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Original Research

Gut Microbial Similarity Analysis in Mono and Dizygotic Twins Discordant for Down Syndrome

Pierre-Yves Maillard [1, †], Sima Tokajian [2, †], Marie Vilaire-Meunier [1], Charbel Al Khoury [2], Cybel Mehawej [3], Eliane Chouery [3], Marie-Anne Caillaud [1], Louise Maillebouis [1], Ines Ben Smida [1], Sophie Durand [1], Aimé Ravel [1], Cecile Cieuta-Walti [1], Marie-Noëlle Ungeheuer [1], Clotilde Mircher [1], André Mégarbané [1, 3, *]

1. Institut Jérôme Lejeune, Paris, France; E-Mails: pierre-yves.maillard@institutlejeune.org; marie.vilaire@institutlejeune.org; marie-anne.caillaud@institutlejeune.org; louise.maillebouis@institutlejeune.org; ines.bensmida@institutlejeune.org; sophie.durand@institutlejeune.org; ravel.pediatre@wanadoo.fr; cecile.cieuta-walti@institutlejeune.org; ungeheuer.marienoelle@gmail.com; clotilde.mircher@institutlejeune.org; andre.megarbane@institutlejeune.org
2. School of Art and Sciences, Lebanese American University, Byblos, Lebanon; E-Mails: stokajian@lau.edu.lb; charbel.alkhoury@lau.edu.lb
3. Department of Human Genetics, Gilbert and Rose-Marie Chagoury School of Medicine, Lebanese American University, Byblos, Lebanon; E-Mails: cybel.mehawej@lau.edu.lb; eliane.choueiry01@lau.edu.lb; andre.megarbane@lau.edu.lb

† These authors contributed equally to this work.

* **Correspondence:** André Mégarbané; E-Mails: andre.megarbane@institutlejeune.org; andre.megarbane@lau.edu.lb

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Abstract

To investigate the potential impact of the additional chromosome 21 on the gut microbiome in patients with Down syndrome (DS), two monozygotic (MZ) and two dizygotic (DZ) twin pairs discordant for DS were studied. Whole-genome shotgun sequencing was conducted to analyze the taxonomic and functional profiles of the gut microbial community in the twins and assess whether the gut microbiome composition influences the development of psychological pathologies. Sequencing generated a total of 2,338,132 reads, combined across the four samples. The number of contigs ranged from 98,521 to 122,000, and the GC content was 46.34-47.15%. The Simpson index at the species level in the DS of our study was distinctly higher in the DZ-DS but not in the MZ-DS. Taxonomic classification revealed a Western diversity profile in the twins, where the dominant bacterial phyla were Firmicutes, Bacteroidetes, and Actinomycetota. Each sample had a diversity of ten families at abundance >0.5%, of which *Prevotellaceae, Lachnospiraceae*, and *Bacteroidaceae* were the most prevalent. The *Prevotellaceae* family was detected at a high level in all samples, except in the DZ-DS, where *Lachnospiraceae* was the most abundant family. At the genus level, marked differences were observed between the DZ-DS twins compared to the others. *Prevotella* was not detected in the DZ-DS twin whereas it was the dominant genus in the MZ-DS and both healthy twins. The variation in predicted metagenomes between DS and non-DS showed that the average relative abundances for DS were comparable to those for non-DS. The two MZ twins discordant for DS appear to have relatively similar microbiomes, suggesting that the added chromosome 21 did not have a significant impact on gut microbiome composition. Identifying biomarkers of DS that modulate the gut composition and affect the overall health needs to be further studied, along with the potential role of *Prevotella*.

Keywords

Down syndrome; microbiome; chromosome 21; trisomy 21; twin studies

1. Introduction

The composition of the human gut microbiome is now well established as a crucial factor in various human health conditions. Human gut microbiome composition is shaped by genetics, diet, and environmental factors. It plays a vital role in human health; its composition varies substantially between individuals and evolves throughout the lifetime, with dysbiosis being linked to a variety of diseases [1, 2]. Bacteria secrete enzymes that modulate various host functions, including the metabolism of dietary carbohydrates, the release of microbial-mediated gut hormones, the production of vitamins, the regulation of the immune system, and the maintenance of intestinal homeostasis [3-5].

Several studies have shown that the gut microbiome can be shaped by environmental factors, such as diet and drugs, and is correlated with anthropometric measurements. However, the extent to which host genetics plays a role in microbiome diversity remains to be identified [6-10]. Indeed, it is challenging to distinguish between environmental and genetic inheritance in humans, and sometimes, only twin studies can help better understand the link by comparing microbiome similarity among monozygotic (MZ) and dizygotic (DZ) twins, assuming that variations can be attributed to genetic effects [6]. As such, twin studies have been conducted, showing associations between heritable traits and genes related to diet, metabolism, olfaction, barrier defense, and self/non-self recognition [11, 12].

Down syndrome (DS), resulting from the presence of all or part of an extra chromosome 21, is one of the most frequent forms of intellectual disability, occurring in around one out of every 700 to 2000 newborns [13]. Patients with DS present with a wide range of clinical manifestations, including physical, medical, and psychological features [14, 15].

Here, we used whole-genome shotgun sequencing (WGS) to study taxonomic and functional profiles of the gut microbial community in MZ and DZ twins discordant for DS to investigate the possible impact of the additional chromosome 21 on the gut microbiome and determine if the gut microbiome composition modulates the onset of any psychological pathology.

