Changes in the intestinal bacterial community, short-chain fatty acid profile, and intestinal development of preweaned Holstein calves.

1. Effects of prebiotic supplementation depend on site and age

J. J. Castro,* A. Gomez,† B. A. White,*† H. J. Mangian,‡ J. R. Loften,§ and J. K. Drackley*¹

*Department of Animal Sciences,

†Institute for Genomic Biology, and

‡Department of Food Science and Human Nutrition, University of Illinois, Urbana 61801

§Milk Specialties Global Animal Nutrition, Eden Prairie, MN 55344

ABSTRACT

Digestive disorders are common during the first few weeks of life of newborn calves. Prebiotics are nondigestible but fermentable oligosaccharides that modulate growth and activity of beneficial microbial populations, which can result in enhanced gut health and function. Galactooligosaccharides (GOS) have demonstrated such prebiotic potential. In this study, the effect of GOS supplementation on intestinal bacterial community composition and fermentation profiles; intestinal health, development, and function; and growth was evaluated in dairy calves fed for high rates of growth. Eighty male Holstein calves were assigned either to a control treatment consisting of commercial milk replacer or to a GOS-rich (i.e., 3.4% of dry matter) milk replacer treatment. After 2 and 4 wk, 8 calves per treatment were slaughtered at each age. Samples of intestinal digesta and tissue were collected for assessment of bacterial communities, short-chain fatty acid concentrations, *in vitro* measurement of nutrient transport and permeability, histomorphology, and gastrointestinal organ size. The remaining 48 calves continued to wk 8 to measure body growth, nutrient intake, and fecal and respiratory scores. Calves fed GOS displayed greater *Lactobacillus* and *Bifidobacterium* relative abundance and more developed intestinal epithelial structures, but also had greater fecal scores presumably related to greater colonic water secretion. Control calves showed slightly better growth and milk dry matter intake. Size of intestinal organs, intestinal nutrient transport, and epithelium paracellular resistance were not affected by treatment. Excessive GOS supplementation had both prebiotic and laxative effects, which led to slightly lower growth performance while promoting commensal

bacteria population and greater intestinal epithelium growth.

Key words: calf, gut health, prebiotic, permeability

INTRODUCTION

Neonatal calves are faced with a variety 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). 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 common 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 represented ~80% of US dairy operations, reported a death rate for preweaning heifers of 7.8%. Scours (diarrhea) or other digestive problems accounted for 56.5% of this mortality. Although somewhat better than the 60.5% found in the previous survey (NAHMS, 1996), the incidence of intestinal illness is still 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 (**GI**) tract. To achieve this, different types of prebiotics have been developed and their potential health benefits have been evaluated 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, *Bifidobacterium* and *Lactobacillus* are the bacterial genera targeted by prebiotics such as fructooligosaccharides and galactooligosaccharides (**GOS**; Macfarlane et al.,

Received February 8, 2016.

Accepted August 13, 2016.

¹Corresponding author: drackley@illinois.edu

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 short-chain fatty acids (SCFA) that lower intestinal pH, and stimulating the immune system (Van Loo and Vancraeynest, 2008).

The main source of GOS is whey permeate, which is a byproduct of cheese manufacturing. Briefly, the residual whey left after cheesemaking is rich in whey proteins (e.g., β -LG and α -LA) and lactose. This whey can be ultrafiltered by membrane technology to isolate the protein fraction, which is of high nutritional value to humans. A subsequent byproduct from this filtration is whey permeate, which is nearly devoid of whey proteins and very rich in lactose (Torres et al., 2010). Lactose in this whey permeate can then be crystallized and subjected to enzymatic digestion with β -galactosidases that attack the o-glucosyl group of lactose. Through a sequence of reactions between galactose and multiple di- and oligosaccharides other than lactose, β -galactosidase activity yields a variety of compounds collectively named GOS. Lactose hydrolysis to form GOS also results in an increase in free sugars (Torres et al., 2010). Commercially available GOS are generally mixtures of lactose, glucose, galactose, and oligosaccharides. The oligosaccharide profile of GOS is extremely variable and depends on the specific β -galactosidase used in the manufacturing process, among other processing conditions (Angus et al., 2005). Galactooligosaccharides have the chemical structure G-Gal_n with G = glucosyl moiety, Gal = galactosyl moiety, and n = number of galactosyl units linked together (Frank, 2008; Torres et al., 2010). Bound to the Gal-Glu unit in the reducing end of the oligosaccharide can be additional galactose units but also N-acetyl-glucosamine, fucose, or sialic acid derivatives (Urashima et al., 2009). The degree of GOS polymerization ranges from 2 to 8 residues, with an average of about 3 (Frank, 2008).

The objective of this experiment was to evaluate the prebiotic potential of a β -galactosidase treated whey permeate as a source of GOS in milk replacer (MR) on fore and distal gut bacterial communities, fermentation profiles, health, intestinal development and function, and growth of preweaned calves fed for high rates of growth. The milk-derived oligosaccharides reported here fit within the definition of GOS as described in the previous paragraph.

MATERIALS AND METHODS

Animals, Feeding, and Treatment Allotment

All experimental procedures involving animals were approved by the Institutional Animal Care and Use

Committee (protocol #122109) of the University of Illinois at Urbana-Champaign. Eighty newborn male Holstein calves were purchased from sale barns. Upon arrival to the University of Illinois research facilities, calves received an electrolyte solution (Electrolyte System Base plus Add Pack, Land O'Lakes Animal Milk Co., Arden Hills, MN), as well as vaccines and prophylactic antimicrobial treatment (2 mL of BoSe, Shering-Plough, Madison, NJ; 1 mL of vitamin A and D; 1.1 mL of Draxxin, Zoetis, Kalamazoo, MI; 50 mL of antibody serum (Bovisera), Colorado Serum, Denver, CO; 20 mL of C and D antitoxin, Boehringer Ingelheim, St. Joseph, MO; and 2 mL of INFORCE-3, Zoetis). Navels were treated with povidone iodine once daily for the first 3 d. Calves were weighed and a sample of blood from a jugular vein was used to estimate plasma protein score by refractometer. Arrival BW and plasma protein scores ranged from 36.3 to 51.3 kg and 4.6 to 7.2 g/dL, respectively. Individual age of calves was not known but was estimated to be between 1 and 4 d of age. Because colostrum intake also was unknown, calves were sorted by arrival BW and plasma protein score, then blocked by BW, and within block, treatments were assigned randomly to obtain treatment groups of calves as homogeneous as possible.

Treatments were (1) a commercial MR (CON; Excelerate, Milk Specialties Global Animal Nutrition, Eden Prairie, MN) and (2) a similar MR containing whey permeate rich in GOS (Milk Specialties Global Animal Nutrition). The production practices and conditions for GOS manufacture and incorporation were proprietary but generally followed the procedures described in the Introduction. Final measured concentration of oligosaccharides for the GOS treatment was 3.35% of MR DM and 0.06% of MR DM for CON (Table 1). The amount of GOS in the treatment MR was the maximum that could be produced by the technique and incorporated into the MR. This dosage was consistent with previous studies with pigs where ~4.8% of the diet DM as GOS supplementation resulted in a clear prebiotic effect (Smiricky-Tjardes et al., 2003). Analysis showed that CON contained ~44% of DM as lactose versus ~20% in GOS (Table 1), which reflects the treatment with β -galactosidase that converted lactose into a variety of oligo- and free monosaccharides.

Milk replacers were formulated to be isoenergetic and isonitrogenous and to meet or exceed NRC daily nutrient allowance recommendations (NRC, 2001). Protein was provided exclusively by dried whey and whey protein concentrate. Fat was a proprietary blend of tallow, lard, and coconut oil. Milk replacers were reconstituted with water to achieve 13% solids. Although higher intakes of nondigestible oligosaccharides cause changes in the osmotic gradient within the large intestine (Binder,

Table 1. Nutrient composition of experimental milk replacers

Nutrient	Treatment ¹	
	CON	GOS
DM, %	95.9	95.0
CP, % of DM	28.8	29.6
Crude fat, % of DM	15.7	15.1
Ash, % of DM	8.27	7.76
NE _M , Mcal/kg	2.67	2.67
NE _G , Mcal/kg	1.91	1.90
Ca, % of DM	1.05	1.12
P, % of DM	0.68	0.63
Total disaccharides, mg/g of DM	437.3	197.3
Lactose	436.3	197.3
Sucrose	1.0	0
Total oligosaccharides, mg/g of DM	0.6	33.5
2'-Fucosyl-lactose	0	3.7
Lacto- <i>N</i> -fucopentanose	0	28.8
Lacto- <i>N</i> -neotetraose	0.2	0
Lacto- <i>N</i> -tetraose	0	0.8
Sialic acid	0	0
Lactosialyltetraose-tetrasaccharide	0	0.2
6'-Sialyl-lactose	0	0
3'-Sialyl-lactose	0.4	0
Free sugars, mg/g of DM	7.7	101.1
Arabinose	0	0.01
Fructose	0	0.49
Fucose	0.05	0.04
Galactose	6.05	35.01
Glucose	1.55	65.37
Total hydrolyzed monosaccharides, mg/g of DM	487.2	473.0
Fucose	0	0
Galactose	260.8	188.6
Glucose	223.3	281.4
Mannose	3.1	3.0
Total hydrolyzed minus free sugars, mg/g	479.5	371.9

¹CON = control milk replacer; GOS = galactooligosaccharide-supplemented milk replacer.

2010), no attempt was made to make MR iso-osmotic as we were interested in assessing the overall effects of the GOS milk formula. Milk replacers were fed twice daily (0500 and 1700 h) at a rate of 1.25% of BW daily on a DM basis for the first 2 d, 1.5% of BW daily until d 10, and then 2% of BW daily through d 42. From d 43 to 49, MR were fed at 1.5% of BW daily and from d 50 to 56 at 1% of initial BW only once daily. Calf DMI was adjusted individually on a BW basis each week. Calves were fed only the MR treatments from arrival to d 30 with no grain offered so that effects of GOS could be isolated. On d 31, starter grain (22.9% CP, DM basis) was introduced and calves were fully weaned at d 56. Calves had free access to clean water at all times and daily intake was recorded.

After allocation to treatments, any calves undergoing severe diarrhea and dehydration received electrolyte solution every 3 to 4 h, during which time milk feeding was restricted to half the daily amount until diarrhea receded, per veterinary staff direction. Amount of electrolyte consumption was not recorded. Dex-

trose present in electrolyte was not expected to affect hindgut fermentation (i.e., to interfere with GOS) as it is readily absorbed throughout the small intestine. Whether tubed electrolyte flowing into rumen affected the microbiota or their activity is unknown and was not able to be discerned from our measurements. Nevertheless, as described later, ruminal populations remained unstructured throughout the first 4 wk of life, indicating that not enough substrate, whether from MR or electrolyte, was present to induce bacterial community development.

