

Association of Housing Location and Sex with Cultivable Gut Microorganisms in Iraqi Infants

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ABSTRACT: Background: The infant gut microbiota is essential for immune function, neurobehavioral development, and overall health. Therefore, studying how infants' intestinal flora develops and what influences it is important. Early gut flora is affected by various factors, including internal ones such as maternal and infant-related aspects, and external environmental factors like geographic location. **Objective:** To determine the diversity of the gut microbiota of Iraqi infants under two years of age by gender and by housing location (urban and rural). **Methods:** We collected 248 fecal specimens from infants under two years; 291 microbial isolates were identified by traditional methods (Gram staining, microscopy, biochemical tests), and bacterial growth was achieved using multiple culture media; the definitive diagnosis was then performed by sequence analysis. **Results:** The study shows a diverse intestinal microbiota, with fourteen species identified for the first time in Iraqi infants as newly identified strains in this community. Significant differences between urban and rural housing areas were found: the chi-square statistic is 19.541, $p < 0.005$, and the result is significant at $p \leq 0.05$. For the gut microbiome and related to the housing location, the chi-square statistic is 53.68. The $p < 0.0001$. The result is significant at $p < 0.05$, and the differences between females and males have a chi-square statistic of 48.22. The $p < 0.0001$. The result is significant at $p < 0.05$. **Conclusions:** Significant differences were revealed between the culture-dependent microbial isolates and strains across the regional study, with variation associated with the housing location factor and the infant's sex. These findings suggest that the infant intestinal microbiota is dynamic and that its diversity is shaped by a combination of biological sex-related factors and environmental conditions linked to the place of residence.

KEYWORDS: Infants; Intestinal flora; Infant sex; Housing location; Microbiome diversity

INTRODUCTION

Human gut microbial diversity is complex, with the intestinal microbiota playing a key role in human health and intestinal health, and also affecting the ability to metabolize nutrients [1]; it works as an important metabolic and immunological organ, maintaining host physiological homeostasis, synthesizing important vitamins, and strengthening the intestinal barrier against pathogenic threats, controlling systemic inflammation, and guiding the maturation of the host immune system [2], [3], [4]. Numerous factors affect the arrangement of the gut microbiome. Individual lifestyle, socioeconomic, gender, medications, genetics, age, and geographic location [5], baby's delivery mode, which is the first meeting with the outside, where the cesarean delivery differs from the vaginally one [6], [7]. Dietary habits, the quality of food, and the consumption of artificial dietary additives are important factors [8]. Probiotics, prebiotics, dietary modifications, and fecal microbiota transplantation help to enhance the gut-brain axis, stabilizing the microbiota balance and early-life microbial colonization [9], [10]. Environmental factors alter the gut microbiome, leading to transient microbial communities, and the early-life intestinal microbiota is frequently manipulated by these factors, which impact the composition and function of the gut microflora and hence confer short- and/or long-term health outcomes extending to adulthood [11].

Pediatric health and diseases that affect the early-life gut microbiome have been increasingly recognized as a causative factor; some pediatric diseases are directly linked with the imbalance of the microbiome, like inflammatory bowel disease [1]. Microbe-host communication is necessary for maintaining vital activities of a healthy host, and its dysbiosis has been linked to various illnesses [12]. The microbiome community was wide, involving lactic acid bacteria, which have the ability to produce several organic acids that work as an antibacterial effect against pathogenic bacteria and maintain health [5]. *Lactobacillus fermentum* (*L. fermentum*) has an anti-diarrhea effect [11]. The gut microbial diversity is characterized by a transition toward increasing taxonomic complexity during the first three years of life [13]. A thorough evaluation of how differences between urban and rural areas affect the diversity and composition of the gut microbiota across varying geographical settings reveals the complex relationship between lifestyle, environment, and the gut microbiome [14], [15]. Investigate the diversity and functional characteristics of gut microbiota in two different cities, and study the geographical locality [16]. Gut microbes exhibit flexible adaptive strategies shaped by host and environmental conditions [17].

Among the different factors that affect the gut microbial diversity, the study focuses on the sex factor and geographic housing in Baghdad and its outlying areas and different worldwide locations to understand the population habits' differences in diet, hygiene, crowding, antibiotic exposure, animal contact, and environmental microbial exposure, all of which can affect gut colonization, which would advance the current knowledge to understand the variations of the gut microbial diversity.

MATERIALS AND METHODS

Ethics Clearance and Participation Consent

The ethics paper presented to all Iraqi family participants in the study, seeking help to obtain the infants' data, informed them about the follow-up study before their enrollment, and each individual provided informed consent before the information was gathered.

Study Specimens

Stool specimens were collected from apparently healthy infants meeting the following inclusion criteria: under two years of age. Exclusion criteria included infants who were older than two years, infants who had a history of long-term antibiotic use, newborns with congenital abnormalities, newborns at high risk, and infants who had suffered from diarrhea; specimens from infants' diapers were definitely contaminated with urine and diaper materials. The stool should be collected as soon as possible after a bowel movement using a clean, sterile, leak-proof container. Ideally, the sample should come from the middle portion of the stool, and if the study requires microbiological analysis, it should be transported to the laboratory quickly and stored according to the study protocol. For the sample transport, stool samples were immediately placed in a cooled box (0-4°C). Samples were transported to the laboratory in less than 30 minutes after collection for further laboratory processing. Samples not immediately processed were kept at 4 °C. The collection period covers January to June 2025.

