Environmental, Biochemical, and Dietary Factors that Influence Rumen Development in Dairy Calves

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ABSTRACT

The dairy industry today is beginning to dedicate more focus on the growth of the calf from birth to first breeding to better improve the milk production as well as the overall performance of the individual cows. While the development of the rumen is one of the most vital contributors to the performance of the calf, it remains unknown what molecular mechanisms are responsible for the development of the rumen, and more specifically the proliferation of rumen epithelial cells. The objectives of this study were to investigate the existing data on rumen development through meta-analysis and to explore the effects of sodium butyrate and lipopolysaccharide (**LPS**) on rumen development in calves through experiment.

In the first study a meta-analysis was performed to summarize the literature on calf performance and derive equations that relate rumen (e.g., rumen pH, reticulorumen weight, papillae area) and non-rumen factors (e.g., feed composition, form of feed, housing) to animal performance (e.g., intake of milk replacer (**MR**), starter, and forage; average daily gain (**ADG**); and feed efficiency). We looked at four different relationships to further investigate the connections between rumen, non-rumen, and performance factors. In the first and second relationships of interest, the effect of dietary and environmental variables on rumen variables and performance variables were examined, respectively. The third relationship of interest was how rumen variables influenced performance variables. The final relationship of interest was investigating the additive effects of the rumen, dietary, and environmental variables on the performance variables. Forward selection, multiple regression was used to derive equations to

select variables that explained variation in the response variable in each model. Results showed that the variation in calf ADG was explained by daily forage intake, calves that were weaned, total starter intake, and total MR intake (concordance correlation coefficient (**CCC**) = 0.976). The variation in feed to gain ratio was explained by the weight of the ruminal contents, daily forage, MR, and starter intakes, percent of starter in the diet, and total starter intake (CCC = 0.992). The variation in daily forage intake was explained by the percent of the diet that was starter or MR (CCC = 0.998). The variation in daily starter intake was explained by the percent of acid detergent fiber in the starter, a pelleted starter (versus a texturized), diets including starter and forage (versus a milk replacer only diet), and the percent of the diet that was MR (CCC = 0.998). The variation in daily MR intake was explained by the percent of the diet that was starter, final body weight, ruminal propionate concentration, and daily starter intake (CCC = 0.918). Based on these analyses, although dietary and environmental factors are closely associated with calf performance, ruminal factors such as volatile fatty acid (**VFA**) concentration and ruminal contents appear to have additional, additive influences on calf performance.

In the second study, 24 Holstein bull calves were challenged with oral doses of LPS and sodium butyrate. The hypothesis here was that LPS and sodium butyrate would instigate rumen cell proliferation independently and additively. Calves were assigned to one of four treatments: control (**CON**; n=5), butyrate (**BUTY**; n=5), LPS only (**LPS-O**) (n=6), or LPS plus butyrate (**LPSB**; n=6). All treatments were administered orally twice daily consisting of either: 0.9% saline (CON); 11 m*M* sodium butyrate (BUTY); LPS ranging from 2.5 to 40 μg/kg metabolic body weight (**BW**$^{0.75}$, LPS), or both butyrate and LPS (LPSB). Calves were fed milk replacer (22% CP, 20% fat, as-fed) and starter (20% CP, 3% fat, as-fed) based on metabolic BW, or about 12% BW of MR and 3% BW of starter. Feed intake, fecal and respiratory scores, and rectal temperature

were recorded daily. Calf BW, hip height, jugular blood samples, and rumen content samples (via oroesophageal tube) were collected weekly. Calves were weaned at 6 wk of age and euthanized at 8 wk of age, whereupon ruminal weights and ruminal samples for papillae area and epithelial thickness were collected. Blood and rumen samples were analyzed for concentrations of beta-hydroxybutyrate, glucose, LPS-binding protein, and VFA. Data were analyzed as a 2x2 factorial with the repeated effect of week. Three non-orthogonal contrasts (CON versus the average of all other treatments; LPS-O versus LPSB, and LPSB versus BUTY) were investigated. Feed intake, health measures, and blood metabolites did not differ by treatment. Calf BW increased by week ($P < 0.0001$). Irrespective of week, LPS calves weighed more and had higher ADG than BUTY calves ($P = 0.020$). Irrespective of week, withers height was greater in LPS compared to CON ($P = 0.006$). Rumen pH and rumen VFA concentrations did not differ by treatment but did decrease and increase, respectively, with week in conjunction with increased starter intake. Total empty forestomach ($P = 0.014$) and reticulorumen weights ($P = 0.012$) were greater in LPSB compared to BUTY. Overall, LPS and sodium butyrate appeared to have synergistically affected some, but not all rumen measurements without affecting calf growth, intake, or health.

Results from the meta-analysis emphasize the importance of continuing to focus on the solid feed intake of the calf from birth through weaning. Implications from the LPS study are imperative to other dairy scientists who will attempt to further study the effects of LPS on the rumen.

Environmental, Molecular, and Dietary Factors that Influence Rumen Development in Dairy Calves

Carrie A. Ceh

GENERAL ABSTRACT

Dairy calves are born with an under-developed stomach. The stomach has four compartments: the rumen, reticulum, omasum, and abomasum. The rumen is the largest component where finger-like projections called papillae grow to absorb nutrients for the calf. It is vital to the calf that the rumen develops not only the papillae to absorb nutrients but also to foster a microbe-rich environment so the microbes can act as a defense mechanism for the calf to aid in fighting disease. While it is known that things like solid feed support the development of the rumen, the mechanism behind how that is happening still remains unclear in the literature.

The objective of this study was first to better understand the relationships that exist in the literature between dietary, environmental, and ruminal factors, and second to investigate the claim that certain components of the bacteria in the rumen are stimulating rumen development independently and additively with sodium butyrate.

In order to investigate the relationships amongst the dietary, environmental, and ruminal parameters, a computer program called R Studio was used to analyze over 30 different models that extracted data from a database that included a collection of 36 studies from the literature. This is also known as a meta-analysis. The associations of interest that we found were: average daily gain (**ADG**) of the calf was associated with daily forage intake, calves that were weaned, total starter intake, and total MR intake. Feed efficiency of the calf was associated with the weight of the ruminal contents, daily forage, milk replacer (**MR**), and starter intakes, percent of the diet composed of starter, and total starter intake. Daily forage intake was associated with the percent

of the diet that was starter or MR. Daily starter intake was associated with acid detergent fiber in the starter, a pelleted starter (versus a texturized starter), diets including starter and forage (versus a MR only diet), and the percent of the diet that was MR. Daily MR intake was associated with the percentage of the diet that was starter, final body weight (**BW**), ruminal propionate concentration, and daily starter intake. These relationships emphasized that although dietary and environmental factors are more closely associated with calf performance, ruminal factors such as rumen contents and volatile fatty acid concentrations appear to have additional, additive influences on calf performance.

The second part of the study objective was to explore an idea that, to our knowledge, has not been published in the literature. In the second study, 24 dairy calves were challenged with oral doses of a gram-negative bacteria lipopolysaccharide (**LPS**), and a short-chain fatty acid sodium butyrate. The hypothesis in this study was that the LPS and sodium butyrate would trigger metabolic pathways on the rumen cell membranes to a greater extent together, versus independently, to increase the amount of cells growing. Calves were assigned to one of four treatments: control (**CON**), butyrate (**BUTY**), LPS only (**LPS-O**), or LPS plus butyrate (**LPSB**). To study this effect, each treatment group was administered their respective treatment orally as a liquid twice daily. To measure the results, the following data was collected: feed intake, fecal and respiratory scores, rectal temperature BW, hip and withers height, blood samples, rumen content and pH samples, papillae area, epithelial thickness, and organ weights. Blood and rumen samples were analyzed for blood metabolites and volatile fatty acids concentrations respectively. Data were analyzed and results showed no difference amongst feed intake, health measures, rumen pH, rumen VFA concentration, and blood metabolites by treatment. Calves on the LPS treatment weighed more and had higher ADG than BUTY treatment calves. Withers height was higher in the LPS

group when compared to CON. Stomach weights were higher in the LPSB group when compared to the BUTY group.

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LIST OF ABBREVIATIONS

ADF – Acid detergent fiber
ADG – Average daily gain
AIC – Akaike information criterion
AREG – Amphiregulin
a.u. – arbitrary unit
BrdU – 5-bromo-2'-deoxyuridine
BUTY – Sodium butyrate treatment
BW – Body weight
CB – treatments that contained butyrate
CCC – Concordance correlation coefficient
CL – treatments that contained LPS
CON – Control treatment
CP – Crude protein
DM – Dry matter
EGFR – Epidermal growth receptor
F:G – Feed to gain ratio
IRAK – IL-1R associated kinase
LBP – LPS binding protein
LPS – Lipopolysaccharide
LPSB – Lipopolysaccharide plus butyrate treatment
LPS-O – LPS only treatment
MAMP – Microorganism–associated molecular patterns
MBW – Metabolic body weight
MCT1 – Monocarboxylate transporter 1
MCT4 – Monocarboxylate transporter 4
MR – Milk replacer
MyD88 – Myeloid differentiation primary response 88
NDF – Neutral detergent fiber
NFC – Non-fiber carbohydrates
NFκB – Nuclear factor kappa-light-chain-enhancer of activated B cells
SARA – Subacute ruminal acidosis
SCFA – Short chain fatty acids
SEM – Standard error of the mean
TIR – Toll-interleukin-1 domain
TLR4 – Toll-like receptor 4
TRAF6 – TNR receptor-associated factor 6
VFA – Volatile fatty acid
VIF – Variance inflation factor

INTRODUCTION

Ruminants are unique because their multi-compartment stomach has extensive capabilities not found in non-ruminants, namely pre-gastric fermentation and ability to absorb fermentation end products. Absorption of fermentation end products contributes 65-75% of ruminant metabolizable energy needs (Bergman, 1990). The ingestion of solid feeds such as hay and starter and subsequent anaerobic fermentation of them by rumen microbes can aid in the development of the rumen. However, underlying mechanisms responsible for rumen papillae growth are not well understood. Conceivably, diet factors and rumen factors, such as volatile fatty acids (**VFA**) and components of rumen bacteria, might both contribute to rumen papillae growth.

Rumen papillae, the absorptive structures within the rumen, are present at birth in rudimentary form. As the ruminant animal grows and consumes dry feed and the feed undergoes anaerobic fermentation, papillae will further develop and the rumen organ will become the equivalent of a bio-fermentation chamber, complete with a diverse microbiome. This microbiome will consist of microorganisms that ferment feed into VFA. The VFA will then be absorbed by rumen papillae and in the case of butyrate, contribute to rumen papillae cell proliferation. We theorize that the microbial cell wall component lipopolysaccharide (**LPS**) works along with butyrate, in a synergistic manner, to affect cell proliferation in the rumen.

The **long-term goal** is to advance our knowledge about rumen development and the mechanisms and factors that are involved. The **overall research objectives** are to: 1) use a statistical modeling approach to evaluate what dietary, management, and environmental factors explain variation in rumen development, and 2) test whether or not LPS can directly affect rumen growth when orally administered by itself or in combination with butyrate.

CHAPTER 1: LITERATURE REVIEW

Dairy Calf Growth and Performance

Contemporary Dairy Calf Feeding Practices

A calf's protein and energy requirements for maintenance, as expressed per kg of BW, are highest at birth and decline with age. This is due to environmental stresses (e.g. thermal regulation) and newly required functions outside in utero conditions (e.g. functioning tissue for nutrient digestion) being major shifts the calf must adjust to after birth (Davis & Drackley, 1998). In order to meet the calf's protein and energy demands for maintenance and growth the calf must consume a diet that is easily digestible for their underdeveloped digestive tract.

A common goal for raising dairy calves is to double birth weight by 56 d of age (DHCA Gold Standards, 2016). For Holstein heifers, this amounts to about 41 kg of gain over 56 d, or an ADG of 0.732 kg/d (DHCA Gold Standards, 2016). If such growth is desired, it is imperative that the diet of the calf allows for such gains. In calf nutrition, common metrics to assess when evaluating calf rations are metabolizable energy (**ME**) allowable gain and apparent digestible protein (**ADP**) allowable gain. Metabolizable energy consists of the portion of energy consumed by the calf that is available for maintenance and growth, with the rest lost in feces, urine, or rumen fermentation (Moran, 2012). Apparent digestible protein is said to be 75% of the ingested crude protein (**CP**) (NRC, 2001). The average daily gain (**ADG**) of a calf can be predicted using ME allowable gain and ADP allowable gain to more closely reflect actual calf performance in most cases. For example, if the ration formulation software calculates ME allowable gain to be 0.86 kg/d for a given ration and ADP allowable gain to be 1.26 kg/d for that same ration, it would be expected that the observed ADG of the calf more closely resembles the lower of the two, in this

case 0.86 kg/d. Calf body weight gains diets are therefore limited by either energy intake or intake of apparent digestible protein.

There are three nutritionally relevant phases that exist for dairy calves. These include the liquid, transition, and ruminant phase (NRC, 2001). Within phase, diet components and intake will determine whether a calf's maintenance requirements and requirements for desired body weight gain are satisfied. Definitions of each phase and examples of diets within each growth phase are discussed next.

Liquid Phase.

The liquid phase consists of the time period from birth until calves begin voluntarily consuming dry feeds such as calf starter. Even if offered from birth, most calves do not start consuming appreciable amounts of concentrate feed (>50 g/d as-fed) until the third week of life. For the first 3 wk of life the liquid phase contributes 100% of the calf's maintenance and growth needs by milk or MR. The unique part of this phase is that when the calf drinks milk, their reticular groove will close and milk will shunt past the rumen into the abomasum, preventing the milk products from fermenting in the rumen (Wise and Anderson, 1984; Baldwin et al., 2004). This anatomical feature helps contribute to the efficiency of nutrient use for maintenance and growth. Growth during this phase is the most efficient of the three for dairy calves; less food is required to achieve a given targeted body weight gain per day.

Given that nearly 50% of dairy farms in the United States feed some type of milk replacer (**MR**) in the liquid phase, it is vital to implement a structured calf feeding protocol to ensure the needs of the calf are being met (NAHMS, 2014). The 2016 DHCA gold standards state that in order to meet the nutrient, ME, and ADP requirements of the calf, no less than 3.8 L of milk replacer should be fed a day. In general, MR should contain at least 22% CP (NRC, 1989) and at

least 10% fat (Davis and Drackley, 1998). Studies show that feeding a higher crude protein percentage in the MR improves calf performance measures such as ADG, starter intake, final body weight (**BW**), and feed efficiency (Hill et al., 2008; Hill et al., 2009). When allowed to nurse or feed from an automatic calf feeder until satiated, Holstein calves will consume about 10 L/d of whole milk, or of the given MR, respectively (de Passille and Rushen, 2016; Jorgensen et al., 2017; Knauer et al., 2017). As shown in the recent USDA survey (NAHMS, 2014), many US farms offer calves less than this, with a daily offering of 3 to 5 L. This practice originated to encourage consumption of cheaper starter feed as opposed to whole milk or MR. Steele et al. (2017) demonstrated the importance of higher MR feeding in calves that were fed up to 20% MR of their BW having an improved overall health status compared to limit fed calves. A study by Vasseur et al. (2010) collected data from over 100 herds and showed that the average amount of MR fed was 4 L per day during the first week of life, 5.5 L between the first and last week of weaning, and 3 L in the last week of weaning. With the standard MR feeding of 12.5% solids, the volumes would equate to 0.5 kg MR powder during the first week of life, 0.7 kg of MR powder from 2 to 7 wk of age, and 0.4 kg of MR powder the week of weaning. When feeding a calf in the first 8 wk of life, whether the calf is consuming a restricted or enhanced milk diet, the transition period for weaning is crucial to the health of the calf.

Transition Phase.

The transition period is a time when calves begin consuming solid feed (e.g. from concentrates) in addition to milk or MR, meaning nutrient supply shifts from a milk/MR only diet to a diet that supports utilization of nutrients from both milk/MR and concentrates. This is the second-most efficient of the three growth phases. Adding concentrate to the calf's diet at an early age has shown to help develop the rumen due to the presence of rapidly fermentable feeds

(Lengemann & Allen, 1959; Khan et al., 2016). However, the practice of limiting MR intake can cause the calf to experience hunger and increased risk of disease (Kahn et al., 2011). Abrupt transitions from milk to solid feeds can reduce starter intakes, retard growth, decrease volatile fatty acid (**VFA**) concentration in the rumen, and disrupt calf behavior (Jasper et al., 2008; Sweeney et al., 2010; Steele et al., 2017). In the US, most farmers currently offer concentrate feed to calves for the first time at 11 d of age (NAHMS, 2014). The transition phase of calf growth ends with weaning. The average age for weaning in the US is currently about 9 wk (NAHMS, 2014).

Ruminant Phase.

The ruminant phase can be considered the time period when the calf is weaned and consuming a diet that does not contain milk. This phase is focused on the development of the rumen and ruminant metabolism. As such, growth in this phase is the least efficient of the three phases. Growth of rumen papillae and increased rumen mass are important in this phase of growth because rumen fermentation must supply the majority of the animal's energy and protein requirements.

There are many factors that contribute to the development of the rumen and ruminant metabolism. These include morphological, metabolic and microbial changes, which will be discussed next.

Morphological Rumen Development

At birth the rumen is small in size and the rumen tissue (epidermis containing papillae + dermis + smooth muscle) is thin and slightly transparent due to the absence of fermentation in the rumen (Heinrichs, 2005; Baldwin et al., 2004; Khan et al., 2016). As a calf consumes MR, the reticular groove closes and directs all liquid to the omasum, omitting most products from entering the rumen for fermentation. If concentrate is not offered and the animal remains on a milk/MR

diet, physical development of rumen will not progress to a fully developed rumen like one on a diet containing solid feed (Xie et al., 2013). The progression of tissue development within the rumen happens partly due to a shift in nutrient absorption (Baldwin et al., 2004). A diversified diet allows nutrients to deliver to the intestine as the liver shifts away from a glucose-dependent source of energy, resulting in rumen tissue development. As mentioned above, at birth rumen tissue (epidermis containing papillae + dermis + smooth muscle) is underdeveloped. The rumen epidermis has obvious projections called papillae which are made up of 4 layers of keratinized, stratified, squamous cells, which distinguish the rumen from intestinal lining that has columnar epithelial cells (Heinrichs, 2005; Garcia et al., 2017). The rumen epidermis serves two main functions: 1) absorption of VFA and 2) physical barrier between rumen contents and the animal's bloodstream. These aspects will be discussed in more detail in later sections.

Metabolic Rumen Development

It is widely believed that volatile fatty acids largely contribute to papillae development (Baldwin et al., 2004; Sander et al., 1959). Specifically, the VFA butyrate and propionate, are associated with thickened stratum corneum of the rumen papillae (Gilliland et al., 1962), which are the two outermost layers of cells in the epidermis that separate the epidermis from rumen contents (Garcia et al., 2017). Górka et. al (2009) did a study showing that supplementation of sodium butyrate to both MR and starter resulted in increased rumen development when compared to the control calves. Sodium butyrate has a faster absorption rate in the rumen than acetate and propionate (Sander et al., 1959). The introduction of solid feed into the diet increases rumen VFA concentrations. Rumen microbes can utilize fermented products like VFA for rumen development (Quigley et al., 1990). Butyrate is absorbed by rumen papillae and converted into BHBA and acetoacetate in the rumen cell to be further transported to the blood (Baldwin et al., 2004; Bergman,

1990). The fate of BHBA in young dairy calves is involved with rumen epithelial metabolic activity by providing an energy substrate for the rumen in the form of acetoacetate (Khan et al., 2007). Once in the bloodstream in the form of a ketone, the calf can utilize this substrate for energy through ketogenesis to meet ATP demands other than those of the rumen. It is necessary to ensure a balanced diet with adequate volumes of milk and solid feeds is met to develop a complete and functional rumen.

Microbial Rumen Development

While physical development of the rumen is vital to the growth of the calf, the rumen must also foster an environment that the microbes can thrive in in order to function at their highest capacity. The microorganisms function to make the metabolic processes for calf growth and health possible (Hungate, 1966). By the second day of life, calves will have a strictly anaerobic bacterial population present in the rumen with over 200 species of bacteria at a concentration of about 10^{11} bacteria/mL (Fonty et al., 1988). A major shift in the microbial populations can result in compromised feed efficiency and potentially disease (Hungate, 1966). The microbes not only produce metabolites but microorganism-associated molecular patterns (**MAMP**), which are parts of the cell membranes, as well (Neish, 2009). A few common examples of MAMP that exist include flagellin, β-glucan, and LPS which are recognized by specific receptors that are recognized in the same pattern within the organism. The MAMP are critical for microbes to survive and are thought to be highly conserved (Hungate, 1966). Protozoa make up the second largest class, after bacteria, to be found in the rumen. While protozoa are not required for ruminal function, they play a vital role in fermentation products (Hungate, 1966). Lastly, anaerobic fungi are also present in the normal rumen microbial population, however their presence is in much lower concentrations (Dehority and Orpin, 1997; Hungate, 1966). Although there are endless factors that can play a role

in enhancing or compromising the rumen, diet is said to be one of the most influential factors (Dehority and Orpin, 1997; Hungate, 1966). Suarez et al. (2007) showed that inadequate diet transition and management can lead to reduced VFA concentrations and therefore slow microbial activity. In turn this can lead to decreased nutrient absorption and rumen development resulting in poor calf health and performance. It is therefore important to understand the morphological, metabolic, and microbial properties of the rumen to provide the best calf raising protocol possible.

Rumen pH

Calf growth and performance can be impacted by pH of the rumen. A major challenge with restricting MR intake and increasing starter intake at an earlier age is the resulting decrease in rumen pH from the fermentation of the starter in the rumen (Anderson et al., 1986). While starter will promote rumen development, rapid rumen fermentation will lower the pH if more protons are being produced than removed (Laarman and Oba, 2011). Adding hay into the diet of the weaned calf can assist in buffering the pH to not drop as low as a diet with no supplemental forages (Castells et al., 2013; Kim et al., 2016). Pre-weaned calves typically have a rumen pH in the range of 6.0-7.0 (Lesmeister et al., 2004). The threshold for rumen pH is 5.5 and anything below that can be considered subacute ruminal acidosis (**SARA**) (Kleen et al., 2003; Laarman and Oba, 2011). It is fairly common for calves that are going through weaning to have rumen pH drop below 5.5 (Suarez et al., 2006; Kim et al., 2016). The form of the feed can also play a role in changing rumen pH, a finely ground starter has a more rapid fermenting rate then a pelleted feed, therefore lowering the pH in an accelerated manner (Imani et al., 2017).

