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Gut Microbiota structure and ESBL-*Enterobacteriaceae* colonization in hospitalized versus healthy children

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DEDICATION

I dedicate this thesis to my beloved parents, whose unconditional love, endless sacrifices, prayers, and unwavering support have always been the source of my strength and success.

To my beloved husband, for his patience, encouragement, understanding, and continuous support throughout this journey.

This achievement is dedicated to you with all my love and gratitude.

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ABSTRACT

Background: The intestinal microbiota plays a critical role in host health, particularly during early life when microbial colonization and immune development are still evolving. Disruption of this ecosystem (dysbiosis) has been increasingly linked to gastrointestinal disease and the emergence of antimicrobial-resistant organisms. Among these, extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBL-EB) are of particular concern due to their capacity for persistent gut colonization and transmission.

Methods: This study investigated the intestinal microbiota composition of young children (≤3 years) with acute gastroenteritis compared with healthy controls. Fecal samples were analyzed using 16S rRNA gene–based approaches to characterize key bacterial taxa and assess differences in microbial diversity, structure, and abundance between groups.

Results: Hospitalized children exhibited marked alterations in gut microbiota composition, including depletion of beneficial commensal taxa such as *Faecalibacterium* and members of the phylum *Bacillota*, alongside changes in *Bacteroides*-associated populations. Opportunistic and potentially pathogenic bacteria, particularly within the *Enterobacteriaceae* family, were consistently enriched. In contrast, probiotic-associated genera, including *Bifidobacterium and Lactobacillus,* showed variable patterns across samples without a consistent directional change, suggesting inter-individual variability in their abundance. ESBL colonization was associated with compositional differences in the microbial community, characterized by shifts toward facultative anaerobes and reduced representation of taxa associated with colonization resistance.

Conclusion: These findings indicate that indicate that alterations in the gut microbiota in early childhood is closely associated with susceptibility to both gastrointestinal disease and colonization by antimicrobial-resistant organisms. Changes of key butyrate-producing and barrier-supporting taxa may facilitate the persistence of ESBL-producing *Enterobacteriaceae*. This study provides insights into the compositional and ecological differences of the pediatric gut microbiome during hospitalization and highlights the potential relevance of microbiota-targeted approaches, including dietary modulation and probiotic interventions, in the context of antimicrobial resistance.

Keywords:

Gut microbiota; Extended spectrum Beta Lactamase (ESBL); *Enterobacteriaceae*; Gastroenteritis; Dysbiosis, Multi drug resistant bacteria (MDR).

الملخص

المقدمة :تلعب الميكروبيوتا المعوية دورًا حاسمًا في الحفاظ على صحة العائل، لا سيما خلال مرحلة الطفولة المبكرة حيث لا تزال عملية الاستعمار الميكروبي وتطور الجهاز المناعي في طور التشكّل. وقد ارتبط اضطراب هذا النظام البيئي المعقّد (المعروف باختلال التوازن الميكروبي) بشكل متزايد بالأمراض المعوية وبظهور الكائنات المقاومة للمضادات الحيوية. ومن بين هذه الكائنات، تُعد البكتيريا المعوية المنتجة لإنزيمات البيتا-لاكتاماز واسعة الطيف (ESBL-EB) مصدر قلق صحي عالمي نظرًا لقدرتها على الاستعمار المستمر في الأمعاء وإمكانية انتقالها.

الاهداف :هدفت هذه الدراسة إلى تحليل تركيب الميكروبيوتا المعوية لدى الأطفال الصغار (≥3 سنوات) المصابين بالتهاب المعدة والأمعاء ومقارنتها بأقرانهم الأصحاء، من خلال فحص عينات البراز باستخدام تقنيات التسلسل المعتمدة على جين S rRNA16 لتوصيف المجتمعات البكتيرية المعوية الرئيسية، وتقييم الاختلافات في التنوع الميكروبي والتركيب البنيوي والوفرة النسبية للأنواع البكتيرية بين المجموعتين، بهدف فهم التغيرات المرتبطة بالمرض ودورها المحتمل في صحة الجهاز الهضمي لدى الأطفال.

النتائج :أظهرت النتائج حدوث تغيّرات ملحوظة في تركيب الميكروبيوتا المعوية لدى الأطفال المرضى في المستشفى، تمثلت في انخفاض البكتيريا المفيدة مثل*Faecalibacterium*وأعضاء شعبة*Bacillota*، إلى جانب زيادة عدم الاستقرار في تجمعات .Bacteroides كما لوحظ ارتفاع مستمر في البكتيريا الانتهازية والممرضة المحتملة، خاصةً ضمن عائلة .Enterobacteriaceae في المقابل، أظهرت الأجناس المرتبطة بالبروبيوتيك مثل*Bifidobacterium*و*Lactobacillus*أنماطًا متباينة، مما يشير إلى استجابات بيئية معقّدة بدلاً من تأثيرات وقائية بالكائنات مباشرة. وارتبط الاستعمار ببكتيريا ESBL باضطراب في البنية الميكروبية، تميّزت بانخفاض مقاومة الاستعمار وزيادة سيطرة البكتيريا اللاهوائية الاختيارية.

الاستنتاج: تُظهر هذه النتائج أن اختلال توازن الميكروبيوتا في مرحلة الطفولة المبكرة يرتبط بشكل وثيق بزيادة القابلية للإصابة بالأمراض المعوية والاستعمار بالبكتيريا المقاومة للمضادات الحيوية. كما أن فقدان الأنواع البكتيرية المنتجة للبيوتيرات والداعمة لحاجز الأمعاء قد يسهم في استمرار بقاء البكتيريا المنتجة لإنزيمات .ESBL وتوفر هذه الدراسة فهمًا أعمق للديناميكيات البيئية للميكروبيوم المعوي لدى الأطفال، وتدعم تطوير استراتيجيات علاجية تستهدف تعديل الميكروبيوتا، بما في ذلك التدخلات الغذائية واستخدام البروبيوتيك، للحد من انتشار مقاومة المضادات الحيوية.

LIST OF ABBREVIATIONS

Abbreviation	Full Term
3CG	Third generation cephalosporin
C°	Celsius degree
CFU	Colony forming unit
CTX	Cefotaxime
EB	Enterobacteriaceae
ESBL	Extended-spectrum beta-lactamases
FOS	Fructo-oligosaccharides
G	Gram
Hr	Hour
J	Junaidi
L.B	Luria-Bertani (LB) broth
LAB	Lactic acid bacteria
Mac	MacConkey
MDR	Multiple drug resistance
Min	Minute
Ml	Milliliter
µl	Microliter
OD	Optical density
ONPG	O-nitrophenyl-β-D-galactopyranoside
P	Probiotics
SCFA	Short-chain fatty acids
SM	Skim milk
T	Titer

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CHAPTER ONE: INTRODUCTION

1.1 Overview of the Human Gut Microbiome

The human gut microbiota represents a highly complex and dynamic microbial ecosystem that is continuously adapting to intrinsic and extrinsic influences, including age, diet, health status, and environmental influences, and composed of bacteria, viruses, fungi, and archaea, collectively known as the gut microbiota. It plays a central role in maintaining host physiological homeostasis, including digestion, metabolic activity, immune system maturation, and protection against pathogenic colonization (Zeng et al., 2025).

In healthy individuals, the gut microbiota is characterized by high diversity and a functional stability, with dominant bacterial phyla, primarily *Bacillota (formerly Firmicutes*) and *Bacteroidota (formerly Bacteroidetes*), alongside contributions from *Actinobacteriota, Proteobacteria, and Verrucomicrobiota* (Almeida et al., 2019). The balance between these microbial communities is essential for maintaining intestinal health. Disruptions of this equilibrium, known as dysbiosis, is associated with multiple pathological conditions, including increased susceptibility to infection and colonization by antimicrobial-resistant organisms. Dysbiosis may result from antibiotic exposure, dietary changes, infections, or environmental and lifestyle factors. Therefore, understanding these microbial dynamics is essential for elucidating host–microbiota interactions in health and disease (Lynch & Pedersen, 2016).

The intestinal microbiota of healthy children constitutes a progressively maturing ecosystem that is central to immune development, nutrient metabolism, and maintenance of intestinal homeostasis. In early life, microbial communities undergo rapid succession before stabilizing into a more complex and resilient adult-like configuration dominated by obligate anaerobes such as Bifidobacterium and Bacteroides, together with members of *Bacillota* (Lynch & Pedersen, 2016; Thursby & Juge, 2017). These taxa contribute to fermentation of indigestible carbohydrates and production of short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, which support epithelial barrier integrity and immune regulation (Buffie & Pamer, 2013). A key feature of this ecosystem is colonization resistance, which

prevents pathogen expansion through nutrient competition, niche exclusion, antimicrobial metabolite production, and host immune modulation (Lozupone et al., 2012).

1.2 Development of the Infant Gut Microbiota

The establishment of the infant gut microbiota is a dynamic, sequential process shaped by perinatal and early-life environmental exposures, including delivery mode, feeding practices, antibiotic exposure, surrounding environmental conditions, and social and cultural practices (Chichlowski, 2023; Pantazi, 2023) (**Figure 1**).

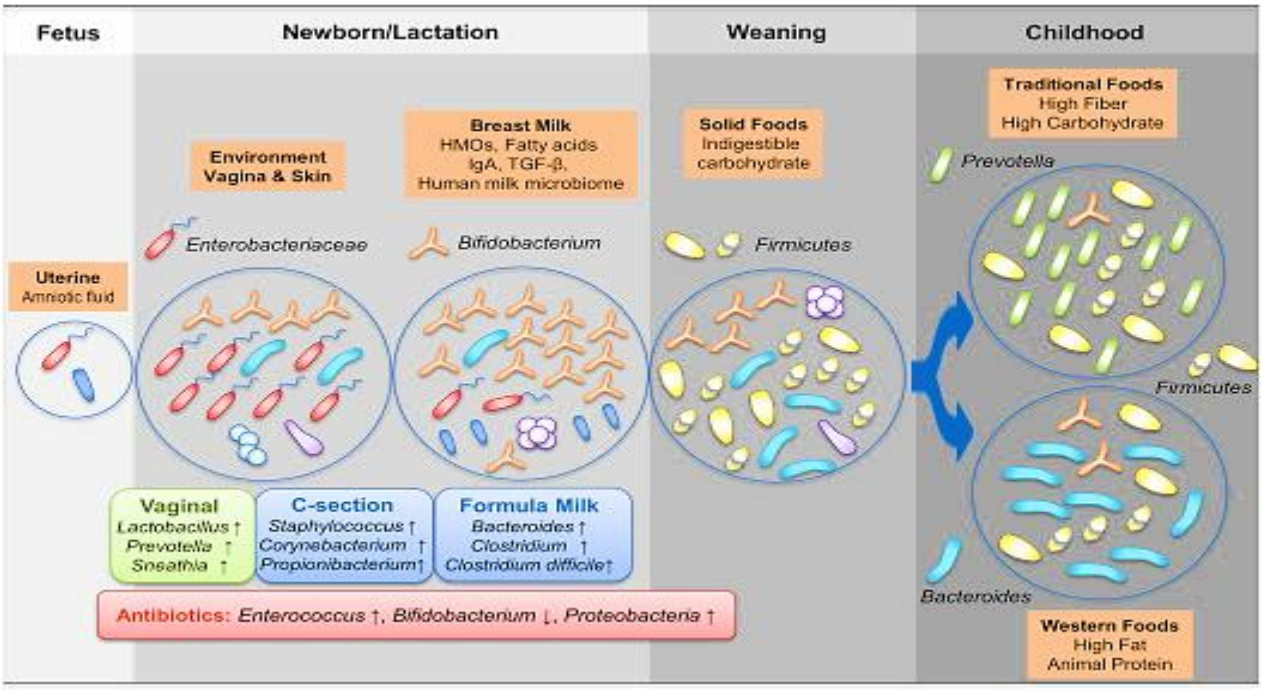

Figure 1. Timeline illustrating the development and succession of major gut microbiota taxa in healthy infants during the first two years of life.

At birth, microbial colonization is strongly influenced by delivery mode. Vaginally delivered infants acquire maternal vaginal and fecal microbiota, dominated by *Bifidobacterium and Lactobacillus* species, whereas cesarean-delivered infants are initially colonized by skin-associated taxa such as *Staphylococcus and Corynebacterium* (Chichlowski, 2023).

Feeding practices further shape microbial development. Breastfeeding promotes the expansion of *Bifidobacterium* through human milk oligosaccharides (HMOs), enhancingshort-chain fatty acids (SCFAs) production, supporting intestinal barrier integrity, and suppress pathogens expansion. In contrast, formula feedingis associated with increased microbial diversity but higher abundance of facultative anaerobes, including *Enterobacteriaceae* (Wu et al., 2025).

With the introduction of solid foods, the microbiota undergoes further maturation. characterized by increased abundance *of Bacteroidota and Bacillota* and the emergence of key functional taxa such as *Faecalibacterium, Akkermansia, and Prevotella*. This transition supports metabolic maturation, immune development, and microbial stability. Disruption during this critical window, particularly through antibiotic exposure or inadequate nutrition, may result in persistent dysbiosis and long-term impairment of immune and metabolic homeostasis (Pantazi, 2023; Hickman et al., 2024).

Early-life microbial development is highly sensitive, and disturbances during this period may lead to long-lasting alterations in microbial resilience, reduced colonization resistance, and increased susceptibility to antimicrobial-resistant organisms (Reyman et al., 2022).

1.3 Common taxa involved in the human health

1.3.1 *Lactobacillus*

Lactobacillus species are aerotolerant, lactic acid–producing bacteria that contribute to gut homeostasis by producing lactic acid, hydrogen peroxide, and bacteriocins, thereby limiting pathogen growth (Ouwehand et al., 2002). These bacteria also interact with epithelial and immune cells, enhancing mucosal immunity and modulating host responses (Zheng et al., 2020). Certain *Lactobacillus* strains inhibit colonization by extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae through competitive exclusion and microbiota restoration following antibiotic exposure (Djukovic et al., 2022).

1.3.2 ***Bacteroides***

Members of the genus *Bacteroides* (phylum *Bacteroidota*) are among the most abundant obligate anaerobes in the human gut and play important roles in carbohydrate metabolism, short-chain fatty acid (SCFA) production, and immune modulation. Through these activities, *Bacteroides* species contribute substantially to metabolic homeostasis and the maintenance of intestinal barrier integrity. Although generally regarded as beneficial commensals, some Bacteroides species harbor antimicrobial resistance genes, including β-lactamases, and may serve as reservoirs of resistance determinants within the gut ecosystem (Zhu et al., 2021). *Bacteroides* also are thought to contribute to gut ecosystem functioning by maintaining anaerobic conditions and competing for dietary substrates. Reduction of *Bacteroides* populations has been associated with changes in colonization resistance and with increased abundance of *Enterobacteriaceae* (Palleja et al., 2018).

In dysbiotic states associated with colonization by multidrug-resistant organism, significant reductions in *Bacteroides* and *Faecalibacterium* have been observed. Such depletion is thought to impair colonization resistance, thereby facilitating the persistence of ESBL colonization (Palleja et al., 2018). *Prevotella* species, along with other members of the *Bacteroidota* phylum, are commonly associated with high-fiber diets and are specialized in fermenting complex polysaccharides. Through this activity, they produce metabolites such as succinate and acetate, which can influence both local immune responses and systemic metabolic pathways (Jiang et al., 2022).

Their role in colonization resistance against *Enterobacterales* is complex and context-dependent. On one hand, competition for carbohydrate substrates may help limit the growth of *Enterobacterales*. On the other hand, excessive succinate production, particularly by *Prevotella copri*, has been linked to pro-inflammatory signaling, including interleukin 18activation, which may favor pathogen colonization (Iljazovic et al., 2021; Abdelsalam, 2023). Therefore, the overall impact of Prevotella depends on dietary composition, host immune status, and interactions with existing microbial taxa.

1.3.3 ***Muribaculum***

Muribaculum, within the family *Muribaculaceae*, is Obligate anaerobes bacteria that contributes to the degradation of complex polysaccharides and production of SCFAs such

as propionate and butyrate (Zhu et al., 2024). These metabolites support epithelial integrity and immune regulation. *Muribaculum* interacts with taxa such as *Bifidobacterium and Faecalibacterium* through cross-feeding and may limit *Enterobacteriaceae* expansion by competition for nutrients. Antibiotic exposure, particularly β-lactams, reduces *Muribaculum* abundance, potentially facilitating colonization by resistant Enterobacteriaceae (Zhu et al., 2024).

1.3.4 ***Akkermansia***

Akkermansia muciniphila is a highly specialized anaerobic bacterium that predominantly inhabits the mucus layer of the large intestine, where it plays a vital role in maintaining intestinal homeostasis. By degrading host derived mucin, *A. muciniphila* generates metabolites that serve as a substrate for other commensal taxa, reinforce epithelial barrier function, modulate immune signaling pathways, and influence host metabolic regulation (Gao et al., 2025). Collectively, these activities support the stability of the gut ecosystem, and enhance colonization resistance against opportunistic pathogens.

Emerging studies indicate that alterations in *Akkermansia* abundance may be associated with susceptibility to colonization by multidrug-resistant bacteria, including ESBL-producing *Enterobacterales.* Reduced levels of *A. muciniphila* have been associated with changes in mucosal integrity and microbial competition, potentially creating conditions that may support persistence and expansion of resistant strains (Zhang et al., 2025). Although the underlying mechanisms remain incompletely defined*, Akkermansia* may indirectly be involved in the colonization dynamics of ESBL-producing bacteria by contributing to gut ecosystem homeostasis and interacting with host defensive processes.

This role is particularly relevant in clinical and early-life contexts. In hospitalized patients or individuals exposed to antibiotics, disruptions to gut microbial communities often coincide with decreased *A. muciniphila* abundance, which may exacerbate vulnerability to colonization by resistant pathogens. Similarly, in infants, the establishment and stability of *Akkermansia* populations contributes to the maturation of mucosal barrier and the development of microbial resilience, potentially influencing long-term susceptibility to opportunistic and antibiotic-resistant organisms. Together, these observations highlight the importance of preserving *Akkermansia* to maintain gut integrity and mitigate the risks posed by ESBL-producing *Enterobacterales* (Zhang et al., 2025).

