Published in final edited form as:

JPEN J Parenter Enteral Nutr. 2016 September ; 40(7): 1042–1049. doi:10.1177/0148607115585353.

Bombesin preserves goblet cell resistin-like molecule beta during parenteral nutrition, but not other goblet cell products

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Abstract

Introduction—Parenteral nutrition (PN) increases the risk of infection in critically ill patients and is associated with defects in gastrointestinal innate immunity. Goblet cells produce mucosal defense compounds including mucin (principally MUC2), trefoil factor 3 (TFF3,) and resistin-like molecule β (RELM β). Bombesin (BBS), a gastrin-releasing peptide analogue, experimentally reverses PN-induced defects in Paneth cell innate immunity. We hypothesized that PN reduces goblet cell product expression and PN+BBS would reverse these PN-induced defects.

Methods—2 days after IV cannulation, male Institute of Cancer Research mice were randomized to Chow (n=15), PN (n=13), or PN+BBS (15 ug TID) (n=12) diets for 5 days. Defined segments of ileum and luminal fluid were analyzed for MUC2, TFF3, and RELM β by qRT-PCR and Western blot. Th2 cytokines IL-4 and IL-13 were measured by ELISA.

Results—Compared to Chow, PN significantly reduced MUC2 in ileum ($p<0.01$), and luminal fluid ($p=0.01$). BBS supplementation did not improve ileal or luminal MUC2 compared to PN ($p>0.3$). Compared to Chow, PN significantly reduced TFF3 in ileum ($p<0.02$) and luminal fluid ($p<0.01$). BBS addition did not improve ileal or luminal TFF3 compared to PN ($p>0.3$). Compared to Chow, PN significantly reduced ileal RELM β ($p<0.01$). BBS supplementation significantly

increased ileal RELM β to levels similar to Chow ($p < 0.03$ vs. PN; $p > 0.6$ vs. Chow). Th2 cytokines were decreased with PN and returned to Chow levels with BBS.

Conclusion—PN significantly impairs the goblet cell component of innate mucosal immunity. BBS only preserves goblet cell RELM β during PN, but not other goblet cell products measured.

Keywords

Parenteral nutrition; enteric nervous system; small intestine; innate immunity; goblet cells; mucin 2; trefoil factor 3; resistin-like molecule beta; bombesin

INTRODUCTION

Parenteral nutrition (PN) clinically allows gut rest while preventing progressive malnutrition and starvation. While PN remains a life-saving therapy for patients unable to be fed via the gut for prolonged periods, critically-ill PN-fed patients exhibit increased risk of infectious complications compared to those fed enterally.^{1–4} Data from our laboratory implicate impairments in both the adaptive and innate immune systems in this nutrition-associated septic morbidity.^{5–8}

Our data implicate both route and type of nutrition in the deleterious effects of parenteral feeding on mucosal immunity. In the adaptive (acquired) arm of mucosal immunity, these effects primarily occur through endpoint reductions in secretory immunoglobulin A (sIgA),⁹ the main effector molecule of that system. PN with decreased enteral stimulation results in decreased gastrointestinal associated lymphoid tissue (GALT) lymphocyte mass⁹ and correlative decreases in lamina propria IgA-stimulating Th2 cytokines interleukin-4 (IL-4), IL-13 and IL-10.^{10, 11} Together these changes significantly reduce sIgA availability at multiple mucosal surfaces resulting in loss of anti-viral¹² and anti-bacterial immunity,⁵ providing a cogent explanation for increases in pneumonia observed in PN-fed patients.