Environmental Factors and Their Role in Calf Health

Environmental factors additionally play a vital role in the overall well-being and health of the dairy calf. For every 1°F increase in a calf's body temperature, the average daily gain can drop

up to 15% (Bateman and Hill, 2012). While it is believed that temperature is the primary environmental influence, factors such as humidity, ventilation, wind, etc. should be considered for the proper development and welfare of the calf (NRC, 1981; Davis and Drackley, 1998). The thermoneutral zone is the range of temperature in which the animal is not expending any extra energy to meet normal maintenance requirements (Jones and Heinrichs, 2013). For dairy calves, the thermoneutral zone is 15-25°C (NRC, 1981). The lower critical temperature is the temperature below the thermoneutral zone at which the calf will increase its heat production (Davis and Drackley, 1998). As with protein requirements, the lower critical temperature for a calf decreases with age. As the calf builds thicker skin and increases fat stores, the calf can better withstand extreme temperatures. The calf can withstand extremities to a certain extent, although the body of the animal is more insulated, the energy reserves used for maintenance can be depleted quickly; therefore, it is important to supplement with extra MR powder during these times (Davis and Drackley, 1998). Aside from temperature effects, Lago et al. (2006) did a study evaluating ventilation, bedding, pen area, etc. and the relationship between airborne bacterial counts. Authors found that barn alley bacterial counts decreased with increased ventilation. If not properly managed, environmental conditions such as heat stress can lead to increased respiration and body temperature (Scibilia et al., 1987; Spain and Spiers 1996) and decreased ADG and starter intake (Broucek et al., 2008). Environmental factors are necessary to evaluate to best benefit the growth and performance of the calf, but could continue to be further investigated in research.

Lipopolysaccharide

Lipopolysaccharide (**LPS**) is an endotoxin present in the outer membrane of Gram-negative bacteria. It has two main constituents known as lipid A and O-antigen (**Figure 1.1**). Lipid

A is a glucosamine-based phospholipid that contains six lipid chains and is the instigator of immunological responses, which are activated through TLR4. The O-antigen is a distal polysaccharide that can be important to generate structural diversity (Raetz & Whitfield, 2002 & Park et al., 2009).

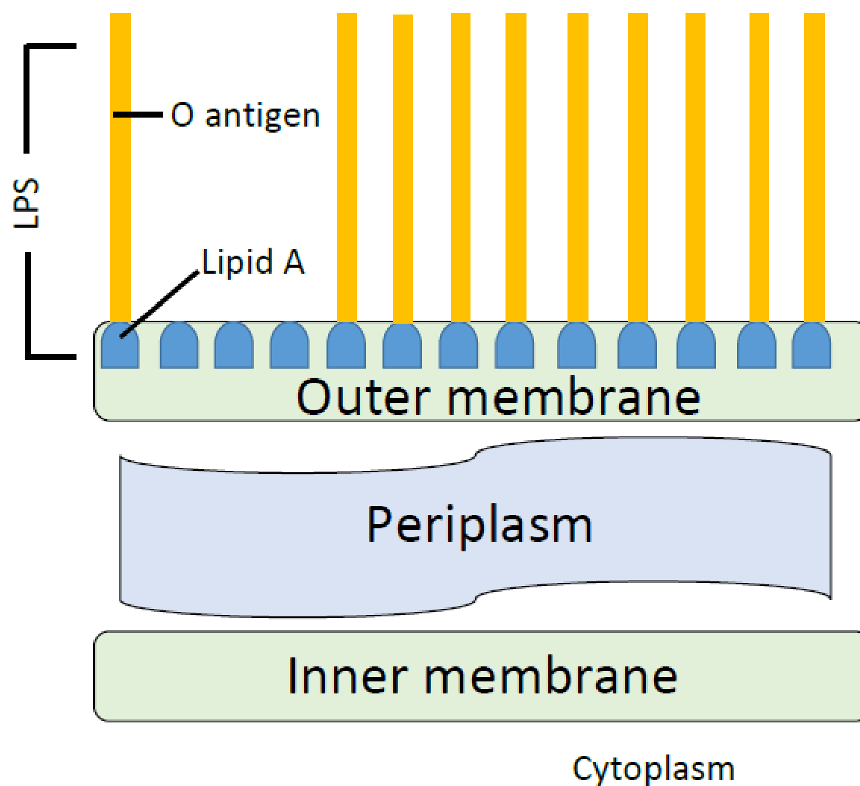

Figure 1.1. Two major constituents of lipopolysaccharide (LPS) are the lipid portion and the O-antigen portion. The lipid is incorporated into the membrane of gram-negative bacteria while the O-antigen is exposed.

Lipopolysaccharide is separated from the membrane and binds to TLR4 via the aid of CD14 and LPS binding protein (**LBP**) (Park et al. 2009; Lu et al. 2008). The new configuration of the LPS-LBP-CD14 complex then has the ability to attach to the TLR4-myeloid differentiation

factor 2 (**MD-2**) complex (**Figure 1.2**) (Lu et al. 2008; Park et. al 2009). The attachment happens at the MD-2 site and not actually the TLR4 site. The MD-2 has a folded structure that creates a pocket for ligand binding, where the lipid A chains of the LPS bind (Park et. al, 2009). In order for the complexes to dimerize, the TLR4 will form hydrophobic and hydrophilic bonds directly with the LPS as well as some beta strands of the MD-2.

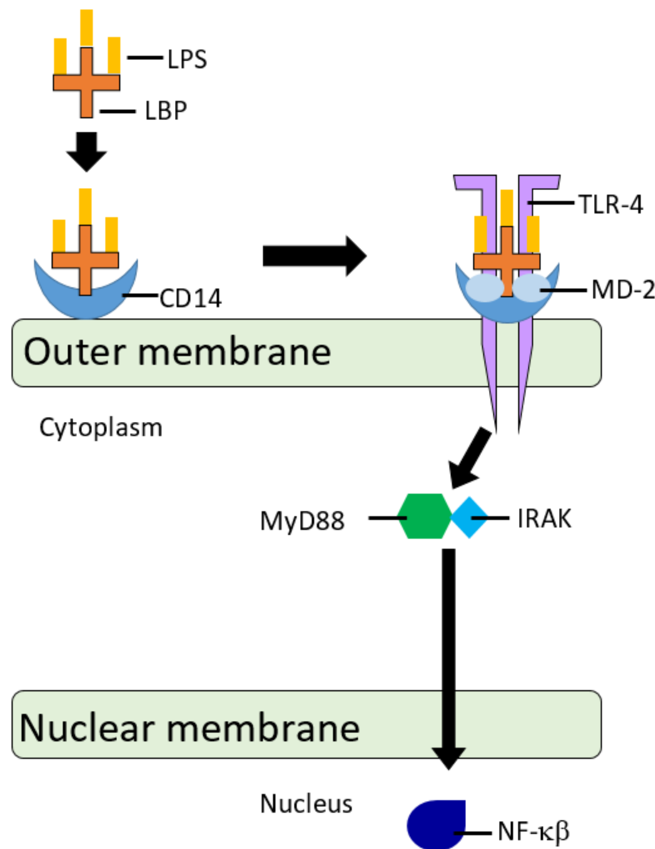

Figure 1.2. Lipopolysaccharide (LPS) signaling pathway, starting with the binding of the LPS-LPS binding protein (LBP) complex to CD14 which then docks on Toll-like receptor-4 (TLR4) to indirectly signal nuclear factor-kappa B (NF-kB) in the nucleus.

Different variations of LPS from different bacteria can contain different numbers of lipid chains. An LPS molecule with five lipid chains instead of six is less active, while even fewer with

four lipid chains are completely inactive (Park et al., 2009). *Escherichia coli* LPS does in fact contain six lipid chains which was our source of LPS for experimental purposes (Park et al., 2009).

The next step in the cascade is known to be the activation of the Toll-interleukin-1 receptor domain **(TIR)**. This domain consists of five parts, including MyD88. The cascade can either follow an MyD88 independent pathway or an MyD88-dependent pathway (**Figure 1.3**).

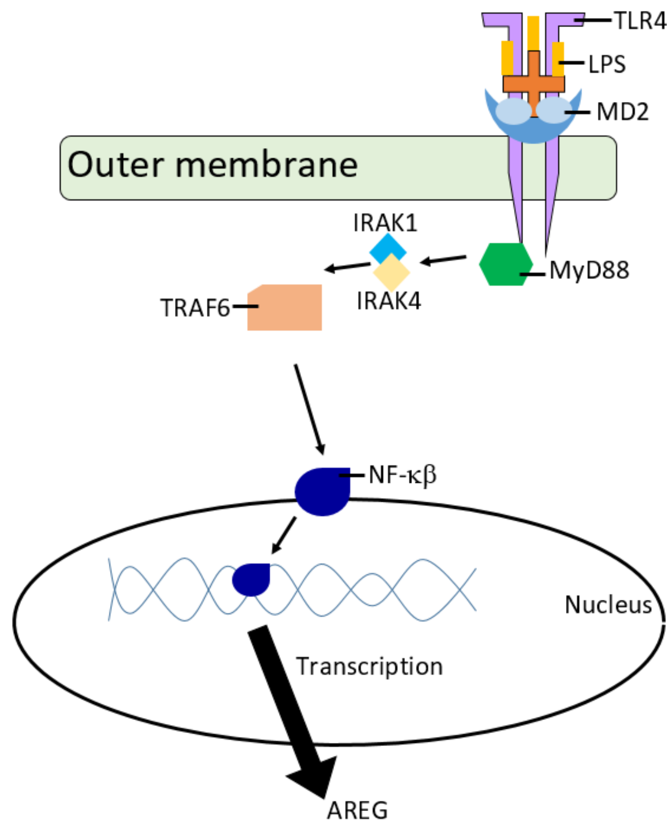

Figure 1.3. Proposed mechanism for the downstream effects of the Toll-like receptor-4 (TLR4) and its cascade within the cell. Nuclear factor-kappa B (NF-κB) will lead to the expression of amphoregulin (AREG) via transcription of the DNA in the cell.

The MyD88 will then activate proteins in the IL-1R-associated kinase (**IRAK**) family which will then activate TNR receptor –associated factor 6 (**TRAF6**). This interaction will activate nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-κB**) (Fitzgerald et al., 2003). The MyD88 independent pathway will activate NF-κB as well but through different means (Fitzgerald

et al., 2003). Streicher et al. (2007) showed that the binding of NF-κB to DNA will signal the activation of epidermal growth factor receptor (**EGFR**) when NF-κB creates a positive feedback loop with interleukin-1 alpha (**IL-1**). EGFR and amphiregulin (**AREG**) create an autocrine loop that increases cell proliferation (**Figure 1.4**), as shown in human cancer studies (Streicher et al., 2007; Silvy et al., 2001).

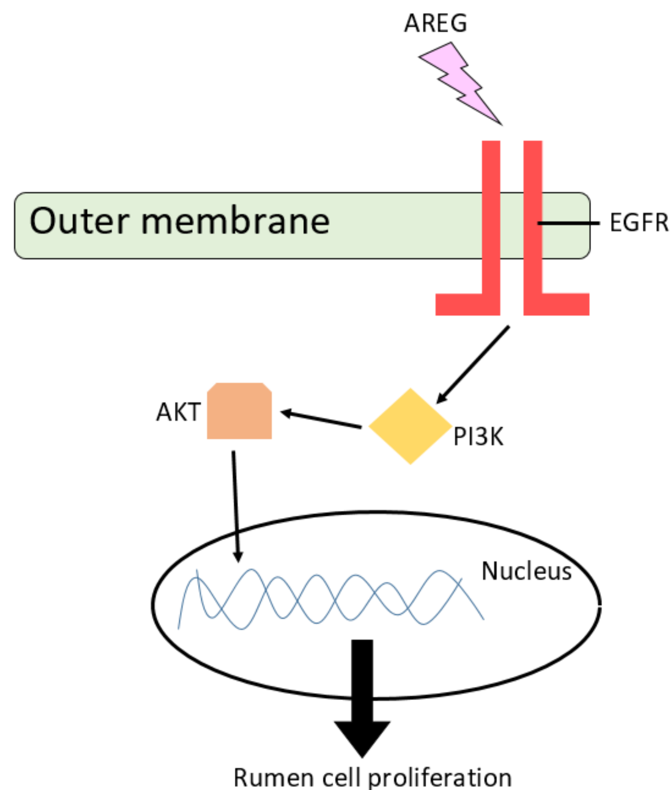

Figure 1.4. Amphoregulin (AREG) will bind to epidermal growth factor receptor (EGFR) and stimulate the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway. The PI3K/AKT pathway regulates cell proliferation.

TLR4 mediates LPS signaling in many cell types across species (Nagai et al., 2002). In mice the mutation of the gene encoding for TLR4 diminishes the responsiveness of LPS (Nagai et al., 2002). Authors Khafipour et al. (2009a) demonstrated that the concentration of LPS in the rumen was elevated when cows were in a state of SARA, however changes in the levels of LPS in

the blood were not detected. Elevated LPS levels are observed in a state or SARA (Khafipour et al., 2009a) and MCT1 likely improves absorption of VFA (Castells et al., 2013). Based on the knowledge of this previous evidence, we believe LPS is working with MCT. We propose that the relationship is that of a synergistic one to further activate rumen cell proliferation since TLR4 will indirectly upregulate the PI3K/AKT proliferation cascade.

In order to effectively measure LPS activity, previous studies have measured inflammatory markers and acute phase proteins like serum amyloid A (**SAA**), LPS-binding protein (**LPSB**) and haptoglobin (Nagai et al., 2002; Gozho et al., 2006; Ametaj et al., 2012).

Previous studies have performed LPS challenges by injecting the endotoxin into the bloodstream and measuring blood samples following the injection (Anderson et al., 1996, Eicher et al., 2006). Although LPS is naturally present in the gram-negative bacteria in the rumen (Plaizier et al., 2008), dosing additional LPS can cause diseases associated with acidosis and even death if not cautious (Gozho et al., 2005). It is for this reason that we need to be very liberal with the monitoring of the LPS-treated calves in this study.

Host-Microbe Interactions

Host-microbe interactions can be symbiotic or pathogenic, resulting in a healthier or disease-born host. An example of a symbiotic host-microbe relationship can be one that protects the host from disease (Medzhitov, 2007). The mechanism through which the host will detect this signal from the microbes is through pattern recognition receptors (**PRR**) (Medzhitov, 2007). The PRR will detect pathogens and trigger a defense in response. The TLR4 is an example of a PRR (Park and Lee, 2013) that is activated when LPS binds to the receptor. The calf responds to excess LPS through that of an immune inflammatory response (fever, decreased appetite, lethargic, etc.) to prevent against further infection (Benzaquen et al., 2007). The inflammatory response of the

calf can be diminished over time as a host can become immune to LPS (Jacobsen et al., 2005; Novoa et al., 2009).

If the immune response is diminished over time from tolerance, we propose in this thesis that the LPS will eventually take the path of the PI3K/Akt pathway to stimulate rumen cell proliferation. We are also interested to see what other factors outside the body of the calf can be influencing the development of the rumen.

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CHAPTER 2: RUMINAL, DIET, AND ENVIRONMENTAL FACTORS THAT AFFECT DAIRY CALF PERFORMANCE

ABSTRACT

Although impacts of dietary and environmental factors on calf performance are investigated, no studies have investigated how rumen development, independent of diet and environment, influences dairy calf performance. Our objective was to summarize the literature on calf performance and derive equations that relate rumen (e.g., rumen pH, reticulorumen weight, papillae area) and non-rumen factors (e.g., feed composition, form of feed, housing) to animal performance (e.g., intake of milk replacer (**MR**), starter, and forage; average daily gain (**ADG**); and feed efficiency). 146 treatment means from 36 trials were obtained under the following selection criteria: study reported dairy calves only; calf between 0 to 24 wk of age; calves had to be fed MR for some part of the study; study reported one or more rumen variables; and ADG > 0.2 kg/d. Forward selection, multiple regression was used to derive equations to select factors that influenced the response variable in each model; models were weighted by the inverse of the standard error of the mean. Models were evaluated based on root estimated variance and concordance correlation coefficients (**CCC**). Calf ADG was associated with daily forage intake, calves that were weaned, total starter intake, and total MR intake (CCC=0.976). Feed to gain ratio was associated with the weight of the ruminal contents, daily forage, MR, and starter intakes, percent of the diet composed of starter, and total starter intake (CCC=0.992). Daily forage intake was associated with the percent of the diet that was starter or MR (CCC=0.998). Daily starter intake was associated with acid detergent fiber in the starter, a pelleted starter, diets including starter and forage, and the percent of the diet that was MR (CCC=0.998). Daily MR intake was associated with the percentage of the diet that was starter, final body weight (**BW**), ruminal propionate concentration, and daily starter intake (CCC=0.918). Although dietary and

environmental factors are closely associated with calf performance, ruminal factors appear to have additional, additive influences on calf performance.

Key words: dairy calf, rumen development, diet, meta-analysis

INTRODUCTION

Dairy calves, like all animals, must ingest and metabolize feed in order to meet nutrient requirements for maintenance and growth. The diet of the typical dairy calf changes drastically from birth to 10 wk of age, a period of time that encompasses weaning (Baldwin et al., 2004). Briefly, dairy calves are born as functional non-ruminants and become true ruminants in roughly 10 wk. Diet (Warner et al., 1956; Coverdale et al. 2004) and digestive physiology (Lane et al., 2000) both factor into this change. Understanding the fundamental aspects of calf nutrition is of vital importance for enhancing efficiency of calf growth. In preweaned dairy calves, dietary nutrients needed for maintenance and growth are primarily supplied by a liquid diet of either milk or milk replacer (**MR**) (Drackley, 2008).

Voluntary starter intake typically commences at around 2 wk of age in dairy calves (Kertz et al., 1979; Yohe et al., 2015; Moore et al., 2017). Increased solid feed intake, commonly in the form of starter, is needed for the rumen tissue and the rumen microbiome to develop (Warner et al., 1956; Meale at al., 2017; Steele et al., 2017). For these reasons, calf starters typically contain large amounts of starch (40% starch [DM basis] for texturized starters and 25% starch [DM basis] for pelleted starters) (Quigley et al., 2018), from grain sources such as: corn, barley, oats, and wheat. Calf starters are fed to calves in a variety of forms – pelleted, ground, or texturized; and the degree of processing is linked to overall starter digestibility (Porter et al., 2007; Nejad et al., 2012). Historically, consumption of high concentrate diets (compared to high-forage diets or diets with no dry feed) in dairy calves are associated with: heavier rumens (Stobo et al., 1965), larger rumen

papillae (Heinrichs et al., 2005), increased molar proportions of VFA (Anderson et al., 1987), and lower rumen pH (Suarez-Mena et al., 2016).

In the United States, forage provision to dairy calves prior to weaning is not currently recommended (Dairy NRC, 2001), nor is it currently a common practice (NAHMS, 2016). The rationale for not including forage in pre-weaned calf diets was due to observations that forage can limit metabolizable energy intake in young ruminants and because capacity for ruminal cellulose degradation is generally limited in young calves (Dairy NRC, 2001).

A recent meta-analysis on the subject of forage feeding to dairy calves concluded that forage consumption was associated with: increased starter intake, increased rumen pH (which the authors equated to rumen health), increased molar proportion of acetate, and increased acetate to propionate ratio in the rumen, along with positive associations with calf body weight, and average daily gain (**ADG**) (Imani et al., 2017). Consensus has not yet been obtained on the merits of including forage in contemporary calf diets. Further, although impacts of dietary and environmental factors on calf performance are investigated (Imani et al., 2017), to our knowledge no retrospective studies have investigated how rumen development, independent of diet and environment, influences dairy calf performance. The objective was to summarize the literature and derive equations that model four main relationships. These relationships include: 1) relating rumen (e.g., rumen pH, reticulorumen weight, papillae area) and non-rumen (dietary and environmental) factors to animal performance (e.g., intake of MR, starter, and forage; ADG; and feed efficiency) 2) relating only dietary and environmental factors to calf performance 3) relating only ruminal factors to calf performance and 4) relating dietary and environmental factors to rumen factors.

MATERIALS AND METHODS

Literature Search, Inclusion Criteria, and Data Cleaning

Literature Search. Data were collected from published, peer-reviewed articles in between September of 2016 and January of 2018. Key words used to search for relevant articles were: "calf rumen development", "calf performance", "weaning on rumen development", and "dairy calf rumen". Queried online databases used to return relevant articles were: Google Scholar (http://www.scholar.google.com/) and PubMed (https://www.ncbi.nlm.nih.gov/pubmed) as well as the search function on the Journal of Dairy Science website (http://www.journalofdairyscience.org/). References contained in every article recovered were screened for suitability of inclusion. To make results more readily applicable to the field, only articles originally published from 2000 to 2018 were considered for inclusion.

Inclusion Criteria and Data Cleaning. In order to remain in this meta-analysis, returned articles must have used dairy breed calves between the ages of 0 and 24 wk-old, with at least some of that time spent on a diet that contained either milk or milk-replacer (**MR**). All calves must have been individually housed and fed at least twice daily in the pre-weaning period in the original study. Additionally, calf diet, rumen characteristics, calf performance, and housing environment must have been reported.

Three additional databases were formulated regarding calf diets, which included the composition of individual feed components in MR, starter, and forage (if applicable). Values reported in these databases included dry matter (**DM**), crude protein (**CP**), neutral-detergent fiber (**NDF**), acid-detergent fiber (**ADF**), Ash, crude fat (**CF**), Starch, and non-fiber carbohydrates (**NFC**). Due to a lack of reporting of individual milk replacer components across the majority of papers, only crude protein and fat values were used to reflect milk replacer composition. If the

nutritive value was not listed for the composition of individual diet ingredients, the NRC for dairy cattle was used to obtain an average composition estimate (Dairy NRC, 1989; Dairy NRC, 2001). For any forage nutritive values and any missing values for the starter ingredients, the Dairy One feed database (http://dairyone.com/analytical-services/feed-and-forage/feed-composition-library/interactive-feed-composition-library/) was used. When calculating NFC any negative values were accepted as zero.

The total DM starter and milk replacer intake were calculated by multiplying the average intake per day by the number of days the trial lasted if total intake was not specifically stated in the paper. The MR, CP, and CF were scaled to be all on a DM basis. If VFA concentrations were reported on a weekly basis, values at the latest date were recorded in the database. For the Azevedo et al. (2016) publication, tropical grass intake on the pasture was not accounted for, but the study was retained in the database because of the similarities within other studies in terms of body weight and intake numbers.

Each study had to report on two or more of the following rumen characteristics: papillae length; papillae width; rumen wall thickness; reticulorumen weight; rumen pH; and ruminal VFA concentrations. Reticulorumen weights were converted to kg. Papillae lengths and widths, and rumen wall thickness measurements were converted to mm. Papillae areas were converted to mm^2. Rumen VFA concentrations (e.g., acetate, propionate, and butyrate) were converted to m*M*. The VFA units in Lesmeiester et al. (2004a) were reported as µM but should have been to m*M* (personal communication A. J. Heinrichs). We assumed that VFA values in Muya et al., (2015), Khan et al. (2008), and Santos et al. (2015) were also in error and changed µM values to m*M*. We assumed that reported papillae lengths were off by a factor of 10 in four articles (Wang et al., 2017, Steele et al., 2017, Khan et al. 2011, Khan et al., 2008), and adjusted our database values accordingly.

For Lesmeister et al. (2004a), Lesmeister et al. (2004b), and Lesmeister et al. (2005), reported units for papillae length were changed to millimeters after conversing with the corresponding author A.J. Heinrichs (Pennsylvania State University, State College, PA, personal communication). Papillae measurements for Suarez-Mena et al. (2011a) came from the published erratum in JDS (Suarez-Mena et al., 2011b) as opposed to the original work.

Required calf performance entries were treatment ADG > 0.2 kg/d, feed intakes, and feed efficiency (Feed:gain; F:G). The ADG cutoff was set to exclude extremely poor-growing calves (Dairy Calf and Heifer Association Gold Standards, 2016; Dairy NRC, 2001). Initial calf body weights for Khan et al. (2008; 2011) and Canon et al. (2010b) were obtained from companion papers Khan et al. (2007) and Canon et al. (2010a) respectively that are not explicitly cited in the database. If ADG was not directly reported, then we calculated ADG from given calf body weights and given days enrolled in study. If F:G was not directly reported, we calculated F:G from given feed intakes (kg DM) and given calf body weights (kg).