2. Materials and Methods

2.1 Ethics Statement

This study and all experimental protocols were approved and authorised by the French review board: CPP - "Comité de Protection des Personnes" under the number: SI number: 22.01708.000098#1. This committee safeguards the rights and welfare of human research subjects who are recruited to participate in research activities.

Written informed consent for analysis and publication of anonymized data was obtained from the participants' legal representatives, in compliance with French regulations and the Declaration of Helsinki. At least two weeks before inclusion, parents or legal guardians received a child-friendly illustrated information sheet and informed consent forms.

2.2 Subjects

Recruitment began in December 2022. Two pairs of male twins were included in this study: one pair was monozygotic (MZ; monochorionic-diamniotic), and the other was dizygotic (DZ; dichorionic-diamniotic). In each pair, one twin had karyotype-confirmed DS (47,XY,+21), and the co-twin had a normal karyotype (46,XY), with no evidence of mosaicism or translocation in any of the participants.

Inclusion criteria were: no special diet or medication in the three months preceding stool collection, and no history of chronic gastrointestinal disease or current antibiotic therapy. Exclusion criteria included incomplete genetic or clinical data, the presence of metabolic, neurological, or immune disorders unrelated to DS, and any recent acute illness.

The monozygotic (MZ) twins were delivered by cesarean section at 34 weeks of gestation following an uncomplicated pregnancy. The twin with DS showed delayed growth parameters, including reduced length, weight, and head circumference compared to his co-twin (Table S1). He was diagnosed with a ventricular septal defect, which was surgically corrected, and later with Hirschsprung disease, for which he underwent surgical resection of the affected bowel segment at the age of one. At the time of inclusion in the study, the twins were four years old (Table S1). The child with DS presented with moderate intellectual disability. His developmental milestones were within the average range typically observed in individuals with DS.

The dizygotic (DZ) twins were born at 37 weeks of gestation after an uncomplicated pregnancy. The twin with DS had lower birth measurements (Table S1) and was diagnosed with both ventricular and atrial septal defects, which were surgically treated. At the time of inclusion, the twins were seven years old (Table S1). The child with DS exhibited mild intellectual disability, while his co-twin was receiving medication for epilepsy.

The developmental milestones of both children with DS were consistent with the typical range observed in individuals with the condition.

The four patients live in the same area in France. They shared a similar environment and Western diet, and none of them followed any specific dietary regimen. Families were not asked to change their lifestyle, except to avoid collecting stool samples close to any event that might alter their usual diet. In our questionnaire, we collected information on the meals consumed the day before sample collection for all four patients.

2.3 Genotyping

Genotyping by microsatellite analysis of 27 different short tandem repeat (STR) markers was performed using the PowerPlex® Fusion 6C System (Promega). This includes 23 autosomal STRs, 3 Y-STRs, and Amelogen—a comparison between twins, followed by genotyping.

2.4 Analysis of Gut Microbiota by Shotgun Sequencing

Fecal samples were collected in DNA/RNA Shield Fecal Collection Tubes (Zymo Research) and stored according to the manufacturer's guidelines. DNA was extracted with the ZymoBIOMICS DNA Miniprep Kit (Zymo Research) following the manufacturer's instructions. Libraries were prepared with the Illumina DNA Prep (M) Tagmentation library kit with 100 ng DNA and sequenced on Illumina NovaSeq 6000 (2 × 101 bp) at CeGaT GmbH, 72076 Tubingen, Germany.

2.5 Bioinformatic Analysis of Sequencing Data

Raw reads were demultiplexed using Illumina bcl2fastq (v2.20). Adapters were trimmed with Skewer (version 0.2.2) [16]. Read quality was assessed with FastQC (v0.11.5-cegat) [17]. Assembly of the metagenomes was performed with metaSPAdes v3.15.3 [18]. Taxonomic placement, functional classification, and all downstream analyses were performed using OmicsBox (Version 3.0.30) [19].

Taxonomic and functional data analysis was conducted by first aligning 10 million of the adapter-trimmed raw forward reads to the filtered RefSeq protein database (version 94) using Diamond in BLASTX mode [20]. Taxonomic placement was performed using the Lowest Common Ancestor (LCA) algorithm implemented in MEGAN6 Ultimate Edition (version 6.18.7) [21]. Only Taxa with relative sequence abundances above 0.01% were considered. Functional classification was carried out in MEGAN6 Ultimate Edition (version 6.18.7) by assigning the reads to KEGG, SEED, VFDB, and Interpro identifiers. Relative sequence abundances for taxonomic units and functions as well as diversity indices and Bray-Curtis dissimilarities were calculated using R (version 4.0.4) (R Core Team 2015). Plots were created using ggplot2 (Wickham 2009) in R (version 4.0.4) (R Core Team 2015).

2.6 Shannon and Simpson Diversity Index

To assess the diversity of the bacterial species in the gut microbiome, Shannon and Simpson's diversity indices were calculated at the species level to measure species richness and evenness. The Shannon diversity index increases with evenness. Simpson's diversity index (1-D) is a value between 0 and 1 that represents the probability that two organisms randomly selected from a sample will belong to different species.

2.7 KEGG Pathway and Functional Enrichment

Functional abundance profiles were compared between DS and normal individuals across four KEGG levels. The five most abundant KEGG functions per level were visualized in scaled heatmaps. Pathways related to the immune, endocrine, and nervous systems were highlighted. Only tasks with relative abundance >0.01% were included. P-values and effect sizes were calculated descriptively due to the small sample size.

