EFFICACY OF BUTYRATE-PRODUCING THERAPIES FOR USE IN PEDIATRIC
INTESTINAL FAILURE

BY

JENNIFER LYNN BARNES

DISSERTATION

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Doctoral Committee:

      Professor Emeritus George C. Fahey Jr., Chair
      Professor Kelly A. Tappenden, Director of Research
      Assistant Professor Michael J. Miller
      Assistant Professor Margarita Teran-Garcia

ABSTRACT

In 2011, preterm births accounted for approximately 12% of all births in the United States (1). Preterm infants have an immature gastrointestinal tract and are at increased risk of developing necrotizing enterocolitis, which may result in intestinal resection, intestinal failure (IF) and dependence on parenteral nutrition (PN). Exclusive PN therapy results in intestinal atrophy (2, 3) and long-term use increases risk for serious complications. PN supplemented with butyrate, a 4-carbon short chain fatty acid (SCFA) produced *in vivo* by bacterial fermentation, stimulates structural and functional adaptation of the residual gastrointestinal tract following small bowel resection (4). Intestinal adaptation is critical for successful PN weaning; however, butyrate is not currently added to PN formulations due to its undesirable chemical properties. The central hypothesis of this research is that strategic pre- and probiotic supplementation can be a clinically feasible means of delivering butyrate and stimulating intestinal adaptations in a pediatric IF model.

Our first experiment aimed to determine whether prebiotic and/or probiotic supplementation could serve as a clinically feasible means of providing butyrate to the residual intestine and assess structural and functional adaptations. Neonatal piglets (48 hours old, n=87) underwent placement of a jugular catheter, an 80% jejunoileal resection and were randomized to one of the following treatment groups: 1) **control** (20% standard enteral nutrition (EN)/80% standard PN); 2) control plus **prebiotic** (10 g short chain fructooligosaccharides (scFOS)/L EN); 3) control plus **probiotic** (1 x 10^9 CFU *Lactobacillus rhamnosus* GG (LGG)), or; 4) control plus **synbiotic** (scFOS + LGG) (5). Animals received infusions for 24 hours to assess acute adaptations and 3 or 7 days to assess chronic adaptations. Dependent variables of intestinal

adaptation including gross morphology, cellular composition, mucosal architecture, epithelial cellular kinetics, disaccharidase activity, nutrient transport, and glucagon-like peptide-2 (GLP-2) were assessed. Prebiotic treatment increased ileal mucosa weight compared to all other treatments (P= 0.017) and ileal protein compared to control (P= 0.049), regardless of day. Ileal villus length increased in the prebiotic and synbiotic groups (P= 0.011), regardless of day, specifically due to an increase in epithelial proliferation (P= 0.003). Epithelial proliferation was also increased with prebiotic and synbiotic treatments in the duodenum (P= 0004), residual jejunum (P< 0.0001), and colon (P= 0.0007). Apoptosis was decreased with prebiotic and synbiotic treatments in the jejunum (P= 0.030) and ileum (P= 0.004). Cdx-2 mRNA expression, a marker of differentiation, was unchanged in the jejunum but increased with scFOS in the ileum (P= 0.033). In the 7 day prebiotic group, peptide transport was up-regulated in the jejunum (P= 0.026) whereas glutamine transport was increased in both the jejunum and colon (P= 0.001 and 0.003, respectively). Disaccharidase activity was not affected by treatment in any intestinal segment. Glucagon-like peptide-2, a hormonal mediator of intestinal adaptation (6), was not impacted by treatment. We concluded that prebiotic and/or synbiotic supplementation resulted in enhanced structure and function throughout the residual intestine but that LGG was ineffective. In addition, the signaling mechanism mediating these adaptations was not identified.

Our next experiment aimed to investigate possible signaling mechanisms through the intestinal epithelium resulting in intestinal adaptations. We hypothesized that prebiotic supplementation would increase colon luminal butyrate concentration, SCFA transporters MCT1 and SMCT1 and G-protein SCFA receptors FFAR2 and FFAR3 mRNA expression in the ileum and colon of IF piglets. Prebiotic supplementation was associated with increased butyrate concentrations compared to control, independent of time (P= 0.050), while other SCFA or lactate

did not differ. Total acetate, propionate, and butyrate concentration tended to increase after 3 days of synbiotic treatment (P= 0.078). Ileal MCT1 and SMCT1 mRNA increased in prebiotic, probiotic, and synbiotic groups compared to control after 24 hours of treatment (P= 0.012 and 0.014). Ileal FFAR2 and FFAR3 mRNA were greatest in the prebiotic and probiotic group compared to control after 24 hours of treatment (P= 0.013 and 0.008). After 3 days of treatment, synbiotic-supplemented animals had greater mRNA expression of MCT1, FFAR2, and FFAR3 in the ileum (P= 0.004, 0.017, and 0.039). On day 7, ileal MCT1 mRNA was greater in control, pre-, and probiotic treatments compared to synbiotic treatment (P= 0.004). Seven days of treatment was also associated with greater expression of ileal FFAR2 and FFAR3 with probiotic treatment compared to prebiotic and synbiotic but similar to control (P= 0.025 and 0.004). Colon mRNA levels were not as significantly impacted by treatment. This investigation suggests that treatment may acutely influence expression of SCFA transporters and receptors and is a potential regulatory mechanism for butyrate-associated intestinal adaptation.

Butyrate-stimulated intestinal adaptation is also associated with an increase in expression of the facilitative glucose transporter 2, GLUT2 (7-9). We hypothesized that scFOS administration would be associated with an increased mRNA expression of GLUT2 and the sweet taste receptor T1R3. Jejunal GLUT2 mRNA expression was greater after control and probiotic treatments compared to prebiotic treatment on day 3 (P= 0.034), while ileal GLUT2 mRNA was not different at any individual time point. On day 3, jejunal T1R3 mRNA was increased (P= 0.049) in control animals compared to prebiotic and synbiotic treatments but was similar to probiotic. The 24 hour and 7 day time points were not statistically different in the jejunum. In the ileum, 24 hours of prebiotic or probiotic treatment tended to result in greater T1R3 mRNA compared to control (P= 0.054). On day 3 in the ileum, control piglets expressed

greater T1R3 mRNA compared to probiotic treatment (P= 0.056). By day 7, probiotic piglets

had greater ileal T1R3 expression compared to prebiotic and synbiotic groups while control was

greater than synbiotic (P< 0.001). Jejunal T1R3 mRNA expression did not differ after 24 hours

or 7 days of treatment. After 3 days of treatment, control piglets expressed a significantly greater

amount of T1R3 mRNA in the jejunum compared to synbiotic piglets (P= 0.049). Main

treatment effects for T1R3 did not differ in the jejunum (P= 0.239) or the ileum (P= 0.120).

Jejunal and ileal GLUT2 mRNA was not statistically different when time points were pooled

within treatment (P= 0.166 and 0.446, respectively). Overall, GLUT2 mRNA expression was

not impacted by treatment while T1R3 expression was associated with treatment but this was not

reflected in GLUT2 mRNA expression. This suggests that T1R3 expression was not regulating

GLUT2 expression in this model.

In summary, scFOS but not LGG, administration is associated with increased butyrate

concentrations and intestinal adaptation in a neonatal piglet model of IF. SCFA receptor and

transporter gene expression may an important acute signaling mechanism for these adaptations.

Identification of a synergistic prebiotic and probiotic combination may enhance the promising

results obtained with prebiotic treatment alone.

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CHAPTER 1

LITERATURE REVIEW

INTESTINAL FAILURE

Introduction and Etiology

Intestinal failure (IF) is defined as a decrease in functional intestinal mass below the amount required for digestion and absorption to meet the body's needs (10, 11), and often results in dependence on parenteral nutrition (PN) (12). In an adult, energy and nutrient requirements are based on maintenance whereas a pediatric patient must receive adequate energy and nutrients to support growth. Pediatric IF can be secondary to many diseases, which Goulet and colleagues stratify into 3 primary groups 1) anatomical reduction in intestinal mass; 2) neuromuscular disease of the intestine; and 3) congenital diseases (10). The anatomical reduction in mass is called short bowel syndrome (SBS) and is most commonly the result of surgical resection due to atresia, volvulus, gastroschisis, necrotizing enterocolitis (NEC), thrombosis, or trauma. NEC is of particular concern in premature infants. Recent estimates suggest that NEC occurs in 5.1% of infants born less than 33 weeks of gestation (13) and 11% of infants born between 22-28 weeks of gestation and weighing 401- 1,500 g (14). Of these infants who develop NEC, the greatest estimates indicate 50% of cases require surgery (15), which may result in SBS. IF due to neuromuscular disease in the pediatric patient is usually secondary to Hirschsprung's disease or pseudo-obstruction whereas congenital diseases include enterocyte disorders such as microvillus inclusion disease. IF due to SBS will be the primary focus of discussion.

Prognosis

SBS is characterized by malabsorption resulting in growth retardation in children, weight loss, diarrhea, dehydration and electrolyte imbalances (11, 16). Patients are also at risk for bone loss, renal oxalate stones, gallstones, lactic acidosis, and bacterial overgrowth. In 2005, Goulet reported a 15-year survival rate of 89.7% based on 87 children with SBS (17). Of the surviving children, 11.5% were dependent on PN. These PN-dependent children also had less than 40 cm remaining intestine and/or no ileocecal valve (ICV). Another 12 children were dependent on PN for an average of almost 4 years, weaned, and after proving unable to support growth through oral nutrition alone, required additional enteral nutrition (EN) or PN intervention 4 years later. These children had a mean of 35 cm remaining intestine and 50% retained the ICV. The remaining 57 patients retained a mean of 57 cm of intestine, 81% with an ICV, and these children weaned from PN after an average of 16 months.

Children with IF are at risk for several life-threatening complications. Unfortunately, many infants succumb to these complications, which highlight the need for PN-weaning therapies. Wales and associates followed infants requiring a laparotomy for a number of reasons including NEC, atresia, meconium ileus, and others, and separated the subjects into those with SBS, defined as requiring PN for greater than 42 days and less than 25% of predicted intestinal length for age, and those without SBS (18). Infants with SBS had a 2-year mortality rate of 37.5% compared to 13.3% mortality rate in those without SBS. In addition, 67.5% of infants with SBS experienced central venous catheter complications, and 25% developed PN-associated liver disease (PNALD). A retrospective study of SBS infants reports a 30% mortality rate with 6 infants succumbing to liver failure, 2 with sepsis and 1 dying from cardiac arrest (19). Residual intestine length, but not the presence of an ICV, was correlated with PN duration (R= -0.475).

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Angsten and colleagues report a SBS mortality rate as high as 50% with 33% of deaths due to PNALD (20). Most recently, the Pediatric Intestinal Failure Consortium reported on 272 infants with IF, whom they followed for at least 2 years (21). In these subjects, 9 catheter-related blood stream infections (CRBSI) were reported per 1,000 catheter days, with 68% of infants experiencing at least 1 septic episode. In total, 26% of patients received an intestinal transplant, 47% achieved enteral autonomy and 27% died over the follow-up period.

From these data, the primary causes of death in pediatric SBS are PNALD and CRBSI. In addition, long-term PN carries a large financial burden, reported to be up to $1,619,851 for 5 years of care for a child with SBS, and reduces quality of life (22, 23). Therefore, the primary nutritional goals for pediatric IF patients are to support growth and wean from PN. The best predictors of the ability to achieve PN independence are length and anatomical location of residual intestine (17, 18, 24). A remaining ICV is often cited as a determining factor for PN independence, but remains controversial (25-27). It is well established that the ileum maintains greater potential for adaptation compared to the jejunum (28-30) and improves prognosis.

Nutritional Management

The majority of data on PN management for pediatric patients with SBS and/or IF is in the form of case studies or retrospective reviews of medical documents. Many of the current recommendations are extrapolated from adult patients and based on expert opinion. In infants with IF, it is vital to initiate PN as soon as possible (31). Preterm infants have approximately 110 kcal/kg non-protein energy reserves, equivalent to 4 days of energy, making them especially at risk for malnutrition with delay of PN initiation (32). The Guidelines on Pediatric Parenteral Nutrition of the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition

(ESPGHAN), the European Society for Clinical Nutrition and Metabolism (ESPEN), and the European Society of Pediatric Research (ESPR) provide detailed, evidence-based recommendations for the macro- and micronutrient content of PN for premature and term infants (31).

At least small amounts of EN are recommended whenever possible to reduce intestinal atrophy (3, 33, 34), encourage intestinal adaptation (35, 36), and reduce incidence of PNALD (19). Expressed breast milk is recommended as the EN of choice for infants when available and should be slowly increased as tolerated with corresponding decreases in PN (19, 31). However, a recent report indicated that only 19% of 272 infants with IF received breast milk as their EN source (21). The ability of a patient to achieve EN autonomy is influenced by the length and anatomical locations of remaining intestine, age, health, and degree of adaptation. The focus of this review will now shift to strategies aimed at enhancing intestinal adaptation.

INTESTINAL ADAPTATION

Structural Adaptations in Animal Models

Intestinal adaptation is well characterized in animal models, but is less studied in humans. Animal and human data indicate an increase in structure and function of the residual intestine following intestinal resection as compensation for the missing functional tissue. Structural adaptations evidenced in the literature include increases in residual intestine length and weight, dilation, and alterations in cell epithelial proliferation and apoptosis. Structural remodeling also includes enhanced mucosal architecture such as increased villus length, crypt depth and, therefore, absorptive surface area.

The animal studies examining the outcomes of intestinal adaptation are frequently conducted in rodent models, although some investigators utilize piglets. When examined in piglets with a 75% jejunoileal resection, the residual intestine dilated in circumference by 86% and increased in length by 74% at 16 weeks after surgery (37). Resection in a rat model resulted in a 55% increase in residual mass (38) while Vanderhoof and associates observed a 250%, 150%, and 125% increase in mucosal weight in the jejunum, mid-ileum, and distal ileum, respectively (39). Dowling and Booth examined the effect of proximal and distal small intestine resection in adult rats (28). Resection of the jejunum resulted in a 33% increase in ileal diameter and 53% increase in villus length. Alternatively, resection of the ileum showed more modest, but statistically significant, structural adaptations including 14% increases in both jejunal diameter and villus length. These data support the greater potential for adaptation in the ileum compared to the jejunum. Similar effects on villus length following resections in rats, mice, and pigs are reported (40-43). These alterations in mucosal architecture often are accompanied by an increase in epithelial cell proliferation. Sacks reports a 4-fold increase in proliferating cells in ileal crypts 24 hours after a 70% proximal resection in 21-day old rats (44). A 2-fold increase in ileal crypt proliferation also was seen in 4-week old piglets 2 weeks after a 75% small bowel resection, which preceded an increase in villus length by 6 weeks post- resection (41). In addition to enhanced residual mass, mucosal surface area, and proliferation, resection also influences cell composition. Rats with a 60% proximal resection did not have an increase in DNA, RNA, or protein at 3 weeks, but by 8 weeks DNA increased by 192% in the jejunum, 168% in the mid-ileum, and 158% in the distal ileum (39). The distal ileum also showed 137% increases in RNA and protein. Similar results were reported in 20-day old rats following a 50% distal resection of the ileum and ICV where jejunal DNA increased by 1.4-fold, RNA by 1.7 and

protein by 1.6-fold 7 days after surgery (45). Colonic RNA (1.6-fold) and protein (1.3-fold), but not DNA, also increased. These structural adaptations provide evidence for robust remodeling of the residual intestine to compensate for the loss in surface area.

Functional Adaptations in Animal Models

While an increase in structure is desirable, a corresponding increase in function must be present to decrease the malabsorption characteristic of SBS. Indeed, evidence suggests that the increase in tissue results in greater functional capacity of the residual intestine, often assessed through glucose absorption. Glucose absorption was increased by 19% in the jejunum after ileum resection and 96% in the ileum after jejunum resection in rats, again displaying greater ileal adaptation (28). In fact, O'Connor and others did not observe an increase in glucose absorption in the proximal intestine following distal resection (38). Distal glucose absorption was not increased when normalized for tissue mass but increased 2-fold when normalized for tissue length. This corresponds to the increase in mucosal weight per length described above. Glucose transport following resection was further investigated by Iqbal et al. in a rat 70% proximal resection model (46). No changes in sodium-dependent glucose transporter-1 (SGLT-1) or GLUT2 mRNA or protein were detected when normalized per weight, despite a 126% increase in ileum glucose transport after 1 week. However, the increase in glucose transport corresponded with the increase in villus length, suggesting that the increase in glucose transport was due to hyperplasia rather than an upregulation in glucose transporters. Disaccharidase activity is also utilized as a marker of functional adaptation although many studies do not report differences (39, 45). The increases in functional capacity just described should decrease malabsorption, which is common in SBS. Turner and associates utilized a piglet model of SBS,

with both proximal small intestine and distal small intestine resections represented (47). Piglets with jejunal resections increased fat absorption by 24% from day 5 to day 15 post-surgery, while piglets with ileal resections increased 41%, suggesting a decrease in malabsorption. It is important to note that after 5 days, the piglets with jejunal resection were malabsorbing approximately 10% of enterally provided fat while piglets with ileal resections were malabsorbing 20%. Clearly, the residual intestine, particularly the ileum maintains the potential for not just structural, but also functional, adaptations following resection.

Adaptation in Humans

Studies characterizing the extent of intestinal adaptation following resection in humans are far less plentiful compared to those using animal models. In fact, much of the human data is presented as case studies or retrospective analysis and rarely controlled. Human reports do not often include end points such as histology, cellular composition, or directly measured nutrient transport but rather ability to achieve PN independence (as discussed in *Prognosis* section) or malabsorption through measurement of macro- and micronutrient fecal output. Early work in humans measured jejunal glucose, water, and sodium absorption. Dowling and Booth showed 26% greater glucose absorption, in the jejunum of patients with SBS compared to healthy controls (29). Similarly, individuals with SBS showed a 54% and 59% increase in water and sodium absorption respectively, compared to controls (48). Recently, a retrospective analysis of villus length and crypt depth in infants with NEC was performed (49). Biopsies were taken at time of resection at the ostomy site and again at ostomy closure, an average of 74 days later. McDuffie reported a 32% and 22% increase in villus length and crypt depth, respectively. These limited studies in humans suggest that the residual intestine undergoes similar adaptations as

those seen in animal models, although more investigation is needed. When considered in terms of the IF infant mortality reports, there is an urgent need to develop strategies to optimize intestinal adaptation with the goal of achieving PN independence.

Butyrate and Intestinal Adaptation

Short chain fatty acids (SCFA) are a potential mediator of intestinal adaptation. SCFA are products of bacterial fermentation of non-digestible carbohydrates in the distal intestine. The 3 primary SCFA produced in humans are acetate, propionate, and butyrate and are generated at an *in vivo* molar ratio of approximately 60:25:15 (50). Butyrate is the 4-carbon SCFA and stimulates sodium and water absorption and serves as a primary energy source for colonocytes (51, 52).   In addition, butyrate has been shown to be intestinotrophic, but due to its volatile and putrid chemical properties, butyrate administration is not well studied in humans.   SCFA were first added to PN by Koruda and others in 1988 (53). After 7 days, these SBS, SCFA-supplemented rats experienced a 1.5 to 2-fold increase in jejunal mucosa weight, DNA, RNA, and protein concentrations. Ileal mucosa responded similarly for mucosa weight and DNA. In non-SBS rats, both SCFA in PN and intracecal infusion of SCFA for 7 days inhibited the PN-induced atrophy observed without SCFA supplementation (54). Specifically, SCFA supplementation resulted in greater jejunal and ileal mucosa weight, and ileal DNA, RNA, and protein concentrations compared to a TPN control, but less than animals consuming a standard rat chow. These SCFA effects were confirmed in an adult rat model of SBS (55) wherein ileal mucosa weight increased by 57%, protein by 29%, DNA by 37%, and RNA by 36% after 7 days compared to control without SCFA supplementation. Importantly, these findings were confirmed in a piglet model of SBS, a highly relevant model for the premature infant, with an

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additional treatment group with butyrate-supplemented PN (4). From this study, it was concluded that butyrate was the SCFA responsible for the increases in structural adaptation. Specifically, Bartholome et al. report a 17% increase in ileal DNA in response to butyrate-supplemented PN corresponding with a 4-fold increase in the number of proliferating cells after 7 days of treatment. Villus length increased by 27% and 36%, respectively, in the jejunum and ileum in the butyrate group, regardless of treatment duration ranging from 4 hours to 7 days. The adaptations outlined here are critical considering the increased adaptation compared to a resection control. These adaptations associated with butyrate beyond those seen in control represent a potential therapy for infants with IF as this will increase the chances of achieving enteral autonomy, the ultimate goal in these patients.

Functional Adaptations

The functional adaptations previously described are further enhanced with SCFA supplementation. Reported functional adaptations attributed to SCFA include an increase in glucose uptake and levels of glucose transporter mRNA and protein. Glucose uptake increased in the residual ileum of rats following a jejunoileal resection in response to SCFA-supplemented PN (7). D-glucose uptake increased similarly after 3 and 7 days of treatment with a 1.5-fold increase in V_{max}. Ileal GLUT2 mRNA increased 3-fold after 3 days of SCFA treatment, but was not sustained at 7 days. There was also a trend for an increase in SGLT-1 mRNA, but no differences were seen in GLUT5 mRNA or disaccharidase mRNA. Similarly, GLUT2 mRNA increased 13 to 18-fold in rats with SBS after receiving SCFA-supplemented TPN for 6, 12, and 24 hours compared to TPN control but not SCFA-TPN for 72 hours (8). Increases in jejunal mRNA GLUT2 also were reported in normal rats receiving SCFA-PN in addition to an

approximately 5-fold increase in ileal GLUT2 protein after 72 hours of supplementation (9). These adaptations provide critical evidence for the efficacy of SCFA and butyrate in IF, but also pose the question of signal mediation.

One suggested mechanism for butyrate action is the intestinotrophic hormone glucagon-like peptide 2 (GLP-2), which is cleaved from the proglucagon protein. SBS rats infused with SCFA-supplemented PN experienced a 38% increase in ileum proglucagon mRNA abundance 3 days after surgery and was sustained at 7 days (55). This increase in ileal proglucagon mRNA abundance was mirrored in the same model after SCFA-PN for 6, 12, and 24 hours, but not 72 hours (8). At 12 hours, there was a doubling of plasma GLP-2 concentration. In normal rats receiving SCFA-TPN, a doubling of ileal proglucagon mRNA was seen after 24 hours of treatment; however, there was no effect on plasma GLP-2 (9). Neonatal SBS piglets, receiving SCFA or butyrate supplemented-PN, increased plasma GLP-2 concentrations, which corresponded with structural adaptations (4). Increased GLP-2 plasma concentration are, therefore, a potential signaling mechanism for butyrate, induced adaptations that warrant further investigation.

PREBIOTICS, PROBIOTICS AND SYNBIOTICS

Butyrate is capable of stimulating both structural and functional adaptations of the residual intestine in animal models of SBS. The reported effects are similar to the native response of the remaining intestine, but to a greater extent. It is likely that GLP-2 is involved in the signal transduction resulting in these adaptations. Despite its proven efficacy, butyrate is not easily added to PN or EN formulas due to its unpalatable and volatile properties. An alternative

delivery method must be identified. One strategy is through strategic provision of pre- and probiotics to increase luminal fermentation and butyrate production.

Prebiotics

Prebiotics are digestion-resistant food ingredients fermented by the host microbiota resulting in selective growth and/or activity of beneficial bacteria (56). In the pediatric population, prebiotics are of interest for their ability to bring the microbiota composition of formula-fed infants closer to that of the breast-fed infant, considered the nutritional gold standard. Combinations of galactooligosaccharides (GOS) and fructooligosaccharides (FOS), chosen for the similarities in molecular weight to human milk oligosaccharides, are the most studied prebiotic combination. These prebiotics were selected for their ability to alter the microbiota composition to a more bifidogenic population. However, it is important to note that approximately 200 different human milk oligosaccharides have been identified, making it unreasonable to replicate breast milk oligosaccharide composition at this time (57). Randomized, double-blind, placebo-controlled trials of prebiotics are utilized to assess the safety, tolerance and ability to promote growth of both preterm and full-term infants. The prebiotic composition of studied formulas varies in total oligosaccharide (4-10 g/L), but are generally 9:1 GOS:FOS. Most frequently, GOS is in the form of short chains, while FOS is usually long chain. Regardless, prebiotic formulas are shown to support growth and to not increase adverse events in both preterm (58, 59) and full term infants (60, 61). These formulas also are shown to increase the proportion and/or absolute number of bifidobacteria as measured in fecal samples. Arslanoglu reports an 18% increase in bifidobacteria colony forming units (CFU) in the feces of full term infants after 6 months on a formula containing 8 g/L oligosaccharides (62). Other

studies corroborate these results with a doubling in bifidobacteria on a percentage of total bacteria basis after 12 weeks of an 8 g/L prebiotic formula, while control remained constant (61). In fact, Holscher et al showed that after 6 weeks of 4 g/L prebiotic formula, the bifidobacteria abundance was similar to that of the breastfed controls (63). A dose response was observed by Moro and others where bifidobacteria increased by 2.1 log CFU/g feces after 4 g/L formula while an 8 g/L formula resulted in a 2.5 log CFU/g feces increase (64). An increase in lactobacilli also was reported with both formulas. Similar effects were shown in premature infants (58, 65). Recently, several studies have been published that investigate a new prebiotic blend. This blend includes polydextrose rather than long-chain FOS, with the rationale to more similarly emulate the range in human milk oligosaccharide molecular weights. In two randomized controlled trials, a formula containing 4 g/L polydextrose and 4 g/L GOS was well tolerated by healthy term infants and supported equivalent growth compared to the control formula while altering stool consistency to be more similar to a breastfed infant (66, 67). Similar to the reports for GOS:FOS formulas, the polydextrose:GOS formula also increased fecal bifidobacteria populations (66).

Other outcomes investigated in prebiotic infant formula trials include incidence of infection and fecal SCFA concentrations. When measured in the feces of preterm infants, the abundance of pathogens included were slightly (0.43 log CFU/g feces) but significantly lower in the prebiotic supplemented group (68). Westerbeek reports a trend toward a decrease in the incidence of serious infections in premature infants with prebiotic supplementation (69), while Arslanoglu observed half the reported number of infections with prebiotic supplementation in a similar population (19, 62). Finally, several studies show an increase in the fecal acetate content with prebiotic formula. Knoll found that the predominant fecal SCFA in preterm infants

consuming a prebiotic formula with 8 g/L oligosaccharides was acetate at 85.2% of total SCFA compared to 77.2% in the control group (70). Modi et al showed 50% greater concentration of acetate after 5 days of 9 g/L oligosaccharide formula in preterm infants compared to a control formula (59). The literature on prebiotics in the human infant indicates that GOS and FOS were able to increase the number of bifidobacteria in the microbiota. In addition, prebiotics were shown to decrease the incidence of infection, which is critical for the premature infant. Infants with IF may experience even greater benefit from prebiotic supplementation.

Probiotics

Probiotics are live microorganisms that, when consumed in adequate amounts, confer a health benefit to the host (71). A multitude of probiotic studies in both premature and full term infants exist in the literature, particularly utilizing lactobacilli and bifidobacteria species. *Lactobacillus rhamnosus* GG (LGG) has been extensively investigated in premature and low birth weight (LBW) or very low birth weight (VLBW) infants. A double-blind, randomized controlled trial of infants born <33 weeks gestation or VLBW (<1,500 g) were supplemented with 6×10^9 CFU LGG/day or placebo (72). Although the primary outcomes of urinary tract infection and NEC incidence were not significantly different, infants receiving probiotics grew at the same rate as controls and did not experience any adverse events attributed to the probiotic. A retrospective review of 6 years of clinical charts for VLBW infants receiving LGG for 4-6 weeks confirmed safety of the supplementation through no adverse events, including no septic events associated with LGG (73). LBW infants, 1,500-1,999 g at birth, received 10^9 CFU LGG/day in a randomized manner (74). After 8 days of supplementation, LGG in the feces was confirmed in 47% of supplemented infants. A similar study compared infants born <32 weeks and birth

weight >1,000 g receiving 6 x 10^9 CFU LGG/day or placebo for 42 days (75). Greater amounts

of LGG were detected in the stool of supplemented infants at days 7 and 21, but was no longer

different at 42 days. *Bifidobacterium lactis* Bb12 has been investigated in similar studies. A

randomized controlled trial of 1.2 x 10^{10} CFU Bb12/kg/day did not reduce incidence of

nosocomial infections in infants weighing less than 1,500 g and born less than 30 weeks

gestation, but again was well tolerated (76). Mohan et al. showed that Bb12 are present in fecal

samples after supplementation in the preterm infant (77), while Holscher et al. confirmed

tolerance and fecal presence of Bb12 in the full term infant (78).

The evidence of efficacy for decreasing the incidence of NEC in preterm infants is

strong; however the different probiotic species and combinations used in RCTs make

recommendations difficult. In a randomized controlled trial, only 4% of preterm, VLBW infants

supplemented with 10^9 CFU /day of *Bifidobacterium infantis, Streptococcus thermophiles,* and

Bifidobacterium bifidum developed NEC compared to 16% of their control counterparts (79).

The infants receiving probiotics with NEC had less severe cases as measured with the 3 stage

Bell's Criteria. Three NEC-related deaths occurred during the study, all in the control group.

Lin and others used the combination of *Lactobacillus acidophilus* and *B. infantis*, administered

twice daily, or control in infants weighing <1,500 g at birth (80). A decrease from 5% to 1% was

reported in NEC incidence with probiotic supplementation. There were 6 cases of severe NEC

evidenced by a stage 3 score from the Bell's criteria, all from the control group. Similar findings

were reported in preterm infants receiving *Lactobacillus reuteri* DSM 17938 where probiotic

supplementation nonsignificantly decreased NEC incidence by 40% and nosocomial pneumonia

by 52% (81). The same probiotic, *L. reuteri* DSM 17938, was reported to decrease NEC in

extremely low birth weight infants (ELBW; <1,000 g at birth) from 15.1% to 2.5% in a

retrospective review of medical records (82). Several recent meta-analyses corroborate this reduced incidence of NEC with probiotic supplementation. A meta-analysis included 11 RCTs of infants that were preterm (<34 weeks of gestation) and/or VLBW (<1,500 g) (83). The included trials utilized various bifidobacteria or lactobacilli species alone or in combination. *S. thermophilus* and the probiotic yeast *Saccharomyces boulardii* also were utilized. The primary outcomes show a 30% decreased incidence of NEC with probiotic supplementation, a relative risk (RR) of 0.42 for death from NEC, and no difference in the incidence of sepsis. Similarly, a meta-analysis of 9 RCTs including preterm infants (<37 weeks) and/or LBW (<2,500 g) report a RR of 0.32 for incidence of NEC with probiotic supplementation and 0.43 for mortality (84). Indeed, in 2011, a Cochrane meta-analysis concluded that probiotic therapy should be used in preterm infants to decrease incidence of NEC, but more data are needed to make conclusions regarding ELBW infants (85). Although not a conclusion of the Cochrane review, the inconsistencies in probiotic species makes clinical standards difficult and more work is required to identify the optimal probiotic formulation. However, the strong evidence for use in this critical population suggests that IF may benefit from probiotic supplementation and should be investigated.

There are few randomized, controlled trials in humans with probiotic supplementation as the intervention for stimulating adaptation in SBS and/or IF. Animal models of SBS suggest that probiotics may be efficacious in enhancing adaptation and controlling small intestinal bacterial overgrowth although this is controversial. ABT-4 is a probiotic supplement containing *L. acidophilus,* bifidobacteria*,* and *S. thermophilus* and was recently investigated in SBS rats. ABT-4 resulted in 16% greater jejunal villus length and 77% greater crypt depth compared to SBS rats not receiving ABT-4 although ileal epithelial architecture was not impacted (86).

Similar adaptations were observed by Mogilner and colleagues in SBS rats receiving LGG wherein ileal crypt depth increased by 15% and the number of jejunal and ileal apoptotic cells decreased by 1.75 and 2-fold respectively (87). Other measures such as mucosal weight, DNA, protein, and villus length in both the jejunum and ileum failed to reach significance when compared with SBS rats not receiving LGG.   Mogilner also evaluated bacterial translocation to lymph nodes, liver, portal vein, and peripheral circulation. While all SBS rats exhibited bacterial translocation to the lymph nodes, the number of rats with bacterial translocation to the liver, portal vein, and peripheral circulation was numerically decreased by probiotic supplementation. Bacterial translocation of lactobacilli specifically was not observed. Eizaguirre et al., utilizing a SBS rat model to study the effect of *B. lactis* on bacterial translocation, reported that 87% of control SBS rats experienced bacterial translocation compared to 50% of those in the probiotic group (88). A double-blind, placebo-controlled, crossover clinical trial was performed in pediatric SBS patients investigating the effect of LGG on intestinal permeability; however no differences were observed (89). The probiotic yeast *S. boulardii* also was investigated in rat SBS, but intestinal adaptation measures such as mucosal mass, DNA, protein, and sucrase activity in rat SBS were not impacted by supplementation (90). These studies suggest that probiotic treatment alone may not be effective in inducing intestinal adaptation in animal models; however, it is difficult to reach broad conclusions due to the many different probiotic possibilities and their unique effects and mechanisms.

Synbiotics

Synbiotics, the combination of pre- and probiotics, are investigated in various formulations in the pediatric population for their safety and ability to promote beneficial bacteria

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colonization while decreasing incidence of infection. In healthy, full term infants, synbiotic

supplementation of *Bifidobacterium longum* and *L. rhamnosus* plus 4 g/L of a 9:1 ratio

GOS:scFOS or *B. longum* and *Lactobacillus paracasei* plus 4 g/L of a 9:1 ratio GOS:scFOS

supported similar growth, length, and head circumference compared to a placebo formula (91).

