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Study protocol

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Integrating dietary assessment and shotgun metagenomics to characterise diet–gut microbiome associations (GUTDIET-PT): Protocol for a multicentre cross-sectional study

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Abstract:

Background: The gut microbiome plays a fundamental role in human health, influencing immune, metabolic, and nutritional functions. Diet is a key factor in shaping microbiota composition, with dietary patterns like the Mediterranean Diet (MD) and Plant-Based Diets (PBD) associated with greater microbial diversity and beneficial bacteria. In contrast, the Western Diet (WD) has been associated with reduced diversity and increased risk of disease.

Methods: The GUTDIET-PT study is a multicentre, cross-sectional observational study aiming to recruit 2,500 healthy participants aged 40 to 74. It is part of the GUTBIOME-PT (NCT06741293), a larger project designed to improve colorectal cancer (CRC) screening in Portugal. Sociodemographic, clinical, anthropometric, and lifestyle information is collected through a purpose-built, GDPR-compliant digital platform, enabling structured and standardised participant-level data capture. Dietary intake will be assessed using two non-consecutive 24-hour dietary recalls, and principal component analysis (PCA) will be used to identify and characterise dietary patterns. Additionally, adherence to the MD and PBD will be evaluated using validated tools such as the Mediterranean Diet Adherence Score and the Plant-Based Dietary Index, while the inflammatory potential of the participants' diet will be

assessed through the Dietary Inflammatory Index. Gut microbial composition and functional capacity will be determined by shotgun metagenomic sequencing of faecal samples.

Results: Recruitment for GUTBIOME-PT began in November 2023 and is ongoing. As of September 2026, 488 participants had been enrolled. Recruitment is expected to be completed by November 2029, with publication of the study findings anticipated by the end of 2030.

Discussion: By combining advanced microbiota analysis with detailed dietary assessments through structured nutritional interviews, we aim to provide novel insights into the relationship between dietary patterns and gut microbiota composition and function. Additionally, focusing the study on healthy individuals will allow us to characterise the microbiota in the absence of disease, offering a clearer understanding of the features of a “healthy” microbiome. This study will establish a foundational framework for identifying dietary patterns that promote microbial diversity and functionality, contributing to tailored nutritional recommendations and public health strategies aimed at improving gut health.

Trial Registration: NCT06741293

Keywords: Gut Microbiota, Dietary Patterns, Mediterranean Diet, Western Diet, Plant-based Diet, Dietary Inflammatory Index

Background

Gut microbiota and its role in health and disease

The gastrointestinal microbiota is a complex ecosystem composed of trillions of microorganisms that play a fundamental role in maintaining health and preventing disease. This community is largely dominated by two major bacterial phyla: Bacteroidota (Bacteroidetes), including genera such as *Bacteroides* and *Prevotella*, and Bacillota (Firmicutes), comprising genera like *Clostridium*, *Enterococcus*, *Lactobacillus*, and *Ruminococcus*. Together, these phyla account for approximately 90% of the gut microbial community. Other phyla include Pseudomonadota (Proteobacteria), Actinomycetota (Actinobacteria), with genera like *Bifidobacterium*, and Verrucomicrobiota (Verrucomicrobia) [1,2].

Gut microorganisms synthesise, transform, and metabolise essential nutrients and bioactive molecules, including lipids, bile acids, short-chain fatty acids (SCFAs), amino acids, and vitamins. Emerging evidence suggests that microbiota composition and metabolic activity exert substantial influence over immune regulation, nutritional homeostasis, and metabolic health [3]. Numerous diseases have been associated with gut microbiota imbalances, including gastrointestinal disorders like inflammatory bowel disease and irritable bowel syndrome [4,5], metabolic conditions such as type 2 diabetes Mellitus [6], various types of cancer [7,8], and neurological disorders like Parkinson's and Alzheimer's disease [9]. Moreover, the gut microbiota may serve not only as a marker of current health status but also as a predictor of long-term health outcomes and longevity [10].

