h of incubation. After recording this value at each time point, the gas pressure was reset to cero. The values of pressure were then converted to volume of gas (mL/g DM of substrate); to do so, a standard curve was first generated by injecting known volumes of CO_2. The equation of this standard curve was generated by adding a linear regression line, and this equation was: Gas volume (mL/g of substrate) = pressure (kg/cm^2)* 39.46 + 0, with an R^2 of 0.94. This standard curve was generated at room temperature. The use of this technique has also been recently reported by other investigators[29].

The accumulated volume of gas at each time point was used to estimate the parameters of the fermentation kinetics: maximum volume of gas (*Vm*; mL/g), *lag* phase (*L*; h), and the rate of gas production (*S*; h^{-1}). This was conducted using a logistic model described by Schofield *et al*[37]:

$$\text{Volume of gas} = \frac{Vm}{1+exp^{(2-4\times S\times (t-L))}} \qquad \text{(Equation 1)}$$

Where:

Vm is maximum volume;

S is the rate of gas production;

t is the time point of measurement;

L is the *lag* phase.

In addition to parameters of fermentation kinetics, the IVDMD of substrates was determined at 24 and 72 h fermentation ($IVDMD_{24}$, and $IVDMD_{72}$, respectively). At each time point, the content of corresponding fermentation bottles was filtered through Whatman filter paper No. 4. The residue was dried at 100 ºC for 12 h in a forced air oven and the dry weight was recorded. Then, IVDMD was calculated relative to the amount of original sample used.

Statistical analysis

The GLM procedure of SAS[38] was used. In each experiment (n =3), the mean values within each fermentation substrate were considered as the experimental unit. Given the controlled experimental conditions, significant effect was declared at $P<0.01$; this level of significance may also contribute to reduce the type I error risk. Only when first order interaction was not significant, mean separation for main effects was conducted by Tukey; otherwise, paired mean separation by *t-test* was performed. Dispersion parameter reported is the largest standard error of the mean (SEM).

Data from Exp 1 were analyzed as a completely randomized experimental design with a 4 × 4 factorial arrangement of treatments (4 inoculum types and 4 fermentation

substrates). Data from Exp 2 were analyzed according to a completely randomized experimental design with a 2 × 4 factorial arrangement of treatments (2 inoculum types and 4 fermentation substrates). In both experiments, the main effects of inoculum type and fermentation substrate were analyzed. The interaction of inoculum type × fermentation substrate was also evaluated. The statistical model for the analyses was:

$$Y_{ijk} = \mu + \tau_i + \beta_j + \tau\beta_{ij} + \varepsilon_{ijk}$$

Where:

$\mathbf{Y_{ijk}}$ represents the observation of the ijk treatment;

$\boldsymbol{\mu}$ represents the overall mean;

$\boldsymbol{\tau_i}$ represents the inoculum type i;

$\boldsymbol{\beta_j}$ represents the fermentation substrate j;

$\boldsymbol{\tau\beta_{ij}}$ represents the interaction effect of the inoculum type i and the fermentation substrate j.

The residual term ε_{ijk} was assumed to be normally, independently, and identically distributed, with variance σ^2_e.

