Impact of dietary induced precocious gut maturation on cecal microbiota and its relation to the blood-brain barrier during the postnatal period in rats

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Funding information
Direktör Albert Påhlsson Foundation

Abstract

Background: Precocious maturation of the gastrointestinal barrier (GIB) in newborn mammals can be induced by dietary provocation, but how this affects the gut microbiota and the gut-brain axis remains unknown. The objective of this study was to investigate effects of induced GIB maturation on gut microbiota composition and blood-brain barrier (BBB) permeability.

Methods: Suckling rats were studied at 72 h after gavage with phytohemagglutinin (PHA) or microbial protease (PT) to induce maturation of GIB. For comparison, untreated suckling and weaned rats were included (n = 10). Human serum albumin (HSA) was administered orally and analyzed in blood to assess permeability of the GIB, while intraperitoneally injected bovine serum albumin (BSA) was measured in the brain tissue for BBB permeability. The cecal microbial composition, plasma lipopolysaccharide-binding protein (LBP) levels and short-chain fatty acids in serum and brain were analyzed.

Key Results: Cessation of HSA passage to blood after PHA or PT treatment was similar to that seen in weaned rats. Interestingly, concomitant increases in cecal Bacteroidetes and plasma LBP levels were observed after both PHA and PT treatments. The BBB passage of BSA was surprisingly elevated after weaning, coinciding with lower plasma LBP levels and specific microbial taxa and increased acetate uptake into the brain.

Conclusions & Inferences: This study provides evidence that the gut microbiota alteration following induced precocious GIB maturation may induce low-grade systemic inflammation and alter SCFAs utilization in the brain which may also play a potential role in GIB-BBB dysfunction disorders in neonates.

KEYWORDS
blood-brain barrier, dietary factors, gastrointestinal barrier permeability, gut microbiota, inflammation, short-chain fatty acids

Abbreviations: GIB, gastrointestinal barrier; BBB, blood-brain barrier; PHA, phytohemagglutinin; PT, microbial protease; HSA, human serum albumin; BSA, bovine serum albumin; LPS, lipopolysaccharides; LBP, lipopolysaccharide-binding protein; SCFAs, short-chain fatty acids; CNS, central nervous system; BW, body weight; OTUs, operational taxonomic units; PLS, Partial least squares analysis; LDA, Linear discriminant analysis; LEfSe, LDA effect size analysis; ANOSIM, Analysis of similarities; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; PCoA, Principal Coordinates analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.
INTRODUCTION

Mammals are born with an immature gastrointestinal barrier (GIB) function, with relatively high permeability to macromolecules. The timing of GIB maturation varies among species. In rats, this occurs at weaning around the age of 21 days, while it has been suggested to be completed within 20 days after birth in humans. The process of GIB maturation can also be influenced by gastric intrinsic factors, hormones, growth factors, as well as early colonizing gut microbiota.

Several studies have found associations between an imbalanced gut microbial community, GIB dysfunction, and many metabolic disorders such as diabetes and obesity, or even brain-related disorders, such as autism, and Parkinson's and Alzheimer's disease. Leakage of lipopolysaccharides (LPS), bacterial endotoxin derived from Gram-negative bacteria, from the gut is usually recognized as a key event triggering low-grade inflammation. LPS-binding protein (LBP) is increasingly being used as a relevant marker for metabolic endotoxemia. Meanwhile, the gut microbial metabolites, short-chain fatty acids (SCFAs), have been suggested to affect the host beneficially. Specifically, butyric acid serves as an important substrate for the colonic epithelium and helps to maintain GIB integrity. Moreover, acetic acid readily crosses the blood-brain barrier (BBB) and is used as an alternative energy source for the brain in form of acetyl-CoA. Although it is well documented that the BBB is well-established from early life, systemic inflammation may lead to changes in many elements of brain development. The emerging links between the gut microbiota and the central nervous system (CNS) suggest that its influence on host barrier and its metabolites are important nodes within the gut-brain axis. Hence, it is important that the gut microbial community is properly established from early life.