2.8 Insulin Resistance Markers

Functional gene groups associated with insulin resistance (e.g., KO4621 – insulin signaling) were extracted from KEGG annotations. Relative sequence abundance differences between DS and non-DS samples were reported. While formal hypothesis testing was limited by sample size, notable trends were highlighted and interpreted in the context of published metabolic dysfunctions in trisomy 21.

3. Results

3.1 Genotyping

The genetic profile in the discordant MZ twins revealed that they share the same genetic profile for the studied STR markers, confirming that both patients are monozygotic twins. Among the analyzed STRs, three are located on chromosome 21, which helped further confirm the trisomy 21 in the affected twin. They were classified as monozygotic heterokaryotic, discordant for DS; an infrequent event.

3.2 Gut Microbial Diversity and Abundance

Sequencing generated a total of 2,338,132 reads, combined across the four samples. The number of contigs ranged from 98,521 to 122,000, and the GC content was 46.34-47.15%. The richness and evenness of the species present in a sample were assessed using Shannon's index, whereas diversity was measured using Simpson's Diversity Index, which takes into consideration both the number of species present and the relative abundance of each species. The Shannon and Simpson diversity indices were highest in the DZ-DS twin.

3.2.1 Firmicutes and Bacteroidetes were the Two Most Abundant Phyla Detected in the Twins

The dominant bacterial phyla were Firmicutes (52–63% of total sequences), Bacteroidetes (28–47%), Actinomycetota (3–8%), and Pseudomonadota (2–6%) (Figure 1A; Table S2). At the family level, *Prevotellaceae* and Oscillospiraceae were the most abundant (Figure 1B, 1C). Each sample contained at least ten families with relative abundance greater than 0.5%, with *Prevotellaceae*, *Lachnospiraceae*, and *Bacteroidaceae* being the most prevalent (Figure 1B). *Prevotella* (except in the DZ-DS twin), *Faecalibacterium*, and *Blautia* were the most common genera. *Faecalibacterium prausnitzii* was the only organism consistently detected at comparable levels across all twins (Table S2), and *Prevotella copri* was absent in the DZ-DS twin but was dominant in all other samples (Figure 1C; Table S2).

