

Fructan Supplementation and Infection Affect Food Intake, Fever, and Epithelial Sloughing from Salmonella Challenge in Weanling Puppies¹⁻³

Carolyn J. Apanavicius,⁴ Kristy L. Powell,⁵ Brittany M. Vester,⁴ Lisa K. Karr-Lilienthal,⁴ Lynda L. Pope,⁴ Nathan D. Fastinger,⁴ Matthew A. Wallig,⁶ Kelly A. Tappenden,⁵ and Kelly S. Swanson^{4*}

⁴Department of Animal Sciences, ⁵Department of Food Science and Human Nutrition, and ⁶Department of Veterinary Pathobiology, University of Illinois, Urbana, IL 61801

Abstract

Our objective was to examine the effects of fructan supplementation on the immune response of weanling puppies subjected to bacterial challenge. Previous studies in bacterial challenged neonatal piglets have reported benefits of fructan supplementation. Thirty hound-cross puppies (12 wk of age) were used in a 2 × 3 factorial randomized complete block design. Following a 7-d baseline period, puppies were assigned to diets containing: 1) no prebiotic, 2) 1% short-chain fructooligosaccharides (scFOS), or 3) 1% inulin. After 14 d on treatment diet, dogs received an oral gavage of: 1) *Salmonella typhimurium* DT104 (5 × 10⁸ colony forming units) or 2) 0.9% saline. Food intake, fecal and activity scores, body temperature, body weight, blood chemistry, intestinal nutrient transport, intestinal morphology and pathology, and gut microbiota were measured. Food intake decreased ($P < 0.01$) and body temperature increased ($P < 0.05$) in infected puppies. However, the decrease in food intake was less ($P < 0.05$) in those consuming fructans. Infected puppies consuming fructans also had decreased ($P = 0.05$) severity of enterocyte sloughing than those fed the control diet. Ileal Na⁺-dependent glucose transport was decreased ($P = 0.02$) in infected vs. noninfected puppies consuming CON, whereas no changes occurred in fructan-supplemented animals. Puppies consuming inulin also had increased fecal acetate ($P = 0.03$) and total short-chain fatty acid ($P = 0.06$) concentrations than scFOS-fed puppies and controls. Finally, puppies fed inulin had an increase ($P = 0.05$) in *Lactobacillus* concentrations compared with scFOS and CON. In summary, fructan supplementation appeared to attenuate some of the negative responses associated with Salmonella challenge and may provide protection against infection in weanling puppies. J. Nutr. 137: 1923–1930, 2007.

Introduction

Salmonella contamination of raw pet foods and treats has been associated with several salmonellosis outbreaks among both humans and pets in recent years (1). Infection in dogs is most often caused by consumption of infected food, contact with infected feces or other environmental components, and scavenging. The potential for exposure to infected food may be due in part to an increased popularity of feeding raw diets, such as the bones and raw food diet. However, feeding raw meat to dogs increases their exposure to certain types of bacteria that would not be present in processed dog foods. A previous study analyzed 112 samples of commercial raw meat used in greyhound diets and reported that 66% of the samples contained Salmonella, with *Salmonella*

typhimurium being the most prevalent (2). Another source of exposure may be through contaminated pet treats. Multiple studies have found >25% of animal-derived treats, such as pig ears, to contain Salmonella (3–5).

Although most adult dogs are able to recover from salmonellosis, young and stressed puppies are more susceptible to severe infection or death. As a result, weanling puppies are at high risk of illness because of the stressors associated with weaning and the lack of a fully developed immune system. One study estimated that weanling puppies infected with Salmonella have up to a 100% morbidity and 40% mortality rate (6).

Previous experiments have shown that nondigestible fructans have a prebiotic effect in dogs (7,8). Fructans are linear chains of fructose units linked by bonds (2,1) with or without a terminal glucose unit. In the current study, 2 fructan sources, short-chain fructooligosaccharide (scFOS)⁷ and inulin were tested. scFOS

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³ Supplemental Table 1 is available with the online posting of this paper at jn.nutrition.org.

* To whom correspondence should be addressed. E-mail: ksswanso@uiuc.edu.

⁷ Abbreviations used: CFU, colony forming units; CON, control; DGGE, denaturing gradient gel electrophoresis; DM, dry matter; FOS, fructooligosaccharide; INF, infected; INU, inulin; NON, noninfected; SCFA, short-chain fatty acids; scFOS, short-chain FOS.

contains 2–5 fructose units, whereas inulin is a long-chain FOS with a chain length of 10–60 units. As a result of its shorter chain length, scFOS is rapidly fermented in distal ileum and proximal colon and is thought to have its greatest effect in this region of the gut. In comparison, inulin's longer chain length results in a slower fermentation, allowing it to be fermented throughout the entire colon. Fructan supplementation increases the prevalence of beneficial bacteria such as bifidobacteria and lactobacilli while decreasing potential pathogens such as *Clostridium perfringens* and *Escherichia coli* (9). Fructan supplementation also increases fecal short chain-fatty acid (SCFA) concentrations. Such studies in dogs have been performed using healthy adult dogs, but there is still a lack of knowledge pertaining to young canine microbiota.

A previous experiment using neonatal piglets demonstrated protective effects of scFOS during a pathogen (*S. typhimurium*) challenge (10). In that experiment, scFOS reduced the prevalence of diarrhea and inflammatory responses associated with infection. Because dogs and pigs are both appropriate models for humans and often respond similarly to dietary intervention, we hypothesized that the addition of fructans to puppy diets would result in similar beneficial effects, decreasing the incidence and severity of clinical signs associated with *Salmonella* infection.

