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SHORT COMMUNICATION

Fecal Microbial Transplantation impact on gut microbiota composition and metabolome, microbial translocation and T-lymphocyte immune activation in recurrent *Clostridium difficile* infection patients

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SUMMARY

This short communication reports the preliminary results of Fecal Microbial Transplantation (FMT) impact on microbiota, microbial translocation (MT), and immune activation in four recurrent *Clostridium difficile* infection (R-CDI) patients. After FMT a restore of gut microbiota composition with a significant increase of fecal acetyl-putrescine and spermidine and fecal acetate and butyrate, a decrease of immune activation of T cells $CD4^+$ and $CD8^+$levels, and of LPS binding protein (LBP) level, were observed. Preliminary results indicate that FMT seems to be helpful not only as a CDI radical cure, with an impact on fecal microbiota and metabolome profiles, but also on MT and immune activation.

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Fecal microbiota transplantation (FMT) is the most effective therapy for refractory, recurrent *Clostridium difficile* infection (R-CDI) (Li YT *et al.*, 2016; Cammarota *et al.*, 2017). The initial effect of FMT is the reconstitution of a healthy microbial ecosystem in the gut (Khanna *et al.*, 2016; Weingarden *et al.*, 2014) that, by reducing levels of pro-inflammatory cytokines such as TNF-α, IL1β or IL-8 (Konturek *et al.*, 2016), determines a rapid attenuation of colonic mucosa inflammation. However, the mechanisms leading to FMT R-CDI clinical remission of extra-intestinal inflammatory reactions are more complex and not fully clarified. Namely, microbial translocation (MT) and their products through the CDI-damaged intestinal mucosa, expressed by different cytokines, such as soluble CD14 (sCD14), lipopolysaccharide (LPS), LPS binding protein (LBP) in the plasma, and associated with multiple infective and non-infective diseases (Brenchley JM *et al.*, 2012), has not been investigated in R-CDI patients submitted to FMT. Because bacteria regulate host metabolic and immune functions (Kim D *et al.*, 2017) by their metabolites, such as short chain fatty acids (SCFAs) and polyamines, it could be possible that the restore of gut microbial ecosystem induced by FMT positively affects circulating immune cells, T-lymphocyte activation and differentiation. Acetate that enters the systemic circulation has an effect on the immune function of monocytes, T cells, and neutrophils (Sivaprakasam S *et al.*, 2017) as well as on antioxidant-oxidant balance and cytokines production in several immune cells (Kamp ME *et al.*, 2016; Nadeem A *et al.*, 2017; Björkman L *et al.*, 2016;Vinolo MA *et al.*, 2011). Butyrate offers an energy reserve to colon epithelial cells and might compensate for its CDI-induced reduction, which is associated with increased barrier permeability and MT (Peng L *et al.*, 2009). Polyamines take part in cellular functions at different levels. Increased concentration of putrescine in the colon, and of spermidine and spermine in the blood, after oral administration of arginine in mice, represses inflammatory cytokines production (Kibe R *et al.*, 2014). *Clostridium difficile* infection (CDI) might therefore be associated with a certain grade of MT and immunity adaptation.

The present on-going study aims to evaluate whether FMT had an impact on modulation of MT and immunity, and the changes in metabolites profiles that could occur after FMT. To this purpose gut microbiota composition, short-chain fatty acids (SCFAs) and polyamine profiles, MT soluble markers and systemic T-cell activation in R-CDI be-

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fore and after one week from FMT, were assessed. The results presented in this short communication relate to the first four R-CDI patients (3F/1M; median age 72y, range 62-83y). The study was approved by Sapienza University Ethical Committee (n. 3336), and written informed consent was obtained from the patients in our center.

