Journal of Animal Prod., *35(3),* https://rpa.reduc.edu.cu/index.php/rpa/article/view/e4588

Husbandry and Nutrition

Review article

In vitro Gas Production Using Ruminal Fluids or Feces as Inoculum, a Useful Technique for Evaluating Ruminant Feeds

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Received: October 2023; Accepted: December 2023; Published: January 2024.

ABSTRACT

Background: The aim of this paper was to provide general information about the technique used for *in vitro* gas production and its uses today, due to its relevance to evaluate digestibility in feeds for ruminants. **Development:** This technique dates back to 1979. Since then, its application has undergone several changes, though keeping the main procedure of measuring the gas produced following inoculation of the sample using ruminal fluid, feces, or enzymes, in a buffered mineral medium with bicarbonates releasing a large amount of gas, which is proportional to the volume of volatile fatty acids produced. The procedures for gas measurements have changed until today, with the utilization of computer-linked pressure transducers. This technique has been used in multiple studies cited in this paper, along with other works on improvements based on previous flaws. **Conclusions:** The *in vitro* technique for gas production is probably the most versatile measuring tool for digestibility. It has didactic and accessible procedures, it is less costly, and it constitutes more compatible with animal welfare legislation since it uses feces. However, there is still much to do in terms of greater accuracy and precision, and its possible application for the evaluation of animal feeds.

Keywords: digestibility, animal ethics, feed evaluation, deposited feces (*Source*: *AIMS*)

INTRODUCTION

What best shows the nutritional value of feeds is linked to the animal response. Traditionally, it has depended mostly on factors like consumption, chemical composition, and digestibility (Baumont *et al.*, 2000).

Feed evaluation must define the characteristics that help predict the animal' s productive performance, such as weight gain, milk production, and wool production. In that sense, laboratory techniques are increasingly used instead of animals, thanks to the quickness, and

Citations (APA) Martínez Sáez, S., Espinosa Sifontes, E., Palacio Collado, D., & Pedraza Olivera, R. (2024). *In vitro* Gas Production Using Ruminal Fluids or Feces as Inoculum, a Useful Technique for Evaluating Ruminant Feeds *Journal of Animal Prod*, *35*(3). https://rpa.reduc.edu.cu/index.php/rpa/article/view/e4588

relatively low cost of technology (Posada and Noguera, 2005). Often, the term digestibility has been used to show the amount of dry matter and/or nutrients absorbed in the intestinal tract by the animal.

Both ruminal or *in sacco* degradability and *in vitro* digestibility techniques, particularly gas production, have become important (Ayasan *et al*., 2018). This paper aims to collect information about some general aspects of the technique and refer to its current extent.

DEVELOPMENT

A brief historical review

The technique called *in vitro* gas (IVG) was developed by Menke *et al*. (1979) and remained in active as a standard method adopted in Germany for feed evaluations. The incubation receptacles, syringes, or bottles are placed in a lukewarm water bath at 39±0.5 °C. Gas is produced by inoculating ruminal fluid, feces, or enzymes in a buffered mineral medium with bicarbonates releasing a large amount of gas, which is proportional to the volume of volatile fatty acids produced. For forages, the measuring time is generally after 3, 6, 12, 24, 48, 72, and 96 hours, though in feedstuffs, measurements are more frequent during the first 24 hours. Measurements every hour can increase accuracy remarkably, which provides a more precise description of the lag phase (Mauricio *et al*., 2001; Sobalvarro *et al*., 2020; Juraci *et al*., 2023).

Moreover, as early as 1974, there was a different criterion for measuring *in vitro* gas production (Wilkins, 1974), consisting of keeping the fermented product in a sealed container and measuring pressure in the vacuum space. The principle of measuring pressure using the proper sensor has been widely adopted using several variants. The simplest was described by Theodorou *et al*., (1994), and it consists of measuring pressure manually using a pressure transducer. The authors suggested a semi-automatic system in which the substrates are incubated in sealed flasks where the gases from fermentation gather on the top, and then a syringe/pressure transducer is used to measure and release the accumulated gas until the atmospheric pressure is restored in the container. The gas accumulated on the top is measured to estimate the existing volume of gas.