1.3.5 ***Bacillota (Firmicutes)***

The phylum *Bacillota* includes a diverse array of bacteria with critical roles in gut health, notably those capable of producing butyrate and acetate. Butyrate- producing members of this phylum provide essential energy substrates for colonocytes, promote regulatory T-cell differentiation, and maintain anti-inflammatory intestinal conditions that suppress the expansion of opportunistic *Enterobacterales* (Lenoir et al., 2020; Fusco et al.,2023).Loss or reduction of butyrogenic *Bacillota* is frequently observed in individuals with elevated ESBL carriage, highlighting the importance of these bacteria in maintaining ecological barriers against multidrug-resistant organisms. The phylum *Bacillota* comprises a diverse group of bacteria that play a fundamental role in gut health, particularly through the production of short chain fatty acids such as butyrate and acetate.

Key butyrate-producing taxa of this phylum are predominantly found within the families *Ruminococcaceae, Lachnospiraceae, and Eubacteriaceae*, including well-characterized genera such as *Faecalibacterium, Roseburia, and Eubacterium*.

These bacteria supply essential energy to colonocytes, support regulatory T-cell differentiation, and contribute to intestinal anti-inflammatory environment, which suppress the expansion of opportunistic *Enterobacterales*. (Hertli& Zimmermann, 2022; Snodgrass & Velayudhan, 2026). Loss or reduction of butyrogenic *Bacillota* is frequently observed with increased carriage of extended-spectrum β-lactamase (ESBL)-producing organisms, highlighting the importance of these bacteria in maintaining ecological barriers against multidrug-resistant organisms (Buffie & Pamer, 2013; Litvak et al., 2018) (Hertli& Zimmermann, 2022; Snodgrass & Velayudhan, 2026).

1.3.5.1. *Faecalibacterium*

Faecalibacterium prausnitzii is anaerobic butyrate producing bacterium and a well-established marker of a healthy, anti-inflammatory gut microbiome. Its metabolic outputs enhance epithelial health, promote regulatory immune pathways, and suppress inflammation, thereby reinforcing colonization resistance against opportunistic *Enterobacter*ales (Lenoir et al., 2020; Zheng et al., 2025). Consistent reductions in *Faecalibacterium* abundance have been observed in dysbiotic microbiomes associated with ESBL carriage, reinforcing its protective role against expansion of against resistant pathogens (Lenoir et al., 2020; Zheng et al., 2025).

*1.3.5.2.**Enterococcus***

Enterococcus species are facultative anaerobic bacteria that constitute natural members of the gastrointestinal microbiota of humans and animals*. T*, but their role is highly context-dependent. Under balanced conditions, commensal strains contribute to colonization resistance through the production of bacteriocins and lactic acid, which inhibit Gram-negative bacteria, including members of the *Enterobacteriaceae* family (Foulquié-Moreno et al., 2020). Additionally, *Enterococcus faecium* has been shown to modulate mucosal immunity and enhance IgA production, supporting health defenses. (Hanchi et al., 2018).

Conversely, hospital-associated *Enterococcus,* particularly vancomycin resistance *Enterococcus faecium* (VRE), represent major nosocomial pathogens, and important reservoir of transferable resistance genes (Arias & Murray, 2012). *Enterococcus* can disseminate resistance genes to *Enterobacteriaceae* through horizontal gene transfer mechanisms such as conjugative plasmids; and increase the resistance burden. (Lebreton et al., 2017; Cattoir& van Tyne, 2020).

1.3.6 ***Bifidobacterium***

Species of the genus *Bifidobacterium* species represent hallmark commensals of the infant gut. *Bifidobacterium* spp. are anaerobic to aerotolerant anaerobic bacteria that efficiently metabolize human milk oligosaccharides (HMOs), producing acetate and other metabolites that lower intestinal pH and inhibit the growth of opportunistic and pathogenic

bacteria. Infant-associated *Bifidobacteria* species, including B. *longum* subspecies, enhance SCFA production, strengthen mucosal defenses, and are consistently associated with reduced abundance of antimicrobial resistance genes in infants.

Conversely, early life depletion of *Bifidobacterium*, such as that induced by antibiotic exposure, correlates with impaired vaccine responses and increased risk of ESBL colonization (De Bruyn et al., 2024; Yang et al., 2024). These findings emphasize the foundational role of *Bifidobacterium* in shaping immune competence and colonization resistance during early development.

1.3. 7. ***Enterobacteriaceae***

The family *Enterobacteriaceae* comprises a wide spectrum of bacteria ranging from commensal *Escherichia coli* strains to highly pathogenic species like *Klebsiella pneumoniae* and *Enterobacter cloacae*. Under homeostatic conditions, their abundance is tightly regulated by colonization resistance mediated by obligate anaerobes and by the acidic environment generated by SCFAs (Gay et al., 2023; Liu et al., 2024).

Antibiotic exposure and other environmental stressors disrupt this balance, leading to dysbiosis and uncontrolled expansion of *Enterobacteriaceae*. This bloom is considered a hallmark of microbial imbalance and is strongly associated with increased carriage of ESBL-producing strains (Gay et al., 2023; Liu et al., 2024). The clinical and epidemiological relevance of *Enterobacteriaceae* is amplified by their exceptional capacity for horizontal gene transfer (HGT). ESBL genes, including *bla_CTX-M*, *bla_SHV*, *bla_TEM* that are frequently carried on mobile genetic elements such as plasmids and transposons, enabling rapid dissemination within the gut microbiome and across humans, animals, and the environmental reservoirs (Gay et al., 2023; Liu et al., 2024). Consequently, high *Enterobacteriaceae* abundance serves not only as biomarker of dysbiosis but also as a functional driver of antimicrobial resistance spread.

Given that intestinal colonization with ESBL-producing *Enterobacteriaceae* often precedes invasive infection, strategies aimed at reducing their carriage are of major public health importance. Effective approaches must integrate antimicrobial stewardship with microbiota-targeted interventions, such as prebiotics and probiotics, to restore ecological balance and eliminate niches that support resistant population (Gay et al., 2023; Liu et al., 2024).

1.3.7.1. Escherichia coli

Escherichia coli is one of the earliest and most persistent colonizers of the human gastrointestinal tract. As a facultative anaerobe, E. coli can switch between aerobic respiration, anaerobic respiration, and mixed-acid fermentation depending on oxygen availability. Although pathogenic lineages such as enterotoxigenic or ESBL-producing strains are widely recognized, the vast majority of *E. coli* strains inhabiting healthy individuals are non-pathogenic commensals. These commensal *E. coli* strains coexist with other members of the gut microbes and play critical ecological roles, including nutrient metabolism, facilitation of microbial cross-feeding, and competitive exclusion of enteric pathogens (Tenaillon et al., 2010; Schlager et al., 2021).

During early infancy, *E. coli* frequently dominates the gut microbial community, particularly in formula-fed infants, where it exploits the availability of simple carbohydrates and residual oxygen availability of the immature intestinal environment (Milani et al., 2017). As gut maturation progresses, obligate anaerobes such as *Bifidobacterium* and *Bacteroides* expand, leading to decline in the relative abundance of *E. coli*. Nevertheless, *E. coli* persists as a stable and integral member of the intestinal microbiota throughout the human lifespan (Rosenberg & Zilber-Rosenberg, 2018).

Beyond it is commensals presence, *E. coli* may exert protective effects within the gut ecosystem. Commensal strains compete with pathogenic bacteria for ecological niches and nutritional resources, and certain lineages produce bacteriocins, such as colicins, that inhibit closely related bacterial competitors (Czárán et al., 2018). Moreover, commensal *E. coli* stimulates mucosal immunity by inducing IgA responses and maintaining a low level of tonic activation of the innate immune system, which enhances readiness against pathogens (Leimbach et al., 2013).

Despite these beneficial roles, the dual nature of *E. coli* warrants careful consideration. Commensal strains serve as reservoirs of genetic material and can acquire resistance determinants via horizontal gene transfer. This genomic plasticity underlies the emergence of ESBL-producing phenotypes from previously benign lineages under antibiotic selective pressure (Touchon et al., 2020). For this reason, commensal *E. coli* represents both a marker of a healthy gut ecosystem and as a potential source of clinically significant resistance traits, particularly in the context of microbial dysbiosis.

1.3.7.2. Klebsiella sp

Klebsiella species, particularly *Klebsiella pneumoniae*, are facultative anaerobes opportunistic pathogens capable of establishing long-term colonization in the gastrointestinal tract, where they act as a reservoir for extended-spectrum β-lactamases (ESBLs). Colonization by ESBL-producing *Klebsiella* is frequently associated with a reduction in gut microbial diversity, weakening colonization resistance and creating environment conditions that favor the persistence and proliferation of resistant strains. This pattern often coexists with ESBL-producing *Escherichia coli*, underscoring the complex interactions multi drug resistant organisms within the gut ecosystem (Microbe cohort study, 2023). These clinical dynamics are particularly relevant in hospitalized patients, who frequently harbor high levels of ESBL-producing *Klebsiella*, sometimes accompanied by to carbapenem resistance, posing major challenges for treatment and infection control

1.4 Colonization Resistance

Colonization resistance refers to the collective ecological and immunological functions of the gut microbiota that prevent the establishment and expansion of pathogenic organisms. Colonization resistance depends on a diverse anaerobic microbiota enriched in *Bifidobacterium, Bacteroides, and Bacillota*, which collectively maintain ecological stability and suppress *Enterobacteriaceae* expansion (Hickman et al., 2024). Commensal bacteria inhibit pathogen colonization through competition for nutrients and epithelial adhesion sites along the intestinal mucosa. In addition, they produce antimicrobial compounds, including bacteriocins and short-chain fatty acids (SCFAs), which suppress the growth of pathogenic microbes (Caballero & Pamer, 2015; Hickman et al., 2024; Zhang et al., 2025). The (SCFAs) lowering gut PH, direct antimicrobial affects, protect the intestinal barrier and supporting immune function.

Beyond direct microbial competition, the gut microbiota plays a key role in modulating host immune responses. Commensal organisms promote the production of secretory immunoglobulin A (IgA) and regulate inflammatory signaling pathways, thereby maintaining mucosal immune homeostasis. Disruption of colonization resistance, particularly following antibiotic exposure, impairs these protective mechanisms and

increases susceptibility to colonization by multidrug-resistant *Enterobacteriaceae*, including ESBL-producing *Escherichia coli and Klebsiella* species (Caballero & Pamer, 2015; Hickman et al., 2024; Zhang et al., 2025).

1.5 Antibiotic-Induced Dysbiosis

Antibiotic exposure is a major factor associated with alterations in the gut microbiota, particularly during early life. Even short antibiotic courses have been shown to reduce microbial diversity and deplete key beneficial taxa such as *Bifidobacterium* and *Faecalibacterium* (Korpela et al., 2020). These changes may result in prolonged shifts in microbial community structure and have been associated with increased relative abundance of *Enterobacteriaceae,* likely reflecting reduced microbial competition and altered metabolic interactions within the gut ecosystem (Francino, 2021).

A critical consequence of antibiotic-induced dysbiosis is the depletion of short-chain fatty acid (SCFA)-producing bacteria. SCFAs are essential for maintaining intestinal epithelial integrity, regulating immune responses, and inhibiting pathogen expansion through luminal acidification. Their depletion promotes a gut environment that is more permissive for colonization by Multi drug resistant bacteria, including ESBL-producing *E. coli* and *Klebsiella* species (Francino, 2021).

Experiments demonstrate that antibiotic exposure induces a marked expansion of *Enterobacteriaceae*, whereas microbiota restoration strategies such as prebiotic and probiotic supplementation or SCFA administration can partially restore colonization resistance (Zhang et al., 2018). In infants, early antibiotic exposure is associated with reduced microbiota resilience and increased carriage of multidrug-resistant *Enterobacteriaceae* (Reyman et al., 2022). Collectively, these findings indicate that antibiotic-induced dysbiosis represents a sustained ecological shift rather than a transient perturbation.

1.6 Antimicrobial Resistance

Antimicrobial resistance (AMR), particularly among extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL-EB), represents a major global health threat due to it is ability to hydrolyze third-generation cephalosporins and severely limit

treatment options. The prevalence of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* has increased significantly in both community and hospital settings, highlighting the urgent need for effective surveillance and preventive strategies (Suvvari et al., 2025; Zhang et al., 2023).

In infants, the gut microbiota serves as an important reservoir for multidrug-resistant organisms. Asymptomatic carriers contribute to silent transmission within households and healthcare systems (Li et al., 2024; Wu et al., 2025).

1.7 Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* (ESBL-EB): Characteristics and Implications

Extended-spectrum β-lactamase–producing *Enterobacteriaceae* are Gram-negative bacteria capable of producing enzymes that hydrolyze abroad range of β-lactam antibiotics, including third-generation cephalosporins and monobactams. These enzymes significantly reduce therapeutic options and contribute to the global burden of antimicrobial resistance (Paterson & Bonomo, 2005).

ESBL production is most commonly observed in *Escherichia coli* and *Klebsiella pneumoniae*, which are associated with both community- and hospital-acquired infections. Their global dissemination is largely driven by plasmid-mediated horizontal gene transfer, facilitating rapid spread of resistance determinants (Castanheira et al., 2021).

Clinically, ESBL infections are associated with increased morbidity, mortality, and healthcare costs due to limited treatment options (Pitout&Laupland, 2008). Furthermore, the emergence of additional resistance mechanisms, including carbapenemase production, further threatens the effectiveness of the effectiveness of critical antimicrobial agents (Queenan & Bush, 2007). The emergence of additional resistance mechanisms, including carbapenemase production, further threatens the effectiveness of last-line antibiotics (Queenan & Bush, 2007; Brolund, 2014).

1.7.1 **Digestive Colonization by Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* (ESBL-EB)**

The human gastrointestinal tract represents a major reservoir for ESBL–producing *Enterobacteriaceae* (ESBL-EB). Intestinal colonization refers to the asymptomatic persistence of these organisms within the gut microbiota. Although clinically silent, intestinal carriage has significant epidemiological importance, as carriers represent a key source of transmission of ESBL-producing organisms in both community and healthcare settings (Pitout&Laupland, 2008). Colonization is increasingly detected among healthy individuals worldwide, reflecting the global dissemination of antimicrobial resistance.

Successful persistence of ESBL-EB in the guts influenced by complex host–microbiota interactions, and is facilitated under conditions of reduced colonization resistance, particularly following antibiotic-induced dysbiosis (Pitout&Laupland, 2008). Importantly, intestinal carriage of ESBL-EB is a major risk factor for subsequent invasive infections, including urinary tract infections, bloodstream infections, and other systemic diseases caused by multidrug-resistant Enterobacteriaceae (Pitout&Laupland, 2008).

1.7.2 ***Prevalence of ESBL-EB in the Gut Microbiota***

The human gastrointestinal tract serves as a major reservoir for ESBL–producing *Enterobacteriaceae* (ESBL-EB), with increasing colonization rates reported globally. Colonized individuals are often asymptomatic, enabling silent transmission of resistant bacteria and increasing infection risk.

A systematic review and meta-analysis including 28,909 individuals estimated a global colonization prevalence of 14%, with marked regional variation. The highest prevalence was reported in the Western Pacific region (46%), followed by Southeast Asia and Africa (22% each), while lower rates were observed in Europe (4%) and the Americas (2%) (Karanika et al., 2016).

Similarly, global surveillance data indicate a substantial increase in intestinal carriage of ESBL-producing Escherichia coli over recent decades. Meta-analyses have consistently confirmed these rising global trends. Bezabih et al. (2021) reported a

pooled prevalence of 16.5% among healthy individuals, with the highest rates observed in Southeast Asia (27%). Temporal analysis revealed a marked increase from 2.6% (2003–2005) to 21.1% (2015–2018), indicating rapid global expansion.

Comparative analyses across healthcare and community settings further show higher colonization rates among hospitalized individuals (21.1%) compared to healthy populations (17.6%), with increasing trends observed in both groups (Bezabih et al., 2022). Collectively, these findings highlight the growing epidemiological importance of intestinal ESBL carriage worldwide.

1.8 Strategies for ESBL-EB Decolonization

1.8.1. Fecal Microbiota Transplantation (FMT)

Disruption of the gut microbiota facilitates colonization by multidrug-resistant organisms. Consequently, restoration of microbial diversity has emerged as a promising strategy for ESBL decolonization. A range of microbiome restoration strategies have been investigated to reduce intestinal colonization by resistant organisms. These therapeutic approaches aim to re-establish a stable and diverse microbial community capable of suppressing the growth and persistence of multidrug-resistant bacteria. Such approaches focus on restoring microbial balance within the gastrointestinal tract and strengthening colonization resistance against pathogenic microorganisms (Gopalsamy et al., 2018).

Fecal microbiota transplantation (FMT), which involves the transfer of processed fecal material from healthy donors, has demonstrated the ability to restore colonization resistance and reduce carriage of resistant bacteria. Clinical studies suggest that FMT can reduce intestinal colonization by ESBL-producing *Escherichia coli*, particularly in vulnerable patient populations (Lagier et al., 2015; Bilinski et al., 2017).

Additional case reports and clinical trials indicate that microbiota restoration following antibiotic exposure may reduce carriage of multidrug-resistant *Enterobacteriaceae* (Singh et al., 2014; Huttner et al., 2019). Experimental models further support these findings, demonstrating significant reductions in intestinal colonization by pathogens or ESBL following microbiota transfer (Mahieu et al., 2017).

1.8.2. Bacteriophage Therapy

Bacteriophage therapy represents a targeted antimicrobial strategy that utilizes viruses capable of infecting and lysing specific bacterial hosts. This approach offers the advantage of selectively reducing pathogenic bacterial populations while preserving commensal microbiota (Javaudin et al., 2021). Preclinical studies have demonstrated that bacteriophages can significantly reduce intestinal *Escherichia coli* in animal models (Sheng et al., 2006). Emerging evidence further suggests potential efficacy against multidrug-resistant strains (Javaudin et al., 2021). However, robust clinical evidence for the efficacy of bacteriophages in humans remains limited, and its routine application for intestinal decolonization is not yet established.