Identifying microbes initially

Stool specimens were collected from apparently healthy Iraqi infants under two years of age. The General Stool Examination (GSE) was performed on all specimens to detect indicators of gastroenteritis. Specimens were obtained from healthy infants of both sexes in urban and rural areas. General and selective media were used to isolate a single, purified colony for diagnosis and identification [18]. Isolation conditions were selected to target anaerobic bacteria, aerobic bacteria, and facultative anaerobes. Several bacterial types require different artificial conditions to achieve growth (Blood, MacConkey, Salmonella-Shigella, Mannitol Salt Agar (MSA), Chocolate, MRS (De Man, Rogosa, and Sharpe), MRS with 0.05 L-cystine (Sigma-Aldrich/USA), TSA (Tryptone Soy Agar), and Chrome agar for *Candida* species, all media from Himedia, India). All plates were incubated aerobically and anaerobically using an anaerobic jar with the Gas-Pak (Himedia/India). For facultative and microaerophilic bacteria, an anaerobic jar with a candle was used. Plates were incubated at (37-44) °C for (1-5) days, depending on the incubation time required for the bacterial type and its conditions. Diagnosis of the isolated, purified single colony began with traditional biochemical tests (oxidase, catalase), supported by direct microscopic examination to detect cell shape, arrangement, and Gram reaction.

16S rRNA identification

16S rRNA was the basic method for definitively diagnosing the study isolates by using a universal primer. The isolated sequence was obtained to identify the unknown isolates. Sanger sequencing was performed on the polymerase chain reaction (PCR) products [19] of several isolates using ABI3730XL automated DNA sequencers by Macrogen Corporation in Korea. The sequence analyses by the bioinformatics tools were the NCBI (National Center for Biotechnology Information) within BLAST (Basic Local Alignment Search Tool) nucleotide to match the local sequence with the most related species, depending on the percentage identity, the query coverage, and the accession length, the decision was made. The most specific and definite diagnosed isolates were inserted with their information to get a new name through the NCBI submission portal and were confirmed as a new strain.

The 850 bp oligonucleotide primers used in the study of 16S rRNA techniques have a forward sequencing direction (5'-GTGCCAGCAGCCGCGCTAA-3') and a reverse sequencing direction (5'-AGACCCGGGAACGTATTCAC-3'). They were first dissolved and diluted in free nuclease-distilled water in their lyophilized form (amount according to Alpha DNA Company, USA). The PCR technique was used to detect the gene in the amplification process. The final volume of the PCR mixture was 25 μ L (12.5 μ L of Master Mix 2 $\times$, 5 μ L template DNA, 1 μ L primers for each of the forward and reverse primers, and finally, 5.5 μ L nuclease-free water) [20]. The amplification for the PCR program is shown in Table 1.

Table 1. PCR program steps

Steps	°C	Min:Sec	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60	00:30	30
Extension	72	01:00	30
Final extension	72	07:00	1
Hold	10	10:00	1

About the new strain submission procedure, it follows the steps in <https://submit.ncbi.nlm.nih.gov/>. Regarding the verification of the isolates' authenticity, the NCBI submission portal assigns a new serial number to an isolate, and it becomes a strain only after confirming its reality and establishing that it is distinct and unique in terms of its genetic sequence.

Statistical Analysis

The total sample size was calculated using the G*Power 3.1.9.4 program and was 134. The researcher sought to increase the sample size as time and sample availability allowed, while maintaining impartiality and sample homogeneity, to reach a total of 248, as documented in the research. For the statistical analysis, p-values marked "significant," "vastly significant," and "extremely significant," corresponding to 0.05, 0.01, and 0.001, respectively, were considered statistically significant, while p-values greater than 0.05 were considered not statistically significant. For the communicative analysis, the chi-square test was applied. The statistical analyses were performed using SPSS version 20. The Shannon Diversity Index (H) is a mathematical measure of species diversity within a community or dataset. It accounts for both species richness (the total number of different species) and species evenness (the uniformity of individual distribution among those species).

The standard equation for the Shannon index is:

$$H = - \sum_{i=1}^S p_i \ln p_i \quad (1)$$

where H represents the Shannon diversity index, S is the total number of species (species richness), i is an individual species, p_i refers to the proportion of all individuals in the community that belong to species i (calculated as $\frac{n_i}{N}$) and $\ln$ represents the natural logarithm. A value closer to 0 indicates lower diversity, while a higher value signifies greater diversity and a more balanced distribution.

RESULTS AND DISCUSSION

Microbial Identification

A total of 248 fecal samples involved in the study were obtained from apparently healthy infants, with numerous isolates from each specimen. The distribution of initial growth results across the study samples was positive growth (199/248), negative growth (40/248), and not identified (9/248) for each infant's stool specimens. It is worthy to mention that each single stool specimen resulted in more than one microbe due to the use of different culture media for each sample; the total isolate was 291 from 248 fresh stool specimens. Because the study isolates its culture-dependent, many intestinal microorganisms are difficult or impossible to culture under routine laboratory conditions. Culture-based analysis provides useful information on viable and recoverable bacteria, but it has intrinsic limitations compared with next-generation sequencing. In particular, it may miss uncultivable or fastidious taxa.

