Romania-Republic of Moldova
ENI-CROSS BORDER COOPERATION

RESEARCH REPORT

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A project implemented by
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i. Project identification data	
Title of the Action:	Changes in human colonic microbiome in antibiotic generated stress
Project acromym:	COLONSTRESS
Lead Beneficiary:	PMSI Institute of Oncology, Republic of Moldova
EMS - ENI:	2 SOFT/1.2/105
Priority:	1.2 Promotion and support for research and innovation
Total implementation period:	24 months
Start Date:	10.10.2020
ii. Information concerning the cross-border partnership	
Beneficiary no.1:	Regional Institute of Oncology Iasi
iii. Work plan by group of activities	
GA 3: Activity 3.9	Evaluation of changes induced by antibiotics and reporting the effect of various antibiotic regimens on microbiome balance
Type of deliverable:	Report

CONTENT

BACKGROUND

Cross-border cooperation between the PMSI Institute of Oncology and the Iasi Regional Institute of Oncology focused on comparing side effects and opportunistic infections related to antibiotic use, with evaluation of antibiotic therapy protocols influencing the colonic microbiome using paired samples from the same individuals, including the development of two research and higher molecular diagnostic laboratories in oncology capable of further training staff in molecular biology.

The results of data and information from the research project "Changes in the human colonic microbiome in antibiotic generated stress" demonstrate the impact of antibiotic abuse on the colonic microbiome. These data will be used to alert hospital managers and public authorities about the implementation of antibiotic use restrictions.

As oncology medical institutions in the cross-border cooperation region, we have focused our oncology care with access to similar patient populations and statistical data demonstrating different habits in prophylactic and therapeutic use of antibiotics in patients.

While Iasi Regional Institute of Oncology uses a standard regimen based on a single dose of intravenous second-generation cephalosporin preoperatively, PMSI Institute of Oncology has adopted a liberal policy about antibiotic diversity and long periods of use in non-prophylactic regimens, caused by:

- Lack of sterile air in operating rooms;
- Traditional concepts of antibiotic prophylaxis and antibiotic therapy in colorectal surgery, considered contaminated (infected) from the start;
- Lack of a unified institutional protocol on antibiotic prophylaxis and antibiotic therapy.

The broader antibiotic spectrum and longer period of use, the greater is impact of the antibiotic on the human microbiome. Thus, we aimed to compare side effects and opportunistic infections between healthcare institutions (partners), how antibiotic use is changing and to promote better, including cost-effective, treatment. We also aimed to evaluate antibiotic therapy protocols that influence the colonic microbiome, using paired samples from the same individuals, in the same population - a cohort of approximately 400 patients from whom stool samples were collected and aimed to assess the capacity and compare the ability of the microbiome to self-regulate. Microbiome assessment was performed by DNA sequencing, of the 16s RNA gene (V2, V3, V4 and V8 variable region) using the next generation sequencing method. Based on the new technology, we aimed to produce a cross-border cooperation of excellence group for parallel DNA sequencing in two centers. Both medical institutions (partners) used DNA sequencing equipment, so we increase the level and quality of expertise. As a result - creating the common platform for synergistic work and compatible protocols.

The aim of the project *was to compare differences between the microbiome of two populations that are exposed to different antibiotic protocols to support the development of a centre of excellence in molecular biology that addresses a different geographical region, thereby extending the diagnostic value of particular techniques to other development areas.*

The overall objective of the project is to create a common network of cross-border cooperation (CBC) in molecular research, focused on establishing the impact of antibiotic treatment on the human colonic microbiome, to create a measurable evidence base of antibiotic-induced changes and to promote a new restrictive protocol for antibiotic use as a way to reduce costs and limit the development of multidrug-resistant germs.

The project is based on two main components of actions: comparative analysis of colon microbiome changes after different perioperative antibiotic regimens in two similar study groups, and

the creation of a joint centre of excellence capable of transferring molecular research expertise and generating new research approaches to stimulate economic development and innovation.

This final research report of the project " Changes in the human colonic microbiome in antibiotic generated stress " improves the condition of the innovation framework for cross-border human microbiome research actions.

GENERAL ISSUES ADRESSED BY IRO IASI

The gut microbiota is essential for human health and has a significant impact on a variety of biological processes, including inflammation and pathogen resistance. Antibiotic use has a significant impact on bacterial diversity, can enhance antibiotic resistance, and disrupts the balance between bacterial species. The key to understanding post-antibiotic impacts on the gut microbiome is the use of a good technique for isolating microbial DNA and the careful assessment of experimental sequencing artifacts. We present the bacterial community dynamics of 200 surgical oncology patients before and after intravenous administration of cefuroxime, an antibiotic commonly used in surgical antibioprophylaxis that has been shown to be effective against both gram-positive and gram-negative bacteria. In this investigation, we used a high-throughput sequencing technique that targets the V3-V4 region of the 16S rRNA gene to examine patient fecal samples collected via rectal examination before and after cefuroxime medication.

The initial obstacle in implementing the study design was extracting an appropriate amount of DNA from the microbiota samples, which required the use of both mechanical and chemical lysis. The richness and makeup of the gut microbiota differed significantly between the two groups; however, the majority of the variations were determined by additional perioperative treatments rather than antibioprophylaxis. Intestinal microbiota composition did not differ significantly one week after cefuroxime treatment when compared to pre-treatment conditions in individuals without mechanical bowel preparation, however there was some loss in taxonomic variety. Cefuroxime, when used in conjunction with other perioperative therapies, does not increase long-term dysbiosis in postoperative patients.

The human colon microbiome is made up of intricate bacterial communities, fungi, archaea, viruses, and eukaryotic parasites, with a structure that is difficult to measure and quantify [1,2]. The gut microbiota has a significant impact on critical human processes such as digestion, metabolism, and inflammation through modifying the host's immunological, endocrine, and neurological pathways. The human gastrointestinal system is home to over 1000 endemic bacterial species, which play an important role in determining the host's health or illness status, as well as maintaining microbial-host balance. The colonic mucosa, as a natural barrier of the human body, constantly interacts with this bacterial population, influencing and effecting the equilibrium of a diverse variety of bacterial species [3].

Despite differences in richness and complexity across individuals and across different locations of the gut [4] the microbiome ensures a certain level of resilience to exogenous disruptions. Changes in food and the consumption of substances that can serve as antibiotics target specific bacterial populations, resulting in dysbiotic changes that are mirrored in the overall distribution of bacterial species. Changes in food habits have been shown to produce a favorable environment for opportunistic microorganisms to proliferate and become prevalent [5,6]. Clostridium difficile proliferation and infection, which can be called a new epidemic caused by antibiotic overuse, is a well-known example of a catastrophic result of disturbed microbiome balance. Short-term antibiotic therapy may have long-term dysbiotic effects on the gut microbiota, which might aid in illness progression and aggravate it [7]. Dysbiosis occurs when gut bacterial equilibrium is interrupted and has been associated to a variety of diseases such as type 2

diabetes, obesity, inflammatory bowel disease, asthma, rheumatic disorders, neurodegenerative diseases, and colorectal cancer. The dysbiotic period, which occurs after antibiotic medication, provides a window of opportunity for disease-causing bacteria to infiltrate the human intestine. The dysbiosis caused by antibiotic treatment, which is expected to result in a loss of stability in the species composition of the gut microbiota and perhaps the extinction of distinct species, could be the cause of variation in antibiotic resistance's effects [8].

Advances in high-throughput sequencing technologies have tremendously aided research into the function of the microbiome in health and illness. Over the last ten years, high-throughput DNA sequencing has dramatically expanded the genomic composition profiling of microbial communities, resulting in numerous discoveries for taxonomic, phylogenetic, and profiling elements of the gut microbiome. Microbial profiling by 16S ribosomal RNA (rRNA) gene sequencing is one of the most popular techniques for examining bacterial phylogeny and taxonomy, even though complex metagenome assembly strategies from shotgun sequencing data are the most accurate in microbiome profiling and novel species discovery and characterization. The small ribosomal subunit (16S rRNA) gene is the most well-established genetic marker used for bacterial identification and classification since it contains both highly conserved and hypervariable areas. The 341F/785R pair corresponding to the V3-V4 hypervariable regions of the 16S rRNA gene, as defined by Klindworth et al. [9], is one of the most widely used primer sets for the investigation of bacterial diversity in various contexts. Another difficult task is to effectively lyse mixed communities of microbial cells without causing genome damage [10-12]. It is especially important to develop a sensitive and repeatable DNA extraction method that enables isolation of microbial DNA of sufficient quantity and purity from all the existing microbial species in order to study the natural microbial community using high-resolution molecular approaches, such as Next Generation Sequencing (NGS).

Preoperative antibiotics should be used routinely in surgical procedures due to the numerous reports on surgical site infections (SSI) as postoperative complications throughout the past few decades. Cephalosporin became the most often used medication for postoperative prophylaxis in general surgeries after proving effective in multiple clinical trials [11-16]. Second-generation cephalosporin cefuroxime, which can be used in conjunction with other antibiotics, if necessary, is effective against both gram-positive and gram-negative bacteria. It is also the most stable -lactam antibiotic used to lower the risk of post-operative surgical site infections, sepsis, or abscesses, making it a safe and economical medication [17-19].

The influence of the bowel preparation method itself on gut microbiota composition is still being debated, with publications frequently failing to reach consensus for a variety of reasons, including a lack of analytical depth. A recent study found minor changes in gut microbiome composition in non-surgical patients undergoing BP, but a significant impact of BP combined with oral antibiotics in surgery patients' gut microbiome, with variations persisting in the early postoperative period and later repopulation to baseline.

High-throughput sequencing of the V3-V4 region of the 16S rRNA gene in patient feces samples was used to compare changes in gut microbiome composition before (M) and after (T) perioperative antibiotic therapy in a group of 200 surgical oncological patients. Given the possibility that even short-term antibiotic treatment may result in long-term dysbiotic conditions, we must recognize the importance of a proper, gut-friendly preoperative empiric antibiotic program.

CHAPTER I THE CHANGES INDUCED IN THE COLON MICROBIOME INSIDE EACH OF THE GROUPS (STANDARDIZED PROPHYLAXIS AND LIBERAL USE) WITH RESPECT TO THE TIMEFRAMES DESCRIBED

THE IMPACT OF THE COLON MICROBIOME ON HEALTH

Surgical infections are an important public health problem, ranking according to statistical data from different countries 2-3rd among healthcare-associated diseases, reaching a rate of 15-20% in intra-abdominal operations. This increases the time spent in intensive care units, the risk of readmission and postoperative mortality. To combat postoperative infections antibiotic prophylaxis is crucial, but its negative effects on the colon microbiome and host immunity have been shown to be of greatest importance. Several studies have recognised the adverse consequences of antibiotic use on the colon microbiome in adults and neonates, causing colon dysbiosis. Such changes can create a favourable environment for the evolution of opportunistic bacteria, which may subsequently become pathogenic. An example of a dramatic consequence of an abnormal microbiome balance is the proliferation and infection of Clostridium difficile, a new epidemy generated by antibiotic abuse

In general, the microbiome, also called the microbiota, is the collection of commensal, symbiotic, or pathogenic microorganisms that inhabit an ecosystem or habitat (e.g. soil, water, milk, plants, animals, humans). The micro-organisms that make up a microbiome are bacteria, archaea, unicellular algae, protozoa, fungi and viruses. The term "microflora" (= all micro-organisms of a plant nature, e.g. algae) is incorrectly used instead of microbiota to refer to all micro-organisms in a given environment. The term microbiome, which is synonymous with microbiota, also has another meaning: the collective genome of the microorganisms that populate an ecosystem.

Recent scientific research lays the groundwork for a new approach in treatement of various pathologies, bringing to light the interdependent relationship between colon health and antibiotic use. Called the second brain, the colon microbiome has a unique fingerprint for each individual, and its content in terms of number and type of microorganisms dictates the body's well-being. New clinical findings highlight the role the colon microbiome plays in restoring homeostasis.

A 'healthy' colon microbiome is highly diverse; any disturbance can lead to dysbiosis, a critical condition of imbalance between commensal and pathogenic microbes. The human gastrointestinal tract carries a large number of microorganisms associated with complex metabolic processes and interactions, and the action of various stressors, in particular the action of antibiotics, is not well studied, and studies dedicated to this issue are still highly topical

It is well known that antibiotics can cause changes in the host's indigenous microbiome by selecting resistant bacteria that may emerge as opportunistic pathogens. In addition, low antibiotic intake or sub-therapeutic antibiotic treatment (STAT) from food and environment have also been associated with colon dysbiosis. Colon dysbiosis promotes negative effects in many host systems and functions, thus research has focused on the relationship between antibiotics and the colon microbiome. The evidence accumulated by researchers to date has highlighted the contribution of antibiotics to disturbances in the colon microbiome. Although morbidity and mortality due to infectious diseases have been remarkably reduced, antibiotic treatment has been implicated in disruptions of the colon microbiome.

Until recently, the use of classical microbiological techniques limited the amount of information about the human microbiome, but the introduction of new molecular research methods such as next-generation sequencing (NGS) and methodologies such as 16S ribosomal RNA (rRNA) gene sequencing and shotgun metagenomic sequencing have revolutionised scientists' knowledge of these microorganisms. Advanced DNA sequencing technologies have created a new field of research, called

metagenomics, which allows the examination of genomes of microbial communities taken from natural environments without the need for culture. The genes of microorganisms are characterised by their abundance, diversity and characteristics and are collectively known as the human microbiome, which has recently gained an important role in health and disease.