It is known that GIB maturation can be induced by dietary components, or dietary provocative agents, such as phytohemagglutinin (PHA), a lectin from red kidney beans, and microbial protease (PT), used in dairy and other types of foods. Previous studies have focused on the effects of precocious GIB maturation in physiological and immunological aspects, and yet the effects on the gut microbial community have been overlooked. PHA has been known as a T-cell mitogen and a previous study showed that orally administered PHA induced GIB maturation, stimulating increase in mucosal CD19+ and CD3+ cells, which in fact is the same immune activation observed in weaned rats at 21-28 days with natural GIB maturation. Investigation of the change in the gut microbiota following the administration of dietary provocative agents, as opposed to natural GIB maturation, will provide a better understanding whether the immune activation (in induced GIB maturation) might be triggered by the altered gut microbiota toward a more adult-like community.

This study was designed to elucidate how exposure to PHA and PT, as dietary provocative agents of precocious GIB maturation, may affect the gut microbiota and the implication of bacterial changes in the permeability of the BBB. The study was performed in neonatal rats, since they are born with a highly permeable GIB to macromolecules. Albumins were used as macromolecular markers for measuring GIB and BBB permeability. Changes in the gut microbiota, LBP, and SCFA proportions in suckling rats that were gavaged with PHA or PT were investigated at the age of 17 days. Untreated rats at 14, 17, and 28 day old were also included to follow the changes in normal conditions. Understanding these events will help to determine the potential effects of early exposure to dietary components which may contain such gut provocative agents and contribute to improving preventative strategies toward GIB dysfunctions and brain-related disorders later in life.

MATERIALS AND METHODS

Animals and study design

The Malmö-Lund's Ethical Committee on Animal Experiments approved the study according to the European Parliament and Council Directive (2010/63/EU) and the Swedish Animal Welfare Act (SFS 1988:539) (Permit Number: M169-14). Sprague-Dawley rats (Taconic, Denmark) of both genders were studied using a split-litter design. pups remained with their dam throughout the study. To ensure that the pups consumed only maternal milk, wall extenders were used to prevent access to solid chow. At the age of 14 days, rats from 3 litters were orally treated by gavage with PHA (0.1 mg/g body weight [BW]; n = 10) or PT (0.6 mg/g BW; n = 10) to induce GIB closure, while control littersmates were fed water (n = 10). The dissection was performed at 17 days of age, i.e., 3 days after the treatment. Two untreated additional groups were included in the study with dissection at 14 days (n = 10) and 28 days of age (n = 10), in order to assess the conditions before the treatments and after weaning. From day 21, the rats in the last group were separated from their dams (weaned) and had free access to water and standard rodent chow (RM1, SDS) until the day of dissection. On the dissection day, the rats were separated from the dam one hour prior to gavage of human serum albumin (HSA, 1.25 mg/g BW) as a marker for GIB permeability and intraperitoneal injection of bovine serum albumin (BSA, 0.5 mg/g BW) as a marker for BBB permeability. Three hours after marker administration, the rats were anesthetized with Isoflurane (Abbott, Chicago, USA) and blood...
samples were taken from the heart before the rats were terminated via decapitation. Brain samples were immediately collected for assessing the quantity of BSA that had passed from the bloodstream to the brain tissue, as well as for SCFA analysis. Finally, the cecum was collected for gut microbiota and SCFA analyses. A schematic illustration of the study design is shown in Figure S1.

### 2.2 Plasma HSA

Plasma HSA levels were quantitated by electroimmunoassay using rabbit anti-HSA (DAKO A/S, Denmark) as the precipitating antibody. Purified HSA was used as the standard and sample concentrations were interpolated.