Materials and Methods

Animals and diets. Thirty hound-cross puppies (2 blocks of 15 dogs; 12 wk of age at baseline; 15 male and 15 female) were used in this experiment. Three dry extruded kibble diets (Table 1), varying in prebiotic concentration and source were used in this study. The main dietary ingredients (brewer's rice, poultry by-product meal, and poultry fat)

TABLE 1 Ingredient composition of diets fed to weaning puppies

| Ingredient | CON | FOS | INU |
|-------------------------------|-------|-------------|-------|
| | | <i>g/kg</i> | |
| Brewer's rice | 428.7 | 428.7 | 428.7 |
| Poultry by-product | 360.0 | 360.0 | 360.0 |
| Poultry fat | 135.0 | 135.0 | 135.0 |
| Dehydrated egg | 22.0 | 22.0 | 22.0 |
| Beet pulp | 40.0 | 30.0 | 30.0 |
| scFOS | 0.0 | 10.0 | 0.0 |
| Inulin | 0.0 | 0.0 | 10.0 |
| Sodium chloride | 4.5 | 4.5 | 4.5 |
| Potassium chloride | 5.6 | 5.6 | 5.6 |
| Choline chloride ¹ | 1.3 | 1.3 | 1.3 |
| Vitamin premix ² | 1.2 | 1.2 | 1.2 |
| Mineral premix ³ | 1.2 | 1.2 | 1.2 |
| Analyzed composition | | | |
| Dry matter, % | 91.8 | 91.9 | 91.8 |
| Organic matter, % DM | 92.5 | 92.1 | 92.4 |
| Ash, % DM | 7.5 | 7.9 | 7.6 |
| Crude protein, % DM | 31.8 | 31.6 | 30.0 |
| Acid hydrolyzed fat, % DM | 19.0 | 19.1 | 18.6 |
| Total dietary fiber, % DM | 3.3 | 3.4 | 5.3 |
| Gross energy, kJ/g | 21.8 | 21.8 | 21.3 |

¹ Provided per kg of diet: choline, 780 mg.

² Provided per kg of diet: all-*trans* retinol, 4.49 mg; cholecalciferol, 0.02 mg; 2-ambo- α -tocopherol, 59.88 mg; menadione sodium bisulfate complex, 0.60 mg; thiamin, 11.98 mg; riboflavin, 9.58 mg; pantothenic acid, 17.96 mg; niacin, 44.91 mg; pyridoxine, 11.98 mg; biotin, 0.11 mg; folic acid, 0.72 mg; vitamin B-12, 0.02 mg.

³ Provided per kg of diet: Mn (as MnSO₄), 12 mg; Fe (as FeSO₄), 90 mg; Cu (as CuSO₄), 2.4 mg; Zn (as ZnSO₄), 120 mg; I (as KI), 1.5 mg; Se (as Na₂SeO₃), 0.24 mg.

were chosen because they are free of fructans. Diets were formulated to meet all nutrient recommendations by the Association of American Feed Control Officials (11) and contain ~30% crude protein (CP), 20% fat, and minimal dietary fiber. Thus, these diets were formulated to mimic commercially available "premium" dog foods. The control diet (CON) contained 4% beet pulp as a dietary fiber source. Treatment diets consisted of CON with 25% of the beet pulp (1% of diet) being replaced with scFOS (Nutraflora; GTC Nutrition; \geq 95% scFOS; scFOS); or inulin (Ultra-FOS ST; Encore Technologies; 90–94% inulin; INU). Dogs were individually housed in containment suites of the animal facility in the Edward R. Madigan Laboratory at the University of Illinois. Pen sizes were $\sim 2.7 \times 3.6 \times 4.5$ m with rubber coated mesh flooring. Rooms were climate controlled with a 12-h light:12-h dark cycle. The University of Illinois Institutional Animal Care and Use Committee approved all procedures prior to animal experimentation.

Experimental design. Six treatments were evaluated using a 2×3 factorial design. Dogs were allotted by weight to 1 of 6 treatments having different dietary (CON, scFOS, INU) and infection [infected (INF), noninfected (NON)] treatments. With the exception of the scFOS INF group with 4 males and 1 female, there were no major differences in gender among treatments. The CON NON, INU NON, and INU INF consisted of 2 males and 3 females each, whereas the remaining treatments consisted of 3 females and 2 males. Food was consumed ad libitum and food refusals were measured daily to determine total food intake. Rectal temperatures were taken daily prior to the morning feeding on d 0 to d 14. Body weight was measured on d 0, 7, 14, 15, and 16. Blood samples were also collected after a 12-h fast on d 0, 7, 14, 15, and 16 of the experiment. Fresh fecal samples were collected on d 0 and d 14.

After a 7-d adaptation period on CON, puppies were fed their assigned treatment diet from experimental d 0 to d 13. On d 14, puppies received an oral gavage of 5×10^8 colony forming units (CFU) of *Salmonella enterica* serovar *typhimurium* DT104 or 0.9% saline solution as described previously (10). In a previous study using adult beagles, dogs were dosed with 1×10^9 CFU of an attenuated strain of *Salmonella* that produced an infection response (6). In the current study a lower dosage was based on the higher potency of the strain used as well as response seen in neonatal piglets given a similar dose. The *Salmonella* used in the current experiment was a canine-derived strain graciously provided by R. A. Rastall (University of Reading, UK). All puppies continued to receive their assigned diets for 2 d. During the infection period, rectal temperature and activity scores were assessed every 8 h. Body weight and fecal scores were assessed daily. On d 16, puppies were killed by intravenously by administering a lethal dose of Euthasol (active ingredient pentobarbital sodium; 390 mg/L, Delmarva Laboratories) for intestinal tissue and digesta collection.

