CÉSAR ROBERTO VIANA TEIXEIRA

COMPARING THE RESPONSES OF RUMEN CILIATE PROTOZOA AND BACTERIA TO EXCESS GLUCOSE

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa as partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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APPROVED: June 29th, 2018

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This thesis is dedicated to my parents,
who have always been a source of inspiration, stamina and support.

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BIOGRAPHY

CÉSAR ROBERTO VIANA TEIXEIRA, son of Ari Roberto Teixeira de Carvalho and Maria das Dores Viana Teixeira, was born in Viçosa - Minas Gerais - Brazil on August 31st, 1988. In 2007, joined the Universidade Federal de Viçosa, in the Animal Science course. In November of 2012, he earned a Bachelor of Science degree in Animal Science. In this same year he joined the Master degree program in the Department of Animal Science at Universidade Federal de Viçosa, concentrating his studies in Ruminant Nutrition and Production area, concluding this course in February of 2014. In March of 2014 he joined the PhD program in the same area and department. Between August of 2015 and October of 2016, he developed part of research in University of Florida, USA. In order to obtain the Doctor Scientiae degree in Animal Sciences, his thesis has been submitted to the committee in June of 2018.

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ABSTRACT

TEIXEIRA, César Roberto Viana, D.Sc., Universidade Federal de Viçosa, June, 2018. **Comparing the responses of rumen ciliate protozoa and bacteria to excess glucose**. Adviser: Rogério de Paula Lana. Co-adviser: Cristina Mattos Veloso.

Rumen microbes hold a central role in ruminant nutrition. They ferment feed components to produce volatile fatty acids (VFA) and grow (synthesize microbial protein), which supplies the greater part of energy and amino acids required by the animals. However, when given excess carbohydrate, microbes growth efficiency becomes low because microbes direct energy to non-growth functions, instead of using it for growth. Different microorganisms respond to this excess in different ways. Certain species respond by storing energy (synthesizing reserve carbohydrate), but other species respond by dissipating the energy as heat (spilling energy). To determine the relative importance of these responses in the microbial community of the rumen, this study aims to quantify how mixed ciliate protozoa and bacteria respond to glucose. It was hypothesized that ciliate protozoa would direct more glucose to synthesis of reserve carbohydrate and less to energy spilling than would bacteria. Ciliates and bacteria were isolated from rumen fluid using filtration and centrifugation, respectively. Posteriorly, ciliates and bacteria were resuspended in nitrogen-free buffer to limit growth and dosed with 5 mM glucose. Samples were collected over time and were subsequently divided in pellet (cells) and supernatant by centrifugation. Pellet samples were analyzed for reserve carbohydrate and protein, while supernatant sample were analyzed for free glucose, D-/L-lactic acid, acetic acid, propionate and butyrate. Additionally, were analyzed heat production and fermentation gases (H_2, CH_4 and CO_2). Endogenous metabolism, reserve carbohydrate synthesis and energy spilling were calculated from the data obtained from the analysis data. Most data were analyzed using PROC GLIMMIX of SAS. Student's *t*-test was used to separate means or determine if means differed from 100%. Local regression (LOCFIT package of R; Loader, 1999) was used to fit time-series data to smooth curves. Compared to bacteria, ciliates consumed glucose more than 3-fold faster and synthesized reserve carbohydrate 4-fold faster. They incorporated 53% of glucose carbon into reserve carbohydrate, nearly double the value (27%) for bacteria. Energy spilling was not detected for ciliates, as all heat production was accounted by synthesis

of reserve carbohydrate and endogenous metabolism. For bacteria, reserve carbohydrate and endogenous metabolism accounted for only 68% of heat production, thus they spilled large amounts of energy (32% of total heat production). These results suggest that rumen ciliates protozoa alter the course of carbohydrate metabolism in the rumen by consuming glucose more rapidly and outcompeting bacteria for excess carbohydrate. This action of the ciliates in the rumen likely maximizes reserve carbohydrate synthesis while minimizing spilling.

RESUMO

TEIXEIRA, César Roberto Viana, D.Sc., Universidade Federal de Viçosa, junho de 2018. **Respostas de protozoários ciliados e bactérias do rúmen ao excesso de glicose**. Orientador: Rogério de Paula Lana. Coorientadora: Cristina Mattos Veloso.

Os microrganismos ruminais têm um papel central na nutrição de ruminantes. Eles têm a capacidade de fermentar componentes do alimento para produzir ácidos graxos voláteis (AGV's) e crescer (sintetizar proteína microbiana), os quais fornecem a maior parte da energia e aminoácidos exigidos pelos animais. No entanto, quando são fornecidos carboidratos em excesso, a eficiência de crescimento dos microrganismos torna-se baixa porque estes direcionam a energia para outras funções, ao invés de a utilizarem para o crescimento. Diferentes microrganismos respondem a esse excesso de maneiras diferentes. Certas espécies respondem armazenando energia (sintetizando carboidratos de reserva), mas outras espécies respondem dissipando a energia na forma de calor. Para determinar a importância relativa dessas respostas na comunidade microbiana do rúmen, este estudo foi cinduzido com o objetivo de quantificar como os protozoários ciliados e as bactérias responderam à glicose. Teve-se como hipótese que os protozoários ciliados direcionariam mais glicose para a síntese de carboidratos de reserva e desperdiçariam menos energia na forma de calor, em relação as bactérias. Ciliados e bactérias foram isolados do líquido ruminal por filtração e centrifugação, respectivamente. Posteriormente, os ciliados e as bactérias foram suspensos em tampão isento de nitrogênio para limitar o crescimento e dosados com 5 mM de glicose. As amostras foram coletadas ao longo do tempo e, posteriormente, divididas por centrifugação em pellets (células) e sobrenadante. Amostras de pellets foram analisadas quanto à reserva de carboidratos e proteínas, enquanto amostras de sobrenadante foram analisadas para glicose livre, ácido D-L lático, ácido acético, propionato e butirato. Adicionalmente, foi analisado a produção de calor e gases de fermentação (H_2, CH_4 e CO_2). O metabolismo endógeno, a síntese de carboidratos de reserva e o desperdício na forma de calor foram calculados a partir dos dados das análises. A maior parte dos dados foi analisada usando o PROC GLIMMIX do SAS. Teste t de Student foi usado para separar as médias ou determinar se as médias diferiam de 100%. Regressão local

(pacote LOCFIT de R; Loader, 1999) foi usada para ajustar os dados das séries no tempo. Em comparação com as bactérias, os ciliados consumiram três vezes mais glicose e sintetizaram carboidratos de reserva quatro vezes mais rápido. Eles incorporaram 53% da glicose em carboidratos de reserva, quase o dobro do valor (27%) obtido para as bactérias. Desperdício de energia na forma de calor não foi detectado para os ciliados, uma vez que toda a produção de calor foi contabilizada pela síntese de reserva de carboidratos e pelo metabolismo endógeno. Em bactérias, a síntese de carboidratos de reserva e o metabolismo endógeno representaram apenas 68% da produção total de calor, assim, elas desperdiçaram grande quantidade de energia por meio da produção de calor (32% da produção total de calor). Esses resultados sugerem que os protozoários ciliados ruminais alteram o curso do metabolismo de carboidratos no rúmen, consumindo glicose mais rapidamente, limitando o uso do excesso de carboidratos pelas bactérias. Essa ação dos ciliados no rúmen provavelmente maximiza a síntese carboidratos de reserva, enquanto minimiza a ocorrência de desperdício de energia na forma de calor.

Chapter 1: General Introduction

The rumen and its microbes hold an important role in ruminant nutrition (Hackmann, 2014). Owing to the symbiotic interaction with bacteria, protozoa, fungi, and methanogens inhabiting their rumen, ruminants can ferment fiber and other feed components to volatile fatty acids (VFA) and, in the process, generates energy that supply microbial growth (Hackmann and Firkins, 2015).

The VFA produced by fermentation of feed components meet up to 70% of the animal's energy needs (Bergman, 1990) and microbial growth provides 60 to 85% of amino acids (AA) that reach the animal's small intestine (Storm et al., 1983). Thus, ruminants can convert feedstuffs into extremely nutritious meat and milk (Hackmann, 2013). In total, ruminants produce, respectively, approximately 30% and 100% of the world's supply of these products [calculated from 2016 FAO Statistical Databases (http://faostat.fao.org/)].

Although microbial fermentation may be essential to ruminant nutrition and a keystone in the world's food supply, it has been recognized for a long time that microbes grow (synthesize microbial protein) with low efficiency (Stouthamer, 1973). For mixed rumen microbes *in vivo*, microbial growth ranges from only one third to two third of the theoretical maximum (Russell and Wallace, 1997). Production of microbial protein in the rumen has low efficiency because microbes do not direct all ATP toward growth. Rather, microbes direct energy on non-growth functions such as maintenance, synthesis of reserve carbohydrate and spilling (Hackmann and Firkins, 2015).

Improving microbial growth efficiency could increase supply of essential AA, permit feeding of lower protein diets, reducing feed costs and N excretion. Furthermore,

crude protein from feed often has poor content of essential AA (Hackmann, 2013), but rumen microbes can convert feed crude protein into microbial protein, which is rich with essential AA (Hackmann et al., 2013). Thus, changes in efficiency of microbial growth in the rumen can have a dramatic impact on the economics of milk and meat production (Nocek and Russell, 1988).

In addition to improving efficiency of microbial growth, improving prediction of efficiency by diet formulation systems is another strategy to increase confidence in diet formulation, and potentially decrease the amount of protein in diets. According to Hackmann (2013), microbial growth efficiency ranges widely, but it is poorly predicted.

To fill these gaps, one of the first steps is to understand how much energy rumen microbes waste in non-growth functions. Hackmann et al. (2013) evaluated how mixed rumen microbes respond to excess carbohydrate and concluded that cells dosed with small excess of carbohydrate (5 mM of glucose) respond predominantly by synthesis of reserve carbohydrate, without spilling. Nevertheless, under large doses of glucose (20 mM), energy spilling reached nearly 40% of heat production in one incubation.

Although the importance of energy spent in non-growth functions has already been demonstrated in mixed rumen microbes (Hackmann et al., 2013), it is not known exactly how rumen microbial groups respond individually to excess carbohydrate. We hypothesized that ciliates protozoa would respond to excess glucose chiefly by synthesizing reserve carbohydrate, whereas bacteria would spill energy.

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Chapter 2: Literature Review

Introduction

In the early 1940s, Monod (1942) studied the growth of microbe cultures and observed that dry weight of the organisms was directly proportional to the amount of available energy. Thus, by the late 1950s, most of the bacteriologists had accepted the idea that microbial growth was usually equivalent to energy yield (Sokatch and Gunsalus, 1957; Senez, 1962).

In 1960, Bauchop and Elsden (1960) correlated biomass production with ATP availability (Y_{ATP}). Despite the great variation of the data (more than 50%), the average Y_{ATP} value of 10.5 g of cells per mol of ATP was considered as a biological constant (Stanier et al., 1976; Ingraham, 1983; Gottschalk, 1986; Brock and Madigan, 1991).

However, by the 1970s, the notion of a constant Y_{ATP}, was being questioned. Research showed that the correlation between ATP and biomass formation was often weak (Stouthamer, 1979; Tempest and Neijssel, 1984). As it had been defined, the concept of Y_{ATP} assumed that the energy from catabolism could be entirely used for growth. However, this assumption had been contradicted by Buchanan and Fulmer (1928) who observed that low levels of energy were not effective for bacterial growth and suggested that bacteria need some energy to ‘‘maintain’’ the cells.

The bacterial maintenance energy suggested by Buchanan and Fulmer (1928) have not been precisely defined, but is known that this energy supports functions like ion balance across the cell membrane, motility and turnover of macromolecules (Russell and Wallace, 1997).

The addition of maintenance energy to microbial energetics calculations helped explain why microbes did not reach the theoretical value previously proposed. Thus, Stouthamer and Bettenhaussen (1973) introduced a new term (Y_{ATP}/MAX), which Y_{ATP} was corrected for maintenance energy. Stouthamer's calculations indicated that Y_{ATP} should be threefold higher than the value derived by Bauchop and Elsden (1960) (32 versus 10.5 g of cells per mol of ATP) (Stouthamer, 1979). However, DeVries et al. (1970) observed that not even using this correction it could reach Y_{ATP} values of 32 g of cells per mol of ATP.

In the 1980s, by applying the principles of nonequilibrium thermodynamics to the study of bacterial growth, Westerhoff et al. concluded that microbial growth yields were 50% less than their theoretical potential (Westerhoff et al., 1982; Westerhoff et al., 1983; Westerhoff et al., 1984). Thus, it became apparent that many bacteria also expend energy on functions that are not directly related to growth or maintenance (Russell and Cook, 1995).

The terms energy spilling, overflow metabolism, slip reactions, and uncoupling were all used to describe the spent of energy not directly related to growth or maintenance, but the process was not well understood (Stouthamer, 1979).

In 1986, work done by Russell has shown that microcalorimetry provides a sensitive assessment of bacterial growth and heat production (Russell, 1986). Since heat is the ultimate end-product of energy spilling reactions, it seemed that microcalorimetry might provide a more direct method of studying this phenomenon. Thus, Russell (2007) defined energy spilling as the energy dissipated as heat when ATP is in excess, with no protein production.

Using microcalorimetry, Hackmann et al. (2013a) concluded that when mixed rumen microbes were given a large excess of energy, nearly 40% of heat was generated from spilling alone, thus energy spilling may be considered a particularly wasteful process.