The innate immune system acts synergistically with the adaptive immune system to protect mucosal surfaces - in part by providing a physiochemical barrier against mucosal attachment and invasion by pathogens. Paneth and goblet cells are the main effectors of the gastrointestinal innate immune system. Paneth cells generate the natural antibiotics of the intestine, antimicrobial peptides (AMPs), which directly neutralize invading pathogens. Goblet cells secrete a variety of products including MUC2, trefoil factor 3 (TFF3), and resistin-like molecule β (RELM β) within the small intestine which are involved in formation of the intestinal mucus layer, epithelial restitution following injury, and immune maintenance, respectively. Th2 cytokines IL-4 and IL-13 mediate expression of these goblet cell products and stimulate IgA production in the lamina propria.^{13–15} Taken together, the Paneth cell AMPs, along with sIgA produced by the GALT, concentrate in the goblet cell mucus layer to provide a fortified physiochemical barrier between the gastrointestinal epithelium and intestinal microbiome.^{16, 17}

Recent work identifies derangements in Paneth cell AMP production and innate immune function with PN compared to enteral feeding.^{8, 18–24} These PN-associated deficits appear intimately related to decreased enteric nervous system (ENS) stimulation during PN since

supplementation of PN with the neuropeptide bombesin (BBS) restores intracellular AMP levels in PN-fed animals to levels comparable to Chow feeding.²⁵ BBS, a gastrin releasing peptide analogue, stimulates intestinal functions that normally occur in response to enteral feeding such as activation of the ENS. Experimentally, exogenous BBS administration during PN also reverses many of the PN-induced defects in both gut and respiratory adaptive immune function in addition to its effects on the innate immune system.^{25–32}

We previously identified reductions in goblet cell MUC2 with PN.^{33, 34} The effect of BBS on goblet cell products of the innate immune system in a model of decreased enteral stimulation is currently unknown. Since PN reduces innate immune capacity of Paneth cells and the addition of BBS restores Paneth cell products to Chow levels, we hypothesized that PN would globally decrease goblet cell products and supplementation with BBS would restore goblet cell products, MUC2, TFF3, and RELM β toward Chow levels compared with PN alone.

MATERIALS AND METHODS

Animals

All protocols were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison, and the William S. Middleton Memorial Veterans Hospital, Madison. Male Institute of Cancer Research (ICR) mice were purchased from Harlan (Indianapolis, IN) and housed 5 per covered/filtered box under controlled temperature and humidity conditions with a 12:12 hour light: dark cycle in an American Association for Accreditation of Laboratory Animal Care accredited conventional facility. Animals were fed standard mouse chow (Rodent Diet 5001; LabDiet, PMI Nutrition International, St. Louis, MO) and water *ad libitum* for at least 1 week prior to initiation of study protocol.

Experimental Design

Our previous publication describes the experimental detail: samples were stored at -80°C and used in this experiment.²⁵ In brief, male ICR mice, ages 6 to 8 weeks, underwent placement of a silicon rubber catheter (0.012-inch I.D./0.025-inch O.D.; Helix Medical, Inc., Carpinteria, CA) in the vena cava through the right external jugular vein. The catheter was tunneled subcutaneously and exited at the midpoint of the tail. The mice were randomized to receive Chow, PN, or PN with 15 μg exogenous bombesin (Sigma, B4272) three times daily (PN+BBS) delivered IV for 5 days ($n=6-10$ mice/group on two separate runs). Chow and PN animals were also given a saline vehicle control (100 μL) to the BBS treatment vehicle three times daily. Mice were housed individually and partially immobilized by tail restraint in metabolic cages with wire floors to prevent coprophagia and bedding ingestion. This technique produces no signs of physical or biochemical stress.³⁵ The catheterized mice received saline (0.9%) at 4 mL/day and *ad libitum* chow (Rodent Diet 5001) and water for 48 hours. After 48 hours, Chow mice receive 0.9% saline at 4 mL/day with *ad libitum* access to chow and water. PN and PN+BBS mice received PN solution at goal rates of 4 mL/d (day 1), 7 mL/d (day 2) and 10 mL/d (day 3 to 5) as a graded infusion period is necessary for the mice to adapt to the increasing glucose and fluid loads. The PN solution contains 6.0% amino acids, 35.6% dextrose, electrolytes, and multivitamins, containing 1440 kcal/L and a

non-protein calories/ nitrogen ratio of 128:1 to meet the nutrient requirements of mice weighting 25 to 30 g, which were metabolically scaled to the animals in these experiments.³⁶