The role of antibiotics as a stressor on the human microbiome is not yet fully understood. New techniques, state-of-the-art research methods and more sophisticated, randomised control studies are needed to elucidate the stress-microbiome relationship and examine the potential challenges of antibiotic use in combating postoperative complications.

A number of antibiotic prophylaxis guidelines are currently being drawn up by the relevant scientific societies, institutions, universities and reference hospitals, but there is a lack of unification and unequivocal acceptance of these guidelines, and the theoretical and practical research on the problems mentioned is still important and topical. Also of scientific and practical interest are all the studies relating to current evidence on the intestinal microbiome and its changes in relation to antibiotics, their inappropriate use and methods of preventing dysbiosis.

THE COLONIC MICROBIOME - METHODS AND MATERIALS USED

The research is based on patients with surgical oncological pathology admitted for surgery in the partner hospitals PMSI Institute of Oncology (PMSI IO), Chisinau and IRO Iasi - 400 patients, 200 in IRO Iasi and 200 in PMSI IO Chisinau. To validate the DNA sequencing method, samples from 50 patients were double tested, at IRO Iasi and PMSI Institute of Oncology.

METHODS USED IN PMSI INSTITUTE OF ONCOLOGY

DNA Extraction. DNA was extracted from the gut bacteria (filter paper) using a PureLink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific), with the addition of an initial bead beating step. DNA was also extracted using QIAamp DNA Microbiome Kit (Qiagen). Each set of samples was isolated together with a negative control (PBS). The majority of samples were isolated using PureLink Microbiome DNA Purification Kit

Samples were isolated 15 in common with the inclusion of a negative control (The 16th sample). The smeared portions of filter paper were cut in thin strips with sterile scissors in order to avoid contamination. The strips from each sample were added to 1.5 mL sterile Eppendorf tubes. The steps were followed according to the manufacturer's protocol with one modification - at the lysis step, additional proteinase K was added and were incubated overnight at 56°C.

For preparing the lysate, was added to the Bead Tube 800 µL of S1—Lysis Buffer, paper filter, 100 µL of S2—Lysis Enhancer and the mix was incubated at 65°C for 10 minutes. After incubation to the Bead Tube was added 40 µL of proteinase K and left overnight at 56°C. After overnight incubation, the samples were bead beat for 10 minutes at maximum speed on the vortex mixer (horizontally) and then centrifuged at 14,000 × g for 1 minute. 500 µL of the supernatant were transferred to a clean microcentrifuge tube, avoiding the bead pellet and any debris.

To the tube with supernatant were added 900 µL of S4—Binding Buffer and vortexed, after that, 700 µL of the sample mixture were loaded onto a spin column-tube assembly, and centrifugated at 14,000 × g for 1 minute. The flow-through, were discarded and centrifugated one more time.

For washing and elution of the DNA solution the spin column was placed in a clean collection tube in which was added 500 µL of S5—Wash Buffer then centrifugated 2 times at 14,000 × g for 1 minute and for 30 seconds respectively. The spin column was placed in a clean tube and added 50 µL of S6—Elution

Buffer then incubated at room temperature for 1 minute. The spin column-tube assembly were centrifugated at 14,000 × g for 1 minute.

The metagenomic DNA obtained was quantified using the Qubit 3.0 fluorometer in combination with the Qubit dsDNA HS assay kit (Life Technologies, ThermoFisher Scientific).

Library preparation and NGS sequencing. The metagenomic library was constructed using Ion 16S Metagenomics Kit. Three primer sets, one targeting the V2 region, one the V4 region and one primer pairs targeting the V8 region of the 16S rRNA gene. Multiplex PCR assays were done for amplification of this 16S rRNA hypervariable regions (Fig.1) in one single tube.

Figure no.1. Primer set used for 16S gene amplification, hypervariable regions and amplicon length.

16 libraries were constructed simultaneously, including a positive control and a negative control per PCR run. In each tube was added 15 µL of 2X Environmental Master Mix, 10 µL of 16S Primer Set (10X), 2 µL of DNA (sample or diluted E. coli DNA control) and 10 µL of Negative Control (water). All the tubes were incubated in the thermal cycler with the following program: denaturation at 95°C, followed by 22 cycles at 95°C, 58°C and 72°C for 30 s, 30 s and 20s respectively and a final elongation step of 7 min at 72°C. The amplification products were purified with 1.8X Agencourt AMPure XP and 70% ethanol and eluted with 15 µL nuclease free water. The amplicons were quantified using Qubit 3.0 fluorometer in combination with the Qubit dsDNA HS assay kit.

Each 60 ng of the purified amplicons were end repaired in a 1.5-mL Eppendorf LoBind tube with 20 µL of 5X End Repair Buffer and 1 µL of End Repair Enzyme and then incubated at room temperature for 20 minutes. The DNA fragments were purified with 1.8X Agencourt AMPure XP and 70% ethanol and eluted 25 µL of Low TE.

Then purified amplicons were ligated with barcodes and nick-repaired. For this, in each sample were mixed the following components: µL of 10X Ligase Buffer, 2 µL of Ion P1 Adapter, 2 µL of Ion Xpress Barcode (1 to 16), 2 µL of dNTP Mix, 49 µL of Nuclease-free Water, 2 µL of DNA Ligase and 8 µL of Nick Repair Polymerase. The tube was placed in a thermal cycler and run with the following program: 25°C for 15 minutes and 72°C for 5 minutes. The adapter ligated and nick repaired DNA were purified with 1.4X Agencourt AMPure XP and 70% ethanol and eluted 20 µL of Low TE.

Obtained metagenomic library was quantified using 7500 RT-PCR System in combination with Ion Universal Library Quantitation Kit (Thermo Scientific, Thermo Fisher Scientific).

16S rRNA targeted metagenomics sequencing was done using Ion Torrent PGM machine (Thermo Fisher Scientific, USA) as per manufactures' instructions. With each sequencing run negative control (PBS) and positive control (E. coli), was also included.

NGS of the amplified library was done after a round of emulsion PCR using Ion PGM HiQ OT2 Kit (Life technologies, USA) and sequencing was done using Ion PGM HiQ sequencing reagents on a 318 chip. The 318 Chip can generate >1000 Bases (in Mb). Each run has included 16 barcoded sample including Positive Control and Negative Control.

Sequence and bioinformatics analysis

Sequence analysis. Post sequencing base calling and adaptor trimming was performed using the computer program Torrent Suite (Life technologies, USA). The output reads were aligned and mapped using Ion Reporter software v5.18 (USA) and Metagenomics 16S w1.1 algorithm with following parameters:

Read Length Filter: ≥ 150 bp
Minimum Alignment Coverage: ≥ 90 %
Read Abundance Filter: ≥ 10 (Number of unique reads needed for that read to be a valid)
Genus Cutoff**:** 97.0 % (similarity)
Species Cutoff**:** 99.0% (similarity)
Slash ID Reporting Percentage**:** ≥ 0.2% (multiple taxonomy assessments - reads with a conflicting assignment)
Database**:** Curated MicroSEQ(R) 16S Reference Library v2013.1; Curated Greengenes v13.5

Bioinformatic analysis.

DADA2 - DADA2 implements a novel algorithm that models the errors introduced during amplicon sequencing, and uses that error model to infer the true sample composition. DADA2 replaces the traditional "OTU-picking" step in amplicon sequencing workflows, producing instead higher-resolution tables of amplicon sequence variants (ASVs).

Alpha-beta diversity on Ion Report - The Metagenomics 16S analysis workflow in Ion Reporter Software includes alpha diversity calculations and beta diversity calculations. Alpha diversity results describe the diversity in a single sample at the species, genus, and family levels. Beta diversity results describe the diversity between multiple samples at the species, genus, and family levels. Alpha-beta diversity calculations are based on the information that is gathered from the consensus files that are generated by the Metagenomics 16S analysis workflow. Read counts per sample for species, genus, and family are collected. Operational taxonomic unit (OTU) tables are generated.

PCOA Plot/PC Matrix – generate several plot types, including: Eucledian, Manhattan, Chi-Square and Bray Curtis PCOA Plots and PC Matrices.

Clustering methods - microbiota analysis by identifying subgroups for patients' stratification. In our study we used R pheatmap clustering algorithm.

Mean Relative Abundance - Relative Abundance tells us what is the ratio (proportion)/how many percentages of the microbiome are made up of a specific organism, e.g., if E. coli makes up 0.01 (1%) or 0.1(10%) of the total amount of bacteria detected in a sample.

Sample Processing and 16S rRNA Sequencing

DNA extraction

To avoid cross-contamination, the DNA extraction technique was optimized and done in batches of 12 samples. DNA isolation was carried out using the NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) in accordance with the manufacturer's instructions, with the following additional procedures. To avoid contamination, the smeared pieces of filter paper were divided into small strips with sterile scissors. Each sample's strips were placed in 1.5 mL sterile Eppendorf tubes.

NucleoSpin® Soil is suitable for processing 250–500 mg of sample material. However, do not fill the MN Bead Tube Type A higher than the 1 mL mark (including the ceramic beads) to ensure sufficient head space for an efficient mechanical disruption. A thorough mechanical lysis step is essential to break up the soil crumbs, to free the cells within the soil, and to break up cells and spores. Ceramic beads have proven to be most effective in combination with a bead mill, a FastPrep®-24 instrument (MP Biomedicals, set instrument to 5 m/s for 30 s), or an MN Bead Tube Holder, see ordering information. In most cases, however, this kind of equipment is not necessary. The same result can be achieved by taping the lysis tubes horizontally to a standard vortexer.

The lysis time should be as short as necessary to avoid shearing of DNA and to minimize the release of humic acids. Depending on the sample, however, it might be advantageous to increase the lysis time to 10, 20, or 30 min.

Homogenization and cell disruption should be performed at room temperature (18–25 °C) to avoid SDS precipitation in the lysis buffers. Overheating the sample, for example by prolonged bead beating in a bead mill or the FastPrep®-24 instrument, should be avoided to minimize liberation of humic acids.

Quality and Quantification of Extracted DNA

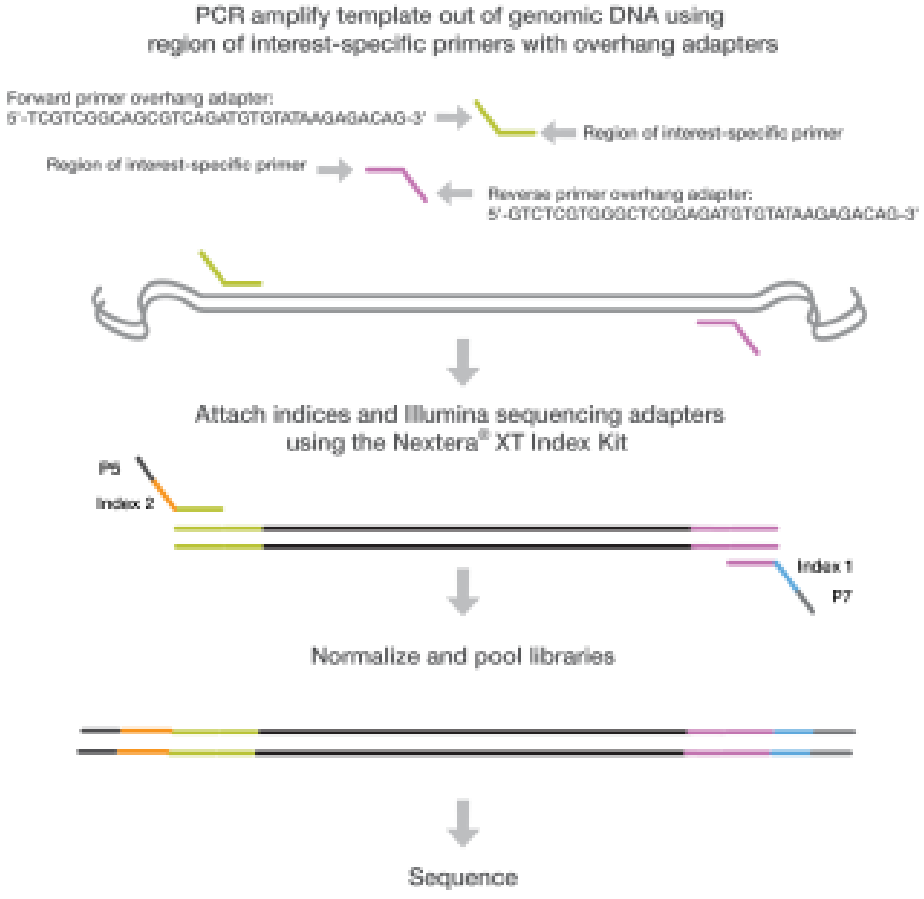
NanoDrop was used to spectrophotometrically assess the content of the extracted DNA (absorbance at 260 nm) and its purity (absorb-ance ratios 260/230 and 260/280). (Thermo Fisher Scientific, Massachusetts, USA). A lack of protein contamination was indicated by ratios between 1.8 and 2.0. To identify contamination with organic chemicals, phenols, and carbohydrates, the ratio of absorbance at 260 and 230 nm was utilized. Gel electrophoresis on a 2% agarose gel (w/v) stained with ethidium bromide and conducted in 1x TAE buffer at 180 V was used to measure the consistency and size of the DNA samples.

