

Influence of Delivery Mode On The Colonization Of Gut Bacteria in Breastfed Infants

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Key words: metagenome, Gut microbiota, delivery mode, Next Generation Probiotics

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Abstract

The human gut harbors a dynamic population of microorganisms, including bacteria and viruses. The dominant gut microbial phyla are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia, while the Actinobacteria make up the majority of infant's gut. Microbial colonization in early life differs between infants and strongly influences their health status and susceptibility to later immune diseases. The microbiome during the first years of an infant's life is influenced by the mode of delivery and the gut bacteria differs between infants born via C-section and vaginally. Infants that are born via C-section get the mother's skin microbiota and hospital-acquired harmful bacteria whereas vaginally born infants will get their bacteria from the mother's birth canal. This project examines the infant microbiome of 174 breastfed infants from the PREVAIL cohort study, comparing C-section to vaginally delivered infants. DNA was extracted from stool samples collected from 6-week-old infants, sequenced using NovaSeq, and the bacterial species abundance in each group was analyzed using R statistical analysis LEfSe analysis. Data analysis revealed that the relative abundance of Firmicutes was significantly higher ($p < 0.0001$) in the infants born via C-section when compared to the infants born vaginally. In infants born vaginally, the Bacteroidetes ($p < 0.0001$) and Actinobacteria ($p < 0.05$) were significantly higher than the other bacterial groups. Another novel finding in this study is the significant increase in the abundance of next generation probiotic bacteria (NGP) such as, *Parabacteroides goldsteinii*, *Bacteroides ovatus*, *Bacteroides dorei* and *Bacteroides uniformis* ($p < 0.0001$) in infants that were born vaginally compared to infants born via C-section.

Introduction

Each individual's gut microbiota is unique and it plays a vital role in maintaining host metabolism, cultivating the integrity of the gut mucosal barrier, and eliciting immunity against pathogens. (Rinninella et al., 2019) The gut bacterial colonization starts quite early and gets shaped as infant transitions occur. The development of gut microbiota during the first years of an infant's life is crucial to balancing the immune system and this is greatly influenced by the mode of delivery (Reyman et al., 2019). Infants born via C-section display differences in gut microbiota composition and immune responses when compared to infants born vaginally (Gensollen & Blumberg, 2017). Vaginally delivered infants get most of their gut bacteria from their mother while infants delivered by C-section get their mother's skin microbiome and other hospital-acquired microbes (Dunn, Jordan & Baker, 2017). So, the colonization of the infant gut is disturbed by C-section delivery (Liu, 2019; Shao, 2019). It is also evident that infants born via C-section lack bifidobacteria and other beneficial microbes when compared to vaginally delivered infants (Shao, 2019; Montoya-Williams, 2018).

The mode of delivery has been shown to substantially alter the gut microbiota composition of newborn infants. A previous study on the fecal samples collected over the first year of life from Dutch children born vaginally showed enrichment of health-associated *Bifidobacterium* spp., and a reduction in the colonization of potentially pathogenic *Enterococcus* and *Klebsiella* spp (Reyman et al., 2019). Bifidobacteria are health-associated microbes well-known for their use as probiotics (Allen, Martinez, Gregorio & Dans, 2010). Babies born via cesarean section had hospital-associated bacteria like *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, and *Clostridium perfringens* which belong to phylum *firmicutes* (Shao et., 2019). They also had higher percentages of opportunistic pathogens than their vaginally born

counterparts. This suggests that exposure to the hospital environment particularly affected the C-section-born infants and could predispose them to opportunistic infections that affect their respiratory system. This leads to the hypotheses that 1. the infants that are born vaginally will have higher bacteroidetes and Actinobacteria abundance compared to infants that are born via Cesarean section (C-section), 2. infants born via C-section will have higher Firmicutes abundance likely from the mother's skin microbiota.

Material and methods

Stool sample collection

This study utilized infant stool samples (n=174) via an established protocol (CDC-funded PREVAIL cohort study and approved by Institutional Review Board at University of Cincinnati) with informed consent in accordance with the current NIH procedures. All patient information was removed prior to use. The infant stool samples collected from 6-week-old infant diapers were stored frozen until used for microbial DNA extraction.

