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"Diet Quality Modulates Gut Microbiota Structure in Blastocystis-Colonised Individuals from Two Distinct Cohorts with Contrasting Sociodemographic Profiles"

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Abstract

Diet and gut microbiota are key determinants of host health, but their interaction in *Blastocystis*-colonised individuals remains poorly understood. We evaluated the relationship between diet quality, faecal microbial diversity, and *Blastocystis* colonisation in two cohorts: university students (FACSA) and institutionalised children with their caregivers (PAVILA), all *Blastocystis*-positive subjects. We assessed diet quality using the Healthy Eating Index-2020 (HEI-2020), and we characterised faecal microbiota by 16S rRNA sequencing. Alpha and beta diversity were analysed, and genus-level transformed data were further evaluated using PERMANOVA, PCoA, and db-RDA.The FACSA cohort presented higher microbial richness and diversity (Shannon and Simpson indexes) than PAVILA ($p < 0.01$), and microbial composition differed significantly between cohorts (PERMANOVA $R^2 = 0.39$, $p = 0.002$). Total diet quality was associated with microbial structure ($R^2 = 0.26$, $p = 0.016$), with protein ($R^2 = 0.23$, $p = 0.017$) and vegetable ($R^2 = 0.17$, $p = 0.044$) components being the main contributors. Multivariate analysis showed higher protein and vegetable intakes were associated with genera such as *Sellimonas*, *Murimonas*, *Alistipes* and *Desulfovibrio* (FACSA group). In contrast, *Hydrogenoanaerobacterium*, V9D2013_group and *Haemophilus* were related to lower-quality diets (PAVILA group). Despite universal colonisation by *Blastocystis*, differences in microbial diversity and structure reflect a modulating effect of diet conditioned by the sociodemographic environment. These findings suggest the relevance of dietary quality as a modulator of the gut microbiota in populations colonised by *Blastocystis* and may guide future nutritional interventions in vulnerable groups.

Keywords: Diet; Microbiota; *Blastocystis*; Cohorts; 16S rRNA.

1. Introduction

The human gastrointestinal tract is inhabited by diverse and dynamic microbial ecosystem [1,2] that greatly contributes to metabolic homeostasis [3], immune modulation [4], and the general wellbeing [5]. Imbalances of this community, called dysbiosis [6], is associated with several diseases such as inflammatory bowel disease [7], obesity, [8,9] and infections [10]. Among the gut inhabitants, the protozoan parasite *Blastocystis* has recently drawn attention, due to its global distribution and the ongoing debate over its role in health and disease [11]. *Blastocystis*, one of the most common eukaryotic microorganisms that colonise the human gut, is highly prevalent, from 10-100%, depending on the population and the diagnostic methods used [12–16]. The clinical significance of

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Blastocystis spp. remains controversial, as several studies have reported a higher prevalence in healthy individuals who typically exhibit greater bacterial richness and diversity in their gut microbiota [17]. This has led to suggestions that *Blastocystis* may be a component of a healthy gut microbiota. However, associations have also been reported between *Blastocystis* and symptoms such as diarrhoea, abdominal pain, and bloating [18]. In addition, some studies have linked its presence to irritable bowel syndrome, intestinal dysbiosis, diabetes, obesity, and depression [19]. The clinical interpretation of *Blastocystis* spp. remains uncertain, mainly due to the wide variety of subtypes that exist [15]. Despite its common prevalence, the role of this protozoan in the disease is a matter of debate.

In contrast, the association between *Blastocystis* and the gut microbiota remains complex and variable [20]. *Blastocystis* colonisation influences bacterial diversity and the composition of dominant phyla, such as Firmicutes and Bacteroidetes [21]. The bacterial diversity of the intestine increases with the colonisation of *Blastocystis*. A study conducted in a rural population in Colombia reported that individuals colonised by *Blastocystis* had a higher abundance of beneficial bacterial genera, such as *Faecalibacterium, Alistipes, and Prevotella*, compared to non-colonised individuals. Depending on the intensity of *Blastocystis* colonisation, distinct microbial profiles are also identified [22]. These connections are known to vary among different groups. Moreover, factors linked to hosts, such as geographical location, age, and dietary habits, are believed to influence the relationship between *Blastocystis* and the gut microbiota. A study of Colombian children found no significant differences in bacterial composition between individuals colonised and non-colonised by *Blastocystis*, although colonised children exhibited greater microbial richness [23].

Diet has a significant role in shaping the structure and function of the gut microbiota [24,25]. High-fibre, predominantly plant-based diets are associated with greater microbial diversity and a greater abundance of beneficial short-chain fatty acid (SCFA)-producing bacteria, such as *Faecalibacterium* and *Roseburia*. These bacteria play a crucial role in mitigating intestinal inflammation. Plant-based dietary patterns contribute to the balance of the gut microbiota and help reducing systemic inflammation [26]. An intervention study demonstrated that the consumption of high-fibre or fermented foods modulates the gut microbiota and enhances immune function in healthy adults, highlighting the role of diet in beneficially modifying microbiome composition [25].In contrast, Western diets characterised by high consumption of saturated fats, refined sugars, and ultra-processed foods have an impact on lower microbial diversity and an increase in pro-inflammatory bacterial taxa. Such diets may promote intestinal dysbiosis and increased gut permeability, thus contributing to chronic low-grade inflammation [3]. Recent studies suggest that they could also influence *Blastocystis* colonisation [22].

In children living in institutional settings or from low socioeconomic backgrounds, the interaction between diet, gut microbiota, and protozoa, such as *Blastocystis*, highlights the need to study this interaction. These children often face heightened risks due to inadequate nutrition and limited access to healthcare services, factors that can affect gut health and microbial resilience. Socioeconomic status (SES) is a key determinant of gut microbiota composition in children. Children from low socioeconomic backgrounds often exhibit distinct microbial profiles compared to those from higher socioeconomic backgrounds. A study conducted in Indonesia revealed that children from low-SES had higher abundances of *Prevotella* and *Escherichia-Shigella*. In contrast, individuals from high-SES showed increased levels of *Bifidobacterium* and *Lactobacillus* [27]. Malnutrition poses a significant challenge to children's health, particularly in low-income regions. A poor diet may alter the gut microbiota, reducing its diversity and promoting the growth of pathogenic bacteria [28]. Parasitosis is characterised by chronic intestinal inflammation and increased permeability, resulting from repeated exposure to enteric pathogens and poor sanitation, which leads to nutrient malabsorption and impaired child growth [29,30]. In this context, *Blastocystis* colonisation is notably prevalent among children with malnutrition in resource-limited settings [31]. Based on these observations, the present study aimed to investigate the relationship between *Blastocystis* colonisation and gut microbiota composition in individuals from two cohorts with different dietary patterns.

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2. Materials and Methods

2.1. Study Population and Design

This cross-sectional study analysed two cohorts: (a) the FACSA cohort, consisting of university students (n = 46) from the Faculty of Health Sciences, and (b) the PAVILA cohort (n = 37), composed of children living in a group home and their adult caregivers. Participants were recruited voluntarily and provided informed consent in accordance with institutional ethical standards. Demographic information, including age, sex, and anthropometric measurements, was collected. Diet quality was assessed using a validated food frequency questionnaire.

For the gut microbiota analysis, a subset of 14 individuals colonised by *Blastocystis* was selected, including eight from the FACSA cohort and six from the PAVILA cohort. Two samples from the PAVILA cohort were excluded due to poor DNA integrity, which prevented successful sequencing. This analysis aimed to evaluate whether diet quality influences gut microbiota composition across different host environments, with a particular focus on individuals colonised by *Blastocystis*.

