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Development of Protocol for Lyophilization of Goat Rumen Fluid

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ABSTRACT

Background: Rumen fluid has been used as microbial inoculum to treat indigestion in ruminant animals and to conduct *in vitro* rumen fermentation experiments. Lyophilization of the goat rumen fluid will provide continuous supply of rumen inoculums either for laboratory studies or for transfaunation in treating digestive disorders sequelae to high grain rations. However, no standard protocol is available for lyophilizing goat rumen fluid. Hence, this study was designed to develop a protocol to lyophilize goat rumen fluid as an alternate source for fresh goat rumen fluid.

Methods: The study was conducted using 5 × 3 × 3 factorial design with four different cryoprotectants *viz.,* 10% skim milk powder, 10% skim milk powder + 5% sodium glutamate, 5% glycerol, 5% DMSO and no cryoprotectant, at three pre freezing (2, 24 and 48 hours) and three freeze drying (8, 24 and 32 hours) time intervals to standardize protocol for lyophilizing goat rumen fluid. The viability of rumen microbes in the "lyophilized goat ruminal inoculum", was determined *via in vitro* gas production study.

Result: Pre freezing (-80°C deep freezer) duration of 48 hours with 32 hours of time duration in lyophilizer (-45°C) was ideal for lyophilizing goat rumen fluid with or without the addition of various cryoprotectants. Glycerol used at 5% as cryoprotectant resulted in significantly (P<0.05) highest gas production at all (12, 24 and 48) incubation hours studied indicating better viability of rumen microbes.

Key words: Cryoprotectant, Goat rumen fluid, Lyophilization, Lyophilised goat rumen inoculum.

INTRODUCTION

Lyophilization or freeze drying is a low temperature dehydration process used to preserve biological materials *viz.,* bacteria, virus and yeast for vaccine production, bio-pharmaceutical products like proteins for therapeutic purposes and fruits and foods as astronaut's meals and military rations. Preservation of microorganisms by freeze drying is the preferred method for long term storage of cultures and it makes the culture stable and transportable at ambient temperature (Morgan *et al*. 2006).

Rumen fluid consists of a wide variety of microorganisms such as bacteria, protozoa and fungi, which ferment the diet consumed by the ruminants in an anaerobic environment. The symbiotic relationship among the different group of diverse microorganisms in the rumen fluid causes microbial fermentation that results in end products such as gases and volatile fatty acids that supply nutrients and energy to the host. Rumen fluid has been effectively used as microbial inoculum to treat indigestion in ruminant animals (DePeters and George, 2014), rumen fluid sampled from live animals is used in laboratory *in vitro* rumen fermentation experiments to evaluate the nutritive value and gaseous emissions from ruminant feeds (Lopez *et al*., 2005 and Yanez-Ruiz *et al*. 2016). Rumen fluid is also used to inoculate continuous fermenters for studies of rumen fermentation (Hiristov *et al*., 2012).

Rumen transfaunation is a common recommended practice of transferring rumen fluid from a healthy donor animal to an animal with digestive disorders so as to treat indigestion or to bring back the normal rumen function in sick animals (DePeters and George, 2014). Generally, rumen fluid is obtained from slaughter house or from live animals through intubation of stomach tube or through surgically implanted rumen fistula for cud transfaunation purposes (Laflin and Gnad, 2008). In order to provide rumen inoculum continuously, either for the purpose of *in vitro* rumen fermentation studies or for transfaunation and reduce the need to frequently collect rumen fluid from live animals, preserving the rumen fluid and creating stocks would be of immense help. The preservation techniques of rumen fluid using low temperatures and the addition of cryoprotectants and freeze-drying have been studied in sheep and cattle (Denek *et al*. 2010 and Chaudhry *et al*. 2012). However, the preservation techniques of rumen fluid obtained from goats needs to be standardized. Standardization of preservation techniques of rumen fluid obtained from goats and development of a protocol for the preparation of lyophilized goat rumen inoculum will ensure availability of goat rumen

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inoculum for transfaunation at the time of sudden emergency conditions like fermentation disorders arising in goats as a sequelae to grain overload. Grain overload giving rise to emergency situations in goats is common in semi-urban and urban regions of Tamil Nadu, India, where feeding rice to livestock is a common practice. Hence, this study was designed to standardize the lyophilization protocol of goat rumen fluid so that it can serve as an alternate source for fresh goat rumen fluid while treating digestive disorders in goats.