Donor (<30 years of age) screening and sample preparation were performed according to Cammarota group (Cammarota *et al*., 2017). A specific control step on the donor's fecal microbiota *eubiosis* status was added to the protocol. For this purpose, the relative abundances of Bacteroitedes, Firmicutes and Enterobacteriaceae were evaluated in donor fecal samples by qPCR with specie specific primer. For total bacteria Fw GTGSTGCAYGGYTGTCGTCA, and Rw ACGTCRTCCMCACCTTCCTC (Maeda H *et al*., 2003); for Bacteroitedes phylum Fw GGARCATGTGGTTTAATTCGATGAT and Rw AGCTGACGACAACCATGCAG; for Firmicutes phylum Fw GGAGYATGTGGTTTAATTCGAAGCA, and Rw AGCTGACGACAACCATGCAC (Guo X *et al.*, 2008); for the Enterobacteriaceae family Fw, and Rw CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC (La-Ongkham O *et al*., 2015). When the relative abundances of Bacteroitedes and Firmicutes phyla accounted for about 80% of the total bacteria, the donor fecal microbial ecosystem was considered in eubiosis status (Mariana X Byndloss *et al*., 2017). FMT was performed via retention enema after oral vancomycin (500 mg every 6 hours) for four days and polyethylene glycol-based colonic lavage (4L) the day preceding the procedure. Loperamide (4 mg) was given 3 hours prior to the infusion in order to facilitate the retention of transplanted material. Fecal samples were collected before (T0) and after one week from FMT (T1).

Gut microbiota composition of fecal samples collected were evaluated by next generation sequences (NGS) through 16S rDNA V3-V4 targeted sequencing by BMR Genomics on the Illumina MiSeq platform. Total DNA was extracted with DNeasy fecal kit (Qiagen, Hilden, Germany) from collected fecal samples according to the manufacturer's instructions. 'FASTQ' sequences obtained were further analyzed to perform study population and correlation among all data collected. Dedicated pipelines for filtering (sequencing errors, chimerae) and grouped into Operational Taxonomical Units (OTUs), at 97% of similarity, using standardized parameters and SILVA rDNA Database v.1.19 for alignment, were used. Raw 'FASTQ' files from NGS analysis filtered were processed through Mothur v1.95 pipeline to perform microbiota compositional analysis along with alfa- and beta-diversity. Statistical analysis for comparisons was performed with the nonparametric Mann–Whitney U-test. Lefse analysis was implemented to reveal discriminant species in all cohorts studied (Segata N *et al*., 2011). LDA score represents the importance of a definite species within the cohort.

Short-chain fatty acids (SCFAs)/polyamine profiles were analyzed, after a solid phase micro-extraction (SPME), by Gas-Chromatography coupled to mass spectrometry GC-MS (Tumanov S *et al*., 2016), before (T0) and after (T1) FMT. Statistical analysis for metabolite profiles were performed using one-way ANOVA with Tukey's post-hoc test.

Lymphocyte surface phenotypes were evaluated before (T0) and after (T1) Fecal Microbiota Transplantation (FMT) by flow cytometry (MACSQuant Analyser with 8 channels) using fresh peripheral blood. Data were analyzed by using the FlowJo V10 Software. Immune activation