To account for potential environmental effects, geographic study location, year the study was conducted, gender, and breed were always included in the database. If it was not specifically stated that calf hutches were temperature controlled or ventilated, it was assumed that calf housing was not temperature controlled or ventilated. Only one study did not report about bedding (Wang et al. 2017) so it was assumed to not use bedding. Temperature controlled, ventilation, and bedding were all input as binary (1= yes, 0 = no) variables into the database. A total of 36 articles with 143 observations met all of our inclusion criteria and were included in our final dataset (S1).

We checked all articles in our final dataset to ensure that standard errors of the mean (**SEM**) were reported. We also determined whether statistical methods in the original studies used fixed-effect models or mixed model. In a few instances, corresponding authors were contacted for

clarification on statistical methodology (e.g., Kato et al. 2011; Suarez et al. 2007; Ragionieri et al. 2016; Mirzaei et al. 2016; Laarman et al. 2012). If the corresponding author did not reply (e.g. Khan et al. 2008; Suarez et al. 2006; Schaff et al. 2018; Xie et al. 2013), then we assumed a fixed effect model if no variable was specifically stated to be random. Standard error of the mean calculations were done for any additionally calculated values using the error propagation method as described in White et al. (2016). Studies that reported low SEM values were truncated to prevent overweighting of those studies. The SEM were truncated at half the mean until <10% of observations were curtailed.

In order to evaluate standard errors for models in an unbiased manner, truncation was done separately for fixed and mixed effects models (Roman-Garcia et al., 2016). The reciprocal was then divided by the mean to normalize both mixed and fixed effects to 1, putting them on the same scale.

Model Permutations and Model Derivation Procedures

Model Permutations. We were interested in evaluating how diet, environment, and rumen factors independently and interdependently affect calf performance (**Figure 2.1**). More specifically, we were interested in knowing: how diet and environmental factors directly affect the rumen (**Figure 2.2**, relationship A); how diet and environmental factors directly affect calf performance (**Figure 2.2**, relationship B); how rumen factors directly affect calf performance (**Figure 2.2,** relationship C); and how diet, environmental, and rumen factors jointly affect calf performance (**Figure 2.2**, relationship D). Hence, we investigated 4 main relationships.

Model Derivation. Models were derived using the lmer package of R version 3.4.3 (R Core Team, 2017). Variables within the diet/environment category (**Figure 2.2**) were only investigated as explanatory variables, whereas variables in the performance category (**Figure 2.2**) were only

investigated as response variables. Variables within the rumen category served as either explanatory variables (**Figure 2.2**, relationship C; see also **Figure 2.2**, models 17 to 24) or response variables (**Figure 2.2**, relationship A; see also **Figure 2.2**, models 1 to 8) depending on the model being investigated.

Forward selection, multiple regression was used to derive all model equations. Regressions for each of the models were weighted in two ways. In the first weighting approach, we weighted all regressions based on 1 / SEM. For the second weighting approach, all regressions were weighted by the number of observations (**n**). For brevity, only models weighted by SEM are reported herein. Models weighted by n are provided as a supplementary file (**S2-S5**) but are not discussed further.

Regardless of weighting approach, in each regression, the variable with the lowest Akaike information criterion (**AIC**) at the significance level ($P \leq 0.05$) was added into the model until no more variables were found to be significant at $P \leq 0.05$. Once a given model was derived that met the above criteria, variance inflation factors (**VIF**) were evaluated on all non-intercept variables (Akinwande et al., 2015). VIF is a measure of collinearity between two variables and 10 was used as the cutoff for linear terms in the model, while 100 was the cutoff for quadratic terms. Following established precedent (Oldick et al., 1999; Roman-Garcia et al., 2016), we always removed variables with VIF >100. In all of our final models, all VIF were <10.

Evaluating Model Performance. Similar to Roman-Garcia and others (2016), we report the root estimated variance as σ_e (i.e., the estimated σ for error) and the root estimated variance due to study, as σ_s. Variance terms are expressed in the same units as the dependent variables. To compare among models of the same response variable, a concordance correlation coefficient calculated without study-specific intercepts (**uCCC**) and concordance correlation coefficient

calculated with these intercepts (**CCC**) were used (Roman-Garcia et al., 2016; White et al., 2016). Model evaluation was mainly based on uCCC based on the description provided in White et al. (2016). All dietary and environmental variables (**S6**) and rumen variables (**S7**) were analyzed for correlation coefficients to address collinearity and error structure in the original data set. Residual plots used data adjusted for the random effect of study and the linear regressions were weighted for standard error of the mean to check for patterns (White et al., 2016). Slope-study interactions were investigated for all variables in every model. When slope-study interactions were identified, graphical analysis of the interacting variables was used to discern why the interaction was occurring and inform on interpretation of the model.

RESULTS AND DISCUSSION

Descriptive statistics for each numerical variable can be found in **Table 2.1**. Data from models found to have a slope-study interaction were plotted by study and further investigated, there were no clear trends seen across studies.

Rumen Response Variable Models

Rumen response variables included empty reticulorumen weight, rumen pH, papillae area, rumen contents, rumen wall thickness, butyrate concentration, acetate concentration, and propionate concentration (**Figure 2.1**, **Table 2.1**). The first relationship of interest was how the diet influenced each of the rumen response variables. Total MR intake was a dietary factor that explained much of the variation in model 1 (empty reticulorumen wt.) and model 5 (rumen wall thickness). Although we would have expected total starter intake to explain more of this variation in model 1 and 5 over MR intake, starter intake and MR intake are closely related (corr = 0.004), and therefore MR intake may be masking any potential starter intake effects. When the calf starts to become a true ruminant the rumen wall thickness and reticulorumen weight will both increase

(Xie et al., 2013). Another plausible explanation for the effect of total MR intake could be indicating that the calf needs a set amount of MR until it then becomes a ruminant. Starter fat percentage was the only factor explaining variation in model 4 (papilla area), indicating that as starter fat decreased, papillae area would increase. This is in agreement with Khan et al. (2008) who demonstrated that a diet higher in fat yields a lower papillae length, width, and concentration (number per cm^2) than a low-fat diet. We expected overall starter intake to explain more variation in regards to papillae area than starter fat, however the relationship between starter fat and papillae area could be indicating an underlying starter intake effect. If there is a strong negative association with starter fat and rumen development parameters like papillae, it is possible that starter intake also has a negative association but because it is not as strong of an association it becomes masked. The form of the calf starter, textured and pelleted, was significant in model 3 (rumen pH). Imani et al. (2017) and Bach et al. (2007) also saw a relationship between the form of the starter and ruminal pH. Daily MR intake explained some variation in models 6,7, and 8, for acetate, propionate, and butyrate respectively. The directionality of the coefficients in models 6 & 8 (n=57) suggests that as daily MR intake decreases, acetate and butyrate concentrations increase. This relationship is not surprising since an increase in butyrate and acetate is observed in diets that contain higher amounts of starter and lower amounts of MR (Quigley et al., 1991).

A second relationship of interest was how the environment influenced the rumen response variables. Model 3 (rumen pH) indicated a temperature controlled environment explained some variation for rumen pH. Previous research has confirmed that a constantly changing environment causes increased stress on the calf (Roland et al., 2016), which could potentially lead to a shift in the calves' dietary intake and therefore cause the rumen pH to fluctuate.

When comparing diet and environment factors, dietary factors explained more variation in rumen response variables. Each of the rumen response variables had at least one dietary factor in the model, while rumen pH was the only model that had an environmental factor explaining variation. Environmental parameters are hard to control for and in research, and as a result are underrepresented in the literature. It is known that environmental factors like humidity, ventilation, or outside temperature (Roland et al., 2016) can play a role in influencing overall calf performance and health, so it can be hypothesized that environmental factors also may play a role in rumen development. There are no studies to our knowledge that report how environmental factors play a role in rumen development, and therefore needs to be further researched. Results from relationship A indicate that dietary variables explain more variation than environmental variables for rumen response variables.

Models 1-5B were analyzed with the additional explanatory variables of each of the VFA concentrations. The models did not change with the addition of acetate, propionate, and butyrate concentration, with the exception of model three (rumen pH). Model 3B indicates that propionate concentration explains some variation in rumen pH. In this scenario, it also masks the environmental variable and indicates that daily starter intake and propionate concentration are the only things that collectively influence rumen pH.

Performance Response Variable Models

Performance response variables included ADG, F:G, and total and daily MR, starter and forage intakes (**Figure 2.1, Table 2.1**). The relationships of interest here were how the rumen factors and dietary and environmental factors both individually and additively influenced performance variables.

The relationship investigating dietary and environmental variables individually (**Figure 2.1** relationship B, **Table 2.3**) found that only dietary variables explained variation in performance variables in models from relationship B. Weaning status was a dietary factor that explained some variation in models 9 (ADG) and model 10 (F:G). The directionality of the coefficients suggests calves that were weaned had a lower ADG and were less feed efficient. This could be due to the growth slump that most dairy calves experience at the beginning of weaning. The growth slump is commonly known to decrease ADG to some extent regardless of weaning method (Sweeney et al., 2010). At the start of weaning calves may also be less feed efficient due to the shift in metabolic activity in the rumen going from a milk diet to a starter-based diet (Khan et al., 2007). The directionality of the coefficients in model 9 also indicate that increased forage, MR, and starter intake will lead to higher ADG, while increased forage intake will result in a less feed efficient animal in model 10. Increased feed intake in general is expected to be associated with increased ADG since the calf consumes more as it grows. There are many studies that indicate a higher forage diet at weaning can lead to a more feed efficient calf (Coverdale et al., 2004; Daneshvar et al., 2015; Castells et al., 2013), however in another meta-analysis, Imani et al. (2017) was in agreement with our findings. This could be explained by the ideal that consumption of rapidly fermentable feeds, like starter, are easier to digest than forages, making the calf more feed efficient on a starter-based diet. A meta-analysis analyzes data from multiple different feedings regimes, so it is possible that it highly depends on what quality and type of forage is being fed. The directionality of the coefficients in model 10 suggest that a diet high in fat will be less feed efficient, while a diet high in protein will be more feed efficient. Authors Hill et al. (2008) demonstrate the same findings. The percent of the diet that was composed of starter partially explained the variation in performance variables in models 13 (total forage intake), 15 (daily MR

intake), and 16 (daily forage intake). It is not surprising to see that in all of these relationships the directionality of the coefficients suggests that with less starter in the diet, more MR and forage is consumed. The percent of the diet that was composed of MR partially explained some variation in performance variables in models 13 (total forage intake), 14 (daily starter intake), and 16 (daily forage intake). These relationships are showing the inverse of the previously stated relationship suggesting that less MR in the diet leads to more starter and forage consumption. Similarly, models 11 and 13 suggest that MR intake exists in an inverse relationship with starter and forage intake. In other words, a higher daily MR intake is associated with a lower total starter intake and daily forage intake. Models 12 and 15 also show the same relationship with total and daily MR as the response variables and daily starter intake having a negative association. In model 14 (daily starter intake) a pelleted (versus texturized) starter explains much of the variation with daily starter intake. This finding is in agreement with Imani et al. (2017). There are studies that show a texturized starter improves starter intake (Franklin et al., 2003; Bach et al., 2007; Omidi-Mirzaei et al., 2018), as well as studies that saw no difference (Terre et al., 2015; Mirzaei et al., 2016), so it seems this is still an inconclusive relationship to define in the literature. Higher starter ADF explains much of the variation in models 11 and 14, indicating an increased total and daily starter intake. In model 15 (daily MR intake) a heavier final body weight explains some of the variation in high daily MR intakes. This finding is in agreement with Khan et al. (2011) who show increased BW in calves across multiple studies that fed a higher MR volume. In model 14 the type feed ingredients in the diet explains some of the variation in daily starter intake. The directionality of the coefficients for model 14 indicates calves on a diet with some solid feed, either forages or starter, will eat more starter on a daily basis then calves on a milk only diet. This relationship is likely picking up on post-weaning data when the calves are no longer on a milk diet.

The relationship investigating rumen variables individually (**Figure 2.1** relationship C, **Table 2.4**) found that only papillae area, rumen contents weight, and empty reticulorumen weight explain the variation in certain performance models. The directionality of the coefficients in models 17 (ADG) and 22 (daily starter intake) indicate a larger papillae area is associated with higher ADG and daily starter intakes. This is consistent with findings from Muya et al. (2015) who showed that a larger papillae density was prevalent in the treatment group that ate more starter and had higher ADG. The directionality of the coefficients in model 18 (F:G) suggests that increased ruminal contents weight results in a less feed efficient animal, while model 23 (daily MR intake) suggests that more ruminal contents are associated with a lower MR intake on a daily basis. As the calf grows and more closely resembles a ruminant, it becomes a little less feed efficient. This explanation could be due to the cycle of the cow regurgitating the food and therefore being less feed efficient than a calf that has not yet begun that process. Lastly, the directionality of the coefficients in model 20 (total MR intake) indicate a heavier empty reticulorumen with smaller amounts of total MR intake. These models seem to be picking up on the early life stages of the calf in its non-ruminant phase.

When looking at these two relationships in an additive manner (**Figure 2.1**, relationship D, **Table 2.5**) no differences were found, with the exception of models predicting F:G and daily MR intake. This indicates that the effect of the dietary variables subsumed the ruminal variables' effects in the model.

CONCLUSIONS

From the relationships observed, it can be concluded that dietary variables explain more variation in rumen development and calf performance than do the environmental and rumen variables. Specifically, the intake parameters seemed to explain much of the variation in models

as opposed to individual feed components (i.e. starter NDF). This could be explained by the idea that it is more imperative the calf receives a certain amount of feed to develop physically and ruminally. While we know composition and quality are contributing factors to the development of the calf, those factors are not as largely influential as the volume ingested. Another speculation could be that the individual components of starter and MR did not have enough variation across studies to pick up on this effect. One interesting finding was that the directionality of the coefficients suggests a pelleted starter increases starter intake. The database evenly represented both a pelleted and a texturized feed, however as previously stated there are studies that make a claim to both sides.

A common challenge in meta-analyses is the limited reporting of standard errors. We recognize this challenge and attempted to optimize both data availability and statistical correctness by fitting models weighted both by SEM and n. Future research can and should be done to include a larger number of papers in the meta-analysis, but the field of dairy scientists are in the process of publishing more papers that include rumen development data.

In summary, this meta-analysis investigated the effect of dietary, environmental, and rumen factors independently and additively. The models resulted in relationships that did not differ much from the two scenarios. From this we can conclude that producers should continue to focus on the dietary needs of the calf first, then evaluate some of the rumen and environmental parameters. The models portray that certain rumen and environment parameters still explain some variation, but it is important to establish standards for the dietary factors first since they explain more of the variation.

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Table 2.1. Descriptive statistics of the numerical variables from the peer-reviewed studies in the data set

Variable	N	Mean	Median	SD	Minimum	Maximum
Diet/Environment[1]						
MR Crude protein, %	140	23.9	22.8	4.48	20.0	41.8
MR Fat, %	140	20.8	20.0	4.35	15.0	30.4
Percent milk, % of total diet	132	57.1	55.0	25.2	0.0	100
Percent starter, % of total diet	138	38.8	41.5	25.6	0.0	100
Final Age, d	143	59.4	56.0	23.1	7.0	150
Initial BW, kg	143	44.7	43.9	7.74	33.4	72.9
Final BW, kg	143	80.3	75.0	29.8	43.7	230
Starter DM, %	787	543	531	303	0.0	1250
Starter CP, % DM	799	132	116	103	0.0	356
Starter NDF, % DM	770	101	90.7	79.9	0.0	317
Starter ADF, % DM	771	49.4	40.1	39.3	0.0	158
Starter Ash, % DM	786	123	124	74.9	0.0	268
Starter Fat, % DM	782	23.5	15.6	29.7	0.0	115
Starter Starch, % DM	769	170	181	103	0.0	383
Forage DM, % DM	140	45.3	0.0	65.0	0.0	276
Forage CP, % DM	140	6.97	0.0	10.3	0.0	42.8
Forage NDF, % DM	140	28.2	0.0	41.0	0.0	164
Forage ADF, % DM	140	18.7	0.0	26.7	0.0	108
Forage Ash, % DM	140	4.36	0.0	6.23	0.0	26.3
Forage Fat, % DM	140	1.44	0.0	2.15	0.0	8.28
Forage Starch, % DM	140	1.77	0.0	4.99	0.0	31.8
Performance[2]						
ADG, kg/d	143	0.592	0.580	0.219	0.240	1.29
Total Starter Intake, kg	132	41.9	24.2	61.2	0.0	501
Daily starter intake, kg/d	98	0.648	0.530	0.570	0.0	2.79
Total MR intake, kg	143	39.1	34.6	36.9	0.0	388
Daily MR intake, kg/d	87	0.919	0.680	0.906	0.0	3.65
Total forage intake, kg	121	3.48	0.0	11.9	0.0	67.8
Daily forage intake, kg/d	121	0.043	0.0	0.135	0.0	0.75
F:G[3]	138	2.32	2.13	0.844	0.90	5.46
Rumen[4]						
Reticulorumen wt., kg	75	1.18	1.05	0.832	0.38	5.55
Rumen content wt., kg	35	6.59	6.70	2.92	1.29	13.8
Rumen pH	97	5.65	5.59	0.557	4.90	7.20
Papillae area, mm^2	75	1.98	1.02	3.03	0.280	20.6
Rumen wall thickness, mm	50	5.30	2.16	5.89	0.111	19.5
Butyrate concentration, mM	105	11.0	11.2	6.35	0.700	27.3
Propionate concentration, mM	105	33.5	33.1	16.8	7.10	75.2
Acetate concentration, mM	105	56.3	57.6	15.9	19.3	97.7

[1]All numerical variables in the database from the diet and environment category.
[2]All numerical variables in the database from the performance category.
[3] Feed to gain ratio was calculated by dividing the total feed intake by the weight gain of the animal.
[4]All numerical variables in the database from the rumen category.

Table 2.2. Parameter estimates and model fit statistics for models predicting rumen response variables (**Figure 2.2**; Relationship A)

	Reticulorumen[1]	Rumen contents[2]	Rumen pH	Papillae area	Rumen wall[3]	Acetate[4]	Propionate[5]	Butyrate[6]
Model no.	1	2	3	4	5	6	7	8
Intercept	2.68	11.2	5.60	2.64	6.01	97.4	53.3	26.5
Variables[7]								
For Fat			0.0749					
Form Pell			-0.265					
Form Text			-0.613					
Temp Yes			-0.657					
Final BW			0.00547					
Daily St	0.920							
Daily MR						-59.6	-34.7	-23.0
St Fat				-0.0248				
Daily For		-132			3.00		-84.2	
Pct MR		-7.97						
Tot MRI	0.00875				-0.00225			
MSF code	-0.286							
MR fat	-0.114							
Tot St.							0.144	
Fit Statistics								
n[8]	46	22	85	61	33	57	51	57
CCC[9]	0.918	0.942	0.954	0.905	0.998	0.701	0.883	0.923
uCCC[10]	0.742	0.801	0.470	0.0915	0.00742	0.249	0.571	0.140
$\hat{\sigma}_s$[11]	0.383	1.27	0.498	3.07	5.89	13.1	11.6	6.57
$\hat{\sigma}_e$[12]	0.473	1.17	0.466	7.15	0.451	19.7	10.2	6.96
CVCCC[13]	--	--	--	0.343 ± 0.340	1.56 ± 3.30	0.626 ± 0.266	0.597 ± 0.173	0.512 ± 0.247

[1] Empty reticulorumen weight.
[2] Rumen contents weight.
[3] Rumen wall thickness (submucosa + muscularis).
[4] Ruminal acetate concentration (in m*M*).
[5] Ruminal propionate concentration (in m*M*).
[6] Ruminal butyrate concentration (in m*M*).
[7] Forage fat (For Fat), pelleted form of starter (Form Pell), texturized form of starter (Form Text), temperature controlled environment (Temp Yes), final body weight (Final BW), daily starter intake (Daily St), daily milk replacer intake (Daily MR), perentage of fat in the starter fed on study (St Fat), daily forage intake (Daily For), percent of the total diet that composed of milk replacer (Pct MR), total milk replacer intake (Tot MRI), diet that consisted of milk replacer, starter, and forage (MSF code), fat content of the milk replacer (MR fat), total starter intake (Tot St.).
[8] n = number of observations.
[9] Concordance correlation coefficients.
[10] Unadjusted concordance correlation coefficients.
[11] Square root of the estimated study variance.
[12] Square root of the estimated residual variance.
[13] Concodrance correlation coefficient for coefficient of variation ± standard deviation.

Table 2.3. Parameter estimates and model fit statistics for models predicting performance response variables (**Figure 2.2**; Relationship B)

	ADG[1]	F:G[2]	Tot St.[3]	Tot. MRI[4]	Tot. For[5]	Daily St.[6]	Daily MR[7]	Daily For[8]
Model no.	9	10	11	12	13	14	15	16
Intercept	0.420	1.77	-52.3	33.0	186	1.00	0.457	1.84
Variables[9]								
For Fat		-0.121						
St ADF			0.0738			0.00142		
MSF code						0.147		
Wean Yes	-0.0914	0.557						
Final BW							0.00744	
Daily St.				-17.4			-0.191	
Daily MR			20.6		-2.81			
MS code						0.0646		
Daily for	0.330	-0.645	32.7					
Pct MR					-184	-1.23		-1.88
Tot MRI	0.00356							
Pct St			89.5		-185		-0.659	-1.83
Tot St.	0.00254							
St. ash			0.0598					
Init. BW			0.369					
Form Pell						0.0189		
For CP		0.0272						
Fit Statistics								
n[10]	108	83	63	21	25	85	39	26
CCC[11]	0.976	0.960	0.989	0.819	0.980	0.985	0.959	0.998
uCCC[12]	0.724	0.399	0.454	0.815	0.980	0.867	0.872	0.998
$\hat{\sigma}_s$[13]	0.164	0.520	27.8	3.22	0.000	0.137	0.112	0.007
$\hat{\sigma}_e$[14]	0.225	0.943	1.93	20.18	7.02	0.266	1.17	0.527
CVCCC[15]	--	--	0.702 ± 0.152	0.473 ± 0.108	0.679 ± 0.353	--	0.653 ± 0.327	0.492 ± 0.447

[1] Average daily gain (ADG); [2] Feed to gain ratio (F:G); [3] Total starter intake (Tot St.); [4] Total milk replacer intake (Tot. MRI); [5] Total forage intake (Tot. For); [6] Daily starter intake (Daily St.);
[7] Daily milk replacer intake (Daily MR); [8] Daily forage intake (Daily For).
[9] Forage fat (For Fat), acid detergent fiber of starter (St ADF), diet that consisted of milk replacer, starter, and forage (MSF code), calves that were weaned (Wean Yes), final body weight (Final BW), diet that consisted of milk replacer and starter (MS code), percent of the total diet that was composed of milk replacer (Pct MR), total milk replacer intake (Tot MRI), percent of the total diet that was composed of starter (Pct St), ash content of the starter (St. ash), initial body weight (Init. BW), pelleted form of starter (Form Pell), forage crude protein (For CP).
[10] n = number of observations.
[11] Concordance correlation coefficients.
[12] Unadjusted concordance correlation coefficients.
[13] Square root of the estimated study variance.
[14] Square root of the estimated residual variance.
[15] Concodrance correlation coefficient for coefficient of variation ± standard deviation.