2.2. Dietary Assessment

A validated Food Frequency Questionnaire (FFQ) developed by Macedo et al. in 2013 was used [32] to evaluate diet quality. Nutritionists administered the FFQ to participants via Google Docs. Reported food group portions were converted into grams per day and analysed for energy and macronutrients using the Evalfinut 2.0 software (Ibero-American Nutrition Foundation, FINUT). This program assesses individual dietary intake based on food frequency and nutrient composition according to the Spanish Food Composition Database (BEDCA 2.0) [32] and the USDA National Nutrient Database for Standard Reference (Version 28) [33].

The Healthy Eating Index (HEI-2020) was calculated for each participant to assess their diet quality [34]. The International Physical Activity Questionnaire (IPAQ) was used to evaluate Physical activity, classifying individuals into low, moderate, or high activity levels based on self-reported time spent in vigorous and moderate activity, walking, and sedentary time. Physical activity levels were expressed in MET minutes per week, in accordance with standard IPAQ scoring protocols [35].

2.3. Sample Collection and DNA Extraction

Stool samples were collected from participants in sterile 2 mL vials and stored at −80 °C until processing. Total bacterial genomic DNA was extracted from 200 mg of stool using the E.Z.N.A.® Stool DNA Kit (OMEGA Bio-Tek, Inc.), following the manufacturer's instructions. DNA integrity was assessed by 1.5% agarose gel electrophoresis, and DNA concentration was measured using a NanoDrop One spectrophotometer.

2.4. Gut Microbiota Profiling

Microbiota profiles were analysed in fourteen faecal DNA samples from *Blastocystis*-colonised subjects: eight from the FACSA cohort and six from the PAVILA cohort. Two samples of the PAVILA cohort were excluded due to low DNA quality. For each sample, the V3 hypervariable region of the 16S rRNA gene was amplified using primers containing Illumina overlap adapters (forward: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACYCCTACGGGRGGCAGCAG-3′ and reverse: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTACCGCGGCTGCTGGCAC-3′). Amplicons were purifed with AMPure XP beads and subsequently index using the Nextera XT v2 kit to add dual indices. DNA concentrations were quantified using a Qubit 3.0 fluorometer. Libraries were then normalised and pooled in equimolar amounts, followed by sequencing on the Illumina MiSeq platform using the MiSeq Reagent Kit V2 (500 cycles) to generate 250 bp paired-end reads [36].

2.5. Bioinformatic Processing

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Initial quality control of raw reads was performed using FastQC. Forward and reverse reads were trimmed to 240 base pairs based on quality scores. The trimmed reads were then merged and denoised using the DADA2 plugin in QIIME2, enabling the generation of high-resolution amplicon sequence variants (ASVs). Taxonomic assignment was conducted using a closed-reference approach with the SILVA reference database (99% similarity threshold). Chimeric sequences were removed using the VSEARCH plugin. Taxonomic classification was carried out with a Naive Bayes classifier trained on SILVA sequences. Phylogenetic reconstruction involved multiple sequence alignment with MAFFT and tree construction using FastTree through the q2-phylogeny module. Alpha diversity was assessed using the Shannon and Simpson indices, while beta diversity was calculated using Bray–Curtis distances.

2.6. Detection of Blastocystis

The presence of *Blastocystis* DNA from faecal DNA extracts was determined using a real-time PCR assay with a specific probe. The primers used were F1: GGTCCGGTGAACACTTTGGATTT and R1: CCTACGGAAACCTTGTTAC-GACTTCA, with the probe FAM-TCGTGTAAATCTTACCATTTAGAGGA-MGBNFQ (Integrated DNA Technologies, IDT). Reactions were performed in a RotorGene® thermal cycler (Qiagen) using the QuantiNova Probe RT-PCR kit in 10 µL volumes containing 5 µL of 2× master mix, 0.1 µL of RT mix, 0.5 µL of primer and probe mix, 3.5 µL of RNase/DNase-free water, and 1 µL of DNA (40 ng). Thermal cycling conditions followed the manufacturer's protocol: 95°C for 5 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds [37].

2.7. Statistical Analysis

Dietary characteristics between the FACSA and PAVILA cohorts were compared using the HEI-2020 Healthy Eating Index, considering both the total score and the scores of its 13 individual components. Quantitative variables were summarised as means ± standard deviations or medians with interquartile ranges, depending on the distribution of the data. Normality was assessed using the Shapiro–Wilk test. Based on data distribution, comparisons between cohorts were conducted using either the Student's t-test or the Mann–Whitney U test. Statistical significance was set at $p < 0.05$, and p-values were adjusted for multiple comparisons using the Benjamini–Hochberg method. For microbiota data, alpha diversity analysis was conducted on genus-level abundance data transformed using the Hellinger transformation. The Shannon and Simpson indices were calculated to evaluate community richness and evenness. Beta diversity was assessed using Bray–Curtis distances, also based on Hellinger-transformed data. PERMANOVA (Permutational Multivariate Analysis of Variance) was used to test whether overall gut microbiota composition differed significantly according to the total HEI-2020 score and its individual components. Additionally, distance-based Redundancy Analysis (db-RDA) was performed to visualise associations between dietary variables and microbial community variation, with particular emphasis on key components such as vegetable and protein intake. The ten taxa most strongly associated with the ordination axes ($p < 0.1$) were highlighted graphically. All statistical and graphical analyses were performed using R (version 4.4.3).

2.8. Ethical Considerations

The Research Ethics Committee of the Faculty of Medicine and Nutrition at Juárez University of Durango, under registration number CEI-FAMEN-36, approved the study. All procedures were carried out according to the ethical principles outlined in the Declaration of Helsinki and compliance with current national regulations governing research involving human subjects.

Adult participants written informed consent prior to inclusion in the study. The legal representatives of the children in the PAVILA cohort signed the consent letters, as well as assent from minors, considering their age and level of understanding. The confidentiality of personal data was

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ensured through the code and the secure storage of biological samples. Participation was voluntary and posed no physical or psychological risks to the subjects.

3. Results

3.1. Dietary Quality and Blastocystis Status by Cohort

We first compared HEI-2020 dietary component scores between individuals with *Blastocystis*-colonisation within each cohort. In the FACSA cohort, colonised participants exhibited higher total HEI-2020 scores, with a tendency toward greater intake of fruits and vegetables (Table 1). In contrast, in the PAVILA cohort, colonised children showed significantly lower vegetable intake compared to non-colonised peers (adjusted $p = 0.018$, Table 2).

Table 1. HEI-2020 component scores by *Blastocystis* status in the FACSA cohort.

