Effect of Substrate to Inoculum Ratio on Outcomes of In Vitro Rumen Fermentation Studies

by
Jon Donald Remy

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Animal Science
(Honors Scholar)

Presented May 15, 2019
Commencement June 2019

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Jon Donald Remy for the degree of Honors Baccalaureate of Science in Animal Science presented on May 15, 2019. Title: Effect of Substrate to Inoculum Ratio on Outcomes of In Vitro Rumen Fermentation Studies

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Abstract approved:_____

Joseph Klopfenstein

The objective of this study was to identify the varying effects of altering the rumen fluid: substrate ratio on the outcomes of in vitro rumen fermentation. In vitro fermentation is a useful tool for evaluating the energy value of feedstuffs and the effect of feed additives on Volatile Fatty Acid (VFA) production in the ruminant. In this study, rumen fluid was collected from 3 mature ruminally cannulated Holstein heifers, combined with a buffer, and incubated with increasing amounts of ground substrate. Increasing the ratio of substrate to inoculum caused an increase in total VFA production (mmol/L; $P < 0.0001$), as well as production (mmol/L) of propionic ($P < 0.0001$), valeric ($P < 0.0001$), acetic ($P < 0.0001$), and butyric acids ($P < 0.0001$). pH decreased as the inclusion rate increased ($P < 0.0001$), as did the ratio of acetic to propionic acid ($P < 0.0001$). The lag time (h) of fermentation was inversely related to the inclusion rate ($P < 0.0001$). Maximum gas production (mL/g DM) was only significantly decreased in the 8.0 g treatment ($P < 0.0001$). Between the ratios of 1.2, and 1.6 g dry substrate: 100 mL inoculum, none of the measured outcomes

were statistically different ($P > 0.05$), and the environment of the fermenter units was comparable to that of the functioning rumen.

Key Words: Rumen, Fermentation, Volatile Fatty Acid, Inclusion rate

Corresponding e-mail address: remyj@oregonstate.edu

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APPROVED:

_____

Joseph Klopfenstein, Mentor, representing Veterinary Medicine

_____

Shelby Armstrong, Committee Member, representing Phibro Animal Health

_____

Jorge Vanegas, Committee Member, representing Veterinary Medicine

_____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Jon Donald Remy, Author

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Introduction

The use of in vitro fermentation to measure gas production is widely considered to be a reliable model for analyzing the energy value of feedstuffs for ruminants (Yánez-Ruiz et al., 2016). The premise of such research is that rumen fluid from a donor cow, along with a buffer, form the inoculum solution. The inoculum solution is combined with a substrate, either a dried feedstuff or dried ration, and gas production is measured over an incubation period. This model accounts for the buffering by saliva, microbial breakdown in the rumen, and the approximate temperature and agitation parameters seen in the functioning rumen. The gas production technique provides strong data without the expense, labor and welfare considerations associated with in vivo feeding trials (Gosselink et al., 2004).

Since the detailed in vitro study of rumen fermentation began looking at gas production in the early 1950's, a wide range of systems and measuring techniques have been employed to study the products of fermentation (Rymer et al., 2005). A review of in vitro rumen fermentation protocols by Yánez-Ruiz et al. (2016) reports a variety of methods used in most aspects of fermentation protocols, and that there seem to be remarkable effects of these variations on the outcomes of fermentation studies. Yánez-Ruiz et al. (2016) found that across accepted methods, variation in rumen fluid handling, substrate processing, and sampling procedure all had impacts on microbial populations, and gas production.

The vast majority of research to date using in vitro rumen fermentation techniques has been associated with assessing the nutrient value of feedstuffs, or the effect of

treatments on gas production, VFA profile, environmental changes or microbiome character.

Outline of concept

In vitro studies offer the opportunity to both avoid the demands and challenges of large in vivo feeding trials, and provide the research team with the opportunity to control each aspect of the fermentation environment. Van Dyne (1962) described the varied methods among in vitro systems, as well as the commonalities in successful methods. At the time of this survey, digestion of cellulose was the primary outcome measured by investigators (Van Dyne, 1962). More recently, technology has allowed for analysis to be completed using computerized modules to monitor gas production over an incubation period (McFarlane et al., 2017). While the techniques used for data collection have developed remarkably, recent studies such as that by McFarlane et al. (2017) retain most of the traditional practices for collecting rumen fluid from fistulated cattle, blending and straining the solution, and mixing with a buffer and feedstuff to perform the incubation. Each of these practices is further explored in the following review of past work.