Although disease-related microbial signatures differ from those found in healthy individuals, there is still uncertainty when defining a 'healthy' microbiome [11]. Certain bacterial species may be associated with either health or disease, and different strains within the same species can exhibit distinct functionalities, which poses a challenge when it comes to associating species with health outcomes [12]. Nevertheless, the recently published consensus from the International Scientific Association for Probiotics and Prebiotics emphasises that gut health

cannot be defined solely by microbiome composition, but rather reflects a broader state of normal gastrointestinal function, integrating microbial, host, and clinical factors. [13].

Interindividual variation in microbiota composition is shaped by genetics, age, diet, lifestyle, and environmental factors such as physical activity, stress, and medication use [14]. Among these, diet stands out as a modifiable factor, with studies showing its major impact in defining the structure and function of the gut microbiota [15]. Understanding the mechanisms through which diet and dietary patterns influence microbial composition and functionality is therefore essential for developing targeted health-promoting strategies.

Diet and the gut microbiota

Diet exerts a major influence on gut microbiota composition, and the microbiota, in turn, modulates nutrient metabolism and bioavailability, forming a bidirectional relationship [3]. Dietary patterns - rather than isolated nutrients - are key determinants of microbial community structure, reflecting the complex combinations in which foods are habitually consumed. Both short-term dietary changes and long-term dietary patterns can substantially alter microbiota composition, although responses are highly individualised and may not lead to uniform community convergence across individuals [16–18].

A plant-based diet (PBD), which encompasses vegetarian, vegan, pescatarian, and flexitarian patterns [19], has been associated with lower risks of cardiovascular disease [20], metabolic syndrome [21], type 2 diabetes Mellitus [22], and CRC [23], as well as improved weight management [24]. A recent large-scale, multi-cohort metagenomic study reported that vegan dietary patterns are associated with the enrichment of several species-level genome bins, including known butyrate producers such as *Butyricicoccus spp.*, *Roseburia hominis*, and members of the *Lachnospiraceae* family that are highly specialised in fibre degradation [25].

The Mediterranean Diet (MD), often considered a PBD, is widely recognised as a leading dietary model for health promotion and disease prevention [12]. Originating from Mediterranean coastal countries, it emphasises nutrient-rich foods such as fruits, vegetables, whole grains and healthy fats (particularly olive oil), moderate consumption of poultry, fish and dairy products, and low intake of red meat. Its high intake of unprocessed foods contributes to

its anti-inflammatory and antioxidant properties, highlighting its role in promoting overall health [26]. Adherence to the MD has been associated with greater microbial diversity and enrichment of beneficial genera, such as *Bacteroides*, *Butyricoccus*, *Lachnoclostridium*, *Parasutterella*, and *Lachnospira* [27,28].

Key components of PBD and MD, particularly dietary fibre, promote the growth of fibre-degrading bacteria that produce SCFAs such as propionate and butyrate, which support gut barrier integrity and intestinal homeostasis, and serve as energy sources for colonocytes and peripheral organs [29,30]. A randomised intervention study showed that adherence to the MD was associated with improved intestinal barrier integrity, reflected by reduced levels of lipopolysaccharide-binding protein and zonulin, alongside increased levels of propionate and butyrate, with these being identified as key mediators of this effect [31]. Polyphenols, abundant in olive oil, wine, fruits, nuts and vegetables, have antioxidant, anti-inflammatory and antibacterial properties and modulate microbiota composition [32]. While polyphenols themselves are poorly absorbed by the human body, they are metabolised by the gut microbiota, providing substrate for bacterial families such as *Bifidobacteriaceae* and *Lactobacillaceae* while reducing pathogenic bacteria like *Escherichia coli* and *Clostridium perfringens* [12,33]. Polyphenols also regulate microbial metabolites, including SCFAs, trimethylamine N-oxide (TMAO), and secondary bile acids, by modulating microbial enzyme activity [33]. Polyunsaturated fatty acids, including omega-3, also prominent in the MD, enhance gut epithelial barrier function, reduce inflammation, increase Bacteroidota, and decrease the Bacillota-to-Bacteroidota ratio [34,35]. Intervention studies have shown MD-associated increases in *Faecalibacterium prausnitzii* and *Roseburia faecis* and reduced levels of *Ruminococcus gnavus*, *Collinsella aerofaciens*, and *Ruminococcus torques* [36,37].