Milk Replacer Sugar and Oligosaccharide Analysis

Carbohydrate analysis was performed by the laboratory of G. C. Fahey at the University of Illinois (Urbana-Champaign). Sugar and oligosaccharide analysis followed procedures described previously (Vester Boler et al., 2013). Free sugars and hydrolyzed monosaccharides were quantified using standards of sugars and monosaccharides in a Dionex ICS-3000 HPLC system with pulsed electrochemical detection (HPLC-PED, Dionex Corp., Sunnyvale, CA). Free monosaccharides were injected in a volume of 25 μ L. All assays were conducted using a CarboPac PA-1 (4×250 mm) column and guard column (4×50 mm) following methods cited by Smiricky et al. (2002). Briefly, an initial degassed mobile phase was 120 mM NaOH increasing linearly to 150 mM NaOH by 10 min and 240 mM by 20 min. At that time, the NaOH concentration was increased to 300 mM for 10 min to clean the column. The column was then re-equilibrated for 15 min with 120 mM NaOH. Flow rate was constant at 1.0 mL/min. Oligosaccharides were quantified using pure standards (V-Labs Inc., Covington, LA) and analyzed by Dionex HPLC-PED using the same methods. Only oligosaccharides for which we could obtain commercial standards and measure accurately are reported. As a result, not all peaks during the HPLC run could be identified and thus oligosaccharide recovery was not complete (i.e., <100%).

Animal Slaughter and Sampling

Eight calves per treatment were euthanized at each of 2 and 4 wk into the experiment (i.e., 32 calves total) to collect tissue and digesta samples. Calves from each treatment were selected for slaughter randomly from those healthy calves that had not received any antibiotic treatments. On each day of euthanasia, 16 calves were transported in groups of 4 to the Diagnostic Laboratory of the University of Illinois at 0430 h and remained unfed. Euthanasia was performed by administering an overdose of sodium pentobarbital intravenously (85 to

150 mg/kg of BW). The 4 calves per batch were simultaneously dissected by 4 teams of trained staff and total processing took approximately 45 to 60 min/calf. Processing of the 16 calves per day took approximately 5 to 6 h. To account for the effect of waiting time in unfed conditions, each group of 4 calves contained 2 calves from each treatment and the group was assigned a slaughter order number (i.e., 1 to 4). Therefore, given that the last meal took place at approximately 1700 h on the previous day, fasting time extended from the normal 12 h daily to as much as 18 h. The possibility existed that such prolonged fasting could have affected bacterial populations or intestinal function. Therefore, a multivariate statistical test was performed comparing the entire global bacterial community composition among treatment, age, slaughter order groups 1 to 4, and the interaction between slaughter order group and treatment. The test failed to reject the null hypothesis of no group difference and no interaction (data not presented), indicating that the short-term fast did not affect the bacterial community structure and this lack of effect did not depend on dietary treatments. Hence, if such fasting conditions did not affect calves differently between treatments, comparison of treatment effects would still be valid. Regardless, we caution readers about this potential limitation in interpreting our results.

After death, the thoracic cavity was opened; the GI tract was removed and ligated between omasum and abomasum, between abomasum and duodenum, and at the ileo-cecal junction as a precaution against postmortem digesta flow due to manipulation. Then, the forestomachs, small intestine, and large intestine were separated for digesta and tissue sample collection. Once samples were collected, all digesta was manually removed and the weight and length of GI organs were recorded.

Performance and Health

Growth data were collected from all 80 calves to wk 2, from the remaining 64 calves to wk 4 (after slaughter of the first 16-calf batch), and from the remaining 48 calves to wk 8 (after slaughter of the second 16-calf batch). Weekly growth measurements included BW, heart girth, body length, withers height, hip height, and hip width. Intakes of MR, water, and starter as well as fecal scores were measured and recorded daily. Reported treatment means for total water intake comprise water content from the milk powder (~5%, Table 1), water consumed with reconstituted MR, and free-choice water consumption. Rectal temperatures were measured twice daily during the first 3 d of the trial. No culture analysis for fecal pathogens was con-

ducted. Health measures included daily assessment of fecal scores using the following guidelines: 1 = dry and consistent; 2 = soft and pasty; 3 = pudding-like and runny but not sifting through bedding; and 4 = watery and sifting through bedding. Also, respiratory scores were recorded daily using the following guidelines: 1 = no coughing and no nasal discharge; 2 = occasional coughing, normal breathing, and minor nasal cloudy discharge; 3 = repeated coughing and bilateral nasal mucus discharge; and 4 = repeated coughing and excessive purulent bilateral nasal discharge.

In Vitro Nutrient Transport and Permeability

To assess intestinal tissue integrity and functionality, nutrient transport and para-cellular resistance (a proxy for intercellular tight junction proteins condition) were measured in modified Ussing chambers (Physiologic Instruments, San Diego, CA) following procedures described by Albin et al. (2007). Within 20 min of euthanasia, pieces about 6 cm² of dorsal rumen and 7 cm long of duodenum (approximately halfway between the duodenal bulb and the duodenojejunal flexure), jejunum (approximately 2 m distal to the duodenojejunal flexure), ileum (proximal of the ileocecal valve), and colon (middle transverse colon) were collected for measurement of nutrient transport and paracellular resistance. Samples were placed in oxygenated (95% O₂:5% CO₂) Krebs' solution (120 mM MgCl₂, 120 mM CaCl₂, 40 mM KH₂PO₄, 240 mM K₂HPO₄, 1.15 M NaCl, and 260 mM NaHCO₃) on ice and transported to the laboratory for immediate electrophysiological and ion transport analysis. Basal short-circuit current (I_{sc}; indicator of nonspecific active ion transport), resistance, and potential difference (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 mM solutions of D-glucose, L-glutamine, or L-arginine to the mucosal medium (Albin et al., 2007). Peak I_{sc} values (point of maximal change) were measured after each nutrient was added, and the difference between basal (measured before each nutrient addition) and peak values was calculated (Albin et al., 2007).

Histology

To assess GI epithelial development, histomorphology measurements of the epithelium were performed. Samples about 6 cm² of rumen (one sample from each of dorsal rumen, caudal ventral sac, and cranial ventral sac) and 7 cm long of duodenum, jejunum, and ileum (located adjacent to the samples collected for nutrient transport), and from middle ascending (approx-

mately halfway between ileocecal valve and the hepatic flexure), transverse, and descending (approximately halfway between the splenic and sigmoid flexure) colon were collected for histomorphology analysis. Tissues were fixed and maintained in 10% formalin solution for 72 h. Afterward, tissue was transferred and dehydrated with ethanol and embedded into paraffin blocks, from which 5- μ m-thick sections were cut and set onto glass slides. Before staining, wax was removed by rinsing in xylene followed by a posterior set of rinses with anhydrous and diluted alcohols. The sections were then stained with hematoxylin in a regressive manner: slides were submerged in hematoxylin for 2 to 4 min and then rinsed with deionized water and acid alcohol to remove unbound hematoxylin. Next, slides were successively submerged in deionized water and a bluing solution for 1 min each to adjust their pH, and then were counterstained with eosin Y for 1 to 3 min. After a final set of washes in alcohol and xylene to remove unbound eosin, the coverslip was applied (Feldman and Wolfe, 2014). The hematoxylin solution used contained 5 g/L of dye, 0.5 g/L of sodium iodate, and 50 g/L of aluminum sulfate, whereas the eosin solution contained 2 g/L of eosin Y dye and 1 g/L of phloxine.

Measurements of papillae length and width were made in the rumen sections. Villus length and width and crypt depth were measured in duodenum, jejunum, and ileum sections. Crypt depth and width were measured in colon sections. Ten measurements were recorded per section per calf per GI segment and their average was used for statistical analysis. Microscopy measurements were performed using digital images obtained with a Nanozoomer scanner and software (Hamamatsu, Japan) at the Institute for Genomic Biology at the University of Illinois.

Digesta SCFA Concentration

Digesta samples were collected from ventral ruminal and colonic (3 mL from middle transverse colon) contents and acidified with a 10 *N* HCl solution. Samples then were centrifuged at $15,000 \times g$ for 15 min at 4°C; the supernatant was recovered, frozen, and sent to the laboratory of V. Fellner (North Carolina State University, Raleigh) for SCFA analysis by GLC (Varian CP 3380/3800). The column, injection port, and detector temperatures were 150, 270, and 300°C, respectively. Helium was used as the carrier gas at a flow rate of 26 mL/min. Digesta sample supernatant (1 μ L) was injected into a column (Nukol fused silica capillary column, 30 m $\times$ 0.25 mm $\times$ 0.25 mm; Sigma-Aldrich, St. Louis, MO) in the splitless mode. A flame ionization detector was used to quantify SCFA (Herfel et al., 2013). L-Lactate was measured using an assay kit from

Eton Bioscience Inc. (San Diego, CA). Although some bacteria produce D-lactate, we did not measure this isomer, which renders our assessment incomplete.

Statistical analysis comparing molar proportions of SCFA among slaughter order groups 1 to 4, and the interaction between slaughter order group and treatment failed to reject the null hypotheses of no difference and no interaction (data not presented), thus reflecting the lack of change in bacterial community composition and suggesting that a short-term fast did not affect the relative activity of those communities as measured by SCFA concentrations.

Colon Digesta DM Percentage

To assess the concentration of luminal water in the hindgut as a proxy measure of water secretion driven by a fermentative osmotic shift that could result from carbohydrate fermentation, digesta samples from middle-ascending and middle-descending colon from each calf were dried in a forced-air oven at 80°C to constant weight (~5 d). Digesta DM% was calculated from the loss of weight during drying.

Bacterial Community Diversity and Structure

To assess compositional changes of the bacterial populations in response to prebiotic treatment across GI sites and calf ages, digesta samples from the ventral rumen and middle transverse colon of slaughtered calves were collected into 5-mL sterile cryovials and snap frozen in liquid N₂ to quickly stop microbial activity. Samples were stored at -20°C until DNA extraction. Samples were submitted to an external laboratory (Research and Testing Laboratory, Lubbock, TX) for bacterial tag-encoded FLX amplicon pyrosequencing.

Total microbial DNA was extracted using a MoBio PowerFecal kit (Mo Bio Laboratories Inc., Carlsbad, CA), which implements mechanical disruption (i.e., bead beating) and has been shown to be a good overall method for DNA extraction from digesta from diverse species (Hart et al., 2015). 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 (i.e., 27Fmod 5'-AGRGTTTGATCMTGGCTCAG-3' and 519Rmodbio 5'-GTNTTACNGCGCKGCTG-3') and the product sequence determined by bacterial tag-encoded FLX (non-titanium chemistry) amplicon pyrosequencing (Roche, Nutley, NJ) as reported previously (Dowd et al., 2008a,b). Afterward, sequences were depleted of barcodes and primers. Sequences that were too short (<200 bp), had ambiguous base calls, or had

homopolymer runs exceeding 6 bp 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) as illustrated elsewhere (Dowd et al., 2008a,b; Edgar, 2010; Capone et al., 2011; Swanson et al., 2011). The OTU 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 1,834 and a maximum of 16,400. The 25th and 75th percentile for the 32 samples were 4,328 and 7,511 quality reads per sample after filtering. Rarefaction curves indicated that our sampling depth yielded sufficient taxa coverage (not shown).

