Fermentable Fibers Enhance Aspects of Innate and Adaptive Immunity in Piglets infected with *Salmonella Typhimurium*

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Objective: To test the hypothesis that fermentable fiber prevents *Salmonella typhimurium* infection-associated symptoms by enhancing innate and adaptive immune system in neonatal pigs.

Methods: Two-d-old-piglets (n=120) were randomized to receive either a nutritionally complete sow milk replacer formula (CON), or supplemented with methylcellulose (MCEL-non-fermentable), soy polysaccharides (SPS-moderately fermentable), or fructooligosaccharides (FOS-highly fermentable). On d7, piglets received an oral gavage of *S. typhimurium*-798, and continued receiving the same diets up to 48h post-infection. Ileal mucosal samples were obtained for further analyses.

Results: A reduction in chloride secretion was observed in FOS when compared to other diets ($p<0.0003$). The number of ileal sulfo-acidomucins was higher ($p<0.05$) in FOS before infection compared with other diets. NFkB was inhibited in FOS following infection ($p<0.05$), when compared with CON. IL-1β expression was increased at 4h post-infection ($p<0.05$) in CON; however, this response was attenuated in the fiber groups. IL-6 expression was higher ($p<0.05$) in CON post- infection, higher in SPS at 24h ($p<0.05$), but unchanged in MCEL and FOS when compared to pre-infection values. FOS had a higher expression of neutrophil-chemoattractant IL-8 before infection ($p<0.05$) compared to other groups.

Conclusion: The reduction in chloride secretion, proinflammatory cytokines expression and NFkB activation, and increased number of sulfo-acidomucins, and IL-8 expression in the fiber groups, indicates that the degree of fermentability impacts the innate and adaptive immune system, and could be the mechanisms by which dietary fibers reduce *S. typhimurium* infection-associated-symptoms in neonatal pigs and apply these results to infants. [*P R Health Sci J 2020;39:311-318*]

Key words: Dietary fiber, Innate immunity, Adaptive immunity, Infection, Neonates

The immune system is the host mechanism of defense against pathogens. Lymphocytes and other components of the adaptive immune response require 4-5 days to be activated; therefore, the host requires an early defense system (1, 2). The innate immune system provides the first line of defense and has components that act as physical barriers. In the intestine, the production of mucus and other secretory products can *wash-out* the bacteria in the lumen, thus acting as physical barrier by preventing pathogens from adhering and entering the intestinal epithelial layer (3, 4). The increased mucus production acts to engulf the bacteria, preventing their entrance. It has been showing that the acidic mucins localized in the villus lining are produced upon bacterial infection making them mucins are more resistant to bacterial degradation (3,4). Within the acidic mucins, the sulfated subtypes can inhibit bacterial growth *in-vitro* (5). Other aspects of the innate immune system include phagocytes, natural killer cells, polymorphonuclear neutrophils, and macrophages.

Some investigators have shown that nutrients, especially fermentable fibers, can enhance intestinal immunity (6,7) through a variety of mechanisms, including reducing bacterial translocation in patients in partial enteral nutrition (8,9) increasing immune cell numbers in dogs (10,11), increasing the number of Peyer's patches (12, 13), altering lymphocyte numbers in intestinal mucosa (14,15), and increasing IgA secretion (16,17). Other studies have shown a reduction of chloride secretion by butyrate, a product of fiber fermentation

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in human colonic cells (18,19). Butyrate decreases pro-inflammatory cytokine expression via inhibition of NFkB activation and IkB degradation in human intestinal isolated colonic cells (20,21) however, whether butyrate exerts a similar action in- vivo is as yet unreported.

We previously demonstrated that the addition to fermentable fiber to infant formula prevented infection-associated symptoms of diarrhea and maintained intestinal structure and function (22). To investigate potential mechanisms underlying the protection against *S. typhimurium* conferred by fermentable fibers, intestinal NFkB activation and cytokine expression were assessed prior to infection and at time points over the first 48h post-infection. Previous studies have shown a rapid response in immune cell infiltration and cytokine expression following *S. typhimurium* infection. For example, an increase of intestinal neutrophils and macrophages and an up-regulation in the expression of the chemoattractant, IL-8, was observed as early as 3h-8h post infection (23,24).

Activation of NFkB precedes the transcription of pro-inflammatory cytokines TNF-α and IL-1β, which are produced by macrophages early in the inflammatory response. Thacker and colleagues (25) reported that the response of pigs to an invading microorganism is divided in three phases: early response (4h), where the innate barriers and neutrophils are activated, the early response to the invasion (4h-96h) and the adaptive response after 96h. Herein, we hypothesized that the absence of diarrhea in the piglets fed fermentable fibers was due to the enhanced immune system acting as a barrier to prevent bacteria attachment to the mucosa by increasing mucus production (26), preventing chloride secretion and inhibiting the production of pro-inflammatory cytokines via the inactivation of NFkB.