The Western Diet (WD) is characterised by high consumption of refined sugars, animal fats, processed meats (especially red meat), refined grains, high-fat dairy products, and fried or pre-packaged foods and low intake of fruits, vegetables, whole grains, meat, fish, nuts, and seeds [38]. This dietary pattern is linked to a higher prevalence of non-communicable diseases, with systematic reviews and meta-analyses showing associations between the WD

and increased risk of breast cancer [39], metabolic syndrome [40], gestational diabetes [41], CRC [23], and overall mortality among cancer survivors [42]. Red meat-derived compounds, such as choline and carnitine, can be transformed into trimethylamine and subsequently into TMAO, an organic compound associated with chronic diseases and cardiovascular risk [43]. Furthermore, nitrates and nitrites used in processed meats can be transformed by bacteria into carcinogenic N-nitroso compounds that are associated with a higher risk of GI cancers [44].

Beyond assessing adherence to specific dietary patterns, it is also important to evaluate the overall inflammatory potential of the diet [45]. Diets rich in fibre, polyphenols, and unsaturated fatty acids generally exert anti-inflammatory effects and are linked to a more favourable gut microbiome profile. In contrast, dietary patterns high in saturated fats, refined carbohydrates, and processed meats are associated with increased systemic inflammation [46]. Given the bidirectional relationship between inflammation and gut microbiota composition [47], assessing the inflammatory potential of the overall diet may provide additional insight into how dietary patterns influence microbial diversity and functional capacity.

In this study, we will investigate how dietary patterns and their inflammatory potential interplay with the gut microbiota. Understanding these relationships is a crucial step, as it helps to shape hypotheses and refine methodologies for future prospective research and can guide the development of public health initiatives, preventive strategies, dietary recommendations, and targeted interventions.

Study Aims

The primary aim of this study is to investigate how different dietary patterns associate with the composition and functionality of the gut microbiota. Specifically, we will (1) characterise the gut microbiota of the study population, (2) identify prevalent dietary patterns within the study sample, and (3) analyse associations between these dietary patterns, specific dietary constituents, and faecal microbiome profiles.

Methods

Study Design

This cross-sectional observational study, conducted at the Gulbenkian Institute for Molecular Medicine, is part of the “Improving Colorectal Cancer Early Screening in Portugal: Identification and Validation of Gut Microbiome Biomarkers (GUTBIOME-PT)” project (NCT06741293). This project aims to recruit 30,000 participants over a period of 6 years. Recruitment started in November 2023 and is planned to be completed by 2029. From the 30,000 participants recruited for GUTBIOME-PT, we aim to recruit 2500 individuals with a negative colonoscopy result for this study.

The project is approved by the Ethics Committee of the Academic Medicine Centre of Lisbon (Ref 111/23). All procedures comply with the principles of the Declaration of Helsinki, ensuring informed consent, respect, integrity, privacy and confidentiality for all participants. Participants retain the right to withdraw from the study at any time without penalty.

Recruitment and eligibility criteria

Participants are recruited at partner hospitals, among patients who are referred for a screening colonoscopy and do not have a clinical diagnosis of CRC or a first-degree family history of CRC. Eligible participants are selected from the control group of the GUTBIOME-PT study, consisting of individuals with negative colonoscopy results. Participants who meet all eligibility criteria (Table 1) receive detailed information about the study and are invited to sign the informed consent form. The overall flow diagram of the study is shown in Fig. 1.