The Shannon index was calculated as a proxy measure of bacterial community 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 described below.

Statistical Analysis

Given the objective of this experiment to evaluate the effect of a GOS-rich MR and its interaction with GI site or calf age on measures of intestinal bacterial community structure, function, development, and calf growth, here we only (with a few exceptions) present and discuss the size of such effects, even though all main effects (treatment, age, GI site) and their interactions (treatment $\times$ age, treatment $\times$ GI site, age $\times$ GI site, and treatment $\times$ age $\times$ GI site) were included in the various statistical models. The results and biological relevance of age, GI site, and their interaction are presented and discussed in a complementary manuscript (Castro et al., 2016).

Bacterial Community Composition. A Bray-Curtis distance matrix to estimate the similarity among each pair of digesta samples originating from different age by treatment by GI tract site groups was built from the OTU or genus level matrix. Then, to visually explore the degree of bacterial composition similarity between samples, nonmetric multidimensional scaling (NMDS) was conducted on the OTU-based 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 bacterial community in a reduced number of dimensions (Borcard et al., 2011). Square root transformed

and nontransformed data provided similar NMDS, thus the latter was used throughout.

To contrast with the NMDS and to statistically test differences in bacterial community composition among treatment, GI site, and calf age groups, analysis of similarities (ANOSIM; Clarke, 1993) and permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) were performed on the same OTU-based Bray-Curtis distance matrix. The ANOSIM procedure statistically tests whether there is a difference between 2 or more groups of sampling units based on the difference of mean distance ranks computed from the Bray-Curtis matrix. The ANOSIM statistic R lies in the range from -1 to 1 . The statistic $R = 1$ if all replicates within a group are more similar to each other than any replicates from different groups, whereas R approaches 0 as similarities between and within different groups become the same on average. Finally, R is rarely less than 0 , but it would indicate possible mislabeling of samples as it implies that samples are more similar between than within groups. 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 sum 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 violation of the homogeneous variance assumption 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, bacterial communities in the colon presented statistically smaller variation (data not shown). Although this can be undesirable for the validity of a multivariate ANOVA test, it has been recently observed that, with balanced designs such as our study (i.e., nearly equal sample number in each treatment combination), heterogeneity is less of an issue for PERMANOVA (Anderson and Walsh, 2013).

Subsequently, based on results from PERMANOVA and ANOSIM indicating that bacterial community composition changes due to prebiotic treatment occurred in colon but not in rumen, similarity percentage analysis (SIMPER; Clarke, 1993) was performed using a genus level Bray-Curtis distance matrix built from colon sample data only, to identify which genera contributed most to the differences in bacterial populations observed between dietary treatment groups. 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 SIM-

PER methodological limitations (Warton et al., 2012), results are complemented with univariate tests as appropriate using the generalized linear model (GLM) framework (Fitzmaurice et al., 2011) to test statistical differences of the identified taxa among groups. The GLM approach also has the advantage of allowing quantification of the effect size of dietary treatment, age, or their interaction.

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

$$\eta_i = X_i' \beta,$$

where η_i is a linear function of the β unknown parameters and the covariates X corresponding to the i th observation, which include dietary treatment (CON, GOS), calf age (2 wk, 4 wk), and their interaction. Because the sequencing data are taxa counts and presented large over-dispersion 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 by zero and errors did not meet normality assumptions (Fitzmaurice et al., 2011). A log link function was used throughout. Maximum likelihood was used to estimate parameters and standard errors.

To aid interpretation of the coefficients in the log linear predictor, the above model for taxa counts at the genus level is expressed by taking the inverse of the natural log (i.e., the exponential), as

$$\frac{\text{Genus}_{ij} \text{ counts}}{\text{Total \# genera counts}} = e^{\beta_0} \times e^{\beta_1 \times X_i} \times e^{\beta_2 \times X_j} \times e^{\beta_3 \times X_i \times X_j}.$$

From here we estimated the expected number of counts of a specific taxa adjusted for the total sample number of sequence reads present in the colon of a calf at a particular age and treatment group. Thus, the exponentiated intercept (β_0) represents the relative taxa abundance at 2 wk of age in the colon, the exponentiated coefficient for X_i (β_1) is the fold change in GOS with respect to CON at 2 wk, the exponentiated coefficient for X_j (β_2) is the fold change in wk 4 relative to wk 2, and the exponentiated coefficient for X_{ij} (i.e., β_3 , the interaction term) is the adjustment to the GOS effect (X_i) for its dependence on calf age (X_j).

The NMDS and ANOSIM analyses were carried out in Primer-E software V.6.0. The SIMPER (Clarke and Gorley, 2006), PERMANOVA, and multivariate heterogeneity of variances test were conducted with the package Vegan (Oksanen et al., 2012). The GLM procedures were performed with the base package in the

open access R language (version 2.14.0, R Development Core Team, Vienna, Austria).

Growth Performance, Nutrient Intake, and Fecal Scores. Average weekly measurements of water and nutrient intake and body size were analyzed as repeated measures. All calves were included in the analysis to wk 2, 64 to 80 calves were included to wk 4 (80 to 2 wk and 64 to 4 wk), and only 48 calves were included to wk 8 because calves consuming grain were analyzed separately to quantify grain contributions to total DM, ME, and CP intakes. 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 and -2 ratio log-likelihood. Residuals were checked for homogeneity of variance and normality. Heterogeneous variances were common and an autoregressive function of first order allowing for heterogeneity alleviated the problem. Statistical difference 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 as the upper bound to perform group means comparisons.

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, Permeability, and Colonic Water. Except for histomorphology, these measurements were considered as spatially repeated measures on the same calf along its GI tract. The rationale was that intestinal measures performed on the same calf are more similar than measures performed among different calves, so we must account for these 2 levels of variability. As such, the same guidelines as those for growth performance were followed. Residual errors were in general normally distributed. Error correlation and variance heterogeneity were dealt with as explained above. Histomorphology measures were analyzed by GI site separately because dimensions from different GI site were considered different variables (e.g., length of intestinal villi is not comparable to length of rumen papillae).

RESULTS

Milk Replacer Composition

The composition of experimental MR is shown in Table 1. As planned, MR had similar concentrations of

Table 2. Permutational multivariate ANOVA for effect of prebiotic treatment, calf age, and gastrointestinal site on microbial community composition

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

¹Treatment = galactooligosaccharide-supplemented milk replacer vs. control milk replacer; week = 2 vs. 4; site = gastrointestinal site (i.e., rumen vs. colon).

²Sum of squared multivariate distances.

³Mean squared multivariate distances.

CP, fat, net energy, Ca, and P (Table 1). The difference 100 – CP – fat – ash indicated that CON and GOS contained similar total carbohydrate concentrations. As expected from treating the whey permeate with β -galactosidases, GOS MR contained lower concentrations of lactose (197.3 vs. 436.3 mg/g) and higher concentrations of total free sugars (101.1 vs. 7.6 mg/g), free galactose (35.0 vs. 6.0 mg/g), and free glucose (65.4 vs. 1.6 mg/g) compared with CON MR (Table 1). At the same time, GOS contained 33.5 mg/g (3.35% of DM) of GOS, whereas CON contained only 0.6 mg/g (0.06% of DM). The predominant oligosaccharides that were identified in GOS were 2'-fucosyl-lactose (3.7 mg/g) and lacto-*N*-fucopentanose (28.8 mg/g); lacto-*N*-tetraose and lactosialyltetraose-tetrasaccharide were present in very small amounts (<1 mg/g). Thus, the GOS formula must have contained at least 3.35% of galactosyl-oligosaccharides compared with at least 0.6% in CON.

The total hydrolyzed monosaccharide concentration, which is the result of acid hydrolysis and so measures the total carbohydrates in the MR, was similar between treatments. The difference between total hydrolyzed monosaccharides and free sugars grossly represents total bound monosaccharides; this number was higher in CON than in GOS (479.5 vs. 371.9 mg/g), reflecting the release of monosaccharides with β -galactosidase treatment.

Concentrations of galactose and glucose in the total hydrolyzed monosaccharide pool were different in CON and GOS MR (Table 1). In theory, assuming that lactose was the only carbohydrate present before β -galactosidase treatment, glucose and galactose contents should be similar. That they were not similar may be related to interconversion of galactose to glucose during enzymatic treatment or acid hydrolysis during analysis. This fact, along with the incomplete oligo-

saccharide recovery, results in inability to account for 100% of carbohydrate fractions.

Bacterial Diversity and Community Composition

Several findings were highlighted by the PERMANOVA analysis (Table 2). First, at 5% type 1 error rate, treatment had a significant effect ($P = 0.032$) on the average relative abundance of one or many of the several hundred organisms identified; however, its contribution to the total variation was small at 2.6%. This small effect is clearly reflected in the wide variation within treatments as shown in the NMDS for dietary treatments presented in Figure 1A, where hardly any difference between CON and GOS can be noticed.

According to PERMANOVA, the 2-way interactions of treatment with week or GI site also were not significant at 5% (Table 2; $P = 0.074$ and $P = 0.133$, respectively), suggesting the effect of prebiotic supplementation was largely independent from the effects of GI location and calf age. Figures 1B and 1C illustrate dissimilarity in the bacterial communities of 2- and 4-wk-old calves and rumen versus colon, respectively. At first sight, it is not readily apparent that the GOS and CON calves group differently within age or GI site groups (i.e., lack of dependence), supporting the nonsignificant PERMANOVA results for the 2-way interactions.

Nevertheless, the significance ($P = 0.016$) of the 3-way interaction among treatment, age, and GI site (Table 2) suggests that the dependence of the GOS effect on a particular GI site was itself dependent on the age of the calf. Yet, the variance explained by this interaction only accounted for 3.1% of the total variation. These significant yet seemingly small effects of such dependency will be dealt with in detail in the present manuscript.

Figure 1. Nonmetric multidimensional scaling ordination plot of digesta samples from control (CON) and galactooligosaccharide (GOS) milk replacer treatments, in rumen and colon, and at 2 and 4 wk of age (2 and 4 CON and 2 and 4 GOS, respectively). Samples that are more similar to one another are located closer together. Axis units and plot orientation are arbitrary. Stress values were approximately 0.18 in all 3 plots.