Results

Chemical composition of forages used as fermentation substrates

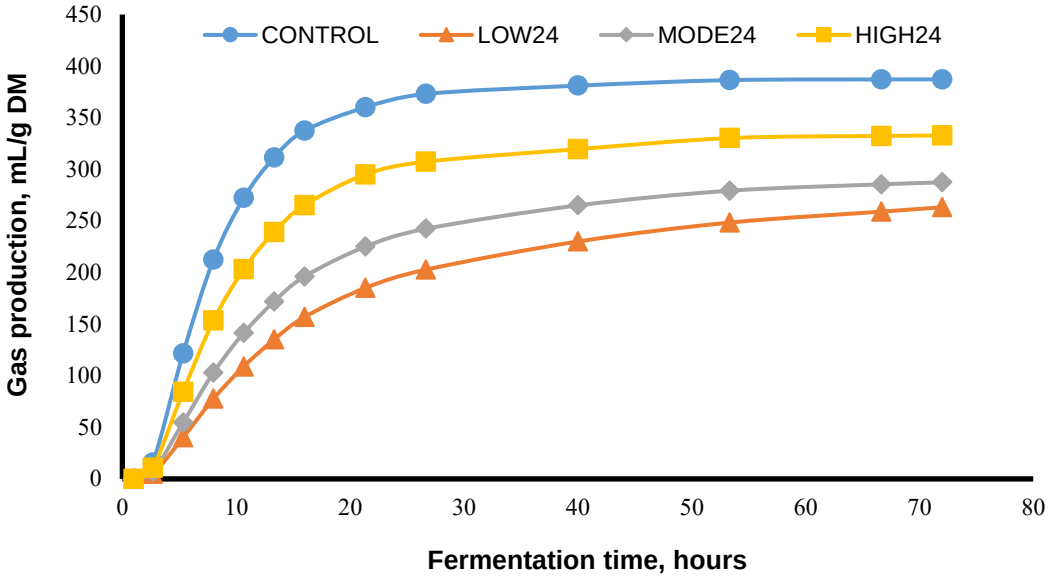
Analyzed chemical composition of the four forages used is listed in Table 1. Guinea grass was low in crude protein and high in fiber contents (65.0 and 779.0 g/kg DM for crude protein and NDF, respectively); whereas alfalfa was high in protein and low in fiber content (206.0 and 442.0 g/kg DM for crude protein and NDF, respectively), with these nutrients having intermediate values for orchardgrass and cocuite.

Fermentation kinetics and IVDMD for inocula reactivated by 24 h pre-incubation

Figure 1 illustrates *in vitro* gas production for the CONTROL and inocula reactivated by pre-incubation for 24 h. CONTROL displayed the fastest and greatest maximum gas production compared to the other treatments. Even with the HIGH24 treatment, fermentation was reduced by around 50 % during the first hours of incubation. Within the preserved inocula, HIGH24 displayed the greatest maximum gas production, followed by MODE24 and by LOW24. In addition, Figure 2 displays gas production for each fermentation substrate. Alfalfa had the greatest and fastest maximum gas production

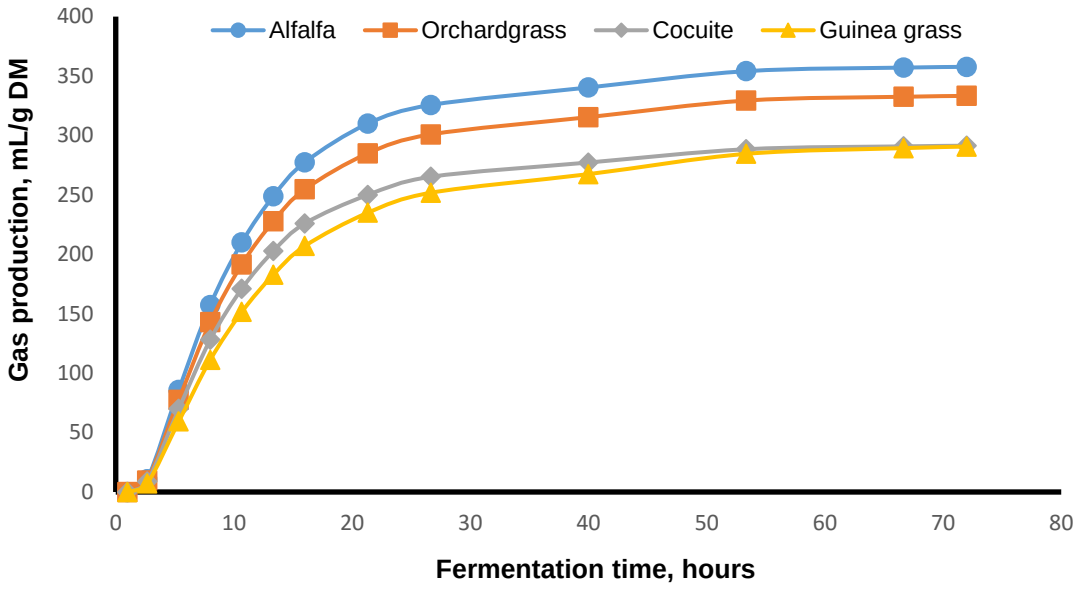
compared to the rest of forages. Orchard grass displayed intermediate values for gas production, with cocuite and guinea grass having the lowest values.

