Curriculum Vitae

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ABSTRACT

Title of Thesis: Insights into the Role of *Bifidobacterium* in Neonatal Intestinal Maturation

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Infant mortality and morbidity rates are impacted by premature birth, a critical issue in neonatal health. The prevalence of preterm births and accompanying disorders, such as necrotizing enterocolitis (NEC), underscore the urgent need for improved remedies. The "leaky gut" phenomenon, characterized by decreased intestinal barrier integrity, lies at the heart of these difficulties. Understanding the role of u in gut health, particularly in metabolizing maternal breast milk, holds potential for intervention. Human Milk Oligosaccharides (HMOs), a key component in breast milk, promote the growth of beneficial *Bifidobacterium* species in the newborn gut, but further research into their genetic features is necessary for a better understanding. This study aims to isolate *Bifidobacterium* strains from preterm infant stool samples, characterize their carbon utilization attributes, and define their genetic content. The findings reveal significant phenotypic and genotypic diversity among strains, illustrating varied genetic content within *Bifidobacterium* species. Differences in carbon source consumption patterns suggest functional versatility across strains. These results emphasize the crucial importance of understanding *Bifidobacterium*'s genetic characteristics and metabolic capabilities in supporting gut health, particularly in preterm infants, and pave the way for

targeted interventions aimed at reducing gastrointestinal complications and improving outcomes in this vulnerable population.

Insights into the Role of *Bifidobacterium* in Neonatal Intestinal Maturation

by
Rebecca A Collins

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CHAPTER 1: INTRODUCTION

Premature birth, a critical issue in neonatal health, is marked by infants being born <37 weeks' gestation. Prematurity affects 1 in every 8 children in the United States, making it the leading cause of death in children under the age of five, according to the New UN Report highlights from 2017. Preterm infants often face various health challenges, including underdeveloped organs and immune systems, necrotizing enterocolitis, late/early-onset sepsis, which significantly elevate their vulnerability to many other complications like Multiple Organ Dysfunction Syndrome (MODS) (Ma et al. 2022). The alarming prevalence and disease severity underscore the unmet medical need to address the complications associated with premature births and their profound impact on the mortalities and morbidities of one of the most vulnerable populations.

Majority of preterm infants are born with a hyperpermeable intestine due to premature intestinal development, with heightened risk of gastrointestinal (GI) disorders with increased intestinal permeability (IP), commonly referred to as "leaky gut" (Ginglen and Butki 2024). Leaky gut is characterized by the compromised integrity of the intestinal barrier, allowing bacteria and their by-products normally confined to the intestinal lumen to translocate to the internal compartment and bloodstream. This condition can lead to microbial invasion, inflammation, severe epithelial damage, necrosis, multi-organ failure, and Necrotizing Enterocolitis (NEC) (Ginglen and Butki 2024). NEC is a significant GI condition characterized by bacterial translocation, primarily affecting preterm neonates, with no effective treatment (Lemme-Dumit et al. 2022). NEC is prevalent in 7-10% of preterm neonatal NICU admissions and carries a high mortality rate, reaching as much as fifty percent (Ma et al. 2022). Detecting leaky

gut early on and intervening promptly to limit increased intestinal hyperpermeability is crucial. No preventive or therapeutic options currently exist for neonatal leaky gut, representing a critical unmet medical need in neonatal care. Addressing these issues at an early stage is important in reducing later complications and lessening the impact of conditions like NEC on the vulnerable preterm neonatal population.

Notably, the full maturation of the intestinal lining typically occurs around 34 weeks (about 8 months) of gestation and continues post gestation, making premature infants particularly vulnerable to GI-related complications (Schoultz and Keita 2020). A well-functioning intestinal barrier incorporates a physical barrier consisting of chemical, immunological, and microbiological components (Ma et al. 2022). The delicate balance of the gut microbiome, which refers to a diverse community of microorganisms living in the gastrointestinal tract, plays a pivotal role in supporting gut health. This microbial community contributes to the maturation of the intestinal barrier, stimulates the immune system, and aids in the digestion process (Ma et al. 2022). Among these bacteria, *Bifidobacterium* is a well-known inhabitant, playing a crucial role in promoting the maturation of the intestinal barrier, enhancing biodiversity, supporting carbohydrate metabolism, fermenting short chain fatty acids to provide energy for mucosal cell lining the GI tract, and contributing to overall gut health (Schoultz and Keita 2020). In recent studies *Bifidobacterium* has also been identified as a microbial biomarker for postnatal intestinal barrier permeability (Ou et al. 2023; Ma et al. 2022). Another key aspect linking *Bifidobacterium* to gut health is its ability to metabolize Human Milk

Oligosaccharides (HMOs), which is otherwise indigestible to human host due to the lack of enzymes in humans. HMOs are complex sugars found in mothers' breast milk and serve as a crucial nutrient source for and host, and metabolized by beneficial gut bacteria, particularly *Bifidobacterium*, a process known as “bifidogenic” (Ma et al. 2022).

Previous studies have indicated that bifidogenic HMO supports the growth of *Bifidobacterium* species, specifically *Bifidobacterium breve, Bifidobacterium longum, and Bifidobacterium infantis* strains in the infant gut. Understanding the genetic characteristics governing *Bifidobacterium*'s role in HMO metabolism can provide insights into its contribution to the development of the intestinal barrier in preterm infants. This knowledge is the prerequisite to develop novel therapeutics to promote postnatal barrier maturation to reduce the incidences of bacterial translocation-associated mortality and morbidities. The significance of this project lies in addressing the critical knowledge gap surrounding the genetic characteristics of *Bifidobacterium* strains in preterm infants and their connection to postnatal intestinal development, facilitating further understanding the specific genetic content underlining *Bifidobacterium*'s role in neonatal barrier development – an imperative need in neonatal care in the United State and worldwide.

CHAPTER 2: LITERATURE REVIEW

Prematurity is a major concern for our most vulnerable population. According to a 2017 UN report from, it presently affects 1 in 8 babies globally, and the percentage has been rising yearly. Prematurity is defined as birth <37 weeks of gestation and it can occur due to several factors, including maternal health conditions/age, infections, lifestyle factors such as smoking or substance abuse, multiple pregnancies (i.e., twin, triplets), etc... It can be divided into subcategories based on gestational age (GA). Infants born 32-37 week are considered moderate to late term, infants born less than 32 but more than twenty-eight are considered very preterm and any infants born earlier than that are extremely preterm. According to the World Health Organization prematurity is the leading cause of death in children under five years of age, due to the many health conditions that can arise due prematurity. The consequence affects not only immediate neonatal health like underdeveloped organ systems, but also long-term outcomes going into adulthood.