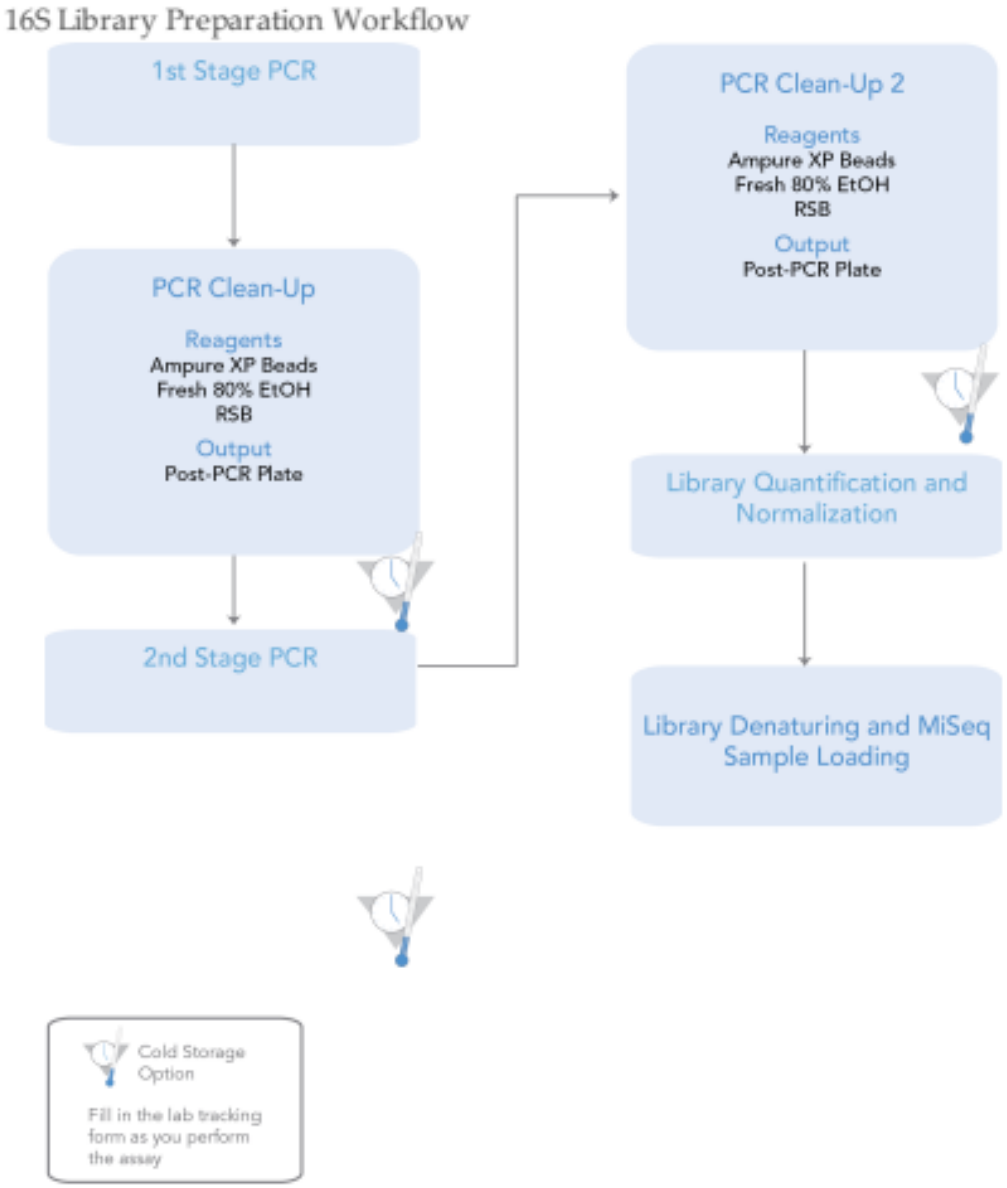
16S metagenomic sequencing / Targeted Metagenomics Sequencing

Metagenomic studies are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions of 16S rRNA are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations. Which 16S rRNA region to sequence is an area of debate, and your region of interest might vary depending on things such as experimental objectives, design, and sample type. This protocol describes a method for preparing samples for sequencing the variable V3 and V4 regions of the 16S rRNA gene. This protocol can also be used for sequencing other regions with different region-specific primers. This protocol combined with a benchtop sequencing system, on-board primary analysis, and secondary analysis using MiSeq Reporter or BaseSpace, provides a comprehensive workflow for 16S rRNA amplicon sequencing.

Workflow Summary:

1. Order amplicon primers–The protocol includes the primer pair sequences for the V3 and V4 region that create a single amplicon of approximately ~460 bp. The protocol also includes overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters.
2. Prepare library–The protocol describes the steps to amplify the V3 and V4 region and using a limited cycle PCR, add Illumina sequencing adapters and dual-index barcodes to the amplicon target. Using the full complement of Nextera XT indices, up to 96 libraries can be pooled together for sequencing.
3. Sequence on MiSeq–Using paired 300-bp reads, and MiSeq v3 reagents, the ends of each read are overlapped to generate high-quality, full-length reads of the V3 and V4 region in a single 65-hour run. The MiSeq run output is approximately > 20 million reads and, assuming 96 indexed samples, can generate > 100,000 reads per sample, commonly recognized as sufficient for metagenomic surveys.
4. Analyze on MSR or BaseSpace–The Metagenomics workflow is a secondary analysis option built into the MiSeq Reporter (on-system software) or available on BaseSpace (cloud-based software). The Metagenomics Workflow performs a taxonomic classification using the Greengenes database showing genus or species level classification in a graphical format. This protocol can be used to sequence alternative regions of the 16S rRNA gene and for other targeted amplicon sequences of interest.





A 16S metagenomic sequencing library was created for each sample in accordance with Illumina's guidelines in order to identify the bacterial species present. Briefly, we used the appropriate primer pair sequences with overhang adapters listed below in a first PCR to target the V3-V4 region of the 16S rRNA gene: F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' We used 2.5 µl of microbial genomic DNA and 5 µl of each forward and reverse 1 M primers. The obtained sequences were then cleaned of free primers and primer dimer species using Agencourt AMPure XP magnetic beads from Beckman Coulter in Brea, California. The dual indexes and Illumina sequencing adapters were then attached using a second PCR from 5 µl of the purified PCR amplicons using the Nextera XT Index Kit V2 set A (Illumina, San Diego, USA) with 2X KAPA HiFi HotStart Ready Mix (Roche Holding AG, Basel, Switzerland) and Nextera XT Primers. The final library was cleaned up once again with AMPure XP XP

magnetic beads (Beckman Coulter, Brea, US) prior to quantification, and the DNA concentration of the PCR products was assessed using a Qubit 4 fluorometer and the QubitTM 1X dsDNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). The Illumina MiSeq platform was used to sequence the V3-V4 region of the 16S rRNA gene libraries using a MiSeq Reagent Kit (Illumina, San Diego, USA) and the 300 paired-end (2 300bp (PE300)) sequencing technique.

Bioinformatics and Statistical Analysis

Taxonomic profiling

The dada2 package v 1.22 built in R (version 4.1.2) was used to handle demultiplexed sequences in accordance with the DADA2 pipeline. There were 120 paired samples remaining from each group (M or T). Out of the T group, 36 samples received PEG BP, 46 samples underwent pre-operative enema, and 38 samples underwent no pre-operative colon cleansing (NO group) (PEG group).

The phyloseq software v 1.38 was used to create phyloseq objects, which included ASV tables, taxonomic classifications, and metadata. Using the phangorn software version 2.9.0 and an ASV multiple sequence alignment, a neighbor-joining phylogenetic tree was created.

Alpha and Beta Diversity

Faith's phylogenetic distance was determined using picante v. 1.8.2, whilst non-phylogenetic alpha diversity indices were obtained using phyloseq v. 1.38. The Wilcoxon signed-rank test was used to determine statistical significance.

To determine the importance of compositional dissimilarity, the Bray-Curtis distance matrix and variance-adjusted weighted UniFrac distances were subjected to permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM).

To visualize microbiota similarity and grouping across samples, dimensional reduction by principal coordinates analysis (PCoA) based on the Bray-Curtis dissimilarity matrix and variance adjusted weighted UniFrac distances was carried out; ggplot2 version 3.3.6 was used to create all of the charts.

Differential Abundance Analysis

Using genus-agglomerated phyloseq items, significance in differential abundance between groups was evaluated using four alternative approaches, in accordance with previous guidelines for the selection of differential abundance methods. The microbiomeMarker software v 1.0.2 was used to do the linear discriminant analysis effect size (LEfSe) on raw. To normalize raw data into trimmed mean of M-values (TMM) counts, edgeR v 3.36.0 was utilized. These counts were then used as input for variance modeling at observational level (voom), which was implemented in limma v 3.50.3.

THE SAMPLING PROCEDURE

1. **Pre-screening**: pre-selection of patients who can be enrolled in the study according to inclusion and exclusion criteria.
   - *Inclusion criteria:*

   a. Adult patient(s) admitted to surgical wards, undergoing surgery involving antibiotic prophylaxis, including patients undergoing neoadjuvant treatment (chemotherapy, radiotherapy) after a break of at least 21 days;
   b. Patient who has not undergone mechanical colon preparation in the last 30 days prior to admission.
   c. Patient who has not administered antibiotics in the last 30 days prior to admission.

d. Patient who agrees to participate in the given study (signed informed consent to collect stool samples for processing).

- *Exclusion criteria:*

a. Systemic and/or oral antibiotic therapy within the last 30 days (for infectious pathologies - e.g. urinary tract infections);
b. History of mechanical colon preparation within the last 30 days;
c. Presence of ileostomy at the time of admission or if the operative protocol requires the formation of an ileostomy;
d. Surgical procedures without antibiotic prophylaxis (e.g. breast surgery, plastic surgery);
e. Late resumption of bowel transit (more than 10 days after surgery) or occlusive syndromes.

2. **Informing the patient about the potential participation in the clinical trial, its phases, the timeframe**.

Once identified, the patient receives informed consent for the COLONSTRESS study, explanations of the steps and processes of participation in the trial, the procedures to be performed. The patient has sufficient time for documentation and questions and answers.

3. **Sign informed consent for participation in the COLONSTRESS study**.

The patient knowingly signs the informed consent in 2 copies: one copy goes to the patient and the second copy to the sample package.

4. **Anonymisation of subjects.**

In the research, general and medical information collected about patients was kept secret and identified by assigning anonymized IDFiecărui pacient inclus în studiu i s-a atribuit un ID anonimizat pentru păstrarea datelor confidențiale a pacientului, după următorul model O-NN-ZZLLAA-P, unde:

O = the city where the samples are taken, the usable letters being C (for the centre in Chisinau) or I (for the centre in Iasi)

NN = initials of the patient's first name and surname (if the patient has more than one name/surname, only the first letter of the first name and surname will be used, according to the order in the identity card)

ZZ = patient's date of birth (if the patient is born on a day with only one digit, this will be preceded by the digit 0 - zero)

LL = month of birth of the patient (if the patient is born in a month with a single digit, this will be preceded by the digit 0 - zero)

YY = year of birth of the patient (the last two digits of the year will be used)

P- sample number (PROBE M/PROBE 0/PROBE T)

example: I-PI-17.02.79-M C-PI-17.02.79-M

5. **Collection, marking and storage of samples.**

Three faecal samples were collected as follows:

1. control sample collected at the time of inclusion in the study (SAMPLE M).
2. intraoperative sample collected before anaesthetic induction (PROBE 0)
3. test sample collected at the end of antibiotic therapy (PROBE T).

Samples were collected from the rectum or colostoma (except ileostoma). Fecal material was collected from the rectum or colostoma using a glove and applied to filter paper (minimum 2cm2 area). Beforehand the filter paper must be annotated on the reverse side with the patient ID and the type of sample (M, 0 or T).

Fecal samples taken on filter paper were allowed to dry for a minimum of 30 minutes at room temperature (positioning of samples in areas with strong air currents was avoided). The filter paper was folded in such a way that the sample was positioned on the inside (reverse side). The folded filter paper, with the dried sample, was stored in a sealable plastic bag, specially designed for transport. The envelopes were stored in a dry storage area at normal temperature.

6. **Sealing of the collected samples, completion of the data sheet prepared after collection of 3 samples**.

After the collection of Sample T, the completed data sheet, together with the plastic envelope with the two previously collected samples, is placed in an A6 paper envelope and the 10-box sticker with the patient's ID is applied.

7. **The envelopes with the samples are sent to the laboratory together with the data sheet**.

As the study batches are completed, the envelopes with the collected samples are sent to the laboratory.

STATISTICAL EVALUATION OF LOTS

Scientific evidence shows that an increasing number of diseases are associated with changes in the colon microbiome, and people with disease have significantly different colon microbiota profiles compared to healthy individuals.

A growing body of scientific evidence shows that the colon microbiota plays an extremely important role in the therapeutic responses of cancer patients to chemotherapy, radiotherapy, immunotherapy and toxicity sensitivity. Thus, improving the health and balance of the colon microbiota could substantially optimise cancer treatment.

Many drugs, including antibiotics, can wreak havoc on the microbiome, killing quantities of bacteria that can no longer be replaced, altering the pH of the colon in a way that favours the growth of pathogenic species, or damaging the colon lining and allowing bacteria to enter areas where they should not normally be.

The analysis is based on data and evidence that ensures the highest standard of analytical validity and clinical utility.

THE CHARACTERISTICS OF THE BATCH IN PMSI INSTITUTE OF ONCOLOGY

In order to analyse DNA fragments from the genetic material of the colon microbiome the research team enrolled 200 patients with three samples (M,O,T) from each according to inclusion and exclusion criteria.

Demographic characteristics of the enrolled subjects were performed based on the parameters: sex, mean age and tobacco consumption. Thus, the demographic profile of the subjects represents 58.5% (n=117) males and 41.5% (n=83) females. The distribution of subjects by age group (see Fig.2) was as follows - age group 60-69 years, which represents 46.5% (n=93), followed by subjects aged 70-79 years is 27.0% (n=54) and age group 50-59 years (17.5%; n=35), age group 40-49 years (5.0%; n=10), age group 30-39 years (2.5%; n=5) and age group 80-89 years (1.5%; n=3).