Figure 1: *In vitro* gas production for the control and the preserved inocula reactivated by pre-incubation for 24 h in different culture media



CONTROL= fresh ruminal fluid; LOW24= inoculum reactivated by 24 h pre-incubation in a basal culture solution; MODE24= similar to LOW24, but included yeast extract and peptone from casein; HIGH24= similar to MODE24, but included carbohydrates. Control: *Vm*=387.15 mL/g, *L*=4.06 h, *S*=0.041 h^{-1}; LOW24: *Vm*=266.11 mL/g, *L*=13.70 h, *S*=0.018 h^{-1}; MODE24: *Vm*=288.22 mL/g, *L*=7.62 h, *S*=0.023 h^{-1}; HIGH24: *Vm*=332.83 mL/g, *L*=8.05 h, *S*=0.32 h^{-1}.

Figure 2: *In vitro* gas production for four forages when values for fresh ruminal fluid and inocula reactivated by pre-incubation for 24 h were averaged



Alfafa: *Vm*=357.90 mL/g, *L*=5.22 h, *S*=0.030 h^{-1}; orchardgrass: *Vm*=333.50 mL/g, *L*=11.77 h, *S*=0.029 h^{-1}; cocuite: *Vm*=291.50 mL/g, *L*=3.83 h, *S*=0.030 h^{-1}; guinea grass: *Vm*=291.20 mL/g, *L*=12.20 h, *S*=0.025 h^{-1}.

More specifically, fermentation kinetics, $IVDMD_{24}$ and $IVDMD_{72}$ were affected by treatment and fermentation substrate (Table 3). Regardless of the nutrient composition of the medium used for reactivating the inoculum, *Vm* decrease ($P<0.01$) when reactivated inoculum was used compared to fresh ruminal fluid, with this difference being greater during the first 24 h (Figure 1). However, treatment HIGH24 displayed greater *Vm* ($P<0.01$) compared to treatments MODE24 or LOW24 regardless of fermentation substrate. In addition, both alfalfa and orchardgrass had the highest ($P<0.01$) *Vm* across treatments with an average of 345.7 ± 14.40 mL/g. The interaction of treatment × fermentation substrate was significant ($P<0.01$) for *L* and *S*. Specifically, *L* was highest ($P<0.01$) for LOW24 when orchardgrass or guinea grass was used as fermentation substrates with an estimate of 22.29 h. However, there was no difference in *L* among treatments when cocuite was used as fermentation substrate. Furthermore, *S* was lowest ($P<0.01$) for treatment LOW24 regardless of fermentation substrates with an average of 0.018 h^{-1}. However, *S* reached highest ($P<0.01$) values in most fermentation substrates when CONTROL was used as inoculum followed by HIGH24.

Table 3: Parameters of the fermentation kinetics (*Vm*, *L* and *S*) and *in vitro* dry matter digestibility at 24 and 72 h ($IVDMD_{24}$, $IVDMD_{72}$) for fresh inoculum or lyophilized inocula reactivated by 24-h pre-incubation in one of three culture media

