CALF INTESTINAL HEALTH: ASSESSMENT AND DIETARY INTERVENTIONS FOR ITS IMPROVEMENT

BY

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DISSERTATION

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ABSTRACT

Digestive problems are common in calves between birth and weaning, and about 25% of pre-weaned heifer population in the US commonly presents diarrhea. Moreover, about 57% of the mortality in pre-weaned heifers is due to diarrheal disease. Costs associated with high mortality and reduced productivity exceed $200 million annually.

Part of the problem is that methods to assess gut health in pre-weaning dairy calves at the farm level are not well defined. Aside from sound facility hygiene protocols, there is a lack of effective preventive gut health schemes and mostly ex-post therapeutic schemes are available. Development of complementary ex-ante quantitative models based on gut health measures at the calf and tissue level could help prevent disease.

Here, results from analysis of calf level data are reported, where quantitative changes in calf gut health throughout the first month of life as measured by fecal scores, and their relationship with thermal variability, blood protein and bodyweight at birth, are described. We also assessed the changes in calf gut health as measured by intestinal paracellular permeability in response to age, failure of passive transfer, and intestinal location.

Analysis of fecal score data indicated that risk for intestinal disease changed hyperbolically from 5 to 35 days with a risk peak around 13 days of age (~0.7), which agrees with previous reports in the literature. The variations in time, however, depended on the calf-specific features noted above, suggesting that variables related to thermal homeostasis, passive immunity obtained by the calf, and variables related to fetal maturity in early life might influence the risk of diarrheal episodes. However, the direction and size of such effects were not consistent and seem to be dependent upon other unknown variables that evolve over time.

On the other hand, gut health as measured by paracellular permeability in Ussing chambers appeared not to be intrinsically related with time but colon appeared to be the tissue having the largest changes in permeability compared to other GIT regions. Also, failure of passive transfer appeared to have no influence on gut health at the tissue level even though it reduced the odds (0.84/day) of scour episodes at the calf level. More data on intestinal permeability should be collected in response to key predictor variables. Furthermore, indicators of gut health other than just permeability need to be evaluated for development and implementation of quantitative models to be feasible. Specific areas of search for risk factors, including calf immune, intestinal pathogen load and metabolomic/metabonomic profiling appear to be promissing for development of calf and tissue level empirical prediction models that support individual calf preventive care protocols.

Another portion of this research related to the assessment of the effect of age and gastrointestinal location (rumen vs colon) on microbiota diversity and composition, and short-chain fatty acid profiles of Holstein pre-ruminant calves. Compared to rumen, higher lactic acid bacteria abundance (e.g. *Lactobacillus*: 51 vs 2 %, $p < 0.001$) and high concentrations of lactate (0.1 vs 1.5 mmol/mL, $p<0.05$) and butyrate (0.4 vs 2.3 mmol/mL, $p<0.05$) were observed in the colon at 2 and 4 weeks of age. Comparatively, ruminal microbiota was not stable and varied more between animals by 4 weeks of age. Such microbiota and fermentation profiles in the hind gut could help prevent diarrheal infections.

In addition, the prebiotic potential of enzymatically treated whey permeate rich in galactooligosacharides was evaluated. Galactooligosacharide (GOS) supplementation at 3.4% of dietary dry matter caused a dramatic increase in the lactobacillus (Control: 39, GOS: 63%) and bifidobacterium (Control: 2.3, GOS: 5.6%) populations along with more developed intestinal

epithelial structures. Nevertheless, greater odds (2.36, $p<0.001$) of higher fecal scores, indicative of diarrhea, were observed as a result of prebiotic supplementation. Because epithelium barrier function was unaffected, we hypothesize that a laxative effect from prebiotic overdosing caused the diarrhea like condition observed in these animals. Definition of the galactooligosacharide dose response curves and its dependence on milk feeding rates is crucial to optimize use of this prebiotic in terms of calf health.

Lastly, another experiment was conducted to test the hypothesis that glucose, non-metabolizable glucose, stevioside and a glutamate source could enhance GLP-2 secretion and thereby increase gut growth or health of newborn Holstein calves. No effect of any treatment on intestinal growth and development, as measured by epithelium histomorphological dimensions, cellular proliferation, gastrointestinal organ size or the mRNA expression of the GLP2 signaling pathway, was observed. Such lack of response was perhaps due to consideration of one single fixed food intake rate and failure to identify supplementation levels that could stimulate incretin release, even though previously published and non-published work with models such as lambs and pigs, respectively, had detected responses at these concentrations. Species-specific sensitivity to sweet flavor or glutamate may render direct dose extrapolation between species inappropriate. Species-specific dose response surfaces, likely in interaction with food intake levels, may provide a better approach to optimize the incretin release response.

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I must thank my parents first of all for putting in all the effort and giving up your own dreams to raise me and get me going. When we were little, you would not eat to feed me and my siblings. I will always owe you. I am what you made of me. Thanks for you unconditional love Ciro and Janeth.

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CHAPTER I

LITERATURE REVIEW

Introduction

Digestive health in pre-weaning calves. Digestive problems are common in calves between birth and weaning. According to the last national survey conducted by The National Animal Health Monitoring System (NAHMS, 2010), about 25% of pre-weaned heifers in the US had diarrheal diseases during 2007. Moreover, 7.8 % of the pre-weaned heifers died during this period and scours or other digestive problems accounted for 57% of this mortality (NAHMS, 2007). Costs associated with high mortality and reduced productivity exceed $200 million annually, excluding expenses of antibiotic treatment, lost feed, labor and overhead costs (Quigley et al., 2005).

Scours (i.e., feces with dry matter below 10%) in the newborn calf involve an interaction between one or more pathogenic bacterial, viral or protozoal microorganisms; the immune status of the calf; and the environment. It is estimated that enterotoxigenic *Escherichia coli* (ETEC), rota- and corona-viruses, and *Cryptosporidium spp*. together account for 75 to 95 % of all cases of neonatal calf diarrhea (Butler and Clarke, 1994).

Neonatal calf diarrhea can take place in calves from 3 to 21 days of age and leads to loss of extracellular fluids and electrolytes (Butler and Clarke, 1994). The onset and duration of a diarrheal episode will depend on the number of pathogens involved and the immune condition of the animal (Butler and Clarke, 1994). In chronic cases, bodyweight loss results from dehydration and muscle wasting due to protein catabolism induced by inadequate caloric intake and poor digestion (Butler and Clarke, 1994).

The resultant growth delay may have major financial consequences in the long run for the farm. A recent report on the impact of pre-weaning growth performance on first lactation yield of dairy cows in a research herd found that, for every 1 kg of pre-weaning average daily weight gain (ADG), heifers, on average, produced 850 kg more milk during their first lactation and 235 kg more milk for every megacalorie of metabolizable energy intake above maintenance; whereas, in a commercial herd, for every 1 kg of preweaning ADG, milk yield increased by 1,113 kg in the first lactation and, further, every 1 kg of prepubertal ADG was associated with a 3,281 kg increase in first-lactation milk yield (Soberon et al., 2012). Moreover, prepubertal growth also determines the age at first breeding and first lactation; while calving below optimal bodyweight (BW) results in lower milk yield (NAHMS, 2010).

Calf diarrhea and intestinal function and integrity

Etiology of diarrhea in calves. Development of microbial populations in the gastrointestinal tract (GIT) of the newborn calf commences soon after birth (Izzo et al., 2011) and while pathogens autochthonous to the gut ecosystem can live in harmony with the host during this process, they can become pathogenic under certain circumstances (Izzo et al., 2011). Some commensal organisms such as *Bifidobacteria* and *Lactobacilli* species have been linked to increased resistance to pathogen infection and diarrheal disease (Izzo et al., 2011) by, for instance, competing for nutrients and attachment sites in the gut epithelium, producing bacteriocins, increasing production of short-chain fatty acids (SCFA) that lower intestinal pH, and stimulating the immune system (Izzo et al., 2011). But under specific calf and environmental circumstances, as of yet not fully understood, pathogens can override the colonization resistance of such commensal organisms, spread and cause infection.

Thus, the most commonly incriminated agents in neonatal calf scours include viral (rotavirus and coronavirus), protozoal (*Cryptosporidium parvum*) and bacterial pathogens (enterotoxigenic *Escherichia coli* and *Salmonella* spp.) (Izzo et al., 2011).

These pathogens colonize and spread through distal small and proximal large intestine (Butler and Clarke, 1994), and can cause secretory or exudative diarrhea. For example, ETEC and *Salmonella spp*. produce toxic peptides that induce secretory diarrhea where electrolyte and water secretion is triggered by the direct effect of the toxin on the cell's secretory system as mediated by adenylyl cyclase and cyclic adenosine monophosphate (cAMP)synthesis. However, like rotavirus and coronavirus, they can also impact the intestinal tissue and cause cell loss and tissue disruption, thereby compromising the epithelial barrier function. This leads to exudative diarrhea with water, electrolytes, mucus, and protein accumulating luminally as a result of permeability loss (Sears, 2000; Field, 2003; Ulluwishewa et al., 2011).

Pathogen-induced secretory diarrhea. Chloride secretion is the principal determinant of luminal water outflow. With Cl^- secretion, paracellular movement of Na^+ follows. The resulting accumulation of luminal NaCl provides an osmotic gradient for the diffusion of water (Field, 2003).

In the case of secretory diarrhea induced by ETEC, a heat labile enterotoxin can bind to a glycolipid in the enterocyte membrane where it is endocytosed into the cell to elicit activation of adenylyl cyclase and synthesis of cAMP, which causes an increase in its cellular concentration [cAMP] (Field, 2003). High [cAMP] induces electrolyte net secretion by: (a) stimulation of Cl^- secretion into the lumen against the electrochemical gradient, and (b) inhibition of NaCl absorption (Field, 2003; Croxen and Finlay, 2010).

High [cAMP] causes Cl^- secretion into the lumen by opening the Cl^- secretory channel (CFTR) on the apical membrane, thereby triggering its dumping into the intestine (Croxen and Finlay, 2010).

At the same time, the high [cAMP] opens a basolateral membrane K^+ channel that enables K^+ efflux, hence re-polarizing the cell and counteracting the depolarizing effect of the opening of the Cl^- channel, which maintains the electrical driving force for the continued Cl^- secretion into the lumen. High [cAMP] also enhances the basolateral influx of Cl^- into the cell through the NaK_2Cl channel to sustain prolonged Cl^- secretion into the intestine. The net result is Cl^- secretive loss (Berkes et al., 2003). As a result, during secretion, the cell's electric potential is ~ -40 to -50 mV and the serosal potential is 5 to 30 mV, both relative to the lumen (Berkes et al., 2003). This transcellular potential difference drives Na^+ movement into the lumen through the lateral paracellular spaces in order to reach electroneutrality. Water follows Na+ and Cl- intestinal secretion osmotically (Berkes et al., 2003; Field, 2003; Croxen and Finlay, 2010).

Pathogen-induced exudative diarrhea. In the case of exudative diarrhea, enteric pathogens disrupt the intestinal barrier either by directly binding to cell-surface molecules and inducing changes in tight junction (TJ) proteins or indirectly by releasing toxins, which cause cell damage, alter epithelial ion transport, and disrupt TJ and the cytoskeleton (Sears, 2000; Groschwitz and Hogan, 2009; Croxen and Finlay, 2010; Ulluwishewa et al., 2011).

Tight junctions are adhesive junctional protein complexes between epithelial cells that form a continuous belt-like ring around the cells. These multiprotein complexes function as a semipermeable paracellular barrier, which facilitates the passage of ions and solutes through the intercellular space while preventing the translocation of luminal antigens, microorganisms, and their toxins. The TJ consist mainly of 3 families of interacting transmembrane proteins: occludin,

claudins, and junctional adhesion molecules (JAM). These proteins interact extracellularly with each other to hold adjacent cells together and intracellularly with adaptor proteins such as zonula occludens 1, 2, and 3 (ZO-1, ZO-2, ZO-3) that link them to the actin cytoskeleton of the cell, thereby tightening the whole structure and maintaining the selective permeability capacity of the tissue (Liu et al., 2005; Turner, 2006). Pathogen disturbance of the TJ with the concomitant loss of selective permeability is illustrated below.

For instance, enteropathogenic *Escherichia coli* (EPEC) attachment and infection stimulates phosphorylation of the myosin light chains (MLC) by MLC kinase, inducing contraction of the perijunctional actomyosin ring. This exerts tension on the cell membrane in the region of the zonula adherens where the perijunctional actomyosin ring inserts. This tension is transmitted to the TJ thereby increasing paracellular permeability. Additionally, EPEC also causes occludin dephosphorylation and relocation from the TJ into the cytosol of the cell, further disrupting the TJ and increasing permeability (Sears, 2000; Groschwitz and Hogan, 2009; Ulluwishewa et al., 2011). Gutmann et al. (2006) showed that in vivo infection with a pathogen capable of attaching to and effacing the microvillus border of the enterocyte, similar to EPEC, caused alteration of theTJ by relocation of the transmembrane TJ proteins claudin 1, 3, and 5. This resulted in increased permeability and colon luminal water content (Guttman et al., 2006).

Another example is the toxin produced by *Clostridium perfringens.* Exposure of intestinal cells to the toxin results in tissue damage followed by fluid and electrolyte secretion and increased tissue permeability. Structurally, this toxin has been shown to induce disruption of TJ fibrils and remove claudin 4 from the TJ in vitro (Berkes et al., 2003).

Also, exposure to the toxins A and B produced by *Clostridium difficile* causes degradation of the cytoskeleton filamentous actin leading to increased permeability and

paracellular flux, possibly due to TJ disruption. Parallel to this is the direct effect on the relocation of TJ proteins such as ZO-1 and occludin away from the TJ and into the cytoplasm of the cell (Berkes et al., 2003).

Furthermore, in vitro, exposure of Caco-2 cells to rotavirus has resulted in increased paracellular permeability and disorganization of occludin as well (Sears, 2000).

Immune status of the calf and its impact on animal health

As mentioned above, the immune status plays a major part in neonatal calf diarrhea (Butler and Clarke, 1994). Immunoglobulins transferred from the cow to the calf through the colostrum are all the immune endowment of the offspring at birth. Poor immunoglobulin transfer is called failure of passive transfer (FPT) and is linked to increased neonatal morbidity and mortality in dairy calves (Robison et al., 1988; Quigley et al., 2005). According to NAHMS (NAHMS, 2010), the estimated prevalence of FPT in US dairy heifer calves is about 20%.

On the other hand, although all essential immune components are present in neonates at birth, many of the components are not functional until calves are at least 2 to 4 weeks of age and may continue to develop until puberty. In part, this is related to immune suppression from placental production of progesterone, prostaglandin E2, and cytokines (e.g., IL-4 and Il-10) that affect the near-term fetus and the dam and suppress cell-mediated and memory (TH1) responses (Chase et al., 2008). Cows also produce estrogen and cortisol before parturition that have immunosuppressive effects. Finally, as part of the parturition process calves produce high levels of cortisol; which remain elevated for the first weeks of life. Thus, the cumulative effect of these hormones is to suppress immune responses and direct the immune response away from the TH1 response (Chase et al., 2008).

Furthermore, stress related suppression of the active immune system has also been related with intestinal infection and pathogen shedding (Vilte et al., 2011). The term "super shedders" has been used to describe animals which shed *E. coli* O157 at higher levels and for a longer duration compared to other animals in the same herd. One possible factor that may influence the presence of "super shedders" is immunosuppression potentially related to stress. Indeed, dexamethasone-treated calves (i.e. corticosteroid immunodepressed) have been shown to increase *E. coli* shedding by ~33% ($p=0.04$) during the days when they are immunosuppressed but not in the long-term. This is accompanied by a reduction of ~70% in the number of lymphocytes and an increase in the number of neutrophil granulocytes of ~47% in blood (Sreerama et al., 2008). However, when calves have been vaccinated against intimin and EspB, the virulence factors of *E. coli* O157, significantly higher levels of IgG (~ 4-7 fold, $p<0.05$) in blood have been induced for about 8 weeks. The greater immune status achieved by vaccination was followed by a ~38% lower pathogen shedding ($p<0.05$) over the course of 2 weeks compared to non-immunized animals (Vilte et al., 2011).

At the farm level, management practices like allowing for the newborn to spend longer than 1 hour with the dam have been associated with 59% greater odds of diarrhea (Trotz-Williams et al., 2007).

Altogether, GIT microbial colonization disturbance, intake and absorption of colostrum immunoglobulins by neonates, degree of perinatal immune suppression induced by hormonal changes in the dam and calf, calf stress and pathogen exposure levels defined by management practices, for instance, could potentially determine a large portion of the risk factors for intestinal infection issues in the pre-weaning calf.

Finally, thorough understanding of those risks factors will provide the opportunity for preventive action aimed at diminishing the effects of diarrhea in newborn calves. Clear definition of risk factors will also allow better evaluation of nutritional interventions to improve intestinal wellness, overall health and even maybe growth performance.

Within this framework, 4 research manuscripts are presented in this document that focus on the following topics:

1. Definition of gut health and how it can be measured for prognostic purposes.
2. Changes in composition and activity of the intestinal microbiome during the calf's first month of life and their potential role in gut health.
3. Evaluation of the prebiotic potential of a galactooligosacharide rich milk replacer on intestinal microbiome composition, short-chain fatty acids, intestinal function and development, and calf growth performance.
4. Evaluation of supplements with secretagogue potential to promote gut development through enhanced release of glucagon like peptide 2.

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CHAPTER II

CALF INTESTINAL HEALTH ASSESSMENT DURING THE BIRTH TO WEANING PERIOD

ABSTRACT

Methods to assess gut health in pre-weaning dairy calves in farms are not well defined. Simultaneous use of subjective methods like scoring systems and objective methods like biomarkers or intestinal function measurements can provide a more integral assessment that leads to targeted therapeutic strategies for intestinal infection. Also, eventual replacement of ex-post diagnostic therapy protocols for quantitative ex-ante forecasting prevention schemes may be desirable to reduce the financial impact of diarrheal disease on farms and improve calf welfare.

Here we have quantified the changes in calf gut health throughout the first month of life as measured by fecal scores and their relationship with thermal variability, blood protein, and bodyweight at birth. We also assessed the changes in calf gut health as measured by intestinal paracellular permeability in Ussing chambers, in response to age, failure of passive transfer, and intestinal location. Analysis of fecal score data indicated risk for diarrhea changed hyperbolically from 5 to 35 days with a risk peak around 13 days of age, which agrees with previous reports in the literature. The variations in time, however, depended on the calf-specific features noted above, suggesting that variables related to thermal homeostasis, passive immunity obtained by the calf, and perhaps variables related to fetal maturity in early life, might influence the risk of diarrheal episodes occurrence. However, the direction and size of such effects was not consistent and seem to be dependent upon other unknown variables that evolve over time.

On the other hand, gut health as measured by paracellular permeability appeared not to be intrinsically related with time but colon appeared to be the tissue to undergo the largest changes in permeability compared to other GIT regions. Also, blood protein (i.e., failure of passive transfer) appeared to have no influence on gut health at the tissue level even though it affected the risk of scour episodes at the calf level.

Finally, while further evaluation of epithelial permeability in response to meaningful predictor variables should be conducted, indicators of gut health other than just permeability need to be evaluated for development and implementation of quantitative models to be feasible. Specific areas of search for gut health risk factors, including calf immune, intestinal pathogen load and metabolomic/metabonomic profiling appear to be promissing to eventually develop complementary calf and tissue level empirical prediction models that support individual calf preventive care protocols.

INTRODUCTION

Digestive problems are common in calves between birth and weaning. According to the last national survey conducted by The National Animal Health Monitoring System (NAHMS, 2010), about 25% of pre-weaned heifers in the US had diarrheal diseases during 2007. Moreover, 7.8 % of the pre-weaned heifers died during this period and scours or other digestive problems accounted for 57% of this mortality (NAHMS, 2010). Costs associated with high mortality and reduced productivity exceed $200 million annually, excluding expenses of antibiotic treatment, lost feed, labor and overhead costs (Quigley et al., 2005).

Neonatal calf diarrhea can take place in calves from 3 to 21 days of age and the onset and duration of a diarrheal episode will depend on the number of pathogens involved and the immune condition of the animal (Butler and Clarke, 1994).

Scours (i.e., feces with dry matter below 10%) in the newborn calf involve an interaction between one or more pathogenic bacterial, viral or protozoal microorganisms, the immune status of the calf, and the environment. It is estimated that enterotoxigenic *Escherichia coli* (ETEC), rota and corona viruses, and *Cryptosporidium spp*. together account for 75 to 95 % of all cases of neonatal calf diarrhea (Butler and Clarke, 1994).

Pathogens colonize and spread through distal small and proximal large intestine (Butler and Clarke, 1994), and can cause secretory or exudative diarrhea. For example, ETEC and *Salmonella spp*. produce toxic peptides that induce secretory diarrhea where electrolyte and water secretion is triggered by the direct effect of their enterotoxins on the cell's secretory system as mediated by adenylyl cyclase and cAMP synthesis. However, like rotavirus and coronavirus, they can also impact the intestinal epithelium at the molecular level disrupting the tight junctions and cytoskeleton architecture causing cell loss and tissue damage, thereby compromising the epithelial barrier function (Sears, 2000; Groschwitz and Hogan, 2009; Croxen and Finlay, 2010; Ulluwishewa et al., 2011). This leads to exudative diarrhea with water, electrolytes, mucus, and protein accumulating luminally as a result of permeability loss (Sears, 2000; Field, 2003; Ulluwishewa et al., 2011).

But even though these mechanisms underlying both types of diarrhea are well documented, assessing overall gut health of calves at the farm level is not possible currently. Like in humans, the expression "gut health" has become commonly used in the literature and by the feed additive and pharmaceutical industry, but it is infrequently measured in the field since it is still unclear exactly what gut health is and how it can be measured (Bischoff, 2011).

For instance, in newborn dairy calves fecal scoring scales (e.g., 1 to 4) where higher scores indicate poorer health are used routinely in the research setting to assess intestinal health.

At the farm level though, these scoring systems are replaced by pure visual appraisal of the calf behavior and fecal consistency. In any case, however, scoring systems provide no information on other aspects related to gut health such the types and abundance of pathogens causing infection, their impact on epithelial integrity, or the intestine mucosal immune response. A practical consequence is that standard antibiotic therapeutic intervention decisions are often limited and blindly taken (e.g., 75% of diarrhea-ill calves in 2007 were treated with antibiotics (NAHMS, 2010). In humans, integral assessment of intestinal health has been recommended to include both subjective measures like scoring systems, but also biological indicators like permeability, histomorphology, mucus secretion, inflammatory and regulatory cytokines, immunoglobulins, bacterial cultures and toxin measurements (Bischoff, 2011).

On-farm decision support tools that provide ex-ante technical guidance based on calf specific characteristics to reduce diarrheal incidence in pre-weaning calves are clearly non-existent at this time. Identification of meaningful and effective risk factors to predict diarrheal episode occurrence based not only on scores at the calf level, but also definition of intestinal barrier function changes at the tissue level will be needed to build complementary quantitative prevention schemes that help forecast and prevent or buffer the effect of diarrhea in newborn calves.

Some potentially useful farm and calf level risk factors such as failure of passive transfer (FPT) (Robison et al., 1988), plasma cortisol level (Sreerama et al., 2008), immunization against virulence factors (Vilte et al., 2011), milk feeding rate (Quigley et al., 2006), calf diarrhea prophylaxis of the cow, maternity facilities type and management or time spent by the newborn with the cow (Trotz-Williams et al., 2007), have been related to diarrheal occurrence or pathogen shedding. For example, at the tissue level, immune profiling of newborn calves may uncover

useful predictors of gut health during the first few weeks of life, as evidence indicates that combining passively acquired antibodies with induced early recruitment of CD4+ and CD8+ T lymphocytes into the intestinal mucosa may stimulate thorough mucosal protection against pathogen infection and shedding (Wyatt, 2000). Newborn age and its apparent interaction with the environment appear also to affect gut permeability in human newborns (Weaver et al., 1984; van Elburg et al., 2003) and infants as well (Lunn et al., 1991).

In clinical practice with humans it is considered that both approaches, subjective (e.g., scores) and objective (e.g., biomarkers and functions) gut health indicators, are complementary, equivalent and cannot replace each other (Bischoff, 2011). The objectives of this work were to: 1) quantify the effect of age, serum protein level (BP), body temperature at the first 24 (BT_24), 48 (BT_48) and 72 hours of life (BT_72), variation in body temperature during the first 72 hours of life (TV), and birth BW on the occurrence of diarrhea, 2) assess the effect age, FPT and gastrointestinal (GIT) site on epithelial paracellular permeability during the first month of life of Holstein calves, and 3) qualitatively contrast observations on diarrheal events at the calf level (i.e., subjective gut health measure) with changes in permeability at the tissue level to identify patterns (i.e., objective gut health measure) and discuss their potential degree of complementarity.

MATERIALS AND METHODS

Effect of Age and Other Newborn Calf Characteristics on the Occurrence of Diarrheal Episodes

Two data sets from studies conducted in our laboratory where fecal scores were measured daily were used to define the relationship of age, BP, BT_24, BT_48, BT_72, TV and birth BW

of the new born calf with the probability of occurrence of a diarrheal episode during the first weeks of life.

To this purpose, a mixed effects logistic regression model containing such effects was fit to this repeated measures dataset. Adding a random intercept to model the calf-to-calf variability is reasonable because two levels of variability must be differentiated, namely, variation between and variation within calves (Schabenberger and Pierce, 2002). This approach also aids in accounting for autocorrelation between observations from the same calf over time (Schabenberger and Pierce, 2002).

In the first experiment (GAM), 93 calves were employed. The objective of that trial was to evaluate a plasma protein based formula for newborn calves on performance, morbidity, and mortality in the pre-weaning period. Feeding rate was 1.5% of BW as DM and grain was fed ad libitum during the first month of life. In the second experiment (GOS), 48 calves were employed. The objective of that experiment was to evaluate the effect of inclusion of prebiotics on intestinal health and development, and performance during the pre-weaning period. Feeding rate was 2% of BW as DM and, unlike GAM, no grain was fed during the first month of life.

Calves from both experiments originated from a sale barn, were ~72 ours old, and were evaluated by the veterinary staff upon arrival to UIUC research facilities. The evaluation included hydration status, alertness, mobility, navel status, and body temperature. A blood sample was obtained by jugular venipuncture and plasma protein was determined by refractometry. Calves were then weighed and body temperatures recorded for the first 5 days.

Fecal scores were recorded daily with a scale from 1 to 4 where: 1= dry, hard; 2 = soft and formed to pudding-like; 3 = mix of liquids with some solids; and 4 = liquid.

Fecal scores were then binary coded as 1 if the fecal score was 3 or 4 and as 0 if the fecal score was 1 or 2. Therefore, the event of a diarrheal episode Y_{ij}, conditional upon the predictor variables $\boldsymbol{X_{ij}}$ and the random effects $\boldsymbol{b_i}$, was considered a Bernoulli distributed random variable:

$$y_{ij}|\boldsymbol{X_{ij}}, \boldsymbol{b_i} \sim Bernoulli(\pi_{ij})$$

Where, Y_{ij} represents the occurrence of a diarrheal episode on the jth day for the ith calf with mean probability π_{ij} to be estimated. The probability of no occurrence is thus 1- π. A logit link function was used to linearly relate the predictor variables in $\boldsymbol{X_{ij}}$ with π_{ij} as:

$$log\ odds = \ln\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right) = ln\left(\frac{P(y_{ij} = diarrhea|\boldsymbol{X_{ij}}, \boldsymbol{b_i})}{1-\left(y_{ij} = diarrhea|\boldsymbol{X_{ij}}, \boldsymbol{b_i}\right)}\right) = \eta_{ij} = \boldsymbol{X_{ij}B} + \boldsymbol{Z_i b}$$

from which the probability π_{ij} was estimated as:

$$P(y_{ij} = diarrhea|\boldsymbol{X_{ij}}, \boldsymbol{b_j}) = \pi_{ij} = \frac{exp^{(\boldsymbol{X_{ij}B}+\boldsymbol{Z_i b})}}{1 + exp^{(\boldsymbol{X_{ij}B}+\boldsymbol{Z_i b})}}$$

And

$$\boldsymbol{b_j} \sim NIID(0, \sigma^2)$$

Thus, $\boldsymbol{X_{ij}}$ contained the fixed effects of study (GAM, GOS), age, BP, BT_24, BT_48, BT_72, TV and birth BW. Birth BW and TV were coded as dummy variables and calves were considered to present "normal" BW if above the 50th percentile (40.7 kg) or "underweight" otherwise, while TV values above and below the 50th percentile (0.69) were deemed as 'High' and 'Low' thermal variability, respectively. $\boldsymbol{B}$ is a vector of regression parameters for the fixed effects; $\mathbf{Z_i}$ is the random effects design matrix which only contained an intercept for the ith calf and **b** is the random effects parameter vector. Descriptive statistics and the rationale to include each independent variable are shown in Table 2.1.

From exploratory plots (Figure 2.1) of the observed log odds for diarrheal episodes (i.e scores 3 and 4), the following specific hypotheses were defined based on non-parametric Loess smoothing with 95% confidence bands: the log odds 1) change quadratically with time (Figure 2.1a), 2) increase with lower BP (i.e. FPT) but this effect may vary with age and depend on the study (GAM vs GOS; Figure 2.1b), 3) are higher for the 50% of the population with lower birth BW but this effect may vary with age and differ among studies (Figure 2.1c), 4) are higher for the 50% of the population whose TV in the first 72 hours of life is larger but this effect may vary with age and differ among experiments (figure 2.1d). Body temperature at 24, 48 and 72 hours showed no relationship with diarrhea and thus no specific hypotheses were formulated. Preliminary model development and selection was based on Wald t-tests, Wald t confidence intervals, deviance tests and corrected Akaike Information Criteria (Bolker et al., 2009; Fitzmaurice et al., 2011).

Three final models are reported (Table 2.2). Model 1 displayed the greatest deviance and AICc reduction compared to an empty model (i.e., only intercept model) and considers, aside from the study effect, the linear and quadratic effect of age along with TV and its interaction with time and experiment. It was, in turn, considered the reduced model against which models 2 and 3 were compared. Model 2 considers the linear and quadratic effect of age as well as the impact of BP and its interaction with age and experiment. Model 3, in turn, considers the linear and quadratic effect of age, the effect of BW and its interaction with age and experiment. Blood protein and BW were considered separately because, when in the same model, autocorrelation of their 3-way interactions with age and experiment would render them non-significant despite their large individual contributions to lower deviance compared to model 1. The BT_24, BT_48, BT_72 did not affect the probability of diarrheal events and thus were discarded.

Inclusion of a random intercept was justified by a reduction in the -2 x log likelihood from 4398 to 4155 with 1 degree of freedom. Variance components for the linear or quadratic effects of age or serum protein were not justified by the deviance test.

Parameters were estimated by approximating the likelihood function using Laplace approximation. To estimate the day j^{th} at which the probability of a diarrheal episode (i.e., π) was maximal for the i^{th} animal, the first derivative of the function containing the coefficients for the linear and quadratic components of age from model 1 was set equal to zero (Figure 2.2):

$$MAX\ \pi_{ij} = \frac{d(\pi_{ij})}{dAge} = \frac{d}{dAge}\left[\frac{exp^{\left(B_0+B_1(Age)+B_2\left(Age^2\right)\right)}}{1+\ exp^{B_0+B_1(Age)+B_2(Age^2)}}\right] = 0$$

Effect of age, GIT region and FPT on epithelium permeability in the calf

Data from two different studies conducted in our lab where electrical conductance as a surrogate for permeability was measured were used here to define the relationship of age and FPT with intestinal paracellular permeability in different regions of the GIT.

In the first experiment ("Experiment 1"), 32 newborn calves were used and 16 calves were slaughtered at 14 and 28 days of age. In the second experiment ("Experiment 2"), 34 calves were used, and 6 calves were slaughtered on each of days 0, 7, 14 and 21; whereas, 9 calves were slaughtered on day 28.

In both experiments, within 20 minutes of euthanasia, pieces about 3 $inch^2$ of rumen and 7 cm long of jejunum, ileum and colon were collected for permeability measurements in modified Ussing chambers (Physiologic Instruments; San Diego, CA) as per Cannon (2009). Samples were placed in oxygenated (95% O_2:5% CO_2) Krebs' solution (120 m*M* $MgCl_2$, 120 m*M* $CaCl_2$, 40 m*M* KH_2PO_4, 240m*M* K_2HPO_4 , 1.15 *M* $NaCl_2$, and 260 m*M* $NaHCO_3$) on ice and

transported to the laboratory for immediate electrophysiological and ion transport analysis. Basal resistance was assessed upon stabilization of tissue incubation for ~20 min (Cannon, 2009).