### 2.3 Brain BSA

Brains were homogenized in physiological saline 1:2 (w/v) and the supernatant was analyzed by electroimmunoassay for quantitation of BSA using rabbit anti-BSA (Sigma-Aldrich, MO, USA) as the precipitating antibody. Purified BSA was used as the standard to estimate sample concentration. Rat’s IgG in the brain was also analyzed by single radial immunodiffusion using rabbit anti-rat-IgG (DAKO A/S, Denmark) as the precipitating antibody, while purified rat IgG (Miles Laboratories Inc., Pennsylvania, USA) was used as the standard. The ratio of BSA (BBB permeability marker) to IgG (endogenous blood marker) in the brain was calculated in order to normalize for the amount of blood from the vessels that may have contaminated the brain samples.

### 2.4 Plasma LBP

Lipopolysaccharide-binding protein (LBP) enzyme-linked immunosorbent assay (ELISA) kit for a wide variety of species (Hycult Biotech, Uden, The Netherlands) was used to quantitate LBP concentration in plasma of the rats. The plasma samples were diluted 1:10 in dilution buffer and the procedures were performed following the protocol from the manufacturer.

### 2.5 Cecal microbiota analysis

Cecal DNA was extracted from 100 to 150 mg of rat’s cecum, both tissue and content, using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The protocol from the manufacturer was followed with an addition of bead beating step using sterile glass beads (1 mm). The V3 and V4 region of 16S rRNA genes were amplified using forward and reverse primers containing Illumina overhang adaptor (Table 1) and unique dual indices, according to 16S sequencing library preparation protocol provided by Illumina. Paired-end sequencing with a read length of 2 × 300 bp using MiSeq V3 reagent kit was carried out on a MiSeq Instrument (Illumina Inc., San Diego, CA, USA). Sequencing data were analyzed using the open-source bioinformatics pipeline Quantitative Insights into Microbial Ecology (QIIME). The sequences were grouped into operational taxonomic units (OTUs) by UCLUST at a minimum of 97% sequence similarity. Representative sequences (most abundant) from each OTU were aligned using Python Nearest Alignment Space Termination (PyNAST). Taxonomy was assigned using Greengenes database (v.13_8). The procedure details are described in Data S1.

### 2.6 SCFA analysis

A maximum weight of 400 mg of frozen cecum was used for the analyses. The protocol from Zhao et al., was followed with some modifications due to limited amounts of cecal samples. A detailed protocol is described in Data S1.

A volume of 240 μL of rats plasma was used following the procedures described by Zhao et al., and with an optimization by Jakobsdottir et al., for analyzing SCFAs in rats plasma. The procedures details are described in Data S1. The same protocol for measuring SCFAs in plasma was applied for analyzing brain samples, 240 μL of rats’ brain homogenates were used in the analysis.

### 2.7 16S metagenomes prediction using PICRUSt

The 16S rRNA gene sequences were used to predict bacterial gene functions using the open-source software, PICRUSt. The OTU table, generated in QIIME from the 16S sequencing data at 6,780 rarefied sequences per sample, was used as an input. The copy number per OTU was normalized before the metagenome was predicted using Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

### 2.8 Statistical analyses

Significant differences in plasma HSA, brain BSA, plasma LBP levels, SCFAs and relative abundance of bacteria at each taxonomic level in rats treated with PHA and PT were evaluated by one-way ANOVA and subsequent Holm-Sidak’s multiple comparisons test to compare results with age-matched control group, while rats at 14 and 28 days were compared with control rats (17 day old) in a separate set of statistical analysis, using GraphPad Prism version 6 for windows (GraphPad Software, Inc., CA, USA, www.graphpad.com). In addition, alpha (α-) and beta (β-) diversities were analyzed after rarefying the OTU table at 6,780 randomly selected sequences per sample for the entire data

### TABLE 1 Sequence of the 16S amplicon primers with Illumina overhang adaptors

| 16S amplicon PCR Forward Primer with Illumina overhang adaptor (underlined) | 5′TCGTGCAGCGTCAAGACGCCTACGGCGGGGCGWGCAG |
| 16S amplicon PCR Reverse Primer with Illumina overhang adaptor (underlined) | 5′GTCTCGTGCCAGATGTATATAAGAGACAG |

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set. Correlations between plasma HSA, brain BSA, plasma LBP, SCFA levels and gut bacterial genera were analyzed with SIMCA-14 software (Umetrics, Umeå, Sweden). A partial least squares (PLS) plot was used to illustrate correlations between different biomarkers and the gut microbiota. Moreover, linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed to identify differentially predicted metagenomes in rats treated with PHA and PT as compared to age-matched controls. Predicted genes with higher LDA score than a threshold of 3 were considered to be enriched in that particular group.

