Bacterial nutrient foraging in a mouse model of enteral nutrient deprivation: Insight into the gut origin of sepsis

Short Title: Bacterial nutrient foraging during TPN-dependence

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Abstract

Background: Total parenteral nutrition (TPN) leads to a shift in small intestinal microbiota with a characteristic dominance of Proteobacteria. This study examined how metabolomic changes within the small bowel support an altered microbial community in enterally deprived mice.

Methods: C57BL/6 mice were given TPN or enteral chow. Metabolomic analysis of jejunal contents was performed by liquid chromatography/mass spectrometry (LC/MS). In some experiments, leucine in TPN was partly substituted with $^{13}$C-leucine. Additionally, jejunal contents from TPN dependent and enterally fed mice were gavaged into germ-free mice to reveal if the TPN phenotype was transferrable.

Results: Small bowel contents of TPN mice maintained an amino acid composition similar to that of the TPN solution. Mass spectrometry analysis of small bowel contents of TPN dependent mice showed increased concentration of $^{13}$C compared to fed mice receiving saline enriched with $^{13}$C-leucine. $^{13}$C-leucine added to the serosal side of Ussing chambers showed rapid permeation across TPN-dependent jejunum, suggesting increased transmucosal passage. Single-cell analysis by fluorescence in situ hybridization (FISH) – NanoSIMS demonstrated uptake of $^{13}$C-leucine by TPN-associated bacteria, with preferential uptake by Enterobacteriaceae. Gavage of small bowel effluent from TPN mice into germ-free, fed mice resulted in a trend toward the pro-inflammatory TPN-phenotype with loss of epithelial barrier function.

Conclusions: TPN-dependence leads to increased permeation of TPN-derived nutrients into the small intestinal lumen, where they are predominately utilized by Enterobacteriaceae. The altered metabolomic composition of the intestinal lumen during TPN promotes dysbiosis.

Keywords: Total parenteral nutrition; microbiota; metabolome; nutrient foraging; epithelial barrier function, NanoSIMS

New & Noteworthy: Using a mouse model of total parenteral nutrition (TPN), this study shows that enteral nutrient deprivation leads to the permeation of TPN-derived nutrients into the gut lumen, where they are preferentially utilized by Enterobacteriaceae. This provides insight into the mechanisms behind the loss of epithelial barrier function and subsequent complications seen in the TPN-dependent state. Dysbiosis related to TPN may be exacerbated by an altered intestinal metabolome.
Introduction

For patients undergoing periods of complete bowel rest, or deprivation of enteral nutrition due to a non-functional gastrointestinal tract, total parenteral nutrition (TPN) provides critical support (4, 13). Each year, approximately 350,000 patients in the U.S. rely on this therapy (34). While life-sustaining, adverse consequences have been observed with TPN. This includes mucosal atrophy and loss of epithelial barrier function (EBF) leading to increased systemic infectious complications (1, 7, 18, 20). The theory of gut-origin sepsis has been discussed since the 1940s to explain these infectious complications (11). In short, the theory states that in times of stress and starvation, epithelial barrier dysfunction allows enteric bacteria and/or their byproducts to translocate, leading to systemic infection or multisystem organ dysfunction.

In order to examine the gut origin of sepsis theory, a mouse TPN model has been used (15, 16, 28, 40, 51). In this model, TPN-dependence leads to a pro-inflammatory state within the intestinal mucosa. This is associated with elevated mucosa-derived inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (16, 17). These changes then drive mucosal atrophy and a loss of EBF (12). Epithelial barrier dysfunction is commonly attributed to enterocyte malnutrition. This concept has led to administration of enteral and perenteral glutamine, the preferred energy source of enterocytes, to maintain barrier function (32, 45). The clinical effectiveness of this strategy is variable, however, with evidence of increased mortality in critically ill patients (22). As further evidence that enterocyte malnutrition alone is not responsible for EBF loss with TPN, mucosal atrophy is not reversed by ensuring complete nutritional needs via TPN (24). Enterocytes are able to obtain nutrition basally from an intravenous (IV) source (39), and basally-fed cultured enterocytes show increased proliferation compared to those receiving only apical nutrition (33). These data suggest that enterocytes are able to adequately utilize IV nutrition. There is an emerging theory for epithelial cell dependence upon luminal nutrients that focuses on the role of intestinal microbiota.