Component	*Blastocystis* Present n=8	*Blastocystis* Absent=38	p-value	Adjusted p-value
Energy intake	2812.75 ± 721.59	2728.89 ± 950.18	0.522	0.642
Caloric activity	2490.50 ± 622.20	2449.11 ± 650.75	0.805	0.882
Fruits	4.01 ± 1.21	2.82 ± 1.65	0.071	0.298
Whole fruits	1.56 ± 0.96	1.38 ± 1.74	0.195	0.390
Vegetables	2.53 ± 1.25	1.81 ± 0.77	0.172	0.390
Legumes	2.83 ± 0.79	3.35 ± 1.42	0.184	0.390
Whole grains	9.03 ± 2.43	7.75 ± 3.41	0.331	0.530
Dairy	4.12 ± 2.39	3.78 ± 2.47	0.503	0.642
Protein foods	3.32 ± 0.41	3.21 ± 0.53	0.485	0.642
Seafood/plant protein	3.28 ± 1.72	3.21 ± 1.64	0.835	0.882
Fatty acid ratio	4.77 ± 1.43	3.16 ± 2.20	0.020*	0.163
Refined grains	9.18 ± 1.25	8.08 ± 2.32	0.262	0.466
Added sugars	1.10 ± 2.03	2.40 ± 2.58	0.193	0.390
Saturated fat	8.33 ± 2.12	6.15 ± 3.03	0.075	0.298
Total HEI-2020	64.03 ± 2.36	56.67 ± 9.91	0.005*	0.079

HEI-2020 component scores (mean ± SD) by *Blastocystis* status in the FACSA cohort. No statistically significant differences were found after p-value adjustment. Adjusted p-value corrected by the Benjamini-Hochberg method.Significance: * ≤ 0.05, ns > 0.05.

Table 2. HEI-2020 component scores by *Blastocystis* status in the PAVILA cohort.

Component	*Blastocystis* Present n=8	*Blastocystis* Absent n=29	p-value	Adjusted p-value
Energy intake	4678.75 ± 2245.32	3175.45 ± 1459.14	0.140	0.681
Caloric activity	2070.25 ± 396.57	2148.70 ± 752.25	0.981	0.981
Fruits	2.67 ± 2.06	3.50 ± 1.45	0.225	0.681

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Whole fruits	2.29 ± 2.06	2.30 ± 1.77	0.961	0.981
Vegetables	**0.55 ± 0.49**	**1.70 ± 0.72**	**0.001****	**0.018***
Legumes	2.65 ± 1.98	3.40 ± 1.68	0.426	0.681
Whole grains	7.86 ± 3.51	6.41 ± 3.71	0.468	0.681
Dairy	4.57 ± 1.79	4.66 ± 3.05	0.788	0.901
Protein foods	2.72 ± 0.66	2.91 ± 0.63	0.654	0.805
Seafood/plant protein	2.62 ± 0.51	1.72 ± 1.82	0.246	0.681
Fatty acid ratio	4.58 ± 3.41	3.43 ± 3.35	0.445	0.681
Refined grains	7.78 ± 3.41	6.48 ± 3.21	0.412	0.681
Added sugars	2.79 ± 2.74	1.37 ± 3.24	0.065	0.520
Saturated fat	6.69 ± 4.80	6.09 ± 3.76	0.607	0.805
Total HEI-2020	57.13 ± 7.20	53.78 ± 11.78	0.434	0.681

HEI-2020 component scores (mean ± SD) by *Blastocystis* status in the PAVILA cohort. A significant difference was observed in the 'Vegetables' component after p-value adjustment. Adjusted p: p value corrected by the Benjamini-Hochberg method. Signif.: ** ≤ 0.01, * ≤ 0.05, ns > 0.05

3.2. Alpha Diversity Analysis Between FACSA and PAVILA Cohorts

To characterise the gut microbiota structure in participants from the two cohorts, FACSA (university students) and PAVILA (children and caregivers in a shelter), we evaluated three alpha diversity metrics: observed richness (number of genera), Shannon diversity index, and Simpson diversity index.

Our results showed that the FACSA cohort exhibited significantly greater richness and diversity than the PAVILA cohort. Specifically, the observed number was higher in the FACSA group ($p = 0.0024$), as was the Shannon index ($p = 0.0013$), suggesting a more diverse and evenly distributed gut microbial community. The Simpson index was also significantly different between cohorts ($p = 0.0013$), further supporting the presence of compositional differences in microbial communities (Figure 1).

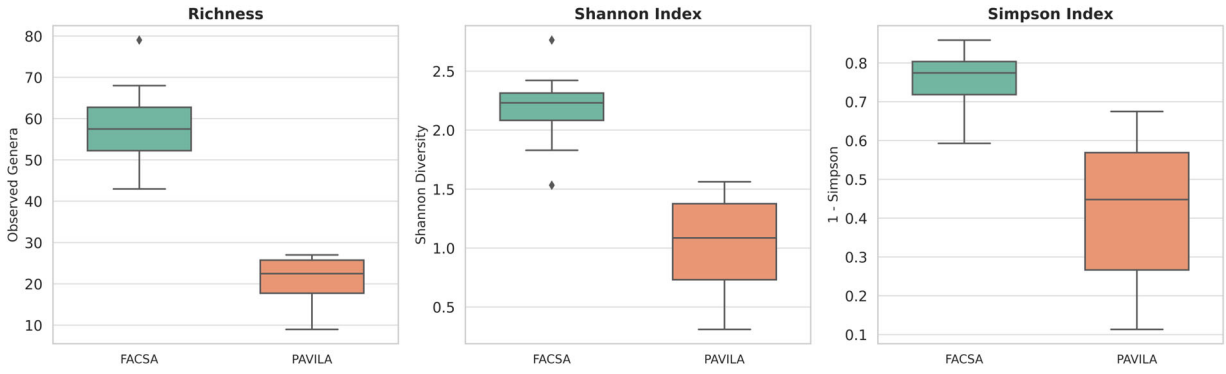

Figure 1. Alpha diversity comparison between cohorts colonised by *Blastocystis* spp. Boxplots show (A) richness (number of observed genera), (B) Shannon diversity index, and (C) Simpson diversity index (1 – D) among individuals from the FACSA (university students) and PAVILA (institutionalised children) cohorts. Points represent individual samples; horizontal lines denote medians. FACSA individuals exhibited significantly higher richness and diversity across all indices compared to PAVILA ($p < 0.05$ for all tests, Wilcoxon rank-sum).

3.3. Beta Diversity Analysis

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Figure 2 illustrates a clear separation in microbiota composition between the FACSA and PAVILA cohorts along the first two principal coordinates. Each point represents an individual sample, while shaded ellipses denote the 95% confidence interval for each group. The first two principal coordinates analysis (PCoA) axes explained 50.76% and 14.35% of the total variance, respectively. The PERMANOVA test confirmed a significant difference between groups ($F = 3.33$, $R^2 = 0.217$, $p = 0.018$), supporting the hypothesis that host-related factors contribute to distinct gut microbial community structures.