1.8.3. Probiotics

Probiotics are live, non-pathogenic microorganisms that confer health benefits when administered in adequate amounts (Wan et al., 2019). These microorganisms are naturally present in fermented food, including yogurt, cheese, fermented milk, cereals, fruit juices, and other traditional food products (Wan et al., 2019). Their growing scientific and clinical relevance stems from their potential to support intestinal homeostasis, enhance host immunity, and improve overall health outcomes. (Plaza-Diaz et al., 2019; Wan et al., 2019)).

Several mechanisms have been proposed to explain the ability of probiotics to limit colonization by multidrug-resistant organisms. These include modulation of the intestinal microbiota, competitive exclusion of pathogens through mucosal adhesion, enhancement of epithelial barrier integrity, and regulation of host immune responses (Plaza-Diaz et al., 2019). Among the wide range of probiotic taxa, *Bifidobacterium* and lactic acid bacteria (LAB) represent the most extensively investigated groups (Plaza-Diaz et al., 2019).

Members of the genus *Bifidobacterium* constitute a dominant component of the human intestinal microbiota, particularly during early life and neonatal period (Tham et al., 2012; Plaza-Diaz et al., 2019). Although colonization is initially high in newborns, it gradually decreases with age (Tham et al., 2012). Several species*, including B.*

bifidum, B. longum, and B. infantis, are widely used as probiotic strains due to their beneficial physiological properties (Khani et al., 2012). *Bifidobacteria* exert their health-promoting effects through modulation of immune responses, competitive inhibition of pathogenic microorganisms, and maintenance of intestinal barrier function. Metabolically, they produce acetate and lactate, which contribute to gut homeostasis (Hoover, 2014). In addition, they synthesize certain vitamins and amino acids and enhance mineral absorption within the gastrointestinal tract (Sharma et al., 2021; You et al., 2022).

Certain probiotic strains such as *Escherichia coli* Nissle 1917 have further been investigated for their ability to restore microbial balance and improve colonization resistance against pathogenic microorganisms (Tannock et al., 2011). However, clinical studies assessing its efficacy in reducing multidrug-resistant *E. coli* carriage, particularly in elderly populations, have reported inconsistent outcomes.

Lactic acid bacteria (LAB) are another major group of probiotics widely distributed in natural environments, fermented foods, and the gastrointestinal and urogenital tracts of humans and animals (Ruiz Rodríguez et al., 2019). LAB exert beneficial effect through facilitating lactose digestion; strengthen gut barrier integrity; and suppressing enteric pathogen (Ruiz Rodríguez et al., 2019).

These bacteria primarily metabolize carbohydrates to produce lactic acid, and contributing to the prevention of certain gastrointestinal infections (Teneva-Angelova et al., 2018). Common probiotic LAB include species such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*, which are widely used as starter cultures in dairy fermentation processes (Gilliland, 1990).

1.8.4 Prebiotics

Prebiotics are non-digestible dietary carbohydrates that selectively stimulate the growth and activity of beneficial gut microorganisms (You et al., 2022). Key prebiotics, including inulin, fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS) resist digestion in the upper gastrointestinal tract and reach the colon intact, where they promote the proliferation of *Bifidobacterium* and *Lactobacillus* species. This fermentation process leads to the production of short-chain fatty acids (SCFAs) and contributes to modulation of colonic pH (Guarino et al., 2020; Jangid et al., 2022).

Inulin, a plant derived-fructan found in sources such as chicory root and Jerusalem artichoke, varies in chain length from oligosaccharides to polysaccharides and promotes SCFA production, bacterial biomass, and stool bulk. It has also been linked to improved bowel function, modulation of serum lipid profiles, and potential immunomodulatory effects (Bărboi et al., 2020; Bosscher et al., 2006).

Similarly, FOS found in onions, garlic, and chicory, selectively stimulates *Bifidobacteria* growth, thereby increasing SCFA production, lowering colonic pH, and supporting immune responses, including mucosal IgA secretion (Jangid et al., 2022). Prebiotics may also contribute to the prevention of colonization by pathogens, including ESBL-producing *Enterobacteriaceae* (Song et al., 2022; Kim et al., 2011).

Through microbial fermentation, they enhance the colonic pH, strengthens the gut barrier, and generate SCFAs with anti-inflammatory and immunomodulatory effect (Habteweld& Asfaw, 2023; Nawaz et al., 2018). By creating a selective ecological niche that favor beneficial microbes, prebiotics can inhibit the establishment and persistence of pathogenic bacteria, including ESBL-producing *Enterobacteriaceae* (Song et al., 2022; Kim et al., 2011).

Despite promising experimental and mechanistic evidence, clinical studies directly evaluating prebiotics as a therapeutic strategy for decolonization of multidrug-resistant bacteria in humans remain limited (Ishnaiwer, 2022; Feehan & Garcia-Diaz, 2020). The concept of synbiotics, which combines prebiotics and probiotics, has therefore gained attention as a strategy to enhance beneficial microbial colonization and achieve synergistic antimicrobial, immunomodulatory, and metabolic effects (Markowiak&Śliżewska, 2017; Panesar & Anal, 2022).

1.9 Dietary and Environmental Determinants of Gut Microbiota Composition

1.9.1 *Yogurt and Fermented Foods*

Diet represent one of the most powerful modulators of gut microbiota composition. Dietary fibers serve as substrates for microbial fermentation, promoting SCFA production and supporting the growth of beneficial anaerobic bacteria. Among dietary components, fermented foods such as yogurt play a significant role in shaping microbial composition and metabolic activity.

Yogurt is a fermented dairy product containing live microorganisms, primarily *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*, which are responsible for milk fermentation. Due to the presence of these live bacteria, yogurt has been extensively studied for its potential effects on intestinal microbiota composition and host health.

Evidence suggests that fermented dairy products can influence both microbial composition and metabolic activity, thereby contributing to improved intestinal homeostasis and host health (Savaiano &Hutkins, 2021). Regular yogurt consumption has been associated with beneficial shifts in gut microbiota, particularly, increased abundance of *Lactobacillus* and *Bifidobacterium*, both of which play key roles in maintaining gut homeostasis, regulating immune responses, and reinforcement intestinal barrier function (Savaiano &Hutkins, 2021).

Probiotic bacteria in yogurt may also transiently colonize the gastrointestinal tract and interact with resident microbiota, influencing microbial diversity and stability within the gut ecosystem (Lisko et al., 2017).In addition, interactions between yogurt-derived microorganisms and the host microbiota can stimulate the production of bioactive metabolites such as short-chain fatty acids (SCFAs), which are essential for maintaining intestinal integrity, regulating inflammation, and supporting metabolic function(González et al., 2019).

Emerging evidence further suggests that functional modifications of yogurt, such as enrichment with flavonoids, may enhance its microbiota-modulating potential. In experimental models, flavonoid-enriched yogurt increased microbial diversity and promoted the abundance of beneficial bacterial taxa, indicating that dietary modification may amplify its biological effects (Li et al., 2021). Overall, current evidence indicates that yogurt consumption supports gut microbial balance by promoting beneficial taxa, enhancing microbial diversity, and stimulating production of functional metabolites.

1.9.2 Early-Life Factors Shaping Infant Gut Microbiota

The establishment of the infant gut microbiota is a dynamic and a highly sensitive process influenced by a range of multiple early-life factors, including delivery mode, feeding practices, antibiotic exposure, and environmental conditions. Vaginal delivery and breastfeeding promote early colonization by beneficial taxa such as *Bifidobacterium* and *Lactobacillus,* which are critical for healthy microbiota development. In contrast, cesarean delivery and formula feeding are associated with delayed microbial maturation and increased abundance of *Enterobacteriaceae*. Environmental exposures, such as siblings and outdoor contact, also significantly impact microbial diversity and community stability (Gao et al., 2025; Hickman et al., 2024).

1.10 Microbiota–Host Interactions and Immune Development

The gut microbiota plays a fundamental role in shaping the immune system, particularly through its interactions with gut-associated lymphoid tissue (GALT). Microbial- derived metabolites including short chain fatty acids (SCFA) such as butyrate and acetate, are key mediators of these interactions. These regulatory T-cell differentiation, enhance intestinal barrier integrity, and modulate local immune responses. Early-life dysbiosis can compromise immune development, increasing susceptibility to colonization by resistant *Enterobacteriaceae* and potentially predisposing infants to long-term health complications (Pantazi, 2023; Wu et al., 2025).

1.11 Knowledge Gaps and Relevance to the Present Study

Current literature indicates that healthy children typically harbor a relatively diverse and stable gut microbiota that contributes to metabolic homeostasis, immune development, and colonization resistance. In contrast, children with gastroenteritis often exhibit microbiota disruption characterized by reduced diversity, increased abundance of Enterobacteriaceae, and greater inter-individual variability. These ecological alterations may create favorable conditions for colonization by ESBL-producing organisms,

particularly in hospitalized settings where antibiotic exposure and healthcare-associated transmission are common.

Despite increasing interest in this field, several important knowledge gaps remain. First, relatively few studies have simultaneously compared healthy controls, hospitalized children, and ESBL carriers within the same pediatric cohort. Second, many investigations have focused primarily on taxonomic abundance while giving limited attention to ecological structure, community variability, and inter-taxa relationships. Third, evidence from low- and middle-income settings remains limited despite potentially higher burdens of antimicrobial exposure and antimicrobial-resistant organism carriage.

The present study was designed to address these gaps by integrating community-level analyses, including principal coordinate analysis (PCoA) and beta-diversity assessment, with taxon-level and correlation-based approaches to characterize microbiota structure, dysbiosis patterns, and ecological interactions. This ecological framework may provide deeper insight into microbial community dynamics and their relationship to antimicrobial resistance in hospitalized pediatric populations.

CHAPTER TWO: PROBLEM STATEMENT AND OBJECTIVES

2.1 Problem Statement

The human gut microbiota plays a central role in maintaining intestinal homeostasis, supporting immune development, and providing colonization resistance against enteric pathogens. In early childhood, the gut microbial ecosystem is highly dynamic and particularly susceptible to disruption by infectious diseases, dietary changes, and antibiotic exposure. Acute gastroenteritis is one of the most common gastrointestinal conditions in children and is strongly associated with transient or prolonged disturbances in gut microbial composition. Such disturbances may lead to dysbiosis characterized by reduced beneficial commensals and altered microbial diversity, potentially affecting key functional properties of the microbiota.

In addition to its role in disease recovery, microbiota composition may influence susceptibility to intestinal colonization by opportunistic and antimicrobial-resistant organisms. In particular, alterations in microbial community structure and reduced colonization resistance may create ecological niches that facilitate colonization by Enterobacteriaceae, including extended-spectrum β-lactamase (ESBL)-producing strains. Despite the clinical relevance of these interactions, limited data exist regarding gut microbiota composition in children with gastroenteritis compared to healthy controls, and even less is known about its relationship with ESBL colonization in pediatric populations. This is particularly relevant in settings with high infectious disease burden and frequent antibiotic exposure.

In Palestine, where pediatric gastroenteritis remains common and antimicrobial use is widespread, no comprehensive studies have systematically investigated differences in gut microbiota between children with gastroenteritis and healthy controls, nor their association with ESBL-producing Enterobacteriaceae colonization.

Accordingly, this study aims to characterize and compare the gut microbiota in children with gastroenteritis and healthy controls, and to assess its association with intestinal colonization by ESBL-producing *Enterobacteriaceae.*

2.2 General objectives

To characterize normal microbiota composition and dysbiosis patterns in Palestinian children, and to investigate their association with intestinal colonization by ESBL-producing *Enterobacteriaceae* colonization among hospitalized and healthy children.

2.3 Specific Objectives

The overall aim of this study is to investigate the intestinal microbiota of children and evaluate ecological factors potentially relevant to colonization by multidrug-resistant *Enterobacteriaceae*, particularly ESBL-producing strains.

The specific objectives are:

1) To characterize the composition and relative abundance of selected bacterial taxa in the fecal microbiota of children hospitalized with acute gastroenteritis and healthy controls using qPCR-based approaches.
2) To evaluate differences in gut microbial community structure, diversity, and ecological dispersion between hospitalized children and healthy controls.
3) To identify the significant bacterial taxa significantly associated with hospitalization status.
4) To determine the prevalence and intestinal load of ESBL-producing Enterobacteriaceae among hospitalized childrenand healthy children (base line).
5) To investigate the relationship between gut microbiota composition, dysbiosis, and ESBL colonization.
6) To identify bacterial taxa correlated with ESBL abundance and assess their potential value as microbial indicators of colonization.

CHAPTER THREE: METHODOLOGY

3.1 Study Design

Stool samples were collected from children hospitalized with gastroenteritis at Al-Ahli Hospital and Alia Governmental Hospital in Hebron, Palestine, alongside age-matched healthy controls from local community. Healthy controls were selected from children attending outpatient clinics or community settings with no history of diarrhea, vomiting, fever, antibiotic use, or gastrointestinal illness during the preceding two weeks. Controls were matched to cases by age as closely as possible to reduce confounding related to age-dependent microbiota variation. The study integrated culture-based detection of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* with molecular profiling of the gut microbiota using genomic DNA extraction and quantitative PCR (qPCR).

A total of 40 stool samples were collected, including 28 from hospitalized children and 12 from healthy controls. Samples were obtained in sterile containers, transported on ice, and processed within two hours of collection. Each sample was aliquoted for ESBL-producing *Enterobacteriaceae* (ESBL-EB) culture and molecular analyses, and DNA extracts were stored at −20 °C until further processing. Clinical metadata were available for a subset of hospitalized children (n = 10). These cases are presented to illustrate the diversity of clinical presentations (**Table 1**). Comprehensive clinical data were not available for the remaining participants**.**

3.2 Ethical Considerations

Ethical approval was obtained from the Deanship of Scientific Research and the institutional review boards of both Palestine Polytechnic University and participating hospitals. Written informed consent was obtained from parents or legal guardians prior to sample collection. All samples were anonymized using unique coded identifiers to ensure confidentiality and compliance with ethical standards.

Table 1. Clinical characteristics of hospitalized children included in the study (subgroup analysis, n = 10 of 28).

Age Group	n	Gender (when available)	Main Complaints	Key Associated Symptoms	Past Medical History	Clinical Examination
1–12 months	3	Female (n=2), Male (n=1)	Diarrhea, vomiting, fever	Poor feeding, hypoactivity, dehydration	NICU admission, incomplete vaccination, surgical history	Signs of dehydration
>12–18 months	1	Female (n=1)	Fever, diarrhea	Hypoactivity	Previous NICU admission	Alert; possible CNS abnormality
>24–36 months	6	Female (n=3), Male (n=3),	Vomiting, diarrhea, abdominal pain	Generalized weakness, variable symptoms	Milk allergy, prior IV therapy, not documented	Clinically stable abdomen; variable findings (including dehydration)
Clinical data unavailable	18	Not documented	Acute gastroenteritis (diagnosis only)	—	—	—

Notes: NICU = Neonatal Intensive Care Unit; **ICU** = Intensive Care Unit; **ORS** = Oral Rehydration Solution; **IV** = Intravenous; **CS** = Cesarean section; **CNS** = Central Nervous System; **"—"** = no additional symptoms reported; "Data not available" = missing or undocumented data.

3.3 ESBL-Producing *Enterobacteriaceae* (ESBL-EB) Screening, Isolation, and Quantification

ESBL-producing Enterobacteriaceae were isolated using ChromID™ ESBL agar (bioMérieux), a chromogenic selective medium designed for the isolation and

differentiation of ESBL-producing Gram-negative bacteria. The medium contains peptones, amino acids, vitamins, and chromogenic substrates that enable presumptive identification based on colony coloration. Selectivity is enhanced by the AC3F supplement (FD278), which contains third-generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone, aztreonam) and fluconazole to inhibit non-target bacteria and fungi.

For preparation, 20 g of dehydrated medium was dissolved in 500 mL of distilled water, heated to boiling until fully dissolved, and sterilized by autoclaving at 121 °C for 15 minutes. After cooling to 45–50 °C, the FD278 supplement was added aseptically. The medium was then poured into sterile Petri dishes (25 mL per plate) and allowed to solidify. Approximately 30–50 mg of stool was suspended in 1 mL sterile distilled water and homogenized by vortexing for 10 minutes. Serial tenfold dilutions (10^{-1} to 10^{-4}) were prepared in sterile saline. From each dilution, 100 µL was inoculated in duplicate onto ChromID™ ESBL agar plates and incubated aerobically at 37 °C for 24 hours.

Colonies were identified presumptively based on manufacturer guidelines: *Escherichia coli* (pink to burgundy), *Klebsiella and Enterobacter* spp. (green to blue-green), and *Proteus* spp. (blue). This color differentiation is based on species-specific enzymatic activities acting on chromogenic substrates incorporated in the medium. *E. coli* produces β-glucuronidase, which hydrolyzes its corresponding substrate, releasing a pink to burgundy chromophore. In contrast, *Klebsiella and Enterobacter* spp. typically express β-glucosidase, resulting in the cleavage of a different substrate and the formation of green to blue-green colonies. *Proteus* spp. exhibit distinct enzymatic pathways, such as tryptophan deaminase-related activity, which interact with specific substrates to produce blue coloration. Colony morphology and coloration were recorded for downstream analysis.

Colony-forming units (CFU) were counted on plates containing 30–300 colonies. Bacterial load was expressed as CFU per gram of stool (CFU/g) using standard dilution-based quantification methods.

$$\mathrm{CFU/g} = \frac{\text{Number of colonies} \times \text{Dilution factor} \times 1000}{\text{Volume plated (mL)} \times \text{Sample weight (mg)}}$$

3.4 DNA Extraction from Stool Samples

Genomic DNA was extracted from 40stoolsamples (28 hospitalized, 12 controls) using the PowerSoil® Pro Kit (Qiagen, Germany) according to the manufacturer's protocol. Approximately50 mg of stool material was transferred into Power Bead tubes containing lysis buffer and beads. Mechanical lysis was performed using bead-beating for 10 minutes to ensure efficient disruption of both Gram-positive and Gram-negative bacteria. This was followed by inhibitor removal, silica membrane–based DNA binding, washing, and elution. DNA was eluted in 50 µL of elution buffer and stored at −20 °C. DNA concentration and purity were assessed using a NanoPhotometer (Implen, Germany)**.** Concentrations ranged from 35–180 ng/µL. Purity was evaluated using 260/A280 ratios, with values between 1.7 and 1.9 considered acceptable. Samples outside this range were re-extracted to ensure data reliability.