Numerous microbes were isolated during the study for infants under two years old, including *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), and *Staphylococcus aureus* (*S. aureus*), *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., and *Candida* spp., which were the most prevalent microbes. Suspected diagnosis decisions depend on the morphological characteristics of the colonies, which contribute to the primary diagnosis and give macroscopic features (color, odor, surface, margin, and height), as shown in Figure 1.

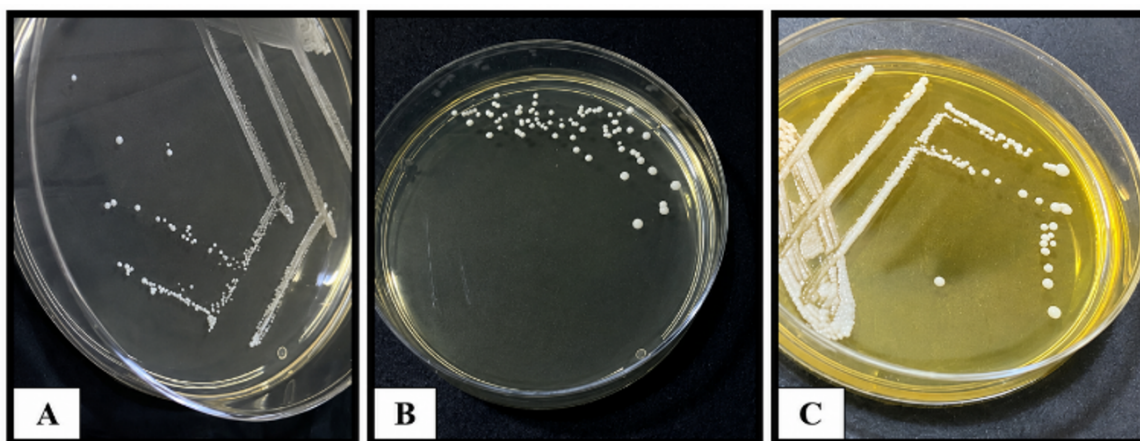


Figure 1. Three culture plates show single isolated colonies: (A) *Bacillus subtilis* (*B. subtilis*) strain RI70, (B) *Limosilactobacillus oris* strain RI118, and (C) *Enterococcus gallinarum* (*E. gallinarum*) strain RI19

The microscopic features (Gram reaction, cell shape, and arrangements) were the main guidelines for reaching the final diagnosis, as seen in Figure 2. It is worth noting that not all the study species were detected by the primary diagnosis. The catalase test, oxidase tests, and coagulase test were done for the isolates to obtain their biochemical properties. Table 2 illustrates some biochemical tests used for the study.

While the definite diagnosis was confirmed by sequencing analysis, single isolates selected colonies from the stool sample; the morphologically distinct colonies were selected based on the differences of the single isolate colony (different morphologies) and microscopically characterized (different shapes), followed by BLAST informatics and submission of some bacterial isolates as a new strain with their own specific characteristics, which are observed clearly in [20]. There were fourteen strains involved, three families, and different species registered, as seen in Table 3.

Recognizing sex as a biological variable is important for study design, statistical stratification, and the interpretation of infant microbiota research. Including sex analyses improves reproducibility and clinical relevance of findings, which describes the microbial species and their distribution between males and females. As seen in Table 4, total infant samples in the study were separated into two sex groups; the samples comprise 111 females (N=111/248) and 137 males (N=137/248). The infant samples were collected across a broad age range, from 1 day of age to 2 years.

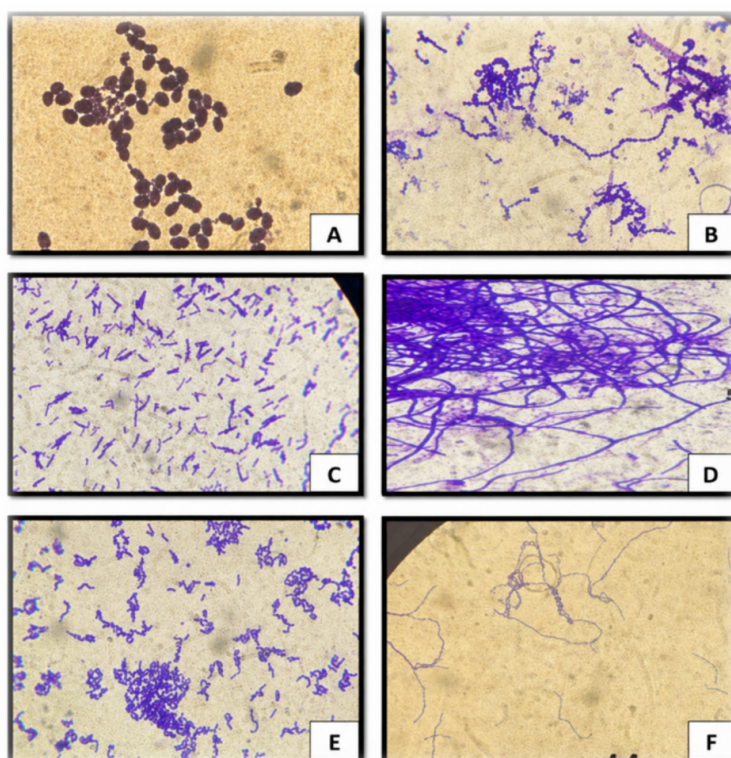


Figure 2. Direct microscope examination reveals the staining, shape, and cell arrangements for some study microorganisms: (A) *Candida albicans* (*C. albicans*), (B) *Enterococcus lactis* strain RI99, (C) *Heyndrickxia coagulans* strain RI48, (D) *Lactobacillus acidophilus* (*L. acidophilus*), (E) *Limosilactobacillus fermentum* strain RI37, and (F) *L. fermentum* strain RI88