The intestinal epithelium serves not only as the site of nutrient absorption, but also as the interface between the host and its resident microbiota. This microbial community is critical in the development of gut immune health (29), and it is highly sensitive to changes in environmental factors such as diet (10). Alterations in the composition of enteral nutrition may impact the microbial population of the GI tract, and subsequently, host pathology (47, 48). With complete enteral nutrient deprivation, striking shifts occur in the composition of the small and large bowel microbiota (38), moving from a Firmicutes-dominated population to a less diverse Proteobacteria-rich mucosa-associated community (21, 23, 31). However, a causal relationship between the altered microbiota and decreased EBF has not been demonstrated. In addition, a critical question remains as to how these bacteria forage for nutrients in a state of complete enteral nutrient deprivation.

The present study examined the interactions between the intestinal microbiota and the metabolomic constituents in the TPN-dependent setting. We demonstrate TPN-associated changes in the luminal metabolome, characterized by an amino acid composition closely resembling that of TPN solution. Importantly, we further demonstrate that the observed shift in microbial community composition may be due to selective utilization of TPN-derived amino acids by Proteobacteria. Finally, we show that TPN-
derived enteric contents produce a similar pro-inflammatory phenotype in fed germ-free mice as conventional TPN-dependent mice.

Methods

Animals and housing. C57BL/6J male, specific pathogen-free mice (10-12 wk old; Jackson Laboratory, Bay Harbor, ME; n = 6 for each experimental group) were maintained under temperature-, humidity- and light-controlled conditions. Mice were initially fed ad libitum with standard mouse chow and water. During the administration of IV solutions, mice were singly housed in metabolic cages to prevent coprophagia. Colonization studies were performed with 10-12 wk old germ-free C57BL/6J mice from a breeding colony established at the University of Michigan germ-free core facility. Mice were housed in sterile isolators with irradiated food, bedding, and water for the duration of the experiments. All animal protocols were approved by the University Committee on the Use and Care of Animals of the University of Michigan (no. PRO00003986).

Operative procedures and PN. Mice underwent jugular venous cannulation and TPN administration as previously described(26). After 24 h, mice were randomized into two groups: 1) enterally-fed conventional mice (FED) which received IV crystalloid solution at 0.2 ml/h and standard laboratory mouse chow; 2) TPN-dependent conventional mice (TPN) which were deprived of all enteric nutrition and received TPN at 0.5 ml/h. Groups were maintained isonitrogenous and isocaloric. All animals were killed at 7 days using CO2.

Effluent gavage in germ-free mice. Small bowel effluent from TPN and FED mice was steriley extracted under an oxygen-free nitrogen hood. This was performed by removing the entire small intestine from ligament of Treitz to the terminal ileum and extracting enteric contents. This was immediately gavaged (200 μL intragastric) into enterally fed germ-free mice (GF) under otherwise sterile conditions. Each treatment group was housed in separate sterile isolators. These mice were allowed a standard germ-free diet and sacrificed after 7 days for investigation of the inflammatory state of the small bowel.

Untargeted metabolomic interrogation. In order to better understand the interaction between the intestinal luminal environment and the host, small bowel effluent from TPN, FED, and GF mice underwent untargeted Liquid Chromatography-Mass Spectrometric (LC/MS) analysis. A small portion of each sample was weighed in a micro-tube. Samples were than dried under the stream of nitrogen for 5 hours, resuspended in 100 μl of water and placed on ice. Extraction buffer was prepared by mixing 50 ml of methanol : chloroform : water (8 : 1 : 1) with 0.45 ml of 2 mg/ml solution of recovery standards (mixture of 13C-labeled amino-acids). Extraction buffer was pre-chilled at -20°C for 30 min and added to each sample at a ratio of 10 μl/mg wet weight. One stainless steel bead was then placed in each test tube and samples were homogenized for 2 min at top speed. Extracts were clarified by centrifugation at 4750 rpm for 10 min (4°C). 200 μl aliquots were then dried under the stream of nitrogen, resuspended in 100 μl of water containing injection standards (tBoc-L-Alanine, tBoc-L-Asparagine, tBoc-L-Phenylalanine), clarified by
centrifugation at 12,000 rpm for 20 min (4°C) and transferred to HPLC glass vials and moved to the instrument for LC/MS analysis as previously described (30).

Stable isotope labeled leucine administration. Stable isotope labeled L-Leucine (2-13C, 99% CLM-2014-1, Cambridge Isotope Laboratories, Andover, MA) was added to a specially formulated TPN (Health Dimensions Inc., Ann Arbor, MI). To maintain an isocaloric and isonitrogenous state, a 50% reduction of leucine in the amine solution was replaced with targeted isotope. Stable isotope-labeled TPN was administered in standard fashion and given either throughout the entire period of enteral deprivation (6 days) or only during the last two days of infusion. A group of FED mice were allowed enteral chow, but underwent jugular venous cannulation and given an IV saline solution with identical concentrations of 13C-leucine either throughout or only during the last two days of the experiment.