3.5 Quantitative PCR (qPCR) Analysis

Quantitative PCR was performed to quantify selected bacterial taxa using 1 µL of extracted DNA per reaction. Target taxa included *Akkermansia muciniphila, Bacteroidota, Muribaculaceae, Bacteroides spp., Bacillota (Firmicutes), Escherichia coli, Enterobacteriaceae, Enterococcus spp., Lactobacillus spp., Lachnospiraceae, Klebsiella pneumoniae, Prevotella spp., and Bifidobacterium* spp. Primer sequences, amplicon sizes, and references are provided in (**Appendix, Table A1**). All primers were selected from previously validated studies to ensure reproducibility.

Primers were selected or designed using comparative genomic analysis of representative bacterial genomes to ensure specificity and coverage.

qPCR reactions were performed using PowerUp™ SYBR Green Master Mix (Applied Biosystems) in a total volume of 20 µL. Each reaction contained 1 µL of template DNA and 19 µL of reaction mixture composed of 10 µL of SYBR Green Master Mix, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), and 7 µL of nuclease-free water. Thermal cycling conditions consisted of an initial denaturation at 94 °C for 3 minutes, followed by 40 cycles of 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds.

For selected taxa (*Bacteroides* spp., *Muribaculaceae*, *Lachnospiraceae*, *Enterobacter*, *Klebsiella*, *Bacillota* (*Firmicutes*, *Bacteroidota* (*Bacteroidetes)*, *Faecalibacterium*, and *Enterobacteriaceae*) the annealing was performed at 59 °C for 1 minute.

Standard curves were generated using purified reference amplicons. DNA concentration was measured using a NanoDrop spectrophotometer, and copy number was calculated based on amplicon length and DNA mass using the appropriate conversion formula (Bustin et al., 2009) :

$$\text{Copy number} = \frac{(\text{DNA mass (g)} \times 6.022 \times 10^{23})}{(\text{amplicon length (bp)} \times 660)}$$

Briefly, purified amplicons were quantified and converted to copy number, then adjusted to a stock concentration of 5 × 10^6 copies/µL for qPCR standard curve preparation. Ten-fold serial dilutions were subsequently prepared to a final concentration of 5 copies/µL.

3.6 Data Processing and Validation

Amplification efficiency for all qPCR standard curves was assessed to ensure methodological reliability. All assays demonstrated efficiencies within the acceptable range of 90–110%, with coefficients of determination (R^2) ≥ 0.99, confirming both linearity and consistency of amplification across the dynamic range. Quantification cycle (Cq) values were automatically generated using the StepOnePlus™ Real-Time PCR System software and exported for subsequent analysis.

Relative abundance of target bacterial taxa was performed using the ΔCq method, whereby target Cq values were normalized to total bacterial 16S rRNA gene Cq values as an internal reference for total microbial load. Where appropriate, absolute bacterial abundance was estimated using standard curves and expressed as gene copy numbers per gram of stool.

Amplification specificity was verified by melt-curve analysis performed at the end of each qPCR run. The presence of a single melting peak was considered indicative of specific amplification, whereas reactions showing multiple peaks or evidence of non-specific products were excluded from further analysis. (Ririe et al., 1997; Wittwer et al., 2001).

Results were expressed either as relative abundance (%) or as absolute copy number per gram of stool, depending on the analytical approach. For illustrative purposes, an example of relative quantification is provided in Supplementary Materials, demonstrating the conversion of ΔCq values into percentage abundance of the target taxon relative to total bacterial load.

Example Calculation

For a given sample:

- $Cq_{\text{target}} = 25$
- $Cq_{16S} = 20$

$$\Delta Cq = 25 - 20 = 5$$

$$\text{Relative abundance} = 2^{-5} = 0.03125$$

3.7 Statistical Analysis

Statistical analyses were performed using R (v4.3.2) and GraphPad Prism 10. Microbial abundances were expressed as medians with interquartile ranges (Q1–Q3). Group comparisons (hospitalized vs control) were performed using the Wilcoxon rank-sum test. Multiple testing correction was applied using the false discovery rate (FDR), with statistical significance defined as adjusted $p < 0.05$.

Spearman's rank correlation was used to assess associations between taxa. Positive correlations ($\rho > 0$) indicated co-occurrence, while negative correlations ($\rho < 0$) suggested potential ecological competition or antagonisms.

3.8 Multivariate Analysis and Data Visualization

Principal Coordinate Analysis (PCoA) was performed using Euclidean distance on log-transformed microbial abundances to assess global community structure**.**

The first three principal coordinates, representing the majority of variance in microbial composition, were retained for interpretation. Group differences in beta diversity (hospitalized vs. control, and ESBL-positive vs. ESBL-negative) were evaluated using

Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations.

Intra-individual variability was evaluated by calculating the distance of each individual's microbiota profile from the spatial median of its respective group. This metric, hereafter referred to as the *dysbiosis index*, quantifies the degree of deviation of an individual microbiota profile from the central tendency of the reference group (healthy controls). Differences in dysbiosis index values between groups were assessed using the Wilcoxon rank-sum test.

Statistical relationships and multivariate patterns within the microbiota were visualized using heatmaps, correlation matrices, and Principal Coordinate Analysis (PCoA) plots. These approaches enabled the identification of both positive and negative associations among key bacterial taxa, including *Lactobacillus* spp., *Enterococcus* spp., *Klebsiella* spp., and *Escherichia coli*. All figures were generated using the ggplot2 and vegan packages in R, ensuring reproducibility and high-quality graphical representation of the data.

CHAPTER FOUR: RESULTS

4.1 Comparison Between Hospitalized and Control Children

Differential abundance analysis using the Wilcoxon rank-sum test identified limited but biologically meaningful differences in gut microbiota composition between hospitalized children and healthy controls (**Table 2, Figure 2, and Appendix Table A2)**. Detailed quantitative PCR measurements of all analyzed bacterial taxa are provided in **Appendix Table A3**.

Table 2. Comparative analysis of fecal microbiota abundance (log_{10} cells/g) in hospitalized versus control children.

Category	Direction pattern	Taxa (number)	Interpretation
FDR-significant increased	↑	*Enterobacteriaceae*	The only taxon showing robust evidence of enrichment associated with hospitalization after multiple-testing correction.
FDR-significant decreased	↓	—	No taxa in this category
Nominal increase (raw p < 0.05, not FDR-significant)	↑	*Lactobacillus, Klebsiella*	Higher abundance observed in hospitalized patients, but significance was not retained after FDR correction.
Nominal decrease (raw p < 0.05, not FDR-significant)	↓	*Bacteroides, Bacteroidota*	Lower abundance observed in hospitalized patients, but evidence did not remain significant after FDR correction.
Trend toward decrease		*Enterococcus, Faecalibacterium, Prevotella*	Suggestive reductions with borderline statistical support and substantial overlap between groups.
No consistent difference between groups		*Akkermansia, Bifidobacterium, Escherichia coli, Enterobacter, Lachnospiraceae, Muribaculum, DNA16S, Bacillota*	No clear directional shift or statistically supported difference.

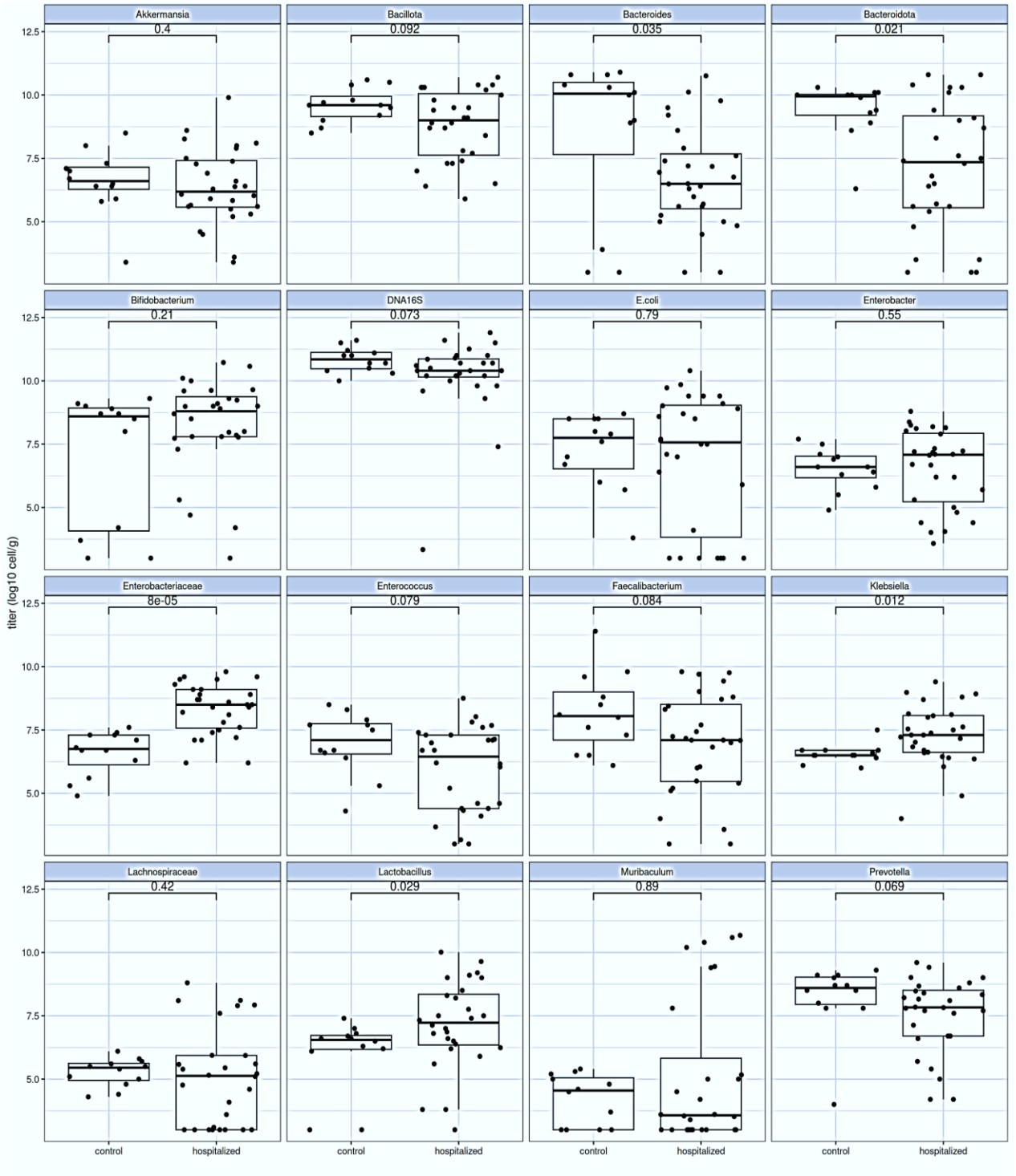

Figure 2. Comparison of selected fecal bacterial taxa between controls and hospitalized children. X-axis: study group; y-axis: bacterial titer (log_{10} cells/g feces). Boxes show Interquartile Range (IQR) and median; points represent individual samples. P values indicate between-group comparisons. Note: Wilcoxon rank-sum test.

Overall, hospitalization was associated with increased inter-individual variability in gut microbiota composition rather than uniform taxon-level shifts. After false discovery rate (FDR) correction, only *Enterobacteriaceae* showed a statistically significant increase in hospitalized children (Figure 3). No taxa showed significant decreases after FDR adjustment. Several taxa showed nominal differences (raw $p < 0.05$) without retaining significance after FDR correction. *Lactobacillus and Klebsiella* were increased in hospitalized children, whereas *Bacteroides and Bacteroidota* were decreased compared with controls.

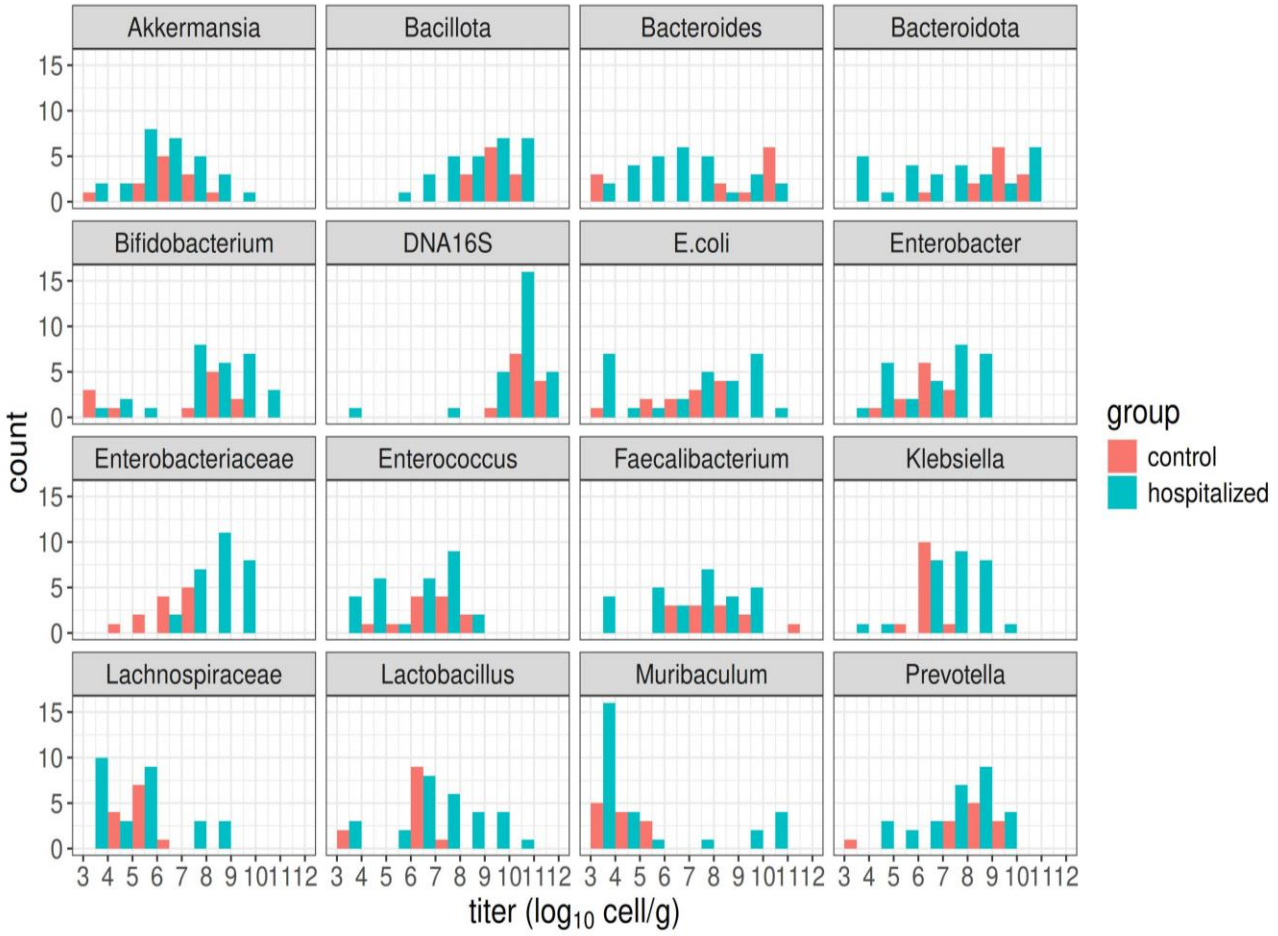

Figure 3: Histograms showing the frequency distribution of bacterial abundances measured by qPCR in fecal samples from hospitalized children and healthy controls. The x-axis represents bacterial abundance ($\log_{10}$ cells/g stool), and the y-axis represents the number of children within each abundance category. Red bars indicate healthy controls, whereas blue bars indicate hospitalized children.

A subset of taxa showed only trend-level changes without statistical significance, including *Enterococcus, Faecalibacterium, and Prevotella*, which showed a tendency toward decreased abundance in hospitalized children. Multiple taxa showed no consistent or statistically supported differences between groups, including *Akkermansia, Bifidobacterium, Escherichia coli, Enterobacter, Lachnospiraceae, Muribaculum, DNA16S, and Bacillota*.

Distributional analysis (Figure 3) showed overlapping abundance patterns between hospitalized and control groups for most taxa, while *Enterobacteriaceae* showed a consistent increase in hospitalized children.

4.2 Beta Diversity and Dysbiosis Assessment

Beta diversity analysis using Principal Coordinate Analysis (PCoA) demonstrated a significant difference in overall microbial community composition between hospitalized children and controls (**PERMANOVA, P = 0.002; Figure 4**), indicating that hospitalization is associated with a distinct shift in gut microbiota structure.

Inter-individual variability was further assessed by calculating the distance of each sample from its group spatial median. Hospitalized children exhibited significantly greater dispersion compared to healthy controls (median distance in hospitalized group: 3.8 [IQR: 3.3–4.5] vs. median distance in control group: 2.2 [IQR: 1.6–3.6]; Wilcoxon test, P = 0.005), indicating greater variability in microbial community composition within the hospitalized group.

In addition, a dysbiosis index based on deviation from the control group centroid was significantly elevated in hospitalized children (P = 0.0006), indicating a greater divergence from a healthy microbial profile. Notably, 25% of hospitalized children (7/28) exceeded the maximum dysbiosis index observed in controls, further supporting substantial microbial disruption of normal microbiota in this group.