Materials and Methods

Experimental design

The experimental design is presented in Figure 1. This study was approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign (IACUC #01223). One hundred twenty (n=120) 48h-old piglets were obtained from the Swine Facility from the University of Illinois at Urbana-Champaign. Piglets (n=30/group) were randomized to receive one of four diets: sow milk replacer formula alone or supplemented with 7.5 g/L of methylcellulose (MCEL: non-fermentable fiber), soy polysaccharides (SPS; moderately fermentable fiber), or fructo-oligosaccharides (FOS; highly fermentable fiber). The diets were isoenergetic and isonitrogenous and provided at 15 ml/kg BW/day via nipple tubing attached by tubing to an enteral nutrition bag (Flexiflow Easyfeed®, Ross Laboratories, Columbus, OH). Piglets were housed in the Edward R. Madigan Laboratory, with a room temperature at 30 °C with a 12h light/dark cycle.

At d7, piglets were further randomized to five groups within each diet group, a non-infected group and four infected groups

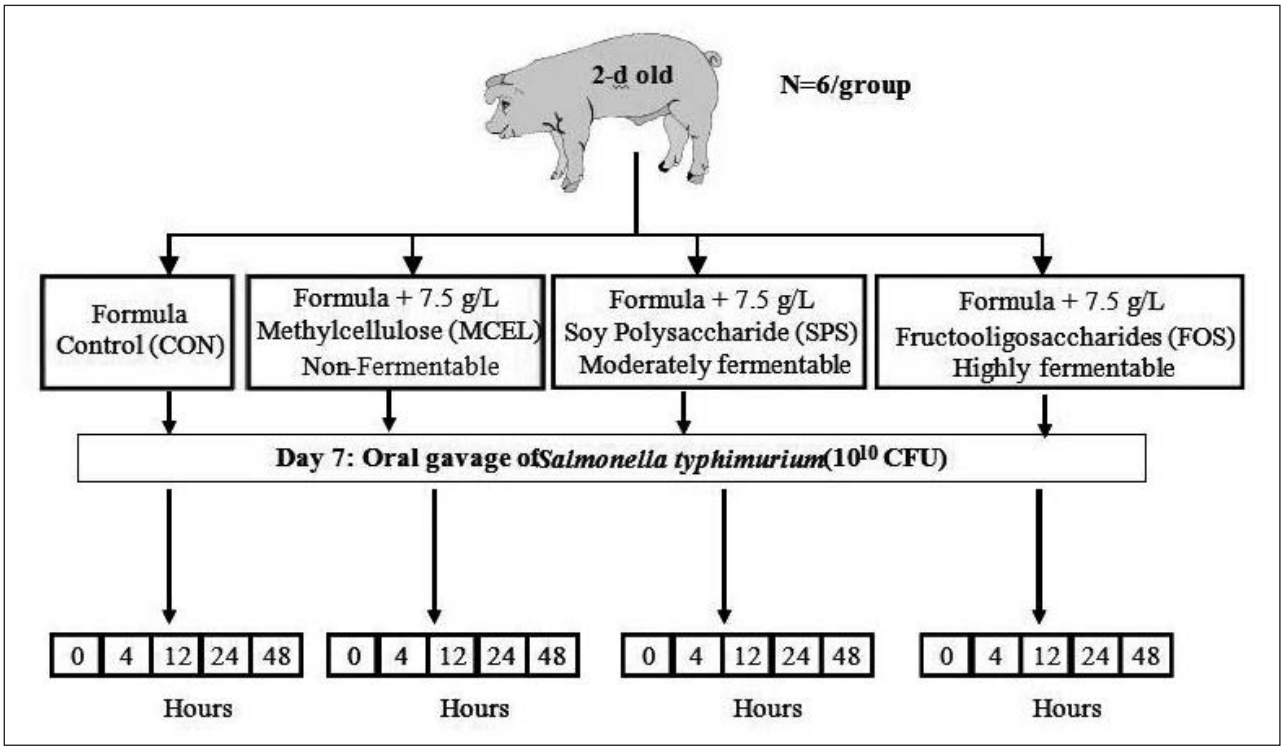

Figure 1. Experimental Design. Two d-old piglets (n=120) were randomized into four diets groups with formula alone (CON) or formula supplemented with methylcellulose (MCEL), soy polysaccharides (SPS) or fructo-oligosaccharides (FOS). At d7, piglets were infected with an oral gavage of S. typhimurium 1010 CFU and were further randomized for euthanasia at 0, 4, 12, 24, and 48h post-infection.

that were euthanized 4, 12, 24 and 48h following infection (n=6/group). The infected piglets received an oral gavage of 10^{10} colony forming units (CFU) of *S. typhimurium*, and also continued receiving their respective diets for the following 4h, 12h, 24h and 48h post infection. Following infection, piglets were housed in the containment area. To prevent cross-contamination, a one-way flow from non-infected area to the infected area was implemented and investigators used disposable coveralls, plastic boots, double gloves and masks.

