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Total Parenteral Nutrition Induces a Shift in the Firmicutes to Bacteroidetes Ratio in Association with Paneth Cell Activation in Rats[1,2]

Caroline M. Hodin,[3] Ruben G. J. Visschers,[3] Sander S. Rensen,[3] Bas Boonen,[3] Steven W. M. Olde Damink,[3,4] Kaatje Lenaerts,[3]* and Wim A. Buurman[3]

[3]Department of Surgery, NUTRIM School for Nutrition, Toxicology, and Metabolism, Maastricht University Medical Center, Maastricht, The Netherlands, and [4]Department of Hepato-Pancreatic-Biliary Surgery and Liver Transplantation, Royal Free Hospital, University College London, London, United Kingdom

Abstract

The use of total parenteral nutrition (TPN) in the treatment of critically ill patients has been the subject of debate because it has been associated with disturbances in intestinal homeostasis. Important factors in maintaining intestinal homeostasis are the intestinal microbiota and Paneth cells, which exist in a mutually amendable relationship. We hypothesized that the disturbed intestinal homeostasis in TPN-fed individuals results from an interplay between a shift in microbiota composition and alterations in Paneth cells. We studied the microbiota composition and expression of Paneth cell antimicrobial proteins in rats receiving TPN or a control diet for 3, 7, or 14 d. qPCR analysis of DNA extracts from small intestinal luminal contents of TPN-fed rats showed a shift in the Firmicutes:Bacteroidetes ratio in favor of Bacteroidetes after 14 d ($P < 0.05$) compared with the control group. This finding coincided with greater staining intensity for lysozyme and significantly greater mRNA expression of the Paneth cell antimicrobial proteins lysozyme ($P < 0.05$), rat α-defensin 5 ($P < 0.01$), and rat α-defensin 8 ($P < 0.01$). Finally, 14 d of TPN resulted in greater circulating ileal lipid-binding protein concentrations ($P < 0.05$) and greater leakage of horseradish peroxidase ($P < 0.01$), which is indicative of enterocyte damage and a breached intestinal barrier. Our findings show a shift in intestinal microbiota in TPN-fed rats that correlated with changes in Paneth cell lysozyme expression ($r_s = -0.75$, $P < 0.01$). Further studies that include interventions with microbiota or nutrients that modulate them may yield information on the involvement of the microbiota and Paneth cells in TPN-associated intestinal compromise. J. Nutr. 142: 2141–2147, 2012.

Introduction

In the intestine, maintenance of the balance between immune tolerance to resident microbiota and defense against invasion of bacteria is crucial. This is achieved by a physical barrier, consisting of enterocytes connected by tight junctions and a mucous layer produced by goblet cells, and an immunologic barrier constituted by the gut-associated lymphoid tissue and specialized cells such as Paneth cells. Paneth cells have been shown to be crucial in the prevention of translocation of commensal and pathogenic bacteria (1,2). These cells, which reside in the crypts of the small intestine, are the main producers of antimicrobial peptides, including lysozyme and α-defensins. The antimicrobial proteins are secreted both constitutively and in response to activation by bacteria or their products (3).

External factors interacting with the host play an important role in upholding the intestinal immune barrier. In this context, it is noteworthy that interaction of the commensal microbiota with the host is crucial in protecting the intestine from epithelial damage and in maintaining mucosal homeostasis (4–7). Changes in the composition of the commensal microbiota can therefore affect intestinal barrier function.

Diet is an external factor that directly affects the composition of microbiota (8). An increase in food intake, such as in obesity, has been shown to be related to a shift in the composition of intestinal microbiota (9–12), Paneth cell compromise (13), a disturbance in tight junction distribution (14), intestinal inflammation, and overall disturbance of intestinal homeostasis (13,15). Conversely, starvation resulted in impaired Paneth cell function, increased intestinal permeability (16), and decreased alkaline phosphatase expression (17) and was also associated with changes in gut microbiota composition (18).

Patients with compromised function of the small intestine, such as critically ill patients, are dependent on total parenteral nutrition (TPN) to meet daily energy needs. Although necessary, this feeding regimen is associated with increased gut wall

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* To whom correspondence should be addressed. E-mail: kaatje.lenaerts@maastrichtuniversity.nl.