One of the most significant consequences of premature birth is the immaturity of their gastrointestinal (GI) tract. The GI tract encompasses a physical, microbiological, and immunological barrier (Ma et al. 2022). Intestinal barrier function, which develops mostly in utero in term infants, can be improved postnatally. The GI tract's main job is to help break down nutrients from food into smaller, more absorbable molecules. Digestion is the process by which the body gets vital nutrients—carbs, proteins, lipids, vitamins, and minerals—that it needs for growth, energy production, and general body functioning (Halloran and Underwood 2019). As food passes through the digestive system, the GI tract uses mechanical and chemical processes, including the actions of enzymes, acids,

and other digestive secretions, to do this. Additionally, in the GI tract, a single monolayer of epithelial cells forms a tight intercellular connection and functions as a selective barrier to stop the passage of toxins and germs from the lumen into the extracellular space (Faziova and Akhmedova 2023). The most critical time for the maturation of the GI tract is during the early neonatal period. Preterm babies have an immature barrier because of reduced mucus production, low IgA levels, and compromised intercellular connection integrity, which raises IP (Lemme-Dumit et al. 2022). As a result, the barrier loses its selectivity and makes it easier for bacterial pathogens and their toxins to enter the extracellular space, which exacerbates tissue damage and inflammation (Mank et al. 2021).

This immaturity leads to a condition known as "leaky gut," where the intestinal barrier is hyperpermeable, allowing bacteria and its products to translocate into the bloodstream leading to systemic infections and inflammatory responses (Mank et al. 2021). Intestinal permeability is the proximate cause for a serious condition known as necrotizing enterocolitis (NEC) (Neu Josef and Walker W. Allan 2011). NEC is a severe GI condition primarily affecting preterm neonates, with a prevalence of 7-10% among NICU admissions and a high mortality rate of up to fifty percent (Mank et al. 2021).

Outside of its physical function, the GI tract also serves microbiological functions. The GI tract is home to many commensal microorganisms that make up the gut microbiome (Westerbeek et al. 2006). These bacteria help break down and absorb nutrients, especially fiber and complex carbohydrates, which the human body is unable to process on its own. Furthermore, some vitamins, including vitamin K and some B vitamins, which are necessary for a number of metabolic functions, are synthesized in

part by the gut bacteria (Faziova and Akhmedova 2023). Additionally, by battling for resources and creating antimicrobial chemicals, the gut microbiota competes with opportunistic pathogens for nutrients. Due to the diverse array of microorganisms in the GI tract, no individual bacterial line persists in the gut. The biodiversity of the gut microbiota is seen in a mature GI tract; however, a significant lack of biodiversity is characteristic of an immature intestinal barrier (Lemme-Dumit et al. 2022). Any imbalance of the gut microbiota is known as gut dysbiosis. Consequently, this dysbiosis has been linked to a higher risk of complications such as the previously stated NEC (Patton, de la Cruz, and Neu 2022). Microbial dysbiosis, characterized by an imbalance in the gut microbial community, is common in preterm neonates Delayed colonization by beneficial bacteria, such as *Bifidobacteria* and *Lactobacillus*, further exacerbates this imbalance leading to more intestinal hyperpermeability and bacterial translocation (Westerbeek et al. 2006). Understanding the dynamics of microbial biodiversity in premature infants is essential for developing targeted interventions to promote gut health and prevent complications like NEC (Lee and O'Sullivan 2010).

Bifidobacteria, a key component of the gut microbiota, has been studied for its potential therapeutic benefits in premature infants (Bottacini et al., 2018). *Bifidobacterium* is a non-sporing, non-motile, gram-positive bacilli that is facultative anaerobe (Gavzy et al. 2023). They belong to the phylum *Actinobacteria*, and they represent one of the dominant early microbial colonizers of the human and animal gut. *Bifidobacteria*, commonly found colonizing the gut, vagina, and oral cavity of humans, are integral to maintaining GI health through many mechanisms (Ma et al. 2022). These beneficial microbes possess the enzymatic capability to break down complex

carbohydrates and ferment dietary fibers, yielding short-chain fatty acids (SCFAs) that serve as essential energy sources for the epithelial cells lining the GI tract (Lemme-Dumit et al. 2022). Furthermore, *Bifidobacteria* plays a pivotal role in modulating the immune response within the gut environment by stimulating the production of secretory IgA antibodies, crucial for mucosal immunity and defense against pathogens (Ma et al. 2022). They compete with opportunistic bacteria like *Proteobacteria* for nutrients (Lemme-Dumit et al. 2022). Among the prevalent species of *Bifidobacteria* are *Bifidobacterium breve, Bifidobacterium longum*, and *Bifidobacterium infantis*, each believed to distinctly aid in infant gut health (Ma et al., 2022). This underscores the significance of *Bifidobacteria* in promoting GI health and highlights their potential therapeutic implications in maintaining gut homeostasis and mitigating disease states. Comparative genomics studies have provided insights into the metabolic capabilities of B*ifidobacteria*, highlighting their ability to utilize specific carbohydrates available in the infant gut environment, which are for development of the infant (Bottacini et al. 2018).

Maternal breast milk, rich in nutrients and bioactive molecules, plays a crucial role in supporting gut health and reducing the risk of complications in premature infants (Mank et al. 2021). Infants who receive enough of their mother's own breast milk exhibit improved intestinal permeability compared to those who rely on alternative feeding methods like exclusively formula (Ma et al. 2022). This protective effect is attributed to human milk oligosaccharides (HMOs), which promote the growth of beneficial bacteria like *Bifidobacteria* in the infant gut. The concentration of human milk oligosaccharides (HMOs) is higher than that of all the human milk proteins put together, making them an important part of human milk (Milani et al. 2017). Complex sugars such as glucose,

galactose, fucose, N-acetylglucosamine, and sialic acid are among them. Though the total quantity and makeup of HMOs varies among women, they are essential in forming the infant's gut microbiota (Milani et al. 2017). HMOs are resistant to breaking down in the developing child's digestive system and make it all the way to the colon, where they function as a substrate to support the growth and colonization of beneficial bacteria like *Bifidobacterium longum subsp. Infantis* (Milani et al. 2017).

This bifidogenic effect of HMOs contributes to the establishment of a healthy gut microbiota in early life, with potential long-term implications for infant health and development. Overall, these findings highlight the importance of mom's breast milk in promoting gut health and reducing the risk of complications in premature infants.