Samples were collected on d 16 (48 h postinfection) because this time was shown to be the peak infection response to *Salmonella*, including body temperature (6). Previous work utilizing *Salmonella*-challenged neonatal piglets reported a similar response (10). In that study, resolution of diarrhea occurred by d 7 postinfection with fructan supplementation having beneficial effects. Those researchers hypothesized that an earlier collection (e.g., 48-h postinfection) may increase the likelihood of observing beneficial effects of fructan supplementation.

Physical activity scores. Visual assessment of the physical activity of the puppies was assessed by the same individuals each morning. Activity score was graded based on the following scale: 1 = weak; 2 = lethargic; 3 = active. Puppies were assessed based on their responses to feeding, weighing, and rectal temperature measurement.

Fecal scores. Visual assessment of stool consistency was assessed by the same individuals for all fecal samples during d 14 to d 16. Feces were graded using the following scale: 1 = hard dry feces; 2 = hard, formed stool; 3 = soft, formed stool; 4 = soft, unformed stool; 5 = watery liquid.

Blood collection. Blood samples were collected following an overnight (12 h) fast via jugular venipuncture for serum immunoglobulin A (IgA), IgM, and IgG and complete blood count measurements. Serum was stored on ice until centrifugation within 1 h of collection. Samples were centrifuged at 4°C at $2000 \times g$ for 10 min. Following centrifugation,

supernatant was collected and stored at -80°C until further analysis for Ig concentration.

Feces and digesta collection. Fresh fecal (d 0 and 14) and digesta (d 16) samples from the proximal colon were collected within 15 min for fermentative end-product concentrations and microbial analyses. Due to lack of sample, digesta was only available for microbial analyses. On d 14, fecal samples were collected prior to *Salmonella* dosing. Approximately 1 g of each sample for microbial analyses was placed into a cryovial and stored at -80°C . Approximately 3 g of each sample was acidified using 10 mL of HCl (2 mol/L) to maintain pH before fermentative end-product analyses. These samples were stored at -20°C until further analyses.

Intestinal sample collection. Intestinal sections were collected from the ileum and colon for intestinal morphology, pathology, and nutrient transport measurements. Ileal and colon samples were rinsed in PBS and placed in phosphate buffered formalin for histomorphological and pathological analyses or in PBS for electrophysical analysis of electrolyte and nutrient transport using modified Ussing chambers. A third portion of each tissue was cut opened longitudinally, and the mucosa was scraped from the luminal surface using a glass microscope slide and snap-frozen for subsequent microbial analyses as described previously (10).

Intestinal histomorphology and pathology. Ileum and colon specimens were embedded in paraffin, sectioned at $5\mu\text{m}$ thickness using a microtome, and stained with hematoxylin and eosin at the University of Illinois at Urbana-Champaign Veterinary Pathology Laboratory. Villus length, villus height, and crypt depth measurements were performed on as many as 15 well-oriented, intact villi using Axiovision AC software and an AxioCam MRc5 (Zeiss, Obercohen).

In addition, histopathology was assessed by a board-certified pathologist (M.A. Wallig) who was unaware of the study design. A scale of 0–3 was used to assess intestinal samples (Table 2).

TABLE 2 Histopathology scale used to assess intestinal samples in weanling puppies fed diets containing short-chain fructooligosaccharides (scFOS) or inulin

| Item | Description of score |
|--|---|
| Enterocyte sloughing | 0: epithelium lifted from <5% of villus tips of the ileum/luminal surface of the colon. |
| | 1: Epithelium lifted from 5–25% of villus tips of the ileum/luminal surface of the colon. |
| | 2: Epithelium lifted from 26–50% of villus tips of the ileum/luminal surface of the colon. |
| | 3: Epithelium lifted from > 50% of villus tips of the ileum/luminal surface of the colon. |
| Neutrophil and macrophage infiltration | 0: None observed in the lamina propria |
| | 1: Scattered, small aggregates (2–5) near laminal surface. |
| | 2: Scattered, small aggregates (2–5) in superficial portions of the lamina propria. |
| | 3: Aggregates in most villi and in the deep lamina propria. |
| Edema | 0: No expansion or spreading apart of cells |
| | 1: <50% of villi, confined to outer third of the tips/multifocal within the luminal half of the lamina propria. |
| | 2: >50% of villi, extending past the outer third of the tips/multifocal, extending entire lamina propria. |
| | 3: >50% of villi, extending the full length of villi intercryptal areas/diffuse extending entire lamina propria |

Nutrient and ion transport measurements using modified Ussing chambers. Ussing chamber nutrient and ion transport was evaluated using methods previously described by Kles et al. (12,13). Briefly, intestinal samples were cut longitudinally along the mesentery, stripped of the muscularis, and mounted in modified Ussing chambers (Physiological Instruments) exposing 0.5 cm^2 of the mucosal and serosal sides to 8 mL of oxygenated (95% O_2 and 5% CO_2) modified Krebs buffer solution maintained at 37°C by use of a circulation water bath (IsoTemp 2006S, Fisher Scientific). After allowing 20–30 min to reach equilibrium, basal transmucosal short-circuit (I_{sc} , $\mu\text{A}/\text{cm}^2$), epithelial resistance (Ωcm^2), and potential difference (PD, mV) were measured. Sodium-dependent nutrient transport was determined by addition of 10 mmol/L glucose, glutamine, serotonin, or carbachol to the mucosal reservoir. Nutrient transport was measured by quantifying the electrogenic gradient change of the short-circuit current. The modified Ussing chambers were connected to dual channel voltage/current clamps (VCC MC2, Physiologic Instruments) with a computer interface that allowed for real time data acquisition and analysis with Acquire and Analyze software (Physiological Instruments).