In experiment 1, the following hypotheses were tested: 1) Overall intestinal permeability differs between 14 and 28 days, and 2) Overall intestinal permeability differs between FPT groups. Permeability data were subjected to multivariate analysis of variance (MANOVA). Before conducting MANOVA variables were log transformed in order to meet multivariate normality assumptions. MANOVA was then performed on the 4 variables to test the effect of age and FPT on overall gut parcellular permeability through Wilks' Lambda, Pillai's Trace, Hotelling-Lawley Trace, and the Roy's Greatest Root hypothesis tests. Subsequently, canonical analysis (Johnson, 1998) was performed to determine which tissue has the most weight to differentiate age or FPT groups in terms of epithelial permeability.

In experiment 2, the following hypotheses were tested: 1) permeability differs among fore stomach, small intestine and hind gut, 2) Statistically significant changes in intestinal permeability occur throughout time during the first month of life in calves, 3) Changes in epithelial permeability throughout time will depend on the GIT location, and 4) Animals positive for FPT will have greater epithelium permeability compared to the negative ones.

The following linear model was assumed to analyze the epithelium permeability data,

$$\boldsymbol{y}_{ij} \sim N(\boldsymbol{X}_{ij}\beta, \boldsymbol{V}_i)$$

Where $\mathbf{y}_i$ is the set of permeability measures from the different regions of the GIT performed on the i^{th} calf. Permeability measures were log transformed to conform to normality. Observations were therefore assumed to be normally distributed with mean $\boldsymbol{X}_{ij}\beta$ and variance $\boldsymbol{V}_i$.

The design matrix $\boldsymbol{X_{ij}}$ contained the independent variable levels for age in days, GIT site, failure of passive transfer status (FPT=yes or no). The vector β contained the respective regression parameters, including an intercept.

The error covariance matrix $\boldsymbol{V}_i$ was first fully parameterized (i.e., unstructured) to gauge the degree of correlation among observations along the GIT belonging to the same calf, but all covariance parameters were non-significant according to a Wald Z and a deviance test. However, variances of permeability measures were heterogeneous among GIT locations and, therefore, a simple heterogeneous compound symmetric structure of the form

$$\boldsymbol{V_i} = \begin{bmatrix} \sigma^2_{rumen} & \rho & \rho & \rho \\ \rho & \sigma^2_{jejunum} & \rho & \rho \\ \rho & \rho & \sigma^2_{ileum} & \rho \\ \rho & \rho & \rho & \sigma^2_{colon} \end{bmatrix}$$

was adopted simply to account for heterogeneity and to avoid pseudo-replication. ρ was estimated to be 0.09 and not different from 0 by a Wald test (p =0.36) and a deviance test comparing against a 0 banded heterogeneous Toeplitz (p > 0.56). Otherwise, errors met normality assumptions as assessed from the Pearson residuals. Permeability means from the different GIT locations were compared using a Bonferroni adjustment to maintain the experiment wise error rate at 5%.

RESULTS

Effect of age and other newborn calf characteristics on the occurrence of diarrheal episodes

Table 2.2 shows the results of the logistic mixed model fitting. Model 1 indicates that there was a significant effect of experiment on the odds of a diarrheal episode, which is obvious and expected. Calves in the GAM experiment had only ~ 0.11 times the odds ($exp^{-2.21} = 0.109$) of presenting diarrhea compared to GOS. Also, as suggested by Figure 2.1a and initially

hypothesized, a quadratic function seems adequate to describe the changes in the log odds of diarrheal events over time. Moreover, the maximal probability for a diarrheal episode to take place was identified at ~13 days of age (Figure 2.2).

On the other hand, TV in the newborn calf affected the quadratic coefficient of age, slowing or accelerating the rate of decay in the probability of a scour event happening. This effect, however, was different between the GOS and GAM experiments and was larger by 1 order of magnitude roughly in the former compared to the latter experiment. In the GAM trial such effect appears to be null (Figure 2.3a). The deviations from the quadratic coefficient of age for the reference group (i.e., -4.62×10^{-3} for GOS /LOW TV) were 6.98×10^{-4} for GOS/HIGH-TV, -2.10×10^{-3} for GAM/LOW, and -1.83×10^{-3} for GAM/LOW; the first of which, although seemingly minuscule, could have a large impact in the longer term. For instance, when the probability functions of HIGH and LOW TV groups in the GOS experiment are compared in Figure 2.3a, after 2 weeks, right after the function maxima at 13 days, the two groups begin to separate and by day 35 the difference in the estimated probability of presenting diarrhea between HIGH and LOW is about 20%.

In regards to BP in model 2, the Wald t-test and confidence intervals indicated non significance at a type 1 error rate of 5% but only a tendency towards an effect; however, the deviance test, the AICc index and a partial F test (not shown) suggested otherwise and hence it was deemed worth including in the model for the sake of discussion. The BP main effect was not significant and depended on the age of the animal instead. Like TV status, this interaction also was different among GOS and GAM experiments. In GAM, the log odds of a diarrheal event increased by 0.012 per unit increase in age per unit increase in BP; whereas, in GOS they decreased by -8.71×10^{-3}. Figure 2.4 illustrates this interaction in terms of probability of a scour

event and depicts a decreasing likelihood of a scour outbreak as the calf ages in both GOS and GAM experiments; nonetheless, per each unit increase in BP, while holding every other variable constant and ignoring the intercepts, there is a further reduction in the probability of diarrhea in the GOS study. In contrast, BP seemed to drive such probability upwards in the GAM study. For instance, from 6 to 13 days of age, when diarrheal episodes appear to exacerbate, and at average BP of 5.6 mg/dL, the estimated probability of a scour outbreak increased from 0.57 to 0.58 in the GOS study; whereas, it went from 0.67 up to 0.72 in the GAM study.

Lastly, model 3 shows that calves with normal BW had lower odds ($exp^{-0.73} = 0.48$) of presenting diarrhea compared to underweight calves (i.e. BW < 40.7 kg). Moreover, BW interacted with the linear component of age affecting the rate of increase of the odds of presenting scours, but this interaction differed between studies as well. Compared to the GOS study, the increase in the odds of a diarrheal episode was faster for calves in the GAM trial ($exp^{0.21,0.16} = 1.23,\ 1.17$). Figure 2.3b illustrates this in terms of probability.

Finally, none of the variables employed in the 3 models could lower the calf specific variance below 0.7 (Table 2.2) even though they improved the fit of the model, indicating that other unknown calf specific variables are responsible for the differences among calves. Yet, the average intra calf correlation (Fitzmaurice et al., 2011) of the score measures for the 3 models was ~ 0.14, indicating rather low variability among calves (14%); this is the proportion of total unexplained variance attributed to variation among calves (i.e., clusters). The remaining 86% is due to unidentified sources of variation at the repeated measure level; these are variables whose values change from one day to the next for a given calf and that remain to be identified.

Effect of age, GIT region and FPT on epithelium permeability in the calf

Regarding the effect of age in experiment 1, all multivariate hypothesis tests were highly significant ($P < 0.01$), indicating a significant effect of age on paracellular permeability. Subsequently, canonical analysis was performed to determine which tissue's epithelial permeability has the most importance in differentiating calves 14 and 28 days old.

The standardized canonical coefficients indicated colon permeability to have the largest influence (6.5) compared to rumen (1.1), jejunum (0.88) and ileum (0.86). The product of these coefficients with the raw permeability data was computed to obtain a linear function so called canonical variable. Naturally, the degree of correlation between permeability and the canonical variable reflects the weight given by the standardized canonical coefficients and thus colon is the most highly correlated followed by ileum. In short, these correlation coefficients (Table 2.3) indicate that changes in colon permeability weigh most in making animals at 14 and 28 days of age different with respect to other tissues.

A subsequent analysis of variance to test the effect of calf age on the estimated canonical variable, which could be seen as a permeability measure weighted by the relative contribution of each tissue, indicates that age explains about 48% of its variation and there is a significant difference between 14 and 28 d of age (Table 2.4). Error assumptions of normality and constancy were checked and met.

The box and scatter plots below (Figure 2.5) illustrate the distribution and distance between the two age groups based on intestinal permeability, and also the relation between colonic permeability and the estimated canonical function, and how calves cluster by age in accordance with gut permeability.

On the other hand, and contrary to our initial hypothesis, FPT had no effect on paracellular permeability. All multivariate hypothesis tests were not significant ($P > 0.6$).

In conclusion, paracellular permeabitity was statistically higher at 2 than at 4 weeks of age and the colon seemed to be the most affected tissue compared to other regions of the GIT. However, the extent to which the decrease in permeability from week 2 to 4 is an animal intrinsic ontogenic change or is rather due to recovery from disturbance is unknown.

With the intent to obtain a better idea of temporal changes in epithelial permeability, we analyzed the experiment 2 data set, which contained measures collected at several time points during the first month of life. Unexpectedly, in contrast with experiment 1, we observed no relationship of age with permeability throughout the first month of life in the calves. Also, no interaction with GIT location was present. Failure of passive transfer showed no effect on permeability either. The GIT location main effect, however, did affect average paracellular permeability and jejunum and colon showed to be the most permeable epithelia while rumen and ileum were least (Table 2.5).

In summary, jejunum and colon presented the highest permeability throughout the first month of life in experiment 2.

DISCUSSION

Effect of age and other newborn calf characteristics on the occurrence of diarrheal episodes

Although rather expected, this analysis confirms through the significant study effect how fecal scores can differ from trial to trial or from farm to farm, due to subjective scorer perception and actual biological unidentified causes (e.g. diets, management, year season, etc.); therefore, the need for complementary underlying information is emphasized. Also, the fact that not only the mean probability of a scour episode differs between studies, but the fact the age regression

coefficients vary from one to the other, highlights the temporal dynamic and interactive nature of TV, BP and BW and other unknown variables that likely assume particular importance at a given trial or farm.

Yet, although GAM and GOS studies differed widely in the frequency of diarrheal episodes, analysis of the joint data set indicates that episodes were maximal around day 13 of age. This observation coincides with previous reports from Canada (Trotz-Williams et al., 2007). Although discrete time frames have been identified for diarrhea incidence from different pathogens like ETEC (4 days of age), *Cryptosporidium parvum* (14 days of age), rotavirus (6 days of age) and coronavirus (4 to7 days of age) (Foster and Smith, 2009) to peak in the early life of the calf, a diarrheal episode is the result of the joint action of several pathogen types (Butler and Clarke, 1994). Thus, the estimated critical point at 13 days of age is likely a sort of weighted average of the abundance and activity of each of various such pathogens. Assuming that the onset and duration of a diarrheal episode depend on the number and type of pathogens involved and the immune condition of the animal (Butler and Clarke, 1994), the prognostic value of a statistical predictive model would then lie on the knowledge of the calf specific pathogen intestinal load and its immune profile very early in life.

In that sense, in the current study, BP and TV were initially reasoned as potentially sound indicators of the immune and inflammatory status of newborn calves, respectively, and perhaps good predictors of diarrheal outbreaks given their wide variability among animals and apparent straightforward biological meaning. However, even though FPT is linked with increased calf morbidity and mortality (Robison et al., 1988), BP level impacted the probability of a scour episode in different direction for the GOS and the GAM trials. Similarly, differential TV effects were seen in the GOS compared to the GAM trial. The significant but inconsistent direction of

these effects and their size throughout time and between studies suggests that other calf specific unknown variables could confound or modulate the effect of BP and TV. For instance, dehydration (i.e., plasma volume) can bias BP concentrations but BP effects could perhaps also be modulated by the pathogen population size and calf stress. Furthermore, BW, although lacking an obvious biological interpretation related to intestinal health, did have a direct effect and smaller animals were more likely to undergo illness, but this effect also varied across studies. At any rate, associations observed here must be cautiously interpreted because these predictor variables are not causal factors and, rather, are only proxy variables for underlying physiological process networks that ultimately define the animal response. For example, low BW animals many not be more prone to scours simply because they are smaller, but perhaps because of an immature gut that is more intolerant to food and susceptible to pathogenic infection due to poor mucosal barrier function as is documented with human infants with extremely low birth weights (Neu, 2007).

Our observations, nonetheless, contribute information on risk factors that with further knowledge could indeed be practical and 'easy to use' predictors to forecast which newborn animals will likely present disease and undertake preventive intervention instead of therapeutic treatment after the fact. Good calf, farm and environmental level predictors such as maternity pen management, time spent by calf with the dam, calf-scours prophylactic dam treatment, calf enteropathogen shedding, birth season or facility cleanliness among others, have already shown promise (Trotz-Williams et al., 2007) and warrant further evaluation in longitudinal studies.

Moreover, measuring other colostrum components, either in the colostrum itself or calf blood such as IgG, cytokines (e.g., IL-4 , IL-6, interferon gamma and IL-10) and leukocyte (e.g., macrophages, lymphocytes and neurophils) concentrations could yield a more effective index of

passive immunity status (Chase et al., 2008). Moreover, calf cortisol and peripartum cow levels of hormones like progesterone, prostaglandin E2, estrogen, and cortisol could provide an index of the immunosuppressive status of the newborn calf (Chase et al., 2008). Although cost prohibitive currently, performing many such tests may become less expensive in the future as a preventive medicine industry develops.

Remarkably, the fact that 86% of unknown variance is due to differences from one day to the next (i.e., among repeated measures), and 14% to differences among calves, suggests that major unidentified risk factors in this trials are evolving over time and frequent monitoring might be required. Empirical model robustness will eventually be achieved upon their iterative refinement and evaluation.

Effect of age, GIT region and FPT on epithelium permeability in the calf

While calf level empirical models can provide specific information about the risk of a calf to become ill, when and how long a scour episode can last, they provide no information on relevant matters like the type of predominant pathogens and intestinal infection under course (i.e., secretory vs. exudative diarrhea) and the concomitant changes of the intestinal barrier.

Although gut barrier is more than simply measuring permeability (Bischoff, 2011), the latter can at least tell us about the integrity of the tight junctions between epithelial cells (Boudry, 2005), and thus hint whether diarrhea is secretory or malabsorptive. For instance, our paracellular permeability measures in experiment 1 show a reduction from 2 to 4 weeks of age, suggesting closure of the tight junctions, but whether this is an ontogenic establishment of a low permeability status or a recovery from a temporal increase caused by pathogenic action in the first 2 weeks of life, is unknown. This dataset also highlights colon as the organ that experiences the largest fluctuations in this time window. On the other hand, experiment 2 identified jejunum

and colon as the organs with highest paracellular permeability. However, since no change throughout the first month of life was evidenced, those values could simply be characteristic of 'healthy' epithelia in those GIT regions. Otherwise, if those calves ever presented intestinal infection, it did not compromise the tight junction structure.

In addition, FPT, as a measure of immune status after birth, did not bear any influence upon the integrity of the intercellular space according to both experiments even though it was associated with the probability of diarrheal occurrence at the calf level. Variations in paracellular permeability (i.e., tight junction integrity) need to be further evaluated in relation to a variety of risk factors.

Although the mechanisms through which pathogens like EPEC, *Cryptosporidium parvum*, rotavirus, and coronavirus damage the brush border and disrupt the tight junctions supporting the epithelial barrier are understood (Sears, 2000; Klein et al., 2008; Foster and Smith, 2009; Ulluwishewa et al., 2011), there is currently no means to predict and prevent infection and the concomitant barrier function loss in farms. At this time, dairy operations can only provide ex-post standard antibiotic and hydration therapy (NAHMS, 2007), regardless of whether infection is bacterial, protozoal, or viral; due partly to lack of knowledge. Thus, sound indicators of gut health and risk factors for intestinal barrier loss must be identified in early life to advance quantitative knowledge to the point where recommendations can be made based on calf specific characteristics during the first few days of life.

A specific example is the realization that the intensity of *Cryptosporidium parvum* infection in the distal bowel depends on the antigen specific antibodies present in colostrum and the timing of calf exposure to the parasite oocytes, which determines the power of the mucosal immune response in terms of $CD4^+$ and $CD8^+$ T lymphocyte recruitment into the intestinal

mucosa (Wyatt, 2000). Thus, for instance, a robust equation that defines the optimal specific antibody concentration and exposure age at which clinical signs and shedding are minimized would prove useful in order to make a technical recommendation to prevent or at least control infection.

Furthermore, technologies such as genomic sequencing and metabolomic/metabonomic profiling are suggested to be the basis for a new kind of personalized medicine in humans (Nicholson, 2006; Bischoff, 2011), in which the interaction between the genetic makeup of the individual, the current metabolic status and exogenous factors, such as exposure to pathogens, is quantified to determine the possible outcomes to pharmaceutical or dietary (i.e., prebiotics) intervention (Nicholson, 2006).

Hopefully, in the future, complementary implementation of models at the calf and tissue level should aid in forecasting when and what type of intestinal infection (i.e., secretory, exudative or both) could occur unless preventive action tailored to the individual calf is taken.

CONCLUSIONS

Methods to assess gut health in pre-weaning dairy calves in farms are not well defined. Joint use of subjective methods like scoring systems and objective methods like biomarkers or intestinal function measures, can provide better assessments in order to properly choose the best therapeutic strategy for intestinal infection and recovery. In addition, because of the financial and animal welfare toll a diarrheal episode takes, it may be desirable to eventually replace ex-post therapeutic protocols for ex-ante preventive schemes. Nevertheless, this approach requires the ability to predict the risk and potential extent of infection at the calf and tissue level in order to provide specific recommendations. Here we have quantified the changes in calf gut health throughout the first month of life as measured by fecal scores and whether this can be modulated

by TV, BP, and birth BW. We also assessed the changes in calf gut health throughout the first month of life as measured by intestinal paracellular permeability and whether this can be modulated by BP and how it changes across GIT regions. Analysis of fecal score data indicated risk for gut health changed hyperbolically from 5 to 35 days with a risk peak around 13 days of age, which agrees with previous reports in the literature. Also, a modulatory effect of TV, BP and birth BW was observed, suggesting that variables related to thermal homeostasis, passive immunity obtained by the calf, and perhaps variables related to fetal maturity in early life influence the risk of diarrheal episodes occurrence. However, the direction and size of such modulatory effects seem to be dependent, in turn, upon other unknown variables. Importantly, 86% of the unknown variance comes from differences between daily measures, suggesting that risk factors that vary over time are involved.

On the other hand, temporal changes in gut health based on paracellular permeability are difficult to reconcile across the two datasets employed here. While one dataset suggested no change from 0 to 4 weeks of age, the other suggested a significant reduction from 2 to 4 weeks of age and colon appeared to undergo the largest changes. More data needs to be collected evaluating permeability in response to other predictor variables to develop permeability response surfaces.

Also, FPT (i.e., serum protein level) appeared to have no influence on gut health at the tissue level even though BP affected the risk of scour episodes at the calf level.

Of course, because fecal score and permeability data reported here arise from different experiments and animals, direct objective comparison is not possible and only qualitative discussion is proposed for consideration.

Finally, more indicators of gut health than just permeability need to be evaluated and implemented and specific areas of search for risk factors, including calf immune, intestinal pathogen load and metabolomic/metabonomic profiling appear to be promissory to eventually develop individual calf preventive care protocols.

TABLES AND FIGURES

Table 2.1. Descriptive statistics for predictor variables

Variable	n	Mean	Std.Dev.	Minimum	Maximum	CV	Rationale
BP	141	5.6	0.82	3.9	9.8	14.8	FPT indicator
BT_24	141	101.7	1.04	99.5	106.0	1.03	Fever-infection indicator
BT_48	141	101.7	0.84	99.7	104.1	0.83	Fever-infection indicator
BT_72	129	101.8	0.89	98.6	103.6	0.87	Fever-infection indicator
TV	141	0.73	0.46	0.06	3.1	63.6	Overall thermal homoestasis
BW	141	41.3	2.83	36.0	48.6	6.8	Non-specific

[1] n: Number of calves
[2] CV: Coefficicent of variation
[3] Std.Dev: Standard deviation

Table 2.2. Estimated fixed effects, standard errors and 95% confidence intervals (CIs) for models predicting occurrence of diarrheal episodes

		Model 1 [1]			Model 2			Model 3		
Effect	Group[1]	**β**	**SE**	**95% CI[6]**	**β**	**SE**	**95% CI**	**β**	**SE**	**95% CI**
Intercept		**1.08**	0.30	0.49, 1.68	**2.74**	0.90	0.96, 4.52	**2.47**	0.44	1.6, 3.4***
Exp [2]	GAM	**-2.21**	0.21	-2.64, -1.78 ***	**-3.18**	0.35	-3.88, -2.47 ***	**-3.80**	0.48	-4.7, -2.85***
Age		**0.13**	0.02	0.08, 0.17 ***	**0.11**	0.04	0.03, 0.18 ***	**0.016**	0.04	-0.05, 0.09
Age^2		**$-4.62x10^{-3}$**	$5.24x10^{-4}$	$-5.65x10^{-3}$, $-3.59x10^{-3}$ ***	**$-3.21x10^{-3}$**	$6.55x10^{-4}$	$-4.49x10^{-3}$, $-1.92x10^{-3}$ ***	**$-2.4x10^{-3}$**	$7.8x10^{-4}$	$-3.9x10^{-3}$, $-9.0x10^{-4}$**
TV [3]	HIGH	**0.02**	0.19	-0.36, 0.40	**-0.02**	0.1960	-0.40, 0.36	**0.026**	0.19	-0.36, 0.41
TV x Age^2x Exp	GAM/HIGH	**$-1.83x10^{-3}$**	$3.67x10^{-4}$	$-2.55x10^{-3}$, $-1.11 x 10^{-3}$***	**$-4.56x10^{-3}$**	$8.55x10^{-4}$	$-6.24 x10^{-3}$, $-2.89x10^{-3}$***	**$-6.3x10^{-3}$**	$1.2x10^{-3}$	$-8.6x10^{-3}$, $-3.9x10^{-3}$***
TV x Age^2x Exp	GAM/LOW	**$-2.10x10^{-3}$**	$3.54x10^{-4}$	$-2.79x10^{-3}$, $-1.40x10^{-3}$ ***	**$-4.93x10^{-3}$**	$8.71x10^{-4}$	$-6.64x10^{-3}$, $-3.22x10^{-3}$ ***	**$-6.6x10^{-3}$**	$1.2x10^{-3}$	$-8.9x10^{-3}$, $-4.2x10^{-3}$***
TV x Age^2x Exp	GOS/HIGH	**$6.98x10^{-4}$**	$2.61x10^{-4}$	$1.85x10^{-4}$, $1.21x10^{-3}$ ***	**$6.58x10^{-4}$**	$2.64x10^{-4}$	1.40 $x10^{-4}$, $1.176x10^{-3}$ **	**$6.8x10^{-4}$**	$2.7x10^{-4}$	$1.7x10^{-4}$, $1.2x10^{-3}$**
BP [4]					**-0.18**	0.14	-0.47, 0.1			
BP x Age x Exp	GAM				**0.012**	$6.39x10^{-3}$	$-3.5x10^{-4}$, 0.02 *			
BP x Age x Exp	GOS				**$-8.71x10^{-3}$**	$6.33x10^{-3}$	-0.02, $3.7x10^{-3}$			
BW [5]	NW							**-0.73**	0.25	-1.2, 0.2**
BW x Age x Exp	GAM/NW							**0.21**	0.05	0.12, 0.31***
BW x Age x Exp	GAM/UW							**0.16**	0.05	0.07, 0.25***
BW x Age x Exp	GOS/NW							**0.02**	0.01	$-4.4x10^{-3}$, 0.04
Variance components										
σ_{calf}		0.73	0.12		0.70	0.12		0.73	0.12	
Fit indices [6]										
-2 LLH		4060			4045			4029		
AICC		4078			4069			4055		

[1]Hypothesis tested concerning specific groups, and as defined by the regression model are explicitly explained in table 2.6 in the appendix.
[2] Exp: Experiment (GAM, GOS)
[3] TV: thermal variation (high or low)
[4] BP: blood protein
[5] BW: body weight (normal weight (NW) or underweight (UW))
[6] -2LLH: deviance, AICC: Corrected Akaike Information Criteria, Wald t test significance: * P < 0.1, **P < 0.01 and *** P< 0.0001

Table 2.3. Correlation coefficient between the log of the permeability measures for different tissues and the canonical function

Variable	Correlation with Canonical function
Log (rumen)	0.48
Log (Jejunum)	0.45
Log (Ileum)	0.54
Log (Colon)	0.71

Table 2.4 Means difference for canonical variable at 14 and 28 days of age in Holstein calves

Age	Mean	Standard error	R^2	$P \geq \|t\|$
14 vs. 28 days	9.3	2.12	0.48	0.0002

Table 2.5. Effect of GIT locations, age and FPT on epithelial permeability

GIT location					P-value	
Rumen	Jejunum	Ileum	Colon		Location	
3.40 ± 0.17[b]	5.10 ± 0.44 [a]	2.71 ± 0.09[c]	3.98 ± 0.11 [a]		< 0.001	
Age						
0d	7d	14d	21d	28d	Age	L x A[1]
3.90 ± 0.27	3.65 ± 0.27	3.78 ± 0.27	3.83 ± 0.27	3.80 ± 0.27	0.98	0.3
Failure of passive transfer						
	No	Yes			FPT	
	3.93 ± 0.15	3.65 ± 0.15			0.11	

[1]L x A: GIT location by age interaction

Table 2.6. Hypothesis or contrasts under test in each model

Effect[1]		Contrast or hypothesis
		MODEL 1
Exp	GAM	Experiment effect, GAM ≠ GOS
Age		Age effect ≠ 0
Age^2		Age^2effect ≠ 0
TV	HIGH	HIGH vs LOW
TV x Age^2x Exp	GAM/HIGH	GAM/HIGH vs GOS /LOW
TV x Age^2x Exp	GAM/LOW	GAM/LOW vs GOS/LOW
TV x Age^2x Exp	GOS/HIGH	GOS/HIGH vs GOS/LOW
		MODEL 2
BP		BP effect ≠ 0
BP x Age x Exp	GAM	BP x Age in GAM ≠ 0
BP x Age x Exp	GOS	BP x Age in GOS ≠ 0
		MODEL 3
BW		BW effect, NORMAL ≠ UNDERWEIGHT
BW x Age x Exp	GAM/NW	BW x Age effect , GAM/NORMAL vs GOS/UNDERWEIGHT
BW x Age x Exp	GAM/UW	BW x Age effect, GAM/ UNDERWEIGHT vs GOS/UNDERWEIGHT
BW x Age x Exp	GOS/NW	BW x Age effect, GOS/ NORMAL vs GOS/UNDERWEIGHT

[1]Effect: Exp= Experiment effect (GAM, GOS), TV=Thermal variability (High, low), BP=Blood protein, and BW=Bodyweight.

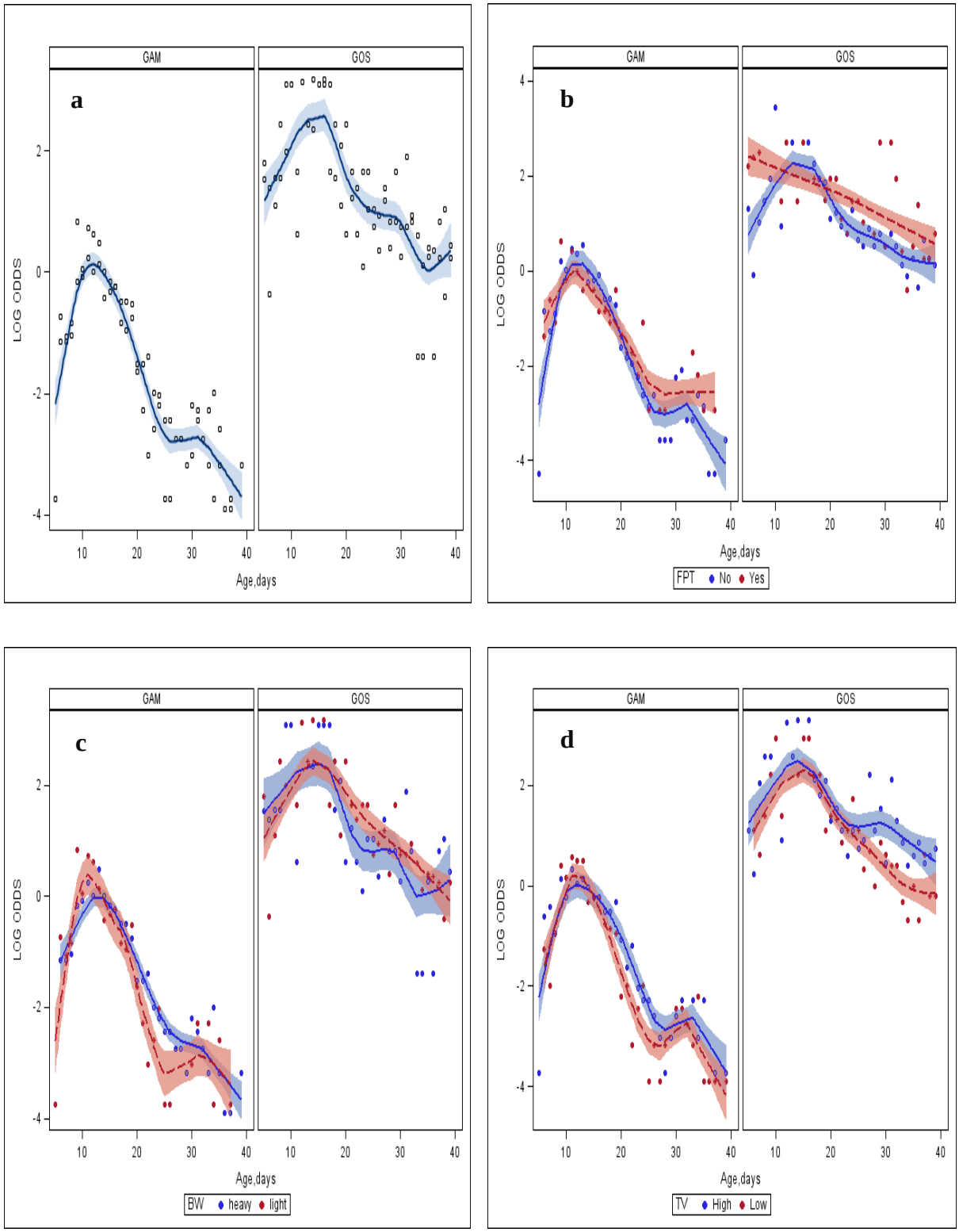

Figure 2.1. Exploratory plots of log odds for GOS and GAM experiments with respect to: a) age; b) Blood protein as categorized into failure of passive transfer groups (FPT: Yes (dashed line), No (solid line)); c) Bodyweight (BW; light (dashed line), heavy (solid line)) and d) Thermal variability (TV; Low (dashed line), high (solid line)).

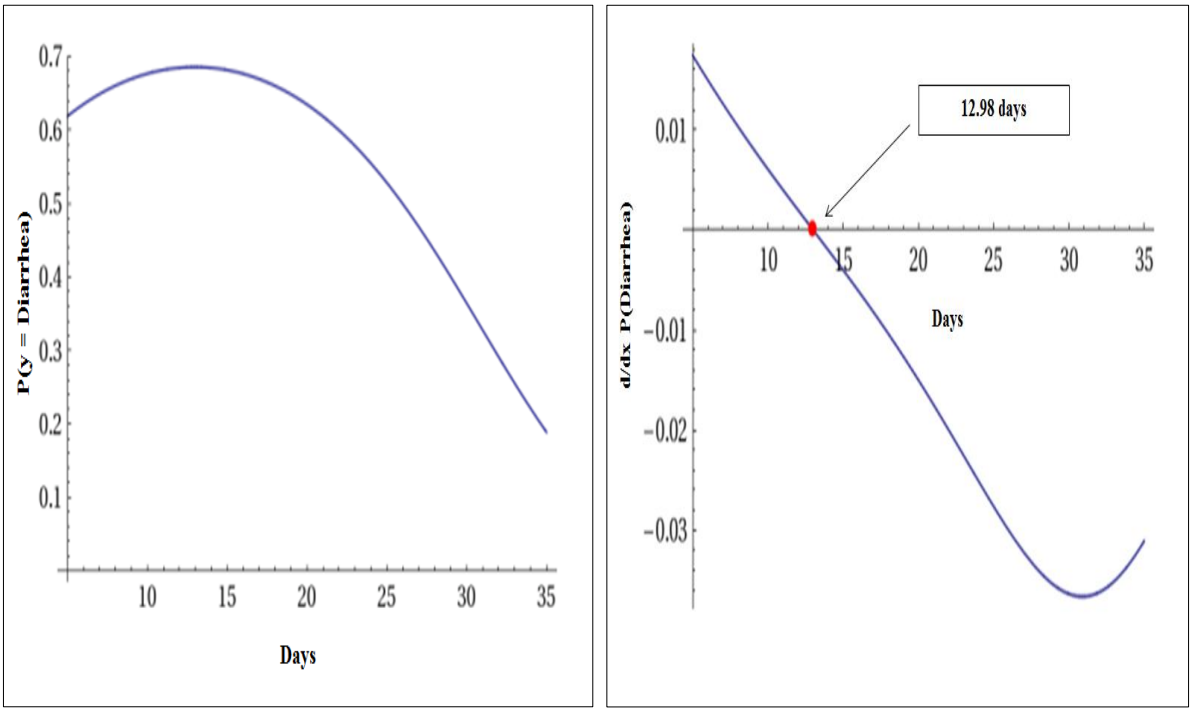

Figure 2.2. Diarrhea episode probability function and age at maximal value.