Isotope collection and analysis. Serum, small bowel effluent, and bile samples were collected after sacrifice. Microbial biomass was extracted by transferring small bowel effluent into 50 ml of wash buffer (50 mM sodium phosphate buffer [pH 8], 0.1% Tween 80). The solution was shaken at 100 oscillations/min for 20 min on a reciprocating horizontal platform at room temperature and subsequently stood upright for 15 min to allow large debris to settle. The supernatant was transferred to a clean 50 ml conical tube and kept on ice. The pellet was resuspended in wash buffer and cellular debris was allowed to settle 3 additional times. Supernatants were pooled and bacteria were collected by centrifugation at 12,000 X g for 15 min at room temperature. After 20 minutes of sonication at 4°C, samples were treated with sarkosyl + Triton X-100 (Sigma-Aldrich; 1.5% + 2%(42). Samples were then centrifuged at 16,000 X g for 15 min, and all pellets were stored at -80°C prior to 13C mass isotopomer flux analysis.

13C mass isotopomer flux analysis. 100 µl aliquots of each sample were extracted with 150 µl of extraction solvent (methanol:chloroform:water) then centrifuged at 15000 X g for 5min. Supernatant containing metabolites were transferred to autosampler vials and dried at 45°C by a vacuum centrifuge. Dried samples are derivatized with 40ul of the 20mg/ml methoxyamine (37°C and 60min) and 40ul of BSTFA (70°C for 30min). Derivatized samples were analyzed by GCMS on an Agilent J&W DB-5 column (250 µm x 0.25µm x 30 m). GCMS conditions: Temperature program: 2 min hold at 70°C, then 70-300°C @ 25 °C/min, then hold 300°C for 2min. Flow rate: 1.1ml/min. Injector temperature: 250°C, 1ul injection at 1:10 split ratio. Data were processed by MassHunter workstation software, version B.06. Isotope enrichment values were normalized to harvested sample mass.

Epithelial Barrier Function measurement. Transepithelial resistance (TER) of full thickness jejunum (0.3-cm²), 6 cm distal to the ligament of Treitz, was assessed using modified Ussing chambers (Physiologic Instruments, Inc., San Diego, CA, USA) as previously described(50). Intestinal permeability was assessed using fluorescein isothiocyanate (FITC)-dextran (4000 kDa at 50 mg ml⁻¹; Sigma-Aldrich, St. Louis, MO)(6). 150 µl FITC-dextran was added to the mucosal compartment after equilibration, and 500 µl was removed from the serosal compartment after 60, 90, and 120 min.

For acute amino acid leakage experiments, tissue was mounted in the Ussing chamber such that the transepithelial current was applied in a serosa-to-mucosa direction. 13C-leucine was dissolved in the
Krebs buffer of the serosal compartment [15 mM]. 500 μl was removed from the mucosal compartment at 0, 10, 30, 60, and 90 minutes and snap frozen for mass isotopomer measurement. Results were expressed as the ratio of $^{13}$C-leucine to $^{12}$C-leucine to account for $^{12}$C-leucine derived from sloughed enterocytes during the Ussing chamber experiment.

**Fluorescence in situ hybridization of small bowel microbiota.** Flushed luminal contents were fixed with 4% formaldehyde for 4 h, sonicated to disrupt cell aggregates, and used for fluorescence in situ hybridization (FISH) and NanoSIMS imaging. FISH was performed with fluorescently-labeled rRNA-targeted oligonucleotide probes specific for all *Bacteria* (S-D-Bact-0338-a-A-18-Cy5, 5'-GCT GCC TCC CGT AGG AGT-3'; S*-BactP-0338-a-A-18-Cy5, 5'-GCA GCC ACC CGT AGG TGT-3'; S*-BactV-0338-a-A-18-Cy5, 5'-GCT GCC ACC CGT AGG TGT-3') or *Enterobacteriaceae* (S*-EBAC-1790-a-A-18-Cy3, 5'-CGT GTT TGC ACA GTG CTG-3'). Using a standard protocol (9), to evaluate potential non-specific FISH probe binding, parallel samples were hybridized with the reverse complement of the bacterial probe (NONEUB-5'-ACTCCTACGGGAGGCAGC-3'). Hybridized samples were imaged and marked on an epifluorescence laser microdissection microscope (LMD, Leica LMD 7000) as previously described (3).