No differences in adverse events or tolerance were observed. While most studies report similar

growth between synbiotic and control, Picaud et al. observed a 1.4 g/d greater increase in weight

in healthy term infants receiving *B. longum*, *S. thermophilus*, and FOS (92). These infants also

had less incidence of infectious disease (31% synbiotic vs. 40.6% control) corresponding with

fewer episodes of acute diarrhea. A decrease in respiratory infections also was found with

probiotic supplementation of the mother for 4 weeks before delivery and the infant with a

synbiotic formulation after birth (93). Importantly, synbiotics' ability to alter the composition of

the microbiota also is evident. A synbiotic supplement consisting of *Lactobacillus plantarum*

and FOS resulted in *L. plantarum* in the feces of 84% of the synbiotic infants and 0% of control

(94). *L. plantarum* colonized one third of the infants as evidenced by fecal detection at 6

months, despite supplementation cessation at 7 days.

In addition to healthy term infants, there is also evidence for efficacy in preterm and

critical pediatric patients. In premature infants, a synbiotic formula containing LGG and inulin

was compared to a formula containing *L. acidophilus, B. longum, B. bifidum, B infantis,* and

inulin vs. control (95). While weight gain was similar, the presence of bifidobacteria in the feces

was impacted by formula. Of the LGG and inulin infants, 18% showed bifidobacteria in the

feces, while 64% of those receiving the more complex supplementation formula were positive

for bifidobacteria compared to 27% of the control. A decrease in fecal bifodobacteria was

observed in children 1-3 years of age on ventilators in the intensive care unit (96). Children

supplemented with *L. paracasei, B. longum,* FOS, inulin, and Acacia gum for 7 days returned to baseline fecal bifidobacteria levels by 14 days while control children remained at the decreased level.

There are no randomized controlled trials investigating the effect of synbiotics in SBS, but there are several case reports. In all instances, the synbiotic combination of *Bifidobacterium breve, Lactobacillus casei,* and GOS was utilized. Seven primarily pediatric SBS patients received probiotics plus 1 g GOS 3 times per day for a minimum of 15 months (97). Due to the uncontrolled nature of this report, differences in patients' remaining bowel lengths, and underlying disease, conclusions based on the synbiotic treatment are difficult. Nevertheless, authors reported a decrease in aerobic bacteria in the feces, a 2-fold increase in fecal SCFA, accelerated weight gain, and decreased nutrition support requirements. Similar results were reported in 4 pediatric SBS patients, again receiving the same synbiotic combination (98).

MECHANISMS OF PRE-, PRO-, AND SYNBIOTIC ACTION

With specific reference to SBS and IF, pre- and probiotics may encourage intestinal adaptations through several possible mechanisms. Microbiota and their fermentation products interact with the epithelium to modulate cytokinetics, resulting in alterations in the structure and function of the intestine. The increase in fermentable substrate with prebiotic supplementation and the corresponding increase in SCFA results in a necessity to increase the capacity to transport and oxidize. Finally, pre- and/or probiotic supplementation may alter the microbiota composition in ways favorable for patients with SBS. There are several known SCFA transporters, with varying sensitivity to butyrate, the primary SCFA of interest for intestinal adaptation. In addition, proteins responsible for sensing luminal composition may be impacted

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by an alteration in SCFA ratios and/or abundance, thereby initiating a cascade of events potentially responsible for signaling intestinal adaptations. These potential mechanisms are examined in detail in the following section.

Microbiota and the Epithelium

The microbiota were first identified as a potential modulator of the intestinal epithelium in the early 1960s when germ-free animals were utilized to assess differences in intestinal structure compared to conventional animals. Gordon and colleagues observed that the small intestine from chicken, rat, and mouse all weighed less in germ-free animals compared to their conventional counterparts (99). In rodents, weights of cecal contents were 4 to 6-fold greater and cecal tissue 2 to 3-fold greater in germ-free animals. These differences disappeared with colonization of the germ-free animals. Further investigation into the proliferative state within the ileum revealed accelerated proliferation in the conventional mice where total cellular transit time from crypt to villus tip was approximately 2-times longer in the germ-free mice (100). The germ-free mice also had fewer lymphocytes and mononuclear cells within the lamina propria and smaller Peyer's patches. A similar phenomenon was described in the duodenum of germ-free and conventional mice (101). Although the villi were longer in germ-free animals, cells migrated approximately 110 µm in 12 hours in germ-free animals and 300 µm in conventional mice. In addition, labeled thymidine appeared in 8 cells in germ-free mice compared to 12.8 cells in conventional animals 12 hours after intraperitoneal injection. Khoury and colleagues also utilized labeled thymidine to assess proliferation differences (102). Twenty-four hours post-injection, 9% of cells were labeled in the ileum of germ-free mice versus 31% in mice with a microbiota. After 96 hours, 92% of germ-free cells were labeled while conventional mice

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exceeded this marker with 97.5% of cells labeled after only 48 hours. These data were further confirmed by Savage and colleagues (103). Based on these investigations, epithelial turnover is approximately 2-times faster in conventional animals with a microbiota compared to germ-free animals. Goodlad and others investigated whether this increased proliferation in conventional rats was due to specific regulatory properties of microbiota or related to the production of fermentation products (104). Conventional and germ-free rats were deprived of food for 3 days followed by refeeding with an elemental diet, elemental + non-fermentable fiber, or the elemental diet + fermentable fiber. Conventional rats receiving fermentable fiber experienced a 1.3-fold increase in crypt cell proliferation in the proximal small intestine, a 2-fold increase in the distal small intestine, and a 7-fold increase in the colon. The germ-free rats receiving fiber were similar to conventional and germ-free rats receiving an elemental diet or an elemental + non-fermentable fiber diet. Interestingly, there is evidence of an increase in functional markers in germ-free animals. Enteric enzymes including alkaline phosphatase, glucose 6-phosphatase, and adenosine triphosphatase are reported to be 2.3, 2.9 and 1.7-fold greater in the small intestine of germ-free rats compared to conventionally raised animals (105). Further, disaccharidase activity in the proximal small intestine including sucrase, maltase, trehalase, and lactase were greater by 1.6, 1.5, 2.3, and 1.8-fold, respectively.

Cytokinetic outcomes also have been investigated following prebiotic, probiotic, and synbiotic supplementation in conventionally colonized animal models and humans. Several supplementation studies assessed the prebiotic protective properties with an intestine-specific challenge. In piglets, inulin supplementation at 60 g/L of sow milk replacer protected against decreased proliferation associated with lactulose challenge (106). Piglets receiving inulin for 7 days prior to lactulose maintained a cecal proliferation rate similar to control piglets who did not

receive lactulose. Apoptosis was not detected in most animals. Five percent oligofructose or long-chain inulin were supplemented in rats for 3 weeks followed by exposure to 1,2-dimethylhydrazine, a carcinogen, and euthanized 24-hours later (107). Prebiotic-supplemented rats showed 3- and 3.5-fold greater colonic crypt cell apoptosis compared to control for oligofructose and long-chain inulin, respectively. This suggests that oligofructose and long-chain inulin may be protective in the early stages of colon cancer, but also that prebiotics can influence colonic apoptosis. However, these same prebiotics were supplemented at 7.5 g oligofructose and 7.5 g inulin daily for 2 weeks in healthy human subjects and no effect on proliferation was observed in cecal, transverse, descending colon, or rectum (108).

Similar effects are reported with probiotic supplementation. Rats consumed an elemental diet and were supplemented with 10^7 CFU *L. casei* or *Clostridium butyricum* or vehicle daily for 7 days (109). Probiotics resulted in an increase in crypt cell proliferation by 25-40% in the jejunum and ileum, 70% in the cecum, and more than 200% in the distal colon. A recent study suggests that the modulation of proliferation may be specific to the strain of probiotic (110). Neonatal mice were provided with 10^8 CFU of 1 of 2 different strains of *L. reuteri*, DSM 17938 or ATCCPTA 6475. At 4 hours post-BrdU injection, migration of labeled cells was similar for the 2 strains. By 24 hours, 17938-treated animals had enterocyte migration 3-times farther up the villus compared to 6475. At 6 days post-BrdU injection, 17938-treated animal enterocytes with BrdU incorporation were no longer detected on the villus, only a few cells were detected for 6475, and control animals showed cell migration approximately 46-72% up the villus. Finally, the effect of *B. breve* with and without raffinose was investigated in a rat model (111). Rats received 1.5x10^7 CFU encapsulated *B. breve* and/or 30 g raffinose/kg diet for 3 weeks. Proliferation increased in the synbiotic group but not with *B. breve* or raffinose alone.

Proliferation was 1.4-times greater in the small intestine with synbiotic treatment compared to control, but there was no effect in the cecum or distal colon. The authors suggest that the probiotic became unencapsulated in the small intestine and fermented the raffinose, producing SCFA and, therefore, impacted proliferation at this relatively proximal location.

In vitro fermentation of different prebiotics under the same experimental conditions produce varying concentrations of SCFA, providing evidence that this also occurs *in vivo*. Depending on the prebiotic, microbiota populations, and other factors, different amounts of SCFA will be presented to the epithelium, therefore impacting proliferation and apoptosis as discussed above. *In vitro* fermentation experiments simulated the gastrointestinal environment and utilize fecal inoculations from healthy donors to assess the SCFA profile of various prebiotics upon fermentation. Van De Wiele and colleagues used a simulator of the human intestinal microbial ecosystem with specific sections simulating each portion of the gastrointestinal tract including the stomach, small intestine, ascending colon, transverse colon, and descending colon to assess the SCFA production of an oligofructose with 2-20 degrees of polymerization (DP) and inulin with 3-60 DP (112). Both oligofructose and inulin increased total SCFA by 30% in each section of the simulated colon. This increase was primarily attributed to an increase in propionate and butyrate production. Inulin fermentation approximately doubled propionate concentrations in the ascending, transverse, and descending colon whereas oligofructose increased butyrate and propionate equally. *In vitro* data from Pompei showed that acetate production increased by approximately 5-fold after 24 hours of fermentation with oligosaccharides composed of primarily 2-9 DP and high solubility inulin composed of 60% 2-9 DP, 28% $\geq$ 10 DP, and 12% sugar (113). Butyrate concentrations increased by 47% with oligosaccharides and 87% with the high solubility inulin at 12 hours of

fermentation but returned to control levels at 24 and 48 hours. When short, medium, and long-chain fructooligosaccharides were compared, the short-chain polymer produced the greatest total SCFA at 4, 8, and 12 hours of fermentation but the authors noted that the long-chain fructooligosaccharide (inulin) likely did not reach its full fermentation potential within the study timeframe (114).

The germ-free animal investigations demonstrate the role of the microbiota in regulating enterocyte cytokinetics including proliferation and apoptosis. However, these effects may be attributed to the microbes themselves or metabolic products such as SCFA. Further evidence suggests that both the microbiota and SCFA drive modifications in the proliferative and apoptotic state within the lumen. These data serve to support pre- and/or probiotic supplementation in a stressed or challenged intestinal environment such as IF, wherein these dietary interventions may beneficially impact repair and adaptation within the injured intestine.

Microbiota Composition

As discussed in the '*Pre-, Pro-, and Synbiotic*' section, pre-, pro-, and synbiotic supplementation can modify the intestinal microbiota in healthy, preterm, and critically ill pediatric populations. Healthy infants are colonized by a large population of bifidobacteria with a report of 1 month old infants, regardless of diet, having as many as 10.7 log CFU/g feces with a total bacteria count of 11.12 CFU/g feces (115). The number of bifidobacteria increase rapidly after birth with 56.5% of vaginally-born infants colonized with bifidobacteria by 3 days after birth compared to 0% of Cesarean section (C-section) infants (116). Vaginally-born term infants increased in bifidobacteria fecal counts from 7.5 log CFU/g feces 8 days after birth to 8.5 log CFU/g feces by 90 days (117). Prior to weaning, breastfeeding versus formula feeding also

influenced the predominance of bifidobacteria, with breastfed infants having approximately 55% bifidobacteria and formula-fed infants with 25% (118). Formula-fed infants also were more often colonized with *E. coli* and *Clostridium difficile* than their breastfed counterparts (115). Within infants receiving formula, 100% of those whose formula contained oligosaccharides were colonized with bifidobacteria compared to the 95-96% consuming non-prebiotic-supplemented formulations.

ELBW infants, <1,000 g at birth, show a strikingly different microbiota compared to healthy infants. Recent studies show that of 29 preterm, ELBW infants, 57% were colonized with *Staphylococcus*, 15.1% with *Clostridium*, 10% *Enterococcus*, 7% *Enterobacteriaceae,* and only 1.2% with bifidobacteria (119). Pyrosequencing from 11 ELBW infants in the first month after birth showed Saccharomycetales yeast to be the most prominent eukaryote (120). Notably, 5 infants also showed sequences matching the genus Trichinella, a parasitic nematode, although infants were without symptoms. Only 16 sequences corresponded to lactobacilli or bifodobacteria, despite exclusive breastfeeding, although 8 of the 11 infants were born by C-section. Infants born ≤32 weeks gestation, or ≤ 1,250 g at birth who developed NEC were compared with age-matched and diet-matched, healthy controls (121). Infants with NEC showed a 34% increase in Proteobacteria and a 32% decrease in Firmicutes between 1 week and 72 hours of NEC diagnosis. In addition, 3 of the 9 infants with NEC provided a sequence related to *Enterobacteriaceae*, but not matching any specific species by greater than 97%. None of the healthy infants contained this sequence. These discrepancies between healthy and high risk infants pose a possible window for pre- and/or probiotic intervention.

The microbiota of a patient with SBS is not well studied. However, a case study of a 4 year old girl with 25 cm of small intestine and colon showed that supplementation with *B.*

longum, L. casei, and 3 g GOS/d resulted in a decrease in aerobic bacteria from 29.7% of total bacteria to 0.81%, an increase in bifidobacteria from 6.91 to 11.04 log CFU/g feces, and undetectable lactobacilli to 9.02 log CFU/g feces after 1 month of supplementation, and essentially maintained these levels at 19 months (122). SBS patients varying in age from 2 to 24 years with differing residual intestinal anatomy also experienced similar changes in the microbiota composition following the same synbiotic treatment for at least 6 months (97). A recent study compared fecal and mucosa-associated bacteria in 11 SBS adults with a jejunocolonic anastomosis with 8 controls (123). In fecal samples, lactobacilli accounted for the majority of bacteria in the SBS parients but only 0.3% in controls. Specifically, *L. mucosae* was detected in 10 of the 11 SBS fecal samples and 7 of 9 biopsies, but was not detected in control feces or biopsies. Certainly, a significant decrease in intestinal length will alter the environment in a way that impacts the microbiota. More studies are needed to better characterize the impact of SBS on microbiota populations in both pediatric and adult patients.

Short Chain Fatty Acid Transporters

SCFA transporters are responsible for moving butyrate and other SCFA from the lumen of the intestine into enterocytes and mediating entrance into portal circulation. Several transporters have been identified in the GI tract. Monocarboxylate transporters (MCT) are abundantly expressed in many cell types and tissues including erythrocytes, muscle, kidney, liver, sperm, and the gastrointestinal tract (124). Monocarboxylates include lactate and pyruvate, which MCTs transport in tissues such as muscle and liver, but in the gastrointestinal (GI) tract, the primary monocarboxylates are the SCFA. MCT1, the most well characterized of the GI MCT family, is an electroneutral anion exchange SCFA transporter and is pH activated (125). In

isolated luminal membrane vesicles from both pig and human large intestine, butyrate uptake was optimized 4 to 5-fold at pH 5.5 compared to pH 8.0. Acetate and propionate inhibit butyrate uptake by 40%, while other monocarboxylates, pyruvate, lactate, and α-ketobutyrate, inhibit butyrate uptake by 50%. Conversely, acetate, propionate, and butyrate all inhibit lactate uptake, ranging from 30-50%, with butyrate resulting in the greatest inhibition (126). Butyrate inhibition was due to an increase in K_m without a corresponding increase in V_{max}, indicating competitive inhibition. Importantly, MCT1 mRNA and protein concentration in the GI tract were shown to be responsive to the concentration of substrate. An *in vitro* study used nontumorigenic human colonic adenoma cell line (AA/C1) to investigate the effect of SCFA concentration on MCT1 (127). Cells treated with 5 mM sodium butyrate for 48 hours increased in MCT1 mRNA by 6.7-fold and protein by 6.2-fold. This response was dose-dependent, with 5 mM butyrate resulting in the greatest increase. A functional increase also was reported where butyrate uptake was increased by exposure to 2 mM butyrate for 30 hours. Treatment with sodium acetate or sodium propionate did not impact MCT1 mRNA or protein levels. Recent *in vitro* analysis revealed a 3-fold difference in butyrate uptake when C2BBe1 cells were incubated with and without a MCT1 inhibitor (128). MCT1 V_{max} doubled in the presence of 10 mM butyrate while K_m did not change. *In vivo* analysis also suggested an increase in MCT1 with fiber supplementation. MCT1 mRNA expression was upregulated by 40% in the cecum and 50% in the colon of piglets fed a 5% oat β-glucan-supplemented diet supplemented for 14 days (129). The potential for an increase in SCFA production with a corresponding increased MCT, particularly MCT1, is important in IF patients for several reasons. Greater transport of SCFA provides fuel for enterocytes, and there may be an increase in butyrate reaching the circulation.

The location of MCT1 on enterocytes and colonocytes is debated in the literature. The studies by Ritzhaupt et al presented above were conducted in isolated luminal vesicle membranes, suggesting presence in the brush border membrane (125, 126). Similarly, Gill and colleagues isolated human luminal vesicle and basolateral membranes from jejunum, ileum, proximal and distal colon human biopsies (130) and found MCT1 to increase moving distally down the GI tract, with negligible protein expressed in the basolateral membranes. The isoforms MCT4 and MCT5 were reported to be present on the basolateral membrane, also increasing down the length of the GI tract. Immunohistochemical staining confirmed the basolateral location of MCT5. There is also evidence for MCT1 localization to the basolateral membrane. One of the first studies characterizing MCT1 in the gastrointestinal tract reported immunofluorescence detection of MCT1 on the basolateral membrane in hamster cecum samples (131). This finding was corroborated in 2006 in mice, rats, and human samples (132). Immunohistochemistry indicated the presence of MCT1 increasing down the length of the GI tract and located basolaterally in mouse and rat samples. This finding was mirrored in mouse cecum electron micrographs. Human samples of the proximal colon also showed basolateral localization of MCT1, primarily expressed in the epithelial colonocytes. Tamai et al. suggested that the cellular location of MCT1 was dependent on the maturity of the cell wherein cells in the crypt expressed MCT1 basolaterally with increased MCT1 in enterocytes brush border (133). No data were presented on the large intestine. Interestingly, MCT1 protein expression was increased in rats after consuming a 2.5% pectin-supplemented diet for 2 weeks (134). MCT1 protein increased by 5-fold in both the small and large intestine after pectin consumption. In the small intestine of control animals, MCT1 protein was primarily expressed basolaterally at the base of villi and crypts. With pectin, the MCT1 immunohistochemical staining increased on the

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brush border of villi. A similar effect was reported in the large intestine where pectin resulted in an increase in apical protein expression of MCT1 in the upper crypt and epithelium; however, basolateral staining was still present. Similarly, Borthakur and colleagues reported an increase in apical immunofluorescence labeling of MCT1 in the colon of rats fed a diet supplemented with 6% pectin for 7 or 14 days (128). These data present an inconclusive story as to the exact localization of MCT1. However, based on these limited studies, it appears that MCT1 is responsive to pectin, a fermentable substrate, and also to SCFA abundance.

Sodium monocarboxylate transporters (SMCT) are another class of SCFA transporter present in the GI tract. SMCT1, also known as SLC5A8, is the best characterized. While MCT1 is electoneutral, SMCT1 transports sodium ions along with SCFA at a debated ratio of 3 to 4 Na^+:1 SCFA (135, 136). SMCT1 is a high affinity transporter with a reported affinity in the following order: butyrate > propionate > lactate > acetate (136). Human and rodent analysis shows that SMCT1 is located on the brush border membrane, with increasing expression from the jejunum to the distal colon (132, 137-139). Importantly, SMCT1 also was shown to be responsive to the microbiota (140). Germ-free mice displayed 85 and 75% less mRNA for SMCT1 in the ileum and colon, respectively, compared to conventional mice. This phenomenon was reflected in protein abundance. Upon initiation of conventional care and, therefore, colonization of the GI tract, SMCT1 mRNA and protein increased to levels consistent with conventional control mice within one week although was not possible to ascertain whether this was due to the establishment of a microbiota or fermentation products. *In vitro* analysis revealed that SMCT1-mediated butyrate transport was increased after incubation with *Lactobacillus plantarum* (2.5-fold) or *L. casei* (1.75-fold) culture supernatants but not *L. acidophilus* or *L. rhamnosus* (141). Incubation with *L. plantarum* culture supernatant also increased SMCT1

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mRNA expression to 300% of control. This is of particular interest, as SMCT1 is reported to be more selective to butyrate compared to MCT1. It can be hypothesized that SMCT1 would be responsive to both prebiotic and probiotic supplementation.

Short Chain Fatty Acid and Taste Receptors

Receptors present in the GI tract are critical in sensing the luminal contents. Several of the receptors described here are localized to enteroendocrine cells and are implicated in signaling for hormone release. The orphan G protein-coupled receptors (GPR) 40-43 were first characterized in 2003 by two different groups with similar findings (142, 143). SCFA were shown to be agonists for two receptors relevant to GI physiology, GPR41 and GPR43. For GPR41 (also known as, and will be referred to, as free fatty acid receptor 3 (FFAR3)), butyrate was reported to have equal or slightly less sensitivity than propionate, followed by acetate. Reports on the SCFA specificity for GPR43 (also known as, and will be referred to, as free fatty acid receptor 2 (FFAR2)) vary. Brown et al reported equal sensitivity, Le Poul et al. observed a greater sensitivity for propionate and equal sensitivity for butyrate and acetate, while Nilsson et al. reported the following observation: acetate = propionate > butyrate (144). mRNA and protein quantification indicated FFAR2 presence in the mucosa of the distal ileum and colon of rats (65, 145). Immunohistochemistry identified the cells expressing FFAR2 as enteroendocrine L cells, with specific co-localization with peptide YY (PYY), but not 5-hydroxytryptamine (5-HT). Similar localization was reported for FFAR3, including co-expression with PYY (146). These findings were mirrored in human colon samples, with FFAR2 expression greater at the crypt epithelium and co-localized with PYY but not 5-HT (147). Finally, FFAR2 also was shown to be responsive to FOS supplementation. In rats receiving a 5% FOS diet for 28 days, FOS

resulted in a non-significant 1.5-fold increase in density of immunoreactive FFAR2 cells in the terminal ileum and a significant 3.3-fold increase in the proximal colon (148). Further evidence of co-expression with GLP-1 but not 5-HT also was presented. In addition to being co-localized with GLP-1, FFAR2 also has been found to mediate its release from enteroendocrine L cells, although a role of FFAR3 also is indicated (149). In primary mouse colon cell cultures, incubation with 1 mM acetate and propionate resulted in an approximately 1.5-fold increase in GLP-1 secretion. This was confirmed with FFAR3$^{-/-}$ and FFAR2$^{-/-}$ mice. Primary cell cultures from FFAR2$^{-/-}$ mice showed a 70% reduction in propionate-induced GLP-1 secretion, while acetate-induced secretion was eliminated. GLP-1 secretion also was reduced in response to SCFA in the FFAR3$^{-/-}$, but was significantly greater than the FFAR2$^{-/-}$. The potential for hormone section mediation is important in IF, as hormonal regulation, particularly GLP-2, is implicated in intestinal adaptation.

Another G protein-coupled receptor, GPR109A, has recently become of interest for butyrate-mediated signal transduction. In 2005, Taggart and others reported that GPR109A was activated by the ketone body, β-hydroxybutyrate, but no other ketones (150). In addition, GPR109A was activated by sodium butyrate at an EC$_{50}$ of 1.6 mM, a luminally-relevant concentration, but not sodium acetate or sodium propionate. Mouse colon samples contain mRNA for GPR109A at increasing abundance from proximal to distal intestine, with immunohistochemistry indicating apical membrane localization (151). Cresci et al. also measured GPR109A mRNA and protein abundance in germ-free mice in comparison to conventional mice with essentially identical results to that of SMCT1 (140). Germ-free mice expressed 90% less GPR109A mRNA in the ileum and 65% less in the colon with similar values for protein. Colonization of the germ-free mice resulted in comparable GPR109A mRNA and

protein to that of conventional mice. Although not as much data are available for GPR109A, its

suspected sensitivity for butyrate is of great interest for IF. As butyrate was previously shown to

result in intestinal adaptation, the effects of pre-and/or probiotic supplementation on GPR109A

must be investigated.

Taste receptors are a relatively new area of research in GI signaling. Taste receptors have

not yet been studied in relationship to SCFA, but research suggests a role in the monitoring of

luminal contents. These sweet and bitter gustatory taste receptors express the transmembrane G

protein, α-gustducin, which may be fundamental in sampling luminal contents (152). Rats

express α-gustducin protein in stomach, duodenum, and colon tissue, primarily on the luminal

side of cells with lesser expression basolaterally (152, 153). mRNA for the family of bitter taste

receptors, type 2 receptors (T2R), have been identified in the duodenum of both mice and rats

and shown to increase intracellular Ca^{2+} concentration in response to bitter compounds *in vitro*

(153, 154). In human colon samples, α-gustducin is localized to enteroendocrine cells

expressing PYY and glucagon-like peptide-1 but not serotonin (5-HT) (155). This suggests that

taste receptors are present on enteroendocrine L cells and are involved in sensing the luminal

chemical composition and regulating levels of these hormones. There are many T2Rs and the

most important in the GI tract have not yet been determined.

Another class of taste receptors is the sweet receptors, or type 1 receptors (T1R). Mouse

mucosal scrapings indicate that mRNA and protein levels of the sweet receptors T1R2 and T1R3

are greatest in the jejunum compared to the duodenum or ileum and that T1R3 levels are

approximately double that of T1R2 (156). The expression of these receptors is greater in the

upper region of the villi compared to crypts. Dyer et al. also showed that T1R2 and T1R3 are

expressed in the enteroendocrine cell line STC-1, but not in enterocyte-type cell lines. NCI-

H716, an enteroendocrine L cell line also was shown to express GLP-1, PYY, α-gustducin, T1R2, and T1R3 (157). Examination of weaning piglet intestinal tissue confirms these findings where T1R2 and T1R3 are expressed in the villi, but not in the crypts, and only a subpopulation of epithelial cells (158). Staining for the enteroendocrine marker, chromogranin A, indicated that T1R2 and T1R3 are localized to enteroendocrine cells, and that not all enteroendocrine cells express T1Rs. Similar to other G-protein-couple receptors, including the GPRs and T2Rs, T1Rs are expressed in enteroendocrine L cells. Human duodenum sample analysis suggests that >90% of duodenal L cells express α-gustducin (157) with co-localization of GLP-1 and T1R3 in duodenum and colon samples (159). Proximal pig intestine shows T1R2 and T1R3 to be co-localized with gastric inhibitory protein (GIP), GLP-1, and GLP-2 (158). A recent examination of T1R expression in germ-free mice showed a 6-fold increase in mRNA and a corresponding increase in T1R3 protein compared to conventional mice (160). Levels of T1R2 were not different. Therefore, the T1R receptors may be a critical component to enteroendocrine sensing and function within the proximal GI tract especially.

Indeed, T1R receptors are implicated in modulating levels of glucose transporters, including SGLT-1 and GLUT2, and also affecting hormone levels following a glucose challenge. Wild-type mice on a high carbohydrate (CHO) diet had 1.6-fold greater mRNA expression for SGLT-1 compared to wild-type mice on a low CHO diet, with similar increases in SGLT-1 protein and glucose transport (161). T1R3 $^{-/-}$ and α-gustducin$^{-/-}$ mice, after consuming the same high CHO diet, maintained SGLT-1 mRNA levels comparable to the low CHO diet wild type mice. This suggests that α-gustducin and/or T1R3 are necessary for the signal transduction resulting in the adaptation to the high CHO diet. Interestingly, the artificial sweetener, sucralose, resulted in the same phenomenon, which also was reported in a piglet study (158). In addition to

impacting SGLT-1, analyses of isolated luminal vesicles from rat jejunum suggested regulation of GLUT2 (162). The artificial sweetener, sucralose (1 mM), in combination with 20 mM glucose, resulted in a 3.4-fold increase in apical GLUT2 levels compared to 20 mM glucose alone, with values similar to that of 75 mM glucose. After a glucose challenge, α-gustducin $^{-/-}$ mice did not experience an increase in plasma GLP-1 as do their wild-type counterparts (157). The impact of T1R signaling on GLP-1 and PYY levels also has been investigated in humans. When an intragastric glucose or whole meal challenge was administered with or without a T1R inhibitor in a double-blind manner, the individuals receiving the inhibitor experienced decreased GLP-1 and PYY levels compared to those not receiving the inhibitor (163). Intragastric administration resulted in a 2-fold greater GLP-1 peak without the inhibitor while PYY levels were 1.5-fold greater. The inhibitor had no effect on the liquid meal and, interestingly, the responses were similar with intraduodenal administration.

In summary, FFAR3, FFAR2, GPR109A, and the taste receptors, T1R2 and T1R3, are localized to enteroendocrine L cells and knockout models and indicate impaired hormone release. The ability of pre- and/or probiotics to modulate GPR and/or T1R signaling are not yet known, but are a promising avenue of research for hormone-mediated intestinal adaptation.

Glucagon-like Peptide 2

The primary hormone implicated in inducing intestinal adaptation is GLP-2. GLP-2 is a 33 amino acid, intestintrophic hormone secreted from the enteroendocrine L cells in the distal intestine. GLP-2 is cleaved post-translationally from proglucagon by the enzyme prohormone convertase 1/3 (164) and has a half-life of approximately 7 minutes. GLP-2's intestintrophic properties were first presented by Drucker and colleagues in 1996, wherein GLP-2 was observed

to increase proliferation and villus length in the jejunum and ileum of mouse intestine (6).

Martin et al. reported similar small intestinal weight, villus length, and proliferation in enterally

fed and total parenteral nutrition (TPN) + GLP-2 SBS rats compared to TPN alone (42).

Similarly, Sigalet et al. showed a 2-fold increase in small intestinal weight and jejunual protein

content corresponding with a 1.5-fold increase in jejunal DNA and villus length in rats with SBS

on TPN + GLP-2 compared to TPN alone (165). In addition to the above-mentioned adaptations

in an SBS rat model, exogenous GLP-2 also was reported to increase ileal GLP-2 receptor (GLP-

2R) mRNA while preserving native proglucagon mRNA (166). However, another investigation

by the same group found that GLP-2 administration in a rat SBS model decreased proglucagon

mRNA with no increase in jejunal GLP-2R mRNA (167). It is important to note that these

investigators performed a jejunocolonic anastomosis and resected the entire ileum. When

animals received GLP-2 for the first 7 days post-surgery, GLP-2 effects including increased

jejunal cellularity, crypt cell proliferation, and decreased apoptosis returned to levels similar to

SBS rats receiving TPN alone by 18 days.

When combined with partial EN, GLP-2 plus partial EN showed a synergistic effect

compared to TPN, partial EN, or GLP-2 alone in rats with SBS (168). A non-resection piglet

model compared the effects of TPN alone, TPN with supplemental GLP-2, and enterally

delivered sow milk (169). Piglet small intestine length, weight, mucosal mass, glucose, and

leucine transport were similar in those receiving TPN with GLP-2 and sow milk, but greater than

TPN alone. The sodium-glucose transporter, SGLT-1, mRNA was increased 3-fold in the distal

small intestine by the EN control and TPN + GLP-2. This increase was reflected in protein

abundance but there was no change in GLUT2 expression. These increases in intestinal weight,

morphology, protein, and DNA also were detected in a non-resection premature piglet model

comparing TPN alone, TPN + GLP-2, and full EN (170). Additionally, these authors noted that GLP-2 decreased intestinal protein breakdown while EN increased protein synthesis. These data indicate that GLP-2 protects against TPN-associated atrophy equally well compared to EN although through potentially different mechanisms. The combination of GLP-2 and EN may produce maximal results.

Human studies of GLP-2, provided by subcutaneous injection, show promising efficacy. Jeppesen and others examined the effect of exogenous GLP-2 administration in 8 SBS patients without a colon (171). After 35 days of GLP-2 treatment, energy absorption increased by 3.5% with a concomitant increase in fluid (11%) and nitrogen absorption (4.7%), although fluid absorption was the only outcome to reach statistical significance. Total body weight increased with lean body mass increasing by 2.9 kg. A longer clinical study of 1 year of active GLP-2 treatment saw a decrease in fecal wet weight by approximately 1 kg/d in 8 SBS patients, but failed to detect a difference in intestinal architecture or body composition (172). These same patients also were assessed for compliance, quality of life and adverse events (172). Overall quality of life increased with GLP-2 treatment and the most commonly reported adverse events were abdominal pain and enlargement of the stoma nipple, which returned to baseline after cessation of GLP-2. Based on potential application in SBS, a GLP-2 analog was developed. This analog, teduglutide (commercially known as GATTEX®), is resistant to degradation by dipeptidyl peptidase IV (173) compared to the native GLP-2 and, therefore, has an extended half-life. A 24-week intervention investigated 0.05 mg/kg/day and 0.1 mg/kg/day teduglutide dose versus control in 83 adult SBS patients (174). Subjects were monitored every 4 weeks for urine output and PN was adjusted accordingly. After 24 weeks, 0.05 mg/kg/day teduglutide resulted in a significant decrease in daily PN requirements from $1,374 \pm 639$ to 354 ± 334 mL. When

comparing the two doses, 45% of subjects in the 0.05 mg/kg/day, 25% in the 0.1 mg/kg/day and 6% of placebo decreased their daily PN by at least 20%. A subset of subjects agreed to provide intestinal biopsies, which showed that the lower dose increased villus length by 45% and the higher dose by 27%. A weight gain of 1.2 to 1.4 kg over the 24 weeks was reported in the teduglutide groups, specifically due to a 2-3.8% increase in lean body mass. There were no differences in the number of adverse events, although 95% of subjects reported at least one occurrence. The most commonly reported adverse events were abdominal pain, nausea, and headache. These same patients were followed and evaluated at 3, 6, and 12 months after discontinuing teduglutide therapy (175). Follow-up data were available for 37 patients, among whom 15 maintained their PN prescription, 7 decreased and 15 increased the amount of PN required. The subjects who increased their PN requirements also experienced a decrease in BMI over the follow-up period and were more likely to not have colon-in-continuity. Teduglutide was approved by the U.S. Food and Drug Administration in December 2012 for use in adult SBS patients with PN dependence.