At the same time, from the PERMANOVA analysis (Table 2) it was readily apparent that GI site ($P = 0.001$; $R^2 = 0.244$), age ($P = 0.001$; $R^2 = 0.044$), and their interaction ($P = 0.006$; $R^2 = 0.04$) explained the greatest amount of the identifiable variation (i.e., the largest portion of the total sum of squares) in the GI bacterial community, with a cumulative R^2 of about 32.8% ($R^2 = 0.244 + 0.044 + 0.04 = 0.328$). The influence of these 2 major nondietary sources of variation on the development of the newborn calf is thoroughly explored in a companion manuscript (Castro et al., 2016).

Thus, to understand the previously mentioned 3-way interaction among prebiotic treatment, GI site, and calf age, ANOSIM was conducted to perform 1-, 2-, and 3-way multivariate pairwise comparisons among treatment groups at each age and GI site (Table 3). In contrast to PERMANOVA, ANOSIM deemed the difference between CON and GOS to be null with the multivariate test statistic R equal to zero and P -value of 1 (Table 3), which agrees with the visual appraisal from NMDS in Figure 1A. However, when the overall treatment effect is broken down into its effects in colon and rumen, ANOSIM indicated that, while no bacterial compositional difference existed between GOS and CON in rumen, there was a small (multivariate test statistic $R = 0.14$) but statistically significant difference in the colon ($P = 0.007$). This subtle difference can be seen in Figure 2A (same as Figure 1C but enhanced with ellipses as a visual aid) where ruminal and colonic bacterial communities are distinctly different, but whereas GOS and CON samples are not distinguishable in the rumen, they appear to differ in spread and location in the colon. When the 2-way comparisons were further broken into 2 and 4 wk (Table 3), the meaning of the 3-way interaction identified by PERMANOVA became readily apparent. Whereas no changes occurred in the rumen in response to GOS treatment at both 2 and 4 wk of age ($R = 0.04$ and $R = 0.05$, $P = 0.24$ and $P = 0.21$, respectively), the bacterial community composition of the colon differed more strongly between GOS and CON groups ($R = 47$, $P = 0.001$) at 2 wk of age, but these differences faded away by wk 4 of age ($R = 0.06$, $P = 0.13$). Because any treatment effects were completely absent in the rumen to this point, we decided to consider only the colonic populations hereafter to quantify the effect of prebiotic supplementation on the bacterial community assemblage. Figure 2B clearly shows that colonic bacterial populations exposed to GOS differ from CON, but only at 2 wk of age, suggesting a very narrow and specific time window of the prebiotic effect. Moreover, this plot visually suggests that the colonic bacterial populations of GOS and CON animals at 4 wk did not differ from those of GOS calves at 2 wk.

Table 3. One, 2, and 3-way pairwise analysis of similarities (ANOSIM) tests between control milk replacer (CON) and galactooligosaccharide-supplemented milk replacer (GOS) groups by gastrointestinal (GI) site and calf age

Treatment comparison	R ¹		P	
1 way: treatment CON vs. GOS	0.0		1.0	
	Colon		Rumen	
	R	P	R	P
2 way: treatment by GI site CON vs. GOS	0.14	0.007	0.0	0.46
3 way: treatment by GI site by age CON vs. GOS at 2 wk	0.47	0.001	0.04	0.24
CON vs. GOS at 4 wk	0.06	0.137	0.05	0.21

¹ANOSIM R test statistic = 1 when group difference is maximal, 0 when group difference is null.

Once we identified that the prebiotic effect occurred primarily over the first 2 wk and was localized to the colon we proceeded to identify the specific taxa responsible for such differences between GOS and CON. To that end, SIMPER analysis was performed at the genus level on the colon samples (Table 4). Out of 393 genera identified, just 8 of them accounted for ~80% of the dissimilarities between CON and GOS. Galactooligosaccharide-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%), *Fecalibacterium* (~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 cutoff threshold of 80% was set arbitrarily because the additional contribution of each individual taxon to the dissimilarity between treatments was less than 2% and over 100 genera represented the remaining 20%.

To assess whether the colonic relative abundance of these genera varied significantly due to calf age or prebiotic treatment, in an independent or interactive fashion, and to quantify such effects, negative binomial regression was performed to analyze the number of counts of DNA sequence reads corresponding to each taxon. Results are presented in Table 5. Estimated *Lactobacillus* relative abundance in the colon of CON calves at 2 wk of age was ~8% (i.e., intercept: $\exp^{(-2.51)} = 0.0809 \times 100 = \sim 8\%$). In the presence of GOS, this increased ($P < 0.0001$) by about 7.37-fold (i.e., $\exp^{(1.994)} = 7.37$). By wk 4, however, this genus increased its relative abundance by 8.68-fold in CON calves (i.e., $\exp^{(2.162)} = 8.68$) compared with wk 2 ($P < 0.0001$). The significant interaction ($P = 0.002$) explains how the GOS effect depended on calf age. Even though GOS-treated calves had numerically and statistically greater amounts of *Lactobacillus* in their colon at 2

wk of age, by 4 wk of age such differences ceased to exist and GOS-supplemented calves presented about 0.96 times (i.e., $\exp^{(1.994)} \times \exp^{(-2.034)} = 7.34 \times 0.13 = 0.96$) the relative abundance of this LAB with respect to 4-wk-old CON calves (Table 5, Figure 3). In other words, at 4 wk CON and GOS calves presented similar relative abundance of *Lactobacillus* in their colon. This finding supports the notion of a very narrow and specific time window of the prebiotic effect as suggested by Figure 2b.

In the case of *Bifidobacterium*, GOS-treated calves at 2 wk of age presented relative abundance approximately 8.9 times higher ($P = 0.02$) than those in CON calves. By 4 wk, however, the GOS effect on *Bifidobacterium* relative abundance went down to about 6% of the GOS effect size at 2 wk of age (i.e., $\exp^{(-2.822)} = 0.06$) as indicated by the significant interaction ($P = 0.03$; Table 5, Figure 3). Thus, the GOS effect on these 2 LAB was transient around the first 2 wk of life and faded away by wk 4 of life when the abundance of such commensal organisms increased sharply in CON calves to match those in the prebiotic-fed calves (Figure 3).

On the other hand, *Streptococcus* was not statistically affected by GOS treatment (Table 5, $P = 0.15$) even though it contributed 9% of the numeric dissimilarity between CON and GOS groups according to the SIMPER analysis. Instead, *Streptococcus* was only significantly affected by age, decreasing to 20% (i.e., $\exp^{(\text{wk } 4)} = 0.20$) of the initial relative abundance by 4 wk 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 (Table 5).

Furthermore, the increase in LAB (*Lactobacillus* + *Bifidobacterium*) in response to GOS supplementation occurred at the expense of *Fecalibacterium*, *Oscillospira*, and *Clostridium*, which were reduced to 1.5 ($P < 0.0001$), 4.1 ($P < 0.0001$), and 9.8% ($P = 0.002$), re-

Figure 2. Nonmetric multidimensional scaling ordination plot of samples from (A) colon and rumen for control (CON) and galactooligosaccharide-supplemented (GOS) milk replacers; (B) only samples from colon for CON and GOS at 2 and 4 wk. Samples that are more similar to one another are located closer together. Axis units and plot orientation are arbitrary. Stress values were approximately 0.18 in all 3 plots.

spectively, of the relative abundance in CON calves at 2 wk of age (Table 5; Figure 3). Similar to LAB, these 3 genera were present at similar relative abundance in CON and GOS by 4 wk of age (Figure 3).

In relation to the bacterial diversity, we observed no changes as measured by the Shannon index due to calf

age ($P = 0.34$), supplementation with GOS ($P = 0.24$), or their interaction ($P = 0.49$) (data not shown).

Overall, considering (1) that the total number of bacterial species and their evenness was not affected, (2) that out of 393 genera just 5 (*Lactobacillus*, *Bifidobacterium*, *Fecalibacterium*, *Oscillospira*, and *Clostridium*) constituted the most significant response to prebiotic treatment, (3) that LAB were ~8- to 9-fold more abundant in GOS than CON, and (4) that all effects were confined to the colon in the first 2 wk of life, it seems that the prebiotic effect of treated whey permeate on the bacterial community composition was very potent, but also anatomically specific and transient. This summation agrees with the low proportion of total variation explained by dietary treatment (Table 2).

Visceral Organ Size

Supplementation with GOS did not affect the average weight (GOS: 0.45 kg, CON: 0.44 kg; SE = 0.013, $P = 0.60$) or length (GOS: 3.87×10^2 cm, CON: 3.84×10^2 cm; SE = 0.12, $P = 0.90$) of the GI tract. In addition, no interaction was found between treatment and age ($P = 0.6$ for weight, $P = 0.8$ for length) or GI organ ($P = 0.8$ for weight, $P = 0.6$ for length). As expected, the size of the GI tract and individual portions increased with age (data not presented).

Colon Digesta DM Percentage

Colon digesta DM concentration was greater ($P = 0.0003$) by ~4 percentage units in CON compared with GOS calves (Table 6). This effect was independent of calf age ($P = 0.80$).

Table 4. Similarity percentage analysis for genera driving dissimilarity between galactooligosaccharide-supplemented milk replacer (GOS) and control milk replacer (CON) groups

Genus	Average relative abundance, %		Dissimilarity, ¹ %
	GOS	CON	
<i>Lactobacillus</i>	63.4	39.2	33
<i>Streptococcus</i>	5.67	9.80	9
<i>Fecalibacterium</i>	0.816	10.9	9
<i>Oscillospira</i>	1.15	8.24	7
<i>Bifidobacterium</i>	5.63	2.27	6
<i>Collinsella</i>	2.80	7.03	6
<i>Clostridium</i>	1.23	6.48	5
<i>Enterococcus</i>	4.08	3.02	4
Total			79

¹Contribution to overall dissimilarity between groups.

SCFA Concentration

The main effect of prebiotic treatment on SCFA concentration in the GI tract was significant (Table 6). Counterintuitively, calves receiving CON showed greater mean concentrations of total SCFA ($P = 0.04$), propionate ($P = 0.01$), and butyrate ($P = 0.001$) than calves receiving GOS; however, this effect depended on the GI site. Indeed, concentrations of total SCFA ($P = 0.0005$), acetate ($P = 0.003$), propionate ($P = 0.0005$), butyrate ($P = 0.0009$), and lactate ($P = 0.0004$) were greater for CON than GOS in the colon but not in the

rumen. This difference is likely explained by the low influx of fermentable matter into the rumen of calves fed only milk where the reticular groove is functional. Isobutyrate, valerate, and isovalerate did not differ between CON and GOS in rumen or colon.