**NanoSIMS imaging of small bowel microbiota.** Nano-scale resolution secondary ion mass spectrometry (NanoSIMS) measurements were performed on an NS50L (Cameca). Data were recorded as images by scanning a finely focused Cs$^+$ primary ion beam (ca. 80 nm spot size with 2 pA beam current) and detection of negative secondary ions and secondary electrons. Recorded images had a 512 x 512 pixel resolution and a field-of-view ranging from 60 x 60 to 70 x 70 μm$^2$. Analysis areas were pre-sputtered to establish a Cs$^+$ dose density of 8E14 or 2E16 at/cm$^2$. All images were recorded with a dwell time of 5-10 ms/pixel/cycle.

NanoSIMS images were processed using the WinImage software package (Cameca). Cells were identified in drift-corrected, stack-accumulated NanoSIMS images and manually verified with aligned FISH images. $^{13}$C/$^{12}$C/$^{12}$C$^{13}$C isotope fractions, designated as atom percent (at%) $^{13}$C throughout the text, were calculated from dead time- and QSA-corrected $^{12}$C$_2$- and $^{12}$C$^{13}$C signal intensities. Summary statistics from each region of interest were calculated for single-cell analysis. Individual cells were considered significantly enriched in $^{13}$C if the mean cellular at% $^{13}$C was 5 standard deviations above the mean at% $^{13}$C of the unlabeled control cells from the gut lumen and if the measurement error (1σ, Poisson) was smaller than the difference between the at% of the labeled cell and the mean at% of unlabeled control cells.

**Cytokine profiling.** Cytokine profiles were measured in tissue lysates MILLIPLEX MAP multiplex kits (Millipore Corporation, Billerica, MA, USA) (17). Mucosal scrapings from 1 cm jejunal segments were used to carry out protein purification and cytokine profiling per manufacturer instructions.

**Immunofluorescence imaging.** To further study EBF, immunofluorescence microscopy of targeted tight and adherens junctions was performed as previously described (16). Primary antibodies were rat monoclonal anti-E-cadherin 1:100, rabbit polyclonal anti-ZO-1 1:100, and mouse monoclonal anti-occludin 1:100 (Invitrogen, Calsbad, CA). Fluorescence was analyzed with an Olympus BX-51 upright light and
fluorescence microscope. Images were stacked using Nikon image software and processed using Adobe Photoshop CC. Statistical analysis. All results are expressed as mean ± standard deviation unless otherwise specified. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Comparison between two groups used the two-tailed, unpaired Student’s T-test. Comparison between >2 groups used analysis of variance (ANOVA). A p value of < 0.05 was considered significant.

Principal components analysis. Principal components analysis (PCA) was used to process untargeted LC/MS metabolomics data. Data analysis was performed using Find by Formula algorithm of the Agilent MassHunter Qualitative analysis software. Library for the analysis was constructed partially from the authentic standards and partially by creating “Known Unknowns” (KU) from mass spectral features consistently present in a large proportion of the samples. Molecular features were identified by Molecular Feature Extractor (Agilent MassHunter component) and processed using in-house software. To evaluate possible biological signal Principal Components Analysis (PCA) and multiple group T-test were performed using all features (except standards) present in > 70% of the samples. Statistical analysis was performed using R (cran.r-project.org) and was done separately for positive and negative mode data.

Results
Survival
All animals survived for 7 days post-intervention until experimental endpoints were reached.

Metabolomic composition of small bowel effluent
We first asked what metabolomic components were present in the intestinal lumina of mice with enteral nutrient deprivation. Untargeted metabolomics was performed with small bowel effluents from TPN, FED, and GF mice by using LC/MS. PCA of mass spectrometric data demonstrated significant separation of experimental groups (Fig 1A). The first principal component (PC1) accounted for 90% of differences between the three study groups (Fig 1B). The presence of bacteria (TPN and FED) led to a significant increase in several luminal amino acids compared to GF mice (Table 1). Seven of the first 10 compounds identified in PC1 (Fig 1C) were amino acids (AA); including increases in leucine, phenylalanine, methionine, valine, tryptophan, tyrosine and proline (note that leucine and isoleucine co-elute and have identical masses and are reported only as ‘leucine’). Interestingly, these amino acids were significantly increased in FED mice compared to GF mice despite both groups receiving identical chow. Even higher concentrations of these amino acids were observed in TPN mice despite a complete lack of enteral nutrient delivery. Each of the amino acids identified in PC1 matched those supplied in the TPN solution in crystalline form. The amino acid that contributed most to the first and the second PC (i.e. observed differences in metabolite composition) was leucine, an essential amino acid that was present in a relatively high concentration in the TPN formulation used in our TPN model (Fig 1D).
Nutrient substrate from the TPN solution is found in the intestinal lumen