Microbial DNA extraction from infant stool samples

Microbial DNA from infant stool samples was extracted using QIAamp PowerFecal Pro DNA kit (cat.# 51804, Qiagen, Germantown, MD, USA). Briefly, ~250 mg of stool sample was taken in a PowerBead Pro tube and 850uL of solution CD1 was added. The samples were vortexed briefly to mix and secured horizontally on a vortex Adapter and vortexed at maximum speed for 10 min. The tubes were spun down at 15,000xg for 1min. The supernatant was transferred to a clean 2 ml microcentrifuge tube. 200uL of solution CD2 was added to the supernatant and vortexed for 5 sec. Centrifuged at 15,000xg for 1 min. Without disturbing the pellet, the clear supernatant was transferred to a clean 2ml microcentrifuge tube. 600uL of solution CD3 was added to the supernatant and vortexed for 5 sec. 650ul of the lysate was loaded onto an MB Spin column provided in the kit and centrifuged at 15,000Xg for 1min. The flow through was discarded and the earlier step was repeated to ensure that all of the lysate has passed through the MB spin column. The MB spin column was placed into a clean 2ml collection tube and 500uL of solution EA was added to the column. Centrifuged at 15,000xg for 1 min. The flow-through was discarded and the MB spin column was placed into the same 2 ml collection tube. 500uL of solution C5 was added

to the column and centrifuged at 15,000xg for 1 min. The flow-through was discarded and the MB spin column was placed into a new 2 ml collection tube. The tubes were centrifuged at 16,000xg for 2 min. After the spin the MB spin column was carefully removed without touching the collection tube and placed into a clean 1.5 ml Elution tube. 100uL of solution C6 was added to the center of the white filter membrane inside the MB spin column. Centrifuged at 15,000xg for 1 min and the MB spin column was discarded. The DNA was used for the downstream applications.

Metagenomic sequencing and data analysis

The microbial DNA samples extracted using QIAamp PowerFecal Pro DNA Kit were sent to DNA sequencing and Genotyping core for Next Generation sequencing using NovaSeq6000 sequencer (Illumina). The sequencing data obtained from the DNA sequencing and Genotyping core were analyzed using R statistical software. LEfSe analysis was performed to detect differences in relative abundance of bacterial taxa between infants delivered via C-section and those delivered vaginally. A non-parametric statistical hypothesis test, Wilcoxon signed-rank test, was used to find statistical significance since the distribution of the microbiome differences between the two samples (Vaginal vs. C-section). The data analyzed using R statistical software were used to generate graphs using Graphpad Prism software (Version 9).

Results

The sequencing data obtained from the DNA sequencing and genotyping core were analyzed using R statistical software. The delivery mode and other clinical data was obtained from the CDC-funded PREVAIL cohort and analyzed in relation to the infant's microbiome data. **Table 1** Shows the subject demographic characteristics of study participants such as number of participants included in this study, their maternal age, economic background and exclusive breastfeeding criteria.

The Linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed to compare the significant changes and relative richness of the microbiotas in the fecal samples of infants born vaginally and via C-section, at phylum and genus levels (**Figure 1a and 1b**). At the phylum level, five phylum communities were enriched and out of them, 2 phylum communities, Bacteroidetes and Actinobacteria, had a higher relative abundance in the fecal samples of infants born vaginally. The phylum community Firmicutes was more enriched in the fecal samples collected from infants born via C-section (**Figure 1a**). A histogram of the LDA scores (Linear Discriminant Analysis) was computed for features that showed differential abundance in the microbiotas at the genus level between infants born vaginally and via C-section. The LDA scores indicated that the relative abundance of microbiotas such as Clostridium, Bacillus and Eubacterium were much more enriched in the fecal samples collected from infants born via C-section than in the fecal samples collected from infants born vaginally (**Figure 1b**). The most differentially abundant bacterial taxon in the fecal samples of infants born via C-section was Clostridium (LDA score $[\log_{10}] > 3$), whereas the fecal samples of infants born vaginally was characterized by a preponderance of Bacteroidetes and Collinsella (LDA score $[\log_{10}] > 3$) (**Figure 1b**).

Among the bacterial phylum, Firmicutes was relatively abundant in the fecal samples of infants born via C-section compared to infants born vaginally ($p < 0.0001$; **Figure 2**). Bacteroidetes ($p < 0.0001$) and Actinobacteria ($p < 0.05$) were significantly higher in abundance in the fecal samples of infants born vaginally compared to C-section (**Figure 2**). At the genus level, bacteria that belong to Clostridium ($p < 0.05$), Bacillus ($p < 0.0001$), and Eubacterium ($p < 0.0001$) were significantly higher in the fecal samples of infants born via C-section compared to infants born vaginally (**Figure 3**). The relative abundance of Bacteroides ($p < 0.0001$) and Bifidobacteria ($p < 0.005$) was higher in the fecal samples of infants born vaginally compared to C-section (**Figure 3**).

The relative abundance of bacterial species that are recently characterized as Next generation Probiotics (NGP) such as *Parabacteroides goldsteinii*, *Bacteroides ovatus*, *Bacteroides dorei* and *Bacteroides uniformis* was significantly higher in the fecal samples of infants born vaginally compared to C-section ($p < 0.0001$; **Figure 4**).

Table 1. Subject demographic characteristics.

	C-section	Vaginal
Number	n=69	n=105
Mean Maternal Age (years)	32.3	29.4
White Mothers	73.9%	56.1%
Non-White Mothers	26.1%	43.9%
Mean Household Income <\$25k	16.5%	24.9%
Exclusive Breastfeeding	32.7%	24.9%

Figure 1. a. LEfSe analysis at the Phylum level, b. LEfSe analysis at the Genus level.