After 5 days of experimental nutritional support (7 days post-catheterization), mice were anesthetized with intraperitoneal ketamine (100 mg/kg) and acepromazine (10 mg/kg), and exsanguinated via left axillary artery transection. The small intestine was removed and the lumen rinsed with 20 mL Hanks Balanced Saline Solution (HBSS, Bio Whittaker, Walkersville, MD). The luminal wash was centrifuged at $2,000 \times g$ for 10 min and supernatant aliquots were frozen at -80°C for analysis. Tissue samples were collected by removing 3 cm ileal segments (excluding Peyer's patches) that were 5 cm proximal to the ileocecal junction. These samples were frozen in either RNA later or snap frozen in liquid nitrogen and stored at -80°C until processing for tissue analysis. Mice with clogged catheters or evidence of dislodged catheters were sacrificed throughout the experiment. Combined runs yielded $n = 15, 13,$ and 12 for diets Chow, PN, and PN+BBS, respectively. The N varies across groups for analysis by qRT-PCR, Western blot, and ELISA however, since some samples were depleted in the previous experiment.²⁵

qRT-PCR for *Muc2*, *Tff3*, and *Relmb* mRNA

RNA Two centimeters of ileal tissue from 3 cm proximal to the ileocecal junction was snap frozen in RNAlater (R0901, Sigma) and stored at -80°C until analysis. The SV Total RNA Isolation System (Z3100, Promega) was used to isolate mRNA from tissue samples per manufacturer instructions as previously described.²⁵ mRNA samples were confirmed as pure and of adequate concentration by nanodrop spectrometry (Thermo Scientific). Complementary DNA (cDNA) was synthesized from isolated mRNA using a high-capacity cDNA reverse transcription kit (4368813, Applied Biosystems, Waltham, MA) with the provided protocol. Relative quantitative gene expression analysis was performed using FastStart Universal SYBR Green Master mix (04913914001, Roche, Basel, Switzerland) on a StepOnePlus real-time PCR System (Applied Biosystems, Waltham, MA) using specific primers designed for *Muc2*, *Tff3*, and *Relmb*, (Table 1). Gene expression of *Muc2*, *Tff3*, and *Relmb* was normalized to gene expression of *beta-actin*. The comparative C_t method was used with PN and PN+BBS groups normalized to the Chow group for analysis.³⁷

Tissue homogenization and Protein Concentration

Small intestinal segments from the ileum were homogenized in RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1% protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO). The homogenates were incubated 20 minutes on ice before centrifugation at $16,000 \times g$ for 10 minutes at 4°C . The supernatants were moved to a clean tube and were stored at -20°C until analyzed. Protein concentrations for each homogenate were determined by using the Bradford dye binding method with bovine serum albumin as a standard.

Western Blot for MUC2, TFF3, and RELM β

Homogenized protein from small intestinal tissue was separated on a 10 % polyacrylamide gel (Ready Gel, Bio-Rad Laboratories, Hercules, CA) under non-denaturing conditions for

MUC2 or denatured at 95 °C for 10 minutes with sodium dodecylsulfate and β -mercaptoethanol for TFF3 and RELM β before electrophoresis at 150 V for 120 minutes on ice (MUC2) or 35 minutes (TFF3, RELM β). After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using transfer buffer (Tris-glycine buffer plus 20% methanol) at 80 V for 60 minutes (MUC2) or 35 minutes (TFF3, RELM β). Membranes were blocked with 5 % nonfat dry milk prepared in TBS-Tween (Tris-buffered saline with 0.5% Tween-20) for 1 hour with continuous agitation, and western blotting was performed as previously described.^{33, 38} The membranes were incubated with the primary antibody mouse anti-human MUC2 (ab-11197, Abcam Inc, Cambridge, MA) diluted 1:2500, mouse anti-rabbit TFF3 (ab-57752, Abcam Inc) diluted 1:500, or rabbit anti-mouse RELM β (ab-11429, Abcam Inc). After washing, membranes were incubated with stabilized goat anti-mouse IgG-HRP conjugate (sc-2005, Santa Cruz Biotechnology, CA) diluted 1:20000 (MUC2) and 1:15000 (TFF3) and goat anti-rabbit IgG-HRP (Pierce) diluted 1:15000 (RELM β). After washing with TBS-tween, membranes were incubated with HRP substrate (Super Signal West Femto maximum sensitivity substrate; Pierce, Rockford, IL) for 5 min and bands were detected using ImageQuant4000 (GE Healthcare). Densitometric analysis of molecular weight bands at 260kDa and 500 kDa were used to measure MUC2 concentration while molecular weight band at 33 kDa and 18 kDa were used to measure TFF3 and RELM β , respectively.