Chemical analyses. Diet samples were analyzed for dry matter (DM) and organic matter (OM)(14). Crude protein was determined using a Leco Nitrogen/Protein Determinator (model FP-2000, Leco Corporation) (15). Fat concentrations were measured by acid hydrolysis (16) followed by ether extraction (17). Gross energy was measured by the use of a bomb calorimeter (Model 1261, Parr Instruments). Total dietary fiber concentration was determined as previously described (18).

Fecal and digesta samples were analyzed for DM (14). Ammonia concentrations were measured according to previously determined methods (19). Short-chain fatty acids were determined via GC (20). Concentrations of acetate, butyrate, and propionate were determined in the supernatant of acidified colonic aliquots using a Hewlett-Packard 5890A Series II gas chromatograph (Palo Alto, CA) and a glass column (180 cm \times 4 mm i.d.) packed with 10% SP-1220.1% H_3PO_4 on 80/100+ mesh Chromosorb WAW (Supelco). Nitrogen was the carrier gas with a flow rate of 75 mL/min. Oven temperatures, detector temperature, and injector temperature were 125, 175, and 180°C , respectively.

Blood analyses. Serum IgA, IgG, and IgM concentrations were measured using Animal Immunoglobulin and Protein RID kits [IgA 646471, IgG 646251, and IgM 646261 (MP Biomedicals, LLC)] according to manufacturer's instructions. Complete blood count was measured using a Cell-Dyn 3500 hematology analyzer (Abbott Laboratories) at the University of Illinois Veterinary Diagnostics Laboratory.

Microbial analyses. Bacterial DNA was purified using QIAamp DNA stool mini kits (Qiagen) according to manufacturer's instructions. Fecal DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Isolated DNA was amplified using polymerase chain reaction (PCR) with primers targeting variable region 3 (V3) of the 16S rDNA as previously described (21,22). The primers used for denaturing gradient gel electrophoresis (DGGE) were 341F ("eubacterial primer") and 534R. Primer 341F has a 40-nucleotide GC-clamp at the 5'-end (5'-CGCCCCCGCGCGCGCGGGGCGGGGCGGGGGCA-CGGGGGG-3') to prevent complete dissociation of the DNA double strand during DGGE. Briefly, the PCR mixture contained 10 ng of genomic DNA, 25 pmol of each primer, and 50 μL Taq PCR Master Mix Kit (Qiagen). The final volume was adjusted to 100 μL with sterile deionized water. The PCR amplification used touch-down cycling (beginning at 65°C) that consisted of lowering the annealing temperature every second cycle until it reached 55°C , at which temperature 9 additional cycles were completed for a total of 29 cycles. Amplification resulted in PCR products of ~ 200 bp in length. Single-stranded DNA remaining from the PCR was degraded using mung bean nuclease (Stratagene) as described previously (21).

To determine population changes within the predominant fecal microflora, bacterial species were separated using parallel DGGE (22) and a Bio-Rad D-Code System. Denaturing gradient gel electrophoresis analysis for V3–16S rDNA variable region products was performed using gels containing a gradient of 29–48% denaturant. A 100% denaturing

solution contains 40% (v:v) formamide and 7 mol/L urea. The polyacrylamide was diluted from a nondeionized 40% acrylamide/bis stock solution 37.5:1 (Bio-Rad). Gradients were formed using a Bio-Rad Gradient Former Model 475 (Bio-Rad) and gels were polymerized onto support film (FMC). Electrophoresis running time was 10 min at 50 V followed by 3 h at 150 V. Additionally, bacterial reference ladders were loaded to allow standardization of band migration and gel curvature among different gels (21). Gels were silver stained and photographed. Dice's similarity index (23) was calculated on pairs of lanes on the DGGE gel to indicate differences among the baseline and treatment periods.

Bifidobacterium genus, *Lactobacillus* genus, *Clostridium perfringens*, *E. coli*, and *Salmonella* were quantified using quantitative real-time PCR (Supplementary Table 1) (24–28). Amplification was performed in triplicate reactions for each bacterial genus within each sample according to the procedures described previously (29). For amplification, 10- μ L final volume containing 2 \times SYBR Green PCR Master Mix (Applied BioSystems), 15 pmol of each primer, and 10 ng of DNA template was used. Pure cultures of each bacterium were utilized to create 5-fold serial dilutions in triplicate. The DNA from each serial dilution was extracted using a QIAamp DNA stool minikit and amplified along with the fecal DNA samples to create triplicate standard curves using an ABI PRISM 7900HT Sequence Detection System (Applied BioSystems). The CFU of each standard curve was determined by plating the *Lactobacillus* genus on Difco Lactobacilli MRS broth (Becton, Dickenson, and Company), *Clostridium perfringens* and *Bifidobacterium* genus on Difco Reinforced Clostridial Medium (Becton, Dickenson, and Company) and *E. coli* and *Salmonella* on Luria Broth. The log of the CFU/L from each serial dilution was plotted against the cycle threshold value to create a linear equation for determining the CFU/g of dry feces. Data analysis was carried out with the sequence detection system software (Applied BioSystems).