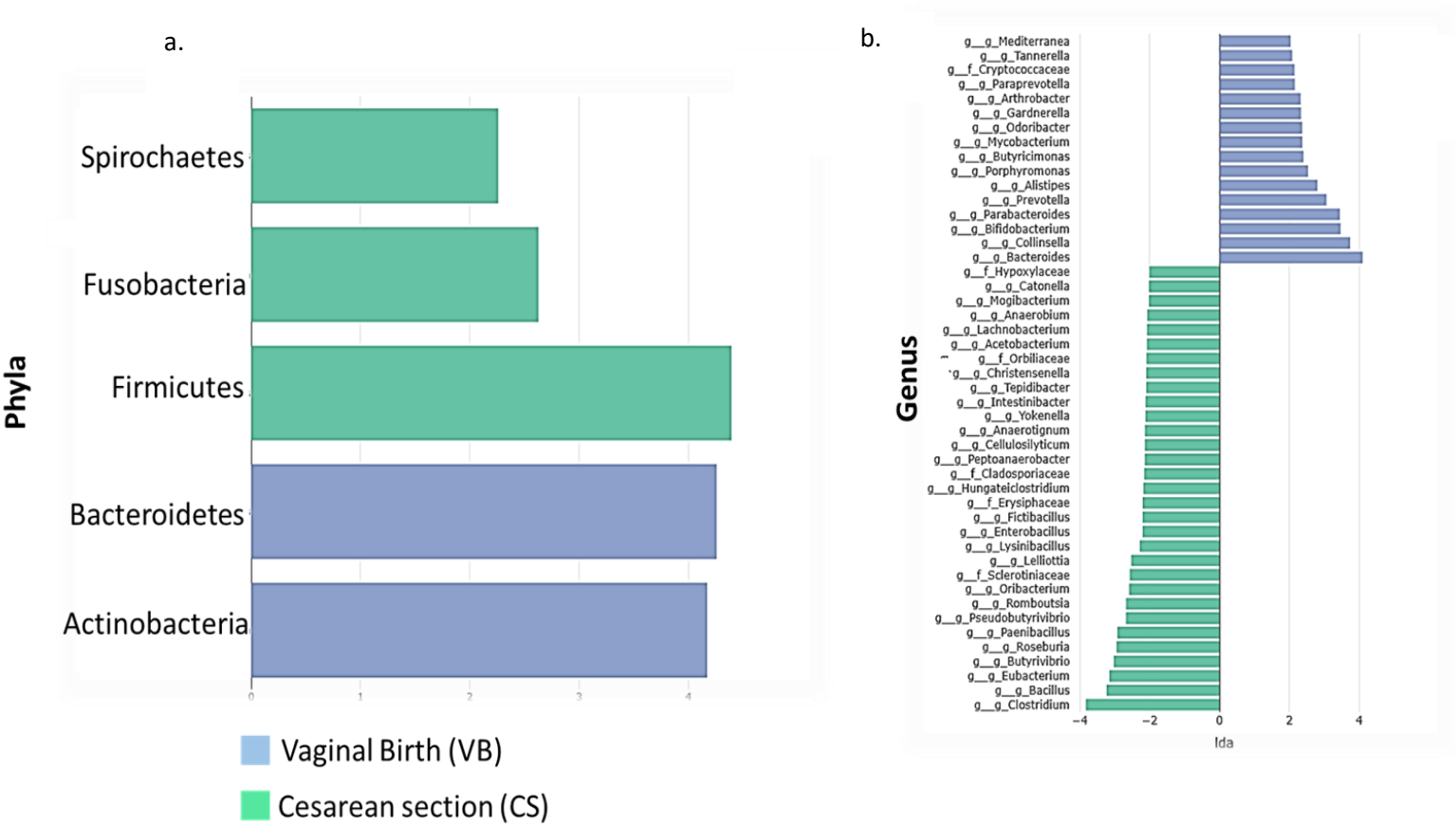


Figure 2. Relative abundance of Bacterial Phyla in the fecal samples collected from infants born via C-section and vaginal birth. Statistical significance was evaluated by Wilcoxon signed-rank test, using $p \leq 0.05$ as the measure of significance.