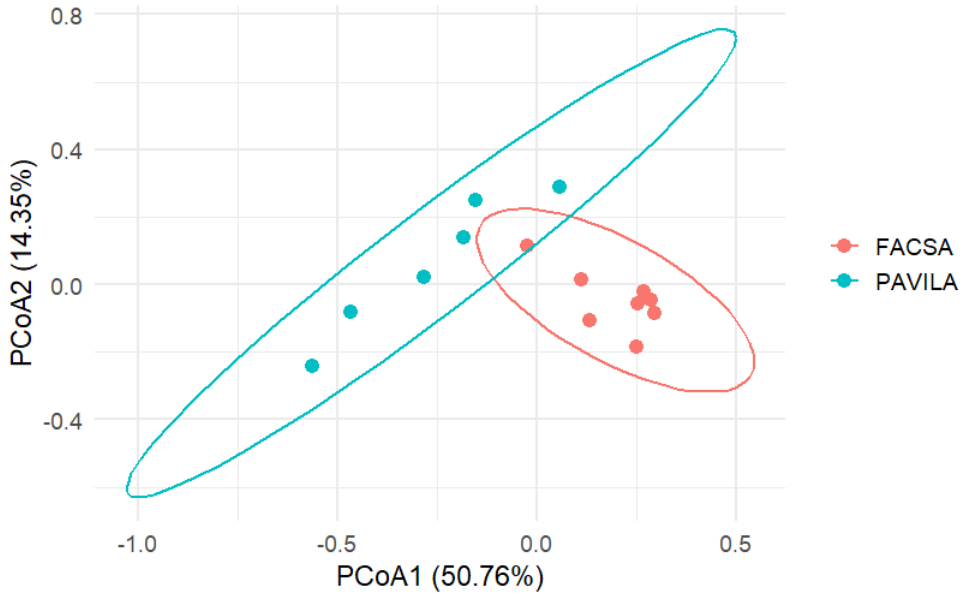

Figure 2. Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarities of genus-level microbiota profiles in FACSA and PAVILA cohorts. Each point represents an individual sample, and ellipses indicate 95% confidence intervals around group centroids. The first two PCoA axes explain 50.76% and 14.35% of the total variance, respectively. Group separation was statistically significant (PERMANOVA, $F = 3.33$, $R^2 = 0.217$, $p = 0.018$).

3.4. PCoA Biplot with Genus-Level Arrows

The PCoA based on Bray–Curtis dissimilarities and Hellinger-transformed genus-level data revealed a clear separation between the two cohorts, despite all individuals being colonised by *Blastocystis* (Figure 3). Samples from the FACSA cohort, with higher HEI-2020 scores and healthier dietary patterns, clustered predominantly toward the right side of the ordination space. In contrast, individuals from the PAVILA cohort, who had lower overall dietary quality, grouped toward the left. Genus-level vectors overlaid on the biplot illustrate the taxa most strongly associated with this compositional variation. Notably, most genera—including *Bacteroides*, *Odoribacter*, *Subdoligranulum*, *Murimonas*, *Faecalibacterium*, and *Parabacteroides*—were projected toward the FACSA cluster, suggesting an enrichment of these taxa in individuals with better diet quality. Surprisingly, only *Segatella* was directionally associated with the PAVILA cluster (Figure 3). This pattern suggests that the microbiota of university students (FACSA), despite colonisation by *Blastocystis*, remains more diverse and enriched in genera typically linked to metabolic health and dietary fiber intake.

These results underscore the modulatory role of diet quality in shaping gut microbial communities, even among *Blastocystis*-colonised hosts, and highlight the ecological impact of contrasting host environments such as institutionalisation and youth in the PAVILA cohort versus independent adulthood in the FACSA cohort.

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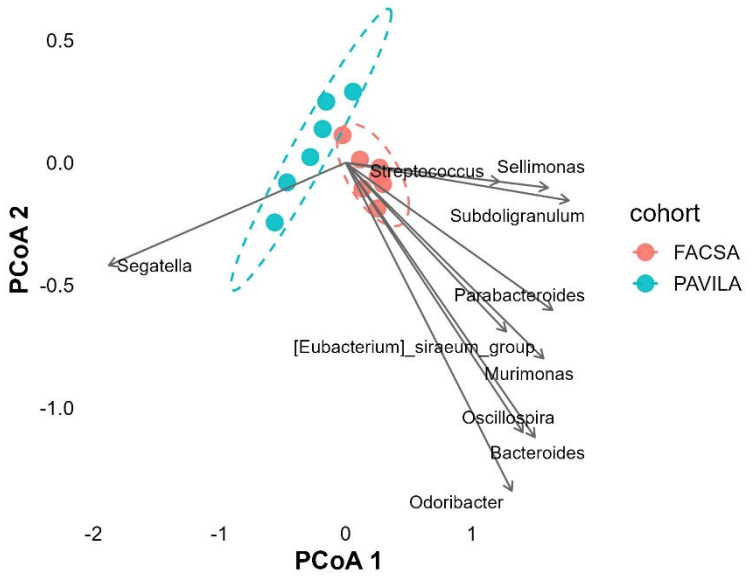

Figure 3. Principal coordinates analysis (PCoA) biplot showing the top 10 bacterial genes that are most aligned with Bray-Curtis dissimilarity axes. Arrows indicate genus direction and magnitude of correlation. Samples are coloured by cohort (FACSA = orange, PAVILA = blue) and enclosed by 95% confidence ellipses.

3.5. Diet–Microbiota Associations within Colonised Individuals

3.5.1. Beta Diversity and Diet Quality Score in Blastocystis-Colonised Individuals

To evaluate whether diet quality influences overall gut microbial composition in individuals colonised by *Blastocystis*, we performed a PERMANOVA using Bray-Curtis dissimilarities. We modelled the total HEI-2020 diet quality score as a continuous predictor variable. The PERMANOVA test revealed a statistically significant association between diet quality and microbiota structure (R^2 = 0.26, F = 4.27, p = 0.016), indicating that dietary quality accounts for approximately 26% of the variance in microbial community composition. Figure 4 illustrates a visible compositional gradient, where individuals with lower diet scores tend to cluster separately from those with higher scores. The first two axes explain 50.76% and 14.35% of the total variance, respectively.

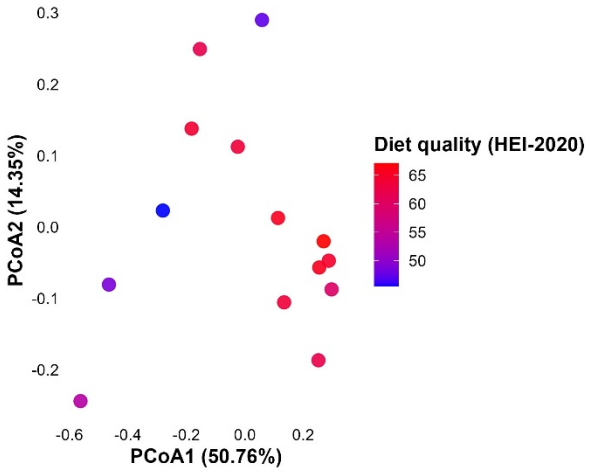

Figure 4. Principal coordinates analysis (PCoA) of gut microbiota profiles at the genus level based on Bray–Curtis dissimilarity among individuals colonised by *Blastocystis* from two cohorts. The HEI-2020 total diet quality score colours points. The first two PCoA axes explain 50.76% and 14.35% of the variance, respectively. A transparent ordination gradient is visible, with samples exhibiting higher dietary quality (red tones) clustering

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toward the right of the PCoA1 axis. In contrast, samples with lower HEI-2020 scores (represented by blue and purple tones) are positioned to the left. PERMANOVA confirmed a significant association between microbial composition and dietary quality ($R^2 = 0.26$, $p = 0.016$), indicating that the host diet strongly influences the gut microbiota structure, even in the presence of *Blastocystis* colonisation.

3.5.2. Microbial Composition Projected onto Dietary Quality Gradient

To analyse the relationship between diet quality and gut microbiota composition, PCoA based on Bray–Curtis dissimilarities were performed and coloured by HEI-2020 scores. The first two axes explained 50.76% and 14.35% of the total variance, respectively. A compositional gradient was observed: individuals with lower HEI-2020 scores (blue hues) were positioned mainly on the left side of the ordination space, whereas those with higher scores (red hues) clustered toward the right, both in the upper and lower quadrants.