Statistical analyses. Data were analyzed using the Mixed Models procedure of SAS (SAS Institute). Fixed effects included diet, infection, and diet \times infection interaction. Block was included as a random effect of the statistical model. Food intake, body temperature, body weight, complete blood count, and serum Ig concentration data were analyzed as a change from baseline. Ussing chamber data were analyzed using 2-way ANOVA analysis in SAS. The fixed effects were diet, infection, and diet \times infection interaction. A probability of $P < 0.05$ was accepted as significant and $P < 0.10$ was accepted as a trend. When significant interactions were observed, contrasts were run to determine the effect of infection within each diet. Main diet and infection effects were compared when the interaction was not significant. Values in the text are means \pm SEM.

Results and Discussion

Food intake, fecal scores, and activity scores. Food intake did not differ among dietary treatments during baseline (Table

3). However, a decrease in food intake occurred in all groups on d 15. This change likely occurred because blood samples were collected and temperatures were taken more frequently, which may have led to these changes. Although food intake on d 15 did not differ among groups, the change from the treatment period was affected by diet ($P < 0.05$).

Fecal scores increased ($P = 0.01$) in infected as compared with noninfected puppies regardless of dietary treatment (Table 3). Greater fecal scores (loose stool) among infected puppies was expected because diarrhea is one of the main symptoms associated with *Salmonella* infection. The intestinal inflammatory response, specifically neutrophil infiltration of the ileum, although serving to eliminate infection also results in damage to the mucosa and subsequent fluid leakage into the intestinal lumen. Salmonellosis also causes damage to the small intestine, decreasing the surface area available for absorption.

Body weight. Body weight did not differ among treatments at baseline (8.09 ± 0.92 kg), d 7 (9.92 ± 1.10 kg), d 14 (10.90 ± 1.58 kg), or d 15 (11.20 ± 1.54 kg). However, the change in body weight from d 14 to d 16 was greater ($P < 0.01$) in infected (-0.70 ± 0.48 kg) compared with noninfected (0.21 ± 0.48 kg) puppies. The decrease in body weight can most likely be attributed to the decrease in food intake.

Body temperature. A body temperature effect occurred at 8 h postinfection, with dogs fed CON having a higher ($P < 0.05$) body temperature than the fructan supplemented puppies (Fig. 1). Infection resulted in greater ($P < 0.01$) body temperatures at 32 and 40 h postinfection. In a previous experiment, purpose-bred adult beagles inoculated with 1×10^9 CFU of *Salmonella enterica* serovar *typhimurium* had a peak in body temperature 2 d postinfection (6). The values noted in the current study agree with their findings, with the body temperatures of infected puppies decreasing within 48 h postinfection. Similar to decreased food intake, increased body temperature is also an early sign of infection. Based on these data, puppies fed inulin may be protected during a bacterial challenge.

Immune characteristics. Baseline and d 14 complete blood count was similar across treatments and within normal ranges for growing puppies (data not shown). Following infection (d 15), however, greater ($P = 0.05$) change in WBC count ($3.27 \times 10^9 \pm 4.01 \times 10^9$ cells/L) and neutrophil concentration ($4.30 \times 10^9 \pm 3.61 \times 10^9$ cells/L) occurred. Conversely, infected puppies had decreased ($P < 0.01$) lymphocyte concentrations ($1.47 \times 10^9 \pm 0.52 \times 10^9$ cells/L). Similar changes occurred on d 16, with greater

TABLE 3 Food intake and fecal scores for weanling puppies fed control (CON), scFOS, or inulin (INU) diets and untreated (NON) or administered *S. typhimurium* DT104 (INF)¹

| Item | CON | | FOS | | INU | | Pooled SEM | P-value | | |
|--------------------------|------|------|------|------|-----|------|------------|-----------------|-------|-------------------|
| | NON | INF | NON | INF | NON | INF | | Diet | INF | Diet \times INF |
| Food Intake, g/d | | | | | | | | | | |
| Baseline ² | 466 | 483 | 427 | 425 | 468 | 394 | 58.4 | NS ³ | NS | NS |
| Day 15 | 296 | 108 | 343 | 136 | 362 | 208 | 55.1 | NS | <0.01 | NS |
| Change ⁴ | -174 | -371 | -122 | -261 | -68 | -214 | 85.8 | 0.05 | <0.01 | NS |
| Fecal score ⁵ | 3.3 | 3.4 | 3.2 | 3.3 | 3.1 | 3.7 | 0.19 | NS | 0.01 | NS |

¹ Values are means; $n = 5$ except INU/NON, $n = 4$.

² Food intake from d 0 to d 14.

³ NS, not significant, $P > 0.05$.

⁴ Food intake from baseline to d 15.

⁵ 1 = hard, dry feces; 2 = hard, formed stool; 3 = soft, formed stool; 4 = soft, unformed stool; 5 = watery liquid.