There is compelling evidence that GLP-2 enhances both structural and functional intestinal adaptation following resection in both animal models and humans. As previously mentioned, SCFA and butyrate specifically are shown to increase plasma GLP-2, suggesting an increase in endogenous GLP-2 production (4). This corresponded with structural adaptations, which may have been mediated by the increase in GLP-2.

SUMMARY

Novel therapies are needed for pediatric IF patients to increase the number who achieve enteral autonomy, thereby improving prognosis and quality of life. The SCFA, butyrate, is a possible nutritional intervention shown to be effective at increasing intestinal adaptation in IF piglet models. Pre- and/or probiotic supplementation is a potential strategy for delivering butyrate to the residual intestine, specifically through increased fermentation and SCFA production. The mechanisms by which butyrate stimulates intestinal adaptation is not currently known; however, GLP-2 is one possibility. Butyrate transport and luminal nutrient sensing are emerging areas of research and may help elucidate a potential mechanism regulating the effects of butyrate. Greater understanding of the impact of pre and/or probiotic supplementation, butyrate production, subsequent intestinal adaptations, and gene expression will help to develop future nutritional therapies for pediatric patients with IF.

CHAPTER 2

RATIONALE AND OBJECTIVES OF RESEARCH

RATIONALE OF RESEARCH

Pediatric intestinal failure (IF) can occur as a result of intestinal resection, most commonly secondary to necrotizing enterocolitis in premature infants (21). These infants are dependent on parenteral nutrition (PN) unless adequate intestinal adaptation and subsequent weaning to full enteral nutrition (EN) is possible. Long-term PN is associated with catheter-related septic events and PN-associated liver disease (21). Patients who develop PN-associated liver disease often require small intestinal transplantation. Until recently, intestinal transplant mortality rates were greater than any other type of transplant but with greater experience, mortality rates are more similar to other organ transplants (176). Nevertheless, 3-year survival rates are reported to be approximately 52%, with the greatest rate of death in patients 5 years and younger (176, 177). Considering these complications, therapies to optimize intestinal adaptation clearly are needed. Previous work from the Tappenden laboratory showed that short chain fatty acid (SCFA)-supplemented PN is associated with increased structural adaptations in a neonatal piglet model of IF, with butyrate being the specific SCFA responsible for the adaptations (4). However, due to butyrate's volatile chemical properties, it is not currently available in PN formulations. Therefore, the objective of this thesis research was to identify a clinically feasible means of providing butyrate to the residual intestine and assess the corresponding intestinal adaptations.

AIMS AND SCOPE OF RESEARCH

The overall **objective** of this thesis research is to examine the effectiveness of prebiotic, probiotic and synbiotic supplementation on intestinal adaptation in a pediatric IF model. Thus, we aimed to assess 1) the structural, functional and cellular kinetic impacts of the prebiotic, short chain fructooligosaccharide (scFOS), probiotic, *Lactobacillus rhamnosus* GG (LGG), and their synbiotic combination; 2) SCFA transporters and receptors as possible mechanisms responsible for mediating adaptations associated with prebiotic and/or probiotic supplementation; 3) prebiotic and/or probiotic supplementation and the impact on gene expression of an enteroendocrine sweet taste receptor and GLUT2. The **central hypothesis** is that strategic prebiotic and probiotic supplementation can be a clinically feasible means of delivering butyrate and stimulating intestinal adaptations in a pediatric intestinal failure model. The following specific aims and hypotheses will be investigated:

Hypothesis 1: Strategic provision of partial enteral nutrition (PEN), specifically formulated to augment butyrate production by the inclusion of pre- and probiotics, will promote structural and functional adaptations in neonatal piglets with SBS by enhancing mucosal surface area and nutrient processing capacity.

Chapter 3 provides evidence that prebiotic scFOS, but not LGG, stimulates structural adaptations in the residual jejunum and ileum, particularly through an increase in proliferation and a decrease in apoptosis resulting in enhanced intestinal architecture and surface area. Nutrient transport was increased in the jejunum but functional adaptations were not observed in the ileum. Glucagon-like peptide-2 was not impacted by treatment. These data raised the

question of possible signaling mechanisms responsible for mediating the intestinal adaptations associated with scFOS.

Hypothesis 2: Butyrate concentrations will increase with prebiotic administration and be associated with an increase in the SCFA transporters, monocarboxylate transporter 1 (MCT1) and sodium-coupled monocarboxylate transporter1 (SMCT1), gene expression and SCFA receptors, free fatty acid receptor 2 (FFAR2) and free fatty acid receptor 3 (FFAR3), gene expression in the residual ileum and colon.

Chapter 4 outlines data indicating an increase in butyrate with scFOS supplementation. Ileal MCT1, SMCT1, FFAR2, and FFAR3 mRNA were increased in piglets receiving pre-, pro- and synbiotic treatment for 24 hours. Colonic mRNA expression of SCFA transporters and receptors were not as significantly impacted in comparison to ileal expression. This suggests that ileal modifications in expression and the corresponding signaling mechanism may be more important for intestinal adaptations. Since G protein-coupled receptors and SCFA transporters were associated with treatment in the distal intestine, we sought to investigate if proximal mRNA expression of receptors and the glucose transporter, GLUT2, were also impacted.

Hypothesis 3: scFOS supplementation will be associated with increased expression of sweet taste receptor T1R3 mRNA in the jejunum and ileum, and a concomitant increase in GLUT2 mRNA.

Chapter 5 presents data showing that jejunal and ileal expression of GLUT2 were minimally impacted by prebiotic and/or probiotic supplementation. Similarly, jejunal T1R3 was not impacted by treatment. Ileal T1R3 expression was not associated with SCFA concentrations but

did change by time. GLUT2 mRNA expression was not associated with the treatment modifications in T1R3 expression.

Chapter 6 summarizes the overall findings of this thesis research and the future directions for this line of research. Particular emphasis is placed on the next steps to increase functional adaptations beyond those observed in this study, especially in the residual ileum. Assessment of whether the intestinal adaptations associated with scFOS therapy are able to support gradual PN weaning will be an important next step. Analysis of the microbiota also will be critical for understanding the ramifications of prebiotic and probiotic supplementation in this model and human infants.

CHAPTER 3

INTESTINAL ADAPTATION IS STIMULATED BY PARTIAL ENTERAL NUTRITION SUPPLEMENTED WITH THE PREBIOTIC SHORT CHAIN FRUCTOOLIGOSACCHARIDE IN A NEONATAL INTESTINAL FAILURE PIGLET MODEL[1]

ABSTRACT

Background: Butyrate has been shown to stimulate intestinal adaptation when added to parenteral nutrition (PN) following small bowel resection but is not available in current PN formulations. We hypothesized that pre- and probiotic administration may be a clinically feasible method to administer butyrate and stimulate intestinal adaptation.

Methods and Materials: Neonatal piglets (48 h old, n=87) underwent placement of a jugular catheter, an 80% jejunoileal resection and were randomized to one of the following treatment groups: 1) control (20% standard enteral nutrition/80% standard PN); 2) control plus prebiotic (10 g/L short chain fructooligosaccharides (scFOS)); 3) control plus probiotic (1 x 10^9 CFU *Lactobacillus rhamnosus* GG (LGG)), or; 4) control plus synbiotic (scFOS + LGG). Animals received infusions for 24 h, 3 or 7 days and markers of intestinal adaptation were assessed.

Results: Prebiotic treatment increased ileal mucosa weight compared to all other treatments (P= 0.017) and ileal protein compared to control (P= 0.049), regardless of day. Ileal villus length

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increased in the prebiotic and synbiotic group (P= 0.011), regardless of day, specifically due to an increase in epithelial proliferation (P= 0.003). In the 7d prebiotic group, peptide transport was upregulated in the jejunum (P= 0.026) whereas glutamine transport was increased in both the jejunum and colon (P= 0.001 and 0.003, respectively).

Conclusions: Prebiotic and/or synbiotic supplementation resulted in enhanced structure and function throughout the residual intestine. Identification of a synergistic prebiotic and probiotic combination may enhance the promising results obtained with prebiotic treatment alone.

CLINICAL RELEVANCY STATEMENT

Optimizing intestinal adaptation following resection is vital to enhancing absorption of enteral nutrients and reducing long-term complications associated with parenteral nutrition (PN). PN supplemented with butyrate is effective, but not commercially available. In a relevant preclinical model for pediatric intestinal failure, these data reveal that the prebiotic scFOS induces intestinal adaptation, whereas the probiotic LGG is less effective. As such, prebiotic supplementation of partial enteral nutrition may be a clinically useful strategy for intestinal rehabilitation, however the pre- and/or probiotic must be carefully selected.

INTRODUCTION

Necrotizing enterocolitis is the most common surgical emergency among infants born preterm (178, 178), which accounted for 12.2% of all births in the United States in 2009 (179). To enable survival of the infant, the necrotic intestine must be removed; however, the infant is often left with inadequate intestinal surface area for digestion and absorption of enteral nutrients

– a state known as intestinal failure (IF) (12). Accordingly, these infants must receive fluid and/or nutrients via parenteral nutrition (PN).

Parenteral nutrition has proven to be life-sustaining for many patients; however, it is believed to result in further loss of intestinal structure and functional capacity (180) and impair the morphological (i.e. dilation, lengthening and thickening) and functional (i.e. digestive and absorptive) adaptations that are known to occur in the remnant intestine following massive small bowel resection (37, 181). If intestinal adaptation is halted or insufficient, dependence on PN may be permanent. With long-term PN dependence, up to 65% of infants develop PN-associated liver disease (PNALD) (182). The only proven treatment for PNALD is PN reduction with increasing enteral feeding made possible by adaptive growth of the remnant bowel (18, 183). Given the immeasurable human suffering and healthcare burden associated with short bowel syndrome (SBS), medical therapies aimed at intestinal rehabilitation are clearly needed.

Short chain fatty acids (SCFA) are a group of intestinal specific fuels produced by anaerobic bacterial metabolism of dietary fiber and resistant carbohydrate. Acetate, propionate and butyrate account for ~85% of formed SCFA and are produced intraluminally in a molar ratio of 60:25:15 (50). Among their properties, SCFA are quickly absorbed by the intestinal mucosa, relatively high in energy, readily metabolized by intestinal epithelium and liver, stimulate sodium and water absorption in the colon, and are trophic to the intestinal mucosa. SCFA have been shown to prevent PN-associated mucosal atrophy (54) and enhance structural indices of adaptation following intestinal resection in rats (53, 55). Our previous work revealed that it is the 4-carbon SCFA, butyrate, that is responsible for these effects (4), which include a critical upregulation in nutrient transport following intestinal resection (7). The concept that butyrate could be used during IF to prepare the intestinal brush-border for effective digestion and

absorption of oral nutrition is particularly attractive; however, butyrate cannot feasibly be added to nutrient formulas because of its volatile nature and putrid properties. Therefore, the use of prebiotics – which are non-digestible food ingredients that selectively stimulate growth and/or activity of a number of potentially health stimulating intestinal bacteria and are fermented to SCFA – provide an attractive clinical alternative (56).

The objective of this work was to explore the efficacy of pre- and probiotics as a clinically feasible method for inducing intestinal adaptation in a pediatric model. We hypothesized that the strategic provision of partial enteral nutrition (PEN), specifically formulated to augment butyrate production by the inclusion of pre- and probiotics, would promote structural and functional adaptations in neonatal piglets with SBS by enhancing mucosal surface area and nutrient processing capacity. To test our hypothesis we utilized a neonatal piglet model of IF. Neonatal piglets are the ideal animal model for human infants as their anatomical, physiological, and metabolic processes are very similar (184, 185). Neonatal piglets are a well-studied clinical model for the PN-fed infant (4, 186) and importantly, show the full clinical spectrum of IF as observed in human neonates following massive small bowel resection (47). Finally, to mimic PEN or 'trickle feeds' aimed at providing enteral stimulation while minimizing malabsorption, 80% of estimated nutritional requirements were provided parenterally, whereas the remaining 20% was provided enterally. As such, our experimental approach allowed for focused investigations in a pre-clinical model of IF in the PN-supported neonatal piglet.

METHODS

Experimental Design

Neonatal piglets (total n= 87; 24 hrs and 3 day piglets, n= 6/treatment; 7 day piglets, n= 9-10/treatment) were obtained from a University of Illinois Urbana-Champaign swine producer within 48 hrs of birth and underwent placement of a jugular catheter and an 80% proximal jejunoileal resection, as described below. Littermate piglets with similar body weights (1.76 ± 0.07 kg) were randomized to one of four treatment groups:

1) **control** (20% standard enteral nutrition (EN)/80% standard PN);

2) control plus **prebiotic** [10 g/L short-chain fructooligosaccharides (scFOS); NutraFlora®, GTC Nutrition, West Chester, IL];

3) control plus **probiotic** [1 x 10^9 CFU *Lactobacillus rhamnosus* GG (LGG); Culturelle®, i-Health Inc., Cromwell, CT], or;

4) control plus **synbiotic** (10 g/L scFOS and 1 x 10^9 CFU LGG).

Within each treatment group, animals were further randomized to receive infusions for various time points following surgery to allow for examination of acute (24 hrs) and chronic (3 or 7 day) adaptations.

Surgical Procedures

All animal procedures were approved by the Illinois Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign. Piglets were fasted for a minimum of 2 hrs preceding surgery. Immediately prior to surgery, piglets were anesthetized with 2% isoflurane (Baxter, Deerfield, IL). Anesthesia was carefully monitored throughout surgery. The incision sites were cleaned with betadine and anaesthetized with a subcutaneous injection of

lidocaine hydrochloride (Sparhawk Laboratories Inc., Lenexa, KS). A subclavian parenteral catheter (3.5 French polyvinyl chloride catheters, Sherwood Medical, St. Louis, MO) was inserted 6 cm through the external jugular and into the vena cava for infusion of PN, as previously described (4). The catheter was tunneled and excised between the scapulae. Piglets were then subjected to an 80% proximal jejunoileal resection leaving 15-cm of jejunum distal to the ligament of Treitz and 75-cm of ileum proximal to the ileocecal junction (4, 7, 9, 55), as measured along the antimesenteric border. Vessels leading to the intestine to be removed were ligated using 5-0 silk sutures (Ethicon, Inc, Somerville, NJ) prior to excision, and measurements of weight and length obtained. Continuity was restored by an end-to-end jejunoileal anastomosis with interrupted 5-0 silk sutures and the laparotomy closed with interrupted ligatures in each of the peritoneum, fascia and skin.

Vital signs and activity levels were monitored in each piglet following recovery from surgery. Buprenorphine analgesic (0.01 mg/kg body weight via I.V.; Henry Schein Inc, Melville, NY) was provided immediately following surgery and every 12 hrs for the next 48 hrs to minimize pain. Ceftiofur sodium antibiotic (1.5 mg/kg body weight via I.V.; Pfizer Inc., New York, NY) was administered to decrease post-operative infection.

Animal Care and Housing

After surgery, piglets were fitted with jackets connected to a swivel tether (Alice King Chatham Medical Arts, Hawthorne, CA) to protect the catheter and infusion lines while allowing free mobility of each piglet. Piglets were housed individually in clean metabolic, clear acrylic glass cages (approximately L 76 cm x W 23 cm x H 46 cm) located at the Edward R. Madigan Laboratory animal care facility at the University of Illinois Urbana-Champaign. HEPA-filtered

airflow to each suite was individually controlled and the room temperature was maintained at 30°C with a 12-hour light/dark cycle. Additional heat was provided by radiant heaters located on top of the cages. Cages were equipped with environmental stimulation and a secured provision of enteral feedings. A full clinical assessment was performed every morning according to the following criteria: weight gain, body temperature, respiration rate, activity level, healing of surgical site, and absence of edema and guarded posture. A partial clinical assessment was performed each evening to re-evaluate piglet condition.

Nutrient Solutions

The prebiotic, probiotic and synbiotic treatments were administered as supplements to the PEN. Nutrient solutions were provided as 20% EN and 80% PN in amounts necessary to meet the daily nutritional requirements of 253 kcal/kg and 12.8 g protein/kg body weight/day, as determined by the National Research Council (187). Volume of formulas provided to each piglet were determined daily based on individual piglet body weight.

Enteral formula was obtained as a polymeric solid sow milk replacer formula (composition outlined in **Table 3.1**, Advance Liquiwean, Milk Specialties Global Animal Nutrition, Eden Prairie, MN). Formula was reconstituted twice daily with deionized water to provide 600 kcal/L composed of 15% (w) solids to 85% (v) deionized water at which time the nutritional treatments were supplemented. Each of the PEN formulas was administered in 3 bolus aliquots each day using bowls within each piglet's metabolic cage. The bowls were tightly screwed into the bottom of each cage, effectively preventing the spillage of formula. Piglet consumption of formula was carefully quantified and, after 24 hours, any remaining formula was orally gavaged to ensure all formula was consumed.

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PN was provided as a 3-in-1 solution (Baxter Clinimix, formulation 2B7740, nutrient composition outlined **Table 3.2** and **3.3**) composed of 5% amino acids and 15% Dextrose in a Dual Chamber 1L bag and 30% Intralipid (Baxter Healthcare Corporation, Deerfield, IL). PN was compounded daily using sterile technique in a laminar flow hood, as outlined in the A.S.P.E.N. Guidelines on Safe Practices for Parenteral Nutrition.(188). PN was continuously infused over a 20 hour period via Flo-Gard 6200 volumetric infusion pumps (Baxter Healthcare Corporation, Deerfield, IL).

Sample Collection

Blood samples were obtained daily and at euthanasia in vacutainers containing EDTA (0.3 mg; Fisher Scientific, Itasca, IL) and 2 U TIU of aprotinin (trypsin inhibitor units; Sigma-Aldrich, St. Louis, MO.). Plasma was separated by centrifugation at 3500 x g for 10 minutes at 4°C and stored at -80°C until further use. At euthanization, 0.25 mL/kg sodium pentobarbital (Veterinary Laboratories, Inc, Lenexa, KS) was administered by IV to each piglet. The intestine was rapidly removed starting with the duodenum excised proximal to the ligament of Treitz and distal of the stomach. The intestine distal to the ligament of Treitz and proximal to the anastomosis was designated jejunum, and ileum included the remaining intestine proximal to the ileocecal valve. The entire colon was removed distal to the ileocecal valve. Segments were weighed and length measured by suspending the intestine longitudinally with a 10 g weight attached to the distal end. Order and location of intestinal sample acquisition was consistent among all piglets to reduce variation due to sampling location. Mucosa was scraped from each segment with a glass slide, snap-frozen in liquid nitrogen and stored at -80°C for analysis of DNA, RNA, protein, and disaccharidase activity. A 1-cm section was scraped free of mucosa

and placed on separate slides for mucosa and submucosa weight. An additional 1-cm section was opened longitudinally, stapled to a square of cardboard at the proximal and distal ends, mucosa side up, and placed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO) for histomorphology and immunohistochemistry analysis. Two 2-cm samples of each intestinal segment were prepared for electrophysiological analysis. Unless otherwise specified, the analysis of all samples was conducted in triplicate.

DNA, Protein, RNA Quantification

Intestinal samples for DNA and protein quantification were homogenized (Tissue Tearor, model 985370, BioSpec Products Inc., Bartlesville, OK) individually for 60 seconds in autoclaved diethyl pyrocarbonate (DEPC; 2 mL/L; Sigma-Aldrich, St. Louis, MO) –treated distilled water at a 1:10 dilution. DNA content of the mucosa samples was determined using the Hoechst microplate method.(189) Mucosal DNA concentration was quantified in sample homogenate using fluorescence at an excitation of 360 nm, emission of 450 nm (SpectraMax Gemini XS fluorometer, Molecular Devices, Sunnyvale, CA), and standard curve methodology with a herring sperm DNA standard (Promega, Madison, WI).

Mucosal protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) with a bovine serum albumin standard (Sigma-Aldrich, St. Louis, MO),(190) using the manufacturer's protocol. Absorbance was measured at 595 nm using a SpectraMax Plus 384 (Molecular Devices LLC., Sunnyvale, CA) to quantify protein.

RNA was isolated from mucosal tissue samples using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD), according to the manufacturers' specifications. An overnight cleaning step was performed by adding 10 µL of 3M sodium acetate at pH 5.2, 250 µL of 100% ethanol,

briefly vortexing and placing samples in a -20°C freezer. The following morning, samples were centrifuged for 15 minutes and the resulting pellet was washed an additional time with 75% ethanol. Total RNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA) at OD260 absorbance, and purity was assessed by determining the OD260/OD280 and OD 260/OD230 ratios.

Morphometric Analysis of the Mucosal Architecture

Intestinal sections were fixed in formalin for less than 24 hrs then transferred to 50% ethanol. Samples were infiltrated with paraffin wax and sectioned to approximately 5 μm thickness with a microtome. A hematoxylin and eosin stain was applied and sections were visualized with Nanozoomer Slide Scanner Digital Pathology System (Hamamatsu, Bridgewater, NJ) and NDP View imaging software. Villus length, mid-villus width and crypt depth was measured in 8-10 vertically well-orientated villi and crypts within each sample.

Epithelial Cell Proliferation

Epithelial cell proliferation was assessed by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). Paraffin was removed from sections by xylene, rehydrated in decreasing concentration ethanol baths then rinsed in tap water for 5 minutes. Antigen retrieval was performed by placing slides in a 95°C citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6.0, Sigma-Alrdich, St. Louis, MO) bath for 10 minutes. After 10 minutes, the citrate bath with slides was placed at room temperature for 20 minutes followed by 7 minutes in room temperature phosphate buffered saline (PBS; 2 mmol/L $Na_2H_2PO_4 \bullet H_2O$, 8.5 mmol/L Na_2HPO_4, 1.5 mmol/L NaCl, pH 7.4). Samples were incubated with 1.5% normal horse serum

(NHS) for 20 minutes to prevent non-specific binding, followed by 60 minute incubation with

primary PCNA antibody (Millipore Billerica, MA) diluted 1:500 in 1% NHS PBS. A

biotinylated universal secondary antibody and ABC complex (Vectastain Elite ABC kit, Vector,

Burlingame, CA) were prepared and utilized according to manufacturers' instructions. Finally,

sections were washed in PBS, stained for 6-8 minutes with VIP (Peroxidase Substrate Kit,

Vector, Burlingame, CA), dehydrated in increasing ethanol baths, finishing with xylene and

cover slipped. Nanozoomer Slide Scanner Digital Pathology System and NDP View imaging

software were used to capture images at 20x magnification. PCNA positive cells in 8-10 well-

oriented crypts of each sample were counted using ImageJ software (National Institutes of

Health, Bethseda, MD).

Epithelial Cell Apoptosis

DNA fragmentation was measuring immuohistochemically with the ApopTag® Plus

Peroxidase *In Situ* Apoptosis Detection Kit (TUNEL assay) (Millipore Billerica, MA) to assess

jejunum and ileum epithelial cell apoptosis. Paraffin was removed from the sections as

described above followed by pretreatment of the tissue with proteinase K, prepared as directed.

Endogenous peroxidase was quenched using a 3.0% peroxide solution, followed by incubation

with the equilibration buffer and TdT enzyme according to kit instructions. Following the

stop/wash buffer and anti-digoxigenin conjugate, the peroxidase substrate DAB was applied. No

counterstain was used as during validation of the assay, methyl green staining overpowered

positive cells. The samples were washed and mounted as instructed in the manufacturer's

protocol.

Epithelial Cell Differentiation

Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to quantify Cdx2 mRNA, a marker of intestinal cell differentiation. Jejunum and ileum mucosal RNA was isolated using the TRIzol method (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) as described above. RNA quantity and quality was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Quantity was calculated from OD260 absorbance and quality was assessed with the OD260/OD280 and OD 260/OD230 ratios. Reverse transcription was carried out using approximately 0.5 μg of isolated RNA, random hexamer primers, and SuperScript® III in a GeneAMP® PCR System 9700 (Life Technologies, Grand Island, NY) to generate cDNA. TaqMan® Universal PCR Master Mix and Cdx2 sus scrofa (Ss033736_m1) were used for the RT-PCR (Applied Biosystems by Life Technologies, Carlsbad, CA). Cdx2 and endogenous 18S controls were measured in separate wells with a TaqMan® ABI 9700. Samples were quantified using the ABI Sequence Detection System software, a pooled cDNA standard curve and normalized to 18S.

Disaccharidase Specific Activity

Intestinal mucosa was homogenized 1:10 in a buffer containing protease inhibitors. All enzyme activity assays were performed in triplicate in addition to a control homogenate without added substrate. Sucrase and lactase specific activities were determined by the method of Dahlquist et al.(191). Liberated glucose was quantified using standard curve methodology after subtracting each sample's internal control for endogenous glucose and normalized to mucosal protein.

Mucosal Ion and Nutrient Transport

The technique utilized for measuring nutrient transport through ion flux in modified Ussing chambers has been described previously by our laboratory (192). Duplicate sections of duodenum, jejunum, ileum and colon were gently stripped of serosa, cut longitudinally and mounted in modified Ussing chambers (Physiologic Instruments, San Diego, CA) to expose 0.5 cm^2 of tissue. The tissue was bathed in 8 mL of oxygenated (95% O2/5% CO2) modified Kreb's buffer maintained at 37°C with a circulating water bath (Fischer Scientific, Itasca, IL). Basal transmucosal short-circuit current, resistance and potential difference were measured after an equilibration period. Active transport was determined by measuring the change in short-circuit current induced by the addition of 10 mmol/L glucose, glutamine, glycyl-sarcosine, proline, arginine or threonine (Sigma-Aldrich, St. Louis, MO) to the medium on the mucosal side. Neurally mediated secretion was measured by the addition of 1 μM serotonin and immune mediated secretion was measured by the addition of 1 μM carbachol (Sigma-Aldrich, Chemical, St. Louis, MO). Dual-channel voltage/current clamps (VCC MC2, Physiologic Instruments) with a computer interface allowed for real time data acquisition and analysis (Acquire & Analyze software, Physiologic Instruments).

Plasma GLP-2 Quantification

Plasma GLP-2 (1-33) was quantified in collaboration with Dr. Jens J. Holst at the Panum Institute at the University of Copenhagen, Denmark. The method utilized has been previously published by Dr. Holst's research team.(193) Briefly, plasma samples were extracted with ethanol, centrifuged, the supernatant lyophilized and reconstituted to the original plasma volume in reaction buffer. Samples were incubated for 24 hrs with GLP-2 antibody raised in Danish

white rabbits to the 11 amino acids of the N-terminus, followed by an additional 24 hour incubation with labeled bovine GLP-2. Free and bound GLP-2 was separated by a plasma-coated charcoal suspension and the supernatant was counted with a gamma counter. GLP-2 was quantified using the standard curve method.

Statistical Analysis

Based on a power analysis using previously obtained SBS neonatal piglet Ussing data, a samples size of 6 per time and treatment combination was determined to be adequate. Upon preliminary analysis, we decided to increase the number of piglets in the 7 day treatment groups to 9-10 per treatment to further increase our ability to detect differences. Results were analyzed as a randomized complete block design. Main fixed effects were time (24 hrs, 3 or 7 days) and treatment (control, prebiotic, probiotic or synbiotic). Differences within day were assessed individually. Litter was included as a random effect. Piglet body weight and GLP-2 data were analyzed using repeated measures. All data was checked for normality by the Shapiro-Wilk statistic. A log 10 transformation was applied to all non-normal data. If a main effect of time or treatment was significant, means were separated using the least squared difference and considered significant at $p \leq 0.05$. Statistical analysis was performed using SAS (Version 9.2; SAS Institute, Cary, North Carolina). All data are reported as mean ± standard error.

RESULTS

Growth and Nutrition Support

Prior to acquiring piglets, each animal was assigned a time point. A total of 94 surgeries were performed with 88 piglets completing the study. The 6 piglets who were not included in final analysis either died due to surgical complications or where euthanized early due to suspected complications. At study start, piglet weights did not differ between treatments (Con= 1.76 ± 0.07; Pre = 1.79 ± 0.07; Pro = 1.76 ± 0.07; Syn= 1.74 ± 0.07 kg, P= 0.937). PN was initiated immediately following surgery and the first EN dose provided that day. Administered EN and PN averaged a total of 204 kcal/day and did not differ between treatment groups (Con= 202 ± 7; Pre = 206 ± 7; Pro = 197 ± 7; Syn= 196 ± 7 kcals, P= 0.630). Daily weight gain did not differ among groups (Con= 0.15 ± 0.02; Pre = 0.16 ± 0.02; Pro = 0.14 ± 0.02; Syn= 0.14 ± 0.02 kg/day, P= 0.855). Final body weight and organ weight, including the stomach, pancreas, liver and kidney did not differ among treatments at each of the time points assessed. Of interest, spleen weight (g/kg body weight) was significantly higher among control piglets than those receiving treatment (Con= 3.14 ± 0.28; Pre = 2.55 ± 0.29; Pro = 2.49 ± 0.28; Syn= 2.49 ± 0.28 g/kg, P= 0.038). This result provokes need for immunological study to the pre- and/or probiotic therapy studied herein as bacterial translocation and infectious susceptibility has been of concern with PN-dependence, albeit controversial (87, 194).

Gross Intestinal Morphology

Prebiotic (P= 0.067) and synbiotic (P= 0.030) treatment increased total jejunal mass, compared to control, regardless of time points studied (pooled treatment means: Con= 1.7 ± 0.2;

Pre = 2.1 ± 0.3; Pro = 1.8 ± 0.2; Syn= 2.2 ± 0.3 g/kg piglet). The prebiotic and synbiotic treatments increased total ileal mass compared to the probiotic group (pooled treatment means: Con= 7.9 ± 0.5; Pre = 8.7 ± 0.5; Pro = 7.1 ± 0.4; Syn= 8.5 ± 0.5, g/kg piglet, P= 0.045). At the mucosal level, ileal mucosal mass (mg/cm) increased with prebiotic administration compared to all other treatments (P= 0.017), regardless of time studied (pooled treatment means: Con= 23.4 ± 2.4; Pre = 31.5 ± 2.4, Pro = 23.9 ± 2.4, Syn= 24.0 ± 2.4). No treatment differences on gross intestinal structure were observed in the duodenum or colon (data not shown).

DNA, RNA, and Protein Concentration

Cellular composition of the intestinal mucosa was assessed through quantification of mucosal DNA, RNA, and protein concentration, as these measures provide insight into mucosal cellularity, transcription and translation. Within the jejunal mucosa, prebiotic and synbiotic treatment resulted in a rapid upregulation of mucosal DNA concentration compared to control (Con= 1.2 ± 0.2; Pre = 2.4 ± 0.2, Pro = 1.8 ± 0.2, Syn= 2.0 ± 0.2 μg DNA/mg mucosa, P= 0.011), likely reflecting an early proliferative response to the stimuli. Mucosal cellularity did not differ among groups in other segments of the intestine. Cellular protein abundance in the jejunal mucosa (**Figure 3.1**) was greater in the prebiotic piglets after 7 days, compared to control and probiotic groups (μg protein/μg DNA, P= 0.017). When examined on a tissue basis, ileal protein abundance (**Figure 3.2**) also increased following 7 days of prebiotic treatment, compared to control (mg protein/cm, P= 0.049).

Crypt-Villus Architecture

Villus height (**Table 3.4**), mid-villus width and crypt depth were measured by histomorphology to assess epithelial surface area. Prebiotic and synbiotic treatment increased ileal villus length compared to control, regardless of treatment duration (**Figure 3.3**; μm, P= 0.011). These remodeled ileal villi were not only longer, but also thinner than those in the control group (Con= 95.0 ± 3.3; Pre = 86.9 ± 3.3; Pro = 90.0 ± 3.3; Syn= 91.7 ± 3.5 μm, P= 0.049). Jejunal villus length also tended (P= 0.054) to increase in response to prebiotic administration, with both the prebiotic and synbiotic groups significantly greater than control. This response to prebiotics also occurred in the duodenum at day 7 (Con= 249 ± 40; Pre = 382 ± 38; Pro = 331 ± 40; Syn= 405 ± 39 μm; P= 0.006). Crypt depth did not differ among treatment groups at any location within the intestine.

Epithelial Cell Proliferation

Epithelial cell proliferation, measured by immunohistochemistry for PCNA-positive cells, increased throughout the intestine in response to prebiotic administration, either alone or in the synbiotic group. Throughout the entire length of the intestine, the prebiotic and synbiotic treatment groups had significantly more PCNA-positive cells/crypt than the control group, regardless of time studied (**Table 3.5**, **Figure 3.4**; duodenum, P= 0.0004; jejunum, P < 0.0001; ileum, P= 0.003; colon, P= 0.0007). Probiotic treatment enhanced epithelial proliferation, regardless of time, in the ileum (P= 0.003), and by day 7 in the colon (P= 0.023).

Epithelial Cell Apoptosis

Apoptosis was measured in the jejunum and ileum by the TUNEL assay for DNA fragmentation (**Figure 3.5**). Both jejunum and ileum experienced a decrease in apoptosis with prebiotic treatment. Specifically, in both segments, the number of apoptotic cells per villus was decreased by the prebiotic and synbiotic treatments, independent of day (**Figure 3.6** and **3.7**; P= 0.030 for jejunum and P= 0.004 for ileum). It should be noted that the difference between the lowest and greatest treatments was approximately 1 apoptotic cell. This is a small quantitative difference, but when considered along the length of the jejunum and ileum, may be biologically relevant.

Mucosal Cell Differentiation

Cdx2 is a marker of cellular differentiation that was quantified in jejunal and ileal mucosa to understand how pre- and probiotics may impact maturation of mucosal cells into distinct, functional units. Pre- and/or probiotic treatment did not alter Cdx2 mRNA abundance in the jejunal mucosa (Con= 0.16 ± 0.01; Pre = 0.15 ± 0.01; Pro = 0.14 ± 0.01; Syn= 0.14 ± 0.01, P= 0.230). In the ileal mucosa, Cdx2 mRNA abundance was increased in the prebiotic group, compared to the control group, regardless of time (**Figure 3.8**, P= 0.033).

Mucosal Disaccharidase Activity

Activity of sucrase and lactase in the intestinal mucosa were not impacted by either treatment or time (data not shown).