In the rumen, the input of energy sources from feed is only occasionally abundant and the alternation between periods of abundance and energy deficiency often happens. Thus, the efficiency of ATP management is an important factor influencing the survival of microorganisms. Under these conditions, rumen organisms have developed strategies to survive in periods of starvation. At these periods, ability to store energy (reserve of carbohydrate synthesis) can be crucial for maintaining viability and to ensure that the organism can respond properly to the next influx of energy to the rumen (Russell and Wallace, 1997).

Reserve carbohydrate synthesis refers to formation of glycogen and other compounds during energy excess (Preiss and Romeo, 1989). Although reserve carbohydrate would be less detrimental than energy spilling because it may be mobilized later for growth (Wilkinson, 1959) or pass from the rumen without being fermented (McAllan and Smith, 1974), storage is still wasteful because ATP is irreversibly spent to synthesize glycogen. This waste represents 20 to 50% of the available ATP in glucose (Stouthamer, 1973).

Ciliate protozoa have been speculated to stabilize rumen fermentation because of its high capacity to consume carbohydrates and synthesize glycogen, preventing rapid fermentation of those substrates by bacteria (Williams and Coleman, 1992). This prevents accumulation of short-chain fatty acids (SCFA), pH drop, and lactic acid acidosis, consequently (Williams and Coleman, 1992; Nocek, 1997).

Although ciliate protozoa are known to accumulate more reserve carbohydrate than bacteria (Williams and Coleman, 1992), quantitative support for this assumption remain sparse by the inability to culture protozoa axenically (Denton et al., 2015).

Therefore, for the above reasons, the efficiency of microbial grow is far from the maximum efficiency (Stouthamer, 1973). That is, microbes spend part of the available energy on non-growth function such as maintenance, energy spilling and energy storage instead use it for growth. Studies demonstrated the magnitude of energy spent in non-growth functions for mixed rumen microbes (Hackmann et al., 2013a). However, it is not known exactly how rumen microbial groups individually respond to excess carbohydrate.

Maintenance

For many years, it was assumed that energy yielding pathways in bacteria were tightly coupled to growth, but it has since been demonstrated that energy-sufficient cultures can continue to metabolize energy sources in the absence of growth (Neijssel and Tempest, 1976). Part of this occurrence can be explained by spending on maintenance energy (Russell and Cook, 1995).

Maintenance functions include mostly maintaining ion balances across the cell membrane, re-synthesis of protein following intracellular turnover, and motility (Russell and Cook, 1995). Ion fluxes across the membranes of growing bacteria have not been measured directly, but because membranes have a ‘leakiness’, this component is probably the most important one (Russell, 2007).

Protein turnover experiments with *E. coli* cells indicated that the rate of protein turnover in exponentially growing ranged from 0.5 to 2.5%/h, and 5%/h during stationary phase (Mandelstam and McQuillen, 1985). Although significant, these rates correspond to a smaller portion of the maintenance in *E. coli*. The glucose consumption rate required to sustain a protein turnover rate of 5%/h is only approximately 13% of the total maintenance rate of *E. coli* (Pirt, 1965).

Although mentioned separately, motility of flagellated bacteria can be viewed as a case of ion turnover because the flagella are driven by proton or sodium motive force (Russell and Cook, 1995). MacNab and Koshland (1972) indicated that up to 1% of the total energy in *E. coli* could be directed to motility.

Thus, unlike spilling and synthesis of reserve carbohydrate, maintenance is required for cell survival and it is an unavoidable expenditure (Hackmann, 2014). Still, maintenance is considered wasteful from the productive perspective because its final product is heat with no microbial protein synthesis (Hackmann, 2014).

Energy directed to maintenance makes up a relatively small proportion of total energy use. However, as the growth rate decreases, maintenance becomes more significant and can reach up more than 30% of the total energy expended (Hackmann, 2014).

There are different alternatives to estimate the value of maintenance energy. In this current study, it was assumed that maintenance functions were equal to endogenous metabolism. Following the definition of Dawes (1985), the rate of heat production generated from endogenous metabolism was measured prior to dosing glucose, during starvation and absence of growth, and then integrated.

An alternative method assume that maintenance functions is equal to energy use of rumen bacteria extrapolated to growth rate = 0, during fed conditions and growth (Pirt, 1965; Cook and Russell, 1994).

Comparing both, the method used in this study estimates the maintenance at approximately 20% greater than the alternative method for mixed rumen bacteria (Hackmann, 2013). Consequently, because energy spilling is calculated by difference, its estimation is approximately 5% lower for rumen bacteria dosed with 5 mM glucose, compared to the alternative method (Hackmann, 2013).

Reserve of carbohydrate

Reserve carbohydrate synthesis is the formation of glycogen and other carbohydrate reserve compounds during energy excess (Preiss and Romeo, 1989).

Rumen microbes are capable of accumulating large amounts of reserve carbohydrate, which may exceed 50% of cell weight (Preiss and Romeo, 1989; Russell, 1998). Although reserve carbohydrate seems to be more evident in ciliate protozoa, it is also present in rumen bacteria and fungi (Hackmann and Firkins, 2015).

At first moment, synthesis of reserve carbohydrate seems to improve growth efficiency. Glycogen, the most common reserve carbohydrate, requires fewer ATP for synthesis than almost all other cell macromolecules (Hackmann and Firkins, 2015). In addition, it may be mobilized later for growth (Wilkinson, 1959) or pass from the rumen to intestine without being fermented (McAllan and Smith, 1974); and its synthesis can prevent ruminal acidosis by limiting the amount of carbohydrates to rapid fermentation after feeding. (Williams and Coleman, 1992).

The facts cited above, makes the synthesis of reserve carbohydrates appear to be economical. However, reserve carbohydrate accumulation is dynamic (Hackmann and Firkins, 2015). Usually, it is accumulated immediately after feeding , when there is a carbohydrate excess, and then mobilized thereafter, when there is a carbohydrate limitation (Jouany and Thiven, 1972; McAllan and Smith, 1974; Williams and Harfoot, 1976; Leedle et al., 1982; Hackmann and Firkins, 2015). Sequential synthesis and degradation of glycogen represents waste of 20 to 50% of the available ATP in glucose, given 1 net ATP is spent on glycogen synthesis (Stouthamer, 1973) and between 2 to 5 ATP are available from glucose fermentation (Russell, 2002). Therefore, despite synthesis of glycogen cost few ATP compared to most other macromolecules, it is quickly outweighed by this sequential synthesis and degradation. Nevertheless, reserve carbohydrate still less detrimental to growth efficiency than energy spilling (Hackmann and Firkins, 2015).

Despite its importance, only recently reserve carbohydrate have been used in efficiency measurements. Most experiments used chemostats under steady-state conditions, which by design prohibit variations between glycogen synthesis and mobilization (Novick and Szilard, 1950; Monod, 1950; Marr et al., 1962; Pirt, 1965; Neijssel and Tempest, 1975; Neijssel and Tempest, 1976; Russell and Baldwin, 1979; Tempest and Neijssel, 1984). Experiments using batch cultures allows the the variation of the synthesis and degradation of glycogen (Russell and Cook, 1995), but they are usually finalized during exponential growth, before reserve carbohydrate degradation typically occurs (Hackmann and Firkins, 2015).

Recently, in a study conducted in batch culture, Hackmann et al. (2013a) observed that when mixed rumen microbes were dosed 5 mM and 20 mM glucose, at

peak accumulation, microbes incorporated 59.5% and 52.6% of glucose carbon in reserve carbohydrate, respectively. After glucose was exhausted, reserve carbohydrate quickly declined (Hackmann et al., 2013a). In a subsequent batch culture study, Denton et al. (2014) performed competition experiments between ciliate protozoa and bacteria for glucose consumption. It was observed that in small excess of carbohydrate (5 mM glucose) ciliate protozoa were responsible for 58.7% of glycogen accumulation, while bacteria accumulated only 1.7% of glucose in reserve carbohydrate. When the cultures were dosed with a high concentration glucose (20 mM), the amounts incorporated were 21.4% for protozoa and 5.0% for bacteria, respectively. Thus, ciliate protozoa would appear the main group of microorganisms accumulating reserve carbohydrate, and consequently, they have been proposed to stabilize rumen fermentation, for preventing rapid fermentation by bacteria (Williams and Coleman, 1992). This prevents accumulation of SCFA, depression of pH, and lactic acid acidosis, which decreases animal performance (Nocek, 1997; Williams and Coleman, 1997).

In addition, the synthesis of reserve carbohydrate by protozoa would deprive substrate for bacteria growth, and this could explain why ciliate protozoa may persist longer in the rumen than bacteria, despite bacteria grow much faster than protozoa, giving protozoa an advantage in competition over bacteria (Russell and Baldwin, 1979; Sylvester et al., 2009).

Although reserve carbohydrate synthesis and energy spilling have been discussed independently, glycogen cycling would bind these functions because it is a form of energy spilling (Hackmann and Firkins, 2015). According to Hackmann et al. (2003a), glycogen cycling have been speculated as the main mechanism of spilling observed for mixed rumen microbes, although it is less detrimental that other energy

spilling mechanisms. Thus, directing more energy to reserve carbohydrate synthesis instead to spilling could improve the efficiency of microbial growth (Hackmann, 2013).

There are many different methods for detecting reserve carbohydrates. It can be detected enzymatically by hydrolyzing glucans and quantifying released glucose (Gunja-Smith et al., 1977; Schulze et al., 1995; Parrou and Francois, 1997) or chemically, such as by the anthrone reaction (Trevelyan and Harrison, 1956; Herbert et al., 1971). However, it has been recognized that different methods detect diverse values of reserve carbohydrate (Rothman and Cabib, 1969; Kaeppeli et al., 1975).

Few studies have investigated which method detects reserve carbohydrate completely and quantitatively. Some authors have suggested that amyloglucosidase hydrolysis completely detects glycogen (Gunja-Smith, et al., 1977; Becker, 1978). However, they did not determine energy, carbon, and cell recoveries to evaluate if detection was quantitative.

Hackmann (2013), in a study with rumen microbes, concluded that the anthrone method detected more carbohydrate and produced larger changes in reserve carbohydrate than did the amyloglucosidase hydrolysis method, with high energy, carbon, and cell recoveries. Based on it the anthrone method appeared to accurately detect the change in reserve carbohydrate.

Energy spilling

Energy spilling by rumen microorganisms is the most common response to excess carbohydrates (Russell, 1998). It refers to energy dissipated as heat when ATP exceeds needs for growth, maintenance functions, and reserve carbohydrate synthesis

(Russell, 2007). Rumen bacteria can ferment glucose up to 10-fold faster when they are spilling energy, confirming that energy spilling may be a significant sink for ATP (Van Kessel and Russell, 1996).

To prove the energy spilling concept, Russell (1986) dosed rumen bacterial cultures with glucose. The bacterial cultures quickly fermented excess glucose, produced insignificant amounts of protein, and spilled energy by producing heat. Since then, spilling has been demonstrated in rumen (*Streptococus bovis*) and non-rumen bacteria (*E. coli, Klebsiella aerogenes*) (Hackman and Firkins, 2015). Although there are few studies, this also probably occurs in protozoa, fungi and methanogens, suggesting broad importance (Hackman and Firkins, 2015).

In addition to glucose dosage, excess carbohydrates have been generated by culturing cells under limitation of anabolic substrates (e.g., N, Mg, P, S, K), ammonia-N replacing amino-N, excess H_2 or CO_2 (for methanogens), and exogenous protonophore (Hackmann and Firkins, 2015).

Energy spilling can occur by three different mechanisms: futile cycles of ions, glycogen or trehalose. The most well-known mechanisms is for *S. bovis* and for *E. coli*, for which spilling happens by futile cycling of protons (Hackmann and Firkins, 2015). This cycling starts with a growth limitation and triggers a cascade of biochemical events (Russell, 2002), which results exclusively in production of heat (Hackmann and Firkins, 2015).

In some other organisms, cycling of glycogen or trehalose may occur and indicate the presence of energy spilling, although more direct evidence has not been reported (Hackmann and Firkins, 2015). Glycogen cycling would link synthesis of reserve carbohydrate and energy spilling (Hackmann and Firkins, 2015), and although

glycogen cycling has not yet been demonstrated for rumen communities, it has been speculated as the main mechanism of spilling (Hackmann et al., 2013a). Trehalose may perform a protective function during thermal or osmotic stress (Arguelles, 2000).

Energy spilling has already been demonstrated in many pure bacterial cultures, but there are few examples of mixed bacteria and protozoa cultures spilling energy. Van Kessel and Russell (1996) concluded that rumen bacteria spilled energy when grown under ammonia-N limitation, but they did not measure reserve carbohydrate, which may have overestimated energy spilling. Chen et al. (2000) induced spilling by including a protonophore to a bacterial culture. However, spilling was not measured under physiological conditions, and this undermined the reliability of the data.

More recently, Hackmann et al. (2013a) quantified spilling for mixed rumen microbes dosed with high concentration of glucose (20 mM) in absence of N. They consumed glucose rapidly, accumulated reserve carbohydrate, spilled energy and did not grow. Energy spilling accounted for as much as 38.7% of heat production in one incubation.

The function of spilling is not completely understood, but it could give to microbe a competitive advantage over other species (Russell, 2007). Some authors have speculated that energy spilling allows microbes to quickly catabolize substrate and dissipate energy to limit the availability of energy to other competing species or accelerate the restart of growth when a growth limiter is supplied (Tempest, 1978; Russell and Cook, 1995). According to Newsholme and Crabtree (1975), some futile cycles also can perform the sensible control of the liquid flow of metabolites . Thus, energy spilling could have the function of avoiding the toxicity caused by metabolites

produced when there are excess carbohydrates, such as methylglyoxal (Russell, 1998; Hackmann, 2013).