3 | RESULTS

3.1 | Gastrointestinal barrier maturation

The passage of HSA from the gut into blood plasma was high in the 14- and 17-day-old rats, while it was not detected in the weaned 28-day-old rats. PHA and PT treatments of the rats decreased plasma HSA levels to nearly zero and zero, respectively, as compared to their age-matched controls (17 day old, P < .0001) (Figure 1A).

3.2 | Blood-brain barrier maturation

Rats at the age of 14 days had higher level of BSA in the brain tissue than 17-day-old rats (P < .05). Rats treated with PHA and PT had similar BBB permeability as their age-matched control rats. Interestingly, 28-day-old rats had higher brain BSA levels compared to 17-day-old rats (P < .0001) (Figure 1B).

To estimate any possible influence of blood contamination in the BSA analysis, the ratio of BSA to IgG in the brain tissue was calculated (Figure S2). The IgG levels were consistent in all groups and comparison of the BSA to IgG ratio in the brain showed a similar pattern even in older rats, with bigger blood vessels. The combination of tests suggested that blood contamination in the brain should have no interference with the results on BBB permeability.

3.3 | Lipopolysaccharide-binding protein

Plasma LBP concentration was high in 14-day-old rats and slightly decreased as the rats aged where the difference was significant in 28-day-old rats (P < .05). PHA tended to increase (P = .09), while PT significantly increased plasma LBP in the rats as compared to their age-matched controls (P < .01) (Figure 1C).

3.4 | Cecal microbiota composition

Bacterial 16S rRNA gene sequencing results revealed shifts in both diversity and composition of the gut microbiota in the different groups of rats.

3.4.1 | Bacterial diversity indices

Estimation of α-diversity of bacterial 16S rRNA genes at the sequence number of 6,780 sequences per sample (Figure 2A) demonstrated that α-diversity increased with age (chao1, P < .01). However, PT treatment decreased α-diversity in the rats as compared to age-matched control rats (chao1, P < .01), while there was a tendency for decreased α-diversity after PHA treatment (chao1, P = .06). ANOSIM analyses of both unweighted (Figure 2B) and weighted (Figure 2C) UniFrac revealed a complete separation of 28-day-old rats (weaned) from the other groups (both P < .01).

3.4.2 | Gut microbiota composition

Gut microbiota composition at phylum and genus levels is shown in Figure 3A-C. The dominant gut microbiota at phylum level in rats at the age of 14 days were Proteobacteria, Bacteroidetes and Firmicutes and they constituted approximately 50%, 35%, and 16% of total bacteria, respectively (Figure 3A). As rats aged, there was a significant decrease in Proteobacteria (P < .0001 for both 17- and 28-day-old rats) and an increase in Firmicutes (P < .001 and P < .0001 in 17- and 28-day-old rats, respectively). Bacteroidetes was increased in 17-day-old rats (P < .0001) but went down to a similar level as in 14-day-old rats in 28-day-old rats, (Figure 3A). Moreover, the ratio of Firmicutes to Bacteroidetes was significantly increased in 28-day-old rats as compared to 14- and 17-day-old rats (P < .0001 and P < .001, respectively)(Figure 3B).