Table 2.4. Parameter estimates and model fit statistics for models predicting performance response variables (**Figure 2.2**; Relationship C)

	ADG[1]	F:G[2]	Tot St.[3]	Tot. MRI[4]	Tot. For[5]	Daily St.[6]	Daily MR[7]	Daily For[8]
Model no.	17	18	19	20	21	22	23	24
Intercept	0.525	1.42		34.8		0.443	0.872	
Variables[9]								
Pap area	0.0302					0.118		
Rumen Cont		0.0902					-0.0510	
Empty RR				-14.7				
Fit Statistics								
n[10]	75	17		23		51	17	
CCC[11]	0.932	0.906		0.268		0.987	0.742	
uCCC[12]	0.330	0.387		0.268		0.020	0.134	
$\hat{\sigma}_s$[13]	0.189	0.277		0.00		0.676	0.167	
$\hat{\sigma}_e$[14]	0.325	0.515		39.7		0.428	1.64	
CVCCC[15]	0.587 ± 0.299	0.342 ± 0.224		0.541 ± 0.262		0.420 ± 0.180	0.660 ± 0.203	

*Shaded boxes represent models with no significant rumen variables.

[1] Average daily gain (ADG); [2] Feed to gain ratio (F:G); [3] Total starter intake (Tot St.); [4] Total milk replacer intake (Tot. MRI); [5] Total forage intake (Tot. For); [6] Daily starter intake (Daily St.); [7] Daily milk replacer intake (Daily MR); [8] Daily forage intake (Daily For).

[9] Papillae area: length times width (Pap area), rumen contents (Rumen Cont), empty reticulorumen (empty RR).

[10] n = number of observations.

[11] Concordance correlation coefficients.

[12] Unadjusted concordance correlation coefficients.

[13] Square root of the estimated study variance.

[14] Square root of the estimated residual variance.

[15] Concodrance correlation coefficient for coefficient of variation ± standard deviation.

Table 2.5. Parameter estimates and model fit statistics for models predicting performance response variables (**Figure 2.2**; Relationship D)

	ADG[1]	F:G[2]	Tot St.[3]	Tot. MRI[4]	Tot. For[5]	Daily St.[6]	Daily MR[7]	Daily For[8]
Model no.	25	26	27	28	29	30	31	32
Intercept	0.420	2.21	-52.3	33.0	186	1.00	0.622	1.84
Variables[9]								
St ADF			0.0738			0.00142		
St Ash			0.0598					
Wean Yes	-0.0914							
Init. BW			0.369					
Daily St		-1.67		-17.4			0.187	
Daily MR		-0.926	20.6		-2.81			
Forage Fat								
Daily For	0.330	6.28	32.7					
Rumen cont		0.0375						
Percent Starter		1.01	89.5		-185		-1.14	-1.83
Total St	0.00254	0.0115						
Pct MR					-184	-1.23		-1.88
Final BW							0.00284	
MS code						0.0646		
MSF code						0.147		
Prop Conc							0.0056	
Form Pell						0.0189		
Fit Statistics								
n[10]	108	17	51	21	25	83	20	26
CCC[11]	0.976	0.992	0.993	0.819	0.980	0.998	0.918	0.998
uCCC[12]	0.724	0.846	0.992	0.815	0.980	0.992	0.918	0.998
$\hat{\sigma}_s$[13]	0.164	0.153	2.42	3.22	0.00	0.0549	0.00	0.00787
$\hat{\sigma}_e$[14]	0.225	0.111	2.28	20.1	7.02	0.177	0.963	0.527
CVCCC[15]	--	1.72 ± 2.86	0.604 ± 0.291	0.304 ± 0.311	0.587 ± 0.345	--	0.831 ± 0.157	0.907 ± 0.095

[1] Average daily gain (ADG); [2] Feed to gain ratio (F:G); [3] Total starter intake (Tot St.); [4] Total milk replacer intake (Tot. MRI); [5] Total forage intake (Tot. For); [6] Daily starter intake (Daily St.); [7] Daily milk replacer intake (Daily MR); [8] Daily forage intake (Daily For).

[9] Acid detergent fiber of starter (St ADF), ash content of the starter (St ash), calves that were weaned (Wean Yes), initial body weight (Init. BW), daily starter intake (Daily St), daily milk replacer intake (Daily MR), Forage fat (For Fat), daily forage intake (Daily For), ruminal contents (Rumen cont), percent of the total diet that was composed of starter (Pct St), total starter intake (Total St), percent of the total diet that was composed of milk replacer (Pct MR), final body weight (Final BW), diet that consisted of milk replacer and starter (MS code), diet that consisted of milk replacer, starter, and forage (MSF code), ruminal propionate concentration (Prop Conc), pelleted form of starter (Form Pell).

[10] n = number of observations.

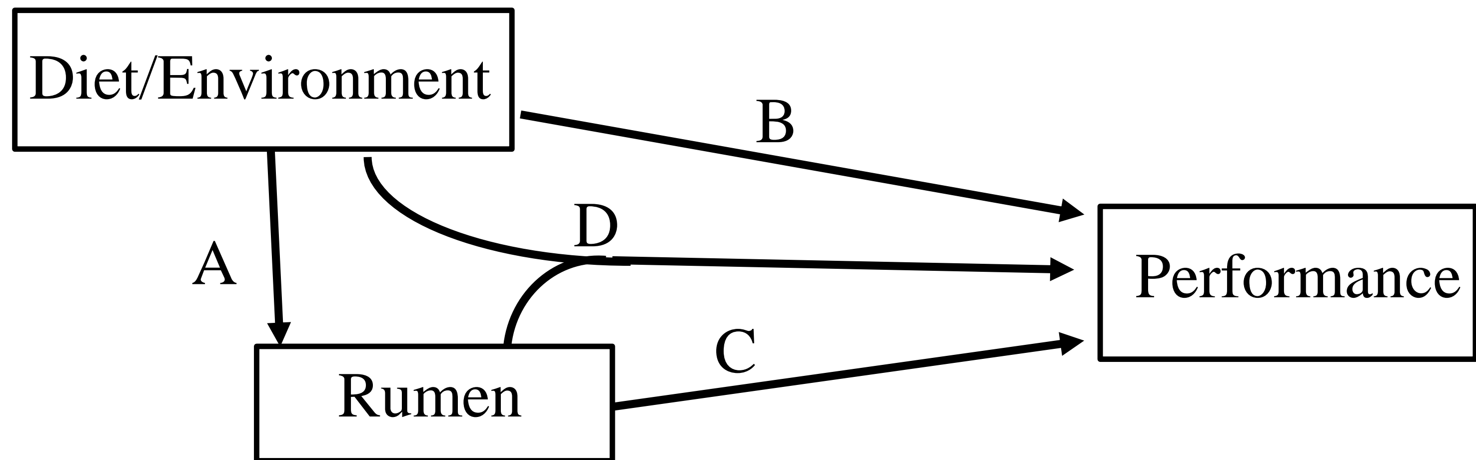
[11] Concordance correlation coefficients.

[12] Unadjusted concordance correlation coefficients.

[13] Square root of the estimated study variance.

[14] Square root of the estimated residual variance.

[15] Concodrance correlation coefficient for coefficient of variation ± standard deviation.



Diet/Environment

- Total milk replacer dry matter intake
- Total starter dry matter intake
- Total forage intake
- Percent of the diet composed of milk
- Percent of the diet composed of starter
- Code
- Pelleted or texturized starter
- Legume or grass forage
- Milk replacer fat content
- Milk replacer protein content
- Continent where study was conducted
- Was there bedding? Yes or no
- Was it temperature controlled? Y o N
- Was it ventilated? Y or N
- Did calves drink from a bucket or teat?
- Were calves weaned? Y o N
- Holstein or mixed breed?
- Bulls or bulls/heifer combination
- Final age at slaughter

- Final body weight at slaughter
- Initial body weight when enrolled in study
- Starter dry matter
- Starter crude protein
- Starter ADF
- Starter NDF
- Starter fat
- Starter ash
- Starter starch
- Forage dry matter
- Forage crude protein
- Forage fat
- Forage NDF
- Forage ADF
- Forage ash
- Forage starch
- Daily milk replacer intake
- Daily starter intake
- Daily forage intake

Performance

- Average daily gain
- Feed to gain ratio
- Total starter dry matter intake
- Total milk replacer dry matter intake
- Total forage intake
- Daily forage intake
- Daily starter intake
- Daily milk replacer intake

Rumen

- Empty reticulorumen weight
- Rumen contents weight
- Rumen pH
- Papillae area (length x width)
- Rumen wall thickness
- Ruminal butyrate concentration
- Ruminal propionate concentration
- Ruminal acetate concentration

Figure 2.1. Schematic diagram representing the three main categories of investigated variables. Parameters reported within those categories are listed in the boxes below. Arrows represent all the relationships that were investigated. An arrow pointing to a box indicates that category was a response variable, while the box it is stemming from indicates the explanatory variables.

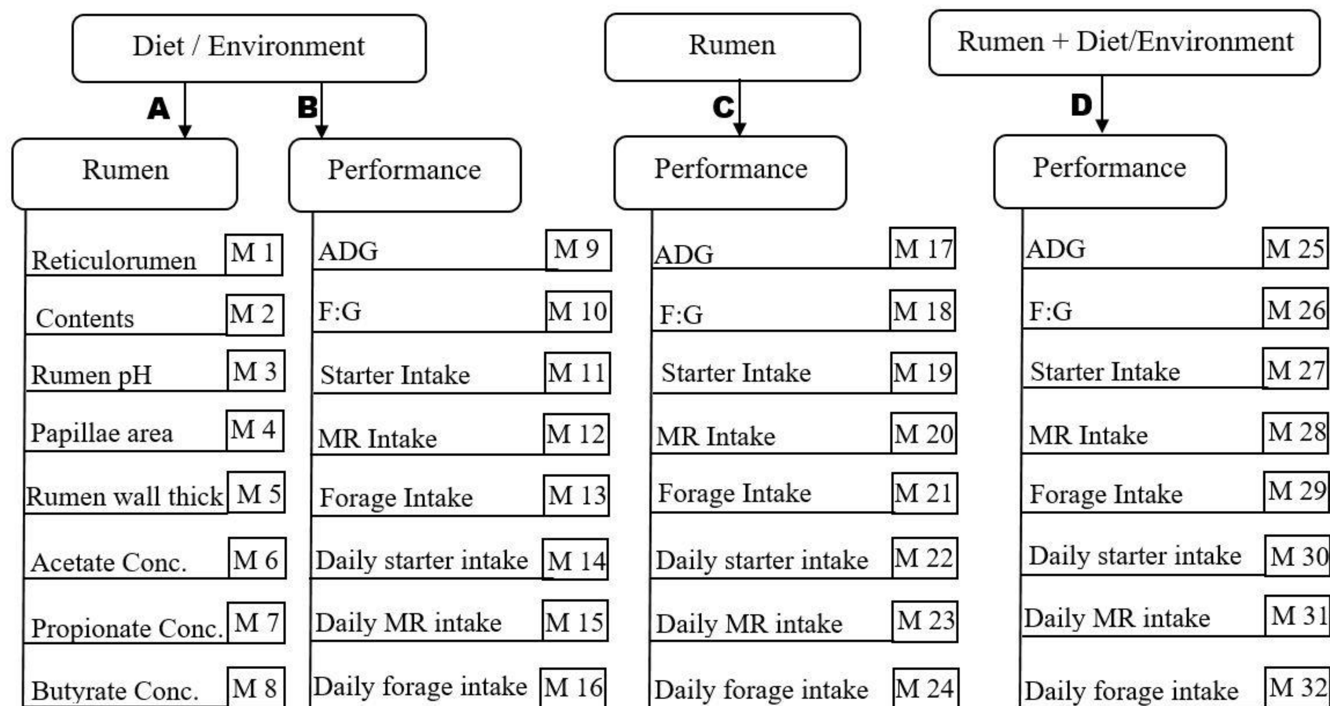


Figure 2.2. Letters A-D represent relationships. All variables within the diet/environment category were only investigated as explanatory variables (relationships A, B, & D) whereas variables within the performance category were only investigated as response variables (relationships B, C, & D). Variables within the rumen category were investigated as both explanatory (relationship C, models 17-24) and response variables (relationship A, models 1-8). Variables with a corresponding model number are response variables.

CHAPTER 3: THE SYNERGISTIC RELATIONSHIP OF LIPOPOLYSACCHARIDE AND BUTYRATE STIMULATING RUMEN DEVELOPMENT

ABSTRACT

The objective was to investigate the effects of orally dosed lipopolysaccharide (**LPS**) and sodium butyrate on rumen cell proliferation in Holstein dairy calves. The hypothesis was that LPS and butyrate synergize to promote rumen epithelial cell proliferation via the binding of LPS to toll-like receptor 4 and mobilization of volatile fatty acids (**VFA**) through the monocarboxylic transporter (**MCT**) pathways. Twenty-four single-sourced purchased bull calves arrived to the Virginia Tech (**VT**) dairy in one of two arrival groups, spaced 2 wk apart, and enrolled on the study for 56 d. Within each group, calves were assigned to one of four treatments: control (**CON**; n=5), butyrate (**BUTY**; n=5), LPS only (**LPS-O**) (n=6), or LPS plus butyrate (**LPSB**; n=6). All treatments were administered orally twice daily at least 30 minutes following the morning meal and consisted of : 0.9% saline (CON); 11m*M* sodium butyrate (BUTY); LPS ranging from 2.5 to 40 µg/kg metabolic body weight (**MBW**) ($BW^{0.75}$, LPS), or both butyrate and LPS (LPSB). Treatment dosage volumes increased as the study progressed, ranging from 10 to 40 mL per dose. Calves were fed milk replacer (22% CP, 20% fat, as-fed) and starter (20% CP, 3% fat, as-fed) twice daily (0600 and 1800) based MBW. Feed intake, fecal and respiratory scores, and rectal temperature were recorded daily. Calf BW, hip height, jugular blood samples, and rumen content samples (oroesophageal route) were collected weekly, 2 h after morning feeding. Calves were weaned at 6 wk of age and euthanized at 8 wk of age, whereupon ruminal weights and ruminal samples for papillae area and epithelial thickness were collected. Blood and rumen samples were analyzed for blood metabolites (BHBA, glucose, LPS-binding protein) and VFA concentrations,

respectively. Data were analyzed as a 2x2 factorial with the repeated effect of week. Three non-orthogonal contrasts (CON versus the average of all other treatments; LPS-O versus LPSB, and LPSB versus BUTY) were investigated. Feed intake, health measures, and blood metabolites did not differ by treatment. Calf BW increased by week ($P < 0.0001$). Irrespective of week, LPSB calves weighed more than BUTY calves (LPSB, 66.9 ± 0.68 kg; BUTY, 64.0 ± 0.75 kg; $P = 0.02$; Table 3.5). Irrespective of week, withers heights of calves in treatments that contained LPS had an overall average of 87.7 ± 0.21 cm and overall withers height for calves on treatments that did not contain LPS were 86.8 ± 0.22 cm ($P = 0.006$; Table 3.6). Rumen pH and rumen VFA concentrations did not differ by treatment but did decrease and increase, respectively, with week in conjunction with increased starter intake ($P < 0.0001$; Table 3.8). Total empty stomach (LPSB, 2.87 ± 0.076 kg; BUTY, 2.48 ± 0.076 kg; $P = 0.01$; Table 3.6) and reticulorumen weights (LPSB, 1.76 ± 0.065 kg; 1.44 ± 0.065 kg; $P = 0.01$; Table 3.6) were higher in LPSB calves when compared to BUTY calves. No treatment differences were detected in transporter intensity, BrdU cell counts, or LPS binding protein (Table 3.9). Proteins EGFR, MCT1 and MCT4 were affected by location ($P < 0.0001$) and LPS binding protein was affected by week ($P < 0.0001$). The lack of effects observed in this study could be indicative of many possibilities including: LPS and/or sodium butyrate did not breech gastrointestinal barriers, calves became tolerant to orally dosed LPS, or the sodium butyrate was so quickly metabolized and therefore not detected in blood and rumen samples 3h after feeding. Future studies are necessary to build on the findings in this paper for a more complete understanding of the role LPS plays in the rumen of young dairy calves.

Key words: dairy calf, rumen development, lipopolysaccharide

INTRODUCTION

Lipopolysaccharide (**LPS**) is a chemical of interest because of its role within the calf when subacute ruminal acidosis (**SARA**) is induced. This disease, SARA, occurs when rumen pH drops due to an increase of rapidly fermentable feedstuff in the rumen (Kleen et al., 2003). The threshold for rumen pH is 5.5 and anything below that can be considered SARA in mature dairy cattle (Kleen et al., 2003; Laarman and Oba, 2011). It is fairly common for calves that are going through weaning to have rumen pH below 5.5 (Suarez et al., 2006; Kim et al., 2016). Under such conditions, LPS can translocate to the bloodstream, causing life-threatening scenarios for lactating dairy cows (Gozho et al., 2005). Nagaraja et al. (1978) demonstrated that steers fed a higher grain-based diet have elevated levels of LPS in the rumen. Gozho et al. (2005) went one step further to confirm that LPS levels in the bloodstream are indeed higher when SARA is induced, but additionally showed an increased inflammatory response by looking at acute phase protein markers in the bloodstream. Considering LPS is linked with grain consumption (Gozho et al., 2005), and grain consumption is linked with rumen epithelial development in calves (Stobo et al., 1966) there are gaps in our knowledge regarding how LPS may be affecting the rumen on a mechanistic level, which is what we sought out to investigate.

Toll-like receptor 4 (**TLR4**) is the receptor that mediates LPS signaling in many cell types across species (Nagai et al., 2002). The receptor is a class one transmembrane receptor that is expressed on the cell surface (Chaturvedi and Pierce, 2009). Harris et al. (2006) demonstrated that TLR4 is present in the GI tract of humans. Additionally, monocarboxylate transporters (**MCT**) are located along the GI tract of ruminants, which are responsible for transporting volatile fatty acids (**VFA**) from the lumen to the blood (Graham et al., 2006). Because MCT and TLR4 function on the same epidermal sheet, it is for this reason we proposed there exists a synergistic relationship

between VFA and LPS (**Figure 3.1**). When the calf increases grain intake around weaning, butyric, propionic, and acetic acid concentrations increase. Because MCT are responsible for the transport of VFA (Kirat et al., 2007), we expect the intensity of MCT and TLR4 to be more abundant when larger amounts of VFA and LPS are present in the rumen lumen, respectively. Further, we expect these differences to coincide with measures of increased rumen growth. Stefanska et al. (2018) established that TLR4 is more abundant in SARA induced versus healthy dairy cows. Sodium butyrate is important for rumen cell proliferation (Sakata and Tamate, 1978). It is with this knowledge that we formulated our hypothesis that twice daily oral doses of either butyrate, LPS, or both will affect abundance of MCT, TLR4, and rumen cell proliferation with overall effects on somatic and rumen growth in young dairy calves.

To our knowledge there are no studies to date that present research on the TLR4 pathway with regards to the synergistic relationship between LPS and VFA in dairy cattle. Previous studies have performed LPS challenges both intravenously and orally in calves and lactating cattle respectively, however, those studies investigated the immune response and not the synergistic mechanism we are proposing here (Anderson et al., 1996; Eicher et al., 2006; Ametaj et al., 2012). We hypothesize: 1) that LPS will further enhance rumen cell proliferation when present in larger quantities and 2) rumen cell proliferation will be even further enhanced when LPS and butyrate are both present in higher concentrations. With a better understanding of the mechanisms behind LPS transport in the rumen, further conclusions can be made to improve performance and health of the calf.

MATERIALS AND METHODS

Animals, Housing, and Treatments

Animals. All experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (protocol #17-040). Twenty-four Holstein bull calves sourced from a single farm located 232 km from Virginia Tech were used. Calves were transported via trailer and arrived in one of two groups of 12 calves each. The first group (5d ± 1d of age; mean ± standard deviation) was on site from May 29, 2018 to July 25, 2018. The second group (6d ± 2d of age; mean ± standard deviation) was on site from June 12, 2018 to August 8, 2018. We verified that farm staff at the farm of origin fed colostrum to all calves by quantifying serum immunoglobulin G (Radial Immunodiffusion Plates, Triple J Farms, Bellingham, WA) of each calf upon arrival to Virginia Tech. Serum immunoglobulin G averaged 2,086 mg/dL ± 354 mg/dL; (mean ± standard deviation); no calf had failure of passive transfer of immunity (range: 1,378 to 2,661 mg/dL IgG, with failure of passive transfer cutoff of 800 mg/dL). Twice-daily meals after colostrum feeding at the farm of origin consisted of milk replacer (20% CP (as-fed), 20% fat (as-fed); 454 g powder (as-fed)/calf per day). No starter was offered at the farm of origin. Each calf received the following vaccinations and injections at the farm of origin:1mL multi-min subcutaneously and 2mL enforce 3 nasally.

Upon arrival to Virginia Tech, all calves were screened for bovine viral diarrhea virus by ear notch test (Snap BVDV Antigen Test, IDEXX, Westbrook, MN); all calves tested negative. Further, body weight (**BW**), withers height (**WH**), and hip height (**HH**) were recorded for each calf. All calves received 1.94 L of oral electrolytes (50g powder; Diaque, Boehringer Ingelheim Vetmedica, INC., Duluth, GA) within 1 h of arrival to Virginia Tech.

Housing. Calves were individually housed in and fed from hutches for the duration of the experiment. Hutches were naturally ventilated and bedded with fresh sawdust on a weekly basis. Nose-to-nose contact was prevented by placing hutches approximately 1m apart. Calves were bottle-fed milk replacer until they were bucket trained; thereafter milk replacer was fed from open 9.5 L buckets. Calves were fed twice daily (600 and 1800h). Prior to weaning, calves were fed at 32% MBW ($BW^{0.75}$), or 12% BW in two equally sized meals. Amount of MR was adjusted weekly. The MR was mixed at 13% solids and fed at 20 g powder DM per kg of MBW and contained 22.8% CP, 20.7% fat DM basis (Performance, Purina Animal Nutrition LLC, Shoreview, MN). Calves had ad libitum access to drinking water and were fed a common medicated calf starter (20% CP, 2% fat, 50 g/ton monensin sodium, 9.1 g/ton diflubenzuron (as-fed); Ampli-Calf, Purina Animal Nutrition LLC, Shoreview, MN) in increasing amounts each week based on MBW. After weaning calves were offered a total of 3% BW of the starter. The MR, water, and starter intakes were recorded daily for each calf by weighing back any refusals.

Calves were weaned starting on d 42 of the experiment (6 wk) with MR reduced to once daily feeding (50% volume reduction) at 1800 h for 5 d. Fresh MR and starter samples were collected weekly for analysis. Weekly samples were pooled into one composite sample for analysis; pooling was deemed appropriate because all MR and starter were from the same manufactured lots. Samples of starter refusals were also collected on a daily basis and pooled into one composite sample per week to ensure the feed composition did not vary between fresh and refused starter samples. All samples were stored at -20°C and underwent analysis at Cumberland Valley Analytical Services (Waynesboro, PA) using the relative feed value package with additional starch, ether extract, and ash options (**Table 3.1**). Dry matter percentages of refused

starter were determined by heating the composited sample to 100°C for 24 h. This information was used to correct starter intake data.