		Fermentation parameters[A]						
Inoculum type	**Fermentation substrate**	***Vm* (mL/g)**	***L* (h)**	***S* (h^{-1})**	**$IVDMD_{24}$ (g/kg)**	**$IVDMD_{72}$ (g/kg)**	**% gas at 72 h**	**% IVDM at 24h/72h**
CONTROL	Alfalfa	435.75a	2.80d	0.043a	558.0a	622.0a	100.0	89.7
	Orchardgrass	390.25a	4.89d	0.041ba	538.0a	618.0a	100.0	87.1
	Cocuite	346.25a	2.46d	0.044a	466.0ba	508.0dc	100.0	91.7
	Guinea grass	376.35a	6.09dc	0.036cb	398.0c	550.0cb	99.9	72.4
LOW24	Alfalfa	309.30b	7.31bc	0.019e	432.0cb	590.0b	94.9	73.2
	Orchardgrass	303.20b	20.2a	0.017e	314.0d	624.0a	87.1	50.3
	Cocuite	207.85c	2.89d	0.020e	324.0d	454.0e	97.1	71.4
	Guinea grass	244.10c	24.38a	0.016e	180.0f	424.0e	94.0	42.5
MODE24	Alfalfa	319.45b	5.14dc	0.025d	482.0ba	674.0a	99.1	71.5
	Orchardgrass	307.75b	10.32bc	0.018e	402.0c	636.0a	92.0	63.2
	Cocuite	288.80c	4.91d	0.025d	382.0c	516.0c	99.1	74.0
	Guinea grass	236.90c	10.12bc	0.021ed	252.0e	484.0d	96.1	52.1
HIGH24	Alfalfa	367.15a	5.64dc	0.033cb	524.0a	630.0a	99.9	83.2
	Orchardgrass	333.00b	11.68bc	0.039ba	502.0ba	656.0a	99.9	76.5
	Cocuite	323.25b	5.04dc	0.030dc	404.0c	512.0c	99.8	78.9
	Guinea grass	307.75b	9.85bc	0.025d	324.0d	554.0b	98.5	58.5
Inoculum means	CONTROL[y]	387.15a	4.06c	0.041a	490.0a	574.5a	100.0	85.3
	LOW24[x]	266.11c	13.70a	0.018d	312.5d	523.0b	90.0	59.8
	MODE24[w]	288.22c	7.62b	0.023c	379.5c	577.5a	98.1	65.7
	HIGH24[v]	332.83b	8.05b	0.032b	438.5b	588.0a	99.8	74.6
Forage means	Alfalfa	357.90a	5.22b	0.030a	499.0a	629.0a	99.8	79.3
	Orchardgrass	333.50a	11.77a	0.029a	439.0b	633.5a	99.3	69.3
	Cocuite	291.50b	3.83b	0.030a	394.0c	497.5b	99.8	79.2
	Guinea grass	291.20b	12.61a	0.025b	288.5d	503.0b	98.1	57.4
SEM[B]		14.40	1.290	0.0012	10.80	10.50		
P-values	Inoculum	0.001	0.001	0.001	0.001	0.001		
	Forage	0.001	0.001	0.001	0.001	0.001		
	Inoculum × forage	0.0766	0.001	0.0002	0.0012	0.0001		

[A]*Vm*: maximum volume of gas; *L*: *lag* phase; *S*: rate of gas production.

[B]SEM: the largest standard error of the mean is reported.

a-f: Means in the same column with different superscripts are different ($P<0.01$).

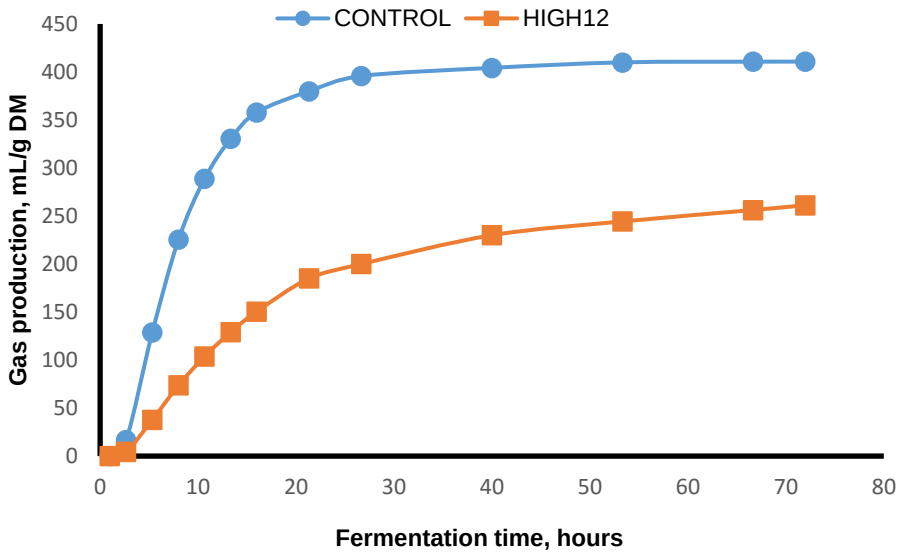
The treatment × fermentation substrate interaction was significant ($P<0.01$) for $IVDMD_{24}$ and $IVDMD_{72}$. Specifically, when alfalfa was used as fermentation substrate, $IVDMD_{24}$