MATERIALS AND METHODS

The study was carried out during the year of 2020 at Institute of Animal Nutrition, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India. The study was designed to develop a protocol to lyophilize goat rumen fluid for preparation of lyophilized goat rumen inoculum. The study was executed using 5 × 3 × 3 factorial design with six replications in three runs. Four different cryoprotectants *viz.,*10% skim milk powder, 10% skim milk powder + 5% sodium glutamate solution, 5% glycerol solution, 5% DMSO solution and no cryoprotectant, at three prefreezing time intervals *viz.*, 2, 24 and 48 hours, at three freeze drying time intervals *viz.,* 8, 24 and 32 hours were tested.

The rumen fluid for the study was collected from healthy goats that were maintained in the farm on a standard ration of 70:30 roughage to concentrate ratio. The rumen fluid was collected using a motor-powered stomach tube. The tube consisted of a 150 cm long polyvinyl chloride orogastric tubing with a 1000 mL air tight container attached with an electric vacuum pump with 0.08 MPa of maximum continuous pressure. During rumen liquor collection, the head of the animal was restrained and ruminal fluid was collected by passing the tube using an oral speculum down the esophagus into the rumen. Ruminal fluid collected in the container was transferred into an airtight flask previously flushed with CO_2 to maintain an anaerobic condition during transit. The collected ruminal fluid was strained through four-layer cheese cloth with continuous flushing of CO_2, pH was checked and only ruminal fluid having pH of 6.7 and above was chosen for lyophilization. The strained ruminal fluid 20 ml per vial was transferred into glass vials added with different cryoprotectants *viz.,*10% (w/v) skim milk powder, 10% skim milk powder + 5% (w/v) sodium glutamate solution, 5% (v/v) glycerol solution, 5% (v/v) DMSO solution. A control group was maintained wherein no cryoprotectant was added. For pre freezing the containers were stored at -80°C in a deep freezer adopting three prefreezing time intervals *viz.*, 2, 24 and 48 hours. The containers were then transferred immediately to the freeze dryer so as to prevent the formation of effervescence inside the pre freezed glass containers during vacuum creation in the lyophilization process. Three freeze drying time intervals *viz.,* 8, 24 and 32 hours were adopted at -45°C. At the end of the respective time period the containers were removed and visually examined for complete lyophilization. The containers that had revealed complete lyophilization were selected to document lyophilization yield.

The yield of the lyophilized goat ruminal inoculum was calculated as per the formula given below.

$$\text{Lyophilization yield (\%)} = \frac{\text{Wt of lyophilized powder in vial- Empty vial wt}}{\text{Volume of ruminal flluod taken for lyophilization (ml)}} \times 100$$

To determine whether the ruminal microbes in the "lyophilized ruminal inoculum" was alive post thawing an *in vitro* gas production study was carried out as per the method described by Menke *et al*. (1979). On the postulation that if the ruminal microbes in the "lyophilized goat ruminal inoculum" are viable then they will bring about fermentation of the substrate into which they are inoculated and result in the production of gases. Prior to the *in vitro* gas production study, the lyophilized goat ruminal inoculum was reconstituted with Mcdougall's artificial saliva (Mcdougall, 1948). The volume of Mcdougall's artificial saliva used for the lyophilisation process was 20 ml (the same volume that was used during lyophilisation), after its addition the contents were kept for one hour in room temperature. This reconstituted "lyophilized goat rumen inoculum" was used for the *in vitro* gas production studies. The following were the treatments.

T_1 - Rumen inoculum (RI) with 10% skim milk powder.
T_2 - RI with 10% skim milk powder + 5% sodium glutamate solution.
T_3 - RI with 5% glycerol solution.
T_4 - RI with 5% DMSO solution.
T_5 - RI without cryoprotectant.
Each treatment had six replicates.