Luminal fluid was also evaluated for MUC2 and TFF3 protein content using a similar technique. MUC2 antibody concentrations for luminal fluid were the same as for tissue whereas primary antibody was diluted 1:1500 and secondary antibody was diluted 1:20,000 for TFF3. Relative protein density was compared amongst 15 μ L of luminal fluid samples.

Analysis of Tissue IL-4 and IL-13

Concentrations of IL-4 and IL-13 were measured in the small intestinal tissue homogenate using a solid phase sandwich ELISA (BD Biosciences, San Diego, CA) as previously described per manufacturer provided instructions. Briefly, 96-well plates (Costar, 9018) were coated with 100 μ L coating buffer containing 0.1 M sodium carbonate coating (pH 9.5) with anti-mouse IL-4 or IL-13 in a 1:250 dilution. After overnight incubation at 4°C, plates were washed and blocked for 1 hour at room temperature. 100 μ L of tissue homogenate or cytokine standard (BD Biosciences) was added to respective wells to incubate for 2 hours at room temperature. After washing, 100 μ L of a 1:250 dilution of either IL-4 or IL-13 secondary antibody was added and incubated for 1 hour at room temperature. After another series of wash steps, streptavidin-horseradish peroxidase (SAv-HRP) conjugate was added to the wells and incubated for 30 minutes at room temperature. Reactions were stopped by adding 50 μ L of 2N H₂SO₄, and the absorbance was read at 450 nm in a Vmax Kinetic Microplate Reader. The mass amounts were determined by plotting sample absorbance values on a 4-parameter logistic fit standard curve, as calculated with SOFTmax PRO software (Molecular Devices).

Statistical analysis

The data are expressed as mean $\pm$ standard error of the mean. Statistical significance was determined using ANOVA with Fisher's protected least significant difference *post hoc test*

or Student's *t*-test. Differences were considered to be statistically significant at $p < 0.05$. All statistical calculations were performed with StatView (Abacus Concepts, Berkeley, CA).

RESULTS

The pre-experiment body weight of mice in all groups was similar ($p > 0.05$), (Table 2). PN and PN+BBS mice lost significantly more weight than Chow animals throughout the experiment, but there was no difference in weight change between PN and PN+BBS animals ($p = 0.68$).

MUC2

There was no statistical difference in *Muc2* mRNA transcription between Chow and PN mice ($p = 0.42$) or Chow and PN+BBS mice ($p = 0.06$). Transcription of *Muc2* mRNA was significantly decreased in PN+BBS mice compared to PN alone ($p = 0.009$), (Figure 1A). PN significantly decreased ileal tissue and luminal levels of MUC2 compared to Chow ($p = 0.008$ and $p = 0.01$, respectively), (Figure 1B and 1C). PN+BBS tissue and luminal levels of MUC2 remained decreased compared to Chow ($p = 0.08$ for both) and neither was different from PN ($p = 0.3$).

TFF3

There was no statistical difference in transcription of *TFF3* mRNA among Chow, PN, or PN+BBS although transcription trended to increase in both PN and PN+BBS compared to Chow ($p = 0.07$ vs. PN; $p = 0.09$ vs. PN+BBS), (Figure 2A). PN significantly decreased ileal tissue and luminal levels of TFF3 compared to Chow ($p = 0.02$ and $p = 0.003$, respectively), (Figure 2B and 2C). Tissue and luminal levels of TFF3 remained significantly decreased when BBS was added to PN compared to Chow, ($p = 0.04$ and $p = 0.03$, respectively) and were not different from tissue or luminal levels of TFF3 with PN alone, ($p = 0.91$ and $p = 0.34$, respectively).