for treatments MODE24 and HIGH24 was similar ($P \geq 0.1$) to CONTROL with an average of 521.3 ± 10.8 g/kg. However, $IVDMD_{24}$ for alfalfa was lower ($P<0.01$) for treatment LOW24 compared to CONTROL with estimates of 432.0 and 558.0 ± 10.8 g/kg for LOW and CONTROL, respectively. Likewise, $IVDMD_{72}$ for treatments MODE24 and HIGH24 were similar ($P \geq 0.1$) to CONTROL with an average of 642.0 ± 10.5 g/kg. However, $IVDMD_{72}$ for alfalfa was lower ($P<0.01$) for LOW24 compared to CONTROL24 with estimates of 590 and 622.0 ± 10.5 g/kg for LOW24 and CONTROL, respectively. The lowest ($P<0.01$) $IVDMD_{72}$ was observed for treatment LOW24 when cocuite was used as fermentation substrate with an average of 439.0 ± 10.5 g/kg. Overall, regardless of fermentation substrate, there was a depression ($P<0.01$) in IVDMD for LOW24 compared to any of the other treatments.

Fermentation kinetics and IVDMD for inoculum reactivated by 12 h pre-incubation

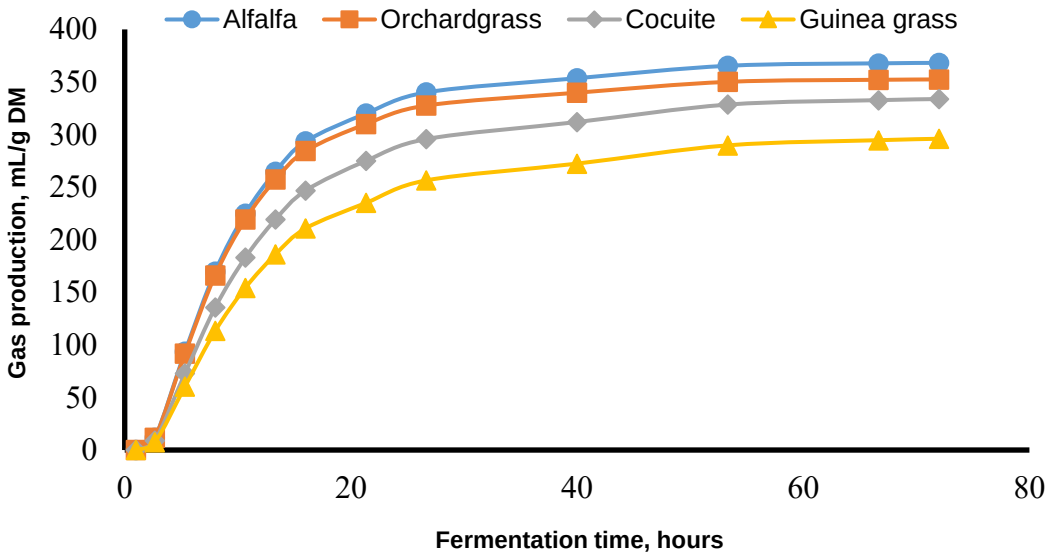
Figure 3 illustrates *in vitro* gas production for the CONTROL and inoculum reactivated by incubation for 12 h. CONTROL displayed a faster and greater maximum gas production compared to HIGH12. In addition, Figure 4 illustrates gas production for each fermentation substrate. Alfalfa displayed the greatest and fastest maximum gas production compared to the rest of forages, with cocuite and guinea grass having the lowest values.