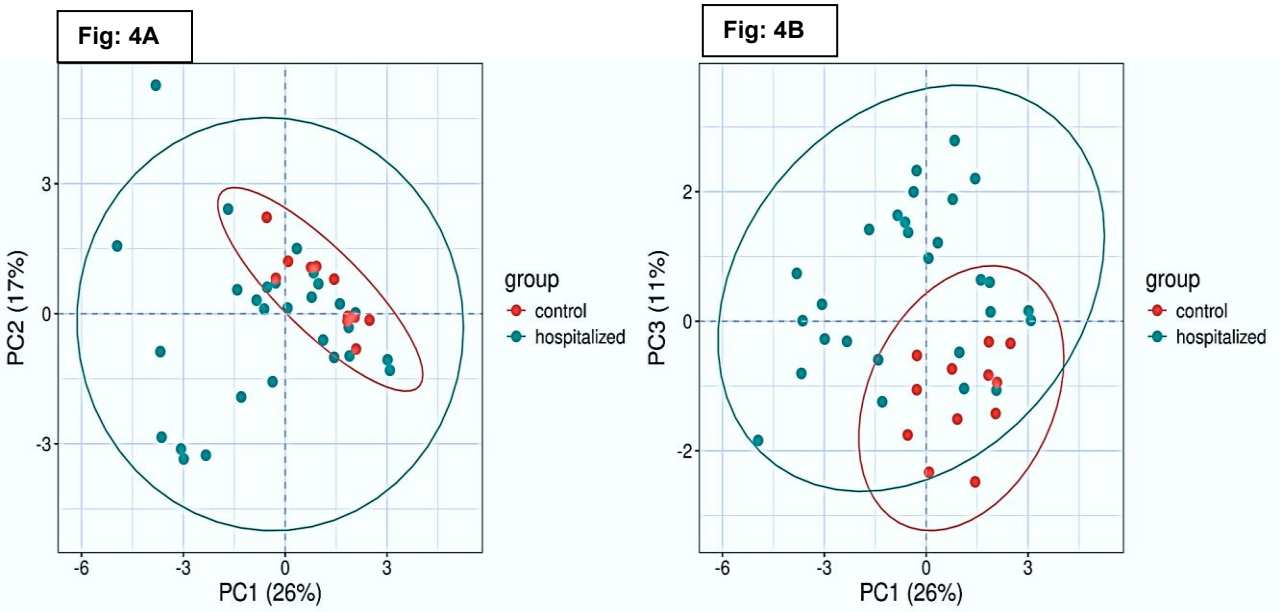

Figure 4: **Principal coordinate analysis (PCoA) of fecal microbiota profiles comparing hospitalized children and healthy controls.** (A) Distribution of samples along PC1 and PC2. (B) Distribution of samples along PC1 and PC3.Distribution of samples illustrates group-level separation based on Euclidean distance of standardized microbiota data.

4.3 Overall Architecture of the Fecal Microbiota

The overall structure of fecal microbiota communities was assessed using PCoA based on standardized quantitative titers and Euclidean distance metrics (**Figure 5**). The first three principal coordinates explained 54% of total variance (26%, 17%, and 11%, respectively). Members of the *Enterobacteriaceae* family did not form a unified ecological cluster. Interestingly, *Klebsiella* occupied a distinct position from *E. coli and Enterobacter*, indicating ecological divergence despite taxonomic relatedness. Interestingly, *Klebsiella* showed closer proximity to *Lactobacillus and Muribaculaceae.* In contrast, *Enterococcus* clustered more closely with *E. coli*, suggesting partial ecological co-association within the gut environment.

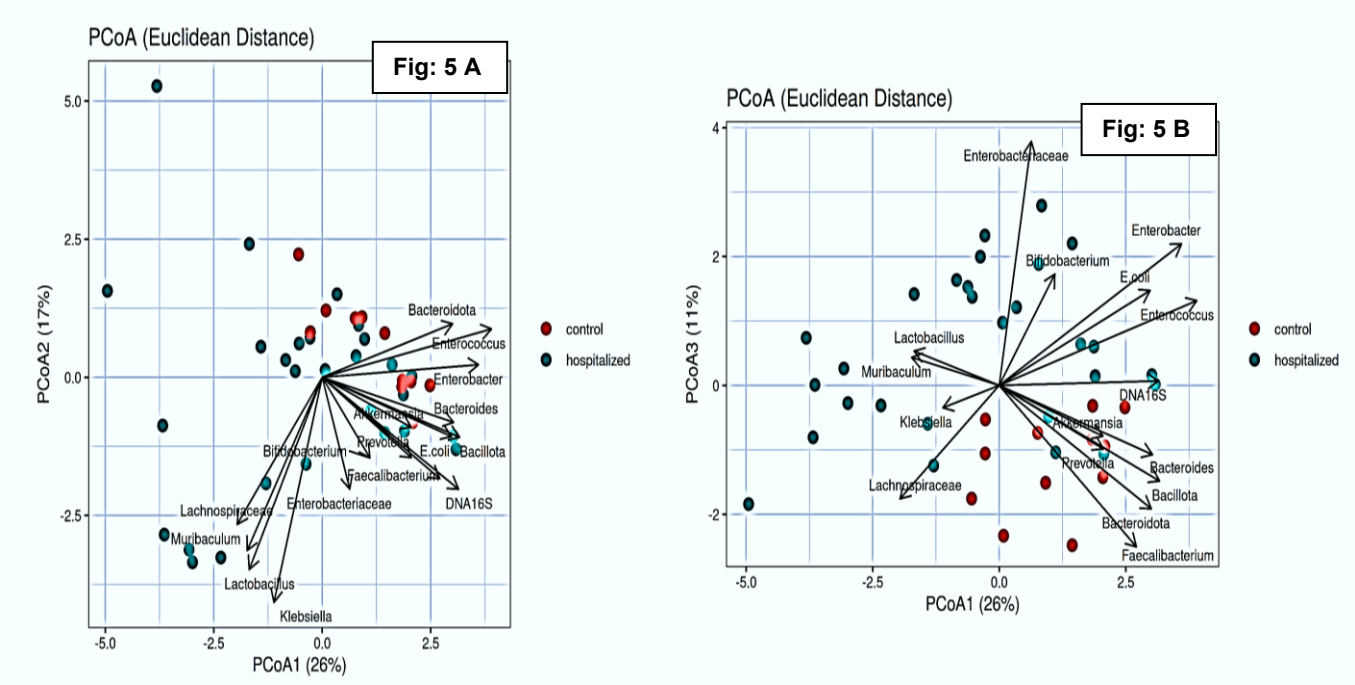

Figure 5. Principal coordinate analysis (PCoA) of fecal microbiota profiles illustrating variance structure and taxon-level contributions in controls and hospitalized children. (A) PC1 versus PC2. (B) PC1 versus PC3. Arrows indicate taxon loadings contributing to sample separation. The analysis was based on Euclidean distance using standardized quantitative microbiota data. Percentages on axes represent the proportion of variance explained by each principal coordinate.

4.4 Correlation Analysis of E*nterobacteriaceae*

Spearman's correlation analysis was performed to evaluate associations between members of *Enterobacteriaceae* family and other bacterial taxa across the full cohort (**Figure 6**). Data from hospitalized and control children were analyzed together to describe global co-occurrence patterns. Correlation strength was interpreted according to Spearman's ρ coefficient as follows: 0.00–0.19 (very weak), 0.20–0.39 (weak), 0.40–0.59 (moderate), 0.60–0.79 (strong), and 0.80–1.00 (very strong), with the sign indicating direction of association.

At the family level, *Enterobacteriaceae* abundance was positively associated with *Escherichia coli, Klebsiella spp., and Enterobacter* spp., reflecting coordinated variation among these taxa. No significant negative correlations were observed between *Enterobacteriaceae* and the taxa examined.

At the genus level, *E. coli and Enterobacter* exhibited a moderate positive correlation, in contrast, *Klebsiella* showed no significant associations with either taxon. *Enterococcus* was positively correlated with *E. coli* and *Enterobacter,* but negatively correlated with *Klebsiella,* consistent with potential niche differentiation or competitive interactions.

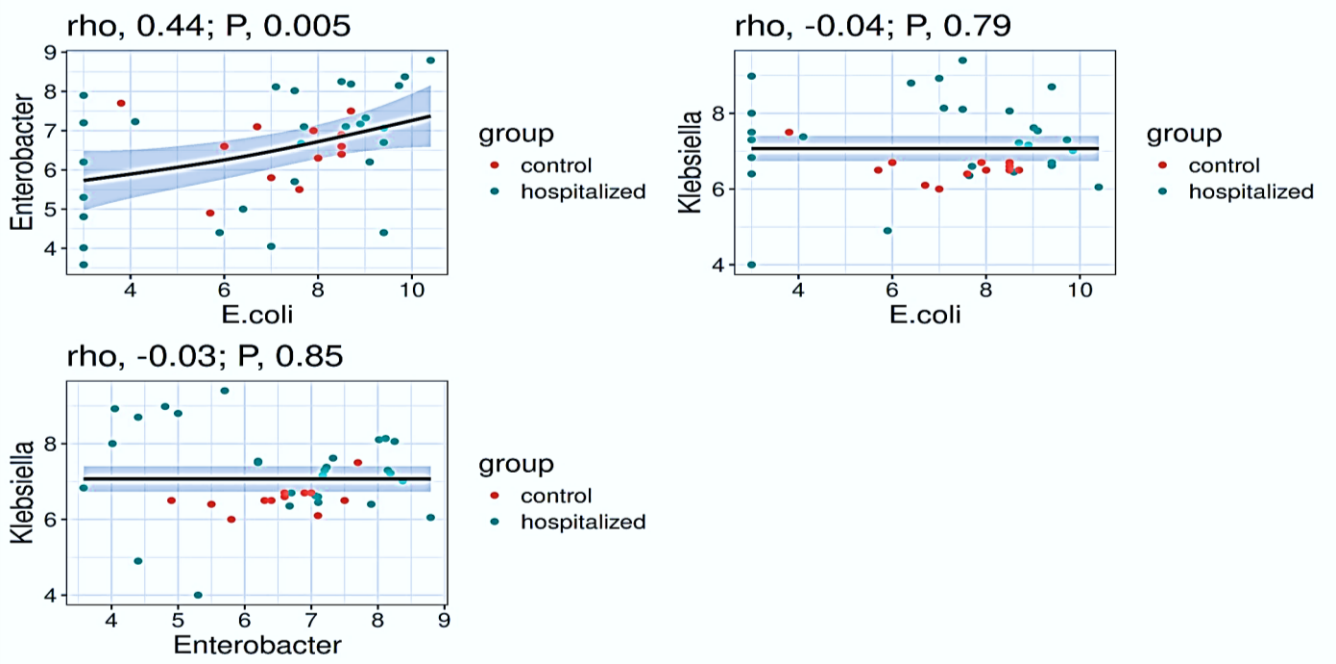

Figure 6: Spearman Correlation Network of *Enterobacteriaceae* and Associated Bacterial Taxa.

4.5 Correlations Involving *Enterococcus*

Spearman's rank correlation analysis was conducted to examine the associations between *Enterococcus* titers and other bacterial taxa (**Figure 7; Table 3**). *Enterococcus* showed positive correlations with total bacterial load (16S rRNA), *Bacteroidota, Bacteroides, Bifidobacterium, E. coli, and Enterobacter*.

In contrast, only weak associations were observed with *Prevotella*, suggesting limited co-variation within this dataset rather than a strong ecological linkage. *Enterococcus* also showed negative correlations with *Lactobacillus and Klebsiella*.

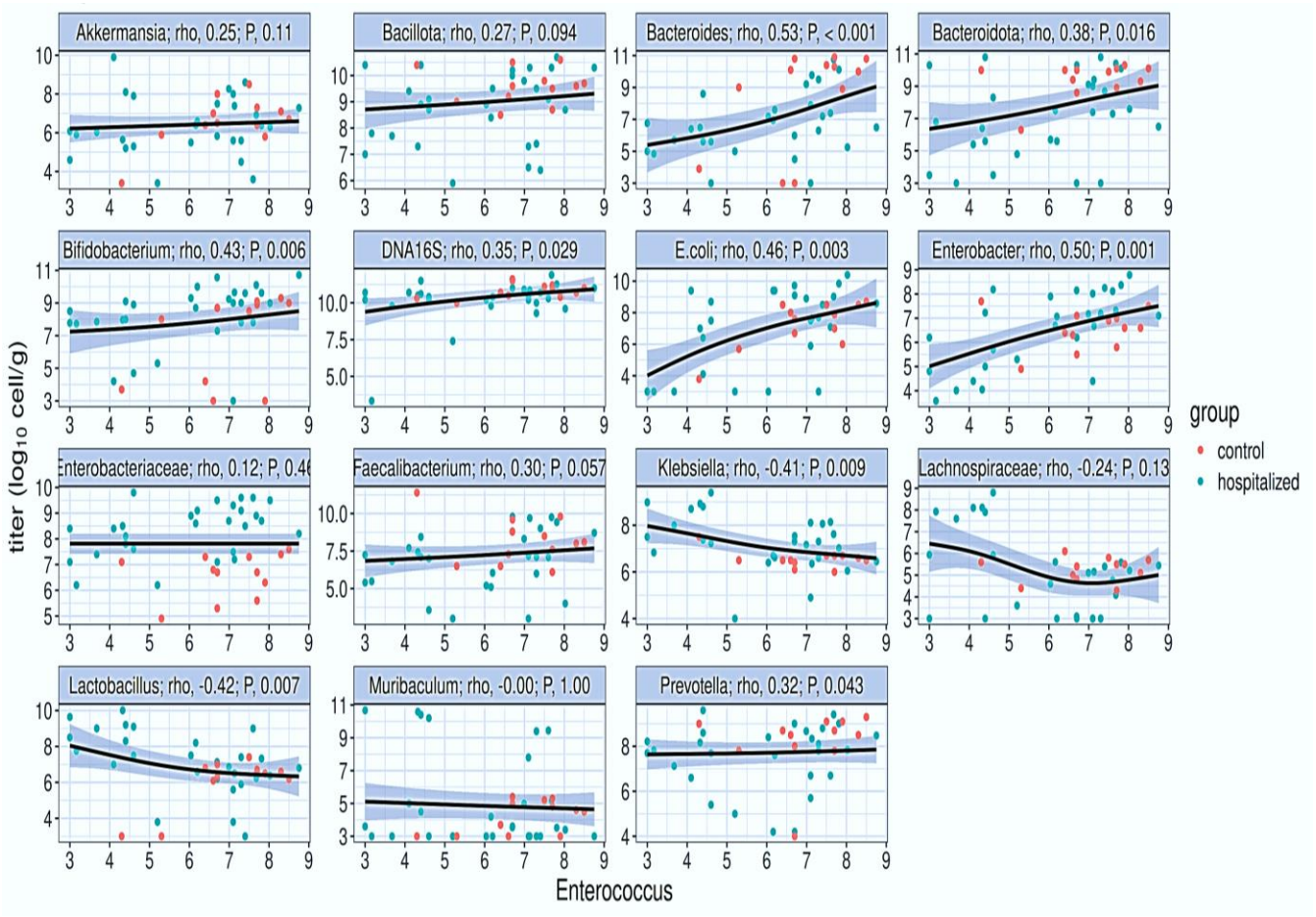

Figure 7: Associations between *Enterococcus* abundance and selected gut microbiota taxa. Scatterplots showing correlations between *Enterococcus* and bacterial taxa *abundance* (log_{10} cells/g). Points represent individual samples (control, red; hospitalized, blue). The solid line indicates the fitted trend, and the shaded area represents the 95% confidence interval. Spearman's correlation coefficient (ρ) and P-value are shown in each panel.

Table 3: Spearman Correlation Analysis of *Enterococcus* with Major Bacterial Taxa in the Fecal Microbiota

Taxon	Spearman's ρ	P-value	Interpretation
Akkermansia	0.25	0.11	Weak positive, not significant
Bacillota	0.27	0.094	Weak positive, trend only
Bacteroides	0.53	<0.001	Moderate positive, significant
Bacteroidota	0.38	0.016	Weak–moderate positive, significant
Bifidobacterium	0.43	0.006	Moderate positive, significant
Total bacteria (16S)	0.35	0.029	Weak–moderate positive, significant
Escherichia coli	0.46	0.003	Moderate positive, significant
Enterobacter	0.50	0.001	Moderate positive, significant
Enterobacteriaceae	0.12	0.46	Very weak positive, not significant
Faecalibacterium	0.30	0.057	Weak positive, borderline
Klebsiella	-0.41	0.009	Moderate inverse, significant
Lachnospiraceae	-0.24	0.13	Weak inverse, not significant
Lactobacillus	-0.42	0.007	Moderate inverse, significant
Muribaculum	0.00	1.00	No association
Prevotella	0.32	0.043	Weak positive, significant

4.6 Correlations of Lactobacillus and Bifidobacterium

Given their frequent association with dietary intake and probiotic supplementation, the relationships of *Lactobacillus and Bifidobacterium* with other microbial taxa were further analyzed (**Figures 8 and 9**). Correlation strength was interpreted according to

the absolute Spearman's rho (ρ) values as follows: 0.00–0.19 very weak, 0.20–0.39 weak, 0.40–0.59 moderate, 0.60–0.79 strong, and 0.80–1.00 very strong.

Several taxa demonstrated positive associations with *Lactobacillus*, although only some reached statistical significance (**Figure 8; Table 4**). Significant but weak positive correlations were observed between *Lactobacillus and Bacteroidota* (ρ = 0.37, P = 0.019), and *Klebsiella* (ρ = 0.42, P = 0.006), *Lachnospiraceae* (ρ = 0.42, P = 0.006), and *Muribaculum* (ρ = 0.35, P = 0.026), showing significant positive associations within the microbial community.

A very weak positive correlation trend was observed between *Lactobacillus and Escherichia coli* (ρ = 0.11, P = 0.052); however, this association did not reach statistical significance. Similarly, *Akkermansia* (ρ = −0.19, P = 0.25), *Bacillota* (ρ = 0.03, P = 0.85), DNA16S (ρ = 0.02, P = 0.91), *Enterobacterales* (ρ = 0.22, P = 0.17), *Enterobacteriaceae* (ρ = 0.22, P = 0.10), and *Prevotella* (ρ = −0.08, P = 0.64) demonstrated non-significant correlations with *Lactobacillus* abundance.

In contrast, although the association was not statistically significant, *Enterococcus* showed a weak negative correlation with *Lactobacillus* (ρ = −0.24, P = 0.14).

