Qualitative and Quantitative Comparison of Gut Bacterial Colonization in Enterally and Parenterally Fed Neonatal Pigs

Roger B. Harvey[1]*, Kathleen Andrews[1], Robert E. Droleskey[1], Ketan V. Kansagra[2], Barbara Stoll[3], Douglas G. Burrin[3], Cynthia L. Sheffield[1], Robin C. Anderson[1], and David J. Nisbet[1]

[1]Food and Feed Safety Research Unit, Southern Plains Agricultural Research Center, USDA-ARS, 2881 F&B Road, College Station, TX 77845, USA
[2]St. Peter's University Hospital, Division of Neonatology, New Brunswick, NJ 08901, USA
[3]USDA-ARS-Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA

Abstract

Total parenteral nutrition (TPN) has been associated with mucosal atrophy, impaired gut barrier function, and translocation of luminal bacteria with resultant sepsis in preterm human infants. Currently, we examined the effects of enteral (ENT) or TPN treatments on translocation events in neonatal pigs and on colonization and composition of microbiota in the neonatal gut. Newborn, colostrum-deprived pigs (<24 hours old) were fitted with intravenous catheters and were fed either ENT (n = 13) or TPN (n = 13) for 7 days. After 7 days of treatment, pigs were euthanized and samples were collected for bacterial culture from the blood, intestinal tract and organs. ENT pigs had increased numbers of bacterial genera isolated, higher concentrations of bacteria (CFU/g), and increased colonization of all segments of the intestinal tract compared to the TPN pigs. Translocation of bacteria from the intestinal tract to tissues or blood was similar (8 of 13) for both groups. The ENT group had 1/13 positive for *Clostridium difficile* toxin A whereas the TPN group had 5/13. We concluded that ENT favored increased bacterial concentrations comprised of more speciation in the gastrointestinal tract compared to TPN, and that TPN-treated piglets were at higher risk of colonization by toxin-expressing strains of *C. difficile*.

Introduction

Total parenteral nutrition (TPN) in preterm human infants has been associated with increased incidence of sepsis and it has been suggested that sepsis may occur due to translocation of gut luminal bacteria (Alverdy *et al*., 1988; Berg, 1995; Lemons *et al*., 2001; Pierro *et al*., 1996). Studies in neonatal animals and human infants show that TPN induces gut mucosal atrophy and impaired gut barrier function with increased gut permeability in the neonatal pig (Burrin *et al*., 2000; Rossi *et al*., 1993; Kansagra *et al*., 2003). In an earlier study, Kansagra *et al*., (2003) reported that while TPN increased gut permeability when compared to enteral (ENT) feeding, no differences were observed

*For correspondence: harvey@ffsru.tamu.edu

between treatment groups for bacterial translocation from the intestinal tract to blood or organs. In another study, TPN was shown to favor the selective growth of mucolytic bacteria, including *Clostridium perfringens* (Deplancke *et al*., 2002). *C. perfringens* and *C. difficile* concentrations were shown to be increased in neonatal necrotizing enterocolitis, a gastrointestinal disease of premature infants (Butel *et al*., 2002). The etiology of necrotizing enterocolitis is multi-factorial, but has been associated with prior TPN administration, antibiotic usage, aggressive enteral feeding, and reduced bacterial colonization of the neonatal gut (Butel *et al*., 2002). We hypothesized that TPN would negatively impact the early colonization of the neonatal gut by commensal bacteria, thus predisposing the neonate to colonization by pathogenic bacteria. In the present study, we sought to identify the composition of microbiota in the neonatal pig gut, their relationship to translocation events, and to examine the effects of TPN versus enteral (ENT) feeding on early gut colonization.

Results and discussion

Bacterial translocation from the intestinal tract to tissues or blood was not different on the basis of treatment and was reported earlier: each treatment group had 8 of 13 pigs with translocation events (Table 1) (Kansagra *et al*., 2003). However, in that study, the authors did not elaborate on the population characteristics of translocation bacteria. In this study, the number of bacterial genera and the number of translocation sites were greater for the ENT group compared to the TPN (Table 1). Except for one *Clostridium*, all of the TPN translocation isolates were *Enterococcus* species and the TPN group had only one translocation genus per pig (Table 1). By their nature, these translocation events induced a bacteremia with bacteria in blood and tissues, yet we did not observe clinical signs of septicemia in piglets. We attributed this lack of an inflammatory response to immune tolerance to commensal microorganisms of the gut. Immune tolerance has been described as an aspect of the undeveloped immune system of neonates (Toms and Powrie, 2002).