Figure 3: *In vitro* gas production for the control and the preserved inoculum reactivated by pre-incubation for 12 h in a culture medium



CONTROL= fresh ruminal fluid; HIGH12, inoculum reactivated by 12 h pre-incubation in a basal culture solution, yeast extract, peptone from casein and carbohydrates. Control= *Vm*=410.80 mL/g, *L*=5.42 h, *S*=0.032 h^{-1}; HIGH12= *Vm*=264.97 mL/g, *L*=4.29 h, *S*=0.017 h^{-1}.

Figure 4: *In vitro* gas production for four forages when values for fresh ruminal fluid and inoculum reactivated by pre-incubation for 12 h were averaged



Alfafa: *Vm*=368.30 mL/g, *L*=2.13 h, *S*=0.032 h^{-1}; orchardgrass: *Vm*=352.51 mL/g, *L*=9.19 h, *S*=0.033 h^{-1}; cocuite: *Vm*=334.16 mL/g, *L*=3.79 h, *S*=0.027 h^{-1}; guinea grass: *Vm*=296.56 mL/g, *L*=4.31 h, *S*=0.025 h^{-1}.

Specifically, fermentation kinetics, $IVDMD_{24}$ and $IVDMD_{72}$ were affected by treatment and fermentation substrate (Table 4). *Vm* was greater ($P<0.01$) for CONTROL compared to HIGH12, with estimates of 410.80 and 264.97 ± 13.050 mL/g, respectively. The interaction of inoculum type × fermentation substrate was significant for *L* ($P<0.011$); orchardgrass and alfalfa incubated in HIGH12 had the greatest and lowest ($P<0.01$) *L*, respectively; with estimates of 12.4 and 0.07 ± 0.700 h for orchardgrass and alfalfa. Likewise, an interaction ($P<0.01$) was detected for *S*; alfalfa incubated in CONTROL and cocuite incubated in HIGH12 had the greatest and lowest *S*, respectively; with estimates of 0.047 and 0.013 ± 0.007 h^{-1} for alfalfa and cocuite. Regardless of forage type, the $IVDMD_{24}$ was greater for CONTROL compared to HIGH12 with estimates of 520.6 and 374.3 ± 12.70 g/kg, respectively. There was an inoculum type × fermentation substrate interaction ($P<0.01$) for $IVDMD_{72}$ with alfalfa and orchargrass incubated in CONTROL having the greatest $IVDMD_{72}$, and cocuite and guinea grass having the lowest $IVDMD_{72}$ values.

Table 4: Parameters of fermentation kinetics (*Vm*, *L* and *S*) and *in vitro* dry matter digestibility at 24 and 72 h ($IVDMD_{24}$, $IVDMD_{72}$) for fresh inoculum or lyophilized inoculum reactivated by 12 h pre-incubation in a nutrient-rich medium

		Fermentation parameters[A]						
Inoculum type	Fementation substrate	*Vm* (mL/g)	*L* (h)	*S* (h^{-1})	$IVDMD_{24}$ (g/kg)	$IVDMD_{24}$ (g/kg)	% gas at 72 h	% IVDM at 24 h
CONTROL	Alfalfa	457.33d	4.19b	0.047f	625.3a	673.3d	100.0	92.9
	Orchardgrass	409.96c	6.14b	0.042e	594.6a	678.6d	100.0	87.6
	Cocuite	400.80c	4.36b	0.041e	463.9b	573.3b	100.0	80.9
	Guinea grass	375.10b	7.00b	0.034d	398.6c	551.9b	99.9	72.2
HIGH12	Alfalfa	279.26a	0.07a	0.017b	461.3b	619.9c	94.7	74.4
	Orchardgrass	295.06a	12.24c	0.024c	407.9b	657.3d	97.7	62.1
	Cocuite	267.53a	3.23ab	0.013a	350.6c	478.6a	82.9	73.3
	Guinea grass	218.03a	1.63ab	0.015ab	278.6d	459.9a	90.2	60.6
Inoculum means	CONTROL	410.80b	5.42	0.041b	520.6a	619.3b	100.0	84.1
	HIGH12	264.97a	4.29	0.017a	374.3b	553.9a	93.1	67.6
Forage means	Alfalfa	368.30b	2.13a	0.032	543.3a	646.6b	99.9	84.0
	Orchardgrass	352.51b	9.19c	0.033c	501.3a	667.9b	99.8	75.1
	Cocuite	334.16b	3.79ab	0.027b	407.3b	525.9a	99.5	77.4
	Guinea grass	296.56a	4.31b	0.025a	338.6c	505.9a	99.2	66.9
SEM[B]		13.050	0.700	0.0007	12.70	7.80		
P-values	Inoculum type	0.0001	0.0359	0.0001	0.0001	0.0001		
	Forage species	0.0003	0.0001	0.0001	0.0001	0.0001		
	Inoculum type × forage species	0.1200	0.0001	0.0001	0.0295	0.0006		

