Romania-Republic of Moldova
ENI-CROSS BORDER COOPERATION

REPORT

on antibiotic-induced changes and the response of the human colonic microbiome to various stressful situations

www.ro-md.net

A project implemented by
PMSI Institute of Oncology
Address: 30 Nicolae Testemitanu, street
MD-2025, Chisinau
Tel: + 373 22 852 - 303
Fax: + 373 22 733-363

This project is funded by the European Union

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

CONTENT

INTRODUCTION

The microbiome of the human colon is composed of intricate bacterial communities, fungi, archaea, viruses and eukaryotic parasites, with a structure that is challenging to appropriately evaluate and quantify. The gut microbiota has a major impact on crucial human processes such as digestion, metabolism and inflammation. Approximately 1000 endemic bacterial species populate the human gastrointestinal tract, maintaining microbial-host homeostasis. As a natural barrier of the human body, the colonic mucosa constantly interacts with this bacterial population, being influenced and impacting the equilibrium between broad ranges of bacterial species.

Changes in diet and intake of chemicals that can act as antibiotics target specific bacterial populations, producing dysbiotic alterations that are reflected in the overall distribution of bacterial species. Such changes may create a favorable environment for opportunistic bacteria to develop and become predominant, and dietary habit changes have been demonstrated to do so. Short-term antibiotic therapy may have consequences on the gut microbiota that induce long-term dysbiotic conditions (which persist for more than 30 days), which can assist the progression and worsen the disease. Thus, understanding the physiology of the gut microbiome and how it is affected by antibiotic stress is a crucial milestone for all of patients, physicians and other personnel working in the medical system.

Studies in the role of the microbiome in health and disease have been greatly facilitated by advancements in high-throughput sequencing technologies. During the past ten years, the genomic composition profiling of microbial communities through high-throughput DNA sequencing has increased remarkably and delivered many breakthroughs for taxonomic, phylogenetic and profiling aspects of the gut microbiome. To explore the natural microbial community by high-resolution molecular approaches including Next Generation Sequencing (NGS), it is particularly essential to develop a sensitive and reproducible DNA extraction method that facilitates isolation of microbial DNA of sufficient quantity and purity from all the existent microbial species.

ANTIBIOPROFILAXIE WITH CEFUROXIME (IRO IASI)

The numerous reports on surgical site infections (SSI) as postoperative complications over the past few decades urged to establishing a routine use of preoperative antibiotics in surgical approaches. After demonstrating efficacy in several clinical trials, cephalosporin became the most used drugs for surgical prophylaxis in general surgeries. Cefuroxime, a second-generation cephalosporin, is efficient against both gram-positive and gram-negative bacteria, and can be administered in combination with other antibiotics if needed. Additionally, it represents a safe and affordable drug, and is the most stable β-lactam antibiotic used to reduce the risk of post-operative surgical site infections, sepsis, or abscesses.

Of note, preoperative oral antibiotic treatment in combination with bowel preparation (BP) is recommended in cases of colorectal surgery with the aim of decreasing bacterial density and SSI. A recent meta-analysis of 38 randomized clinical trials revealed that the combination of BP with oral antibiotics resulted in the lowest rate of SSI after elective colorectal surgery, but that the reduction of SSI was not significantly different between the group receiving a combination of BP and pre-operative antibiotic treatment, and the group receiving pre-operative antibiotic only.

ANTIBIOPROFILAXIE WITH DIFFERENT ANTIBIOTICS (PMSI INSTITUTE OF ONCOLOGY OF REPUBLIC OF MOLDOVA)

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.

THE IMPACT OF THE BOWEL PREPARATION METHOD

The impact of the bowel preparation method itself on gut microbiome composition is still under question, with reports often not reaching consensus due to various reasons including lack of analytical depth. A recent report highlights minor shifts in gut microbial composition in nonsurgical patients undergoing BP, but a substantial impact of BP combined with oral antibiotics in the gut microbiome of surgery patients, with compositional changes persisting in the early postoperative period, with a later repopulation to baseline.

STUDY OBJECTIVES

The main objective of the current study was to assess the human microbiome's plasticity following antibiotic challenge, as well as its capacity and degree of recovery after surgical intervention. Shifts in gut microbiome composition before (M) and after (T) perioperative antibiotic treatment were comparatively analyzed in a group of surgical oncological patients, by high-throughput sequencing analysis of the V3-V4 region (IRO IASI) and V2-4-8 (Institute of Oncology of Republic of Moldova) of the 16S rRNA gene in patient stool samples. We must acknowledge the significance of a suitable, gut friendly preoperative prophylactic antibiotic program given the hypothesis that even short term antibiotic treatment might lead to long term dysbiotic conditions.