Treatments. Within 24 h of arrival, calves were randomly allocated to one of four oral treatments: control (**CON**), lipopolysaccharide only (**LPS-O**), butyrate (**BUTY**), or LPS and butyrate (**LPSB**). The CON consisted of 0.9% sodium chloride (Fisher Chemical, Fair Lawn, NJ) dissolved in distilled water. The LPS consisted of increasing weekly concentrations of LPS from *Escherichia coli* O55:B5 (catalog no. L2880; Sigma Aldrich Saint Louis, MO) dissolved in distilled water, as described further below. The BUTY consisted of 11 m*M* of sodium butyrate (Alfa Aesar, Ward Hill, MA) dissolved in distilled water. The LPSB consisted of a combination of the LPS and BUTY solutions based on metabolic BW of the individual calf, as described below. LPS concentrations were selected based on results from a pilot trial in our laboratory (Daniels et al., unpublished) and a study by Iqbal and others (2013) where oral dosages of LPS were administered to lactating dairy cattle. All solutions were mixed and stored (4°C) in non-pyrogenic glassware. Each calf had its own stock vial of its assigned treatment, which was mixed fresh at least once per week. In the first 2 wk of the experiment, calves assigned to a treatment containing LPS received 2.5 μg LPS/kg $BW^{0.75}$. Thereafter, LPS dose doubled every 2 wk so that calves assigned to a treatment containing LPS received a dosage of 20 μg/kg $BW^{0.75}$ in week 7 of the experiment. In the final week of the experiment (wk 8), LPS dosage was doubled again resulting in a dosage of 40 μg LPS/kg $BW^{0.75}$.

All treatments were orally administered twice daily; eqivolume dosages were used in the following manner. In weeks 1 and 2, dosing volume for all treatments was 10 mL. In wk 3 and 4, dosing volume was 20 mL. In weeks 5 and 6, dosing volume was 30 mL. In weeks 7 and 8, dosing volume was 40 mL.

In all cases, treatments were administered at least 30 min after feeding (but before 1h after feeding) through 50 mL dosing syringes (Agri-Pro Dosing Syringe; Valley Vet Supply Marysville, KS) equipped with 0.7 cm tips. For treatment administration, a member of the research team donned a pair of new nitrile gloves, entered the calf's hutch, manually restrained the calf, placed the dosing syringe in the calf's mouth, and manually emptied the complete contents of the dosing syringe. Average time to perform this entire procedure was 1 min per calf. Each calf was observed momentarily afterward to ensure that it swallowed its treatment. The size and style of the dosing syringes allowed us to administer treatments at the juncture between the oral cavity and esophagus. We chose to administer treatments at least 30 min after feeding in hopes that calves' reticular grooves would be open (Wise et al., 1984) due to them no longer anticipating a liquid meal. Admittedly, we did not verify this; nonetheless, we assume that nearly all of each dose went into the rumen rather than the abomasum.

Final number of calves for each treatment were: CON, n=5; LPS-O, n=6; BUTY, n=5; LPSB, n=6. During the first 3 wk of the experiment one CON calf and one BUTY calf were euthanized due to poor prognosis. The BUTY calf had a suspected umbilical mycoplasma infection in its umbilicus that spread to the calf's hind joints. The CON calf presented with severe diarrhea and signs of respiratory infection; it was euthanized upon suspicion of peritonitis. These deaths are not thought to be treatment related. Data from these calves are not reported.

Experimental Measures and Procedures

Rectal body temperatures, respiratory scores, and fecal scores were obtained twice daily for the first 2 wk of the experiment, then once daily for the remainder of the study. Respiratory scores were based on a scale of 1 to 3, where 1 was considered normal breathing and 3 was

considered heavy breathing with or without coughing. The fecal scoring was based on a scale from 0 to 3, where 0 was a solid feces and 3 was watery feces.

Rectal body temperatures were additionally obtained once weekly at measurement time, which was 2 to 3 h after the morning feeding. Weekly measurement of BW, WH, and HH were recorded during this period as well. Likewise, rumen content samples were collected from each calf via the oro-esophageal route (Yohe, 2018); these samples were used to measure pH and small aliquots were frozen for later VFA and LPS analyses. Lastly, jugular blood (~10 mL) was collected into glass vacutainer tubes without anticoagulant, allowed to sit at room temperature for 30 min, centrifuged at 2,000 x *g* for 15 min, processed to serum (BioSource, 2007), and then stored at -80 °C for eventual analysis of BHBA, glucose, and lipopolysaccharide binding protein (**LBP**).

Slaughter Procedure

Five hours before slaughter, calves were injected with 5-bromo-2'-deoxyuridine (**BrdU**) (20 mg BrdU powder/mL of 0.9% NaCl saline; BrdU powder: cat no. B5002, Sigma-Aldrich, St. Louis, MO) for detection of proliferating cells in the rumen epithelium. The dosage of BrdU for each calf was 5 mg BrdU powder/kg of BW (Geiger et al., 2017). The BrdU was injected intravenously into the jugular vein using either a 20 or 50 mL syringe and an 18-gauge needle; to verify placement, ~1 mL of blood was first drawn back into the syringe and then the entire volume was injected slowly into the calf. For slaughter, which occurred when calves were 56 +/-1d on the experiment, calves were transported approximately 14 km from the Virginia Tech Dairy to a necropsy room in the Litton-Reaves building on the Virginia Tech campus. Calves were slaughtered by captive-bolt stunning followed by immediate exsanguination. Feed and water were not withheld prior to slaughter.

Organ Weights and Contents

During harvest, the full forestomach was dissected from the intestines, weighed full, and then dissected at the reticulo-omasal orifice and weighed again. The rumen was then opened, its contents emptied into a container, a subsample of contents was then strained through 2 layers of cheesecloth. Strained samples were stored at -20°C for later VFA and microbiome analysis. The empty rumen was then flushed with cool tap water and re-weighed. The small and large intestine were separately removed and tied off on both ends, and weighed full with contents.

Tissue Collection and Processing

Tissue samples were then collected from the reticulorumen sac for morphological analyses from cranial ventral location using a punch biopsy tool with an internal diameter of 2.54 cm. Samples were stored in phosphate-buffered saline (pH 7.4) and measured for surface muscle: whole sample ratio. For this analysis, the whole tissue section was weighed, then the epithelium was separated from the rumen wall using forceps and weighed. Representative cranial ventral samples were cut and stapled to tongue depressors and placed in 10% neutral buffered formalin. After 24 h of fixation in formalin, new formalin was replaced for another 24 h period. After 48 h in formalin, tissue samples were transferred to 70% ethanol for indefinite storage prior to further analyses. Papillae were then plucked, 10 from each calf, from fixed tissue and attached to 2mm graphing paper and photographed. The area of each papilla was measured using Image-Pro Plus version 7.0 (Media Cybernetics, Inc.; Rockville, MD). Averaged values of the 10 papilla were reported. Tissue samples were also taken for RNA extraction using the same punch biopsy tool. The papillae layer was removed from each section of tissue using two pairs of forceps and sliced into small sections and snap frozen in liquid nitrogen. The small tissue pieces were stored in -80°C for indefinite storage until subsequent RNA extraction.

Fluorescent Immunohistological Analysis

Rumen tissue samples from the cranial ventral section of each rumen were used for fluorescent immunohistology. Samples were first removed from storage in 70% ethanol and then cut into 0.5-cm wide sections. Sections were placed into a labeled histology cassette and routinely processed for embedding in paraffin. Once embedded in paraffin, 5-µm thick sections were mounted onto positively charged microscope slides. Microscope slides were made 1 slide per animal. Tissues were deparaffinized using xylene and hydrated (100% ethanol, then 95% ethanol, then 70% ethanol, then distilled water). After tissues were hydrated they were boiled in 1X citrate buffer at 32°C for 35 minutes, allowed to cool for 30 minutes and then rinsed in 1X phosphate buffered saline (**PBS**). CAS Block (Life Technologies Corporation; Grand Island, NY) was then added to all sections (positive and negative) on the slides and incubated in the dark for 1 h. Immediately after incubation, the primary antibody was added onto positive sections. Primary antibody solutions were prepared by diluting antibody stocks in CAS block. The dilution for TLR4, EGFR, and MCT4 was 1:200 (catalog no. NB100-56580SS, SAB5500096**,** AB3314P; Novus Biologicals, Centennial CO; Sigma Aldrich, St. Louis, MO; EMD Millipore Corp, Billerica, MA); these were rabbit monoclonal antibodies. The chicken MCT1 antibody was used at 1:30 (catalog no. AB1286-I, EMD Millipore Corp, Billerica, MA). The mouse BrdU antibody was used at 1:200 (catalog no. MAB3424, EMD Millipore Corp; Billerica, MA, USA). Individual sections were treated with either 80 µL of the primary antibody solution or 80 µL of CAS Block only (negative control). Slides were incubated with primary antibody overnight at 4°C in a humidified chamber.

After removal of the primary antibody solution via a vacuum aspirator, slides were rinsed in PBS (3 x 5 min). Fluorescent secondary antibody solutions were prepared by diluting in CAS Block (1:200). Secondary antibodies were: Alexa 594 Goat Anti- rabbit IgG (H+L) (No. A21125; Invitrogen, Eugene, OR), which was used to detect TLR4, EGFR, and MCT4. Alexa 488

Goat Anti-mouse IgG 1 (No. A21121; Life Technologies, Eugene, OR) was used to detect BrdU. Alexa 594 goat anti-chicken IgG (H+L) (No. A11042; Invitrogen, Eugene, OR) was used to detect MCT1. All tissue sections, including negative controls, received 80 μL of the secondary antibody mixture and were incubated for 60 min at room temperature. Secondary antibody solutions were then aspirated and slides were rinsed in PBS (2 x 3 min). Coverslips were mounted using Slowfade Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (**DAPI**; catalog no. S36939; Invitrogen, Eugene, OR).

Image Acquisition and Analysis

Within 48 h of cover slipping, 6 images per animal were acquired at 40x magnification using a Nuance FX multispectral imaging system (Perkin Elmer Inc., Waltham, MA) mounted on a Nikon Eclipse E600 (Nikon, Tokyo, Japan) epi-fluorescence microscope, as described in Tucker et al. (2016). Each image was converted into a JPEG file and opened with ImagePro Plus 7 software (Media Cybernetics, Inc., Rockville, MD). Within ImagePro Plus software, layered composite images were created (DAPI, blue; BrdU, green; TLR4, EGFR, MCT1 and MCT4 red; (**Figure 3.3**). Individual areas of interest (AOI) within each layered composite image were manually outlined for evaluation by a single observer blinded to treatment, with AOI defined as basal and suprabasal areas (**Figure 3.4**). Manual outlining of AOI generated image masks, which were saved as TIFF images and used as described below.

Layered composite images of the 6 original photomicrographs per calf and all image mask files pertaining to each image were then imported into CellProfiler software (version 2.20; www. Cellprofiler.org) and organized by image and calf. CellProfiler is open access software; software overviews can be found in papers by Carpenter et al. (2006) and Bray et al. (2015). Briefly, within CellProfiler we constructed an automated analysis "pipeline" that used the component images,

image masks, and internal software algorithms to calculate measures of: integrated object intensity, mean object intensity, area, area adjusted integrated object intensity, and area adjusted mean object intensities within image. Once the analysis pipeline was started, all images were analyzed automatically in the same run.

Resultant data (in arbitrary units) from the single pipeline run were exported to a spreadsheet (Microsoft Excel for Mac, version 16.16.6) and sorted by cell location (basal or suprabasal), and then by treatment (CON, BUTY, LPS-O, or LPSB). For each measurement, data contained within the original spreadsheet were averaged across images within calf and multiplied by 1,000,000 to ease in data visualization.

Statistical Analyses

The statistical analysis was performed using a 2x2 factorial involving presence or absence or LPS (**CL**) and sodium butyrate (**CB**) where calf was the experimental unit. An ANOVA table (**Table 3.2**), displays both the single-point measures as well as the repeated weeks. Combinations of LPS and butyrate were compared using preplanned nonorthogonal contrasts to determine the average effect of treatment CON vs. the average of all other treatments, the effect of LPS-O versus LPSB, and the effect of LPSB versus BUTY. The contrast p-values were increased by a Bonferroni adjustment due to their lack of independence.

All residuals were checked for outliers and normality by evaluating their studentized form, which expressed them with a mean of 0 and a standard deviation of 1. All models were checked for outliers by investigating any observations that were above or below 3.0 and -3.0 respectively. If any observations were above or below these parameters, data was checked to see if it was balanced on both sides, meaning if a point was at -4.0 then it would be expected to have a point at 4.0 as well if it was normally distributed. This procedure ensures no more than 1-2 data points

are removed. In addition, a graphical representation was also compared against the outlier data to ensure data was normally distributed on both the positive and negative end.

Results focused on the least square means with their standard errors. Significance was declared at $P < 0.05$ for all procedures. Single time-point data were analyzed using the GLIMMIX procedure of SAS (SAS Version 9.4, SAS Institute, Cary, NC); LPS-O, BUTY, LPSB, CON, and group were the fixed effects and calf within treatment and group was the random residual. Group interactions with CB and CL were left out of the model as most of them had no significant effect. They became part of the calf variation. The denominator degrees of freedom used a Satterthwaite approximation for variables measured once per calf which included organ weights, papillae area, and rumen biopsy weights.

Additional single-time point data were analyzed using the same analysis design as described above for the single time-point data, but with additional fixed effects of location and its interaction with the treatments (**Table 3.3**). The denominator degrees of freedom used a Satterthwaite approximation for variables measured once per calf which included TLR4, EGFR, MCT1, and MCT4 intensities and areas as well as BrdU positive cells.

Health, intake, and blood metabolite data measured weekly for 8 wk were analyzed using the GLIMMIX procedure of SAS with a repeated measures model. An autoregressive covariance structure [AR(1)] was used under the assumption that adjacent weeks were more correlated than weeks farther apart. In certain models the residual term was removed from [AR(1)] due to a degenerate run. Week zero was used as a covariate since calves had not yet begun treatments on week zero. Week was the repeated measure and calf within treatment and group was the variable used to test LPS-O, BUTY, LPSB, CON, and group. The denominator degrees of freedom for calf used a Kenward-Rogers approximation to accompany the [AR(1)] option.

Data were first averaged in Excel by day if necessary, then by calf as weekly values. Slices by week were analyzed for all interaction with CB and CL if $P < 0.20$. Slices are a more detailed analysis output from SAS that show, in this case, the ls means for treatment combinations by week (SAS/STAT(R) 9.3 User's Guide, 2019).

RESULTS AND DISCUSSION

Feed Intake

Of the 24 calves that were initially enrolled in the study, two calves died, unrelated to the treatments administered, and their data were not included in analyses. Milk replacer intake was not different by treatment, increased over time until the week of weaning (wk 6), and then gradually fell to zero for all calves by 8 wk during weaning (**Table 3.4**). Starter intake was free choice, increased weekly ($P < 0.001$; **Table 3.4**), and was not affected by treatment (**Table 3.4**). Voluntary water intake also increased weekly ($P < 0.001$; **Table 3.4**), and was not affected by treatment (**Table 3.4**). To summarize the feed intake data, total DMI increased weekly ($P < 0.001$; **Table 3.4**), and was not affected by treatment (**Table 3.4**). While these observations are in agreement with authors Kato et al. (2011) who also saw no difference in feed intake with butyrate supplemented calves, authors Górka et al. (2011) observed an increase in feed intake with butyrate supplementation. In the study done by Kato et al. (2011) the sodium butyrate was supplemented in the MR. In the study done by Górka et al. (2011) the differences observed were when the sodium butyrate was incorporated into the starter feed. It is important to note this could be the reason for the observed differences. The milk replacer, starter, and voluntary water intakes we recorded were similar to reported intakes in other experiments that fed similar diets to growing calves (Bach et al., 2007; Yohe et al., 2015; Quigley et al., 2018).

Body and Organ Weights and Health Measures

Body weight and stature measurements and health measures are reported in **Table 3.5**. All body weight, stature, and health data were affected by week (*P* < 0.0001; **Table 3.5**) with an increase observed over time for BW, ADG, withers height, and hip height, a decrease over time for rectal temperature, fecal score, and respiratory score, and an increase in G:F for the first 5 weeks until weaning when G:F then decreased. While no CB x CL effect was detected for ADG (*P* = 0.34; **Table 3.5**), G:F (*P* = 0.43; **Table 3.5**), hip height (*P* = 0.28; **Table 3.5**), withers height (*P* = 0.09; **Table 3.5**), rectal temperature (*P* = 0.43; **Table 3.5**), fecal score (*P* = 0.44; **Table 3.5**) or respiratory score (*P* = 0.18; **Table 3.5**), a CB by CL effect was observed for BW (*P* = 0.02; **Table 3.5**). If a 2-way interaction between CB and CL was detected, non-orthogonal contrasts were used to analyze the relationship. Non-orthogonal contrast LPSB versus BUTY (*P* = 0.04) indicates LPSB calves had higher average BW when compared to BUTY calves (LPSB, 66.9 ± 0.68 kg; BUTY, 64.0 ± 0.75 kg). This data suggests that the additive treatment, LPSB, somehow contributed to the calves growing at a faster rate when compared to the butyrate dosed calves. This could be due to some synergistic relationship between LPS and butyrate, since feed intakes did not differ. Additionally, we detected an LPS effect on withers height (*P* = 0.006; **Table 3.5**) and ADG (*P* = 0.03; **Table 3.5**). Withers heights of calves on treatments that contained LPS had an overall average of 87.7 ± 0.21 cm and overall withers height for calves on treatments that did not contain LPS were 86.8 ± 0.22 cm. Although statistically different, this small difference in withers height is not viewed as biologically important, especially given that a similar difference was not detected for hip height. Regardless, the hip and withers heights we recorded here are in line with recent data from similarly aged Holsteins fed similar diets (Lesmeister et al., 2004; Khan et al., 2007; Yohe et

al., 2015). The ADG of calves on treatments that contained LPS was 0.62 ± 0.02 kg/d while the ADG for calves on treatments that did not contain LPS was 0.55 ± 0.02 kg/d.

Calf organ weights are presented in **Table 3.6**. An interaction between CB and CL was detected for empty stomach and empty reticulorumen weights (P = 0.01; **Table 3.6**), while no interactions were detected for remaining organ weights or when organ weights were analyzed as a percent of final body weight. The non-orthogonal contrast LPSB versus BUTY (P = 0.003; **Table 3.6**) indicates LPSB calves had heavier empty stomach weights when compared to BUTY calves (LPSB, 2.87 ± 0.076 kg; BUTY, 2.48 ± 0.076 kg). Non-orthogonal contrast LPSB versus BUTY (P = 0.003; **Table 3.6**) shows empty reticulorumen weight in LPSB calves was 1.76 ± 0.065 kg at 8 wk and this measurement was 1.44 ± 0.065 kg at 8 wk for BUTY calves. Additionally, empty stomach weight and empty reticulorumen weights were higher in calves dosed with treatments that contained LPS when compared to treatments without LPS (2.8 vs. 2.6 kg empty stomach, P = 0.04; 1.7 vs. 1.6 kg empty reticulorumen, P = 0.05 respectively; **Table 3.6**). No other organ data presented in **Table 3.6** was affected by treatment.

Given the ~3 kg difference in mean body weight between LPSB and BUTY, the empty reticulorumen mass difference was ~320 g between these two treatments. Because stomach fill, empty abomasum + omasum, full small intestine, and full large intestine weights were not affected by treatment when measured at wk 8 (**Table 3.6**), we can infer that the unaccounted for average body weight difference (~2.7 kg) went to lean tissue growth, adipose, or internal organs other than those we specifically measured in LPBS compared to BUTY. At wk 8, LPSB weighed 84.0 ± 1.40 kg and BUTY weighed 78.0 ± 1.53 kg (data not shown). However, we should note that at wk 8, empty reticulorumen mass as a percentage of final body weight was not different by treatment (**Table 3.6**). The interaction observed between CB and CL for BW and empty reticulorumen

weight partially supports our hypothesis that LPS and butyrate work together to synergistically upregulate rumen growth.

Receptor Intensity and BrdU Labeling

Rumen receptor intensity and BrdU labeling index data are presented in **Table 3.9**. No treatment differences or interactions between CB and CL were detected for receptor intensity or BrdU labeling index. The main ligand for TLR4 is LPS. In the rumen epidermis of 8 wk-old calves we detected a tendency for increased TLR4 staining intensity in treatments that contained LPS compared to those that did not (P = 0.07; **Table 3.9**). When sliced by location, there was a CL treatment effect observed within the suprabasal layer for TLR4 staining intensity (P = 0.05; **Table 3.9**), however overall TLR4 staining intensity across suprabasal and stratum basale layers of the rumen epidermis was not affected by location (P = 0.79; **Table 3.9**). The intensity of TLR4 staining in the suprabasal layer of the rumen epidermis averaged 0.04 ± 0.004 a.u. for calves that received LPS, while intensity averaged 0.03 ± 0.004 a.u. for calves that did not receive LPS. At the onset of the experiment, we anticipated detection of higher TLR4 staining intensity in suprabasal layers of the rumen epidermis as compared to the stratum basale. This structural arrangement coincides with the sentinel function of TLR4; suprabasal layers of the epidermis theoretically encounter free LPS more often than cells of the stratum basale. The EGFR was affected by location (P < 0.0001) and had higher intensity in the stratum basale layer. The increased intensity of the EGFR in the basal layer of the rumen epidermis (**Figure 3.2**) is in agreement with the findings of Steele et al. (2015). Rumen epidermal cells are known to function as the main site of absorption of nutrients as well as regulate microbial permeability (Yohe et al., 2016). In order to maintain the layers of rumen epidermal cells, the cells in the stratum basale layer will first proliferate and cells in the above layers will differentiate to perform specialized tasks (Yohe et al., 2016). Baldwin (1999)

demonstrated the importance of the EGFR in rumen cell proliferation and differentiation, while Geiger et al. (2017) and Yohe et al. (2018) used the labeling of BrdU as a measure of the proliferative status of rumen epithelial cells. Given this information, we expected to see a CL treatment effect on the intensity of TLR4, EGFR and BrdU proliferative status, however we only observed this trend with TLR4. These results indicate that the additional LPS dosed for CL treatments was likely binding to the TLR4 receptor, however may have signaled another pathway other than EGFR. Although in this study we proposed that TLR4 indirectly activated EGFR through the activation of NF-κB (**Figure 3.2**), it seems that maybe NF-κB was regulating a pathway other than that of activating EGFR. The NF-κB also regulates immune function and therefore future studies should be done to investigate gene expression of these additional associated proteins.

Sodium Butyrate and Blood Metabolites

Presumably, orally dosed butyrate was quickly absorbed by rumen papillae and partially oxidized to BHBA during absorption. Existing data suggest that VFA entry into ruminal epithelial cells can occur via facilitated transport and passive diffusion (Muller et al., 2000; Connor et al., 2010; Aschenbach et al., 2011) where facilitated transport is used for dissociated VFA (via MCT1 and MCT4) and passive diffusion is used for the undissociated VFA. Transporters MCT1 and MCT4 were affected by location ($P < 0.0001$) and MCT1 had higher intensity in the stratum basale, while MCT4 staining intensity was higher in suprabasal layers in the approximate location of the stratum granulosum (**Figure 3.2**). This is consistent with the known function of MCT4 transporting VFA from the apical ends of rumen epidermal cells into the cell cytoplasm (**Figure 3.2**). The location of MCT4 we observed is in agreement with the location of MCT4 that other authors observed (Graham et al., 2007; Connor et al., 2010; Yohe, 2014). The location of the MCT4 gives

it the ability to transport VFA into the bloodstream (Graham et al., 2007). We also observed higher staining intensity of MCT1 in the basal cell compartment compared to the suprabasal compartment (**Table 3.9**). LPS binding protein data are presented in **Table 3.9**. While no treatment differences or interactions between CB and CL were detected for LPS binding protein, a week effect was observed ($P < 0.0001$).