CHAPTER 3: HYPOTHESIS

Understanding the genetic characteristics governing *Bifidobacterium*'s role in HMO metabolism can provide crucial insights into its contribution to the development of the intestinal barrier in preterm infants. This knowledge gap forms the basis for our hypothesis. As mom's own breastmilk feeding is the greatest clinical factor associated with improved intestinal barrier function post-birth, **we hypothesize that *Bifidobacterium* strains in preterm infant stool samples with improved intestinal barrier functions and having received mom's breastmilk will have potent HMO metabolism capabilities underlined by its specialized genetic content**. By addressing this issue, our study aims to inform strategies for promoting a healthy gut microbiota in preterm infants, therefore reducing the risk of complications associated with intestinal hyperpermeability.

To address this hypothesis, our study will isolate *Bifidobacterium* strains from stool samples of low-IP subjects, characterize carbon utilization attributes of *Bifidobacterium*, and define the genetic content of low IP *Bifidobacterium*. These objectives align with the critical questions surrounding the biological processes connecting *Bifidobacterium* and breast milk in promoting postnatal intestinal barrier development and the genetic features underlying *Bifidobacterium*'s capabilities to metabolize HMOs.

CHAPTER 4: METHODOLOGY

Fecal Specimen Selection and Cultivation

The study involved selecting subjects from a retrospective cohort of preterm neonates admitted to the Neonatal Intensive Care Unit (NICU) at the University of Maryland Medical Center involving 215 preterm (24-32 weeks gestational age) infants (Ma et al. 2022). Stool samples were collected over 21 days for all infants enrolled in study for a microbial community survey, while urine samples were obtained at 7-10 days to measure intestinal permeability. Demographic and clinical data were gathered from medical records at enrollment. Twenty subjects with low intestinal permeability were selected, four stool samples were chosen from different days of life within the first month. Selected samples based on data from previous study conducted in Ma et al. 2022 (Figure 1).

Figure 1: Samples Selection Map (Ma et al. 2022)

Stool samples collected were kept frozen at –80 °C. For stool samples selected, 50mg was homogenization in 1mL *Bifidobacterium* Selective Medium (BSM) broth, followed by dilution (1:10) and streaked onto BSM agar plates. Both broth and agar plates were made from media by Sigma Adrich company and supplemented with BSM supplement, to inhibit the growth of other bacteria. After incubation in the anaerobic chamber by Coy Laboratories, 4-8 colonies with distinct morphologies were selected from each plate and re-streaked to ensure purity. Morphologies of colonies observed include small pink or white colonies, and mucoid or shiny colonies, etc.....

Gram Staining

Isolate cultured were also subjected to Gram staining to detect *Bifidobacterium* morphology. Gram staining is used to quickly and inexpensively classify bacteria based on their cell wall composition. It involves an initial staining with crystal violet, adding iodine as fixative, decolorizing with an acetone, and counterstaining with safranin. Gram-positive bacteria retain the purple coloring of the crystal violet due to their thick peptidoglycan wall, while Gram-negative bacteria lose this staining and are counterstained pink with the safranin (O'Toole 2016). Gram staining *Bifidobacterium*, a Gram-positive bacillus, usually gives it a purple appearance. Under a microscope, they frequently exhibit the characteristic Y shape morphology.

Boil Preparation

"Boil Prep" DNA extraction method was employed for sample processing. This technique involved the collection of "dirty" DNA by boiling 100 µL of the broth in a 100°C heating block for a duration of 10 minutes. Subsequently, the samples were

centrifuged, and the precipitate containing DNA was carefully collected and stored frozen until further analysis via PCR.

Sample Screening using *Bifidobacterium* Primers

For sample screening purposes, a three-tiered approach was employed to target *Bifidobacterium* within the collected samples. Initially, a *Bifidobacterium* genus primer (Matsuki et al. 2004) was utilized as the primary screening tool. This primer was first to cast a wide net, targeting the genus level to detect any presence of *Bifidobacterium* species from isolates in the samples. Following the initial screening, samples that were PCR positive, as visualized through gel electrophoresis, underwent further analysis. Three species-specific primers designed for *Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium infantis* were employed (Matsuki et al. 2004).

Table 1: Bifidobacterium primers used for initial sample screening.

Target	***Primer***	***Sequence***	***Product size***
Bifidobacterium genera	BifG1-F	CTCCTGGAAACGGGTGG	549-563
	BifG1-R	CCGGATGCTCCATCACAC	
Bifidobacterium breve	Bbre2-F	CCGGATGCTCCATCACAC	288
	Bbre2-R	TTCCAGTTGATCGCATGGTC	
Bifidobacterium longum	Blon2-F	TTCCAGTTGATCGCATGGTC	831
	Blon2-R	GGGAAGCCGTATCTCTACGA	
Bifidobacterium infantis	Binf1-F	TTCCAGTTGATCGCATGGTC	828
	Binf1-R	GGAAACCCCATCTCTGGGAT	

DNA extraction, bacterial 16S rRNA gene amplification, and Illumina sequencing analysis

PCR amplification of the 16S rRNA gene V3-V4 hypervariable region was performed using dual-barcoded universal primers 318F and 806R to confirm

Bifidobacterium identification. Confirmatory Sanger sequencing is conducted to verify bacterial species. This process involves the purification of PCR-amplified DNA fragments containing the target gene regions using the Qiagen Purification kit. Subsequently, these purified DNA fragments are subjected to Sanger sequencing. The sequencing reactions involve the incorporation of chain-terminating dideoxynucleotides, fluorescently labeled for detection, which are then resolved by capillary electrophoresis (Jost et al. 2013). The resulting sequencing chromatograms are analyzed using specialized software to determine the nucleotide sequence of the DNA fragments (Jost et al. 2013). After which High-throughput sequencing of the amplicons was performed. Short-read sequencing of samples was conducted using the Illumina NovaSeq 6000 platform. Sequencing was performed by the University of Maryland School of Medicine, Institute for Genome Sciences, Genomics Resource Center with standard operating procedures ("Institute for Genome Sciences").