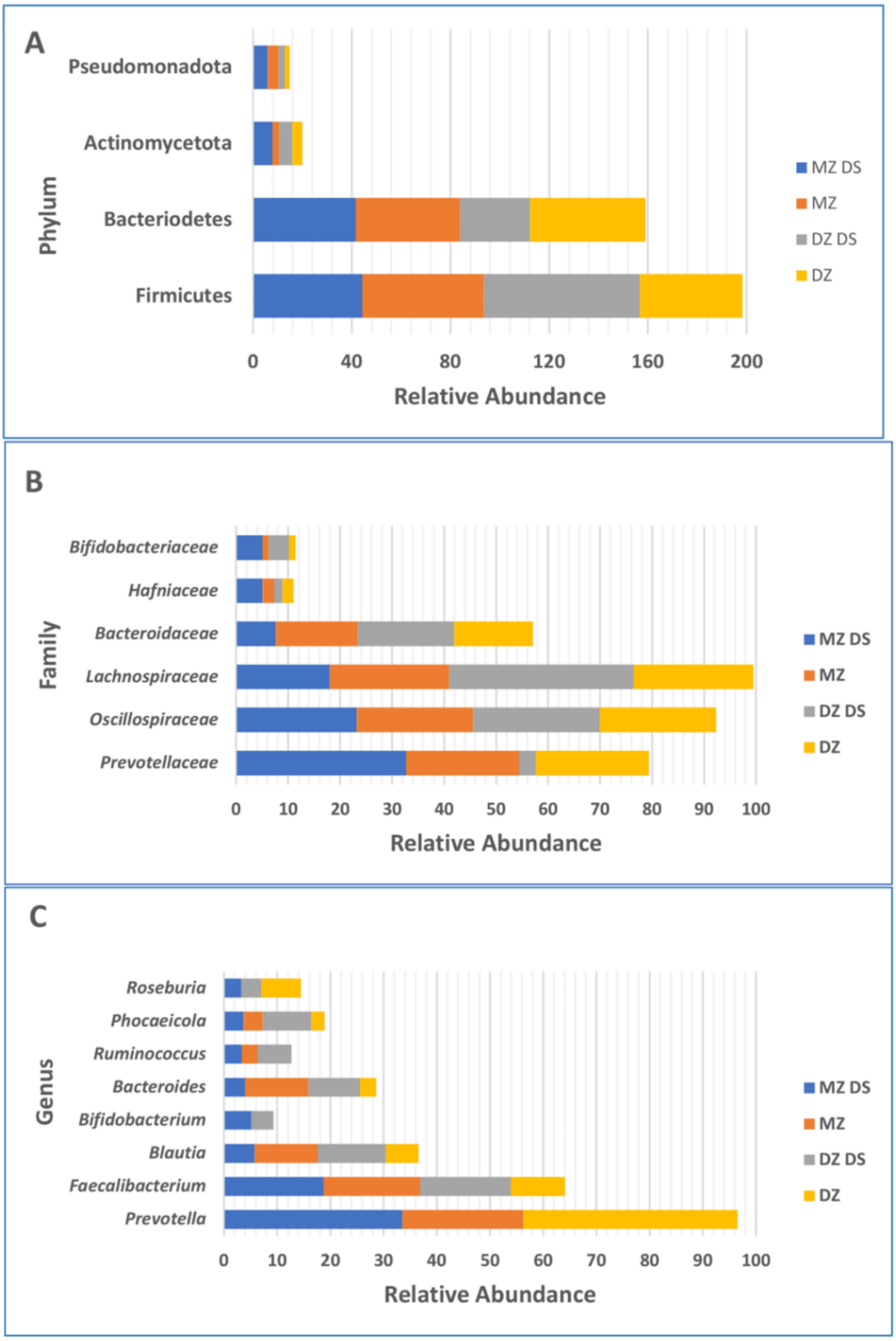

Figure 1 Relative abundance of gut bacterial taxa at the phylum (A), family (B), and genus levels (C).

3.2.2 Metabolic Pathway Enrichment Analyses

Metabolic pathway enrichment analyses showed no substantial differences between individuals with and without DS (Figure 2). However, markers of insulin resistance were higher in non-DS individuals.

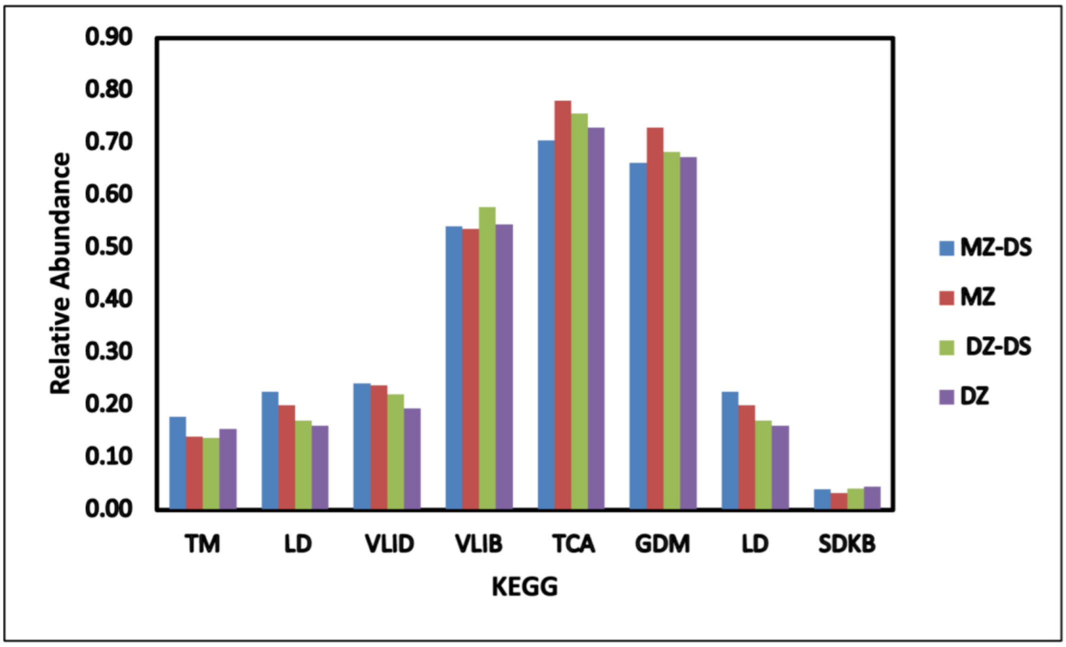

Figure 2 Metabolic pathway enrichment analyses of the intestinal microbiome for valine, leucine, and isoleucine biosynthesis and degradation, synthesis and degradation of ketone bodies, glyoxylate and dicarboxylate metabolism, tyrosine metabolism, lysine degradation, and the citrate cycle (Tyrosine metabolism TM; Lysine degradation TS; Lysine degradation LD; Valine, leucine and isoleucine degradation VLID; Valine, leucine and isoleucine biosynthesis VLIB; Citrate cycle (TCA cycle) TCA; Glyoxylate and dicarboxylate metabolism GDM; Lysine degradation LD; Synthesis and degradation of ketone bodies SDKB).

3.2.3 Microbial Function Prediction and Pathway Enrichment Analysis

We estimated the functional capacity of the twins' gut microbiomes by comparing the average relative abundance of predicted microbial functions between DS and non-DS individuals. Overall, the functional profiles were broadly similar, although some differences were observed (Figure 3, Figure 4, Figure 5). Predicted metagenomic variations were primarily associated with Organismal Systems (Figure 4), including the Immune System (KO4621), Nervous System (KO4724, KO4727), Environmental Adaptation (KO4626), and Excretory System (KO4964). Additional differences were linked to Human Diseases, such as Infectious Diseases: Viral (KO5165), Endocrine and Metabolic Diseases (KO0493), Neurodegenerative Diseases (KO5010, KO5012, KO5016), and Cancer: Specific Types (KO5215), as well as to Cellular Processes (Figure 5), including Cell Motility (KO2030, KO2040), Cell Growth and Death (KO4112), and to Genetic Information Processing, specifically Replication and Repair (KO3440).