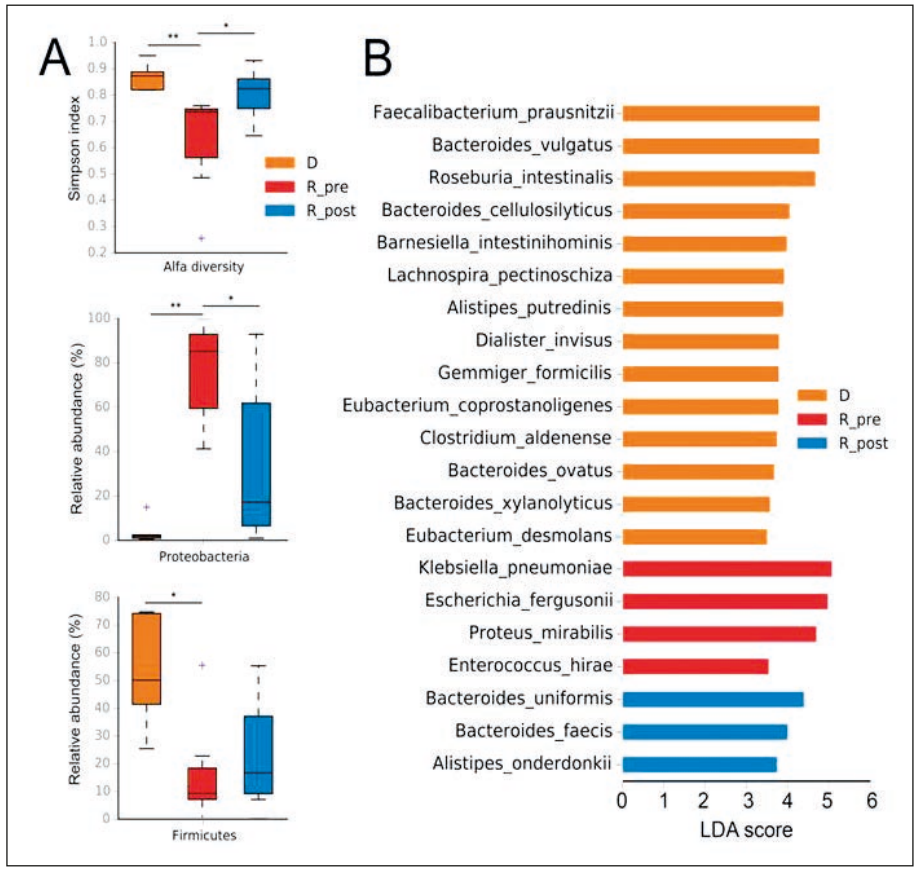

Figure 1 - *Microbial compositional analysis.*
*Panel A reports alfa-diversity among the three cohorts studied (donors - D, pre-transplant - R_pre, post-transplant - R_post), along with differences among Proteobacteria and Firmicutes phyla. Asterisks report significant Mann-Whitney U test comparisons (*P=0.05, **P<=0.01). Lefse analysis reports discriminant species within the three cohorts.*

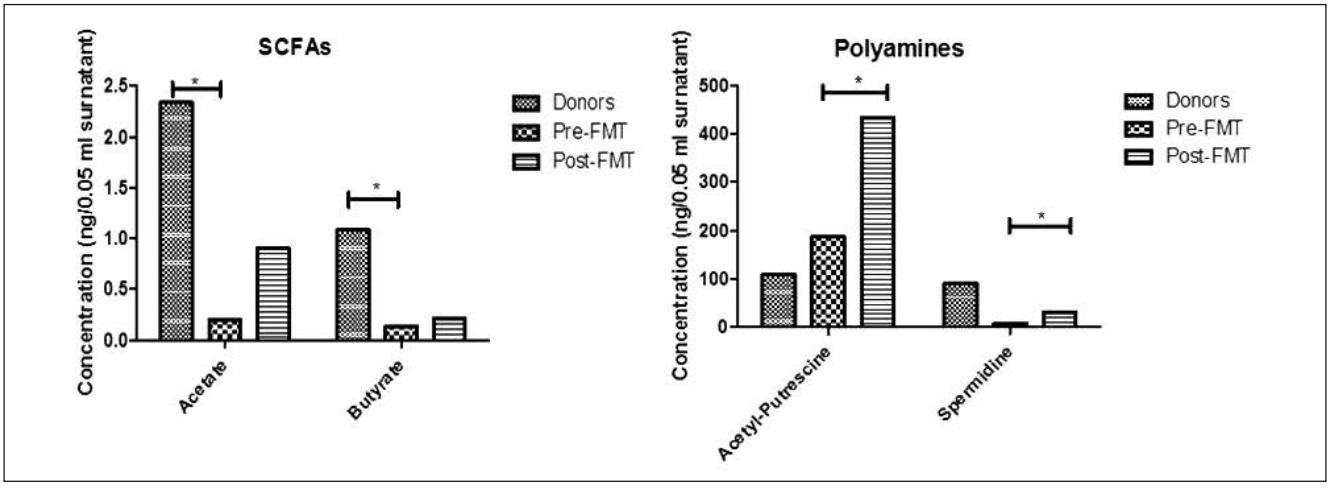

Figure 2 - *Results of SCFAs and polyamines profiles.*
*SCFAs (acetate and butyrate) were significantly reduced in patients with R-CDI compared to donors. Polyamines (acetyl-pututrescine and spermidine) showed a significant increase after FMT. *indicate a statistically significant differences (P<0,05).*

was defined as the presence of HLADR$^+$ CD38+ on T CD4$^+$ and CD8$^+$ cells surface. For MT markers, plasma samples were collected from EDTA whole blood samples within 6 hours of collection with centrifugal force of 1000g for 20 mins, then removed and stored at -80°C until the enzyme-linked immunosorbent assays (ELISA) for sCD14 and LPB were performed. Samples were collected immediately before (T0) and 7 days after transplantation (T1). Experiments were performed in duplicate. Spearman test assessed the correlation between different soluble markers and T-cell CD4$^+$ and CD8$^+$ immune-activation. Quantitative variables were analyzed with Mann Whitney and Wilcoxon tests, as appropriate. Differences with a P value less than 0.05 were considered to be significant.