Phenotype Microarrays

For phenotyping, the Biolog Phenotype Microarray plate, commonly referred to as Biolog, was utilized. The AN (Anaerobic) Biolog plate comprises 96 wells, with 95 wells pre-coated with different sole-carbon sources, and one well containing water serving as the negative control. This plate is specifically designed with various carbon

sources known to be utilized by anaerobic bacteria. Metabolism of the substrate in the wells leads to formazan production, resulting in a color change in the tetrazolium dye present (Frąc et al.). The suspension used for well inoculation in the microplates was prepared as follows: Isolated colonies were cultivated on BSM agar in a lawn for 48-72 hours. Subsequently, approximately 0.1g of growth was collected using a sterile 10uL inoculating loop. Colonies were washed with phosphate buffer saline (PBS) and centrifuged (3700rpm for 3 min at 4 °C). Following centrifugation, the supernatant was discarded, and the pellet was reconstituted with 14mL of inoculating fluid, a minimal growth media provided by Biolog^{TM}. The final concentration of the fluid was adjusted to 0.2-0.3 OD (optical density) using a spectrophotometer ($\lambda = 590$), equivalent to $8x10^8$ cfu/mL (Zhao et al., 2022). Subsequently, 100uL of suspension was inoculated into each of the 96 wells of the Biolog AN Microplates containing the carbon sources (Figure 2). The microplates were then sealed into the anaerobic GasPak EZ anaerobe gas pouch system with an anaerobic indicator and incubated at 37°C. Following incubation, the plates were read 20-24hrs post-inoculation using a spectrophotometer microplate reader, and data acquisition was performed using SoftMax Pro 7 software. To ensure accuracy and consistency, plates were run in triplicates, and after visual confirmation of the negative well being clear/negative to insure validity of protocol.

Statistical Analysis

To ensure the validity of the analysis, the readings from the blank or negative well (Well A1, containing only water), visually observed to be negative, were utilized for normalization purposes to balance the data. The statistical analysis was conducted using the student's t-test, performed in Microsoft Excel spreadsheet software. Specifically, the

mean metabolic activity of the ATCC reference strain was compared to that of our isolated sample, 41-9E. A significance threshold (alpha level) of $p < 0.05$ was established to determine the statistical significance of the results.

AN MicroPlate™

A1 Water	**A2** N-Acetyl-D-Galactosamine	**A3** N-Acetyl-D-Glucosamine	**A4** N-Acetyl-β-D-Mannosamine	**A5** Adonitol	**A6** Amygdalin	**A7** D-Arabitol	**A8** Arbutin	**A9** D-Cellobiose	**A10** α-Cyclodextrin	**A11** β-Cyclodextrin	**A12** Dextrin
B1 Dulcitol	**B2** i-Erythritol	**B3** D-Fructose	**B4** L-Fucose	**B5** D-Galactose	**B6** D-Galacturonic Acid	**B7** Gentibiose	**B8** D-Gluconic Acid	**B9** D-Glucosaminic Acid	**B10** α-D-Glucose	**B11** Glucose-1-Phosphate	**B12** Glucose-6-Phosphate
C1 Glycerol	**C2** D,L-α-Glycerol Phosphate	**C3** m-Inositol	**C4** α-D-Lactose	**C5** Lactulose	**C6** Maltose	**C7** Maltotriose	**C8** D-Mannitol	**C9** D-Mannose	**C10** D-Melezitose	**C11** D-Melibiose	**C12** 3-Methyl-D-Glucose
D1 α-Methyl-D-Galactoside	**D2** β-Methyl-D-Galactoside	**D3** α-Methyl-D-Glucoside	**D4** β-Methyl-D-Glucoside	**D5** Palatinose	**D6** D-Raffinose	**D7** L-Rhamnose	**D8** Salicin	**D9** D-Sorbitol	**D10** Stachyose	**D11** Sucrose	**D12** D-Trehalose
E1 Turanose	**E2** Acetic Acid	**E3** Formic Acid	**E4** Fumaric Acid	**E5** Glyoxylic Acid	**E6** α-Hydroxybutyric Acid	**E7** β-Hydroxybutyric Acid	**E8** Itaconic Acid	**E9** α-Ketobutyric Acid	**E10** α-Ketovaleric Acid	**E11** D,L-Lactic Acid	**E12** L-Lactic Acid
F1 D-Lactic Acid Methyl Ester	**F2** D-Malic Acid	**F3** L-Malic Acid	**F4** Propionic Acid	**F5** Pyruvic Acid	**F6** Pyruvic Acid Methyl Ester	**F7** D-Saccharic Acid	**F8** Succinamic Acid	**F9** Succinic Acid	**F10** Succinic Acid Mono-Methyl Ester	**F11** m-Tartaric Acid	**F12** Urocanic Acid
G1 L-Alaninamide	**G2** L-Alanine	**G3** L-Alanyl-L-Glutamine	**G4** L-Alanyl-L Histidine	**G5** L-Alanyl-L-Threonine	**G6** L-Asparagine	**G7** L-Glutamic Acid	**G8** L-Glutamine	**G9** Glycyl-L-Aspartic Acid	**G10** Glycyl-L-Glutamine	**G11** Glycyl-L Methionine	**G12** Glycyl-L-Proline
H1 L-Methionine	**H2** L-Phenylalanine	**H3** L-Serine	**H4** L-Threonine	**H5** L-Valine	**H6** L-Valine plus L-Aspartic Acid	**H7** 2'-Deoxy Adenosine	**H8** Inosine	**H9** Thymidine	**H10** Uridine	**H11** Thymidine-5'-Monophosphate	**H12** Uridine-5'-Monophosphate

Figure 2: AN Microplate Substrate Map

CHAPTER 5: RESULTS

Sanger Sequencing

The Sanger sequencing analysis was conducted on PCR-positive samples to identify the bacterial strains present in the preterm infant gut samples. The obtained sequencing data were subjected to a Nucleotide Blast Database search for species identification. Interestingly, diverse species of *Bifidobacterium* were isolated from different subjects at various time points. For instance, in subject 55, D.O.L. 14, colony C (55-14C) was identified as *Bifidobacterium longum subsp. infantis*, exhibiting a 98.12% percent identity with an accession length of 5,551 bases (see Table 2). Furthermore, in subject 36, Bifidobacterium was isolated from multiple colonies at four time points (see Table 3). At D.O.L. 6, colony C was identified as *Bifidobacterium pseudolongum subsp. globosum*, whereas at the same time point, colony E was identified as *Bifidobacterium longum subsp. suillum*. These findings underscore the diversity of *Bifidobacterium* species present in the preterm infant gut microbiota across different subjects and time points.

