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In vitro Fermentation Method as a Tool to Assess the Effects of Secondary Metabolites on Rumen Fermentation—A Brief Review

Helio Lima Neto[1] & Edward Okello[2]

[1] Olds College, Centre for Innovation, Technology Access Centre, Canada

[2] Newcastle University, Institute of Neuroscience, UK

Correspondence: Helio Lima Neto, Olds College, Centre for Innovation, Technology Access Centre, 4500 50 St, Olds, AB T4H 1R6, Canada. E-mail: hlima-neto@oldscollege.ca

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Abstract

In vitro fermentation is a useful method to test the effects of secondary metabolites on the rumen fermentation. It can be a noninvasive method when the rumen fluid is collected from slaughterhouses from dead animals. The experimental design for this type of experiment is normally a factorial design where common factors tested are: incubation time, main energy source incubated, and additive concentrations (secondary metabolites). The data gathered from the incubations can be quite extensive and therefore the statistical power of the *in vitro* method can be very good. In this paper we will discuss the main characteristics (how the method works and which parameters are measured) of the *in vitro* fermentation method.

Keywords: *in vitro* fermentation, ruminants, dairy cattle, beef cattle, sheep

1. Introduction

Noninvasive methods to study novel feedstuff to be used in animal agriculture have currently been developed and improved. The *in vitro* fermentation system can be a noninvasive method which allows laboratory testing of novel feedstuff produced secondary metabolites in the rumen. It is derived from the methodology developed by Tilley and Terry (1963), with some adjustments. For a noninvasive approach rumen fluid is pooled from different donors and taken to the laboratory to start the incubation. Once completed, the incubation will yield vast datasets with information such as: pH, ammonia levels, methane concentration, volatile fatty acids, dry matter degradability, total gas production, profile of microbial population, amongst others (Calsamiglia et al., 2007; Makkar et al., 2006; Yang et al., 2010). The analysis of this information can reveal the effects of an individual chemical compound when incubated in the rumen.

2. Rumen Digestive System and Considerations

2.1 Rumen Fermentation

The rumen, which is about one-seventh of the body mass of ruminants (McDougall, 1948), is maintained at relatively constant temperature (39 ^{o}C), buffered by salivary secretion, and is an ideal fermentation site for microbial ecosystems. During fermentation of feedstuffs by microorganisms; VFA, microbial cells, NH3, carbon dioxide (CO_2), CH_4, adenosine triphosphate (ATP), and heat are formed. VFA and ATP are used as the available energy sources for the animal, while microbial cells are the significant source of quality protein entering the small intestine (Demeyer, 1981; Russel & Hespell, 1981). Non-utilized NH3, CH4, and heat production may represent the loss of energy and N for the ruminants (Demeyer, 1981). In order to obtain appropriate knowledge and strategies to manipulate rumen fermentation, it is important to understand the mechanisms of carbohydrate and protein metabolism, methanogenesis, and acetogenesis in the rumen.

Ruminant diets contain substantial amounts of carbohydrate polymers such as cellulose, hemicellulose, starch, pectin, xylan, and water-soluble carbohydrates mainly in the form of fructans (McDonald et al., 2011; Russel & Hespell, 1981). A description of the conversion of carbohydrate polymers to VFA in the rumen is provided: diets containing plant particles are attacked by microorganisms and carbohydrate polymers are then released from plant cell structural matrices. After this, the carbohydrate polymers are hydrolysed to simple sugars such as cellobiose, maltose, xylobiose, hexoses, and pentoses by extracellular microbial enzymes. Cellulose is catalyzed by β-1,4-glucosidases to cellobiose and further converted either to glucose or glucose-1-phosphate. Starch is

initially hydrolyzed by amylases to maltose and iso-maltose, and then by maltose phosphorylases or 1,6-glucosidases to either glucose or glucose-1-phosphate. Fructans are degraded by enzymes involving 2,1 and 2,6 linkages to form fructose. This may be produced, together with glucose by the degradation of sucrose naturally present in plant materials. In hemicellulose, xylan is broken down by enzymes attacking the β-1,4 linkages to give pentoses as the major product, xylose, and uronic acids. Uronic acids are also produced from pectins, which are initially hydrolized to pectic acid and methanol by pectin esterase. The pectic acid is then converted by polygalacturonidases to galacturonic acids to further yield xylose. Xylose may also be obtained from hydrolysis of the xylans, which may be hugely available in forages (McDonald et al., 2011).