According to Russell and Cook (1995), energy spilling is an indicator of the imbalance between anabolism and catabolism and bacteria spill energy when catabolic rate (substrate degradation rate) is faster than the anabolic rate (microbial growth rate). When bacteria have no amino acids limitation, the balance of anabolic and catabolic rates are closely matched. Under this condition, they typically grow faster and spill less energy (Russell and Cook, 1995). Thus, the effect of amino acids on microbial protein yield seems to be correlated to energy spilling (Russell, 2007). The idea that amino acids could decrease energy spilling was demonstrated with rapidly growing continuous cultures of *S. bovis* (Bond and Russell, 1998). Therefore, adequate supply of amino acids, especially of rumen degradable protein (RDP) from feed, may not completely eliminate spilling, but it may reduce the imbalance between anabolism and catabolism and energy spilling consequently (Hackmann, 2013). Additionally, Bacteria grow faster with true protein than with non-protein nitrogen (NPN), this results in less energy spilling and greater microbial growth efficiency when the true protein is used, either *in vitro* (Van Kessel and Russell, 1996) or *in vivo* (Hume, 1970).

In the productive scenario, energy spilling is detrimental because of its potential to reduce growth of rumen microbes, the main source of protein for ruminant livestock (Clark et al., 1992). Thus, spilling may be a important target to improving efficiency because, unlike maintenance, it is not essential to cell survival and it is the main reason for the low efficiency during carbohydrate excess or other growth-limiting conditions (Hackmann, 2013).

As described in Hackmann et al. (2013a), energy spilling is calculated from total heat production, minus heat accounted by endogenous metabolism and heat accounted by reserve carbohydrate synthesis (Figure 2).

Occurrence of excess carbohydrate in the rumen

Synthesis of reserve carbohydrate and energy spilling occur primarily under carbohydrate excess (Hackmann and Firkins, 2015). Usually, excess carbohydrate is present when there is an imbalance between energy and amino acids availability, especially of RDP from feed. However, carbohydrate excesses can also happen in growing cells under limitation of other anabolic substrate different from protein, for example Mg, P, S, and K (Hackmann, 2013).

When animals are fed high grain diet and low N availability, particularly those transitioning to a high-grain diet, carbohydrate excess is further intensified, and glucose can reach concentrations approximately to 5 mM (Ryan, 1964; Mackie et al., 1978). It may also happen for dairy rations with corn silage as the sole source of forage (Vandehaar, 2005), due to the high concentration of sugars; for animals in which N is chiefly in the form of ammonia (Van Kessel and Russell, 1996), because rumen microbes grow slower with ammonia-N than amino-N (Argyle and Baldwin, 1989; Van Kessel and Russell, 1996); and for animals fed low protein forage, such sugarcane and some species of tropical grasses, without adequate addition of RDP. Even higher concentrations of glucose were reported for animals fed dextrose (18 mM) (Piwonka et al., 1994); beet pulp (69 mM) (Clapperton and Czerkawski, 1969); and in microenvironments such as round starch granules (Kajikawa et al., 1997).

Animals fed high-forage or those well adapted to high-grain diets, carbohydrate excess is smaller, and concentration of glucose is hardly greater than 2.5 mM (Kajikawa et al., 1997; Saleem et al., 2012). Energy spilling seems to be less important under these conditions, given that it was not detect spilling in batch cultures with glucose concentrations of 5 mM (Hackmann et al., 2013a). Even still, rumen microbes accumulate reserve of carbohydrate when animals were fed low-quality grass diets (Van Kessel and Russell, 1997), and thus responses discussed above may still have relevance.

Avoiding energy excess

Proper diet formulation may reduce excess carbohydrate in the rumen, and consequently, decrease waste through spilling and storage. Usually, carbohydrate excess occurs when RDP is low, thereby limiting microbial growth (Hackmann, 2014). According to Dairy NRC (2001), RDP requirements ranges between 9.5 to 11.3% of diet dry matter for lactating cow and 8.6 to 10.8% for heifers.

RDP deficit can be corrected by increasing the inclusion of ingredients with high RDP, such as urea and soybean meal. In addition, to reduce excess carbohydrate, part of the carbohydrate of the ration should be decreased by substituting it with fat, which is rich in energy but cannot be fermented (Hackmann, 2014).

Though adequate supply of RDP can reduce energy spilling and reserve carbohydrate synthesis, it may not eliminate them. Some energy waste still occurs even when RDP is apparently adequate, mainly when RDP is offered in the form of NPN from ammonia (Hackmann et al., 2013b; Hackmann, 2014). According to Van Kessel and Russell (1996), bacteria grow slowly with NPN than with true protein, and this

favors the occurrence of energy spilling. This finding corroborate the results observed *in vivo*, where microbial growth efficiency has increased 36% by partially replacing urea (NPN) with casein (true protein) (Hume, 1970).

Prediction of microbial protein

Improving prediction of microbial growth can improve efficiency and reduce dietary protein, which is the most expensive component of the ruminant diet. Approaches for predicting microbial growth currently exist but they are imperfect (Hackmann, 2013). Most of empirical systems predicts efficiency from a simple constant, adjusted for availability of RDP. Therefore, it does not account for energy spilling or reserve carbohydrate synthesis, despite their likely impact on growth efficiency. Furthermore, empirical systems often express growth efficiency in terms of total tract digestible organic matter (OM) (NRC, 2001). However, rumen microbes cannot generate ATP from all OM digested (Dijkstra et al., 1998). As a result, the popular NRC (2001) system can explain only 35% of variation in efficiency (Hackmann, 2013).

Nowadays, there are already more sophisticated mechanistic approaches to predicting growth efficiency which take into account maintenance, energy spilling and reserve carbohydrate expenditures and express growth efficiency in terms of ATP or ruminally-fermented carbohydrate. However, many of their parameter are heuristic values or simple constants, due to the lack of experimental data (Dijkstra et al., 1992; Russell et al., 1992; Dijkstra, 1994; Baldwin, 1995; Dijkstra et al., 1998; Hackmann and Spain, 2010). For example, reserve carbohydrate is assumed as a constant fraction of

microbial biomass for the most of systems, and thus, the application of their models has been restricted only to research (Russell et al., 1992; Baldwin, 1995; Hackmann and Spain, 2010; Hackmann, 2013).

Thus, whereas models need to be improved, experiments on energy spilling and reserve carbohydrate could better parameterize these models, improving their prediction of growth efficiency and guiding efforts to maximize efficiency of microbial production.

Conclusion

Rumen microbes grow with low efficiency because they spend part of the available energy on non-growth function instead of using it for growth. It has been hypothesized that ciliates protozoa waste energy mainly by synthesizing reserve carbohydrate, whereas bacteria spilling energy. However, experiments are necessary to determine how rumen microbial groups individually respond to excess carbohydrate and the relative magnitude of these responses.

In addition, experiments on energy spilling and reserve carbohydrate could supply data to better parameterize models, improving their prediction of growth efficiency; guiding efforts to maximize efficiency of microbial production; and potentially decrease supply of crude protein in the diet.

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Chapter 3: Comparing the responses of rumen ciliate protozoa and bacteria to excess carbohydrate

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Abstract: When given excess carbohydrate, certain microbial species respond by storing energy (synthesizing reserve carbohydrate), but other species respond by dissipating the energy as heat (spilling energy). To determine the relative importance of these responses in the microbial community of the rumen, this study was conducted to quantify how mixed ciliate protozoa vs. bacteria responded to glucose. We hypothesized that ciliates would direct more glucose to synthesis of reserve carbohydrate (and less to energy spilling) than would bacteria. Ciliates and bacteria were isolated from rumen fluid using filtration and centrifugation, resuspended in nitrogen-free buffer to limit growth, and dosed with 5 mM glucose. Compared to bacteria, ciliates consumed glucose >3-fold faster and synthesized reserve carbohydrate 4-fold faster. They incorporated 53% of glucose carbon into reserve carbohydrate, nearly double the value (27%) for bacteria. Energy spilling was not detected for ciliates, as all heat production (104%) was accounted by synthesis of reserve carbohydrate and endogenous metabolism. For bacteria, reserve carbohydrate and endogenous metabolism accounted for only 68% of heat production, and spilling was detected within 11 min of dosing glucose. These

results suggest that ciliates alter the course of carbohydrate metabolism in the rumen by outcompeting bacteria for excess carbohydrate, maximizing reserve carbohydrate synthesis, and minimizing energy spilling.

Keywords: bacteria, ciliate protozoa, rumen, energy spilling, glucose, reserve carbohydrate

Introduction

When the ruminant host is fed large amounts of grain, microbes in the rumen encounter levels of nonstructural carbohydrate that exceed their requirement for growth (Russell, 1998). Rumen microbes respond to this excess carbohydrate in two major ways (Hackmann and Firkins, 2015). Many species of bacteria and ciliate protozoa respond by directing excess carbohydrate into glycogen reserves (Hackmann and Firkins, 2015), which can exceed 50% of cell mass (Russell, 1998). Other, bacterial species instead direct excess carbohydrate towards energy spilling (Russell, 2007; Hackmann and Firkins, 2015), a process that dissipates excess ATP energy as heat via futile cycles. Other responses to excess carbohydrate include releasing metabolic intermediates (overflow metabolites) or methylglyoxal (Russell, 1998), but these responses seem to be minor.

In addition to their physiological significance to microbes themselves, energy spilling and reserve carbohydrate synthesis are important to the productivity of cattle and other ruminants. Rumen microbes account for over 50% of the protein metabolized by the ruminant, and thus increasing the efficiency of the ruminant host in turn (Hackmann and Firkins, 2015). Spilling depresses efficiency because is leads to fermentation of carbohydrate without production of microbial protein; the net products

of spilling are heat, fermentation acids, and fermentation gases alone (Hackmann and Firkins, 2015). Another detrimental effect of spilling is that non-structural carbohydrates that could have been digested by the host are instead lost to fermentation. Reserve carbohydrate depresses efficiency of protein production and leads to loss of carbohydrate also, because feed carbohydrate must be fermented to fuel reserve carbohydrate synthesis (Stouthamer, 1973). Still, reserve carbohydrate synthesis is more economical than spilling because carbohydrate can be later mobilized to fuel microbial protein production (Wilkinson, 1959) or pass from the rumen unfermented (McAllan and Smith, 1974).

Even as reserve carbohydrate synthesis is less detrimental to the host than spilling, few studies have quantified the relative importance of these responses and in mixed communities or groups. For example, Van Kessel and Russell (Van Kessel and Russell, 1996) established that mixed bacteria could spill energy when grown under ammonia-N limitation, but that study did not measure reserve carbohydrate. Many studies have established that mixed bacteria and ciliates can synthesize reserve carbohydrate (Hackmann, 2013, Hackmann and Firkins, 2015), but none has quantified energy spilling.

Recently, it was demonstrated that the whole rumen community responds to a moderate carbohydrate excess by synthesizing reserve carbohydrate. It also spilled energy but only under a large excess (Denton et al., 2015). The current study seeks to extend this work by separately examining responses of mixed bacteria and ciliates, the two largest groups of the mixed community. Based on glucose competition experiments that showed ciliates synthesize much more reserve carbohydrate than bacteria (Denton

et al., 2015), we hypothesized that ciliates would respond to excess glucose chiefly by synthesizing reserve carbohydrate, whereas bacteria would spill energy.

Material and methods

Preparation of isolated ciliates and bacteria

Rumen fluid was collected from two dry Holstein cows (nos 8224 and 8155) of approximately 750 kg live weight. One cow was used per experiment. Animals were housed at the animal physiology unit at University of Florida in Gainesville, FL. The University of Florida Institutional Animal Care and Use Committee approved all animal procedures (Protocol #: 201408363).

Cows were fed 8.1 kg dry matter (DM) of alfalfa (*Medicago sativa)* and orchardgrass (*Dactylis glomerata*) hay and 5.9 kg DM of a concentrate supplement (Hillandale Show Ration; Hillandale Quality Feeds, Lake Butler, FL) once daily. As determined by wet chemistry methods (Cumberland Valley Analytical Services, Hagerstown, MD), chemical composition of the hay was 83.6% organic matter, 54.1% neutral detergent fiber analyzed with heat stable amylase and expressed exclusive of residual ash (aNDFom), 35.3% acid detergent fiber, 12.6% crude protein, 2.5% starch, and 3.1% ether extract (all values expressed on a DM basis). Similarly, the chemical composition of the concentrate supplement was 93.6% organic matter, 27.0% aNDFom, 16.6% acid detergent fiber, 12.3% crude protein, 33.8% starch, and 4.1% ether extract. The ingredient composition of the supplement was 50% corn, 15% soybean hull pellets, 15% cottonseed hulls, 10% distillers dried grains, 0.02% fat, and <10% other ingredients (soybean meal, molasses, extruded cottonseed meal, peanut hulls, Uni-Bond,

vitamins, minerals). Corn was included in both ground and cracked forms, and the other ingredients were included in proprietary amounts.

Preparation of clarified fluid was described previously (Hackmann et al., 2013a). Briefly, rumen contents were strained through cheesecloth, diluted 1:1 with N-free buffer (see composition below), and added to a separatory funnel under O_2-free CO_2. After 45 minutes at 39°C, plant particles rose to the top, and the clarified fluid below was removed by draining through the stopcock. The diet composition had been adjusted to optimize this flocculation, and not all plant particles rose to the top when feeding a low-quality hay (bermudagrass) and less concentrate.