Table 2: Sanger Sequencing Results 1

Sample ID	Scientific Name	E value	Percent ID	Acc. length
55-14C	*Bifidobacterium longum subsp. infantis*	0	98.12%	5551
63-6G	*Bifidobacterium longum subsp. infantis*	0	93.00%	2781
63-17A	*Bifidobacterium breve*	0	100.00%	294
69-16A	*Bifidobacterium longum subsp. infantis*	0	90.05%	4170
69-20B	*Bifidobacterium breve*	0	99.20%	453
70-10A	*Bifidobacterium breve*	0	91.98%	2328
70-19A	*Bifidobacterium longum subsp. infantis*	0	81.14%	1718
75-20D	*Bifidobacterium longum subsp. infantis*	0	97.90%	5285
75-20A	*Bifidobacterium breve*	0	97.88%	407
75-20D	*Bifidobacterium breve*	0	98.41%	442
78-10B	*Bifidobacterium breve*	0	98.41%	442
78-10C	*Bifidobacterium breve*	0	98.02%	435
83-4A	*Bifidobacterium breve*	0	98.41%	438
83-13A	*Bifidobacterium longum*	0	95.44%	1271
88-14A	*Bifidobacterium breve*	0	88.57%	267
116-7A	*Bifidobacterium longum subsp. suillum*	0	92.47%	1120
117-5A	*Bifidobacterium longum*	0	91.48%	1109
117-9A	*Bifidobacterium longum*	0	91%	1215
119-14C	*Bifidobacterium longum*	0	98%	1369

Table 3: Sanger Sequencing Results 2

Sample ID	Scientific Name	E value	Per. Ident	Acc. Len
36-6C	*Bifidobacterium pseudolongum subsp. globosum*	0	98.80%	1526
36-6E	*Bifidobacterium longum subsp. suillum*	0	97.50%	1494
36-10D	*Bifidobacterium pseudolongum subsp. globosum*	0	93.88%	1526
36-16B	*Bifidobacterium longum subsp. suillum*	0	98.51%	1494
36-16C	*Bifidobacterium longum subsp. suillum*	0	97.01%	1494
36-16G	*Bifidobacterium longum subsp. suillum*	0	95.42%	1494
36-20H	*Bifidobacterium longum subsp. suillum*	0	98.45%	1494
36-20A	*Bifidobacterium longum subsp. suillum*	0	96.98%	1494
36-20C	*Bifidobacterium longum subsp. suillum*	5.00E-178	98.35%	1494
36-20F	*Enterococcus faecalis*	0	98.17	1483

Carbon Utilization

The results obtained from the AN Biolog plates revealed distinct metabolic profiles between the ATCC reference strain and the isolated strain 41-9E. The Biolog assay was run in triplicates to ensure that any observed metabolic activity is not due to random chance but is representative of consistent and reproducible patterns. The t-test was conducted using an Excel spreadsheet on the means of the three plates for the ATCC reference *B. breve* strain, comparing it to one of our isolated *B. breve* 41-9E strains, with a p-value threshold of <0.05 set for all included results (Table 3). Amongst the 95 carbon sources, these exhibited the most statistical significance. Additionally, given the visual nature of the test, a cutoff of 0.5 was established based on a comparison of visually negative plates. In the investigation of different carbon source utilization using the AN Biolog plates, out of all the carbon sources examined, 77% of the carbon sources were shared but 9% of the carbon were specific to the isolate sample and 4% were specific to the lab reference strains (Figure 3). The ATCC strain exhibited positive reactions for a range of substrates, including D-Sorbitol, D-Galactose, D-Mannitol, β-Methyl-D-Glucoside, Glycerol, Maltotriose, β-Hydroxybutyric acid, β-Methyl-D-Galactoside, N-Acetyl-D-Galactosamine, Sucrose, Dextrin, Pyruvic Acid Methyl Ester, and Salicin. In contrast, strain 419E displayed some different metabolic activities, with positive reactions observed for D-Trehalose, Uridine, Palatinose, Amygdalin, Gentibiose, N-Acetyl-D-Glucosamine, α-Methyl-D-Glucoside, D-Galacturonic acid, and Glucose-6-Phosphate. These findings indicate variations in substrate utilization and metabolic capabilities between the two strains of *B. breve* species.

Table 4: Carbon Utilization of Reference B. breve ATCC strain vs B. breve Sample XM0800_41-9E strain

carbon source	*B. breve* ATCC	XM0800_41-9E	p value (2 tail)
D-Sorbitol	+	-	0.0019
D-Galactose	+	-	0.0089
D-Mannitol	+	-	0.0094
β-Methyl-D-Glucoside	+	-	0.0096
Glycerol	+	-	0.0117
Maltotriose	+	-	0.0128
β-Hydroxybutyric acid	+	-	0.0164
β-Methyl-D-Galactoside	+	-	0.0168
N-Acetyl-D-Galactosamine	+	-	0.0242
Sucrose	+	-	0.0300
Dextrin	+	-	0.0303
Pyruvic Acid Methyl Ester	+	-	0.0323
Salicin	+	-	0.0532
D-Trehalose	-	+	0.0004
Uridine	-	+	0.0018
Palatinose	-	+	0.0080
Amygdalin	-	+	0.0010
Gentibiose	-	+	0.0119
N-Acetyl-D-Glucosamine	-	+	0.0288
α-Methyl-D-Glucoside	-	+	0.0332
D-Galacturonic acid	-	+	0.0517
Glucose-6-Phosphate	-	+	0.0547