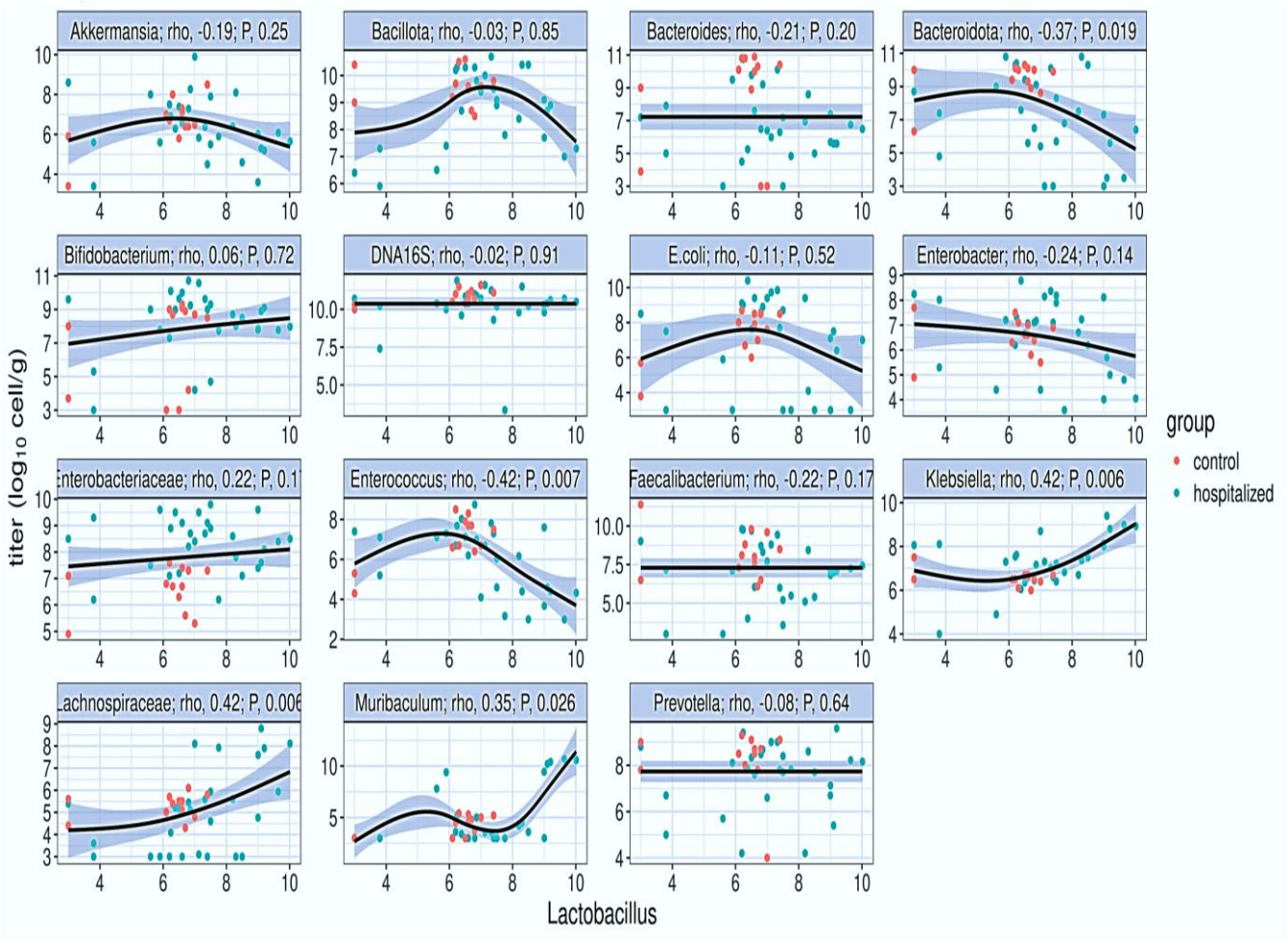

Figure 8: Associations between *Lactobacillus* abundance and selected gut microbiota taxa. Scatterplots showing correlations between *Lactobacillus* and bacterial taxa abundance ($\log_{10}$ cells/g). Points represent individual samples (control, red; hospitalized, blue). The solid line indicates the fitted trend, and the shaded area represents the 95% confidence interval. Spearman's correlation coefficient (ρ) and P-value are shown in each panel.

Table 4*:* Spearman correlation analysis between *Lactobacillus* and gut microbial taxa.

Taxon	Spearman's ρ	P-value	Interpretation
Akkermansia	-0.19	0.25	Very weak inverse, not significant
Bacillota	-0.03	0.85	Very weak inverse, not significant
Bacteroides	-0.21	0.20	Weak inverse, not significant
Bacteroidota	-0.37	0.019	Weak–moderate inverse, significant
Bifidobacterium	0.06	0.72	Very weak positive, not significant
Total bacteria (16S)	-0.02	0.91	No association
Escherichia coli	-0.11	0.52	Very weak inverse, not significant
Enterobacter	-0.24	0.14	Weak inverse, not significant
Enterobacteriaceae	0.22	0.18	Weak positive, not significant
Enterococcus	-0.42	0.007	Moderate inverse, significant
Faecalibacterium	-0.22	0.17	Weak inverse, not significant
Klebsiella	0.42	0.006	Moderate positive, significant
Lachnospiraceae	0.42	0.006	Moderate positive, significant
Muribaculum	0.35	0.026	Weak–moderate positive, significant
Prevotella	-0.08	0.64	Very weak inverse, not significant

Similarly, *Bifidobacterium* was positively correlated with total bacterial load, *E. coli, and Enterococcus* (**Figure 9; Table 5**). *Bifidobacterium* expansion may occurs alongside broader community-level shifts rather than being associated with suppression of opportunistic taxa.

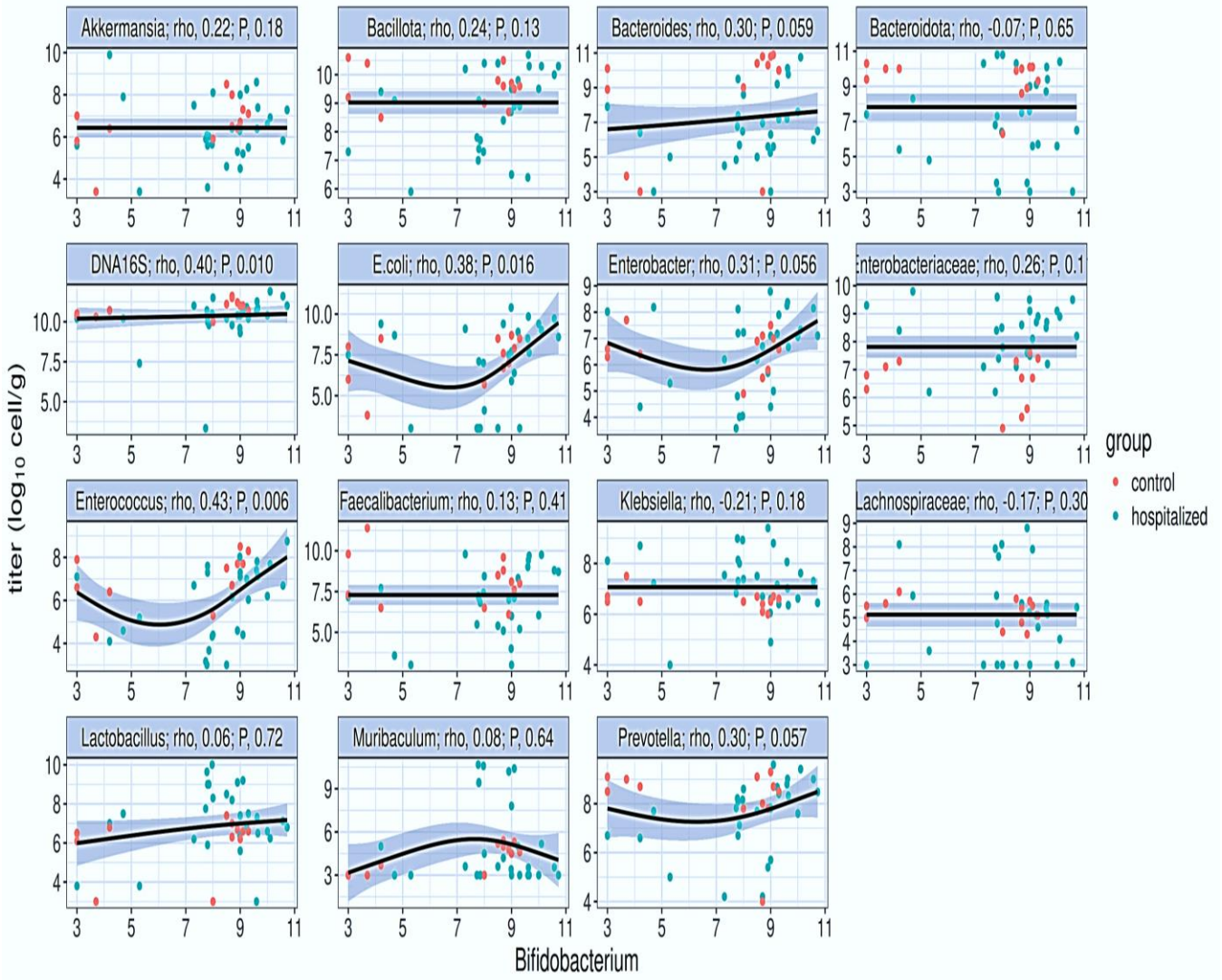

Figure 9: Associations between *Bifidobacterium* abundance and selected gut microbiota taxa. Scatterplots showing correlations between ***Bifidobacterium*** and bacterial taxa abundance (log_{10} cells/g). Points represent individual samples (control, red; hospitalized, blue). The solid line indicates the fitted trend, and the shaded area represents the 95% confidence interval. Spearman's correlation coefficient (ρ) and P-value are shown in each panel.

Table 5: Spearman correlation analysis of *Bifidobacterium* with total bacterial load and gut microbial taxa

Taxon	Spearman's ρ	P-value	Interpretation
Akkermansia	0.22	0.18	Weak positive, not significant
Bacillota	0.24	0.13	Weak positive, not significant
Bacteroides	0.30	0.059	Weak–moderate positive, borderline
Bacteroidota	-0.07	0.65	Very weak inverse, not significant
Total bacteria (16S)	0.40	0.010	Moderate positive, significant
Escherichia coli	0.38	0.016	Moderate positive, significant
Enterobacter	0.31	0.056	Weak–moderate positive, borderline
Enterobacteriaceae	0.26	0.10	Weak positive, not significant
Enterococcus	0.43	0.006	Moderate positive, significant
Faecalibacterium	0.13	0.41	Very weak positive, not significant
Klebsiella	-0.21	0.18	Weak inverse, not significant
Lachnospiraceae	-0.17	0.30	Weak inverse, not significant
Lactobacillus	0.06	0.72	Very weak positive, not significant
Muribaculum	0.08	0.64	Very weak positive, not significant
Prevotella	0.30	0.057	Weak–moderate positive, borderline

4.7 Summary of correlations

Overall, the correlation network showed structured patterns of associations within the gut microbiota. A negative correlation was observed between *Lactobacillus* and *Enterococcus*, while a positive correlation was observed between *Lactobacillus* and *Klebsiella* **(Figure 10).**

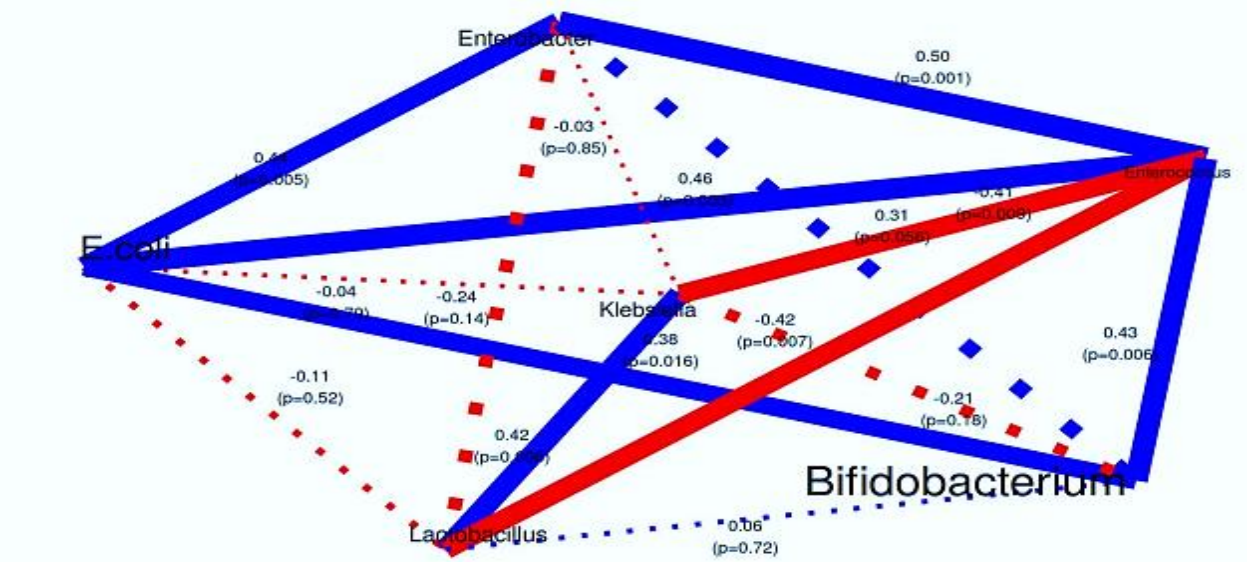

Figure 10. Integrated correlation network of key bacterial taxa. Edges represent significant Spearman correlations between taxa. Blue indicates positive correlations and orange indicates negative correlations. Edge thickness reflects correlation strength (|ρ|). All rho and P values are correctly mapped to corresponding connections, and node labels are displayed with uniform font size for consistency.

4.8 ESBL Colonization

None of the control children were colonized with extended-spectrum β-lactamase–producing *Enterobacteriaceae* (ESBL-EB), whereas ESBL-EB presence was identified in 28.6% of hospitalized children (8/28; 95% confidence interval: 13%–49%) (Appendix, Table A4). ESBL-negative samples were assigned the lower limit of detection value (10^2 CFU/g feces). Among ESBL-positive hospitalized children, fecal ESBL-EB titers ranged from approximately 10^3 to 10^7 CFU/g feces, with a median value of approximately 5.6 $\log_{10}$ CFU/g among quantifiable positive samples.

Overall gut microbiota composition differed significantly among control/ESBL−, hospitalized/ESBL−, and hospitalized/ESBL+ groups (PERMANOVA, P = 0.006). Principal coordinate analysis demonstrated greater inter-individual variability among hospitalized children compared with controls, whereas substantial overlap persisted between ESBL-positive and ESBL-negative hospitalized subgroups (**Figure 11**).

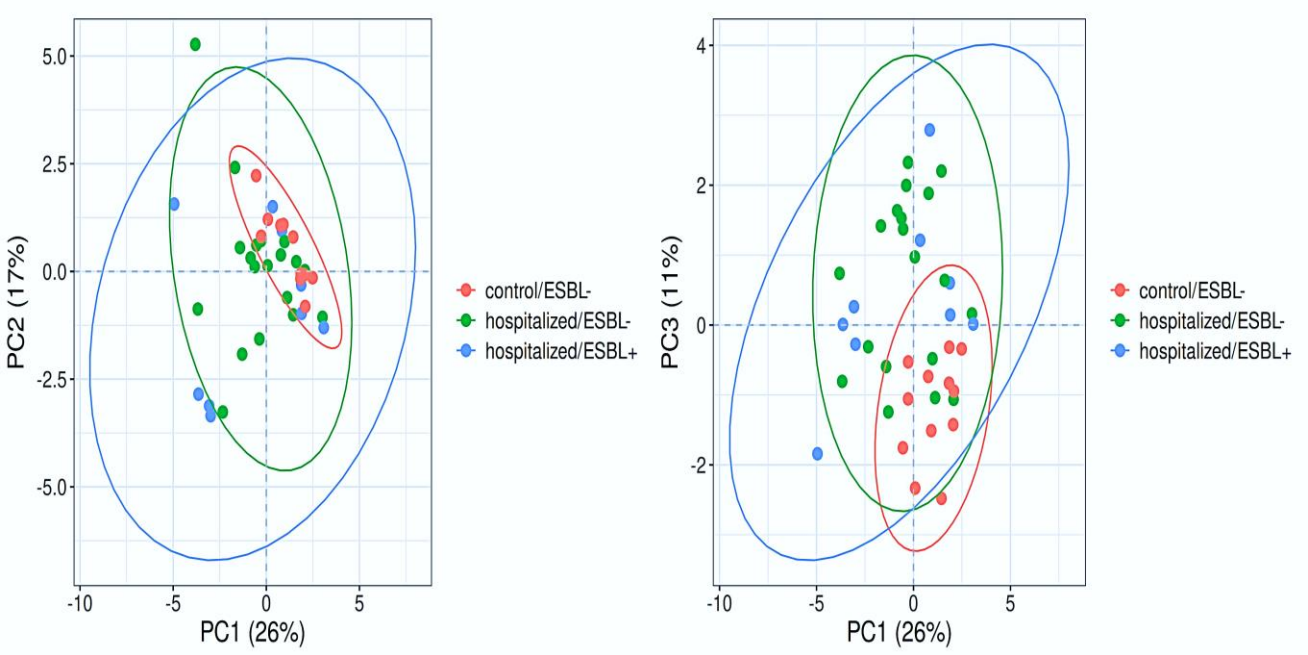

Figure 11. PCoA of microbiota composition: PCoA plots (PC1 vs PC2; PC1 vs PC3) showing clustering of control/ESBL− (red), hospitalized/ESBL− (green), and hospitalized/ESBL+ (blue).

Comparisons of bacterial taxa demonstrated that most compositional differences were associated with hospitalization status rather than ESBL carriage (**Figure 12**). Only limited taxonomic differences were observed between ESBL-positive and ESBL-negative hospitalized children. In particular, *Enterobacteriaceae* and *Klebsiella* showed higher abundances in ESBL-positive children, while no significant differences were observed for the remaining taxa. Overall, bacterial community differences were more pronounced between hospitalized and control groups than between ESBL-positive and ESBL-negative hospitalized subgroups.

