Version 2. 19th September, 2025

ANALYSIS OF THE COMPOSITION OF THE INTESTINAL MICROBIOTA AND ITS EVOLUTION IN PATIENTS POST–INTESTINAL ANASTOMOSIS

Protocol Code: MIDESIN

Version 2, September 19, 2025

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IIS–FJD

Ethics Committee Code: PIC165-25_FJD

SUMMARY

Study Identification

Title: “Analysis of the Composition of the Intestinal Microbiota and Its Evolution in Patients Post–Intestinal Anastomosis”

Code: MIDESIN

Version: 1, July 22, 2025

Study Sites

The study will be conducted at the Hospital Universitario Fundación Jiménez Díaz and the Instituto de Investigación Sanitaria Fundación Jiménez Díaz.

Ethics Committee

The study will be submitted to the CEI/CEIm of the Hospital Universitario Fundación Jiménez Díaz. A

Ethics Committee Code: PIC165-25_FJD

Approved: 2025, 19th September

Hypothesis

Considering natural interindividual variability, the scientific literature suggests that specific microbiological patterns may exist among patients with colorectal cancer. Our primary hypothesis is that within this patient population, the colorectal microbiota differs between patients who develop postoperative anastomotic leakage and those who do not experience postoperative complications.

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Objectives

Primary Objective

To characterize the microbiome of patients undergoing colorectal surgery and evaluate the impact of current surgical preparation on the microbiota of the local population.

Secondary Objectives

- Classification and prediction of postoperative evolution in patients undergoing colorectal anastomosis, through metagenomic characterization of the intestinal microbiota.

- Analyze the relationship between intestinal microbiota and its potential involvement in postoperative anastomotic leaks.

Study Design

Prospective, descriptive, non-interventional research project in which some variables will be analyzed cross-sectionally.

Condition Under Study

Any type of intestinal surgery in which an intestinal anastomosis is performed.

Study Population and Sample Size

A total of 30 patients requiring colorectal anastomosis surgery and meeting the inclusion/exclusion criteria will be enrolled.

Timeline

Start: December 2025

Recruitment: 12 months

Follow-up: 1 month per patient

Total duration: 13 months (until December 2026)

Additional time: 3 months for sample analysis + 1 month for result review.

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PROTOCOL

INTRODUCTION AND STUDY RATIONALE

Recent investigations suggest a significant relationship between the intestinal microbiota and anastomotic leakage (AL) in colorectal surgery. Patients with AL exhibit greater abundance of Lachnospiraceae bacteria and reduced microbial diversity. Factors shaping the microbiota profile include preoperative interventions and surgical variables.

Further evidence regarding the role of microbiota in AL largely comes from animal studies, with limited understanding in surgical patients. Perioperative factors such as diet, radiation, bowel preparation, and medications have been linked to microbiota alterations and increased AL risk. Mechanisms include bacterial adhesion to intestinal mucosa, biofilm formation, and impaired host resistance.

Previous studies also describe involvement of Enterococcus faecalis and Bifidobacterium spp. in AL.

Given the existing data, our objective is to analyze the microbiota of our patient population and subsequently identify microbiome differences between those who develop an anastomotic leak (approximately 9–10%) and those who do not.

REFERENCES

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STUDY HYPOTHESIS AND OBJECTIVES

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Hypothesis

The colorectal microbiota differs between patients with postoperative anastomotic leakage and those without complications.

Primary Objective

To characterize the microbiome of patients undergoing colorectal surgery and evaluate the effects of current surgical preparation.

Secondary Objectives

- Classification and predictive indication of postoperative outcomes in colorectal anastomosis patients using metagenomic characterization.

- Identify preliminary indications of links between intestinal microbiota and postoperative anastomotic leaks.

STUDY TYPE AND DESIGN

Prospective, descriptive, non-interventional project.

SUBJECT SELECTION

Patients will be selected from individuals undergoing abdominal surgery requiring colorectal anastomosis. Eligible patients will be invited to participate and assigned coded identifiers.

Inclusion Criteria:

- Undergoing colorectal anastomosis

- Signed informed consent

- Age ≥18 years

- Availability for postoperative follow-up

Exclusion Criteria:

- Emergency surgery

- Intra-abdominal sepsis or prior leaks

- Severe immunosuppression

- Antibiotic use within 3 months

- Pregnancy or breastfeeding

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STUDY DEVELOPMENT AND OUTCOME EVALUATION

Phase I – Ethics approval

Phase II – Patient selection, consent, coding

Phase III – Sample collection using GutAlive® kits

Phase IV – Sample processing at MicroViable Therapeutics

Phase V – Bioinformatic and clinical outcome analysis

SEQUENCING PROTOCOL

The project presented will be developed according to the following phases:

- Phase I. Authorization of the trial by the HUFJD Ethics Committee.