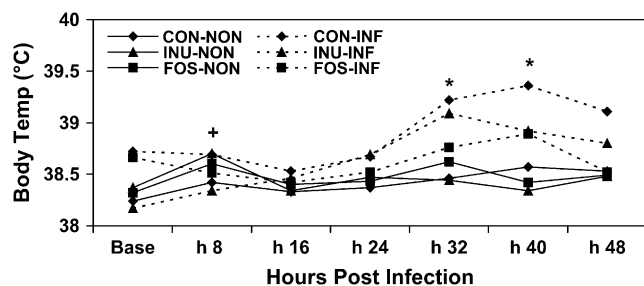


FIGURE 1 Body temperature change in weanling puppies fed diets containing short-chain fructooligosaccharides (scFOS) or inulin. Values are means, $n = 5$. Symbols indicate differences among treatments within hour: $+P < 0.05$, $*P < 0.01$. At 8 h postinfection CON puppies had higher ($P < 0.05$) body temperatures ($38.7 \pm 0.19^\circ\text{C}$) than fructan supplemented puppies ($38.5 \pm 0.19^\circ\text{C}$). Infection resulted in greater ($P < 0.01$) body temperatures at 32 h (NON = $38.5 \pm 0.48^\circ\text{C}$ vs. INF = $39.0 \pm 0.48^\circ\text{C}$) and 40 h (NON = $38.4 \pm 0.50^\circ\text{C}$ vs. INF = $39.1 \pm 0.50^\circ\text{C}$) postinfection.

increases ($P = 0.02$) in neutrophil concentrations ($1.97 \times 10^9 \pm 1.26 \times 10^9$ cells/L) and decreases ($P < 0.01$) in lymphocyte concentrations ($1.57 \times 10^9 \pm 0.55 \times 10^9$ cells/L) among infected compared with noninfected puppies. An increased white blood cell count indicated the presence of infection or inflammation and was an expected response of the immune challenge.

Serum IgA and IgM concentrations were not altered by diet or infection (data not shown). Increased IgA concentrations may be expected in response to an intestinal pathogen, as IgA prevents the adherence of bacteria or toxins to epithelial cells. Although a dramatic IgA response may affect serum concentrations, this was not observed in the current experiment. Maximal IgA production is not reached in dogs until 12 mo of age (30) and may be responsible for the lack of IgA response in this study. Although d 14 IgG concentrations did not differ among dietary treatments (IgG = 2.54 ± 0.37 g/L), a diet \times infection trend existed for serum IgG concentrations on d 15, with a tendency for infected dogs consuming the INU diet (2.03 ± 0.26 g/L) to have lower ($P = 0.10$) IgG concentrations than those consuming CON (2.85 ± 0.26 g/L) and scFOS (2.83 ± 0.26 g/L) diets.

Intestinal histopathology. *Salmonella* infection resulted in increased ($P < 0.01$) neutrophil and macrophage ($P = 0.05$) infiltration and edema ($P = 0.05$) in ileal tissue (Table 4). Neutrophil and macrophage infiltration was expected following infection because of their role in phagocytosis and pathogen killing. Edema was also expected to increase during infection due to active fluid

secretion into the interstitial space resulting from increased permeability of the mucosal venules and capillaries by inflammatory mediators. A diet \times infection interaction ($P = 0.05$) occurred for enterocyte sloughing. Whereas infected puppies fed the CON diet had higher ($P = 0.01$) pathology scores than noninfected CON-fed puppies, the infected and noninfected puppies fed INU and scFOS did not differ from one another. Because enterocyte sloughing indicates epithelial damage, INU- and FOS- fed puppies appeared to be protected during *Salmonella* infection.

Colon pathology was only mildly affected by infection in the current study. The occurrence and severity of colonic edema tended to be higher ($P = 0.08$) among infected puppies, regardless of diet (data not shown). As in the ileum, edema was likely attributed to increased membrane permeability of colonocytes during infection. The lack of pathological changes in the colon compared with the ileum may be due to bacterial competition, decreased pH, or other unknown mechanisms.

Intestinal histomorphology. Ileal villus height ($P = 0.09$), villus area ($P = 0.07$), and villus:crypt ratio ($P = 0.10$) tended to decrease in infected compared with noninfected puppies (data not shown). A diet \times infection interaction occurred for villus width, with infected puppies consuming scFOS tending to have a greater ($P = 0.08$) villus width than in those consuming CON and INU. Reduced villus height, area, and villus:crypt ratio indicate decreased absorptive capacity and may be attributed to the edema and enterocyte sloughing observed in infected puppies. Similar changes to intestinal morphology have been reported in neonatal piglets infected with *Salmonella* (10).

Nutrient transporter activity. Nutrient transporter activity is presented in Table 5. Transmucosal short circuit current (Isc) is a measure of basal active ion transport. Although we expected that Isc would decrease in the ileum due to infection, no changes were expected in the colon. In contrast to our hypotheses, differences did not occur in the ileum. A diet \times infection interaction ($P = 0.04$) occurred for Isc in colonic samples. Although Isc did not differ between infected and noninfected puppies fed CON or scFOS, it did decrease ($P < 0.05$) in infected vs. noninfected puppies consuming INU. Because Isc is a measure of unstimulated ion transport, the apparent decrease in ion flux may be attributed to the same amount of inward and outward flux resulting in circular ion movement (31).

Decreased glucose transport due to epithelial damage was expected in infected dogs. A diet \times infection interaction ($P = 0.04$) occurred for ileal sodium-dependent glucose transport. Whereas glucose transport remained the same in infected and

TABLE 4 Ileal and colonic pathology scores of weanling puppies fed diets containing short-chain fructooligosaccharides (scFOS) or inulin¹

| Item | CON | | FOS | | INU | | Pooled SEM | P-value | | |
|-----------------------------------|------------------|------------------|-----|-----|-----|-----|------------|-----------------|-------|-------------------|
| | NON | INF | NON | INF | NON | INF | | Diet | INF | Diet \times INF |
| Ileal pathology | | | | | | | | | | |
| Enterocyte sloughing ² | 0.1 ^a | 1.4 ^b | 0.2 | 0.4 | 0.6 | 0.1 | 0.44 | NS ³ | NS | 0.05 |
| Neutrophil | 0.1 | 1.1 | 0.3 | 0.7 | 0.3 | 1.1 | 0.46 | NS | <0.01 | NS |
| Macrophage | 0.1 | 0.8 | 0.1 | 0.5 | 0.0 | 0.5 | 0.34 | NS | <0.01 | NS |
| Edema | 0.3 | 1.4 | 0.3 | 0.8 | 0.4 | 0.5 | 0.44 | NS | 0.05 | NS |

¹ Values are means; $n = 5$. Within a group, means in a row without a common letter differ, $P < 0.05$.