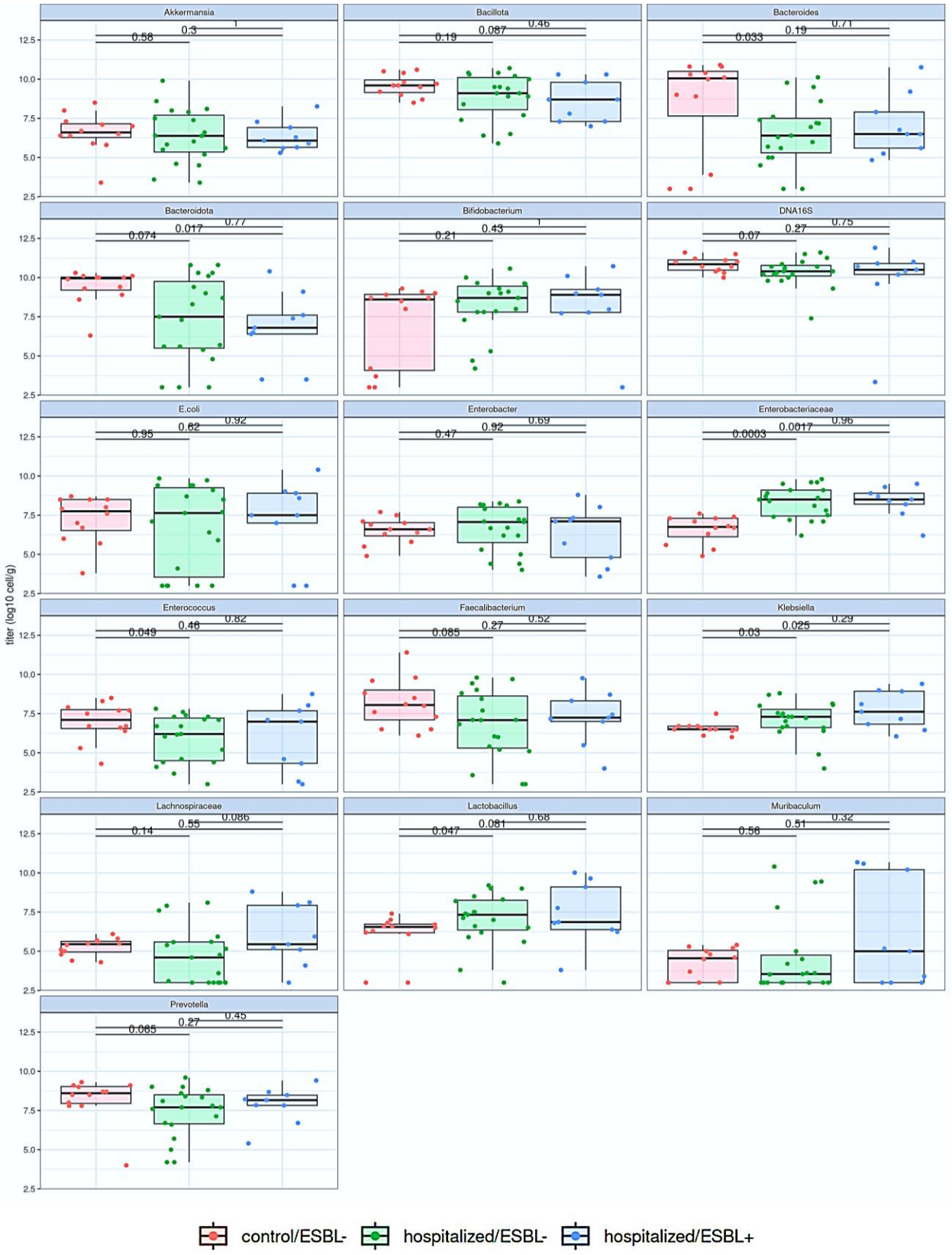

Figure 12. Taxa titers across groups: Boxplots of log_{10} bacterial titers by group (control/ESBL−, hospitalized/ESBL−, hospitalized/ESBL+). Points are individual samples; p-values are shown.

Importantly, correlation analyses restricted to hospitalized children showed weak positive associations between ESBL-EB titers and total bacterial load (16S rRNA gene), *Faecalibacterium, Lachnospiraceae*, and *Prevotella* (Spearman's ρ = 0.38–0.40; **Figure 13**). However, these associations were modest and accompanied by borderline P values, indicating limited statistical robustness.

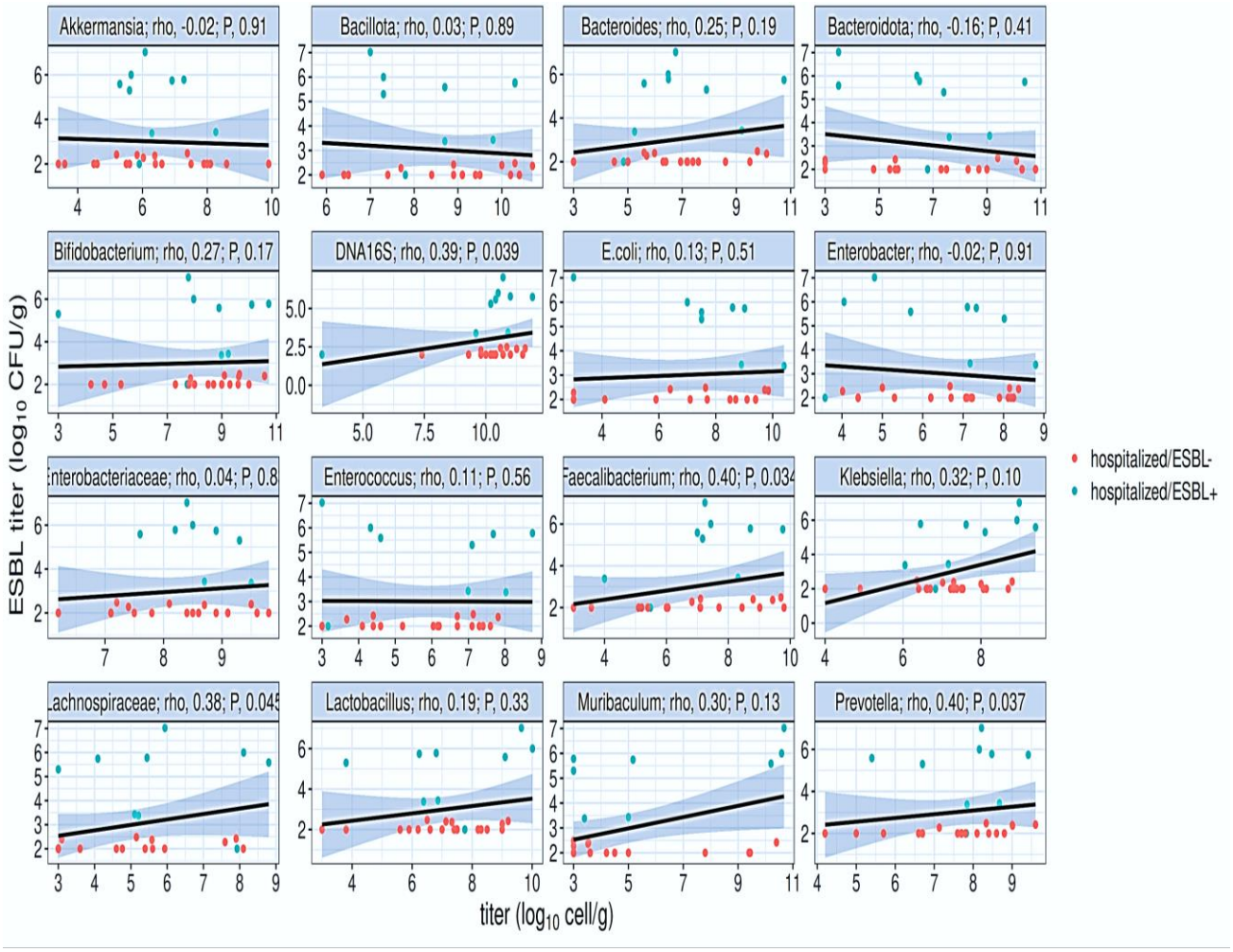

Figure 13. ESBL titer–taxa associations: Scatter plots of ESBL titers vs taxa abundance. Red dots represent ESBL-negative samples and green dots represent ESBL-positive samples. The black line indicates the fitted regression line and the shaded area represents the 95% confidence interval. Spearman's correlation coefficients (ρ) and P values are shown for each taxon. Note: The individual green sample located below the regression line indicates an ESBL-positive sample with a lower ESBL-EB titer than predicted by the overall trend.

CHAPTER FIVE: DISCUSSION

5.1 Ecological Architecture of the Fecal Microbiota

The present study shows that the pediatric gut microbiota is organized along major axes of variation rather than discrete taxonomic clusters. Integration of Principal Coordinate Analysis (PCoA) with correlation-based ecological mapping revealed that microbial community structure in hospitalized children is characterized by both compositional shifts and increased inter-individual variability. The first three principal coordinates accounted for 54% of the total variance, indicating that a limited number of axes capture a substantial proportion of gut microbiota structure.

A key observation is that members of the family *Enterobacteriaceae* do not behave as a unified ecological group. Despite close phylogenetic relatedness, *Escherichia coli, Enterobacter, and Klebsiella* occupied distinct positions in ordination space and exhibited divergent correlation patterns. This finding reinforces the concept that phylogenetic proximity does not necessarily predict ecological similarity in the gut ecosystem. Instead, functional traits—such as metabolic capacity, oxygen tolerance, and adaptation to inflammatory conditions—likely underpin these differences. (Lozupone et al., 2012).

Correlation analyses further supported these differences. *E. coli and Enterobacter* showed moderate positive associations, suggesting partial overlap in ecological drivers, whereas *Klebsiella* consistently displayed a distinct pattern across ordination space and network analyses. Collectively, these findings indicate differential ecological associations within *Enterobacteriaceae* taxa under the observed conditions. Importantly, all observed relationships reflect patterns of co-variation rather than direct biological interactions, and causal inference cannot be established from correlation-based approaches.

From an integrated perspective, three principal observations emerge. First, the fecal microbiota of hospitalized children is characterized by pronounced inter-individual variability. Second, hospitalization is associated with increased abundance of *Enterobacteriaceae* and global shifts in community structure, although these effects are highly heterogeneous across individuals. Third, ESBL-producing *Enterobacteriaceae* colonization is not associated with a distinct or stable microbial signature. Collectively, these findings indicate that pediatric intestinal ecology during hospitalization is defined

primarily by compositional differences and inter-individual variability rather than uniform shifts across all patients.

5.2 Effects of Hospitalization on Microbial Balance

Hospitalized children exhibited significant alterations in overall gut microbiota composition compared with healthy controls, as demonstrated by beta diversity analysis and confirmed by PERMANOVA **(Figure 2).** However, when individual taxa were examined using univariate statistical approaches, only *Enterobacteriaceae* remained significantly different after false discovery rate (FDR) correction **(Table 2).** This discrepancy highlights an important ecological principle: microbiota disturbance often manifests as coordinated restructuring of the microbial community rather than large, unidirectional shifts in individual taxa. Accordingly, multivariate approaches such as beta diversity are generally more sensitive to ecosystem-level perturbations than single-taxon analyses. Similar patterns have been reported in previous microbiome studies, where substantial community reorganization occurs despite limited taxon-level significance (Lozupone et al., 2012; Falony et al., 2016).

The consistent enrichment of Enterobacteriaceae in hospitalized children aligns with established evidence linking intestinal dysbiosis to expansion of facultative anaerobic Proteobacteria. Members of this group, including *Escherichia, Klebsiella, and Enterobacter*, are well adapted to disturbed intestinal environments, particularly those characterized by inflammation, antibiotic exposure, or altered oxygen gradients. Their expansion is widely considered a marker of reduced intestinal homeostasis (Shin et al., 2015; Litvak et al., 2017).

Several taxa showed non-significant directional patterns that did not retain statistical robustness after FDR correction. *Lactobacillus and Klebsiella* displayed nominal increases (raw $p < 0.05$), suggesting possible enrichment in subsets of hospitalized children; however, these findings should be interpreted cautiously as they did not survive correction for multiple testing. Similarly, *Bacteroides and Bacteroidota* showed nominal decreases, but these effects were not statistically robust and therefore cannot be considered conclusive.

A subset of taxa, including *Enterococcus, Faecalibacterium, and Prevotella*, exhibited trend-level reductions with borderline statistical support. These patterns were

characterized by substantial overlap between hospitalized and control groups, indicating marked inter-individual variability and limited separation at the taxon level.

In contrast, several taxa—including *Akkermansia, Bifidobacterium, Escherichia coli, Enterobacter, Lachnospiraceae, Muribaculum*, and DNA16S—showed no consistent or statistically supported differences between groups. This suggests that most components of the pediatric gut microbiota remain stable across hospitalization status in this cohort (Sommer et al., 2017; David et al., 2014).

Distributional analysis further confirmed extensive overlap between hospitalized and control groups for most taxa, while *Enterobacteriaceae* showed the most consistent upward shift. Nevertheless, even for this taxon, inter-individual variability was evident, indicating that hospitalization does not produce a uniform microbial pattern across all patients.

A notable characteristic of the hospitalized group was higher inter-individual variability in microbial composition. Rather than consistent directional changes across taxa, hospitalized children exhibited differences in community composition and variability in abundance profiles across individuals. This pattern suggests that hospitalization is associated with heterogeneous microbial responses rather than a uniform compositional shift across all patients.

Overall, these findings indicate that gut microbiota alterations during hospitalization are characterized by selective enrichment of *Enterobacteriaceae* and pronounced inter-individual variability, while most other taxa show weak, inconsistent, or absent group-level differences.

5.3 ESBL Colonization: Absence of a Specific Microbial Signature

ESBL-producing *Enterobacteriaceae* were detected exclusively in hospitalized children, consistent with established epidemiological evidence linking healthcare exposure to acquisition of antimicrobial resistance organisms (Woerther et al., 2013). However, no distinct microbiota signature differentiating ESBL-positive from ESBL-negative children was identified. Although overall microbial composition differed across control, hospitalized ESBL-negative, and hospitalized ESBL-positive groups, neither differential abundance nor correlation analyses revealed robust ESBL-specific microbial patterns. Observed associations were weak and inconsistent, limiting their biological interpretability.

These findings suggest that ESBL colonization does not depend on a unique microbial community structure but may instead occur opportunistically within a disturbed intestinal ecosystem characterized by increased *Enterobacteriaceae* abundance and reduced colonization resistance. In this context, ESBL carriage may reflect differences in the abundance of resistant strains within an existing microbial community rather than major compositional changes in community structure. This interpretation is consistent with previous studies showing that antimicrobial-resistant colonization is not necessarily associated with major taxonomic shifts but may instead arise from the expansion of resistant strains embedded within existing microbial communities (Taur & Pamer, 2013; David et al., 2017; de Lastours et al., 2020). These findings suggest that ESBL colonization may occur alongside broader compositional variability within the gut microbiota rather than being restricted to a distinct microbial state (Woerther et al., 2013).

These findings also highlight a methodological limitation: taxonomic profiling alone may not be sufficient to identify determinants of antimicrobial-resistant colonization. Future studies incorporating metagenomic sequencing, resistome profiling, and longitudinal sampling will be necessary to better resolve the interaction between microbial community structure and resistance gene dynamics.

5.4 Implications for Microbiota-Modulating Strategies

Lactobacillus and Bifidobacterium are widely considered beneficial commensal bacteria and are commonly used in probiotic and dietary interventions aimed at restoring microbial balance or limiting pathogen colonization. In this study, we evaluated the associations between these genera and multidrug-resistant (MDR) organisms.

A significant inverse association was observed between *Lactobacillus* and *Enterococcus,* suggesting potential antagonistic or competitive interactions **(Figure 8)**. Although correlation analyses do not establish direct biological interactions, this observation is biologically plausible, as *Lactobacillus* species can produce antimicrobial metabolites, including lactic acid, bacteriocins, and other inhibitory compounds capable of suppressing Gram-positive organisms. Previous studies have similarly reported inhibitory effects of *Lactobacillus* strains against *Enterococcus* spp. in intestinal and mucosal environments (Ramia et al., 2008; Tytgat et al., 2016). In contrast, *Lactobacillus* showed a positive association with *Klebsiella* and no consistent inverse relationship with *Enterobacteriaceae*

overall. This indicates that the observed association patterns do not support a uniform relationship between *Lactobacillus* and MDR-associated taxa. Rather than direct interactions, these associations may reflect underlying differences in community composition or shared responses to host and environmental factors (Faust & Raes, 2012; Coyte et al., 2015).

Similarly, *Bifidobacterium* showed positive associations with *Escherichia coli* and *Enterococcus*, indicating that these taxa may co-occur within the same microbial communities **(Figure 9)**. These findings challenge the assumption that enrichment of traditionally beneficial genera necessarily results in suppression of opportunistic or multidrug-resistant organisms. An additional mechanism may involve metabolic cross-feeding. Lactate produced by *Lactobacillus* can serve as a substrate for other microbial members, including opportunistic taxa under specific conditions, potentially contributing to their persistence. Such interactions highlight the complexity of microbial metabolic networks within the gut ecosystem (Heinken& Thiele, 2021).

These findings suggest that microbiota-modulating strategies should be guided by ecological principles rather than generalized assumptions of benefit. Probiotic interventions should be evaluated within a systems-level framework that considers competitive, neutral, and facilitative interactions across microbial networks. While *Lactobacillus* showed an inverse association with *Enterococcus*, it also exhibited a positive association with Klebsiella, indicating that these relationships may be context-dependent. More broadly, these results support a precision-microbiome approach integrating ecological structure, metabolic interactions, and strain-level dynamics. Uniform probiotic administration is unlikely to produce consistent outcomes across heterogeneous populations. Instead, individualized approaches based on baseline microbiota composition and ecological state are likely required. The gut microbiome should therefore be viewed as a dynamic ecosystem in which external interventions may produce non-linear and sometimes unexpected outcomes.

Importantly, all observed associations are correlation-based analyses and do not imply causality. Nevertheless, they provide a useful ecological framework for interpreting microbiota–host–pathogen interactions in hospitalized children. In particular, *Bifidobacterium* showed positive associations with opportunistic taxa such as *Escherichia coli* (as well as total bacterial load and *Enterococcus*), while *Lactobacillus* showed no consistent association with *E. coli* but was positively associated with *Klebsiella*.