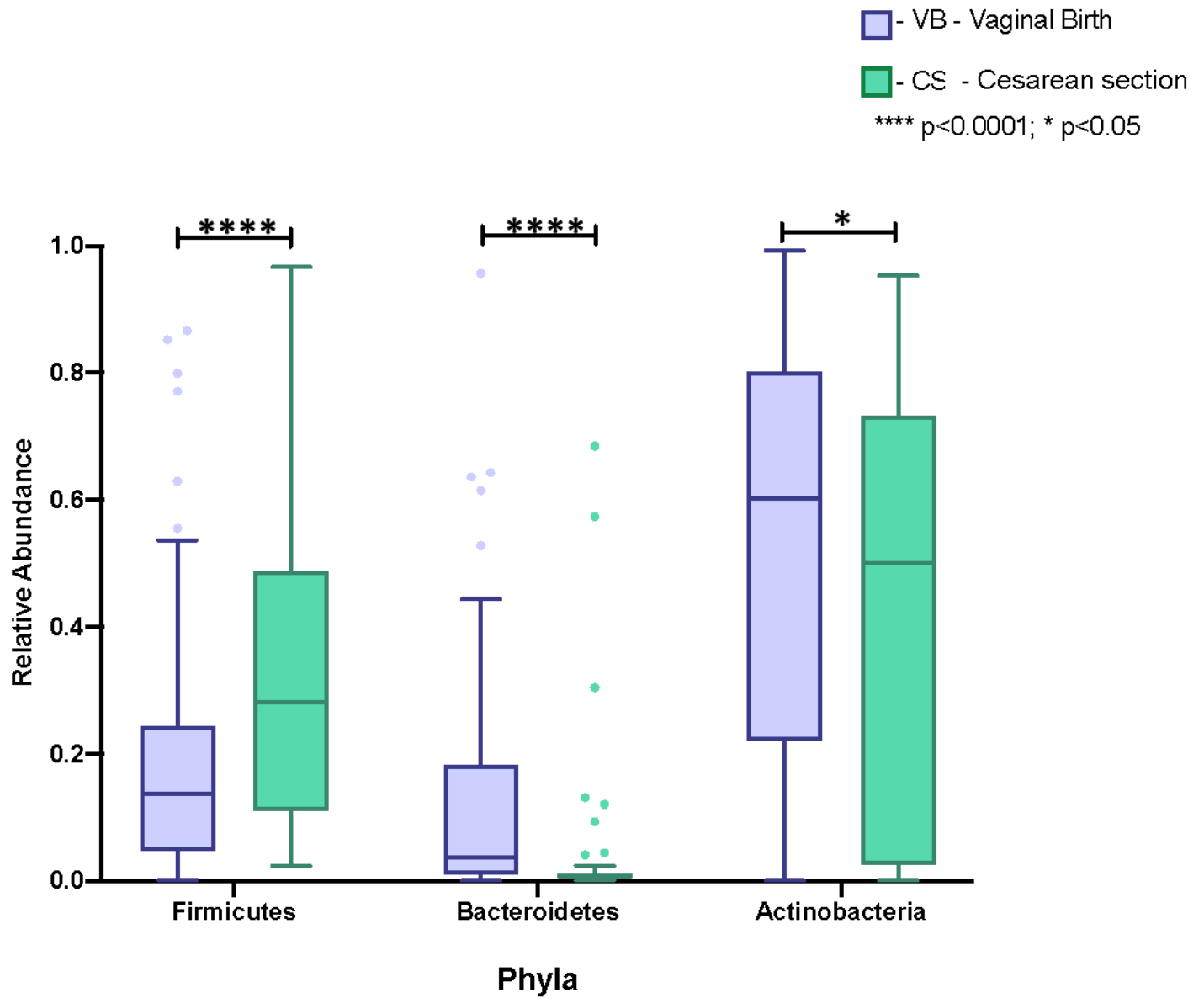


Figure 3. Relative abundance of Bacterial Genera in the fecal samples collected from infants born via C-section and vaginal birth. Statistical significance was evaluated by Wilcoxon signed-rank test, using $p \leq 0.05$ as the measure of significance.