An *envfit* projection was used to identify the top 10 genera most associated with this gradient ($p < 0.1$). *Sellimonas* and *Subdoligranulum* were strongly associated with higher HEI-2020 scores. At the same time, *Acutalibacter*, *Phocea*, *Bilophila*, *Bacteroides*, and *[Eubacterium]_siraeum_group* also pointed in that direction, suggesting a shared ecological niche among participants with higher-quality diets. In contrast, *Segatella* was the only genus whose vector aligned with the cluster of individuals with lower HEI-2020 scores, suggesting a potential association with poorer diet quality (Figure 5).

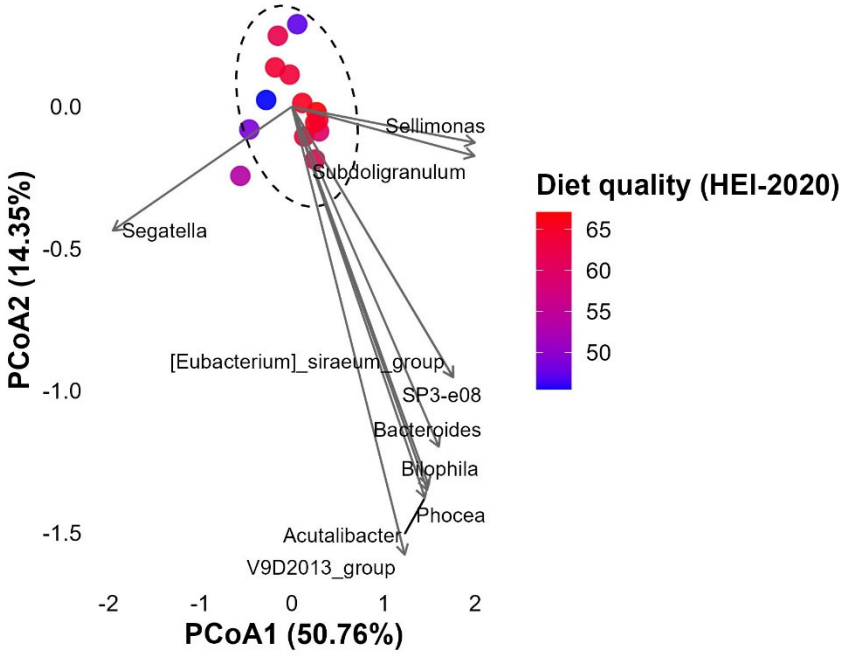

Figure 5. Biplot of the top 10 genera significantly associated with gut microbiota composition based on HEI-2020 diet quality scores (envfit, $p < 0.1$). The plot is based on Bray–Curtis dissimilarities and the first two PCoA axes, which explain 50.76% and 14.35% of the total variance, respectively. Arrows indicate the direction and strength of the association between each taxon and the compositional gradient. Genera such as *Sellimonas* and *Subdoligranulum* were aligned with higher HEI-2020 scores, while *Segatella* was associated with lower-quality diets.

3.5.2. PERMANOVA Results by Dietary Component

We performed PERMANOVA using Bray-Curtis dissimilarities calculated from Hellinger-transformed genus-level microbiota data to assess the influence of each dietary component on microbial community structure. As shown in Table 3, significant associations were observed for the protein intake score ($R^2 = 0.232$, $p = 0.017$) and the vegetable intake score ($R^2 = 0.167$, $p = 0.044$). These

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findings indicate that these two components of diet quality may be key contributors to the observed variation in gut microbiota composition. The remaining components showed weaker and non-significant relationships with microbial structure.

Table 3. PERMANOVA results evaluating the association between each dietary component (HEI-2020) and gut microbiota composition.

Diet Component (HEI-2020)	R^2	F	p-value
Protein foods score	0.232	3.63	0.017*
Total vegetable score	0.167	2.40	0.044*
Total fruit score	0.132	1.82	0.117
Whole fruit score	0.135	1.88	0.117
Legume score	0.097	1.29	0.260
Refined grains score	0.092	1.21	0.279
Whole grain score	0.075	0.97	0.408
Saturated fats score	0.070	0.91	0.421
Sodium score	0.071	0.92	0.464
Seafood and plant protein score	0.064	0.82	0.482

Table 3. PERMANOVA results evaluate the association between individual dietary components from the Healthy Eating Index-2020 (HEI-2020) and gut microbiota composition based on Bray-Curtis dissimilarities of Hellinger-transformed genus-level data. Statistically significant associations ($p < 0.05$) were observed between the protein foods score and the total vegetable score, indicating their influence on microbial community structure among individuals colonised by Blastocystis. R^2 represents the proportion of variance explained by each dietary variable.

3.5.3. db-RDA Analysis: Protein and Vegetable Intake

We conducted distance-based redundancy analysis (db-RDA) using Bray-Curtis dissimilarities of Hellinger-transformed genus-level microbiota data constrained by specific HEI-2020 dietary components.

To analyse the relationship between protein intake and gut microbiota composition in *Blastocystis*-colonised individuals, a distance-based redundancy analysis (db-RDA) was performed using Bray–Curtis dissimilarities of Hellinger-transformed genus-level data, constrained by the protein foods component of the Healthy Eating Index-2020 (HEI-2020). The first canonical axis (CAP1) explained 100% of the constrained variance, while the first unconstrained axis (MDS1) accounted for 32.45% of the residual variance.

A clear cohort-based separation was observed along CAP1: individuals from the FACSA cohort (orange), characterised by higher protein scores, clustered to the right, while those from the PAVILA cohort (green), characterised by lower protein intake, grouped on the left. Environmental vector fitting (*envfit*, $p < 0.1$) identified ten genera strongly associated with the ordination axes. Genera such as *Sellimonas*, *Subdoligranulum*, *Bacteroides*, and *Murimonas* were aligned with higher protein intake and the FACSA cohort. Conversely, *Segatella* was the only genus that projected in the direction of lower protein scores, aligning with the PAVILA group. (Figure 6). These findings suggest a diet-driven structuring of the gut microbiota that reflects differences in protein consumption between cohorts.

To explore the influence of vegetable intake on gut microbiota composition in *Blastocystis*-colonised individuals, a distance-based redundancy analysis (db-RDA) was conducted using Bray–Curtis dissimilarities of Hellinger-transformed genus-level data, constrained by the vegetable component score of the Healthy Eating Index-2020 (HEI-2020). The first constrained axis (CAP1) explained 100% of the variance associated with vegetable intake, while the first unconstrained axis (MDS1) accounted for 36.55% of the residual variance.

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A clear separation between cohorts was observed: individuals from the FACSA cohort (orange), characterised by higher vegetable scores, were positioned on the right side of CAP1, whereas those from the PAVILA cohort (green), with lower scores, clustered on the left. The environmental fit analysis (*envfit*, $p < 0.1$) identified several genera associated with the CAP1 gradient. Taxa such as *Sellimonas*, *Acutalibacter*, *Murimonas*, and *Alistipes* were aligned with higher vegetable intake in the FACSA cohort, while *Segatella* was projected in the direction of lower vegetable intake in the PAVILA group. Figure 7. These findings suggest a compositional shift in the microbiota structure linked to dietary vegetable intake among *Blastocystis*-positive individuals.