Table 1: Inclusion and exclusion criteria for eligibility

Inclusion criteria

1. Provision of written informed consent
2. Residence in the Lisbon metropolitan area, Portugal
3. Age 40-74 years

4. Referral for a screening colonoscopy

Exclusion criteria

1. Age < 40 or ≥ 75 years

2. Active oncological disease

3. Personal or first-degree family history of CRC

4. Intestinal adenomas removed within the previous 24 months

5. Diagnosis of inflammatory bowel disease, irritable bowel syndrome, or recurrent *Clostridioides difficile* infection

6. Severe cardiovascular or heart diseases

7. Severe renal failure requiring hemodialysis

8. Severe lung disease

9. Pregnancy

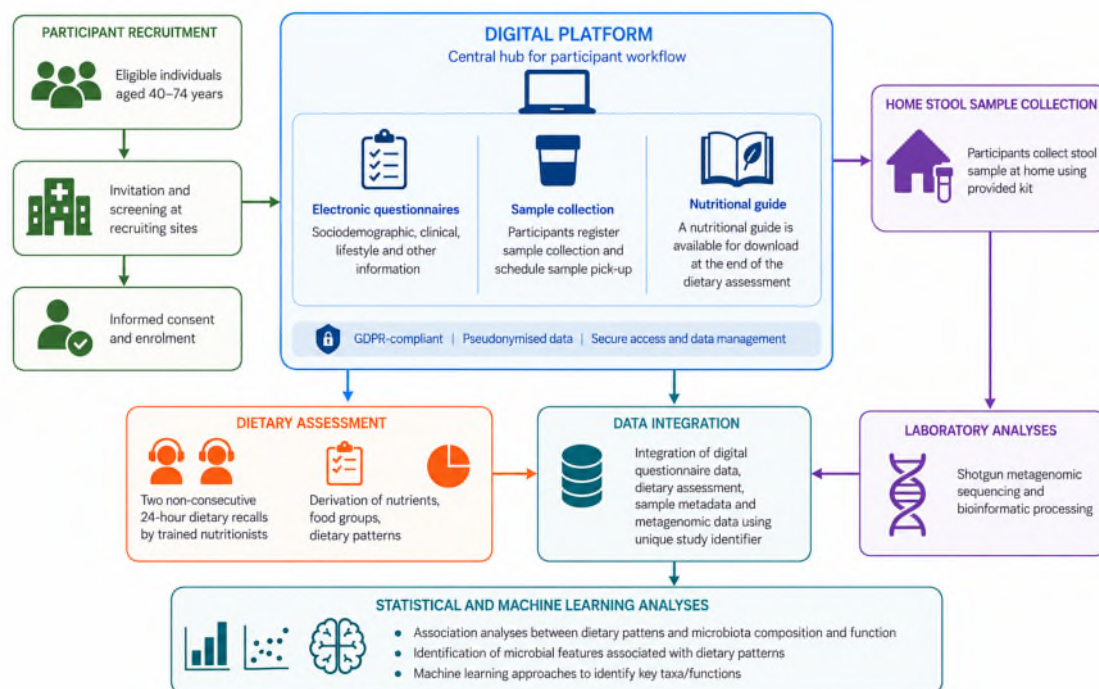


Figure 1: Overview of the GUTDIET-PT study workflow.

Digital study platform

After signing the informed consent, all participants are registered in a purpose-built, GDPR-compliant digital platform developed for the GUTBIOME-PT project that supports participant engagement and study procedures. The platform provides a centralised interface through

which participants complete study questionnaires and manage key steps of their study journey. At the end of the dietary assessment, a nutritional guide on healthy eating and the Mediterranean diet is also made available through the platform for participants to download. Automated reminders can be sent through the platform to encourage completion of pending questionnaires and faecal sample collection. Following home collection of the faecal sample, participants register completion of this step in the platform and can schedule sample pick-up directly through the digital interface. The digital infrastructure was designed to support data collection across multiple recruitment sites while maintaining a harmonised data structure.

Data collection

Sociodemographic, lifestyle, and health-related characteristics: data are collected using self-administered questionnaires completed by participants in the digital platform. The questionnaires are structured into three main sections: sociodemographic characteristics such as education level and economic status, clinical information including any previous or current medical condition, and lifestyle factors such as smoking status, sleep, stress, and physical activity.

Body mass index

Each participant self-reports their weight and height in a health questionnaire following stool sample collection. Body Mass Index (BMI) is calculated using the formula ($BMI = \text{weight (kg)} / \text{height}^2 \text{ (m)}$) and is categorised based on the World Health Organisation's cutoffs [48].