Factors Affecting Fermentation

The environment of the fermentation unit is readily modified in the in vitro system, and can have significant impacts on the outcomes of a fermentation study. Management of these environmental factors is critical to having a study which reflects the reality of a functioning rumen.

pH

In vitro fermentation units require some form of pH management to imitate the buffering effect of ruminant saliva. Healthy rumen pH is necessary for maintenance of

animal health. Sub-acute ruminal acidosis, which is associated with sustained rumen pH below 5.5 has health implications such as laminitis, development of abscesses, and depressed milk production (Kleen, et al. 2003). McDougall (1947) developed a method for producing synthetic ruminant saliva for use in in vitro systems. Developed based on data collected from parotid salivary samples in sheep, McDougall's solution is widely used as the buffer to incubate in the artificial rumen. The incorporation of McDougall's buffer into the fermentation system varies among past experiments. Many procedures involve diluting freshly collected rumen fluid with buffer prior to incubation. Cho et al. (2014) prepared a solution 4-parts McDougall's buffer: 1-part rumen fluid before incubating the units.

Temperature

The purpose of temperature control during the incubation period of in vitro fermentation studies is to maintain the system at physiologic conditions. Depending on time of year, normal rumen temperatures in mature cattle can range from 37.5˚C to 40.2 ˚C (Boehmer et al., 2015). In vitro fermentation studies typically use a water bath, maintained at 39 ˚C to regulate the temperature surrounding each fermentation unit (McFarlane et al., 2017), or an incubator set to maintain the surrounding temperature at 39˚C (Cho et al., 2014).

Agitation

In order to maintain normal feed passage, the functioning rumen contracts to achieve mixing and movement of feed at a rate of 1-5 contractions every 2 minutes (Imran et al., 2011). In order to mirror this quality in the in vitro system, many procedures involve some form of constant agitation. A range of practices exists, from a constant rate of 50

oscillations per minute (McFarlane et al., 2017) to 120 oscillations per minute (Meale et al., 2012).

Substrate character

Feed particle size is an important consideration when feeding cattle for production purposes, as well as when preparing substrate for in vitro studies. Cattle mechanically break down their feed repeatedly during rumination. It is estimated that mastication during eating accounts for about 25% of large particle breakdown in the ruminant, with another 50% large particle breakdown during rumination (McLeod et al. 1988). In order to imitate this mechanical breakdown, procedures typically grind dry feedstuffs through a screen ranging from 1 mm (Meale et al., 2012; Cho et al., 2014) to 2 mm (McFarlane et al., 2017).

Fluid collection and handling

Relatively little variation exists within the procedures used for collecting and preparing rumen fluid for inoculation. The simplest and most common means of collecting rumen fluid is to use an animal which has been fitted with a cannula (Cho et al., 2014; McFarlane et al., 2017). The fluid is kept in a prewarmed thermos while being promptly transported to the laboratory (Cho et al., 2014; Gunun et al., 2017). At the lab, it is common to blend the rumen fluid and collected fiber mat to dislodge microbes, and homogenize the inoculum (Peripolli et al., 2014). CO_2 is typically used to maintain anaerobic conditions (Gunun et al., 2017). There is evidence that collecting fluid between 2 and 4 hours after feeding offers the most productive microbial populations (Brewster et al., 2018)

Measured Outcomes

pH

In an analysis of past work, Maccarana et al. (2016) found that of 30 observed studies, only 17 reported final pH values. Of those, it was clear that a wide array of factors impacts pH of the fermentation unit. Maccarana et al. (2016) suggests that increasing the ratio of substrate to inoculum may, in fact, be associated with a drop in pH, which alters the microbial populations and their functions. It has been established in vivo that altering diet, by increasing concentrate to roughage ratio, leads to a decreased rumen pH. Wanapat et al. (2013) found that increasing concentrate to roughage ratio from 0.2 to 0.8 shifted rumen pH on average from 6.4 to 5.9.