As the TPN mice had no enteral source of amino acids, we hypothesized that the leucine detected in the lumen derived directly from the TPN solution through leakage into the small bowel lumen. To confirm this, we targeted leucine, which was the most significant contributor to PC1, for subsequent studies. Isotope labeled leucine ($^{13}$C-leucine) was added to the TPN (consisting of a 50% substitution of non-isotope leucine) and administered IV during a 6 day period of enteral deprivation. Fed control mice were allowed an enteral diet during $^{13}$C-leucine IV infusion. A significantly higher concentration of $^{13}$C-leucine (expressed as the % $^{13}$C-leucine to total leucine) was present in the small bowel effluent after 6 days of TPN compared to enteral feeding (Fig 2A). In fact, in TPN-dependent mice, 50% of luminal leucine was $^{13}$C-labeled after 6 days, the same proportion as in the TPN solution, which demonstrated that the TPN solution is the main source of these luminal amino acids.

These findings suggested a transepithelial permeation of leucine with TPN administration. To determine if other sources of leucine were contributing the elevated luminal levels, serum (Fig 2B) and biliary (Fig 2D) levels of $^{13}$C-leucine were measured. Biliary and serum $^{13}$C-leucine levels trended toward isotopic enrichment in TPN-dependent mice compared to enterally-fed mice. Possibly, enterally absorbed $^{12}$C-leucine acted to dilute IV $^{13}$C-leucine in the FED group. The increased ratio of effluent:serum $^{13}$C-leucine in TPN versus FED mice suggested that leucine was preferentially concentrated in the intestinal lumen with TPN-dependence (Fig 2C).

TPN-derived leucine becomes incorporated into luminal bacteria

We next examined the content of $^{13}$C-leucine in harvested luminal bacteria. The enrichment of $^{13}$C-leucine in bacterial biomass doubled from 2 days to 6 days of infusion, suggesting progressive bacterial access to and uptake of TPN-derived leucine with time (Fig 2E). Furthermore, $^{13}$C-leucine enrichment was significantly higher in bacteria derived from TPN mice than in bacteria derived from FED mice. These findings support the hypothesis that enteral deprivation drives epithelial barrier breakdown with subsequent permeation of TPN-derived amino acids from the serum into the intestinal lumen. The elevated levels of $^{13}$C in bacterial isolates demonstrate that the intestinal microbiota utilize TPN-derived amino acids as a direct nutrient source, which has not been previously recognized.

Acute transepithelial amino acid leakage

To further demonstrate a transepithelial leakage of amino acids rather than enterocyte uptake and subsequent sloughing, jejunal tissue derived from mice receiving TPN or saline without any enrichment with $^{13}$C-leucine was mounted in Ussing Chambers with current directed toward the mucosal compartment. $^{13}$C-leucine was added to the serosal compartment, and transepithelial movement was assessed by measuring mucosal $^{13}$C-leucine. The transepithelial resistance (TER) of jejunum from TPN mice was significantly lower than that of FED mice at all time points, with a parallel gradual decrease in TER over
time (Fig 3A). The relative concentration of $^{13}$C-leucine progressively rose in the mucosal compartment, and was significantly greater by 90 min (Fig 3B).

### Imaging of TPN-derived $^{13}$C-leucine utilization by gut microbiota at the single cell level

We next examined whether the incorporation of leucine by luminal bacteria correlated with previously reported TPN-associated microbial changes(31). Thus, Enterobacteriaceae were specifically targeted, as they are the dominant Proteobacteria known to bloom in small intestine during TPN(31).

FISH was combined with NanoSIMS to visualize utilization of $^{13}$C-leucine by bacterial cells in the small bowel effluent. In FED (13C leucine IV + FED) and control effluent samples (mice not receiving 13C-leucine; 12C + FED), Enterobacteriaceae were rarely detected and were below the quantification limit of FISH (<0.5% relative abundance) (Figure 4A). In contrast, many Enterobacteriaceae were detected in the luminal content from 13C leucine + TPN mice and comprised 81% of all bacterial cells (Figure 4B).

NanoSIMS imaging was performed on representative fields of view to quantify the uptake of 13C-leucine from the administered TPN by individual bacterial cells. Quantification of the 13C labeling of individual cells revealed that 90% of the cells in the 13C+FED mice were enriched in 13C (12 cells analyzed; Figure 4C and E, center column). Similarly, 99% of the cells in the luminal contents from 13C+TPN mice were enriched in 13C (108 cells measured; Figure 4D and E, right column). Statistical analysis showed that treatment (i.e., 13C+TPN, 13C + FED, and 12C + FED) as well as target group (i.e. Enterobacteriaceae versus other Bacteria) were significant factors determining at% 13C (ANOVA, p<0.001). The enrichment in 13C was greater in cells from 13C+TPN mice than either 13C + FED or 12C + FED controls (Tukey post-hoc test, p<0.001). The difference in 13C enrichment between the 13C+FED and 12C+FED controls, though elevated, was not significant (Tukey post-hoc test, p=0.08), which may be due to too few cells measured in the 13C + FED samples. The marked difference observed in the degree of 13C labeling of cells between the 13C + FED and 13C + TPN mice emphasizes the change in EBF and the permeation of TPN-derived leucine into the small bowel lumen. Additionally, in the TPN mice, Enterobacteriaceae were significantly more enriched in 13C than non-Enterobacteriaceae cells (using a universal FISH probe for all Bacteria; Tukey post-hoc test, p<0.001; Figure 4E, right column). This difference highlights the ability of TPN-derived nutrients to support the previously reported metabolically selective activity of Enterobacteriaceae and possibly other Proteobacteria members (46).