Dietary intake

Dietary intake data is collected through telephone interviews conducted by a qualified clinical nutritionist, following validated protocols by the European Food Safety Authority [49] and the Portuguese National Food and Physical Activity Survey [50]. Two non-consecutive 24-hour dietary recalls are performed, recording all foods, beverages, and dietary supplements consumed during the day - from 00h00 to 23h59 - preceding each interview. Each food item is documented with details on the context of consumption (location and time), quantity

consumed, and preparation and cooking methods. Common volume measures (e.g., cups, tablespoons, teaspoons, palm) are used to estimate portion sizes. If possible, the brand of the product eaten is recorded. The conversion of food into nutrients is made with nutritional information from the Portuguese Food Composition Table (FCT), available on the PortFIR website [51], version 7.0. For foods not included in the Portuguese FCT, the French [52] and British [53] food composition tables are used.

Adherence to the MD

MD adherence is assessed using the 14-Item Mediterranean Diet Adherence Score developed by the PREvención con Dieta MEDiterránea (PREDIMED) study authors [54], which includes 12 questions on food consumption and frequency, and two questions on food intake habits that are key principles of the MD. Responses are scored 0 (condition not met) or 1 (condition met), resulting in a score ranging from 0 to 14. Based on this final score, adherence to the MD is categorised as low (score < 5), moderate (score 6-9), or high (score > 10). This questionnaire is applied to participants during the first 24-hour dietary recall.

Adherence to a PBD

Adherence to a PBD is assessed according to the method published by Satija *et al.* using a plant-based dietary index (PDI), a healthful PDI (hPDI), and an unhealthful PDI (uPDI) [55]. The individual foods collected through the 24-hour dietary recalls are combined into groups based on animal foods, healthy plant foods, and unhealthy plant foods. Based on the intake levels of these food groups, all food groups are divided into quintiles and assigned separate positive scores (i.e., higher intake receives higher scores, with a score range of 1–5) or reverse scores (i.e., higher intake receives lower scores, with a score range of 5–1). For the overall PDI, all plant-based foods are assigned positive scores, whereas animal-based foods are assigned negative scores, and the scores are then summed to obtain an overall PDI score. For the hPDI, positive scores are assigned to healthy plant foods, whereas negative scores are assigned to animal and unhealthy plant foods. For the uPDI, positive scores are assigned

to unhealthy plant foods, whereas negative scores are assigned to animal and healthy plant foods. For all three PDIs, higher scores indicate greater adherence to dietary patterns [55,56].

Dietary inflammatory index

Dietary inflammatory potential is assessed using the Dietary Inflammatory Index (DII), as developed by Shivappa *et al.* [57]. Dietary intake data derived from the 24-hour dietary recalls are used to calculate individual DII scores. Intakes of available dietary components are standardised to a global reference database by calculating z-scores based on global means and standard deviations. These z-scores are converted to centred percentiles to account for skewed intake distributions. Each centred percentile is then multiplied by a literature-derived inflammatory effect score specific to each dietary component, reflecting its pro- or anti-inflammatory association. To account for total energy intake, an energy-adjusted DII (E-DII) is also calculated by expressing dietary intakes per 1,000 kcal and applying energy-adjusted global reference values. Only dietary components available from the dietary assessment are included in the DII calculation, and missing components are not imputed. The resulting component-specific scores are summed to generate an overall DII and E-DII score, with higher positive values indicating a more pro-inflammatory diet [57].

Stool sample characterisation

Participants self-collect a stool sample using a provided kit (EasySampler stool collection kit with DNA stabilisation buffer, Invitek), which preserves microbial DNA integrity at the moment of collection. Stool consistency is assessed using the Bristol Stool Scale (BSS) scores [58], which categorises stool into seven types as a proxy for intestinal transit rate [59]. Stool samples are also characterised by colour, and recent gastrointestinal symptoms are annotated, such as bloating, cramps or abdominal pain, excessive intestinal gas, mucus in the stool, abnormal stool colour, and presence of blood in the stool. Frequency of bowel movements over the past 5 days is also considered.