At genus level, an unclassified genus from Enterobacteriaceae was the main group of bacteria from the Proteobacteria phylum and was found to decrease in rats at 17 and 28 days of age (P < .0001). Among the members in the Firmicutes phylum, Oscillospira (P < .01 in 28-day-old rats), Ruminococcus (P < .01 and P < .0001 in 17- and 28-day-old rats, respectively) and an unclassified genus from Lachnospiraceae (P < .0001 in 28-day-old rats) were found to increase, while an unclassified family from Coriobacteriales (both P < .0001, data not shown) was decreased as rats aged. Moreover, Bacteroides (P < .0001 in 28-day-old rats), Parabacteroides (P < .0001 in 28-day-old rats), Prevotella (P < .01 in 28-day-old rats), and unclassified genera from Bacteroidaceae (P < .001 in 28-day-old rats), Rikenellaceae (P < .001 in 17-day-old rats) and S24-7 (P < .0001 in 28-day-old rats) were among the Bacteroidetes found increased as rats aged. All these bacterial genera were present with more than 5% relative abundance, except for the unclassified family from Coriobacteriales order (Figure 3C). The gut microbiota at genus level, including those presenting less than 5% relative abundance, are shown in Data S2.

When comparing gut microbiota composition in the cecum of rats treated with PHA and PT with age-matched control rats (17-day old) it was found that Proteobacteria was decreased when treated with PHA (P < .0001), and PT (P < .05). Firmicutes was also decreased in both PHA- and PT-treated rats (P < .001 and P < .0001, respectively). Interestingly, phylum Bacteroidetes was increased in both PHA- and PT-treated rats (P < .0001) and became the most dominant phylum with a span of more than 75% in both groups (Figure 3A).

At genus level, an unclassified genus from Enterobacteriaceae was the member from Proteobacteria that decreased in both PHA- and
PT-treated rats \( (P < .001 \text{ and } P < .01, \text{ respectively}) \) and consequently showing the same change as in 28-day-old rats with natural gut closure. Of the members in the Firmicutes phylum, Oscillospira \( (P < .05) \) and Ruminococcus \( (P < .001) \) were found to decrease in PT-treated rats, while an unclassified family from Coriobacteriales decreased in both PHA- and PT-treated rats \( (P < .0001, \text{ data not shown}) \). Bacteroides \( (P < .001 \text{ and } P < .0001 \text{ for PHA- and PT-treated rats, respectively}) \) and Parabacteroides \( (P < .0001) \) were the members in the Bacteroidetes phylum that increased in both PHA- and PT-treated rats. An increase in an unclassified genus of Bacteroidaceae was only significant in PHA-treated rats \( (P < .0001) \). Moreover, a significant decrease in an unclassified genus from Rikenellaceae was observed in PT-treated rats \( (P < .01) \) (Figure 3C).

### 3.5 SCFAs in cecum, plasma and brain

Acetic, propionic and butyric acids were the most frequent SCFAs detected in cecum, plasma and brain of the rats (Figure 4A-C, respectively). The concentration of acetic acid \( (1-2 \text{ mg/g in cecum, } 500-700 \mu\text{mol/L in plasma and } 250-350 \text{ mg/g in brain}) \) was higher than that of butyric acid \( (\text{up to } 1 \text{ mg/g in cecum, } 2-6 \mu\text{mol/L in plasma and } 0.6-0.8 \text{ mg/g in brain}) \), and propionic acid \( (0.1-0.6 \text{ mg/g in cecum, } 10-15 \mu\text{mol/L in plasma and about } 2 \text{ mg/g in brain}) \) at all three sites. Furthermore, the proportion of acetic acid increased to a higher extent than the other SCFAs when absorbed from the cecum to the portal blood and further into the brain. When comparing the different ages of the untreated rats, the cecal concentrations
of the specific SCFAs increased with age, and especially butyric acid increased when solid food was introduced. PHA and PT treatments did not affect the formation of SCFAs in the cecum, plasma, or the brain. The concentration of SCFAs in plasma was related to those formed in the cecum. In the brain, the levels of propionic and butyric acids were similar in all groups, while the levels of acetic acid was higher in 28-day-old rats as compared to the other groups ($P < .05$).