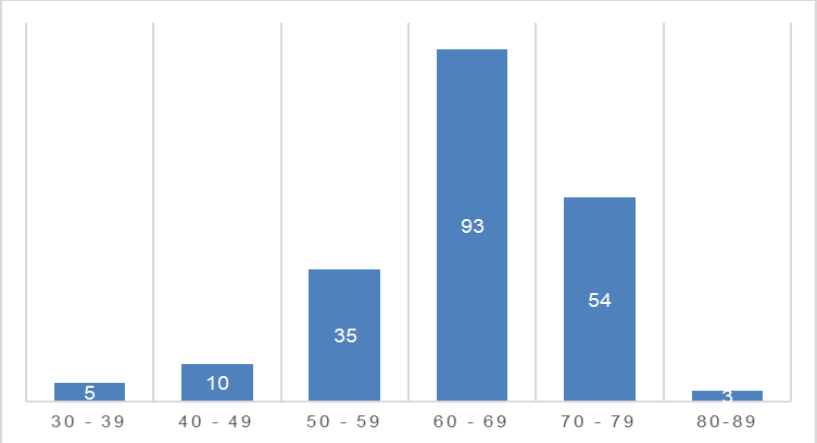


Figure no. 2. Distribution of patients according to age groups

The youngest subject enrolled was 33 years old, the oldest 82 years old, and the average age was 64 years old. (Tab.1).

Table 1. Mean, minimum and maximum age of patients in the study

Average age	64 years
Minimum age	33 years
Maximum age	82 years

Distribution of patients according to tobacco use, out of the total number of 200 patients, 42 are smokers and 158 are non-smokers (Fig. 3).

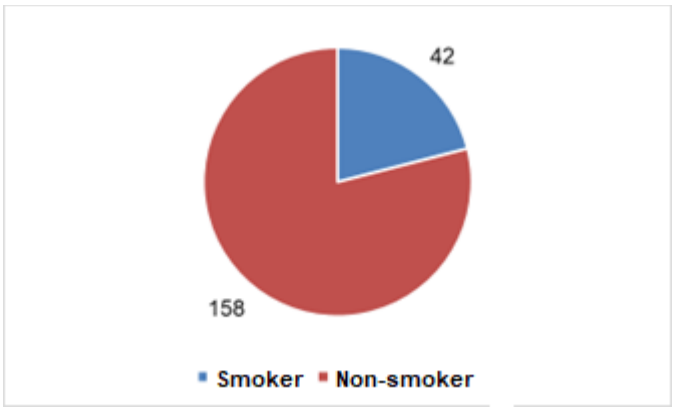


Figure no.3. Distribution according to tobacco consumption.

The study subjects were admitted to the IMSP Oncology Institute for malignant tumours of the colon and rectum for surgical treatment. 82 patients were admitted with the diagnosis of rectal cancer 41.0%, 56 patients with sigmoid colon cancer (28.0%), 17 with ascending colon cancer (8.5%), 11 with descending colon cancer (5.5%), 9 with liver flexure colon cancer (4.5%), 8 with rectosigmoid junction cancer (4.0%), 7 with transverse colon cancer (3.5%), 6 with cecal cancer (3%), 2 with colon flexure

cancer (1.0%) and 1 patient each with ascending colon cancer + sigmoid colon cancer and gastric cancer (0.5%). (Fig. 4)

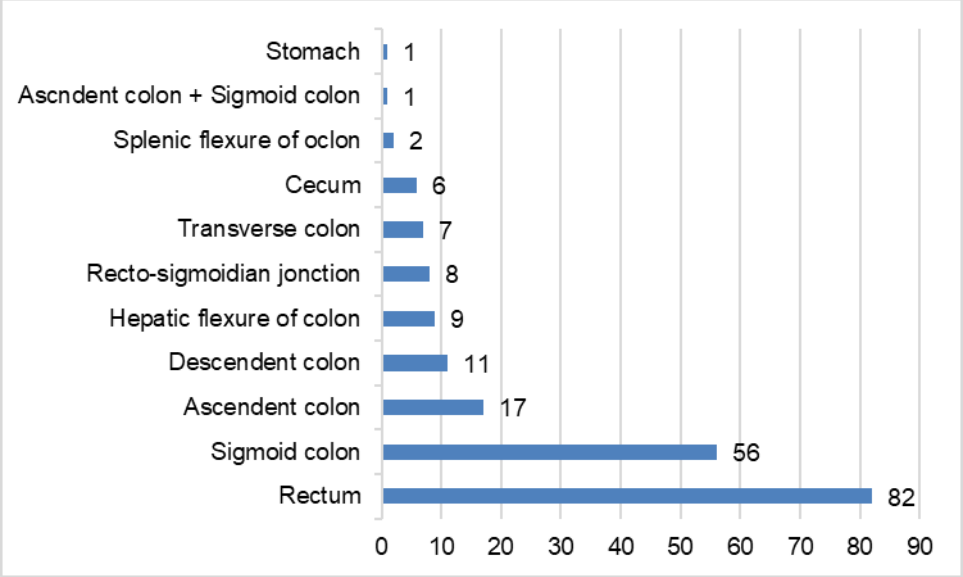


Figure no. 4. Distribution by malignant tumor site.

The analysis data reflects that 79% (n= 158) of patients received voiding enema in combination with laxatives, 15.5% (n=31) received only voiding enema, 5% (n=10) received no preoperative preparation and 0.5% (n=1) received only laxatives (Tab. 2).

Table 2. Pre-operative colon preparation

Type of pre-operative training	abs.	**%**
Laxatives	1	0,5%
Evacuative enema	31	15,5%
Evacuative enema + laxatives	158	79%
Without preparation	10	5,0%

Data analysis was also carried out depending on the surgeries performed. According to table No. 3 the most frequently performed surgery was resection of sigmoid colon – 43 cases (21,5%), followed by anterior rectal resection - 41 operations (20.5%), 37 palliative laparotomy with colostomy (18.5%), 30 right hemicolectomies (15.0%), 16 abdominoperineal resection (8.0%), 10 left hemicolectomies (5.0%), 8 reconstructive operations (4, 0%), 6 exploratory laparotomies (3.0%), 2 laparotomies with enterocolostomy (1.0%), 2 abdominoperineal endoanal pull-through resection (1.0%), and 1 of each: metastasis excision, obstructive rectal resection, right hemicolectomy + sigmoid resection, laparotomy + biopsy, tumor excision (each 0.5%).

Table 3. Profile of surgical interventions performed.

No.	Type of surgery	abs.	%
1	Resection of sigmoid colon	43	21,5%
2	Anterior rectal resection	41	20,5%
3	Laparatomy, colostomy	37	18,5%
4	Right hemicolectomy	30	15,0%
5	Abdominoperineal resection	16	8,0%
6	Left hemicolectomy	10	5,0%
7	Reconstructive surgery	8	4,0%
8	Exploratory laparatomy	6	3,0%
9	Laparatomy, enterocolostomy	2	1,0%
10	Abdominoperineal endoanal pull-through resection	2	1,0%
11	Excision of metastases	1	0,5%
12	Obstructive resection of the rectum	1	0,5%
13	Right hemicolectomy + sigmoid resection	1	0,5%
14	Laparatomy, biopsy	1	0,5%
15	Tumour excision	1	0,5%

Analysis of the batch in terms of antibiotic treatment shows a varied distribution of antibiotics used (Tab. 4).

Table 4. Antibiotics administered

No	Name of Antibiotic	No. of Uses	%
1	Sol. Metronidazol 0,5% - 100 ml	177	88,5%
2	Sol. Cefoperazon 1g + Sulbactam 1g	148	74,0%
3	Sol. Cefazolin 1g	93	46,5%
4	Sol. Cefoperazon 1g	46	23,0%
5	Sol. Ceftriaxon 1g	24	12,0%
6	Sol. Cefotaxim 1g	9	4,5%
7	Sol. Obrocin 500 mg	9	4,5%
8	Sol. Amikacin 500 mg	8	4,0%
9	Sol. Cefuroxim 750 mg	2	1,0%
10	Sol. Imipenem 500 mg + Cilastatina 500 mg	1	0,5%

Sol. Metronidazole 0.5% - 100 ml i/v drip. twice a day was administered to 177 patients, which constitutes 88.5%. Cefoperazone 1 g + Sulbactam 1g i/v twice a day was administered to 148 patients (74%). Sol. Cefazolin 1 g i/v twice a day was administered to 93 patients (46.5%). Sol. Cefoperazon 1g i/v twice a day was administered to 46 patients (23.0%). Sol. Ceftriaxon 1g i/v twice a day was administered to 24 patients (12.0%). Sol. Cefotaxime 1g i/v twice a day was administered to 9 patients (4.5%). Sol. Obrocin 500 mg i/v twice a day was administered to 9 patients (4.5%). Sol. Amikacin 500 mg i/v twice a

day was administered to 8 patients (4.0%). Sol. Cefuroxime 750 mg i/v twice a day was administered to 2 patients (1.0%). Sol. Imipenem 500 mg + Cilastatin 500 mg i/v twice a day was administered to 1 patient (0.5%).

The average duration of antibiotic use (Tab. 5) was as follows: Metronidazole 4.41 days, Cefoperazone + Sulbactam 3.81 days, Cefazolin 5.47 days, Cefoperazone 1.28 days, Ceftriaxon 5 days, Cefotaxime 5.88 days, Obrocin 5.88 days, Amikacin 4.62 days, Cefuroxime 7 days, Imipenem + Cilistatin 10 days. For the total number of patients, the average duration of antibiotic use was 5.34 days.

Table 5. Average duration of antibiotic therapy

No.	Antibiotic	Average no. of days
1	Sol. Metronidazol 0,5% - 100 ml	4,41
2	Sol. Cefoperazon 1g + Sulbactam 1g	3,81
3	Sol. Cefazolin 1g	5,47
4	Sol. Cefoperazon 1g	1,28
5	Sol. Ceftriaxon 1g	5,00
6	Sol. Cefotaxim 1g	5,88
7	Sol. Obrocin 500 mg	5,88
8	Sol. Amikacin 500 mg	4,62
9	Sol. Cefuroxim 750 mg	7,00
10	Sol. Imipenem 500 mg + Cilastatină 500 mg	10,00
	Medium	5,34

In Table No. 6, we can see that there was a gap between the minimum and maximum duration of antibiotic administration, thus for Sol. Metronidazole 0.5% - 100 ml the minimum duration was 1 day and the maximum 9 days; for Sol. Cefoperazone 1g + Sulbactam 1g - minimum 1 day, maximum 12 days; for Sol. Cefazolin 1g minimum 1 day, maximum 10 days; for Sol. Cefoperazon 1g minimum 1 day, maximum 5 days; for Sol. Ceftriaxon 1g minimum 1 day, maximum 8 days; for Sol. Cefotaxime 1g minimum 3 days, maximum 9 days; for Sol. Obrocin 500 mg minimum 5 days, maximum 7 days; for Sol. Amikacin 500 mg minimum 2 days, maximum 8 days; for Sol. Cefuroxim 750 mg minimum and maximum were 7 days and for Sol. Imipenem 500 mg + Cilastatin 500 mg minimum and maximum 10 days (only one patient was administered).

Table 6. Minimum and maximum duration of antibiotic use

No	Antibiotic	Min. days	Max. days
1	Sol. Metronidazol 0,5% - 100 ml	1	9
2	Sol. Cefoperazon 1g + Sulbactam 1g	1	12
3	Sol. Cefazolin 1g	1	10
4	Sol. Cefoperazon 1g	1	5
5	Sol. Ceftriaxon 1g	1	8
6	Sol. Cefotaxim 1g	3	9
7	Sol. Obrocin 500 mg	5	7

8	Sol. Amikacin 500 mg	2	8
9	Sol. Cefuroxim 750 mg	7	7
10	Sol. Imipenem 500 mg + Cilastatină 500 mg	10	10

CHARACTERISTICS OF THE LOT IN IRO IAȘI

The study group consisted of 200 surgical oncology patients who were consecutively sampled from April 2021 to July 2022 at the Regional Institute of Oncology (IRO) Iași, Romania, and who were eligible for prophylactic antibiotic administration (aged 30-94 years, average age 62.96, median age 66). These patients had been admitted for noninfectious issues and had not received antibiotic treatment in the previous three months. According to clinical practice recommendations for antimicrobial prophylaxis in surgery, all patients underwent a standard prophylactic antibiotic regimen before surgery: a single dose of 1.5g cefuroxime intravenous administration before incision, up to a maximum of two doses in 12 hours, based on the duration of the surgery.

In order to minimize potentially deceptive microbiome changes, patients who were already on antibiotics were not included in the study. For the molecular characterization of the microbiome, paired samples from before treatment (M) and seven days after treatment (M) were collected (T). Sample M was acquired before bowel preparation (BP), if it was employed in addition to systemic antibioprophylaxis. 39 patients received polyethylene glycol (PEG), 48 received enema, and 41 received no mechanical BP (NO).

CHAPTER II THE OBSERVATION OF THE DIFFERENCES MET BETWEEN THE 2 GROUPS

RESULTS FROM PMSI INSTITUTE OF ONCOLOGY

Were made 30 sequencing runs, 7 runs with 6 barcoded samples, 7 runs with 8 barcoded samples and 5 runs with 14 barcoded sample of which 2 runs were made with the samples from IRO Iasi. In addition to this, 11 negative controls and 13 positive controls were sequenced.