Mucosal Ion and Nutrient Transport

Mucosal resistance, a measure of passive ion transport, in the jejunum was decreased by prebiotic treatment, compared to control as a treatment main effect (Con= 44.0 ± 3.2; Pre = 31.0 ± 3.2; Pro = 38.2 ± 3.4; Syn= 38.8 ± 3.5; P= 0.026). In contrast, mucosal resistance in the ileum underwent a transient increase due to prebiotics, that was not maintained at days 3 and 7 (Con= 54.0 ± 15.9; Pre = 101 ± 17.3; Pro = 42.5 ± 17.3; Syn= 61.8 ± 15.6; P= 0.050). Probiotic treatment reduced mucosal resistance in the colon, regardless of time studied (Con= 71.5 ± 6.7; Pre = 57.7 ± 6.6; Pro = 47.1 ± 6.9; Syn= 61.9 ± 6.9; P= 0.048). Potential difference and basal short-circuit current, measures of total and active ion transport respectively, did not differ.

Seven days of prebiotic administration enhanced electrogenic glutamine (**Figure 3.9**, P= 0.001) and peptide (**Figure 3.10**; P= 0.023) transport in the jejunal mucosa compared to control, with similar stimulation of glutamine absorption induced in the synbiotic group. Peptide transport, measured by glycyl-sarcosine, increased in the 7 day prebiotic group in the jejunum (P= 0.026). Ileal threonine transport was greatest in the probiotic group, compared to all others (Con= 2.5 ± 0.6; Pre = 2.2 ± 0.6; Pro = 4.3 ± 0.6; Syn= 1.3 ± 0.6, P= 0.002). Interestingly, electrogenic glutamine transport in the colon was increased by the prebiotic and synbiotic treatments, compared to control (**Figure 3.11**, P= 0.001 and P= 0.003, respectively). No differences were observed in mucosal proline or arginine transport, nor was serotonin- or carbachol-induced chloride secretion impacted in this study (data not shown).

Plasma GLP-2 Concentration

Plasma GLP-2 concentration was not impacted by the nutritional treatments in this study (data not shown); however, plasma GLP-2 concentration increased by 48 hrs following surgery and this concentration was maintained throughout the remainder of the study (24 hrs = 20 ± 5.5 pmol GLP-2/ L plasma; day 2 = 57 ± 5.1; day 3 = 61 ± 6.4; day 4 = 60 ± 5.7; day 5 = 56 ± 7.3; day 6 = 63 ± 7.5; day 7 = 47 ± 7.1; euthanasia = 59 ± 6.4; $P < 0.0001$).

DISCUSSION

The objective of this study was to determine if the provision of pre- and/or probiotics enhance structural and functional aspects of intestinal adaptation in a pediatric IF model. The neonatal piglet supported by PN and partial EN following 80% proximal jejunoileal resection was chosen as it represents a rapidly growing (184), clinically relevant model of pediatric intestinal failure (47). The anthropometric data obtained in this study confirm the adequacy of our nutrition support protocol wherein changes in body weight did not differ between treatment groups at any point in the study, piglets grew at an appropriate rate compared to sow-reared piglets, and the energy and nitrogen content administered, either parenterally or enterally, was equal among groups.

The experimental treatments were carefully selected for their relevance to infants with IF. scFOS was selected as the prebiotic due to its rapid fermentation profile and was administered at a dose (10 g/L) known to produce appropriate butyrate concentrations within the distal ileum and colon (195, 196). This prebiotic has clinical relevance as it is the most studied in human infants, with no adverse outcomes but demonstrated beneficial effects, including reduction of diarrhea

and increased fecal butyrate concentration (197, 198). We specifically chose to use scFOS alone, rather than in combination with trans-galactooligosaccharides (GOS), as found in many commercial infant formulas with prebiotics, due to the extended fermentation profile of GOS that renders it less useful in SBS wherein transit time is greatly reduced. LGG was selected as a clinically-relevant, commercially available probiotic that is well studied in human infants (74, 199). LGG administration is safe and well tolerated with demonstrated benefits even in high risk infants, such as very low birth weight infants and those with advanced necrotizing enterocolitis (73, 200, 201). Of particular importance to this study, LGG consumption augments colonization of the commensal microbiota in both human *and* piglet intestine at the dose included within this study (202, 203). Further, the synbiotic effects of scFOS and lactobacilli species is documented in the literature (204, 205).

Previous work in our laboratory revealed that butyrate-supplemented PN induces intestinal adaptation rapidly after intestinal resection in the neonatal piglet receiving total PN (4). Similar to results obtained with prebiotic therapy in the current study, butyrate-supplemented PN increased epithelial cellularity, villus length, and epithelial cell proliferation in the remnant intestine of piglets. These similarities support our experimental rationale that prebiotic-supplemented partial EN may be a clinically feasible strategy to stimulate intestinal adaptation. The results also show great promise for the inclusion of prebiotics within a comprehensive intestinal rehabilitation program; however the mechanistic relationship to butyrate production awaits further study and the role of other factors, such as the evolving microbiota, inflammatory state, altered luminal contents, and responsive epithelial receptors, should not be overlooked.

The intestinal-specific responses to prebiotic therapy in the current study, varied by time and intestinal segment. It is well established that the ileum undergoes more extensive structural

and functional adaptation following enterectomy than does the jejunum, albeit adaptation does occur in both segments (28, 30). In the residual jejunum, prebiotic therapy, either alone or as part of the synbiotic supplement, resulted in modest structural adaptation, as evidenced by epithelial hyperplasia, a decrease in apoptosis, and a trend toward increased villus length. Athough Cdx2 mRNA abundance did not increase in the jejunum, prebiotic treatment resulted in a doubling of cellular protein abundance and a robust increase in glutamine and peptide transport, providing clear evidence of more mature, functional cells. Although the exact timing cannot be determined, it is possible that the differentiation process occurred between the 3 and 7 day time points, resulting in well- differentiated cells by day 7. Jejunum and ileum intestinal adaptations are summarized in **Figure 3.12** and **3.13**.

In contrast to the functional adaptations that were prominent in the jejunum, the ileum responded with robust structural adaptations. Villus height was increased in the prebiotic and synbiotic treatment group independent of time point, with parallel shifts in epithelial proliferation and a reduction in apoptosis. Unlike the jejunal adaptations, nutrient transport did not differ among treatments. However, ileal Cdx2 mRNA was more abundant with scFOS consumption throughout the study. This indicates an increase in cellular differentiation in the ileum, which may result in functional adaptations at later time points than those studied here. Together, effects in the jejunum and ileum are characteristic of adaptation profiles previously reported. Within the 7 days studied, the residual jejunum increased in function with fewer structural responses occurring following scFOS treatment. On the other hand, the residual ileum underwent marked structural adaptations in response to scFOS supplementation, but functional adaptations had not yet occurred. The increased differentiation marker provides evidence of the molecular sequelae necessary to induce downstream functional adaptation.

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Passive ion transport or resistance measured in modified Ussing chambers was altered by treatment. In the jejunum and colon, the control treatment resulted in the greatest resistance. Interestingly, these are also the intestinal segments that displayed a change in nutrient transport by treatment. Glutamine and peptide transport in the jejunum and glutamine transport in the colon was enhanced by prebiotic treatment. To interpret this dichotomy it is important to note that Na+- dependent nutrient transport is known to cause a dilation of the epithelial tight junctions, leading to a decrease in resistance (206, 207). The current data are consistent with this knowledge wherein the control piglets had the lowest nutrient transport, but the greatest resistance. Prebiotic, probiotic, and/or synbiotic treatments enhanced the activity of various Na^+-dependent nutrient transporters, thereby inducing dilation of the epithelial tight junctions and lowering resistance.

The colon's importance in SBS is established in terms of energy salvage, SCFA production, and reduced dependence on PN (208-212). However, the colon is not generally regarded as a location of carrier-mediated nutrient transport in the adapting intestine; therefore, the increase in colonic amino acid absorption after pre- and/or probiotic treatment should be noted. Recently, Joly and colleagues investigated the colonic adaptations of 7 SBS adults and found no differences in PepT-1 mRNA or protein abundance (213) whereas Ziegler et al. reported an increase in Pept-1 mRNA in 33 SBS adult patients (214). In this study, colonic peptide transport did not differ by treatment. However, colonic absorption of glutamine was enhanced by pre- and/or probiotic treatment. These results do support Ziegler's hypothesis that the colon can adapt to take on absorptive functions in individuals with short-bowel syndrome, however a definitive answer awaits further study.

GLP-2 is an intestinotrophic hormone secreted from the enteroendocrine L cells in the distal intestine and has been shown to enhance intestinal adaptation in both animal models and human SBS patients (165, 168, 169, 172, 174, 215, 216). Previous reports indicate SCFA increase circulating GLP-2 and proglucagon, the peptide from which GLP-2 is cleaved, and has been proposed as the potential mechanism whereby butyrate induces intestinal adaptation (4, 7, 9, 55, 217, 218). Studies providing fiber show inconsistent GLP-2 effects where GLP-2 is increased in diabetic or healthy rats (217, 219) but no change in juvenile SBS piglets (220). In this study, SCFA and specifically butyrate delivery was through the prebiotic scFOS and no GLP-2 differences were found due to treatment. This suggests that structural and functional adaptations reported here is the result of a mechanism independent of GLP-2. Nevertheless, a hormonal mechanism of action is implicated as the distal ileum and colon are the sites of fermentation, but changes in both structure and function were seen in the residual jejunum and proximal ileum prior to the intraluminal fermentation of scFOS.

The potential impact of probiotics, and especially prebiotics in terms of modulating intestinal adaptation require much investigation. Several studies document benefit following probiotic administration (86-88), but prebiotic investigation is limited to a series of reports on the synbiotic combination of *Bifidobacterium breve, Lactobacillus casei,* and the prebiotic GOS (97, 98, 122). In the current study, the probiotic, LGG, and even synbiotic administration was associated with a modest and significantly lower level of adaptation than that of scFOS. Indeed, if LGG were simply inactive within the current model, the synbiotic effect would equal that of the scFOS group. However, the fact that LGG actually negated the scFOS effect in certain outcomes was unexpected. It is possible that LGG administration resulted in remodeling of the microbial community to that which produced less intraluminal butyrate and/or provided less

stimulation/support to the adapting intestine via other mechanisms. Vast alterations in the microbial composition of the fecal and mucosa-associated microbiota occur in adult SBS subjects, wherein a shift between dominant and sub-dominant microbial groups, and an atypical prevalence of *L. mucosae* are reported (123). No data have been obtained in children with SBS, but it is hypothesized that the role of pre- and probiotic therapy in an infant with IF may be more important that in its healthy counterpart (221).

The current data also emphasize the need to determine the optimal synbiotic for maximizing intestinal adaptation in children with IF. Central to this objective is the recognition that fermentable substrates, such as prebiotics, vary greatly regarding their rate and extent of fermentation and each of the probiotics available obviously differ in species and strain. Selection based evidence supporting use in specific disease states is critical. We postulate that a better option would be a combination of scFOS and a probiotic known for its ability to produce butyrate. Further, the infant microbiota differs from that of an adult due to the predominance of bifidobacteria species. As such, use of a bifidobacteria probiotic may be more appropriate for the developing intestine, rather than LGG, despite its common availability within hospital formularies. This knowledge is necessary to select the optimal enteral nutrient formula for therapeutic use in individuals with IF.

Future directions include investigating the effect of SBS, pre- and/or probiotics on the microbiota and SCFA production in the piglet model of IF. The lack of a GLP-2 suggests other mechanisms are at work and warrants further study. Also, the ability of these adaptations to support growth and development during weaning from PN must be studied. In particular, 20% EN may not be great enough to fully stimulate functional adaptation in the ileum, which would

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likely be seen with greater luminal nutrient concentrations. These data will provide important clinical insight into the efficacy of the prebiotic treatment.

In summary, this study indicates that the prebiotic scFOS was highly effective at inducing adaptation in the residual jejunum, ileum and colon. Essentially all significant findings were due to either prebiotic and/or synbiotic treatments. Based on this, we determine that scFOS is responsible for the induction of adaptation with little to no additional benefit, at least in the variables assessed, of the probiotic LGG. Thus, a rapidly fermented prebiotic such as scFOS is a good candidate for intestinal rehabilitation programs but further knowledge is necessary to identify the optimal probiotic and to further elucidate the mechanisms of adaptation. Ultimately, this information will be an important step toward enhancing the nutrition support currently provided to children with IF.

TABLES AND FIGURES

Table 3.1. Nutrient composition of enteral nutrition solution[1]

Nutrient[2,3]	Quantity
Crude Protein[4] (%)	25.0
Crude fat[5] (%)	13.0
Lactose (%)	44.6
Crude fiber (%)	0.00
Calcium (%)	1.07
Phosphorus (%)	0.98
Sodium (%)	0.92
Magnesium (%)	0.10
Potassium (%)	2.23
Copper (ppm)	12.2
Cobalt (ppm)	1.05
Iron (ppm)	114
Manganese (ppm)	29.2
Zinc (ppm)	125
Selenium (ppm)	0.30
Vitamin A (IU/lb)	10,000
Vitamin D_3 (IU/lb)	2,000
Vitamin E (IU/lb)	50.0
Vitamin K (ppm)	3.83
Thiamine (ppm)	5.23
Riboflavin (ppm)	22.0
Vitamin B_6 (ppm)	5.47
Vitamin B_{12} (ppm)	71.0
Pantothenic Acid (ppm)	63.6
Niacin (ppm)	29.9
Folic Acid (ppm)	2.97
Ascorbic Acid (ppm)	89.9
Biotin (ppm)	0.67
Choline (ppm)	2,157

[1]Milk Specialties LiquiWean® (Eden Prairie, MN).
[2]Nutrient composition provided by Milk Specialties.
[3]% composition based on diet wet weight.
[4]Whey protein.
[5]Animal and vegetable fat, mono and diglycerides of vegetable oil.

Table 3.2. Composition of parenteral nutrition solution[1]

Nutrient	Quantity/ 1 L Parenteral Nutrition
Dextrose (g/L)	137
Amino Acids (g/L)	45.7
Lipid (g/L)	28.5
Leucine (g/L)	3.34
Isoleucine (g/L)	2.74
Valine (g/L)	2.65
Lysine (g/L)	2.65
Phenylalanine (g/L)	2.56
Histidine (g/L)	2.19
Threonine (g/L)	1.92
Methionine (g/L)	1.83
Tryptophan (g/L)	0.82
Alanine (g/L)	9.47
Arginine (g/L)	5.26
Glycine (g/L)	4.71
Proline (g/L)	3.11
Serine (g/L)	2.29
Tyrosine (g/L)	1.83
Sodium (mmol/L)	32.0
Potassium (mmol/L)	27.4
Magnesium (mmol/L)	9.15
Calcium (mmol/L)	2.01
Chloride (mmol/L)	35.7
Phosphate (mmol/L)	13.7

[1]Parenteral nutrition was provided as a 3-in-1 solution composed of 5% amino acids with electrolytes and 15% dextrose in a Dual Chamber 1L bag (Baxter Healthcare Corporation, Deerfield, IL Clinimix, formulation 2B7740). 30% Intralipid (Baxter) was injected into each Dual Chamber bag. Final solution provided 0.91 kilocalories per mL. Overall macronutrient composition was 51.5% carbohydrate, 20.2% protein, and 28.3% lipid.

Table 3.3. Intravenous micronutrient solution[1]

Micronutrient	Quantity/ day
Vitamin A[2] (IU)	2,000
Vitamin D[2] (IU)	300
Vitamin E (IU)	1.20
Vitamin K (mg)	0.04
Thiamin (B1)[3] (mg)	0.51
Riboflavin (B2)[3] (mg)	0.08
Niacin (B3)[3] (mg)	0.51
Pantothenic Acid (B5)[3] (mg)	0.20
Vitamin B6[3] (mg)	0.20
Biotin (B7) (mg)	0.81
Folic Acid (B9) (mg)	0.03
Cobalamin (B12)[3] (µg)	0.20
Vitamin C (mg)	16.2
Chromium[4] (ng)	0.32
Copper[4] (ng)	32.4
Manganese[4] (ng)	24.0
Selenium[4] (ng)	1.60
Zinc[4] (µg)	0.65
Iron (mg)	2.25

[1]Micronutrients were provided once daily through the jugular catheter.
[2]From a combination solution containing 500,000 IU vitamin A/ mL and 75,000 IU vitamin D/mL.
[3]B vitamin complex contains 12.5 mg/mL thiamin hydrochloride, 12.5 mg/mL niacinamide, 5 mg/mL, 5 mg/mL pyridoxine hydrochloride, 5 mg/mL p-panthenol, 2 mg/mL riboflavin, and 5 µg/mL cyanocobalamin.
[4]From MTE-5 containing 0.3 µmol/L chromium, 25.2 µmol/L copper, 21.6 µmol/L manganese, 1.0 µmol/L selenium, and 492 µmol/L zinc.

Table 3.4. Small intestine villus length[1-3]

	Treatment				P Value	Effect[4]
	Con	**Pre**	**Pro**	**Syn**	**Txt**	
DUODENUM						
Mean (pooled within txt)	340 ± 28	405 ± 28	361 ± 27	371 ± 31	0.100	
JEJUNUM						
Mean (pooled within txt)	361 ± 25	431 ± 23	370 ± 23	430 ± 26	0.054	
ILEUM						
Mean (pooled within txt)	375 ± 28	474 ± 28	408 ± 28	487 ± 30	**0.011**	**Pre, Syn > Con**

[1]Data are expressed as mean ± SEM.

[2]Unit of measure is microns.

[3]Abbreviations used: Con - control; Pre - prebiotic treatment; Pro - probiotic treatment; Syn - synbiotic treatment; Txt –treatment.

[4]Treatment groups not listed in the effect column do not differ from the other treatment groups.

Table 3.5. Epithelial proliferation, as assessed by quantifying proliferating cell nuclear antigen-positive cells per crypt[1-3]

	Con	Pre	Pro	Syn	Txt	Effect[4]
		Treatment			P Value	
DUODENUM						
7 days	17.7 ± 0.8	20.8 ± 0.8	17.7 ± 1.0	18.7 ± 0.8	**0.024**	**Pre > Con, Pro, Syn**
Mean (pooled within txt)	16.3 ± 0.6	19.3 ± 0.6	17.4 ± 0.6	18.3 ± 0.6	**0.0004**	**Pre, Syn > Con**
JEJUNUM						
7 days	18.5 ± 0.6	23.0 ± 0.6	20.4 ± 0.6	22.1 ± 0.7	**<0.0001**	**Pre, Syn > Con**
Mean (pooled within txt)	16.4 ± 0.6	20.4 ± 0.5	17.8 ± 0.5	18.8 ± 0.5	**<0.0001**	**Pre, Syn > Con**
ILEUM						
7 days	17.6 ± 1.2	21.9 ± 1.2	17.9 ± 1.2	21.9 ± 1.2	**0.0003**	**Pre, Syn > Con, Pro**
Mean (pooled within txt)	16.0 ± 0.8	18.9 ± 0.7	17.3 ± 0.7	19.0 ± 0.7	**0.003**	**Pre, Pro, Syn > Con**
COLON						
7 days	26.2 ± 1.5	32.4 ± 1.6	30.6 ± 1.3	30.9 ± 1.6	**0.023**	**Pre, Pro, Syn > Con**
Mean (pooled within txt)	25.9 ± 0.8	30.3 ± 0.8	27.3 ± 0.8	29.5 ± 0.8	**0.0007**	**Pre, Syn > Con, Pro**

[1]Data are expressed as mean ± SEM.
[2]Unit of measure is the number of positively stained proliferating cell nuclear antigen cells per crypt.
[3]Abbreviations used: Con - control; Pre - prebiotic treatment; Pro - probiotic treatment; Syn - synbiotic treatment; Txt –treatment.
[4]Treatment groups not listed in the effect column do not differ from the other treatment groups.
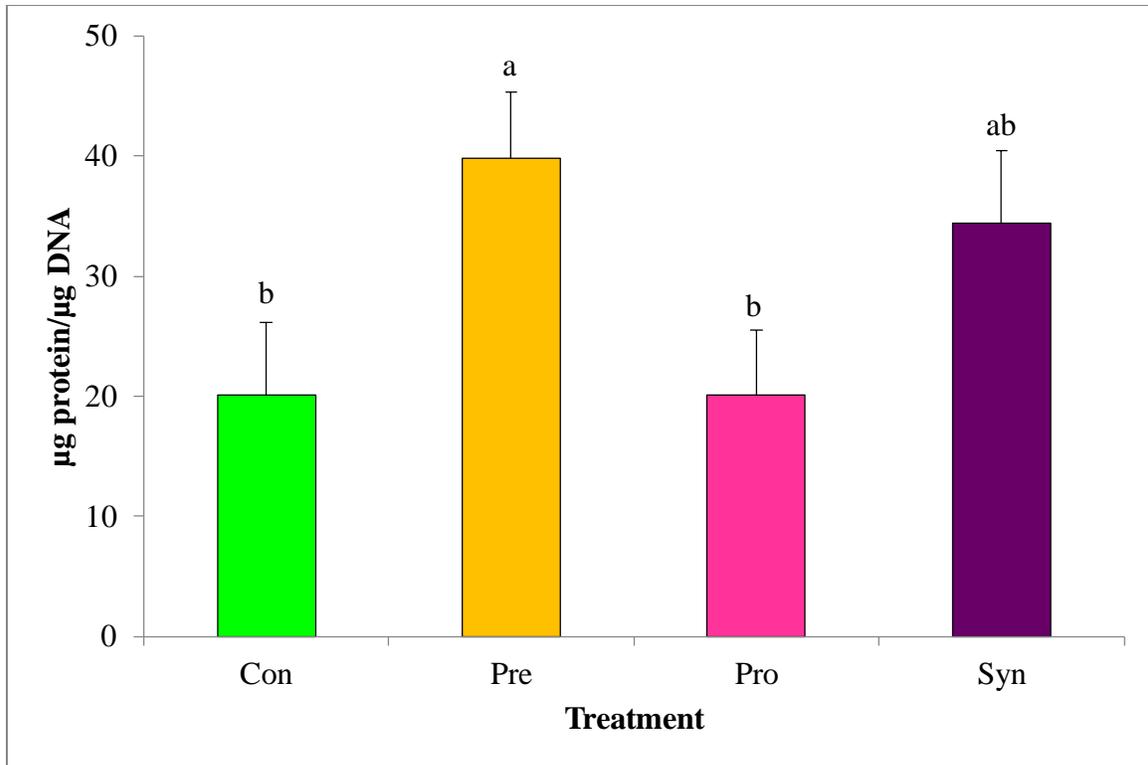
Figure 3.1. Jejunum mucosa protein content after 7 days of treatment. Data are expressed as pooled treatment means ± SEM. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. Different letters over bars within each panel indicate a statistically significant difference. In the jejunum, prebiotic treatment resulted in greater protein per cell compared to control and probiotic treatment (P= 0.017).
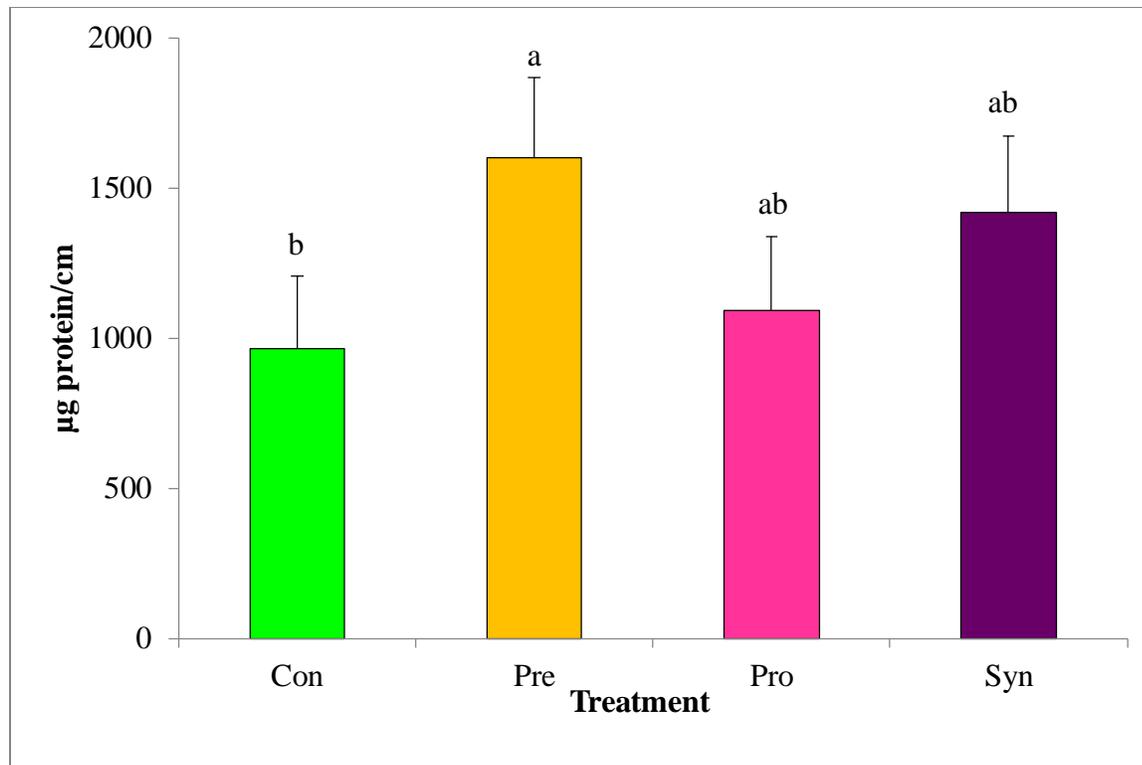
Figure 3.2. Ileum mucosa protein content after 7 days of treatment. Data are expressed as pooled treatment means ± SEM. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. Different letters over bars within each panel indicate a statistically significant difference. In the ileum, prebiotic treatment resulted in greater protein per length compared to control (P= 0.049).
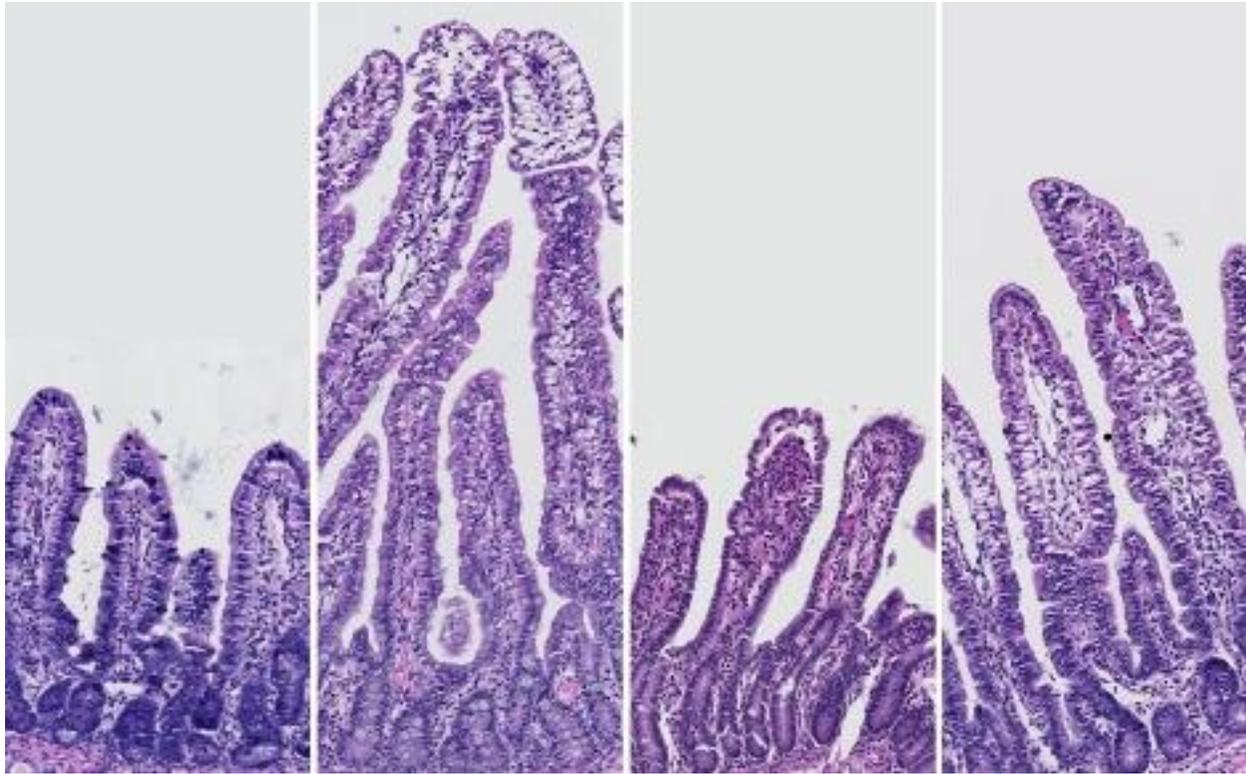
| Control | Prebiotic | Probiotic | Synbiotic |

Figure 3.3. Ileal villus height after 7 days of control, prebiotic, probiotic or synbiotic treatment where prebiotic and synbiotic treatments result in longer villi than control or probiotic treatments. Magnification of 10X with hematoxylin and eosin stain. Prebiotic and synbiotic treatments resulted in greater villus height compared to control and probiotic treatments, regardless of time (P= 0.011).
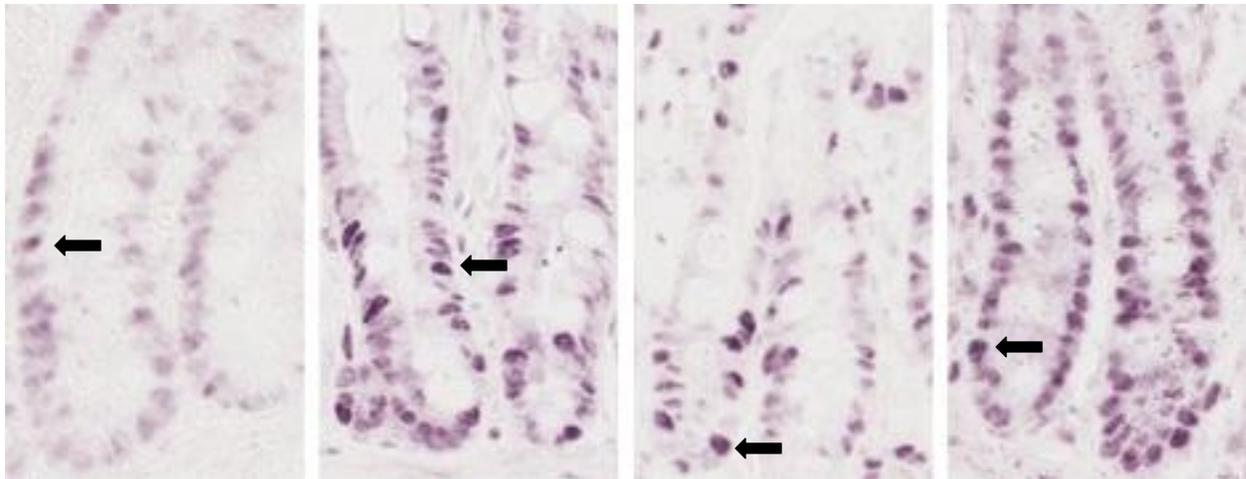
| Control | Prebiotic | Probiotic | Synbiotic |

Figure 3.4: Ileal epithelial cell proliferation after 7 days of control, prebiotic, probiotic, or synbiotic treatment, as measured by quantifying the number of proliferating cell nuclear antigen (PCNA)-positive cells per crypt.   Black arrows indicate PCNA positive cells. Magnification of 20X with VIP stain (Peroxidase Substrate Kit, Vector Burlingame, CA). When treatment means are pooled, prebiotic, probiotic, and synbiotic treatments resulted in significantly more PCNA-positive cells than control (P= 0.006).
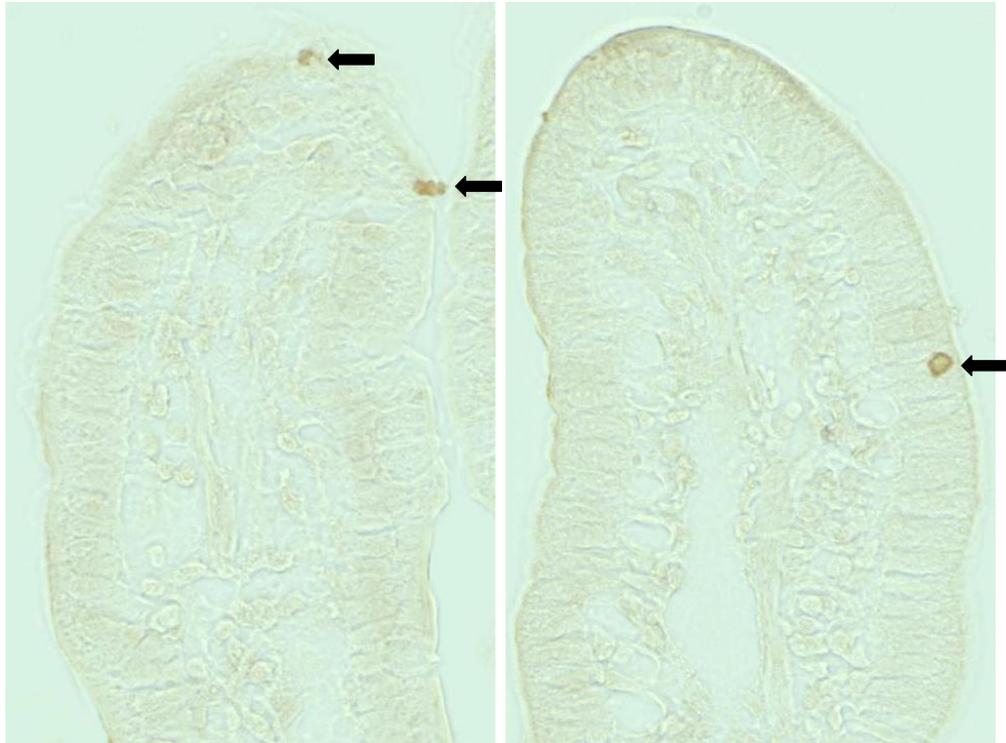
Control   Prebiotic