Gas production

Gas production within fermentation units can be modelled using the Gompertz Gas model (Peripolli et al., 2014). There are three distinct phases to gas production in this model of fermentation. The lag phase is the first period of fermentation after the incubation begins. In this phase microbial populations are colonizing the substrate particles and gas production is relatively slow. In the second phase, gas production is rapid, and the microbes are at their most active. This phase ends with the asymptotic phase, at which point gas production slows and microbial activity diminishes (Lutakome et al., 2017). The total gas production is effectively considered a measure of the energy extracted from the feedstuff (Yánez-Ruiz et al., 2016).

Surveys of existing work have demonstrated that a tremendous number of factors affect gas production in the fermenter unit (Maccarana et al., 2016). Since procedural variation exists between researchers, comparison of gas production values between

experiments is of little value. Gas production can be used to model rate of fermentation over time within a fermenter unit, and can be used for relative comparison within a particular experiment. Modern technology allows for measuring gas production at short intervals and then releasing pressure to maintain consistent head space pressure (McFarlane et al., 2017). This technology generally reports higher total gas production than techniques allowing for a buildup of gas pressure, as increasing dissolved CO_2 concentrations in the fluid reduces the measure of total gas production (Maccarana et al., 2016).

Volatile Fatty Acid (VFA) Profile

Beyond measuring the total volume of gas produced by a given fermentation unit, use of High-Performance Liquid Chromatography (HPLC) allows for characterization of VFA profiles. The VFA profile has significance in understanding ruminant energy utilization (Cho et al., 2014). As the diet characteristics of the ruminant shift, so does the profile of VFA's produced. The rumen produces 3 major VFA's: Acetic, Butyric, and Propionic which are used by the ruminant for different aspects of physiologic function. Understanding the VFA profile resulting from a particular diet offers insight into the potential production outcomes of the animal. Acetic acid, for example, which is associated with the slow degradation of fibrous feeds, is responsible for 65% of milk fat synthesis in the cow (Schmidt, 2011). Propionic acid is associated with rapid fermentation of starches, and is a precursor for protein synthesis and gluconeogenesis (Schmidt, 2011). It has been established that butyric acid plays a significant role in gastrointestinal health (Scheppach et al., 1992; Tagang et al., 2010). There are several isoacids (valeric, isovaleric, isobutyric) which are produced in much lower concentrations, and are understood to a much lesser extent, but seem to play a role in milk production (O'Callaghan et al., 2018). Because the

characteristics of a given diet dramatically alter the microbial population in the rumen, and the microbial population controls the production of VFA's, there is a direct connection between dietary character and productivity of the ruminant.

A relative increase in concentrates in the diet shifts the microbial population to favor amylolytic bacteria and increases relative production of propionic acid (Wanapat et al., 2013). Conversely, a relative increase in roughage content in the ruminant diet shifts microbial populations by increasing cellulolytic bacteria, and increases acetic acid production (Wanapat et al., 2013).

VFA concentrations can be analyzed using samples from the headspace of the fermentation unit (Gunun et al., 2018) or from a sample of fluid at the end of the fermentation period (McFarlane et al., 2017). Fluid sampling has been considered to be more reliable than headspace sampling due to the varying solubilities of organic gasses and challenges with sampling procedures (Molofsky et al. 2016). Considering the VFA profile allows researchers to evaluate the relative availability of each VFA to the ruminant.

Substrate: Inoculum Ratio

It is suggested that increasing mass of substrate relative to volume of inoculum increases total gas production with relatively low variation in actual rate of fermentation (Rymer et al., 2005). This effect has not, however, been the primary focus of a study using modern laboratory equipment and techniques.

As rumen volume and dry matter intake vary significantly among animals and diets, and the dynamic nature of feed passage is not well replicated in the in vitro system, estimation of the in vivo substrate: rumen fluid ratio is difficult to replicate in vitro. Because of this challenge, there is no accepted standard for this ratio. In vitro rumen

fermentation studies have been completed using as little as 200 mg substrate in 50 mL of buffered inoculum (Lopez et al., 1998), and as much as 1.0 g substrate in 60 mL of buffered inoculum (Theodorou et al., 1994).