- Phase II. Selection of patients by the Surgery team and signing of the informed consent form by the patients. Once the patient has been included in the study, they will be coded so that only the two people involved in this procedure from the research team can associate the medical record number with the code. This code will be marked on the GutAlive® devices using labels that are resistant to deep freezing.

- Phase III. Sample collection: Patients will be given three GutAlive® kits, and one kit will be given to the surgical team.

GutAlive® is an anaerobic stool collection device that guarantees sample stability and microbiota viability, preserving its original composition and diversity by creating an anaerobic atmosphere inside. It also preserves DNA integrity thanks to the viability of the bacterial cells. It is currently the stool collection system included in the protocols of the Spanish Association of Gastroenterology for Fecal Microbiota Transplant procedures.

- Once the samples have been collected by the patients, they will be sent to the IIS-FJD New Therapies Laboratory within 48 hours of collection. Samples received at the New Therapies Laboratory will be stored in the GutAlive® device, frozen at -80°C, until they are processed. This coding will be marked on the GutAlive® devices using labels that are resistant to ultra-freezing.

- Phase IV. Sample processing. The collected and coded samples will be transported refrigerated to MicroViable Therapeutics, using dry ice to prevent them from thawing. At Microviable, the bacterial DNA will be extracted from the samples and massively sequenced using Illumina technology. Subsequently, also at Microviable, the metagenomic and statistical bioinformatic analysis of the sequencing data will be carried out (see protocol).

- Phase V. Analysis of results. Once the coded metagenomic results have been obtained, the research team will compare the clinical evolution of the patients in an anonymized manner with these results and then extract patterns from the microbiota of the study population. To

handle large amounts of data, we have a cloud computing platform with unlimited processing capacity that also guarantees data security.

Protocol Sequencing and analysis of the microbiota present in the samples

1. Collection of a stool sample using the GutAlive® device (https://www.microviable.com/gutalive), which facilitates, sanitizes, and standardizes sample collection.

2. Storage and anonymization of samples in the advanced therapies laboratory of the Jiménez Díaz Foundation's Health Research Institute.

2. Shipping and processing of anonymized samples to MicroViable Therapeutics facilities.

3. Extraction of total DNA from samples using commercial kits such as: DNeasy PowerSoil Pro Kit (Qiagen) for the extraction of microbial DNA from stool samples.

4. Quantification of DNA using the Qubit dsDNA BR Assay kit.

5. Creation of libraries and DNA sequencing using NGS with Illumina technology, 2x300 bp, paired reads, 20-50 million reads per sample.

6. Response evaluation.

VARIABLES AND OUTCOME EVALUATION

Patient-Related Variables:

- Postoperative clinical evolution (1 month)

- Clinically or surgically diagnosed anastomotic leaks

Sample-Related Variables:

- Bacterial sequence comparison

- Taxonomy and abundance

Outcome Evaluation:

- Microbiota characterization

- Association between taxonomy and clinical evolution

- Preliminary indications regarding anastomotic leaks

PRIMARY ANALYSIS

Bioinformatic analysis of sequencing results, including:

- Quality analysis with barcode and sequence filtering.

- Taxonomic identification of microorganisms at the phylum, order, class, family, genus, and species levels through comparison with databases.

- Quantification of relative abundance for each identified bacterial taxon.

- Alpha diversity analysis using different indices (Shannon, Simpson, Fisher, Observed OTUs)

- Beta diversity analysis using different indices and matrices (Bray-Curtis, Jaccard, UniFrac), representation of figures (PCoA, NMDS, etc.) and comparison between groups.

• Statistical analysis using specific algorithms such as ANCOM, Simper, Adonis, Anosim, PERMANOVA, and others.

- Identification of metabolic pathways predicting the functionality of the microbiota.

- Calculation of relative abundances of each identified metabolic pathway.

- Statistical analysis of the differential presence of metabolic pathways using specific algorithms (Maaslin2 and LEFSE)

Assignment of metabolic pathways to the corresponding microbial groups

BIOLOGICAL SAMPLES

Four GutAlive® samples per patient:

1. Before surgical preparation

2. Intraoperative

3. At discharge (~day 5)

4. 1-month follow-up

ADVERSE EVENT MANAGEMENT

No adverse events expected.