Faecal DNA extraction and sequencing

Once collected, the stool samples are transported to the laboratory, where they are subdivided into aliquots and stored at -80°C until further analysis. Samples are processed for DNA extraction within a maximum of 6 months. For faecal microbiome characterisation, samples undergo genomic DNA extraction using the ZymoBIOMICS 96 MagBead DNA Kit (Zymo Research), compatible with the KingFisher™ Flex automatic extractor (Thermo Fisher Scientific). DNA concentrations are measured using a Qubit 1X dsDNA HS assay kit (Invitrogen) on a Qubit Flex Fluorometer (Life Technologies). The composition and genetic content of the faecal microbiota are analysed through shotgun metagenomic sequencing, performed on an Illumina platform (NovaSeq X Plus), with a minimum of 3 million reads per sample.

Data management

Participant withdrawal

In line with the Declaration of Helsinki, participants are informed that they may withdraw from the study at any time. Reasons for withdrawal are reported in the source documents and on the case report form. Samples from participants who withdraw are preserved and may still be analysed, as long as the participant does not request the destruction of their data.

Missing Data

If a sample is missing, associated data (e.g., diet-related questionnaires) is excluded from the analysis, and the participant is considered withdrawn to maintain data integrity, as biological samples are essential for the study. Additionally, participants who do not complete mandatory questionnaires are considered excluded from the study, and their sample is not considered for analysis. If the participant completes only one 24-hour dietary recall, this data is included in the analysis. Although multiple interviews are preferred for accuracy, a single interview can still provide relevant insights into dietary habits [49].

288 **Data codification**

289 Participant data is confidential and identified only by a unique participant code ("Participant
290 ID") on both faecal samples, questionnaires, and the electronic database. Data from
291 questionnaires is recorded on a secure online platform, with sample results stored in a
292 separate database linked through the participant's code to ensure participant anonymity.
293 Personal data is processed based on the participant's explicit consent, strictly for scientific
294 research, study-related communication, and compliance with legal requirements and
295 regulatory obligations.

296 **Data Storage**

297 Personal data is stored with restricted access in compliance with the General Data Protection
298 Regulation and relevant legislation. All data remains within the originating institution in
299 compliance with the data management plan, which outlines protocols for data handling,
300 storage, and sharing. To increase accessibility, pseudonymisation is employed to make the
301 data more widely available. Due to intellectual property constraints, data may not be fully open
302 access. Data and document ownership are clearly assigned, and a designated individual will
303 manage data transfer at project completion. Data will be archived in cold storage for long-term
304 preservation. This approach will help ensure the data remains comprehensible and reusable
305 over time.

306 Personal information is processed in a pseudonymised format, with access to decryption keys
307 restricted to authorised personnel. This project is being conducted in collaboration with multiple
308 institutions under a formal collaboration agreement that outlines data access permissions, with
309 ongoing updates as needed.

310 **Data analysis**

311 **Sample size**

Sample size estimation was informed by power analyses using the Shannon Diversity Index as the primary outcome. Based on the effect size reported by Malinowska *et al.* [60] (mean difference $\approx$ 0.08 units; Cohen's $d \approx$ 0.17), balanced two-group comparisons achieved $\geq 90\%$ power with 1500-2000 participants. However, we acknowledge that power will be reduced under unbalanced group allocations.

To ensure adequate statistical power while accounting for attrition, we applied a 20% anticipated dropout rate. Therefore, to retain an effective sample size of 2000 participants, the study will aim to recruit approximately:

$$2000 \div 0.80 = 2,500 \text{ participants}$$

Thus, the target recruitment number is 2,500 participants, allowing sufficient power for primary comparisons even with expected loss to follow-up or incomplete data.

Software

Statistical analyses will be performed using R (R Foundation for Statistical Computing) and Python (Python Software Foundation). RStudio (Posit) will be used as an integrated development environment for R, with $p < 0.05$ considered statistically significant. R is used for statistical testing, data analysis, and visualisation, while Python supports advanced data analysis and statistical modelling to enhance the robustness of the findings and enable in-depth biological interpretation.