Figure 3.5: Jejunum epithelial cell apoptosis after 24 hours of control or prebiotic treatment, as measured by TUNEL staining for DNA fragmentation. Black arrows indicate apoptotic cells. Magnification of 40X with DAB stain. When treatment means are pooled, prebiotic and synbiotic treatments resulted in significantly less apoptotic cells per villus than control (P= 0.030).
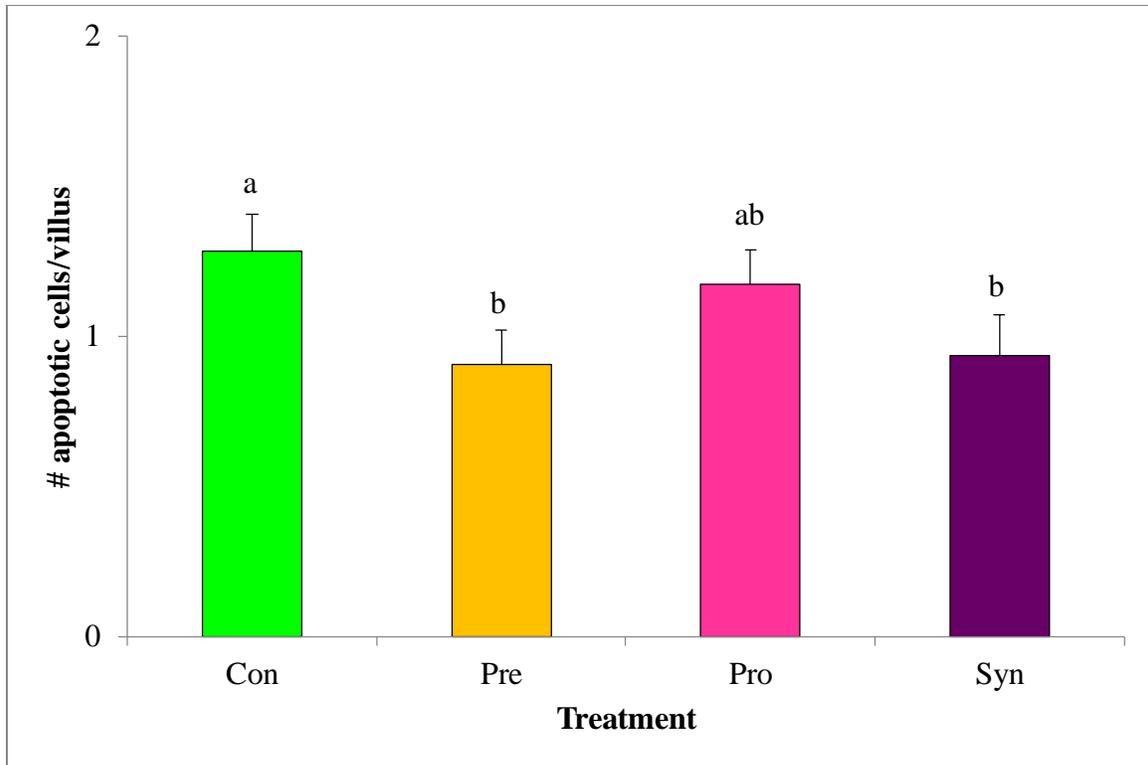
Figure 3.6. Jejunum mucosal cell apoptosis, as measured by DNA fragmentation. Data are expressed as pooled treatment means ± SEM. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. Different letters over bars within each panel indicate a statistically significant difference. In the jejunum, prebiotic and synbiotic treatments resulted in fewer apoptotic cells per villus compared to control (P= 0.030).
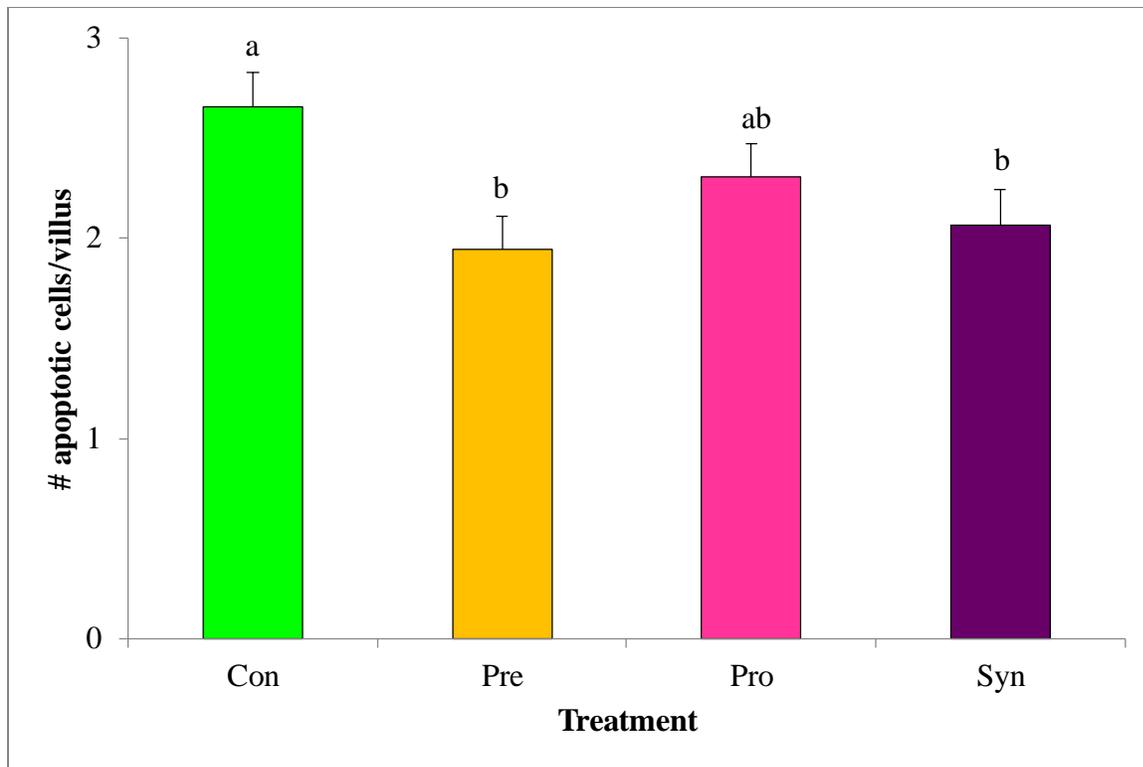
Figure 3.7. Ileum mucosal cell apoptosis, as measured by DNA fragmentation. Data are expressed as pooled treatment means ± SEM. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. Different letters over bars within each panel indicate a statistically significant difference. In the ileum, prebiotic and synbiotic treatments resulted in fewer apoptotic cells per villus compared to control (P= 0.004).
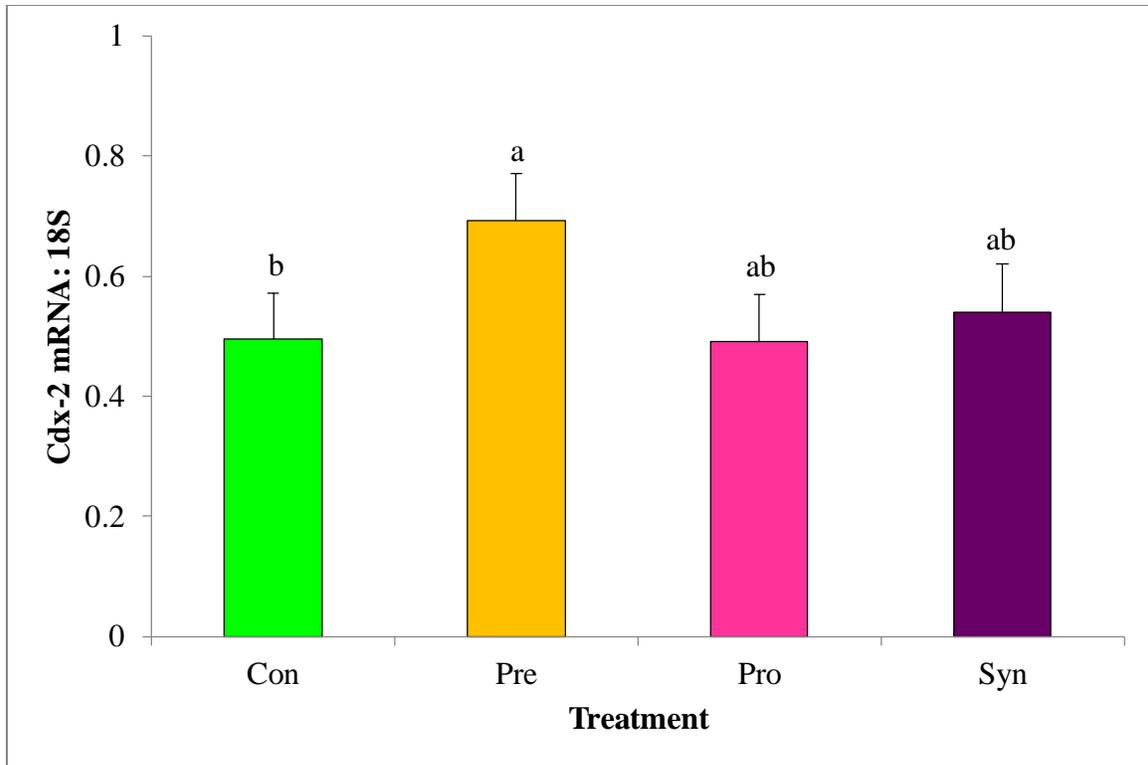
Figure 3.8. Ileal mucosal cell differentiation, as measured by Cdx2 mRNA abundance. Data are expressed as pooled treatment means ± SEM. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. Different letters over bars indicate a statistically significant difference. Prebiotic treatment pooled by time resulted in greater differentiation compared to control treatment (P= 0.033).
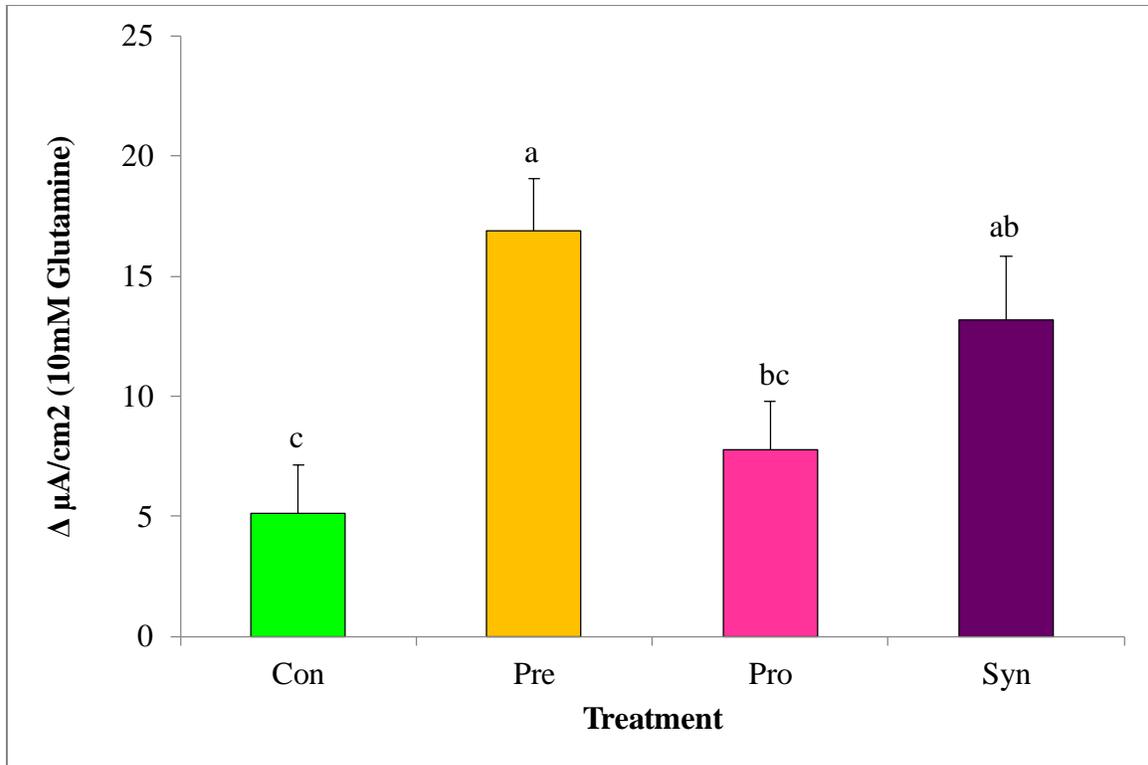
Figure 3.9. Glutamine transport in the jejunum measured by modified Ussing chambers after 7 days of treatment. Data are expressed as 7 day treatment means ± SEM. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. Different letters over bars within each panel indicate a statistically significant difference. Glutamine transport was increased in the jejunum by prebiotic and synbiotic treatment after 7 days (P= 0.001).
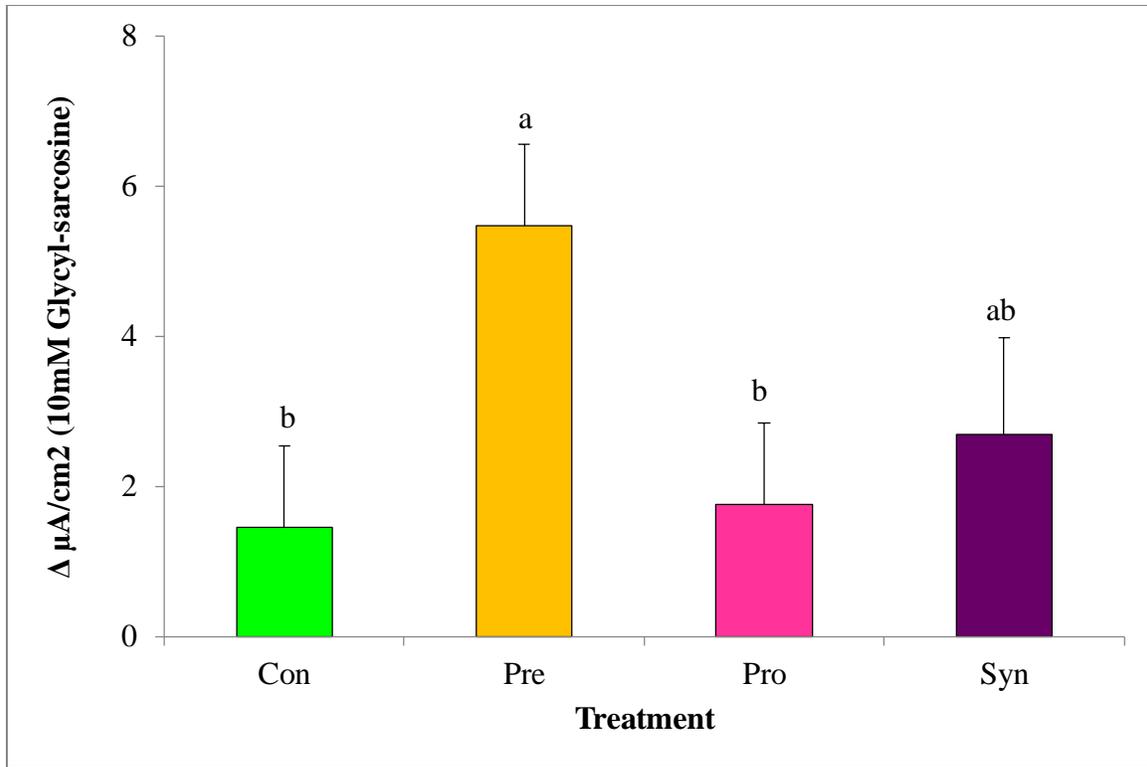
Figure 3.10. Glycyl-sarcosine transport in the jejunum measured by modified Ussing chambers after 7 days of treatment. Data are expressed as 7 day treatment means ± SEM. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. Different letters over bars within each panel indicate a statistically significant difference. Glycyl-sarcosine transport, a measure of peptide transport, increased in the jejunum after 7 days of prebiotic supplementation (P= 0.026).
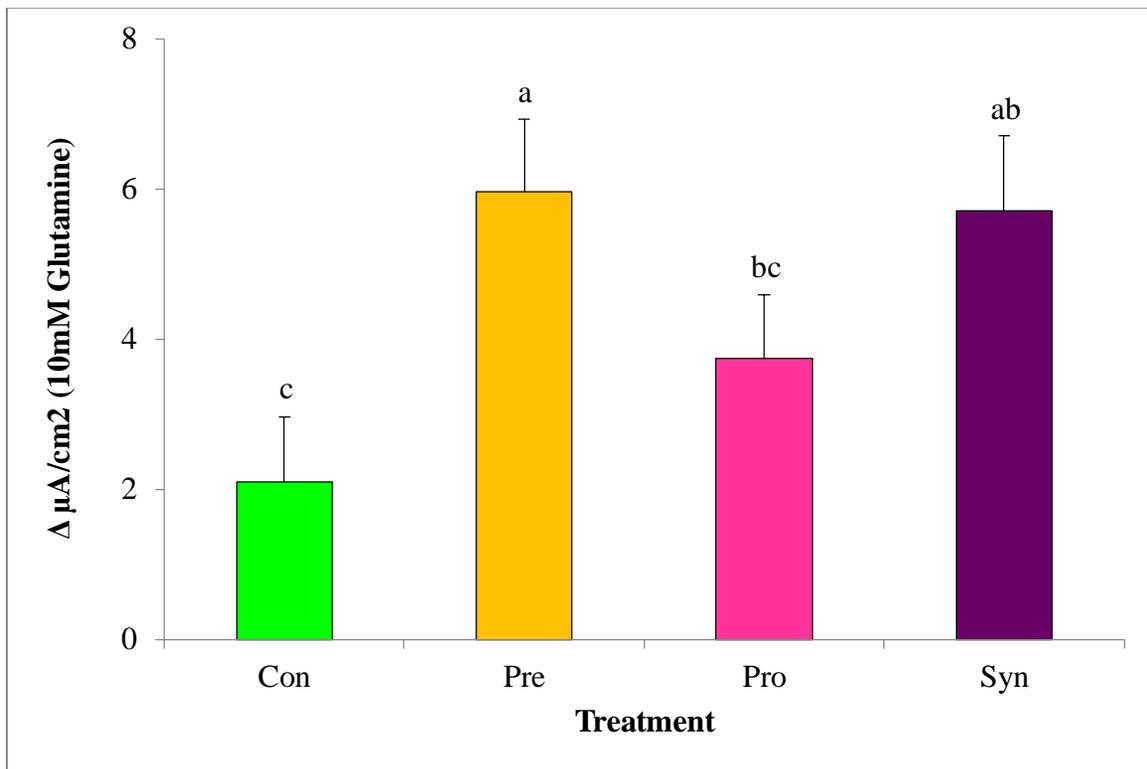
Figure 3.11. Glutamine transport in the colon measured by modified Ussing chambers after 7 days of treatment. Data are expressed as 7 day treatment means ± SEM. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. Different letters over bars within each panel indicate a statistically significant difference. Prebiotic and synbiotic treatment increase glutamine transport in the colon after 7 days (P= 0.003).
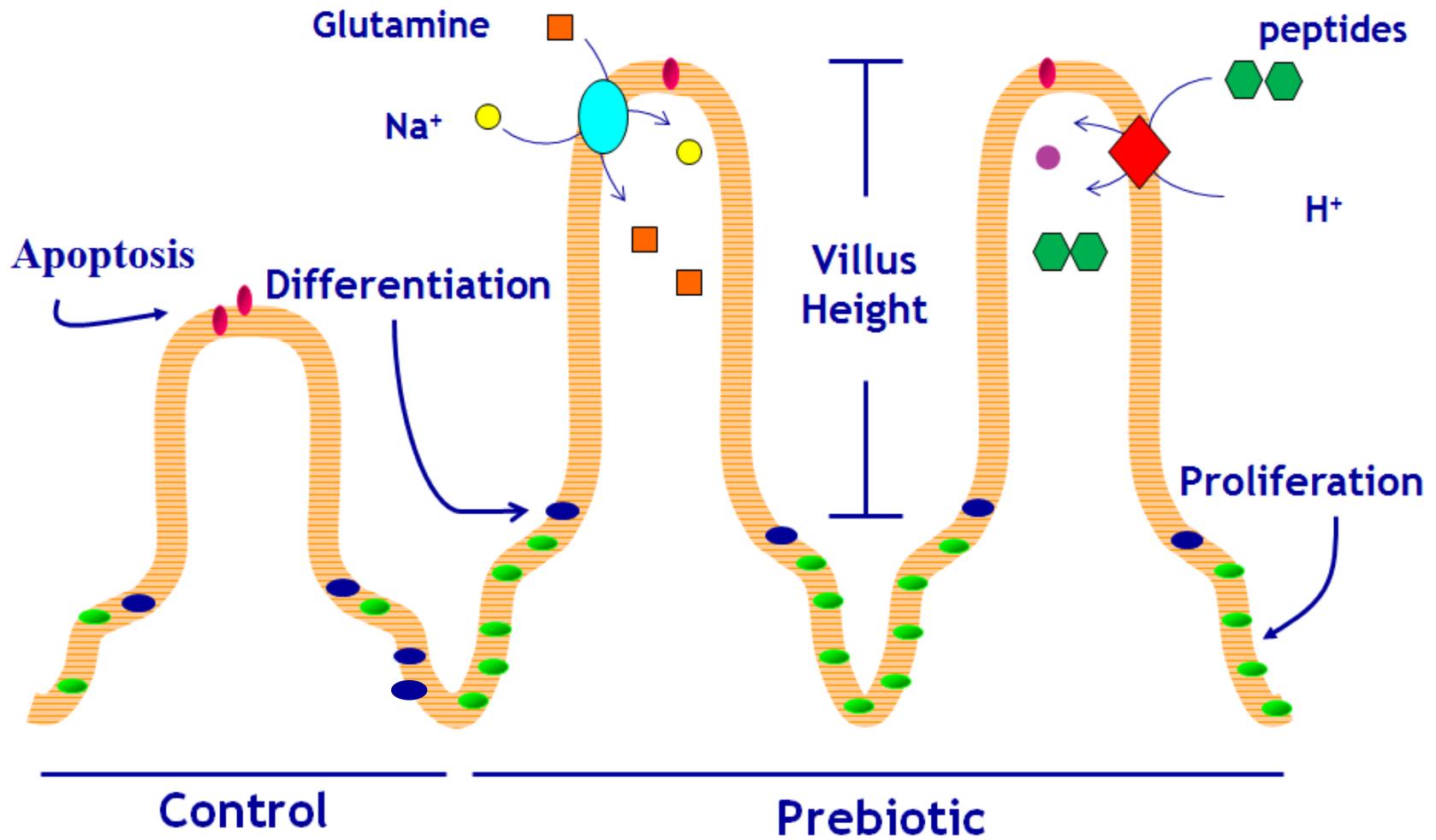
Figure 3.12: Summary of adaptations in the residual jejunum. Prebiotic treatment was associated with decreased apoptosis, increased proliferation and an increase in villus length. Glutamine and peptide transport was increased.
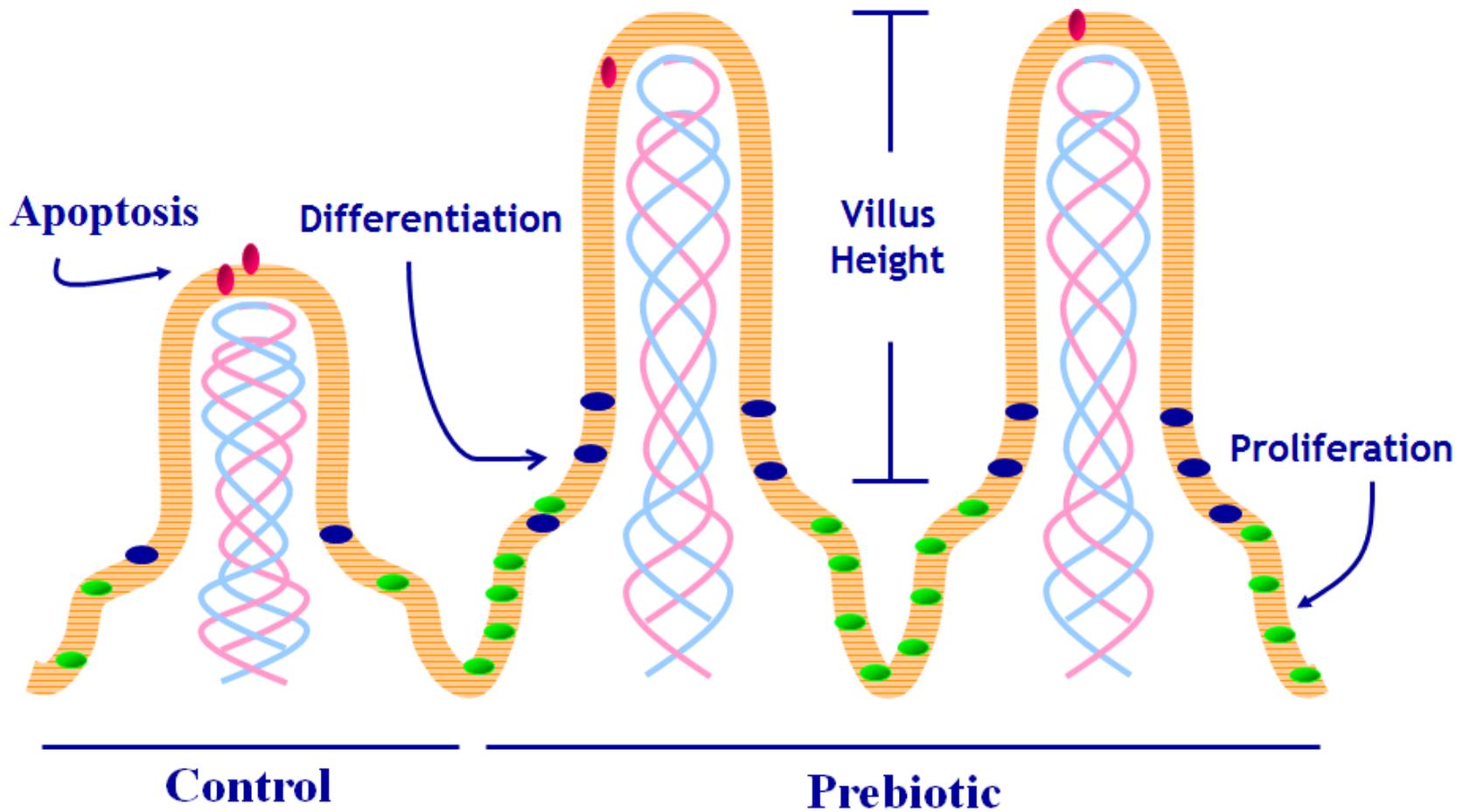
Figure 3.13: Summary of adaptations in the residual ileum. Prebiotic treatment was associated with decreased apoptosis, increased proliferation and an increase in villus length. Differentiation was also increase but there was no measurable increase in functional markers.

CHAPTER 4

PREBIOTIC SHORT CHAIN FRUCTOOLIGOSACCHARIDES INCREASE BUTYRATE AND ARE ACUTELY ASSOCIATED WITH EXPRESSION OF SHORT CHAIN FATTY ACID TRANSPORTERS AND RECEPTORS

ABSTRACT

Butyrate, a 4-carbon short chain fatty acid (SCFA), increases intestinal adaptation in intestinal failure (IF) but the mechanism by which butyrate interacts with the intestinal epithelium is unknown. SCFA transporters present on enterocytes and SCFA receptors present on enteroendocrine cells are possible signaling mechanisms through which butyrate may initiate intestinal adaptation. We hypothesized that prebiotic supplementation would increase butyrate concentration in the proximal colon, resulting in greater monocarboxylate transporter 1 (MCT1), sodium-coupled monocarboxylate transporter 1 (SMCT1), and free fatty acid receptors (FFAR2 and FFAR3) mRNA expression in an IF piglet model. To test our hypothesis, we used a neonatal piglet model of IF. Piglets (n= 87, 48 h old) underwent an 80% jejunoileal resection and placement of a jugular catheter. Piglets received 80% parenteral (PN) and 20% enteral nutrition (EN) for 24 h, 3 or 7 d. **Control** piglets received unsupplemented EN, **prebiotic** piglets received 10 g short chain fructooligosaccharides (scFOS)/L EN, **probiotic** piglets received 1×10^9 CFU *Lactobacillus rhamnosus* GG per day, and **synbiotic** piglets received scFOS plus LGG. Data were analyzed as a randomized block design and considered significant at $P \leq 0.05$. Prebiotic supplementation was associated with increased butyrate concentration in proximal colon digesta compared to control treatment, independent of time (P= 0.050), while other SCFA or lactate did not differ. Total acetate, propionate, and butyrate concentrations tended to increase after 3 d of synbiotic treatment (P= 0.078). Ileal MCT1 and SMCT1 mRNA increased in

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prebiotic, probiotic, and synbiotic treatments compared to control after 24 h (P= 0.012 and 0.014, respectively) while FFAR2 and FFAR3 mRNA were greatest in the prebiotic and probiotic groups vs. control after 24 h (P= 0.013 and 0.008). Three days of synbiotic treatment was associated with greater ileal MCT1 mRNA compared to control (P= 0.004), ileal FFAR2 mRNA compared to prebiotic, and probiotic treatment (P= 0.017), ileal FFAR3 mRNA compared to probiotic treatment (P= 0.039), and colonic FFAR2 mRNA compared to control, prebiotic, and probiotic treatment (P= 0.009). On day 7, ileal MCT1 mRNA was greater in control, prebiotic, and probiotic treatments vs. synbiotic treatment (P= 0.004). FFAR2 and FFAR3 ileal mRNA expression was greatest in probiotic compared to prebiotic and synbiotic treatments but similar to control (P= 0.025 and 0.004). Colon mRNA levels were not as significantly impacted by treatment. This investigation suggests that acute upregulation of MCT1, SMCT1, FFAR2, and FFAR3 mRNA may be an important signaling component of intestinal adaptations. In particular, upregulation of FFAR2 and FFAR3 on enteroendocrine cells in the ileum may be mechanistically responsible for more proximal jejunal and also residual ileum adaptations. Enhanced expression of these SCFA transporters and receptors also were associated with greater SCFA concentrations in the colonic lumen with 3 d of synbiotic treatment.

INTRODUCTION

Intestinal failure (IF) results from a decrease in functional intestinal mass below the amount required for digestion and absorption to meet the body's needs (10, 11). IF results in dependence on parenteral nutrition (PN) in order to ensure adequate fluid and nutrients to support growth of pediatric patients (12). While PN must be utilized long-term in IF, there are life-threatening complications associated with long-term PN dependence including catheter

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infections, PN-associated liver disease as well as decreased quality of life (18, 19, 21, 23). Therefore, therapies to support adaptation of the residual intestine, PN-weaning and enteral autonomy are needed. Short chain fatty acids (SCFA) are products of bacterial fermentation of non-digestible carbohydrates in the distal intestine. The three primary SCFA produced in humans are acetate, propionate, and butyrate generated at an *in vivo* molar ratio of approximately 60:25:15 (50). Butyrate is a 4-carbon SCFA, stimulates sodium and water absorption and serves as a primary energy source for colonocytes (51, 52). In addition, butyrate has been shown to be intestinotrophic. This makes butyrate of interest for application in short bowel syndrome (SBS) and IF.

SCFA have been investigated as a possible strategy for stimulating intestinal adaptation following resection. SCFA-supplemented PN was first demonstrated to increase structural adaptations in rodent models on PN with and without intestinal resection (53-55). More recently, butyrate has proven to be the SCFA responsible for the enhanced intestinal adaptations (4, 222). Administration of prebiotics or digestion-resistant food ingredients fermented by the host microbiota resulting in selective growth and/or activity of beneficial bacteria (56), are a possible strategy for supplying butyrate to the intestinal epithelium. We originally hypothesized that prebiotic supplementation would be associated with intestinal adaptation but that a concomitant probiotic, or live microorganisms that when consumed in adequate amounts confer a health benefit to the host (71), may be necessary to augment colonization of the immature microbiota in a model of pediatric IF. However, when this hypothesis was tested in a neonatal IF piglet model, the prebiotic, short chain fructooligosaccharides (scFOS), proved to be more effective at stimulating intestinal adaptation following resection compared to the probiotic *Lactobacillus rhamnosus* GG (LGG) or their synbiotic combination (5). Further investigation is needed to

better understand the mechanism through which prebiotics and probiotics influence intestinal adaptation.

Butyrate transporters and receptors are potential mediators of luminal sampling and signal transduction in butyrate-stimulated intestinal adaptation. SCFA transporters in the distal intestine include monocarboxylate transporter 1 (MCT1) and sodium-coupled monocarboxylate transporter 1 (SMCT1) (125, 126, 136). SCFA receptors are expressed in enteroendocrine cells within the distal intestine and include free fatty acid receptor 2 (FFAR2, also known as G protein-coupled receptor 43) and free fatty acid receptor 3 (FFAR3, also known as G protein-coupled receptor 41) (142, 143, 145). To the best of our knowledge, mRNA expression of these SCFA transporters and receptors have not been previously investigated in the dynamically adapting luminal environment following intestinal resection.

The objective of this investigation was to quantify the effect of prebiotic and/or probiotic supplementation in 20% enteral nutrition (EN) on SCFA and lactate concentrations in the proximal colon and the corresponding gene expression of MCT1, SMCT1, FFAR2, and FFAR3 in a neonatal piglet model of IF. We hypothesized that prebiotic scFOS supplementation would be associated with increased butyrate concentrations in the proximal colon and greater mRNA expression of MCT1, SMCT1, FFAR2, and FFAR3.