All patients experienced resolution after a single treatment, expect for one patient who required two FMTs. As already reported by other studies, after one week, FMT Proteobacteria relative abundance was significantly decreased (P=0.005) and beneficial phylum such as Firmicutes, was increased (*Figure 1*, panel A). Simpson index increased significantly in the post-transplant group compared to the pre-transplant group (P<0.05 for Simpson index) (*Figure 1*, panel A). Lefse analysis, performed to determine the most important species for characterizing the two groups, evidenced that species belonging to Proteobacteria phylum were characterizing in pre-transplant groups, while the post-transplant group was characterized by species belonging mostly to Bacteroitedes phylum (*Figure 1*, panel B)**.** With respect to donors, acetate (P=0.0054) and butyrate (P=0.0362) were significantly reduced in R-CDI patients fecal samples collected at T0, while acetyl-pututrescine (P=0.04) and spermidine (P=0.0337) were significantly increased after FMT in patients (*Figure 2*).

The levels of immune activation of T cells CD4$^+$ and CD8$^+$ were elevated at T0 and decreased, albeit not statistically significantly, after FMT. LBP at T0 (median 17335 ng/mL, range 14950-63100) were higher, though not statistically significant, than at T1 (median 15885 ng/mL, range 13050-61030, p=0.37), whereas sCD14 values were similar at T0 and T1 (median 1890 ng/mL, range 1351-2274 versus median 1783 ng/mL, range 1239-2754), p=0.99. There was a trend for a direct correlation between LBP and CD4$^+$ HLA-DR$^+$ CD38$^+$ at T0 (p=0.08) and for an inverse correlation between LBP and CD8$^+$ HLA-DR$^+$ CD38$^+$ at T1 (p=0.08), whereas no relationship was found between sCD14 and T-cell immune activation.

Any therapeutic protocol should be screened to observe the effect not only on the direct cause of the disease, but with a broad-spectrum vision. This is the first study to assess the effect–of FMT in R-CDI patients, not only on gut microbiota composition and the presence of the infecting agent (*C.difficile*), but also on MT, immune activation of T lymphocytes and metabolomics profiles of fecal samples, collected before and after the procedure. In these preliminary observations, conducted in four R-CDI patients receiving FMT, the reduction of LBP appears to be a marker of MT. The levels of immune activation of T cells CD4+ and CD8+were elevated at T0 and decreased after FMT. Furthermore, a trend for a direct correlation between LBP and CD4+ HLA-DR+ CD38+ at T0 (p=0.08), and for an inverse correlation between LBP and CD8+ HLA-DR+ CD38+ at T1 (p=0.08) was also observed. The intestinal mucosal alteration, triggered by CDI, can induce some degrees of MT (expressed as LBP plasma level), and a subsequent T-CD4+ immune-activation. A correlation would seem to exist between the degree of MT and the immune response. The FMT impact on microbiota composition and ecological indices, measuring the effectiveness and estimating the unwanted outcomes on the microbial community (Lemon KP *et al.*, 2012), indicates this correlation in our patients. Fecal metabolomics profiles reveal a strong increase of acetyl-putrescine and spermidine, and of Acetate and Butyrate in fecal samples after FMT. Polyamines, such as spermidine, are involved in regulation of the immune response and are useful for inhibition of chronic inflammation due to CDI infection (Babbar N *et al.*, 2007; Larqué E *et al.*, 2007). On the other hand, polyamines are also increased in solid tumor cells as well as in various precancerous manifestations, and the increase observed could be an alarm bell that should not be underestimated (Wallace HM *et al.*, 2003; Kingsnorth AN *et al.*, 1984; Milovic V *et al.*, 2003). Further investigations are necessary to evaluate the real significance of the polyamine increase after FMT. The SCFAs increase observed in all fecal samples after FMT indicate the positive effect on human health (Wong JM *et al.*, 2006). FMT is not only helpful

as a radical cure of CDI and to rebuild gut microbiota, but also seems to have an effect as an immunomodulatory mediator able to positively affect the degree of MT and T-cell immune-activation. Results reported in this short communication require further confirmation on a larger population, but evidenced the importance of carrying out studies that take into account the different effects (positive or negative) that FMT can have, in order to better estimate the results of therapy.

Conflicts of interest

The authors declare that they have no competing interests.

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