As such a range exists in accepted methods, this study aimed to identify the significance of the variations in substrate to inoculum ratio. The objective of this study was to compare the effects of adjustments in mass of substrate relative to volume of inoculum on the kinetics of gas and volatile fatty acid production. It is expected that increasing the ratio of substrate to inoculum will, to some extent, increase the total gas production, without substantially changing the rate of gas production. Understanding the ratio of substrate to inoculum that optimizes gas production offers the ability to study the rumen environment when the microbes are at their most productive physiologic stage.

Materials and Methods

All research trials and analysis were completed at Phibro Animal Health Corvallis Research Center in Corvallis, OR. All animals were cared for according to the Phibro Animal Health Corporation Animal Care and Use Guidelines. Three ruminally cannulated heifers were used as fluid donors. Animals were adapted to a basal diet (Table 1) for at least 14 days before the trial began. A small batch of feed (Table 1) was mixed in the lab, dried, and ground to pass through a 2 mm screen. The dried, ground diet acted as the substrate in the fermentation unit. Each fermentation unit was assigned to 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 4.0, or 8.0 g of substrate. Just prior to collection of rumen fluid, fermentation units containing the assigned amount of substrate and 75 mL of McDougall's Buffer were introduced to water baths (39° C) in order to have the solution warmed prior to inoculation with rumen fluid. Incubations were carried out in 250 mL Ankom RF bottles.

Rumen fluid, along with a sample of fiber mat, was collected 4 hours after feeding the heifers. Samples were pooled into a pre-warmed thermos and transported immediately to the lab. The fluid sample and fiber mat were blended, then filtered through 4 layers of cheese cloth into a warmed flask. A stir plate was used to keep the solution homogenous and warmed to approximately 39° C. CO_2 was used to keep the flask void of O_2. The initial pH of the rumen fluid was recorded just prior to inoculation and 25 mL of rumen fluid was added to each of the Ankom bottles. Bottles were purged using CO_2, then quickly replaced in water baths for incubation. They were incubated at 39°C for 24 hours under constant agitation (60 rpm). Gas production was measured using the Ankom Gas Monitoring System (ANKOM, cat no #7056; Macedon, New York) using 15-minute intervals. Gas production was recorded from the time the last bottle was placed in the water bath. At the end of the

24-hour incubation period, samples of fluid were collected for VFA analysis (Dairyland Laboratories Inc. Arcadia, WI). Final pH and temperature were recorded immediately after removal from the water bath.

Each incubation day included treatments performed in triplicate. Bottles containing only inoculum were incubated and analyzed in triplicate as controls for gas production calculations. Three separate incubation days were included in the trial.

Statistical Analysis

Resulting pressure curves were calculated using the ideal gas law, corrected by dry matter and analyzed by non-linear regression in Graph Pad Prism 8.0 (Graphpad Software, San Diego, CA). Data were analyzed as a randomized complete block design using PROC GLIMMIX of SAS (SAS Institute Inc., Cary, NC) with donor as the experimental unit and day as the random blocking factor. Curves were fit according to France et al. (2000) and the Gompertz equation. Both equations describe three parameters: lag time (time before fermentation begins), fractional rate of fermentation (per hour) and asymptotic gas production. All tests were two-sided. Significance was defined as $P \leq 0.05$, tendency as $0.05 < P < 0.10$.

Results

Fermenter Environment

Increasing substrate relative to inoculum decreased the pH slightly, but the change was not significant ($P > 0.05$) between 1.2 g of substrate and 2.0 g of substrate (Figure 1). Above 2.0 g of substrate, the pH dropped significantly ($P < 0.0001$).

VFA Profile

As the ratio of substrate to inoculum increased, the average total VFA production increased from 88.34 mmol/L at the 1.0 g treatment to 263.96 mmol/L at the 8.0 g treatment ($P < 0.0001$; Figure 2). Production of propionic acid (mmol/L; $P < 0.0001$), valeric acid (mmol/L; $P < 0.0001$), acetic acid (mmol/L; $P < 0.0001$), NH_3 (mmol/L; $P < 0.0001$) and butyric acid (mmol/L; $P < 0.0001$) was favored by those fermentation units with higher ratios of substrate to inoculum (Table 2). This difference was not significant between the 1.2-1.6 g treatments ($P > 0.05$). As the inclusion rate of substrate increased, the ratio of acetic to propionic acid decreased ($P < 0.0001$; Table 2). Isovaleric acid production (mmol/L) was highest at 2.0 g of substrate, with sharp declines in the blank fermenter units, and at treatments beyond 2.0 g ($P < 0.0001$; Table 2). Isobutyric acid production (mmol/L) was at its highest over the range 1.0-1.4 g, with decreases below and above this range ($P < 0.0001$; Table 2).