Overall, in colon, concentrations of total SCFA, acetate, propionate, butyrate, and lactate were between ~66 and 240% greater for CON than for GOS (Table 6). Nevertheless, when SCFA concentrations were adjusted for colonic water concentration (i.e., expressed per unit of colonic DM), total SCFA ($P = 0.93$), acetate ($P = 0.6$), and propionate ($P = 0.5$) were not different

Table 5. Regression coefficients for effects of dietary treatment, calf age, and their interaction on relative abundance of identified genera¹

Effect	Coefficient (β)	SE	P	exp ⁽³⁾ 2
<i>Lactobacillus</i>				
Intercept	-2.515	0.327	<0.0001	0.0809
GOS	1.994	0.462	<0.0001	7.3417
Wk 4	2.162	0.462	<0.0001	8.6878
Wk 4 $\times$ GOS	-2.034	0.654	0.002	0.1308
<i>Bifidobacterium</i>				
Intercept	-4.544	0.656	<0.0001	0.01
GOS	2.183	0.926	0.02	8.87
Wk 4	1.184	0.926	0.2	
Wk 4 $\times$ GOS	-2.822	1.309	0.03	0.06
<i>Streptococcus</i>				
Intercept	-1.812	0.436	<0.0001	0.163
GOS	-0.889	0.617	0.15	
Wk 4	-1.605	0.618	0.01	0.201
Wk 4 $\times$ GOS	0.1583	0.873	0.16	
<i>Fecalibacterium</i>				
Intercept	-1.567	0.566	<0.0001	0.2086
GOS	-4.181	0.804	<0.0001	0.0153
Wk 4	-3.049	0.802	<0.0001	0.0474
Wk 4 $\times$ GOS	4.464	1.136	<0.0001	86.8183
<i>Oscillospira</i>				
Intercept	-2.020	0.566	<0.0001	0.1327
GOS	-3.186	0.802	<0.0001	0.0413
Wk 4	-1.417	0.800	0.07	0.2424
Wk 4 $\times$ GOS	2.582	1.133	0.02	13.2241
<i>Collinsella</i>				
Intercept	-2.590	0.668	<0.0001	0.075
GOS	-1.085	0.945	0.25	
Wk 4	-0.134	0.945	0.88	
Wk 4 $\times$ GOS	0.323	1.336	0.80	
<i>Clostridium</i>				
Intercept	-2.136	0.517	<0.0001	0.1181
GOS	-2.327	0.732	0.002	0.0976
Wk 4	-2.329	0.733	0.002	0.0974
Wk 4 $\times$ GOS	2.459	1.036	0.02	11.69
<i>Enterococcus</i>				
Intercept	-3.116	0.517	<0.0001	
GOS	0.544	0.731	0.42	
Wk 4	-1.014	0.732	0.17	
Wk 4 $\times$ GOS	-1.649	1.036	0.11	

¹n = 8 per treatment $\times$ week combination, 16 per week, and 16 per treatment. GOS = galactooligosaccharide-supplemented milk replacer.

²exp^(Intercept) represents expected taxa relative abundance for CON at 2 wk of age; exp^(GOS) represents fold change in taxa relative abundance with GOS treatment at 2 wk with respect to CON at 2 wk; exp^(wk 4) represents fold change in taxa relative abundance with CON treatment at 4 wk with respect to CON at 2 wk; exp^(GOS) $\times$ exp^(wk 4) $\times$ exp^(wk 4 $\times$ GOS) represents fold change in taxa relative abundance with GOS treatment at 4 wk with respect to CON at 2 wk.

Figure 3. Distribution of relative abundance of taxa driving dissimilarity between control (CON) and galactooligosaccharide-supplemented (GOS) milk replacer groups at 2 and 4 wk of age. CON.2 = CON at 2 wk; GOS.2 = GOS at 2 wk; CON.4 = CON at 4 wk; GOS.4 = GOS at 4 wk. *Faecalibacterium* relative abundance square root presented for ease of visualization. Relative abundance scale ranges from 0 to 1. Lower and upper most bars are maximum and minimum values, respectively, excluding outliers; upper and lower box sides are 25th and 75th percentiles and solid horizontal line is the median or 50th percentile.

Table 6. Least squares means $\pm$ SE for concentrations (mmol/L) of short-chain fatty acids (SCFA) and lactate in rumen and colon, and colonic digesta DM content (%)

Variable	CON ¹		GOS ¹		P-value		
	Rumen	Colon	Rumen	Colon	TRT ²	TRT $\times$ GI site	TRT $\times$ week
Total SCFA	34.86 $\pm$ 3.67 ^B	47.0 $\pm$ 3.27 ^A	34.86 $\pm$ 3.67 ^B	26.64 $\pm$ 3.27 ^C	0.04	0.0005	0.90
Acetate	25.10 $\pm$ 3.50 ^B	35.0 $\pm$ 2.40 ^A	29.8 $\pm$ 3.50 ^B	21.6 $\pm$ 2.4 ^C	0.40	0.003	0.90
Propionate	3.75 $\pm$ 0.34 ^B	8.2 $\pm$ 0.74 ^A	4.23 $\pm$ 0.34 ^B	3.6 $\pm$ 0.74 ^B	0.01	0.0005	0.30
Butyrate	0.30 $\pm$ 0.43 ^C	4.50 $\pm$ 0.40 ^A	0.54 $\pm$ 0.38 ^C	1.5 $\pm$ 0.47 ^B	0.001	0.0009	0.60
Isobutyrate	0.09 $\pm$ 0.20	0.30 $\pm$ 0.17	0.09 $\pm$ 0.20	0.8 $\pm$ 0.24	0.30	0.50	0.42
Valerate	0.02 $\pm$ 0.3	0.30 $\pm$ 0.22	0.02 $\pm$ 0.3	0.4 $\pm$ 0.23	0.60	0.90	0.30
Isovalerate	0.20 $\pm$ 0.16	0.22 $\pm$ 0.15	0.2 $\pm$ 0.14	0.25 $\pm$ 0.18	0.50	0.40	0.90
Lactate	0.13 $\pm$ 0.07 ^C	2.30 $\pm$ 0.31 ^A	0.21 $\pm$ 0.07 ^C	0.81 $\pm$ 0.31 ^B	0.10	0.0004	0.90
Digesta DM, ³ %	—	14.82 $\pm$ 0.86	—	10.45 $\pm$ 0.87	0.0003		
Adj. total SCFA ⁴	—	367.7 $\pm$ 45	—	362.7 $\pm$ 45	0.94		
Adj. acetate ³	—	265.6 $\pm$ 34	—	289.8 $\pm$ 34	0.61		
Adj. propionate ³	—	65.4 $\pm$ 9	—	57.5 $\pm$ 9	0.54		
Adj. butyrate ³	—	28.9 $\pm$ 5	—	14.4 $\pm$ 5	0.05		
Adj. lactate ³	—	24.2 $\pm$ 3	—	13.4 $\pm$ 3	0.01		

^{A-C}Within a row, means without a common superscript differ ($P < 0.05$).

¹CON = control milk replacer, GOS = galactooligosaccharide supplemented milk replacer. n = 8 per treatment $\times$ gastrointestinal site combination, 8 per treatment $\times$ week combination, 16 per week, 16 per treatment, and 32 per gastrointestinal site.

²TRT = dietary treatment effect (CON vs. GOS).

³Average of proximal and distal colon digesta DM. Adj. = adjusted.

⁴Colonic DM-adjusted SCFA and lactate concentrations [mmol/L/(DM/100)].

between CON and GOS, whereas the differences for butyrate ($P = 0.05$) and lactate ($P = 0.01$) remained significant but were smaller (Table 6).

Nutrient Transport and Permeability

There was no effect of treatment or its interaction with GIT location or age on epithelium paracellular resistance (Table 7). Similarly, from a functional standpoint, no effect of treatment or its interaction with GI site or age was detected on glucose, glutamine, or arginine transport (Table 7). Due to the lack of interactions, only marginal treatment means are shown.

Histology

With respect to the length of the villi in the duodenum and ileum (Table 8), we observed that the effect of prebiotic supplementation depended on calf age ($P = 0.048$ in duodenum, $P = 0.06$ in ileum). In CON calves, the length of duodenal villi decreased from wk 2 to 4, whereas in GOS animals it increased from wk 2 to 4. At the same time, villus length in jejunum ($P = 0.034$) and crypt depth in the medial colon ($P = 0.021$) were greater on average for GOS calves than CON calves at either age. Treatment, age, or their interaction did not affect any other histological measures in these GI sites

Table 7. Least squares means $\pm$ SE for epithelial resistance (Ω/cm^2) and change in short circuit current (Isc; Δ mv) in response to glucose, glutamine, or arginine addition to media

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

¹R = resistance measured in ohms (Ω). Δ mv = change in short circuit current in millivolts upon addition of respective nutrient. Surrogate measure of nutrient transport.

²TRT = dietary treatment effect (CON vs. GOS). CON = control milk replacer, GOS = galactooligosaccharide supplemented milk replacer. GI = gastrointestinal. n = 8 per treatment $\times$ GI site combination, 8 per treatment $\times$ week combination, 16 per week, 16 per treatment, and 32 per GI site.

Table 8. Least squares means for histomorphology measures from duodenum, jejunum, ileum, and colon at 2 and 4 wk of age

Measurement	Age × treatment ¹										P-value			
	2 wk					4 wk								
	CON	SE	GOS	SE	CON	SE	GOS	SE	CON	SE	GOS	SE	TRT ²	TRT × Wk
Duodenum														
Villus length, μm	498	45.1	426	52.1	434	41.1	496	37.5	456	22.7	461	24.1	0.87	0.048
Jejunum														
Villus length, μm	653	99.5	716	110.3	460	96.8	660	91.0	556	68.9	688	68.8	0.034	0.23
Ileum														
Villus length, μm	283	47.1	207	51.8	388	42.2	408	40.8	336	36.7	307	37.5	0.24	0.06
Medial colon														
Crypt depth, μm	461	34.3	498	34.3	403	32.4	464	32.4	432	30.2	481	30.2	0.021	0.54

¹CON = control milk replacer, GOS = galactooligosaccharide supplemented milk replacer.
²TRT = dietary treatment effect (CON vs. GOS). n = 8 per treatment × gastrointestinal site combination, 8 per treatment × week combination, 16 per week, 16 per treatment, and 32 per gastrointestinal site.

or in rumen, proximal colon, or distal colon (data not shown).

Intakes of DM, Nutrients, and Water

Average milk DMI was 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 interaction ($P = 0.005$) between dietary treatment and age up to 2 wk of age (Table 9). Otherwise, from 0 to 4 wk ($P = 0.016$) and 0 to 8 wk ($P = 0.0001$) of age, average milk DMI (Table 9) was consistently greater for CON than GOS and somewhat independent from calf age (i.e., treatment by week interaction to 4 and 8 wk, $P = 0.06$ and $P = 0.08$, respectively). Grain DMI as measured in the 48 calves remaining was not different between CON and GOS ($P = 0.99$) and was affected only by age, but total DMI was similar between treatments ($P = 0.75$). The lack of reflection of lower intake of MR for GOS in the total DMI resulted from the fact that starter intake introduced large variation for total DMI (SE = 0.39) compared with MR DMI (SE = 0.005). This highlights the relevance and meaning of statistical and biological significance being dependent on both effect size and sample size.