2.2 In vitro Method

The incubation method works as follows: about 1 g of ground energy source sample (for example: straw or hay or silage), plus the secondary metabolite under test in different inclusion levels according to experimental design, are put into 50-ml polypropylene tubes and 40 ml of the buffered inoculum (32 ml of buffer solution plus 8 ml of filtered rumen fluid) dispensed into each tube, purged with CO_2 to maintain anaerobic conditions, sealed with rubber stoppers fitted with gas pressure release valves, and incubated in a temperature controlled shaking water bath (39 ^{o}C) (Figure 1). During incubations, each tube is manually mixed for few seconds, three times a day (morning, afternoon, and night). The fermentation modules are then collected at specific times according to experimental design (for example: 24 h and 72 h) from the water bath and placed into an ice box (-10 ^{o}C) to stop further fermentation. After that, the liquids and residues are separated by centrifuging each tube at 2500 rpm for 10 min. The supernatant of each tube is then collected to determine VFA concentrations while residues are dried for IVDMD determinations. A separate 2 ml sample of each supernatant is also pipetted into a capped-container and mixed with 0.5 ml of deproteinising solution containing 10 mmol/L of crotonic internal standard solution for VFA determination added and kept in a freezer (-20 ^{o}C). The fermentation modules can have pipes attached to them that will allow the harvest of the fermentation gases in 50 ml glass syringes. Gas chromatography analysis methodology for methane and volatile fatty acid will be discussed in another publication.

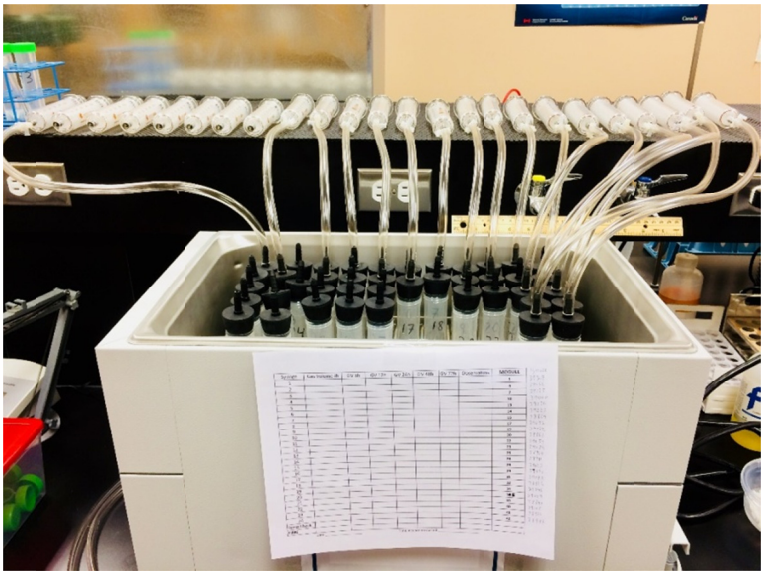

Figure 1. Shaking water bath with the fermentation modules and the harvesting pipes connected to the syringes to collect the fermentation gases

2.3 Plant Extracts (Secondary Metabolites) and the Ruminal Fermentation

Plant extracts containing secondary metabolites (*e.g.* saponins, solanine, solasodine, tannins, essential oils) are of increasing interest as alternative feed additives for humans because of their chemical properties and for ruminants because they can potentially inhibit rumen ciliate protozoa. Defaunation often lowers rumen methanogenesis because about 9 to 25% of ruminal methanogens live in association with protozoa (Newbold et al., 2008). Therefore, reducing methanogenesis is beneficial from the point of view that it can increase energy efficiency of the ruminant (Alexander et al., 2008) and from the reduction in the impact it can have towards the

environment (Makkar & Vercoe, 2007). These groups of natural "growth promoters" constitute a good opportunity to help farmers to increase animal production. Also the consumer can benefit because of the reduction of the potential transmittance of resistance or harmful effects caused by traditional antibiotics, which are an alternative. Practical effects of supplementing these chemicals are described in the literature ranging from metabolic effects which optimize rumen fermentation through to changes in milk composition.