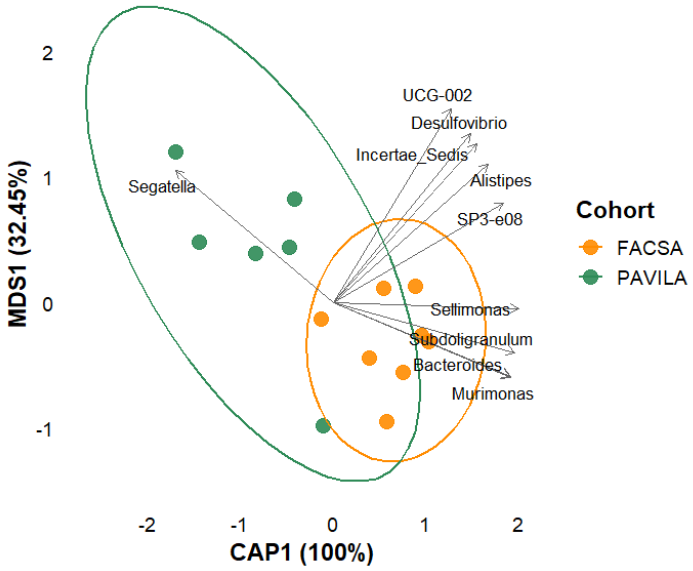

Figure 6. db-RDA constrained by Protein Foods Score. Arrows represent the 10 most correlated genera aligned with Bray-Curtis ordination. Ellipses represent 95% confidence intervals by cohort.

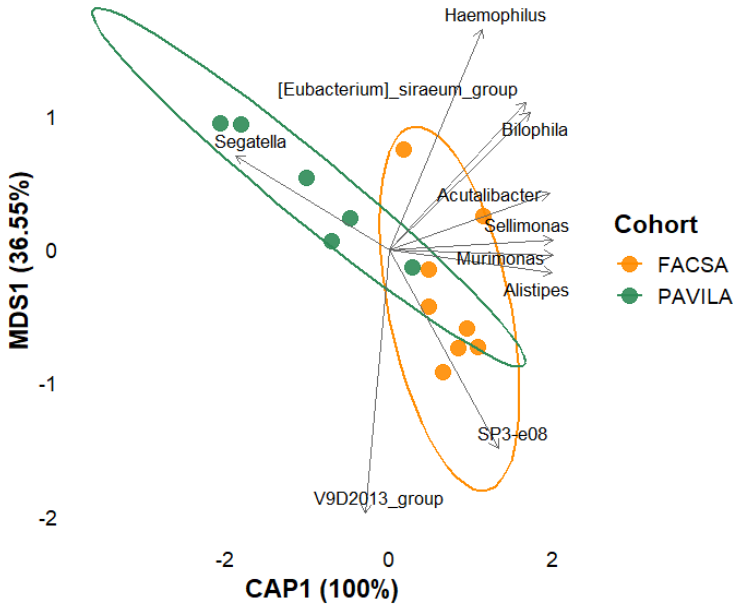

Figure 7. Distance-based redundancy analysis (db-RDA) of genus-level microbiota profiles in *Blastocystis*-colonised individuals, constrained by the vegetable intake component of the HEI-2020. Bray–Curtis dissimilarities were calculated after the Hellinger transformation. The first canonical axis (CAP1) explained 100% of the constrained variation. It separated the two cohorts, with FACSA (orange) projecting to the right, indicating higher vegetable intake, and PAVILA (green) to the left. Arrows represent the ten genera most strongly associated with the ordination axes (envfit, $p < 0.1$). Genera such as *Sellimonas*, *Aculatibacter*, *Murimonas*, and

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Alistipes were associated with higher vegetable intake, whereas *Segatella* was associated with lower scores in the PAVILA cohort.

3.5.4. Multivariate analysis of the combined effect of protein and vegetable intake on gut microbiota composition in individuals colonised by Blastocystis

A multivariate model incorporating protein and vegetable dietary scores demonstrated a significant impact on gut microbiota composition ($R^2 = 0.31$, $F = 2.50$, $p = 0.025$). This result suggests that, in *Blastocystis*-colonised individuals, specific dietary components collectively contribute to explaining more than 30% of the variability in microbial structure, reinforcing their role as key ecological determinants.

Table 4. PERMANOVA Model Summary (Protein and Vegetables).

Term	Df	Sum of Squares	R^2	F	p-value
Model (Protein + Vegetables)	2	0.66195	0.31268	2.5021	0.025
Residual	11	1.45506	0.68732		
Total	13	2.11701	1.0		

Table 4. Results of the PERMANOVA model assessing the joint effect of protein and vegetable scores on gut microbiota composition in individuals colonised by *Blastocystis*. For the analysis, we calculate Bray–Curtis distances from Hellinger-transformed data. The model explained 31.3% of the total variation and was statistically significant ($p = 0.025$, 999 permutations).

We observed a clear separation between the FACSA and PAVILA cohorts in the db-RDA analysis along the CAP1 axis based on the combined protein and vegetable dietary score. The model was statistically significant ($p = 0.025$), indicating that these variables explain a important proportion of the variation in the gut microbiota of individuals colonised by *Blastocystis*. The projected vectors showed that genera such as *Sellimonas*, *Murimonas*, *Alistipes*, and *Desulfovibrio* were associated with better dietary scores and oriented toward the FACSA cohort. In contrast, genera such as *Hydrogenoanaerobacterium, V9D2013_group*, and *Haemophilus* aligned with individuals from the PAVILA cohort, who present lower vegetable and protein intakes. This pattern supports that diet exerts a structuring effect on the microbiota, modulating the microbial community even in the common presence of *Blastocystis.*

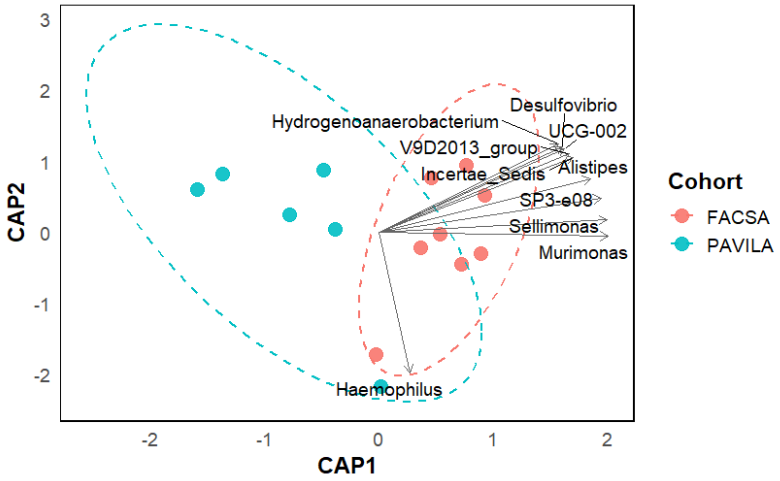

Figure 8. Distance-based redundancy analysis (db-RDA) using protein and vegetable dietary scores as explanatory variables. We perform The analysis using Bray–Curtis distances of Hellinger-transformed data. The points represent *Blastocystis*-colonised individuals from the FACSA (red) and PAVILA (blue) cohorts, and the ellipses indicate the 95% confidence intervals of each group. The ten taxa most associated with the canonical axes are included, projected by vectors (envfit, $p < 0.1$). The separation between cohorts suggests that the

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combined effect of these dietary components differentially modulates gut microbial composition depending on the host context.

4. Discussion

In this study, we examined dietary modulation of gut microbiota composition in *Blastocystis*-colonised individuals from two distinct cohorts: university students (FACSA) and institutionalised children/caregivers (PAVILA). Our results highlight how ecological context and diet may shape microbial patterns.