Table 2. The initial diagnosis for the study of microbial isolates

No.	Catalase test	Oxidase test	Coagulase test	Culture media	Culture conditions	Microscopic features	Suspected diagnosis
1	+	+	-	MacConkey agar	Aerobic	G-ve, rod, single cell	<i>E. coli</i>
2	+	+	-	MacConkey agar	Aerobic	G-ve, rod, single cell	<i>K. pneumoniae</i>
3	-	-	-	MRS	Microaerophilic	G+ve, rod, single cell	<i>Lactobacillus spp.</i>
4	-	-	-	MRS+ L-cystine	Anaerobic	G +ve, irregular shape	<i>Bifidobacterium spp.</i>
5	-	-	-	MRS	Facultative anaerobic	G +ve, Cocci, pair or short chain	<i>Enterococcus spp.</i>
6	+	+	-	MRS	Aerobic, facultative anaerobic	G +ve, rod, single cell	<i>Bacillus spp.</i>
8	+	-	-	Candida Chrome agar	Facultative anaerobic	Oval or round with a nucleus	<i>Candida spp.</i>
9	+	-	+	MSA	Facultative anaerobic	G +ve, Spherical with grape arrangement	<i>S. aureus</i>

Note: *MRS = De Man, Rogosa, and Sharpe, G+ve= Gram positive, G-ve= Gram negative

Table 3. Local bacterial isolates after registration in NCBI

No.	Diagnostic bacterial isolates	Bacterial strains name	Accession No.
1	<i>E. gallinarum</i> is similar to strain LES-SNN-S-17	<i>E. gallinarum</i> strain RI19	PZ262244
2	<i>E. lactis</i> is similar to strain C35	<i>E. lactis</i> strain RI99	PZ262247
3	<i>L. fermentum</i> similar to strain CAU6354	<i>L. fermentum</i> strain RI98	PZ276908
4	<i>Heyndrickxia coagulans</i> similar to strain CACC 834	<i>Heyndrickxia coagulans</i> strain RI48	PZ262245
5	<i>B. subtilis</i> subsp. <i>inaquosorum</i> similar to strain OUN26RKSP	<i>B. subtilis</i> strain RI70	PZ262246
6	<i>Limosilactobacillus fermentum</i> is similar to strain M2L2	<i>L. fermentum</i> strain RI66	PZ295995
7	<i>Limosilactobacillus fermentum</i> is similar to strain S24808	<i>L. fermentum</i> strain RI24	PZ255429
8	<i>Limosilactobacillus fermentum</i> is similar to strain RM106	<i>L. fermentum</i> strain RI40	PZ268721
9	<i>L. fermentum</i> is similar to strain CAU5875	<i>L. fermentum</i> strain RI11	PZ268215
10	<i>Limosilactobacillus fermentum</i> , similar to strain 20489	<i>L. fermentum</i> strain RI32	PZ268611
11	<i>Limosilactobacillus fermentum</i> is similar to strain 2859	<i>L. fermentum</i> strain RI37	PZ269511
12	<i>Limosilactobacillus fermentum</i> , similar to strain ADMH 18	<i>L. fermentum</i> strain RI88	PX762961
13	<i>L. fermentum</i> strain HBUAS63088	<i>L. fermentum</i> strain RI71	PZ268861
14	<i>Limosilactobacillus oris</i> was similar to strain S1	<i>Limosilactobacillus oris</i> strain RI118	PZ262248

Table 4. Distribution of the study sample based on sex and birth

Gender	Total	%
Female	111	44.7%
Male	137	55.3%
Total	248	100.0%
$P < 0.0001$		

A total of 291 single, positively diagnostic microbial isolates were recorded (N=170; 52.84%) for females, while (N=121; 41.58%) for males. Significant differences were the chi-square statistic of 48.22. The $p < 0.0001$. The result is significant at $p < 0.05$, with a Shannon diversity index ($H' \approx 3.85$) as seen in Table 5. The study demonstration of microbial isolates for males and females, were in the females' group, *Enterococcus faecalis* (*E. faecalis*), *Haemophilus parahaemolyticus* (*H. parahaemolyticus*), *Halalkalibacterium halodurans* (*Hala. halodurans*), *Limosilactobacillus mucosae* (*Li. mucosae*), *Peplostreptococcus anaerobius* (*P. anaerobius*), *Propionibacterium lymphophilum* (*Prop. lymphophilum*), and *Stenotrophomonas maltophilia* (*Sten. maltophilia*), which occupied the same percentage (N=1; 0.34%); *Candida parapsilosis* (*C. parapsilosis*) (N=2; 0.69%); and *Candida glabrata* (*C. glabrata*) (N=4; 1.37%), while *L. fermentum* strain RI71 seemed to be in males only (N=2; 0.69%).