Piglets were sedated with an intramuscular injection in a dose of 6-9 mg / kg BW (Telazol®; Fort Dodge, Iowa) and euthanized with 0.25 ml/kg body weight of sodium pentobarbital (Fatal Plus; Veterinary Laboratories, Inc., Lenexa, KS). The small intestine was removed from the ligament of Treitz to the ileocecal valve and divided into equal thirds by length (proximal jejunum, mid-jejunum and ileum) and flushed with 0.9% ice-cold saline solution. Ileal samples were taken for electrophysiological analysis of electrolyte and nutrient transport by modified Ussing chambers. Other samples were snap-frozen with liquid nitrogen and stored at -80 °C for further analyses of RNA, DNA, nuclear factor NFkB and pro-inflammatory cytokines mRNA abundance. Samples were fixed in either 10% formalin or Bouin's solution for histomorphological and goblet cell analyses, respectively.

Clinical measurements

Visual assessments of stool consistency and physical activity levels, as well as the assessment of body weight and rectal temperature were recorded as previously (12). Measurements of body weight (kg), physical activity levels (1=lethargic, 2=week and 3=active), and stool consistency (1=solid 2=semisolid 3=loose 4=watery) were assessed and recorded on a daily basis, whereas the rectal temperature (°C) observations were recorded every other day before infection, and then after infection, the data was recorded daily.

Mucin and Goblet Cell analyses

Bouin's-fixed intestinal samples from ileal segments were embedded in paraffin, sectioned at 5 µm thickness with a microtome at the University of Illinois Urbana-Champaign Veterinary Pathology Laboratory and mounted on glass microscope slides. These sections were stained in three different ways to identify total goblet cells, acidic mucin-secreting Goblet cells and sulfated mucin-secreting goblet cells.

Alcian Blue pH 2.5 staining

The use of the Alcian blue solution (Sigma, St. Louis, MO) was used to distinguish between acidic and neutral mucins. The copper-containing phthalocyanin basic dye produces a blue color in acidic mucins (both sialated and sulfated) in the presence of 3% acetic acid (21). Harris hematoxylin (Sigma) was used to detect neutral mucins, which produce a red color. The combination of acidic and neutral mucins is the indicator of the total goblet cells numbers. Using Image-Pro plus software (Media Cybernetics, Silver Spring, MD) and a Nikon Optiphot-2 microscope (Nikon, Melville, NY), the number of alcian blue-stained cells per villus height and crypt depth was measured on 8-10 well-oriented intact villi and crypts for each sample. All measurements were taken using a 10X objective and expressed as cell numbers normalized by villus height or cypt depth in microns (µm).

High-Iron Diamine-Alcian Blue staining

High-iron diamine-alcian blue was used to stain for acidic sulfo-mucins. Two diamine salts (N, N-dimethyl-meta-phenylenediamine diamine and N, N-dimethyl-para-phenylenediamine dihydrochloride diamine) were mixed with ferric chloride to produce a high iron diamine solution. The slides were bathed in this solution for 18h and further treated with Alcian Blue (pH 1.0). The sulfated mucins produce a brown color and the carboxylated mucins produce a blue color. The stained cells were counted in the microscope, using a 10X objective and expressed as cell numbers normalized by villus height or crypt depth in microns (µm).

Chloride secretion

The methods for electrophysiological measurement of nutrient and ion transport were done previously (22, 27). Briefly, intestinal samples were cut longitudinally along the mesentery, stripped of the muscularis and mounted in modified Ussing chambers (Physiologic Instruments, Inc., San Diego, CA) exposing 0.5 cm^2 of both the mucosal and serosal sides to 4 ml of oxygenated (95 % O_2, 5 % CO_2) modified Krebs buffer solution maintained at 37 °C with a circulating water bath (IsoTemp 2006S, Fisher Scientific, Itasca IL). Mannitol and glucose were added at a final concentration of 10 mM to the mucosal and serosal chambers, respectively, to maintain an osmotic balance. Following an equilibration period of 20-30 min, basal transmucosal short-circuit current (Isc; $\mu A/cm^2$), epithelial resistance ($\Omega \bullet cm^2$), and potential difference (Pd; mV) were measured and chloride secretion was quantified by measuring the change in short-circuit current following the addition of the secretagogues serotonin (0.1 mM) or carbachol (0.1 mM) to the serosal medium, respectively. The modified Ussing chambers were connected to dual channel voltage/current clamps (VCC MC_2, Physiologic Instruments, Inc) with a computer interface allowing for real time date acquisition and analysis using Acquire and Analyze software (Physiologic Instruments, Inc.).

Assessment of NFkB activation

The presence of activated NFkB in ileal mucosal tissue samples of infected piglets fed the CON or FOS diets were assayed using the NFkB Assay Family ELISA kit (ActiveMotif: Carlsbad, CA). The assay specifically targets the p50/p65 heterodimers, which are the most common dimers found in the NFkB signaling pathway. The activated region of NFkB binds to the consensus sequences and is detected by an NFkB

p65 primary antibody (which has cross-reactivity with human, mouse and rat), and a horseradish conjugated secondary antibody. The use of *Raji nuclear extract* at a concentration 5 µg/well of *Complete Lysis Buffer* (Active Motif; Carlsbad, CA) provided the strongest signal of the NFkB activation, and was used as a positive control to validate the assay. The addition of the developing solution for this assay provided a dark blue color. The readings in the spectrophotometer (U-2000, Hitachi, High-Technologies Corporation; Tokyo, Japan) were at 450 nm. Values were quantified using the standard curve method, and expressed as the concentration of activated NFkB protein in the sample.