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permeability (19), a compromised immune system, and bacterial translocation (20–25). We hypothesized that the disturbed intestinal homeostasis in TPN-fed individuals results from an interplay between a shift in microbiota composition and alterations in host immune factors such as Paneth cells, due to enteral food deprivation. In this study, the effect of TPN on factors regulating intestinal homeostasis, including bacteria and defense systems, especially Paneth cells, was investigated.

Materials and Methods

Materials. All reagents were purchased from Sigma unless mentioned otherwise.

Animal model and tissue collection. Male Sprague-Dawley rats from Charles River, initially weighing 300–350 g, were housed under controlled environmental conditions in separate cages. Experiments were approved by the Animal Care Committee of Maastricht University.

For TPN administration, the right jugular vein was canulated. Rats were anesthetized with isoflurane (3–4% induction, 2.5% maintenance), and buprenorphine (0.1 mg/kg) was used for analgesia. A small incision was made above the right clavicle in craniocaudal direction. The right jugular vein was identified and ligated cranially, and a loop was put loosely around the caudal part. Hereafter, a silastic drainage catheter was inserted for 2–3 cm and was fixed and tunneled subcutaneously to the skull. Subsequently, the catheter was connected with a metal stent, which was attached to the skull with dental cement. The metal stent was connected with a swivel by a second catheter (polyurethane), which was completely protected by a metal spring. The distal end of the swivel was used for continuous infusion of TPN. All catheters were flushed with saline before insertion and/or connection to prevent air embolism.

Rats in the experimental group received TPN [205 kcal/(kg · d) continuously] for 3, 7, or 14 d (26). TPN contained 450 mL amino acid mixture (Travasol 10%; Baxter), 360 mL glucose 50% (Glucose50%; Braun), and 140 mL lipids (Intralipid 20%; Fresenius Kabi) per liter of solution. Electrolytes and trace elements (Adamel; Fresenius Kabi) were added as well. Finally, vitamins were added as lipid-soluble (Vitintra; Fresenius Kabi) and nonsoluble (Soluvit; Fresenius Kabi) solutions. Electrolytes, trace elements, and vitamin concentrations are presented in **Supplemental Table 1**.

Rats receiving TPN were compared with control rats (total of 6 groups with 6–8 rats/group) at multiple time points. Control rats were treated according to the same protocol without incision and cannulation of the jugular vein and TPN administration and received nonpurified diet (Ssniff; Soest; **Supplemental Table 2**) ad libitum. All rats had unlimited access to water.

Rats were weighed, and the external catheters were flushed daily. After 3, 7, or 14 d rats were anesthetized in order to obtain arterial blood samples, ileal luminal content, and ileal biopsies, after which they were killed by aortic puncture while under general anesthesia. Rats were killed early in the morning after which samples were immediately processed to exclude diurnal variation and effects of food deprivation. Tissue samples were fixed and stored for paraffin embedment or snap frozen and subsequently stored at −80°C.

Immunohistochemistry. Ileum samples for immunohistochemical analysis were immersed immediately in 4% formaldehyde fixative (Unifix; Klinipath) and embedded in paraffin, after which 3-μm tissue sections were cut. Sections were incubated with specific antibodies to human lysozyme (cross-reactive with rat; DakoCytomation). Next, a biotin-conjugated secondary antibody was applied (swine anti-rabbit; DakoCytomation). Immunoreactivity was detected by using the Vectastain ABC system (Vector Laboratories) and 3-amino-9-ethylcarbazole. Nuclei were counterstained with hematoxylin. Stained sections were mounted in aqueous mounting medium (DakoCytomation). No staining was detected in sections incubated with control serum instead of the primary antibody. The histology shown is representative of all tissue samples studied. Lysozyme staining was quantified by scoring slides, on the basis of color intensity, from 0 to 5 by 2 independent blinded observers at a magnification of ×200. Average scores for each group are presented. Alcian blue staining was performed to study goblet cells. The results of goblet cell count are presented as the mean of the amount of goblet cells per 100 μm villus length.

RNA extraction. RNA was extracted from snap-frozen ileal tissue by using TRI reagent according to the manufacturer's protocol. RNA samples were treated with DNAse (Promega) to ensure the removal of potential contaminating genomic DNA, and RNA concentration was determined by spectrophotometry (Nanodrop). A total of 750 ng of RNA was used as the template for reverse transcription in a cDNA synthesis reaction using the iScript cDNA synthesis kit (Bio-Rad).