ETHICAL CONSIDERATIONS

Compliance with the Declaration of Helsinki, GCP, GDPR, Spanish data protection law. Coded database. No insurance required.

PRACTICAL CONSIDERATIONS

GCP compliance, data recording procedures, patient rights, data retention for 10 years.

The information disseminated and obtained through the implementation of this project is considered confidential and will be treated at all times in accordance with current data protection regulations (Regulation (EU) 2016/679 of the European Parliament and of the Council of April 27, 2016, on Data Protection (GDPR), on the Protection of Personal Data, and Organic Law 03/2018 on Data Protection). An Excel database will be created specifically for the study, in which patients will be identified with a binary code (letter-number) assigned in order of recruitment, and which will not contain any personal information about the patients.

STATISTICAL ANALYSIS

Sample Size

Based on AL incidence (9–10%), n=30 deemed sufficient.

Statistical Methods

Statistical analysis of microbiota correlations and clinical evolution

All statistical comparisons will be made taking into account the grouping of patients according to their clinical evolution, for which a matrix of possible comparisons will be made based on the clinical metadata provided by researchers at the Jiménez Diaz Foundation.

Both longitudinal analyses, which will allow us to see the temporal evolution of the patients' microbiota and how surgical preparation influences it, and cross-sectional analyses will be performed, taking into account variables related to the incidence of dehiscence and the clinical evolution of each group of patients, in search of intestinal microbiota parameters that serve as biomarkers to predict suture failure after surgery.

These analyses will be performed using the R-studio statistical packages for Windows 11 and include, among others, the following packages: dplyr, ggplot2, ggpubr, pheatmap, readxl, RColorBrewer, remotes, stringi, stringr, tibble, tidyr, vegan, ANCOM-BC, Maaslin2, microbiome, microbiomeMarker, phyloseq, and Adonis.STUDY PUBLICATION

Results will be published and made publicly available, following ethical obligations.

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PARTICIPANT INFORMATION SHEET

PROJECT TITLE: Analysis of the composition of the intestinal microbiota and its evolution in post–intestinal anastomosis patients

PROJECT CODE: MIDESIN

PRINCIPAL INVESTIGATOR: Dr. Mariano García Arranz, New Therapies Laboratory / Surgery. Fundación Jiménez Díaz Health Research Institute.

Ethical committee approved Code: PIC165-25_FJD (2025, 19th September)

INTRODUCTION

We are writing to inform you about a research study in which you are invited to participate. The study has been approved by the corresponding Clinical Research Ethics Committee in accordance with current legislation and complies with the principles set out in Biomedical Research Law 14/2007 and the Declaration of Helsinki in its most recent update (75th World Medical Assembly, held in Helsinki in October 2024).

Our intention is that you receive accurate and sufficient information so that you can decide whether or not you wish to participate in this study. To this end, please read this information sheet carefully; afterwards, we will clarify any questions that may arise. You may also consult with anyone you consider appropriate. Please be informed that this work is an independent initiative of the study investigators, has public funding for its execution, and the participating investigators will not receive any financial compensation.

OBJECTIVE OF THE STUDY

The rationale for proposing this study is based on recent research suggesting a relationship between the intestinal microbiota and anastomotic leakage (dehiscence) in colorectal surgery. Patients who develop leaks show a greater abundance of intestinal bacteria from the families Lachnospiraceae, Enterococcaceae, and Bifidobacteriaceae, as well as lower microbial diversity. In addition, various perioperative factors influencing bacterial diversity have been described and linked to a higher risk of post-surgical dehiscence. Therefore, considering the available literature, our main interest is to analyze the composition of the intestinal microbiotaí to analyze the composition of the intestinal microbiota of patients in our social environment and subsequently look for differences at the microbiota level between those who have experienced an anastomotic dehiscence and those who have not.

The primary objective of this study is to determine the composition of the intestinal microbiota (defined as the set of microorganisms residing in our intestine) and the effect that current surgical preparation has on it in the population of our environment.

Likewise, a secondary objective is to carry out a taxonomic classification of the bacteria present in the intestinal microbiota to determine which bacteria are present and in what proportion or abundance. This will generate data to support future studies involving a larger number of patients and to help predict the likelihood of suture dehiscence associated with postoperative evolution in patients undergoing colorectal anastomosis surgery.

DESCRIPTION OF THE STUDY

This study involves the collection of stool samples at four key time points for analysis:

1. Before the intestinal preparation recommended prior to surgery,
2. On the day of surgery,
3. 5–7 days after surgery, and
4. At the one-month postoperative follow-up visit.