[A]Gas production model was Schofield et al. (1994): $\text{Volume of gas} = \frac{Vm}{1+exp^{(2-4\times S\times(t-L))}}$

where Vm is maximum volume of gas; *L* is the *lag* phase, and *S* is the rate of gas production.

[B]SEM: the largest standard error of the mean is reported.

a-f: Means within column with at least 1 letter in common are not different ($P<0.01$).

Discussion

Ruminal fluid sampling and the use of glycerol as a cryoprotectant

Studies[31,32,33] have found differences in *in vitro* fermentation patterns between ruminal fluid from different donors. The source of ruminal fluid can influence *in vitro* fermentation and digestibility trials[31,39]. Differences in fermentation patterns among animals observed by those researchers can be partially attributed to differences in the composition of the established bacterial community among host animals[40,41]. Therefore, in the present study, ruminal fluid samples from three donors were pooled to obtain a representative sample to prevent bias due to ruminal fluid source.

The use of glycerol improves the preservation of the ruminal bacterial community[12,13,14]. Benefits of glycerol may be explained by peripheral vitrification providing protection to the bacterial cytoplasmic membranes from potential damage that can be caused by ice crystal formation[42]. More specifically, glycerol penetrates the cells, which can then protect them from damage by maintaining a semi-fluid state[43,44]. Consequently, the use of glycerol not only protects the integrity and viability of the ruminal bacterial cells, but may also prevent degradation of the microbial DNA[13].

Fermentation kinetics and IVDMD of preserved inocula

In vitro fermentation kinetics and IVDMD revealed differences between fresh and lyophilized ruminal fluid. When compared to fresh ruminal fluid, the greatest depression in fermentation kinetic parameters was observed when lyophilized inocula were reactivated in media without sugars or growth promoters. These observations are in line with other studies[15], indicating a depression on fermentation parameters with frozen inocula, which can be explained by a decrease in microbial activity due to microbial death or nutrient limitation[9]. In addition, researchers have reported[8] that protein degradation rates with preserved ruminal microorganisms were 4 to 8 times slower than when using fresh ruminal fluid. Furthermore, the use of inoculum preserved through freezing affects fermentation parameters during the first hours of fermentation[30], and deep freezing may represent a better preservation method compared to freezing at –20 °C. Consequently, in agreement with recent reports, the reactivation of preserved bacteria is one of the most critical steps in obtaining active and effective microorganisms for *in vitro* fermentation trials[21,22,23].