After preparation of the clarified fluid, ciliates or bacteria (one group per experiment) were isolated following earlier methods [Denton et al., 2015) and Figure 1]. Briefly, ciliates were collected on a nylon cloth (20-μm pore size) under CO_2, and most bacteria were removed by washing with 10 volumes of N-free buffer (Figure 1. A). Bacteria were isolated by centrifugation on a pre-warmed rotor at 13,000 *g* for 10 min (1720 rotor and Rotina 38R centrifuge; Hettich, Beverly, MA) after removing protozoa in an earlier centrifugation step at 2,500 *g* for 5 min (Figure 1. B).

After isolation, ciliates or bacteria were anaerobically re-suspended and diluted in N-free buffer and then transferred to a culture bottle at 39°C. The target concentration of cell protein was 2 g L^{-1}, using optical density of the undiluted culture and preliminary experiments with each cow as guides. If the concentration was inadvertently <1 g L^{-1}, the experiment was repeated to obtain ≥1 g L^{-1}.

Ciliates or bacterial cells were dosed with glucose (5 mM final concentration). In early experiments, the volume of glucose dosed was based on the nominal volume of culture transferred to and sampled from the bottle, but in later experiments, the culture

was weighed to determine the exact volume at time of dosing (assuming a culture density of 1 g mL^{-1}).

At intervals, cell suspension (0.5 or 1-mL per time point) was sampled and harvested aerobically by centrifugation (10,000 *g*, 10 min, 4°C; 75003424 rotor and Legend Micro 21R centrifuge; Thermo Scientific, Rockford, IL). Intervals were chosen to give three sample points prior to dosing glucose, at least two points during glucose excess, and at least two points after glucose was exhausted. Previous studies showed complete recovery of protozoa and bacteria when harvesting under aerobic conditions (Hackmann et al., 2013a; Denton et al., 2015). The resulting cell pellets were aerobically washed once in 0.9% NaCl and stored at -20°C. Cell-free supernatant (from the first centrifugation step) was likewise stored at -20°C. Additional aliquots of cell suspension (2 mL) were taken at the start and end of the experiment, fixed in 5% formalin (final concentration), and stored at 4°C for cell counts and microscopic imaging.

The N-free buffer was modified Simplex type (pH = 6.8) and contained 6.35 g K_2HPO_4, 5 g KH_2PO_4, 650 mg NaCl, 90 mg $MgSO_4 \cdot 7H_2O$, 60 mg $CaC1_2 \cdot 2H_2O$, 7.5 g $NaCHO_3$, and 0.5 g cysteine hydrochloride per liter (Tao et al., 2016). It was prepared anaerobically with water boiled and distilled under O_2-free CO_2.

Chemical analyses

Cell pellets were analyzed for reserve carbohydrate using the anthrone method (Hackmann et al., 2013a; Hackmann et al., 2013b) and protein with the Pierce BCA Assay kit (product #23227; Thermo Scientific, Rockford, IL).

Cell-free supernatant was analyzed for free glucose using glucose oxidase-peroxidase (Karkalas, 1985) after adding 2 moles N-ethylmaleimide per mole cysteine in samples (Haugaard et al., 1981). D-/L-lactic and acetic acids were analyzed with enzymatic kits (product code 11112821035 and 10148261035, R-Biopharm, Marshall, MI).

Propionate and butyrate were determined by HPLC after acidifying cell-free supernatant with H_2SO_4 (0.5% final concentration) and filtering through a 0.22 μm cellulose acetate membrane. The HPLC was a LaChrom Elite (Hitachi, Tokyo) equipped with an Aminex HPX-87H ion-exchange column (Bio-Rad Laboratories, Hercules, CA). Sample volume was 20 μL, eluent was 0.015 N H_2SO_4, flow rate was 0.7 mL min^{-1}, column temperature was 48°C, and UV detection was at 210 nm. Valerate was not detected, and isovalerate and isobutyrate could not be quantified due to unidentified interfering peaks. For mixed rumen microbes, these three fermentation products were minor and accounted for only 2% of carbon recovered from a 5 mM glucose dose (data from Hackmann et al., 2013a). Peaks for lactic and acetic acids could be detected with HPLC, but concentrations were quantified enzymatically, as mentioned, because of the lower detection limit of this approach.

Measurement of heat production and fermentation gases

Heat production (W L^{-1}) was measured on 1-mL cell suspension by isothermal calorimetry (μRC, Thermal Hazard Technology, Piscataway, NJ) as previously described (Hackmann et al., 2013a), except the calorimeter was placed in an incubator (30°C) to minimize baseline drift.

Fermentation gases (H_2, CH_4) were measured using a Micro-Oxymax instrument (Columbus Instruments, Columbus, OH). Cell suspension (5 or 10 mL) was added to a water-jacketed vessel (100 mL nominal volume), gassed with CO_2, maintained at 39°C, and continuously stirred (to promote vaporization of gas from liquid to gas phase). The instrument sampled the gas phase of the vessel at 12 min intervals and circulated sample through CH_4, H_2, and CO_2 sensors. The CH_4 sensor (0 to 0.25%) and CO_2 sensor (0 to 100%) measured gases by infrared absorption, and H_2 sensor (0 to 4%) measured concentrations electrochemically. Room air and CO_2 were circulated as references to correct for sensor drift. Before each experiment, sensors were calibrated using primary standard gas, and the volume and leak rate of the vessel was checked by pressurizing with gas. Production of CH_4 and H_2 was determined by the instrument software from changes in gas concentrations over sampling intervals. Production of CO_2 was also reported, but because of CO_2 evolved from protonation of buffer (and because of the large working range of the sensor), these values were not meaningful or accurate.

Calculation of energy spilling

As described in Hackmann et al. (2013a) and Figure 2, energy spilling (kJ L^{-1}) was calculated as total heat production (kJ L^{-1}) minus (i) heat accounted by endogenous metabolism (kJ L^{-1}) and (ii) heat accounted by reserve carbohydrate synthesis (kJ L^{-1}).

Heat accounted for by endogenous metabolism was calculated by integrating the rate of heat production before dosing glucose. This rate averaged 0.031 [standard error of the mean (SEM) = 0.077] W g protein^{-1} L^{-1} for ciliate experiments and 0.025 (SEM = 0.076) W g protein^{-1} L^{-1} for bacterial experiments. The rate was assumed to hold

constant through the experiment, as any decline impacted calculation of energy spilling only minimally with mixed rumen microbes (Hackmann et al., 2013a).

Heat accounted by reserve carbohydrate synthesis was calculated by integrating the product of (i) rate of reserve carbohydrate synthesis (mM min^{-1}) and (ii) molar heat of reserve carbohydrate synthesis (kJ mol^{-1}). The latter value was calculated using Eq. 4 in Hackmann et al. (2013a) and averaged -61.3 (SEM = 4.6) kJ mol^{-1} for ciliate experiments and -58.4 (SEM = 10.2) kJ mol^{-1} for bacterial experiments. Fermentation products included in this calculation were acetate, butyrate, propionate, lactate, and CH_4.

Other analyses and calculations

Direct counts of ciliates were determined as described previously (Hackmann, et al., 2013a). Direct counts of total bacteria were determined by 4,6-diamidino-2-phenylindole staining and epifluorescence microscopy (Kepner and Pratt JR., 1994). Formalin-fixed cells were imaged by differential interference contrast (DIC) microscopy using an Axioplan 2 microscope (Zeiss, München, Germany), x40 (Plan-Neofluar, numerical aperture, 0.75) or x100 oil (Plan-Apochromat, numerical aperture, 1.40) objectives, and a Retiga 4000R camera (QImaging, Surrey, Canada). Samples were prepared as either standard wet mounts (x40 objective) or wet mounts on a thin layer of 2% noble agar (x100 objective). QCapture Pro 7 (QImaging) was used for image acquisition. Photoshop (Adobe, San Jose, CA, USA) was used to linearly adjust brightness and contrast. The adjustment was applied to whole images and equally to isolated bacteria and protozoa.

To estimate the mass of bacteria contaminating isolated ciliates, bacterial counts in the isolated ciliates were determined and then multiplied by bacterial protein mass. Bacterial protein mass was 8.60 (SEM = 0.85) × 10^{-14} g $cell^{-1}$, which was measured for isolated bacteria at the beginning of experiments (n = 6 total for both cows). A similar approach was used to estimate ciliate contamination of isolated bacteria. Ciliate protein mass was 2.65 (SEM = 0.24) × 10^{-8} g $cell^{-1}$, which was measured for isolated ciliates (n = 6).

Initial rate of glucose consumption was calculated from the linear decrease in concentration over the first 5 min (ciliates) or 20 min (bacteria) after dosing glucose. Initial rates for other variables (e.g. reserve carbohydrate accumulation) were calculated analogously over the same period.

Carbon and energy recovery from the dosed glucose was calculated as described previously (Hackmann et al., 2013a). Briefly, the increase in carbon or energy after dosing glucose was calculated from the concentration of glucose, reserve carbohydrate, fermentation products (acetate, propionate, butyrate, lactate, CO_2, CH_4, H_2, H_2O), and heat production. This increase was compared with that expected from the carbon or energy in glucose. CO_2 and H_2O were determined from reaction stoichiometry (Hackmann et al., 2013). Hydrogen recovery was calculated analogously.

Experiments with lactating cow

Experiments with the dry cows above were replicated with a lactating Jersey cow (no. 490) at the Waterman Dairy Center at The Ohio State University in Columbus, OH, USA. The Ohio State University Institutional Animal Care and Use Committee

approved all animal procedures. The cow was fed a lactation diet *ad libitum* in two equal meals. The ingredient composition was 45.3% corn silage, 13.8% legume silage, 12.5% ground corn, 8.6% soybean meal, 6.4% whole cottonseed, 3.8% distillers grains, 2.8% wheat middlings, 2.1% Amino Plus (Ag Processing Inc. Hiawatha, KS, USA), 1.0% MEGALAC (Church and Dwight, Princeton, NJ, USA), 0.3% direct-fed microbial product (XP DFM, Diamond V, Cedar Rapids, IA, USA), and 3.3% vitamins and minerals. The chemical composition was not determined.

Experimental methods were similar to those used with dry Holstein cows, except the bacteria were isolated at 10,000 *g* for 10 min (JA-17 rotor and J2-21 centrifuge; Beckman, Brea, CA, USA) after removing ciliates in an earlier centrifugation step at 1,000 *g* for 5 min.

Statistics

Most data were analyzed using PROC GLIMMIX of SAS University Edition (SAS Institute, Inc., Cary, NC, USA) using the model

$$Y_{ij} = \mu + G_i + c_j + \varepsilon_{ij} \quad (1)$$

where Y_{ij} is the observation, μ is overall mean, G_i is fixed effect microbial group (i is bacteria or ciliates), c_j is random effect of cow (j is 8155 or 8224), and ε_{ij} is residual error. Data for the purity, recovery, and composition of cells were analyzed similarly, but G_i was removed from the model and groups were analyzed separately. Data for the lactating cow were analyzed similarly, but c_j was removed from the model. Student's *t*-test was used to separate means or determine if means differed from 100%.

To determine if protein differed from its initial value or recoveries differed from 100%, data were analyzed using the model

$$Y_{ijk} = \mu + G_i + c_j + T_k + G_i \times T_k + \varepsilon_{ijk} \quad (2)$$

where T_k is the fixed effect of time k (k is glucose exhaustion or end of experiment) and $G_i \times T_k$ is the interaction between G_i and T_k. A t-test was used to determine if means differed from 100%.

Energy spilling was calculated at 1-min intervals and then analyzed using the model

$$Y_{ijk} = \mu + c_i + E_j + T_k + \varepsilon_{ijk} \quad (3)$$

where E_j is the fixed effect of experiment j (j is experiment 1 for ciliates, experiment 2 for ciliates, or experiment 6 for bacteria) and k is minutes relative to glucose dosing. A t-test was used to determine if means differed from 0. Though these data follow a time series, they were not analyzed as repeated measures because the model did not solve with most variance-covariance structures (as a result of the large number of time intervals). Data were analyzed from time of glucose dosing until the end of the experiment (for the shortest experiment). Data were analyzed separately by microbial group because of the shorter length of ciliate vs. bacterial experiments. When spilling was compared between microbial groups (e.g. at time of glucose exhaustion), model (1) was used instead of (3).

Molar heat of reserve carbohydrate synthesis and ATP yield from fermentation were analyzed using model (3), but data were analyzed from time of glucose dosing to time of peak reserve carbohydrate.

Local regression (LOCFIT package of R; Loader, 1999) was used to fit time-series data to smooth curves as described previously (Hackmann et al., 2013a; Hackmann et al., 2013b). Original data have been presented alongside the smooth curves in figures, and smooth curves were used for calculations (e.g. energy spilling, accumulation of reserve carbohydrate, recoveries) and statistical analysis.

Results

Glucose, reserve carbohydrate, and fermentation products

Immediately after dosing glucose, ciliates and bacteria responded by consuming the glucose, accumulating reserve carbohydrate, forming fermentation products, and producing heat (Figure 3). Ciliates consumed glucose rapidly, with the initial rate 3.2-fold higher than that for bacteria (Figure 4). They also accumulated reserve carbohydrate rapidly, with the initial rate 4.0-fold higher than bacteria (Figure 4). Fermentation acids and gases were formed from hexose (glucose or reserve carbohydrate) included acetate, propionate, butyrate, lactate, H_2, and CH_4 (Figure 3. G, H, I and J). Assuming 6 moles carbon in fermentation products per mole hexose fermented, we calculated that ciliates fermented hexose at least as fast as bacteria; numerically, they fermented hexose at a 1.4-fold higher rate than bacteria, though this value did not differ (P = 0.139) from unity (Figure 4). Of all fermentation products, lactate was formed fastest by the ciliates and at a 4.7-fold higher rate than bacteria (Figure 4).