ANTIBIOTIC-INDUCED CHANGES AND THE RESPONSE OF THE HUMAN COLONIC MICROBIOME (IRO IASI)

The average bacterial composition at phylum level in the M group, according to relative abundances, is 52.3% Firmicutes, 34.3% Bacteroidetes, with a smaller representation of Actionobacteria (3%), Proteobacteria (6.2%), Verrucomicrobia (2%) and other taxa (Figure 4a), as would be expected in the human gut. The T group is less abundant in Firmicutes taxa (45.3%), but more abundant in Proteobacteria (11%) and Campylobacterota (1.4%), suggesting that bacterial composition is indeed impacted by antibiotic treatment. However, when taking into account the preoperative bowel cleansing preparation in the case of gastrointestinal surgical interventions, a bias in compositional shift could be observed for samples from patients which had undergone preoperative PEG BP. As such, patients without any BP had comparable relative abundances in all major phyla both pre-treatment (M group) and 7 days post-treatment (NO group), suggesting that pre-operative BP impacts bacterial composition in addition to antibiotic treatment.

Both groups (M and T) present high inter-sample variations, with no clear segregation, although M type samples cluster together more readily, suggesting that the M group has a more homogenous composition than the T group. Intra-group dispersion was significantly different between M and T groups. A larger dispersion in the T group suggests that samples which have received cefuroxime treatment tend to have a more individualized community composition than samples which have not.

Differential abundance analysis allowed for the identification of taxa for which abundance differed significantly between groups. After clustering at genus level, the phyloseq object which was subjected to analysis contained 359 unique genera. Notably, intersection of genera identified, resulted in 7 differentially abundant genera between M and T, which could be further discriminated in the T group by the applied preoperative bowel cleansing method

Our results found significant intra-group dispersion differences between before (M) and 7 days post-antibiotic treatment (T) groups. In particular, our study found that three genera are most likely to still be depleted 7 days post-cefuroxime treatment, namely Faecalibacterium,

Ruminococcus_2 and Ruminococcae_UCG-002. All three are gram-positive and belong to the Firmucutes phylum, Clostridia class and Ruminococcaceae family, and Faecalibacterium in particular has been shown to promote short fatty acid chain production which can influence intestinal homeostasis through anti-inflammatory cytokine production increase and pro-inflammatory cytokine production decrease. In addition, species belonging to the genus Ruminococcus seem to be consistently present in the healthy human gut, suggesting that they play a significant role in maintaining a normal environment in the gut, and as such, it is not surprising that they are impacted to a certain extent by antibiotic administration. Cefuroxime is also known to cause lower rates of sensitivity in cases of Eneterobactericeae and Streptococcus-induced peritonitis. Our results show that cefuroxime administration permits the repopulation of gut microbiome 7 days post-antibiotic treatment, but only in cases where patients did not undergo a perioperative mechanical bowel preparative procedure. It has been previously reported that bowel cleansing can induce temporary changes in the gut microbial composition, particularly in the relative abundance of Proteobacteria, which corroborated with a loss in overall species richness, can suggest a more efficient repopulation of depleted niches by bacteria of this phylum. Indeed, a recent study showed a significant increase in Proteobacteria and decrease in Firmicutes immediately after colon cleansing with PEG, but recovery of the microbiota to resemble pre-BP condition one month after BP. As such, the observed increase in Proteobacteria for our samples could indicate that the colon has not yet had the time to repopulate to its pre-BP state, and not necessarily an infection caused by the identified differentially abundant opportunistic bacteria. However, the lack of data regarding later post-intervention timepoints urges for future investigations in order to determine if the identified differentially abundant taxa indeed cause SSI or are outcompeted by commensal bacteria in time.

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 constitute 0.15% and 2.16%, respectively, of abundance in cohort M. (***Fig…***).

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 genus (cohort M) out of 210 exceed a 1% presence and 18 genus 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.

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.

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. 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.

CONCLUSIONS

Antibiotics have significant and occasionally long-lasting impact on the intestinal microbiota, inducing a decrease in the quantity of commensals that are beneficial and an increase in commensals that could become harmful. The use of probiotics and antibiotic treatment can be tailored to reduce this "collateral damage" if these effects are better understood.

The choice of DNA extraction technique plays a crucial role in the observed microbial diversity in microbiome studies. Employing a protocol that involves both mechanical and chemical lysis will lead to obtaining utmost species diversity and abundance in all samples.

The added benefit of a metagenomic approach to gut microbiome analysis is that it not only characterizes the microbial community, but also sheds light on its possible physiological effects on the human host. Additionally, metagenomic sequencing allows the detection of dysbiosis events in samples, as well as measurement of the abundance of different bacterial taxa inside a sample.

In our study group of surgical oncological patients, intestinal microbiota com-position was not significantly changed one week post cefuroxime treatment when com-pared to pretreatment condition for patients without mechanical bowel preparation, but some loss in taxonomic variety could be observed. Taken together, cefuroxime does not promote long-term dysbiosis in surgical patients without any additional perioperative procedures.

In samples collected in Institute of Oncology of Republic of Moldova 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.