Statistical methods

An analysis of variation (ANOVA) using PROC-MIXED was used to analyze the effect of diet (n=4), infection time (n=5) and the interaction of diet*time. Computations were performed using the SAS (Version 6.04; SAS Institute, Cary, NC). Statistical significance was defined as $p < 0.05$ and statistical trends were defined as $p < 0.1$. Data are means ±SEM.

Results

Clinical measurements

All piglets consistently gained weight throughout the study independent of diet or infection (Table 1). The body temperature of all piglets was maintained within the normal range for neonatal piglets (38.4-39.8 °C) and was unaffected by diet or infection. Physical activity was reduced ($p<0.05$) at 24h and 48 h after infection in the CON, MCEL and SPS groups, but not the FOS piglets (Table 1). Piglets fed FOS showed softer stools before infection compared to the other diets ($p<0.05$). Following infection, the piglets fed CON, MCEL and SPS had softer stools compared to pre-infection values ($p<0.05$) (Table 1), but piglets fed FOS showed similar stool consistency as pre-infection.

Chloride secretion

Significant main effects of both diet ($p<0.05$) and time ($p<0.05$) was observed on ileal serotonin-induced chloride secretion. Secretion was increased ($p=0.0001$) at 24h and 48h, irregardless of diet. However, within diet, SPS produced the highest ($p< 0.05$) secretion when compared to other diets (Figure 2).

Mucin production

The numbers of mucin-secreting goblet cells were counted within each villi and this number was then normalized by the villus height. Following infection, the total number of villus goblet cells increased in piglets fed CON by 48h post-infection, however, this difference was not statistically significant due to the great inter animal variability in the response to the infection (Table 2). Within the acidic mucins, the sulfated mucins in villus and the crypt were higher in FOS group compared to other diets

Table 1. Clinical Parameters in non-infected piglets and at 24 and 48h after infection with *Salmonella typhimurium* on d7 postnatal age.[1,2]

	Postnatal age (d)								
	1	2	3	4	5	6	7	8	9
	Body weight (kg)[3]								
CON	1.61 ± 0.01	1.69 ± 0.02	1.82 ± 0.02	1.99 ± 0.02	2.10 ± 0.02	2.33 ± 0.02	2.49 ± 0.02	2.51 ± 0.03	2.60 ± 0.05
MCEL	1.55 ± 0.01	1.62 ± 0.01	1.77 ± 0.01	1.92 ± 0.01	2.09 ± 0.01	2.26 ± 0.01	2.39 ± 0.01	2.42 ± 0.03	2.63 ± 0.05
SPS	1.53 ± 0.01	1.68 ± 0.01	1.81 ± 0.01	1.99 ± 0.01	2.14 ± 0.02	2.33 ± 0.02	2.52 ± 0.02	2.66 ± 0.03	2.82 ± 0.03
FOS	1.73 ± 0.02	1.62 ± 0.02	1.73 ± 0.02	1.89 ± 0.02	2.06 ± 0.02	2.20 ± 0.02	2.35 ± 0.03	2.51 ± 0.04	2.51 ± 0.13
	Physical activity level (1=lethargic 2=weak 3=active)[4]								
CON	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	2.93 ± 0.07^a	2.53 ± 0.03^b	1.75 ± 0.13^b
MCEL	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	2.35 ± 0.04^b	2.17 ± 0.15^b
SPS	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	2.33 ± 0.04^b	2.00 ± 0.13^b
FOS	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	3.00 ± 0.00^a	2.77 ± 0.05^a	2.80 ± 0.05^a
	Rectal temperature (°C)								
CON	38.27 ± 0.06	NA	38.22 ± 0.08	NA	38.56 ± 0.07	NA	38.75 ± 0.06	38.92 ± 0.08	38.57 ± 0.06
MCEL	38.17 ± 0.04	NA	38.21 ± 0.04	NA	38.79 ± 0.06	NA	38.68 ± 0.04	38.98 ± 0.02	38.32 ± 0.23
SPS	38.33 ± 0.04	NA	37.99 ± 0.08	NA	38.63 ± 0.06	NA	38.72 ± 0.06	38.97 ± 0.07	38.32 ± 0.26
FOS	38.30 ± 0.04	NA	37.11 ± 0.04	NA	38.68 ± 0.05	NA	38.70 ± 0.06	38.30 ± 0.04	38.67 ± 0.16
	Stool consistency (1=solid 2=semisolid 3=loose 4=watery)[5]								
CON	1.42 ± 0.02^a	1.15 ± 0.01^a	1.06 ± 0.01^a	1.12 ± 0.01^a	1.09 ± 0.01^a	1.09 ± 0.01^a	1.36 ± 0.02^a	2.50 ± 0.05^b	2.87 ± 0.09^b
MCEL	1.37 ± 0.02^a	1.23 ± 0.02^a	1.067 ± 0.01^a	1.10 ± 0.01^a	1.13 ± 0.01^a	1.13 ± 0.01^a	1.20 ± 0.01^a	1.82 ± 0.05^b	2.33 ± 0.16^b
SPS	1.42 ± 0.02^a	1.27 ± 0.02^a	1.17 ± 0.02^a	1.20 ± 0.02^a	1.66 ± 0.01^a	1.67 ± 0.01^a	1.23 ± 0.02^a	2.24 ± 0.06^b	2.67 ± 0.10^b
FOS	2.57 ± 0.02^b	2.40 ± 0.02^b	2.03 ± 0.02^b	1.87 ± 0.02^b	1.73 ± 0.02^b	1.76 ± 0.02^b	1.77 ± 0.02^b	2.05 ± 0.04^b	2.50 ± 0.21^b