Diet quality and cohort differences

Diet quality, as quantified by HEI-2020 scores, differed significantly between the two cohorts, with FACSA participants demonstrating higher overall diet quality. FACSA participants consumed higher amounts of fruits, healthier fats (as reflected in favourable ratios of unsaturated to saturated fatty acids), and lower levels of added sugars patterns known to support gut microbiota diversity and metabolic health [38]. In contrast, PAVILA children exhibited lower vegetable intake, which aligns with studies in diverse populations showing that insufficient consumption of vegetables is associated with reduced microbial richness and functional metabolites like SCFAs [39]. These observations support the assertion that while *Blastocystis* colonisation can occur across a range of dietary backgrounds, the broader dietary environment and host setting critically modulate microbiota interactions. Similar findings have emerged in multi-ethnic cohorts, where HEI scores especially components related to fruit and vegetable intake explained the largest variance in gut microbial community structure [39–41].

Alpha diversity insights

In our analysis focusing exclusively on *Blastocystis*-colonised individuals, those from the FACSA cohort exhibited significantly higher microbial richness and evenness compared to PAVILA participants. This pattern is consistent with multiple high-impact studies demonstrating that *Blastocystis* colonisation is associated with increased bacterial diversity in healthy hosts [42,43]. For instance, Tito et al., reported that colonisation was positively correlated with greater microbial richness, enriched in Clostridiales and Ruminococcaceae taxa [44], while Audebert et al. found that *Blastocystis*-positive individuals had a higher abundance of *Prevotella*- or Ruminococcus-driven enterotypes and greater alpha diversity compared to negative subjects [42].

Furthermore, it is well established that gut microbiota alpha diversity increases substantially during early childhood and continues to mature through early school age. A longitudinal study by Stewart et al. demonstrated that bacterial taxonomic diversity increases significantly from infancy to approximately 36 months, stabilizing thereafter [45]. Similar findings were reported by Roswall et al., indicating gradual diversity gains during the first decade of life [46].

These patterns suggest that the lower alpha diversity observed in the younger, institutionalised PAVILA cohort can be attributed to the ongoing maturation of the microbiome, a process delayed compared to adult levels, rather than external speculation. The evidence underscores that age and colonisation status together influence microbial ecological outcomes.

Beta diversity and ecological context

When analyses were confined to *Blastocystis*-colonised individuals, we observed significant differences in community composition between cohorts (PERMANOVA p = 0.001; Figure 2). This suggests that factors such as lifestyle, and institutional living exert independent effects on gut microbiota structure. Longitudinal cohort studies demonstrate that beta diversity evolves across the lifespan, with adult-like microbiota profiles typically emerging by the end of the first decade of life [46]. Additionally, lifestyle factors such, socioeconomic conditions, and dietary diversity are well documented to influence microbial composition substantially, independent of age; institutional environments and constrained dietary options have also been associated with reduced microbial diversity and shifts in community composition in preschool-age children [47–51].

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Exploratory visualisation of diet–microbiota interactions in *Blastocystis*-colonised individuals

A principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarities and coloured by HEI-2020 total scores revealed a structured distribution of samples, despite all individuals being colonised by *Blastocystis*. Notably, individuals with higher dietary quality (as indicated by red hues) clustered toward one side of the ordination space, while those with lower scores (blue/purple hues) were positioned on the opposite end (as shown in Figure 5). This gradient-like pattern suggests that even within a uniformly colonised population dietary quality may drive compositional shifts in gut microbiota. These findings suggest that *Blastocystis* colonisation can occur in diverse dietary contexts and that host diet plays a modulatory role in shaping microbial ecology. It should be noted that the visualisation of microbiota composition constrained by HEI-2020 scores revealed taxa-specific associations along the dietary quality gradient. Notably, *Segatella* was enriched in individuals with low-quality diets, which contrasts with previously reported role in SCFA production and metabolic health [52,53]. The positioning of *Segatella* along the HEI-2020 gradient revealed a strong association with individuals exhibiting the lowest overall dietary quality. Interestingly, this genus was also detected in the PAVILA cohort, where colonised individuals had notably lower vegetable intake. This apparent paradox suggests that *Segatella* may respond to broader ecological conditions influenced by multiple dietary components, rather than isolated vegetable consumption. Indeed, *Segatella* isolates have been shown to ferment plant polysaccharides into diverse short-chain fatty acids (formate, lactate, succinate, propionate, acetate) [54], indicating sensitivity to complex dietary substrates. This finding is consistent with reports suggesting that *Segatella* may increase in environments where diets are high in sugars and low in protein, which are patterns commonly observed in resource-limited settings. As noted by Xiao et al. (2024) point out, *Segatella* dominance does not necessarily indicate good nutritional quality; rather, it may reflect a monotonous diet rich in plant-based carbohydrates but lacking in variety and essential nutrients [55]. The coexistence of Sega*tella* with *Blastocystis* in PAVILA cohort may suggest a standard ecological configuration associated with institutional diets that have high starch content but low in nutritional diversity. This highlights the importance of understanding microbial profiles within the specific social and dietary contexts of each group.

On the other hand, principal coordinates analysis (PCoA) also revealed a transparent gradient, where individuals with higher-quality diets occupied a different region in the ordination space. Projecting microbial genera using envfit analysis further supported this pattern: genera such as *Sellimonas* and *Subdoligranulum* were enriched in participants with higher HEI-2020 scores, consistent with their potential role in beneficial ecological setups. Interestingly, other taxa, such as *Acutalibacter* and *Bacteroides*, also aligned with this higher-quality dietary group. In a systematic review of dietary interventions in individuals with type 2 diabetes, Bock et al. (2022) reported an increase in the abundance of Bacteroides and *Alistipes* following intervention with Mediterranean or high-fibre diets. *Subdoligranulum* exhibited variable responses, depending on the type of intervention and the basal microbiota; these findings partially align with our results. In our cohort of healthy individuals colonised by *Blastocystis*, we also observed that Bacteroides and *Alistipes* align with patterns of higher dietary quality. Although the metabolic context differs, these observations suggest that specific genera may serve as common functional indicators in response to dietary improvements [56]. A previous study analysing colonic mucosal samples found that participants with lower HEI scores had significantly reduced

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abundances of *Subdoligranulum* and Parabacteroides compared to those with better diet quality. Furthermore, they observed that *Alistipes* tended to be more abundant in individuals with greater adherence to healthy dietary patterns, characterised by higher consumption of fruits, fibre, and greater dietary diversity [57].

Dietary components shape microbiota

The db-RDA constrained by the HEI-2020 protein component suggest that protein intake modulates gut microbiota composition in individuals colonised by *Blastocystis*. The observed separation of cohorts along CAP1 is consistent with dietary records showing higher protein food scores in the FACSA group relative to PAVILA. Notably, the genera *Sellimonas*, *Subdoligranulum*, and B*acteroides*, which projected toward the FACSA cluster, have been previously associated with greater dietary diversity and fibre-protein co-fermentation (ref).

Interestingly, *Segatella* was again aligned with the PAVILA group and lower protein intake, consistent with its prior association with lower vegetable scores in this same population. This recurring pattern suggests that *Segatella* may represent a microbial marker of low-nutrient dietary profiles [55]. Together, these findings highlight the relevance of individual dietary components beyond total diet quality in shaping gut microbial ecology under *Blastocystis* colonisation, potentially modulating colonisation dynamics or host-microbe interactions.