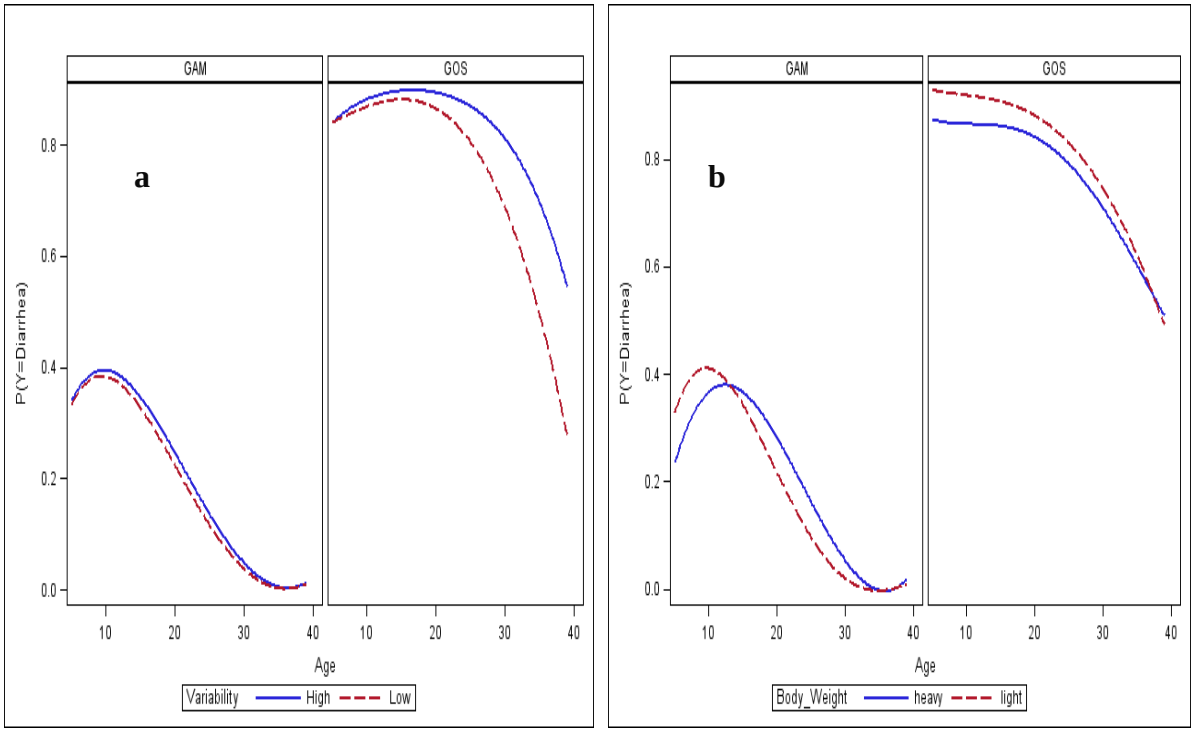

Figure 2.3.Estimated probability of a diarrheal episode for a) Thermal variability (TV: High/Low) and b) Bodyweight (BW: Heavy/Light) groups.

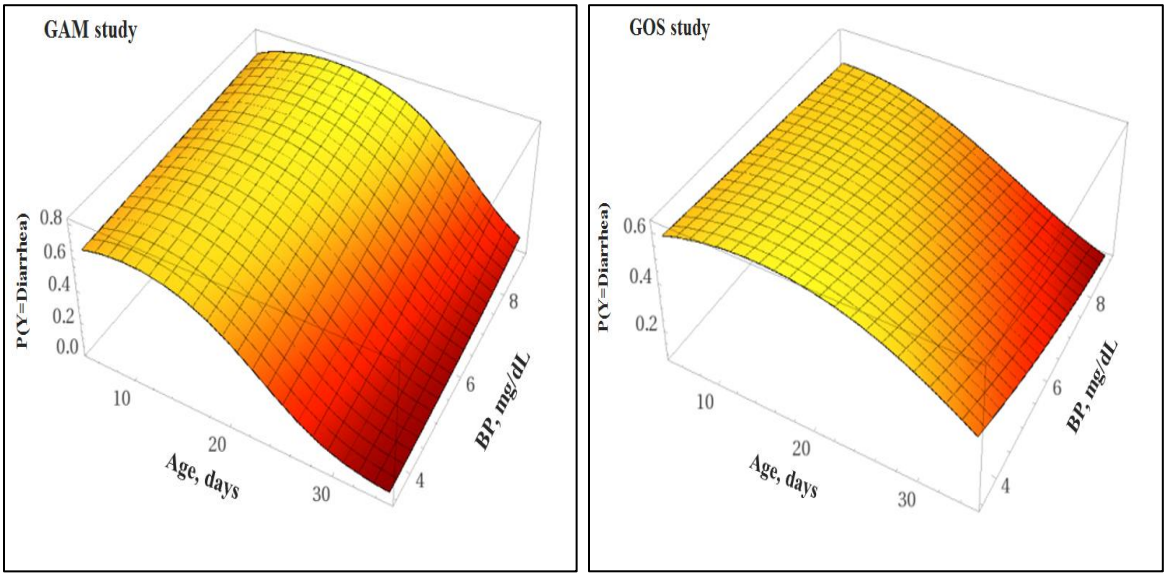

Figure 2.4.Estimated probability of diarrhea occurrence across studies (GAM/GOS) in function of age and blood protein (BP).

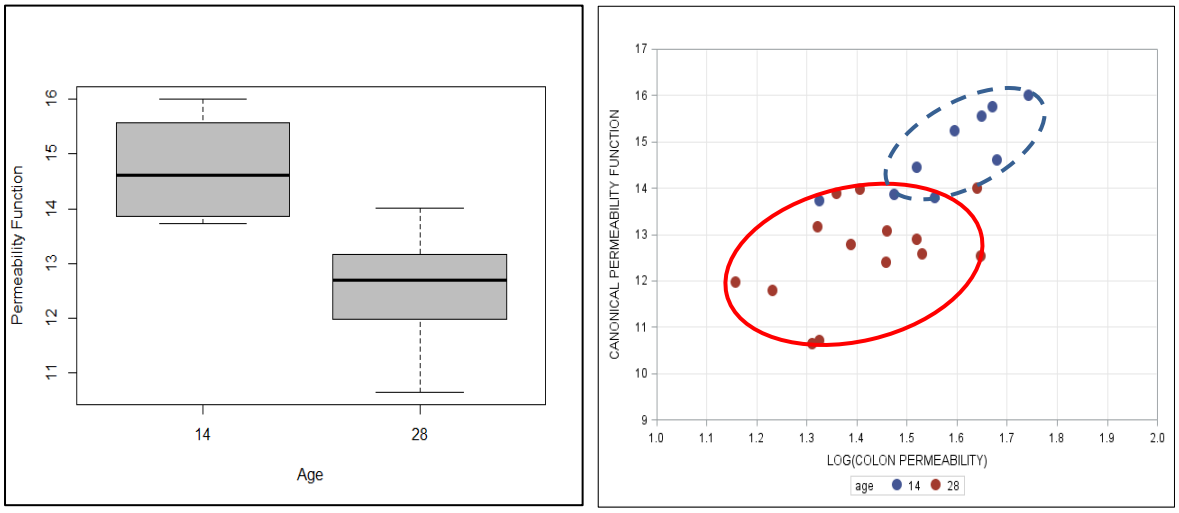

Figure 2.5. Permeability canonical variable distribution by age groups. Permeability Function is unit less (See Canonical discriminant analysis in materials and methods). Dashed ellipse: 14 days, Solid ellipse: 28 days.

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CHAPTER III

MICROBIOTA COMPOSITION AND ACTIVITY IN THE GASTROINTESTINAL TRACT OF PRE-RUMINANT HOLSTEIN CALVES AND THEIR POTENTIAL ROLE IN GUT HEALTH

ABSTRACT

The objective of this work was to assess the effect of age and gastrointestinal location (rumen vs colon) on microbial community diversity and composition, SCFA profiles and predicted metabolic functionality, of Holstein pre-ruminant calves set on an intensive milk feeding program. Thirty-two calves were fed at 2% of their bodyweight on DM basis from day 10 until slaughter. Sixteen calves were euthanized at 2 and 4 weeks of age post-arrival to collect digesta samples from the rumen and colon. The rumen and colon of pre-ruminant calves showed a similar degree of diversity (i.e. Shannon index) ($p = 0.44$) whereas composition differed ($p=0.001$) appreciably. Colonic microbiota was characterized by the dominance of lactic acid bacteria such as *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Bifidobacterium*. In addition, colonic SCFA and lactic acid concentrations were between 50 and 850% higher compared to rumen indicating greater fermentative activity. On the other hand, in the rumen, no genus showed to over-dominate and more variation among animals was present according to a multivariate Levene test ($p<0.05$). Therefore, microbial and fermentative development of fore and hind gut in calves fed only milk seems to present different timings. The authors believe that, because of an active esophageal groove and low grain intake during first 1 to 3 weeks of life, ruminal fermentation may not take preponderance until after 4 to 6 weeks of life, when significant metabolizable energy contributions will start to take place. Until then, high lactic acid

bacteria, lactate, and butyrate concentrations could bring about beneficial effects for the intestinal health of the calf during the first of life.

INTRODUCTION

Development of microbial populations in the gastrointestinal tract (GIT) of higher animals commences soon after birth (Mackie et al., 1999). Some observations indicate that the rumen anaerobic microbiota quickly attains concentrations as high as 10^9 cells ml^{-1}, thus becoming predominant by the second day after birth (Stewart et al., 1988); whereas, bacteria that are essential for mature rumen function can be detected as early as 1 day after birth, long before the rumen is active or even before ingestion of plant material occurs (Jami et al., 2013). Moreover, in only milk fed calves all major types of rumen microorganisms and a stable wide array of microbial metabolic functions have been identified before introducing solid feed, suggesting that the rumen of the young bovine is all but rudimentary (Li et al., 2012).

Once fermentable substrate, especially starch, is supplied to the calf, microbial fermentation develops progressively and the resulting SCFA like acetate, propionate, and butyrate directly stimulate growth and function of the rumen epithelium (Flatt et al., 1958; Sutton et al., 1963b) and will eventually provide over 90% of the energetic requirement of the animal (Beever and Mould, 2000); thereby rendering the calf energetically independent from an external milk source, be it the dam's milk or milk replacer. However, fermentation sufficiency, adequate rumen size, and full absorptive capability are not achieved until after about 4 to 6 weeks of age (Davis and Drackley, 1998) and the calf thus relies mostly on milk as a nutrient source for the first 1 to 4 weeks of life.

Given that during this period the diet is largely milk based, that the esophageal groove is fully functional and that all digestion is performed by the host enzymes, the newborn calf can be

considered a monogastric (Orskov, 1992). Since 10 to 15% of milk dry matter (DM) consumed passes undigested (Petit et al., 1989; Hill et al., 2010) through ileum into the cecum and colon, an amount in the range of 40 to 120 g DM/day, depending on the feeding rate, is likely readily available for colonic fermentation in a calf of 40 kg BW. Comparatively, for the same calf, less than 10 g of milk DM would be expected to enter the rumen for fermentation (Toullec and Guiloteau, 1989). Despite this apparently higher supply of fermentable nutrients into the hindgut of the pre-ruminant calf, little information is available on how microbial activity can impact its development and health (Oikonomou et al., 2013) as compared to the developing rumen (Warner et al., 1956; Sutton et al., 1963a; Sutton et al., 1963b; Warner and Flatt, 1965; Huber, 1969);

Early observations using colon digesta samples indicated that *Escherichia coli*, *Clostridium welchii* and *Streptococci* were the first organisms to be found in milk fed calves shortly after birth while *bacteroides* was found after 2 days of age in the large intestine and by day 12 it was competing with the *lactobacilli* for predominance (Smith, 1965). Human infants have also been observed to be initially colonized by large numbers of *E. coli* and *Streptococci* (Mackie et al., 1999) and it has been proposed that these bacteria are responsible for the creation of a reduced environment favorable for the establishment of facultative anaerobes like *lactobacilli* or the anaerobic genera *Bacteroides*, *Bifidobacterium*, and *Clostridium* by day 4 to 7 after birth (Mackie et al., 1999). But while pathogens are autochthonous to the gut ecosystem and can live in harmony with the host, they can become pathogenic under certain circumstances (Mackie et al., 1999), and organisms like enterotoxigenic *E. coli* and *Salmonella spp*. are indeed responsible for a large proportion of cases of neonatal calf diarrhea (Butler and Clarke, 1994; Izzo et al., 2011), which affects about 25% of pre-weaned heifers and accounts for nearly 50% of their annual mortality in the US (NAHMS, 2010).

On the other hand, *Bifidobacterium* and *Lactobacillus* species have been directly linked to increased resistance to infection and diarrhoeal disease, and stimulation of the mucosal immune system (Macfarlane et al., 2008). For instance, *Bifidobacterium longum* has inhibited bacterial translocation by *E. coli* through a lymphocyte activation mechanism (Yamazaki et al., 1985). *Lactobacillus plantarum* and *L. rhamnosus* have been able to sensitize epithelial cells to respond against enteric *Salmonella typhymurium*, apparently through up regulation of toll like receptors which recognize flagelin (Vizoso Pinto et al., 2009). In addition, *L. rhamnosus* GG and *Lactobacillus casei* DN-114-001 can also protect epithelial barrier function against *Escherichia coli*-induced redistribution of the tight junction proteins (Parassol et al., 2005; Johnson-Henry et al., 2008; Cani et al., 2009).

Also indirectly, through butyrate production, colonic microbiota can exert positive effects such as suppression of over-inflammation in epithelial cell lines (Macfarlane et al., 2008), improvement of gut barrier function and increased tight junction protein expression and lower endotoxemia (Cani et al., 2009).

Therefore, during the pre-ruminant stage, microbial colonization and fermentation establishment in the hindgut appear to be comparatively equally or more relevant than in the foregut for the health and survival of the newborn. The objective of this work was to assess the effect of age and gastrointestinal location (rumen vs. colon) on microbial diversity, community composition, SCFA profiles and predicted metabolic functionality, in intensively milk fed Holstein calves. A grasp of the relative degree of ruminal and colonic microbial development in the pre-ruminant calf shall be attained.

MATERIALS AND METHODS

Animals, Feeding and Treatment Allotment

Thirty-two newborn calves were purchased from Stone Ridge Dairy farm (Manheim, IL). Upon arrival to the University of Illinois research facilities, animals received an electrolyte solution (Land O Lakes, Electrolyte System - Add Pack), vaccines and prophylactic antibiotic treatment (2 mL, Muse, MERCK; 2 mL BoSe, Shering-Plough; 1mL vitamin A and D; 1.1mL Draxxin, ; Bovisera (50mL antibody serum, 20 mL C and D antitoxin, Behringer Ingelheim; 2 mL INFORCE-3, Pfizer). Milk replacers were fed at a rate of 1.25% of BW on a dry matter (DM) basis for the first 2 days, 1.5% of BW until day 10, and then 2% of BW through day 30. Calves were fed the milk replacer from arrival to day 30 with no grain offered.

Animal Slaughter and Sampling

Sixteen calves were euthanized at 2 and 4 weeks after arrival to collect digesta samples from the rumen and colon. Euthanasia was performed by administering an overdose (85-150 mg/kg BW) of sodium pentobarbital intravenously according to IACUC procedures.

Digesta samples from rumen and proximal colon were collected into 5 ml sterile cryovials and snap frozen in liquid nitrogen to quickly stop microbial activity. Samples were submitted to an external laboratory for bacterial tag encoded FLX amplicon pyrosequencing (Research and Testing Laboratory, Lubock, TX).

Microbial DNA Amplicon Sequencing and Taxonomic assignment

Total microbial DNA was extracted using a QIAamp stool DNA mini (Qiagen Inc., Valencia, CA) as previously reported (Dowd et al., 2008a; Dowd et al., 2008b). Sample DNA was quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). Upon extraction, the V1-V3 hyper variable regions of the 16-S ribosomal DNA gene were amplified by

PCR using barcoded primers and the product sequence determined by bacterial tag-encoded FLX (non-titanium chemistry) amplicon pyrosequencing (Roche, Nutley, New Jersey) as reported previously (Dowd et al., 2008a; Dowd et al., 2008b). Afterwards, sequences were depleted of barcodes and primers. Sequences that were too short (< 200bp), that had ambiguous base calls or homopolymer runs exceeding 6bp were removed. Chimera removal and sequence de-noising were then performed. The remaining sequence reads were clustered at 97% similarity to determine operational taxonomic units (OTU's) as illustrated elsewhere (Dowd et al., 2008a; Dowd et al., 2008b; Edgar, 2010; Capone et al., 2011; Swanson et al., 2011). The OTUs were subsequently classified using BLASTn against a curated database (DeSantis et al., 2006) and compiled into the different taxonomic levels such as phyla and genera. The final average number of sequence reads per sample was 6,203 with a minimum of 1834 and a maximum of 16400. The 25^{th} and 75^{th} percentile for the 32 samples were 4328 and 7511 quality reads per sample after filtering.

Predicted Metagenome

A new robust algorithm named "PICRUSt" was used, which predicts metagenomes from 16S data and reference genome databases (Langille et al., 2013). The accuracy of such technique strongly depends on the availability of reference genome sequences for organisms present in a sample. When reference genome sequences for taxa present in a sample are well established, the correlations between predicted and metagenomically measured gene content can reach 0.8 to 0.9 (Langille et al., 2013).

To quantify the availability of a reference genome representative of the overall microbiome in a sample, the nearest sequenced taxon index (NSTI) is estimated (Langille et al., 2013). The NSTI is the sum of phylogenetic distances for each organism in the OTU table to

their nearest relative with a sequenced reference genome. The closer to zero such distance is, the more amenable the sampled OTU matrix is for metagenome prediction. Although very variable, the estimated NSTI was in general very close to zero for our OTU matrix, thus rendering the algorithm suitable to apply (Table 4.1).

Inferred gene contents produced by PICRUSt were annotated against the KEGG Orthology (KOs) classification scheme to obtain a functional gene abundance matrix.

Digesta SCFA Concentration

Ruminal and colonic digesta samples were collected and acidified with a 10-N hydrochloric acid solution. Afterward, samples were centrifuged at 15,000 × g for 15 min, the supernatant extracted, frozen and send to North Carolina State University to the laboratory of Dr. Vivek Fellner for SCFA analysis by gas liquid chromatography. Briefly, digesta samples supernatant was injected (1 μL) into a column (Varian CP 3380/3800 with a Nukol Fused Silica Capillary Column, 30 m × 0.25 mm × 0.25 mm; Sigma-Aldrich, St. Louis, MO). A flame ionization detector was used to quantify VFA elution (Herfel et al., 2013).

Data Analysis

Microbial Community Diversity. The total number of species present (i.e., species richness) and the Shannon index were calculated as proxy measures of diversity. The Shannon index takes into account not only richness but also the abundance evenness among species (Tuomisto, 2010). Richness and Shannon index values estimated for each sample were used as dependent variables in function of GIT site and calf age. To account for the lack of independence between samples from rumen and colon from the same calf (i.e., possibly auto-correlated errors), a generalized linear model with a random intercept was fitted to the data to induce a compound symmetry correlation structure among observations from the same calf (Zuur et al., 2009).

Penalized quasi-likelihood was used to estimate parameters and standard errors. Statistical inference followed guidelines by others (Bolker et al., 2009). Richness data were analyzed under a negative binomial (NB) distribution to account for over-dispersion in species counts; whereas, diversity data were analyzed under a gamma distribution because a normal distribution did not meet its error distribution assumptions.

A log link linear predictor for modelling richness was specified as:

$$\eta_{ij} = \log\left(\frac{\mu_{ij}}{total\#reads}\right) = B_0 + B_1\left(site_{ij}\right) + B_2\left(week_j\right) + B_3 * (site * week)_{ij} + b_0 \ ,$$

where, μij = expected number of species in a given week and GIT site, total # reads= total number of sequence reads per sample included as an offset variable, B_0 = intercept, B_1= regression coefficient for GIT site, B_2= regression coefficient for calf age in weeks and b_0= calf random effects. Because the total number of sequence reads varies from sample to sample due to technical matters, it was used as an offset variable to express abundance in relative terms (Zuur et al., 2009). Calf random effects are assumed to be normally distributed and independent from each other.

For the sake of interpretability of the coefficients for richness, the above model is equivalent to:

$$\frac{\mu_{ij}}{total\#reads} = (\exp^{B_0}) * (\exp^{B_1 * site_{ij}}) * (\exp^{B_3 * week_j}) * (\exp^{B_3 * site * week_{ij}})$$

to estimate the expected number of reads of a specific taxon adjusted for the average total number of sequence reads present in a particular age by GIT site group. The exponentiated intercept represents richness at 2 weeks of age in colon, the exponentiated coefficient for site is the fold change in rumen with respect to colon and the coefficient for week is the fold change in week 4 relative to week 2.

Microbial Community Composition. The sequence read counts of each of the most predominant bacterial taxa were analyzed in the same fashion as richness. Since large overdispersion was present, a Poisson distribution was not suitable and a negative binomial distribution was used (Fitzmaurice et al., 2011).

For multivariate analysis, a Bray-Curtis distance matrix that provides a measure of the similarity among each pair of samples across ages and GIT sites was built. To visually explore the degree of microbial composition similarity between samples collected at 2 and 4 weeks of age from rumen and colon, non-metric multidimensional scaling (NMDS) was conducted on the above Bray-Curtis distance matrix (Borcard et al., 2011). Square root transformed and non-transformed distances provided similar output, thus the latter was used throughout.

To statistically test any visually apparent microbiome group differences from the NMDS plots, Analysis of Similarities (ANOSIM) (Clarke, 1993) and Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) were conducted with sites nested within week. The ANOSIM statistically tests whether there is a significant difference between two or more groups of sampling units based on the difference of mean distance ranks computed from the matrix. The ANOSIM statistic R ranges from -1 to 1 with 0 indicating no grouping of samples. The statistical significance (i.e., P-value) of the observed R statistic is assessed by permutation. The PERMANOVA partitions the total sums of squares estimated from the distance matrix among sources of variation and uses a permutation test with pseudo-F ratios to compute a P-value.

Multivariate homogeneity of variances (Anderson, 2006) was tested by calculating the distance of each sample to the group median (i.e., centroid) in multivariate space and then testing if the average distance among groups is different through parametric ANOVA. Six pairwise

comparisons of group mean dispersions were carried out by calculating Tukey's Honest Significant Differences between groups with a specified family-wise error probability of 5% to control the type 1 error rate.

Subsequently, in order to identify which genera contributed most to the microbiome observed differences among groups, Similarity Percentage Analysis (SIMPER) (Clarke, 1993) was run separately across weeks of age and GIT sites. Due to SIMPER methodological limitations (Warton et al., 2012), some of these results are complemented by univariate tests as appropriate using the generalized linear mixed model framework (Fitzmaurice et al., 2011) to test statistical differences among groups as explained above for richness data.

Besides the intergroup abundance criteria, it was also of interest to detect the assemblage of organisms that best characterizes calves of a certain age or a GIT site based on their affinity for that niche. Thus, Species Indicator Analysis (INDVAL) (Dufrene and Legendre, 1997) was performed. This analysis provides a species indicator index that ranges between 0 and 1. If a genus is uniquely present in one group of calves and it is present in each individual calf belonging to that group, then the indicator index takes a maximum value of 1 (Legendre, 2013). A permutational p-value is used to assess statistical significance of the indicator value.

The NMDS and ANOSIM were carried out in Primer-E software V.6.0, SIMPER (Clarke and Gorley, 2006), PERMANOVA and multivariate heterogeneity of variances tests were conducted with the package Vegan (Oksanen et al., 2012), INDVAL was carried out with the package Labdsv (Roberts, 2012), and generalized linear models were performed with the package MASS (Venables and Ripley, 2002) in the open access R language version 2.14.0.

Predicted Metagenome. This abundance data matrix was subjected to multivariate analysis methods similar to the microbial composition data.

Intestinal SCFA Concentrations. Colon and rumen SCFA data were analyzed as repeated measures on the same calf. A compound symmetric error structure with heterogenous variances was implemented in PROC MIXED of SAS (SAS) (SAS Institute Inc. Cary, NC.,). Residuals were normally distributed.

RESULTS

Microbial Community Diversity

Figure 3.1 visually illustrates the distributions for richness and diversity. While the rumen may visually appear to host a greater total number of species, groups appear more similar in terms of the Shannon index.

Table 3.2 displays the regression coefficients describing the effect of age and GIT site on richness and Shannon diversity indices.

Richness. The effects of age and site were significant. Calves at 4 weeks of age are expected to have 1.4 times as many species in the GIT as those at 2 weeks ($\exp^{0.34} = 1.4$); whereas, compared to colon, there were 1.56 times as many species in the rumen ($\exp^{0.45} = 1.56$). There was no interaction effect between week and site and thus it was removed from the model.

Shannon diversity index. The coefficients for age and site were not different from zero and therefore the mean Shannon diversity index value is about 3.74 ($\exp^{1.32} = 3.74$) across ages and sites.

Taking richness and the diversity indices together, it appears that although calves harbor a 1.56 the number of species in the rumen at 4 weeks, the number of equally abundant species [i.e. effective number of species (Tuomisto, 2010)] does not really differ across ages or GIT sites.

Microbial Community Composition

Phyla level. Out of 18 phyla identified, only 5 of them were found to make up ~98.5% of the total (fig.4.2). Firmicutes was the most abundant with 70%, followed by Bacteroidetes (12%), Actinobateria (8%), Tenericutes (5%) and Proteobacteria (3.5%). Phyla distribution is illustrated in Figure 3.3.

Regression coefficients for the statistical assessment of the effects of age and GIT site on relative abundance of each phylum are displayed below (Table 3.3). Firmicutes abundance was affected by the calf age and GIT site. At 4 weeks calves presented 1.36 times the abundance of this phylum compared to calves 2 weeks old. In addition, rumen contained only ~68% the Firmicutes abundance observed in colon. For Bacteroidetes, there was no effect of age on its abundance, but this phylum was 510% statistically more abundant in rumen than in colon. Actinobacteria abundance was not different between ages or GIT sites. For Tenericutes, both age and GIT site affected its abundance. It was found to be more abundant at 2 than at 4 weeks of age and largely more abundant (i.e., ~4 times) in rumen than in colon. Proteobacteria was also significantly more abundant in rumen than in colon. Unfortunately, because of multicollinearity problems between the coefficients for the main effects and the interaction term, the latter must be removed from the model and only main effects could be considered. Overall, it thus appears that Firmicutes is stronger in colon; whereas, Bacteroidetes, Proteobacteria and Tenericutes exist in larger quantities in rumen.

Genus Level. The NMDS plot below displays the samples belonging to colon and rumen at 2 and 4 weeks of age (Figure 3.4a). Whereas rumen samples from 2 and 4 weeks of age appear to group apart, colon samples from 2 and 4 weeks of age display overlap suggesting that the composition of microbiota is more similar at weeks 2 and 4 in the colon than in the rumen. At the same time, rumen and colon form different clusters (Figure 3.4a) and when considered across

ages, there is a visually evident dissimilarity between ruminal and colonic microbial communities (Figure 3.4b). When comparing weeks 2 and 4, only moderate separation of clusters can be appreciated suggesting that calves 2 and 4 weeks of age share more of their microbial community composition than GIT sites do (Figure 3.4c).

To statistically test these compositional differences, ANOSIM and PERMANOVA were conducted (Tables 3.4 and 3.5). The ANOSIM indicates that there is a large and significant dissimilarity between rumen and colon but none between 2 and 4 weeks of age. Like ANOSIM, PERMANOVA allows evaluation of the main effects of age and GIT site, but in addition, it also allows testing their interaction. Results of PERMANOVA analysis are shown in Table 3.2.

Contrary to ANOSIM, PERMANOVA indicates a significant effect of week. In addition, it indicates a significant interaction between age and GIT site on the microbiota composition of calves meaning that the effect of age depends on the gastrointestinal site. Indeed, it is evident from figure 1a that whereas samples collected at 2 and 4 weeks from colon do not appear to differ greatly, samples from rumen do seem to be different. Still, the sum of squares for GIT site is by far the largest, explaining ~20% of the total variation in microbiota composition compared to age or their interaction.

A variance homogeneity test was conducted and indicated that the microbial community composition in the colon of calves at 4 weeks of age is much less variable than that in the rumen at 2 and 4 weeks or colon at 2 weeks. This is illustrated in Figure 3.5 and agrees with the visual appraisal from Figure 3.4a. Tukey's Honest Significant Differences test between groups shows the variances of the other groups do not differ (Table 3.6). While this observation warns that the variances homogeneity condition is not strictly met, it also raises an interesting question as to

why and how the microbial population in the colon becomes so much more consistent after 4 weeks as compared to the rumen.

We next tried to identify the genera that contributed most to the observed differences between calves at 2 and 4 weeks of age and between colon and rumen. To that end, SIMPER was carried out. Table 3.7 lists the most influential genera in differentiating the 2 age groups in terms of abundance. Also indicated is the anatomical location, the average abundance in each age group and the proportion they contribute to the dissimilarity between 2 and 4 week old calves. Only 14 genera, which represent ~3.6% of the total 393 genera identified, accounted for 60% of the microbial compositional difference between 2 and 4 weeks of age. For instance, *Lactobacillus*, *Streptococcus* and *Faecalibacterium* in the colon contributed about 17, 4 and 4 %, respectively, while *Bacteroides* and *Oscillospira* in rumen contributed about 5 and 4% each to the overall dissimilarity between 2 and 4 week old calves. There was a seemingly remarkable increase of the colonic *Lactobacillus* population from ~34 to 69% and a reduction of *Streptococcus* genus from ~12 to 4% in the time course between 2 and 4 weeks. During the same time span, in the rumen, *Bacteroides* and *Porphyromonas* populations dropped from ~14 to 3 and from ~9.5 to 0.7%, respectively.

Regarding GIT sites (Table 3.8) and similarly surprising, only 7 out 393 genera identified (~1.7%) accounted for 60% of the compositional difference between rumen and colon. Table 3.8 indicates that *Lactobacillus* is by far the main contributor to the abundance differences between colon and rumen, being ~50% more abundant in the hindgut and adding 28% of the total dissimilarity. Furthermore, *Streptococcus*, *Faecalibacterium*, *Clostridium*, *Ruminococcus*, *Oscillospira*, and *Bacteroides* were able to determine the remaining 32% dissimilarity in microbial community composition across GIT sites. Thus, not only did these 7 genera account

for most of the abundance difference between rumen and colon, but their population size also changed so drastically over time that they could explain up to ~46% of the dissimilarity between 2 and 4 week old milk fed calves (Tables 3.7 and 3.8).

Table 3.9 below shows results for univariate tests complementary to SIMPER for those genera whose additive contributions to age and GIT site dissimilarities add to 49 and 60%, respectively. *Lactobacillus* and *Streptococcus* were statistically greater in colon than in rumen, while *Oscillospira*, *Bacteroides* and *Porphyromonas* were statistically in greater numbers in rumen than in colon. Unlike *Lactobacillus*, which increased by 270% from week 2 to 4, all other genera statistically remained at the same level or diminished. No statistical effect of age or GIT site was observed for *Faecalibacterium*, *Oscillospira*, *Clostridium* or *Ruminococcus*.