The difference in DMI resulted in a marginally lower daily ME intake for GOS calves to wk 2 ($P = 0.0006$) and wk 4 ($P = 0.005$), from which point the difference was no longer significant ($P = 0.65$; Table 9). Despite the difference in DMI from MR during the first 4 wk, mean daily CP intake did not differ between CON and GOS perhaps due to the fact that GOS MR was about 0.8 percentage units CP greater than CON. A significant treatment by age interaction (means not shown) to wk 2 indicated that treatment differences varied throughout time. Afterward, no difference was observed (Table 9). Average daily total water intake at 2, 4, or 8 wk was not affected by dietary treatment overall (Table 9).

Growth

Body growth was slightly although significantly affected by dietary treatment (Table 10). The CON diet consistently supported better performance than GOS treatment. The main effect of treatment was significant on BW, heart girth, body length, withers height, hip height, and hip width (all $P < 0.01$), whereas the interaction of treatment with time was only significant for body length ($P = 0.02$). The CON calves on average were 2.5 kg heavier ($P = 0.002$) and heart girth, body length, withers height, hip height, and hip width were about 1.3, 1.5, 0.8, 0.9, and 0.3 cm greater for CON than GOS. These differences between treatments are

Table 9. Least squares means $\pm$ SE for average feed, nutrient, and water intakes

Variable and age interval	N ¹	Treatment ¹		P-value	
		CON	GOS	TRT ³	TRT $\times$ week
Milk replacer DMI, kg/d					
0 to 2 wk	80	0.68 $\pm$ 0.003	0.66 $\pm$ 0.003	0.001	0.004
0 to 4 wk	80, 64	0.82 $\pm$ 0.005	0.79 $\pm$ 0.005	0.016	0.061
0 to 8 wk	48	0.91 $\pm$ 0.005	0.87 $\pm$ 0.005	0.0001	0.07
Grain DMI, kg/d					
4 to 8 wk	48	1.15 $\pm$ 0.44	1.15 $\pm$ 0.44	0.99	0.97
Total DMI, ⁴ kg/d					
4 to 8 wk	48	2.11 $\pm$ 0.39	2.09 $\pm$ 0.39	0.75	0.91
ME intake, ⁴ Mcal/d					
0 to 2 wk	80	2.92 $\pm$ 0.06	2.86 $\pm$ 0.06	0.0006	0.003
0 to 4 wk	80, 64	3.53 $\pm$ 0.06	3.42 $\pm$ 0.06	0.005	0.23
0 to 8 wk	48	8.13 $\pm$ 1.22	7.94 $\pm$ 1.22	0.65	0.87
CP intake, ⁴ kg/d					
0 to 2 wk	80	0.19 $\pm$ 0.004	0.19 $\pm$ 0.004	0.65	0.01
0 to 4 wk	80, 64	0.24 $\pm$ 0.004	0.24 $\pm$ 0.004	0.68	0.18
0 to 8 wk	48	0.52 $\pm$ 0.08	0.51 $\pm$ 0.08	0.77	0.96
Average daily water intake, ⁵ kg/d					
0 to 2 wk	80	7.47 $\pm$ 0.23	7.57 $\pm$ 0.23	0.56	0.89
0 to 4 wk	80, 64	8.32 $\pm$ 0.28	8.37 $\pm$ 0.28	0.62	0.56
0 to 8 wk	48	9.61 $\pm$ 0.16	9.66 $\pm$ 0.16	0.70	0.67

¹Sample size included all 80 calves in analysis from wk 0 to 2, 80 calves to wk 2 and 64 calves (80 – 16 slaughtered at wk 2) between wk 2 and 4 in analysis from wk 0 to 4, and only the 48 calves that received grain (80 – 32 slaughtered at wk 2 and 4) in analysis from wk 0 to 8 for milk DM, ME, CP, and water intake. For grain and total DMI, sample size included only the 48 calves that actually received starter feed between wk 4 and 8.

²CON = control milk replacer, GOS = galactooligosaccharide-supplemented milk replacer.

³Dietary treatment effect (CON vs. GOS).

⁴Total DM, ME, and CP intake includes both milk replacer and grain supplies.

⁵Total water intake consists of water content from the milk powder, water consumed with reconstituted milk replacer, and free choice water consumption.

small and probably of questionable importance under commercial conditions. The possibility of identifying such small differences indicates that the sample size employed was large enough to obtain very small standard errors.

Health

Table 11 shows that the odds of having a fecal score of 4 for GOS calves was 1.36 times that of CON calves ($P < 0.001$). Moreover, the number of days with fecal scores >2 was greater for the GOS treatment than CON at 2 ($P < 0.001$), 4 ($P < 0.001$), and 8 ($P < 0.001$) wk of age, when the CON group was exceeded by ~ 10 d by the GOS calves.

DISCUSSION

In the current experiment, feeding a GOS-rich MR potently boosted LAB such as *Lactobacillus* and *Bifidobacterium* in the colon at the expense of other genera such as *Fecalibacterium*, *Clostridium*, and *Oscillospira*. This prebiotic effect was characterized by LAB relative abundance ~ 8 to 9 times higher in GOS than in CON calves at 2 wk of age, which disappeared by 4 wk as the

CON calves reached the same profile as GOS calves. Therefore, as per definition, enzymatically treated whey permeate did indeed show prebiotic activity by allowing specific changes in the composition of GI bacterial communities (Gibson and Wang, 1994) in this study. *Bifidobacterium* 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 MR from whey permeate treated with β -galactosidases might provide a means to support calf intestinal health during the first weeks of life.

Before further discussion, however, some caveats must be made. Calves fed the GOS MR had slightly less DMI from MR (20 to 40 g/d) and final BW and stature measurements were lower. The lower DMI in the GOS treatment may be the result of more than one cause. Most likely, the lower amount (approximately 50% less) of readily digestible lactose in the GOS MR resulted in a shortage of digestible energy to support lean tissue growth. Because MR allowance was updated weekly on a BW basis, the marginally slower growing GOS calves began to receive an increasingly smaller supply of MR powder. On average, GOS calves were

2.5 kg of BW less than CON calves, which at a feeding rate of, for example, 1.5% of BW as DM, results in the MR DM offered being about 37 g/d less, thus seemingly accounting for most of the actual observed DMI difference between MR treatments. However, the possibility exists that the marginally lower MR DMI in GOS calves was partly attributable to more days with high fecal scores on which intestinally upset animals may have had reduced appetite. On days with severe diarrhea and dehydration, milk offered was decreased by half and electrolyte therapy was administered. Unfortunately, our records of electrolyte consumption were incomplete, and therefore, we cannot definitively determine whether treatments differed in total amounts of electrolyte administered. Overall, however, we do not think it likely that electrolyte administration affected our results for bacterial community composition, but the potential effects cannot be entirely discounted.

The sharp increase in *Lactobacillus* relative abundance to almost 60% of the colonic bacterial population

by wk 4 of life in nonprebiotic-fed calves is remarkable. This observation contrasts strongly with observations in young calves where other genera such as *Bacteroides*, *Prevotella*, or *Fecalibacterium* were fairly abundant at this age (Edrington et al., 2012; Li et al., 2012; Jami et al., 2013; Malmuthuge et al., 2014). We speculate that the high MR intakes employed in this trial may have led to an increased flux of lactose into the hind gut, which in itself could provide a prebiotic effect. This hypothesis warrants future testing.

Unexpectedly, the increase in LAB resulting from GOS supplementation was not reflected in the fermentation profile as CON calves presented much larger concentrations of total SCFA, acetate, propionate, butyrate, and lactate in the colon compared with GOS calves, which might suggest an enhanced fermentative state in the distal bowel of CON animals. Along with lower SCFA concentrations, calves fed GOS exhibited 136% greater odds of presenting high fecal scores and spent a total of approximately 10 more days with fe-

Table 10. Least squares means $\pm$ SE for body measurements

Variable	Treatment ¹		N ²	P-value ³	
	CON	GOS		TRT	TRT $\times$ week
BW, kg					
Overall (1 to 8 wk)	62.9 $\pm$ 1.9	60.4 $\pm$ 1.9	80	0.002	0.60
2 wk	48.4 $\pm$ 2	46.8 $\pm$ 2	80	0.91	
4 wk	59.9 $\pm$ 2	57.0 $\pm$ 2	64	0.12	
8 wk	81.2 $\pm$ 2	78.6 $\pm$ 2	48	0.53	
Heart girth, cm					
Overall (1 to 8 wk)	90.7 $\pm$ 0.7	89.4 $\pm$ 0.7	80	<0.0001	0.12
2 wk	83.3 $\pm$ 0.8	82.1 $\pm$ 0.8	80	0.43	
4 wk	89.8 $\pm$ 0.8	87.8 $\pm$ 0.8	64	0.07	
8 wk	100.1 $\pm$ 0.8	98.4 $\pm$ 0.8	48	0.48	
Body length, cm					
Overall (1 to 8 wk)	68.7 $\pm$ 0.5	67.2 $\pm$ 0.5	80	<0.0001	0.02
2 wk	64.0 $\pm$ 0.6	63.6 $\pm$ 0.6	80	0.90	
4 wk	67.3 $\pm$ 0.6	65.7 $\pm$ 0.6	64	0.20	
8 wk	75.3 $\pm$ 0.6	73.1 $\pm$ 0.6	48	0.02	
Withers height, cm					
Overall (1 to 8 wk)	84.0 $\pm$ 0.5	83.2 $\pm$ 0.5	80	0.001	0.10
2 wk	80.5 $\pm$ 0.5	80.3 $\pm$ 0.5	80	0.60	
4 wk	82.7 $\pm$ 0.5	81.2 $\pm$ 0.5	64	0.01	
8 wk	89.4 $\pm$ 0.6	88.2 $\pm$ 0.6	48	0.005	
Hip height, cm					
Overall (1 to 8 wk)	88.3 $\pm$ 0.3	87.4 $\pm$ 0.3	80	0.005	0.65
2 wk	84.5 $\pm$ 0.3	83.4 $\pm$ 0.3	80	0.97	
4 wk	87.1 $\pm$ 0.4	86.2 $\pm$ 0.4	64	0.70	
8 wk	93.9 $\pm$ 0.4	92.6 $\pm$ 0.4	48	0.40	
Hip width, cm					
Overall (1 to 8 wk)	19.2 $\pm$ 0.07	18.9 $\pm$ 0.07	80	0.009	0.5
2 wk	17.6 $\pm$ 0.09	17.5 $\pm$ 0.09	80	0.90	
4 wk	18.6 $\pm$ 0.1	18.3 $\pm$ 0.1	64	0.39	
8 wk	21.2 $\pm$ 0.1	20.8 $\pm$ 0.1	48	0.60	

¹CON = control milk replacer; GOS = galactooligosaccharide-supplemented milk replacer.