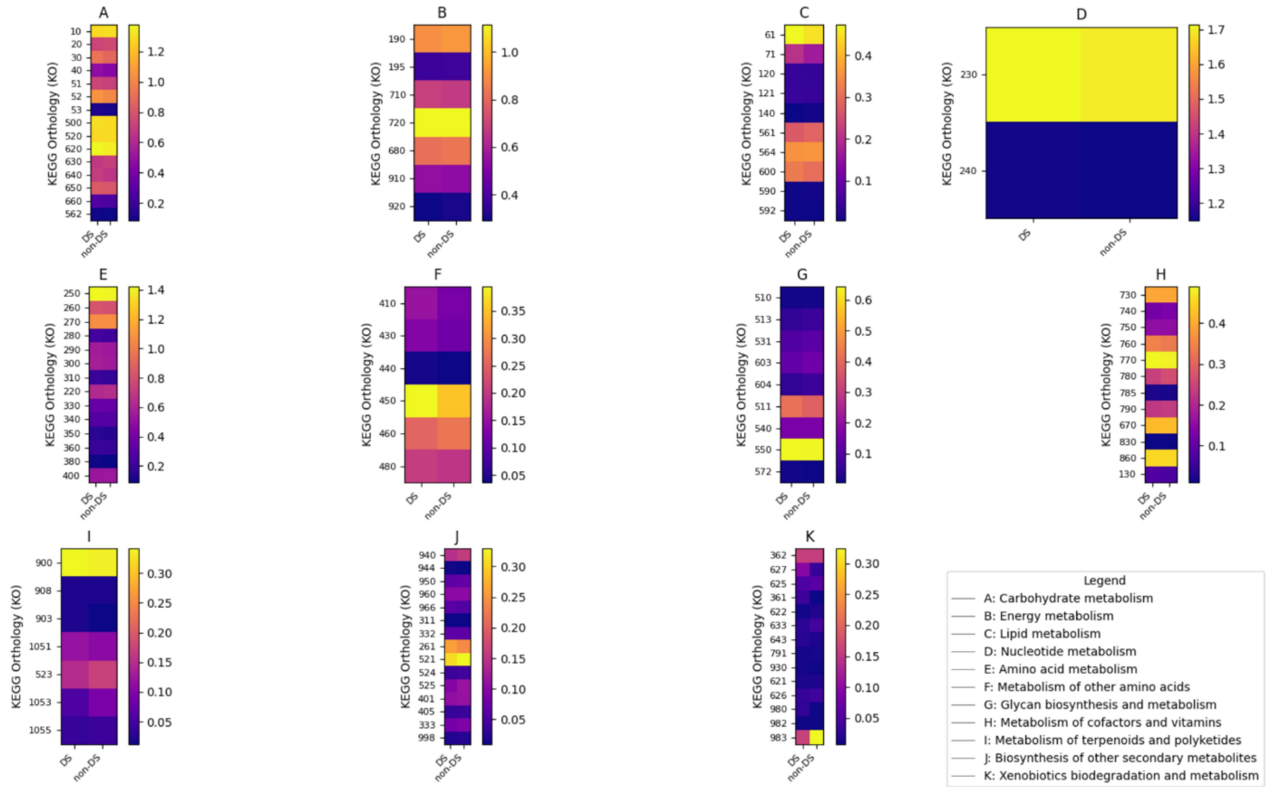

Figure 3 Average relative abundance of the predicted gene of the metagenome related to KEGG pathways/Metabolism at levels 1, 2, and 3.