Bacterial concentration, number of bacterial species isolated, and the number of intestinal segments colonized were greater in the ENT group when compared to the TPN-treated pigs (Table 2). Bacterial concentrations, number of bacterial species, and frequency of isolation progressively increased from the J to the C. These results are similar to others in which a more diverse bacterial population was observed in the C of the ENT gut compared to the TPN gut (Deplancke *et al*., 2002). The majority of enteric bacterial isolates from the ENT group were comprised of *Enterococcus, Pediococcus, Enterobacter, Staphylococcus, Klebsiella,* and *Clostridium* from the J, I, and C. Most of the TPN isolations were *Clostridium* and *Enterococcus* from the C. By far, the most commonly isolated bacterial genus from both treatment
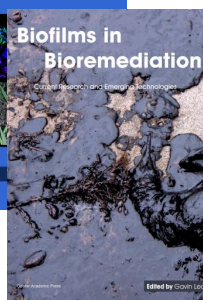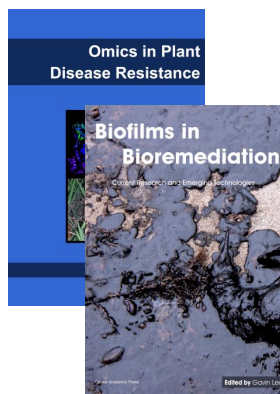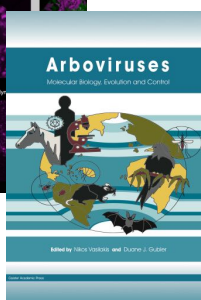
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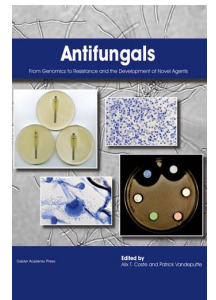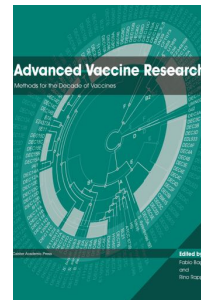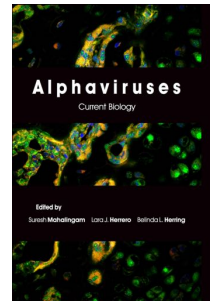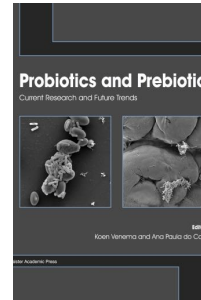
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Table 1. Genera of bacteria translocating from the intestinal tract to organs or tissues of Ent vs. TPN-treated neonatal pigs.

Treatment group	Pig no.	Bacterial genera	Translocation site
ENT (*n* = 13)	1	*Enterobacter, Enterococcus, Pediococcus, Staphylococcus*	Liver, spleen
	2	*Enterococcus, Klebsiella*	Blood, liver, lymph node, spleen
	3	*Klebsiella*	Blood, liver, lymph node, spleen
	4	*Klebsiella*	Blood
	5	*Enterococcus, Staphylococcus*	Blood, liver, lymph node, spleen
	6	*Enterococcus, Klebsiella*	Blood, liver, lymph node
	7	*Enterococcus*	Blood, lymph node
	8	*Enterococcus, Klebsiella*	Blood, lymph node
TPN (*n* = 13)	1	*Clostridium*	Spleen
	2	*Enterococcus*	Spleen
	3	*Enterococcus*	Blood, liver
	4	*Enterococcus*	Blood
	5	*Enterococcus*	Blood
	6	*Enterococcus*	Blood
	7	*Enterococcus*	Blood
	8	*Enterococcus*	Blood
ENT, enterally fed; TPN, total parenteral nutrition.			

groups in our study was *Enterococcus*, and the authors concluded that this group of bacteria is an early colonizer of the gastrointestinal tract. Due to the frequency and prevalence of *Enterococcus*, we used this genus as an example to demonstrate the differences in colonization between ENT and TPN-treated pigs and we quantified the concentrations of *Enterococcus* in various segments of the intestinal tract (Fig. 1). On average, the ENT group had approximately 2 to 3 higher $\log_{10}$ colony-forming units/g of *Enterococcus* than the TPN group, particularly in the J and I (Fig. 1). Oftentimes there were no isolations of *Enterococcus* in the J or I of TPN pigs (as may be inferred from the data on Table 2). Colonization rates and concentrations of other bacterial genera from ENT and TPN pigs generally followed the same patterns as those seen with *Enterococcus* (data not shown).