Luminal content collection and DNA extraction. Luminal content from the ileum was collected for DNA extraction and immediately snap frozen until further processing. DNA was extracted with the QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's instructions, and DNA concentration was determined by spectrophotometry (Nanodrop).

qPCR. qPCR reactions were conducted as previously described (13) in a volume of 20 μL containing 10 ng of tissue cDNA or 1 ng of luminal DNA. 150 nmol/L of gene-specific forward and reverse primers were used (Eurogentec). For the detection of total bacterial DNA and DNA of Bacteroidetes and Firmicutes, 150 nmol/L of 2 pairs of 16S rRNA gene-targeted primers were used (27,28). (The sequences of the primers used are provided in **Supplemental Table 3.**) Host gene expression levels were determined with iQ5 software (Bio-Rad) by using a Δ-Ct relative quantification model. The geometric mean of the expression levels of 2 reference genes, hypoxanthine guanine phosphoribosyl transferase (*Hprt*) and *Rplp*, was calculated and used as a normalization factor. Results on total amount of bacteria were corrected for weight of luminal content. Semiquantitative data on Firmicute and Bacteroidetes abundance are presented relative to the total bacterial abundance in the luminal content.

Enterocyte damage. Ileal lipid-binding protein (I-LBP) is a small cytosolic protein primarily expressed by enterocytes of the ileum that is rapidly released from enterocytes upon cell damage, similar to intestinal fatty acid binding protein (29,30). I-LBP concentrations were measured in plasma by means of a commercially available ELISA according to the manufacturer's instructions (Hycult Biotech). The detection limit was 0.002 ng/L.

Intestinal permeability. Intestinal permeability was assessed by measuring permeability to HRP in isolated segments of ileum as previously described (30). Segments of 8 cm terminal ileum were resected, washed, inverted, filled with 1 mL of Tris buffer (125 mmol/L NaCl, 10 mmol/L fructose, 30 mmol/L Tris; pH 7.5), and ligated at both ends. Filled segments were then incubated in Tris buffer containing 0.040 μg/L of the 44-kDa enzyme HRP. After incubation at room temperature for 45 min, the content of the ligated ileal segment was carefully collected and HRP concentration was measured spectrophotometrically at 450 nm with tetramethylbenzidine as a substrate.

Statistical analysis. Statistical analysis was performed by using Prism 5.02 for Windows (GraphPad Software, Inc.). Gaussian distribution was tested by using the Kolmogorov-Smirnov test. A 2-tailed Student's *t* test was used for between-group comparisons when values were distributed normally. Otherwise, a 2-tailed Mann-Whitney *U* test was used. Differences in time within a group were tested with 2-way ANOVA. When significant effects were found, post hoc comparisons were carried out by using the Bonferroni test. Data are represented as means ± SEM. Spearman's correlation coefficient was determined to study associations between variables. Differences were considered to be significant at $P < 0.05$.

Results

TPN is associated with a shift in small intestinal microbiota composition. First, the influence of TPN administration on small intestinal microbiota in rats was investigated. Analysis