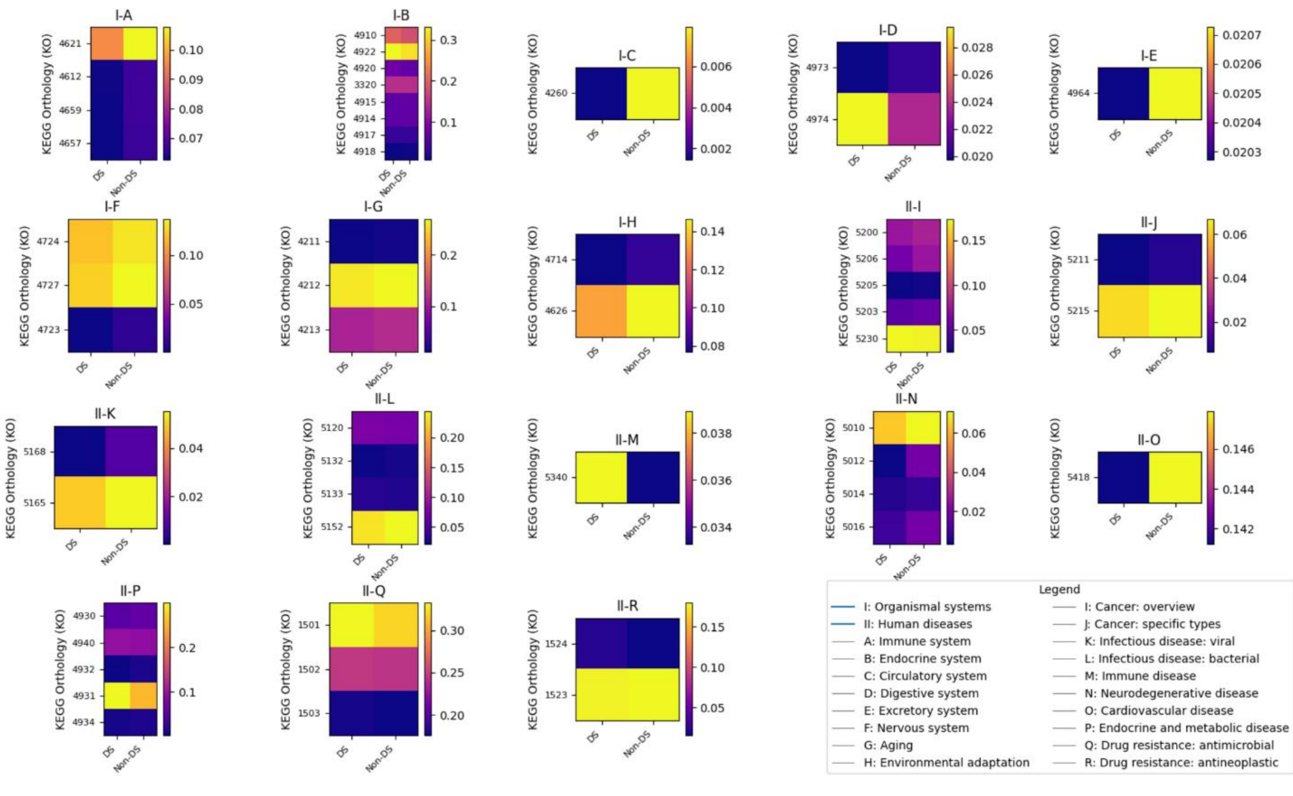

Figure 4 Average relative abundance of the predicted gene of the metagenome related to KEGG pathways/Organismal Systems and Human Diseases at levels 1, 2, and 3.

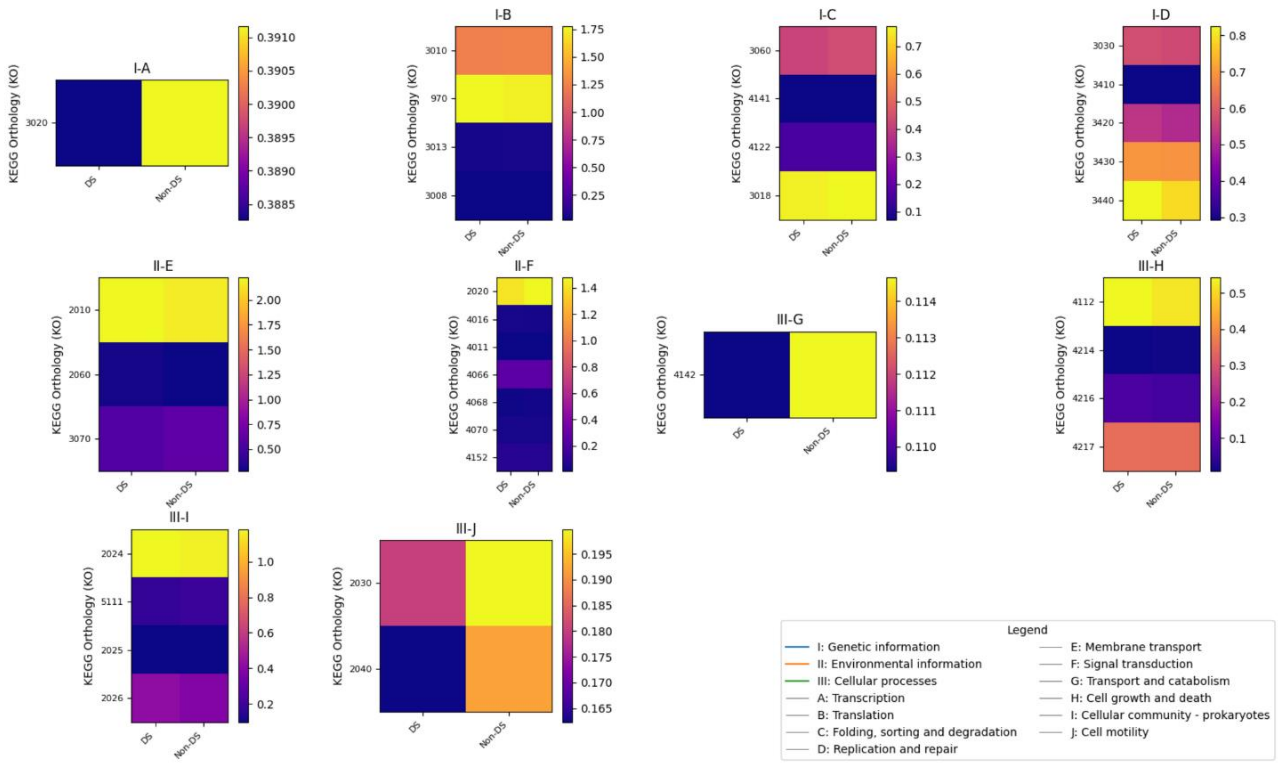

Figure 5 Average relative abundance of the predicted gene of metagenome related to KEGG pathways/Genetic Information, Environmental Information, Cellular Processes, and Human Diseases at levels 1, 2, and 3.

4. Discussion

The relationship between the gut microbiome and DS has been previously investigated, highlighting the impact of this complex interaction on cognitive functions and behavioral features, and revealing the abundance of certain microbial families while emphasizing the role of dietary factors and maternal gut microbiota [22, 23].

Here, we investigated the taxonomic and functional profiles of the gut microbial community in MZ and DZ twins discordant for trisomy 21 to examine the potential impact of the additional chromosome 21 on the gut microbiome. The patients were in the first decade of age. They had the exact geographical location and dietary habits, ruling out as many factors as possible that may influence the gut microbiome's composition and functional characteristics.