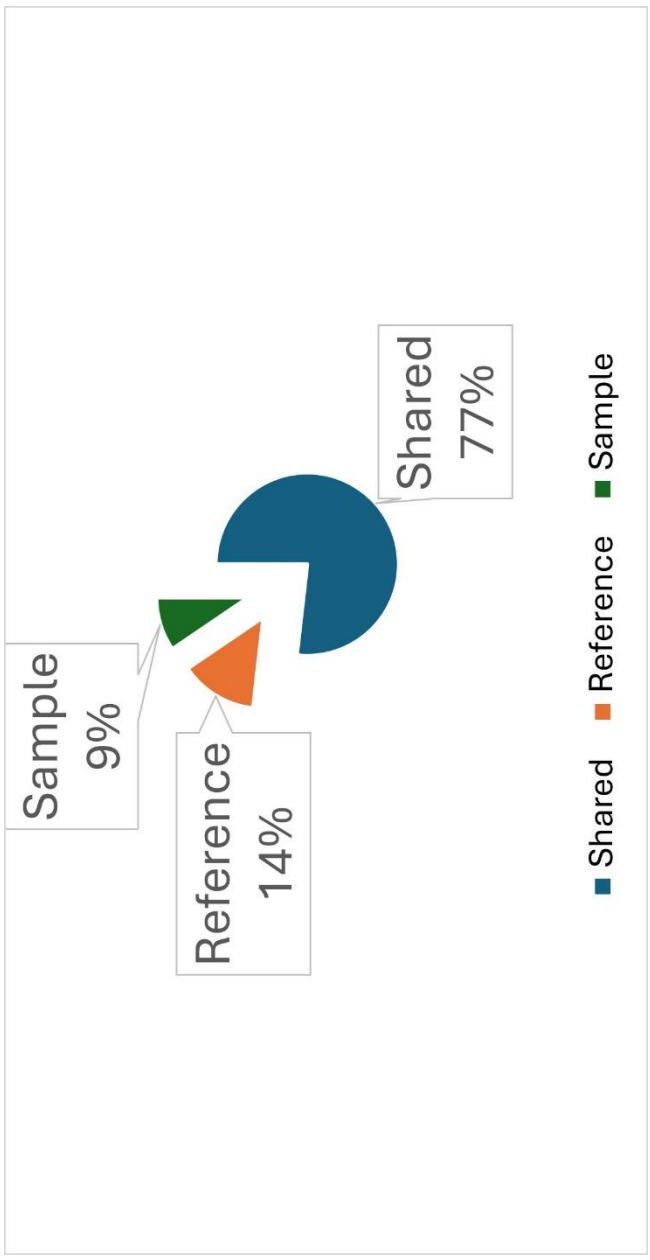

Figure 3: B. breve Strain-specific Carbon Source Utilization Summary

Whole Genome Sequencing

Using standard operating protocols, the sequencing was conducted at the Institute for Genome Sciences, University of Maryland School of Medicine, Genomics Resource Center (http://www.igs.umaryland.edu/resources/grc) on Illumina nova seq 6000 platform. After which the sequencing data was compiled and simplified into tables. The samples analyzed in this study exhibit diverse genomic characteristics. The reference sample, *Bifidobacterium longum* ATCC 15707, possesses a genome consisting of 2,385,164 bases with a GC content of 60.5%, obtained from the NCBI Taxonomy Database. Comparable features were observed in our isolated samples: XM0006_36_6 contains 77 contigs spanning 2,399,575 bases with a GC content of 59.96%, XM0558_36A has 83 contigs covering 2,401,720 bases with a GC content of 59.97%, and XM0558_36D comprises 82 contigs spanning 2,400,410 bases with a GC content of 59.96%. These genomes contain various coding sequences (CDS), genes, tRNA, and 16s rRNA (Table 4), determined through standard sequencing and annotation methods.

Similarly, for *Bifidobacterium breve* genomes, using *Bifidobacterium breve* ATCC 15700 as reference, genomic comparisons were conducted. XM0404_44D's genome consists of 2 contigs totaling 2,351,221 bases with a GC content of 58.78%, while XM0404_44F comprises 38 contigs spanning 2,343,350 bases with a GC content of 58.8%. XM0404_44G's genome encompasses 2,607 contigs covering 4,116,118 bases with a GC content of 53.57%. XM0800 comprises 22 contigs totaling 2,402,749 bases with a GC content of 58.75%, and XM0880_42E contains 33 contigs spanning 2,406,297 bases with a GC content of 58.78%. Other samples, such as XM1446, XM1446_20231220, XM1948_63I, XM1950, XM1950_2, XM5232_116A2,

XM6340_134B2, XM5232_116D2, and XM6822_142A2, display similar diverse genomic features (See Table 5).

Table 5: Whole genome assembly stats for B. longum samples

Sample	**Length/Bases**	**Contigs**	**N50**	**%GC**	**CDS**	**Genes**	**tRNA**	**16s rRNA**
***B. longum* ATCC 15707**	2,385,164	2	1	60.5	1,940	2033	77	4
XM0006_36_6	2399575	77	10	59.96 %	2,001	2,074	59	2
XM0558_36A	2,401,720	83	10	59.97 %	2,002	2,075	59	2
XM0558_36D	2,400,410	82	10	59.96 %	2,005	2,078	59	2

Table 6: Whole genome assembly stats for B. breve samples

Sample	Length/Bases	Contigs	N50	%GC	CDS	Genes	tRNA	16 rRNA
***B. breve* ATCC 15700**	2,275,660	1	1	58.89 %	1928	2004	54	2
XM0404_44D	2351221	2	2	58.78 %	2011	2082	54	2
XM0404_44F	2343350	38	2	58.8%	1995	2067	55	2
XM0404_44G	4116118	2607	4	53.57 %	3425	3533	77	5
XM0800	2402749	22	3	58.75 %	2059	2128	54	2
XM0880_42E	2406297	33	3	58.78 %	2069	2138	54	2
XM1446	2358717	70	3	58.55 %	1950	2020	53	2
XM1446_20231220	2349961	32	2	58.73 %	1952	2021	52	4
XM1948_63I	2489605	59	5	58.7%	2174	2250	58	3
XM1950	2486029	48	6	58.69 %	2172	2248	58	3
XM1950_2	2492192	53	5	58.66 %	2178	2254	58	3
XM5232_116A2	2398769	35	3	58.71 %	2072	2142	55	2
XM6340_134B2	2313581	38	2	58.86 %	1930	2000	53	2
XM5232_116D2	2398520	39	3	58.4%	2075	2146	56	2
XM6822_142A2	2417643	45	4	58.01 %	2071	2140	54	2

Whole Genome Alignment

Using Mauve, a program for visualizing contigs in genomic sequences, we were able to obtain a better understanding of the genome's composition. First, we independently aligned each genome sequence using the reference strains from the ATCC lab (*B. breve* ATCC 15700 and *B. longum* ATCC 15707) because each genome sequence

included a disorganized order of contigs. Next, we concurrently compared all our alignments. Contigs are separated by vertical red line. Interestingly, notable differences were found in the *B. breve* strain alignments between subjects as visualized in Figure 4. On the other hand, contig patterns were identical in the alignments of *B. longum* strains, all from subject 36 and sampled at various times (XM0006 on day of life 6 and XM0558 on day of life 15) seen in Figure 5.