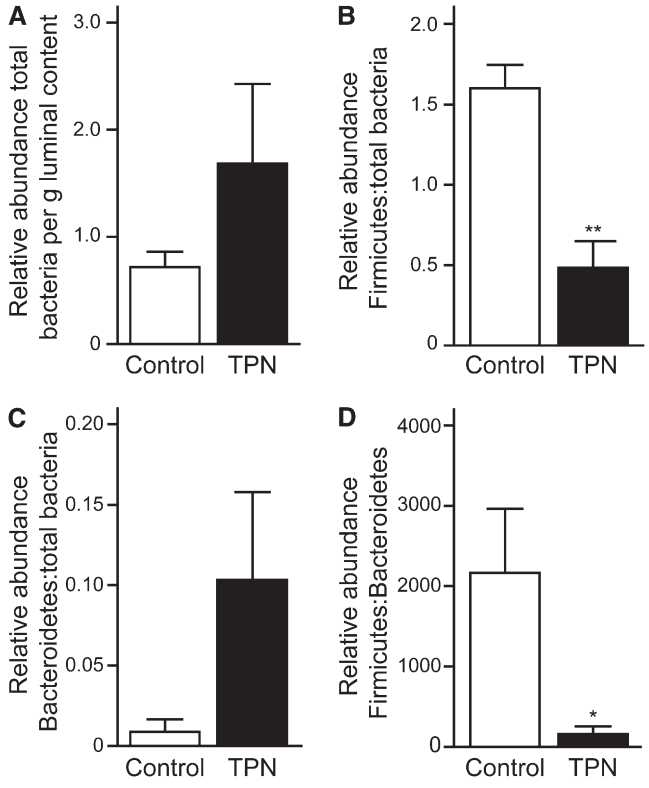

FIGURE 1 Relative abundance of total bacteria per gram of luminal content (*A*) and ratios of Firmicutes:total bacteria (*B*), Bacteroides:total bacteria (*C*), and Firmicutes:Bacteroides (*D*) in the small intestinal luminal contents of control and TPN-fed rats after 14 d. Values are means ± SEM, n = 6–8. Asterisks indicate different from control: *$P < 0.05$, ** $P < 0.01$. TPN, total parenteral nutrition.

showed total bacterial numbers per gram of luminal content did not differ between TPN-fed and control rats after 14 d (**Fig. 1***A*). Next, the prevalence of the most abundant phyla of commensal bacteria in the intestine, Bacteroidetes and Firmicutes (31,32), was assessed after 14 d of nonpurified diet or TPN. In contrast to the total bacteria number, the composition of the bacterial population was significantly changed. Firmicute dominance was less in TPN-fed rats than in controls ($P < 0.01$; Fig. 1*B*), whereas the abundance of Bacteroidetes did not differ between the groups (Fig. 1*C*). Furthermore, the proportional representation of these phyla was significantly shifted in the TPN-fed rats, in favor of Bacteroidetes ($P < 0.05$; Fig. 1*D*). These data indicate the presence of dysbiosis, a condition characterized by qualitative and quantitative changes in the intestinal flora (33), in the small intestine of TPN-fed rats.

Increased mRNA and protein expression of Paneth cell antimicrobial proteins in response to TPN. To examine potential Paneth cell alterations associated with the observed dysbiosis, the expression of Paneth cell–produced antimicrobial proteins was assessed. First, the expression of lysozyme was studied. As expected, staining showed substantial expression of lysozyme in Paneth cell granules of the ileum in control rats (**Fig. 2***A*, *upper left panel*). Interestingly, staining intensity was greater in Paneth cells of TPN-fed rats (Fig. 2*A*, *upper right panel* and *lower panels*). Scoring of the staining showed significant greater staining intensity in rats fed TPN for 14 d compared with controls ($P < 0.05$; Fig 2*B*). Corresponding results were obtained at the mRNA level (Fig. 2*C*). Here also, significant changes in lysozyme mRNA expression were achieved after 14 d of TPN administration compared with controls ($P < 0.05$; Fig 2*C*). Lysozyme mRNA expression increased from 3 to 14 d in the TPN group ($P < 0.05$). To rule out that greater expression of lysozyme was due to increased Paneth cell counts, the number of Paneth cells present in the ileum of TPN-fed rats and control rats was quantified. The number of Paneth cells (4.3 ± 0.2) was the same in both groups and did not change over time.

Next, the expression of α-defensins was studied. The quantification of mRNA expression of rat α-defensins 5 and 8 showed a similar increase in expression over time from 3 to 14 d in the TPN group ($P < 0.01$; **Fig. 3**). Significant increases were observed for *Rd5* in rats that received TPN for 14 d ($P < 0.01$; Fig. 3*A*) and for *Defa8* in rats that received TPN for 7 and 14 d ($P < 0.01$; Fig. 3*B*) compared with the control group. Together, these results showed that TPN was associated with greater expression of Paneth cell antimicrobial transcripts and proteins.