Table 1. Some of the potential effects of secondary metabolites from plants found in the literature

Described Effect	Author
Increased digestibility of feedstuffs	Yang et al., 2007
pH stabilizing effect	Benchaar et al., 2006
Potential to manipulate milk fatty acid content	Benchaar & Chouinard, 2009
Increased propionate production	Poungchompua et al., 2009
Increased nitrogen retention	Wanapat et al., 2008
Increased efficiency in converting food to milk	Lovett et al., 2006
Increased milk yield and lactose content	Benchaar et al., 2007
In vivo and *in vitro* reduction of methane production	Wang et al., 2009
Increased microbial protein synthesis	Makkar et al., 2006
Reduced protein and energy losses	Calsamiglia et al., 2007
Increased feed intake	Yang et al., 2010
Enhanced acetate/propionate ratio in the rumen	Benchaar & Chouinard, 2009
Milk composition with beneficial properties which have potential anticarcinogenic agents (isoprenoids from plants)	Parodi, 1999

2.4 The Utilization of the in vitro Fermentation Method and Some Literature Findings

Alexander et al. (2008) completed an *in vitro* study to identify plant extracts that modulate partitioning of degraded organic matter (OM) towards microbial protein synthesis, at the expense of gas production, and decrease protein degradation in the rumen. They found that that aqueous methanol extract of *M. oleifera* seed and aqueous extract of *P. kurroa* root may have potential as feed additives to increase the efficiency of utilization of energy and nitrogen in ruminant diets. In addition, a review of the potential of some chemicals to manipulate rumen fermentation, utilizing publications that used *in vitro* fermentation method, have indicated that garlic oil, cinnamaldehyde (the main active component of cinnamon oil), eugenol (the main active component of the clove bud), capsaicin (the active component of hot peppers), and anise oil, among others, may increase propionate production, reduce acetate or methane production, and modify proteolysis, peptidolysis, or deamination in the rumen (Calsamiglia et al., 2007).

Another work utilizing the *in vitro* fermentation method, found that a substantial amount (71-93%) of tannins soluble in aqueous acetone was released from leaves of some trees and shrubs on incubation in the *in vitro* medium for 48 h. It also concluded that he rumen liquor was not capable of degrading oligomeric condensed tannins (Makkar et al., 2006). Newbold et al. (1995) studied the importance of methanogenic bacteria associated with ciliate protozoa either by removing protozoa from whole rumen fluid (using defaunated rumen fluid to correct for the effects of centrifugation on bacteria) or by isolating the protozoa. Rumen fluid was withdrawn from sheep inoculated with either *Polyplastron multivesiculatum*, a co-culture of Isotricha prostoma plus *Entodinium* spp. or a mixed type B fauna of *Entodinium*, *Eudiplodinium* and *Epidinium* spp. They found that methanogenesis was highest in rumen fluid containing a mixed protozoal population of the following genera: *Entodinium*, *Eudiplodinium* and *Epidinium*, was lower in defaunated rumen fluid and lowest in rumen fluid containing either I. prostoma plus *Entodinium* or *P. multivesiculatum*. Also, methanogenic bacteria associated with rumen ciliates were apparently responsible for between 9 and 25% of methanogenesis in rumen fluid.

Lima Neto (2012) investigated the effects of plant fractions (*Solanum lycocarpum* St. Hil.) on the rumen fermentation utilizing the *in vitro* fermentation system. The leaf fraction of the shrub (10 g/Kg DM inclusion) had the strongest effect when added to the Lucerne and increased ruminal the Acetate: Propionate ($P < 0.05$). The author also found that when SL fractions were evaluated with incubated ryegrass hay, root and stem fractions decreased Ace: Prop ($P < 0.05$).

3. Conclusions

The *in vitro* fermentation method is a powerful tool to study secondary metabolites in the rumen ecosystem. The method can generate a vast quantity of data, and the results can be validated with a subsequent *in vivo* trial, where similar treatments are tested. Secondary metabolites can definitely be useful to manipulate rumen fermentation and future studies will shed some light on the best ways to utilize them.

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