Together, these patterns suggest that the presence of genera commonly considered beneficial does not necessarily correspond to lower abundance of multidrug-resistant organisms. Instead, the observed associations may reflect broader patterns of microbial co-occurrence within the gut microbiota rather than direct inhibitory or protective relationships.

5.5 Study Limitations

Several limitations should be considered when interpreting the findings of this study. First, the cross-sectional design precludes assessment of temporal changes in the gut microbiota and does not allow causal relationships to be inferred between microbiota composition, hospitalization, and ESBL colonization. The observed associations therefore reflect patterns of co-occurrence and community composition rather than direct biological interactions or causal ecological mechanisms.

Second, the study was conducted within a specific pediatric population and healthcare setting, which may limit the generalizability of the findings to other populations, geographic regions, age groups, or clinical contexts. Differences in antibiotic exposure, dietary practices, underlying medical conditions, and healthcare environments may influence microbiota composition and antimicrobial-resistant colonization patterns.

Third, several host and environmental factors that may affect gut microbiota structure, including prior antibiotic exposure, diet, hospitalization characteristics, and other clinical variables, could not be fully controlled. Consequently, residual confounding cannot be excluded.

Finally, the analyses were based primarily on taxonomic composition and correlation-based approaches. While these methods provide valuable insights into microbial community structure and associations among taxa, they do not directly assess microbial function, metabolic activity, or strain-level variation. Future longitudinal studies incorporating metagenomic, metabolomic, and functional analyses are needed to clarify the mechanisms underlying the observed associations and to evaluate their clinical significance.

CHAPTER SIX: CONCLSUIONS

The present study shows that the pediatric gut microbiota of hospitalized children differs globally from that of healthy controls, primarily through increased relative abundance of *Enterobacteriaceae*, including *Klebsiella*. These alterations were not uniformly distributed across individuals, with marked changes observed in a subset of hospitalized children. Overall, hospitalization is associated with compositional differences in the gut microbiota and increased inter-individual variability rather than a single consistent microbial signature. At the ecological level, *Enterobacteriaceae* genera did not behave as a coherent ecological group. *Escherichia coli* and *Enterobacter* were positively associated with each other and with *Enterococcus*, whereas *Klebsiella* exhibited a distinct pattern, including a negative association with *Enterococcus* and separation within multivariate analyses. These findings indicate that taxonomic relatedness does not necessarily reflect ecological similarity within the gut microbiome.

No consistent or robust differences were observed between ESBL-positive and ESBL-negative hospitalized children, indicating that ESBL colonization is not linked to a distinct microbial signature in this cohort. Instead, ESBL carriage appears to occur within a broader microbial community context characterized by Enterobacteriaceae expansion and compositional variability among individuals**.**

From a translational perspective, the observed microbial association patterns suggest that increasing *Lactobacillus* or *Bifidobacterium* abundance may not uniformly correspond to lower abundance of Enterobacteriaceae or multidrug-resistant organisms. Although *Lactobacillus* showed an inverse association with Enterococcus**,** its positive association with *Klebsiella* highlights the possibility that microbial associations may be context-dependent. These findings emphasize the need for cautious, evidence-based interpretation of microbiota-modulating interventions.

Overall, this work highlights compositional differences in the pediatric gut microbiota during hospitalization. It further suggests the importance of considering microbial community structure and inter-taxa relationships—rather than individual taxa alone—when investigating antimicrobial-resistant colonization or designing future microbiome-based therapeutic strategies.

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APPENDIX

Table A1. Sequences of qPCR primers, including target taxa/genes, primer names, nucleotide sequences (5′–3′), expected amplicon sizes, amplification conditions, and corresponding references.

Target Taxon / Gene	Primer Name	Sequence (5′→3′)	Reference
Total bacteria (16S)	Uni331-F	TCCTACGGGAGGCAGCAGT	Nadkarni et al. 2002
	Uni797-R	GGACTACCAGGGTATCTAATCCTGTT	
***Enterococcus* spp. (16S)**	cocF	CCCTTATTGTTAGTTGCCATCATT	Blake et al., 2019
	cocR	ACTCGTTGTACTTCCCATTGT	
***Lactobacillus* spp. (16S)**	Lact-F	AGCAGTAGGGAATCTTCCA	Galanis et al., 2015
	Lact-R	CACCGCTACACATGGAG	
***Muribaculum* sp.**	Mb-F	GAGAGTACCTGAAGAAAAAGC	Le Guern et al. 2019
	Mb-R	ACGCATTCCGCATACTTCT	
***Lachnospiraceae* (MGBC164943)**	L164-1F	TATTGCCGCCTGCTCAGGAGCAAC	Custom ; from UBA7160 (spo03618585)
	L164-1R	GCGCATCGCACACAAATGCAGAAG	
Adlercreutziacaeci muris	ACA-F	AGTCACGCACCCCCGTATTCTC	Ishnaiwer et al. 2024
	ACA-R	CGCGCCATTCGATGATGCTTCC	
Klebsiella pneumonia	ZKIR-F	CTAAAACCGCCATGTCCGATTTAA	Barbier et al., 2020
	ZKIR-R	TTCCGAAAATGAGACACTTCAGA	
Akkermansia ARN16S	AM-F	CAG CAC GTG AAG GTG GGG AC	Collado et al., 2007
	AM-R	CCT TGC GGT TGG CTT CAG AT	
E. coli	gadE-F	TGCCCCATAAGAATTCACAA	Tillman et al., 2015
	gadE-R	GTGACGATGTCGCTCATACG	
Prevotella	Prevo-F	CACRGTAAACGATGGATGCC	Scher et al., 2013
	Prevo-R	GGTCGGGTTGCAGACC	

Bacteroides spp. (16S)	Bdes-F	AAGGTCCCCCACATTGG	Manz et al.
	Bdes-R	GAGCCGCAAACTTTCACAA	–
***Bifidobacterium* spp. (16S rRNA)**	Bif-F	TCGCGTCYGGTGTGAAAG	Blake et al., 2019
	Bif-R	CCACATCCAGCRTCCAC	–
***Enterobacter* spp. (23S rRNA)**	E23SF	GTTAGTGGAACGGTCTGGAAAG	Patel et al., 2016
	E23SR	TCGGTCAGTCAGGAGTATTTAGC	–
Bacillota (Firmicutes)	Firm934F	GGAGYATGTGGTTTAATTCGAAGCA	Guo et al., 2008; Samudio-Cruz et al., 2025;Lutsiv et al., 2022
	Firm1060R	AGCTGACGACAACCATGCAC	–
***Enterobacteri*aceae (16S)**	Ecol457-F	CATTGACGTTACCCGCAGAAGAAGC	Bartosch et al.; Hakansson et al., 2015
	Ecol652-R	CTCTACGAGACTCAAGCTTGC	–
***Faecalibacterium* spp.**	Faec-F	GAAGGCGGCCTACTGGGCAC	Blake et al., 2019; Hirasaki et al., 2025
	Faec-R	GTGCAGGCGAGTTGCAGCCT	–
***Bacteroidota* (Bacteroidetes)**	Bact934F	GGARCATGTGGTTTAATTCGATGAT	Guo et al., 2008
	Bact1060R	AGCTGACGACAACCATGCAG	–

Table A2. Quantitative Details of fecal microbiota abundance (log_{10} cells/g) in hospitalized versus control children.

Category	Taxa	Pattern in Hospitalized Children	Key Quantitative Details (log_{10} cells/g)
FDR-significant increase	*Enterobacteriaceae*	Consistently higher	20 children > 7.6
Nominal (not FDR-significant)	*Bacteroidota*	Reduced in subset	10 children < 6.3
	Bacteroides	Variable / lower trend	High variability in controls
	Klebsiella	Mixed (increase and decrease)	11 > 7.5; 2 < 6.0
	Lactobacillus	Increased in subset	12 > 7.4
Reduced in subset	*Faecalibacterium*	Decreased	10 < 6.1
	Enterococcus	Decreased	5 < 4.3
	Lachnospiraceae	Decreased in majority; increased in subset	11 < 4.3; 6 > 6.1
Increased in subset	*Bifidobacterium*	Increased	7 > 9.3
	Muribaculum	Increased	7 > 5.4
Mixed / bidirectional response	*Escherichia coli*	Both increased and decreased	9 > 8.7; 7 < 3.8
	Enterobacter	Both increased and decreased	8 > 7.7; 6 < 4.9
No significant difference	*Akkermansia*	Stable	Similar in both groups
	Prevotella	Stable	Similar in both groups

Table A3. Quantitative PCR profiling of fecal microbiota in hospitalized (H) and control (C) children. Values are expressed as $\log_{10}$ bacterial abundance (CFU-equivalents/g feces), including total bacterial load (16S rRNA gene) and selected microbial taxa.

ID	Fecal mass (mg)	Total bacteria (16S rRNA)	*Bacillota*	*Faecalibacterium*	*Enterococcus*	*Lactobacillus*	*Lachnospiraceae*	*Bacteroidota*	*Bacteroides*	*Prevotella*	*Muribaculum sp.*	*Enterobacteriaceae*	*Escherichia coli*	*Enterobacter*	*Klebsiella (genus)*	*Akkermansia*	*Bifidobacterium*
H1	86	10.9	10.3	9.7	7.1	6.5	5.2	9.4	9.8	8.3	3.0	7.2	7.6	6.7	6.4	7.4	9.6
H2	176	9.8	7.7	6.8	3.7	9.0	7.6	3.0	5.7	7.1	3.0	7.4	3.0	4.0	8.0	6.0	7.9
H3	92	11.3	10.7	9.4	7.8	7.3	5.6	10.1	10.1	9.0	3.5	8.7	9.9	8.4	7.0	6.4	9.6
H4	100	10.4	8.7	7.0	4.6	9.1	8.8	3.5	5.6	5.4	10.2	7.6	7.5	5.7	9.4	5.3	8.9
H5	50	11.0	10.3	8.7	8.8	6.8	5.4	6.5	6.5	8.5	3.0	8.2	8.6	7.1	6.4	7.3	10.7
H6	129	3.3	7.8	5.5	3.2	7.8	7.9	6.8	4.8	7.8	3.0	6.2	3.0	3.6	6.8	5.9	7.7
H7	103	10.5	7.3	7.4	4.3	10.0	8.1	6.4	6.5	8.2	10.6	8.5	7.0	4.1	8.9	5.6	8.0
H8	125	10.7	7.0	7.2	3.0	9.6	5.9	3.5	6.8	8.2	10.7	8.4	3.0	4.8	9.0	6.1	7.8
H9	99	9.6	8.7	4.0	8.0	6.4	5.2	7.6	5.2	7.8	3.4	9.5	10.4	8.8	6.1	6.3	9.0
H10	100	10.6	8.9	7.1	4.4	9.2	7.9	5.6	5.6	9.6	10.4	8.1	6.4	5.0	8.8	5.2	9.1
H11	83	11.9	10.3	9.8	7.7	6.2	4.1	10.4	10.8	9.4	5.2	8.9	9.0	7.3	7.6	6.9	10.1
H12	119	11.6	10.0	8.8	6.7	7.1	3.1	2.8	6.0	9.0	3.5	9.5	9.7	8.1	7.3	5.8	10.6
H13	123	10.9	9.8	8.3	7.0	6.9	5.1	9.1	9.2	8.7	5.0	8.7	8.9	7.2	7.2	8.3	9.2
H14	137	10.7	9.4	7.7	4.1	7.0	8.1	5.4	6.4	6.6	5.0	8.4	9.4	4.4	8.7	9.9	4.2
H15	159	10.2	8.9	5.2	6.0	7.5	4.6	5.7	7.2	8.4	3.0	8.9	3.0	7.9	6.4	5.5	9.3
H16	119	9.8	8.4	5.1	6.2	8.2	5.6	7.5	6.9	4.2	4.2	8.6	9.4	6.7	6.7	6.4	8.7
H17	150	10.7	6.4	9.0	7.4	3.0	5.4	8.7	7.2	8.8	3.0	8.5	8.5	8.3	8.1	8.6	9.6
H18	103	7.4	5.9	2.3	5.2	3.8	3.6	4.8	5.0	5.0	3.0	6.2	3.0	5.3	4.0	3.4	5.3
H19	105	10.2	9.1	3.6	4.6	7.5	5.9	8.3	3.0	7.7	3.0	9.8	8.7	8.2	7.2	7.9	4.7
H20	114	10.2	7.3	7.2	7.1	3.8	3.0	7.4	7.9	6.7	3.0	9.3	7.5	8.0	8.1	5.6	3.0
H21	103	10.0	7.4	7.1	7.3	5.9	3.0	10.8	9.5	8.1	9.4	9.6	3.0	7.2	7.3	5.6	7.8
H22	103	10.3	9.1	7.1	7.6	9.0	4.8	7.3	7.4	6.7	9.4	9.6	7.1	8.1	8.1	3.6	7.8
H23	109	11.5	10.4	8.4	4.4	8.3	3.0	10.8	8.6	8.6	4.5	7.8	4.1	7.2	7.4	8.1	8.0
H24	107	10.2	10.4	5.4	3.0	8.5	3.0	10.3	5.0	7.7	3.6	7.1	3.0	6.2	7.5	4.6	8.5
H25	107	11.0	10.2	9.8	6.7	6.2	3.0	10.3	4.5	4.2	3.6	7.1	9.1	6.2	7.5	7.5	7.3
H26	100	9.3	9.5	6.0	7.3	7.4	3.0	3.0	6.3	7.8	3.0	9.1	7.7	7.1	6.6	4.5	9.0
H27	107	10.4	9.5	6.0	6.2	6.6	3.0	5.6	7.6	7.6	3.0	9.1	9.4	7.1	6.6	6.6	10.0
H28	106	10.4	6.5	3.0	7.1	5.6	3.0	9.0	3.0	5.7	7.8	7.5	5.9	4.4	4.9	8.0	9.0
C1	30	10.0	9.0	6.5	5.3	3.0	4.4	6.3	9.0	7.8	3.0	4.9	5.7	4.9	6.5	5.9	8.0
C2	36	10.7	8.5	6.5	6.4	6.8	6.1	10.0	3.0	8.7	3.7	7.3	8.5	6.4	6.5	6.4	4.2
C3	30	10.5	9.2	7.3	6.6	6.1	5.0	9.4	10.1	8.5	3.0	6.8	8.0	6.3	6.5	7.0	3.0
C4	30	11.0	9.5	7.6	7.7	6.6	5.5	10.1	10.9	8.7	5.3	6.7	7.9	7.0	6.7	7.3	9.1
C5	92	10.3	10.4	11.4	4.3	3.0	5.6	10.0	3.9	9.0	3.0	7.1	3.8	7.7	7.5	3.4	3.7
C6	100	11.6	9.6	9.6	6.7	7.0	4.8	8.6	3.0	4.0	5.0	5.3	7.6	5.5	6.4	6.5	8.7
C7	72	10.4	10.6	9.8	7.9	6.5	5.5	10.3	8.9	9.1	3.0	6.3	6.0	6.6	6.7	5.8	3.0
C8	96	11.5	10.5	8.8	6.7	6.3	5.4	10.0	10.8	8.0	5.4	6.7	6.7	7.1	6.1	8.0	8.7
C9	88	11.2	8.7	6.1	7.7	6.7	4.3	8.9	10.3	7.8	4.8	5.6	7.0	5.8	6.0	6.4	8.9
C10	92	11.1	9.8	8.5	7.5	7.4	5.8	9.9	10.4	9.1	5.2	7.3	8.5	6.9	6.7	8.5	8.5
C11	100	10.7	9.6	8.0	8.3	6.6	5.1	9.3	10.0	8.5	4.6	7.4	8.5	6.6	6.6	7.1	9.3
C12	86	11.0	9.7	8.1	8.5	6.2	5.7	10.1	10.8	9.3	4.5	7.6	8.7	7.5	6.5	6.7	9.0

Table A4. Detection and quantitative fecal titers of ESBL-producing *Enterobacteriaceae* (ESBL-EB) in hospitalized (H) and control (C) children, including fecal sample mass, presence of ESBL-producing Klebsiella spp. and Escherichia coli, and calculated bacterial load (CFU/g feces).

Identifier	faeces.mass.mg	ESBL. *Klebsiella*	ESBL. *E. coli*	(ESBL. EB) titer CFU/g
H1	86	negative	negative	3.03E+02
H2	176	negative	negative	1.89E+02
H3	92	negative	negative	2.33E+02
H4	100	positive	negative	3.85E+05
H5	50	negative	positive	6.06E+05
H6	129	negative	negative	1.00E+02
H7	103	negative	positive	1.00E+06
H8	125	positive	negative	1.06E+07
H9	99	negative	positive	2.38E+03
H10	100	negative	negative	2.63E+02
H11	83	negative	positive	5.56E+05
H12	119	negative	negative	2.50E+02
H13	123	positive	positive	2.70E+03
H14	137	negative	negative	1.00E+02
H15	159	negative	negative	1.00E+02
H16	119	negative	negative	1.00E+02
H17	150	negative	negative	1.00E+02
H18	103	negative	negative	1.00E+02
H19	105	negative	negative	1.00E+02
H20	114	negative	positive	2.00E+05
H21	103	negative	negative	1.00E+02
H22	103	negative	negative	1.00E+02
H23	109	negative	negative	1.00E+02
H24	107	negative	negative	1.00E+02
H25	107	negative	negative	1.00E+02
H26	100	negative	negative	1.00E+02
H27	107	negative	negative	1.00E+02

H28	106	negative	negative	1.00E+02
C1	30	negative	negative	1.00E+02
C2	36	negative	negative	1.00E+02
C3	30	negative	negative	1.00E+02
C4	30	negative	negative	1.00E+02
C5	92	negative	negative	1.00E+02
C6	100	negative	negative	1.00E+02
C7	72	negative	negative	1.00E+02
C8	96	negative	negative	1.00E+02
C9	88	negative	negative	1.00E+02
C10	92	negative	negative	1.00E+02
C11	100	negative	negative	1.00E+02
C12	86	negative	negative	1.00E+02