### TPN-derived small bowel effluent drives a pro-inflammatory response in germ-free mice

Because of the differences in luminal metabolome and microbiome compositions between GF and the FED and TPN mouse groups, we then asked whether transplantation of small bowel content from TPN into GF mice could invoke the pro-inflammatory state and associated loss of EBF seen with TPN-dependence. Intestinal effluent was harvested under microaerophilic conditions from TPN and FED mice, and then sterilely gavaged into a group of GF mice. Gavaged mice were examined after one week of sterile feeding. The introduction of bacteria significantly decreased TER compared to non-gavaged GF mice (Fig...
Furthermore, TER decreased slightly more, albeit not significantly (p=0.057), in GF mice receiving TPN small bowel content compared to GF mice receiving FED mice small bowel content.

Immunofluorescence staining was performed to evaluate junctional proteins in the gavaged mice. Representative images of ZO-1, occludin, and E-cadherin are shown in Fig 5C. The mice gavaged with FED effluent displayed a co-localization of ZO-1 and occludin at the cell membrane along inter-cellular junctions. There was a decline in the intensity of this staining in the TPN effluent - gavaged mice, indicating a decrease in localized tight junction expression with exposure to TPN effluent. This pattern was seen on slides stained for E-cadherin as well. Small bowel mucosal morphology was not significantly altered; crypt depth and villus height of TPN-gavaged and FED-gavaged mice (48±2 vs 55±4 μm; p=0.06 and 135±5 vs 151±7 μm; p=0.06, respectively). These histologic trends are similar to those previously found with conventional TPN-dependent mice (16). TPN effluent also drove a trend toward higher immune activity, with increases in inflammatory cytokines. Although only IL12p40 was statistically significant, TPN-gavaged GF mice trended toward higher levels in several other pro-inflammatory cytokines (Fig 5B, full list of cytokines in Table 2). These findings demonstrate that intestinal contents from TPN-dependent mice, including TPN-associated microbiota, recapitulate the TPN phenotype in fed germ-free mice.

Discussion

Intestinal inflammation and dysfunctional EBF have been associated with TPN administration. However, the precise mechanism which drives these changes is unknown. A concurrent change in the intestinal microbial community to a less diverse population has been reported, characterized by a loss of *Firmicutes* and a relative expansion of *Proteobacteria* (14, 21, 23, 31, 37). Whether the altered microbiota results in or is the product of inflammation has never been answered. In this study, we demonstrated several novel findings. First, we showed that the route of nutrient administration results in a significant change in the metabolomic composition of the intestinal lumen. In the absence of enteral nutrients, a time-dependent change in intestinal permeability occurred which allowed passage of leucine and other TPN nutrients to efflux directly into the gut lumen. This was associated with an alteration in nutrient foraging by luminal bacteria. This is consistent with the work of others, which has shown that altered nutrient composition within the intestinal lumen influences the relative dominance of microbiota based on their specific metabolic potential (19). Using a 13C-leucine isotope tracer in TPN, we demonstrated a significant uptake of IV-delivered leucine by small bowel bacteria in the TPN-dependent mice. By using a novel FISH-NanoSIMS approach, we further show that primarily *Enterobacteriaceae* (phylum *Proteobacteria*), the dominant bacterial family in the small bowel lumen of TPN mice, have a selective advantage by metabolizing leucine. Foraging of IV-delivered leucine by this family of bacteria was significantly increased compared to other bacteria present in the lumen and far higher than that seen in enterally-fed groups. Finally, we demonstrated that the introduction of small bowel effluent from TPN mice into GF
mice led to a phenotype similar to that observed in TPN dependent mice. Taken together, this study indicates that the pathophysiology of the loss of EBF in the state of complete TPN-dependence may be driven by a complex interaction between an altered metabolome and the luminal microbiota.