Negative controls contained an average of 22087 reads after all filtering steps. This number of reads is due to the fact that the negative control was subjected to isolation together with the biological samples, while, the protocol provided for the inclusion of the negative control at the stage of the genomic libraries' preparation. The aim was to identify the degree of contamination during isolation. The taxa that occurred most often in the negative controls were: *Propionibacterium, Arthrobacter, Staphylococcus* and *Ralstonia*. Due to the abundance of less than 1% (four genus listed) in the biological samples of the patients, these reads were not excluded from the analysis.

The positive controls used (according to the protocol) included *E. coli* DNA (30 µg/mL) and after all filtering steps were detected an average of 200355 reads. The mean relative abundance of positive controls shows a 56,57% of *Escherichia*, 42,04% of *Escherichia/Shigella* and 1,26% of *Shigella* (*Shigella* are genetically closely related to E. coli). In addition to these genera, they have also been identified *Salmonella* (0,05%), *Enterobacter* (0,03%), *Microbacterium* (0,02%), *Arthrobacter* (0,01%), *Bifidobacterium* (0,01%) and *Erwinia* (0,01%). The abundance of these genus is negligible.

After the quality analysis of the reads of patient's samples, their length was found to meet the quality criteria to be aligned to the hypervariable regions in the reference databases. Metagenomic DNA sequence libraries consist of sequence reads ranging from 145 to 266 bp in length, Mean Read Length (bp) = 237, standard deviation (SD) = 18.258 and Interquartile range (IQR) = 21.25 (Fig.5).

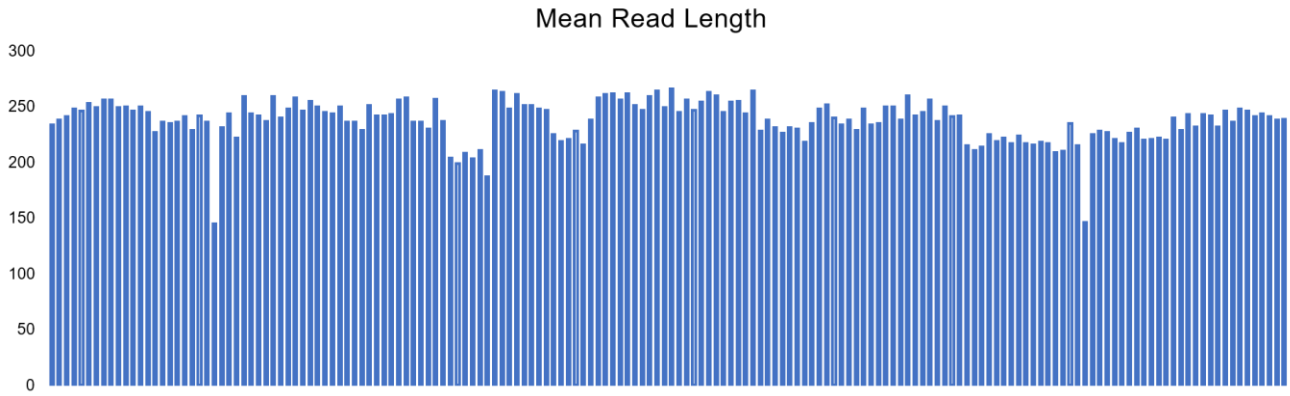


Figure no 5. Mean Read Length (bp) of reads = 237, standard deviation (SD) = 18.258, Interquartile range (IQR) = 21.25, min = 145 and max = 266.

In the same way, were calculated the parameters for the number of reads per sequenced sample: mean = 225643, SD = 142106, IQR = 160503, min = 639 and max = 1338484 (Fig. 6). The samples with a to low number of reads were excluded from data analysis because don't meat quality criteria for bacteria genus identification. Samples excluded from data analysis due to low read counts were repeated starting with the construction of the metagenomic libraries, using a larger amount of DNA in the first step. If, after

repeating the sequencing, the number of reads corresponded to the quality criteria, they were subjected to further bioinformatic analyses, otherwise it was considered that the amount of microbial DNA was very small, thus making it impossible to obtain quality results.

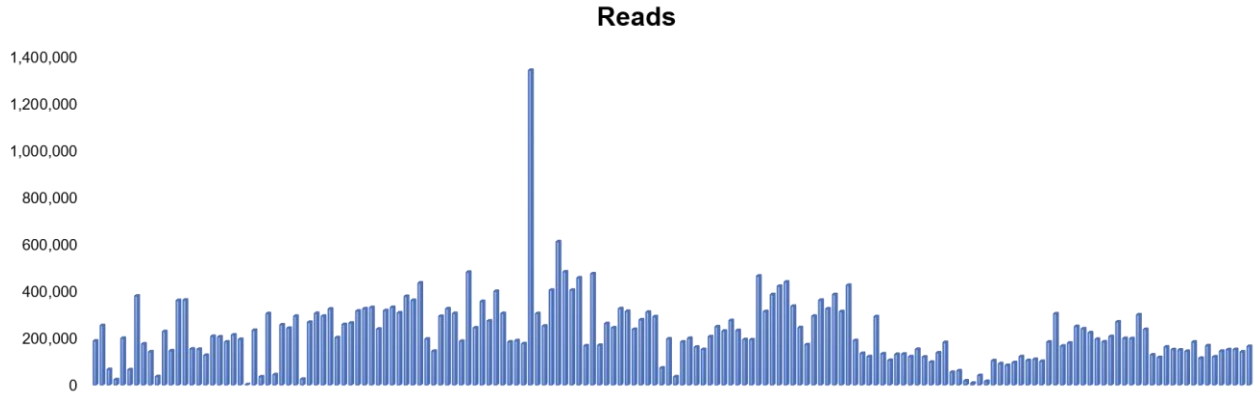


Figure no. 6. Number of reads per sample. mean = 225643, SD = 142106, IQR = 160503, min = 639 and max = 1338484

Top Taxa Genus Level

Joint analysis of samples collected before treatment and samples collected at the end of antibiotic treatment highlighted 26 taxa (Genus taxonomic level) that have an average relative abundance >0.01 (1%) (Tab. 7).

Table 7. Genus taxonomic level that has an average relative abundance >0.01 (1%). The table shows 26 genus of bacteria that were identified in both cohorts (M and T).

No	Name	No	Name
1	*Prevotella*	14	*Peptoniphilus*
2	*Bacteroides*	15	*Anaerococcus*
3	*Faecalibacterium*	16	*Acinetobacter*
4	*Sutterella*	17	*Streptococcus*
5	*Bifidobacterium*	18	*Sporobacterium*
6	*Finegoldia*	19	*Alistipes*
7	*Porphyromonas*	20	*Parvimonas*
8	*Fusobacterium*	21	*Clostridium*
9	*Ruminococcus*	22	*Raoultella*
10	*Corynebacterium*	23	*Morganella*
11	*Desulfovibrio*	24	*Actinomyces*
12	*Campylobacter*	25	*Pseudomonas*
13	*Parabacteroides*	26	*Enterococcus*

Alpha diversity or its richness (number of taxonomic groups) and Chao index showed a smaller difference between the pre-treatment samples and a larger difference between the post-treatment samples (Fig.7). M samples represent an abundance between 20 and 60 taxa (genus taxonomic level), while in T samples an abundance of taxa between 10 and 70 is observed.

If the samples reach to the plateau, that is an indicator that all potential genus have been detected. We can see that in the alpha diversity plot, samples from cohort M plateau reach to the plateau (except for one), whereas, in the T cohort, not all samples reach the plateau, meaning that not all potential genus were identified in those samples.

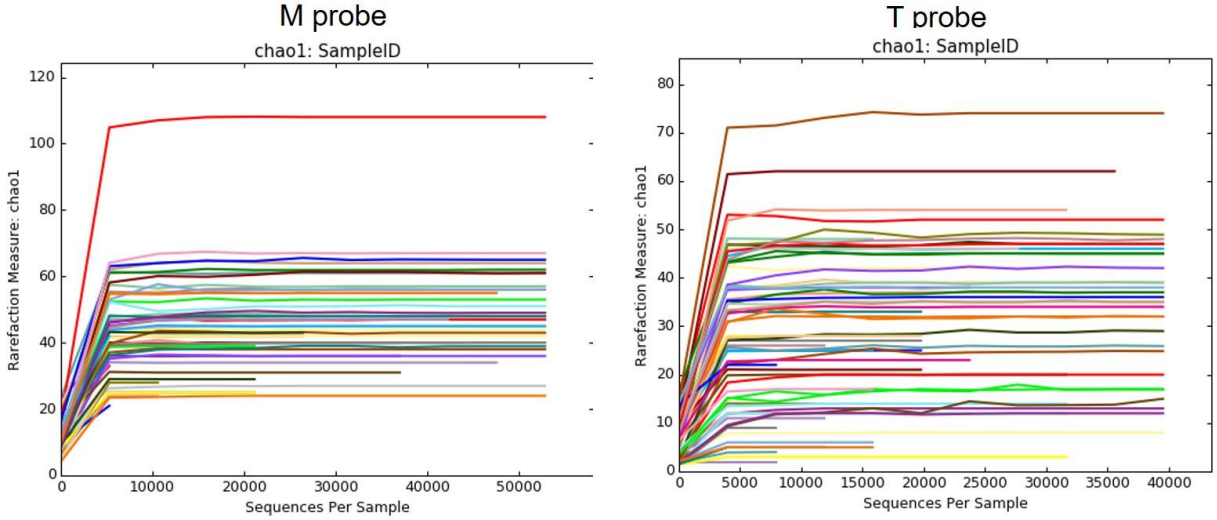


Figure no.7. Alpha diversity (α-diversity) of samples collected and sequenced in IO RM. Rarefaction curves using Chao 1 model. All samples in cohort M (except one) reach to the plateau, which is an indicator that all potential genus have been detected.

Thirtieth biological samples, collected at IRO Iasi, were sequenced in parallel, both using Illumina and Ion Torrent technology. This includes both pre- and post-treatment biological samples. We can se that all the samples (sequenced with Ion Torrent Technology) reach to the plateau.

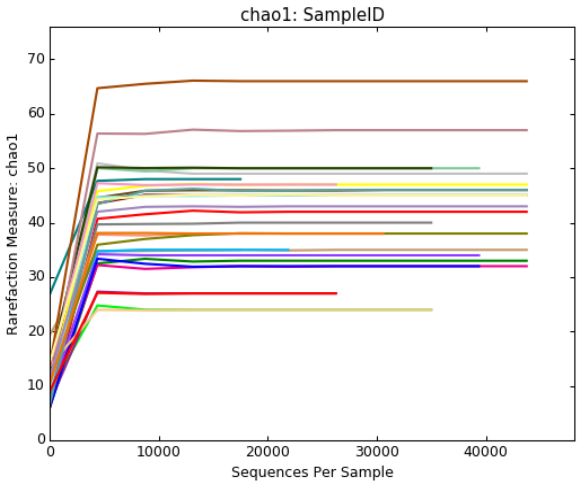


Figure no.8. Alpha diversity (α-diversity) of samples collected in IRO Iasi and sequenced in IO RM and IRO Iasi. Representation of result obtained from Ion Torrent Technology. All samples (M and T) reach to the plateau.

Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity matrix of bacterial taxa shows clustering pattern between M and T samples (Fig. 9). In this representation, relatively representative clustering of the number of taxa in the M samples (big balls) and a large distribution of taxa in the T samples (medium balls) are observed.

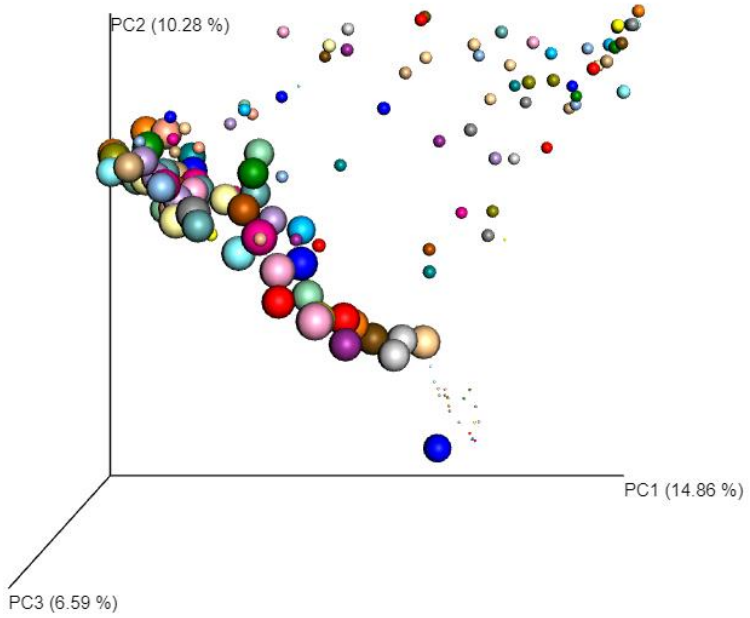


Figure no.9. β-diversity using Bray-Curtis dissimilarity index. T samples (medium balls) are uniformly distributed along de spectrum - is an indirect indicator of intersubject variability. M samples (big balls) are a bit more concentrated.

The most abundant genus ones in M cohort were *Prevotella* (14.73%) and *Bacteroides* (14.24%). The following most common genus were *Faecalibacterium* (7.51%), *Sutterella* (5.25%) and *Bifidobacterium* (4.60%) and the mean relative abundance decreases in T samples: *Prevotella* (4.60%), *Bacteroides* (6.51%), *Faecalibacterium* (1.01%), *Sutterella* (3.06%) and *Bifidobacterium* (1.78%), while, the most abundant genus in T cohort were *Enterococcus* (38.83%) and *Corynebacterium* (11.32%). This genus constitutes 0.15% and 2.16%, respectively, of abundance in cohort M. (Fig.10).