²Sample size included all 80 calves in repeated measures analysis from wk 1 to 8; 80 calves for analysis of wk 2 data, 64 calves (80 – 16 slaughtered at wk 2) for analysis of wk 4 data and 48 calves for analysis of wk 8 data, in week-specific contrasts. Week effect was significant in all cases but is not shown.

³Probability from ANOVA for main effects of treatment (TRT) and the interaction of treatment by week including data from wk 1 to 8. The effect of week was significant for all variables but is not shown.

cal scores >2 over the 8-wk experiment. The challenge, therefore, is to reconcile the higher intake of nondigestible but fermentable oligosaccharides (i.e., GOS) and the greater abundance of LAB in the hindgut with the lower colonic SCFA and overall diarrhea-like condition of prebiotic-supplemented calves.

Carbohydrate malabsorption, even from fermentable sources, can cause osmotic diarrhea to different extents as colonic fermentation progressively exceeds the absorptive capacity of the epithelium for SCFA (Binder, 2010). Indeed, excess nondigestible fermentable carbohydrates arriving into the colon are fermented to SCFA plus CO_2 and H^+ . The SCFA are normally absorbed by the colonocytes, but when their production rate exceeds the absorption rate, the luminal accumulation of SCFA and H^+ causes colonic pH to decrease and water to be secreted into the lumen, leading to diarrhea (Binder, 2010). At an approximate BW of 55 kg, with a DMI of 2% on a BW basis, and with a GOS concentration of $\sim 3.4\%$, the average GOS consumption by supplemented calves must have been about 39 g/d as compared with ~ 1 g/d for CON calves. In humans, inulin oligosaccharides, fructooligosaccharides, 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 was ingested by healthy adults, defecation frequency and flatulence increased (Teuri, 1998). Similarly, when healthy adults were dosed with 10 g/d of GOS, def-

ecation frequency, feces softness, bowel movement, and flatulence 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 was supplemented to human adults, the daily ingestion of 10 g increased the sensation of fullness, and flatulence and abdominal pain had a tendency to increase (Ito et al., 1990). Although BW was not reported in these studies, it is unlikely that an average human adult weighs less than a newborn calf. On a per kilogram BW basis, GOS intake by our GOS calves would have been ~ 4 times higher than in adult human subjects with a hypothetical BW of 55 kg (i.e., the same as an average experimental calf) used in the aforementioned studies.

Based on these reports, we speculate that a reversed osmotic gradient with a concomitant increase in luminal water content in the colon provides an explanation for the lower SCFA concentrations and diarrhea-like condition in GOS-fed calves. Our colonic digesta DM data support this speculation with GOS colonic samples containing ~ 4 percentage units more water than CON. In fact, after correction for colonic digesta DM, colonic concentrations of total SCFA, acetate, and propionate were not different between GOS and CON, and whereas butyrate and lactate differences remained significant, they were smaller. This hypothesis could be tested by feeding large amounts of oligosaccharides to boost colonic fermentation and orally supplying an isotopically labeled oligosaccharide to measure SCFA appearance

Table 11. Effect of milk replacer enriched with galactooligosaccharides on the odds of presenting fecal scores of 4 (watery feces) and number of days with scores >2 (runny and diarrhea-like feces) in calves

Treatment comparison	Logistic estimate of odds to present fecal scores of 4			
	Log odds ratio	SE	Odds ratio ¹	P
GOS vs. CON ²	0.86	0.153	2.36	<0.001
Treatment and age ³	Poisson estimate for days with fecal scores >2			
	Log (d >2)	SE	No. of days >2 ⁴	P
2 wk (n = 80)				
CON	2.49	0.021	12.1	0.0002
GOS	2.60	0.021	13.5	
4 wk (n = 64)				
CON	2.93	0.031	18.7	<0.001
GOS	3.17	0.030	23.8	
8 wk (n = 48)				
CON	3.3	0.044	27.1	<0.001
GOS	3.6	0.040	36.6	

¹Exponentiation of the log (odds ratio).

²CON = control milk replacer; GOS = galactooligosaccharide-supplemented milk replacer.

³n = 48 nonslaughtered calves used in logistic regression to avoid using all 32 nonrandomly missing observations (i.e., slaughtered calves at 2 and 4 wk). Separate analyses were made for number of days with high fecal scores (>2) with n = 80, 64, and 48 for wk 2, 4, and 8, respectively, to fairly compare only animals with the same life span.

⁴Exponentiation of the log (number of days with fecal scores >2).

rates in blood (Kien et al., 1996). In conjunction, body water could be labeled with deuterium to steady state and its fecal excretion subsequent to a fermentation surge could be determined (Shields et al., 1968).

Another measure that supports the idea of osmotic rather than infectious diarrhea is the similarity in the epithelium colonic resistance between CON and GOS. Animals infected with pathogens such as enterohemorrhagic *Escherichia coli* or enteropathogenic *E. coli* 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 GI tract, no statistically significant differences were found between GOS-supplemented and CON calves, suggesting that the increase in luminal water for GOS calves did not result from leak flux due to pathogenic disruption of tight junctions. Two more direct measures that could have provided additional information, colonic luminal pH and fecal pathogen shedding, were not measured in our experiment.

On the other hand, GOS calves presented longer villi in the duodenum, jejunum, and ileum from 2 to 4 wk of age, and deeper crypts in the colon compared with CON calves. 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 such as glucagon-like peptide 2 (Cani et al., 2009), which can act in a paracrine or endocrine way to elicit a powerful systemic trophic effect on the mucosa throughout proximal and distal gut (Burrin et al., 2003). Although in this case GOS-supplemented calves presented smaller luminal SCFA concentrations, it is important to keep in mind 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 calves.

Regarding functionality, the transport of glucose, glutamine (i.e., neutral AA), and arginine (i.e., basic amino acid) was unaffected by GOS supplementation, indicating that these nutrient transport systems were indifferent to prebiotic mediated enhancement of LAB populations. At the animal level, we observed only slightly lower growth in GOS calves. This could have been due to a ~20 percentage unit lower dietary lactose content. Because feeding rates were individually adjusted weekly according to BW, the slower growing GOS calves received less feed than CON as reflected in an average reduction of ~90 kcal/d of milk ME intake

by wk 4 of life. However, these differences in body size were small and likely of little commercial relevance.

Reported research with mannan and fructan-oligosaccharides in calves has often (Heinrichs et al., 2003; Van Loo and Vancraeynest, 2008; Ghosh and Mehla, 2012) 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, the much lower dosage used (~4 g/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 nondigestible oligosaccharides, but also lactose and protein that enter colonic fermentation and ultimately affect 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 mixture (4.9 g/d) containing mainly $\beta 1 \rightarrow 4$ linkages with some $\beta 1 \rightarrow 6$ linkages, after 1 wk of intake by healthy humans (Depeint et al., 2008).

Therefore, 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 build a reliable response surface. Although potent enrichment of LAB during the first 2 wk of life of dairy calves might occur and have a positive effect 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, decreased growth, or laxative effects.

CONCLUSIONS

Substitution of untreated for enzymatically treated whey permeate as a source of GOS resulted in potent growth promotion of LAB in the colon during the first 2 wk of age. This was accompanied by greater development of epithelial structures in small and large intestine. However, supplementation also resulted in lower intestinal SCFA concentrations and increased fecal scores. A possible reason for this would be an over-flux of GOS into the large bowel exceeding the fermentative capacity of the microbiota or the ability to absorb SCFA by this organ, resulting in water secretion. In addition, slightly lower nutrient intake and growth were observed

in GOS-supplemented calves. Taking advantage of enzymatically treated whey permeate as a source of GOS to improve intestinal health in preweaning calves will require optimization of supplementation amounts and MR feeding rates to maximize LAB populations, fermentation, and calf health responses while maintaining calf growth.

ACKNOWLEDGMENTS

Supported by Milk Specialties Global (Eden Prairie, MN) and State of Illinois and USDA-CREES funds appropriated to the Illinois Agricultural Experiment Station. The authors thank K. A. Tappenden (Department of Food Science and Human Nutrition, University of Illinois, Urbana) for providing materials, expertise, and personnel support in measurement of gut nutrient transport, barrier function, and histology. The technical help of the many graduate and undergraduate students in the Department of Animal Sciences (University of Illinois) in care of calves, sampling, and slaughter processing of calves was indispensable to success of the project. The authors appreciate the partial financial support and MR manufacturing providing by Milk Specialties Global Animal Nutrition (Eden Prairie, MN). The authors thank Land O'Lakes Animal Milk Products (Arden Hills, MN) for donation of the electrolyte products.