These data are consistent with the previously identified loss of EBF in the TPN-dependent setting, predominantly due to a pro-inflammatory response via a Myd88-dependent pathway (31). This study is the first to indicate a potentially causal relationship in that the transfer of enteral effluent from TPN-dependent mice resulted in EBF disturbances in previously germ-free mice. This study supports the theory that a major source of the adverse outcomes with enteral deprivation and TPN-dependence may be epithelial barrier dysfunction driven by altered luminal microbiota. The microbiota forages substrates originating from the host serum, including the TPN-derived nutrients, which are increasingly accessible to the gut microbiota as breakdown of EBF occurs. As the microbial population shifts, the invasive nature of *Proteobacteria* may be responsible for, or promote, epithelial barrier breakdown. Alternatively, the loss of EBF-promoting populations dependent on enteral nutrition may be responsible for epithelial breakdown.

Future work should investigate the specific virulence factors of expanded bacterial strains in the TPN-dependent setting to understand specifically how these bacteria induce or contribute to barrier breakdown (2). Once the inflammatory bacterial community is established and EBF is broken down, the leak of PN-derived nutrients from the serum into the intestinal lumen serves to provide nutrients to those bacteria, which in turn appear to preferentially forage upon nutrients which are being intravenously supplied to sustain the host. This results in a self-perpetuating phenomenon. A schematic representation of these changes is summarized in Fig 6.

With further breakdown of EBF, bacteria may translocate, possibly resulting in the increased incidence of infectious complications associated with TPN-dependence (18). The detrimental impact of the loss of EBF has been well described in animal and human models alike (25-27). Although it is thought that changes in barrier function occur in humans, far less is known about alterations in barrier function with enteral nutrient deprivation on a clinical level (5, 7, 8, 41, 44, 49). Recent human studies, however, have suggested a similar pathophysiology, as enteral nutrient deprivation led to changes in the microbiota of the small bowel as well as to a loss of bacterial diversity in humans (36). Using a set of matched human small bowel specimens, whereby one segment was receiving enteral nutrients and the other enterally deprived, a loss of barrier function was found in the unfed intestine, as well as an increase in the abundance of tumor necrosis factor alpha and Toll-like receptor 4 (35). The findings of the current mouse model may have significant translational applications. For instance, future work might investigate the permeation of other IV-delivered medications into the gut lumen of unfed patients, and potential effects these substrates might have on intestinal microbiota and subsequent clinical outcomes.

The present study had some limitations. First, we investigated a single amino acid in the current study – leucine. Future studies looking at alteration of other TPN-derived nutrients may offer better insight into the nutrient requirements of this altered microbiome. Further, we targeted only one *Proteobacteria* family in this study. While prior studies have identified *Enterobacteriaceae* as the dominant
Proteobacteria in the TPN-dependent state, it is quite possible that other microbial populations may have relevance to other physiologic changes in this TPN model. As we saw an expansion of Bacteroides and Akkermansia (to a lesser extent) in our TPN model (12), such organisms may also have important relevance to the pathophysiology of TPN-associated adverse clinical events. Finally, this study was limited by the lack of a viable model of germ-free TPN-dependent mice. Unfortunately, sterile vascular cannulation without introduction of bacteria was not possible; therefore, fed mice were used as the sole germ-free control group.

Concluding Remarks

This study provides support to the theory that the changes in the intestinal environment and microbiota are responsible for the decline in EBF seen with TPN administration. Through untargeted metabolomics and targeted stable isotope labeling analysis, we demonstrated a previously unrecognized mechanistic explanation as to how potentially harmful microorganisms exploit TPN-derived nutrients in the intestinal lumen in a state of complete host enteral nutrient deprivation. This could explain the clinical finding that patients receiving TPN have a higher perioperative complication rate. In the future, therapies directed towards maintenance of a EBF-promoting intestinal microbiota via modulation of the luminal metabolome may lead to a decrease in infectious complications in TPN-dependent patients.
**Figure and Table Legends**

**Figure 1.** Metabolomic composition of small bowel effluent. (A) Weighted principal coordinate analysis of metabolite composition of small bowel samples from TPN, enterally fed (Fed), and germ-free (GF) mice. (B) The first principal component (x-axis) and second principal component (y-axis) account for 90.1% and 5.4% of overall differences, respectively. (C) List of identifiable compounds making up PC1 as determined by untargeted metabolomic analysis via LC/MS. (D) Crystalline AA composition of the TPN formulation administered to TPN-dependent mice. Circled AAs are those that comprise PC1 of analyzed enteral contents.

**Table 1.** Untargeted metabolomics results. Fold-change in abundance of each compound as identified in the first principal component (PC1) of LC/MS results between small bowel effluent from fed germ-free (GF), enterally fed conventional (Fed), and TPN-dependent conventional mice (TPN). P-values determined via ANOVA.