Although we did not detect differences in mean intensity of rumen MCT1 and MCT4 by treatment at 8 wk of age (**Table 3.9**), we did note a CB x CL effect ($P = 0.01$) and an LPS effect ($P = 0.05$) on empty reticulorumen weight (**Table 3.6**). This difference in tissue mass might account for increased total number of MCT1 and MCT4 in the rumen of calves administered either LPS alone, or LPS in combination with sodium butyrate, but not sodium butyrate alone. Rumen epithelium core biopsy and papillae measures are presented in **Table 3.7**. No interactions between CB and CL, or treatment differences were observed for epithelium weight of biopsy cores, rumen wall weight of biopsy cores, epithelial percentage of biopsy cores, or two-dimensional papillae area. Our findings are in agreement with Górka et al. 2009 who also observed no change in rumen muscle layer thickness with supplemented sodium butyrate. We expected, at minimum, to see a CB effect on rumen papillae area as other authors have observed increased papillae growth with supplemental sodium butyrate (Sakata and Tamate, 1978; Lesmeister and Heinrichs, 2005; Górka et al., 2009; Malhi et al., 2013). This could be due to error in administration of the treatment, if the reticular groove was closed then the butyrate did not deliver all the way to the rumen. Although we tried to account for this by giving treatments at least 30 min after a meal to allow for the reticular groove to be open, the calves may have expected this routine as it was given at the same time every day and therefore closed the groove. Although unlikely, if the butyrate was to bypass the rumen and end up in the abomasum, we would expect to see greater abomasum + omasum

weights in those calves as previously observed by Górka et al. (2018) with dietary butyrate supplementation increasing abomasum and omasum epithelium thickness. We did not observe any difference in abomasum + omasum empty or full weights, so if butyrate is active in stimulating gut tissue growth, there is also no evidence that it was shunted to the abomasum.

Blood metabolite, rumen pH and VFA, and LPS binding protein data are presented in **Table 3.8**. Our data show that both rumen pH and individual rumen VFA concentrations, measured within 3 h of treatment dosing, were not different, suggesting to us that administered sodium butyrate was absorbed within 3 h (**Table 3.8**). In defense of that point, our data show that circulating BHBA, measured within 3h post-administration in jugular blood, depended on the combination of CB x CL ($P = 0.03$; **Table 3.8**), but not CL ($P = 0.49$; **Table 3.8**), or CB alone ($P = 0.95$; **Table 3.8**). Although an effect of the interaction between CB x CL was detected for BHBA, no non-orthogonal contrasts were observed to be significant. It remains possible that the sodium butyrate we used had slightly different rumen absorption kinetics than either H^+butyrate or butyrate$^-$. We suspect that the Na+ dissociated once in the rumen milieu, but do not have further support of what happened to the Na+ we introduced. We can conclude that it did not drive thirst (**Table 3.4**). In future studies, analyses should be done to investigate the impacts of Na+/H+ exchangers (**NHE**) as well as epithelial blood flow. There are multiple isoforms of NHE including NHE1, NHE2, and NHE3 identified in the rumen (Graham and Simmons, 2005), and authors Storm et al. (2011) identified an association with butyric acid and disappearance rate of VFA that was correlated with epithelial blood flow. Measuring the effects of treatments on NHE and epithelial blood flow would provide a better measure of sodium absorption kinetics and VFA disappearance in the rumen. The circulating BHBA values we report here are approximately 6 times higher than BHBA values reported by Yohe et al. (2018a) and Khan et al. (2007), twice as

high as BHBA values reported by Górka et al. (2011), and similar to BHBA values reported by Lesmeister et al. (2004). With elevated levels of BHBA in comparison to other studies, this BHBA should have been available for further metabolism. The fate of BHBA in young dairy calves is involved with rumen epithelial metabolic activity by providing an energy substrate for the rumen in the form of acetoacetate (Khan et al., 2007). Once in the bloodstream in the form of a ketone, the calf can utilize this substrate for energy to meet ATP demands other than those of the rumen. Authors Nemati et al. (2015) demonstrate that this additional ATP demand rises in the state of transition from liquid to solid feed for the calf, and therefore BHBA is shifted to become a fuel source for the calf in that time period. This could potentially explain where that the excess sodium butyrate went during this time period. Most dairy calves experience a period in time where ADG and BW are slightly suppressed around and during weaning, also known as the growth slump. It could be possible that the excess butyrate from oral treatments provided enough energy for calves to not experience reduced growth rates. Results from this study indicate no treatment effect was detected to indicate calves supplemented with butyrate were heavier then calves that did not receive butyrate supplementation.

Blood glucose was lower in calves treated with LPS compared to calves that did not receive LPS ($P = 0.008$; **Table 3.8**; 123.1 ± 1.62 mg/dL versus 117.1 ± 1.47 mg/dL in non-LPS containing treatments and LPS-containing treatments, respectively). We did not detect a decrease in blood glucose as calves aged and were weaned onto a diet of 100% starter. This was a bit unexpected but is similar to our own recent findings (Yohe et al., 2018a). Normal blood glucose concentrations of mature ruminants range from about 65 to 75 mg/dL (Kvidera et al., 2017; Baumgard et al., 2011). At wk 8, regardless of treatment, blood glucose averaged 95.67 ± 2.7 mg/dL in our experiment (**Table 3.8**). Our higher values indicate that despite being weaned, calves on our experiment did

not achieve blood glucose concentrations typical of mature cows. In young ruminants, blood glucose is derived from three primary sources, either directly from lactose hydrolysis, from liver or muscle glycogenolysis, or from gluconeogenesis from propionate. At the time we sampled jugular blood (within 3 h of last feeding), lactose hydrolysis is the suspected major source of blood glucose for pre-weaned calves and gluconeogenesis is the suspected major source of blood glucose during wk 7 and 8.

Orally dosing treatments that contained sodium butyrate to calves did not increase ruminal butyrate concentration, compared with calves that received no sodium butyrate (5.4 vs. 5.5 m*M* respectively; *P* = 0.95; **Table 3.8**). We expected to see, at minimum, a higher butyrate concentration in the CB calves since they were dosed with additional 11 m*M* sodium butyrate on a daily basis, but the molar proportion of ruminal butyrate we observed never exceeded what we would normally expect for young dairy calves (10 to 15 m*M* rumen butyrate; Bergman, 1990; Sutton et al., 2003; Wang and Jiang, 2010; Zanton and Heinrichs, 2016; Yohe et al., 2018a) and molar proportion of rumen butyrate followed a similar pattern as reported by Yohe et al. (2018b). Likewise, our rumen acetate and propionate data for newly weaned calves (**Table 3.8**; wk 8) are in line with an expected range of 60 to 80 m*M* for acetate and 15 to 65 m*M* for propionate, respectively (Bergman, 1990; Sutton et al., 2003; Wang and Jiang, 2010; Zanton and Heinrichs, 2016; Yohe et al., 2018).

Although the trend in BW mentioned earlier may suggest that the extra butyrate may be involved in growth and maintenance of the calf, there was only a difference when comparing BUTY to LPSB calves, which would suggest the synergistic effect of the two components are the contributing factor. There was also no difference when looking at the CON versus the average of all other treatments. Additionally, if butyrate was the contributing factor to BW gain, we would

expect a similar observation in BHBA data, but the BHBA data did not differ by treatment. There were no treatment effects of calf health (respiratory and fecal scores or rectal temperature), so we would hesitate to say the metabolized butyrate was directed into immune function. With these speculations in consideration, we propose the metabolized butyrate went into the energy needs of urea recycling based on what authors Agarwal et al. (2015) saw in sheep. Urea recycling would be categorized in the digestive processes of endogenous losses and therefore would not be expected to play a role in regular maintenance growth observed in BW gain, immune function, feed intakes, or rumen development.

LPS Effects

LPS binding protein is a natural ligand for LPS and is upregulated in the presence of LPS. We detected LPSBP in the blood of all calves at both wk1 and wk 8. The amount of LPSBP decreased with age but was not affected by treatment (**Table 3.9).** This could be a result of the calves building a tolerance to the LPS, which in turn can suppress cell activation in immune cells (Novoa et al., 2009). Future studies should investigate gene expression of NF-κB since it is said to be involved with LPS tolerance (Uhrig et al., 2005). If the expression of NF-κB was responsible for regulating such tolerance of LPS, this gene is further up the proposed mechanistic cascade (**Figure 3.2**) and could potentially be suppressing the effects we were expecting to see in EGFR.

The findings of authors Khafipour et al. (2009a) report increased levels of LPS in the rumen, but not in the blood, in SARA. These findings confirm that LPS can still be elevated in the rumen but not undergo the process of translocating into the blood. This could partially explain the lack of effects we observed in the blood metabolite data. Given that one major role of TLR4 is to recognize LPS and regulate an inflammatory response (Chen et al., 2012), future studies should be done to

investigate inflammatory markers and acute phase proteins serum-amyloid A (**SAA**) and haptoglobin.

Tolerance to LPS has proposed in cows (Jacobsen et al., 2005) and noted in other animals (Uhrig et al., 2005; Novoa et al., 2009). This is one reason we increased the dose over time. In the study by Ametaj and others (2012), oral doses of LPS were administered at 0.01, 0.05, and 0.1 µg LPS/kg of BW for a period of 28d. A dosage of 0.01 µg LPS/kg was given twice on day -14 and -10 prepartum, a dosage of 0.05 µg LPS/kg twice on d -7 and -3, and 0.1 µg LPS/kg twice on day +3 and +7 postpartum. Those authors observed no clinical signs (e.g., elevated body temperature, decreased feed intake, or abnormal rumen contraction rates) in LPS treated cows (Ametaj et al., 2012). This prompted us to be more liberal with LPS dosing in the current experiment and to modify our laboratory methods to include use of non-pyrogenic glassware.

In the pilot trial no clinical signs (e.g., elevated body temperature, decreased feed intake, lethargic behavior) were observed in LPS-treated calves, and, as hoped, rumen epidermis was thicker and the empty reticulorumen weighed more (**Figure 3.1**) in LPS-treated compared to non-LPS treated calves. However, we acknowledge that LPS solutions were made up in plastic bottles in our pilot trial, so it is conceivable that some of the LPS clung to the plastic in those bottles (Kitchens et al., 1992), lowering the effective LPS dose.

Although we used a more liberal dosing method of LPS, this study did not prove to have effects on rumen development like initially expected.

CONCLUSIONS

Results from this study indicate orally dosed butyrate and LPS did not impact rumen development as expected. Papillae area and BHBA levels were not different among treatments, which are both indicative of rumen activity and development. Similarly, EGFR intensity was not

different amongst treatments, which was our expected marker for rumen cell proliferation. Although the BW and empty reticulorumen weights were higher in treatments containing LPS, the control calves were not different from the LPS or BUTY treated calves. If oral treatments were not reaching the rumen this would partially explain these lack of ruminal effects, but this should have resulted in a gut growth response downstream from the rumen, which was not observed. If the treatments successfully reached the rumen, then we propose the additional butyrate may have been used up in the metabolic process of urea recycling while additional LPS was involved in a different immune function signaling pathway.

To our knowledge, there are no present studies, other than the current, that provide scientific data on orally dosing LPS to neonatal dairy calves from birth to weaning. No growth responses were observed from a butyrate treatment effect, and thus this work does not support prior observations from studies suggesting a butyrate effect on rumen development. Further research is necessary in this field to draw more conclusive implications about LPS stimulating rumen epithelial cell proliferation, however this study is essential to provide standards for dosing concentrations of LPS as well as introducing new methods of administration.

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Table 3.1. Chemical composition of milk replacer and pelleted calf starter diets

Composition	Milk Replacer	Pelleted Starter	Starter Refusals
Dry Matter, % as-fed	96.4	87.9	85.0
Chemical composition, % of DM			
CP	22.8	23.1	23.3
ADF	-	15.7	16.3
NDF	-	26.5	28.5
NFC	46.5*	39.2	38.0
Sugar	46.5*	ND	ND
Starch	-	21.7	20.8
Ether extract	21.3	2.9	2.8
Ash	9.4	8.2	7.8

*Calculated as: 100 – CP - ether extract – ash. Assumed to be nearly 100% lactose.
ND = not determined.

Table 3.2. Analysis of variance table for single-time point and repeated measures data

Model		Source[1]	df	Effect Type	Error Term
	Repeated	Week0	1	Covariate	Calf (CB*CL*group)
Single Time-Point[1]	Repeated	CB	1	Fixed	Calf (CB*CL*group)
Single Time-Point[1]	Repeated	CL	1	Fixed	Calf (CB*CL*group)
Single Time-Point[1]	Repeated	CB*CL	1	Fixed	Calf (CB*CL*group)
Single Time-Point[1]	Repeated	Group	1	Fixed[2]	Calf (CB*CL*group)
Single Time-Point[1]	Repeated	Calf (CB*CL*group)	16	Random	
	Repeated	Week	7	Fixed	Residual
	Repeated	CB*Week	7	Fixed	Residual
	Repeated	CL*Week	7	Fixed	Residual
	Repeated	CB*CL*Week	7	Fixed	Residual
	Repeated	Residual	126	Random	
		Total	175		

[1]Trial began with 24 calves; total degrees of freedom exclude 2 calves that died during the trial.
[2] Group is a fixed effect because there were only two groups. Group interactions with treatments are in the calf variation.

Table 3.3. Analysis of variance table for intensity data

Model		Source[1]	df	Effect Type	Error Term
Treatment Effects	Transporter Intensity	CB	1	Fixed	Calf (CB*CL*group)
		CL	1	Fixed	Calf (CB*CL*group)
		CB*CL	1	Fixed	Calf (CB*CL*group)
		Group	1	Fixed[2]	Calf (CB*CL*group)
		Calf (CB*CL*Group)	16	Random	
Repeated Measures		Location	1	Fixed	Residual
		CB*Locatiureaon	1	Fixed	Residual
		CL*Location	1	Fixed	Residual
		CB*CL*Location	1	Fixed	Residual
		Residual	19	Random	
		Total	43		

[1]Trial began with 24 calves; total degrees of freedom exclude 2 calves that died during the trial.
[2]Group is a fixed effect because there were only two groups. Group interactions with treatments are in the calf variation.

Table 3.4. Least squares means of milk replacer (MR), and starter, total dry matter (DMI), and voluntary water intake of 8 wk old Holstein bull calves

Item	Week[1]								Test of Fixed Effects[2], *P*-Value			
	1	2	3	4	5	6	7	8	SEM[3]	CB[4]	CL[5]	CB x CL
MR intake, DM (kg/calf per d)										0.61	0.85	0.56
CON	0.68	0.73	0.75	0.84	0.85	0.92	0.47	-	0.02	-	-	-
BUTY	0.70	0.68	0.75	0.83	0.86	0.93	0.47	-	0.02	-	-	-
LPS-O	0.68	0.71	0.75	0.83	0.85	0.92	0.48	-	0.02	-	-	-
LPSB	0.68	0.74	0.76	0.84	0.86	0.94	0.48	-	0.02	-	-	-
Starter intake, DM (kg/calf per d)										0.97	0.44	0.24
CON	0.005	0.04	0.11	0.19	0.34	0.71	1.29	1.69	0.11	-	-	-
BUTY	0.02	0.03	0.097	0.19	0.36	0.55	1.31	1.69	0.10	-	-	-
LPS-O	0.01	0.04	0.10	0.16	0.32	0.59	1.19	1.57	0.11	-	-	-
LPSB	0.03	0.02	0.09	0.19	0.36	0.64	1.41	1.88	0.10	-	-	-
Total DMI, (kg/calf per wk)										0.93	0.56	0.15
CON	0.69	0.77	0.87	1.03	1.20	1.64	2.23	1.69	0.12	-	-	-
BUTY	0.71	0.70	0.83	1.00	1.21	1.47	2.24	1.67	0.11	-	-	-
LPS-O	0.71	0.78	0.87	1.01	1.19	1.53	2.16	1.61	0.12	-	-	-
LPSB	0.70	0.75	0.84	1.02	1.21	1.57	2.36	1.87	0.11	-	-	-
Voluntary water intake, (kg/calf per d)										0.82	0.72	0.35
CON	1.77	2.11	3.05	2.31	3.33	3.64	6.10	7.20	0.77	-	-	-
BUTY	1.08	1.70	2.68	2.25	2.97	2.70	6.50	7.45	0.70	-	-	-
LPS-O	1.09	1.23	2.21	1.95	2.57	3.53	5.78	7.66	0.77	-	-	-
LPSB	1.20	1.35	2.34	1.62	2.51	3.02	6.77	7.90	0.70	-	-	-

[1]Week 0 consisted of the first 3 days of the trial, week 1 started on d 4 of the study and week 8 consisted of d 52-56.
[2]Significance declared when $P \leq 0.05$; three-way interaction not shown. Week interactions were all $P < 0.0001$ for each dependent variable with the exception that gain:feed had a week interaction of P=0.0581. Treatments were either: Control (CON), butyrate (BUTY), lipopolysaccharide only (LPS-O), or butyrate + LPS (LPSB). All treatments were administered orally twice per day and consisted of either 0.9% saline (CON), 11m*M* sodium butyrate (BUTY), LPS ranging from 2.5 to 40 μg/kg $BW^{0.75}$ (adjusted weekly), or both butyrate and LPS (LPSB). Dosage volume increased as experiment progressed and ranged from 10 to 40 mL per dose.
[3]Standard error of the mean was recorded based on the average across weeks.
[4] Treatments contained butyrate.
[5]Treatments contained LPS.

Table 3.5. Growth and health measures of 8 wk old Holstein bull calves

Item	Week[1]									Test of Fixed Effects[2], *P*-Value		
	1	2	3	4	5	6	7	8	SEM[3]	CB[4]	CL[5]	CB x CL
BW (kg)										0.88	0.17	0.02†
CON	50.1	53.9	59.3	62.4	68.5	73.6	79.4	81.1	1.53			
BUTY	49.8	52.5	57.3	63.0	68.2	71.9	78.2	81.1	1.40			
LPS-O	50.0	52.2	56.8	61.1	67.2	70.9	76.5	78.0	1.53			
LPSB	50.9	53.6	58.9	63.3	69.6	74.3	81.1	84.0	1.40			
ADG (kg/d)										0.47	0.03*	0.34
CON	0.16	0.56	0.78	0.46	0.88	0.74	0.84	0.26	0.15			
BUTY	0.23	0.38	0.67	0.81	0.74	0.53	0.89	0.67	0.14			
LPS-O	0.25	0.31	0.64	0.61	0.86	0.51	0.79	0.21	0.15			
LPSB	0.27	0.39	0.77	0.63	0.90	0.68	0.97	0.41	0.14			
Gain:feed										0.93	0.19	0.43
CON	0.24	0.73	0.90	0.45	0.74	0.45	0.37	0.14	0.14			
BUTY	0.32	0.50	0.78	0.78	0.60	0.35	0.38	0.36	0.12			
LPS-O	0.37	0.41	0.78	0.63	0.76	0.34	0.37	0.13	0.14			
LPSB	0.39	0.51	0.94	0.61	0.76	0.44	0.42	0.23	0.13			
Withers Height (cm)										0.13	0.006*	0.09
CON	81.9	82.2	84.1	86.0	87.2	89.7	91.3	91.8	1.06			
BUTY	82.8	83.5	85.5	87.5	89.8	91.2	92.5	93.2	0.97			
LPS-O	82.6	82.7	84.3	86.1	87.6	88.9	90.6	93.4	1.06			
LPSB	82.7	83.4	83.9	86.0	87.2	89.8	91.7	93.0	0.97			
Hip Height (cm)										0.81	0.53	0.28
CON	86.5	86.5	89.5	91.1	93.7	95.7	96.1	86.7	1.15			
BUTY	86.7	87.3	88.8	91.2	93.5	94.3	96.5	96.4	1.04			
LPS-O	87.0	86.9	88.4	91.2	92.3	93.2	95.6	97.1	1.14			
LPSB	86.0	86.9	87.4	90.9	92.9	93.0	96.2	96.7	1.04			
Rectal temperature (°C)										0.42	0.39	0.43
CON	39.2	38.6	38.6	38.5	38.5	38.4	38.5	39.0	0.16			
BUTY	39.2	38.6	38.6	38.5	38.5	38.4	38.5	39.0	0.15			
LPS-O	39.1	38.5	38.6	38.4	38.4	38.3	38.4	39.0	0.16			
LPSB	39.2	38.5	38.6	38.5	38.6	38.4	38.6	39.0	0.15			
Fecal Score[6]										0.24	0.73	0.44
CON	2.4	2.0	1.3	1.5	1.1	0.9	0.9	1.0	0.3			
BUTY	2.4	2.2	1.3	1.2	0.9	0.9	1.0	0.9	0.3			
LPS-O	1.8	2.0	1.5	1.2	0.8	0.8	0.9	0.8	0.3			
LPSB	2.2	2.0	1.5	1.2	0.7	0.9	0.9	0.9	0.3			
Respiratory Score[7]										0.53	0.37	0.18
CON	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1			
BUTY	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1			
LPS-O	0.3	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.1			
LPSB	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1			

[1] Week 0 consisted of the first 3 days of the trial, week 1 started on d 4 of the study and week 8 consisted of d 52-56.
[2] Significance declared when $P \leq 0.05$; three-way interaction not shown. Week interactions were all $P < 0.0001$. Treatments were either: Control (CON), butyrate (BUTY), lipopolysaccharide only (LPS-O), or butyrate + LPS (LPSB). All treatments were administered orally twice per day and consisted of either 0.9% saline (CON), 11 m*M* sodium butyrate (BUTY), LPS ranging from 2.5 to 40 µg/kg $BW^{0.75}$ (adjusted weekly), or both butyrate and LPS (LPSB). Dosage volume increased as experiment progressed and ranged from 10 to 40 mL per dose.
[3] Standard error of the mean was recorded based on the average across weeks.
[4] Treatments contained butyrate. [5] Treatments contained LPS. [6] On a scale from 0-3, where 0 = solid feces and 3 = watery feces. [7] On a scale from 0-2, where 0 = normal breathing and 2 = heavy breathing with or without coughing. †Non-orthogonal contrast LPSB versus BUTY ($P = 0.01$); LPSB 67.0 ± 0.69; BUTY 64.1 ± 0.76. *LS means ± SEM: Contains LPS 87.7 ± 0.21; Does not contain LPS 86.8 ± 0.22.