Gas Kinetics

The lag time (h) was inversely related to the ratio of substrate to inoculum ($P < 0.0001$; Table 3). There was no significant change in the fractional rate of fermentation (K_D) among treatments ($P > 0.05$; Table 3). The maximum gas produced V_M was not significantly different ($P > 0.05$) across treatments with the exception of the highest level

of substrate inclusion (8.0 grams per 100mL inoculum), which had a lower V_M (mL/g DM; $P < 0.0001$; Table 3). None of the gas kinetics data was significantly different ($P > 0.05$) between 1.0 and 2.0 grams of substrate.

Discussion

Fermenter Environment

Since the pH decreased with increasing ratio of substrate to inoculum, it appears that increased inclusion of substrate drives increased acid production. As acid is a major byproduct of fermentation, the decreased pH would suggest that total fermentation was greater when more substrate was available to the population of microbes. Total VFA production was greatest in the highest treatments, which suggests that these acid products were driving the pH shift. This is similar to the findings of literature reviews, where a greater ratio of substrate to inoculum is associated with lower pH (Maccarana et al., 2016). Within the range 1.2-2.0 grams of substrate, where no significant pH change was observed, the buffer was likely at its most effective. Maccarana et al. (2016) suggested that the lower substrate to inoculum ratio allows for more effective buffering, and better maintenance of sustained microbial activity. Beyond this range (1.2-2.0 g), where the acid production was greatest, the buffer likely began to lose some efficacy. This is similar to the decrease in rumen pH observed in vivo when more rapidly fermented feedstuffs are included in the diet (Wanapat et al., 2014).

VFA Profile

The VFA profiles were remarkably influenced by the treatment applied. Environmental conditions such as pH and temperature favor particular microbial populations, which are responsible for observed VFA profiles. It has been well established that type of diet alters rumen conditions and VFA profile (Wanapat et al., 2014). Not unexpectedly, there were observed shifts in the VFA profile at different inclusion rates. The nature of the diet did not change between treatment groups, only inclusion rate was

adjusted. Some VFA's were produced with inverse relationship to the inclusion rate. This suggests that the microbial populations responsible for the production of these VFA's thrive in the conditions seen in the treatments with lower substrate inclusion. The most remarkable of these situations is the concentration of Isovaleric acid. The populations responsible production of this VFA must thrive in the higher pH (7.0 > pH > 6.5; Table 2) environment found in the lower ratio treatments. Most of the other VFA profiles indicate that the microbes favored the conditions in the higher ratio treatments. The change in ratio of acetic to propionic acid suggested that the populations favoring forage digestion, which are primarily responsible for acetic acid production, are more productive in higher pH (7.0 > pH > 6.5; Table 2) conditions while the populations producing propionic acid prefer the lower pH (5.3 < pH < 6.5; Table 2) environment. This aligns with the commonly observed reduction in rumen pH when diets shift to increased levels of concentrate inclusion. (Wanapat et al., 2014).

Increasing the inclusion rate caused a decrease in NH_3 production in this study. NH_3 production is influenced by many factors, including substrate variety and use of additives such as Monensin (Eschenlauer et al., 2002). It is possible that since there was more microbial activity in the high ratio treatments, there was more utilization of NH_3 and reduced free nitrogen available. Further investigation into factors affecting the production of NH_3, such as inclusion of urea in the diet, fermenter environment, and dietary character is needed. As efficient NH_3 production, and protein utilization, is economically important, exploring rate of production and utilization over time, and the significance of dietary character on production would be valuable.

Gas Kinetics

Based on the results of this study, the ratio of substrate to inoculum had no significant effect on the rate of fermentation during the second phase of fermentation, and had no significant impact on the maximum rate of gas production (mL/g DM) across the treatment range of 1.0 g-4.0 g DM per 100 mL inoculum. At the 8.0 g treatment, where the ratio of substrate to inoculum was highest, the maximum rate of gas production (mL/g) decreased significantly. This suggests that this treatment level reached a ratio at which the efficiency of microbial fermentation was hindered by the environment of the fermentation unit. This is likely the combined effect of lowered pH, relatively fewer microbes available for the given substrate, and potentially the accumulation of other detrimental fermentation byproducts in higher concentrations than in the other treatment groups. The survey by Maccarana et al. (2016) found a similar trend between ratio of substrate to inoculum and gas production.