The results of Table 5 demonstrate *Lactobacillus spp.* isolates from healthy infant feces, with an overall large percentage of the rest of the species in definitive diagnosis by 16S rRNA, mirroring the findings of [21], who reported a 75% *Lactobacillus* recovery from infants under 12 months in Iraq using MRS plating and 16S rRNA. The *L. fermentum* was found in human milk and neonatal feces more frequently than any other isolate [22], which agrees with the study finding. *Bacillus sp.*, *Pseudovibrio denitrificans* (*Pseudo. denitrificans*) (N=2; 0.69%), and (N=1; 0.34%) for females and

males, respectively. For *Enterococcus lactis* (*E. lactis*) (N=2; 0.69%), there is an equal percentage in males and females. The total number was similar for *Li. fermentum* strain RI66., *Lactobacillus plantarum* (*L. plantarum*) in females (N=1; 0.34%), (N=3; 1.03%), respectively, and (N=3; 1.03%), (N=1; 0.34%) for males, respectively.

Li. fermentum strain RI32, *B. subtilis* strain RI70, *E. gallinarum* (*E. gallinarum*) strain RI19, *E. gallinarum*, *Heyndrickxia coagulans* (*Heyn. coagulans*) strain RI48, *Li. oris* strain RI118, *Li. fermentum* strain RI88, *Candida dubliniensis* (*C. dubliniensis*), (N=3; 1.03), (N=1; 0.34%), (N=2; 0.69%), (N=2; 0.69%), (N=1; 0.34%), (N=3; 1.03), (N=2; 0.69%), (N=4; 1.37%) for females, respectively, and (N=2; 0.69%), (N=4; 1.37%), (N=3; 1.03), (N=3; 1.03), (N=4; 1.37%), (N=2; 0.69%), (N=3; 1.03), and (N=1; 0.34%) for males, respectively. Two isolates indented the same total number (N=7), *Li. fermentum* strain RI40 were (N=4; 1.37%) for females, and (N=3; 1.03%) for males, while *Li. fermentum* strain RI98, with (N=2; 0.69%) for females, and (N=5; 1.72%) for males. The total number of *C. albicans* (N=8) was (N=4; 1.37%) for both males and females, *Li. fermentum* strain RI37 was for females (N=2; 0.69%) and males (N=6; 2.06%); *Li. oris* (N=5; 1.72%) and (N=3; 1.03%) were for females and males, respectively. *Candida* spp. are commonly found in infant feces, with studies reporting a high incidence of *C. albicans* and other non-albicans species in neonates. These fungi are frequently present in the gut microbiome, where they contribute to both commensal and pathogenic processes. Colonization patterns vary, influenced by factors such as delivery mode and antibiotic administration [23].

Li. fermentum, *Li. fermentum* strain RI24, *E. durans*, *K. pneumoniae*, *L. acidophilus*, *Li. fermentum* strain RI11, *E. lactis* strain RI99, *Lactococcus lactis* (*La. Lactis*), *Bifidobacterium* spp., *Enterococcus faecium* (*E. faecium*), *S. aureus*, and *E. coli* were (N=6; 2.06%), (N=3; 1.03%), (N=8; 2.75%), (N=6; 2.06%), (N=9; 3.09%), (N=8; 2.75%), (N=9; 3.09%), (N=11; 3.78%), (N=10; 3.44%), (N=7; 2.41%), (N=14; 4.81%), (N=21; 7.22%) for females, respectively, and (N=3; 1.03%), (N=7; 2.41%), (N=2; 0.69%), (N=5; 1.72%), (N=2; 0.69%), (N=3; 1.03%), (N=4; 1.37%), (N=6; 2.06%), (N=8; 2.75%), (N=13; 4.47%), (N=9; 3.09%), (N=6; 2.06%) for males, respectively. Emerging evidence suggests that these longitudinal community shifts are compounded by external factors, including feeding practices and delivery mode, which interact uniquely with biological sex [24]. The current findings indicate that male infants frequently exhibit lower gut microbiota diversity files at birth compared to their female equivalents, that's deal with the study of [25], who also mentions that genetic factors may play a potential role in the establishment of infant intestinal microbiota and that the mechanism of sex differences in intestinal microbiota community is still unclear. In the study of [26], [27] the alteration of gender related differentiation microbial patterns is characterized by a lower abundance of Clostridiales and a higher prevalence of Enterobacteriales in male infants. These alterations are believed to influence variance in health outcomes throughout early development, as male infants demonstrate a heightened sensitivity to prenatal exposures.

The study on the development of the microbiota and its associations with several factors, based on the analysis of stool samples collected from infancy through early childhood, showed that males were represented (N=223; 50.7%) and females (N=217; 49.3%) [28]. According to the study, members under six months were males and females, showing the percentages (48.8%) and (51.2%) for males and females, respectively [29]. These results were discordant with previous findings, as they showed a higher percentage of males. Regarding the distribution of microbial isolates and strains by housing location within the study sample, the total sample size was 248 (100%). It involves urban, which is on the rise: 182 (73.4%), and 66 (26.6%) for rural. Regarding the microbial culture depending results, the distribution showed a higher percentage of positive bacterial growth in the infants in urban area 157 (78.8%). In the case of negative growth for the cultures present, it was approximately similar between the urban infant's specimens and rural ones, 21 (52.5%) and 19 (47.5%), respectively. The unidentified group marks the lowest presence (44.5% and 55.5%) for urban and rural, respectively. Positive growth means growth seen in culture and identified with NCBI nucleotide BLAST analysis, while negative growth means no growth seen on the culture media, and not identified significance indicates isolates that grew in culture but could not be definitively assigned a taxonomic identity, even after sequencing and NCBI nucleotide BLAST analysis; therefore, the final result was reported as not identified. The results are statistically significant, with a chi-square statistic of 19.541. The result is significant at $p \leq 0.05$, as seen in Table 6.