Table 3.10 shows the species indicator index values at a cut-off of 0.8 for the genera showing greatest affinity for an age group and their GIT location. These results show that *Escherichia* and *Enterococcus* from the colon, and *Actinomyces* and *Porphyromonas* from the rumen are present almost exclusively and highly frequently in 2 week old calves while rarely or in lesser amount in 4 week old calves. On the other hand, at 4 weeks of age, *Prevotella* and *Lactobacillus* from the rumen are almost an exclusive feature of this organ with respect to the younger calves.

The index values for best indicator genera of colon and rumen are shown next (Table 3.11). While only 3 genera are highly specific to the colon, about 9 genera are so to the rumen. *Lactobacillus*, *Streptococcus* and *Bifidobacterium* for instance, have an extremely high affinity for the colon; whereas, *Actynomices*, *Prevotella* and *Eubacterium* do so for the rumen.

Contrary to SIMPER, INDVAL can eventually, but does not necessarily, identify the most abundant genera overall, and instead identifies those that are most specific to each group.

While abundant taxa like *Lactobacillus* could be expected to impact nutrient fluxes and fementation product pools, it is intriguing whether highly specific low abundance taxa could play an important functional role. In this sense, the exclusive but transitory presence of *Escherichia* and *Enterococcus* in the colon at ~1.5 and 6 %, respectively, and of *Actinomyces* and *Porphyromonas* in the rumen at ~6.3 and 9.5%, respectively, during the first 2 weeks of life, raises the question of what their role could be.

Predicted metagenome

Another objective of this work was to gain insight into the potential metabolic function pool of the microbiota by using a recently published algorithm that allows predicting the metagenome structure based on 454 Pyrosequencing data (Langille et al., 2013). After annotation against the KEGG database and removing all possible host functions, 104 pathways were identified.

The NMDS was applied to all 104 pathways identified to visualize the similarities in the predicted metagenome of samples from colon and rumen at 2 and 4 weeks of age. Figure 3.6 (a and b) suggests that unlike the microbial community composition, metabolic functions do not differ as clearly between 2 and 4 week old calves. Nevertheless, there is a seemingly stronger separate clustering of rumen and colon (Figure 3.6.c), suggesting that microbiome functionality differs to a larger extent from forestomach to hindgut than throughout time during the first month of life of pre-ruminant calves.

The ANOSIM confirmed the NMDS ordination indicating only a moderate difference between rumen and colon and the absence of difference in the metagenome between 2 and 4 weeks of age. Table 3.12 below illustrates these results.

The PERMANOVA results (Table 3.13) agreed with NMDS and ANOSIM, and the test for interaction between calf age and GIT site was not significant. These results indicate that GIT site was more important in defining the microbial metabolic potential in milk fed calves even though the variation it explained is very small according the sums of squares.

Based on these findings we tested for significant differences between GIT sites for the most abundant pathways. The most predominant metabolic potential functions are displayed below (fig.3.7). These variables accounted for ~25% of all potential functions identified, and the remaining ~75% was comprised by functions representing less than 0.2% each and are not displayed. The observed average percentage of each function for colon and rumen seem very similar at eye sight.

Indeed, except for galactose metabolism and bacterial motility proteins, at 5% type 1 error rate, no difference was observed for any of these variables (data not shown) between colon and rumen. Galactose metabolism and bacterial motility protein pathways were present at ~1.5 and 0.9, and 0.2 and 0.7 % in colon and rumen, respectively.

Subsequently, INDVAL was performed to identify signature functions of each GIT location. Table 3.14 shows those KEGG pathways with indicator index values above 0.75. Remarkably, there were 5 pathways of high characteristic value for the colon; whereas, there was none for rumen.

SCFA Concentrations

In addition, SCFA concentrations were measured to gauge metabolic activity in each GIT site at 2 and 4 weeks of age. Figure 3.8 displays the distribution of SCFA in each week by GIT site group. Except for acetate and isovalerate, all other SCFA had higher concentrations in the colon than in the rumen. Propionate, isobutyrate, butyrate and lactate were on average about 1.5,

4.4, 5.2 and 8.5-fold, respectively, higher in colon than in rumen suggesting colon as a much more metabolically active organ in milk fed pre-ruminant calves. These differences did not depend on time; in fact, calf age had no effect at all on SCFA concentrations (table 3.15).

DISCUSSION

In spite of the fact that rumen at 4 weeks displayed the greatest total number of species, using a diversity index that accounts for the evenness among them indicated that both rumen and colon were just as diverse at 2 and 4 weeks of age. The physiological importance of the difference between the absolute and the effective number of species is unknown.

A remarkable observation was the significantly higher homogeneity of the colonic microbiota composition among calves by week 4. This has been observed before by Jami et al (2012) to occur in the rumen of preweaning calves fed starter in an age dependent fashion in which similarity between calves increased to plateau by 6-months of age. Unlike colon, the ruminal bacterial community remained more heterogenous among calves at 4 weeks in our study, maybe due to the absence of fermentation substrate for the microbial community to develop and structure.

In this work we also observed Firmicutes and Actinobacteria as the most abundant phyla in the calf hindgut making up >90% of the whole microbial community, while Firmicutes and Bacteroidetes dominated in the forestomach accounting for ~80%.

In general, at 2 weeks the calf colon was characterized by large numbers of *Lactobacillus* (33%), *Streptococcus* (12%), *Faecalibacterium* (11%), *Clostridium* (6.5%), *Enterococcus* (6%) and *Bifodobacteria* (5%). Two weeks later, *Lactobacillus* increased its primacy to ~69%, and along with *Streptococcus* (~4%), *Collinsella* (~5%) and *Bifidobacterium* (~2.5%), remained dominant and highly specific to the colon comprising ~80% of the microbial population.

In contrast, the rumen underwent many more compositional changes from week 2 to 4 of age and no overwhelming dominance by a particular genus was observed. At 2 weeks of age, *Bacteroides*, *Oscillospira*, *Porphyromonas*, *Bulleidia*, *Faecalibacterium* and *Actinomyces* were characteristically abundant and specific to this niche at ~14, 12, 9, 7, 6 and 6% of the population, respectively. By 4 weeks, *Oscillospira* and *Faecalibacterum* became the most abundant taxa by hardly increasing to 16 and 10%, correspondingly, while the others declined sharply. At the same time, other genera like *Ruminococcus*, *Prevotella*, *Clostridium*, *Blautia* and *Eubacteria* increased to be in the range of 4 to 8%. However, unlike in the colon, these dominating genera altogether barely accounted for ~55% of the whole community and thus the remaining ~45% must have been composed by many multiple organisms.

The minor compositional changes and the consolidation of the initially dominating microbiota in the colon suggests there was greater colonization resistance by lactic acid bacteria (Macfarlane et al., 2008) compared to rumen where no dominant taxa were observed and many more compositional changes occurred. It suggests that colonic populations stabilized earlier than those in rumen (Rolfe, 1996).

On the other hand, our findings differ with some previous observations in pre-weaning calves fed milk or milk plus grain where the phyla Bacteroidetes predominated over Firmicutes and the genera Bacteroides and Prevotella consistently made up the majority of the population in ruminal, colonic or fecal samples at ages between 1 and 6 weeks of life (Edrington et al., 2012; Li et al., 2012; Jami et al., 2013; Malmuthuge et al., 2014). A possible explanation could be that we fed no grain and used an intensified milk replacer feeding program with feeding rates of 2% of BW as DM. This translates to a dietary supply of ~1 kg of DM/d for a 45-kg BW calf. Assuming an average milk replacer total DM digestibility of ~80% (Petit et al., 1989; Hill et al.,

2010), an actual dietary concentration of 28% protein and 43% lactose and assuming each nutrient fraction is at least 80% digestible; then, at least 56 g of protein and 86 g of lactose should have passed undigested into the cecum and colon on a daily basis. In contrast, because the esophageal groove is nearly fully functional in newborn milk fed calves (Orskov, 1992; Davis and Drackley, 1998), milk flow into the rumen, and hence provision of any fermentable substrate may not have exceeded 10 g/d (Toullec and Guiloteau, 1989).

Because representative species in the *Lactobacillus*, *Streptococcus*, *Enterococci* and *Bifidobacteria* genera are known to grow well or be able to adapt and perform well with lactose or galactose as a substrate (Özen and Özilgen, 1992; Burgos-Rubio et al., 2000; Cabral et al.,2007), and are considered major lactic acid bacteria (Smith and Sherman, 1942; Macfarlane et al., 2008), we speculate that colonic supply of endogenous or lactose derived milk di- or mono-saccharides led to their overabundance and the resulting ~ 850% higher lactate concentrations observed in the hindgut compared to the rumen. At the same time, high colonic acid molarities may have caused acidic conditions for bacteria such as *Bacteroides spp* to grow, establish and compete since they prefer a pH of 6.5 (Belenguer et al., 2007). Indeed, low pH is thought to be the major mechanism by which lactic acid bacteria, primarily *Lactobacillus*, *Bifidobacteria* and *Streptococcus*, inhibit growth of various facultative and anaerobic bacteria (Rolfe, 1996).

Moreover, although propionate and butyrate production is not consistent with Bifidobacterial and Lactobacillus metabolism (Macfarlane et al., 2008); it has been shown that propionate producing species such as *Veillonella spp* and *Megasphera elsdenii*, and butyrate-producing species such as *Anaerostipes caccae* and *Eubacterium halli* can cross-feed on lactate produced by *Bifidobacterium adolescentis* (De Vuyst and Leroy, 2011). Therefore, some of the increased propionate, isobutyrate and butyrate could possibly have been the end products of

lactate metabolism through extensive cross-feeding in the colon (Belenguer et al., 2006; De Vuyst and Leroy, 2011).

Lactobacillus and Bifidobacteria have been linked to increased resistance to infection and diarrheal disease, and stimulation of immune system activity, possibly due to the chemical composition and structure of their cell wall components. Indeed, some *Lactobacillus*, *Bifidobacteria* and *Enterococci* species form substances that are antagonistic to other organisms, such as organic acids, hydrogen peroxide, diacetyl and bacteriocins (Macfarlane et al., 2008; Vizoso Pinto et al., 2009). For example, *Bifidobacterium Infantis* can produce substances that are inhibitory to *E.Coli* and *Clostridium perfringens*, whereas other bifido species can inhibit including *Listeria*, *Shigella*, *Campylobacter*, and *Vibrio cholerae* (Gibson and Wang, 1994). Given the high colonic abundance and specificity observed here, lactic acid bacteria and *Bifidobacterieum* may have had a role to play in the intestinal health of calves in this study.

In addition to such direct microbial effects, SCFA, as the major fermentation end products, also have a role in colonic function and integrity. While acetate and propionate affect the epithelium's energy and the host's glucose homeostasis, respectively (Topping and Clifton, 2001), butyrate production in the hindgut can improve epithelium permeability by up regulating tight junction protein expression (Cani et al., 2009), reduce epithelium inflammation (Macfarlane et al., 2008) and promote cell differentiation (Topping and Clifton, 2001).

From this standpoint, our observations would suggest a key role for the colonic microbiota of the newborn pre-ruminant calf, which was reflected, in this case, by propionate, isobutyrate, butyrate and lactate concentrations between 50 and 850% higher compared to the rumen. Consequently, colonic fermentation may appear to be more preponderant to potentially support intestinal health and integrity during the first few weeks of life, while ruminal

fermentation would develop and become increasingly important for the metabolic energy supply of the calf after ~4 to 5 weeks of life (Davis and Drackley, 1998).

Functionally, the potentially higher supply of milk protein and lactose, galactose and glucose into the colon, and the resulting enhanced fermentation could provide a possible explanation for the specificity and unique presence of microbial functions such as alanine and galactose metabolism, and glycosyltransferase activity, with the latter two potentially related to mono and di-saccharide hydrolysis and sugar groups transfer and metabolism (Garrett and Grisham, 2010). Also, the augmented abundance of the glutathione metabolism pathway is perhaps related to the large abundance of the lactic acid bacteria, some of whose members are known to possess parts of the glutathione synthesis or utilization machinery (Pophaly et al., 2012).

Still, it is surprising that out 104 metabolic pathways identified only 4 of them could modestly differentiate the fore and hindgut; whereas, the most abundant ones related to transport, DNA maintenance, or translation were at the same level in both organs throughout time in spite of the fact that ruminal fermentation was rather inoperative. These observations coincide with those of Li et al. (2012) where milk fed calves presented a wide array of microbial metabolic potential functions including transport and metabolism of carbohydrates and amino acids, energy production, and DNA integrity maintenance. Like in our experiment, those authors noticed that calves at 2 and 6 weeks presented very similar metabolic ruminal potential in spite of major phylogenetic composition fluctuations over time. Hence, although the rumen appeared much less active metabolically in our experiment, probably because of minimal substrate availability, it did show to have all the bacterial and metabolic endowment to begin operation upon fermentative substrate delivery.

CONCLUSION

The rumen and colon of intensively milk fed pre-ruminant calves had a similar number of effective bacterial species during the first month of life. The inter-calf variation in colonic microbiome composition diminished significantly by 4 weeks of age compared to the rumen. Lactic acid bacteria such as Lactobacillus, Streptococcus, Enterococcus and Bifidobacteria overly dominated the microbial community in the hindgut. In addition, SCFA concentrations were between 50 and 850% higher compared to rumen. These high concentrations may have inhibited the Bacteroides genus from expanding, which appears atypical in calves and other species. These microbial community and SCFA profiles could bring about beneficial effects for the intestinal health of the calf during the first 2 to 4 weeks of life. On the other hand, in the rumen, no genus showed to over-dominate and butyrate and lactate concentrations were much lower than in colon, presumably due to lack of fermentable substrate. It is reasonable to think that, because of an active esophageal groove and very low grain intake during first 2 to 3 weeks of life, ruminal fermentation may not take preponderance until after 4 to 6 weeks of life, when significant metabolizable energy contributions start to take place. Research studying potential nutritional management means to optimize hindgut fermentation during early life to support intestinal health should prove worthy.

TABLES AND FIGURES

Table 3.1. NSTI values for sampled 16S sequences

GIT[1] site	Week	Mean	S.E
Colon	2	0.06	0.04
Rumen	2	0.08	0.04
Colon	4	0.06	0.06
Rumen	4	0.12	0.06

[1] GIT: Gastrointestinal tract

Table 3.2. Regression coefficients for effect of calf age and GIT site on diversity measures

	Coefficients (β)	S.E	P-value	$exp^{(\beta)}$ [1]
	Species number			
Intercept	-3.28	0.12	<0.00001	0.038
Week 4	0.34	0.13	0.017	1.40
Rumen	0.45	0.13	0.0019	1.56
	Shannon index			
Intercept	1.32	0.03	<0.00001	3.74
Week 4	0.036	0.04	0.34	
Rumen	0.029	0.04	0.44	

[1] $exp^{(Intercept)}$: represents expected value for colon at 2 weeks of age; $exp^{(Week4)}$: represents fold change at 4 weeks with respect to 2 weeks; $exp^{(Rumen)}$: represents fold change in rumen with respect to colon

Table 3.3. Regression coefficients for effects of calf age and GIT site on abundance of predominant phyla

	Coefficient (β)	S.E	P-value	exp$^{(\beta)}$ [1]
		Firmicutes		
Intercept	-0.36	0.08	0.0001	0.69
Week 4	0.313	0.09	0.0022	1.36
Rumen	-0.38	0.08	0.0001	0.68
		Bacteroidetes		
Intercept	-3.17	0.51	< 0.0001	0.042
Week 4	-0.21	0.59	0.7	
Rumen	1.63	0.59	0.01	5.1
		Actinobacteria		
Intercept	-2.19	0.23	<0.0001	0.11
Week 4	-0.51	0.28	0.08	
Rumen	-0.40	0.28	0.15	
		Tenericutes		
Intercept	-3.32	0.4	<0.001	0.0362
Week 4	-1.31	1.39	0.01	0.27
Rumen	1.39	0.5	0.01	4.01
		Proteobacteria		
Intercept	-4.60	0.35	<0.0001	0.01
Week 4	0.06	0.44	0.9	
Rumen	1.0	0.30	0.002	2.71

[1] exp $^{(Intercept)}$: represents expected value for colon at 2 weeks of age; exp $^{(Week4)}$: represents fold change at 4 weeks with respect to 2 weeks; exp $^{(Rumen)}$: represents fold change in rumen with respect to colon

Table 3.4. ANOSIM among samples from rumen and colon and from weeks 2 and 4 of age for microbiota composition

Contrast	R Statistic[1]	P-value
Rumen vs. Colon	0.75	0.001
15 vs. 30 days of age	0	0.67

[1] Strength of groups difference

Table 3.5. PERMANOVA for effect of calf age and GIT site on microbial community composition

Source of variation	df[1]	SS[2]	MS[3]	Pseudo-F	P-value
Site	1	4.8363	4.8363	21.4576	0.001
Week	1	0.8797	0.8797	3.9029	0.001
Site x Week	1	0.8034	0.8034	3.5647	0.004
Residual	59	13.2978	0.2254		
Total	62	19.8172			

[1] Degrees of freedom
[2] Sum of squared multivariate distances
[3] Mean squared multivariate distances

Table 3.6. Pairwise comparison of age by GIT site group variances

Variance pairwise comparison	P-value
2 weeks-rumen vs 2 weeks-colon	0.95
4 week-colon vs 2 week-colon	<0.0001
4 week-rumen vs 2 week-colon	0.61
4 week-colon vs 2 week-rumen	<0.0001
4 week-rumen vs 2 week-rumen	0.9
4 week-rumen vs 4 week-colon	<0.0001

Table 3.7. Contribution by specific genera to overall dissimilarity between the microbial community of 2 and 4 week old calves

		Average abundance %		
Genus	GIT location	2 weeks	4 weeks	Dissimilarity %
Lactobacillus	colon	33.60	68.90	17
Bacteroides	rumen	14.40	3.01	5
Oscillospira	rumen	12.00	15.90	4
Streptococcus	colon	12.30	3.95	4
Faecalibacterium	colon	8.91	1.15	4
Porphyromonas	rumen	9.49	0.74	3
Faecalibacterium	rumen	5.71	9.57	3
Bulleidia	rumen	7.23	1.87	3
Ruminococcus	rumen	2.63	8.27	3
Clostridium	rumen	4.12	8.16	3
Oscillospira	colon	6.89	2.49	3
Eubacterium	rumen	3.67	4.24	3
Enterococcus	colon	6.43	1.07	3
Collinsella	colon	4.53	4.81	2
Total				60

Table 3.8. Contribution by specific genera to overall dissimilarity between the microbial community of rumen and colon

	Average abundance %		
	Colon	Rumen	Dissimilarity %
Lactobacillus	51.30	2.16	28
Oscillospira	4.70	14.0	8
Faecalibacterium	5.87	7.71	6
Bacteroides	1.40	8.52	5
Clostridium	3.86	6.21	5
Streptococcus	7.73	1.26	4
Ruminococcus	2.56	5.54	4
Total			60

Table 3.9. Regression coefficients for effect of calf age and GIT site on abundance of differentiator genera

	Coefficient (β)	S.E	P-value	$exp^{(\beta)}$ [1]
		Lactobacillus		
Intercept	-1.34	0.31	< 0.0001	0.26
Week 4	1.31	0.36	0.0012	3.7
Rumen	-3.52	0.36	< 0.0001	0.029
		Oscillospira		
Intercept	-3.1	0.33	< 0.0001	0.05
Week 4	-0.32	0.39	0.42	
Rumen	1.3	0.38	0.002	3.66
		Faecalibacterium		
Intercept	-3.26	0.50	< 0.0001	0.038
Week 4	-0.29	0.55	0.60	
Rumen	0.86	0.56	0.13	
		Bacteroides		
Intercept	-4.37	0.33	< 0.0001	0.012
Week 4	-1.69	0.41	< 0.0001	0.18
Rumen	2.07	0.29	< 0.0001	7.92
		Streptococcus		
Intercept	-2.26	0.29	<0.0001	0.10
Week 4	-0.85	0.34	0.01	0.42
Rumen	-1.71	0.34	<0.0001	0.18
		Porphyromonas		
Intercept	-6.51	0.44	<0.0001	0.001
Week 4	-1.44	0.52	0.001	0.23
Rumen	3.73	0.52	< 0.0001	41.6
		Clostridium		
Intercept	-3.76	0.59	<0.0001	0.02
Week 4	-0.12	0.62	0.84	
Rumen	0.93	0.63	0.15	
		Ruminococcus		
Intercept	-4.61	0.70	<0.0001	0.009
Week 4	0.49	0.64	0.45	
Rumen	1.34	0.67	0.06	3.82

[1] $exp^{(Intercept)}$: represents expected value for colon at 2 weeks of age; $exp^{(Week4)}$: represents fold change at 4 weeks with respect to 2 weeks; $exp^{(Rumen)}$: represents fold change in rumen with respect to colon

Table 3.10. Indicator index values for best identifier genera of calf age groups

Genus	GIT location	Indicator value		P-value
		2 weeks	4 weeks	
Escherichia	colon	0.93	0.07	0.001
Actinomyces	rumen	0.87	0.13	0.015
Porphyromonas	rumen	0.87	0.13	0.002
Enterococcus	colon	0.86	0.14	0.01
Prevotella	rumen	0.12	0.88	0.015
Lactobacillus	rumen	0.13	0.87	0.043

Table 3.11. Indicator index values for best identifier genera of rumen and colon

Genus	Indicator index		P-value
	Colon	Rumen	
Lactobacillus	0.96	0.04	0.001
Streptococcus	0.86	0.14	0.002
Bifidobacterium	0.83	0.17	0.001
Actinomyces	0.06	0.94	0.001
Prevotella	0.09	0.91	0.002
Eubacterium	0.1	0.9	0.001
Anaerotruncus	0.12	0.88	0.001
Porphyromonas	0.14	0.86	0.001
Bacteroides	0.14	0.86	0.003
Blautia	0.16	0.84	0.001
Bulleidia	0.16	0.84	0.003
Alistipes	0.19	0.81	0.001

Table 3.12.ANOSIM among samples from rumen and colon and from weeks 2 and 4 of age for predicted metagenome

Contrast	R Statistic[1]	P-value
Rumen vs. Colon	0.41	0.001
15 vs. 30 days of age	0	1

[1] Strength of groups difference

Table 3.13. PERMANOVA for effect of calf age and GIT site on metagenome composition

Source of variation	df[1]	SS[2]	MS[3]	Pseudo-F	P-value
Site	1	0.54	0.54	3.45	0.035
Week	1	0.02	0.02	0.002	0.49
Site x Week	1	0.10	0.10	0.66	0.52
Residual	59	9.28			
Total	62				

[1] Degrees of freedom
[2] Sum of squared multivariate distances
[3] Mean squared multivariate distances

Table 3.14. Indicator index values for best identifier microbial functions

	Indicator index		P-value
	Colon	Rumen	
D.Alanine metabolism	0.81	0.19	0.001
Galactose metabolism	0.78	0.22	0.001
Glutathione metabolism	0.78	0.22	0.001
Glycosyltransferases	0.77	0.23	0.001
Metabolism of cofactors and vitamins	0.75	0.25	0.001

Table 3.15. Short chain fatty acid and lactate concentrations in rumen and colon at 2 and 4 weeks of age

	Rumen		Colon		P-value		
	2	4	2	4	site	age	site x age
	mmol/ml						
Acetate	23.5	31.3	26.9	29.6	0.74	0.14	0.33
Propionate	3.9	4.1	5.65	6.22	0.004	0.43	0.78
Isobutyrate	0.11	0.15	0.64	0.42	0.03	0.59	0.39
Butyrate	0.43	0.45	2.44	2.15	0.001	0.71	0.66
Isovalerate	0.25	0.27	0.16	0.44	0.53	0.15	0.08
Lactate	0.09	0.25	1.65	1.41	0.0001	0.85	0.37

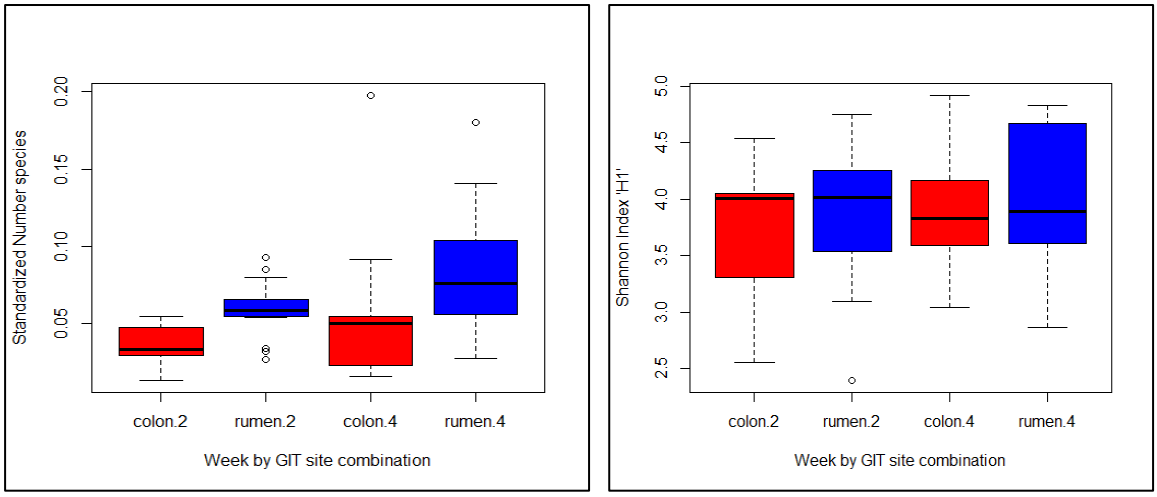

Figure 3.1. Richness and Shannon Index H1 diversity measures across GIT sites and calf ages (2.colon = colon at 2 weeks; 2.rumen = rumen at 2 weeks; 4.colon = colon at 4 weeks; 4.rumen = rumen at 4 weeks).

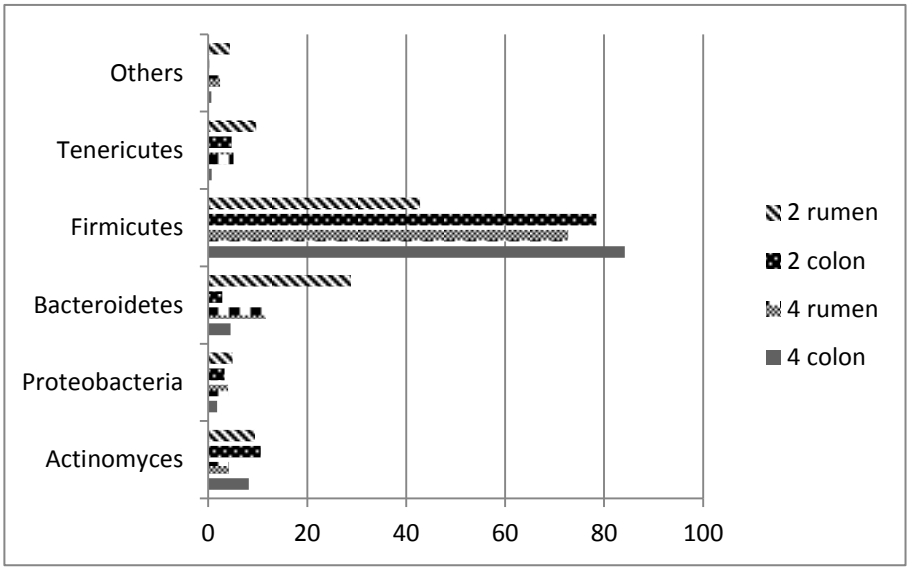

Figure 3.2. Predominant phyla abundance across calf ages and GIT sites

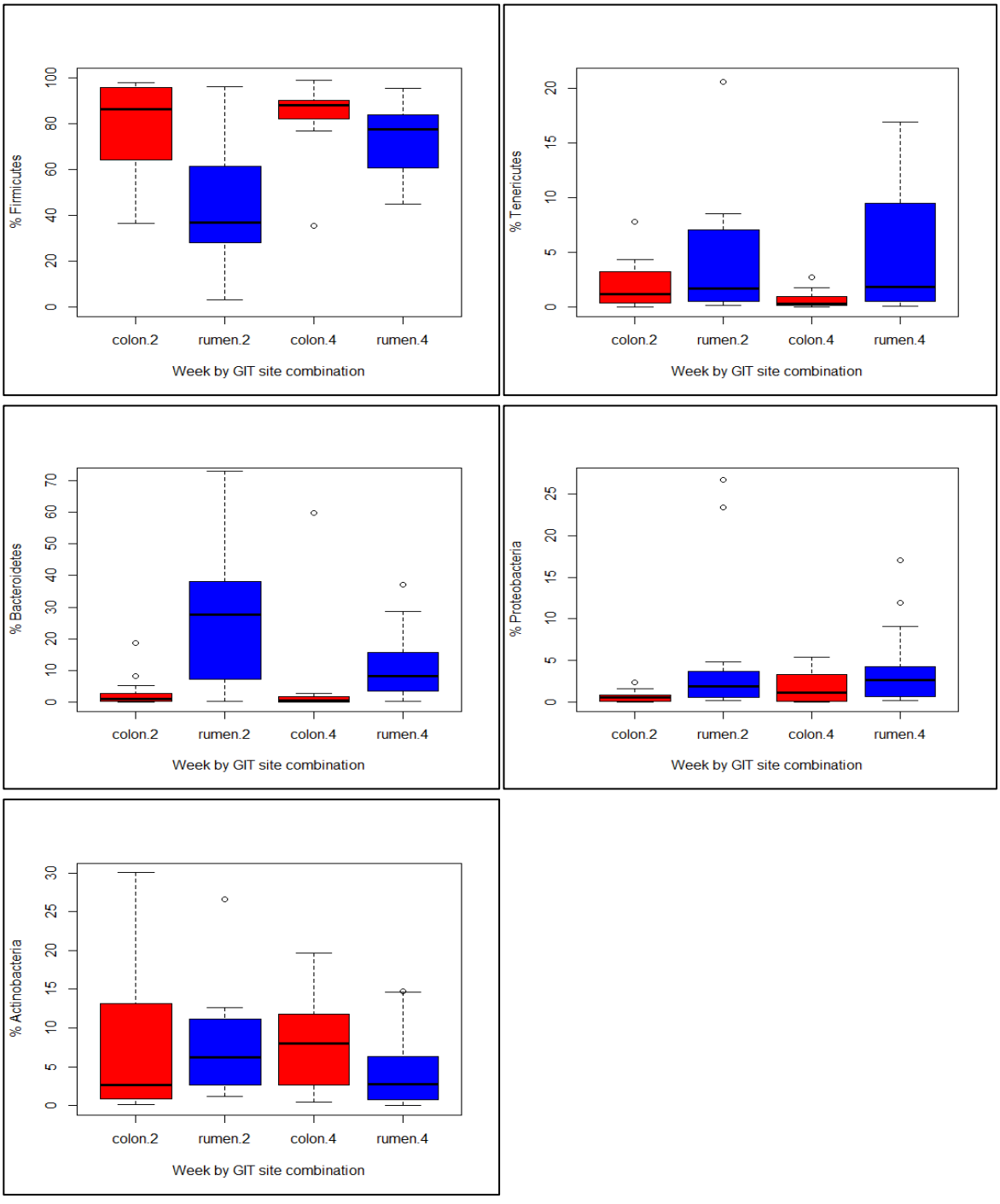

Figure 3.3. Distribution of predominant phyla across calf ages and GIT sites (2.colon = colon at 2 weeks; 2.rumen = rumen at 2 weeks; 4.colon = colon at 4 weeks; 4.rumen = rumen at 4 weeks).