The analysis of the treatment groups with a higher ratio of substrate to inoculum revealed a negative value for the lag phase. This is likely an artifact of the extremely high availability of substrate for the microbes. Since fermentation started so much faster than the other treatment groups, it is also possible that the lag phase elapsed before the system began recording gas production data. Clearly the challenge of this shortened, or non-existent lag phase makes these high ratios less attractive for in vitro studies.

Relevance

The outcomes of this study are likely trends that would apply to a variety of diets, but as the composition of substrate changes, it is likely that the observed VFA profiles and pH measurements would shift. It is well documented that increasing the relative

concentrate portion of a diet will lower pH, lower acetate production, and increase propionate production (Wanapat et al., 2014). With this in mind, it is clear that the trends observed in this study could readily shift between diets. Further work should delve into the effect of the substrate to inoculum ratio in diets of both very high and low concentrate composition.

Conclusion

For every parameter measured in this study there was a range, between 1.2 g and 1.6 g of substrate per 100 mL of buffered inoculum, in which there were no significant differences ($P > 0.05$). Treatment groups outside of this range generally had significantly different outcomes in most parameters. Using a ratio within the range 1.2 g-1.6 g substrate per 100 mL buffered inoculum provides reasonable values for gas production, and maintains environmental conditions consistent with those observed in the living rumen. This ratio should be used in future studies to achieve the highest quality data.

Ingredient	% of Diet DM
Corn Silage	**49.68**
CRC[1] Alfalfa	**7.04**
CRC[1] Hay	**1.77**
Steam Flake Corn	**17.09**
Corn Distillers Ethanol	**7.95**
Soybean Meal	**4.92**
Amino Plus[2]	**4.81**
Energy Booster 100[3]	**1.93**
Urea 281 CP	**0.69**
DCAD Plus[4]	**1.17**
Sodium Bicarbonate	**0.99**
Calcium Carbonate	**0.99**
Payback 12-6[5]	**0.59**
Magnesium Oxide	**0.28**
Dynamate[6]	**0.11**

1-CRC Corvallis Research Center Hays procured by the unit for all 2018 Ruminant Studies

2- Ag Processing Inc. Omaha, Nebraska

3-Milk Specialties Global. Eden Prairie, Minnesota

4- Arm and Hammer. Ewing Township, New Jersey

5-CHS Payback vitamin premix contains: Calcium 12-13%, Phosphorus 6%, Salt 18-21.5%, Magnesium 6.75%, Sulfur 1.0%, Copper 3500 ppm, Iodine 195 ppm, Manganese 3300 ppm, Selenium 53-58 ppm, Zinc 7500 ppm, Vitamin A 250000IU/lb, Vitamin D 25000 IU/lb, Vitamin E 250 IU/lb.

Table 1. Diet used for donor animals as well as substrate for fermenter units.