You will be provided with three GutAlive® kits. On the day of surgery, your surgeon will collect the sample. GutAlive® is a stool collection device (container) that ensures sample stability while preserving its original composition and diversity. The sample should be kept at room temperature and sent or delivered to the New Therapies Laboratory at Fundación Jiménez Díaz within a maximum of 48 hours after collection. To do so, you should contact phone number 915 504 800, extension 3398 (Dr. Mariano García Arranz). GutAlive® is currently the stool collection system included in the protocols of the Spanish Association of Gastroenterology for Fecal Microbiota Transplant procedures.

Once the samples are received at the laboratory, they will be stored inside the GutAlive® device, frozen at −80°C, until processing. At this point, the samples will be coded using four associated digits (PnºMnº, where P = patient and M = sample collected). For example, P1-M1 would be patient 1, first sample; P1-M2 would be patient 1, second sample, and so on. This coding will be labeled on the GutAlive® devices using labels resistant to ultra-freezing.

Subsequently, the collected samples will be transferred to the company Microviable Therapeutics, where high-resolution microbiota analysis will be performed using next-generation sequencing and advanced bioinformatic (metagenomic) analysis. Likewise, periodic meetings will be held between the New Therapies Laboratory of IIS-FJD and the clinical team to collect, in an anonymized manner, data on clinical outcomes associated with your treatment during the first 30 days after surgery.

Finally, once the coded metagenomic results have been obtained, the research team will compare the clinical evolution of patients, in a coded manner, with these results and subsequently extract microbiota patterns from the study population. To manage large

volumes of data, we use a cloud computing platform with unlimited processing capacity that also guarantees data security.

STUDY ACTIVITIES

Once you have signed the informed consent, your physician will carry out the usual tests proposed for your treatment outside the study. During this visit, you may ask the specialist any questions and you will be asked to authorize the selected procedure.

If you meet the criteria to participate in the study and give your approval, you will be included and provided with a GutAlive® device to collect your stool at home, always before starting the intestinal preparation recommended prior to surgery. Once collected, the sample should be kept at room temperature according to the GutAlive® system instructions and sent to the hospital within a maximum of 48 hours after bowel movement. These samples will be coded and stored by members of the New Therapies Laboratory.

On the day of surgery, the responsible surgeon, who is also a member of the research team, will collect any fecal content present in the colon during the procedure and transfer it to the New Therapies Laboratory for coding and storage. Upon hospital discharge or at the first follow-up visit (between days 5–7), you will be asked to collect another sample. Finally, one month after surgery, you will be asked for an additional sample. For this purpose, you will be provided with two additional GutAlive® devices upon discharge.

It is expected that the proposed sample collection will not extend your hospital stay or alter the usual tests or treatment process. Otherwise, you will continue with treatment and follow-up as determined by your physician. Please consult the study physician before taking any prescription or over-the-counter medication until the last stool sample associated with the study has been collected.

You should be aware that you may be excluded from the study if the investigators consider it appropriate, either for safety reasons (e.g., your condition does not respond adequately, any adverse event that may alter the intestinal microbiota, etc.) or because they consider that you are not complying with established procedures. In any case, you will receive an appropriate explanation for the reason for your withdrawal from the study.

By signing the attached consent form, you agree to comply with the study procedures described above.

WHAT WILL HAPPEN IF YOU DECIDE NOT TO PARTICIPATE IN THIS STUDY?

Your participation in this study is completely voluntary. You may refuse to participate or withdraw from the study at any time. This will not affect in any way the care you receive or your relationship with your physician. In addition, the study physician may decide to discontinue your participation (for example, if they believe it is better for you not to participate or if you fail to comply with necessary procedures). If this occurs, you will receive an appropriate explanation.

BIOLOGICAL SAMPLES / GENETIC ANALYSES

Possibility of unexpected findings

When analyzing your samples, information with implications for your health may be obtained, including unexpected findings, which you have the right to know if you so wish. You may indicate your preference at the end of this document. If you decide to be informed and the information also has implications for your biological relatives, you are advised to inform them personally. In any case, you should be aware that, as required by regulations, if the information is deemed by the responsible physician to be necessary to avoid serious harm to your health or that of your biological relatives, a close family member or representative will be informed, after consultation with the hospital's Healthcare Ethics Committee (CEAS).