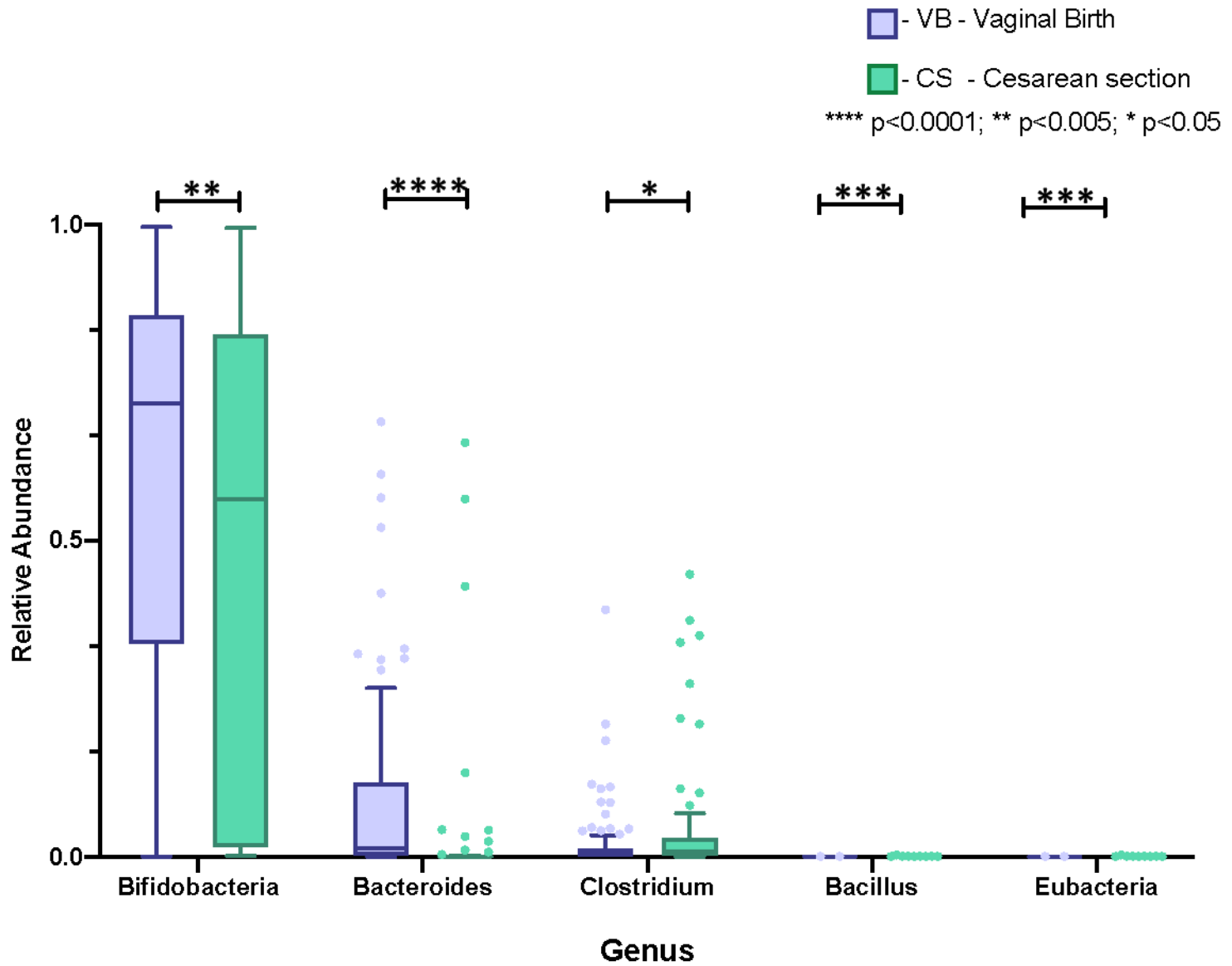
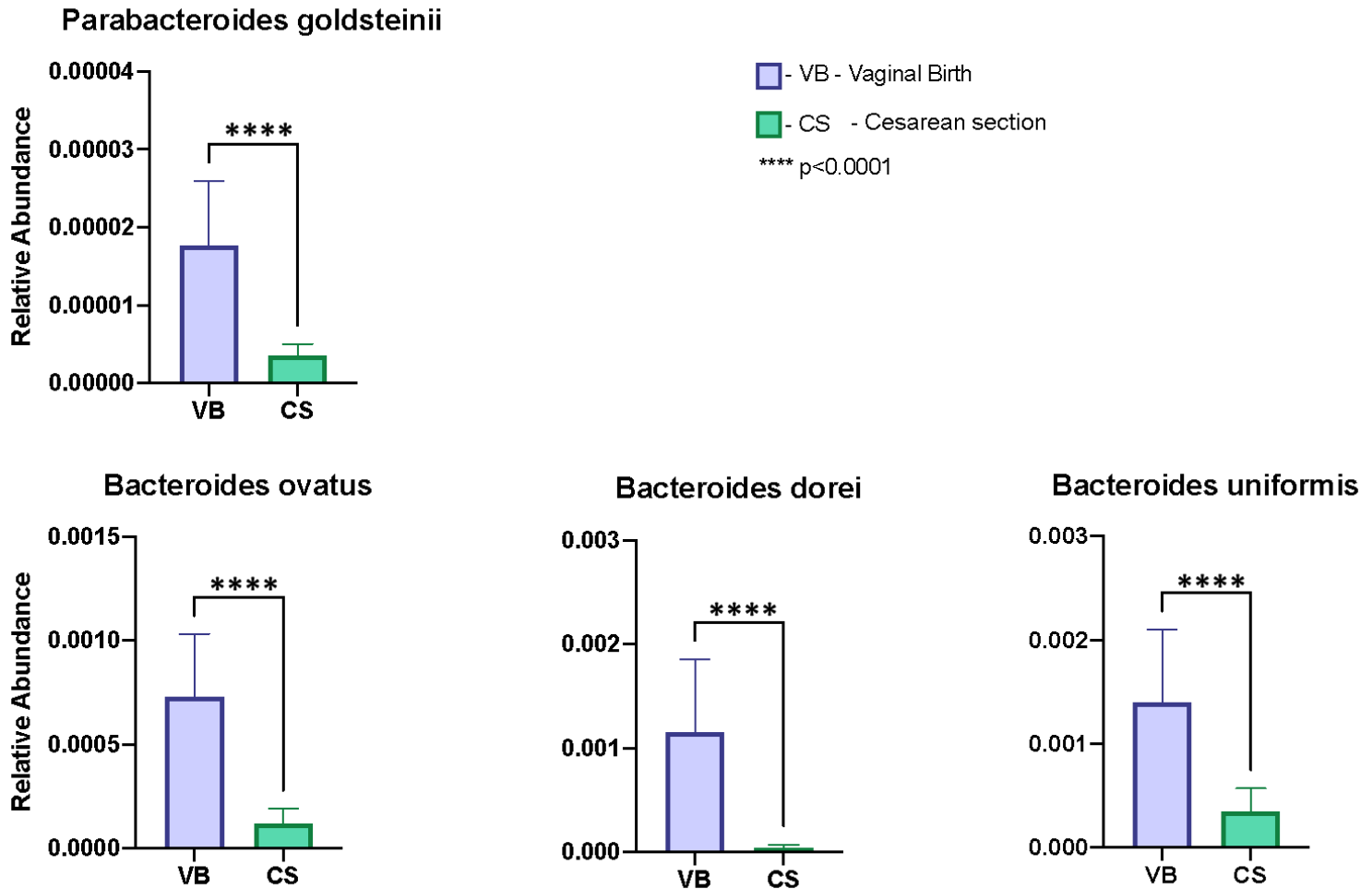