Ciliates and bacteria continued to accumulate reserve carbohydrate as they consumed glucose (Figure 3). At time of peak reserve carbohydrate accumulation,

ciliates had accumulated 2.66 mM glucose equivalents of this carbohydrate, which is 2.0-fold higher than the 1.34 mM accumulated by bacteria (SEM = 0.18 mM; $P < 0.001$). Further, ciliates reached peak reserve carbohydrate in only 25 min after dosing glucose, whereas bacteria required 87 min to reach this peak (SEM = 13 min; $P = 0.007$).

Glucose was exhausted after reserve carbohydrate reached peak values (see Figure 3 and Table 1). From the time of glucose dosing to its exhaustion, ciliates had accumulated more reserve carbohydrate than bacteria (Table 1), as expected from earlier time points. Ciliates generally more formed more H_2 and less CH_4 than bacteria (Table 1), though results varied across individual experiments (see Figure 3. I and J). Formation of lactate and butyrate did not differ between ciliates and bacteria (Table 1). Ciliates formed less acetate and far less propionate than did the bacteria (Table 1). In sum, ciliates responded to glucose by consuming it more rapidly and accumulating more reserve carbohydrate than did bacteria, with the formation of certain fermentation acids and gases also differing between microbial groups.

Heat production and energy spilling

To determine if ciliates or bacteria responded to excess glucose by also spilling energy (dissipating excess ATP as heat), we measured heat production of each microbial group (Figure 3, K and L). Before dosing glucose, ciliates and bacteria produced heat at low rates, but the rate spiked shortly after glucose was dosed (Figure 3. K and L) and reserve carbohydrate began to accumulate (Figure 3. C and D).

To determine if spilling caused this increase in heat production, we calculated how much heat production was accounted by (i) endogenous metabolism and (ii) reserve carbohydrate synthesis (see 'Materials and Methods' and Figure 2). Energy spilling is heat production not accounted by these two functions.

For ciliates, endogenous metabolism and reserve carbohydrate synthesis accounted for all heat production (Figure 5). Across experiments and time points, these two functions accounted for 103.9% (SEM = 7.8%) of total heat production. This value was not different from 100% (P = 0.638) and indicated minimal energy spilling by ciliates.

For bacteria, endogenous metabolism and reserve carbohydrate synthesis accounted for far less heat production (Figure 6). Across experiments and time points, the two functions accounted for only 67.6% (SEM = 3.2%) heat production, which was less than 100% ($P < 0.001$). This implies spilling, and when examined at individual time points, spilling became detectable (>0 kJ L^{-1}; $P < 0.05$) by only 11 min after dosing glucose. At time of glucose exhaustion, spilling grew to 0.464 (SEM = 0.037) kJ L^{-1}, or 50.2% (SEM = 7.9%) of total heat production. This was higher ($P < 0.001$) than the corresponding values for ciliates [-0.012 (SEM = 0.038) kJ L^{-1} or -5.3% (SEM = 8.0%) of total heat production]. The values for the ciliates themselves did not differ from 0 (P = 0.752 and 0.527). Thus, both ciliates and bacteria responded to glucose by producing heat, but this increase in heat was accounted for by energy spilling only for the bacteria.

Purity, recovery, and composition of isolated microbial groups

For experiments above, ciliates were isolated by collecting on a cloth (20 μm pore size) and washing with N-free buffer to remove bacteria. Visually, isolated ciliates were of high purity (contaminated with few bacteria) (Figure 7. A and C). When formally quantified with direct counts, bacterial contamination accounted for 0.221% (SEM = 0.027%) of total microbial protein at the start of the experiment, which rose slightly to 0.334% (SEM = 0.064%) by the end of the experiment.

Bacteria were isolated by centrifugation, with an initial step to remove ciliates. Isolated bacteria appeared to be pure (Figure 7. B and D). From direct counts, ciliate contamination accounted for 0.60% (SEM = 0.18%) and 0.28% (SEM = 0.15%) microbial protein at the start and end of the experiment.

Some ciliate cells were lost through the cloth during washing, and recovery of cells at the start of the experiment was 38.9% (SEM = 6.7%) that of clarified fluid. Recovery of *Entodinium*, which includes the smallest species, was lowest at 31.8% (SEM = 4.0%). Recovery of *Isotricha*, which includes the largest species, was highest at 86.2% (SEM = 6.6%) and did not differ ($P = 0.091$) from 100%.

Ciliate cells were also lost during the experiment (due to death and lysis), and the concentration of ciliates at the end was only 75.9% (SEM = 4.2%) that of the start. The concentrations of *Isotricha* and *Dasytricha* declined the most and were only 51.9% (SEM = 10.6%) and 55.7% (SEM = 5.5%) of their starting value. The concentration of *Entodinium*, by contrast, reduced little and remained at 91.2% (SEM = 4.6%) of the starting value; this did not differ ($P = 0.112$) from 100%.

Some bacteria were lost during centrifugation, particularly during the first step to remove ciliates. Recovery of cells after centrifugation was 21.7% (SEM = 3.0%) that of clarified fluid. Concentration of cells at the end of the experiment was 87.5% (SEM = 4.0%) that of the start, which was relatively high but still less (P = 0.027) than 100%.

Isolated ciliates included diverse types (Figure 7. A and C). At the start of the experiment, the composition was 57.2% (SEM = 5.6%) *Entodinium*, 9.3% (SEM = 1.5%) *Isotricha*, 30.3% (SEM = 4.0%) *Dasytricha*, 3.1% (SEM = 1.0%) *Ophryoscolex*, and 0.091% (SEM = 0.087%) other species belonging to family Ophyroscolecidae (ophyroscolecids). *Epidinium* was not detected. The composition at the end of the experiment was similar, except the proportion of *Isotricha* and *Dasytricha* reduced (as a consequence of lysis mentioned) and *Entodinium* increased. The composition of isolated ciliates in the clarified fluid was 73.2% (SEM = 3.2%) *Entodinium*, 4.5% (SEM = 1.1%) *Isotricha*, 19.7% (SEM = 2.1%) *Dasytricha*, 1.6% (SEM = 0.4%) *Ophryoscolex*, and 0.92% (SEM = 0.34%) other ophryoscolecids.

The composition of bacteria was not formally quantified, but diverse morphologies were present visually (Figure 7. B and D). Fungi and flagellate protozoa were not formally quantified. Protozoa-associated methanogens were not quantified, and free-living methanogens would have been detected as bacteria and included in their direct counts. Visually, fungi and flagellates appeared to contaminate each group negligibly (Figure 7). Methane production by isolated ciliates and bacteria indicated that some methanogens were present in both groups.

Cell protein and recoveries

As was our aim, the initial concentration of protein did not differ (P = 0.443) between experiments with ciliates [2.12 (SEM = 0,29) g L^{-1}] and bacteria [1.81 (SEM = 0,29) g L^{-1}]. By the time of glucose exhaustion, the concentration changed by <5% for both ciliates and bacteria (Table 2). By the end of the experiment, it reduced by nearly 15% of the initial value for ciliates (Table 2).

Recoveries of carbon, hydrogen and energy were near 100% through the time of glucose exhaustion (Table 2). For ciliates, recoveries reduced by the end of the experiment and numerically were less than 100%, though none differed statistically (P = 0.284) from 100% (Table 2).

Experiments with lactating cow

The experiments above were completed with two dry cows fed a hay-grain supplement diet and housed in Florida. These experiments were partly replicated with a cow that was lactating, fed a silage-based lactation diet, and housed in a different location (Ohio). Three experiments were performed for ciliates and two for bacteria. As for experiments with the dry cows, ciliates responded to the dose of 5 mM glucose by consuming it more rapidly and accumulating more reserve carbohydrate than did bacteria (Figure 8). At time of peak reserve carbohydrate accumulation, ciliates had accumulated 2.94 (SEM = 0.20) mM glucose equivalents of this carbohydrate, which is more (P = 0.014) than the 1.28 (SEM = 0.25) mM accumulated by bacteria. Composition of isolated ciliates was 87.2% (SEM = 4.0%) *Entodinium*, 5.04% (SEM = 0.19%) *Isotricha*, 5.8% (SEM = 3.2%) *Dasytricha*, 1.19% (SEM = 0.75%) *Epidinium*,

and 0.70% (SEM = 0.27%) other ophryoscolecids. *Ophryoscolex* was not detected. Fermentation acids, gases, and heat production were not measured. Consequently, energy spilling could not be calculated.

Discussion

Our results demonstrate that mixed rumen ciliates respond to excess carbohydrate by synthesizing reserve carbohydrate and spilling minimal energy, whereas bacteria spill large amounts of energy. Though spilling and reserve carbohydrate synthesis were first demonstrated in rumen microbes decades ago (Oxford, 1951; Hobson and Mann, 1955; Russell, 1986; Russell and Strobel, 1990), their relative importance in mixed microbes has seldom been quantified (Hackmann et al., 2013a). Though both reserve carbohydrate synthesis and spilling are detrimental to host efficiency (Hackmann and Firkins, 2015), spilling is more detrimental and represents a complete loss of carbohydrate for microbial protein production and host digestion.

Importance of carbohydrate synthesis vs. spilling

Two recent studies from our lab have begun to quantify the relative importance of these responses and for mixed microbes. In one study (Hackmann et al., 2013a), we dosed the whole community (principally ciliates and bacteria) with excess glucose after washing with N-free buffer. For a moderate excess of glucose (5 mM), the community responded by incorporating 60% of glucose carbon into reserve carbohydrate, but spilling was detected only when the community was given a larger excess (20 mM). In a

second study (Denton et al., 2015), we dosed glucose to 1:1 mixtures of bacteria and ciliates, then separated cells to determine reserve carbohydrate accumulation by each group. Ciliates incorporated 59% of glucose carbon in reserve carbohydrate when given a moderate excess (4.62 or 5 mM), while bacteria incorporated negligible amounts (<2%). This second study suggested that ciliates responds to glucose by synthesizing reserve carbohydrate, and this accounts for most synthesis in the whole community. However, the response by bacteria was not made apparent. Further, spilling was not measured for either group, as it cannot be distinguished between two groups in a mixture.

By examining ciliates and bacteria in isolation, the current study directly tested how these two groups respond to glucose and if they spill energy. After ciliates were isolated by filtration and dosed with a moderate excess of glucose (5 mM), they rapidly took up glucose and incorporated 53% of the carbon into reserve carbohydrate, mirroring results with the whole community and ciliates from the 1:1 mixture. Previous studies (Heald and Oxforf, 1953; Willians and Harfoot, 1976; Prins and Van Hoven, 1977; Van Hoven and Prins, 1977) have found isotrichids (ciliates belonging to family Isotrichidae) incorporate >50% glucose carbon into reserve carbohydrate. As with the whole community given this dose, ciliates did not spill energy; heat production was fully accounted by endogenous metabolism and synthesis of reserve carbohydrate.

Bacteria incubated under similar conditions took up glucose, but at one-third the rate of ciliates. Additionally, bacteria incorporated only 27% of glucose carbon into reserve carbohydrate. Only 68% of total heat production was accounted for by endogenous metabolism and synthesis of reserve carbohydrate, indicating spilling. Indeed, spilling accounted for >50% of heat production by time of glucose exhaustion.

The capacity of mixed bacteria to spill energy is consistent with the study of Van Kessel and Russell (1996), which showed energy spilling when bacteria were grown under ammonia N limitation.

Energy spilling cannot be directly measured, but instead must be calculated as heat production not accounted by endogenous metabolism and reserve carbohydrate synthesis. As explained in Hackmann et al. (2013a), this calculation requires a number of assumptions regarding ATP yield from fermentation, glucose transport, reserve carbohydrate, thermodynamic properties, and endogenous metabolism. For example, our calculations assumed that fermentation yields 2 moles ATP per mole glucose during glycolysis. However, an unusual glycolytic enzyme (diphosphate-fructose-6-phosphate 1-phosphotransferase) could make the yield as high as 3 ATP for at least one rumen ciliate species (*Isotricha prostoma*) (Mertens et al., 1989). When assuming that mixed ciliates in our experiments yielded 3 ATP, endogenous metabolism and reserve carbohydrate synthesis accounted for only 88.9% (SEM = 5.4%) of total heat production. This value did not differ statistically (P = 0.093) from 100%, even it was numerically less than 100%, and indicated that spilling numerically accounted for 11.2% of heat production. Changes to other assumptions, as with mixed rumen microbes (Hackmann et al,. 2013a), had little impact on energy spilling (data not shown).

Though the current study did not detect spilling by the ciliates, some may in reality occur. As mentioned, spilling was detected numerically, but not statistically, when we assumed the extreme case that ciliates yield 3 ATP from glycolysis. Further, previous studies have demonstrated that isotrichids simultaneously synthesize and degrade glycogen (Heald and Oxford, 1953; Prins and Van Hoven, 1977; Van Hoven and Prins, 1977). This is a phenomenon known as glycogen cycling (Portais and Delort,

2002) and a mechanism of energy spilling (Hackmann and Firkins, 2015). These previous studies thus imply energy spilling, though no study has detected spilling directly from heat production or growth yield data (Hackmann and Firkins, 2015).

In sum, we found that ciliates rapidly consume glucose and incorporate large amounts in reserve carbohydrate, whereas bacteria consume glucose more slowly and spill large amounts of energy. With our earlier studies, these results suggest that ciliates would outcompete bacteria for glucose in the rumen, maximize reserve carbohydrate synthesis, and minimize energy spilling, at least under the moderate excess of glucose (5 mM) examined in this study. Performing the current study with a larger excess of glucose (20 mM) might explain why spilling was observed for the whole community with this dose.