Dried cumbu napier fodder grass (CO_4) was used as substrate. The grass was shade dried and ground in a mill to pass through 1 mm sieve and stored in air tight containers for it to be used as substrate for the *in vitro* gas production study. To each of the 100 ml glass syringe used in the study, substrate of 0.200±0.01 g was accurately weighed and transferred. The pistons of the syringes were lubricated with Vaseline prior to the study. The reconstituted "lyophilised goat rumen inoculum" was mixed with buffer solution (Menke *et al*., 1979) in the ratio of 1:2 (v/v). The buffered reconstituted lyophilized rumen fluid (30 ml) was transferred into the syringe containing dried Cumbu Napier fodder grass (CO_4) as substrate. The syringes were kept in water bath at 39°C, for incubation periods of 6, 12, 24 and 48 hours. At the end of each incubation period, total gas production was recorded and blank corrected. To ensure that gas production was not from the cryoprotectants used during the lyophillisation process, syringes containing only buffered reconstituted lyophilised rumen inoculums (with various cryoprotectants) without the substrate were also incubated in water bath at 39°C, for incubation periods of 6, 12, 24 and 48 hours and the gas measured were used as respective blank correction values. Data were analysed with analysis

of variance (ANOVA) using IBM® SPSS® Statistics version 20.0 for Windows® software as per the Snedecor and Cochran (1989). The critical difference between the groups was analysed by Duncan's multiple range test.

RESULTS AND DISCUSSION

The presence or absence of complete lyophilization of goat rumen fluid as influenced by different cryoprotectants *viz.,*10% (w/v) skim milk powder, 10% skim milk powder + 5% (w/v) sodium glutamate solution, 5% (v/v) glycerol solution, 5% (v/v) DMSO solution, pre freezing time durations *viz.*, 2, 24 and 48 hours and lyophilization time durations *viz.,* 8, 24 and 32 hours is presented in Table 1.

Pre freezing (-80°C deep freezer) duration for two and twenty-four hours, followed by 8, 24 and 32 hours in lyophilizer at -45°C were not optimum for lyophilizing the goat rumen fluid with or without the addition of various cryoprotectants. Pre freezing (-80°C deep freezer) duration of 48 hours with 8 and 24 hours of time duration in lyophilizer (-45°C) was also not ideal for lyophilizing goat rumen fluid with or without the addition of various cryoprotectants. However, pre freezing (-80°C deep freezer) duration of 48 hours with 32 hours of time duration in lyophilizer (-45°C) was ideal for lyophilizing goat rumen fluid with or without the addition of various cryoprotectants.

The freeze dried goat rumen fluid hereinafter to be referred as "lyophilized goat rumen inoculum" obtained at the end of lyophilization process adopting prefreezing (-80°C) duration of 48 hours and freeze drying (-45°C) duration of 32 hours were visually examined. It was observed that when cryoprotectants *viz.,*10% skim milk powder or 10% skim milk powder + 5% sodium glutamate solution were used it resulted in complete lyophilization with a very good quality "lyophilized goat rumen inoculum" compared to when 5% DMSO was used as cryoprotectant. When the goat rumen fluid was lyophilized using glycerol at 5% as cryoprotectant it resulted in the complete lyophilization but the "lyophilized goat rumen inoculum" was sticky in nature. Abadias *et al*. (2001) also had reported sticky product when glycerol at high concentration (10%) was used as cryoprotectant.

Based on this study pre freezing (-80°C deep freezer) duration of 48 hours with 32 hours of time duration in lyophilizer (-45°C) was the selected pre freezing and lyophilizing time durations for further documenting the yield of "lyophilized goat rumen inoculum" with the use of different cryoprotectants *viz.,*10% (w/v) skim milk powder, 10% skim milk powder + 5% (w/v) sodium glutamate solution, 5% (v/v) glycerol solution, 5% (v/v) DMSO solution (Table 2).

Significantly (P<0.05) highest yield was documented in goat rumen fluid with 10% skim milk powder + 5% Sodium glutamate. Significantly (P<0.05) lowest yield was documented in goat rumen fluid with 5% DMSO which was comparable with goat rumen fluid without cryoprotectant.

The results of the *in vitro* gas production at different incubation hours using lyophilized goat rumen inoculum prepared with different cryoprotectants (Table 3).

The results revealed that in treatments where skim milk powder was used as a cryoprotectant the fermentation of the cryoprotectant itself lead to gas production at all

Table 1: Presence or absence of complete lyophilization of goat rumen fluid as influenced by different cryoprotectants, pre freezing and lyophilization time durations.