	Grams of Substrate per 100 mL Inoculum										
PARAMETER	**0**	**1**	**1.2**	**1.4**	**1.6**	**1.8**	**2**	**4**	**8**	**SE**	***P*-VALUE**
ACETATE	31.61^{a}	55.26^{b}	63.37^{bc}	65.41^{bcd}	67.59^{cd}	72.39^{cd}	75.29^{d}	101.71^{e}	124.61^{f}	2.75	<0.0001
PROPIONATE	7.24^{a}	19.17^{b}	22.42^{c}	23.77^{c}	24.98^{cd}	27.61^{de}	29.68^{e}	47.72^{f}	78.05^{g}	1.04	<0.0001
BUTYRATE	9.01^{a}	13.04^{b}	15.22^{c}	15.75^{cd}	16.63^{cd}	18.02^{de}	19.51^{e}	33.21^{f}	57.64^{g}	1.07	<0.0001
ISOBUTYRATE	0.48^{ab}	0.50^{a}	0.52^{a}	0.51^{a}	0.48^{ab}	0.39^{ab}	0.32^{abc}	0.16^{bc}	0.00^{c}	0.072	<0.0001
VALERATE	1.67^{a}	2.26^{b}	2.46^{bc}	2.54^{bcd}	2.65^{cde}	2.81^{de}	2.95^{e}	4.20^{f}	5.79^{g}	1.034	<0.0001
ISOVALERATE	0.55^{a}	0.76^{bc}	0.86^{bc}	0.88^{bc}	0.85^{bc}	0.89^{bc}	0.92^{b}	0.74^{c}	0.13^{d}	0.074	<0.0001
ACETIC: PROPIONIC	6.05^{a}	4.01^{b}	3.96^{b}	3.86^{bc}	3.81^{bcd}	3.69^{cd}	3.63^{d}	3.17^{e}	2.61^{f}	1.04	<0.0001
TOTAL VFA	48.48^{a}	88.34^{b}	102.89^{c}	106.58^{cd}	111.09^{cde}	119.41^{de}	126.76^{e}	185.69^{f}	263.96^{g}	1.04	<0.0001
NH_3	0.0413^{a}	0.0397^{ab}	0.0391^{bc}	0.0385^{bcd}	0.0379^{cd}	0.0371^{de}	0.0360^{e}	0.0294^{f}	0.0255^{g}	$7.46x10^{-4}$	<0.0001
Concentration (mmol/L)											

Table 2. VFA and NH_3 production (mmol/L) at each treatment level. Values with the different letter are significantly different ($P < 0.05$).

		Grams of Substrate per 100 mL Inoculum									
PHASE	**UNIT**	**1**	**1.2**	**1.4**	**1.6**	**1.8**	**2**	**4**	**8**	**SE**	***P*-VALUE**
LAG	h	1.758[a]	1.149[a]	0.901[ab]	0.271[ab]	0.448[ab]	0.556[ab]	-0.353[b]	-1.896[c]	0.5516	<0.0001
K_D	mmol/ (h·g DM)	0.176[a]	0.167[a]	0.160[a]	0.148[a]	0.153[a]	0.149[a]	0.151[a]	0.151[a]	0.0175	0.088
V_M	mL	203.17[a]	210.7[a]	212.1[a]	193.1[a]	215.03[a]	216.57[a]	183.2[a]	123.0[b]	11.24	<0.0001

Table 3. Kinetics of gas production; all data are LS Means ± SE. LAG corresponds to the initial lag phase of fermentation (h). K_D corresponds to the fractional rate of fermentation (mmol/ (h·g DM)). V_M corresponds to the asymptotic phase of fermentation(mL). Values with different letter are significantly different. Gas production was modelled using Gompertz gas equation: $F(t) = e^{-e^{(1-r(t-L))}}$ where F(t) is the cumulative gas production function, L is the lag value r is a rate constant, and t is the time of incubation (Pitt et al. 1999).

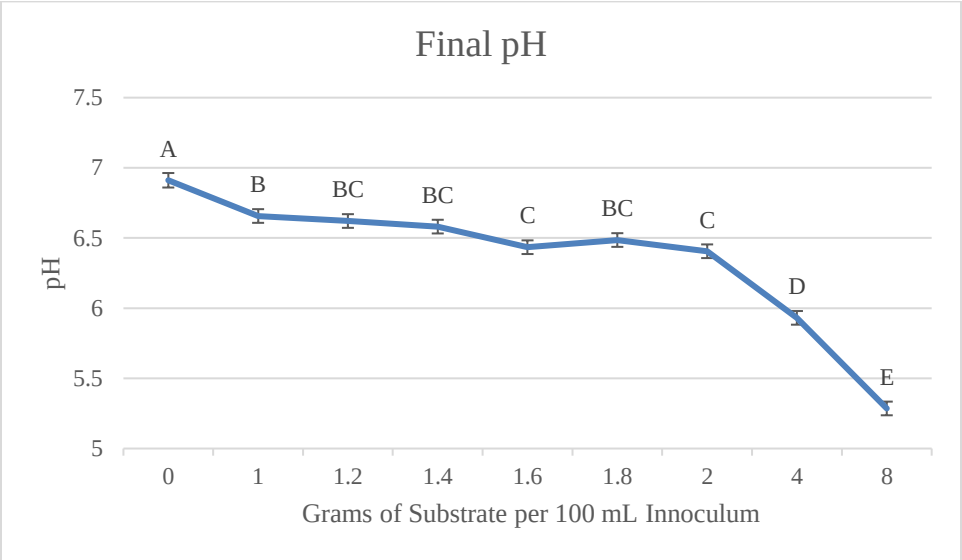

Figure 1. Final pH (average ± SE) of fermenter units is altered by the ratio of substrate to inoculum ($P < 0.0001$). Values with different letters are significantly different ($P < 0.05$).