Figure 4. Relative abundance of Next Generation Probiotic (NGP) bacteria in the fecal samples collected from infants born via C-section and vaginal birth. Statistical significance was evaluated by Wilcoxon signed-rank test, using $p \leq 0.05$ as the measure of significance.



Discussion

The present study has demonstrated that the bacterial communities that belong to the Phyla Firmicutes, Fusobacteria and Spirochaetes were enriched in the fecal samples collected from infants that were born via C-section, as analyzed by metagenomic approach using next-generation sequencing (NGS). The bacterial communities that belong to the phyla Bacteroides, Actinomycetes and Bifidobacteria were enriched in the fecal samples collected from infants that were born vaginally when compared to C-section. In a Previous study, the gut of the infants born via Cesarean section had high-level colonization by healthcare-associated opportunistic pathogens such as *Enterococcus*, *Enterobacter* and *Klebsiella* species (Shao et al., 2019). In a Canadian Healthy infant Longitudinal Development study, maternal Intrapartum Antibiotic Prophylaxis (IAP) has been reported to affect the infant gut microbiome at 3 and 12 months of age. This study reported that the phyla Enterococcus and Clostridium were over-represented whereas Bacteroides and Parabacteroides were underrepresented in the gut of infants at 3 months of age following maternal IAP (Azad et al., 2016). In the study that I performed, abundance of Firmicutes, Fusobacteria and Spirochaetes was detected in the fecal samples of infants born via C-section compared to fecal samples of infants born vaginally, which may be due to the hospital environment the infants were exposed to, antibiotic use, elective vs. emergency C-section and the time point when the samples were collected. Further analysis of the samples based on antibiotic use, elective vs. emergency C-section and the samples collected at different time points such as earlier and later timepoints of 6-weeks of age may be helpful in understanding the microbial colonization. Also, my results corroborate with the finding that the bacteroides were underrepresented in the fecal samples of infants born via C-section. A study in the United Kingdom reported that the samples collected from infants delivered vaginally were enriched with Bifidobacterium, Escherichia and

Bacteroides species comprising 68.3% of the neonatal gut microbial communities. This study detected an overabundance of Bacteroides and Bifidobacterium in the fecal samples of infants born vaginally compared to C-section. The result suggested that Bacteroides and Bifidobacterium spp. were mostly transmitted from mothers to infants through vaginal birth which is in agreement with previous observation in smaller cohorts (Wampach, 2018; Ferretti, 2018; Yassour, 2018).

Bacteroides is one of the predominant genera that colonizes the human gut and plays an important role in maintaining the stability of a healthy gut ecosystem (Wexler & Goodman, 2017). The other predominant phylum of bacteria in the human gut is Firmicutes. The Bacteroidetes to Firmicutes ratio has been implicated in several diseases. It has been reported that the microbiota of obese children had an elevated Firmicutes-to-Bacteroides ratio compared to the microbiota of lean children (Bervote, et al., 2013). Changes in the gut microbial composition (dysbiosis) leads to various pathologic conditions affecting the gastrointestinal tract (diarrhea, irritable bowel syndrome) (Carding, Verbeke, Vipond, Corfe, & Owen, 2015), the immune system (allergy, multiple sclerosis, type 1 diabetes, rheumatoid arthritis) (Carding, 2015; Bravo, 2012) and the central nervous system (Alzheimer and Parkinson disease) (Bravo, 2012; Belkaid, 2014). In my study, an increase in the ratio of bacteroides to Firmicutes suggests a better gut environment when compared to C-section infants. This balance can be reversed in the C-section infants via mother's milk, diet and by administering probiotics. While a C-section birth cannot be completely avoided, it should only be an option when it is medically necessary. In cases where a C-section delivery is required for the health and safety of the child and the mother, a dietary intervention can help modulate the dysbiosis that occurs after a C-section delivery.