RELM β

RELM β mRNA transcription was significantly decreased in PN and PN+BBS groups compared to Chow ($p = 0.003$ vs. PN; $p = 0.03$ vs. PN+BBS). There was no difference in *RELM β* mRNA transcription in PN versus PN+BBS ($p = 0.26$), (Figure 3A). Tissue levels of RELM β were significantly decreased in PN compared to Chow ($p = 0.02$) while the addition of BBS to PN significantly increased tissue levels of RELM β back toward Chow levels ($p = 0.03$ vs. PN; $p = 0.67$ vs. Chow), (Figure 3B).

IL-4

Tissue levels of IL-4 were not statistically different between Chow and PN in this experiment ($p = 0.08$), (Figure 4A). PN+BBS significantly increased IL-4 compared to PN alone ($p = 0.006$) and was not different from Chow levels ($p = 0.29$).

IL-13

PN significantly decreased tissue levels of IL-13 compared to Chow ($p = 0.009$), (Figure 4B). The addition of BBS significantly increased tissue levels of IL-13 compared to PN

alone ($p = 0.001$). There was no difference between Chow and PN+BBS tissue levels of IL-13 ($p = 0.57$).

DISCUSSION

PN is a lifesaving strategy that prevents progressive malnutrition in patients with contraindications to enteral nutrition. Unfortunately, lack of enteral feeding is associated with increased risk of infectious complications, suspected to be due in-part to altered mucosal immune integrity. Our previous work identifies BBS, an ENS neuropeptide analogue, as an active stimulus for maintenance of adaptive immune function in the setting of PN with decreased enteral stimulation.^{27, 28, 30–32} Recently our laboratory evaluated the effects of PN and PN+BBS on the production and release of AMPs by Paneth cells, an important component of innate immune function.²⁵ PN with lack of enteral stimulation reduced Paneth cell AMP production and secretion while reducing bactericidal activity of stimulated secretions released from isolated intestinal segments. PN also impaired intestinal resistance to enteroinvasion by pathogenic *E. coli*. Exogenous BBS administered with PN normalized these parameters, indicating that the ENS plays a role in innate as well as adaptive immune function. The close proximity of intestinal goblet cells to enteric nerves and immune cells (within 13 μm),³⁹ provided an initial rationale for ENS-dependent immune function. Given the significant effects on Paneth cell AMPs in our prior work, the current work examined the effects of PN with decreased enteral stimulation on innate mucosal barrier properties of goblet cell products both with and without BBS supplementation in expectation of beneficial BBS effects.

PN with decreased enteral stimulation negatively affects the levels of several goblet cell products including MUC2, TFF3, and RELM β . Generalized mucosal atrophy also occurs with PN in animals and humans.^{9, 40–42} Morphological changes include decreases in intestinal wet weight, villus height, total protein, and lymphocyte populations. Since measures of MUC2, TFF, and RELM β are normalized to total intestinal protein, our findings likely underestimate the detrimental impact of PN on intestinal immune product levels since they all decrease in the context of a decreased total protein. The addition of BBS to PN prevents PN-associated atrophy of gut-associated lymphoid tissue,⁹ but it did not rescue tissue or luminal levels of MUC2 or TFF3 in these experiments contrary to our hypothesis. As TFF3 levels in the ileal tissue dropped with PN, *Tff3* mRNA expression in the ileal tissue increased although the results failed to reach statistical significance when compared to Chow. However, BBS significantly increased RELM β intestinal protein levels consistent with our hypothesis.