METHODS AND MATERIALS

Experimental Design

The experimental design, surgical procedures, animal care, nutrient solutions and sample collection have previously been outlined in detail (5). Briefly, neonatal piglets (n= 87; 24 h and 3 d piglets, n= 6/treatment; 7 d piglets, n= 9-10/treatment), 48 h old, underwent placement of a

jugular catheter, an 80% jejunoileal resection and were randomized to one of four treatment groups: 1) **control** (20% standard EN/80% standard PN); 2) control plus **prebiotic** (10 g scFOS /L EN); 3) control plus **probiotic** (1 x 10^9 CFU (LGG)); 4) control plus **synbiotic** (10 g scFOS/L EN and 1 x 10^9 CFU LGG). Piglets were further randomized to receive treatments for 24 h to represent acute adaptations or 3 or 7 d to represent chronic intestinal adaptations. Following the predetermined treatment length, piglets were euthanized with a 0.25 mL sodium pentobarbital/kg (Veterinary Laboratories, Inc, Lenexa, KS) dose administered by the jugular catheter. The abdomen was carefully opened and the entire gastrointestinal tract gently excised. Distal to the stomach and proximal to the ligament of Treitz was considered duodenum, distal to the ligament of Treitz and proximal to the anastomosis was designated jejunum, distal to the anastomosis and proximal to the cecum was designated ileum, and proximal to the cecum was considered colon. Proximal colonic digesta samples were immediately collected, snap-frozen in liquid nitrogen, and stored at -80°C for analysis. For each intestinal segment, mucosa was scraped with a glass slide, snap-frozen in liquid nitrogen and stored at -80°C.

SCFA and Lactate Concentrations

SCFA concentrations were measured by gas chromatography according to the method of Erwin et al (223, 224). Triplicate samples of proximal colonic contents were acidified and diluted (1:4 w/v) with 25% meta-phosphoric acid and distilled water, vortexed, and incubated at room temperature for 30 min. The samples then were placed in a -20°C freezer overnight. The next morning, samples were thawed and centrifuged for 10 min at 12,000 x g. The clear supernatant was collected and placed in a gas chromatography vial for processing in the gas chromatogram (Hewlett-Packard 5890A series II gas chromatograph, Palo Alto, CA) and glass

column (180 cm × 4 mm i.d.; packed with 10% SP-1200/1% H_3PO_4 on 80/100+ mesh Chromosorb WAW, Supelco Inc., Bellefonte, PA). Samples were stored at 4°C for less than 1 month. Nitrogen was used as the carrier gas with a flow rate of 75 mL/min. Lactate was quantified using a protocol adapted from Barker and Summerson (225).

Quantification of MCT-1, SMCT-1, FFAR2, FFAR3 mRNA abundance

RNA was isolated using the TRIzol method (Life Technologies, Inc., Gaithersburg, MD), following manufacturers' instructions with the addition of an overnight cleaning step. Specifically, after completion of the TRIzol protocol, 10 µL of 3M sodium acetate at pH 5.2, and 250 µL of 100% ethanol were added followed by vortexing, and placement at -20°C. The next day, samples were centrifuged for 15 min, washed one additional time with 75% ethanol and suspended in autoclaved diethyl pyrocarbonate (DEPC)-treated distilled water. RNA integrity was assessed with RNA gel electrophoresis by running 10 µg RNA on a denaturing agarose gel at 100 V until the bromophenol blue dye reached the end of the gel. The 18S and 28S rRNA bands were examined using a UV illumination system (Fotodyne Foto/Analyst Image Capture System; Fotodyne, Hartland, WI). Total RNA was quantified with a NanoDrop 1000 spectrophotometer at OD260 absorbance, and purity further assessed by determining the OD260/OD280 and OD 260/OD230 ratios that were deemed acceptable ≥1.8.

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to quantify mRNA. MCT1, SMCT1, FFAR2, and FFAR3 mRNA were quantified in ileum and colon mucosa samples. Reverse transcription of ileum and colon mucosal RNA was carried out with approximately 0.5 µg of isolated RNA, random hexamer primers, and SuperScript® III in a GeneAMP® PCR 9700 System to generate cDNA. TaqMan® Universal PCR Master Mix

and MCT1 (Ss03374095_m1) and FFAR2 (Ss03374174_s1) sus scrofa primer/probes were used

for RT-qPCR (Applied Biosystems by Life Technologies, Carlsbad, CA). Sus scrofa

primer/probes were not available for SMCT1 and FFAR3. These primer/probes were designed

based on rodent sequences compared to the Sscrofa9 pig genome sequence using Primer Express

2 software (Applied Biosystems). The primer used for SMCT1 was composed of the following

sequences: forward AATCAAACCTCGCTTCAGATCTCT; reverse

CACTCATTCCTTGGGAAATCCA; with probe TCAGAAAGGTCCCTCTC. The primer used

for FFAR3 was composed of the following sequences: forward

AGAGGGTGGCAGGACTTGTG; reverse GCCCAAAGCAGACGAGGAA; with probe

AGCCACGCTGCTC. All primer/probes were validated by assessing the efficiency and R^2 of

the pooled cDNA standard curve. Each sample was run in triplicate, normalized to endogenous

18S and quantified using a pooled cDNA standard curve.

Statistical Analysis

Statistical analysis was conducted with SAS version 9.2 (SAS Institute, Cary, North

Carolina). The data were analyzed as a randomized complete block design with main effects of

time and treatment and blocked by the random litter effect. Differences within time points were

assessed individually. A log 10 transformation was applied if the data proved to be non-normal

according to the Shapiro-Wilk test. Means were separated by least squared difference when

main effect p-values were ≤ 0.05. All data are presented as mean ± standard error.

RESULTS

SCFA Concentrations

SCFA concentrations in proximal colon digesta were quantified to assess the contribution of prebiotic and/or probiotic supplementation to fermentation products and potential regulatory impact on SCFA transporter and receptor mRNA (**Table 4.1**). Prebiotic supplementation, pooled by time, was associated with increased butyrate concentration compared to control while synbiotic treatment increased the concentration of isobutyrate, a branched chain fatty acid (BCFA), compared to control (µmol/mg dry matter, P= 0.048 and 0.032 respectively, **Figure 4.1** and **4.2**). Acetate, propionate, total SCFA (the combination of acetate, propionate and butyrate), BCFAs valerate, and isovalerate were not statistically different by treatment (**Table 4.1**). In addition, the molar ratio of actetate: propionate: butyrate was not statistically impacted by treatment (Con= 71.3 ± 2.23: 19.8 ± 1.97: 8.85 ± 0.99; Pre = 69.2 ± 2.57: 20.0 ± 2.36: 10.9 ± 1.13; Pro = 72.4 ± 2.03: 18.2 ± 1.82: 9.54 ± 0.90; Syn= 68.2 ± 2.84: 21.2 ± 2.39: 10.6 ± 1.25; P= 0.296 acetate; 0.367 propionate; 0.248 butyrate). Acetate, propionate, and butyrate concentrations did not differ at individual time points (data not shown). Total SCFA including acetate, propionate, and butyrate did not differ at 24 hours or 7 days (data not shown). However, after 3 days, synbiotic treatment was associated with the greatest concentration of total SCFA (acetate, propionate, and butyrate) within the proximal colon digesta (P= 0.078, **Figure 4.3**). Isobutyrate concentrations were statistically significant at day 3, with the greatest isobutyrate concentrations with synbiotic treatment compared to control and probiotic treatments (P= 0.028, **Figure 4.4**).

Lactate Concentrations

Lactate concentrations, when analyzed as a main effect of treatment, did not differ between control, prebiotic, probiotic, and synbiotic piglets (P= 0.101, **Figure 4.5**). Likewise, treatment did not differ within individual days (24 hours, P= 0.310; 3 days, P= 0.136; 7 days, P= 0.139, data not shown).

SCFA Transporter and Receptor mRNA Expression: Main Effects of Treatment

Ileal and colon MCT1 (ileum: P= 0.233; colon P= 0.222), SMCT1 (ileum: P= 0.170; colon: P= 0.233), FFAR2 (ileum: P= 0.220; colon: P= 0.138), and FFAR3 (ileum: P= 0.114; colon: P= 0.085) mRNA expression did not differ as main effects of treatment (data not shown).

SCFA Transporter and Receptor mRNA Expression

After 24 hours of treatment, ileal SCFA transporter mRNA was greater with pre-, pro-, and synbiotic treatment compared to control (MCT1 P= 0.012; SMCT1 P= 0.014, **Figure 4.6**). Ileal SCFA receptor mRNA also was increased with prebiotic and probiotic treatment after 24 hours. Specifically, FFAR2 (P= 0.013) and FFAR3 (P= 0.008) mRNA was significantly greater in ileum mucosa with prebiotic and probiotic treatment compared to control (**Figure 4.7**).

SCFA transporter and receptor mRNA expression patterns differed after 3 days of treatment compared to 24 hours. In the ileum, 3 days of treatment was associated with greater MCT1 mRNA expression in control and synbiotic piglets compared to probiotic piglets (P= 0.004, **Figure 4.8**). There were no significant treatment differences detected in ileal SMCT1 after 3 days of treatment (P= 0.080, **Figure 4.8**). Ileal FFAR2 mRNA expression was significantly greater in synbiotic treatment animals compared to prebiotic and probiotic but was

similar to control (P= 0.017, **Figure 4.9**). Three days of synbiotic treatment was associated with increased FFAR3 mRNA in ileum mucosa compared to the probiotic treatment but did not differ from control or prebiotic administration (P= 0.039, **Figure 4.9).**

Seven days of treatment corresponded with significantly less ileal MCT-1 mRNA with synbiotic treatment compared to all other treatments (P= 0.004, **Figure 4.10**). SMCT1 mRNA expression was not statistically different by treatment in the ileum (P= 0.113, **Figure 4.10**). By day 7, piglets receiving probiotic treatment had greater FFAR2 mRNA expression in the ileum compared to pre- and synbiotic piglets (P= 0.025, **Figure 4.11**). Ileal FFAR3 expression was greatest in probiotic piglets compared to prebiotic and synbiotic treatment (P= 0.004, **Figure 4.11**). Control piglets expressed greater ileal FFAR3 mRNA than synbiotic treatment but did not differ from probiotic treatment.

Colon MCT1 (P= 0.123), SMCT1 (P= 0.051) and FFAR2 (P= 0.089) mRNA expression were not statistically different by treatment after 24 hours while FFAR3 mRNA was significantly greater with probiotic treatment compared to pre- and synbiotic treatment (P= 0.005, **Table 4.2).** After 3 days of treatment, colonic MCT1 (P= 0.242) and SMCT1 (P= 0.356, **Table 4.2)** expression did not differ by treatment. Increased colonic FFAR2 mRNA expression was associated with synbiotic treatment compared to control, prebiotic, and probiotic (P= 0.009, **Table 4.2**) while colonic FFAR3 expression did not differ by treatment after 3 days (P= 0.290, **Table 4.2**). Colonic MCT1 (P= 0.386), SMCT1 (P= 0.057), FFAR2 (P= 0.090), and FFAR3 (P= 0.170) mRNA levels were did not differ by treatment after 7 days (**Table 4.2**).

DISCUSSION

The objective of this study was to establish possible mechanisms regulating prebiotic scFOS-associated structural and functional adaptations in a neonatal piglet model of pediatric IF. We hypothesized that colonic luminal butyrate concentrations would increase with prebiotic administration and be associated with an increase in butyrate transporter and receptor mRNA. Indeed, butyrate concentration increased in the prebiotic group, but total SCFA and molar ratios of acetate: propionate: butyrate remained unchanged. Other groups also have shown an increase in butyrate and/or SCFA production with scFOS both *in vitro* and *in vivo* (195, 196, 226, 227). A likely explanation for the modest increase in butyrate and lack of increase in overall SCFA production is the amount of scFOS supplemented in this study. The design was intended to mimic clinical practice in pediatric IF. Infant formulas on the market generally contain 4-10 g/L of a galactooligosaccharide and fructooligosaccharide mix. This study utilized 10 g scFOS /L, but enteral feedings only provided 20% of nutrient needs, similar to a trickle feed in a neonatal intensive care unit. As a result, piglets received approximately 1-2 g scFOS daily, depending on body weight. However, the increase in butyrate suggests that as enteral intake and, therefore, scFOS increased, butyrate production would also increase. Finally, it should be noted that quantifying SCFA concentrations from colonic contents provides a single time point for measurement and does not take into account production and absorption/oxidation rates. Interpretation of SCFA concentration data must be made with these caveats. Significant changes may be due to an increase in production or a decrease in oxidation. Likewise, a lack of difference may either be a true finding or the result of modified absorption and/or oxidation rates.

Specific increases in other SCFA including acetate and propionate, BCFA including valerate and isovalerate, and the fermentation intermediate lactate were not different as a main effect of treatment or within individual time points. The lack of difference in lactate was not anticipated given that two of the treatment groups were receiving lactate-producing LGG. However, lactate is a fermentation intermediate rather than a fermentation-end product. Therefore, any lactate produced would be rapidly metabolized to SCFA (228). Although lactate concentrations among treatments did not reach significance, there was a trend for lactate to be greater with prebiotic supplementation. This is in-line with the increase in butyrate concentrations seen with prebiotic treatment. Although the prebiotic piglets did not receive a lactic acid-producing probiotic, the limited research in microbial communities and SBS suggests that lactobacilli populations may be increased (123, 229). Additionally, lactate can be further fermented by the microbiota to produce butyrate that provides a possible rationale for the increase in both lactate and butyrate with scFOS administration (230).

In vitro evidence by other groups indicates that MCT1 mRNA expression is mediated by physiologically relevant butyrate concentrations (127), suggesting that MCT1 is critical in butyrate transport. In particular, an *in vitro* investigation using luminal membrane vesicles showed a dose-dependent increase in MCT1 mRNA and protein after 48 hours of incubation with 0, 1, 2, or 5 mM sodium butyrate (127). An *in vivo* investigation showed that MCT1 was upregulated in the small and large intestine of rats with 2.5% pectin supplementation (134). Likewise, SMCT1 had a high affinity for butyrate and was affected by the microbiota with impaired expression in germ-free mice and a return to conventional levels with colonization (136, 140). The orphan G protein-coupled receptors (GPR) 40-43 were first characterized in 2003 and were involved in luminal sensing of the distal gastrointestinal tract (142, 143). FFAR3

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(GPR41) was reported to have the greatest sensitivity for butyrate followed by propionate and acetate. Reports on the SCFA specificity for FFAR2 (GPR43) vary, but may be approximately equal for acetate, propionate, and butyrate (142, 143). Similar to MCT1, FFAR2 was responsive to FOS supplementation. Rats receiving a 5% FOS diet for 28 days experienced a non-significant 1.5-fold increase in the density of immunoreactive FFAR2 cells in the terminal ileum and a significant 3.3-fold increase in the proximal colon (148). These investigations laid the ground work for our hypothesis that increased luminal butyrate concentrations with scFOS supplementation would be associated with increased mRNA expression of MCT1, SMCT1, FFAR2, and FFAR3 in the residual ileum and colon. While these SCFA transporters have been investigated in *in vivo* animal models, there were no studies available in the literature investigating their potential role in SCFA-mediated intestinal adaptation following intestinal resection. This model presents a greatly altered luminal environment with additional variables including transit time, substrate provision, and microbial remodeling that may play a role in expression of these specific genes and the corresponding signaling mechanism.

Twenty-four hours of prebiotic, probiotic, and synbiotic treatment was associated with 2 to 3-fold greater mRNA expression of both SCFA transporters MCT1 and SMCT1 in the ileum compared to control. Similar increases were apparent in ileal SCFA receptors FFAR2 and FFAR3 after 24 hours of treatments. This acute increase in gene expression represents a possible signaling mechanism through which intestinal adaptations occurring at later time points may be initiated. This is particularly pertinent for enhanced adaptations observed in the jejunum and ileum with prebiotic and synbiotic treatment. The increase in FFAR2 and FFAR3 expression represent a possible initial step in elevated signaling and hormone release through activation of these G-protein coupled receptors. These statistically significant findings are particularly notable

considering that RNA was isolated for total mucosal preparations and enteroendocrine cells make up only a small percent of the total cells. Colonic expression of MCT1 was not statistically significant after 24 hours. However, there were trends for an increase in colonic expression with probiotic treatment in SMCT1, FFAR2, and FFAR3. This suggests that the luminal environment created by LGG alone, in contrast to that of the synbiotic combination of LGG + scFOS, may be stimulating increased expression in the colon; however, these increases in mRNA expression were not associated with increased structural or functional adaptations with probiotic treatment. In addition, MCT1 expression in the colon was approximately 2 times that of SMCT1 and in potentially adequate quantities for SCFA absorption through that mechanism. These increases in expression and, potentially, hormonal signaling may be the initiating action resulting in structural adaptations that were evident at earlier time points, main effects of treatment, and also functional adaptations that were not detected until 7 days of treatment. **Figure 4.12** outlines a working model for intestinal adaptations initiated through FFAR2, FFAR3, MCT1, and SMCT1.

Three days of synbiotic treatment was associated with a trend for increased total acetate, propionate, and butyrate concentrations and significantly increased isobutyrate concentrations in proximal colon digesta. This increase in SCFA and isobutyrate were reflected in significantly greater mRNA expression of MCT1, FFAR2, and FFAR3 in the ileum and FFAR2 in the colon after 3 days of synbiotic treatment. There was also a trend for greater SMCT1 mRNA expression with synbiotic administration in the ileum. Interestingly, the greater SCFA concentrations with synbiotic treatment as measured in the proximal colon were more closely associated with increased SCFA transporter and receptor mRNA in the ileum. Specific SCFA concentrations were not measured in the ileum but, given the rapid fermentation of scFOS and well-established presence of microbiota in the ileum, SCFA were likely produced in this location

as well. Increases in both FFAR2 and FFAR3 mRNA occurred at 3 days in the ileum whereas only FFAR2 was increased in the colon. FFAR2 was hypothesized to have approximately equal sensitivity for acetate, propionate and butyrate, which corresponds to the increase in colonic SCFA concentrations and FFAR2 mRNA as measured at 3 days. The ileum experienced an increase in both FFAR2 and FFAR3 that may be due to preferential oxidation of butyrate and/or propionate at this more proximal location. Finally, after 7 days of treatment, synbiotic treatment mRNA levels for ileal MCT1 and FFAR3 decreased below that of control, prebiotic and probiotic treatments. SMCT1 in the ileum and both SCFA receptors in the colon did not differ by treatment at this chronic experimental time point. This further suggests that the acute response in these SCFA transporters and receptors are an early signaling mechanism for butyrate-stimulated adaptations that are detected at the chronic time points.

The more profound differences in ileal mRNA expression of the SCFA transporters and receptors are associated with greater overall adaptation occurring within this tissue. Specifically, prebiotic and synbiotic treatments were associated with increases in ileal mucosal mass and protein, villus height, proliferation, and decreased apoptosis as previously described (5). The jejunum also experienced functional adaptations after 7 days of treatment. As the jejunum is proximal to the primary location of fermentation, the alterations in SCFA receptor mRNA in the ileum serve as a potential signal for hormone secretion resulting in these proximal adaptations. Treatment group was not as greatly associated with colonic expression of these receptors and transporters as compared to ileal mucosa. In fact, MCT1 and SMCT1 mRNA levels did not reach significance by treatment in the colon. Interestingly, colonic tissue did not undergo the same extensive remodeling as seen in the residual ileum. Specifically, there was no difference in gross morphology, cellular composition, or crypt depth among treatments.

Overall, these data indicate that supplementation of the prebiotic, scFOS, in an IF piglet model increases luminal butyrate concentration. This is a critically important step in stimulating structural and functional adaptations as previously reported (4, 7-9, 53-55). Acute administration of scFOS, LGG and the synbiotic combination were associated with greater ileal expression of SCFA transporters MCT1 and SMCT1, and SCFA receptors FFAR2 and FFAR3. This represents a possible signaling mechanism responsible for the adaptations detected in the jejunum and ileum.

Table 4.1. SCFA Concentrations[1,2]

| | Treatment | | | | P Value | | |
	Con	Pre	Pro	Syn	Txt	Time	Effect[3]
Acetate						0.011	**Day 1 < 3, 7**
Mean (pooled within txt)	89.0 ± 26	160 ± 47	107 ± 25	112 ± 34	0.181		
Propionate						0.219	
Mean (pooled within txt)	23.9 ± 7.9	43.5 ± 17	24.1 ± 7.4	34.3 ± 14	0.214		
Butyrate						0.026	**Day 1 < 3**
Mean (pooled within txt)	14.3 ± 5.1	32.1 ± 5.9	18.8 ± 4.7	20.1 ± 6.1	0.048		**Pre > Con**
Total SCFA[4]						0.027	**Day 1 < 3, 7**
Mean (pooled within txt)	174 ± 55	277 ± 64	181 ± 51	239 ± 66	0.233		
Isobutyrate						<0.001	**Day 1, 7 < 3**
Mean (pooled within txt)	4.10 ± 1.0	6.55 ± 1.1	4.60 ± 0.9	7.28 ± 1.1	0.032		**Syn > Con**
Valerate						0.250	
Mean (pooled within txt)	2.08 ± 0.9	4.05 ± 1.0	2.80 ± 0.9	3.38 ± 1.0	0.150		
Isovalerate						0.028	**Day 1, 7 < 3**
Mean (pooled within txt)	5.56 ± 1.3	7.58 ± 1.5	5.26 ± 1.2	8.21 ± 1.5	0.144		

[1]Data are expressed as mean ± SEM, μmol SCFA/ mg dry matter.

[2]Abbreviations used: Con - control; Pre - prebiotic treatment; Pro - probiotic treatment; Syn - synbiotic treatment; Txt –treatment.

[3]Treatment groups not listed in effect column are statistically similar to all treatments.

[4]Total SCFA= acetate + propionate + butyrate.

Table 4.2. Colon mRNA expression of SCFA transporters and receptors[1,2]

	Con	Pre	Pro	Syn	P value	Effect[3]
	Treatment					
MCT1						
24 hours	0.148 ± 0.049	0.077 ± 0.028	0.150 ± 0.050	0.079 ± 0.029	0.123	
3 days	0.071 ± 0.014	0.082 ± 0.014	0.079 ± 0.014	0.092 ± 0.019	0.242	
7 days	0.079 ± 0.016	0.070 ± 0.014	0.092 ± 0.019	0.084 ± 0.018	0.386	
SMCT1						
24 hours	0.004 ± 0.001	0.003 ± 0.001	0.008 ± 0.002	0.002 ± 0.001	0.051	
3 days	0.002 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.002	0.356	
7 days	0.008 ± 0.003	0.002 ± 0.001	0.004 ± 0.001	0.004 ± 0.002	0.057	
FFAR2						
24 hours	0.080 ± 0.019	0.062 ± 0.016	0.081 ± 0.019	0.043 ± 0.011	0.089	
3 days	0.044 ± 0.008	0.045 ± 0.007	0.050 ± 0.008	0.083 ± 0.013	**0.009**	**Syn > Con, Pre, Pro**
7 days	0.062 ± 0.009	0.047 ± 0.007	0.059 ± 0.009	0.041 ± 0.007	0.090	
FFAR3						
24 hours	0.052 ± 0.012	0.029 ± 0.007	0.064 ± 0.014	0.023 ± 0.006	**0.005**	**Pro > Pre, Syn**
3 days	0.022 ± 0.007	0.030 ± 0.008	0.028 ± 0.008	0.037 ± 0.010	0.290	
7 days	0.039 ± 0.012	0.026 ± 0.008	0.035 ± 0.011	0.019 ± 0.016	0.170	

[1]Data are expressed as mean ± SEM, mRNA: 18S.

[2]Abbreviations used: Con - control; Pre - prebiotic treatment; Pro - probiotic treatment; Syn - synbiotic treatment.