The ENT group of our study had 4/13 positive for *C. perfringens* and 1/13 positive for *C. difficile* toxin A production (Table 3). There were multiple clostridial isolations in the ENT group; in fact, 35 different isolates were differentiated by ribotype analysis as being *Clostridium* spp. The TPN pigs had 0/13 positive for *C. perfringens*, 5/13 isolates positive for *C. difficile* toxin A ($P < 0.1$), and a total of 16 different *Clostridium* recovered (Table 3). On this basis, it would appear that ENT treatment favors increased clostridial colonization when compared to TPN treatments. However, we are of the opinion that increased clostridial isolations were merely a

reflection of increased bacterial concentrations (including numerous commensal *Clostridium* spp.) in the gut of ENT pigs. The results of our study are somewhat at odds with that of Deplancke *et al.* (2002) in which TPN favored the growth of *C. perfringens* over that associated with ENT. However, some of the differences between our study and that one could be attributed to dissimilar methods of sample collection and processing. Furthermore, in that study (Deplancke *et al.*, 2002) the authors only tested for *C. perfringens* and not other *Clostridium*. In the present study, TPN treatment allowed the growth of toxin-expressing strains of *C. difficile*. *C. difficile* has been associated with pseudomembranous colitis and sepsis as a sequel to TPN and/or high dosages of antibiotics in hospitalized patients (Knoop *et al.*, 1993; Larson *et al.*, 1978). *C. difficile* is an opportunistic organism that is able to grow in the absence of endogenous microbiota. Individual bacteria, mixtures of commensals, or yeasts have been shown to decrease the presence of, and the mortality associated with, *C. difficile* in mice, hamster, hares, and humans (Dubos *et al.*, 1984; Wilson *et al.*, 1981; Wilson and Freter, 1986). In our study, the authors hypothesize that *C. difficile* was able to colonize TPN pigs due to the reduced presence of commensals in the gastrointestinal tract of TPN pigs. In the case of ENT pigs, the presence of nutrients in the gastrointestinal tract encouraged a more diverse microbiota with a corresponding decrease in *C. difficile* colonization. Similarly, when the autochthonous

Table 2. Colonization of segments (qualitative recovery) of the intestinal tract by bacteria in ENT vs. TPN treated neonatal pigs.

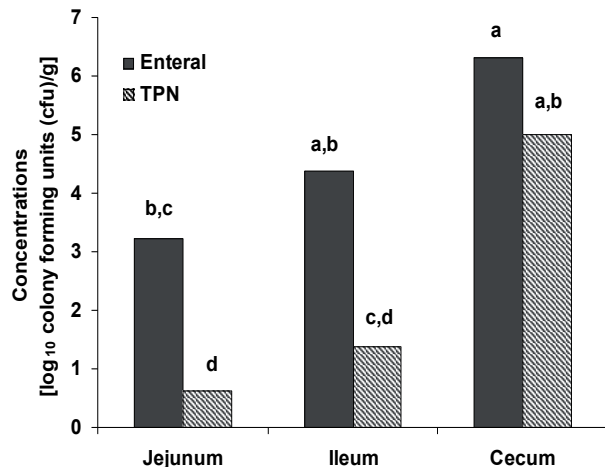| Pigs colonized (number of bacterial genera)			
Treatment	Jejunum	Ileum	Cecum
ENT (*n* = 13)	11 (6)	13 (7)	13 (7)
TPN (*n* = 13)	2 (1)*	5 (3)**	13 (4)
Ent = enterally fed; TPN = total parenteral nutrition. *P = 0.0012; **P = 0.0016 by Fisher's Exact Test.			

Fig. 1. Concentrations [$\log_{10}$ colony-forming units (cfu)/g] of *Enterococcus* in the jejunum, ileum and cecum of ENT vs. TPN-treated neonatal pigs. [a–d]Columns without common superscripts are significantly different based on Tukey's means comparison test ($P< 0.05$, SEM = 0.51).

microflora is absent, increased *C. perfringens* and *C. difficile* have been observed with cases of necrotizing enterocolitis of preterm infants (Butel *et al*., 2002).

We acknowledge the bias of using cultivation techniques to identify and enumerate the presence of gut bacteria; however, under the conditions of our study and on the basis of our results, we concluded that TPN was not positively associated with translocation, that translocating bacteria were a reflection of the concentrations of those bacteria in the gastrointestinal tract, that ENT favored increased bacterial concentrations comprised of a more diverse speciation in the gastrointestinal tract, that TPN retarded the colonization of the intestinal tract, and that TPN-treated neonates were at a higher risk of colonization by toxin-expressing strains of *C. difficile*.

Experimental procedures

Animals and study design
The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals [DHHS publication no. (NIH) 85–23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. For the study, pregnant, crossbred sows (Large White x Hampshire x Duroc) were obtained from the Texas Department of Criminal Justice (Huntsville, Texas) approximately one week prior to estimated date of farrowing. They were housed, fed, and kept under surveillance in the animal facility at the Children's Nutrition Research Center

(Houston, TX) until delivery. The sows delivered piglets vaginally. The newborn piglets were kept in heated cages and fed only water until surgery.