My study is unique from previously reported studies since this cohort (PREVAIL Cohort of Cincinnati) has not been investigated before. However, one limitation of this study is that the

samples were collected only from 6 weeks old infants. Microbial colonizations may be different at earlier or later time points so further studies are needed to validate the results that were observed in this study. Also, a longitudinal study is needed to better understand the human gut-microbiome interaction.

Another finding in my study is an increase in the abundance of bacteria that belong to Next Generation Probiotics (NGP) in the fecal samples of vaginally born infants compared to the fecal samples of C-section infants. This is a novel finding since these bacteria have only been reported in mouse model but not in infant fecal samples so far. Next generation probiotics are revealed as new preventative and therapeutic tools (Chang et al., 2019). These probiotic bacteria are known to reverse obesity, diabetes and positively modulate the immune system. Administering next generation probiotics is a potential therapeutic strategy for C-section infants to restore normal gut homeostasis. My long-term goal is to generate a tailored probiotic diet that can reconstitute an individual's microbiome rather than the "one-size-fits all" diet that is available in the market today.

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I would also like to thank Grace Adkins, David Newburg and Tejeswini Siva Sathya for assisting me in the lab.

Influence of Delivery Mode on the Colonization of Gut Bacteria in Breastfed Infants

Left Panel

Sanchita Paranthaman
12th grade

Abstract

The human gut harbors a dynamic population of microorganisms, including bacteria and viruses. The dominant gut microbial phyla are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia, while the Actinobacteria make up the majority of infant's gut. Microbial colonization in early life differs between infants and strongly influences their health status and susceptibility to later immune diseases. The microbiome during the first years of an infant's life is influenced by the mode of delivery and the gut bacteria differs between infants born via C-section and vaginally. Infants that are born via C-section get the mother's skin microbiota and hospital-acquired harmful bacteria whereas vaginally born infants will get their bacteria from the mother's birth canal. This project examines the infant microbiome of 174 breastfed infants from the PREVAIL cohort study, comparing C-section to vaginally delivered infants. DNA was extracted from stool samples collected from 6-week-old infants, sequenced using NovaSeq, and the bacterial species abundance in each group was analyzed using R statistical analysis LEfSe analysis. Data analysis revealed that the relative abundance of Firmicutes was significantly higher ($p < 0.0001$) in the infants born via C-section when compared to the infants born vaginally. In infants born vaginally, the Bacteroidetes ($p < 0.0001$) and Actinobacteria ($p < 0.05$) were significantly higher than the other bacterial groups. Another novel finding in this study is the significant increase in the abundance of next generation probiotic bacteria (NGP) such as, *Parabacteroides goldsteinii*, *Bacteroides ovatus*, *Bacteroides dorei* and *Bacteroides uniformis* ($p < 0.0001$) in infants that were born vaginally compared to infants born via C-section.

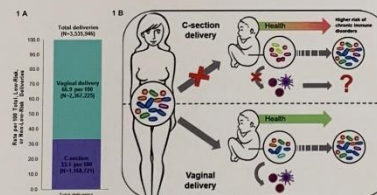


Figure 1A. Centers for Disease Control and Prevention data released May 15, 2019 show that about one-third of all births were by cesarean delivery in the United States. Figure 1B. Cesarean section babies are statistically more prone to develop allergies, chronic inflammatory diseases and metabolic diseases. NeuroscienceNews.com; Image credit: Linda Wampach

Dominant Gut Bacteria

Bacteroidetes:

➤ Includes *Bacteroides*, *Prevotella*, and *Parabacteroides*

Actinobacteria:

➤ Includes *Bifidobacteria*, *Collinsella*, and *Streptomyces*

Firmicutes:

➤ Includes *Bacillus*, *Clostridium*, *Eubacteria*, and *Streptococcus*



Middle Panel

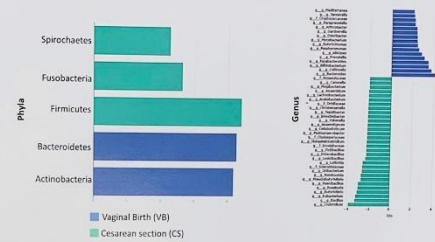
Hypotheses

1. Infants born via vaginal delivery will have a higher Bacteroidetes and Actinobacteria abundance compared to infants born via Cesarean section.
2. Infants born via cesarean section, will have a higher Firmicutes abundance likely from the mother's skin microbiota.