[3] Treatment groups not listed in effect column are statistically similar to all treatments.
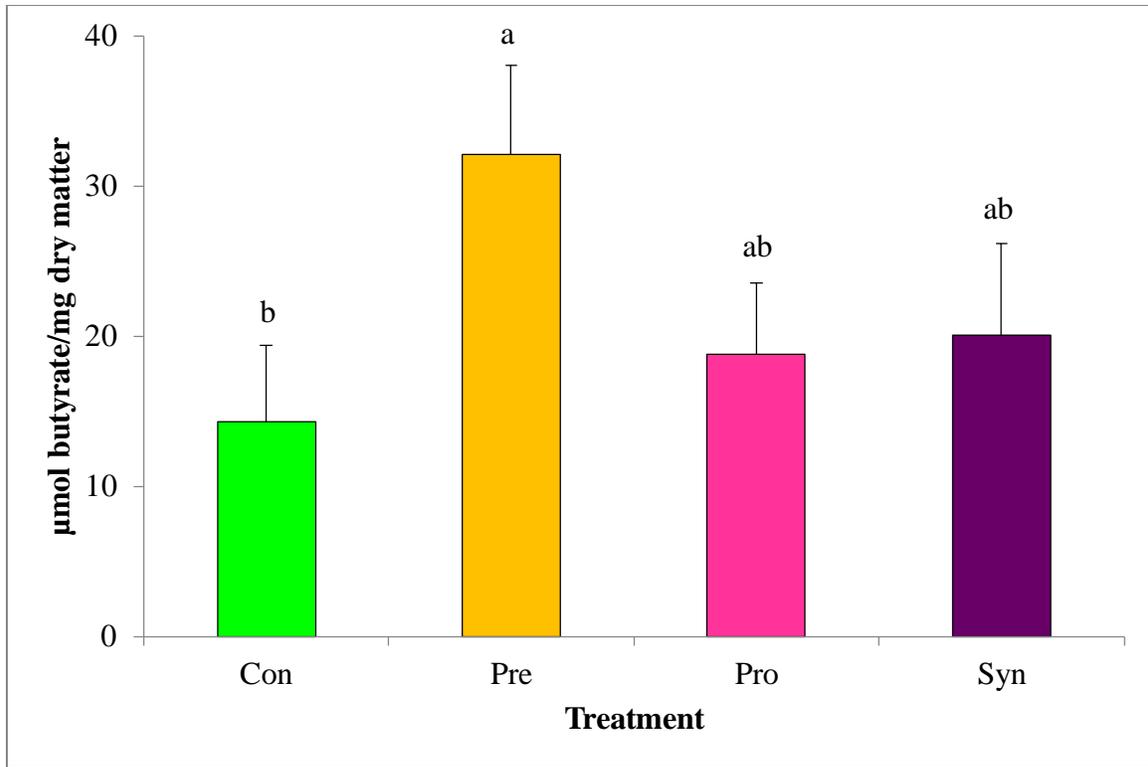
Figure 4.1: Butyrate concentrations with data expressed as pooled treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters over bars indicate a statistically significant difference. Butyrate concentration was greater with prebiotic treatment compared to control but similar to probiotic and synbiotic treatments (P= 0.050).
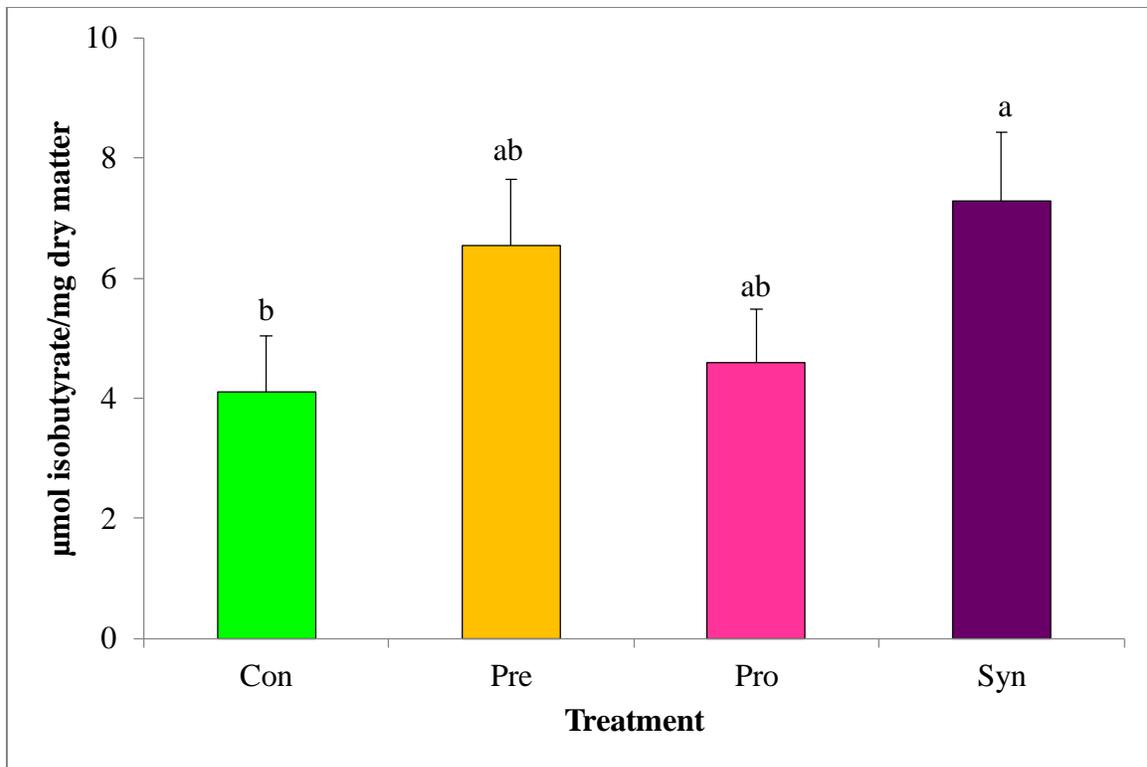
Figure 4.2: Isobutyrate concentrations with data expressed as pooled treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters over bars indicate a statistically significant difference. Isobutyrate concentration was greater with synbiotic treatment compared to control but was similar to prebiotic and probiotic treatments (P= 0.032).
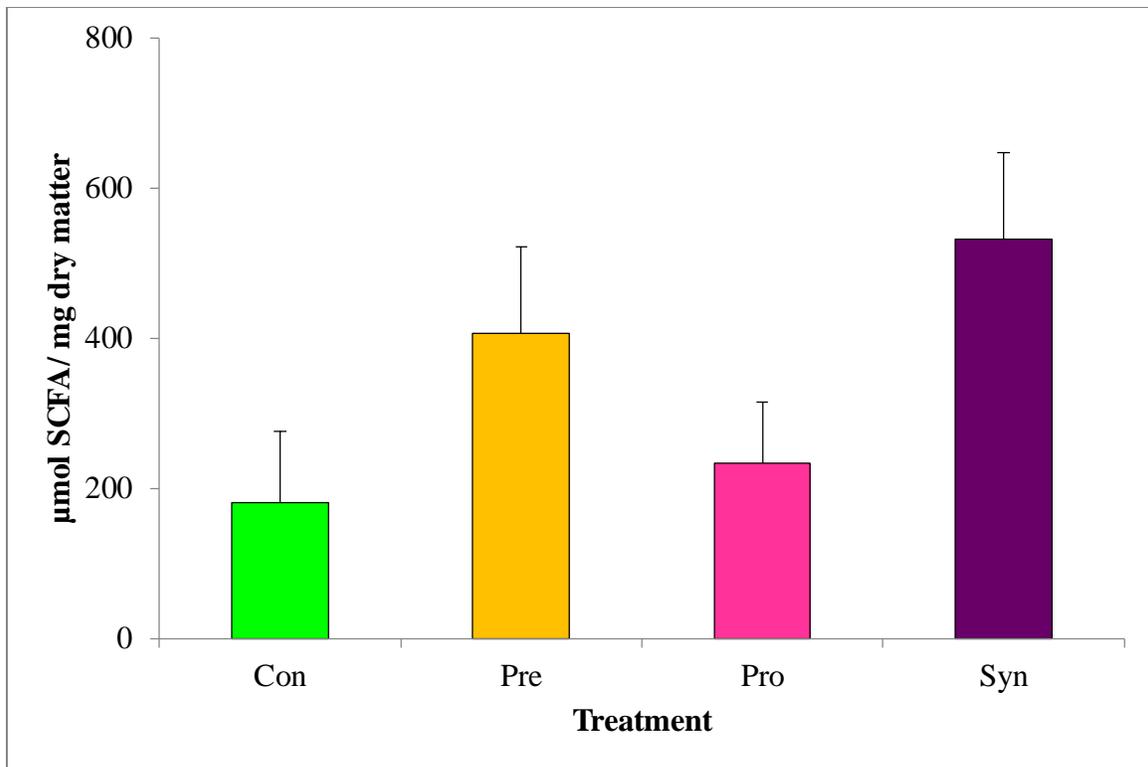
Figure 4.3: Total acetate, propionate and butyrate concentrations after 3 days of treatment with data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Total SCFA concentration tended to be greatest with synbiotic treatment (P= 0.078).
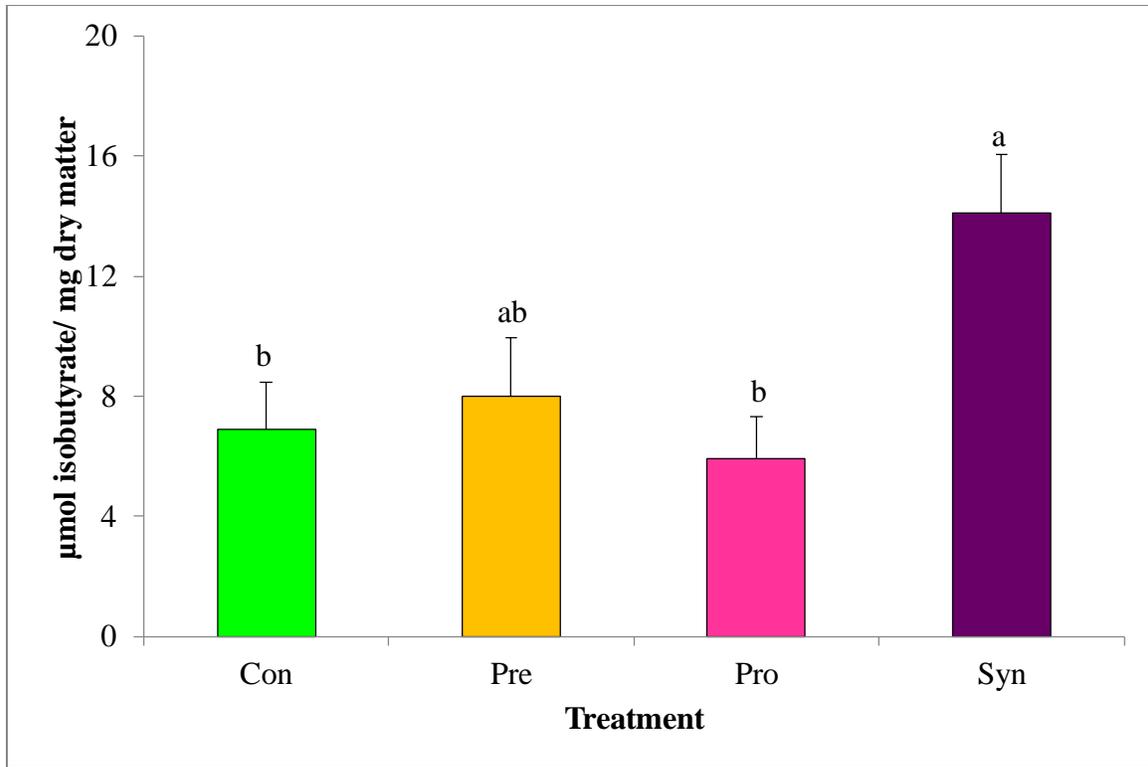
Figure 4.4: Isobutyrate concentrations after 3 days of treatment with data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters over bars indicate a statistically significant difference. Isobutyrate concentration was greater with synbiotic treatment compared to control and probiotic but was similar to prebiotic treatment (P= 0.028).
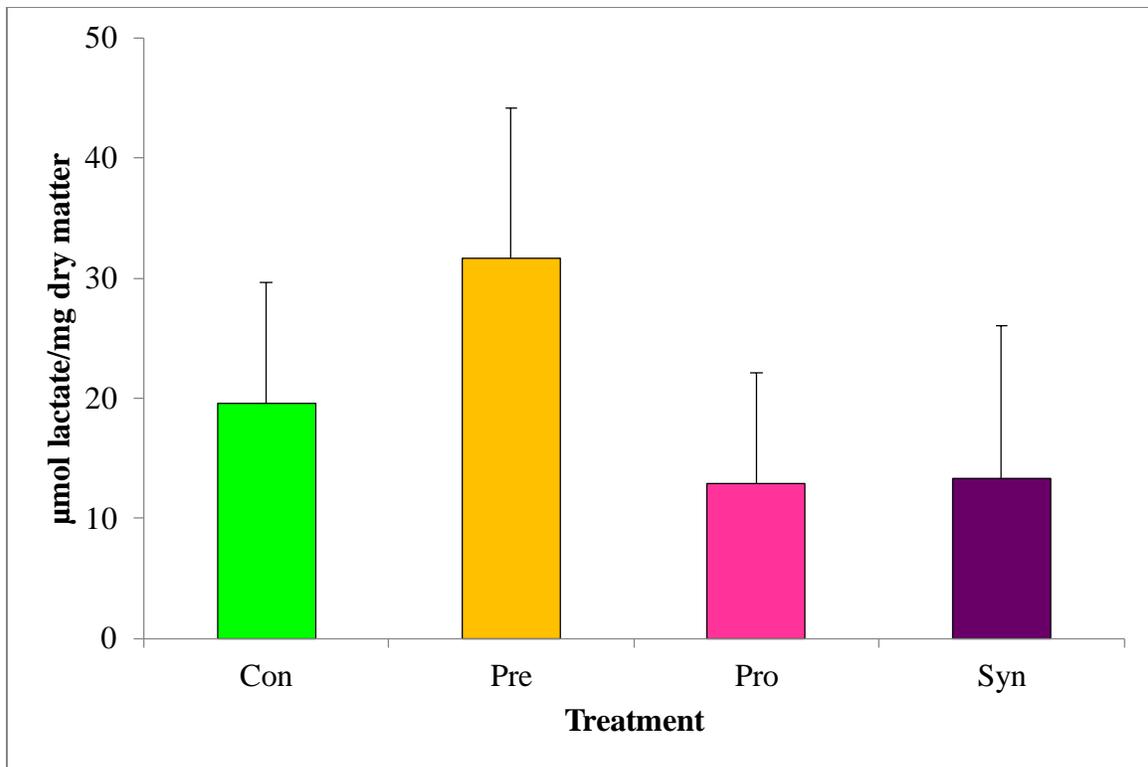
**Figure 4.5**: Lactate concentrations with data expressed as pooled treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Lactate concentration did not differ by treatment when pooled by time point (P= 0.101).
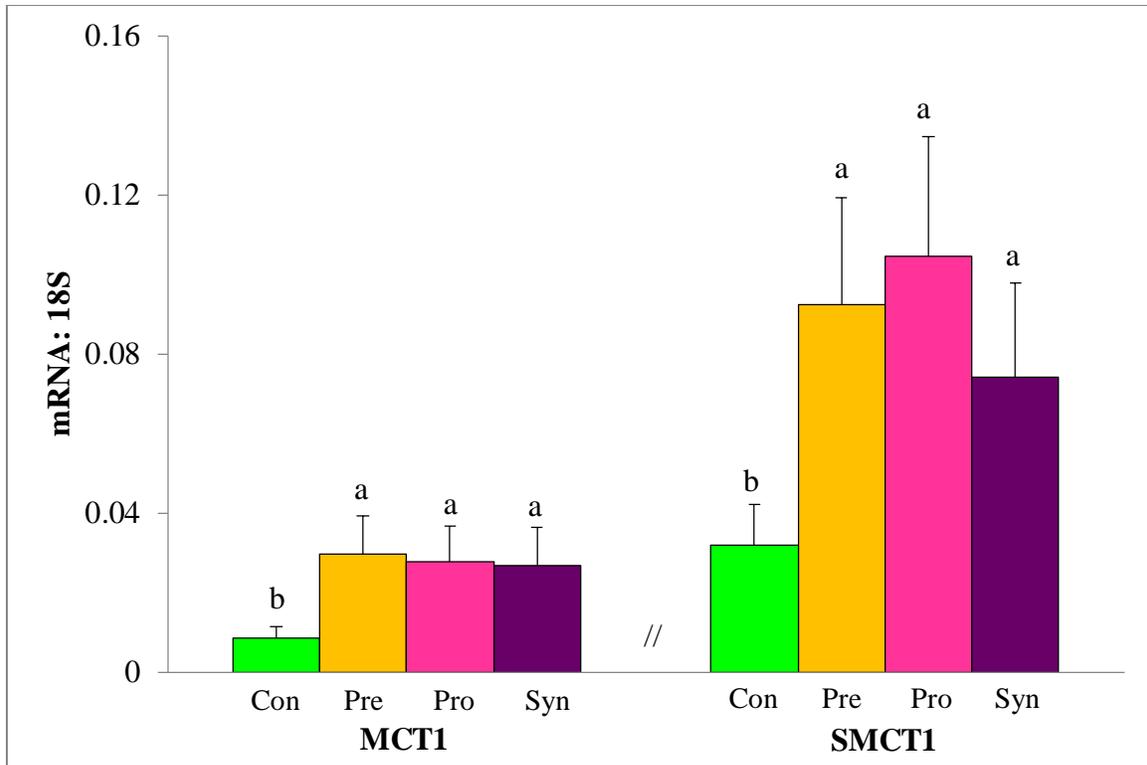
Figure 4.6: Ileal SCFA transporter mRNA expression after 24 hours of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. MCT1: Pre-, pro-, and synbiotic treatment were associated with greater MCT1 mRNA expression compared to control (P= 0.012). SMCT1: Pre-, pro-, and synbiotic treatment were associated with greater SMCT1 mRNA expression compared to control (P= 0.014).
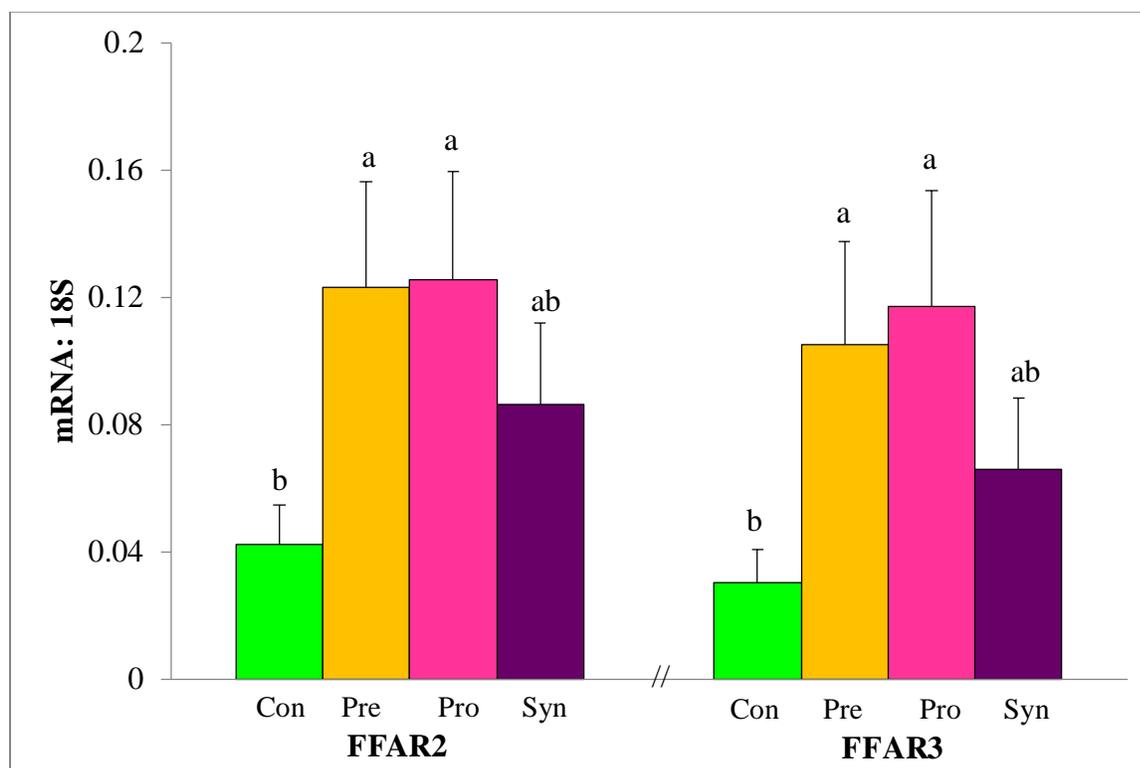
Figure 4.7: Ileal SCFA receptor mRNA expression after 24 hours of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. FFAR2: Pre- and probiotic treatment was associated with greater FFAR2 mRNA expression compared to control (P= 0.013). FFAR3: Pre- and probiotic treatment was associated with greater FFAR2 mRNA expression compared to control (P= 0.008).
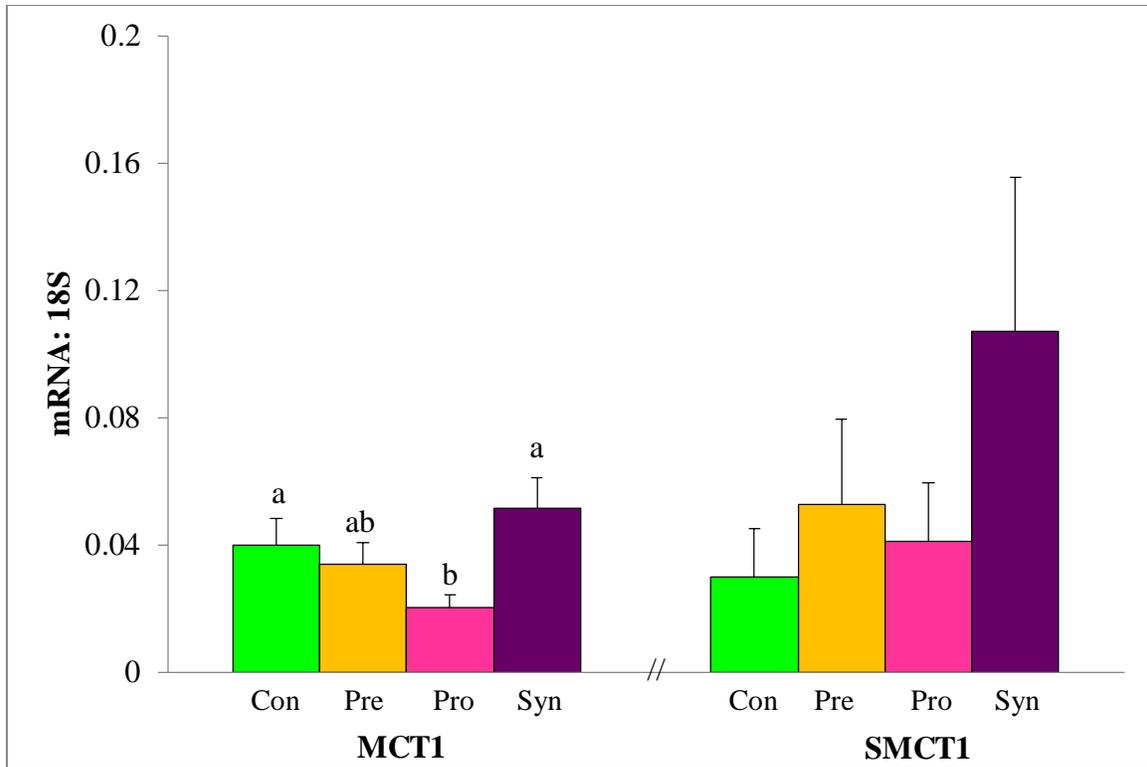
Figure 4.8: Ileal SCFA transporter mRNA expression after 3 days of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. MCT1: Control and synbiotic treatments were associated with greater MCT1 mRNA expression compared to probiotic (P= 0.004). SMCT1: Expression did not differ after 3 days of treatment (P= 0.080).
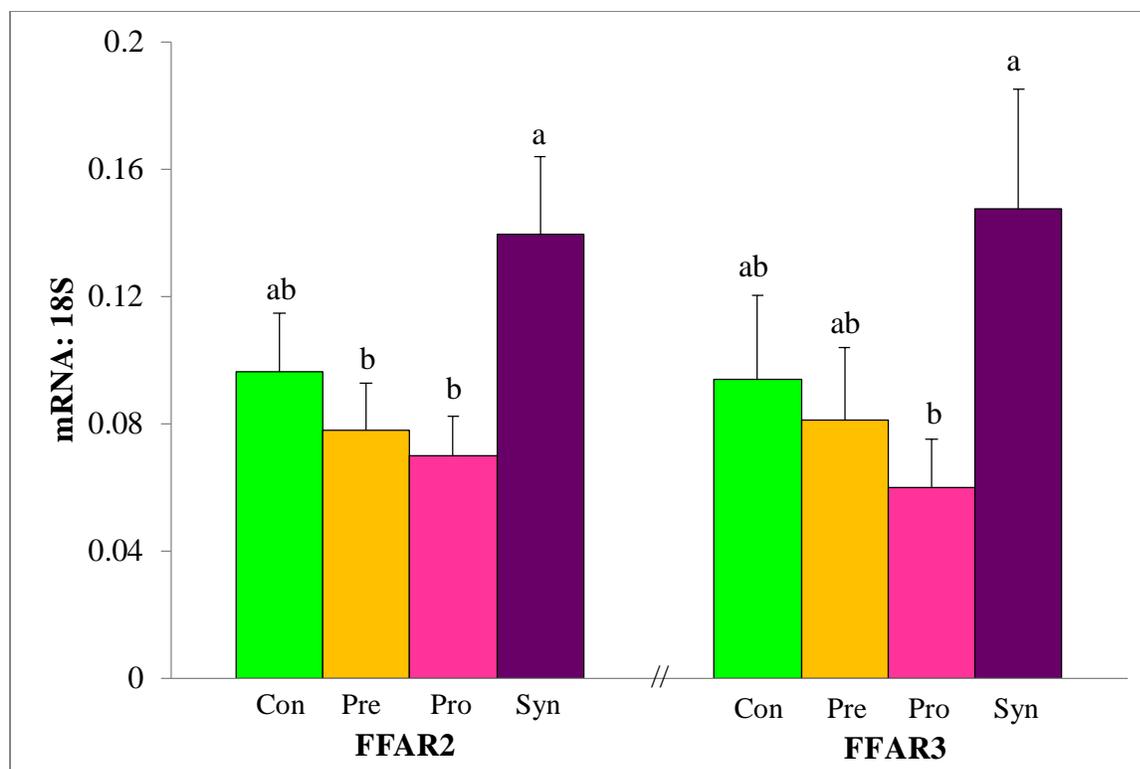
Figure 4.9: Ileal SCFA receptor mRNA expression after 3 days of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. FFAR2: Synbiotic treatment was associated with greater FFAR2 mRNA expression compared to prebiotic and synbiotic treatment (P= 0.017). FFAR3: Synbiotic treatment was associated with greater FFAR2 mRNA expression compared to probiotic treatment (P= 0.039).
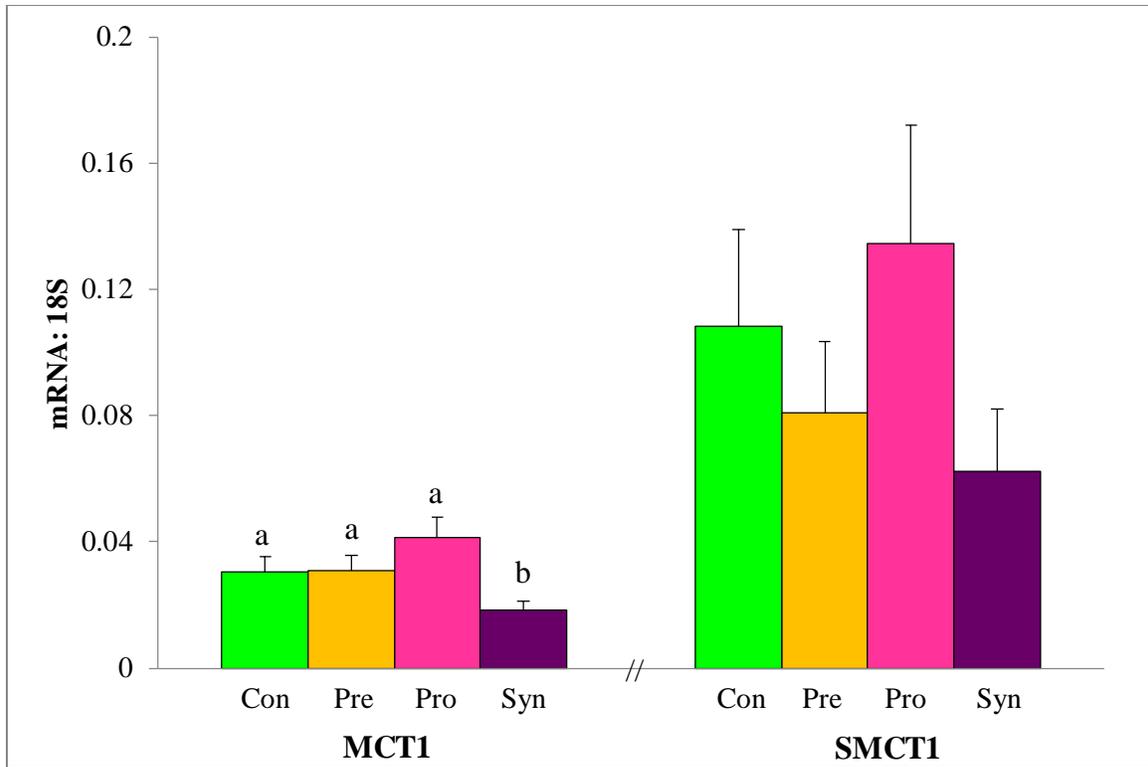
Figure 4.10: Ileal SCFA transporter mRNA expression after 7 days of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. MCT1: Control, pre-, and probiotic treatment were associated with greater MCT1 mRNA expression compared to synbiotic treatment (P= 0.004). SMCT1: Expression did not differ after 7 days of treatment (P= 0.113).
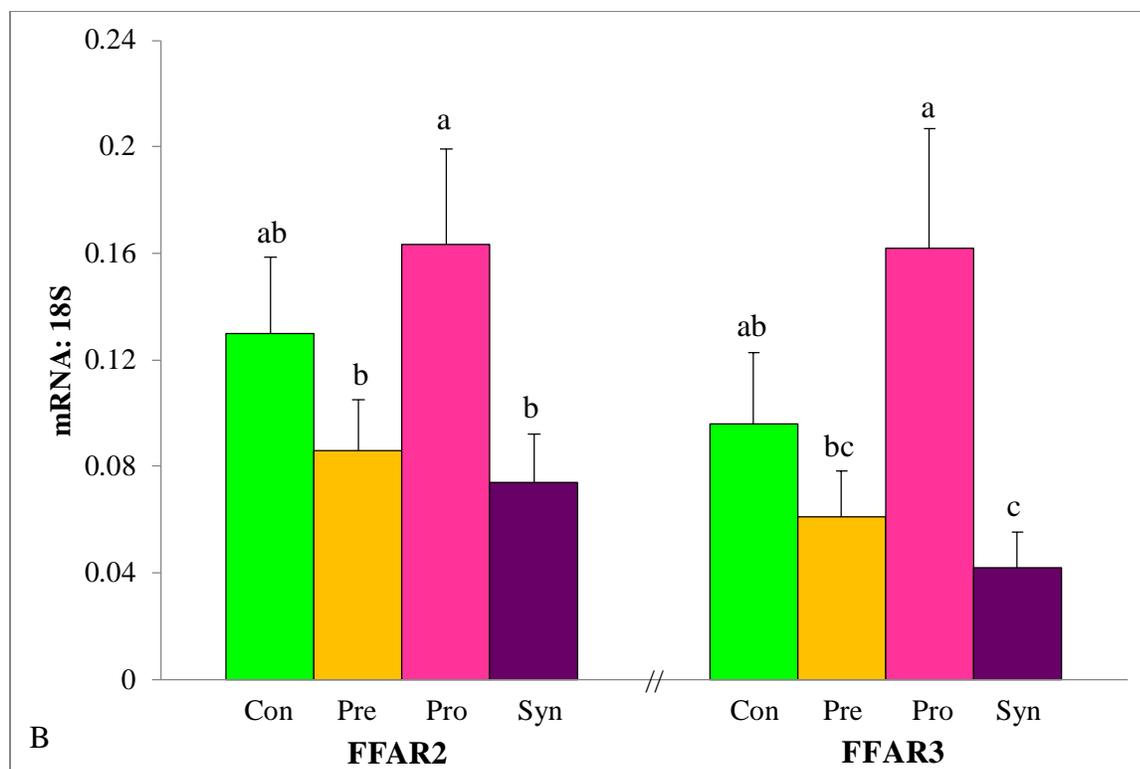
Figure 4.11: Ileal SCFA receptor mRNA expression after 7 days of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. FFAR2: Probiotic treatment was associated with greater FFAR2 mRNA expression compared to prebiotic and synbiotic treatment (P= 0.025). FFAR3: Probiotic treatment was associated with greater FFAR3 mRNA expression compared to pre- and synbiotic treatment while control was greater than synbiotic treatment (P= 0.004).
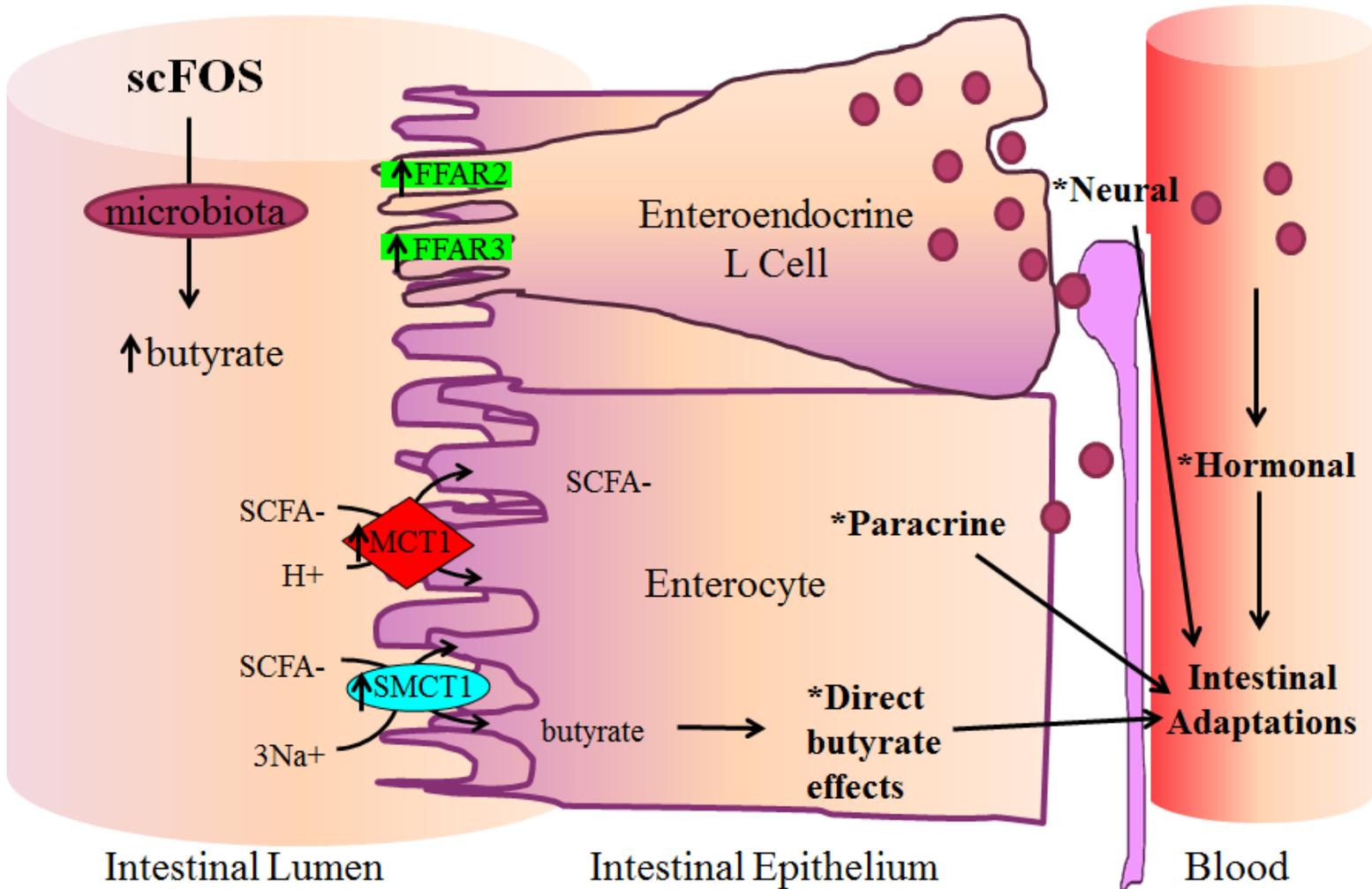
Figure 4.12: Summary of SCFA receptor and transporter mRNA expression in the ileum. Prebiotic supplementation was associated with an increase in butyrate concentrations and mRNA expression of FFAR2, FFAR3, MCT1 and SMCT1. Increased butyrate transport through MCT1 and SMCT1 and increased hormonal signaling through activation of FFAR2 and FFAR3 may stimulate intestinal adaptations through direct butyrate effects, paracrine, hormonal, and/or neural signaling.

CHAPTER 5

GLUT2 AND THE SWEET TASTE RECEPTOR T1R3 mRNA ARE DIFFERENTIALLY IMPACTED BY SHORT CHAIN FRUCTOOLIGOSACCHARIDE AND *LACTOBACILLUS RHAMNOSUS* GG SUPPLEMENTATION IN AN INTESTINAL FAILURE PIGLET MODEL

ABSTRACT

An increase in nutrient transport is a critical component of functional adaptation in intestinal failure. Butyrate, a short chain fatty acid and product of bacterial fermentation, has been shown to increase expression of the facilitative glucose transporter 2, GLUT2, but the signaling mechanism in intestinal failure is unknown. The sweet taste receptor T1R3 is expressed in enteroendocrine cells in the proximal small intestine and represents a possible mechanism through which GLUT2 is up-regulated. We hypothesized that prebiotic short chain fructooligosaccharide (scFOS) administration would increase mRNA expression of GLUT2 and T1R3. Neonatal piglets (n=87) were obtained 48 hours after birth and immediately underwent placement of a jugular catheter and an 80% jejunoileal resection. Piglets were randomized to one of four treatment groups: 1) **control** (20% standard enteral nutrition /80% standard parenteral nutrition); 2) control plus **prebiotic** (10 g scFOS/L enteral nutrition); 3) control plus **probiotic** (1×10^9 CFU *Lactobacillus rhamnosus* GG (LGG)), or; 4) control plus **synbiotic** (scFOS + LGG). Piglets were further randomized to receive treatment for acute adaptations (24 hours) or chronic adaptations (3 or 7 days). GLUT2 and T1R3 mRNA expression were analyzed as a randomized complete block design and considered significant at $P \leq 0.05$. Prebiotic and probiotic treatments were associated with greater ileal T1R3 mRNA compared to control after 24 hours ($P= 0.054$); however, by day 3, control expressed the numerically greatest amount of T1R3

mRNA in the ileum (P= 0.056). By day 7, probiotic piglets had greater T1R3 ileal mRNA

expression compared to prebiotic and synbiotic groups while control was greater than synbiotic

(P< 0.001). Jejunal T1R3 mRNA was greatest in control animals compared to synbiotic

treatments but was similar to probiotic and prebiotic after 3 days of treatment (P= 0.049).

Jejunum GLUT2 mRNA expression was significantly greater in control and probiotic animals

compared to prebiotic piglets on day 3 (P= 0.040), but did not differ at 24 hours or 7 days.

Jejunal and ileal GLUT2 mRNA was not different when time points were pooled within

treatment (P= 0.166 and 0.119, respectively). Ileal GLUT2 mRNA was not different at any

individual time point. Overall, GLUT2 mRNA expression was not impacted by treatment. T1R3

was associated with treatment at each time point, but did not correspond with GLUT2 mRNA

changes. This suggests that the treatment-associated changes in T1R3 mRNA expression were

not impacting GLUT2 expression.

INTRODUCTION

Intestinal failure (IF) occurs when functional intestinal mass decreases below the amount

required for digestion and absorption to meet the body's needs (10, 11). These patients are

dependent on parenteral nutrition (PN) until the residual intestine adapts to compensate for the

decrease in functional capacity (12). After intestinal resection, nutrient transport in the

remaining intestine increase in both animal models (28, 38, 46, 47) and human patients (29, 48).

Butyrate is a short chain fatty acid (SCFA) produced in the distal gastrointestinal tract as a

product of bacterial fermentation of fiber and resistant carbohydrates. Short-chain

fructooligosaccharides (scFOS) are a rapidly fermented prebiotic, or digestion-resistant food

ingredient fermented by the host microbiota resulting in selective growth and/or activity of beneficial bacteria (56). As outlined in Chapter 4, scFOS supplementation resulted in increased colonic luminal butyrate concentrations in neonatal piglets with IF.

Butyrate has been shown to further increase intestinal adaptation, including increases in mRNA expression of glucose transporter 2 (GLUT2) (7-9). GLUT2 is a facilitative glucose transporter that is present in various tissues including the intestine, liver, pancreas, kidney, and brain (231). GLUT2 is present in the basolateral membrane but evidence suggests that it can translocate to the apical membrane to augment active glucose absorption through the function of sodium-dependent glucose cotransporter-1 (SGLT-1) (232-235). An *in vitro* investigation shows that butyrate is specifically responsible for initiating transcription of GLUT2 (222). However, the signaling mechanism by which functional adaptations are initiated is not known.

Sweet taste receptors are present in the small intestine and express a transmembrane G protein, α-gustducin hypothesized to monitor luminal nutrients (152). The sweet taste receptors include T1R3 and are expressed on enteroendocrine L cells in the small intestine (156-158). Examination of proximal pig intestine shows T1R3 to be co-localized with gastric inhibitory protein (GIP), glucagon-like peptide-1, and 2 (GLP-1 and GLP-2) and may be important for enteroendocrine sensing and the resulting hormone release (158). Recent research indicates that sweet taste receptors including T1R3 are integral in regulating the expression of both glucose transporters SGLT-1 and GLUT2 and secretion of hormones originating from the gastrointestinal tract (158, 161, 162). In fact, GLP-1 and PYY concentrations are decreased in α-gustducin -/- mice or when a T1R inhibitor was provided to human subjects (157).

The objective of this experiment was to determine the impact of prebiotic and/or probiotic administration on GLUT2 and T1R3 mRNA expression in the jejunal and ileal mucosa of neonatal piglets with IF. We hypothesize that scFOS will be associated with an increase in the sweet taste receptor T1R3 mRNA in the jejunum and ileum, and a concomitant increased in GLUT2 mRNA.

METHODS

The experimental design, surgical procedures, animal care, nutrient solutions and sample collection are outlined in detail in Chapter 3 (5). Briefly, 48-hour old neonatal piglets (n= 87; 24 hours and 3 day piglets, n= 6/treatment; 7 day piglets, n= 9-10/treatment) were subjected to placement of a jugular catheter and an 80% jejunoileal resection. The piglets were randomized to one of four treatment groups: 1) **control** (20% standard enteral nutrition (EN)/80% standard PN); 2) control plus **prebiotic** (10 g scFOS/L EN); 3) control plus **probiotic** (1 x 10^9 CFU *Lactobacillus rhamnosus* GG (LGG)); 4) control plus **synbiotic** (10 g scFOS/L EN and 1 x 10^9 CFU LGG). Piglets were further randomized to receive treatments for acute or chronic time points. Twenty-four hours of treatment administration represented acute adaptations while treatment for 3 or 7 days was representative of chronic intestinal adaptations. At the end of the designated treatment duration, piglets were euthanized with 0.25 mL/kg intravenous dose of sodium pentobarbital (Veterinary Laboratories, Inc, Lenexa, KS). The entire gastrointestinal tract was carefully removed, measured and weighed, and separated into duodenum, jejunum, ileum, and colon by previously described designations (4, 5). For each intestinal segment,

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mucosa was carefully scraped with a glass slide, snap-frozen in liquid nitrogen, and stored at -80°C.

mRNA Quantification of GLUT2 and T1R3

RNA was isolated using the TRIzol method (Life Technologies, Inc., Gaithersburg, MD) following manufacturer's instructions with the addition of an overnight cleaning step. Specifically, after completion of the TRIzol steps, 10 µL of 3M sodium acetate at pH 5.2 and 250 µL of 100% ethanol was added followed by vortexing and placement at -20°C. The next day, samples were centrifuged for 15 minutes, washed one additional time with 75% ethanol, and suspended in autoclaved diethyl pyrocarbonate (DEPC)-treated distilled water. RNA integrity was assessed with RNA gel electrophoresis by running 10 µg RNA on a denaturing agarose gel at 100 V until the bromophenol blue dye reached the end of the gel. The 18S and 28S rRNA bands were examined using a UV illumination system (Fotodyne Foto/Analyst Image Capture System; Fotodyne, Hartland, WI). Total RNA was quantified with a NanoDrop 1000 spectrophotometer at OD260 absorbance, and purity was further assessed by determining the OD260/OD280 and OD 260/OD230 ratios. Ratios ≥1.8 were accepted.