[1]Data are means ±SEM. [2]Significant differences are expressed as P<0.05, trends as P<0.1. [3]Within this dependent variable, a trend (p=0.08) for the main effect of day was observed regardless of diet. [4]Within this dependent variable, the interaction diet*day is significant. Values with different letter superscripts are different from each other. [5]Within this dependent variable, the main effect of diet is significant. Values with different letter superscripts are different from each other. NA= Data not available

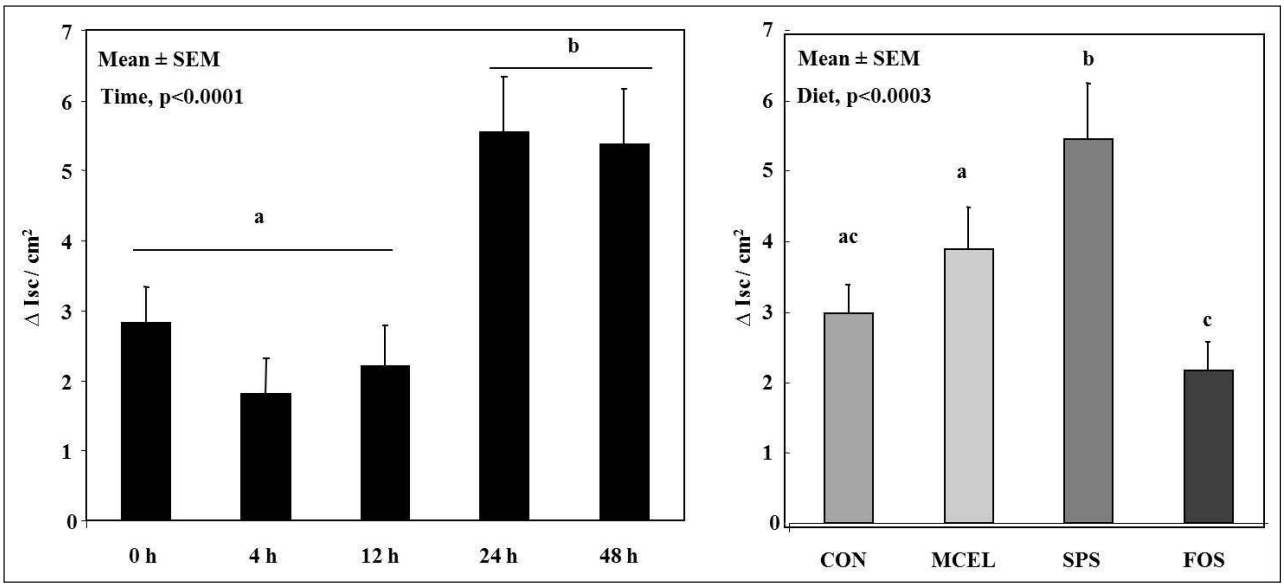

Figure 2. Ileal Serotonin-Induced Chloride Secretion in Non-Infected Piglets and Piglets 4, 12, 24 and 48 Hours After Infection with Salmonella Typhimurium. Bars with different letter superscripts within each panel are significantly different from each other. The highest chloride secretion was observed at 24 and 48h post-infection, regardless of diet (p<0.0001; panel A). A main effect of diet also existed indicating an increase in chloride secretion in the piglets fed-SPS (p<0.0003; panel B) when compared to the other diets. Data are means ± SEM.

Table 2. Number of total, neutral and acidic mucins in the ileal villus of non-infected piglets and piglets infected at 4,12, 24 and 48h with *Salmonella typhimurium*[1,2].