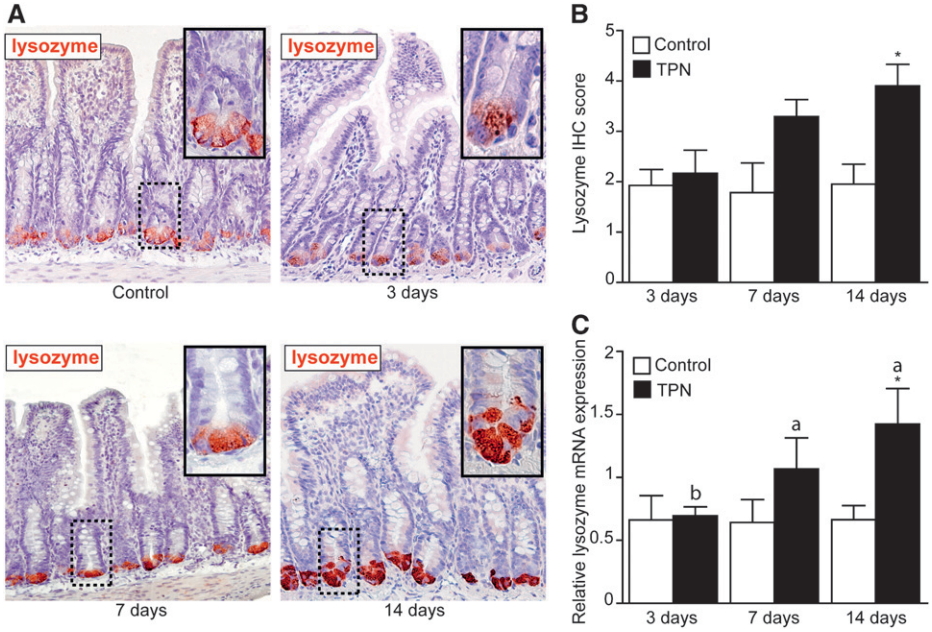

FIGURE 2 Lysozyme staining (*A*), quantification (*B*), and mRNA expression (*C*) in the small intestine of control (*upper left panel*) and TPN-fed rats over 14 d. (*A*) Magnification, ×200; inserts, ×400. (*B*, *C*) Values are means ± SEM, n = 6–8. Asterisks indicate different from control: *$P <$ 0.05. Labeled means within a group without a common letter differ, $P <$ 0.05. IHC, immunohistochemistry; TPN, total parenteral nutrition.

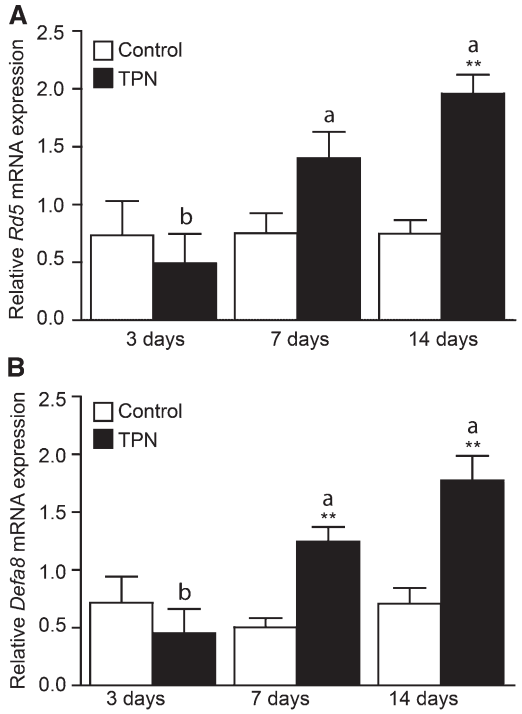

FIGURE 3 *Rd5* (*A*) and *Defa8* (*B*) mRNA expression in the small intestine of control and TPN-fed rats over 14 d. Values are means ± SEM, n = 6–8. Asterisks indicate different from control: $^{**}P < 0.01$. Labeled means within a group without a common letter differ, $P < 0.01$. TPN, total parenteral nutrition.

Lysozyme expression correlates inversely with Firmicute abundance in TPN-fed and control rats. The relation between the observed shift in small intestinal microbiota composition and the greater antimicrobial protein expression in Paneth cells in TPN-fed animals was studied further. A strong and significant inverse correlation was found between Firmicute abundance and lysozyme mRNA expression after 14 d ($P < 0.01$, $r_s = -0.75$; **Fig. 4**), strongly suggesting a link between Paneth cell function and composition of the small intestinal microbiota.

TPN does not affect goblet cells in the small intestine. The putative effects of TPN on goblet cells and their products were investigated next. Quantification of total goblet cell numbers per 100 μm villus showed comparable amounts in the small intestine of both study groups at all time points (**Fig. 5***A, B*). Next, the mRNA expression of *Muc2*, *Muc3*, and *Fcgbp* was assessed. Results showed no differences in mRNA levels of any of the 3 assessed goblet cell products in the TPN group when compared with control rats at any of the time points (Fig. 5*C–E*). These findings indicate that goblet cells and their products are not affected by TPN administration.