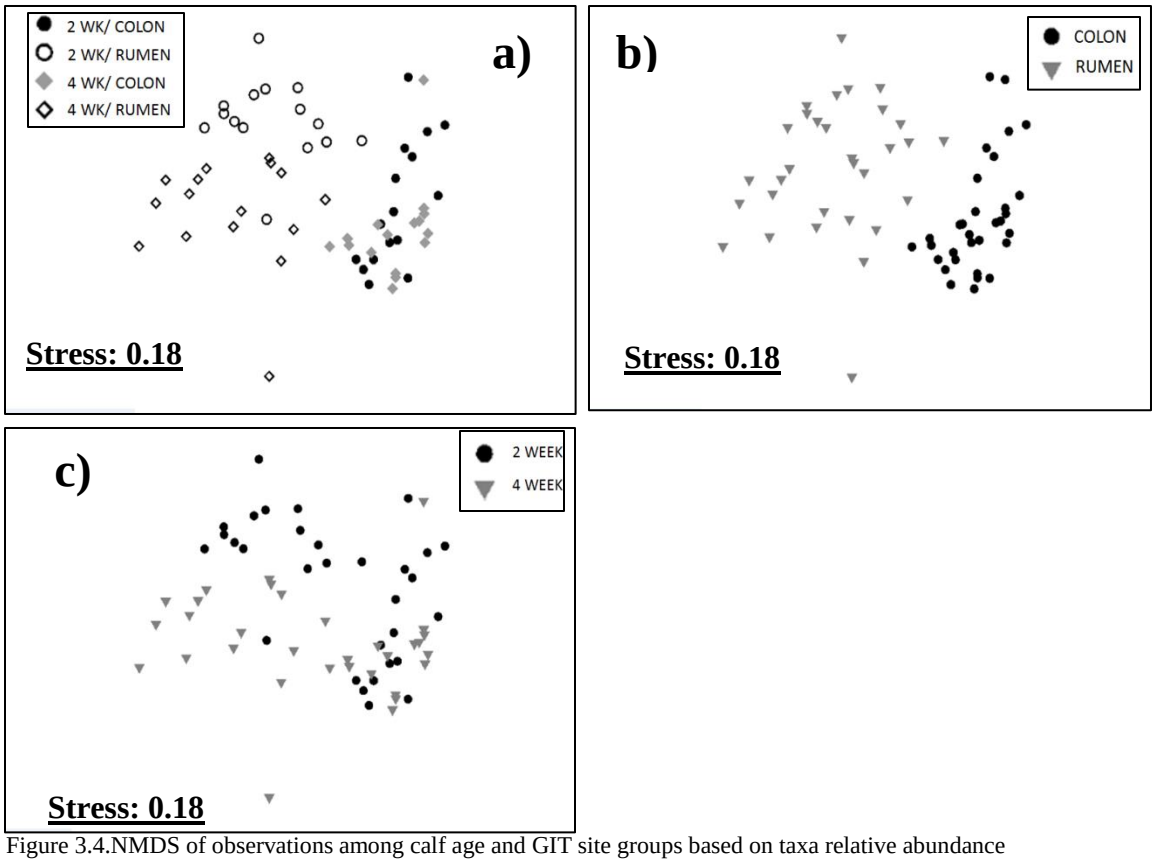

Figure 3.4.NMDS of observations among calf age and GIT site groups based on taxa relative abundance

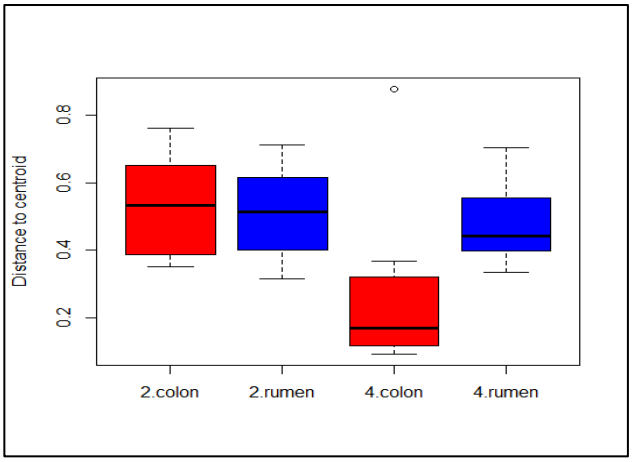

Figure 3.5.Variances distribution for calf age and GIT sites (2.colon = colon at 2 weeks; 2.rumen = rumen at 2 weeks; 4.colon = colon at 4 weeks; 4.rumen = rumen at 4 weeks).

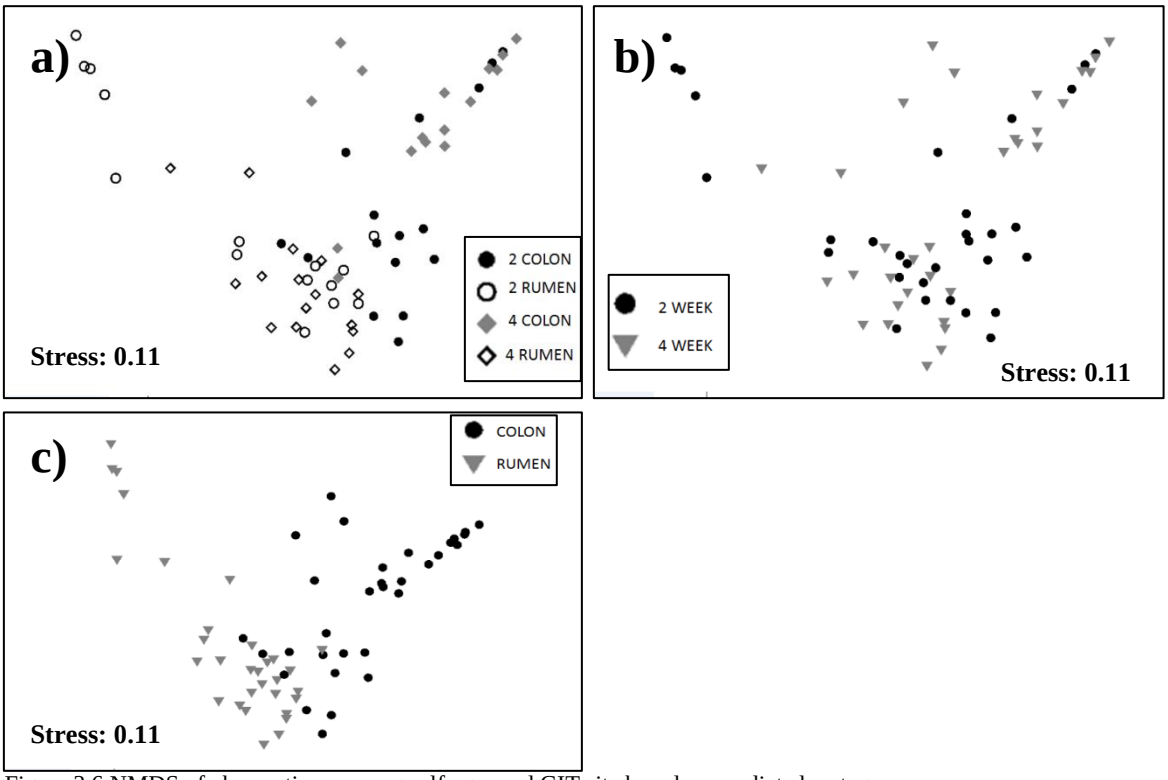

Figure 3.6.NMDS of observations among calf ages and GIT site based on predicted metagenome.

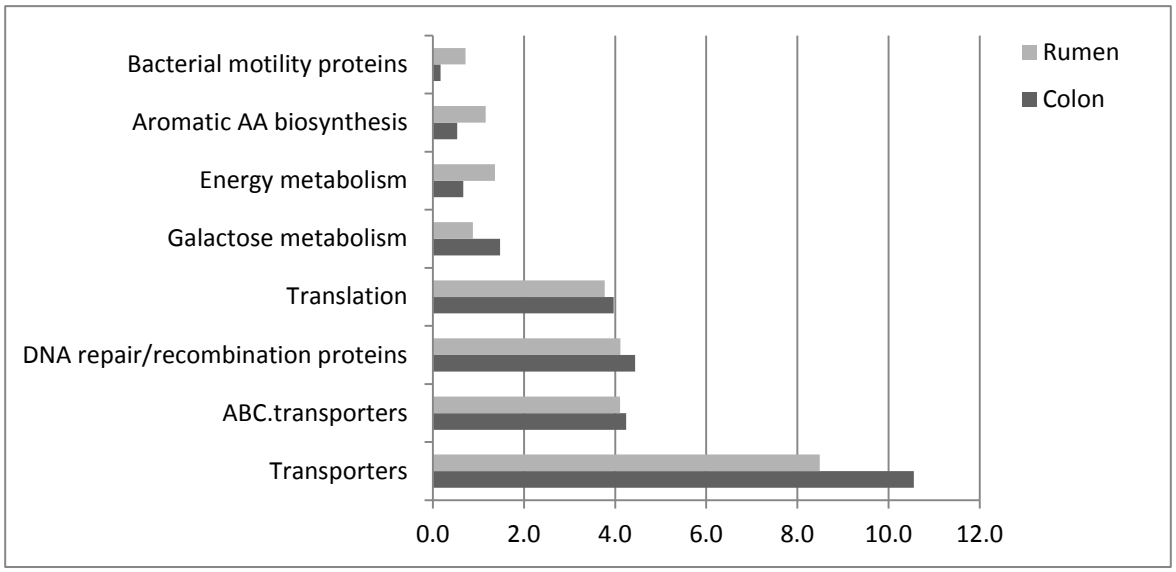

Figure 3.7. Most abundand predicted microbial functions in rumen and colon.

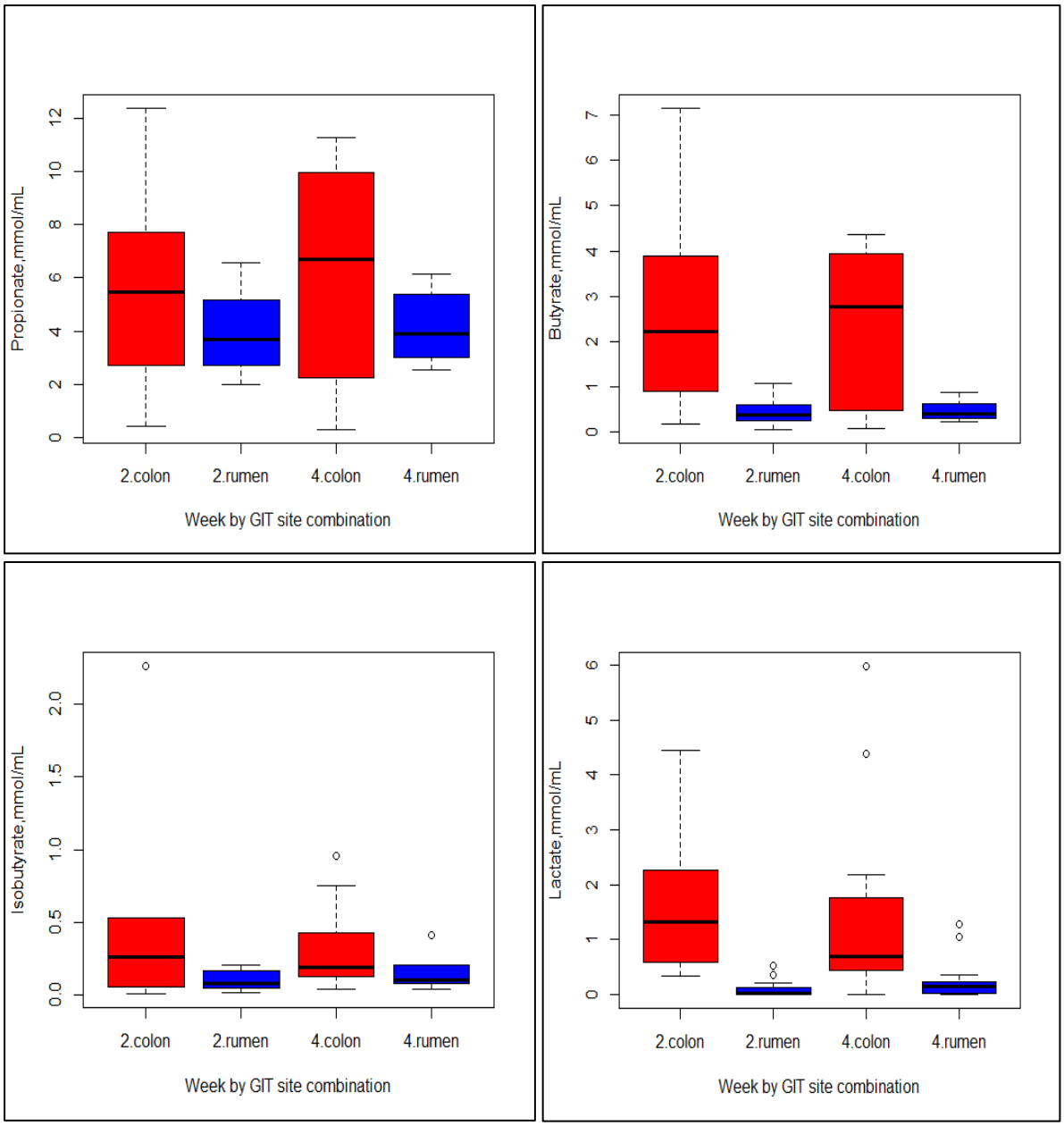

Figure 3.8.Distribution of SCFA at 2 and for weeks of age in colon and rumen. (2.colon = colon at 2 weeks; 2.rumen = rumen at 2 weeks; 4.colon = colon at 4 weeks; 4.rumen = rumen at 4 weeks).

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CHAPTER IV

ASSESSMENT OF THE PREBIOTIC POTENTIAL OF A GALACTOOLIGOSACCHARIDE RICH WHEY PERMEATE IN MILK REPLACERS FOR HOLSTEIN CALVES ON THE INTESTINAL MICROBIOTA COMPOSITION, FERMENTATION PROFILE, GROWTH AND HEALTH

ABSTRACT

Digestive disorders are common during the first few weeks of life of the newborn calf. Prebiotics are non-digestible oligosaccharides with good fermentable potential that modulate growth and activity of beneficial microbial populations which can result in enhanced gut health and function. Galactooligosacharides (GOS) have demonstrated such prebiotic potential. In this study, the effect of GOS supplementation on intestinal microbiota composition and fermentation profiles; intestinal health, development and function; and on the performance of dairy calves was evaluated under accelerated feeding conditions. Eighty calves were assigned either to a control treatment consisting of commercial milk replacer or to a GOS rich milk replacer treatment (i.e. 3.4% of dry matter). After 2 and 4 weeks in trial, 8 calves per treatment were slaughtered. Samples of intestinal digesta and tissue were collected for assessment of microbiota composition, short-chain fatty acid concentrations, in vitro measurement of nutrient transport and permeability, histomorphology, and gastrointestinal organs size. The remaining 48 calves continued in trial up to week 8 to measure body growth, nutrient intake and fecal and respiratory scores. Animals in the control treatment showed significantly better growth ($p < 0.05$), milk dry matter intake ($p < 0.05$) and fecal scores ($p < 0.001$) and spent less days with scours ($p < 0.001$)

than GOS supplemented animals. Size of intestinal organs, nutrient transport and epithelium permeability were not affected by treatment and, although digesta short chain fatty acid concentrations were higher for control ($p < 0.05$), villi height and width and crypt depth were larger in animals receiving GOS ($p < 0.05$). In conclusion, high GOS supplementation levels may have a laxative effect leading to slightly lower performance while promoting greater intestinal epithelium growth.

INTRODUCTION

Neonatal calves are faced with different types of stressors and promoting an early balance among the animal, its environment and etiological agents is necessary to minimize the probability of illness episodes (Davis and Drackley, 1998). At this stage, the gut has a central role, not only in nutrient assimilation, but also in preventing direct contact between pathogens and the internal body as mediated by commensal microbial colonization (Martin et al., 2010).

Intestinal illness is not uncommon in newborn calves. Diarrhea is one of the most frequent health problems in young dairy calves (Svensson et al., 2003). The last National Animal Health Monitoring System survey (NAHMS, 2010), which represents ~ 80% of U.S. dairy operations, reported a death rate for pre-weaning heifers of 7.8 %. Scours, diarrhea, or other digestive problems accounted for 56.5 % of this mortality. This appears somewhat better compared to 60.5% in the previous survey (NAHMS, 1996) but still is too high.

A potential alternative to promote gut health in newborns is to prevent infection by fostering growth of lactic acid bacteria (LAB) to inhabit the gastrointestinal tract (GIT). To achieve this, different types of prebiotics have been developed and their potential health benefits shown experimentally in different species. Prebiotics have been defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the

GI microbiota that confers benefits upon host well-being and health" (Gibson et al., 2004). Usually, bidifobacteria and lactobacilli are the microbial populations targeted by prebiotics such as fructooligosacharides (FOS) and galactooligosaccharides (GOS) (Macfarlane et al., 2008). These microbes can potentially displace pathogenic bacteria by, for instance, competing for nutrients and attachment sites in the gut epithelium, producing bacteriocins, increasing production of SCFA that lower intestinal pH and stimulating the immune system (Van Loo and Vancraeynest, 2008).

A potential source of GOS is whey permeate, which is a byproduct of whey protein concentrate manufacture and contains much of the whey lactose. By using enzymes, it is possible to synthesize galactosyl-oligosaccharides from the lactose, with some net hydrolysis of lactose resulting in an increase in monosaccharides as well (Torres et al., 2010).

The objective of this experiment was to evaluate an enzyme treated whey permeate as a source of GOS in milk replacer for pre-weaning calves and assess its potential prebiotic effects on gut health, development and function, and the performance of such animals under accelerated feeding conditions.

MATERIALS AND METHODS

Animals, Feeding and Treatment Allotment

Thirty-two newborn calves were purchased from Stone Ridge Dairy farm (Manheim, IL). Upon arrival to the University of Illinois research facilities, animals received an electrolyte solution (Land O Lakes, Electrolyte System - Add Pack), vaccines and prophylactic antibiotic treatment (2 mL, Muse, MERCK; 2 mL BoSe, Shering-Plough; 1mL vitamin A and D; 1.1mL Draxxin, ; Bovisera (50mL antibody serum, 20 mL C and D antitoxin, Behringer Ingelheim; 2 mL INFORCE-3, Pfizer). Calves were blocked by arrival bodyweight (BW) and plasma protein

score and, within block, treatments were assigned randomly. Treatments were: 1) commercial milk replacer (CON) and 2) commercial milk replacer containing whey permeate rich in galactooligosacharides (GOS). Final concentration of galactooligossaccharides was 3.4% of dry matter (DM). Also, CON contained ~44% as DM of lactose versus ~20% in GOS. This dosage was set based on previous observations with pigs where ~4.8% of the diet DM as galactooligossacharide supplementation resulted in a clear prebiotic effect (Smiricky-Tjardes et al., 2003).

Milk replacers were formulated to be isoenergetic and isonitrogenous and to meet or exceed NRC daily nutrient allowance recommendations (NRC, 2001). Milk replacers were fed at a rate of 1.25% of BW on a DM basis for the first 2 days, 1.5% of BW until day 10, and then 2% of BW through d 42. For days 43 to 49, milk replacer was fed at 1.5% of BW and from days 50 to 56 at 1% of initial BW only once daily. Calf DMI was adjusted individually on BW basis every week. Calves were fed the milk replacer treatments from arrival to day 30 with no grain offered. On day 31 grain was introduced and calves were weaned at day 56.

Performance and Health

Intakes of milk replacer and starter were measured and recorded daily. Weekly growth measurements included body weight, heart girth, body length, withers height, hip height, and hip width. Fecal scores and respiratory scores were recorded daily.

Animal Slaughter and Sampling

Eight calves per treatment were euthanized at 2 and 4 weeks into the experiment to collect tissue and digesta samples. The animals remained unfed until euthanasia took place. Euthanasia was performed by administering an overdose of sodium pentobarbital intravenously (85-150 mg/kg BW).

After euthanizing the animal, the thoracic cavity was cut open, the GIT was removed and ligated between omasum and abomasum, abomasum and duodenum and at the end of the ileum to prevent digesta flow due to manipulation. Then, the fore stomachs, small intestine and large intestine were separated for digesta and tissue sample collection. Once samples were collected all digesta was manually removed and GI organs weight and length were recorded.

In vitro Nutrient Transport

Within 20 minutes of euthanasia, pieces about 3 $inch^2$ of rumen and 7 cm long of jejunum, ileum and colon were collected for nutrient transport and permeability measurements in modified Ussing chambers (Physiologic Instruments; San Diego, CA) following procedures by Cannon (2009). Samples were placed in oxygenated (95% O_2:5% CO_2) Krebs' solution (120 m*M* $MgCl_2$, 120 m*M* $CaCl_2$, 40 m*M* KH_2PO_4, 240m*M* K_2HPO_4 , 1.15 M $NaCl_2$, and 260 m*M* $NaHCO_3$) on ice and transported to the laboratory for immediate electrophysiological and ion transport analysis. Basal short-circuit current (Isc; indicator of non-specific active ion transport), resistance (R; permeability), and potential difference (Pdo; total ion transport) were established during an initial stabilization period of ~20 min. Nutrient transport was assessed by changes in short-circuit current induced by addition of 10 m*M* D-glucose, L-glutamine and L-arginine to the mucosal medium (Cannon, 2009). Peak Isc values (point of maximal change) were measured after each nutrient was added, and the difference between basal (measured prior to each nutrient addition) and peak levels was calculated (Cannon, 2009).

Histology

Samples about 2 $inch^2$ of rumen and 7 cm long of jejunum, ileum and colon were collected for histomorphology analysis. Tissues were fixated and maintained in 10% formalin solution. Afterwards, tissue was dehydrated with ethanol and embedded into paraffin blocks,

from which 5 µm thick sections were cut and set onto glass slides. The sections were then stained with hematoxylin and eosin. Measurements of papillae length and width were made from dorsal rumen, caudal ventral sac and cranial ventral sac of rumen. Villi length, width and crypt depth were measured for duodenum, jejunum and ileum. Crypt depth and width were measured in colon. Microscopy measurements were performed using a Nanozoomer scanner and software (Hammatsu, Japan) at the Institute for Genomic Biology at the University of Illinois. Ten measurements were recorded per animal per GI segment and their average used for statistical analysis.

Digesta SCFA Concentration

Ruminal and colonic digesta samples were collected and acidified with a 10 N hydrochloric acid solution. Afterward, samples were centrifuged at 15,000 × g for 15 min, the supernatant extracted, frozen and sent to North Carolina State University to the laboratory of Dr. Vivek Fellner for SCFA analysis by gas liquid chromatography. Briefly, digesta samples supernatant was injected (1 µL) into a column (Varian CP 3380/3800 with a Nukol Fused Silica Capillary Column, 30 m × 0.25 mm × 0.25 mm; Sigma-Aldrich, St. Louis, MO). A flame ionization detector was used to quantify SCFA elution (Herfel et al., 2013).

Microbial Community Diversity and Structure

Digesta samples from rumen and colon were collected into 5ml sterile cryovials and snap frozen in liquid nitrogen to quickly stop microbial activity. Later on, samples were stored at -20 °C until DNA extraction. Samples were submitted for for bacterial tag encoded FLX amplicon pyrosequencing (Research and Testing Laboratory, Lubock, TX).

Total microbial DNA was extracted using a QIAamp stool DNA mini (Qiagen Inc., Valencia, CA) as previously reported (Dowd et al., 2008a; Dowd et al., 2008b). Sample DNA

was quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). Upon extraction, the V1-V3 hyper variable regions of the 16S ribosomal DNA gene were amplified by PCR using barcoded primers and the product sequence determined by bacterial tag-encoded FLX (non-titanium chemistry) amplicon pyrosequencing (Roche, Nutley, New Jersey) as reported previously (Dowd et al., 2008a; Dowd et al., 2008b). Afterwards, sequences were depleted of barcodes and primers. Sequences that were too short (< 200bp), that had ambiguous base calls or homopolymer runs exceeding 6bp were removed. Chimera removal and sequence de-noising were then performed. The remaining sequence reads were clustered at 97% similarity to determine operational taxonomic units (OTU's) as illustrated elsewhere (Dowd et al., 2008a; Dowd et al., 2008b; Edgar, 2010; Capone et al., 2011; Swanson et al., 2011). The OTUs were subsequently classified using BLASTn against a curated database (DeSantis et al., 2006) and compiled into the different taxonomic levels such as phyla and genera. The final average number of sequence reads per sample was 6,203 with a minimum of 1834 and a maximum of 16400. The 25^{th} and 75^{th} percentile for the 32 samples were 4328 and 7511 quality reads per sample after filtering.

The Shannon index was calculated as a proxy measure of diversity. Shannon index takes into account the total number of species present in a niche but also the abundance evenness among them (Tuomisto, 2010). Shannon index values were estimated for each sample and used as a dependent variable in function of dietary treatment and calf age as illustrated below.

Statistical analysis

Microbial Community Composition. A Bray-Curtis distance matrix to estimate the similarity among each pair of digesta samples originating from different age by treatment by GIT site groups, was built from the OTU or genus level matrix.

Afterwards, to visually explore the degree of microbial composition similarity between samples, non-metric multidimensional scaling (NMDS) was conducted on the above Bray-Curtis distance matrix (Borcard et al., 2011). The stress index was always below 0.2 indicating NMDS provided a good multidimensional representation of the microbial community in a reduced number of dimensions (Borcard et al., 2011). Square root transformed and non-transformed data provided similar NMDS, thus the latter was used throughout.

To contrast with NMDS and statistically test differences in microbiome composition between treatment, GI site and calf age groups, Analysis of Similarities (ANOSIM) (Clarke, 1993) and Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) were conducted. The ANOSIM statistically tests whether there is a significant difference between two or more groups of sampling units based on the difference of mean distance ranks computed from the matrix. The ANOSIM statistic R ranges from -1 to 1 with 0 indicating no grouping of samples. The statistical significance (i.e. p-value) of the observed R statistic is assessed by permutation. The PERMANOVA, on the other hand, partitions the total sums of squared Bray-Curtis distances among sources of variation and uses a permutation test with pseudo-F ratios to compute a p-value. The PERMANOVA is considered more robust to the homogenous variance assumption violation and to have greater statistical power to detect differences (Anderson, 2001; Anderson and Walsh, 2013).

The multivariate homogeneity of variances (Anderson, 2006) assumption was tested and found to be partially unmet. Consistently, microbial communities in the colon presented statistically smaller variation (data not shown, Fig.4.2a for visual appraisal). Although, this can be undesirable for the validity of a multivariate analysis of variance test, it has been recently

observed that, with balanced designs (i.e., our study), heterogeneity is less of an issue for PERMANOVA (Anderson and Walsh, 2013)).

Subsequently, in order to identify which genera contributed most to the microbiome observed differences among dietary treatment groups, Similarity Percentage Analysis (SIMPER) (Clarke, 1993) was performed. The SIMPER computes the contribution of individual genera to the overall Bray-Curtis dissimilarity. Only genera contributing up to 80% of the dissimilarity between CON and GOS are reported here. Due to SIMPER methodological limitations (Warton et al., 2012), results are complemented by univariate tests as appropriate using the generalized linear model framework (Fitzmaurice et al., 2011) to test statistical differences among groups. This approach also has the advantage that allows estimating the effect size of dietary treatment, age or their interaction.

Relative abundance of specific taxa and diversity were thus analyzed through the linear predictor:

$$\eta_i = \boldsymbol{X}_i'\boldsymbol{\beta}$$

where η_i is a linear function of the β unknown parameters and the covariates X corresponding the i^{th} observation, which include, dietary treatment (CON, GOS), calf age (2 weeks, 4 weeks) and their interaction. Due to presence of large over dispersion in taxa counts with respect to a Poisson density, a negative binomial distribution was used to model these. Shannon index was analyzed under a gamma distribution because it is bound from below at zero and errors did not meet normality assumptions (Fitzmaurice et al., 2011). A log link function was used all throughout. Maximum likelihood was used to estimate parameters and standard errors.

For the sake of interpretability of the coefficients in the log linear predictor, the above model for taxa counts at the genus level is expressed as:

$$\frac{Genus_{ij}}{total\#genera} = (\exp^{B_0}) * (\exp^{B_1 * X_i}) * (\exp^{B_3 * X_j}) * (\exp^{B_3 * X_{ij}}) ,$$

to estimate the expected number of reads of a specific taxa adjusted for the average total number of sequence reads present in a particular age by GIT site group. The exponentiated intercept represents the relative abundance at 2 weeks of age in colon, the exponentiated coefficient for X_i is the fold change in GOS with respect to CON at 2 weeks, the exponentiated coefficient for X_j is the fold change in week 4 relative to week 2, and the exponentiated coefficient for X_{ij} (i.e. the interaction term) is the adjustment to the GOS effect for its dependence on time.

The NMDS and ANOSIM were carried out in Primer-E software V.6.0, SIMPER (Clarke and Gorley, 2006), PERMANOVA and multivariate heterogeneity of variances test were conducted with the package Vegan (Oksanen et al., 2012), and generalized linear models were performed with the base package in the open access R language version 2.14.0.

Growth performance, nutrient intake and fecal scores. Average weekly measurements of nutrient intake and body size were analyzed as repeated measures. The error covariance matrix was modeled to choose the one with the least number of parameters and that would minimize the adjusted Akaike Information Criteria (AAIC) and -2 ratio log-likelihood. Residuals were checked for homogeneity of variance and normality. Heterogenous variances were common and an autoregressive function of first order allowing for heterogeneity (ARH1) handled well the problem. Significance was declared at $P < 0.05$ and trends were declared at $P < 0.1$. When interactions were significant, an experiment wise error rate of 5% was set to compare group means.

Repeated fecal score measures, in a scale from 1 to 4, were analyzed by fitting a marginal ordinal logistics regression model (Fitzmaurice et al., 2011) in PROC GENMOD of SAS to estimate the odds of observing diarrheal episodes (i.e., fecal score 4) in GOS with respect to

CON. Each calf was considered a cluster. Also, the number of days with fecal scores over 2 were analyzed under a Poisson distribution with a variance inflation factor to account for over dispersion.

SCFA concentrations, histology, nutrient transport and permeability. All these measurements were considered repeated measures on the same calf. As such, same guidelines as those for growth performance we followed. Residual errors were in general normally distributed. Error correlation and variance heterogeneity were dealt with as explained above.

RESULTS

Milk Replacer and Grain Mix Composition

The composition of experimental milk replacers and the concentrate is shown in Table 4.1. Milk replacers had similar concentrations of crude protein (CP), fat, net energy (NE), calcium (Ca) and phosphorus (P) (Table 4.1). They also contained different concentrations of lactose and GOS (table 4.2). The CON contained ~44% of lactose versus ~20% in GOS. At the same time, CON contained only 0.6 mg/g (0.06% of DM) of galactooligosacharides compared to 33.5 mg/g (3.35% of DM) in GOS. Therefore, the remainder should have been present in the form of monosaccharides. The main galactosyl-oligosaccharides in milk replacer containing treated whey permeate were lacto-N-fucopentanose (~2.9%) and 2'-Fucosyl-lactose (~1 %).

This implies that less than half of the carbohydrates in the enzymatically treated whey permeate were available for host enzymatic digestion and the remainder was substrate for fermentation in the hindgut.

Microbiota Diversity and Community Composition

Various things are highlighted by the PERMANOVA analysis (Table 4.3). First, at 5 % type 1 error rate, treatment can be considered to have a significant effect on the average

abundance of one or many of the several hundred organisms identified; however, its contribution to the total variation is small at 2.6%. This is clearly reflected in the wide variation within treatments as shown in the NMDS for dietary treatments presented in Figure 4.1a where hardly any difference between CON and GOS can be noticed.

The 2-way interactions with week and GI site were not significant, suggesting the effect of prebiotic supplementation is independent from the effects of GI location and calf age. Figure 4.1b and 4.1c show the lack of clearly cut associative patterns of the microbiota between dietary treatments at each age and GI site.

Nevertheless, the significance of the 3-way interaction suggests that the dependence of the GOS effect on a particular GIT site, depends itself on the age of the calf. Yet, the variance explained by this interaction barely accounts for 3 % of the total variation. These significant, yet small effects of such dependency, will be dealt with in detail in a later section.

Also, from the PERMANOVA analysis it is immediately apparent that GIT site, age and their interaction explain most of the variation in the GI microbiota composition of these pre-weaning calves with a cumulative R^2 of 32.8%. Because these latter sources of variation account, by and large, for the majority of the variation and appear to act fairly independently from dietary treatment effects, as evidenced by the non-significance of the 2-way interactions, their influence on the microbiota composition was thoroughly explored elsewhere (i.e., previous chapter).

In order to understand the previously observed 3 way interaction, exploratory ANOSIM was conducted to perform 1, 2 and 3 way multivariate pairwise comparisons for treatment, age and GI site.