	0h	4h	12h	24h
Total mucins (number of cells/villus height)				
CON	32.16 ± 1.13	28.10 ± 1.28	31.22 ± 2.77	24.97 ± 2.63
MCEL	39.99 ±17.58	36.81 ± 11.00	28.89 ± 4.25	30.29 ± 6.01
SPS	36.84 ± 2.19	26.19 ±2.01	51.70 ± 7.30	38.18 ± 2.49
FOS	36.98 ± 3.19	38.46 ± 5.80	19.72 ± 1.34	27.65 ± 4.77
Neutral mucins (number of cells/villus height)				
CON	12.03 ± 0.92	14.44 ± 0.89	16.33 ± 1.69	16.23 ± 1.45
MCEL	18.22 ± 10.18	20.53 ± 4.77	17.25 ± 2.83	17.23 ± 3.45
SPS	14.39 ± 1.01	17.45 ± 1.60	23.47 ± 4.92	14.18 ± 2.06
FOS	10.32 ± 0.83	13.36 ± 0.84	13.35 ± 0.86	15.65 ± 4.46
Acidic mucins (number of cells/villus height)				
CON	20.12 ± 1.77	13.65 ± 0.89	14.89 ± 1.53	18.74 ±2.49
MCEL	21.771 ± 7.40	16.28 ± 0.27	11.63 ± 1.47	13.06 ± 2.76
SPS	22.44 ± 2.37	8.74 ± 0.72	28.23 ± 3.32	24.00 ± 2.41
FOS	26.67 ± 3.04	25.10 ± 5.11	6.37 ± 0.52	12.0 ± 1.45
Ileal villus height (microns)[3]				
CON	775.39 ± 33.24[a]	792.13 ± 25.56[a]	802.95 ± 36.66[a]	584.72 ± 26.81[b]
MCEL	677.59 ± 68.06[a]	708.04 ± 95.89[a]	602.81 ± 24.47[a]	721.50 ± 87.5[b]
SPS	748.38 ± 35.6[a]	837.30 ± 17.20[a]	566.50 ± 101.5[a]	594.49 ± 32.00[b]
FOS	735.90 ± 45.48[a]	744.83 ± 40.87[a]	836.50 ± 53.21[a]	648.17 ± 47.85[b]

[1]Data are means ±SEM. [2]Significant differences are expressed as P<0.05, trends as P<0.1.[3]Within this dependent variable, the main effect of day is significant. Values with different letter superscripts are different from each other.

regardless of infection (p<0.05). Following infection, sialated mucins in the crypt decreased in all groups by 48h (p<0.05) (Table 3). When comparing the ratio of neutral to acidic mucins, each normalized by villus height, we found that piglets fed the CON diet showed an increase (p<0.05) in sulfo-acidic mucin positive goblet cells after 12h when compared to the other time points. SPS showed a significant increase in these mucins at 48h (Figure 3).

NFkB activation

A higher (p=0.02) concentration of activated NFkB was observed in the CON group than the FOS piglets, which had non detectable NFkB activation (not shown). CON piglets showed biphasic activation of NFkB, with an increase at 4h, a decline at 12h and 24h and an secondary increase at 48h post-infection (p=0.06).

Discussion

Results from this study confirm our hypothesis that fermentable fiber exerts its protective effects upon *S. typhimurium* infection in neonatal piglets at least in part by enhancing the innate immune response. However, different fibers provided different mechanisms of protection from *S. typhimurium*. An increase in chloride secretion, which is important for flushing the bacteria from the intestine, was observed in piglets fed SPS at 24h post infection. This observation was temporarily associated with the histological data presented by Stephen et al. (28,29) where the intestinal villi were blunted at that particular time point. In contrast, the FOS diet inhibited chloride secretion, which could be an underlying mechanism of reduced diarrhea observed previously (22).

Table 3. Numbers of of ileal acidic mucins in villus and crypt of non-infected piglets and piglets infected at 4,12, 24 and 48h with Salmonella typhimurium[1,2].

	0h	4h	12h	24h	48h
Sulfoacido mucins in villus (number of cells/villus height)[3]					
CONb	16.84 ± 0.60	16.24 ± 1.97	17.48 ± 1.17	10.48 ± 1.25	9.60 ± 0.95
MCEL[a]	21.14 ±1.22	22.28 ± 0.15	21.97 ± 0.49	16.00 ± 1.81	14.48 ± 1.04
SPS[a]	24.67 ± 0.97	17.54 ± 1.39	16.58 ±1.14	20.45 ± 0.96	9.64 ± 1.20
FOS[a]	23.88 ± 1.01	15.68 ± 1.59	16.97 ± 1.24	20.40 ± 1.39	13.62 ± 1.02
Sialated acidomucins in villus (number of cells/villus height)					
CON	1.11 ± 0.61	1.96 ± 0.46	1.28 ± 0.15	2.40 ± 0.38	0.50 ± 0.11
MCEL	1.20 ± 0.23	1.04 ± 0.23	1.15 ± 0.16	1.33 ± 0.20	1.10 ± 0.31
SPS	2.08 ± 0.30	2.06 ± 0.45	1.68 ± 1.14	1.38 ± 0.18	0.20 ± 0.11
FOS	0.72 ± 0.17	12.37 ± 5.64	0.35 ± 0.56	1.46 ± 0.17	0.67 ± 0.13
Sulfoacido mucins in crypt (number of cells/crypt depth)[3]					
CONb	1.73 ± 0.27	1.86 ± 0.30	1.75 ± 0.22	2.40 ± 0.53	3.99 ± 0.49
MCEL[a]	2.08 ±0.31	3.16 ± 0.36	4.27 ± 0.35	2.48 ± 0.34	2.77 ± 0.63
SPS[a]	3.37 ± 0.30	2.06 ± 0.54	1.45 ± 0.30	3.40 ± 0.43	4.72 ± 0.26
FOS[a]	4.66 ± 0.49	4.85 ± 0.36	3.45 ± 0.32	5.00 ± 0.42	6.18 ± 0.48
Sialated acidomucins in crypt (number of cells/crypt depth)[4]					
CON	5.73 ± 0.20[a]	6.16 ± 0.72[a]	6.75 ± 0.55[a]	6.22 ± 0.39[a]	3.67 ± 0.33[b]
MCEL	7.26 ± 0.31[a]	6.20 ± 0.20[a]	6.20 ± 0.31[a]	6.00 ± 0.40[a]	3.30 ± 0.51[b]
SPS	5.75 ± 0.08[a]	5.62 ± 0.73[a]	6.60 ± 0.30[a]	5.75 ± 0.01[a]	3.00 ± 0.17[b]
FOS	5.12 ± 0.37[a]	4.96 ± 0.37[a]	5.27 ± 0.36[a]	8.08 ± 0.69[a]	3.31 ± 0.29[b]