### 3.6 Associations between gut and BBB permeability, plasma LBP levels, SCFA proportions and the gut microbiota

A PLS plot of all investigated parameters (Figure 5A) revealed associations between a high plasma HSA level, i.e., an immature GIB (lower left component), and increases in *Plesiomonas*, *Escherichia*, *Morganella*, *Haemophilus*, *Aggregatibacter* and an unclassified genus from *Enterobacteriaceae* (Gram-negative bacteria from Proteobacteria), as well as *Streptococcus*, *Allobaculum*, *Enterococcus*, *Granulicatella*, rc4-4 and unclassified genera from *Lactobacillaceae*, *Clostridiaceae* and *Peptococcaceae* families and an unclassified family from *Lactobacillales* order (Gram-positive bacteria from Firmicutes). Cecal acetic, propionic, and butyric acids were located in the opposite component (upper right component), indicating negative correlation with the bacteria mentioned above, and therefore with GIB closure. Furthermore, plasma LBP was associated with increases in some Gram-negative bacteria from Bacteroidetes such as *Bacteroides* and *Parabacteroides* (upper left component), and a decrease in brain BSA, the BBB permeability, as well as decreases in *Ruminococcus*, *Oscillospira*, *Dehalobacterium*, unclassified genera from *Lachnospiraceae*, *Ruminococcaceae* and

**FIGURE 2** Bacterial diversity indices. Alpha rarefaction curves (chao1) are showing differences (A) within-community richness, and in (B) unweighted, and (C) weighted UniFrac PCoA plots for comparing phylogenetic distance matrices between the different groups of rats

**FIGURE 3** Distribution at phylum and genus levels of the gut microbiota. Data is presented as mean relative abundance of the gut microbial taxa in the cecum of rats at (A) phylum level, (B) Firmicutes-to-Bacteroidetes ratio and (C) genus level, only including those with a relative abundance of >5%
(A) Phylum level

(B) Ratio F/B

(C) Genus level
Clostridiales (Gram positive from Firmicutes) and brain acetic acid (lower right component) (Figure 5A).

Throughout the normal development from 14, to 17 and 28 days, the pattern of all the investigated parameters moved from the lower left component (high GIB permeability and slightly high BBB permeability) toward the right component (low GIB permeability and high BBB permeability) where 28-day-old rats are situated. Interestingly, PHA- and PT-treated rats also moved toward the upper left component (low GIB and BBB permeability). A clear separation of the 28-day-old rats, having access to solid food, from the suckling rats was also observed (Figure 5B).

### 3.7 Predicted bacterial gene functions using PICRUSt

A total of 15 and 11 genes were enriched in the PHA- and PT-treated group, respectively, (LDA ≥ 3) as compared to 17-day-old control (Figure 6A, B). Interestingly, these genes are mostly involved in
pathways for carbohydrate and energy metabolism as well as for glycogen biosynthesis and metabolism.

4 | DISCUSSION

Dietary components, like PHA and PT, have been shown to induce precocious maturation of the GIB in young animals\cite{24,28,47} and have been used to supplement feed in young mammals to improve gut health and performance at weaning, for example, in pig production.\cite{29,30} Since all previous studies have been focused on the gastrointestinal effects and immunity in precociously induced maturation of the GIB, their effects on the gut microbiota and the implication of bacterial induced changes in the BBB had not been investigated. This study revealed that a single gavage of PHA or PT was enough to induce GIB maturation and precocious closure in suckling rats, causing drastic changes in the gut microbiota. The gut microbial community changed due to the treatments toward a more imbalanced microbial composition that appeared to be associated with an increased low-grade systemic inflammation, which was different from the community observed in rats with natural GIB development. Furthermore, the BBB permeability seemed to be functional already after 14 days post-natally but was possibly affected by changes in LBP and SCFA levels.