For the microbial diversity, a total of 291 culture-derived isolates were identified, including 189 isolates from urban infants (64.95%) and 102 from rural infants (35.05%). The distribution of microbial species differed significantly between the two settings, indicating that microbial diversity was not uniform across housing locations. The chi-square statistic is 53.68. The analysis revealed a statistically significant difference, $p < 0.001$. The Shannon diversity index was high ($H' \approx 3.98$), suggesting

Table 5. Distribution of microbial isolated species according to the child's sex

Microbial species	N	Female	%	Male	%
<i>Enterococcus faecalis</i>	1	1	0.34	0	0.00
<i>Haemophilus parahaemolyticus</i>	1	1	0.34	0	0.00
<i>Halalkalibacterium halodurans</i>	1	1	0.34	0	0.00
<i>Limosilactobacillus mucosae</i>	1	1	0.34	0	0.00
<i>Peptostreptococcus anaerobius</i>	1	1	0.34	0	0.00
<i>Propionibacterium lymphophilum</i>	1	1	0.34	0	0.00
<i>Stenotrophomonas maltophilia</i>	1	1	0.34	0	0.00
<i>L. fermentum</i> strain RI71	2	0	0.00	2	0.69
<i>Candida parapsilosis</i>	2	2	0.69	0	0.00
<i>Bacillus</i> sp.	3	2	0.69	1	0.34
<i>Pseudovibrio denitrificans</i>	3	2	0.69	1	0.34
<i>E. lactis</i>	4	2	0.69	2	0.69
<i>L. fermentum</i> strain RI66	4	1	0.34	3	1.03
<i>Lactobacillus plantarum</i>	4	3	1.03	1	0.34
<i>Candida glabrata</i>	4	4	1.37	0	0.00
<i>L. fermentum</i> strain RI32	5	3	1.03	2	0.69
<i>B. subtilis</i> strain RI70	5	1	0.34	4	1.37
<i>E. gallinarum</i> strain RI19	5	2	0.69	3	1.03
<i>E. gallinarum</i>	5	2	0.69	3	1.03
<i>Heyndrickia coagulans</i> strain RI48	5	1	0.34	4	1.37
<i>Limosilactobacillus oris</i> strain RI118	5	3	1.03	2	0.69
<i>L. fermentum</i> strain RI88	5	2	0.69	3	1.03
<i>Candida dubliniensis</i>	5	4	1.37	1	0.34
<i>L. fermentum</i> strain RI40	7	4	1.37	3	1.03
<i>L. fermentum</i> strain RI98	7	2	0.69	5	1.72
<i>C. albicans</i>	8	4	1.37	4	1.37
<i>L. fermentum</i> strain RI37	8	2	0.69	6	2.06
<i>Lactobacillus oris</i>	8	5	1.72	3	1.03
<i>L. fermentum</i>	9	6	2.06	3	1.03
<i>L. fermentum</i> strain RI24	10	3	1.03	7	2.41
<i>Enterococcus durans</i>	11	8	2.75	2	0.69
<i>K. pneumoniae</i>	11	6	2.06	5	1.72
<i>L. acidophilus</i>	11	9	3.09	2	0.69
<i>L. fermentum</i> strain RI11	11	8	2.75	3	1.03
<i>E. lactis</i> strain RI99	13	9	3.09	4	1.37
<i>La. lactis</i>	17	11	3.78	6	2.06
<i>Bifidobacterium</i>	18	10	3.44	8	2.75
<i>E. faecium</i>	20	7	2.41	13	4.47
<i>S. aureus</i>	23	14	4.81	9	3.09
<i>E. coli</i>	27	21	7.22	6	2.06
Total	291	170.00	58.42	121.00	41.58

Note: Not identified group; it was excluded (N=9).

Table 6. The distribution of housing location and the cultural growth within the study sample

Total	Urban N(%)	Rural N(%)
Total 248	182 (73.4)	66 (26.6)
Positive growth 199	157 (78.8)	42 (21.2)
Negative growth 40	21 (52.5)	19 (47.5)
Not identified 9	4 (44.5)	5 (55.5)

considerable diversity among the recovered culture-dependent isolates, and demonstrating a rich and relatively well-distributed microbial population.