Microbiome Analysis

The faecal DNA obtained from participants undergoes shotgun metagenomic sequencing [61], generating approximately 3 to 4 gigabytes of data per sample. Paired-end sequencing is performed, and the resulting raw sequences are processed to ensure high-quality data. Fastp is employed to trim and remove low-quality reads [62], and bowtie2 is used to eliminate host contamination by aligning the sample reads to the human genome reference (GRCh38) [63].

To quantify the abundance of microbial taxa, a hybrid approach combining k-mer analysis and mapping techniques is applied to the preprocessed sequences, utilising publicly available genome databases such as GTDB [64] and UHGG [65] for reference support. The functional potential of the microbiomes is inferred by directly mapping the metagenomic data to functional databases such as UniProt [66], KEGG [67], or CAZy [68] and performing gene annotation and characterisation of the assembled metagenomes.

The resulting high-dimensional metagenomic datasets will be integrated computationally with dietary and participant-level phenotypic information, enabling species- and functional-level investigation of diet-microbiome relationships at population scale.

Statistics

The statistical analysis focuses on evaluating the associations between dietary patterns and gut microbiota composition and function. Major dietary patterns present in the study sample will be identified and characterised using PCA with varimax rotation, an *a posteriori* (data-driven) that is a variable reduction procedure based on correlation or covariance matrices of the original variables, creating linear combinations such as patterns [69]. The individual foods collected through the 24-hour dietary recalls will be combined into groups based on their nutritional components- similarity and culinary uses- and these groups will serve as variables for the PCA, as previously described [70]. Alternatively, clustering techniques (e.g., k-means) may be employed to classify participants into distinct dietary groups.

Microbial alpha diversity (within-sample diversity) will be assessed using Shannon, Simpson, and observed richness indices, while beta diversity (between-sample differences) will be calculated using Bray-Curtis dissimilarity and weighted/unweighted UniFrac metrics. Differences in alpha diversity between dietary groups will be evaluated using the Kruskal-Wallis test, and beta diversity will be compared using PERMANOVA (Permutational Multivariate Analysis of Variance), with visualisation through Principal Coordinate Analysis (PCoA) plots.

Differential abundance analysis will be conducted to identify taxa and functional pathways associated with specific dietary patterns while controlling for multiple comparisons with the Benjamini-Hochberg correction. Correlation analyses (Spearman or Pearson) will be performed to explore the relationships between dietary intake (e.g., macronutrient composition) and the relative abundance of microbial taxa and functional features. Multivariable models, including linear or logistic regression, will adjust for potential confounders such as age, sex, BMI, and physical activity to ensure robust associations. Additionally, machine learning approaches, such as Random Forests, will be applied to identify key microbial taxa or functions predictive of dietary patterns. Statistical significance will be set at $p < 0.05$ unless otherwise stated.

Results

Recruitment for GUTBIOME-PT began in November 2023 and is ongoing. As of September 2026, 488 participants had been enrolled. Recruitment is expected to be completed by November 2029, with publication of the study findings anticipated by the end of 2030.

Potential outcomes and clinical impact

To our knowledge, this is the first large-scale observational study to examine how distinct dietary patterns relate to gut microbiota composition and function in the Portuguese population using shotgun metagenomic sequencing. The sample size and methodological rigour provide a robust foundation for characterising diet–microbiota interactions and generating evidence that may inform future research, nutritional guidance, and public health strategies.

Shotgun metagenomic sequencing offers higher taxonomic and functional resolution than 16S rRNA amplicon sequencing, allowing the detection of low-abundance taxa and enabling detailed functional profiling [71,72]. This methodology enhances the capacity to identify structural variation and metabolic pathways within the microbiome [61]. Combined with detailed dietary information obtained through structured nutritional interviews, this approach

enables a comprehensive assessment of the relationship between dietary patterns and microbiota composition and function.