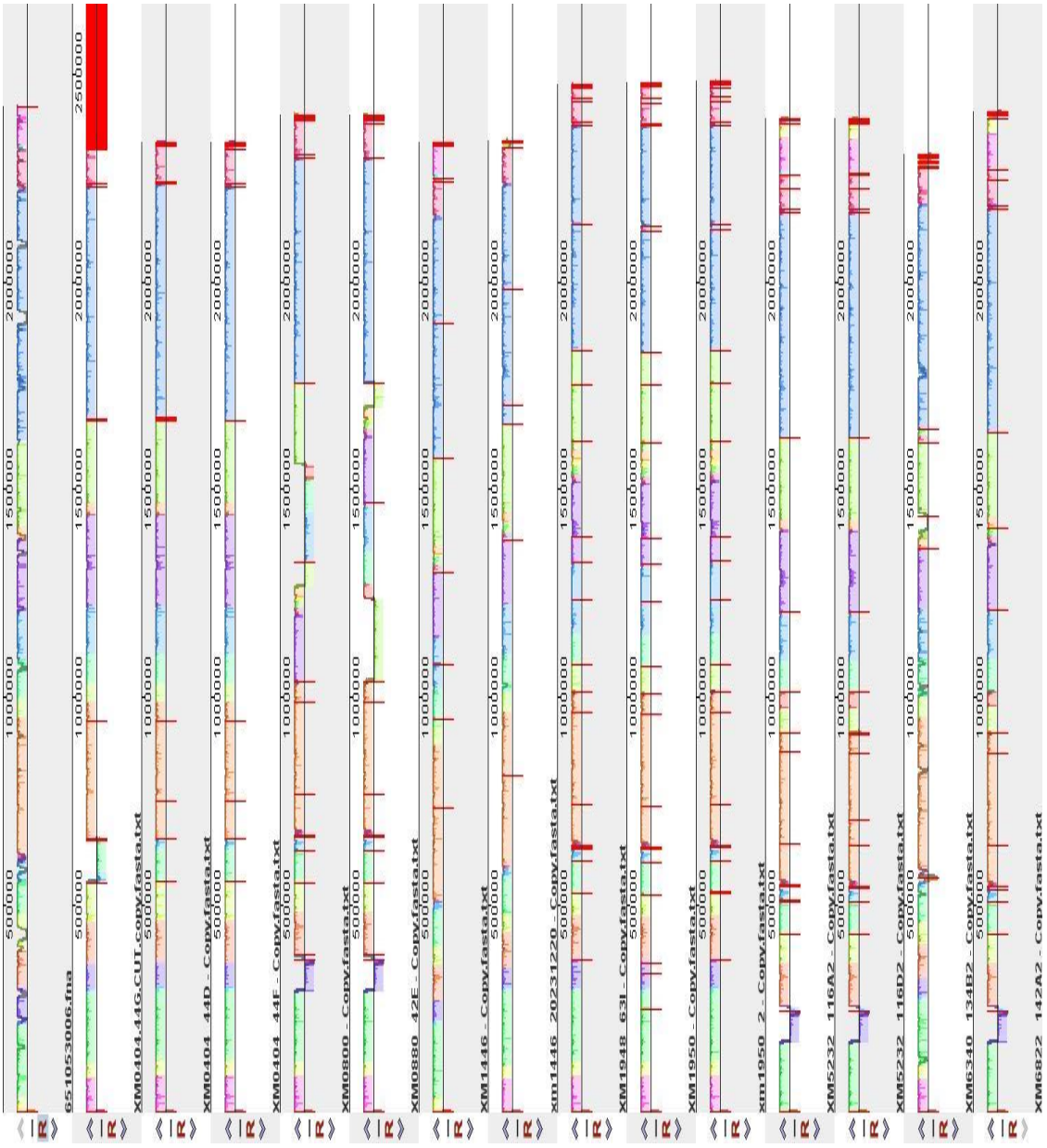

Figure 4: B. breve Alignments

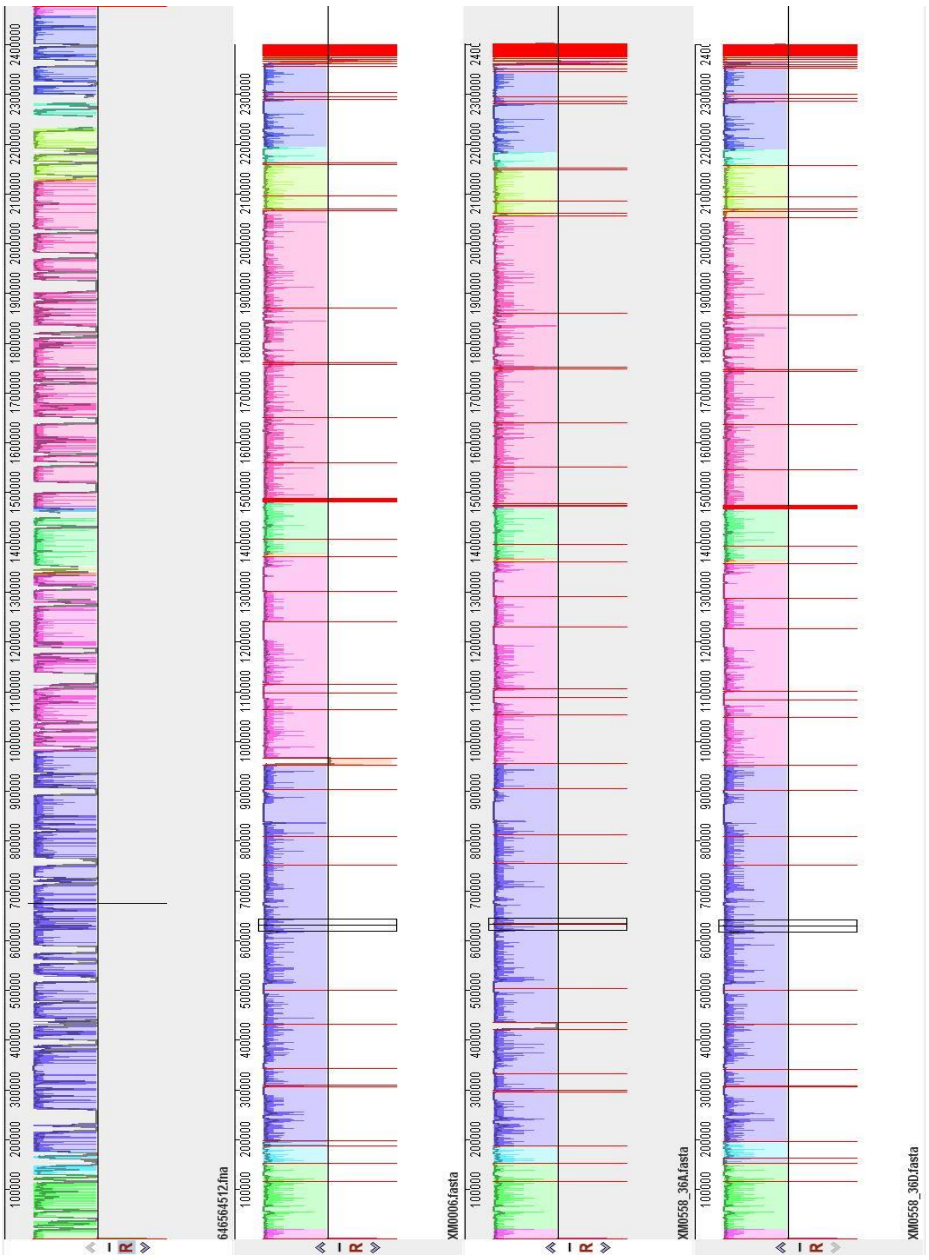

Figure 5: B. longum Alignments

Function Annotation of Protein Coding Genes

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database provides the BlastKOALA tool for functional annotation of protein-coding genes. This was applied to gain a better understanding of the protein coding genes. Protein sequences for each of our samples were submitted and compared to a database of known proteins from various prokaryotes using BLAST. BlastKOALA can indicate what functions certain genes may have based on similarity with known genes. A summary of the data acquired can be found in Table 8. Additionally functional categories than had 15 and less along with unclassified ones were group together in the final row of Table 8.

Table 7: Functional Categories Summary

Functional Category	***Bl* ATCC 15707**	***Bl* XM0006_36**	***Bb* ATCC 15700**	***Bb* XM0800_41-9E**	***Bb* XM0404_44G**
Protein families: signaling and cellular processes	136	150	147	145	184
Genetic information processing	146	142	139	147	181
Protein families: genetic information processing	132	129	132	134	177
Carbohydrate metabolism	114	118	130	136	188
Environmental information processing	71	104	90	91	107
Amino acid metabolism	82	80	79	79	97
Nucleotide metabolism	49	50	51	51	58
Unclassified metabolism	52	47	43	44	57
Metabolism of cofactors and vitamins	38	46	39	39	46
Protein families: metabolism	28	29	27	29	36
Energy metabolism	22	22	24	22	26
Cellular processes	20	21	26	32	23
Glycan biosynthesis and metabolism	16	19	18	19	23
Unclassified/other	102	104	106	101	137

CHAPTER 6: DISCUSSION

The investigation into the microbial composition of samples obtained at different time points revealed intriguing patterns. Notably, there was a substantial increase in *Bifidobacterium* growth during the early days of infant life, aligning with previous findings. To achieve our first objective of isolating *Bifidobacterium* in our low-IP associated samples, we employed a comprehensive screening procedure. Initially, selective media, including BSM broth and BSM agar, were utilized to grow and isolate *Bifidobacterium*. However, due to the non-specificity of the medium, occasional growth of other bacteria like *Enterococcus* and *Streptococcus* occurred. Subsequently, a 3-tier primer test and gram stain analysis were employed. A *Bifidobacterium* genus primer was initially utilized, followed by species-specific *Bifidobacterium* primers for further investigation. The accurate identification of individual colonies through subsequent DNA analysis using species-specific primers revealed the diversity of *Bifidobacterium* species in our sampling population, including *Bifidobacterium breve* and *Bifidobacterium longum*. Sanger sequencing confirmation testing validated these results, confirming the presence of several *Bifidobacterium* species. Despite our efforts some non-*Bifidobacterium* were isolated and sequenced. The exact cause of DNA contamination remains unknown but is speculated to be due to poor isolation.

The utilization of Biolog technology allowed us to achieve the second objective of understanding the carbon utilization attributes of *Bifidobacterium*. Variations in the phenotypic profile between the ATCC reference strain and the isolated strain 41-9E provided valuable insights into the physiological and adaptive characteristics of *Bifidobacterium* strains from diverse environments. These variations were observed even