**Figure 2.** TPN-derived leucine accumulates in the small bowel and is incorporated into luminal bacteria. 13C mass isotopomer flux analysis of small bowel effluent (A), serum (B), bile (D), and small bowel intraluminal bacterial biomass (E) from fed and TPN-dependent mice. (C) The ratio of [13C]-leucine concentration in small bowel effluent to serum in fed and TPN-dependent mice. Intravenous infusions were supplemented with 13C-leucine for the entire six days of treatment or for the final two days only. Bile and bacteria samples were pooled prior to analysis. *p<0.05, **p<0.01

**Figure 3.** Acute intraluminal passage of stable isotope labeled leucine in TPN-dependent mice. (A) Transepithelial resistance of fed and TPN-dependent jejunum. (B) 13C mass isotopomer flux analysis of consecutive samples derived from the mucosal compartment after addition of 13C-leucine to the serosal compartment. *p<0.05

**Figure 4.** NanoSIMS/FISH analysis of small bowel microbiota from enterally fed and TPN-dependent mice receiving intravenous 13C-leucine. (A and B) Small bowel effluents were hybridized with FISH probes targeting all Bacteria (EUB338 I-III, green) and Enterobacteriaceae (Ebac1790, red). NanoSIMS at% 13C distribution maps of the same fields of view for fed (C) and TPN-dependent (D) samples show Enterobacteriaceae significantly enriched in 13C (white arrows) compared to non-Enterobacteriaceae cells from fed mice (scale bar = 5 µm). (E) A summary of cellular carbon isotope composition from single cells. The dashed line indicates the threshold value for considering a cell 13C enriched over cells from 12C+Fed control effluent (mean ± 5 SD). The atom % (at%) threshold for calling a cell enriched in 13C was +5 standard deviations of the mean 13C isotopic composition of cells from control 12C+Fed mice (1.122 at%
$^{13}$C; 104 cells analyzed). Asterisks indicate significant differences between treatments and/or target populations (Tukey post-hoc test, p<0.001).

**Figure 5.** TPN-derived small bowel contents induce inflammatory changes and loss of EBF in axenic mice. Effluent from TPN-dependent (TPN Eff.) and enterally-fed (FED Eff.) small bowel was gavaged into germ-free mice (GF). (A) Epithelial barrier function, was examined using two methods, transepithelial resistance of full-thickness jejunal specimens ($\Omega^*\text{cm}^2$, left panel) and permeation of tracer molecule FITC-dextran (4KD, right panel). (B) Small bowel mucosal cytokine results are measured with using a multiplex protein assay (Millipore Corp, Billerica, MA). (C) Representative images of immunofluorescence staining of small bowel tight and adhesion junction proteins was performed and co-stained with a DAPI nuclear counterstain. Transepithelial resistance (TER), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin 1 beta (IL1-β), interleukin 12p40 (IL-12p40), zonula occludens 1 (ZO-1). *p<0.05

**Table 2.** Cytokine expression in germ-free mice gavaged with small bowel effluent from enterally-fed or TPN-dependent mice. n = 6 for each group. Values expressed as concentration (pg/ml). *p<0.05

**Figure 6.** Summary of effects of TPN on the luminal metabolome and microbiome. With TPN-dependence, an altered microbiome is associated with a loss of epithelial barrier function. TPN-derived serum nutrients are supplied to the epithelium while also permeating into the intestinal lumen via a transepithelial route, further supporting the altered intraluminal bacterial population.
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A: % $^{13}\text{C}$-Leucine in Effluent

B: % $^{13}\text{C}$-Leucine in Serum

C: Intraluminal Accumulation of $^{13}\text{C}$-Leucine

D: % $^{13}\text{C}$-Leucine in Bile

E: % $^{13}\text{C}$-Leucine in Bacteria
Mucosal Passage of $^{13}$C-Leucine

Mucosal $^{13}$C-L: $^{12}$C-L ratio

TPN vs FED: *p<0.05

A Transepithelial Resistance

Minutes after $^{13}$C-L addition to serosal compartment

B Mucosal Passage of $^{13}$C-Leucine

Minutes after $^{13}$C-L addition to serosal compartment

**
Release of nutrients to epithelium

Permeation of nutrients into intestinal lumen

Parenteral Nutrition

Enterally-fed, Firmicutes-dominant

TPN-dependent, Proteobacteria-dominant

TPN
<table>
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<th>Compound</th>
<th>PC1</th>
<th>P-value</th>
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<th>TPN / GF</th>
<th>TPN / FED</th>
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<td>IFN-γ</td>
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