Previous studies have demonstrated that specific nutrients, such as fibre or saturated fat, and foods, such as red meat, are associated with distinct microbiota compositions [43,73]. However, focusing only on individual nutrients or foods may fail to explain their overall effect on health, since it does not take into account the interaction between nutrients and foods. Studying dietary patterns provides a more holistic and comprehensive approach. Research has analysed the associations between dietary patterns - such as MD, PBD, and WD - and microbiota composition, but these studies often focus on specific diseases, such as cancer or metabolic disorders [3,74]. By contrast, the present study focuses on healthy adults, allowing us to characterise microbiota composition in the absence of disease and to identify dietary behaviours that support microbial diversity and functional capacity. This approach contributes to a clearer understanding of microbiome characteristics associated with health and provides a foundational framework for identifying dietary patterns that promote microbial diversity and functional capacity.

Furthermore, large population-based metagenomic studies remain limited in Mediterranean countries. By examining the Portuguese population within its specific cultural and dietary context, this study helps address a national knowledge gap while also expanding the evidence base for microbiome research. This contributes to a broader and more nuanced understanding of microbiota variability across Mediterranean populations.

Dietary intake will be assessed using two non-consecutive 24-hour dietary recalls, a validated method that captures detailed information on consumed foods and beverages [49]. Compared with food frequency questionnaires, which may lack precision and restrict responses to predefined items, 24-hour recalls allow for more accurate, flexible assessment of dietary behaviours, including the detection of non-listed foods [75,76]. This short-term resolution is particularly relevant given the rapid responsiveness of the gut microbiota to recent dietary

intake, which long-term dietary tools may not accurately capture [77]. Although recall-based methods are subject to reporting bias [75,78], this limitation will be mitigated through validated protocols [49], structured interviews conducted by trained nutritionists, and statistical adjustments for potential confounders. While some participants may provide only a single recall, the large sample size of the study is expected to reduce variability and support reliable dietary characterisation.

Altogether, this study is expected to generate valuable insights into the complex interactions between diet and gut microbiota. By clarifying how dietary patterns relate to microbial composition and function, the findings may inform evidence-based public health and nutritional strategies aimed at promoting gut health. In the longer term, this work has the potential to support precision nutrition approaches tailored to individual dietary habits and microbiota profiles, ultimately enhancing the effectiveness of dietary interventions designed to improve health and well-being.

Abbreviations

BMI - Body Mass Index

BSS - Bristol Stool Scale

CRC - Colorectal Cancer

DII - Dietary Inflammatory Index

hPDI - Healthful Plant-Based Dietary Index

MD - Mediterranean Diet

PBD - Plant-Based Diet

PDI – Plant-Based Dietary Index

435 PREDIMED - PREvención con Dieta MEDiterránea

436 SCFA - Short-Chain Fatty Acids

437 TMAO - Trimethylamine N-oxide

438 uPDI - Unhealthy Plant-Based Dietary Index

439 WD - Western Diet

440 **Declarations**

441 **Ethics approval and consent to participate**

442 The project was approved by the Ethics Committee of the Academic Medicine Centre of Lisbon
443 (Ref 111/23) in November 2023. All procedures comply with the principles of the Declaration
444 of Helsinki, ensuring informed consent, respect, integrity, privacy and confidentiality for all
445 participants.

446 **Consent for publication**

447 Unless specified by local laws or regulations, the Sponsor will be responsible for ownership of
448 the data, results, reports, conclusions, or findings related to this study. Consequently, the
449 Sponsor reserves the right to ownership and use of the data from this study for the purpose
450 described in the project. Access to the data obtained may be granted to other qualified
451 researchers proposing valid scientific analyses, always respecting the confidentiality of the
452 data and the anonymity of the participants. Information regarding the identification of
453 participants will never be shared, respecting the data confidentiality mentioned above.

454

455 **Availability of data and materials**

456 Not applicable

457

458 **Competing interests**

The authors declare that they have no competing interests.

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Authors' contributions

CSG, IS and ASA contributed to the conception and design of the study. MMM and JS drafted the manuscript, and all authors critically read and revised it. All authors approved the final version of the manuscript.

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