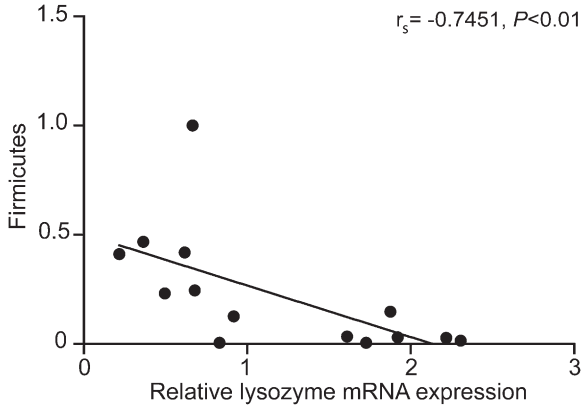

FIGURE 4 Correlation of lysozyme mRNA expression and Firmicute abundance in the small intestine of TPN-fed and control rats after 14 d. n = 6–8. TPN, total parenteral nutrition.

TPN administration results in disturbance of small intestinal integrity. Finally, the effects of TPN-associated changes on small intestinal integrity were studied. TPN resulted in markedly greater amounts of I-LBP after 14 d ($P < 0.05$; **Fig. 6***A*) compared with controls. Also, permeability of the small intestine was determined. After 14 d of TPN administration, HRP amounts were significantly greater in the TPN rats compared with control rats ($P < 0.01$; Fig. 6*B*). Combined, these results suggest a considerable impact of TPN administration on the maintenance of small intestinal homeostasis.

Discussion

The use of TPN in the treatment of critically ill patients has been the subject of debate because its administration is associated with disturbances in intestinal homeostasis and decreased gut barrier function (19–25). During the past years, intestinal microbiota have been increasingly acknowledged as an important factor in the maintenance of mucosal homeostasis (4–7), also through their interplay with Paneth cells, epithelial cells that are crucial participants of the host intestinal immune system (1,34). In this study, a potential contribution of changes in microbiota and Paneth cells to TPN-related intestinal imbalance was investigated.

A number of dietary and nondietary factors influence the composition of intestinal microbiota (35). Changes in the ratio between the 2 phyla that are most dominant in the intestine have especially been the focus of attention. Extreme changes in food intake were shown to result in a shift in the ratio between Firmicutes and Bacteroidetes, with increased feeding favoring Firmicute abundance and lack of feeding tipping the balance toward increased Bacteroides loads (9,18). In this study, the administration of TPN, a situation in which the enteral lumen remains deprived of food whereas cells are systemically supplied with nutrients, was shown to result in a significant decrease in Firmicutes:Bacteroidetes ratio. Hence, the changes in microbiota composition in starvation and TPN, conditions similar in terms of luminal dietary content, correspond. Interestingly, this change in microbiota is in contrast with the shift observed in patients with obesity (9), which carries an excess of luminal energy content. These findings show a close link between luminal exposure to nutrients and the phylum that dominates the intestinal bacterial population. The underlying cause for the shift in microbiota composition in response to changes in diet remains as yet unidentified. In this context, it is interesting to note that endogenous carbohydrate composition influences the gut microbial community by selecting organisms capable of consuming these glycans (36). Consequently, in TPN feeding, the absence of luminal feeding might favor survival of bacteria able to use mucus as a glycan source. Bacteroidetes are shown to rely on host glycans in the absence of dietary input (36), possibly explaining the increased prevalence of this phylum in our TPN-fed rats. In accordance with our findings, Deplancke et al. (37) showed positive selection for mucolytic bacteria in the intestine