Unlike completely automated systems, which combine a bottle and its pressure transducer, it utilizes a single pressure transducer for all the fermenting containers. The advantages of the system are its high capacity, low cost, and easy maintenance. Transducer-based methods with certain automation degrees have been described by several authors (Van Gelder *et al.,* 2005; Van Laar *et al.,* 2006; Rodríguez *et al*., 2017).

The greatest technical IVG innovation is that the sample' s gas, rather than its degradation, is measured (Posada and Noguera, 2005). The same postulate continues today, even after simplifying, improving, and automating the method through computers (García *et al*., 2022)

Feces as inoculum

The search for alternatives to ruminal fluid inoculum, such as feces has garnered relevance (Bauer *et al.,* 2004; Youssef, and Kamphues, 2018).

Earlier, Jones and Barnes (1996) did dry matter digestibility *in vitro* studies in tropical leguminous plants, using ruminal fluid and cattle feces as sources. The digestibility values found using the fecal fluid were directly correlated to the ones from the ruminal fluids (r=0.98).

In comparative studies between cattle feces and ruminal fluids, Akhter *et al.* (1999) concluded that the former could be an alternative to the latter to evaluate forage digestibility *in vitro*. Similar results were published by Martínez *et al.;* Martínez *et al.* (2008); Sáenz *et al*. (2022).

Cattle and sheep feces have been used as alternatives for inoculum, and their potential for *in vitro* gas techniques have been established (Varadyová *et al*., 2005; Martinez *et al*., 2005; Posada *et al*., 2012; Pandian *et al*., 2016; Sáenz *et al*., 2022).

This effort has been motivated mostly due to bioethical matters associated with surgically modified animals.

The influence of the inoculum

Among the factors to be considered for IVG production either using ruminal fluid (RF) or feces, controlling inoculum variability is determining (Martínez *et al.*, 2005).

In that sense, a common practice is to mix the inocula from different animals and introduce reference samples to correct differences caused by the influence of the inoculum (Mauricio *et al*., 2001; Posada, and Noguera, 2005; Martínez *et al*., 2010).

The generally embraced criteria are that just like the target (syringes or transducers without samples), standards, and reference samples should be corrected in each experiment. Each reference strain must have a known gas profile determined by the average from many samples. If the reference sample in a run produces between 90 and 110% of gas regarding the average value, then the ruminal fluid is considered " normal" , and all the gas volume measurements are corrected by the factor " reference sample volume average/volume of the run sample." If, otherwise, the volume of the reference sample in the run is outside this range, the inoculum is considered " altered" , and the data from the run are often discarded (Schofield, 2000).

Quite stable and homogeneous reference sample preparation is a requirement. The internal standard use has a noteworthy shortcoming, since the sample used as reference must have a similar profile to the one analyzed so that the results can be extrapolated. Moreover, it is important to always prepare fresh samples when there is enough replacement material, to make a reliable association between them. External calibration will depend on having a sufficient number of samples whose *in vivo* or *in sacco* digestibility has been well determined (Kamalak *et al*., 2005; Martínez *et al*., 2008; Sharifi *et al*., 2019). The production of such samples is costly, so collaboration among laboratories is essential (Rymer *et al*., 2005).

Experiments conducted by Mauricio *et al*. (1998, 2001) to compare ruminal fluid and feces as sources of inoculum, showed that the fecal matter produces greater lag phases and a lower fermentation capacity. In later works, Martínez *et al.*, (2005 and 2008), and Sharifi *et al*., (2019) concluded the same; the former authors used feces deposited, which had the advantage of not dealing with the animal.