Maybe, were detected higher levels of bacteria belonging to the group Bacteroides-Prevotella because the all patients included in the study have colorectal cancer. The species of *Prevotella* genus may cause anaerobic infections and inflammation of the colon mucosa, but *Bacteroides spp.* are involved in immunity by activation of CD4+ T cells. Some species exclude potential pathogens from the human gut; however, others are opportunistic human pathogens.

Some species of *Corynebacterium,* which has a high mean relative abundance in T samples, can cause diseases, such as diphtheria and the resistant Enterococci (which also has a high abundance in T samples) densely colonize the gut following antibiotic treatment, which can deplete the GI tract of large swaths of protective commensals. Possibly, due to the multiresistance of Enterococci, after administration of antibiotic treatment and disruption of the colonic flora, their abundance increases significantly.

A total of 262 OTU were found in in both cohorts, 210 OTU (Genus) in samples before treatment and 219 OUT after treatment, from which 167 OTU was detected in both research groups, 43 OTU only in M samples but not in T and 52 OTU only in T samples but not in M.

Only 22 genera (cohort M) out of 210 exceed a 1% presence and 18 genera out of 219 exceed a 1% presence in cohort T. From 167 OTU detected in both research groups, 22 OTU have a mean relative abundance > 1.00% in group M and 18 OTU in group T. 14 OTU exceed 1% in both cohorts M and T as follows: *Prevotella* – 14,73%:4,60%, *Bacteroides* – 14,24%:6,51%, *Faecalibacterium* – 7,51%:1,02%, *Sutterella* – 5,25%:3,07%, *Bifidobacterium* – 4,60%:1,78%, *Finegoldia* – 3,52%:3.00%, *Porphyromonas* – 3,49%:1,15%, *Fusobacterium* – 2,95%:1,45%, *Ruminococcus* – 2,80%:1,17%, *Corynebacterium* – 2,16%:11,33%, *Parabacteroides* – 1,91%:2,87%, *Peptoniphilus* – 1,85%:7,72%, *Acinetobacter* – 1,63%:1,14% and *Streptococcus* – 1,54%:1,11%. As we observe, abundance of the genus *Finegoldia, Parabacteroides, Acinetobacter* and *Streptococcus* does not exceed 1% difference in M and T cohort, possibly, these taxa are not influenced by the antibiotics used, the enema or polyethylene glycol.

All 43 OTU detected only in M samples but not in T and all 52 OTU detected only in T samples but not in M do not exceed 1% abundance and does not significantly influence the overall abundance.

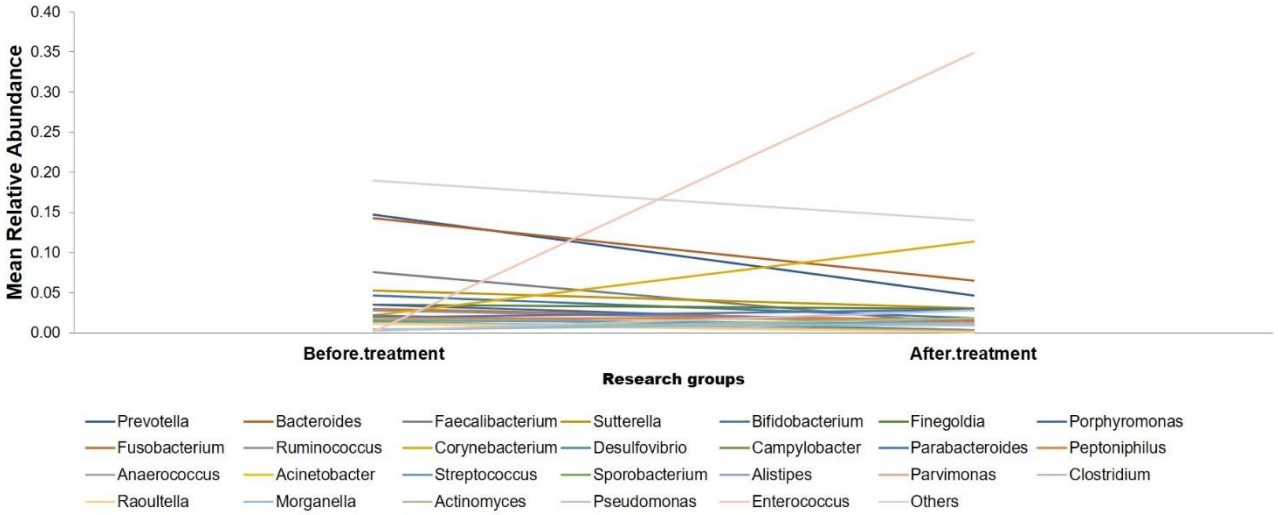


Figure no.10. Mean Relative Abundance of Top Taxa (>0.01) (Genus taxonomic level). The most abundant genus in M cohort is Prevotella (14.73%) and Bacteroides (14.24%) and in T cohort are Enterococcus (38.83%) and *Corynebacterium* (11.32%).

After clusterization of genus Mean Relative Abundance using heatmap representation method the data shows a super warm area in the upper left corner of the heatmap. It corresponds to a bunch of T samples and includes *Enterococcus* and *Corynebacterium* OTUs. The heatmap is clustered in 6 clusters (*Fig 11*). Similarity is observed between these samples which are shown close to each other. The first cluster include the samples that have a high abundance of *Enterococcus* and the second cluster represents the samples with a high abundance of *Corynebacterium.* These two clusters largely include T samples, i.e., samples collected after treatment. The next 4 clusters include samples with relatively high abundance of bacterial genus as follows: *Prevotella* (high abundance in M samples), *Bacterioides, Sutterella* and *Faecalibacterium* and the sixth cluster which includes the other genus.

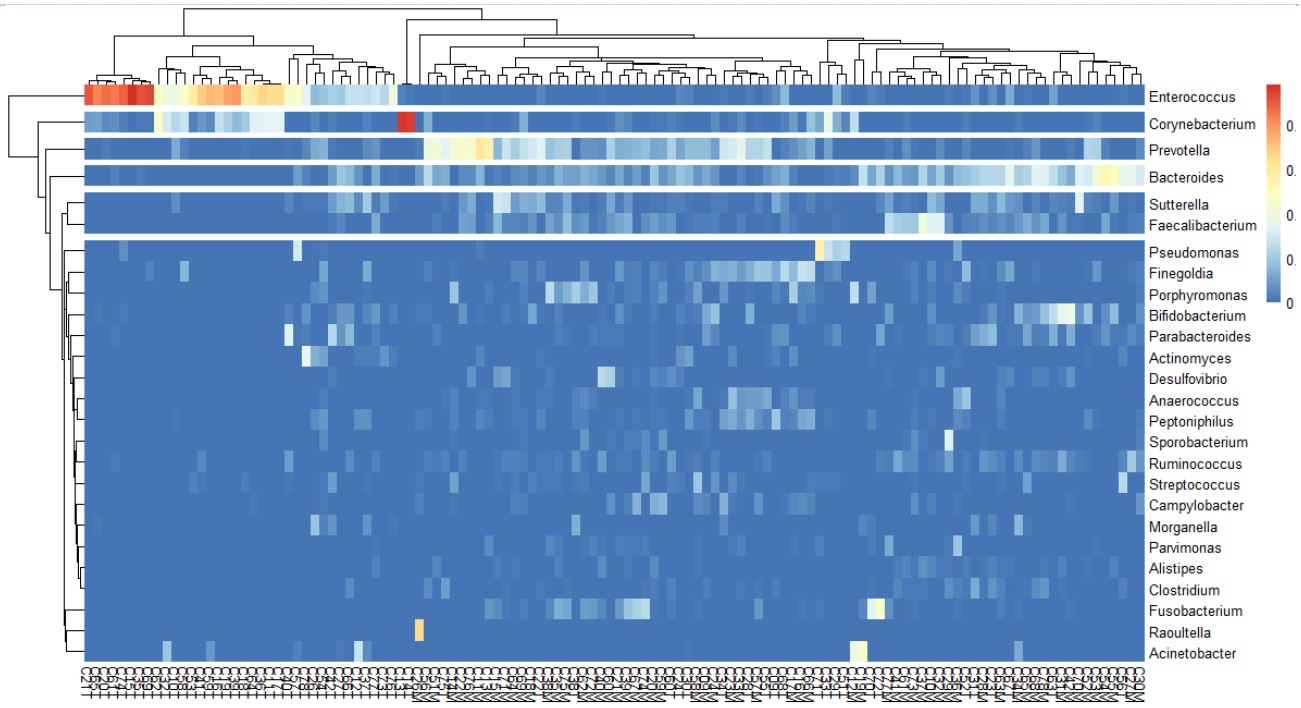


Figure no.11. The heatmap clusterization of research groups. Each row represents a genus while each column represents a sample. 1st cluster – Enterococcus, 2nd – Corynebacterium, 3rd – Prevotella, 4th – Bacterioides, 5th – Sutterella and Faecalibacterium, 6th - the other genus.

Antibiotics used to treat patients whose samples were sequenced were grouped into 16 different groups. The only similarity of the antibiotic groups was Metronidazole (Tab. 8).

Table 8. Groups of antibiotics

Group	Antibiotics	Group	Antibiotics
Group 1	Metronidazole	Group 8	Metronidazole
	Cefoperazone + Sulbactam		Obrocin
Group 2	Metronidazole		Cefoperazone + Sulbactam
	Cefoperazone + Sulbactam	Group 9	Metronidazole
	Cefazolin		Cefoperazone
	Ceftriaxone		Obrocin
Group 3	Metronidazole	Group 10	Metronidazole
	Cefazolin		Cefoperazone + Sulbactam
	Cefoperazone + Sulbactam		Cefoperazone
Group 4	Metronidazole	Group 11	Metronidazole
	Cefoperazone + Sulbactam		Cefazolin
	Ceftriaxone		Cefoperazone + Sulbactam
Group 5	Metronidazole		Cefoperazone
	Cefazolin	Grou 12	Metronidazole
	Cefoperazone		Cefazolin

Group 6	Metronidazole		Cefoperazone
	Cefazolin		Imipenem + Cilistatina
	Cefoperazone	Group 13	Metronidazole
	Ceftriaxone		Obrocin
Group 7	Metronidazole	Group 14	Metronidazole
	Cefazolin		Cefazolin
	Cefoperazone	Group 15	Metronidazole
	Obrocin		Ceftriaxone
		Group 16	Metronidazole
			Cefuroxim

No change in the composition of the microbiome is observed that correlates with any particular group of antibiotics used, but it is observed that the uncontrolled and high-dose use of antibiotics leads to the dysregulation of the colonic microbiome (Fig. 12).

The use of enema or polyethylene glycol was also included in the same analysis. In a small group of patients, who were not administered enema and polyethylene glycol, an increase in the abundance of *Corynebacterium spp.* and *Enterococcus spp*. taxa (Fig. 12).

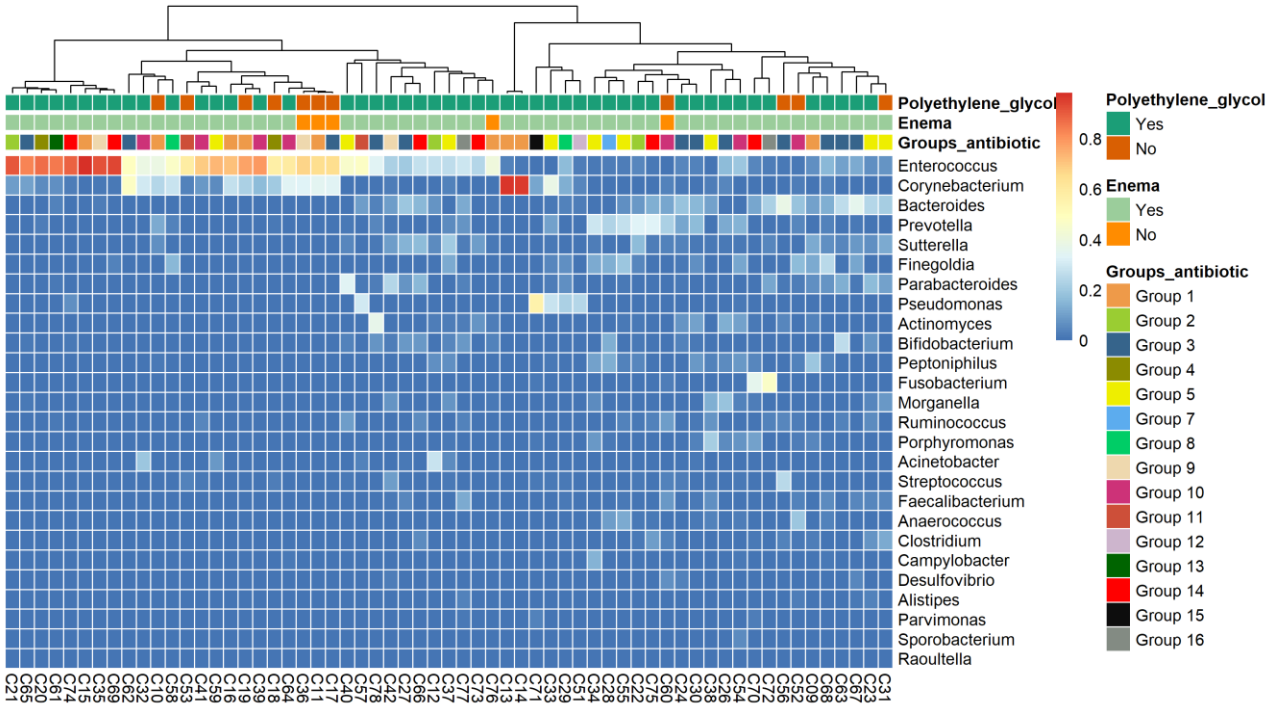


Figure no.12. Representation of interactions between taxa, antibiotics, enema and polyethylene glycol.