REFERENCES

- Albin, D. M., J. E. Wubben, J. M. Rowlett, K. A. Tappenden, and R. A. Nowak. 2007. Changes in small intestinal nutrient transport and barrier function after lipopolysaccharide exposure in two pig breeds. *J. Anim. Sci.* 85:2517–2523.
- Anderson, M. J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26:32–46.
- Anderson, M. J. 2006. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* 62:245–253.
- Anderson, M. J., and D. C. I. Walsh. 2013. PERMANOVA, ANOSIM, and the mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecol. Monogr.* 83:557–574.
- Angus, F., S. Smart, and C. Shortt. 2005. Prebiotic ingredients with emphasis on galacto-oligosaccharides and fructo-oligosaccharides. Pages 120–137 in *Probiotic Dairy Products*. A. Tamime, ed. Blackwell Publishing Ltd., Oxford, UK.
- Bartholome, A. L., D. M. Albin, D. H. Baker, J. J. Holst, and K. A. Tappenden. 2004. Supplementation of total parenteral nutrition with butyrate acutely increases structural aspects of intestinal adaptation after an 80% jejunoileal resection in neonatal piglets. *J. Parenter. Enteral Nutr.* 28:210–222.
- Binder, H. J. 2010. Role of colonic short-chain fatty acid transport in diarrhea. *Annu. Rev. Physiol.* 72:297–313.
- Borcard, D., F. Gillet, and P. Legendre. 2011. *Numerical Ecology with R*. 1st ed. Springer, New York, NY.
- Burrin, D. G., B. Stoll, and X. Guan. 2003. Glucagon-like peptide 2 function in domestic animals. *Domest. Anim. Endocrinol.* 24:103–122.
- Cani, P. D., S. Possemiers, T. Van de Wiele, Y. Guiot, A. Everard, O. Rottier, L. Geurts, D. Naslain, A. M. Neyrinck, D. M. Lambert, G. G. Muccioli, and N. M. Delzenne. 2009. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* 58:1091–1103.
- Capone, K. A., S. E. Dowd, G. N. Stamatas, and J. Nikolovski. 2011. Diversity of the human skin microbiome early in life. *J. Invest. Dermatol.* 131:2026–2032.
- Castro, J. J., A. Gomez, B. A. White, H. R. Mangian, J. R. Lofton, and J. K. Drackley. 2016. Changes in the intestinal bacterial community, short-chain fatty acid profile, and intestinal development of preweaned Holstein calves. 2. Effects of gastrointestinal site and age. *J. Dairy Sci.* 99:9703–9715. <http://dx.doi.org/10.3168/jds.2016-11006>.
- Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* 18:117–143.
- Clarke, K. R., and R. N. Gorley. 2006. *PRIMER v6: User Manual/Tutorial*. PRIMER-E, Plymouth. Massey University, Auckland, New Zealand.
- Davis, C. L., and J. K. Drackley. 1998. *The Development, Nutrition, and Management of the Young Calf*. 1st ed. Iowa State Univ. Press, Ames.
- Deguchi, Y., K. Matsumoto, A. Ito, and M. Watanuki. 1997. Effects of B 1–4 galactooligosaccharides administration on defecation of healthy volunteers with constipation tendency. *Jpn. J. Nutr.* 55:13.
- Depeint, F., G. Tzortzis, J. Vulevic, and G. Gibson. 2008. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* NCIMB 41171, in healthy humans: A randomized, double-blind, crossover, placebo-controlled intervention study. *Am. J. Clin. Nutr.* 87:785–791.
- DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72:5069–5072.
- Dowd, S. E., T. Callaway, R. Wolcott, Y. Sun, T. McKeehan, R. Hagevoort, and T. Edrington. 2008b. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiol.* 8:125.
- Dowd, S. E., Y. Sun, R. D. Wolcott, A. Domingo, and J. A. Carroll. 2008a. Bacterial Tag-Encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: Bacterial diversity in the ileum of newly weaned *Salmonella*-infected pigs. *Foodborne Pathog. Dis.* 5:459–472.
- Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461.
- Edrington, T. S., S. E. Dowd, R. F. Farrow, G. R. Hagevoort, T. R. Callaway, R. C. Anderson, and D. J. Nisbet. 2012. Development of colonic microflora as assessed by pyrosequencing in dairy calves fed waste milk. *J. Dairy Sci.* 95:4519–4525.
- Feldman, A., and D. Wolfe. 2014. Tissue processing and hematoxylin and eosin staining. Pages 31–43 in *Histopathology: Methods and Protocols/Methods in Molecular Biology*, vol. 1180. C. E. Day, ed. Springer-Verlag, New York, NY.
- Fitzmaurice, G. M., N. M. Laird, and J. H. Ware, ed. 2011. *Applied Longitudinal Analysis*. 2nd ed. John Wiley & Sons, Hoboken, NJ.
- Frank, A. 2008. Food applications of prebiotics. Pages 437–446 in *Handbook of Prebiotics*. G. R. Gibson, ed. CRC/Taylor and Francis Group, Boca Raton, FL.
- Ghosh, S., and R. K. Mehla. 2012. Influence of dietary supplementation of prebiotics (mannanoligosaccharide) on the performance of crossbred calves. *Trop. Anim. Health Prod.* 44:617–622.
- Gibson, G. R., H. Probert, J. Van Loo, R. Rastall, and M. Roberfroid. 2004. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutr. Res. Rev.* 17:259–275.
- Gibson, G. R., and X. Wang. 1994. Regulatory effects of *Bifidobacterium* on the growth of other colonic bacteria. *J. Appl. Bacteriol.* 77:412–420.
- Guttman, J., Y. Li, M. Wickham, W. Deng, and A. Vogl. 2006. Attaching and effacing pathogen-induced tight junction disruption in vivo. *Cell. Microbiol.* 8:634–645.
- Hart, M., A. Meyer, P. Johnson, and A. Ericsson. 2015. Comparative evaluation of DNA extraction methods from feces of multiple host

- species for downstream next-generation sequencing. *PLoS One* 10:e0143334.
- Heinrichs, A. J., C. M. Jones, J. A. Elizondo-Salazar, and S. J. Terrill. 2009. Effects of a prebiotic supplement on health of neonatal dairy calves. *Livest. Sci.* 125:149–154.
- Heinrichs, A. J., C. M. Jones, and B. Heinrichs. 2003. Effects of mannan oligosaccharide or antibiotics in neonatal diets on health and growth of dairy calves. *J. Dairy Sci.* 86:4064–4069.
- Herfel, T., S. Jacobi, X. Lin, E. van Heugten, V. Fellner, and J. Odle. 2013. Stabilized rice bran improves weaning pig performance via a prebiotic mechanism. *J. Anim. Sci.* 91:907–913.
- Ito, M., Y. Deguchi, A. Miyamori, K. Matsumoto, H. Kikuchi, K. Matsumoto, Y. Kobayashi, T. Yajima, and T. Kan. 1990. Effects of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. *Microb. Ecol. Health Dis.* 3:285–292.
- Jami, E., A. Israel, A. Kotser, and I. Mizrahi. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7:1069–1079.
- Johnson-Henry, K. C., K. A. Donato, G. Shen-Tu, M. Gordanpour, and P. M. Sherman. 2008. *Lactobacillus rhamnosus* strain GG prevents enterohemorrhagic *Escherichia coli* O157:H7-induced changes in epithelial barrier function. *Infect. Immun.* 76:1340–1348.
- Kien, C., R. Murray, A. Ailabouni, P. Powers, J. Kepner, L. Powers, and H. Brunengraber. 1996. Stable isotope model for assessing production of short chain fatty acids from colon-derived sugar: Application in pigs. *J. Nutr.* 126:3069–3076.
- Kleessen, B., L. Hartmann, and M. Blaut. 2003. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated *Bifidobacteria* in gnotobiotic rats. *Br. J. Nutr.* 89:597–606.
- Li, R. W., E. E. Connor, C. Li, R. L. Baldwin VI, and M. E. Sparks. 2012. Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. *Environ. Microbiol.* 14:129–139.
- Macfarlane, G. T., H. Steed, and S. Macfarlane. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J. Appl. Microbiol.* 104:305–344.
- Malmuthuge, N., P. J. Griebel, and L. L. Guan. 2014. Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tract of pre-weaned calves. *Appl. Environ. Microbiol.* 80:2021–2028.
- Martin, R., A. J. Nauta, K. Ben Amor, L. M. J. Knippels, J. Knol, and J. Garssen. 2010. Early life: Gut microbiota and immune development in infancy. *Benef. Microbes* 1:367–382.
- NAHMS. 1996. National Animal Health Monitoring System. Part 1: Reference of 1996 dairy management practices. USDA:APHIS:VS, CEAH, Fort Collins, CO.
- NAHMS. 2010. National Animal Health Monitoring System. Heifer calf health and management practices on U.S. dairy operations in 2007. USDA:APHIS:VS, CEAH, Fort Collins, CO.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.
- Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. Stevens, and H. Wagner. 2012. Vegan: Community ecology package. R package version 2.0–5. <http://CRAN.R-project.org/package=vegan>.
- Parassol, N., M. Freitas, K. Thoreux, G. Dalmasso, R. Bourdet-Sicard, and P. Rampal. 2005. *Lactobacillus casei* DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic *Escherichia coli*-infected T84 cells. *Res. Microbiol.* 156:256–262.
- Shields, R., J. Miles, and C. Gilbertson. 1968. Absorption and secretion of water and electrolytes by the intact colon in a patient with primary aldosteronism. *BMJ* 1:93–96.
- Smiricky, M. R., C. M. Grieshop, D. M. Albin, J. E. Wubben, V. M. Gabert, and G. C. Fahey. 2002. The influence of soy oligosaccharides on apparent and true ileal amino acid digestibilities and fecal consistency in growing pigs. *J. Anim. Sci.* 80:2433–2441.
- Smiricky-Tjardes, M. R., C. M. Grieshop, E. A. Flickinger, L. L. Bauer, and G. C. Fahey. 2003. Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations, and ileal fermentative characteristics. *J. Anim. Sci.* 81:2535–2545.
- Svensson, C., K. Lundborg, U. Emanuelson, and S. Olsson. 2003. Morbidity in Swedish dairy calves from birth to 90 days of age and individual calf-level risk factors for infectious diseases. *Prev. Vet. Med.* 58:179–197.
- Swanson, K. S., S. E. Dowd, J. S. Suchodolski, I. S. Middelbos, B. M. Vester, K. A. Barry, K. E. Nelson, M. Torralba, B. Henrissat, P. M. Coutinho, I. K. O. Cann, B. A. White, and G. C. Fahey. 2011. Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *ISME J.* 5:639–649.
- Teuri, U. 1998. Increased fecal frequency and gastrointestinal symptoms following ingestion of galacto-oligosaccharide-containing yogurt. *J. Nutr. Sci. Vitaminol. (Tokyo)* 44:465–471.
- Torres, D. P. M., J. Teixeira, and L. Rodrigues. 2010. Galacto-oligosaccharides: Production, properties, applications, and significance as prebiotics. *Compr. Rev. Food Sci. Food Safety* 9:438–454.
- Tuomisto, H. 2010. A consistent terminology for quantifying species diversity? Yes, it does exist. *Oecologia* 164:853–860.
- Urashima, T., M. Kitaoka, S. Asakuma, and M. Messer. 2009. Chapter 8. Pages 295–349 in *Milk Oligosaccharides*. Collection: Advanced Dairy Chemistry/Volume 3: Lactose, Water, Salts and Minor Constituents. P. L. H. McSweeney and P. F. Fox, ed. Springer-Verlag, New York, NY.
- Van Loo, J. V., and D. Vancraeynest. 2008. Prebiotics and animal nutrition. Pages 421–431 in *Handbook of Prebiotics*. G. R. Gibson, ed. CRC/Taylor and Francis Group, Boca Raton, FL.
- Vester Boler, B. M., M. C. Rossoni Serao, F. A. Trevor, L. L. Bauer, J. M. Chow, M. R. Murphy, and G. C. Fahey. 2013. In vitro fermentation characteristics of select nondigestible oligosaccharides by infant fecal inocula. *J. Agric. Food Chem.* 61:2109–2119.
- Vizoso Pinto, M. G., M. Rodriguez Gómez, S. Seifert, B. Watzl, W. H. Holzapfel, and C. M. A. P. Franz. 2009. Lactobacilli stimulate the innate immune response and modulate the TLR expression of HT29 intestinal epithelial cells in vitro. *Int. J. Food Microbiol.* 133:86–93.
- Warton, D. I., S. T. Wright, and Y. Wang. 2012. Distance-based multivariate analyses confound location and dispersion effects. *Methods Ecol. Evol.* 3:89–101.
- Yamazaki, S., K. Machii, S. Tsuyuki, H. Momose, T. Kawashima, and K. Ueda. 1985. Immunological responses to monoassociated *Bifidobacterium longum* and their relation to prevention of bacterial infection. *Immunology* 56:43–50.