The Shannon and Simpson diversity indices detected in this study were highest in the DZ-DS twin. Even though patients with DS and healthy adults were shown to have a comparable level of gut microbiome [24], the current findings are consistent with those of Shaoli *et al.*, who revealed that species and Shannon indices were higher in the DS group compared to the non-DS group. Dysbiosis is frequently observed in patients with DS [25]. The observed difference, which indicates stability and adaptability of the microbiome, could be linked to immune response, environmental, and genetic diversity [26, 27].

Taxonomic classification revealed a Western diversity profile in the twins, with the dominant bacterial phyla being Firmicutes (52-63% of total sequences), Bacteroidetes (28-47%), and Actinomycetota (3-8%). It is worth noting that the twins were seven years old. While a child's microbiota is comparable to that of adults in terms of the number of detected species, the relative abundances of specific genera may differ. In our cohort (Figure 1C), we observed higher abundances of *Bifidobacterium spp.*, *Faecalibacterium spp.*, and members of *Lachnospiraceae*, whereas *Bacteroides spp.* are typically more abundant in the gut microbiota of adults.

Our data showed that Bacteroidetes was the dominant phylum in the healthy DZ twin. Differences in intestinal microbiota composition and diversity between individuals with DS and non-DS volunteers have been previously documented by Shaoli *et al*. [25], who also reported dysregulation in serum cytokine levels and fecal metabolites in DS cases. Their study further demonstrated a significantly elevated Firmicutes-to-Bacteroidetes ratio in the DS group compared to the non-DS group, which aligns with our findings in the DZ-DS twin. The abundance of Firmicutes and Bacteroidetes is highly variable and influenced by several factors, including diet, food additives, antibiotic use, and physical activity [28]. Previous reports suggest that an increase in Firmicutes abundance in the intestinal microbiota of DS individuals could be linked to cognitive function [25].

Each of the tested samples had a diversity of ten families at abundance >0.5%. *Lachnospiraceae* was the abundant family detected in the DZ-DS. *Lachnospiraceae* are strictly anaerobic and can ferment a variety of substrates, producing a range of metabolites, including short-chain fatty acids (SCFAs), such as butyric acid [29]. Butyric acid is a preferred energy source for colonocytes. Butyrate may therefore be necessary for the maintenance of the normal homeostasis since it enables the turnover of the colonic epithelium [30]. On the other hand, *Prevotellaceae*, previously considered a characteristic intestinal bacterium of patients with DS [25], was detected at a high level in all samples except for the DZ-DS.

At the genus level, marked differences were observed between the DZ-DS twin and the others, which partly agreed with previous findings that reported apparent differences in intestinal microbiota diversity in DS compared to non-DS [25].

For example, *Prevotella* was absent in the DZ-DS twin but was the dominant genus in both the MZ and DZ healthy twins. This observation aligns with findings by Cai *et al*. [25], who reported reduced abundance of several genera, including *Collinsella, Coprobacillus, Klebsiella, Megamonas, Prevotella, Ruminiclostridium, Slackia,* and *Tyzzerella*, in individuals with DS. Analysis of bacterial 16S rRNA gene sequences from fecal samples of 17 individuals with DS and 23 non-DS controls also showed that *Prevotella*, along with *Escherichia/Shigella, Catenibacterium*, and *Allisonella,* were significantly more abundant in the DS group. These bacteria were positively correlated with elevated levels of pro-inflammatory cytokines.

P. copri, the most abundant organism detected in this study (Table S2), is a well-studied and common intestinal species, and its abundance in the human gut has been linked to xylan metabolism [30] and hemicellulose metabolism [31]. *P. copri* was also shown to be associated with behavioral manifestations in patients with DS and with impairment of motor function, with chronic inflammation observed in cases with DS being linked to changes in intestinal microbiota and fecal metabolome [25]. Mice treated with fecal microbes from individuals with DS or with *P. copri* showed notable changes in behaviors commonly associated with DS, including signs of depression and reduced motor function [25].

Faecalibacterium prausnitzii, on the other hand, is detected at a relatively comparable level in the studied twins (10-18%, lowest in DZ-DS) (Table S2), constituting up to 15% of the total bacteria within the human gut microbiome. *F. prausnitzii* has a crucial role in maintaining gut physiology and host wellbeing [32]. Decreased levels of *F. prausnitzii*, which exhibits anti-inflammatory properties, were linked to the progression of Crohn's disease (CD) and ulcerative colitis (UC), colorectal cancer (CRC), and type 2 diabetes [33-36].

Surprisingly, in contrast to the findings previously reported by Shaoli *et al*., which revealed a characteristic pattern in the intestinal microbiome of individuals with DS [25], metabolic pathway enrichment analyses did not help uncover apparent differences between individuals with DS and those without DS in this study (Figure 2).

Metabolic dysregulations, including defects in glucose and lipid metabolism, mitochondrial defects, and increased oxidative stress levels, all lead to cellular dysfunctions and decreased energy production, have been reported in patients with DS [37]. Besides, diabetes, obesity, and non-alcoholic fatty liver disease (NAFLD) were shown to occur at a considerably high frequency in patients with DS [38, 39]. The gut microbiome was implicated in the pathophysiology of insulin resistance. The difference observed in the insulin resistance markers in our study between the DS and the non-DS, being higher in the latter, needs to be further verified, taking into consideration that the twins were seven years old and therefore this difference could further develop with age. Moreover, a greater abundance of *Actinobacteria* (phylum) and a lower abundance of *Lachnospiraceae* (family) have been previously linked to insulin resistance by Price *et al*. [40], which partly agrees with our results. However, other factors, such as the interaction between gut microbiota, the host immune system, and metabolism, also contribute to inflammation and insulin resistance [41].