In contrast to PERMANOVA, ANOSIM deems the difference between CON and GOS to be null with R statistic equal to zero and P-value of 1 (table 4.4) which agrees with the visual appraisal from NMDS in Figure 4.1a. However, when the overall treatment effect is broken down into the effect in colon and rumen, ANOSIM indicates that, while no microbiota compositional difference exists between GOS and CON in rumen, there is a very small (R=0.14) but highly significant difference in the colon (P = 0.007). This can be seen in Figure 4.2a where ruminal and colonic microbial communities are distinctly different, in agreement with PERMANOVA, but while GOS and CON groups are seemingly equal in the rumen, they would appear to differ in variability or location in the colon.

When the 2-way comparisons are further broken into 2 and 4 weeks, the significance of the 3-way interaction identified by PERMANOVA becomes apparent. Whereas no changes occurred in the rumen in response to GOS treatment at both 2 and 4 weeks of age (R=0.04 and R=0.05, P=0.24 and P=0.21, respectively), the microbiota composition of the colon differed fairly strongly between GOS and CON groups (R=47, P=0.001) at 2 weeks of age, but these differences faded away by week 4 of age (R=0.06, P=0.13). Because any treatment effects were completely absent in the rumen up to this point, we decided to consider only the colonic populations hereafter. Figure 4.2b clearly shows how colonic microbial populations exposed to GOS differ from CON, but only at 2 weeks of age, highlighting a very narrow and specific time window of the prebiotic effect.

To identify which specific taxa were responsible for the observed differences between GOS and CON in colon, SIMPER analysis was performed at the genus level (Table 4.5). Interestingly, out of 393 genera identified, only 8 of them accounted for ~80% of the dissimilarities between CON and GOS. Galactooligosachharide supplemented calves were

characterized by more abundant *Lactobacillus* (~63 vs 39 %), *Bifidobacterium* (~5.6 vs 2.3 %) and *Enterococcus* (~4 vs 3 %). Meanwhile, *Streptococcus* (~5.7 vs 9.8 %), *Faecalibacterium* (~0.8 vs 11 %), *Oscillospira* (~1.2 vs 8.2 %), *Collinsella* (~2.8 vs 7 %) and *Clostridium* (1.2 vs 3 %) were present in greater amounts in CON than in GOS. The cut off threshold of 80% was set arbitrarily since the additional contribution of each individual taxon to the dissimilarity was less than 2% and over 100 genera were to represent the remaining 20%.

To assess whether the colonic abundance of these genera varied significantly due to age or prebiotic treatment, in an independent or interactive fashion, and to quantify such variation, negative binomial regression was performed to analyze the number of counts of DNA sequence reads corresponding to each taxon. Results are presented in Table 4.6.

Estimated *Lactobacillus* relative abundance in the colon of calves at 2 weeks of age was ~ 8% (Intercept = 0.0809 * 100= ~8%). In the presence of GOS it increased by about 7.37 fold ($\exp^{(1.994)} = 7.37$). By week 4, this genus increased its abundance by 8.68 fold ($\exp^{(\text{week 4})}=8.68$) compared to 2 weeks in CON animals. The significant interaction explains how the GOS effect depended on the week. Even though GOS treated calves had numerically and statistically greater amounts of *Lactobacillus* in their colon at 2 weeks of age, by 4 weeks such difference ceased to exist and GOS supplemented calves presented only ~ 0.96 times ($\exp^{(1.994)}*\exp^{(-2.034)} = 7.34 * 0.13 = 0.96$) the abundance of this lactic acid organism with respect to CON calves 4 weeks old (Table 4.6, Figure 4.3).

In the case of *Bifidobacterium*, GOS treated calves at 2 weeks of age presented levels ~8.9 times as high as those in CON calves. By 4 weeks, however, the GOS effect on *Bifidobacterium* levels went down to about 6% the GOS effect size at 2 weeks of age ($\exp^{(\text{week 4 x GOS})} = 0.06$).

Thus, it appears that the GOS effect on these 2 lactic acid bacteria was transient during the first 2 or 3 weeks of life and faded away by the 4th week of life of these calves fed only milk replacer (Figure 4.3).

Streptococcus on the other hand was not statistically affected by GOS treatment even though it contributed 9% of the dissimilarity between CON and GOS groups according to the SIMPER analysis. Instead, it was only significantly affected by age, decreasing to 20% ($exp^{(week\ 4)} = 0.20$) of the initial abundance levels by 4 weeks of age. Similarly, *Collinsella* and *Enterococcus* were not significantly affected by GOS treatment despite jointly contributing 10% of the dissimilarity between GOS and CON calves.

Furthermore, the increase in LAB (*Lactobacillus* + *Bifidobacterium*) in response to GOS supplementation occurred at the expense of *Faecalibacterium*, *Oscillospira* and *Clostridium*, which were reduced to 1.5, 4.1 and 9.8% (Table 4.6), respectively, of the abundance levels present in CON animals at 2 weeks of age. Similar to LAB as well, these 3 genera were present at similar levels in both CON and GOS by 4 weeks of age.

On a different note, we did not observe any changes in diversity as measured by the Shannon index, due to calf age or supplementation with GOS, or their interaction (Table 4.7).

Overall, considering that the total number of bacterial species and their evenness was not affected at all, that out of 393 genera only 5 (i.e. *Lactobacillus*, *Bifidobacteria*, *Faecalibacterium*, *Oscillospira* and *Clostridium*) constituted the most significant response to prebiotic treatment, that LAB were ~8-9 fold more abundant in GOS than CON, and that all effects were confined to the colon and to the first 2 weeks of life, it seems as though the prebiotic effect of treated whey permeate on the microbiota composition was very potent, specific and transient.

Visceral Organs Size

Supplementation with GOS did not affect the weight or length of the GI tract. Neither was there an interaction between treatment and age or GI organ (Table 5.8).

SCFA Concentration

Main effect of prebiotic treatment on SCFA concentration in the GIT was significant (Table 4.9). Counterintuitively, animals receiving CON showed a higher average total SCFA, propionate and butyrate concentrations than animals receiving GOS; however, this effect depended on the GI site. Total SCFA, acetate, propionate, butyrate and lactate concentrations were significantly larger for CON than GOS in the colon but not in the fore stomach or small intestine. This is not unexpected given the low influx of fermentable matter into the rumen of calves fed only milk where the esophageal groove is functional, and agrees with the absence of any significant shifts in the microbiota composition of the rumen at either 2 or 4 weeks under either diet.

Overall, in colon, total SCFA, acetate, propionate, butyrate and lactate concentrations were between ~66 and 240 % greater for CON versus GOS.

Analysis of SCFA concentrations expressed as molar proportion yielded similar results (data not shown).

Nutrient Transport and Permeability

There was no effect of treatment or its interaction with GIT location on epithelium permeability (Table 4.10). Similarly, no effect of treatment or its interaction with GI site on glucose, glutamine or arginine transport was observed (Table 4.10).

Histology

Considering the length of the papillae and the villae in rumen and small intestine, we observed that the effect of treatment depended on the age of the calf. While the average villae/papillae length remained unchanged in the CON group, they increased significantly in the GOS group from 2 to 4 weeks of age. The effect of the interaction between dietary treatment and GIT site was not significant, meaning that the effect size of GOS supplementation was similar in both fore stomach and small intestine. In addition, there was a significant effect of treatment on the mean depth of the crypts in small and large intestine (Table 4.11) and this effect did not depend on GI site or calf age. On the other hand, treatment or its interaction with GI site or age did not affect the width of the papillae in the rumen, the villae in the small intestine or the crypts in the colon (Table 4.11).

Dry Matter, Nutrient and Water intake

Daily milk DMI was significantly affected by dietary treatment. The magnitude of such effect ranged between 20 and 40 g/d and was dependent on time as illustrated by the significant interaction between treatment and age. At 2, 4 and 8 weeks of age, average milk DMI was greater for CON than GOS and this difference slightly increased from week 2 through week 8. Grain DMI intake was not different between CON and GOS and was affected only by age, similar to total DMI meaning that GOS animals could not compensate the reduced milk DMI by increasing grain DM consumption (Table 4.12).

The difference in DMI resulted in a marginally lower daily energy intake for GOS animals up to week 4 at which point the difference was not significant any further (Table 5.13).

Daily crude protein intake differed between CON and GOS only between weeks 0 and 2 as shown by a significant treatment by week interaction (means not shown). Afterwards, no difference was observed (Table 4.13).

Average daily water intake at 2, 4 or 8 weeks was not affected by dietary treatment overall (Table 4.14).

Growth

Body growth was slightly although significantly affected by dietary treatment and the CON diet consistently supported a better performance than GOS treatment. The CON animals presented between 0.64 and 2 kg higher bodyweights than GOS animals from week 2 to 8 (Table 4.15). Heart girth and hip height also were greater in CON than in GOS animals at weeks 2, 4 and 8 but the magnitude of the difference varied across weeks. Similarly for body length and wither height as shown by the significant interaction between week and treatment but only from week 4 through week 8. Hip width was significantly greater for CON versus GOS at 8 weeks of age.

Health

In Table 4.16 it can be seen that the odds of having fecal score 4 for GOS animals were 2.36 times those of CON animals (Figure 4.4). Moreover, the number of days with fecal scores over 2 was statistically greater for the GOS treatment than CON at 2, 4 and 8 weeks of age when the latter was exceeded by about 10 days.

DISCUSSION

In the current experiment, feeding a GOS rich milk replacer potently boosted LAB such as *Lactobacillus* and *Bifidobacteria* in the colon at the expense of other genera like *Faecalibacterium*, *Clostridium* and *Oscillospira*. This prebiotic effect was characterized by LAB levels ~8-9 times higher in GOS than in CON, which lasted ~2 weeks and then disappeared afterwards. Therefore, enzymatically treated whey permeate has indeed shown to bear prebiotic

activity by allowing specific changes in the composition in the GI microbiota (Gibson and Wang, 1994) in this study.

Bifidobacteria and *Lactobacillus* species have been shown to provide resistance against diarrheal disease and to stimulate the immune system (Yamazaki et al., 1985; Gibson and Wang, 1994; Parassol et al., 2005; Johnson-Henry et al., 2008; Vizoso Pinto et al., 2009). Hence, GOS rich milk replacer from whey permeate treated with B-galactosidases may certainly provide a means to support calf intestinal health during the first weeks of life.

It is also remarkable that while *Lactobacillus* levels remained high in GOS by week 4 of life (~55%), the lack of difference with CON at that age was due to the sharp increase of this LAB in non-supplemented animals, reaching almost 60% of the colonic microbiota. This contrasts strongly with observations in young calves where other genera like *Bacteroides*, *Prevotella* or *Faecalibacterium* were fairly abundant at this age (Edrington et al., 2012; Li et al., 2012; Jami et al., 2013; Malmuthuge et al., 2014).

Unexpectedly, the increase in LAB was not reflected in the fermentation profile as CON animals presented much larger total SCFA, acetate, propionate, butyrate and lactate concentrations in the colon compared to GOS, which might suggest an enhanced fermentative state in the distal bowel of CON animals. Alongside, calves on GOS exhibited 82% higher odds of presenting diarrhea and spent a total of ~10 more days with fecal scores over 2 by week 8.

The challenge is thus to reconcile the higher intake of non-digestible but fermentable oligosaccharides and the greater abundance of LAB in the hindgut with the lower colonic SCFA and overall diarrhea-like condition of prebiotic supplemented calves.

There is evidence that carbohydrate malabsorption, even from fermentable sources, can eventually cause osmotic diarrhea to different extents as the colonic fermentative and VFA

absorptive capacities are progressively exceeded (Binder, 2010). Indeed, excess non-digestible fermentable carbohydrates arriving into the colon are fermented to SCFA plus CO_2 and H^+. SCFA are normally absorbed by the colonocytes, but when SCFA production rate exceeds the absorption rate, the luminal accumulation of SCFA and H cause colonic pH to decrease and water to be secreted into the lumen, leading to diarrhea (Binder, 2010). Let us gauge whether an osmotic gradient reversal due to an excessive colonic influx of GOS is a plausible explanation to our observations. At a hypothetical BW of 55 kg, with a given DM intake of 2% on a BW basis, and with a GOS concentration of ~ 3.4%, the average GOS consumption by supplemented animals must have been about 39 g/d as compared to ~1 g/d for CON animals. In humans, inulin oligosaccharides, FOS and GOS at lower dosages have shown to be mildly laxative and, if supplied in large amounts, can result in flatulence and osmotic diarrhea (Macfarlane et al., 2008). When 15 g/d were ingested by healthy adults, defecation frequency and flatulence increased (Teuri, 1998). Similarly, when healthy adults were dosed with 10 g/d of GOS, defecation frequency, feces softness and bowel movement and flatus symptoms were found to increase (Deguchi et al., 1997). In one other study in which 0, 2.5, 5.0 or 10.0 g of GOS per day were supplemented to human adults, the daily ingestion of 10 g increased the sensation of fullness, and there was a tendency for flatulence and abdominal pain to increase (Ito et al., 1990). Although BW was not reported in these studies, it is unlikely that average human adults weigh less than a newborn calf. On a per kilogram BW basis, GOS intake here was ~ 4 times higher compared to adult human subjects with a hypothetical calf BW of 55 kg and the aforementioned dosages for humans. Based on this evidence, we deem a reversed osmotic gradient with the concomitant increased luminal water content in the colon a reasonable although speculative explanation for the lower SCFA concentrations and diarrhea-like condition.

Another measure that supports the idea of osmotic rather than infectious diarrhea is the similarity in the epithelium colonic resistance between CON and GOS. It has been shown that animals infected with pathogens such as enterohemorrhagic *E. coli* (EHEC) or enteropathogenic *E. coli* (EPEC) suffer from serious diarrhea-like conditions due to an increase in paracellular permeability from tight junction breakdown in the gut epithelium (Guttman et al., 2006). This leak-flux mechanism is exaggerated during *Clostridium difficile* and *Vibrio cholera* infection where toxins lyse the tight junctions (Guttman et al., 2006). However, when we measured intestinal epithelial resistance to assess gut barrier functionality in the different regions of the GIT, there were no statistically significant differences between GOS supplemented and CON animals, suggesting that the increase in luminal water for GOS animals did not result from leak flux due to pathogenic tight junction disruption.

On the other hand, GOS was associated with a greater increase in the size of the villi in duodenum, jejunum and ileum from 2 to 4 weeks of age, and with deeper crypts in the colon and small intestine compared to CON animals. These effects of prebiotics have been shown before and appear to be mediated by a greater SCFA yield, especially butyrate (Kleessen et al., 2003; Bartholome et al., 2004), apparently through release of potent intestinal peptides like glucagon like peptide 2 (Cani et al., 2009), which can act in a para or endocrine way to elicit a powerful systemic trophic effect on the mucosa of both the proximal and distal gut. Although in this case GOS supplemented animals presented smaller luminal SCFA concentrations, it is important to take into account that only by measuring SCFA yields instead of molar concentrations could we be certain whether fermentation was in fact greater in CON or GOS fed animals.

Regarding functionality, the transport of glucose, glutamine (i.e., neutral amino acid) and arginine (i.e., basic amino acid) was unaffected by GOS supplementation even in the colon, indicating that these transport systems were indifferent to prebiotic mediated LAB enhancement.

At the animal level, a slightly lower growth performance in GOS animals was observed. This could have been due to a ~20% lower dietary lactose availability. Since feeding rates were individually adjusted weekly according to BW, the slower growing GOS calves received less food than CON as reflected in an average reduction of ~90 kcal/d of milk metabolizable energy intake by week 4 of life.

Reported work with mannan and fructan-oligosacharides in calves has often (Heinrichs et al., 2003; Van Loo and Vancraeynest, 2008; Ghosh and Mehla, 2011) but not always (Heinrichs et al., 2009) resulted in consistent beneficial effects on fecal scores, commensal bacterial populations, feed intake and efficiency. Besides the difference in oligosaccharide types studied, a much lower dosage used (~ 4g/d) and feeding rates ranging from 1.4 to 1.7 % of BW make it difficult to draw solid comparisons and conclusions against our observations.

Feeding rate and prebiotic concentration determine the amount of not only non-digestible oligosaccharides, but also lactose and protein that that enter colonic fermentation and ultimately impact the microbiota composition and activity in the large bowel. Moreover, depending on oligosaccharide composition, GOS products will vary in their physiological effects (Torres et al., 2010). For instance, the administration of a GOS mixture (3.6 g/d) containing mainly $\beta 1 \rightarrow 3$ linkages with some $\beta 1 \rightarrow 4$ and $\beta 1 \rightarrow 6$ linkages proved to have a better bifidogenic effect than a GOS mixture (4.9 g/d) containing mainly $\beta 1 \rightarrow 4$ linkaages with some $\beta 1 \rightarrow 6$ linkages, after 1 week of intake by healthy humans (Depeint et al., 2008).

To optimize and standardize the response to GOS supplementation in terms of microbial community composition, fermentation products and host health, the above 3 factors (i.e., feeding rate, GOS dosage and type) and their interactions will have to be taken into account to a build a reliable response surface.

Therefore, even though potent enrichment of LAB during the first 2-3 weeks of life of dairy calves can have a positive impact on intestinal health (Macfarlane et al., 2008), inclusion of enzymatically treated whey permeate as a source of GOS must be optimized to avoid undesired collateral effects such as lower nutrient intake, performance or laxative effects.

CONCLUSION

One to one substitution of untreated for enzymatically treated whey permeate as a source of GOS resulted in very potent growth promotion of LAB in the colon for ~2 weeks of age. This was accompanied by greater development of intestinal epithelium structures in small and large intestine. However, GOS supplementation also resulted in lower intestinal SCFA concentrations and increased fecal scores. A possible reason for this would be a GOS over-flux into the large bowel exceeding the fermentative capacity of the microbiota or the ability to absorb SCFA by this organ, resulting in water secretion.

Also, slightly lower nutrient intake and growth were observed in GOS supplemented animals. Taking advantage of enzymatically treated whey permeate as a source of GOS to improve intestinal health in pre-weaning calves will require optimization of supplementation levels and milk replacer feeding rates to maximize LAB populations, fermentation and calf health responses while maintaining performance.

TABLES AND FIGURES

Table 4.1. Nutrient composition of experimental milk replacers and grain mix

Nutrient	CON	GOS[1]	Grain
DM, %	95.9	95.0	88.2
CP[2], % DM	28.8	29.6	22.9
Crude fat, % DM	15.7	15.1	N/A
Ash, % DM	8.27	7.76	N/A
NEm[3], Mcal/kg	2.67	2.67	2.12
NEg[4], Mcal/kg	1.91	1.90	1.44
Ca, % DM	1.05	1.12	1.00
P, % DM	0.68	0.63	0.54

[1] GOS: Galacto-oligosacharide supplemented treatment.
[2]CP: Crude protein.
[3]NEm: Net energy of maintenance.
[4]NEg: Net energy of gain.

Table 4.2. Mono, di and oligosaccharides profile of CON and GOS milk replacer

	CON	GOS[1]
Total disaccharides, mg/g	***437.3***	***197.3***
Sucrose	1.0	0
Lactose	436.3	197.3
Total oligosaccharides, mg/g	***0.6***	***33.5***
2'-Fucosyl-lactose	0	3.7
Lacto-N-fucopentanose	0	28.8
Lacto-N-neotetraose	0.2	0
Lacto-N-tetraose	0	0.8
Sialic acid	0	0
LS-tetrasacharide c	0	0.2
6'-Sialyl-lactose	0	0
3'-Sialyl- lactose	0.4	0
Total hydrolyzed monosacharides, mg/g	***487.2***	***473***
Fucose	0	0
Galactose	260.8	188.6
Glucose	223.3	281.4
Mannose	3.1	3.0

[1] GOS: Galacto-oligosacharide supplemented treatment.

Table 4.3. PERMANOVA for effect of prebiotic treatment, calf age and GI site on microbial community composition

Source of variation	df [1]	SS [2]	MS [3]	R-square	P-value
Treatment	1	0.51	0.51	0.026	0.032
Week	1	0.88	0.88	0.044	0.001
Treatment x Week	1	0.41	0.41	0.021	0.074
Site	1	4.84	4.84	0.244	0.001
Treatment x Site	1	0.32	0.32	0.016	0.133
Site x Week	1	0.79	0.79	0.040	0.006
Treatment x Site x Week	1	0.62	0.62	0.031	0.016
Residual	55	11.46	0.21	0.578	
Total	62	19.82			

[1] Degrees of freedom
[2] Sum of squared multivariate distances
[3] Mean squared multivariate distances

Table 4.4. ANOSIM between CON and GOS by GI site and calf age

	1 way: Treatment	
	R[1]	P-value
CON vs GOS	0.0	1.0

	2 way: Treatment by GI site			
	Colon		Rumen	
	R	P-value	R	P-value
CON vs GOS	0.14	0.007	0.0	0.46

	3 way: Treatment by GI site by age			
	Colon		Rumen	
	R	P-value	R	P-value
CON vs GOS at 2 weeks	0.47	0.001	0.04	0.24
CON vs GOS at 4 weeks	0.06	0.137	0.05	0.21

[1] R statistic = 1 when groups difference is maximal, 0 when groups difference is null

Table 4.5. SIMPER for genera driving dissimilarity between GOS and CON groups

	Average abundance %		
Genus	GOS	CON	Dissimilarity % [1]
Lactobacillus	63.4	39.2	33
Streptococcus	5.67	9.80	9
Faecalibacterium	0.816	10.9	9
Oscillospira	1.15	8.24	7
Bifidobacterium	5.63	2.27	6
Collinsella	2.80	7.03	6
Clostridium	1.23	6.48	5
Enterococcus	4.08	3.02	4
Total			79

[1] Contribution to overall dissimilarity between groups

Table 4.6. Regression coefficients for effects of dietary treatment, calf age and GI site on abundance of identified genera

	Coefficient (β)	S.E	P-value	$exp^{(\beta)}$ [1]
	Lactobacillus			
Intercept	-2.515	0.327	< 0.0001	0.0809
GOS	1.994	0.462	< 0.0001	7.3417
Week 4	2.162	0.462	< 0.0001	8.6878
Week 4 x GOS	-2.034	0.654	0.002	0.1308
	Bifidobacterium			
Intercept	-4.544	0.656	< 0.0001	0.01
GOS	2.183	0.926	0.02	8.87
Week 4	1.184	0.926	0.2	
Week 4 x GOS	-2.822	1.309	0.03	0.06
	Streptococcus			
Intercept	-1.812	0.436	< 0.0001	0.163
GOS	-0.889	0.617	0.15	
Week 4	-1.605	0.618	0.01	0.201
Week 4 x GOS	0.1583	0.873	0.16	
	Faecalibacterium			
Intercept	-1.567	0.566	< 0.0001	0.2086
GOS	-4.181	0.804	< 0.0001	0.0153
Week 4	-3.049	0.802	< 0.0001	0.0474
Week 4 x GOS	4.464	1.136	< 0.0001	86.8183
	O***scillospira***			
Intercept	-2.020	0.566	< 0.0001	0.1327
GOS	-3.186	0.802	< 0.0001	0.0413
Week 4	-1.417	0.800	0.07	0.2424
Week 4 x GOS	2.582	1.133	0.02	13.2241
	Collinsella			
Intercept	-2.590	0.668	< 0.0001	0.075
GOS	-1.085	0.945	0.25	
Week 4	-0.134	0.945	0.88	
Week 4 x GOS	0.323	1.336	0.80	
	Clostridium			
Intercept	-2.136	0.517	< 0.0001	0.1181
GOS	-2.327	0.732	0.002	0.0976
Week 4	-2.329	0.733	0.002	0.0974
Week 4 x GOS	2.459	1.036	0.02	11.69
	Enterococcus			
Intercept	-3.116	0.517	< 0.0001	
GOS	0.544	0.731	0.42	
Week 4	-1.014	0.732	0.17	
Week 4 x GOS	-1.649	1.036	0.11	

[1] $exp^{(Intercept)}$: represents expected taxa relative abundance for CON at 2 weeks of age; $exp^{(GOS)}$: represents fold change in taxa relative abundance with GOS treatment at 2 weeks with respect to CON at 2 weeks; $exp^{(week\ 4)}$: represents fold change in taxa relative abundance with CON treatment at 4 weeks with respect to CON at 2 weeks ; $exp^{(GOS)} * exp^{(week\ 4)} * exp^{(week\ 4 \times GOS)}$: represents fold change in taxa relative abundance with GOS treatment at 4 weeks with respect to CON at 2 weeks.

Table 4.7. Regression coefficients for effect of calf age, dietary treatment and GI site on colonic microbiota diversity

	Coefficients (β) [1]	S.E	P-value
	Shannon index		
Intercept	1.30	0.05	<0.0001
Week 4	0.06	0.07	0.34
GOS	0.08	0.07	0.24
Week 4 x GOS	-0.05	0.08	0.49

[1] Intercept: represents the natural logarithm of the expected value for CON at 2 weeks of age; Week4: represents natural log of fold change for CON at 4 weeks with respect to CON at 2 weeks; GOS: represents natural log of fold change for GOS at 2 weeks with respect to CON at 2 weeks; (Week 4) + (GOS) + (week 4 x GOS): represents natural log of fold change for GOS at 4 weeks with respect to CON at 2 weeks.

Table 4.8. Least squares means ± standard error for organ size for CON and GOS treatments

	Treatment		P-value		
	CON	GOS	TRT[1]	TRT x GI site	TRT x week
Length, m	3.87 ± 0.12	3.84 ± 0.12	0.90	0.60	0.80
Weight, kg	0.45 ± 0.013	0.44 ± 0.013	0.60	0.80	0.60

[1] TRT: dietary treatment effect (CON vs GOS)

Table 4.9. Least squares means ± standard error for SCFA and lactate concentrations for CON and GOS in rumen, jejunum and colon

	CON			GOS			P-value		
	Rumen	Jejunum	Colon	Rumen	Jejunum	Colon	TRT[1]	TRT x GI site	TRT x week
	mmol/mL								
Total SCFA	34.86 ± 3.67	2.39 ± 0.74	47.0 ± 3.27 [A]	34.86 ± 3.67	3.57 ± 0.74	26.64 ± 3.27 [B]	0.04	0.0005	0.90
Acetate	25.10 ± 3.50	2.20 ± 0.70	35.0 ± 2.40 [A]	29.8 ± 3.50	3.2 ± 0.70	21.6 ± 2.4 [B]	0.40	0.003	0.90
Propionate	3.75 ± 0.34	0.23 ± 0.10	8.2 ± 0.74 [A]	4.23 ± 0.34	0.37 ± 0.10	3.6 ± 0.74 [B]	0.01	0.0005	0.30
Butyrate	0.30 ± 0.43	NA[2]	4.50 ± 0.40 [A]	0.54 ± 0.38	NA[2]	1.5 ± 0.47 [B]	0.0014	0.0009	0.60
Isobutyrate	0.09 ± 0.20	NA[2]	0.30 ± 0.17	0.09 ± 0.20	NA[2]	0.8 ± 0.24	0.30	0.50	0.42
Valerate	0.02 ± 0.3	NA[2]	0.30 ± 0.22	0.02 ± 0.3	NA[2]	0.4 ± 0.23	0.60	0.90	0.30
Isovalerate	0.20 ± 0.16	0.17 ± 0.46	0.22 ± 0.15	0.2 ± 0.14	0.07 ± 0.30	0.25 ± 0.18	0.50	0.40	0.90
Lactate	0.13 ± 0.07	0.19 ± 0.79	2.30 ± 0.31 [A]	0.21 ± 0.07	1.78 ± 0.79	0.81 ± 0.31 [B]	0.10	0.0004	0.90

[1] TRT: dietary treatment effect (CON vs GOS)
[2]NA: undetectable level

Table 4.10. Least squares means ± standard error for epithelial resistance (Ω//cm2) and change in Isc (Δ mv) in response to glucose, glutamine and arginine addition

	Treatment		P-value		
Epithelium function	CON	GOS	TRT[1]	TRT x GI site	TRT x week
R, Ω//cm^2 [2]	141.1 ± 7.06	143 ± 6.79	0.80	0.96	0.72
Glucose, Δ mv [3]	1.39 ± 0.35	2.1 ± 0.35	0.20	0.40	0.40
Glutamine, Δ mv	2.5 ± 0.37	2.79 ± 0.37	0.60	0.60	0.14
Arginine, Δ mv	1.83 ± 0.42	2.21 ± 0.44	0.54	0.40	0.80

[1] TRT: dietary treatment effect (CON vs GOS)
[2] R: resistance measured in ohlms (Ω)
[3] Δ mv = change in circuit current in millivolts upon addition of respective nutrient. Surrogate measure of nutrient transport.

Table 4.11. Least squares means ± standard error for histomorphology measures for CON and GOS at 2 and 4 weeks of age

	CON		GOS		P-value		
	2	4	2	4	TRT[1]	TRT x GI site	TRT x week
Rumen, duodenum, jejunum and ileum							
Villae/Papillae length, μm	407.2 ± 16.3	384.23 ± 14.7	362.7 ± 16.1 ^{B}	429.2 ± 14.3 ^{A}	0.90	0.20	0.01
Rumen, duodenum, jejunum, ileum and colon							
Villae/Papillae/crypt width, μm	119.5 ± 2.9	124.4 ± 2.6	118.3 ± 2.9	126.4 ± 2.6	0.90	0.80	0.40
Duodenum, jejunum, ileum and colon							
Crypt depth, μm	407.2 ± 14.6 ^{B}	394.9 ± 12.5 ^{B}	419.7 ± 14.5 ^{A}	439.0 ± 12.4 ^{A}	0.04	0.60	0.24

[1] TRT: dietary treatment effect (CON vs GOS)

Table 4.12. Least squares means ± standard error for average milk, grain, and total dry matter intakes

	TREATMENT		P-value		
	CON	GOS	TRT[1]	week	TRT x week
Milk DMI ,kg/d					
0-2 wk	0.68 ± 0.01	0.66 ± 0.01	0.0005	<0.0001	0.005
0-4 wk	0.82 ± 0.01	0.79 ± 0.01	0.016	<0.0001	0.089
0-8 wk	0.91 ± 0.01	0.87 ± 0.01	0.0001	<0.0001	0.077
Grain DMI ,kg/d					
4-8 wk	1.15 ± 0.44	1.15 ± 0.44	0.34	<0.0001	0.43
Total DMI, kg/d					
4-8 wk	2.11 ± 0.39	2.09 ± 0.39	0.67	<0.0001	0.36

[1] TRT: dietary treatment effect (CON vs GOS)

Table 4.13. Least squares means ± standard error for energy and protein intakes

	TREATMENT		P-value		
Nutrient	CON	GOS	TRT[1]	week	TRT x week
ME[2], Mcal/d					
0-2 wk	2.92 ± 0.06	2.86 ± 0.06	0.0006	<0.0001	0.0026
0-4 wk	3.53 ±0.38	3.42 ±0.38	0.005	<0.0001	0.23
0-8 wk	8.13 ± 1.22	7.94 ± 1.22	0.16	<0.0001	0.32
CP[3] , kg/d					
0-2 wk	0.19 ± 0.004	0.19 ±0.004	0.24	<0.0001	0.03
0-4 wk	0.24 ± 0.002	0.24 ± 0.002	0.30	<0.0001	0.18
0-8 wk	0.52 ± 0.08	0.51 ± 0.08	0.54	<0.0001	0.43

[1] TRT: dietary treatment effect (CON vs GOS)
[2] ME: Metabolizable energy.
[3] CP: Crude protein.