² Pathology scores described in materials and methods section.

³ Not significant, $P > 0.05$.

TABLE 5 Ileal and colonic nutrient and ion transport of weanling puppies fed diets containing short-chain fructooligosaccharides (scFOS) or inulin¹

| Item | CON | | FOS | | INU | | Pooled SEM | P-value | | |
|---|------------------|------------------|-------|-------|------------------|-------------------|------------|-----------------|------|------------|
| | NON | INF | NON | INF | NON | INF | | Diet | INF | Diet × INF |
| Transmucosal resistance, Ωcm^2 | | | | | | | | | | |
| Ileum | 235.1 | 257.4 | 261.4 | 261.8 | 224.4 | 223.7 | 20.75 | NS ² | NS | NS |
| Colon | 173.5 | 215.0 | 175.1 | 200.4 | 194.3 | 180.8 | 15.86 | NS | NS | NS |
| Isc, ³ $\mu\text{A}/\text{cm}^2$ | | | | | | | | | | |
| Ileum | 4.4 | 4.1 | 3.9 | 3.4 | 5.5 | 4.6 | 1.42 | NS | NS | NS |
| Colon | 3.2 | 3.5 | 2.2 | 4.6 | 6.4 ^b | 0.2 ^a | 1.80 | NS | NS | 0.04 |
| PD, ⁴ mV | | | | | | | | | | |
| Ileum | 1.0 | 1.0 | 1.0 | 0.9 | 1.1 | 1.0 | 0.35 | NS | NS | NS |
| Colon | 0.6 | 0.7 | 0.4 | 1.0 | 1.3 ^b | -0.1 ^a | 0.36 | NS | NS | 0.02 |
| Glucose transport, $\Delta\text{Isc}/\text{cm}^2$ | | | | | | | | | | |
| Ileum | 2.4 ^b | 0.6 ^a | 1.5 | 1.7 | 1.0 | 1.5 | 0.53 | NS | NS | 0.04 |
| Colon | 1.0 | 3.5 | 1.7 | 1.8 | 1.9 | 2.7 | 0.72 | NS | 0.03 | NS |

¹ Values are means; $n = 5$. Within a group, means in a row without a common letter differ, $P < 0.05$.

² Not significant; $P > 0.05$.

³ Basal transmucosal short-circuit.

⁴ Potential difference.

noninfected puppies consuming INU and scFOS, it decreased ($P < 0.02$) in infected puppies consuming CON when compared with noninfected puppies. In the colon, sodium-dependent glucose transport was greater ($P < 0.05$) in infected puppies than in noninfected puppies, regardless of diet. These are very interesting findings, as they suggest that fructan supplementation attenuated the decreased glucose transport resulting from infection. Our data suggest that by attenuating epithelial sloughing, fructan supplementation may minimize the reduction of nutrient transport observed during bacterial infection. Our data also demonstrate increased colonic glucose transport during infection, which may be a means of compensating for decreased ileal transport.

Fecal fermentative end products. Fecal or digesta DM did not differ among groups. Individual and total fecal SCFA did not differ at baseline. By d 14, total SCFA concentrations tended to be higher ($P = 0.06$) in puppies consuming INU than in those consuming CON or scFOS. The change in fecal acetate concentration from d 0 to d 14 was greater ($P = 0.03$) in puppies fed INU ($85.5 \pm 30.47 \mu\text{mol/g}$) than in those consuming CON ($-37.7 \pm 30.47 \mu\text{mol/g}$) and scFOS ($13.8 \pm 30.47 \mu\text{mol/g}$). The change in total fecal SCFA concentrations from d 0 to d 14 was also greater ($P = 0.03$) in puppies fed INU ($145.5 \pm 49.18 \mu\text{mol/g}$) than in those consuming CON ($-53.5 \pm 49.18 \mu\text{mol/g}$) and scFOS ($25.4 \pm 49.18 \mu\text{mol/g}$).

SCFA are the primary energy source of colonocytes and have a trophic effect on the colonic epithelium. They also decrease colonic pH, which aids pathogenic resistance. Fermentable carbohydrates, including fructans, are the primary source of SCFA because they are readily fermented by the colonic bacteria. Fecal SCFA concentrations are often not altered when puppies are fed scFOS (7,8). However, increased fecal butyrate concentration was observed in neonatal piglets supplemented with scFOS (10). Although differences may be expected in SCFA production due to fructan consumption, these acids are rapidly absorbed by colonocytes. Thus, fecal SCFA concentrations do not always correlate with SCFA production. In our experiment, increased acetate and total SCFA concentrations were noted among puppies consuming the INU diet but not among those consuming the CON and scFOS diets. These differences are likely due to longer chain length of INU. In contrast to scFOS that are rapidly fer-

mented in the proximal colon, INU is fermented throughout the entire colon, increasing the likelihood of affecting fecal SCFA concentrations. Our study suggests that supplementation with INU may have a beneficial effect on intestinal health and may provide protection in puppies during *Salmonella* infection.