Glucose fermentation

By comparing glucose fermentation by ciliates and bacteria, the current study sheds light into the claim that ciliates stabilize rumen fermentation of sugars. According to this claim, ciliates rapidly consume free sugars, store them as reserve carbohydrate, and prevent their rapid fermentation to lactate by bacteria (Williams and Coleman, 1992). The claim has been similarly applied to starch (Abou and Howard, 1960; Whitelaw et al., 1970; Mackie et al., 1978; Coleman, 1992), which ciliates can store in its original form or as reserve carbohydrate (Belzecki et al. 2017).

Few controlled experiments have determined if ciliates indeed ferment sugars more slowly and produce less lactate than bacteria. Results from Williams and Morrison (1982) would indicate that mixed isotrichids ferment hexose at rate 66% that

of bacteria [calculated assuming stoichiometry of Hackmann et al. (2013a)], and they formed lactate at 68% of the rate. The authors of that study, however, collected ciliates and bacteria at different times after feeding, and they did not report that cell protein concentrations were made equal for ciliate and bacterial experiments. Under the more controlled conditions in the current study, ciliates fermented hexose at a rate at least equal to that of bacteria. Further, ciliates in the current study formed lactate 5-fold faster than bacteria, which is consistent with lactate being a major fermentation product of both isotrichid (Williams and Harfoot, 1976; Prins and Van Hoven, 1977; Van Hoven and Prins, 1977; Ellis et al., 1991b) and ophryoscolecid species (Ellis et al., 1991a; Ellis et al., 1991c). Thus, our study provides direct evidence against the claim that ciliates stabilize fermentation and prevent rapid fermentation of sugars to lactate.

One reason why ciliates rapidly fermented hexose in the current study was to synthesize reserve carbohydrate. We calculate ciliates in the current study must have fermented 35% of the glucose dose solely to fuel reserve carbohydrate synthesis. We made this calculation given (i) 53% of glucose carbon incorporated into reserve carbohydrate, (ii) 2 moles ATP per mole glucose incorporated into reserve carbohydrate (glycogen) (Stouthamer, 1973), and (iii) 3.0 moles ATP per mole hexose fermented [the average for ciliates in the current study; see Hackmann et al. (2013a) for calculation]. Similar calculations for isotrichid species (Prins and Van Hoven, 1977; Van Hoven and Prins, 1977) show reserve carbohydrate is a sink for roughly 70% or more ATP generated by glucose fermentation. Thus, reserve carbohydrate synthesis drives fermentation to generate ATP (or fermentation drives reserve carbohydrate synthesis to act as a sink for ATP). In sum, the current study undermines the claim that ciliates

stabilize fermentation of sugars in the rumen. It shows their rapid synthesis of reserve carbohydrate drives (or is driven by rapid fermentation).

Purity and composition of microbial groups

Our methods for isolating ciliates and bacteria resulted in preparations of high purity but low yield. For ciliates, contamination with bacterial cells was ≤0.3% of microbial protein, but recovery of ciliates was only 39% due to loss of small ciliates through the cloth. In previous studies, our recoveries ranged between 34% [Trial D samples of Fessenden (2016)] and 71% (Denton et al., 2015). Recoveries are dictated by (i) the relative difficulty of each sample and (ii) force needed to wash bacteria through the cloth.

For bacteria, contamination with ciliate cells was ≤0.6% of microbial protein, but recovery of bacteria was only 22% due to loss during removal of ciliates. Composition of bacteria was not measured, but it is unlikely to be fully representative of liquid-associated bacteria of the rumen. We sought high purity so we could compare responses of isolated ciliates and bacteria unambiguously.

Isolated ciliates from both dry cows had an unusually high proportion of *Dasytricha* and *Isotricha* (40% of total ciliates), and the same was observed for clarified rumen fluid (24%). These isotrichids are thought to consume more sugar and accumulate more reserve carbohydrate than the ophryoscolecids (Williams and Coleman, 1992; Dehority, 2003). Isolated ciliates from a lactating cow, by contrast, had a lower and more typical proportion of the isotrichids (7%), and yet still incorporated 59% of glucose carbon into reserve carbohydrate. Our earlier competition study (Denton

et al., 2015), in which ciliates incorporated 59% glucose carbon into reserve carbohydrate, also included samples with a typical proportion of isotrichids. In sum, mixed ciliates with a high proportion of isotrichids accumulate large amounts of reserve carbohydrate, but so do ciliates with lower and more typical proportions.

Ciliate lysis

Cilliates appeared to die and lyse after glucose exhaustion. Between the start of the experiment and glucose exhaustion, protein had declined by <5% of its initial value. By the end of the experiment, protein and cell concentrations had declined by 14% and 24%, respectively. The isotrichids (*Dasytricha*, *Isotricha*) lysed most extensively, and cell concentrations declined by 44% or more. Cells belonging to the genus *Entodinium*, by contrast, declined by 9%. Carbon, energy and hydrogen recoveries reduced after glucose exhaustion also, suggesting release of an unmeasured product from lysed cells.

Because ciliates did not die and lyse until after glucose exhaustion, we presume that ciliates were metabolically normal before that point. However, we cannot rule out that metabolic activity and thus our measurements of reserve carbohydrate, energy spilling and other variables had already been impacted before lysis was apparent.

Lysis or damage of isotrichids under carbohydrate excess is well known (Sugden and Oxford, 1952; Williams and Harfoot, 1976; Prins and Van Hoven, 1977). It has been attributed to uncontrolled uptake of sugar (Williams, 1979) and subsequent accumulation of lactic acid intracellularly (Prins and Van Hoven, 1977), but experiments that directly test this idea have not been described in detail (see Prins and Van Hoven, 1977).

Magnitude of carbohydrate excess

As in our earlier studies (Hackmann et al., 2013a; Hackmann et al., 2013b; Denton et al., 2015), we generated a carbohydrate excess that is large, mainly for proof-of-concept, but still representative of the rumen under certain cases. Glucose concentrations in the rumen can reach 5 m*M* for unadapted animals fed large amounts of grain (Ryan, 1964; Mackie et al., 1978). Nitrogen is never completely absent in the rumen, as it was after we washed cells with N-free buffer. Still, for grain-fed animals, N in the rumen is low and primarily in the form of ammonia-N, which limits growth (Argyle and Baldwin, 1989; Van Kessel and Russell, 1996). Though our large carbohydrate excesses are illustrative, N-limitation (carbohydrate excess) reduces glucose uptake in prokaryotes and eukaryotes (Doucette et al., 2011) and at least one rumen bacterium (Russell and Strobel, 1990), and future experiments should explore more modest excesses.

Conclusions

Mixed ciliates responded to excess carbohydrate by rapidly consuming glucose, incorporating >50% carbon into reserve carbohydrate, and spilling minimal energy. Bacteria respond to excess carbohydrate by synthesizing some reserve carbohydrate, but they spilled large amounts of energy (>30% of total heat production). Because ciliates took up glucose more rapidly in the current study and outcompeted bacteria for glucose in an earlier study, the action of the ciliates in the rumen likely maximizes reserve

carbohydrate synthesis while minimizing spilling. To fuel reserve carbohydrate synthesis, ciliates must rapidly ferment substrate. They fermented hexose at least as fast as bacteria in the current study and this undermines the idea that ciliates stabilize fermentation of sugar in the rumen.

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TABLES

Table 1. Net formation of fermentation products for protozoa or bacteria (between dosing and exhaustion of 5 mM glucose)

		Formation						
Group	Time	Reserve carbo-hydrate	Acetate	Propionate	Butyrate	Lactate	CH_4	H_2
	min	---mmol L^{-1}---						
Protozoa	33	3.21	0.76	0.15	0.66	1.43	0.275	1.36
Bacteria	121	1.17	2.86	2.30	1.04	1.15	0.588	0.22
SEM	15	0.24	0.23	0.56	0.26	0.35	0.087	0.33
P-value	0.003	0.006	<0.001	0.0232	0.320	0.582	0.031	0.021

Table 2. Protein and recoveries for protozoa or bacteria dosed with glucose

		% value at experiment start	% recovery from glucose dose		
Time	Fraction	Protein	Carbon	Hydrogen	Energy
Glucose exhaustion	Protozoa	95.6	95	101	92
	Bacteria	104.5	109	107	107
End of experiment	Protozoa	86.4*	90	95	87
	Bacteria	105.8	113	109	109
	SEM	6.4	11	13	12

*Different from 100% ($P = 0.048$).

FIGURES

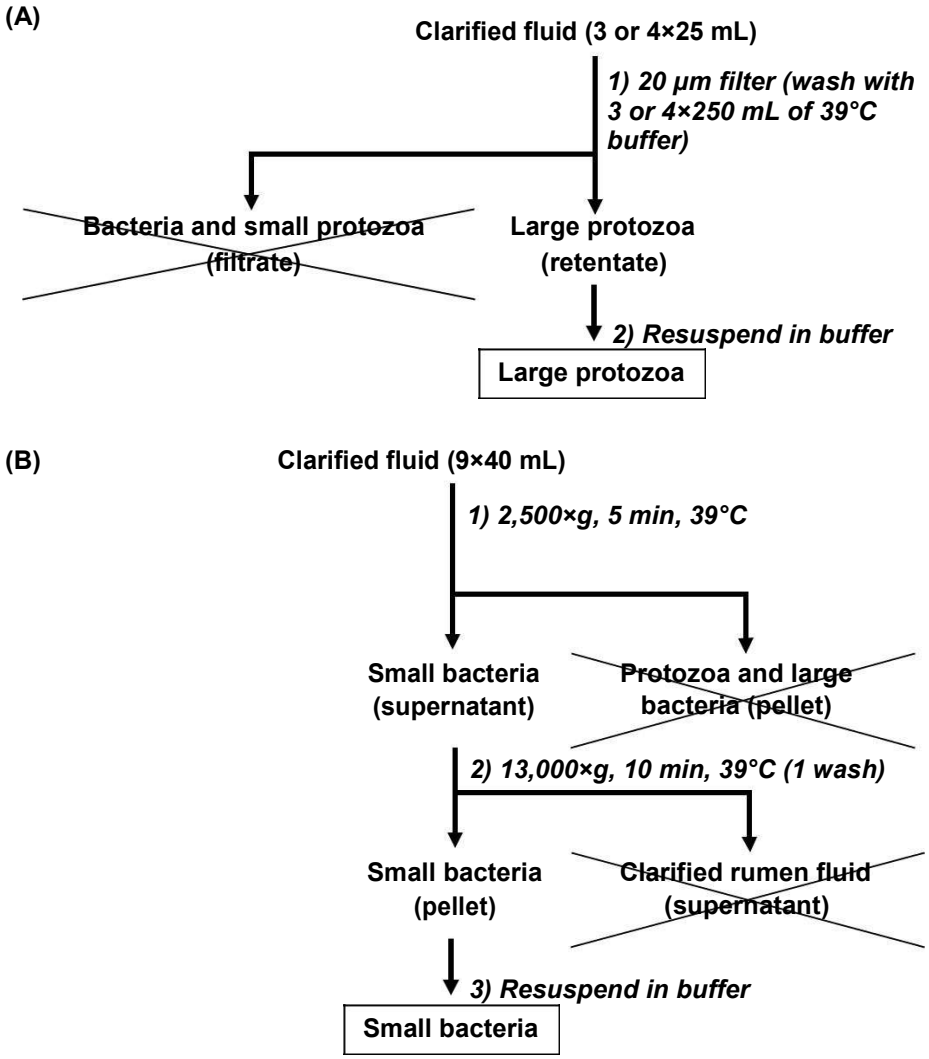

Figure 1. Flowchart for isolation of (A) protozoa and (B) bacteria. Fractions used for experiments are marked by stippled boxes, and fractions discarded are crossed out.