Table 4.14. Least squares means ± standard error for average water intake

	TREATMENT		P-value		
	CON	GOS	TRT[1]	week	TRT x week
Average daily water intake ,kg/d					
0-2 wk	7.47 ±0.23	7.57 ±0.23	0.56	<0.0001	0.89
0-4 wk	8.32 ± 0.28	8.37 ± 0.28	0.62	<0.0001	0.56
0-8 wk	9.61 ± 0.16	9.66 ± 0.16	0.70	<0.0001	0.67

[1] TRT: dietary treatment effect (CON vs GOS)

Table 4.15. Least squares means ± standard error for body measurements growth

Variable[2]	Treatment		P-value		
	CON	GOS	TRT[1]	week	TRT x week
BW, kg					
2 wk	45.40 ±0.66	44.76 ±0.66	0.0264	<0.0001	0.18
4 wk	50.03 ±0.33	49.00 ±0.33	0.014	<0.0001	0.10
8 wk	61.16 ±2.24	59.02 ±2.24	0.0304	<0.0001	0.34
HG, in					
2 wk	32.06 ±0.17	31.85 ±0.17	0.0107	<0.0001	0.0144
4 wk	33.10 ±0.30	32.77 ±0.30	0.0005	<0.0001	0.0014
8 wk	35.27 ± 0.22	34.80 ± 0.22	<0.0001	<0.0001	0.042
BL, in					
2 wk	24.79 ± 0.05	24.70 ± 0.05	0.13	<0.0001	0.16
4 wk	25.33 ± 0.15	25.04 ± 0.15	0.0002	<0.0001	0.0264
8 wk	26.74 ± 0.17	26.18 ± 0.17	<0.0001	<0.0001	0.0147
WH, in					
2 wk	24.79 ± 0.08	24.70 ± 0.08	0.14	<0.0001	0.18
4 wk	25.32 ± 0.11	25.05 ± 0.11	0.0475	<0.0001	0.0056
8 wk	26.71 ±0.16	26.24 ±0.16	0.0058	<0.0001	<0.0001
HH, in					
2 wk	83.59 ± 0.17	83.45 ± 0.17	0.14	<0.0001	0.025
4 wk	84.76 ±0.38	84.40 ±0.38	0.09	<0.0001	0.045
8 wk	87.80 ±0.39	87.07 ±0.39	0.0216	<0.0001	0.08
HW, in					
2 wk	6.83 ± 0.03	6.81 ± 0.03	0.20	<0.0001	0.17
4 wk	6.99 ± 0.02	6.95 ± 0.02	0.09	<0.0001	0.015
8 wk	7.48 ± 0.04	7.40 ± 0.04	0.0165	<0.0001	0.23

[1] TRT: dietary treatment effect (CON vs GOS)
[2] BW: Body weight, HG: Heart girth, BL: Body length, WH: Wither height, HH: Hip height and HW: Hip width.

Table 4.16. Effect of milk replacer rich in galacto-oligosaccarides (GOS) on the odds of presenting fecal scores of 4 and number of days with scores over 2 in calves.

	Logistic estimate of odds to present fecal scores of 4			
	Log odds ratio	S.E	Odds ratio[1]	P-value
GOS vs CON	0.86	0.153	2.36	<0.001
	Poisson estimate for number of days with fecal sores over 2			
	Log (days over 2)	SE	Number of days over 2[1]	P-value
	2 weeks			
CON	2.49	0.021	12.0	0.0002
GOS	2.60	0.021	13.5	
	4 weeks			
CON	2.93	0.031	18.7	< 0.001
GOS	3.17	0.030	23.9	
	8 weeks			
CON	3.3	0.044	26.6	< 0.001
GOS	3.6	0.040	36.9	

[1]Exponentiation the of log (odds ratio) and the log (number of days with fecal scores over 2)

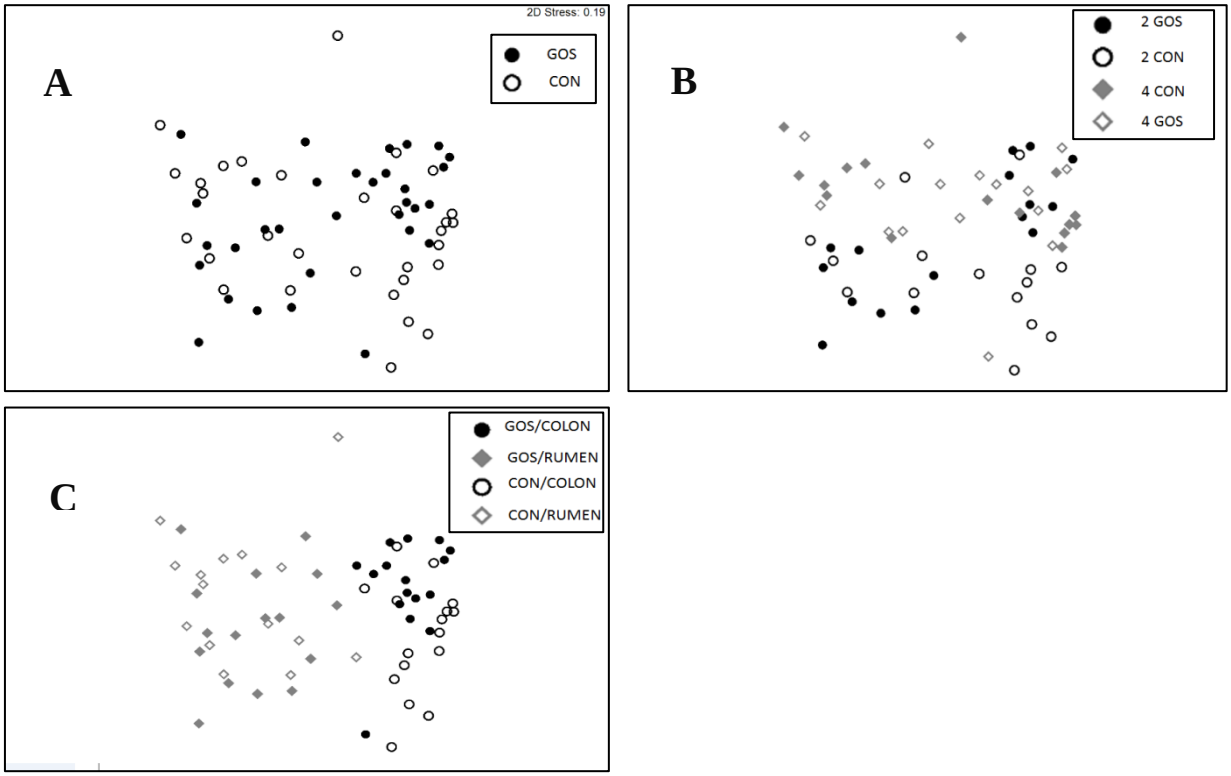

Figure 4.1. NMDS of digesta samples from CON and GOS treatments, in rumen and colon, and at 2 and 4 weeks of age

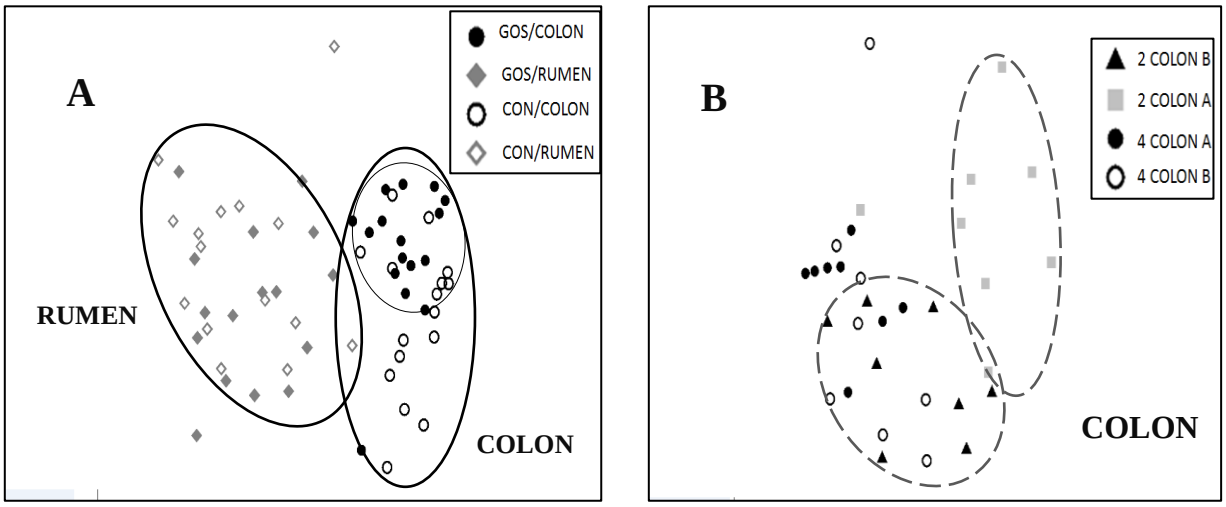

Figure 4.2.NMDS of samples from: a) colon and rumen for CON and GOS; b) Only samples from colon for CON and GOS at 2 and 4 weeks

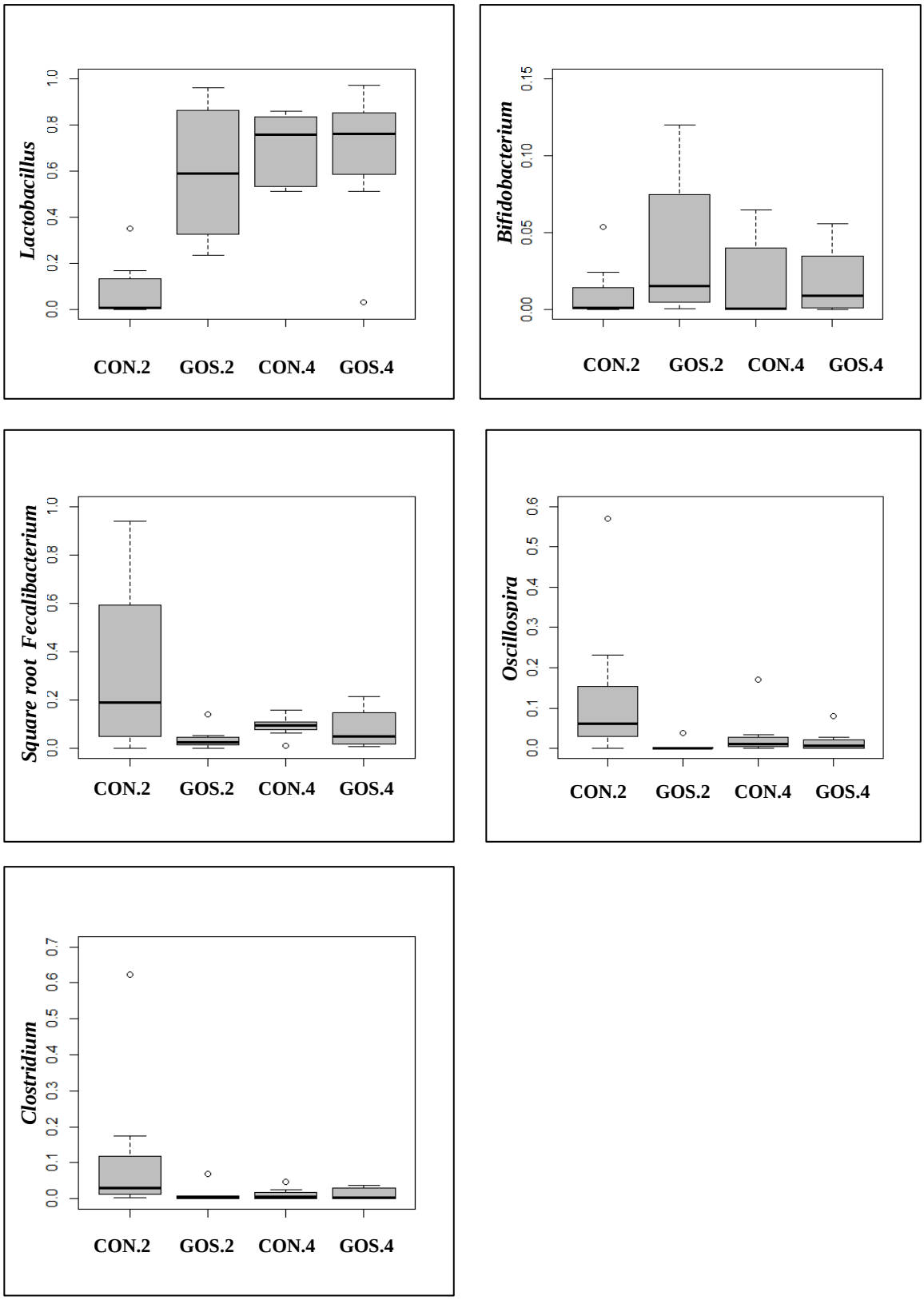

Figure 4.3. Distribution of relative abundance of taxa driving dissimilarity between CON and GOS groups at 2 and 4 weeks of age. CON.2 = CON at 2 weeks; GOS.2 = GOS at 2 weeks; CON.4 = CON at 4 weeks; GOS.4 = GOS at 4 weeks. Faecalibacterium relative abundance square root presented for ease of visualization.

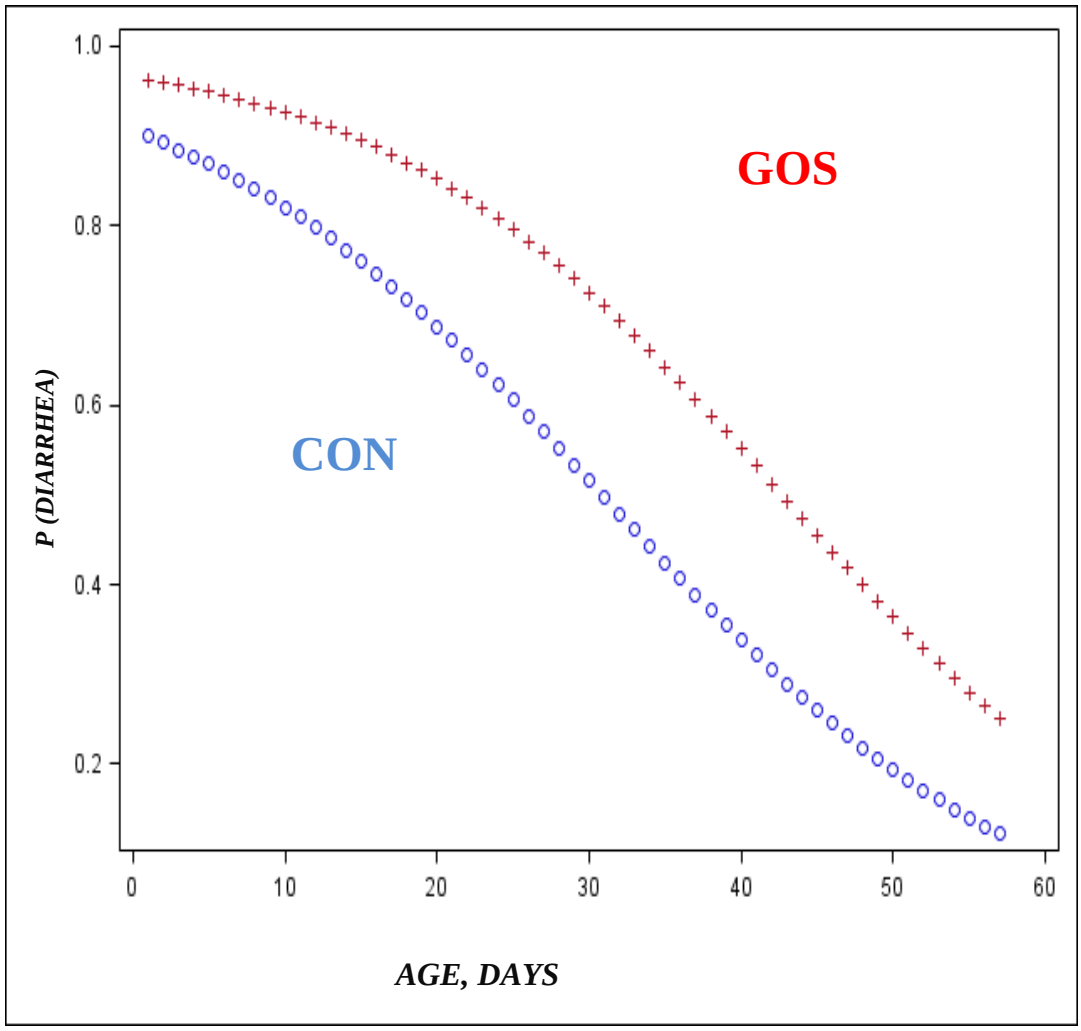

Figure 4.4. Probability of occurence of diarrhea for CON (circles line) and GOS (crosses line) treatments.

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CHAPTER V

EVALUATION OF VARIOUS ADDITIVES AS STIMULATORS OF GLP-2 SECRETION AND GUT DEVELOPMENT

ABSTRACT

Glucagon like peptide 2 (GLP-2) is a trophic factor secreted by endocrine L cells in the distal intestine and is responsible for adaptational changes such as mucosal growth and enhanced intestinal permeability under stressful circumstances, and has been proposed to have therapeutic potential to help young calves in circumstances such as weaning and diarrheal disease. Previous research indicates that GLP-2 secretion can be stimulated by substances like glucose, sweeteners and umami flavor. The objective of this experiment was compare a non-supplemented milk replacer diet against 1) glucose (220 mg/kg of BW/day), 2) stevioside (10 mg/kg of BW/day), 3) non-metabolizable glucose (6 mg/ kg of BW/day) and 4) glutamic acid source (GS) (i.e.,umami flavor) (20 mg/kg of BW/day) supplemented diets in regards to GLP-2 secretion, GI organ size, cellular proliferation, GLP-2 signaling pathway mRNA expression, and intestinal epithelium morphometric dimensions; as indicators of intestinal development in newborn Holstein calves.

No difference among treatments in GLP-2 secretion or any of the intestinal development measurements was observed. Two possible reasons for this could be that at moderate to high milk feeding levels, like those used here, GLP-2 secretion may not be as responsive; and also possible failure to identify the optimal dose to achieve stimulation.

INTRODUCTION

Neonatal calves are faced with different types of stressors. Promoting an early balance among the animal, its environment and etiological agents is necessary to minimize the probability of illness episodes (Davis and Drackley, 1998). Diarrhea is a common condition in the newborn calf and is most incident in the first few weeks of life (NAHMS, 2010). Development of nutritional strategies for the improvement of gut health and development during this life stage is desirable.

Glucagon like peptide 2 (GLP-2) is a trophic factor secreted by endocrine L cells in the distal ileum and proximal colon and is responsible for adaptational changes such as mucosal growth and enhanced intestinal barrier function under stressful circumstances, and has been proposed to have therapeutic potential to help young calves in circumstances such as weaning and diarrheal disease (Burrin et al., 2003). Enteral food intake is the primary stimulus for GLP-2 secretion in neonatal animals (Burrin et al., 2000; Taylor-Edwards et al., 2010). In ruminants, ileal proglucagon and GLP2 receptor (GLP-2R) mRNA abundance and GLP-2 plasma concentrations were up-regulated in response to increased energy intake in steers, suggesting that GLP-2 does play a role in the trophic response of the ruminant gastrointestinal tract to increased energy intake (Taylor-Edwards et al., 2010).

But beyond food or energy as a whole entity, specific nutrients like glucose and certain peptides have shown potential to stimulate GLP-2 secretion in monogastric animals via chemical sensing in the gut. Direct application of glucose to the luminal surface of the ileum stimulates GLP secretion from L cells in rats and humans (Brubaker and Anini, 2003), possibly by signaling through the sweet taste receptor T1R2/R3 located in the luminal membrane (Burrin et al., 2003). Similarly, it was recently shown that perfusion of free glutamate, a common element

of the umami flavor, into the duodenum increased portal venous concentrations of GLP-2 in rats, possibly via the bitter taste receptor T1R1/R3 (Wang et al., 2011). Glucose and glutamate-containing peptides are thus potential GLP-2 secretagogue candidates to evaluate in the young calf.

Some artificial sweeteners like sucralose, saccharin, and acesulfame K have been proven to taste sweet in humans, mice, and pigs. In fact, sweeteners can induce in vivo increased expression of T1R2/R3 and its signal transducing membrane protein, Gα-gustducin, probably by a similar mechanism as that of simple sugars (Margolskee et al., 2007; Moran et al., 2010). If glucose can indeed elicit GLP-2 secretion through the sweet taste receptor, then it is possible that sweeteners bear a similar potential to promote GLP-2 secretion (Shirazi-Beechey et al., 2011), which renders them research candidates of interest as well.

GLP-2 secretion also takes place upon endocrine and neural stimulation of the L cells in the ileum and proximal colon via glucose dependent insulinotropic peptide (GIP) released from enteroendocrine K-cells in the duodenum, and neural reflexes involving gastrin-releasing peptide (GRP) released from vagus nerve terminals innervating the stomach, prior to arrival and actual direct contact between dietary nutrients and the L cells in the distal intestine (Roberge and Brubaker, 1993; Roberge et al., 1996; Burrin et al., 2003). After GLP-2 release, the intestinotropic actions of GLP-2 on the colon are mediated through the actions of keratinocyte growth factor (KGF) and insulin-like growth factor (IGF)-2, whereas small intestinal growth has been linked to IGF-1, IGF-2, and ErbB ligands, as well as the IGF-1 receptor and ErbB (Rowland and Brubaker, 2011). The cellular source of these mediators remains unclear, but it likely includes the intestinal subepithelial myofibroblasts (Rowland and Brubaker, 2011). Conversely, the anti-inflammatory and blood flow effects of GLP-2 are dependent on vasoactive

intestinal polypeptide released from submucosal enteric neurons and nitric oxide, respectively (Rowland and Brubaker, 2011).

A critical aspect of GLP-2 bioactivity after secretion is its rapid degradation by dipeptidyl peptidase IV (DPP4), whose rapid action provides GLP-2 with a relatively short half-life of ~7 minutes in blood circulation (Burrin et al., 2003).

Given its trophic and protective effects on the intestine, GLP-2 activity in calves may lead to improve intestinal health or development in the period between birth and weaning (Burrin et al., 2003).

The hypothesis of this experiment was that, compared to a non-supplemented milk replacer diet, glucose, stevioside, non-metabolizable glucose and umami flavor supplemented diets will enhance GLP-2 secretion, GI organ size, cellular proliferation, GLP-2 signaling pathway mRNA expression, and intestinal epithelium morphometric dimensions; thereby improving overall intestinal development of newborn Holstein calves.

MATERIALS AND METHODS

Animals and Treatment Allotment

Seventy five newborn Holstein male calves were purchased from an outside vendor. Upon arrival to UIUC facilities, calves were weighed and their plasma protein level measured. Complete blocks of calves were made based upon BW and within each block the following five treatments were randomly assigned to each calf: 1) Control: commercial milk replacer, 2) control + glucose (220 mg/kg of BW/day), 3) control + sweetener (stevioside, 10 mg/kg of BW/day), 4) control + 3-O-M glucose (i.e., non metabolizable glucose) (6 mg/ kg of BW/day) and 5) control + a glutamate source (GS) (i.e. umami flavor) (20 mg/kg of BW/day).

Dosing of glucose was based on previous studies and made large enough to ensure induction of physiological response. Studies on expression of the sodium-glucose co-transporter 1 (SGLT-1) in adult sheep infused with 1.5 liters per day of 30 m*M* D-glucose solutions successfully achieved a 40 to 80 fold increase in the rate of glucose transport (Shirazi-Beechey et al., 1991). One and a half liters of a 30 m*M* glucose solution equals about 8 to 9 g of glucose daily. We decided to dose ~ 10 g/d.

3-O-M-glucose has been used at doses that range from 200 to 300 mg/d in humans to cause sustained measurable levels in plasma (Shirazi-Beechey et al., 1991). In-house research at LUCTA (Barcelona, spain) has also used about 300 mg/day in pigs which equals about 6 mg/kg of BW in 50-kg pigs, to reach measureable levels in plasma. Such concentration has been sufficient to up-regulate SLGT-1 (personal communication with Dr. I. Ipharraguerre).

Sweetener and GS dosage was set following levels previously used in pig research at LUCTA as well (personal communication with Dr. I Ipharraguerre).

After arrival, calves were fed commercial milk replacer twice daily at a rate of 1.25 % of BW daily divided into 2 feedings for the first 2 days. On day 3, the amount of milk replacer was increased to 1.75% of BW and then held constant through the end of the experiment. On day 7, treatments were introduced and continued until the end of experiment at day 22 of age.

Milk replacers contained 28% crude protein and 16% fat on a DM basis. Milk replacer composition and feeding rate was set to meet or exceed NRC daily nutrient allowance recommendations (NRC, 2001).

Plasma GLP-2

On day 12, animals were fitted with catheters in the jugular vein for venous blood sampling. On day 13, jugular vein samples were taken at -30, 0, 5, 15, 30, 60, 120, and 240 min

relative to feeding for measurement of GLP-2 plasma concentrations. Blood samples were collected into tubes containing EDTA plus a pyrrolic acid based DPP4 inhibitor. All blood samples were centrifuged immediately after collection, the plasma was extracted and frozen at -20 °C until analyses. Plasma GLP-2 concentrations were determined in the laboratory of Jens Juul Holst at the University of Copenhagen, Denmark.

Animal Slaughter and Sample Collection

On each of days 21 and 22 of age, all calves were euthanized for experimental measurements. Samples from fore stomach, small intestine and hind gut were collected immediately for various analyses as described below. Digesta was thoroughly removed and intestinal organs size was recorded.

Histomorphology

Sections of rumen, duodenum, jejunum, ileum, and proximal, middle, and distal colon were collected and fixed in 10% formalin for 24 h and then embedded in paraffin blocks and stained with hematoxylin and eosin. Paraffin sections 5-μm thick were cut and mounted onto glass slides and these were then scanned through a Nanozoomer scanning microscope (Hamamatsu, Japan) at 20 X to obtain digital microscopic scans on which papillae and villae height and width, and crypt width and depth were measured afterwards. These analyses were performed at the Institute for Genomic Biology at the University of Illinois.

Cellular Proliferation

Abundance of labeled cell nuclei with the protein marker Ki67 by immunohistochemistry was used as a measure of cellular proliferation. Paraffin-embedded tissue sections from jejunum, ileum, and colon were incubated for 1 h at 60^{o}C, deparaffinized, and rehydrated. Sections were treated in EDTA antigen retrieval for 25 min. Endogenous peroxide was blocked with methanol-

3% hydrogen peroxide (15 min). Sections were incubated in 2% normal mouse/2% normal goat in PBS/Tween for 20 min at room temperature. Serum was taped off and the primary antibody (Vector, VP-k451) was added at 1:1000 for 1 hr. Subsequently, sections were incubated in goat anti-rabbit secondary antibody (Jackson Immuno) for 30 min and Vector Elite ABC kit for 45 min. AEC was used for the choice of chromogen. Slides were counterstained with haematoxylin. The abundance of Ki-67 was assessed by scanning the slides on a Nanozoomer microscope (Hamamatsu, Japan) at 40 X to obtain digital microscopic scans. The digital image processing software AXIOVISION (ZEISS, Germany) was used to compute the Ki-67 stained area based on a pre-defined pixel intensity on the microscopic scans. This method is useful to avoid error from miscounting overlapping stained cells.

mRNA Expression

To assess treatment effects on mRNA expression of the GLP-2 signaling pathway in the distal gut, ileum and colon scrapings were collected into RNALater reagent (Qiagen, Hilden, Germany).

For extraction, tissue was homogenized in ice-cold Trizol in a bead beater (Invitrogen Corp., Carlsbad, CA, USA). Afterward, extraction with acid-phenol chloroform was performed (Ambion, Austin, TX, USA). RNA purification and genomic DNA removal was carried out through a miRNAeasy mini spin column (Qiagen, Hilden, Germany) and incubation with DNase. The RNA purity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Upon extraction, complementary DNA was synthesized and subjected to Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, CA, USA) following methodology previously reported by Khan (2013).

Briefly, for cDNA synthesis 100 ng RNA, 1 µg dT18 (Operon Biotechnologies, Huntsville, AL, USA), 1 µL 10 mmol/L dNTP mix (Invitrogen Corp., CA, USA), 1 µL random primer p(dN)6 (Roche®, Roche Diagnostics GmbH, Mannheim, Germany), and 10 µL DNase/RNase free water were mixed and incubated at 65 °C for 5 min and cooled down on ice for 3 min. Then, 6 µL of a mixture of of 4.5 µL 5X First-Strand Buffer, 1 µL 0.1 M dithiothreitol, 0.25 µL (50 U) of SuperScriptTM III RT (Invitrogen Corp.CA, USA), and 0.25 µL of RNase Inhibitor (10 U; Promega, Madison, WI, USA) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient. Complementary DNA was then diluted 1:4 (v:v) with DNase/RNase free water. Afterwards, quantitative PCR was performed using 4 µL diluted cDNA (dilution 1:4) combined with 6 µL of a mixture composed of 5 µL 1 × SYBR Green master mix (Applied Biosystems, CA, USA), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA, USA).

Triplicate runs of each gene were made for each sample. Relative quantitation of RT-qPCR product was performed using relative standard curve method with 6 points plus a non-template control, and expression normalized to the reference gene mRNA expression. The linear range of target quantification was established to determine the appropriate amount of cDNA template to be used in the PCR reaction.

Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA, USA). Final abundance values for each gene were normalized using the geometric mean of 3 internal control genes: Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-2-microglobulin (*B2M*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (Vandesompele et al., 2002).

Genes selected to represent the GLP-2 pathway were proglucagon (*GCG*) and GLP-2 receptor (*GLP2R*) (Connor et al., 2010; Rowland and Brubaker, 2011). Genes thought and selected to represent the GLP-2 paracrine mediator network included keratinocyte growth factor (*FGF7*), endothelial nitric oxide synthase (*NOS3*), insulin like growth factor 1 and 2 (*IGF1*, *IGF2*), IGF1 receptor (*IGF1R*), vasoactive intestinal peptide (*VIP*), epidermal growth factor receptor (*EGFR*), heparin binding EGF like growth factor (*HBEGF*), early growth response 1 (*EGR1*), c FOS proto-oncogene (*FOS*) and neuregulin 1 (*NRG1*) (Rowland and Brubaker, 2011). Genes previously suggested to be involved in the GLP-2 cellular signaling cascade (Rowland and Brubaker, 2011) and tested here were murine thymona viral oncogene (*AKT1*), phosphatidylinositol 3 biphosphate kinase (*PIK3CA*) (Rowland and Brubaker, 2011). In addition, a few previously reported functional marker genes were evaluated (Connor et al., 2010). For peptide transport, apoptosis and proliferation, the genes corresponding to the solute carrier family (*SLC15A1*), caspase 6 (*CASP6*), and proliferating cell nuclear antigen (*PCNA*) and cyclin D1 (*CCND1*), respectively, were assessed. Internal control genes included ATP synthase (*ATP5B*), B2 microglobulin (*B2M*) and hypoxanthine phosphoribosyltransferase (*HPRT1*) (Connor et al., 2010).

Primers were designed using the NCBI (National Center Biotechnology) primer design online facility. Reference intestinal cDNA was used to test primers running them in a 4% agarose gel. If a band of expected size was present in the gel, PCR amplification product was submitted for sequencing to the core DNA sequencing facility at Roy J. Carver Biotechnology Center at the University of Illinois to verify the primer ability to amplify the desired gene sequences (tables 6.1, 6.2). Alignment was performed using Chromas software (CHROMAS, South Brisbane, Australia).

Gene PCR amplification efficiency (table 6.3) was calculated from the standard curve as:

$$Efficiency = -10^{\left(-\frac{1}{slope}\right)}$$

Afterwards, mRNA abundance was computed as:

$$mRNA\ abundance = \frac{1}{(Efficiency)^{\Delta Ct}},$$

Where

$$\Delta \mathrm{Ct} = (\mathrm{C_{t}}_{sample} - (geometric\ meanof\ internal\ control\ genes)) .$$

Feed Intake and Health Check Scores

Milk replacer intake was measured and recorded daily. Average daily milk and water intake, and cumulative milk refusal were calculated for analysis. Fecal scores were recorded daily. Milk replacer samples were taken twice weekly for nutrient composition analysis.

Statistical Analysis

For measurements of nutrient intake, the analysis of variance model included treatment, week as a repeated measure, and their interaction (T*W) as fixed effects. Initial bodyweight and plasma protein concentration were tested as covariates. Different error structures were evaluated and the one that minimized the adjusted and unadjusted Akaike Information Criteria (AAIC and AIC), the Bayesian Information Criteria (BIC) and -2 Ratio Log-likelihood, with the least number of parameters was chosen. The model was:

$$Y_{ij} = \mu + treatment_i + E_{ij} + week_k + (T * W)_{ik} + e_{ijk}$$

Where E_{ij} and e_{ijk} are the error between calves within the same treatment and the error between repeated measures for the same calf, respectively.

Similarly, for GLP-2 concentrations over time, the model included treatment, hour as a repeated measure and their interaction (T*H) as fixed effects. Initial bodyweight and plasma

protein were tested as covariates. Error structure was evaluated as mentioned above. The model was:

$$Y_{ijklmnop} = \mu + treatment_i + E_{ij} + hour_k + (T * H)_{ik} + e_{ijk}$$

Where E_{ij} and e_{ijk} are the error between calves within the same treatment and the error between repeated measures for the same calf, respectively.