T1R3 and GLUT2 mRNA were quantified in jejunal and ileal mucosa RNA samples. Reverse transcription of jejunal and ileal RNA was carried out for T1R3 with approximately 0.5 µg of isolated RNA, random hexamer primers, and SuperScript® III in a GeneAMP® PCR 9700 System to generate cDNA. GLUT2 proved to be in low abundance in these samples, so 5 µg RNA was reverse transcribed for GLUT2 mRNA quantification. Real time quantitative polymerase chain reaction (RT-qPCR) was carried out with TaqMan® Universal PCR Master

Mix and T1R3 (Ss03387204_u1) or GLUT2 (Ss03385240_u1) sus scrofa primer/probes (Applied

Biosystems by Life Technologies, Carlsbad, CA). Both primer/probes were validated by

assessing the efficiency and R^2 of the standard curve and each sample was run in triplicate.

T1R3 was normalized to endogenous 18S and quantified using a pooled cDNA standard curve.

GLUT2, because of the low abundance, was quantified using the $\Delta\Delta$Ct method (236).

Statistical Analysis

The data were analyzed using SAS version 9.2 (SAS Institute, Cary, NC). A randomized

complete block was used as the study design including main effects for treatment and treatment

duration. Litter served as the random block effect. The data were tested for normality with the

Shapiro-Wilk test and if found to be non-normal, a log 10 transformation was applied. T1R3

data was expressed as means ± standard error. For GLUT2, control piglet mRNA expression was

set to 1 and prebiotic, probiotic and synbiotic treatment values were expressed as fold change

from control. Means were separated by least squared difference when main effect p-values were

P≤ 0.05.

RESULTS

After 3 days of treatment, control and probiotic treatments were associated with

significantly greater GLUT2 expression in the jejunum compared to prebiotic treatment (P=

0.040, **Figure 5.1**). However, jejunal GLUT2 mRNA did not differ among treatments at 24

hours or 7 days (P= 0.320 and 0.232, respectively). In the both the jejunum and ileum, GLUT2

mRNA expression did not differ as a main effect of treatment (P= 0.116 and 0.119, respectively,

Table 5.1). Ileal GLUT2 did not differ within individual time points (24 hours, P= 0.276; 3 days, P= 0.175; 7 days, P= 0.392).

T1R3 expression in the jejunum was associated with greater mRNA expression after 3 days of control treatment compared to synbiotic treatment (P= 0.049, **Figure 5.3**). Jejunal T1R3 mRNA expression did not reach statistical significance as a main effect of treatment (P= 0.239, **Table 5.2**), after 24 hours of treatment (P= 0.301, **Figure 5.2**), or after 7 days (P= 0.387, **Figure 5.4**). In the ileum, T1R3 mRNA expression tended to be greater with prebiotic and probiotic treatment compared to control after 24 hours (P= 0.054, **Figure 5.2**) and control alone after 3 days (P= 0.056, **Figure 5.3**). Seven days of treatment was associated with statistically significant increased T1R3 mRNA expression with probiotic treatment compared to prebiotic and synbiotic groups (P< 0.001, **Figure 5.4**).

DISCUSSION

Nutrient transporters such as GLUT2 are critical for normal intestinal function and glucose transport. In IF, the decreased surface area necessitates that the remaining intestine compensate to account for the decrease in surface area. These adaptations are both structural and functional and include increases in nutrient transporters. As previously outlined in Chapter 3, supplementation with the prebiotic, scFOS, resulted in profound structural adaptations within the residual jejunum, ileum, and colon and functional adaptations primarily in the jejunum (5). Further analysis revealed an increase in luminal butyrate concentration with scFOS administration. Early investigations showed that adaptation following intestinal resection included an increase in glucose transport in rodent models and humans (29, 46). The increase in

glucose transport in a rodent model was not associated with an increase in GLUT2 mRNA when normalized to intestinal weight, however; the residual ileum showed an increase in epithelial architecture, suggesting an increase in functional surface area accounting for the increase in glucose transport. These increases in glucose transport and GLUT2 mRNA are further enhanced with SCFA, and specifically, butyrate supplementation (7-9).

In this investigation, GLUT2 mRNA expression was not impacted by scFOS, LGG, or their synbiotic combination. Despite the increases in structural adaptation, functional adaptations were not as robust. The lack of GLUT2 effect is in-line with other functional analyses in these studies. Upregulation of expression and function of SGLT-1 has also been shown to increase with SCFA and butyrate administration (7, 46). While SGLT-1 mRNA abundance was not assessed, functional capacity of SGLT-1 was measured with modified Ussing chambers as previously reported (5). Glucose transport was not increased with any of the treatments in any intestinal segment. While the jejunum showed increases in glutamine and peptide transport with prebiotic treatment, glucose transport was not increased compared with control. The ileum did not increase in nutrient transport with pre- and/or probiotic treatment. One possible explanation for this finding is that the 20% EN and corresponding 10 g scFOS/L was not great enough to stimulate enhanced nutrient transport beyond that which was observed in the control group. Specifically, the relatively small quantity of enteral nutrients presented to the intestinal epithelium may not have been great enough to stimulate significant increases in nutrient transport and GLUT2 expression. In a piglet model with normal intestinal anatomy on PN, enteral nutrition must reach 40-60% of estimated nutrient needs to produce hypertrophic effects in the small intestine (237). In addition, ileal adaptation relies on the provision of nutrients in excess of the usual concentrations arriving after digestion and absorption in the more

proximal small intestine (28-30). In a normal intestinal anatomy scenario, the majority of nutrients, particularly digestible carbohydrates, will be absorbed before reaching the ileum. In short bowel syndrome (SBS) with a jejunoileal resection, the residual jejunum is likely inadequate to absorb the amount of nutrients necessary when a full enteral diet is consumed, resulting in increased nutrient presentation to the ileum and, therefore, functional adaptation. In this model, the 20% EN divided into 4 smaller bolus feedings through the day may not have provided adequate luminal stimulation for detectable increases in GLUT2 and other functional markers of nutrient transport beyond that of the control treatment.

Jejunal GLUT2 mRNA reached statistical significance after 3 days of treatment administration wherein control piglets expressed greater levels compared to prebiotic treatment. These relative proportions are an inverse of that observed for proliferation in the same segment after 3 days of treatment as outlined in Chapter 3. Specifically, jejunal crypt cell proliferation approached significance at 24 hours and 3 days of treatment ($P= 0.055$ and $P= 0.057$) where prebiotic and synbiotic tended to have greater proliferation compared to control. The residual ileum also experienced this same effect but did not reach significance until 7 days of treatment. Therefore, the significant increase in GLUT2 mRNA expression in the jejunum with control and probiotic treatments for 3 days may be an effect of a slower proliferation rate and a greater overall proportion of mature cells compared to that of the prebiotic and synbiotic treatments. Jejunal expression of T1R3 was also significant after 3 days of treatment but not 24 hours or 7 days and presented a similar pattern.

GLUT2 has also been shown to have diurnal mRNA and protein expression with 2 to 3-fold increases reported in duodenal and jejunal mRNA several hours prior to the onset of normal feeding time in non-resection rodent models (238-240). These changes were not detected in the

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ileum, likely because the duodenum and jejunum serve as the primary locations for glucose absorption under normal anatomic conditions. However, in an SBS model, the decrease in proximal small intestine surface area should result in functional adaptation in the ileum. Therefore, in this model, an increase in GLUT2 would be expected although the EN provision may not have been sufficient for extensive functional adaptation as discussed above. In addition, in this experiment, animals received their first enteral feeding around 8:00 AM. According to the investigations in rodents, this would correspond to an increase in GLUT2 mRNA at approximately 3:00 AM. It is possible that an increase in GLUT2 mRNA may have been missed. Finally, GLUT2 mRNA expression proved to be of low abundance in these samples, likely due to the relatively small amount of enterally provided nutrients and timing of sample collection.

T1R3 is expressed in enteroendocrine cells in the proximal intestine and is hypothesized to be involved in sensing luminal nutrients and regulating hexose transporters including SGLT-1 and GLUT2 in response to increased luminal nutrient concentrations (155-162). In this investigation, changes in T1R3 mRNA abundance were not reflected in GLUT2 mRNA abundance. However, T1R3 mRNA expression was associated with treatment, particularly in the ileum. Ileal T1R3 expression followed a similar pattern as the G-protein SCFA receptors discussed in Chapter 4 wherein prebiotic or probiotic treatment exceeds control T1R3 expression after 24 hours of treatment. This may correspond with increased hormonal signaling at this acute time point, but this was not assessed. Regardless, any upregulated signaling secondary to an increase in T1R3 mRNA expression in the ileum was not specifically associated with increased GLUT2 expression in the same segment or in the jejunum. Similarly, after 3 days of treatment, there was a strong trend for increased T1R3 mRNA expression in control and synbiotic

treatments in the ileum. While the elevated expression with synbiotic treatment is associated with the increase in total SCFA and isobutyrate at the same time point (described in chapter 4), the control group also was increased without a corresponding SCFA increase. These increases in expression are not associated with an increase in GLUT2 mRNA or an increase in active glucose transport as measured by the Ussing chambers. By 7 days of treatment administration, jejunal T1R3 mRNA concentrations were very similar across treatments. However, ileal expression continues to be more greatly associated with treatment although not with previously documented adaptations or GLUT2 expression. Specifically, in the ileum, probiotic treatment was associated with the greatest T1R3 expression compared to prebiotic and synbiotic treatment. This suggests that the evolving microbiota composition or another factor is influencing the expression of T1R and that the differing expression levels are not influencing GLUT2 mRNA in this model. In this experiment, fermentation products do not seem to be driving the evolving gene expression of T1R3.

In conclusion, GLUT2 mRNA expression was minimally impacted by pre- and/or probiotic supplementation in an IF piglet model while ileal T1R3 mRNA expression was impacted differently depending on acute or chronic treatment conditions. The lack of GLUT2 effect may be secondary to the 20% EN provided and the consequently low volume of luminal nutrients. Increased enteral feeding may result in GLUT2 upregulation. Ileal T1R3 mRNA expression may be affected by the evolving microbiota composition but is not associated with the SCFA concentrations in the proximal colon. Greater enteral feeding, particularly carbohydrates, may further stimulate T1R3 expression and/or signaling, potentially leading to an upregulation of GLUT2 expression and nutrient transport.

TABLES AND FIGURES

Table 5.1. Jejunal and ileal expression of GLUT2 mRNA[1,2]

| | Treatment | | | | | P Value | |
	Con	Pre	Pro	Syn	Txt	Time	Effect[3]
Jejunum						0.028	**Day 1 > 3**
Mean (pooled within txt)	1.00 ± 0.27	0.54 ± 0.15	0.70 ± 0.20	0.66 ± 0.20	0.116		
Ileum						0.119	
Mean (pooled within txt)	1.00 ± 0.25	0.78 ± 0.22	0.89 ± 0.25	0.85 ± 0.24	0.466		

[1]Data are expressed as mean ± SEM, fold change from control.
[2]Abbreviations used: Con - control; Pre - prebiotic treatment; Pro - probiotic treatment; Syn - synbiotic treatment; Txt –treatment.
[3]Treatment groups not listed in effect column are statistically similar to all treatments.

**Table 5.2. Jejunal and ileal expression of T1R3 mRNA[1,2]**

	Treatment					P Value	
	Con	Pre	Pro	Syn	Txt	Time	Effect[3]
Jejunum						0.024	**Day 1 > 7**
Mean (pooled within txt)	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.239		
Ileum						0.071	
Mean (pooled within txt)	0.09 ± 0.02	0.07 ± 0.01	0.11 ± 0.02	0.06 ± 0.01	0.120		

[1]Data are expressed as mean $\pm$ SEM, T1R3 mRNA: 18S.
[2]Abbreviations used: Con - control; Pre - prebiotic treatment; Pro - probiotic treatment; Syn - synbiotic treatment; Txt –treatment.
[3] Treatment groups not listed in effect column are statistically similar to all treatments.
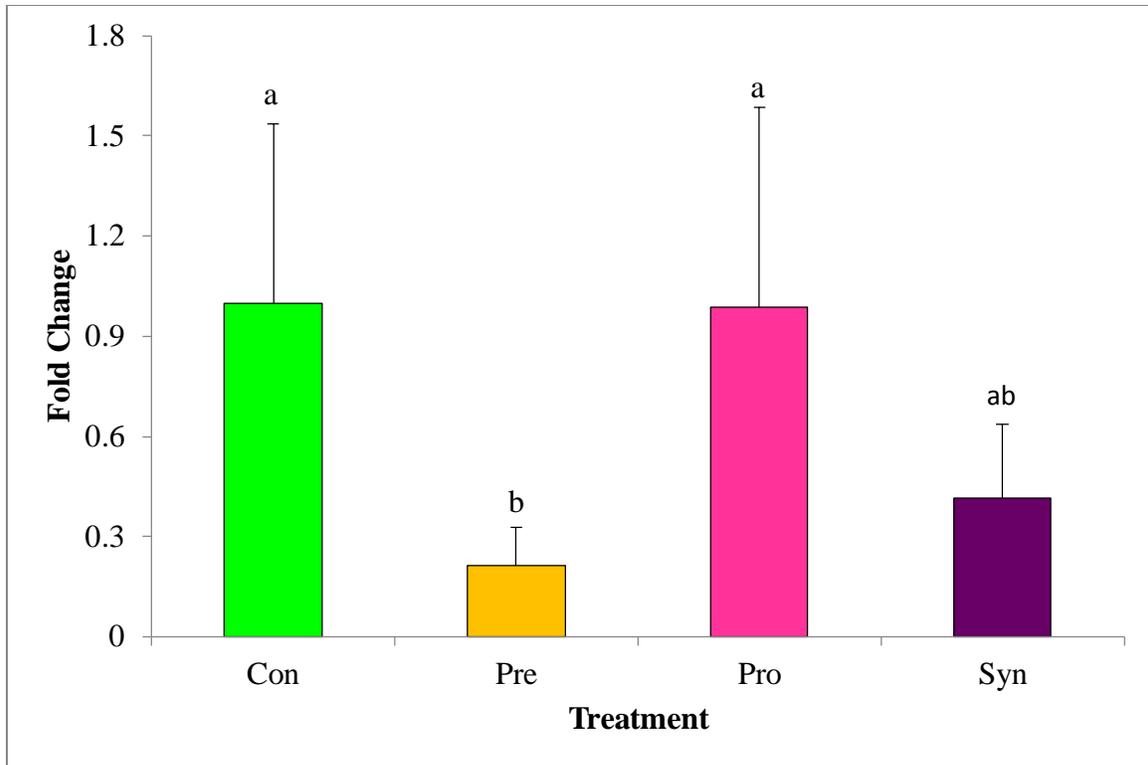
Figure 5.1: Jejunal GLUT2 mRNA expression after 3 days of treatment. Data are expressed as treatment means relative to control ± standard error. Con: control; Pre: prebiotic treatment; Pro: probiotic treatment; Syn: synbiotic treatment. In the jejunum, control and probiotic treatments were associated with significantly greater GLUT2 mRNA expression compared to prebiotic treatment after 3 days (P= 0.040).
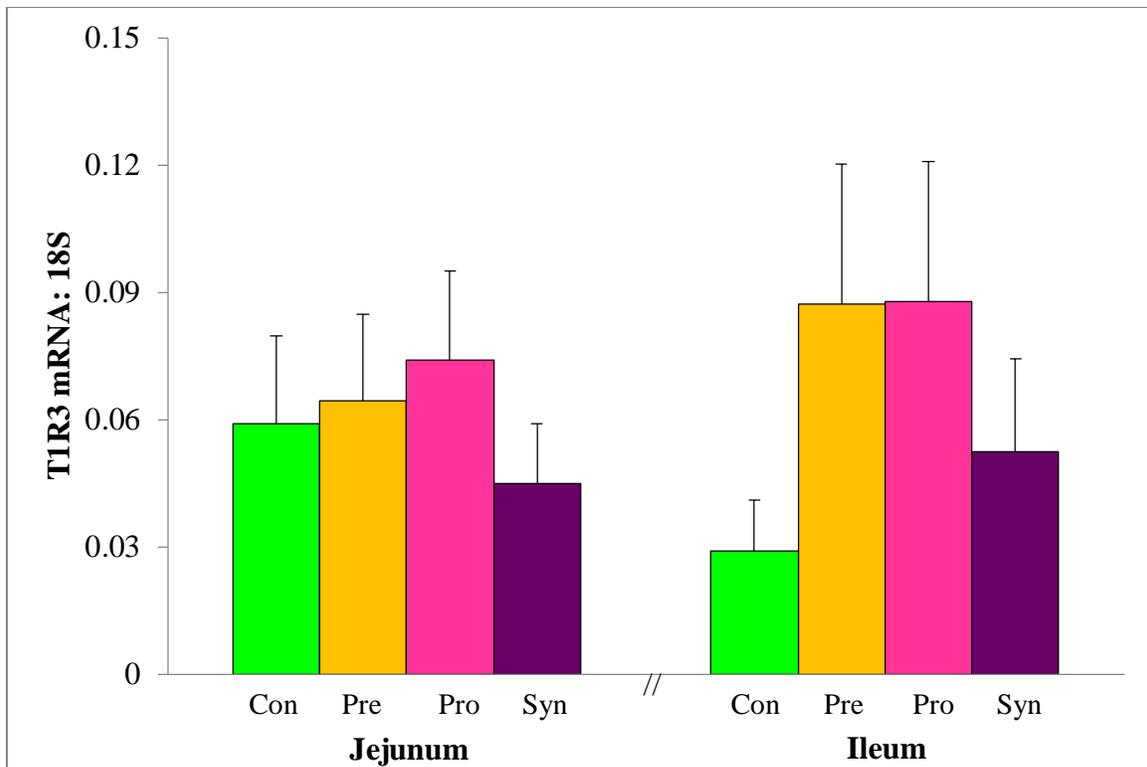
Figure 5.2: Jejunal and ileal T1R3 expression after 24 hours of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. Jejunum: T1R3 mRNA expression did not differ after 24 hours of treatment (P= 0.301). Ileum: T1R3 mRNA expression did not differ after 24 hours of treatment (P= 0.054).
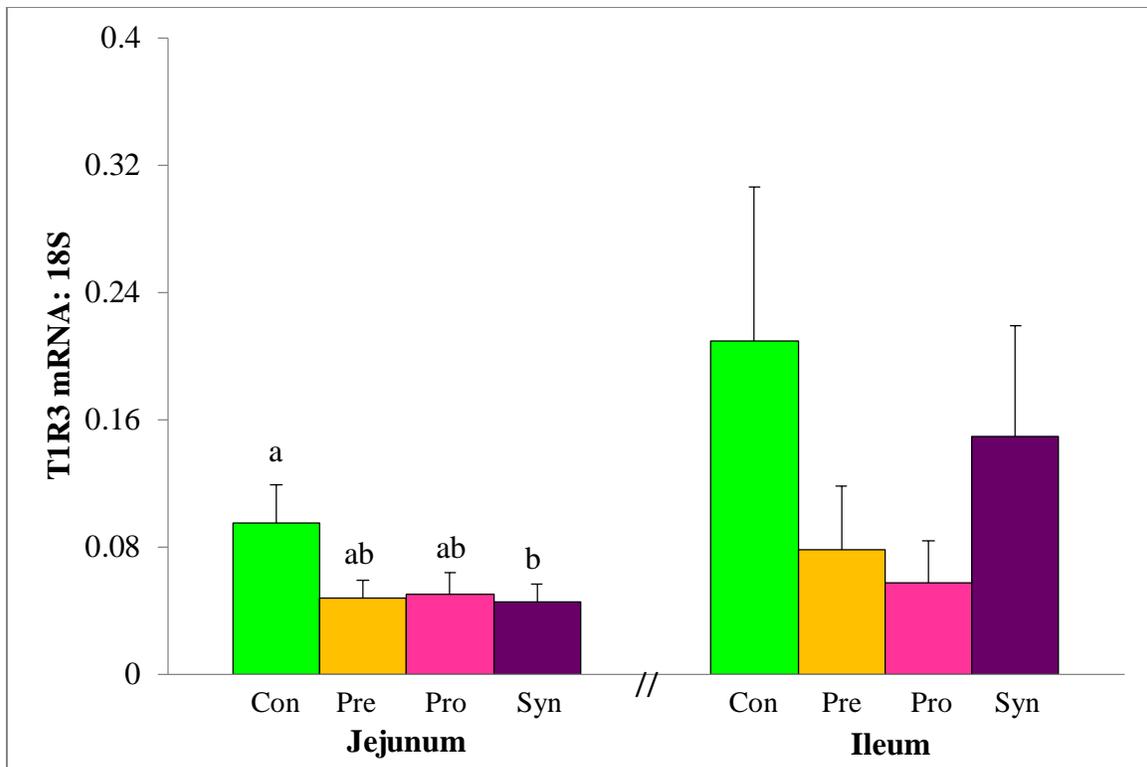
Figure 5.3: Jejunal and ileal T1R3 expression after 3 days of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. Jejunum: Control treatment was associated with greater T1R3 mRNA expression compared to synbiotic treatment (P= 0.049). Ileum: T1R3 mRNA expression did not differ after 3 days of treatment (P= 0.056).
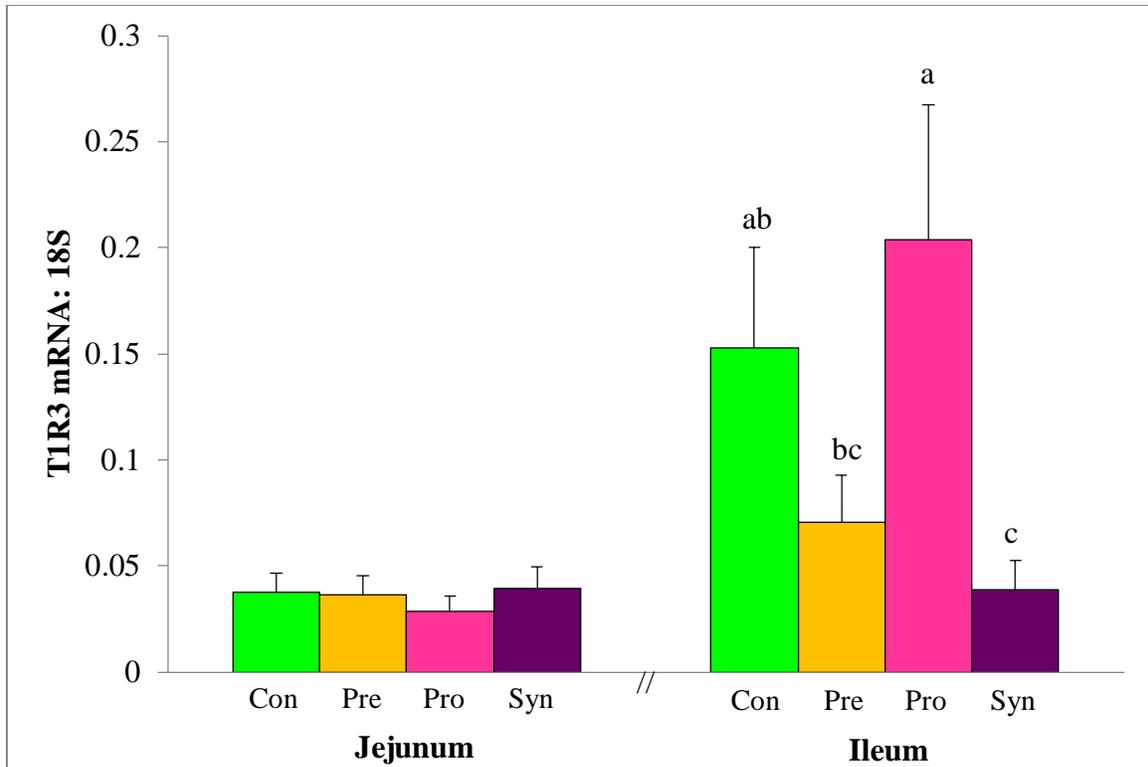
Figure 5.4: Jejunal and ileal T1R3 expression after 7 days of control, prebiotic, probiotic or synbiotic treatment. Data expressed as treatment means ± SEM. Con, control; pre, prebiotic; pro, probiotic; syn, synbiotic. Different letters above bars within each gene indicate a significant difference among treatments. Jejunum: T1R3 mRNA expression did not differ after 7 days of treatment (P= 0.387). Ileum: Probiotic treatment was associated with greater FFAR3 mRNA expression compared to pre- and synbiotic treatment while control was greater than synbiotic treatment (P< 0.001).

CHAPTER 6

DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

Intestinal failure (IF) results in dependence on parenteral nutrition (PN) to support growth, and maintain hydration and micronutrient status. PN is a life-saving measure but long-term administration is associated with serious complications. Enteral autonomy is the ultimate goal for all pediatric patients that are reliant on PN. In order to achieve enteral autonomy, the residual intestine must undergo structural and functional adaptations to increase digestion and absorption to a level that can support growth. Short chain fatty acids (SCFA), specifically butyrate, have been shown to increase intestinal adaptation but are not currently supplemented in intestinal failure. Therefore, the specific aims of this thesis research were to investigate 1) the structural, functional and cellular kinetic impacts of the prebiotic, short chain fructooligosaccharide (scFOS), probiotic, *Lactobacillus rhamnosus* GG (LGG) and their synbiotic combination; 2) SCFA transporters and receptors as possible mechanisms responsible for mediating adaptations associated with prebiotic and/or probiotic supplementation; and 3) prebiotic and/or probiotic supplementation and the impact on gene expression of an enteroendocrine sweet taste receptor T1R3 and GLUT2.

Hypothesis 1: Strategic provision of partial enteral nutrition (PEN), specifically formulated to augment butyrate production by the inclusion of pre- and probiotics, will promote structural and functional adaptations in neonatal piglets with SBS by enhancing mucosal surface area and nutrient processing capacity.

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In a neonatal piglet model of IF, piglets receiving prebiotic scFOS experienced structural adaptations in the jejunum, ileum, and colon and functional adaptations in the jejunum (summarized in **Figure 3.12** and **3.13**). Probiotic LGG was not effective and occasionally the synbiotic group combining both scFOS and LGG had less significant adaptations compared to scFOS alone. This suggests that LGG was involved in decreasing the signal responsible for the adaptations observed with scFOS, likely due to altering the microbiota composition in a manner resulting in decreased butyrate production. Indeed, the literature on probiotic supplementation in short bowel syndrome (SBS) is conflicting wherein there is both no effect (87) and increased adaptation reported (86). Further work is required to identify the optimal probiotic in SBS, which includes examining bacteria that are not classically considered probiotics. The limited research in microbiota profiles in SBS suggests that lactobacilli populations are increased compared to healthy controls (123, 229). In addition, we report a trend for increased lactate concentrations with scFOS supplementation. Therefore, a probiotic known to ferment lactate to butyrate may be an option for future investigation. These species include *Eubacterium hallii*, *Anaerostipes caccae,* and *Anaerostipes coli* (230, 241) but are still being identified and characterized. Also, the microbiota populations of these piglets must be analyzed to contribute to the small body of literature in SBS microbiota populations. Specifically, microbiota analysis from this study will provide unique insight into the timeline of microbiota population shifts occurring in SBS.

As previously discussed, the ileum has greater potential for adaptation compared to the jejunum (28-30). However, the intestinal adaptations documented in chapter 4 (5) show increased functional outcomes with scFOS, specifically nutrient transport in the jejunum, but a lack of effect in the ileum. Burrin and colleagues found that in piglets on PN, enteral nutrition

(EN) provision must reach 40-60% of total nutrient needs in order to observe adaptations within the small intestine suggesting that greater enteral stimulation may be required for ileal functional adaptations (237). Evidence also suggests that structural adaptations occur prior to functional adaptations (242-246). It is likely that the jejunum experienced functional adaptations with scFOS supplementation because there was less potential for structural expansion. Ileal mucosa underwent expansive structural adaptations and expressed significantly greater Cdx2 mRNA, a marker of enterocyte differentiation, with prebiotic supplementation. Given a longer study period and/or greater EN, ileum functional adaptations may occur. Therefore, a future direction for this line of research is to perform a weaning trial in the same neonatal piglet model where EN provision gradually increases with corresponding decreases in PN. The purpose of such a trial would be two-fold: 1) investigate if intestinal adaptations associated with scFOS therapy are able to support gradual PN weaning; and 2) evaluate ileal functional adaptations with greater EN provision.

Hypothesis 2: Butyrate concentrations will increase with prebiotic administration and be associated with an increase in SCFA transporters, monocarboxylate transporter 1 (MCT1) and sodium-coupled monocarboxylate transporter1 (SMCT1), gene expression and SCFA receptors, free fatty acid receptor 2 (FFAR2), and free fatty acid receptor 3 (FFAR3), gene expression in the residual ileum and colon.

This investigation revealed greater butyrate concentrations in the colonic lumen of piglets receiving scFOS. MCT1, SMCT1, FFAR2, and FFAR3 mRNA expression were greater with probiotic, prebiotic and synbiotic supplementation in the ileum compared to control after 24

hours of treatment (summarized in **Figure 4.12**). Colonic expression levels were not as

significantly impacted by treatment at the acute time point. These data provide an association for

a possible signaling mechanism for the adaptations outlined under hypothesis 1. However,

mRNA expression is not the only factor involved in increased or decreased expression of a given

protein and the corresponding signal. The current literature suggests that these SCFA

transporters and receptors are transcriptionally regulated where an increase in mRNA expression

corresponds with a similar magnitude increase in protein (127, 140, 247, 248). Nevertheless,

protein abundance was not assessed in this study and examination may provide guidance for

further investigation into the most likely signaling route. The half-life of mRNA also can be

impacted to result in changes in translation and protein abundance. scFOS administration,

subsequent fermentation and butyrate production may decrease degradation of SCFA

transporters and receptors, potentially resulting in an increased mRNA half-life and protein

abundance.

FFAR2 and FFAR3 are G protein-coupled receptors expressed on enteroendocrine L

cells. These receptors are involved in sampling luminal contents and initiating a signaling

cascade resulting in hormone release. FFAR2 and FFAR3 have been implicated in PYY, GLP-1,

and GLP-2 secretion (146-149, 249). The specific role of the SCFA MCT1 and SMCT1 in

butyrate-mediated intestinal adaptation is not known. One possibility is that MCT1 and SMCT1

interact with the SCFA receptors to increase this signal and, therefore, hormone release. Another

option is that MCT1 and SMCT1 mediate adaptations stemming directly through contact with

butyrate rather than an endocrine signaling mechanism. Interestingly, MCT1 and SMCT1

mRNA quantities were not impacted by treatment in the colon after 24 hours. The colon would

be the primary fermentation location in an anatomically normal piglet without scFOS

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supplementation. More proximal fermentation may be increased in this SBS model, particularly with the rapidly fermented scFOS. Therefore, in future studies, it would be appropriate to measure SCFA concentrations both in the proximal colon but also in the ileum. This would provide data regarding the luminal environment more proximally, where scFOS treatment was associated with greater alterations in SCFA transporter and receptor mRNA expression.

SCFA transporters and receptors are an emerging area of research. The debate on which specific proteins are most important for butyrate sensing, transport and the cellular orientation of transporters (apical versus basolateral) is ongoing in the literature (250). GPR10A is a newly identified SCFA receptor expressed in the distal gastrointestinal tract with reported specificity for butyrate (151). GPR109A was recently implicated as the signaling mechanism for butyrate-induced increases in MCT1 apical protein (128). Although not yet identified in pigs, GPR109A may be an important SCFA receptor for future investigation.

Hypothesis 3: scFOS supplementation will be associated with increased expression of sweet taste receptor T1R3 mRNA in the jejunum and ileum, and a concomitant increase in GLUT2 mRNA.

The final investigation of this thesis research showed that treatment was not associated with changes in mRNA expression of the glucose transporter GLUT2 in the jejunum or ileum. Ileal T1R3 mRNA expression was associated with treatments at individual time points but was not associated with corresponding changes in GLUT2 mRNA expression. As outlined in chapter 5 discussion, greater enteral stimulation may be required to detect a difference in GLUT2 expression. The future directions discussed under hypothesis 1 are also appropriate next steps

for hypothesis 3. Specifically, a SBS piglet trial using the same scFOS treatment with gradual PN weaning with corresponding increases in EN may be associated with an increase in GLUT2 mRNA expression and overall glucose transport.

Ileal mRNA expression of the sweet taste receptor followed a similar pattern to that of the SCFA transporters and receptors in the ileum. T1R3 is also co-localized with gastric inhibitory protein (GIP), PYY, GLP-1, and GLP-2 (157, 158). GLP-2 plasma concentrations were assessed in this thesis research, but did not differ by treatment. Other hormone concentrations were not measured. Measurements of these gastrointestinal hormones are an appropriate next step and would provide insight into the possible change in signaling with increases in T1R3 mRNA expression. It is plausible that the combined stimulation of FFAR2, FFAR3, and T1R3 may result in an overall signal through one or more of the hormones mentioned above.

Sweet taste receptors are not the only form of taste receptor expressed in the gastrointestinal tract. Bitter taste receptors have also been identified in the intestine of mice, rats, and humans (153, 154, 251). This is an emerging area of research and the relationship to SCFA is not yet known. In addition, the most important bitter taste receptors expressed in the intestine have not yet been established. As more data become available for this class of bitter sensing proteins, these taste receptors will be an important avenue for future research.

Summary

In summary, this thesis research establishes scFOS as a potential therapy for use in pediatric IF, specifically through increasing luminal butyrate concentrations and the associated intestinal adaptations. It further provides insight into possible mechanisms mediating the observed adaptations. The information presented herein contributes to the body of knowledge in

pediatric IF and emerging targets for nutritional intervention for use not only in IF but also colon

cancer prevention and inflammatory bowel disease.

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