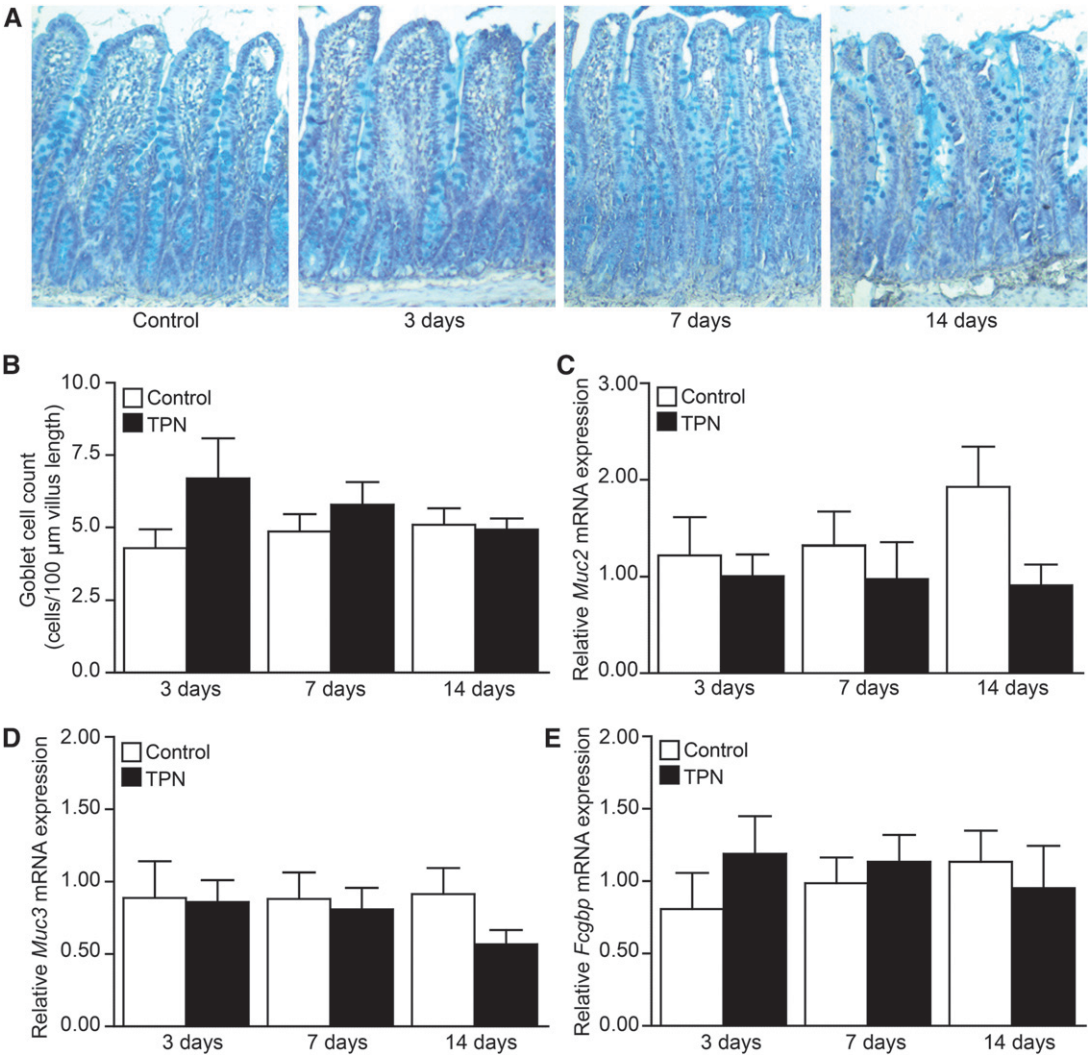

FIGURE 5 Alcian blue staining (*A*), histologic quantification of the number of goblet cells (*B*), and gene expression levels of *Muc2* (*C*), *Muc3* (*D*), and *Fcgbp* (*E*) in the small intestine of control and TPN-fed rats over 14 d. (*B–E*) Values are means ± SEM, $n = 6–8$. TPN, total parenteral nutrition.

of TPN-fed piglets. Although in this study, analysis of microbiota composition was focused on the phylum level, it would be interesting to specify the analysis to a lower level in the future.

The functional relevance of a shift in certain microbial species is as yet unclear because not all species belonging to these phyla and their corresponding metabolic activities have been identified (38). In addition, results on changes in microbiota composition do not necessarily provide information on the metabolic activity of these bacteria. Although the consequence of a decrease in Firmicutes:Bacteroidetes ratio in TPN-fed rats remains a subject for further study, we here postulated that excessive consumption of mucus by adapting bacteria may result in an impaired mucous layer and as a result in a more intimate interaction between microbiota and host enterocytes (39). Consequently, an increased adhesion of bacteria to the intestinal epithelial cells increases the pressure of bacteria invading host tissue. Even though goblet cells also are important mediators in intestinal immunity and function in close association with the intestinal microbiota (40), changes in the latter did not seem to influence goblet cell function.