Treatments with different cryoprotectant	Different time duration adopted in deep freezer (-80°C)								
	2 hrs			24 hrs			48 hrs		
	Different time duration adopted in Lyophilizer (-45°C)								
	8 hrs	24 hrs	32 hrs	8 hrs	24 hrs	32 hrs	8 hrs	24 hrs	32 hrs
Goat rumen fluid with 10% skim milk powder	×	×	×	×	×	×	×	×	√
Goat rumen fluid with 10% skim milk powder + 5% sodium glutamate	×	×	×	×	×	×	×	×	√
Goat rumen fluid with 5% glycerol	×	×	×	×	×	×	×	×	√
Goat rumen fluid with 5% DMSO	×	×	×	×	×	×	×	×	√
Goat rumen fluid without cryoprotectant	×	×	×	×	×	×	×	×	√

Note: √ - Lyophilized; × - Not lyophilized.

Table 2: Yield (Mean*±SE) of lyophilized goat rumen inoculum with different cryoprotectants lyophilized adopting 48 hours deep freezing (-80°C) and 32 hours lyophilizing (-45°C).

Treatment	Yield (%)
Goat rumen fluid with 10% skim milk powder	16.82^{c}±1.55
Goat rumen fluid with 10% skim milk powder + 5% sodium glutamate	21.56^{d}±1.19
Goat rumen fluid with 5% glycerol	8.09^{b}±0.41
Goat rumen fluid with 5% DMSO	4.71^{a}±0.25
Goat rumen fluid without cryoprotectant	5.85^{a}±0.84

*Mean of eighteen observations.

Mean values bearing different alphabetical superscript within column differ significantly (P<0.05).

Table 3: *In vitro* gas production (ml/0.2 g substrate) at different incubation hours using lyophilized goat rumen inoculum prepared with different cryoprotectants (Mean*±SE).

Lyophilized goat rumen inoculum prepared with	Incubation hours			
	6[NS]	12	24	48
10% skim milk powder[#]	-11.38±8.47	-0.63±10.72	-6.25±11.19	-24.63±11.94
10% skim milk powder + 5% sodium glutamate[#]	-9.38±7.55	-12.25±7.58	-15.88±7.36	-37.13±12.00
5% glycerol	1.50±0.63	4.83[b]±1.52	10.50[b]±3.55	11.00[b]±3.59
5% DMSO	1.21±0.44	1.92[a]±0.64	3.35[a]±0.88	7.78[ab]±1.72
Without cryoprotectant	1.07±0.43	1.76[a]±0.62	2.53[a]±0.65	4.53[a]±0.82

*Mean of eighteen observations.

Mean values bearing different alphabetical superscript within column differ significantly (P<0.05).

[#]Negative values were not included for statistical analysis.

Gas production values were corrected for the gas produced by respective lyophilized rumen inoculums with cryoprotectant without substrate.

incubation hours under *in vitro* condition, thus resulting in negative values for these treatments *viz.,* 10% (w/v) skim milk powder, 10% skim milk powder + 5% (w/v) sodium glutamate solution. The effect of inoculum self-fermentation is a possibility, soluble nutrients in the rumen inocula could be used by microorganisms during the incubation of inocula without adding substrate (Prates *et al*., 2010), leading to gas production. Hence, in this study skim milk powder present in the inocula as cryoprotectant had self fermented producing gas, which was of a larger volume than the gas production from fermentation of the substrate.

Significantly (P<0.05) higher gas production at 12, 24 and 48 hours incubation was 5% glycerol was used as cryoprotectant, indicating better viability of rumen microbes when 5% glycerol was used as cryoprotectant. During cryopreservation process, cryoprotectants are added to minimize the cell injury that occurs during freezing (Bircher *et al*., 2018). The authors reported that glycerol can prevent the cell injury by reducing the excessive increase in salt concentration of unfrozen part of water thereby reducing the cell damage by increased osmotic pressure (Lovelock, 1953; Fowler and Toner, 2005). Belanche *et al*. (2018) reported that freezing rumen inoculums without cryoprotectant delayed the fermentation process and caused low microbial activity that affected the *in vitro* fermentation of fibrous feed stuff. Spanghero *et al*. (2019) reported that type of preservation of rumen inoculums *viz.,* warm, refrigeration, chilled and freeze dried had significant impact in gas production and freeze dried rumen inoculum generated lowest gas yield compared to other preservation methods.

Conclusion

The preservation technique of rumen fluid obtained from goats was standardized through lyophilization. Pre freezing (-80°C deep freezer) duration of 48 hours with 32 hours of time duration in lyophilizer (-45°C) was the ideal pre freezing and lyophilizing time durations for goat rumen fluid. Better viability of rumen microbes in lyophilized goat rumen inoculum was when 5 per cent glycerol was used as cryoprotectant.

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