We could not detect any marked differences that could be linked to genetics or DS when examining the relative abundances of KEGG functions. According to previous findings, the gut microbiome and host genetics are mainly independent. Environmental factors seem to be the primary determinant of the human gut microbiome. The analysis of microbiome data and genotypes from 1,046 healthy individuals of distinct ancestral origins, who shared a relatively similar environment, showed that host genetics does not make a significant contribution to microbiome composition [8], which also appears to be the case in trisomy 21.

The microbiome-association index quantifies the overall association between the microbiome and a host phenotype, after accounting for the host's genetic background. Goodrich *et al*. revealed that host genetics most likely shape the microbiome through diet preference, which was also heritable [42]. The heritability of the gut microbiome was previously estimated by assessing the effect of genotype and early shared environment in MZ and DZ twin pairs, which did not show a significant difference between MZ and DZ twins [12]. The three most dominant bacterial families were the *Lachnospiraceae* and *Ruminococcaceae* (Firmicutes) and *Bacteroidaceae,* and this partly agreed with our results (Figure 1B; Table S2). However, it is essential to emphasize the age factor, as the twins in this study were seven years old, whereas the participants in Goodrich *et al*.'s study ranged from 23 to 86 years [12]. More recently, González-Parra *et al*. [43] proposed that sex-specific pathways may connect gut microbiota composition to behavior in the DS mouse model, highlighting the potential of microbiota-targeted approaches to influence social impairments in neurodevelopmental conditions.

5. Conclusion

Examining discordant MZ twins provides a unique opportunity to control for numerous potential confounders that are commonly encountered in general population studies, such as variations in age, gender, environmental exposures, and genetic background.

One of the most interesting findings of the study was that the two MZ twins appeared to have relatively similar microbiomes, suggesting that trisomy 21 did not have a significant impact on gut microbiome composition. In contrast, the DZ twins exhibited much larger differences in gut microbiome composition, which may be related to genetic differences. Identifying biomarkers of DS that may be associated with gut and overall health, particularly with brain function, behavior, and inflammatory response, requires further investigation, along with the potential role of *Prevotella*.

This study presents a rare and scientifically valuable opportunity to investigate the gut microbiome in a monozygotic twin pair discordant for Down syndrome (DS). While the primary limitation lies in the sample size, due to the exceptional rarity of such twin pairs, the unique design controls for genetic background, intrauterine environment, and postnatal exposures. This allows us to attribute observed differences largely to the presence of the extra chromosome 21, offering a powerful model to explore early biological signatures associated with DS.

Our findings are based on a single pair of prepubescent male twins, which inherently limits generalizability and emphasizes the hypothesis-generating nature of this work. Additionally, the young age of the participants introduces developmental factors that may influence gut microbiome composition, further complicating extrapolation to broader DS populations, especially older individuals. Future studies should aim to include age-matched control groups across different developmental stages to distinguish DS-specific effects from age-related variations in the microbiome.

Although our results suggest associations between DS and specific microbial taxa—some with known roles in immune and metabolic modulation—these do not currently justify the use of probiotic or prebiotic interventions. Similarly, while links between the gut microbiome and neurodevelopmental or neurodegenerative processes are plausible, our study was not designed to directly explore such mechanisms. Longitudinal research integrating microbiome, cognitive, and neuroimaging data will be necessary to further investigate these connections.

The clinical utility of microbiome profiling in DS remains speculative but promising. With further validation, microbial signatures could eventually complement conventional clinical assessments to inform risk stratification, immune or gastrointestinal status, or neurodevelopmental outcomes.

In summary, despite the limited sample size, our study provides novel insights and highlights the potential of microbiome analysis in understanding DS pathophysiology. By sharing these findings now, we hope to stimulate collaboration, attract similar case reports, and facilitate larger-scale studies that can build upon this foundational work.

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Author Contributions

PYM, MVM, MAC, SD and AM designed the study, performed genetic studies. ST, CAK, IBS, CM, EC, AM interpreted data and wrote the manuscript. PYM, MVM, LM, AR, CCW, MNU, CM, AM contributed to the follow-up and clinical evaluation of the patient. All authors approved the manuscript.

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Competing Interests

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest.

Data Availability Statement

The data are from human patients: So, these cannot be publicly available to preserve individuals privacy under the European General Data Protection Regulation, along with the details of article/section of the regulation. The data are available from the corresponding author upon request or by contacting the Data Protection Officer (DPO) at the “Institut Jerome Lejeune”: https://www.institutlejeune.org/en/.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1: Clinical characteristics of monozygotic (MZ) and dizygotic (DZ) twin pairs discordant for Down syndrome (DS). (ID: Intellectual deficiency).
2. Table S2: Proportional Distribution of Bacterial Phyla Identified in the twins (S7757Nr1: MZ DS twin; S7757Nr2: MZ normal twin; S7757Nr3: DZ DS twin; S7757Nr4: DZ normal twin).

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