4.1 | PHA and PT altered GIB maturation and modified the colonizing gut microbiota

Proteobacteria was found to be the most dominant phylum in the young rats and its abundance decreased as they aged from 14 to 28 days (normal GIB development). In the 14-day-old rats, half of the

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**FIGURE 5** Partial least squares (PLS) loading and score plots of associations between gut and blood-brain barrier (BBB) permeability, plasma lipopolysaccharide-binding protein (LBP), short-chain fatty acid (SCFA) profile and the gut microbiota in the rats. (A) Loading plot showing associations between gastrointestinal barrier (GIB; lower left component) and BBB (lower right component), plasma LBP (upper left component), cecal (c-), plasma (p-), and brain (b-) SCFAs (right component) and the gut microbiota. (B) Score PLS plot shows how each rat behaves corresponding to the biomarkers shown in loading plot. In (A), bacterial names are colored by the phylum they belong to, the sizes of the circles are relative to the mean relative abundance of each bacteria and biomarkers (plasma human serum albumin (HSA), brain BSA, plasma LBP, and SCFAs in cecum, plasma, and brain) are shown in grey stars. In (B), each triangle represents each rat and is colored by the group they belong to. A clear separation between suckling and weaned rats is seen.
The gut microbial population consisted of bacteria in Enterobacteriaceae family, which are mainly derived from the mother’s vaginal and fecal microbiota, and has previously been observed in neonates along with a low diversity and high abundance of Actinobacteria. With time, higher diversity was observed, and Firmicutes and Bacteroidetes were dominant in 28-day-old rats. Thus, the changes went toward an adult-like microbiota, including an increase in the Firmicutes-to-Bacteroidetes ratio.

PHA and PT altered the gut microbiota very similarly, but the change was not in the same direction as observed in 28-day-old rats. A single gavage of such small doses of PHA (0.1 mg/g BW) or PT (0.6 mg/g BW) may not reach the lower part of the gut to be used as substrate for the gut microbiota; if that was the case, differences in the gut microbiota and SCFAs would be expected, given the distinguished nutritional properties of PHA and PT. Therefore, results from this study suggest that the altered gut microbiota might be a consequence, rather than a cause, of the changes in intestinal morphology.

Previously, a few studies have shown an increase in Gram-negative bacteria, particularly *E. coli*, following PHA treatment. However, these studies were performed before the comparative metagenomics methodologies to analyze microbiota composition were available and thus only specific species were investigated. Here, 16S gene sequencing revealed significant increases in the relative abundance of Bacteroidetes, particularly *Bacteroides* and *Parabacteroides*, in both PHA- and PT-treated rats. Bacteroidetes mostly comprises Gram-negative bacteria, whose membranes contain LPS. Increased plasma LBP levels reflect leakage of LPS across the GIB into the blood circulation, causing low-grade systemic inflammation. In fact, this has previously been observed in suckling rats after PHA exposure, where the rats had elevated levels of haptoglobin, an acute-phase inflammatory protein, in peripheral blood 3 days after the gut provocation, while release of pro-inflammatory cytokines in the small intestine was acutely observed. A high relative abundance of Bacteroidetes in PHA- and PT-treated rats was associated with elevated levels of plasma LBP and predicted genes involved in glycan biosynthesis and metabolism, a main component of LPS. Bacteroidetes genomes have also shown a presence of numerous carbohydrate-active enzymes, which correspond with the carbohydrate metabolism related genes enriched in the rats treated with PHA and PT. In fact, previous studies have reported an alteration of intestinal disaccharidase activities after both PHA and PT treatments, with reduced intestinal lactase activity and increased sucrase and maltase, which probably has a significant
impact on the breakdown of milk lactose that also serves as a substrate for certain bacteria. This might be a reason that the diversity of the gut microbiota tended to decrease in PHA-treated rats and significantly decreased in PT-treated rats, where Bacteroidetes dominated (up to 75% relative abundance) in both groups.