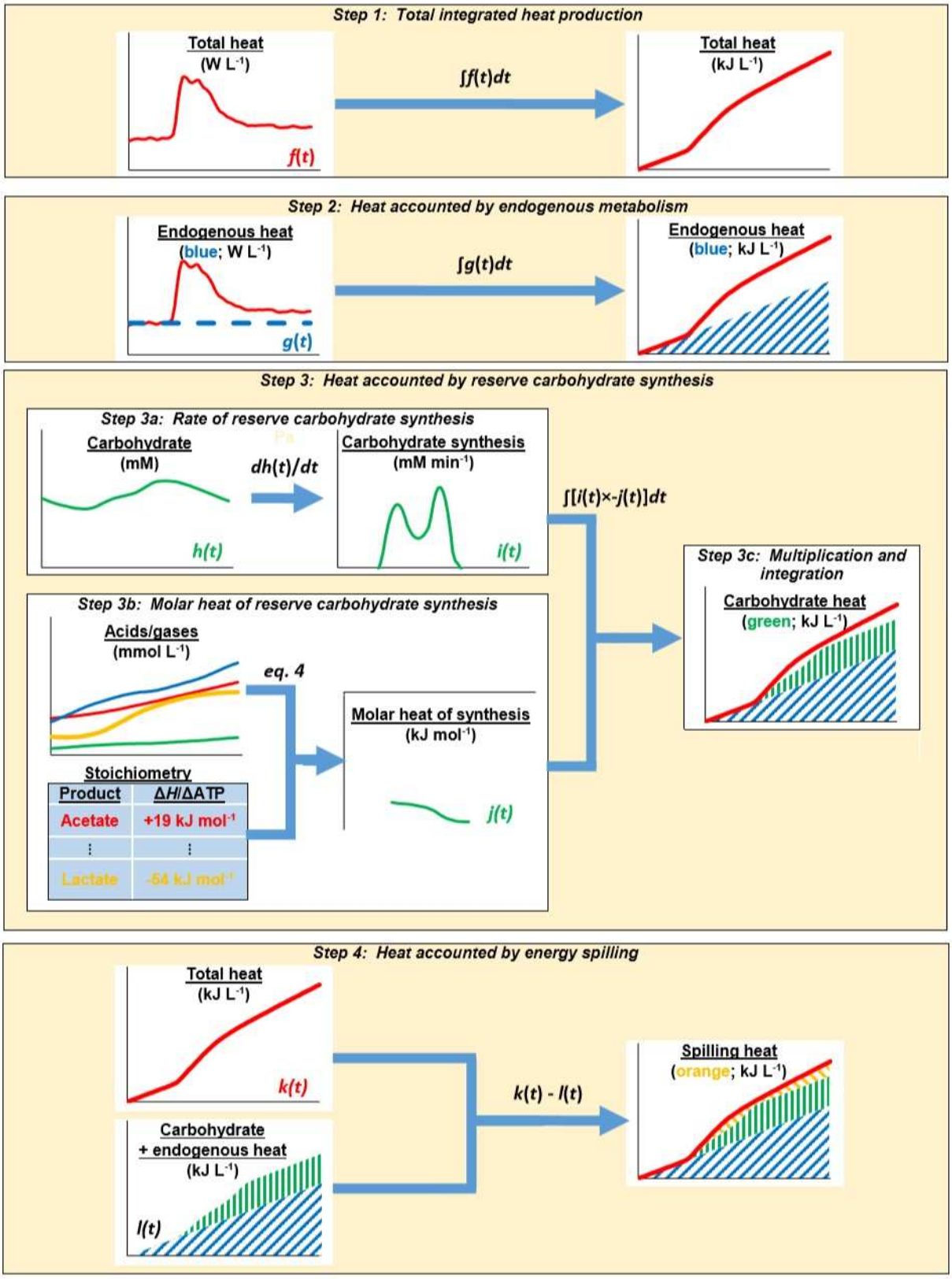
Step 1: Total integrated heat production
Total heat
(W L-1)
f(t)
∫f(t)dt
Total heat
(kJ L-1)
Step 2: Heat accounted by endogenous metabolism
Endogenous heat
(blue; W L-1)
g(t)
∫g(t)dt
Endogenous heat
(blue; kJ L-1)
Step 3: Heat accounted by reserve carbohydrate synthesis
Step 3a: Rate of reserve carbohydrate synthesis
Carbohydrate
(mM)
h(t)
dh(t)/dt
Carbohydrate synthesis
(mM min-1)
i(t)
∫[i(t)×-j(t)]dt
Step 3b: Molar heat of reserve carbohydrate synthesis
Acids/gases
(mmol L-1)
eq. 4
Stoichiometry
Product
ΔH/ΔATP
Acetate
+19 kJ mol-1
Lactate
-54 kJ mol-1
Molar heat of synthesis
(kJ mol-1)
j(t)
Step 3c: Multiplication and integration
Carbohydrate heat
(green; kJ L-1)
Step 4: Heat accounted by energy spilling
Total heat
(kJ L-1)
k(t)
Carbohydrate
+ endogenous heat
(kJ L-1)
l(t)
k(t) - l(t)
Spilling heat
(orange; kJ L-1)

Figure 2. Overview of calculation of heat accounted by endogenous metabolism, reserve carbohydrate synthesis, and energy spilling.

Step 1: Calculation of total integrated heat production. Rate of heat production (W L^{-1}) was integrated (using rectangle rule) to give total integrated heat production (kJ L^{-1}).

Step 2: Calculation of heat production accounted by endogenous metabolism. Rate of endogenous heat production (W L^{-1}) was defined as heat production prior to dosing glucose (stippled blue line). This rate was integrated to give heat accounted by endogenous metabolism (kJ L^{-1}).

Step 3: Calculation of heat accounted by reserve carbohydrate synthesis.

Step 3a: Reserve carbohydrate (mM) is differentiated to give rate of reserve carbohydrate synthesis (mM min^{-1}).

Step 3b: Fermentation acids and gases (mmol L^{-1}) are combined with fermentation stoichiometry according to eq. 4 of Hackmann *et al*. (2013) to give molar heat of reserve carbohydrate synthesis.

(kJ mol^{-1}). $H/\Delta ATP$ is the enthalpy change per ATP generated from fermentation, where a negative value indicates heat production. Though not shown, molar heat of reserve carbohydrate synthesis also includes a small enthalpy change (+4.4 kJ/mol) from synthesis itself [formation of (α1→4) and (α1→6) linkages between incorporated glucose and reserve carbohydrate].

Step 3c: Rate of reserve carbohydrate synthesis (mM min^{-1}) and the absolute value of molar heat of reserve carbohydrate synthesis (kJ mol^{-1}) are multiplied and integrated to give heat accounted by reserve carbohydrate synthesis (kJ L^{-1}).

Step 4: Calculation of heat accounted by energy spilling. Spilling is total integrated heat production (kJ L^{-1}) minus the sum of heat accounted by endogenous metabolism (kJ L^{-1}) and reserve carbohydrate synthesis (kJ L^{-1}). Calculation described in detail in Hackmann *et al*. (2013).

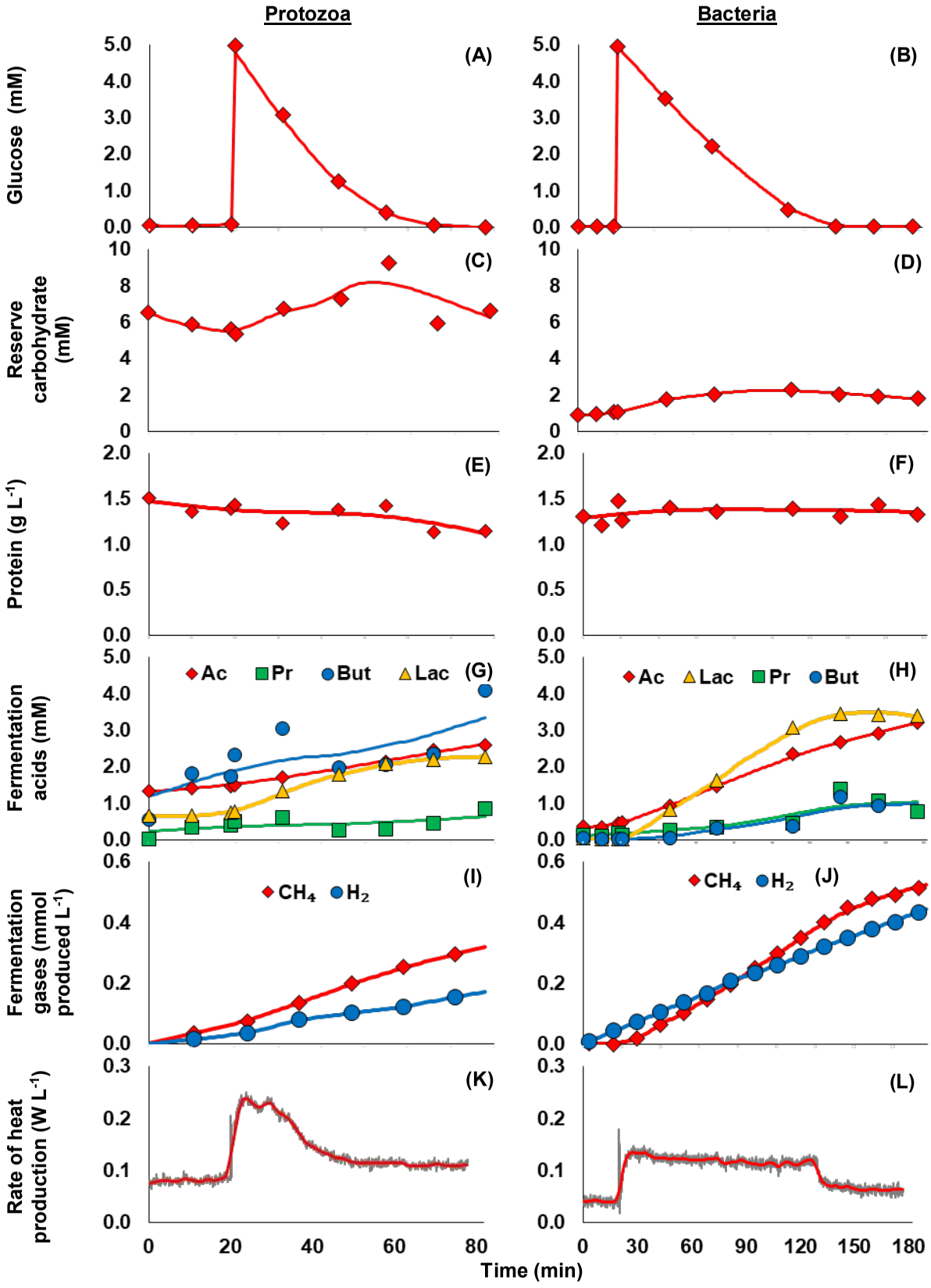
Protozoa
Bacteria
Glucose (mM)
Reserve carbohydrate (mM)
Protein (g L-1)
Fermentation acids (mM)
Fermentation gases (mmol produced L-1)
Rate of heat production (W L-1)
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)
(K)
(L)
Ac
Pr
But
Lac
CH4
H2
Time (min)

Figure 3. Response of protozoa or bacteria to dose of 5 mM glucose at c. 20 min. (A, C, E, G, I, K) Protozoa. (B, D, F, H, J, L) Bacteria. (A, B) Glucose in media. (C, D) Reserve carbohydrate. (E, F) Cell protein. (G, H) Fermentation acids, including acetate (Ac), propionate (Pr), butyrate (But), and lactate (Lac). (I, J) Production of fermentation gases. (K, L) Rate of heat production. Reserve carbohydrate was expressed in mM monomeric glucose equivalents to be in same units as glucose in media. Values for fermentation gases refer to mmoles produced per liter of culture, not their concentration in the culture. Heat production was measured in c. 1-s intervals as described in the text. Data are from one cow (#8224) and represent one experiment each for protozoa and bacteria. Between two cows, experiments were replicated a total of six times per microbial group.

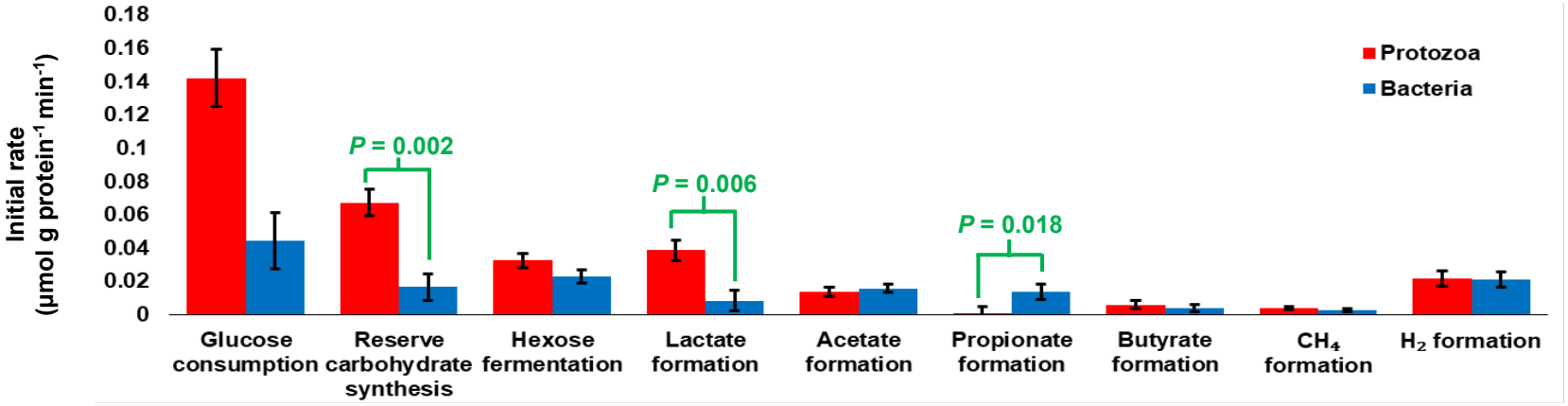

Figure 4. Initial rate of glucose consumption (and other variables) for protozoa vs. bacteria after dosing 5 mM glucose. Values are means ± SEM for six experiments per microbial group. *P*-values not reported in figure are ≥0.139.