[1]Data are means ±SEM. [2]Significant differences are expressed as P<0.05, trends as P<0.1.[3]Within this dependent variable, the main effect of diet is significant. Values with different letter superscripts are different from each other. [4]Within this dependent variable, the main effect of time is significant. Values with different letter superscripts are different from each other.

The intestinal mucous barrier provides another exclusionary mechanism is to prevent binding of the pathogen to the intestinal epithelial cells. This study showed, *in- vivo*, for the first time, the pattern of mucin production following infection and the effects of different fibers on the pattern of mucin expression. In the CON group, the number of sulfo-acidic mucin-secreting goblet cells were increased over 4-fold at 24h and 48 h post-infection. Piglets consuming the MCEL diet showed no increase in the number of acidic-mucin secreting goblet cells following infection and the rise was delayed until 48h and was somewhat diminished in the piglets fed the SPS-containing diet. Piglets fed the FOS diet showed a different pattern than the other groups, in that the number of sulfo-acidomucins secreting goblet cells was elevated prior to infection in this group, declined at 4h and 12h, then returned to pre-infection level at 24h and 48h. The negative charges from the sulfate group in the mucins repulse gram- negative bacteria (30,31), thus, greater expression of sulfo-acidomucins in the FOS group before infection, may have protected these piglets against the bacterial infection by preventing the initial attachment of *S. typhimurium*.

We previously demonstrated that SCFA and butyrate concentrations were greater in the colonic contents of piglets fed the SPS and FOS diets (22). Other studies have shown that *S. typhimurium* grown in medium supplemented with butyrate has reduced infectivity due to the down-regulation of the genes involved in the *Salmonella* Pathogenicity Island 1 (SPI-1) (32). These data suggest another mechanism highly fermentable fiber (such as FOS) may reduce the infectivity of *Salmonella* and prevent its ability to invade the epithelial cells. Future studies should investigate whether this mechanism occurs *in- vivo*.

This study also confirmed previous findings of NFkB modulation by butyrate in- vitro (11). When NFkB activation was assessed in ileal tissue of piglets consuming the CON (no-fiber) or the highly fermentable fiber (FOS) diets, we observed NFkB activation in CON, but not FOS piglets following infection .Y de P and collaborators (20) showed that butyrate inhibited NFkB activation by stabilizing IkB levels, which prevented NFkB translocation to the nucleus to initiate the transcription of pro-inflammatory cytokine and subsequent inflammation. These findings provide a potential underlying mechanism for the reduced diarrhea and inflammation observed in the FOS group.