LEfSe Analysis at the Phylum Level

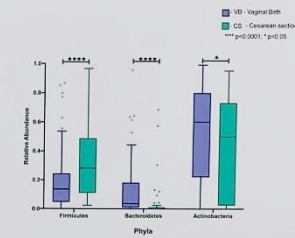
LEfSe Analysis at the Genus Level



Methods

- The delivery mode and other related data was obtained from the CDC-funded PREVAL cohort and analyzed in relation to the infant's microbiome data.
- Stool samples from 174 breastfed (8-week-old) infants were collected by swabbing their diapers.
- DNA from infant stool samples were extracted using a PowerPrep DNA kit.
- Extracted DNA samples were subjected to the CHOCVIA-DNA Sequencing and Genotyping Core (Cincinnati Children's Hospital) for metagenomics sequencing using Illumina MiSeq.
- R statistical software was used to analyze the microbiome of each group.
- LEfSe analysis was performed to analyze the differences between bacterial taxa.
- The analyzed data were graphically represented using GraphPad Prism software.
- Pairwise Wilcoxon signed-rank test was used for statistical significance.
-
- ```
graph TD; A[Feeding sample] --> B[Add Human Cytokines
+ Add Human IgG]; B --> C[Alpha-1 Antitrypsin Adapter
+ Adapter Ligation]; C --> D[Library Amplification
+ Library Purification]; D --> E[Add Illumina BBK12
+ Load onto Ion Spline Cluster]; E --> F[Run with Ion Torrent PGM
+ Base Calling]; F --> G[Data Analysis];
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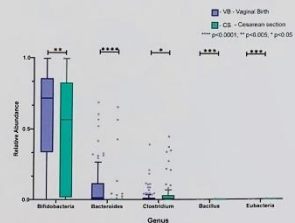
### Relative Abundance of Bacterial Phyla



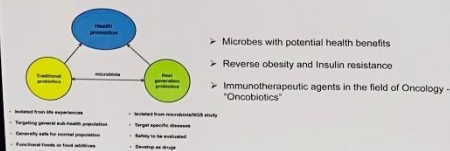
### Subject Demographic Characteristics

|                              | C-section | Vaginal |
|------------------------------|-----------|---------|
| Number                       | n=69      | n=105   |
| Mean Maternal Age (years)    | 32.3      | 29.4    |
| White Mothers                | 73.9%     | 56.1%   |
| Non-White Mothers            | 26.1%     | 43.9%   |
| Mean Household Income <\$25k | 16.5%     | 24.9%   |
| Exclusive Breastfeeding      | 32.7%     | 24.9%   |

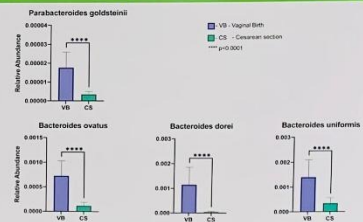
### Relative Abundance of Bacterial Genera



### Next Generation Probiotics (NGP)



Relative Abundance of Next Generation Probiotic (NGP) Bacteria



## Right Panel

### Results

- Phylum Bacteroidetes and Actinobacteria abundance was significantly higher in infants born vaginally compared to infants born via Cesarean section.
- At the genus level, *Bacteroides* and *Bifidobacteria* were significantly higher in infants born vaginally compared to infants born via Cesarean section.
- Phylum Firmicutes abundance was significantly higher in infants born via Cesarean section compared to vaginal delivery.
- At the genus level, *Clostridium*, *Bacillus*, and *Eubacterium* were significantly higher in infants born via Cesarean section compared to infants born vaginally.
- The next generation probiotic (NGP) bacterial species namely, *Parabacteroides goldsteinii*, *Bacteroides ovatus*, *Bacteroides dorei*, and *Bacteroides uniformis* were significantly higher in infants born vaginally compared to infants born via Cesarean section.

### Conclusion

- The Bacteroidetes and Actinobacteria abundance is higher in infants born vaginally compared to infants born via Cesarean section which supported my hypothesis. This suggests that these bacteria were acquired from the mother's birth canal.
- The relative abundance of Firmicutes was higher in infants born via Cesarean section compared to infants born vaginally due to the hospital environment and the mother's skin microbiome which also supports my hypothesis.

#### Other Findings:

- The relative abundance of Bifidobacteria dominates the gut of 6-week old, breastfed infants.
- The abundance of next generation probiotic bacteria (NGP) *Parabacteroides goldsteinii*, *Bacteroides ovatus*, *Bacteroides dorei* and *Bacteroides uniformis* was higher in infants born vaginally compared to infants born via C-section. This is a novel finding and reflects the presence of well-equilibrated microbiota in vaginally delivered infants.

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2. Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life  
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### Future Experiments

- To analyze the colonization of Bifidobacteria at an earlier and later time point (2 weeks to the infants' first year of life).
- To investigate the role of next generation probiotic bacteria in reversing obesity and insulin resistance.
- To generate a tailored probiotic diet that can reconstitute an individual's microbiome rather than the "one-size-fits all" diet that is available in the market today.