GLP-2 secretion area under the curve was also assessed and compared among treatments.

A similar statistical linear model was used for gastrointestinal organ size, histomorphometry and cellular proliferation measures since repeated measures were performed on various anatomical regions of the same calf.

For mRNA abundance, because 21 genes were measured, a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was conducted on a Euclidean distance matrix built from the standardized variables (mean=0, SD = 1) to test the hypothesis of an overall effect of treatments on any of the dependent variables. In addition, nonmetric multidimensional scaling (NMDS) plotting was used to visually identify treatment differences taking all genes into account simultaneously (Borcard et al., 2011).

Residuals were checked for homogeneity of variance and normality assumptions all throughout by residual plots, extended Levene test and Shapiro-Wilk test. Heterogenous variances were handled by implementing error structures that allow heterogenous variances (Littell et al., 2006). If the normality assumption was not met, transformation of the dependent variable proceeded and normality and constancy of errors was verified again. Dunnett's one sided lower bound adjustment would be used to test treatment mean differences. Experiment wise error rate was maintained at 5%. When significance is declared for any Pos-Hoc

comparison, a Tukey adjustment was used to compare treatment means maintaining the experiment-wise error rate at 5%.

For fecal scores, a generalized linear model with a cumulative logistic multinomial link function and a random intercept was fitted to the data (Fitzmaurice et al., 2011). The linear predictor was:

$$\eta_{ij} = \boldsymbol{X} * \mathrm{B} + \boldsymbol{Z} * b,$$

where **X** is a matrix containing the fixed effects of treatment, day and their interaction, and **Z** is a matrix containing a random effect for the intercept which accounts for the clustering of the observations from the same calf.

Repeated measure analyses were performed using SAS (SAS Institute Inc. Cary, NC.,), whereas multivariate gene expression data analysis was carried out through specific packages (Oksanen et al., 2012) in R open source software.

RESULTS

GLP-2 Secretion

There was no effect of treatment or its interaction with time post feeding on the average plasma GLP-2 concentration with respect to the control diet. Also, no treatment effect on area under the GLP-2 secretion curve was observed (Table 5.4). Figure 5.1.a visually illustrates the large overlap among confidence intervals for all treatment means.

GI Organ Size

There was no effect of treatment on organ weight expressed in kilograms or on a BW percentage basis, or organ length. The interaction between treatment and GI organ was also non-

significant. Table 5.5 and Figure 5.1.b show the similar treatment means and their confidence intervals.

Intestinal Epithelium Morphometric Dimensions

There was no effect of treatment or its interaction with GI organ on the length or width of the rumen papillae, the length or width of the villae in the duodenum, jejunum or ileum or the crypt depth or width in duodenum, jejunum, ileum or colon (Table 5.5, Figure 5.2).

Cellular Proliferation

There was no effect of treatment or its interaction with GI organ on the average abundance of ki-67 stained nuclei with respect to the control diet (Table 5.5). Figure 5.1.c displays the similarity between confidence intervals for treatment means.

GLP-2 Signaling Pathway mRNA Expression

PERMANOVA (Table 5.6) indicated that overall expression of the GLP-2 signaling pathways in ileum and colon was not affected by treatments. Figure 5.3 displays a NMDS plot that simultaneously considers information from all genes evaluated on all experimental units and it is clear that all treatment groups cluster together, thus corroborating PERMANOVA findings of no difference among treatments. In contrast, an analysis of similarities identified a very strong difference in the pathway gene expression between ileum and colon (Figure 5.3b).

Water and Milk Replacer Intake and Refusals

Average weekly milk liquid and dry matter intake and total refusal was not affected by treatments or their interaction with time. Mean water intake was also unaffected by treatment or its interaction with time. Table 5.7 and Figure 5.4 display treatment means and their confidence intervals.

Fecal Scores

No effect of treatment or the interaction with time had a significant effect on the odds of calves presenting diarrheal episodes with respect to the control treatment.

DISCUSSION

The fact that no treatment elicited any response in at least GLP-2 secretion indicates that either these compounds (i.e., glucose, non-metabolizable glucose, sweetener, GS) do not actually have any effect or that we failed to properly identify the supplementation level required to induce such response.

In the case of glucose and non-metabolizable glucose, there is evidence that infusing mature sheep with ~8 g/d can increase the monosaccharide transport from 40 to 80 fold as a result of an increase in the number of SGLT1 transporters (Shirazi-Beechey et al., 1991). Mature sheep of the racial background reported in the cited study can be expected to present a similar BW to that of a calf (~50 kg); therefore the dosage per unit of BW would have been similar to the one used in our study. Because GLP-2 modulates expression of SGLT1 (Cheeseman, 1997; Ramsanahie et al., 2003; Cottrell et al., 2006; Sangild et al., 2006), we made the key assumption that if the above dosage could elicit such large changes in transporter expression and activity, it probably should have been through stimulation of the GPL-2 pathway (Shirazi-Beechey et al., 2011).

Regarding sweeteners, unlimited access to 2 m*M* sucralose, 10 m*M* acesulfame K and 20 m*M* saccharin solutions as the water source increased SGLT1 mRNA and protein expression, and glucose absorption in mice (Margolskee et al., 2007). Unfortunately, the authors provided no information to allow calculation of the absolute amount of sweetener consumed by the animals. In this experiment we supplied stevioside at 10 mg/kg of BW/day for a total of about 500 mg/day

(average calf BW = 50 kg), which, for an intake volume of 2.9 L, results in a 0.54 m*M* solution. Although, on a molarity basis it seems a low stevioside supplementation level, it is hard to ascertain whether this is actually low comparatively because different sweeteners can elicit response at different levels as evidenced above (Margolskee et al., 2007). On a milligram/milliliter basis, Margolskee et al.(2007), supplied sucralose, acesulfame K and saccharin at ~ 265, 67 and 122 mg/mL/kg of BW, respectively; whereas, we supplied stevioside at 0.003 mg/kg of BW. On a mass basis our stevioside supplementation level would appear low as well, but again, it is impossible to compare on an absolute amount basis. Consequently, this impedes a more precise statement of how far we might have been from an optimal supplementation level for inducing a response in GLP-2 secretion.

Nevertheless, sweetener and umami flavor dosage levels were set following levels previously determined to trigger response in pigs at LUCTA (personal communication with Dr. I. Ipharraguerre). Recently, it has been shown that the sweet-taste sensitivity varies widely among species and even strains within species. For example, unlike humans, rats do not perceive aspartame, cyclamate, neotame, monellin, thaumatin or alitame as sweet (Pepino and Bourne, 2011). Also, although both mice and rats show some preference for stevia extracts over water, they differ in their responses to sucralose (Pepino and Bourne, 2011). Moreover, even though some animal studies have found that sweeteners activate gut sweet taste-pathways that control incretin release and up-regulate glucose transporters, human studies have found that the sole interaction of sweeteners with sweet-taste gut receptors is insufficient to elicit incretin responses (Pepino and Bourne, 2011). These observations should be considered in the future when designing studies that examine the effects of sweeteners on incretin responses.

But even if sensitivity to stevioside or GS were similar in pigs and calves, it is possible that dosage does not scale linearly from the former to the latter. That is, the number of milligrams per day per kilogram of BW may not be constant across species. Indeed, even though dosage scaling based on BW is commonly used for practical reasons, interspecies differences in the dose–response relationship can be large (Sharma and McNeill, 2009). Thus, this may have been another limitation in this study and perhaps running species specific dose-response curves may be more fruitful.

Lastly, enteral food intake level is known to control GLP-2 secretion powerfully, indeed it perhaps is the single variable with the greatest secretory effect (Burrin et al., 2000). These calves were fed a fairly high feeding rate (1.75% of BW as DM). It is possible that incretin secretion responses to secretagogues like stevioside or umami are dependent upon enteral nutrient intake level. If so, this interaction shall be evaluated and the response surface of enteral intake level by secretagogue supplementation level be defined.

CONCLUSION

The objective of this experiment was to test the hypothesis that glucose, non-metabolizable glucose, stevioside and the umami flavor could enhance GLP-2 secretion and thereby increase gut growth or health of newborn Holstein calves. No effect of any treatment on intestinal growth and development was observed. Such lack of response was due to failure to identify supplementation levels that could stimulate incretin release, even though previously published work and LUCTA's in-house research with models such as lambs and pigs, respectively, had detected responses at these concentrations. Species specific sensitivity to sweet flavor and GS may render direct dose extrapolation between species inappropriate and; therefore,

species specific dose response surfaces may provide a better approach to optimize the incretin release response.

TABLES AND FIGURES

Table 5.1. RT-qPCR performance for all genes measured in ileum and colon

Gene	Ct median[1]	Median ΔCt	Slope[2]	r^2 [3]	Efficiency
AKT1	21.3291	1.341842	-2.91824	0.970876	2.201267
ATP5B	19.96596	0.123137	-3.26196	0.9907	2.025651
CASP6	21.43135	1.347451	-3.29044	0.990168	2.013311
CCND1	24.93846	4.82878	-3.5273	0.991	1.920892
EGFR	24.8108	4.709171	-3.27129	0.994946	2.021573
EGR1	24.14954	4.137083	-3.29501	0.991319	2.011357
FGF7	27.63172	7.378138	-2.87	0.995	2.230653
FOS	24.42071	4.390363	-3.31165	0.98124	2.004306
GCG	23.76609	3.520812	-3.28305	0.981486	2.016486
GLP2R	25.31576	5.181228	-2.92008	0.993242	2.20017
HBEGF	27.75724	7.798241	-2.96694	0.980093	2.172937
IGF1	26.16876	6.125472	-3.01111	0.9937	2.148342
IGF1R	24.88765	4.692747	-3.29146	0.987224	2.012875
IGF-2	23.90864	3.731504	-3.10092	0.996313	2.101284
NOS3	25.71666	5.452764	-3.07289	0.986515	2.115567
NRG1	26.24303	6.147391	-3.20279	0.989386	2.052238
PCNA	22.74805	2.539459	-3.35044	0.98995	1.988238
PIK3CA	24.49259	4.416382	-3.42971	0.981818	1.956904
SLC15A1	22.81702	2.987484	-3.07992	0.980693	2.11195
VIP	29.58792	9.459522	-2.95473	0.985911	2.179919

[1] Median from all ileum and colon samples from 45 calves
[2] Slope of the standard curve
[3] Determination coefficient from standard curve

Table 5.2. Gene ID, accession number, and corresponding forward and reverse verified primers

GENE	NCBI ACCESSION #	FORWARD	REVERSE
AKT1	NM_173986.2	GGATTACCTGCACTCGGAAAAG	TCCGAAGTCGGTGATCTTGAT
ATP5B	BC116099.1	AGCATTTGGGTGAGAGCACA	GTCTCAGGGCCAACAGGAAT
B2M	NM173893.3	TCCAGCGTCCTCCAAAGATT	CCCATACACATAGCAGTTCAGGTAA
CASP6	NM_001035419.2	CACAGGACAGACACGCCAGAT	AAGTCGGCTCCAGCAGGAA
CCND1	BC112798.1	GATGCCAACCTCCTCAACGA	GGCAGGATCTCCTTCTGCAC
EGFR	AY486452.1	AACTGTGAGGTGGTCCTTGG	CATATCCGGCAACCTCCTGG
EGR1	BC118328	TGTGATGATGCTGTGACGATTC	ACTCTGACACATGCTCCGAT
FGF7	XM_005211861.1	AAGTTGCACAGGGCAGACAA	GTTGCTGAGATGCTGTTTGCT
FOS	BT029837	GCAAAACGCATGGAGTGTGT	AAAAGAGACGCAGACCCAGG
GAPDH	NM_001034034	TTGTCTCCTGCGACTTCAACA	TCGTACCAGGAAATGAGCTTGAC
GCG	NM_173916.3	CCCTTCAGAACACAGAGGAGAAA	GAGTGGCGCTTATCTTCATTGA
GLP2R	XM_589370.4|	TTCTGGTGCAGTCTGTAATGGG	CCCTTCCTGAGTTCTCTTCGC
HBEGF	NM_001144090.1	GCCATCTAGATTGGGCCTCC	ACCCAGATCCCACTGGACAT
HPRT1	NM_001034035.2	GCTGGCTCGAGATGTGATGA	TCCAACAGGTCGGCAAAGAA
IGF1	NM_001077828.1	CCAATTCATTTCCAGACTTTGCA	CACCTGCTTCAAGAAATCACAAAA
IGF1R	NM_001244612.1	CGACATCCACAGCTGTAACCAT	AGGAATGTCATCTGCTCCTTCTG
IGF2	NM_174087.3	CCTCCAGTTTGTCTGTGGGG	GGGTGGCACAGTAAGTCTCC
NOS3	NM_181037.3	CGGAACAGCACAAGAGTTACAAGA	TGTGTTGCTGGACTCCTTTCTC
NRG1	XM_005226076.1	CGGTCAGTCCCTTCGTGGAA	GGTTGCAGTGGAACGAGTTGA
PCNA	BC103068	GAACCTCACCAGCATGTCCA	ACGTGTCCGCGTTATCTTCA
PIK3CA	NM_174574	ATTGGCCTCCAATCAAGCCT	CACCGAACAGCAAAACCTCG
SLC15A1	BC140526	ACTCCGTCTCCCAGACACAA	CCACCCAACAGAGTGTCCTG
VIP	AF503910.1	CTTCTTGTGTTCCTGACGCTGTT	AGAGCCGAAGGTGCTCCAA

Table 5.3. Sequence of amplicons obtained from primers and verified against NCBI primer BLAST.

AKT1	CTCAGCTGAGACTCATGCTGGACAGGACGGGCACATCAAGATCACCGACTTCGGA
ATP5B	TGGTTAGAGGTCAGAAAGTCCTGGATTCTGGTGCACCAATCAGAATTCCTGTTGGCCCTG
B2M	GCAATGAACACCACAGAAGATGGAAGCAATTACTGAACTGCTATGTGTATGGGA
CASP6	TTATACACTTCCGTGCTGGAGCCGACTT
CCND1	GGAGGAACGTACGCGCCCTCGGGTATCCTACTTCAAGTGTGTGCAGAAGGAGATCCTGC
EGFR	ACCTTTCTTTTCTCAGACCATCCAGGAGGTTGCCGGATATG
EGR1	TCTTGGTATTATCGGAGCATGTGTCAGAGT
FGF7	GTATCAGCGTGTTATCACAGACAAAGTCAAGTAGCAAACAGCATCTCAGCAAC
FOS	CATCGTAGAGCGTTAGTTAGTAGCATGTGGAGCAGGCCTGGGTCTGCGTCTCTT
GAPDH	CACTCTTCTCTCGAGTCTGGGGGCGTGCATTGCCTCACGACCACTTTGTCAAGCTCATTTCCTGGTACGAAAT
GCG	CTCGGCGATCCAGATCAGATCAATGAAGATAAGCGCCACTC
GLP2R	TGTGTGCTGCCTCATCACTCTGGATGTCTCTGTCCCTGCCTTCCTACTTACCTTGGTGGCGCGAAGAGAACTCAGGAAGGGC
HBEGF	AACAGAATATGTCCAGTGGGATCTGGGT
HPRT1	CCTCGTGTTGCTCAGGGGGGCTATAAGTTCTTTGCCGACCTGTTGGA
IGF1	AATCAGCAGTCTTCACCCAATTATTTAAGTGCTGCTTTTGTGATTTCTTGAAGCAGGTG
IGF1R	ACTTTGTCTTTGCAAGAACCATGCCTGCAGAAGGAGCAGATGACATTCCT
IGF2	CGACATCAGCCGCATACCGACGCAGCCTGTGCATCGATGGAGAGTGTTGCTTCCGAAGCTGCGACCTGGCCCTGCTGG AGACTTACTGTGCCACCC
NOS3	CAGCGTCTCTGCTCAGACCCGCTGTGTCCTCCTGGCGGCGGAGAGAAGGAGTCCAGCAACACA
NRG1	TCCTTGTGTACGGCACACGGCGTCGGGAGAGTATGACCACCACGCCCAGCAATTTCAACTCGTTCCACTGCAACC
PCNA	AGACAATCATTACCTTAAGAGCTGAGATAACGCGGACACGT
PIK3CA	CATTACCCAGATCCTATGGTTCGAGGTTTTTGCTGTTCGGTG
SLC15A1	CAGGTCTGTCCTGGGGGCAGGACACTCTGTTGGGTGGA
VIP	GCTCTTTTTGGAGCACTTC

Table 5.4. Treatment means for plasma GLP-2 concentrations and AUC

	Treatment[1]						P-value	
	CON	Gluc	3-o-M-G	Sweet	GS	S.E.	Trt[2]	Trt x min.[3]
[GLP-2], pmol/ml	38.3	47.4	35.6	38.5	39.7	7.1	0.8	0.47
AUC, nmol[4]	8.6	11.9	8.1	8.7	9.6	2.3	0.76	NA

[1]CON: control; Gluc: glucose; 3-o-M-G: 3-O-Methyl glucose; Sweet: sweetener, GS: glutamate source.
[2]Trt: treatment effect
[3]Trt x min.: treatment by minute interaction effect
[4]AUC: Area under the GLP-2 secretion curve

Table 5.5.Treatment means for organ size, histomorphology and proliferation

	Treatment[1]						P-value	
	CON	Gluc	3-o-M-G	Sweet	GS	S.E.	Trt[2]	Trt x GI organ[3]
GIT organ size								
Weight, kg	0.32	0.34	0.32	0.31	0.32	0.01	0.55	0.94
Weight, % of BW	0.7	0.75	0.71	0.71	0.72	0.025	0.64	0.91
Length, cm	336	351	339	335	335	7.9	0.60	0.86
Histomorphology								
Villae length, µm	515.4	510.0	499.0	491.1	507.0	17.6	0.88	0.69
Villae width, µm	176.0	179.6	171.8	183.0	176.3	6.4	0.76	0.8
Crypt depth, µm	361.0	376.3	376.8	383.3	367.2	9.0	0.48	0.79
Crypt width, µm	61.7	64.3	60.0	61.2	63.7	1.4	0.15	0.59
Proliferation								
ki-67 abundance, %	6.9	6.2	6.0	6.3	6.2	0.3	0.38	0.16

[1]CON: control; Gluc: glucose; 3-o-M-G: 3-O-Methyl glucose; Sweet: sweetener, GS: glutamate source.
[2]Trt: treatment effect
[3]Trt x GI organ: treatment by GI organ interaction effect

Table 5.6.PERMANOVA test for treatment effects on the GLP-2 pathway gene expression

Source	DF[1]	SS[2]	MS[3]	F[4]	R^2	P-value
Treatment	4	0.1	0.025	0.81	0.08	0.68
Residuals	38	1.2	0.031			
Total	42	1.3				

[1] Degress of freedom
[2] Sum of squared distances
[3] Mean squares
[4] F statistic

Table 5.7.Treatment means for milk intake and water intake

	Treatment					P-value		
	CON[1]	Gluc	3-o-M-G	Sweet	GS	S.E.	trt[2]	trt x week[3]
Milk intake, kg/d	2.98	2.92	2.98	2.93	2.96	0.05	0.85	0.99
Milk DMI, kg/d	0.34	0.33	0.34	0.34	0.34	0.005	0.85	0.99
Milk refusal, kg[4]	1.35	1.80	0.69	1.39	1.19	0.34	0.21	NA
Water intake, kg/d	1.07	0.95	1.40	1.12	1.34	0.15	0.07	0.72

[1]CON: control; Gluc: glucose; 3-o-M-G: 3-O-Methyl glucose; Sweet: sweetener, GS: glutamate source.
[2]Trt: treatment effect
[3]Trt x week: treatment by week interaction effect
[4] Cumulative liquid milk refusal over the entire trial (square root transformation to meet normality assumptions)

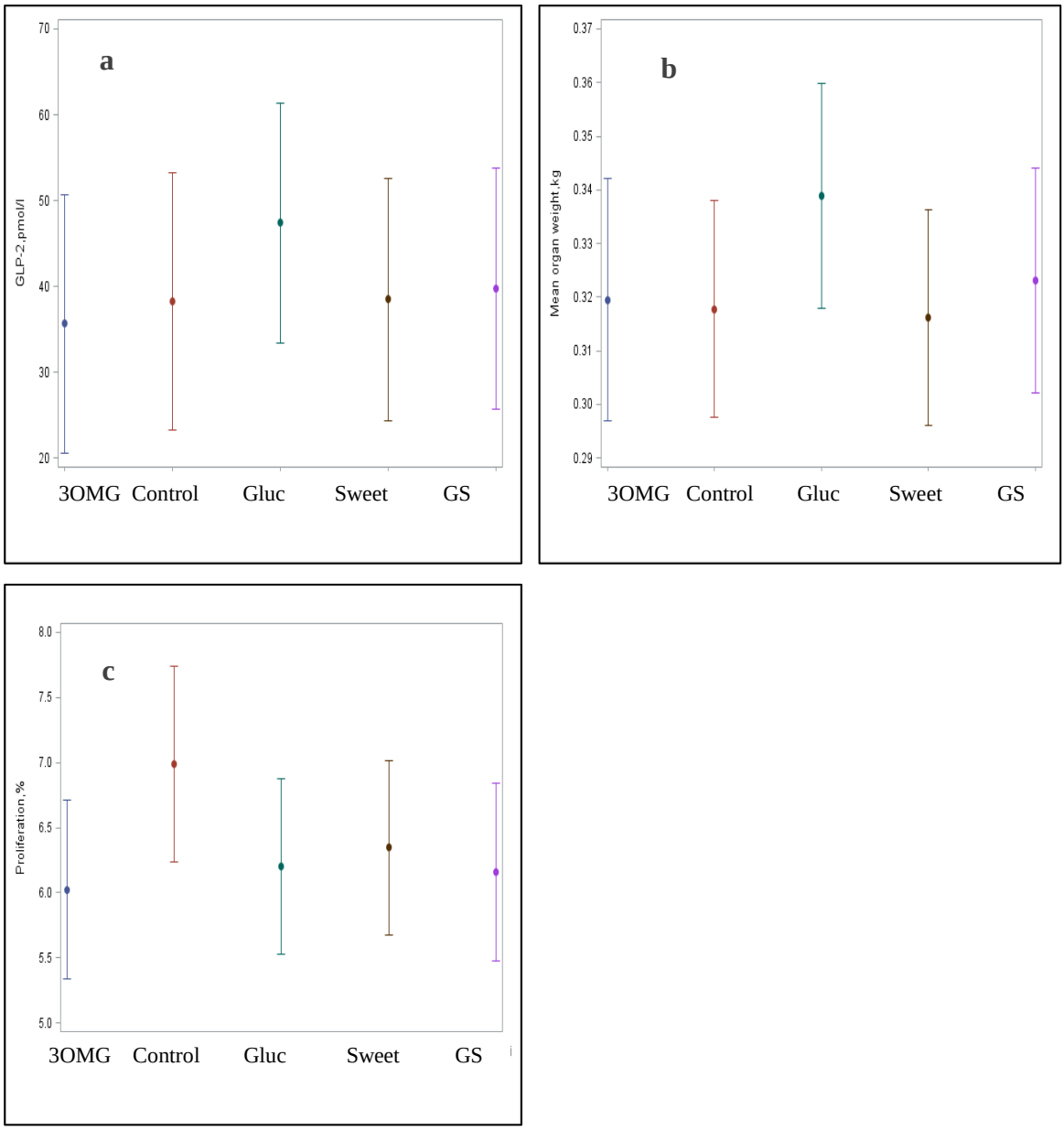

Figure 5.1. Confidence intervals for treatment means of a) plasma GLP-2, b) organ size and c) proliferation. 3OMG: 3-o-Methyl glucose, Gluc: glucose, Sweet: Stevioside, GS: Glutamate source.

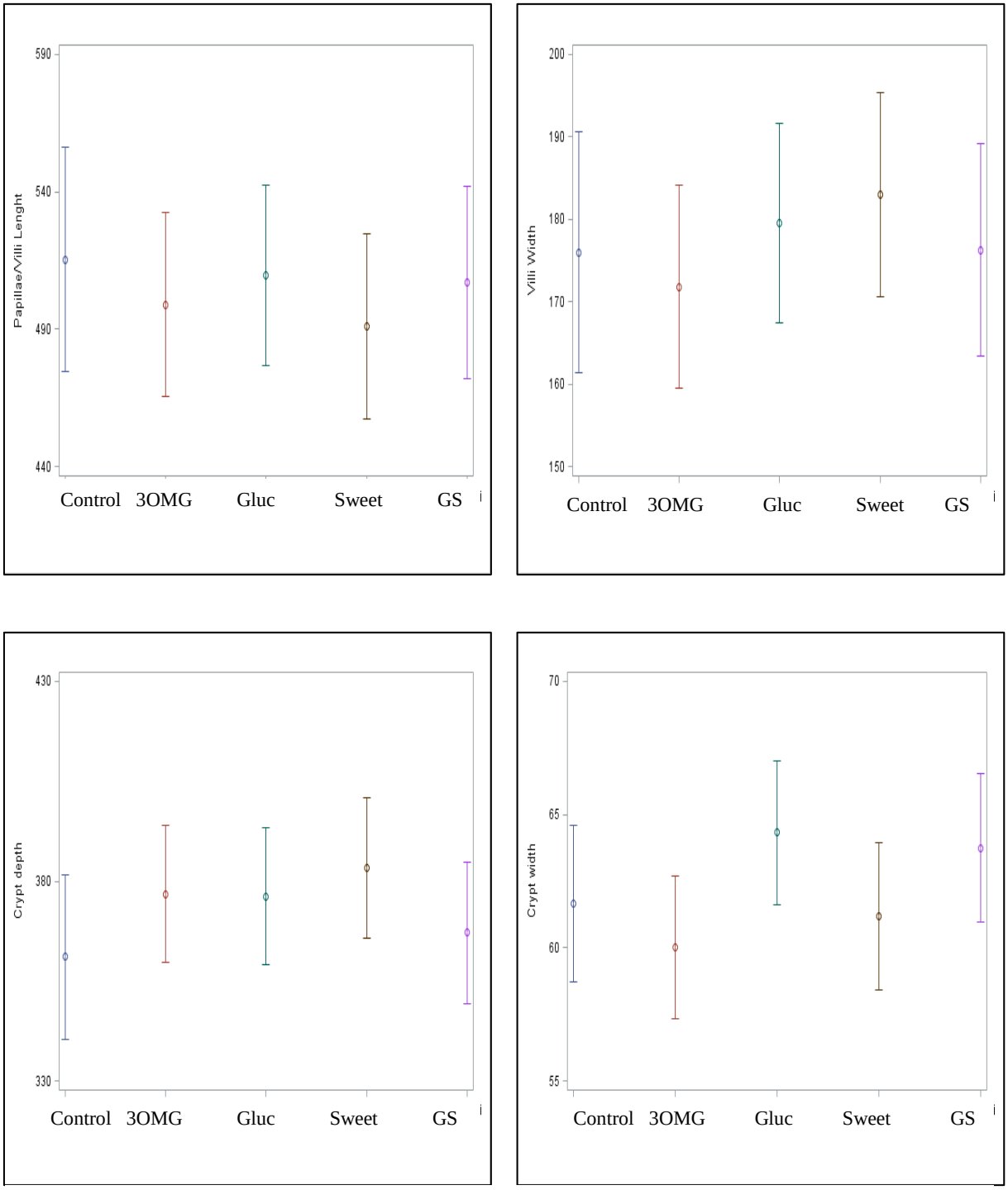

Figure 5.2. Confidence limits for treatment means of histomorphometry measures. 3OMG: 3-o-Methyl glucose, Gluc: glucose, Sweet: Stevioside, GS: Glutamate source.

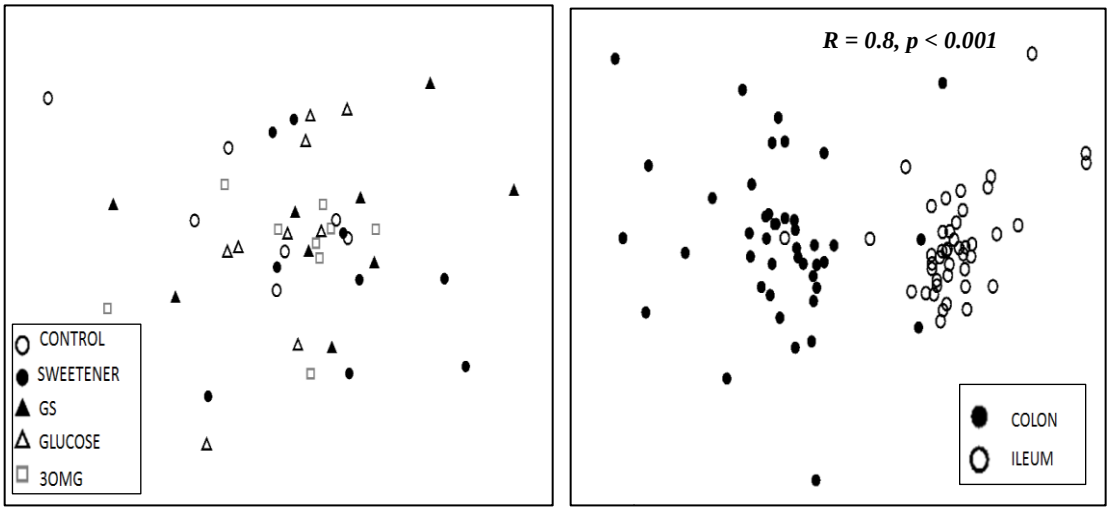

Figure 5.3. NMDS plot of observations and treatments. a) Treatment groups, b) Ileum and colon groups (ANOSIM, R=0.8, p<0.001)

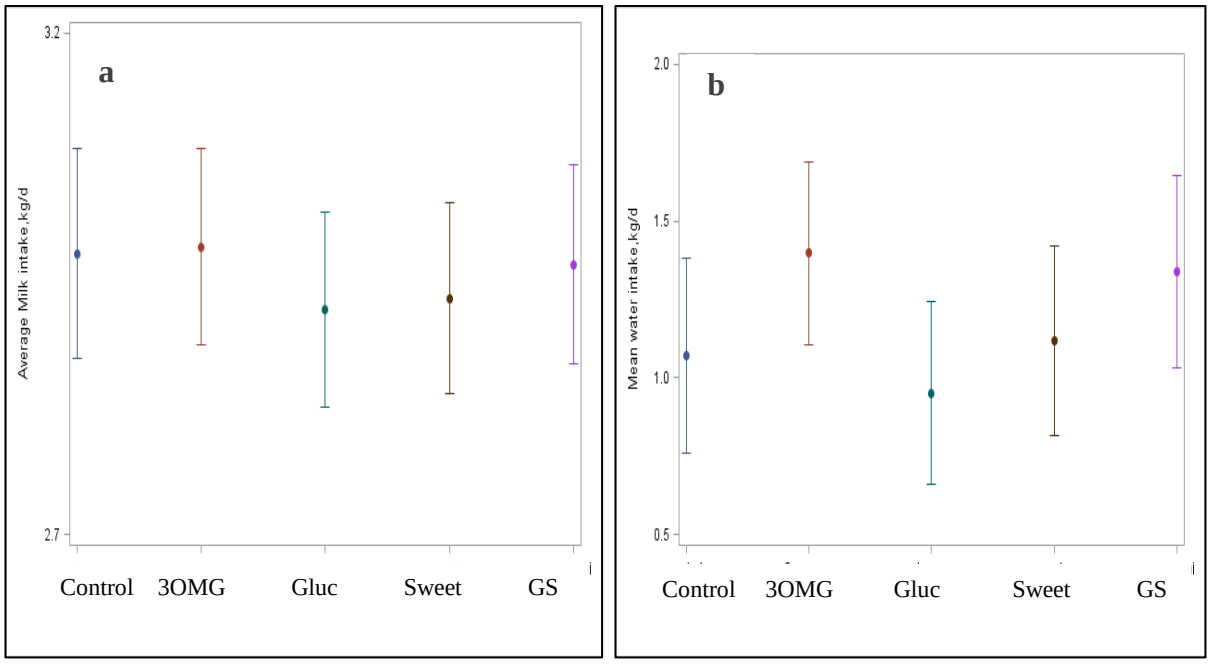

Figure 5.4. Confidence limits for treatment means of a) milk and b) water intake

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