This decoupling of RELM β expression from the other goblet cell products when BBS was added to PN was unexpected and warrants discussion. A variety of mechanisms exist for MUC2 and TFF3 protein production. *Muc2* uses two main pathways for transcription activation: 1) activation of NF- κ B through the Ras MAPK pathway stimulated by LPS⁴³ or the Th2 cytokines IL-4 and IL-13⁴⁴, and 2) activation of transcription factors CREB/ATF1 mediated by MAPK and p38 pathways induced by PGE2 or neuropeptide vasoactive intestinal peptide.⁴⁵ Both Ras MAPK or PI3K/Akt pathways direct TFF3 transcription

following toll receptor 2 (TLR2) stimulation but TLR2 stimulation fails to activate *Muc2* transcription despite use of the common Ras MAPK pathway.⁴⁶

Multiple pathways common to production of MUC2 and TFF3 induce RELM β . In particular, production of IL-4 and IL-13 by intestinal epithelial cells is the most well described stimulator of RELM β and coincides with host protective immunity.^{47, 48} Interestingly, induction of RELM β occurs much more readily with Th2 cytokines (IL-4 and IL-13) than either MUC2 or TFF3,⁴⁷ potentially providing an explanation for our findings. Although the IL-4 reduction during PN failed to reach significance in this study, this is likely the result of decreased power in this analysis since our prior work^{30, 49} demonstrated significant decreases in both IL-4 and IL-13 during PN. However, BBS significantly increased both IL-4 and IL-13 toward Chow levels consistent with prior work.³⁰ While IL-4 and IL-13 levels mediate mucous, TFF3, and RELM β expression in intestinal goblet cells (as well as IgA production in the lamina propria),^{13–15} RELM β is disproportionately affected by Th2 cytokine signaling compared to MUC2 and TFF3. Thus the increase in IL-4 and IL-13 levels during BBS treatment may explain the increases in RELM β production without corresponding changes in MUC2 or TFF3. Further studies are needed to confirm this hypothesis.

The primary limitation of this study is failure to define specific mechanisms for our findings. Many studies show different mechanistic pathways for MUC2, TFF3, and RELM β production, but it remains unclear whether BBS acts directly on goblet cells or indirectly through signaling cascades. Given the relationship between BBS, the Th2 cytokines IL-4 and IL-13, and RELM β , it seems likely that BBS generates an indirect effect, but this hypothesis was not confirmed. While, this study does not report functional differences of BBS related to goblet cell products of innate immunity, we recently reported improvements in bactericidal activity and resistance to bacterial invasion in mice treated with BBS in the same model.²⁵ These improvements were related to restoration of Paneth cell AMP production but it is now clear that goblet cell products, particularly RELM β may contribute to these findings. Unfortunately it is not possible to isolate individual components of the innate immune system or segregate their function from effects on the adaptive immune system *in vivo* since BBS is known to increase intestinal IgA levels to normal in PN-fed mice. Systematic evaluation of these pathways requires decoupling of contributions from the two arms of mucosal immunity which is impossible in an *in vivo* model. Examination of immunohistochemistry might be of value to provide visual confirmation of BBS effects but was not performed in these experiments.

In summary, this study supports the hypothesis that PN with lack of enteral stimulation induces defects in measures of innate immunity including reduced goblet cell products MUC2, TFF3, and RELM β in the small intestine. Th2 cytokines IL-4 and IL-13 are reduced by PN and likely contribute to the PN mediated decreases in MUC2, TFF3, and RELM β . Production of these cytokines is stimulated with exogenous administration of BBS to PN. While the ENS neuropeptide improves innate immune function that is lost during PN,²⁵ the ENS appear only minimally involved in goblet cell function.

Acknowledgments

The project described was supported by Award Number I01BX001672 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development.

The contents of this article do not represent the views of the Veterans Affairs or the United States Government.

This material is also based upon work supported by the National Institute of Health (NIH) Grant R01 DK102598 (MEK) and NIH Training Grants: T32 CA090217 (RAB), T32 DK007074 (JFP), and T32 AG000213 (JCN).