Table 3.6. Least squares means of organ weights of 8 wk old Holstein bull calves

Item	Treatment[1]				SEM[2]	Test of Fixed Effects[3], *P*-Value		
	CON (n=5)	BUTY (n=5)	LPS-O (n=6)	LPSB (n=6)		CB[4]	CL[5]	CB x CL
Weight (kg)								
Full Stomach	9.38	8.07	9.46	9.36	0.687	0.32	0.33	0.39
Empty Stomach*	2.77	2.48	2.73	2.87	0.076	0.36	0.04[α]	0.01[β]
Full Reticulorumen	7.63	6.15	7.52	7.52	0.680	0.30	0.37	0.29
Empty Reticulorumen*	1.72	1.44	1.68	1.76	0.065	0.14	0.05[φ]	0.01[λ]
Rumen Contents*	7.13	5.63	6.73	7.05	0.613	0.36	0.42	0.16
Full Abomasum + omasum†	1.74	1.90	1.92	1.83	0.197	0.85	0.78	0.54
Empty Abomasum + omasum	0.988	1.04	1.04	1.09	0.061	0.43	0.37	0.96
Full Small Intestine	3.93	3.92	3.82	4.22	0.254	0.45	0.71	0.42
Full Large Intestine	1.77	1.82	1.73	2.02	0.153	0.30	0.60	0.44
Weight (% of live weight)								
Full Stomach	11.6	10.4	11.4	11.0	0.6	0.24	0.79	0.60
Empty Stomach*	3.3	3.2	3.3	3.3	0.09	0.64	0.47	0.52
Full Reticulorumen	9.4	7.9	9.1	8.9	0.6	0.24	0.68	0.37
Empty Reticulorumen*	2.0	1.8	2.0	2.0	0.08	0.23	0.28	0.18
Rumen Contents*	8.6	7.2	8.1	8.2	0.6	0.33	0.73	0.30
Full Abomasum + omasum	2.2	2.4	2.3	2.1	0.2	0.92	0.81	0.41
Empty Abomasum + omasum	1.2	1.3	1.2	1.3	0.06	0.29	0.92	0.56
Full Small Intestine	4.9	5.1	4.7	5.0	0.2	0.35	0.58	0.88
Full Large Intestine	2.2	2.3	2.1	2.4	0.1	0.26	0.94	0.73

[1] Treatments were either: Control (CON), butyrate (BUTY), lipopolysaccharide only (LPS-O), or butyrate + LPS (LPSB). All treatments were administered orally twice per day and consisted of either 0.9% saline (CON), 11m*M* sodium butyrate (BUTY), LPS ranging from 2.5 to 40 µg/kg $BW^{0.75}$ (adjusted weekly), or both butyrate and LPS (LPSB). Dosage volume increased as experiment progressed and ranged from 10 to 40 mL per dose.

[2] Standard error of the mean, for treatment, was recorded based on the average value for the interaction CB x CL.

[3] Significance declared at $P \leq 0.05$; tests for fixed effect not shown.

[4] Treatments contained butyrate.

[5] Treatments contained LPS.

*Because of missing data, CON (n=4), LPSB (n=5).

†Group effect, $P = 0.0537$; group one 1.65 ± 0.14; group two 2.05 ± 0.14.

[α]Contained LPS 2.8 ± 0.05; Did not contain LPS 2.6 ± 0.06.

[β]Non-orthogonal contrast LPSB vs BUTY ($P = 0.003$).

[φ] Contained LPS 1.7 ± 0.04; Did not contain LPS 1.6 ± 0.05.

[λ] Non-orthogonal contrast LPSB vs BUTY ($P = 0.003$).

Table 3.7. Least squares means of rumen and papillae measures of 8 wk old Holstein bull calves

Item	Treatment[1]				SEM[2]	Test of Fixed Effects[3], *P*-Value		
	CON (n=5)	BUTY (n=5)	LPS-O (n=6)	LPSB (n=6)		CB[4]	CL[5]	CB x CL
Rumen biopsy weight[6] (g)								
Epithelium†	1.80	1.73	1.83	1.83	0.206	0.86	0.76	0.87
Wall	3.10	2.90	3.12	3.08	0.297	0.70	0.74	0.78
Epithelium (% of wall)	59.8	56.9	57.3	59.5	3.08	0.91	0.99	0.43
Rumen papillae[7] (mm^2)								
Area	38.9	34.7	45.5	41.1	5.50	0.48	0.23	0.94

[1]Treatments were either: Control (CON), butyrate (BUTY), lipopolysaccharide only (LPS-O), or butyrate + LPS (LPSB). All treatments were administered orally twice per day and consisted of either 0.9% saline (CON), 11m*M* sodium butyrate (BUTY), LPS ranging from 2.5 to 40 µg/kg $BW^{0.75}$ (adjusted weekly), or both butyrate and LPS (LPSB). Dosage volume increased as experiment progressed and ranged from 10 to 40 mL per dose.
[2] Standard error of the mean, for treatment, was recorded based on the average for the interaction of CB x CL.
[3] Significance declared when $P \leq 0.05$.
[4] Treatments contained butyrate.
[5] Treatments contained LPS.
[6] Rumen epithelium and wall (muscle + submucosa) weights are from 2.54-cm-diameter rumen punch biopsies of ventral sac location.
[7] Measured from ventral sac location.
†Group effect, $P = 0.0254$; group one 1.65 ± 0.14; group two 1.65 ± 0.14.

Table 3.8. Blood metabolite, LPS binding protein assay, and ruminal VFA concentrations from 8 wk old Holstein bull calves

	Week[1]									Test of Fixed Effects[2], *P*-Value		
Item	1	2	3	4	5	6	7	8	SEM[3]	CB[4]	CL[5]	CB x CL
Rumen pH										0.19	0.38	0.10
CON	7.0	5.7	5.9	5.8	5.3	5.4	5.4	-	0.34			
BUTY	6.6	5.9	6.1	5.8	5.6	5.5	5.3	-	0.31			
LPS-O	6.7	6.3	6.4	6.0	6.0	5.6	5.5	-	0.34			
LPSB	6.8	6.1	6.0	5.7	5.3	5.4	5.4	-	0.31			
Blood glucose (mg/dL)										0.34	0.008*	0.11
CON	127	141	115	142	147	112	111	101	7.7			
BUTY	117	126	116	128	131	102	97	94	7.0			
LPS-O	134	130	127	131	136	113	109	96	7.5			
LPSB	126	129	134	128	143	112	99	93	7.1			
Blood BHBA (m*M*)										0.95	0.49	0.03†
CON	0.26	0.26	0.25	0.27	0.28	0.33	0.52	0.31	0.02			
BUTY	0.26	0.24	0.24	0.27	0.27	0.34	0.39	0.30	0.02			
LPS-O	0.26	0.24	0.26	0.27	0.26	0.33	0.42	0.31	0.02			
LPSB	0.26	0.23	0.28	0.29	0.28	0.36	0.44	0.31	0.02			
LPS binding protein										0.33	0.20	0.87
CON	37.7	-	-	-	-	-	-	10.0	5.5			
BUTY	40.3	-	-	-	-	-	-	18.9	5.0			
LPS-O	41.1	-	-	-	-	-	-	15.6	5.5			
LPSB	52.6	-	-	-	-	-	-	13.1	5.0			
Rumen VFA [6]												
Acetate (m*M*)										0.84	0.57	0.90
CON	6.5	16.4	22.1	31.1	33.8	38.1	61.2	75.3	7.5			
BUTY	8.8	17.2	21.5	35.2	42.8	43.1	59.9	84.9	7.2			
LPS-O	5.1	10.0	18.6	32.0	31.5	39.9	52.9	72.1	7.5			
LPSB	12.5	18.3	24.1	34.3	41.5	40.7	55.9	79.6	7.0			
Propionate (m*M*)										0.30	0.28	0.66
CON	1.7	7.1	14.0	17.9	18.5	23.4	35.6	51.3	5.9			
BUTY	1.7	7.1	12.5	21.7	26.5	25.2	34.6	53.3	5.4			
LPS-O	0.4	2.6	7.8	16.5	14.7	21.7	30.6	43.9	5.9			
LPSB	2.7	5.5	9.1	20.3	22.8	23.4	34.7	51.4	5.4			
Butyrate (m*M*)										0.95	0.14	0.11
CON	0.2	1.7	3.2	3.6	4.5	6.5	10.6	13.6	1.8			
BUTY	0.5	1.4	3.9	5.2	6.5	5.8	8.8	11.3	1.6			
LPS-O	0.2	0.7	2.9	4.6	5.7	5.4	8.7	9.4	1.8			
LPSB	0.9	1.5	3.4	6.4	7.3	6.4	9.5	13.7	1.6			

[1] Week 0 consisted of the first 3 days of the trial, week 1 started on d 4 of the study and week 8 consisted of d 52-56.
[2] Significance declared when $P \leq 0.05$; three-way interaction not shown. Week interactions were all $P < 0.0001$. Treatments were either: Control (CON), butyrate (BUTY), lipopolysaccharide only (LPS-O), or butyrate + LPS (LPSB). All treatments were administered orally twice per day and consisted of either 0.9% saline (CON), 11m*M* sodium butyrate (BUTY), LPS ranging from 2.5 to 40 µg/kg $BW^{0.75}$ (adjusted weekly), or both butyrate and LPS (LPSB). Dosage volume increased as experiment progressed and ranged from 10 mL to 40 per dose.
[3] Standard error of the mean, for treatment, was recorded based on the average across weeks. [4] Treatments contained butyrate. [5] Treatments contained LPS. [6] VFA concentrations were measured from ruminal contents that were sampled using oro-esophageal tubes. *Contains LPS, 117.1 ± 1.47 mg/dL; does not contain LPS, 123.1 ± 1.62 mg/dL.† No non-orthogonal contrasts significant.

Table 3.9. Least squares means of transporter and receptor intensity and BrdU positive cell counts from rumen tissue and serum in 8 wk old Holstein bull calves

	Treatment					Test of Fixed Effects[1], *P*-Value			
	CON	BUTY	LPS-O	LPSB					
Measurement	(n=5)	(n=5)	(n=6)	(n=6)	SEM	Location	CB[2]	CL[3]	CB x CL
TLR4 Mean Intensity						0.79[4]	0.85	0.07	0.54
Suprabasal	0.030	0.026	0.035	0.037	0.004	--	0.85[6]	0.05[6*]	0.24
Basal	0.029	0.027	0.035	0.036	0.004	--	0.87[5]	0.09[5]	0.35
EGFR Mean Intensity						<0.0001[4]	0.61	0.23	0.17
Suprabasal	0.028	0.020	0.017	0.020	0.004	--	0.47[6]	0.15[6]	0.22
Basal	0.024	0.018	0.015	0.019	0.004	--	0.79[5]	0.34[5]	0.47
BrdU Positive Cell Counts						--			
% Positive cells	23.8	21.3	22.4	24.9	1.63	--	0.98	0.52	0.14
MCT1 Mean Intensity						<0.0001[4]	0.64	0.92	0.96
Suprabasal	0.013	0.013	0.014	0.015	0.004	--	0.78[6]	0.77[6]	0.98
Basal	0.031	0.033	0.031	0.033	0.004	--	0.56[5]	0.90[5]	0.95
MCT4 Mean Intensity						<0.0001[4]	0.33	0.15	0.49
Suprabasal	0.015	0.020	0.021	0.023	0.003	--	0.24[6]	0.13[6]	0.26
Basal	0.009	0.013	0.015	0.015	0.003	--	0.45[5]	0.19[5]	0.45

[1]Significance declared when $P \leq 0.05$; three-way interaction not shown. Week interactions were all $P < 0.0001$. Treatments were either: Control (CON), butyrate (BUTY), lipopolysaccharide only (LPS-O), or butyrate + LPS (LPSB). All treatments were administered orally twice per day and consisted of either 0.9% saline (CON), 11m*M* sodium butyrate (BUTY), LPS ranging from 2.5 to 40 µg/kg $BW^{0.75}$ (adjusted weekly), or both butyrate and LPS (LPSB). Dosage volume increased as experiment progressed and ranged from 10 mL to 40 per dose.
[2]Treatments contained sodium butyrate.
[3]Treatments contained LPS.
[4]*P*-value for model effects.
[5]*P*-value for Basal when sliced by location.
[6]*P*-value for Suprabasal when sliced by location.
[7]*P*-value for week one sliced by week
[8]*P*-value for week eight sliced by week
*Contained LPS 0.04 ± 0.003; Did not contain LPS 0.03 ± 0.003.

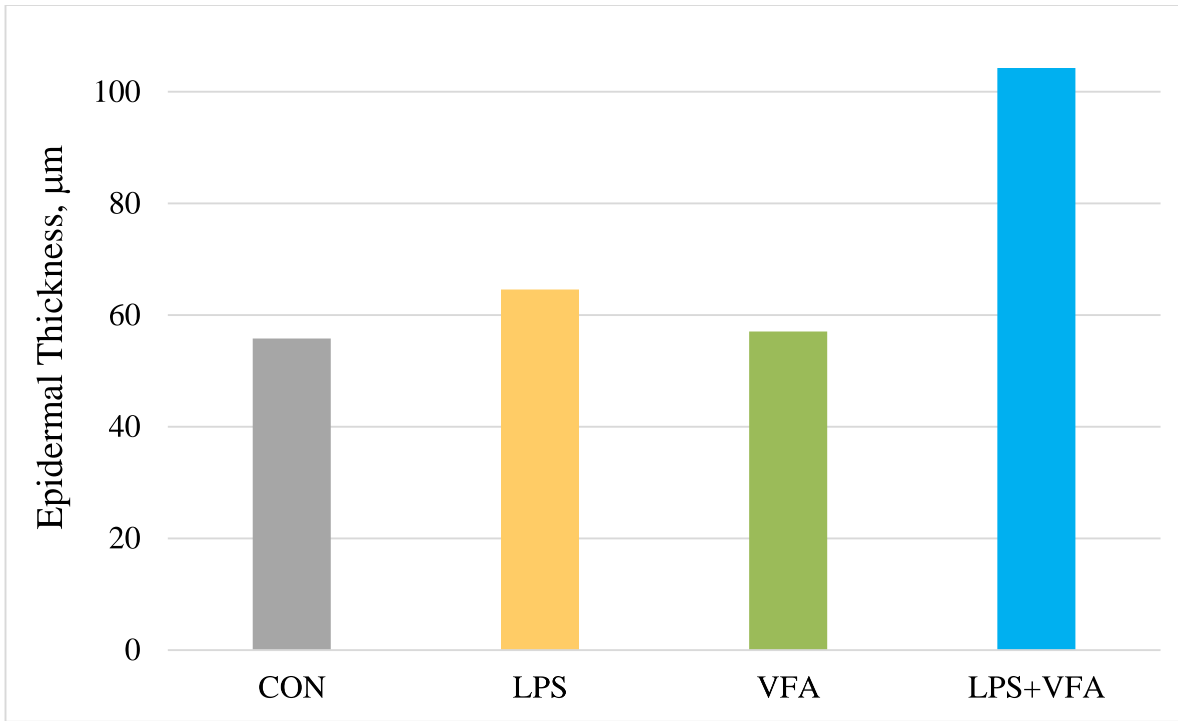

Figure 3.1. Each bar represents rumen epidermal thickness in a single calf that received 200mL per day of an oral treatment containing: 0.9% saline (CON); 20-40 µg/kg body weight of lipopolysaccharide (LPS); 11 m*M* sodium butyrate, 60 m*M* sodium acetate, 40 m*M* sodium propionate (VFA); or a combination of LPS and VFA (LPS+VFA) in a pilot trial. Epidermal thickness was highest for a calf administered LPS+VFA (Daniels et al., unpublished).

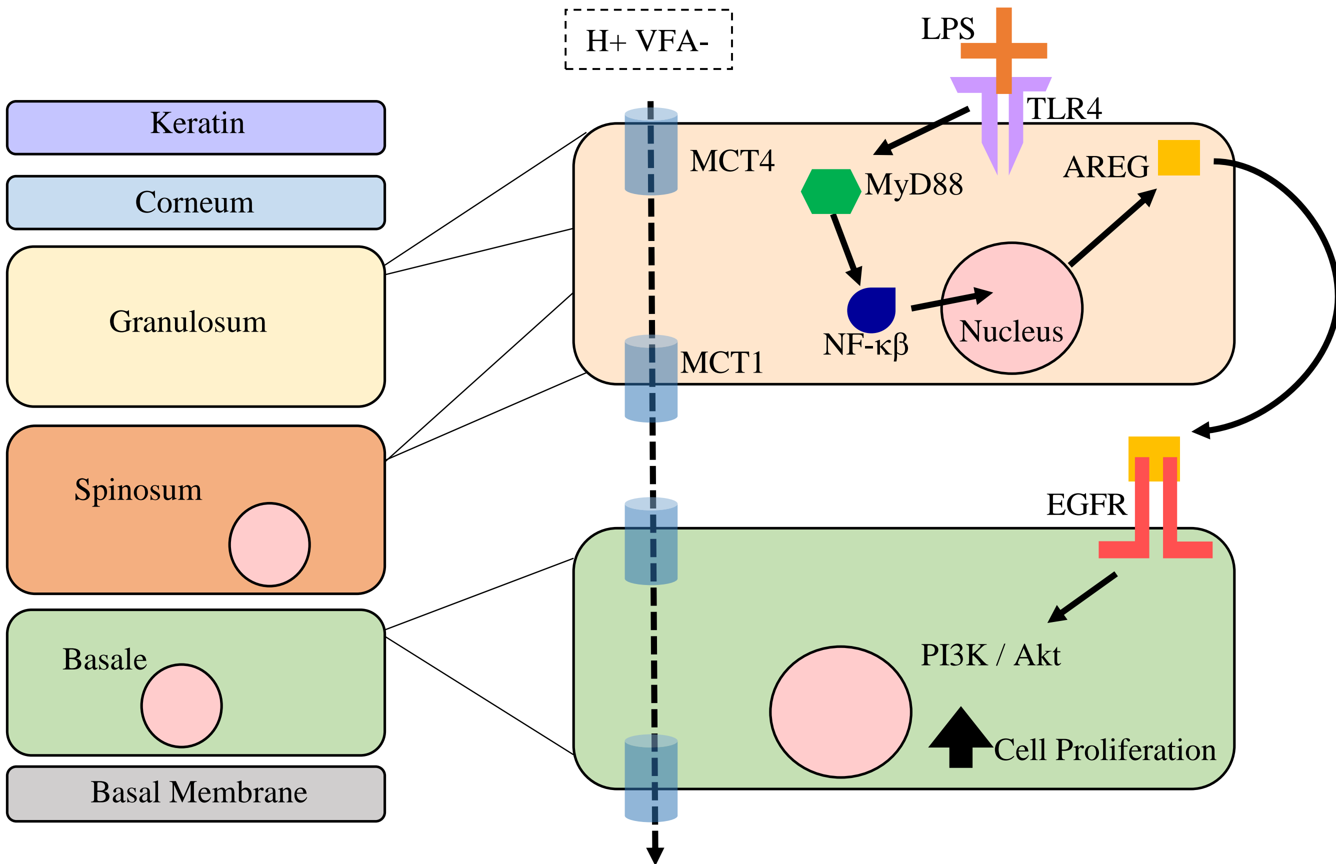

Figure 3.2. The layers of the rumen wall are shown from the lumen side starting with the keratin layer, down to the basal membrane before entering the bloodstream. The monocarboxylic transporter 4 (**MCT4**) is located on the first layer of living cells, the granulosum. This transports the volatile fatty acids (**VFA**) into the spinosum where monocarboxylic transporter 1 (**MCT1**) is responsible for transporting further to the bloodstream. Lipopolysaccharide (LPS) binds to the toll-like receptor 4 (**TLR4**) on the same membrane as MCT4. Once bound, TLR4 will regulated myeloid differentiation factor 88 (**MyD88**) which will then activate nuclear factor kappa B (**NF-κB**). The activation of NF-κB will start transcription in the nucleus and result in upregulation of amphoregulin (**AREG**). The protein AREG then binds to epidermal growth factor (**EGFR**) to stimulate the phosphoinositide 3-kinase/protein kinase B (**PI3K/Akt**) proliferation pathway. It is through this proposed mechanism that we suggest there lies a synergistic relationship between VFA and LPS.

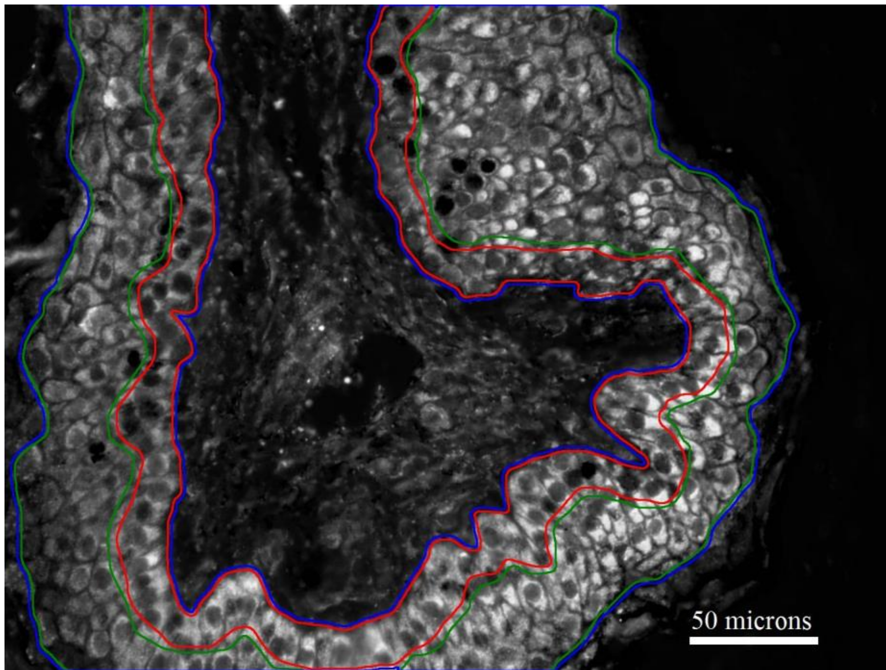

Figure 3.3. Rumen epithelium tissue from an 8 wk old Holstein calf at harvest. Manual tracing of the basal (between the pink and red lines) and suprabasal (between the green lines) layers of the rumen epithelium tissue was done to identify the separate layers. The suprabasal layer was defined as the corneum, granluosum, and spinosum, while the basal layer was simply just the basal layer alone.

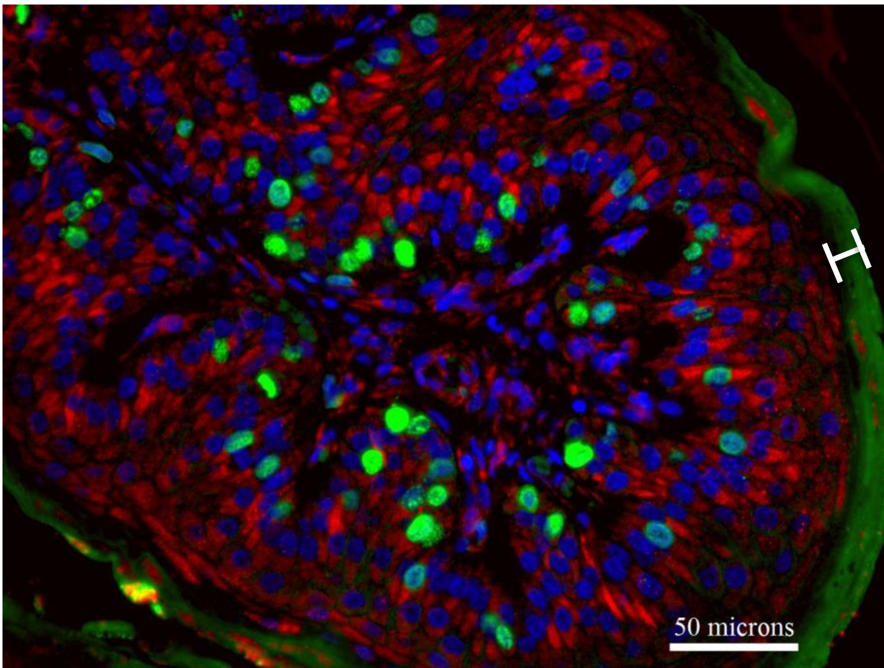

Figure 3.4. Representative pseudo-colored rumen papilla tip from an 8 wk old Holstein calf. Keratin is visible (white brackets; green color). Underneath the keratin layer is the stratum corneum and the stratum granulosum; cells in these layers are non-proliferative; note absence of nuclear proliferation marker bromo-deoxyuridine (BrdU; green color) staining. Based on detected staining patterns, both cells within the stratum spinosum and stratum basale have DNA synthetic capacity (BrdU; green color), with most DNA synthesis in the stratum basale, as predicted. Also shown in this image is representative localization of the pattern recognition receptor toll-like receptor 4 (TLR4; red color). The natural ligand for TLR4 is lipopolysaccharide from the cell wall of Gram-negative bacteria.