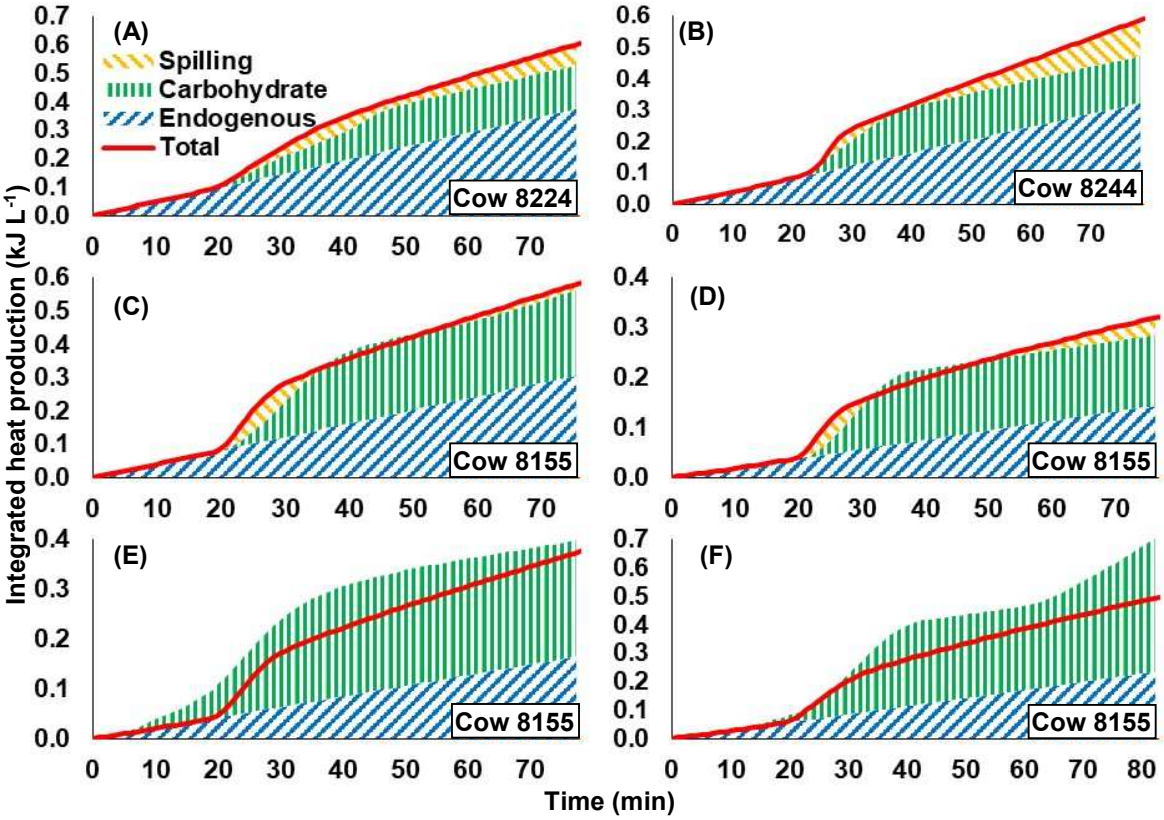

Figure 5. Integrated heat production of protozoa following dose of 5 mM glucose at 20 min. Each panel represents one experiment for cow no. 8224 (A, B) or cow no. 8155 (C, D, E, F). Shown are heat production accounted by energy spilling, synthesis of reserve carbohydrate, and endogenous metabolism. Panel (A) corresponds to protozoal experiment shown in more detail in Figure 3. Methods of calculation are described in text and Figure 2.

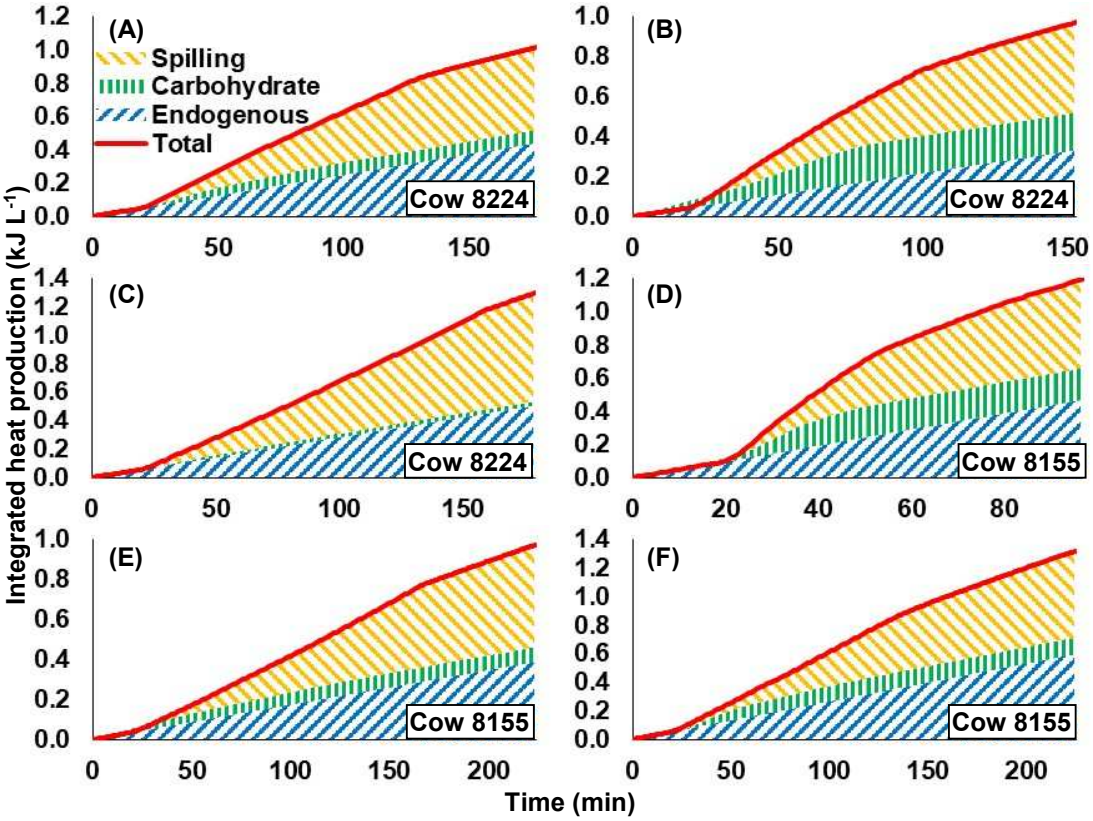

Figure 6. Integrated heat production of bacteria following dose of 5 mM glucose at 20 min. Each panel represents one experiment for cow no. 8224 (A, B, C) or cow no. 8155 (D, E, F). Shown are heat production accounted by energy spilling, synthesis of reserve carbohydrate, and endogenous metabolism. Panel (A) corresponds to bacterial experiment shown in more detail in Figure 3. Methods of calculation are described in text and Figure 2.

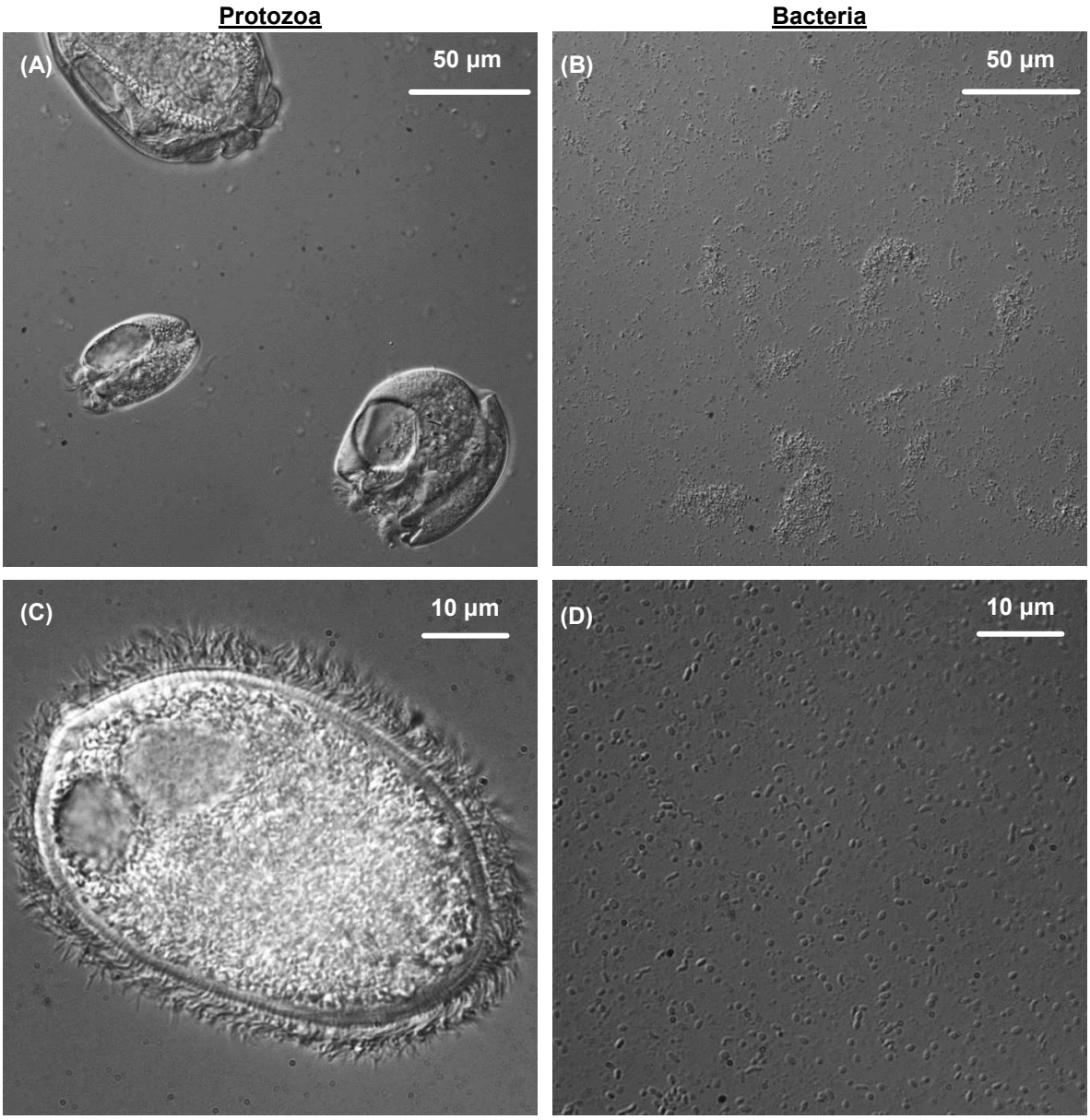

Figure 7. Images of isolated protozoa (A, C) and bacteria (B, D) at the start of experiments dosing 5 mM glucose. Images were taken by differential interference contrast (DIC) microscopy using a 40x (A, B) or 100x objective (C, D). Cells imaged correspond to protozoal and bacterial experiments shown in Figure 3.

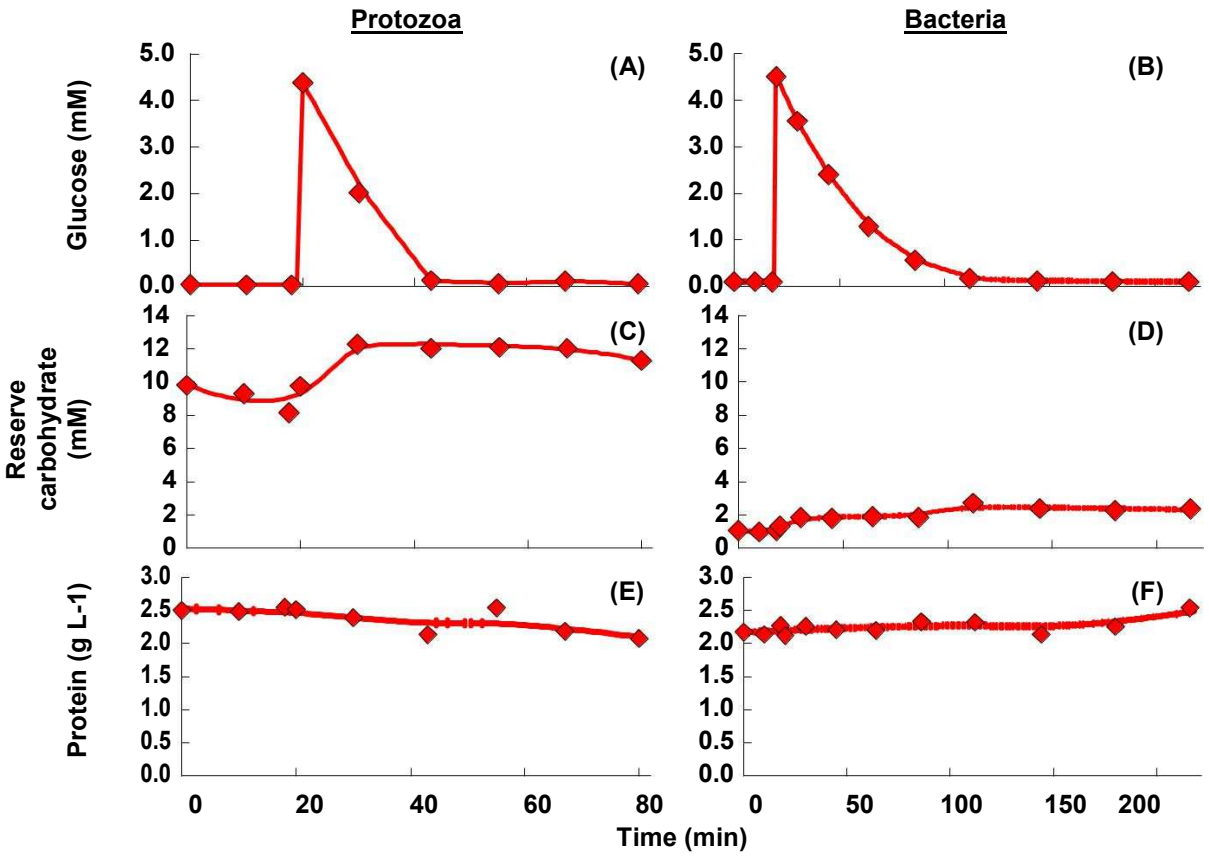

Figure 8. Response of protozoa or bacteria to dose of 5 mM glucose at c. 20 min. Experiment as in Figure 3 but using one cow (no. 490) that was lactating, fed a silage-based lactation diet, and housed in a different location (Ohio). (A, C, E) Protozoa. (B, D, F) Bacteria. (A, B) Glucose in media. (C, D) Reserve carbohydrate. (E, F) Cell protein. Fermentation acids, fermentation gases, and heat production were not measured. Data represent one experiment each for protozoa and bacteria. Experiments were replicated three times in total for protozoa and twice in total for bacteria.

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