Fate of the sample after use in this research project

Once the research is completed, surplus samples may remain. In this case, Microviable will handle the coded samples according to your authorization. You are offered the following options:

- Destruction of the surplus sample.
- Storage for future research use, in accordance with applicable legislation:

o FJD Biobank, Registration No. B.0000647 (if deposited in a biobank, the scope of use will be defined by what you have accepted in the informed consent document for transfer to the biobank, including the clause for donation of research surplus to the FJD Biobank).

BENEFITS AND RISKS OF PARTICIPATION

You are unlikely to benefit directly from participating in this study. However, we hope that the results will contribute to a better understanding of the effect of the microbiota on anastomotic dehiscence. Your participation does not involve any risk, as there are no extraordinary clinical tests other than stool collection. You will be informed if any relevant information arises during the study that could affect your continued participation.

All procedures related to this study are non-invasive and consist solely of four stool collections over the one-month study period.

ECONOMIC ASPECTS

This is a non-profit study that does not have direct funding; however, an agreement has been reached with Microviable to process the samples based on scientific interest. Neither the investigators nor the center receive any financial compensation for participating. Your participation will not incur any costs, nor will you receive financial compensation.

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CONFIDENTIALITY AND DATA PROTECTION

Data Controller

Both the center where the clinical research is conducted and the Sponsor are responsible for processing your personal data, each fulfilling their respective obligations under data protection regulations.

The center where the research is conducted processes the data obtained, including clinical history data when applicable, for the purpose of conducting the research. For any issues related to data processing, you may contact our DPO at DPO@fjd.es.

The Sponsor is also responsible for processing the data generated in the research but will only process coded data and therefore will not know your identity. For any data protection queries, you may also contact DPO@fjd.es.

Legal basis for data processing

The legal basis is compliance with legal obligations under biomedical research regulations, based on the general interest in scientific research and the improvement and assurance of quality and safety once informed consent has been given.

Purposes of data processing

- Promotion and quality of biomedical research
- Monitoring and control of research studies
- Inspections and control measures
- Evaluation of participants' health status
- Obligation to report research results
- Reuse of data for related research areas

Coding process

The Sponsor accesses only coded data. Coding is performed by the research team, specifically by Dr. Rocío Olivera Salazar of the IIS-FJD New Therapies Laboratory, by assigning each participant a unique alphanumeric code. The link between the code and participant identity is securely and confidentially retained exclusively by the IIS-FJD research team.

Who has access to your data?

Access may be granted to the Principal Investigator and their team, CROs (if applicable), monitors, auditors, and other service providers involved in the research, all under confidentiality obligations.

Data disclosure

Data may be disclosed to Ethics Committees and Health Authorities as required by law, and in the event of adverse reactions, to health authorities and insurance companies.

Retention

Data will be retained during the study and for up to five years thereafter to comply with regulatory obligations.

Rights

You have the right to access, rectify, delete, or restrict processing of your data, subject to legal limitations. To exercise these rights, contact the Principal Investigator with the reference “data protection rights,” providing identification and specifying the right you wish to exercise. You may also file a complaint with the competent supervisory authority.

PUBLICATION OF RESULTS

The results of this study will be made public through channels accepted by the scientific community, always maintaining participant confidentiality. No identifying data will be published.

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WRITTEN INFORMED CONSENT FOR THE PATIENT

PROJECT TITLE: Analysis of the composition of the intestinal microbiota and its evolution in post–intestinal anastomosis patients

PROJECT CODE: MIDESIN

PRINCIPAL INVESTIGATOR: Dr. Mariano García Arranz, New Therapies Laboratory / Surgery. Fundación Jiménez Díaz Health Research Institute.

Ethical committee approved Code: PIC165-25_FJD (2025, 19th September)

I, ……..,

declare that:

- Dr. …… has informed me about this study
- I am of legal age
- I have read the information sheet provided to me
- I have been able to ask questions about the study
- I have received satisfactory answers
- I have received sufficient information
- I understand that my participation is voluntary
- I understand that I may withdraw from the study at any time, without explanation and without affecting my medical care
- I freely consent to participate in the study
- I understand that analysis of my samples may reveal health-related information, including unexpected findings.

o I wish to be informed ____ I do not wish to be informed ____

- If surplus samples remain, I acknowledge having been informed of the available options:

o I request destruction of the surplus sample ____

o I request storage in an authorized biobank for future research use under the conditions explained

o Transfer to the FJD Biobank

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In Madrid, on …… of ……., 20……

Name and Signature of the Participant

Date

Name and Signature of the Investigator

Date