The utilization of more a concentrated inoculum or greater volume of fecal matter, and improvements in fermenting microorganisms have also been studied (Cubillas, 2010; Cantet *et* *al*., 2020). The latter found that pre-treating the inoculum was not a viable solution to raise its strength.

Other inocula

Appropriate microorganic cultures could, in theory, be used to produce standard inocula without using experimental animals. These cultures could be prepared through a broad range of activities and create the conditions to keep them whole. Rymer *et al*. (2000) studied the inclusion of bacterial cultures, such as the mixture of several microorganisms, and compared the gas production patterns of several feeds. They found that although there were differences in the profiles using bacterial cultures, which produce gas at much lower speeds than using the ruminal fluid-based inoculum, the correlation between parameters was high. There is still much to do in that direction

Elías *et al*. (2005) reported a methodology for the successful determination of dry matter and organic matter digestibility *in vitro* using " washed cells" (bacteria extracted from ruminal fluid by centrifugation). This alternative may be tested for *in vitro* gas production. Accordingly, Cubillos *et al.* (2010) suggested measuring the strength of an inoculum from deposited cattle feces and using an inversely proportional amount to that strength.

Besides, feces from horses (Elghandour *et al.*, 2016; Franzan *et al.*, 2018) and goats (Martínez, 2014) have been used.

Extent of the technique

The table below shows the versatility of the *in vitro* gas technique.

Topic	**Contributors**
Related to feces - RF	Martinez *et al*. (2005); Posada *et al*. (2012); Pandian *et al*. (2016); Sáenz *et al*. (2022).
Metabolizable energy	Leon (2012); Muizzu and Rizca (2021); Sáenz *et al*. (2023).
Forage arrangement	Martinez *et al*. (2009).
Effect of polyphenols PEG	Martínez (2008); Putri *et al*. (2021).
Prediction of consumption	Rodríguez (2017).
Ensilage evaluation	Ligoski (2020).
Methane production	Pal *et al*. (2015); Molho-Ortiz *et al*. (2019); Ligoski (2020); Sucu (2020); Ellis *et al*. (2020); Kusuma (2022); Wangui *et al*. (2022).
Probiotic use,	Besharati *et al*. (2009); Elghandour *et al*. (2016); Sucu (2020).
Prebiotic use (mannan oligosaccharides)	Zheng *et al*. (2019).
Hay from alternative materials	Sari *et al*. (2021).
Utilization of phytochemicals	Molho-Ortiz *et al*. (2019).
Utilization of fiber, amyloid, and proteolytic enzymes	Freiria *et al*. (2018).
Feedstuff use	Ayasan *et al*. (2018); Amanzougarene and Fondevila (2020).
Use of multi-nutritional blocks	Herrera-Torres *et al*. (2022).

As seen, in recent years, there has been a predominance of techniques for new ways that enable methane control as the principal greenhouse gas produced by ruminants.

Remarkably, the study of the relation between the ruminal fluid and feces has been present since the technique was developed until today.

CONCLUSIONS

The *in vitro* technique for gas production is probably the most versatile measuring tool for digestibility. It has didactic and accessible procedures, it is less costly, and it constitutes a more compatible alternative with animal welfare legislation since it uses feces as inoculum. It has been successfully used to serve several purposes associated with ruminant feed evaluation, including organic matter digestibility estimations, measurements of metabolizable energy of feeds, and fermentation kinetics. Determining how the value of feeds is being influenced by antinutritional factors and additives, among others.

This technique may be used to estimate gas volume at a constant atmospheric pressure and a fixed volume, or using a combination of the two procedures, through manual, semi-automatic, and automatic techniques.

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AUTHOR CONTRIBUTION STATEMENT

Research conception and design: SM, EE, DP, RP; data analysis and interpretation: SM, EE, DP, ML; redaction of the manuscript: SM, EE, DP, RP.

CONFLICT OF INTEREST STATEMENT

The authors state there are no conflicts of interest whatsoever.