within the same species of *Bifidobacterium* from various sources, evident in the comparison between the lab reference strain and our strain isolated from our sample stool, which is often a nutrient-deficient environment of preterm infants. The comprehensive analysis of metabolic activities laid the groundwork for further investigations into the metabolic pathways and adaptive mechanisms employed by microbial communities, contributing to our understanding of *Bifidobacterium*'s niche adaptive capability. Significant differences were observed in both the carbon utilization and genomic content of our isolated samples compared to the reference strains.

We utilized whole genome sequencing to establish our third and final objective of defining the genomic content of *Bifidobacterium***.** The sequencing unveiled variation in our different strain's genomes, highlighting genetic variations among distinct species of *Bifidobacterium* and even among different strains of the same species, as evidenced by differing contigs present in our samples. This highlights a distinctive feature of *Bifidobacterium*, indicating a notable plasticity enabling them to effectively colonize and function within specialized niches, such as those observed in preterm infants. Further investigation is warranted to delve deeper into the insights from genomic contents, paving the way for a better understanding of the role of *Bifidobacterium* in neonatal gut health.

CHAPTER 7: CONCLUSION

Our study confirms the need for different methods to isolate *Bifidobacterium* and shows how diverse they are in their genotypic and phenotypic traits. Notably, our data suggest that these variations extend beyond species-level distinctions to differences at the strain level. We found that each strain has its own unique ability to use different carbon sources. This suggests that their genetic makeup makes them well-suited to their specific environments, even within the same species. Our speculation suggests that these adaptations help them survive in various places, like the guts of premature babies where nutrients are limited, and antibiotics may be common. By elucidating the mechanisms underlying the niche adaptative capabilities of *Bifidobacterium*, our study enhances our understanding of the complex relationship between *Bifidobacterium* and its environment but also holds promise in understanding how it can metabolize breast milk and promote health in preterm infants. Moving forward, continued research efforts aimed at unraveling the genomic and metabolic intricacies of *Bifidobacterium* will be essential for advancing neonatal care and improving outcomes for this vulnerable populations.

CHAPTER 8: LIMITATION

While this study offers valuable insights, several limitations must be acknowledged. A primary challenge arose in isolating pure *Bifidobacterium* samples from stool specimens. Despite our efforts, many colonies presumed to be pure were found to contain traces of other bacterial DNA, such as *Enterococcus faecalis,* rendering them unsuitable for analysis. Implementing additional measures, such as conducting multiple streaking for isolation protocols (>4 plates) may enhance this process.

Furthermore, the small sample size utilized in this study may limit the generalizability of the findings. Future investigations would benefit from a larger sample size to bolster statistical robustness. Lastly, while genomic statistics and comparisons provides valuable insights into the capabilities of *Bifidobacterium,* the functional aspect of the protein coding genes present in the genome need further elucidation to fully understand their role in promoting intestinal barrier maturation and metabolizing HMOs. Despite these limitations, this study serves as a foundational step towards better understanding the role of *Bifidobacterium* in neonatal intestinal maturation.

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