PERMANOVA results revealed that protein intake ($R^2 = 0.23$, $p = 0.017$) and vegetable intake ($R^2 = 0.17$, $p = 0.044$) were significantly associated with microbiota composition. This aligns with evidence that dietary protein strongly influences gut microbial community structure and metabolic outputs: a high-protein diet can modulate bacterial taxa involved in proteolytic fermentation, affecting SCFAs and potentially driving changes in gut health [58]. Plant-based components, including vegetables rich in fibre, have been shown to promote the growth of SCFA-producing bacteria such as Lachnospiraceae and Bacteroidetes and support microbial diversity and functional capacity [59,60].

Multivariate analysis revealed that dietary components of protein and vegetables differentially modulate faecal microbiota composition across cohorts, even in *Blastocystis*-colonised individuals. Some studies have shown that higher total protein intake (animals plus vegetables) correlates with higher microbial diversity and changes in bacterial profiles. Furthermore, evidence from human cohorts indicates that vegetable-rich diets promote greater bacterial richness and the presence of functional genera [61,62]. Furthermore, studies on dietary patterns have shown that plant-based diets are strongly associated with increased genera such as *Alistipes [57]* and *Sellinomas* [63]. In contrast, dietary styles characteristic of more restrictive environments favor distinct bacterial profiles [64]. The association of our proposed genera *Sellimonas* and *Murimonas* with healthier diets [65] and *Haemophilus* with more limited diets [66], reinforces the notion that *Blastocystis* may be shaped by host-specific ecological contexts. This supports the idea that its role should not be regarded as universally pathogenic. Consequently, its role should be analysed while considering nutritional quality and social and environmental determinants.

Strengths and Limitations

This study presents several notable strengths. First, it focuses on individuals colonised by *Blastocystis spp.*, allowing for a targeted analysis of dietary and ecological factors that shape the gut microbiota independent of colonisation status. By controlling for this key variable, we were able to examine microbiota structure within a relatively homogeneous colonisation profile, thereby reducing confounding effects. Second, we integrated detailed dietary quality assessment using the HEI-2020, enabling the exploration of specific

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components (e.g., protein and vegetable intake) and their multivariate associations with gut microbiota. Third, the inclusion of two contrasting populations—university students and institutionalised children/caregivers—provided an opportunity to examine the impact of diverse socioeconomic and dietary environments within a shared colonisation context. Lastly, our use of Hellinger-transformed genus-level data and Bray–Curtis dissimilarity in multivariate frameworks (PERMANOVA, db-RDA) ensured robust ecological interpretations.

However, several limitations must be acknowledged. The sample size was relatively small, particularly within each subgroup, limiting statistical power and the generalizability of results. Although our focus on *Blastocystis*-colonised individuals strengthens internal validity, it precludes direct comparisons with non-colonised controls. Furthermore, we did not perform subtyping of *Blastocystis*, which may be a relevant limitation given the reported variation in host responses and microbial associations for subtypes such as ST1, ST4, and ST7. Our cross-sectional design also limits causal inference, and while dietary data were collected using validated methods, reliance on self-reported intake may introduce recall bias. Finally, microbiota profiling was based on 16S rRNA sequencing at the genus level, which, while informative, lacks resolution on strain-specific or functional dynamics that could be captured through metagenomics.

Future studies should incorporate *Blastocystis* subtyping, expand to larger and more diverse cohorts, and integrate metagenomic and metabolomic analyses to further disentangle the diet–microbiota–*Blastocystis* triad.

5. Conclusions

Our findings highlight the critical role of diet in shaping gut microbiota composition among *Blastocystis*-colonised individuals from different social and dietary environments. Despite universal colonisation, microbial diversity and structure differed markedly between cohorts, reflecting the influence of dietary quality, age, and institutional living. Notably, protein and vegetable intake emerged as key dietary components associated with microbiota variation, underscoring their ecological relevance in modulating gut microbial communities.

Moreover, our results contribute to the growing body of evidence suggesting that *Blastocystis* acts more as an ecological indicator than a pathogen, particularly in individuals with diverse and metabolically active microbiota. These findings support a context-dependent interpretation of *Blastocystis* colonisation and reinforce the need to consider both dietary and host ecological factors in gut microbiome research.

Future research integrating *Blastocystis* subtyping, functional microbiome profiling, and immune markers will be essential to clarify causal pathways and to explore the potential of dietary interventions to modulate the microbiota in colonised populations.

Supplementary Materials: The following supporting information can be downloaded at: guangorena, janeth (2025), "DIET_MICROBIOTA_BLASTOCYSTIS", Mendeley Data, V1, doi: 10.17632/r6x9vthwpx.1

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Institutional Review Board Statement: The study was conducted by the Declaration of Helsinki and was approved by the Research Ethics Committee of the Faculty of Medicine and Nutrition with registration number CEI-FAMEN-36, approved on September 22, 2022.

Informed Consent Statement: We obtained informed consent from adult participants and from the parents, guardians, or legal representatives of minor participants, as well as a letter of assent from minor participants.
Data Availability Statement: The data presented in this study are available in the article and supplementary materials. Supplementary files include anonymised dietary intake and microbiota metadata (n = 14), genus-level microbiota abundance, and R scripts used for statistical analysis. We provide all files to support transparency and reproducibility by the study's ethical approval (CEI-FAMEN-36).

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Conflicts of Interest: "The authors declare no conflicts of interest."

Abbreviations

The following abbreviations are used in this manuscript:

PERMANOVA	Permutational Multivariate Analysis of Variance
HEI-2020	Healthy Eating Index-2020
16S rRNA	16S ribosomal Ribonucleic Acid
db-RDA	Distance-based Redundancy Analysis
PCoA	Principal Coordinates Analysis
R2	Coefficient of Determination
SES	Socioeconomic Status
FFQ	Food Frequency Questionnaire
FINUT	Ibero-American Nutrition Foundation
BEDCA	Spanish Food Composition Database
USDA	United States Department of Agriculture
IPAQ	International Physical Activity Questionnaire
MET	Metabolic Equivalent of Task
E.Z.N.A.	E.Z.N.A. stands for 'Easy Nucleic Acid', a brand of nucleic acid extraction kits
DNA	Deoxyribonucleic Acid
Bio-Tek, Inc	Bio-Tek Instruments, Inc. (a laboratory instrumentation company)
FastQC	Fast Quality Control (a tool for quality checking high throughput sequence data)
DADA2	Divisive Amplicon Denoising Algorithm 2
QIIME2	Quantitative Insights Into Microbial Ecology 2
ASVs	Amplicon Sequence Variants
VSEARCH	Vectorized Search (open-source tool for metagenomics)

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SILVA	SILVA ribosomal RNA gene database project
MAFFT	Multiple Alignment using Fast Fourier Transform
PCR	Polymerase Chain Reaction
IDT	Integrated DNA Technologies
Qiagen	Qiagen (biotech company for DNA/RNA extraction and analysis kits)
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RNase	Ribonuclease
DNase	Deoxyribonuclease
CEI-FAMEN	Comité de Ética en Investigación de la Facultad de Medicina y Nutrición (ESP)
envfit	Environmental Fitting Function (in R vegan package)
CAP1	Canonical Axis 1 (used in constrained ordination analyses)
MDS1	Multidimensional Scaling Dimension 1
F	F-statistic
p	p-value
Df	Degrees of Freedom
SCFAs	Short-Chain Fatty Acids
ST1	Subtype 1 (Blastocystis subtype)
ST4	Subtype 4 (Blastocystis subtype)
ST7	Subtype 7 (Blastocystis subtype)

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