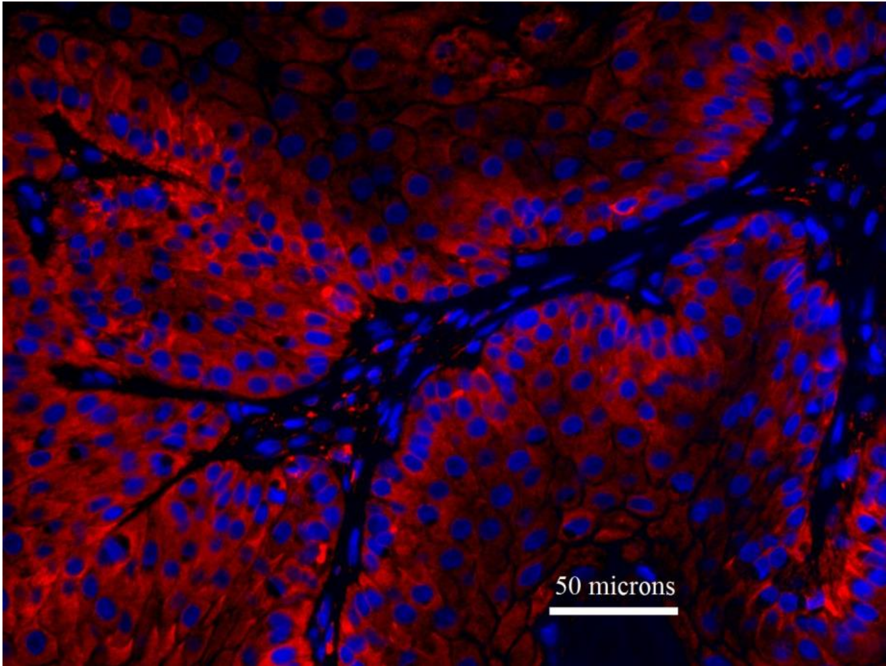

Figure 3.5. Rumen epithelium tissue from an 8 wk old Holstein calf at harvest. The outermost edge of the image represents the suprabasal layers (stratum corneum, stratum granulosum, and stratum spinosum). The cells closest to the center of the image where the black filling is with blue nuclei represents the basale layer. Representative monocarboxylic transporter 4 (MCT4; red color) staining in rumen epidermis. Nuclei are counterstained with DAPI (blue color).

CHAPTER 4: CONCLUSIONS AND IMPLICATIONS

The meta-analysis investigated the effect of dietary, environmental, and rumen factors independently and additively. The models resulted in similar relationships where the addition of rumen variables still resulted in most dietary and environment variables contributing to model relationships. While this does in no way conclude that the rumen variables do not contribute to calf performance, it seems that other environmental and dietary factors play a larger role. Similarly, the LPS study did not observe effects on rumen development as hoped, however looking further into gene expression data could provide more insight on what role LPS does play in rumen development. The data we measured (i.e BW, feed intake, organ weights, etc.) may be too large scale to notice metabolic effects of LPS.

Results from the meta-analysis emphasize the importance of continuing to focus on the solid feed intake of the calf from birth through weaning. Implications from the LPS study are imperative to other dairy scientists who will attempt to further study the effects of LPS on the rumen. This study established a baseline starting point for more research to build from.

This thesis provides novel information to the scientific field of dairy science and more specifically calf rumen development. While these studies may not have direct answers and implications, it is the start to a journey that will eventually provide the answers that scientists are looking for. As scientists continue to develop and test ideas, the metabolic processes that are happening inside the rumen of the calf will begin to become better understood.

APPENDICES

Table S1. List of the featured literature in the meta-analysis database and corresponding characteristics.

Study	Study region	Diet[1]	Number of calves	Weaning status[2]
Azevedo et al., 2016	South America	MS	32	not weaned
Beiranvand et al., 2014	Asia	MS/MSF	42	weaned
Bittar et al., 2017	South America	MS	24	weaned
Cannon et al., 2010	North America	M	27	not weaned
Castells et al., 2012	Europe	MS/MSF	15	weaned
Coverdale et al., 2004	North America	MS/MSF	60	weaned
Daneshvar et al., 2015	Asia	MS/MSF	40	weaned
Harris et al., 2017	North America	MS	60	not weaned
Kato et al., 2011	Asia	MS	24	not weaned
Khan et al., 2008	North America	MS/MSF	30	weaned
Khan et al., 2011	Asia	MSF	32	weaned
Kristensen et al., 2007	Europe	MSF	8	not weaned
Laarman et al., 2012	Asia	MSF/MF	20	weaned
Lesmeister et al., 2004a	North America	MS	92	weaned
Lesmeister et al., 2004b	North America	MS	6	weaned
Lesmeister el al., 2005	North America	MS	75	weaned
Mirzaei et al., 2016	Asia	MS/MSF	48	weaned
Muya et al., 2015	Africa	MS	14	not weaned
Oltramari et al., 2016	South America	MSF	27	weaned
Oltramari et al., 2018	South America	MSF	24	weaned
Ragionieri el al., 2016	Europe	MS	20	not weaned
Saegusa et al., 2017	Asia	MSF	60	weaned
Santos et al., 2015	South America	MSF	30	weaned
Schaff et al., 2018	Europe	MS	28	not weaned
Silper et al., 2014	South America	MSF	42	not weaned/weaned
Steele et al., 2016	North America	MSF	34	weaned
Suarez et al., 2006	Europe	MS/MSF	64	not weaned
Suarez et al., 2007	Europe	M/MS	160	not weaned
Suarez-Mena et al., 2011	North America	MS	34	not weaned
Suarez-Mena et al., 2015	North America	MSF	24	not weaned
Suarez-Mena et al., 2016	North America	MS	17	weaned
Wang et al., 2017	Asia	MSF	56	not weaned/weaned
Wood et al., 2015	North America	MS	14	not weaned/weaned
Xiao et al., 2016	Asia	MS	30	not weaned
Xie et al., 2013	Asia	M/MSF	39	not weaned/weaned
Yohe et al., 2015	North America	MS	52	not weaned/weaned

[1] Diet code included 3 variations: Milk replacer only diet (M), milk replacer and starter diet (MS), or milk replacer, starter, and forage diet (MSF).

[2] Weaning status was based on calves that were weaned or not by the final day of the study.

Table S2. Parameter estimates and model fit statistics weighted by n, for models predicting rumen response variables (**Figure 2.2**; Relationship A)

	Reticulorumen[1]	Rumen contents[2]	Rumen pH	Papillae area	Rumen wall[3]	Acetate[4]	Propionate[5]	Butyrate[6]
Model no.	1b	2b	3b	4b	5b	6b	7b	8b
Intercept	2.36	4.75	4.51	6.15	4.95	59.5	57.4	16.5
Variables[7]								
For Fat	-0.0991							
Form Text	-0.577							
Daily St	0.505		0.675					
Daily MR			1.26			-28.4	-21.6	-12.9
St Starch				-0.0218				
Pct MR		-7.04					-20.4	
Total MRI		0.0576						
MR fat	-0.0695							
Wean YES	0.559							
Tot For					0.0399			
Tot St.						0.311		0.0990
MS Code		2.16						
MSF Code		3.52						
Both Gend	-0.834							
Fit Statistics								
n[8]	38	33	46	54	46	51	51	51
CCC[9]	0.975	0.953	0.946	0.870	0.998	0.867	0.887	0.920
uCCC[10]	0.919	0.745	-0.225	0.426	-0.000	0.408	0.692	0.490
$\hat{\sigma}_s$[11]	0.196	1.81	0.615	3.04	5.45	15.0	6.12	5.07
$\hat{\sigma}_e$[12]	0.333	2.97	0.626	5.68	1.34	24.0	18.2	7.95

[1] Empty reticulorumen weight.
[2] Rumen contents weight.
[3] Rumen wall thickness (submucosa + muscularis).
[4] Ruminal acetate concentration (in m*M*).
[5] Ruminal propionate concentration (in m*M*).
[6] Ruminal butyrate concentration (in m*M*).
[7] Forage fat (For Fat), texturized form of starter (Form Text), daily starter intake (Daily St), daily milk replacer intake (Daily MR), starch content of the starter fed (St Starch), percent of the total diet that composed of milk replacer (Pct MR), total milk replacer intake (Tot MRI), fat content of the milk replacer (MR fat), calves that were weaned by the end of the study (Wean YES), total forage intake (Tot For), total starter intake (Tot St.), diet that consisted of milk replacer and starter (MS code), diet that consisted of milk replacer, starter, and forage (MSF code), gender of calves both heifers and bulls (Both Gend).
[8] n = number of observations.
[9] Concordance correlation coefficients.
[10] Unadjusted concordance correlation coefficients.
[11] Square root of the estimated study variance.
[12] Square root of the estimated residual variance.

Table S3. Parameter estimates and model fit statistics weighted by n, for models predicting performance response variables (**Figure 2.2**; Relationship B)

	ADG[1]	F:G[2]	Tot St.[3]	Tot. MRI[4]	Tot. For[5]	Daily St.[6]	Daily MR[7]	Daily For[8]
Model no.	9b	10b	11b	12b	13b	14b	15b	16b
Intercept	0.607	2.41	52.8	88.3	-23.6	1.33	1.25	-0.209
Variables[9]								
Breed Hols		1.11		-23.3				
Wean YES		0.279			23.4			0.143
Final Age						-0.0074		
Vent YES		-1.52		-16.0				
Init BW				0.341				
Daily St				-13.7	36.1			
Daily MR		-0.481	-37.4		10.0			
For Fat				-1.023		0.0183		0.0158
Daily For	3.24			15.4			0.108	
St Starch			0.056	0.066				
St NDF				0.111				
Pct St					-47.8		-0.321	
Temp YES					15.3			
Starter DM				-0.070				
Form Pell							-0.240	
Form Text							-0.572	
Pct MR						-1.49		
St ADF						0.0010		
Final BW						0.0047		0.0019
Fit Statistics								
n[10]	121	87	68	69	65	77	80	121
CCC[11]	0.961	0.939	0.937	0.994	0.933	0.991	0.998	0.876
uCCC[12]	0.058	0.703	0.188	0.856	0.817	0.825	0.161	0.454
$\hat{\sigma}_s$[13]	0.210	0.512	42.7	10.6	9.82	0.189	0.705	0.103
$\hat{\sigma}_e$[14]	0.182	0.875	33.1	7.51	18.7	0.153	0.115	0.199

[1] Average daily gain (ADG); [2] Feed to gain ratio (F:G); [3] Total starter intake (Tot St.); [4] Total milk replacer intake (Tot. MRI); [5] Total forage intake (Tot. For); [6] Daily starter intake (Daily St.); [7] Daily milk replacer intake (Daily MR); [8] Daily forage intake (Daily For).

[9] Breed of Holstein calves (Breed Hols), calves that were weaned (Wean YES), final age of the calves on the study (Final Age), calves were kept in a ventilated environment (Vent YES), initial body weight (Init. BW), daily starter intake (Daily St), daily milk replacer intake (Daily MR), forage fat (For Fat), daily forage intake (Daily For), starch content of the starter fed (St Starch), neutral detergent fiber of starter (St NDF), percent of the total diet that was composed of starter (Pct St), temperature controlled environment (Temp YES), dry matter of the starter (Starter DM), pelleted form of starter (Form Pell), texturized form of starter (Form Text), percent of the total diet that was composed of milk replacer (Pct MR), acid detergent fiber of starter (St ADF), final body weight (Final BW).

[10] n = number of observations. [11] Concordance correlation coefficients. [12] Unadjusted concordance correlation coefficients.

[13] Square root of the estimated study variance. [14] Square root of the estimated residual variance.

Table S4. Parameter estimates and model fit statistics weighted by n, for models predicting performance response variables (**Figure 2.2**; Relationship C)

	ADG[1]	F:G[2]	Tot St.[3]	Tot. MRI[4]	Tot. For[5]	Daily St.[6]	Daily MR[7]	Daily For[8]
Model no.	17b	18b	19b	20b	21b	22b	23b	24b
Intercept	-0.669	2.10	-177	-88.9	-7.88	0.464	1.01	
Variables[9]								
Papillae area	-0.0257	0.0350				0.108		
Empty Ret	0.258		103		4.33			
Rumen pH	0.182			28.2				
Propionate			2.89		0.094		-0.00550	
Butyrate							-0.0156	
Rumen cont				-3.67				
Fit Statistics								
n[10]	38	70	43	35	42	51	57	
CCC[11]	0.947	0.978	0.962	0.971	0.905	0.987	0.929	
uCCC[12]	0.891	0.039	0.796	-0.230	0.759	0.019	0.272	
$\hat{\sigma}_s$[13]	0.093	0.848	65.5	30.0	2.73	0.674	0.196	
$\hat{\sigma}_e$[14]	0.269	0.601	75.0	19.5	4.42	0.324	0.227	

[1] Average daily gain (ADG); [2] Feed to gain ratio (F:G); [3] Total starter intake (Tot St.); [4] Total milk replacer intake (Tot. MRI); [5] Total forage intake (Tot. For); [6] Daily starter intake (Daily St.); [7] Daily milk replacer intake (Daily MR); [8] Daily forage intake (Daily For).
[9] Area of rumen papillae by multiplying length times width (Papillae area), weight of the empty reticulorumen (Empty Ret), pH of ruminal contents (Rumen pH), propionate concentration (Propionate), butyrate concentration (Butyrate), weight of rumen contents (Rumen cont).
[10] n = number of observations.
[11] Concordance correlation coefficients.
[12] Unadjusted concordance correlation coefficients.
[13] Square root of the estimated study variance.
[14] Square root of the estimated residual variance.

Table S5. Parameter estimates and model fit statistics weighted by n, for models predicting performance response variables (**Figure 2.2**; Relationship D)

	ADG[1]	F:G[2]	Tot St.[3]	Tot. MRI[4]	Tot. For[5]	Daily St.[6]	Daily MR[7]	Daily For[8]
Model no.	25b	26b	27b	28b	29b	30b	31b	32b
Intercept	0.607	0.990	46.1	362	-7.88	1.33	1.25	-1.08
Variables[9]								
Empty Ret			35.8		4.33			
Propionate					0.0945			
Final Age						-0.00741		
Wean YES								0.608
Papillae area		0.0330						-0.0446
Starter CP		0.0144						
For Fat						0.0183		0.0533
Daily For	0.324						0.108	
St Starch		-0.00278		-0.0400				
Daily MR			-181					
Starter DM			0.0967					
Pct St						-0.446	-0.321	
Rumen Cont				-6.15				
MR CP				-10.9				
Form Pell							-0.240	0.513
Form Text							-0.572	0.319
Starter ADF						0.00104		
Pct MR						-1.49		
MSF code		0.404						
Final BW						0.00469		0.00493
Fit Statistics								
n[10]	121	51	30	27	45	98	80	65
CCC[11]	0.961	0.967	0.927	0.983	0.905	0.998	0.998	0.985
uCCC[12]	0.058	0.779	0.924	0.483	0.759	0.985	0.161	0.309
$\hat{\sigma}_s$[13]	0.210	0.352	3.73	23.9	2.73	0.0860	0.705	0.357
$\hat{\sigma}_e$[14]	0.182	0.440	50.1	16.6	4.42	0.116	0.115	0.114

[1] Average daily gain (ADG); [2] Feed to gain ratio (F:G); [3] Total starter intake (Tot St.); [4] Total milk replacer intake (Tot. MRI); [5] Total forage intake (Tot. For); [6] Daily starter intake (Daily St.); [7] Daily milk replacer intake (Daily MR); [8] Daily forage intake (Daily For).

[9] Weight of the empty reticulorumen (Empty Ret), propionate concentration (Propionate), final age of the calves on the study (Final Age), calves that were weaned (Wean Yes), area of rumen papillae by multiplying length times width (Papillae area), crude protein content of the starter (Starter CP), forage fat (For Fat), daily forage intake (Daily For), starch content of the starter (St Starch), daily milk replacer intake (Daily MR), dry matter of the starter (Starter DM), percent of the total diet that was composed of starter (Pct St), weight of the rumen contents (Rumen Cont), crude protein of the milk replacer (MR CP), pelleted form of starter (Form Pell), texturized form of starter (Form Text), acid detergent fiber of the starter (Starter ADF), percent of the total diet that was composed of milk replacer (Pct MR), diet that consisted of milk replacer, starter, and forage (MSF code), total milk replacer intake (Tot MRI),), initial body weight (Init. BW), final body weight (Final BW)

[10] n = number of observations.

[11] Concordance correlation coefficients.

[12] Unadjusted concordance correlation coefficients.

[13] Square root of the estimated study variance.

[14] Square root of the estimated residual variance.

Table S6. Correlation table for numerical dietary variables.

	MR CP	MR fat	Daily St	Tot St	Daily MR	Tot MR	Tot For	Daily For	Pct MR	Pct St	Final age	Init BW	Final BW	St DM	St CP	St NDF	St ADF	St Ash	St Fat	St Star	For DM	For CP	For NDF	For ADF	For Ash	For Fat	For Star
MR CP	1.00																										
MR fat	0.50	1.00																									
Daily St	0.06	0.35	1.00																								
Total St	0.06	0.13	0.97	1.00																							
Daily MR	0.82	0.70	-0.28	-0.22	1.00																						
Tot MR	0.09	0.01	-0.18	0.00	0.45	1.00																					
Tot For	0.28	0.40	0.20	0.20	0.68	0.07	1.00																				
Daily For	0.29	0.41	0.20	0.13	0.70	0.07	0.98	1.00																			
Pct MR	-0.06	-0.18	-0.82	-0.67	0.19	0.23	-0.29	-0.27	1.00																		
Pct St	0.04	0.09	0.81	0.67	-0.39	-0.22	0.00	-0.01	-0.95	1.00																	
Final age	0.17	0.08	0.46	0.63	0.15	0.55	0.33	0.28	-0.49	0.45	1.00																
Init BW	0.40	0.33	-0.17	-0.11	0.94	0.11	0.62	0.65	0.10	-0.26	0.04	1.00															
Final BW	0.31	0.16	0.19	0.53	0.58	0.67	0.42	0.37	-0.19	0.14	0.82	0.30	1.00														
St DM	0.27	-0.08	0.35	0.19	-0.11	0.02	-0.08	-0.08	-0.41	0.38	0.22	0.07	0.18	1.00													
St CP	0.18	-0.16	0.15	0.16	-0.02	0.31	-0.04	-0.04	-0.26	0.28	0.37	0.14	0.32	0.78	1.00												
St NDF	0.12	-0.25	0.19	0.09	-0.06	0.05	-0.03	-0.03	-0.33	0.33	0.28	0.13	0.24	0.88	0.79	1.00											
St ADF	0.14	-0.28	0.29	0.12	-0.01	0.08	-0.01	-0.00	-0.34	0.35	0.31	0.13	0.26	0.86	0.76	0.98	1.00										
St Ash	0.30	0.20	0.31	0.24	-0.01	0.05	-0.02	-0.03	-0.43	0.41	0.22	0.03	0.09	0.76	0.58	0.39	0.33	1.00									
St Fat	-0.01	-0.00	0.15	0.16	-0.10	-0.12	-0.04	-0.03	-0.28	-0.08	-0.12	-0.00	-0.08	0.43	0.15	0.20	0.12	0.23	1.00								
St Starch	0.07	-0.28	0.04	-0.01	-0.33	-0.10	-0.22	-0.22	-0.21	0.23	0.07	-0.11	0.02	0.89	0.61	0.76	0.71	0.48	0.32	1.00							
For DM	0.27	0.25	0.60	0.43	0.34	0.04	0.35	0.41	-0.38	0.34	0.31	0.22	0.28	0.29	0.25	0.27	0.27	0.22	0.09	0.07	1.00						
For CP	0.38	0.42	0.62	0.51	0.54	0.06	0.51	0.55	-0.43	0.35	0.33	0.34	0.36	0.21	0.19	0.15	0.17	0.21	0.16	-0.10	0.91	1.00					
For NDF	0.18	0.12	0.53	0.35	0.17	0.02	0.22	0.28	-0.33	0.32	0.28	0.14	0.21	0.32	0.29	0.32	0.30	0.19	0.06	0.17	0.97	0.81	1.00				
For ADF	0.22	0.18	0.54	0.38	0.24	0.03	0.25	0.31	-0.34	0.32	0.30	0.18	0.25	0.32	0.29	0.31	0.30	0.23	0.07	0.16	0.98	0.84	0.99	1.00			
For Ash	0.31	0.33	0.61	0.47	0.44	0.05	0.43	0.49	-0.40	0.34	0.33	0.28	0.32	0.25	0.22	0.21	0.22	0.21	0.08	-0.01	0.98	0.96	0.93	0.95	1.00		
For Fat	0.14	0.17	0.45	0.32	0.30	-0.01	0.22	0.27	-0.35	0.32	0.26	0.17	0.18	0.27	0.21	0.18	0.16	0.32	0.09	0.06	0.85	0.73	0.88	0.89	0.83	1.00	
For Starch	0.01	-0.07	0.06	0.09	-0.00	0.04	0.02	0.02	-0.10	0.12	0.23	0.05	0.13	0.15	0.33	0.26	0.23	0.00	-0.05	0.19	0.24	0.26	0.33	0.30	0.26	0.34	1.00

*Denotes $P<0.05$

Table S7. Correlation table for numerical rumen variables.

	Reticulorumen[1]	Rumen contents[2]	Rumen pH	Papillae area	Rumen wall[3]	Butyrate[4]	Acetate[5]	Propionate[6]
Reticulorumen[1]	1.000							
Rumen contents[2]	0.459*	1.000						
Rumen pH	0.170	0.028	1.000					
Papillae area	0.742*	-0.333	0.279	1.000				
Rumen wall[3]	-0.084	0.782*	0.439*	-0.040	1.000			
Butyrate[4]	0.303*	0.710*	-0.310*	0.066	0.232	1.000		
Acetate[5]	0.177	0.438*	-0.434*	0.070	-0.457*	0.618*	1.000	
Propionate[6]	0.283*	0.047	-0.267*	0.178	0.115	0.589*	0.712*	1.000

*Denotes $P<0.05$

[1] Empty reticulorumen weight.
[2] Rumen contents weight.
[3] Rumen wall thickness (submucosa + muscularis).
[4] Ruminal butyrate concentration (in m*M*).
[5] Ruminal acetate concentration (in m*M*).
[6] Ruminal propionate concentration (in m*M*).