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CLINICAL RELEVANCY STATEMENT

Parenteral nutrition (PN)-induced defects in the gastrointestinal immune system likely contribute to the increased risk of infection seen in critically ill patients fed parenterally as opposed to enterally. Recent evidence supports that PN impairs gastrointestinal innate immune function in addition to adaptive immune function. PN supplemented with bombesin, an enteric nervous system neuropeptide, experimentally restores gastrointestinal innate immune function associated with Paneth cells. This work investigates the role of bombesin supplementation in the goblet cell component of gastrointestinal mucosal innate immunity during PN.

* $p < 0.05$ versus Chow; # $p < 0.05$ versus PN+BBS

Figure 1. Chow, PN, and PN+BBS effects on MUC2

A) Effects on ileal tissue *Muc2* mRNA (Chow: $n=8$; PN: $n=8$; PN+BBS: $n=10$); **B)** effects on ileal tissue MUC2 relative protein concentration (Chow: $n=7$; PN: $n=12$; PN+BBS: $n=9$); **C)** effects on MUC2 protein in intestinal wash fluid (Chow: $n=15$; PN: $n=12$; PN+BBS: $n=11$). Data are presented as mean $\pm$ SEM. PN, parenteral nutrition; BBS, bombesin.

*p<0.05 versus Chow; #p<0.05 versus PN+BBS

Figure 2. Chow, PN, and PN+BBS effects on TFF3

A) Effects on ileal tissue *Tff3* mRNA (Chow: n=8; PN: n=8; PN+BBS: n=10); **B)** effects on ileal tissue TFF3 relative protein concentration (Chow: n=13; PN: n=11; PN+BBS: n=8); **C)** effects on TFF3 protein in intestinal wash fluid (Chow: n=8; PN: n=12; PN+BBS: n=10). Data are presented as mean $\pm$ SEM. PN, parenteral nutrition; BBS, bombesin.

* $p < 0.05$ versus Chow; # $p < 0.05$ versus PN+BBS

Figure 3. Chow, PN, and PN+BBS effects on RELM β

A) Effects on ileal tissue *Relmb* mRNA (Chow: n=8; PN: n=8; PN+BBS: n=10); **B)** effects on ileal tissue RELM β relative protein concentration (Chow: n=5; PN: n=6; PN+BBS: n=6). Data are presented as mean $\pm$ SEM. PN, parenteral nutrition; BBS, bombesin.

***p<0.05 versus Chow; #p<0.05 versus PN+BBS**

Figure 4. Chow, PN, and PN+BBS effects on Th2 type cytokines

A) Effects on ileal tissue IL-4 (pg/mg), (Chow: n=9; PN: n=11; PN+BBS: n=10); **B)** effects on ileal tissue IL-13 (pg/mg), (Chow: n=10; PN: n=13; PN+BBS: n=12). Data are presented as mean $\pm$ SEM. PN, parenteral nutrition; BBS, bombesin.

Table 1
Specific primers used for qRT-PCR assay

Muc2, mucin 2; *Tff3*, trefoil factor 3; *Relmb*, resistin-like molecule beta.

Goblet cell product	Forward primer (5' to 3')	Reverse Primer (5' to 3')
<i>Muc2</i>	ACAAAAACCCAGCAACAAG	GAGCAAGGGACTCTGGTCTG
<i>Tff3</i>	TAATGCTGTTGGTGGTCCTG	CAGCCACGGTTGTTACACTG
<i>Relmb</i>	GTCAGTGGATGTGCTTGTGG	GTCGAGACCGTGGTTTCATT

Table 2
Animal body weight and body weight change

Weights are mean $\pm$ SEM.

Diet	n	Body Weight Before Feeding, g	Body Weight Change, g
Chow	15	38.0 $\pm$ 1.1	-1.0 $\pm$ 0.4
PN	13	36.2 $\pm$ 1.0	-4.1 $\pm$ 0.2 *
PN+BBS	12	35.8 $\pm$ 0.7	-3.9 $\pm$ 0.5 *

*
p < 0.001 vs. Chow.

PN, parenteral nutrition; BBS, bombesin.