In this study, compared to fresh ruminal fluid, the negative effects of lyophilization on fermentation kinetics and IVDMD was less severe when ruminal inocula were reactivated in a nutrient-rich medium including a basal culture solution, growth promoters and sugars. These observations indicate that growth promoters such as yeast extract and peptone from casein, and carbohydrates such as glucose, cellobiose and starch enhance the reactivation of ruminal microorganisms, thus, improving *in vitro* fermentation. The need for yeast extract in the medium for adequate bacterial reactivation and growth may be attributed to the absence of the genes for the synthesis of some proteinogenic amino acids such as arginine and asparagine in the genome of some ruminal bacterial species[45,46], which indicates that these amino acids contained in the yeast extract need to be included in the medium. Additionally, peptone from casein and carbohydrates provide readily available nitrogen and energy stimulating microbial reactivation, growth and activity[19,47]. It is interesting to note the different patterns (gas production at different time points) among the fermentation curves, which suggests that different microbial populations may be acting on the substrates at each fermentation time point. Further research should aim at investigating shifts in the structure of the microbial community[48,49] using techniques

such as high-throughput DNA sequencing[50,51,52], which allows a broad evaluation of the profile microbial community from highest to lowest taxonomic levels.

It has been found that, in comparison to fresh ruminal fluid, digestibility of alfalfa decreased 17.63 % when using frozen inoculum or lyophilized inoculum reactivated by 24 h pre-incubation in McDougall's solution[9]. In contrast to previous observations[9], in the present study, $IVDMD_{72}$ was not affected when using lyophilized inoculum reactivated by 24 h pre-incubation in a nutrient-rich medium; indicating that, compared to the use of McDougall's solution, using a nutrient-rich medium containing a wider range of nutrients represents a better approach for stimulating the reactivation and activity of ruminal microorganisms.

When averaged across fermentation substrates (i.e. alfalfa, orchard grass, cocuite and guinea grass), the IVDMD for any of the reactivated inocula was negatively affected, compared to the values obtained with the control. However, within lyophilized inocula, the reactivation by 24 h pre-incubation in a nutrient-rich medium displayed the best performance. In addition, when the inoculum was reactivated by 12 h pre-incubation, IVDMD values were lower compared to the control fresh ruminal fluid. It is important to note that, at 72 h fermentation all forages but cocuite reached almost 100 % of the total gas produced. This indicates that 72h-fermentation rates are not suitable for measuring the effectiveness of the treatments, which also suggests that a better approach would be to measure fermentation rates and IVDMD at 24 or 48 h of fermentation.

Effect of fermentation substrate on fermentation kinetics and IVDMD

The use of fermentation substrates with a wide range of nutrient composition facilitated the evaluation of our hypothesis under different scenarios. Overall, our results revealed that forages from temperate zones, namely alfalfa and orchardgrass, had higher *Vm* and IVDMD compared to their counterparts from the tropical regions. These observations were likely due to differences in the structural components of the plant cell-wall existing between forages from temperate and those from tropical zones[53].

Furthermore, alternative inoculum sources have been suggested for *in vitro* fermentations. One of these sources is ruminant feces; however, results have been inconsistent. For example, fecal inoculum has been demonstrated to be effective for *in vitro* gas production studies[54]; nonetheless, fecal inoculum from sheep was not comparable to fresh ruminal fluid when evaluating *in vitro* dry matter digestibility[55]. In addition, other studies, have revealed that fecal inoculum does not perform as good as ruminal fluid in *in vitro* fermentation techniques[55,56], which may be due to differences in the bacterial populations between the rumen and the lower gastrointestinal tract[57].

Conclusions and implications

In vitro fermentation kinetics and IVDMD were affected by lyophilization of ruminal fluid. In most cases, fermentation parameters *Vm*, *L* and *S* were negatively affected when lyophilized ruminal inoculum was used. However, when glycerol was added to the lyophilized ruminal inocula and was reactivated for 24 h in a pre-incubation nutrient-rich medium, including growth promoters and sugars, the negative effects of lyophilization on *in vitro* fermentation kinetics and IVDMD were less severe. As expected, alfalfa and orchardgrass had higher *Vm* and IVDMD compared to cocuite and guinea grass. Results reported in this study should provide new insights into reactivation of preserved ruminal inoculum as well as its utilization in *in vitro* fermentation and digestion trials for laboratories with limited access to fistulated animals or fresh ruminal fluid. Future research should explore changes in rumen microbial populations during *in vitro* fermentations using high-throughput DNA sequencing to understand how shifts in the microbial profiles lead to the different patterns observed among fermentation curves.

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