16S rDNA Sequence Reads Processing

From the input fastq files, a total of 43,041,359 reads from 4 runs were analyzed: 402 paired samples, 2 sample duplicates, 12 negative controls, and 10 positive controls. The number of reads per sample in the sequencing depth varied from 3,283 to 3,681,248. (Average 153,719 reads, median 78,824 reads). 28,619,858 reads were left after all processing stages before taxonomy assignment, or 66.49% of the original reads, with 493 to 2,549,573 merged non-chimeric reads per sample (average 102,213, median 52,612). 15,509 distinct amplicon sequence variants (ASVs), including controls and duplicate samples, were included in the final amplicon sequence variation (ASV) table. 13,795 distinct ASVs were still present in 240 samples following taxonomic assignment, filtering, and the removal of samples with fewer than 5000 reads as well as negative and positive controls. Of these, 720 could be categorized at the species level. Sequences were combined at the species level in order to exclude fictitious ASVs because many distinct ASVs could be categorized as the same species or were left unidentified at the species level. 240 samples yielded a total of 666 distinct ASVs, of which 635 were shared by the M (before antibiotic treatment) and T (seven days after antibiotic treatment) groups and 17 were unique to the M group and 14 unique for the T group.

After all filtering, negative controls had an average of 309 reads. Taxa found in negative controls were not eliminated from samples due to low overall numbers. The biggest bias in variation was found for the gram-negative bacterium Klebsiella pneumoniae in a mock community analysis from three different types of positive controls (CP1, CP2 and CP3) with defined community structure encompassing both gram-negative and gram-positive bacteria (Fig. 13). With the exception of Klebsiella, which showed an 18.65% positive shift, there was an overall mean shift from predicted relative abundances of 5.93%, with a 6.53% mean underrepresentation of gram-positive bacteria, which are more challenging to lyse.

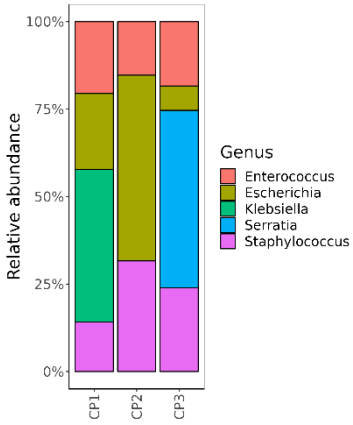


Figure no. 13. Mock bacterial community structure merged at genus level per each type of positive control, in terms of relative abundances.

As expected, 25% of each genus was found in the mean relative abundances for CP1: 43.65% of Klebsiella, 20.53% of Entero-coccus, 21.66% of Escherichia, and 14.16% of Staphylococcus. When compared to the expected values of 45% Serratia, 25% Staphylococcus, 25% Enterococcus, and 5% Escherichia, the mean relative abundances for CP3 were 50.76% Serratia, 23.88% Staphylococcus, 18.38% Enterococcus, and 6.96% Escherichia. Finally, we found that, in the case of CP2, the mean relative abundances of Escherichia, Staphylococcus, and Enterococcus were 52.89%, 31.73%, and 15.37%, respectively (compared to a predicted 50% Escherichia, 25% Staphylococcus, and 25% Enterococcus).

The fact that all of the control bacteria could be correctly identified at the genus level implies that, despite the bias towards gram-negative bacteria that was detected, the DNA extraction process performed well for both gram-positive and gram-negative bacteria. This, however, also demonstrates how the DNA extraction protocol contributes to the overrepresentation of gram-negative bacteria, which may have an impact on how future results are interpreted, particularly if only gram-negative bacteria are discovered to be differentially abundant in particular study groups.

Bacterial Diversity

The T group (7 days post-antibiotic treatment) was less diverse than the M group (before treatment), which suggests that antibiotic treatment had an effect on species richness and that not all of the initial gut microbiota had repopulated after antibiotic treatment. As a whole, inter-group variety was discovered to be statistically distinct, as demonstrated by measured alpha diversity indices (Fig. 14).

While significant differences were found throughout all evaluated performance measures between before-after antibiotic treatment among patients undergoing preoperative BP with PEG, a clear distinction in terms of alpha diversity between groups could be seen if preoperative bowel cleansing method was also taken into consideration (Fig. 15).

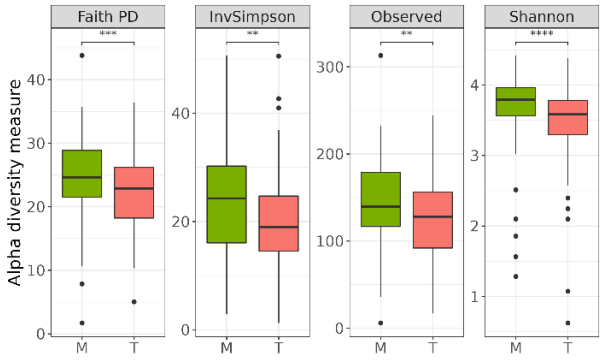


*Figure no. 14. Alpha diversity measures (Faith's Phylogenetical distance, Inverse Simpson's index, Observed ASVs and Shannon Diversity index), before (M)/7-days post antibiotic treatment (T); paired Wilcoxon test: **p-value <0.01, ***p-value <0.001, ****p-value <0.0001*

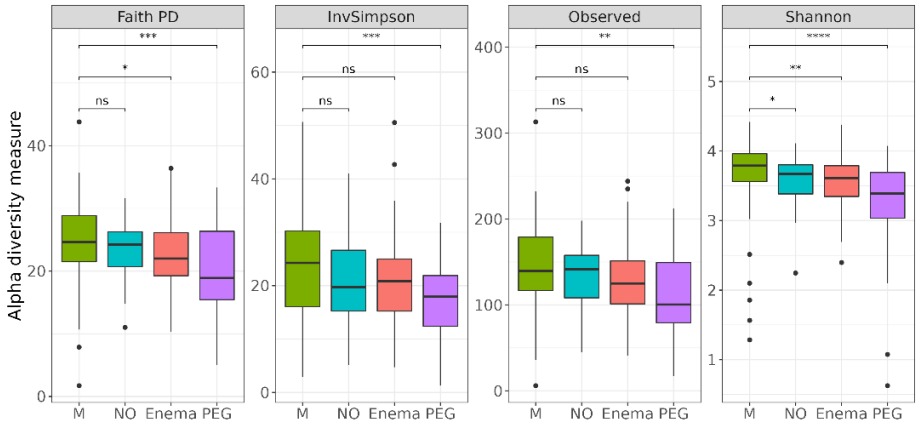


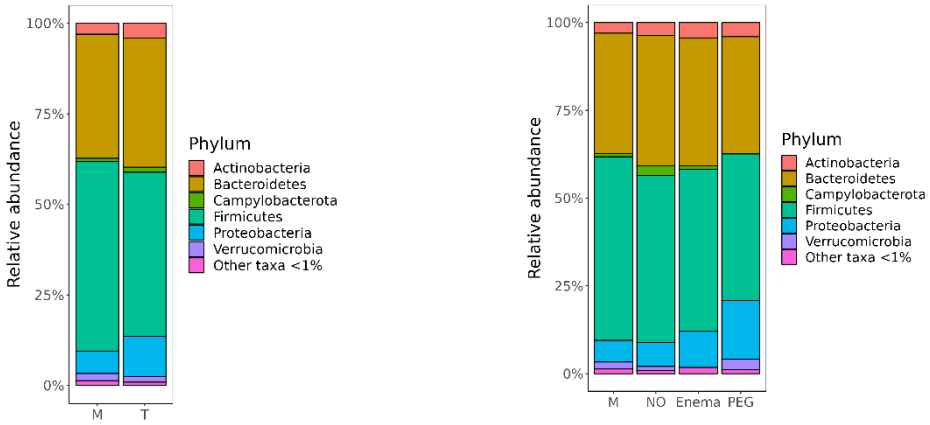
Figure no. 15. Alpha diversity measures (Faith's Phylogenetical distance, Inverse Simpson's index, Observed ASVs and Shannon Diversity index), before (M)/ 7-days post antibiotic treatment, when considering

*BP method: no bowel cleansing (NO); with preoperative enema (Enema); with preoperative PEG BP (PEG); paired Wilcoxon test: **p-value <0.01, ***p-value <0.001, ****p-value <0.0001*

In terms of Faith's phylogenetical distance and the Shannon index, there were less significant changes between pre- and post-antibiotic treatment in patients who had preoperative enema and pre- and post-antibiotic treatment in patients who had no preoperative preparation. This implies that some reduction in species richness happened during antibiotic treatment, independent of previous preparation.

Bacterial Composition

As would be predicted in the human gut, the average bacterial composition at the phylum level in the M group is composed of 52.3% Firmicutes, 34.3% Bacteroidetes, and a lower amount of Actionobacteria (3%), Proteobacteria (6.2%), Verrucomicrobia (2%) and other taxa (Fig. 16a). The T group is less prevalent in Firmicutes taxa (45.3%), whereas it is more prevalent in Proteobacteria (11%) and Campylobacterota (1.4%), indicating that antibiotic treatment does affect the makeup of bacteria. For samples from patients who underwent preoperative PEG BP, a bias in compositional shift could be seen when accounting for the preoperative bowel cleansing preparation in the case of gastrointestinal surgical operations (Fig. 16b). In light of the fact that patients without any BP had comparable relative abundances in all major phyla both prior to treatment (M group) and seven days later (NO group), it is possible that pre-operative BP affects bacterial composition in addition to antibiotic therapy.

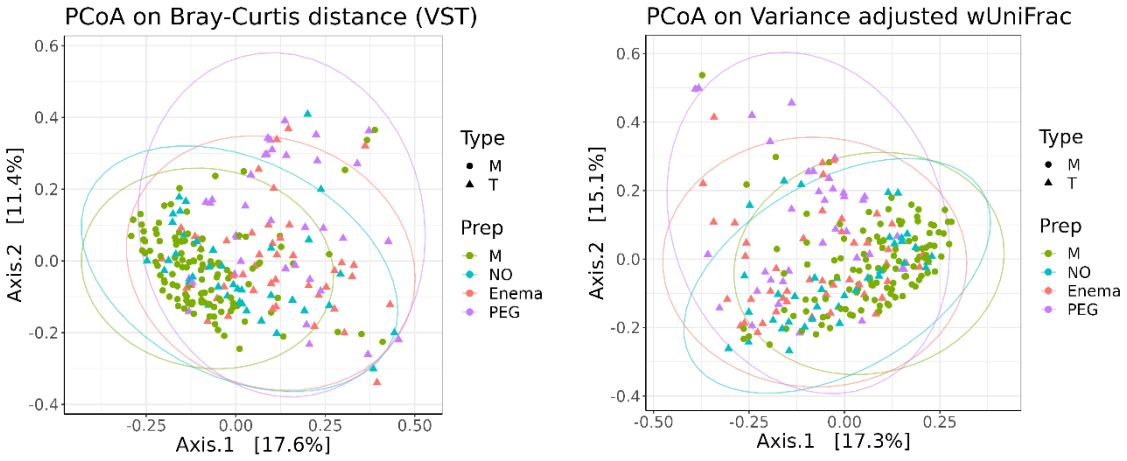


(a) **(b)**

Figure no. 16. Bacterial community structure merged at phylum level per each group, in terms of relative abundances: (a) before (M)/7-days post antibiotic treatment (T); (b) before (M)/ 7-days post antibiotic treatment, when considering BP method: no bowel cleansing (NO); with preoperative enema (Enema); with preoperative PEG BP (PEG); Phyla with lower than 1% relative abundance are grouped together under 'Other taxa <1%'.

Both groups (M and T) exhibit high inter-sample variations with no discernible segregation, according to PCoA analysis using Bray-Curtis dissimilarity and variance-adjusted weighted UniFrac distances, though M type samples cluster together more readily, indicating that the M group has a more homogeneous composition than the T group (Fig. 17). When utilizing Bray-Curtis dissimilarity, there was a significant difference in intra-group dispersion between the M and T groups (P(perm)=0.0001), but not

when using variance-adjusted weighted UniFrac distances (P(perm)=0.068). A greater dispersion in the T group shows that samples treated with cefuroxime had more distinctive communities than samples not treated with it.



(a) **(b)**

Figure no.17. PCoA plots with (a) Bray-Curtis dissimilarity from VST-transformed data and (b) variance-adjusted weighted UniFrac distance, illustrating distances between communities in individual samples (n=240). Point shapes according to treatment type: circles – samples before antibiotic treatment (M); triangles - samples 7 days post-antibiotic treatment (T); Color according to bowel cleansing preparation: green – samples before antibiotic treatment, with no bowel cleansing (M); blue – samples 7 days post-antibiotic treatment, with no bowel cleansing (NO); red – 7 days post-antibiotic treatment, with preoperative enema (Enema); purple – samples 7 days post-antibiotic treatment, with preoperative PEG BP (PEG); Ellipse drawn at 95% confidence level. Percentage of variation explained by the first two dimensions are indicated on respective axes.

The group receiving PEG BP was the most dissimilar in terms of dispersion when preoperative bowel cleansing preparation was considered in terms of Bray-Curtis dissimilarity (P(perm)=0.0001/0.0029/0.0149 for PEG vs. M/NO/Enema groups, respectively), whereas the dispersion for the group receiving no BP was not substantially different from the before antibiotic treatment group (P(perm)=0.306) or treatment group receiving pre-operative The variance-adjusted weighted UniFrac distances did not reveal any notable variations in group dispersions. Therefore, the significance of group centroid differences was evaluated using PERMANOVA on variance-adjusted weighted UniFrac distances.