Several studies have shown that changes in the intestinal bacterial population influence host factors (4–7,41). The current study further extends evidence for this interaction because our results show that the shift in intestinal microbiota composition observed in our TPN-fed rats coincides with changes in Paneth cell antimicrobial protein expression. Our group suggested that a microbial shift as seen in obesity favoring Firmicute abundance would be associated with decreased Paneth cell antimicrobial protein expression (13). Here, we report that a decrease in Firmicutes:Bacteroidetes ratio in TPN administration coincided with increased expression of Paneth cell antimicrobial proteins. The inverse correlation between lysozyme expression and Firmicute abundance show that Paneth cells do not only respond to the extent of intestinal microbial colonization as reported (1,41) but are also under influence of microbiota composition. Conversely, this interplay has also been shown to operate in the other direction. Salzman et al. (34) showed that a deficiency in functionally active α-defensins or overexpression of α-defensins in mice results in a shift in microbiota composition. To recapitulate, microbiota composition and Paneth cells seem to be closely associated, both mutually affecting each other.

Which factor changes first in TPN administration remains a subject for further study. However, we consider that a shift in microbiota, as an adaptive response to the change in nutrients present in the gut lumen, to be prior to altered Paneth cell antimicrobial protein expression because microbiota are capable of rapidly adapting to the changed dietary environment (37,39).

Remarkably, although we and others have shown a shifted Firmicutes:Bacteroidetes ratio in favor of Bacteroidetes in both

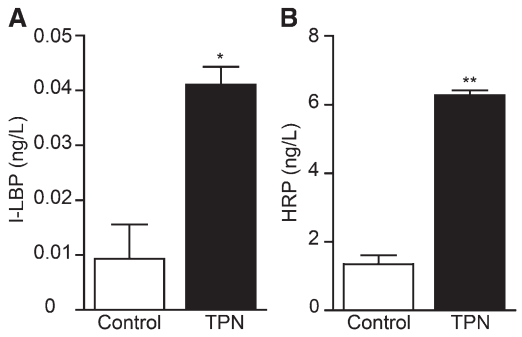

FIGURE 6 Systemic concentrations of I-LBP (*A*) and HRP leakage (*B*) in control and TPN-fed rats after 14 d. Values are means ± SEM, n = 6–8. Asterisks indicate different from control: $*P < 0.05$, $**P < 0.01$. I-LBP, ileal lipid-binding protein; TPN, total parenteral nutrition.

starved (18) and TPN-fed animals (this study), Paneth cell antimicrobial protein expression differs with decreased expression in starvation and elevated expression in TPN-fed animals. We postulate these differences to be attributable to the inability of starved animals to respond to changes in microbiota due to lack of nutrients and the consequent compromise of Paneth cells (16), which is proven for immune activation in general (42), whereas TPN-fed animals retain this ability.

Because both microbiota and Paneth cells play important roles in the maintenance of intestinal homeostasis, the observed changes in these players in this study can have severe consequences for intestinal barrier integrity, which we showed to be disturbed as assessed by increased circulatory concentrations of I-LBP and augmented leakage of HRP through the intestinal wall.

In conclusion, our findings show a shift in intestinal microbiota in TPN-fed animals to correlate with changes in Paneth cell antimicrobial protein expression. Both factors are crucial in the maintenance of intestinal homeostasis. Moreover, the changes described in this study were associated with disturbances in intestinal barrier integrity. Future studies on interventions with microbiota, and nutrients that modulate the microbiota, may significantly improve knowledge on involvement of microbiota and Paneth cells in TPN-associated intestinal barrier compromise.

Acknowledgments

C.M.H., R.G.J.V., S.W.M.O.D., K.L., and W.A.B. designed the research; C.M.H., R.G.J.V., B.B., and K.L. conducted the research; C.M.H., R.G.J.V., S.S.R., B.B., S.W.M.O.D., K.L., and W.A.B. analyzed data and/or performed statistical analysis; and C.M.H. wrote the manuscript and had primary responsibility for final content. All authors read and approved the final manuscript.

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