Enterococcus faecalis, *H. parahaemolyticus*, *H. halodurans*, *L. mucosae*, *P. anaerobius*, *Prop. lymphophilum*, *Sten. maltophilia*, *Li. fermentum* strain RI71, *C. parapsilosis*, *Bacillus* sp., *Pseudo. denitrificans*, *Li. fermentum* strain RI66, *L. plantarum*, *C. glabrata*, *Li. fermentum* strain RI32, *B. subtilis* strain RI70, and *Li. oris* strain RI118 were non-existent in the rural housing location, while (N=1; 0.34%), (N=1; 0.34%), (N=1; 0.34%), (N=1; 0.34%), (N=1; 0.34%), (N=1; 0.34%), (N=1; 0.34%), (N=2; 0.69%), (N=2; 0.69%), (N=3; 1.03%), (N=3; 1.03%), (N=4; 1.37%), (N=4; 1.37%), (N=4; 1.37%), (N=5; 1.72%), (N=5; 1.72%), and (N=5; 1.72%) were present in the urban housing location, respectively.

In *E. lactis* (N=3; 1.03%), (N=1; 0.34%), *E. gallinarum* strain RI19 (N=4; 1.37%), (N=1; 0.34%), *E. gallinarum* (N=4; 1.37%), (N=1; 0.34%), *Heyn. coagulans* strain RI48 (N=1; 0.34%), (N=4; 1.37%), *Li. fermentum* strain RI88 (N=4; 1.37%), (N=1; 0.34%), *C. dubliniensis* (N=1; 0.34%), (N=4; 1.37%), *Li. fermentum* strain RI40 (N=5; 1.72%), (N=2; 0.69%), *Li. fermentum* strain RI98 (N=4; 1.37%), (N=3; 1.03%), *C. albicans* 3 (1.03%), *Li. fermentum* strain RI37 (N=7; 2.41%), 1 (0.34%), *Li. oris* (N=6; 2.06%), (N=2; 0.69%), *Li. fermentum* (N=5; 1.72%), (N=4; 1.37%); and *Li. fermentum* strain RI24 (N=7; 2.41%), (N=3; 1.03%) for urban and rural, respectively.

Although the similarity in the total number of the following infants' feces isolates, the distribution of the microbial isolates between the urban and rural housing communities were not identical: *E. durans* (N=5; 1.72%), (N=6; 2.06%), *K. pneumoniae* (N=10; 3.44%), (N=1; 0.34%), *L. acidophilus* (N=4; 1.37%), (N=7; 2.41%), *Li. fermentum* strain RI11 (N=7; 2.41%), (N=4; 1.37%). The highest total number of *E. coli*, *S. aureus*, *E. faecium*, *Bifidobacterium* spp., *La. lactis*, and *E. lactis* strain RI99 (N=19; 6.53%), (N=5; 1.72%), (N=14; 4.81%), (N=7; 2.41%), (N=11; 3.78%), and (N=9; 3.09%) are from the highest total. The chi-square statistic is 53.68. The results were statistically significant, $p < 0.001$, indicating a significant difference between the two groups and the Shannon diversity index: $H' \approx 3.98$, as shown in Table 7.

The geographic location and the lifestyle correlated with it have an important impact on microbial infant diversity. Urbanization and its impact on diverse ecosystems have garnered significant attention in recent microbial studies. As human populations increasingly migrate toward urban centers, the resulting changes in lifestyle, environment, and health outcomes have profound implications for microbial communities. Understanding these implications is crucial, considering the role of microbiota in human health and ecological balance. Because it changes the variety and diversity of microbes in different habitats, urbanization has a major effect on microbiota. Compared to rural areas, cities often have a lower variety of microbes and a higher prevalence of opportunistic infections, according to studies. It is clear from these shifts that pollution and urban lifestyles have an impact on microbial ecosystems, and it is clear that focused health treatments and policies for urban planning are necessary to lessen these negative consequences [30].

Regional and dietary differences shape the gut microbiota. Several cross-sectional studies indicate that rural African communities consuming predominantly fiber-rich diets exhibit distinct gut microbiota profiles characterized by increased abundance of genera, including Prevotella, Faecalibacterium, and Ruminococcus [31], which don't agree with our study's culture-dependent results, which show that Lactobacillaceae is the most abundant. The quality of urbanization, food habits, and the way of life of a community determine its classification [32]. Rural communities are often smaller than urban regions, which possess a more intricate network. Urban residents often consume more ultra-processed foods than rural residents, who predominantly eat traditional staple cereals and less-processed plant-based foods [33]. That explains the causes of these microbial differences between the two different housing locations, which agrees with the study results.

Table 7. The relationship between the Housing location type and the distribution of isolated microorganisms within the study sample