Newborn, colostrums-deprived pigs (<24 hours old) were fitted with intravenous catheters inserted into the jugular vein and divided into two groups. One group (*n* = 13) daily received 240 ml/kg per day TPN (25 g/kg glucose, 13 g/kg amino acids, and 5 g/kg lipid) through intravenous feeding. The second group (*n* = 13) was enterally fed (ENT) 240 ml/kg per day an antibiotic-free, commercial pig milk replacer (Advance Liqui-Wean, Milk Specialties, Dundee, IL); the nutrient intake did not differ between groups. After 7 days of treatment, pigs were euthanized and jejunum (J), ileum (I), cecum (C), liver, spleen, mesenteric lymph node, and blood were evaluated using restrictive media and serial dilutions to determine the presence and concentrations of enteric bacteria in tissues, blood, and intestinal tract. A translocation event was described as having the same bacterial species present in the blood or organs and the intestinal tract of the same pig.

Microbiological culture techniques
Cultivation of bacteria has been described (Kansagra *et al*., 2003). Blood was obtained by cardiac puncture, organ tissues were collected aseptically at necropsy, and sterile cotton swabs were used to scrape the mucosal surface of intestinal segments. Samples were collected and transported to our laboratory by use of anaerobic transport medium (Anaerobe Systems, Morgan Hill, CA). Tissue samples were macerated and diluted 1:1 with PBS before plating. Gut contents were diluted from log 10^{-2} to log 10^{-5} with PBS before plating, whereas blood was streaked directly onto plates. Both anaerobic and aerobic culture methods were employed. Restrictive media used for aerobic isolations included Rogosa SL Agar for lactobacilli; MacConkey agar for *Escherichia coli*, *Klebsiella*, and *Enterobacter*; enterococcusME agar for *Enterococcus;* and mannitol salt agar for *Staphylococcus* (all media from Difco, Detroit, MI). Plates were incubated at 37°C for 48 hours under aerobic conditions. For anaerobic isolations, Anaerobic Brucella blood agar was used for *Clostridium*, *Bacterioides* Bile esculin agar was used for *Bacterioides*, and cycloserine-cefoxitin fructose agar was used for *Clostridium difficile* (all media from Anaerobe Systems). Plates were incubated under anaerobic conditions for ≤4 days at 37°C.

Bacterial identification was accomplished by observing growth characteristics on restrictive media, by Gram stain and light microscopy examination, and by use of biochemical tests (API Test Strips, bioMerieux, Hazelwood, MO). Suspected *C. perfringens* and *C. difficile* isolates were confirmed by colony morphology, by

Treatment	Site of Isolation			Pigs positive	*C. difficile* (site)	No. of different ribotypes
	J	I	C			
ENT = 13	0	6	13	13	1(C)	35
TPN = 13	5	4	9	11	5 (J, I, C)*	16

Table 3. Isolation of *Clostridium* spp. From the gut of ENT vs. TPN piglets.

J = jejunum, I = ilium, C = cecum.
*Different (P < 0.1) by Fisher's Exact Test.

the ethanol spore test (Allen *et al.*, 1999), and by API Test Strips. Isolates that were identified as *C. difficile* were further tested for the presence of *C. difficile* toxin A by use of an enzyme immunoassay (Post *et al.*, 2002). Clostridial isolates (other than *C. perfringens* or *C. difficile*) that could not be identified as to species were subjected to ribotype analysis.

Ribotype analysis

The RiboPrinter® microbial characterization system (Qualicon, Inc.; Wilmington, DE) characterizes the 5s, 16s, and 23s RNA and flanking regions of a bacterial sample using specified restriction enzymes. The resulting ribosomal RNA pattern (RiboPrint® pattern) is then compared to existing reference patterns in the RiboPrinter database for possible identification. Characterization (inclusion in a ribogroup) requires that the ribopattern be a 90% (similarity number of 0.90) or greater match to an existing ribopattern. Identification requires that the ribopattern be an 85% (similarity number of 0.85) or greater match to an existing static ribopattern located in the standard or the custom libraries to be labeled as that specific bacterium. *Clostridium* isolates were prepared for ribotyping according to the manufacturer's recommendations (Qualicon). Duplicate ribotypes within the same pig and the same treatment groups were eliminated when comparing the number of different isolates between treatment groups.

Statistical analysis

Proportions of animals colonized within each segment (qualitative recovery) of the intestinal tract (Tables 2 and 3) were analyzed for treatment effects using a Fisher's Exact Test (Statistix® 8 for Windows, Analytical Software, Tallahassee, FL). Mean $\log_{10}$ concentrations of *Enterococcus* (Fig. 1) were analyzed for differences using a general analysis of variance and a Tukey's separation of means (Statistix® 8 Analytical Software).

Acknowledgments

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