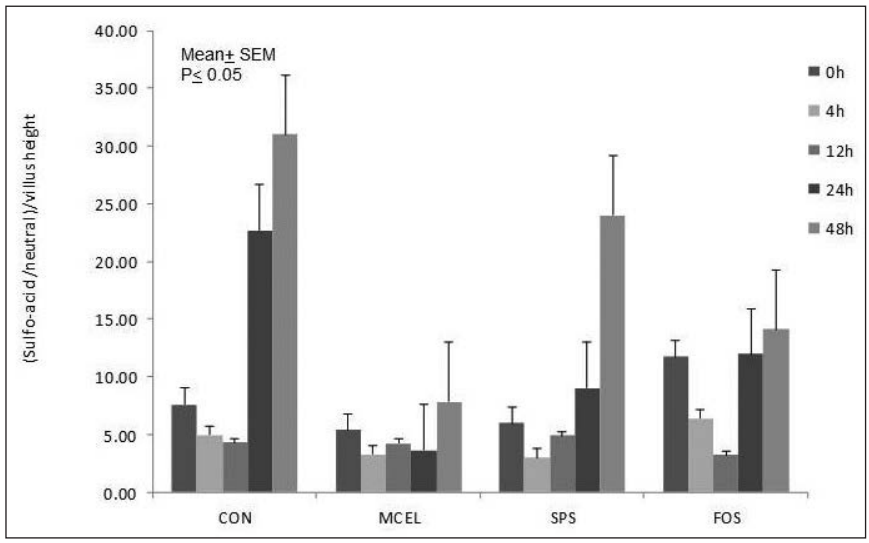

Figure 3. Ratio of Sulfo-Acidic to Neutral Mucins in the Villi in Non-Infected Piglets and Piglets 4, 12, 24 and 48 Hours After Infection with Salmonella Typhimurium. Data was calculated as the number of sulfo-acidic mucins related to neutral mucins normalized by villus height. Bars with different letter superscripts are significantly different from each other. An increase in sulfo-acidomucins was observed in CON at 24 and 48h post infection (compared to the other time points within this diet group (p< 0.05). MCEL showed a muted production of mucins in all time points. Within diets, there were no significant changes in mucins production in the MCEL. SPS showed a significant increase in mucins at 48h (p<0.05). Production of mucins in FOS was reduced at 4 and 12h, and returned to pre-infection values at 24h post infection. Data are means ± SEM.

Interestingly, a biphasic activation of NFkB was observed in the CON piglets, with an increase at 4h post infection and was second increase at 48h post infection. These data suggest that early NFkB activation was a consequence of bacterial invasion, whereas reactivation of NFkB may have occurred as a result of the damage to the intestine, as evidenced by villus blunting (28, 29) and chloride secretion, or an action of pro-inflammatory cytokines feeding back on NFkB. We speculate that early NFkB activation is the result of the innate response, and its reactivation is part of the adapted immunity (33, 34).

It is possible that the expression of other immunomodulatory cytokines also may have been increased, which counteracted the pro-inflammatory cytokines. For example, the addition of FOS in diets of patients with Crohn's disease increased the expression of IL-10, suggesting that fermentable fibers could used as a treatment can reduce inflammation (35). Our studies showed that the provision of fiber prior to infection prevented, or diminished, the rise in pro-inflammatory cytokines following infection.

Our laboratory previously showed that FOS augmented neutrophils migration in the intestine of piglets infected with *S. typhimurium* (36). Herein, FOS also increased the expression of the neutrophil chemoattractant, IL-8, providing a potential signal to target neutrophils to the site of infection. Previous *in- vitro* data using enterocyte-like $CaCO_2$ cells reported a dose-response relationship between butyrate and IL-8 expression; at low doses, butyrate reduced IL-8 mRNA, whereas at a higher dose butyrate increased IL-8 expression (37) and decrease pro-inflammatory cytokines (38). Taken together with our previous findings, we can conclude that the addition of FOS to formula results in the production of butyrate in the colon (22), induces greater neutrophil migration follow infection with *S. typhimurium* (36) and enhances innate immunity, which may be explained by the greater IL-8 mRNA expression in *S. typhimurium*-infected piglets fed the FOS diet.

The results of this study and our previous study (22) provide insight regarding the use of fermentable fiber in the prevention of diarrhea in piglets infected with *S. typhimurium*. The inhibition of NFkB and subsequent reduction in inflammation by inhibiting pro-inflammatory cytokines expression, represent the possible benefits of fermentable fiber in the treatment of diarrheal diseases in infants and adults.

Resumen

Objetivos: Probar la hipótesis de que la fibra fermentable reduce los síntomas asociados a la infección con *Salmonella typhimurium* fortaleciendo el sistema inmunológico innato y adaptativo en cerdos neonatales. Métodos: Ciento-veinte cerdos (n=120) de dos días de nacido, fueron aleatoriamente seleccionados para recibir fórmula sustituta a la leche materna (CON) o recibir la leche suplementada con fibra no-fermentable (MCEL), moderadamente-fermentable (SPS) o altamente-fermentable (FOS). En el día 7, los cerditos recibieron inoculación oral con *Salmonella typhimurium*-798, y la dieta asignada hasta 48-h post infección. Se obtuvieron muestras mucosales del íleo para análisis. Resultados: FOS redujo la secreción de cloruro comparado con las otras dietas ($p<0.0003$). El número de mucinas-ácidas-sulfatadas fueron mayores ($p<0.05$) en FOS pre-infección comparado con otras dietas. NFkB disminuyó en FOS post-infección ($p<0.05$), comparado con CON. IL-1β aumentó a las 4-horas post-infección ($p<0.05$) en CON. La infección aumentó IL-6 en CON y SPS pero no en MCEL y FOS. FOS aumento la expresión de los quimio atrayentes de neutrófilos IL-8 antes de la infección ($p<0.05$) sobre los otros grupos. Conclusión: La reducción en la secreción de cloruro, la expresión de las citoquinas proinflamatorias, la activación de NFkB, el aumento en el número de mucinas- ácidas- sulfatadas y en la expresión de IL-8 en los grupos con fibra, indican que el grado de fermentabilidad impacta al sistema inmunológico innato y adaptativo siendo el posible mecanismo por el cual la fibra dietaria puede reducir los síntomas asociados a la infección con *Salmonella typhimurium* en cerdos neonatales y aplicarlo a infantes.

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