Microbial species	Total	Urban		Rural	
		N	%	N	%
<i>Enterococcus faecalis</i>	1	1	0.34	0	0.00
<i>Haemophilus parahaemolyticus</i>	1	1	0.34	0	0.00
<i>Halalkalibacterium halodurans</i>	1	1	0.34	0	0.00
<i>Limosilactobacillus mucosae</i>	1	1	0.34	0	0.00
<i>Peptostreptococcus anaerobius</i>	1	1	0.34	0	0.00
<i>Propionibacterium lymphophilum</i>	1	1	0.34	0	0.00
<i>Stenotrophomonas maltophilia</i>	1	1	0.34	0	0.00
<i>L. fermentum</i> strain RI71	2	2	0.69	0	0.00
<i>Candida parapsilosis</i>	2	2	0.69	0	0.00
<i>Bacillus</i> sp.	3	3	1.03	0	0.00
<i>Pseudovibrio denitrificans</i>	3	3	1.03	0	0.00
<i>E. lactis</i>	4	3	1.03	1	0.34
<i>L. fermentum</i> strain RI66	4	4	1.37	0	0.00
<i>Lactobacillus plantarum</i>	4	4	1.37	0	0.00
<i>Candida glabrata</i>	4	4	1.37	0	0.00
<i>L. fermentum</i> strain RI32	5	5	1.72	0	0.00
<i>B. subtilis</i> strain RI70	5	5	1.72	0	0.00
<i>E. gallinarum</i> strain RI19	5	4	1.37	1	0.34
<i>E. gallinarum</i>	5	4	1.37	1	0.34
<i>Heyndrickxia coagulans</i> strain RI48	5	1	0.34	4	1.37
<i>Limosilactobacillus oris</i> strain RI118	5	5	1.72	0	0.00
<i>L. fermentum</i> strain RI88	5	4	1.37	1	0.34
<i>Candida dubliniensis</i>	5	1	0.34	4	1.37
<i>L. fermentum</i> strain RI40	7	5	1.72	2	0.69
<i>L. fermentum</i> strain RI98	7	4	1.37	3	1.03
<i>C. albicans</i>	8	3	1.03	5	1.72
<i>L. fermentum</i> strain RI37	8	7	2.41	1	0.34
<i>Lactobacillus oris</i>	8	6	2.06	2	0.69
<i>L. fermentum</i>	9	5	1.72	4	1.37
<i>L. fermentum</i> strain RI24	10	7	2.41	3	1.03
<i>Enterococcus durans</i>	11	5	1.72	6	2.06
<i>K. pneumoniae</i>	11	10	3.44	1	0.34
<i>L. acidophilus</i>	11	4	1.37	7	2.41
<i>L. fermentum</i> strain RI11	11	7	2.41	4	1.37
<i>E. lactis</i> strain RI99	13	9	3.09	4	1.37
<i>La. lactis</i>	17	11	3.78	6	2.06
<i>Bifidobacterium</i>	18	7	2.41	11	3.78
<i>E. faecium</i>	20	14	4.81	6	2.06
<i>S. aureus</i>	23	5	1.72	18	6.19
<i>E. coli</i>	27	19	6.53	7	2.35
Total	291	189	64.95	102	35.05

Several studies have examined the gut bacteria of people in rural areas who eat traditional meals high in resistant starches, raw plant foods, and fiber. In rural areas of South Africa, Nigeria, Tanzania, Cameroon, and Uganda, where fiber-degrading taxa like *Bifidobacterium* spp. are the most common types of bacteria [34], the results agree with our findings, which show that the *Bifidobacterium* spp. is present in a higher percentage than its presence in the urban community. The number of *Prevotella* taxa decreased, suggesting that urbanization, westernized diets, and disease burden may be shifting the microbiota toward proteolytic rather than fiber-digesting taxa. This change fits with a trend of less microbial diversity and possible pro-inflammatory markers, which are often linked to metabolic diseases and gut health problems [35]. Urban cohorts have a narrower variety and lower quantity of SCFA producers and a higher prevalence of inflammatory Enterobacteriaceae strains [36]. This result agrees with the study [37] where urbanization poses a danger to the protective effects of African microbiota profiles. The Westernized gut microbiota, on the other hand, is characterized by a reduced variety of microbes, which makes significant differences.

For rural areas where the people depend on the natural source of feeding, which also includes babies, where the basic feeding behavior was breastfeeding, the *Li. fermentum* was the dominant, in contrast to the study results, and the presence of lactic acid bacteria in breast milk affects both the quantitative and qualitative diversity of the infant gut microbiota, according to [38]. The higher representation of lactic acid bacteria and bifidobacteria in the rural group in the present study is notable, as these taxa are often associated with early-life gut maturation and carbohydrate metabolism [39], which partially agrees with the study results where *Bifidobacterium* spp. is the most prevalent in rural areas, but some of the Lactobacillaceae members are present at higher in urban ones. These fungi are frequently present in the gut microbiota, where they contribute to both commensal and pathogenic processes. Colonization patterns vary, influenced by factors such as delivery mode and antibiotic administration [40].

CONCLUSION

Significant differences were revealed between the culture-dependent microbial isolates and strains across the regional study, with variation associated with the housing location factor and the infant's sex. The identification of fourteen novel local bacterial strains underlines the unique microbial diversity within this population. These findings suggest that the infant intestinal microbiota is dynamic and that its diversity is shaped by a combination of biological sex-related factors and environmental conditions linked to the place of residence. Furthermore, these insights emphasize the necessity of integrating environmental and biological factors when developing targeted pediatric health and probiotic strategies.

SUPPLEMENTARY MATERIAL

None.

AUTHOR CONTRIBUTIONS

Ruqaea Saady Ibraheem: Visualization; Writing – review & editing; Data curation. Eman N. Naji: Conceptualization; Formal analysis; Validation. Intesar N. Khelkal: Methodology, Investigation; Supervision.

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DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF GENERATIVE AI USE

During the preparation of this manuscript, the authors used Grammarly (v1.2.226.1896) for improve the language. After using this tool, the authors thoroughly reviewed and revised the generated content, taking full responsibility for the accuracy and integrity of the final manuscript.

ETHICAL APPROVAL

This study was overseen and authorized by Mustansiriyah University's Ethics Committee (BCSMU/12-21/00052M).

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