According to PERMANOVA, there are compositional differences between the M and T groups (R2=0.03719, P=0.0001), but only 3.71% of this compositional variation can be attributed to antibiotic use; a further 6.13% can be attributed to the pre-operative BP method (R2=0.06137, P=0.0001). Comparisons revealed a weak overall difference in the makeup of the bacteria between the M and T groups (P=0.0001, R=0.109), as well as a stronger influence of the pre-operative BP method on the bacteria (P=0.0001, R=0.158). Given that antibiotic treatment affects bacterial composition regardless of pre-operative preparatory BP, pairwise permutation MANOVAs on variance-adjusted weighted UniFrac distances revealed significant differences in centroid positioning between pre-treatment and all post-treatment conditions (P=0.0039/0.0003/0.0003, respectively). However, distance centroids between the

Enema group and the control group were not statistically different (P=0.0594), indicating that PEG BP has the greatest influence on the bacterial composition following antibiotic therapy.

For VST-normalized abundances, a hierarchical cluster analysis utilizing Bray-Curtis dissimilarity and Ward's clustering algorithm showed that 27 samples from the T group clustered together because they were the most similar to the matching M samples (not shown). Thus, 7 days following antibiotic therapy, the gut microbiota recovered in 22.5% of instances. Only 5.55% of patients with PEG BP experienced microbial repopulation, compared to 19.5% of patients who had pre-operative enema and 42.1% of patients with no BP.

Differential Abundance Analysis

Taxa whose abundance varied considerably between groups could be found using differential abundance analysis. The phyloseq object that was subjected to analysis had 359 distinct genera after clustering at the genus level. Between M and T groups, 12 genera were shown to be differently abundant by LEfSe, 65 by ALDEx2, 85 by ANCOMBC, and 106 by limma voom. Notably, the intersection of the genera discovered by the four approaches revealed seven that were more numerous in the T group than the M group and could be further distinguished in the T group using the preoperative bowel cleansing technique (Fig. 18).

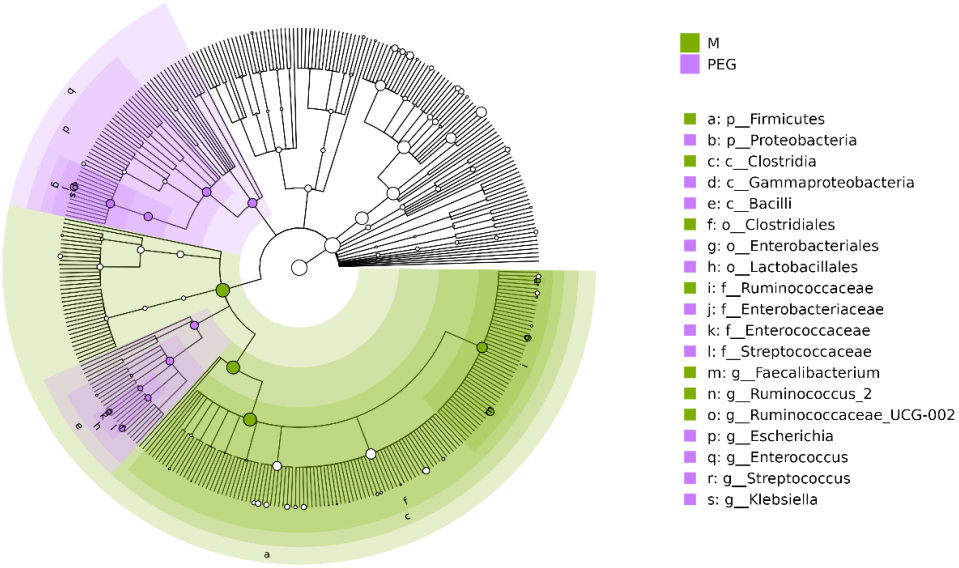


Figure no.18. Circular phylogenetic tree (cladogram) showing the phylogenetic distribution of taxa which differ significantly between M and PEG groups, according to consensus between the ap-plied Differential Abundance Analysis methods; green – taxa which are more abundant in samples of the M group; purple – taxa which are more abundant in samples after antibiotic treatment, with preoperative PEG BP (PEG). Order of hierarchy (from center): Kingdom, Phylum, Class, Order, Family, Genus. Node size is proportional to relative abundance. In the legend, the letter in front of the taxon represents the taxonomy level: p-phylum; c-class; o-order; f-family; g-genus;

Therefore, commensal genera Faecalibacterium, Ruminococcus 2, and Ruminococcae UCG-002 were more prevalent in samples obtained before to antibiotic therapy, indicating that not all microbiota had recovered following antibiotic treatment, regardless of the perioperative preparation techniques used. At the same time, patients receiving PEG BP were more likely to have the opportunistic species Escherichia, Enterococcus, Streptococcus, and Klebsiella in their gut microbiota. ALDEx2 and ANCOMBC also found Ruminococcaceae UCG-014 and Lachnospiraceae UCG-001 to be differentially abundant

between samples taken before treatment (M) and samples taken seven days after treatment from patients who had no bowel preparation, but not LEfSe. This indicates that M and NO groups are the least different in terms of preparation type, and that M and T groups are similar.

The observation of the differences met between the 2 groups

To analyze the composition of the gut microbiome, high-throughput sequencing methods like 16S ribosomal ribonucleic acid (rRNA) gene amplicon sequencing are often used. Since DNA extraction is known to have a considerable negative impact on metagenomic investigations, 16S rRNA gene sequencing must be utilized carefully and should include a detailed analysis of experimental artifacts.

Understanding the human gut microbiota in both health and disease requires accurate and dependable microbial data gathering. A critical step and ongoing challenge consist in selecting the appropriate approach to isolate representative microbial DNA of the sampled microbial community. The efficacy of cell lysis, as opposed to DNA recovery, has been shown to have a bigger impact on the reviewed microbial composition in earlier studies [20-23]. Since gram-positive bacteria typically have substantial peptidoglycan coatings in their cell walls, they are more resistant to lysis than gram-negative bacteria, which frequently leads to poor representation in the observed relative abundance data. We found that extraction processes that included a bead beating mechanical lysis and an enzymatic lysis step generated substantially better illustrations of the bacterial community configuration than procedures omitting either of these phases. Despite the fact that gram-positive bacteria were underrepresented in our investigation, further optimization is still required for accurate stool sample microbiota characterization. Therefore, an improved lysis efficiency offers a more thorough and consistent profile of the microbial diversity. Cell lysis is not very efficient when proteinase K or lysozyme are used alone, particularly for gram-positive bacterial cells. However, a combination of lytic enzymes offered the best representation of microbial diversity for all samples. This is probably because different bacterial species have different peptidoglycan structures, which affect how susceptible they are to lysozyme. A recent study further underlines that, for precise bacterial composition characterization based on the V3-V4 region of the 16S rRNA gene, integrating a bead beating mechanical stage in the DNA extraction technique from stool samples yields better results than simple chemical lysis. Our findings, however, highlight the need for additional DNA extraction protocol optimization in order to more precisely characterize the microbial community in patient stool samples. As a result, our team is currently looking into other bacteriolytic enzymes, as well as chemical lysis for future DNA extraction protocol optimization.

It should be noted that, in order to avoid the preferential isolation of specific bacterial species, the ability of different DNA extraction techniques to accurately represent the microbial diversity in samples cannot be evaluated without the use of a control community with a known composition, also known as a "mock community." This has repeatedly been emphasized in numerous metagenomic research, emphasizing the significance of employing appropriate control communities for accurately defining the examined microbiome [24-26]. Researchers found that antibiotic treatment has a significant impact on the composition of the microbiota and that the dispersion in microbiota composition can occasionally increase after antibiotics treatment using 16S amplicon sequencing and principal coordinate analysis (PCoA) on unweighted UniFrac distances to characterize the gut microbiota composition. These findings are supported by our findings, which showed a substantial difference in intra-group dispersion between the groups before (M) and 7 days after antibiotic treatment (T). In particular, our study discovered that three taxa, Faecalibacterium, Ruminococcus 2 and Ruminococcae UCG-002, are more likely to still be reduced 7 days after cefuroxime treatment. All three are gram-positive and are members of the Firmucutes phylum, the Clostridia class, and the Ruminococcaceae family. Additionally, Ruminococcus species appear to always be present in the healthy human gut, indicating that they play a key role in maintaining a normal environment in the gut, and as a result, it is not surprising that antibiotic therapy has some effect on them. In cases of peritonitis brought on by Eneterobactericeae and Streptococcus,

cefuroxime is also known to reduce sensitivity rates. Our findings demonstrate that cefuroxime administration allows the gut flora to repopulate seven days after antibiotic treatment, but only in patients who did not have a preoperative mechanical bowel preparative surgery. Bowel cleansing has been shown to temporarily alter the composition of the gut's microbes, particularly in the relative abundance of Proteobacteria. When combined with a decline in the diversity of species overall, this finding suggests that Proteobacteria may more effectively fill empty niches in the body. As a result, the observed rise in Proteobacteria for our samples may not necessarily be an infection brought on by the found differentially abundant opportunistic bacteria, but rather a delay in the colon's ability to return to its pre-BP state. Future research is required to ascertain whether the detected differentially abundant species indeed induce SSI or whether they are eventually displaced by commensal bacteria due to a lack of information regarding later post-intervention timepoints.

Antibiotics have a considerable and occasionally long-lasting impact on the gut microbiota, leading to a rise in potentially hazardous commensals and a decrease in helpful commensals. If these effects are better understood, probiotic use and antibiotic therapy can be customized to minimize this "collateral damage".

The microbial diversity that is detected in studies of the microbiome is greatly influenced by the technique of DNA extraction used. For maximum species diversity and abundance in all samples, use a technique that includes both mechanical and chemical lysis.

Metagenomic investigation of the gut microbiome has the added advantage of characterizing the microbial population and shedding insight on potential physiological consequences on the human host. Metagenomic sequencing additionally enables the determination of the abundance of various bacterial taxa inside a sample as well as the detection of dysbiosis events in samples.

When compared to the pretreatment condition for patients without mechanical bowel preparation, the composition of the intestinal microbiota in our study group of 200 surgical oncology patients was not significantly different one week after cefuroxime treatment, but some loss in taxonomic variety could be seen. When considered as a whole, cefuroxime does not encourage long-term dysbiosis in surgical patients who do not have any extra perioperative treatments.

Future research could more effectively address a number of the study's shortcomings. According to a recent study, freezing samples without the addition of cryoprotectants causes changes in the stool microbiota [27]. We solely report data from fresh and frozen stool samples in our analysis and do not take into account any potential alterations brought on by cryopreservation. The gut microbiome is significantly influenced by diet, according to other studies [28], and our study does not take into consideration dietary variations between patients or between before and after surgical intervention. Additionally, because metadata regarding body mass index or diabetes status was not obtained, our investigation does not take into account potential intestinal dysbiosis caused by diabetes [29] or obesity [30].

Nevertheless, our findings imply that the gut microbiota is nearly restored to its pre-treatment state 7 days after cefuroxime antibioprophylaxis, and the degree of repopulation is significantly influenced by other perioperative measures such blood pressure monitoring.

CONCLUSIONS

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1. The most abundant genus of bacteria in sample M (before treatment) are *Prevotella spp., Bacteroides spp., Faecalibacterium, Bifidobacterium spp.* and *Sutterella spp.*
2. The most abundant genus of bacteria in sample T (after treatment) are *Corynebacterium spp., Enterococcus spp*. and *Pseudomonas*
3. Alfa diversity index chao1 (reflects the number of taxa per sample) varies less between samples of the pre-treatment group compared to the samples in the after-treatment group.
4. The samples that are not linked with polyethylene glycol tend to form a cluster that indicates presence of more or less similar taxa abundance.
5. The average number of taxa per sample decreases after antibiotic treatment
6. The more variation of data between the study groups is observed at *Enterococcus, Corynebacterium, Prevotella, Bacteroides, Sutterella* and *Faecalibacterium*
7. Abundance of the genus *Finegoldia, Parabacteroides, Acinetobacter* and *Streptococcus* does not exceed 1% difference in M and T cohort, possibly, these taxa are not influenced by the antibiotics used, the enema or polyethylene glycol.
8. The high abundance of enterococci in the T cohort may be due to their multiresistance acquired following the wide administration of antibiotics in the population of the Republic of Moldova

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1. Cefuroxime does not promote long-term dysbiosis in surgical patients without any additional perioperative procedures.
2. Results suggest that the gut microbiome is repopulated close to pre-treatment condition 7 days post cefuroxime antibioprophylaxis, and the extent of repopulation greatly depends on other perioperative procedures such as BP.
3. The method of DNA extraction chosen has a significant impact on the microbial diversity found in microbiome investigations. For maximum species diversity and abundance in all samples, use a technique that includes both mechanical and chemical lysis.
4. A metagenomic approach to gut microbiome study has the additional advantage of shedding information on the microbial community's potential physiological impacts on the human host in addition to characterizing it. Metagenomic sequencing additionally enables the determination of the abundance of various bacterial taxa inside a sample as well as the detection of dysbiosis events in samples.
5. In our study group of 200 surgical oncology patients, intestinal microbiota composition was not substantially different one week after cefuroxime treatment compared to pretreatment condition for patients without mechanical colon preparation, but some reduction in taxonomic variety may be seen. All things considered, cefuroxime does not encourage long-term dysbiosis in surgical patients without the inclusion of any perioperative therapies.

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