There were no differences in fecal BCFA or ammonia concentrations (data not shown). These compounds are both produced by microbial protein catabolism. Fecal ammonia was previously reported to be present in greater concentrations in adult dogs than those reported in our experiment (7,8). In addition to potential differences in diet composition, a less active or diverse intestinal microbiota in puppies may result in decreased protein catabolism and account for the decreased BCFA and ammonia concentrations in the current study.

Microbial analyses. Fecal *Lactobacillus*, *C. perfringens*, and *E. coli* populations did not differ among groups at baseline or d 14. *Salmonella* was not detected in feces on d 0 or d 14 prior to infection. A greater increase ($P = 0.05$) in *Lactobacillus* occurred from d 0 ($10.5 \pm 0.45 \text{ cfu log}_{10}/\text{g DM}$) to d 14 ($11.2 \pm 0.21 \text{ cfu log}_{10}/\text{g DM}$) in puppies consuming INU than in those fed CON or scFOS. *Lactobacillus* is thought to provide benefits to the host, including the prevention of pathogen colonization by means of nutrient and attachment site competition. These data suggest that INU consumption has a protective role during infection by increasing the amount of lactobacilli present in the gut. This protective role of INU may be attributed to the fact that it is fermented throughout the colon allowing it to have a greater impact on colonic microbial populations than scFOS. Despite these statistical differences, however, only the change from d 0 to d 14 differed (d 14 concentrations were similar). Thus, we are unable to determine the importance of these effects at this time.

Bifidobacteria measurements were also attempted in the current study. However, only 6 of 20 dogs had counts above our detectable limits of $1.81 \text{ log CFU/g DM}$. Additional analyses were performed to verify primer efficiency and DNA integrity, but similar results were generated. Although these results were unexpected, it appears that the population of weanling puppies in the current study had very low concentrations of bifidobacteria.

Previous studies reported an inability to detect bifidobacteria in canine digesta and fecal samples (32,33). One study analyzed

digesta samples collected from puppies at 1, 21, 42, and 63 d after birth, as well as their dams for microbes, including bifidobacteria (32). That study agreed with our results, reporting that bifidobacteria were not detected in any samples by agar plating. In a previous experiment (33), fecal samples from 4 adult Labrador Retrievers were plated and reported no detection of bifidobacteria. These findings suggest the possibility that bifidobacteria is not always a prominent member of the commensal microbial population in dogs.

Previous studies have also found undesirable changes in bacterial populations during the weaning period. Bifidobacteria and lactobacilli were reported to decrease and be replaced with increased number of *E. coli* and *C. perfringens*. Weaning caused a rapid and significant decrease in lactobacilli concentrations and an increase in *E. coli* in piglets 1 d postweaning (34). Our study found high counts of *E. coli* and *C. perfringens*, especially at the beginning of the study when the puppies were likely stressed due to changes in environment and experimental diet. Because the "normal" commensal microbial populations of puppies and changes over time have not been determined, further research is needed to examine these variables.

Lactobacillus, *C. perfringens*, and *E. coli* concentrations in colonic digesta or ileal mucosa were not affected by diet or infection. Values obtained for mucosal microbial populations were lower than those observed for digesta. Evaluation of mucosal vs. luminal microbes in dog intestine has been poorly studied. Our results show that *Lactobacillus* are a prominent species attached to the mucosa, being present in much higher concentrations than *E. coli* or *C. perfringens*. These findings agree with a previous study that reported greater lactobacilli vs. *C. perfringens* populations in the small intestinal mucosal samples of young and adult dogs (32). Surprisingly, *Salmonella* was detected in the ileal mucosa of only 3 of 15 infected puppies. *Salmonella* was detected in the digesta of infected puppies, but was not affected by dietary treatment.

Denaturing gradient gel electrophoresis analysis of fecal samples detected differences in microbial diversity due to diet. Compared with baseline, d 14 Dice's Index scores for puppies consuming scFOS (33%) were lower (greater change; $P = 0.02$) than scores for puppies fed CON (52%) or INU (50%). The findings from our study differ from those observed in a previous work (I. S. Middelbos, N. D. Fastinger, and G. C. Fahey Jr., unpublished data) that evaluated the response of adult dogs supplemented with beet pulp, cellulose, FOS, or yeast cell wall extract. In that study, the researchers reported a high similarity among diets containing fermentable oligosaccharides (Dice's Index scores = 87–88%). Furthermore, beet pulp feeding was similar to FOS treatment (88%). The lack of similar observations due to fructan supplementation in our study may be attributed to the fact that the intestinal microbiota in puppies is not well established and may undergo drastic changes in response to environment and/or diet. This would also explain the lower similarity scores within treatments compared with those previously reported. In the current study, there was a relatively low level of similarity, even among CON puppies, indicating a general change in microbial populations between d 0 and d 14 in our weaning population.

The results of this study suggest that fructan supplementation, including scFOS and inulin, may have beneficial effects on the immune response to a bacterial challenge in weanling puppies. Because weaning is a stressful time for puppies due to changes in diet and environment, they are more susceptible to *Salmonella* infection. In this study, fructan supplementation attenuated the decreased food intake, body weight changes, and

increased body temperature caused by infection. Fructan supplementation also decreased enterocyte sloughing, an indicator of intestinal damage due to infection, suggesting a protective role during infection. However, because enterocyte sloughing was the only histological improvement with fructan supplementation, more research is needed to validate the current results. This protection may have played a role in maintaining ileal glucose transport in fructan-supplemented dogs during infection. The total SCFA and acetate concentration and intestinal microbial populations were also increased by inulin supplementation. Further research will determine whether these outcomes or other mechanisms are responsible for their protective role during a bacterial challenge.

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