In rats treated with PHA and PT, there was a drastic reduction in the GIB permeability to macromolecules, that is, HSA, and thus an improved GIB function. However, the concomitant high plasma levels of LBP reflect a high LPS translocation. Passage of LPS takes place mainly due to paracellular leakage of the intestinal barrier in cases of epithelial dysfunction. Furthermore, transcellular passage of LPS has been documented to be a more efficient pathway for the transfer of LPS when it is packaged into chylomicrons along with nutrient lipids, which indeed are one of the main components of milk.

Before weaning milk oligosaccharides were the only substrates available for the bacteria in the gut, explaining the similar SCFA formation in the cecum of the suckling rats. The availability of solid food after day 21 provided the microbiota with different substrates in the lower gut, causing important changes in the gut microbiota composition as well as a significant increase in cecal butyric acid in 28-day-old rats. The association between increased butyric acid proportion and improved GIB function could be because butyric acid serves as an important substrate for the colonic epithelium. Butyric acid has also been shown to decrease translocation of LPS, through increased expression of tight junction proteins and reduced chylomicron formation. Although butyric acid, and also the other SCFAs formed to some extent, is highly utilized as a substrate for the mucosal cells, the higher amount of SCFAs formed in cecum the higher is the amount passing into the blood. Thus, it is difficult to conclude the effects on GIB maturation since the total amount at the different locations is difficult to quantitate.

4.2 | Increased BBB permeability concomitant with decreased bacterial-linked low-grade systemic inflammation and increased acetic acid levels in the brain after weaning

It has previously been reported that the BBB is well-established during early neonatal development and primed to respond to inflammatory stimuli. In this study, the BBB permeability was high in 14-day-old rats, became lower in 17-day-old rats during the suckling period and increased in the weaned 28-day-old rats, while there was a negative association between BBB permeability and plasma LBP levels. However, this negative association did not seem to fully apply to 14-day-old rats, which could be due to a not fully developed immune response to inflammatory stimuli in very young rats. The higher BBB permeability observed after weaning needs further investigations. However, our findings on SCFA proportions showed an increase of acetic acid in brains of 28-day-old weaned rats. This might suggest that there is a possible need for beneficial substances to pass to the brain, and this seemed to be only allowed in a low systemic inflammation environment. Besides, acetic acid was formed in much higher amount in the cecum and has lower molecular weight (C2), as compared to propionic and (C3) butyric acids (C4), causing more of it to pass into the brain to be used as alternative energy in form of acetyl-CoA.

It may be discussed that although the similar ratio of BSA to IgG in the brain tissue may show that blood contamination was similar in all brain samples the possibility that vascular spaces in the brain may have interfered with the BBB measurement cannot be completely excluded. Therefore, the results obtained on BBB will need further validation in future studies.

In conclusion, this study provides evidence that induced precocious GIB maturation has evident effect on the gut microbiota composition, changing it toward an imbalanced community associated with bacterial-linked low-grade systemic inflammation. The BBB permeability was surprisingly elevated in weaned rats, coinciding with lower levels of LBP and specific microbial taxa colonizing after the natural dietary transition from milk to solid food. The gut microbiota disturbance caused by the gut changes after dietary provocative agents favored low-grade systemic inflammation and altered SCFAs utilization in the brain, which may also play a potential role in GIB-BBB dysfunction disorders in neonates. Thus, we suggest that the use of such dietary provocative agents, including early exposure to solid diets, in neonates deserves careful consideration.

ACKNOWLEDGMENT

This study was funded by the Direktör Albert Påhlsson Foundation, Sweden (grant to FFH).

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTION

FFH and OP designed the experiment. NM, EAS, BW, and OP performed the animal experiment. NM, EAS, AL analyzed the materials. NM, EAS, BW, MN, OP, and FFH participated in results interpretation and manuscript writing. NM wrote the manuscript. All authors read and approved the final version of manuscript.

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Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Marungruang N, Arévalo Sureda E, Lefrançoise A, et al. Impact of dietary induced precocious gut maturation on cecal microbiota and its relation to the blood-brain barrier during the postnatal period in rats. Neurogastroenterol Motil. 2018;30:e13285. https://doi.org/10.1111/nmo.13285