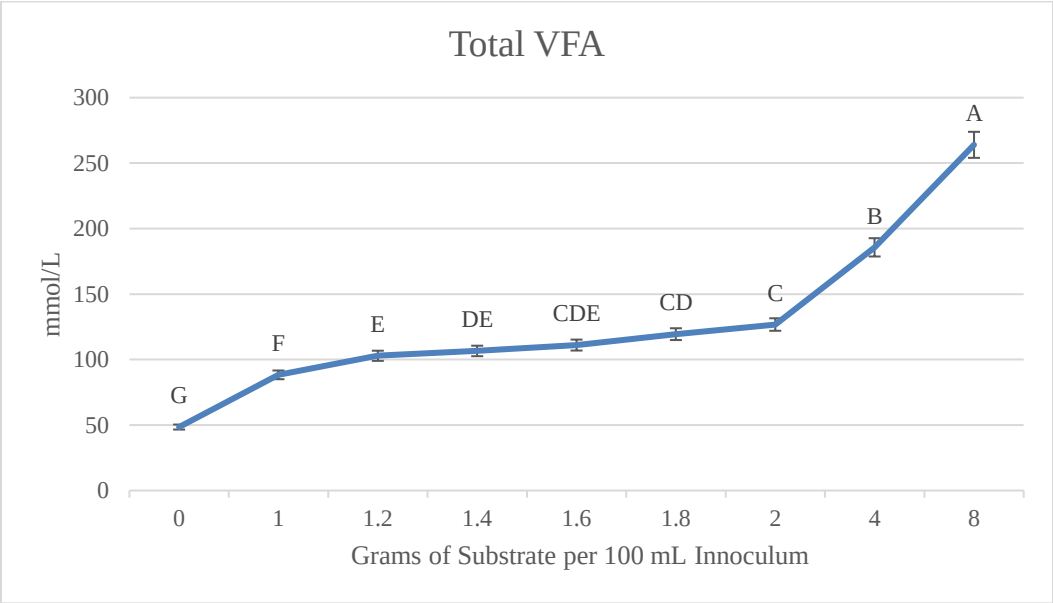

Figure 2. Total VFA production (mmol/L; average ± SE) is altered by the ratio of substrate to inoculum ($P < 0.0001$). Data points with different letters are significantly different ($P < 0.05$).

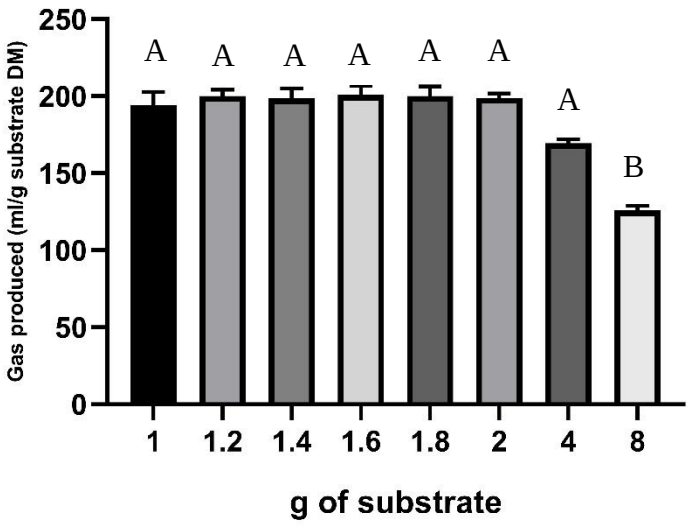

Figure 3. Total gas production (mL/g substrate DM; average ± SE) is significantly impacted by increasing substrate to inoculum ratio (P <0.0001). Values with different letters are significantly different (P <0.05).

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