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METAGENETIC APPROACH TO EXPLORE THE GUT MICROBIOTA AND THEIR SECRETED EXTRACELLULAR VESICLES IN DIARRHEIC AND HEALTHY PATIENTS

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INTRODUCTION

The release of membrane-bound vesicles is a conserved cellular process. Gram-positive and Gram-negative secrete nanometer-scale **extracellular membrane vesicles (EMV)** with important biological functions, including **immune-response regulation**, **long distance transport of virulence factors**, **lateral transfer of antibiotic resistance genes**, or **RNA transfer agents**, among others. For ***Clostridium difficile*** *(C. difficile)*, these vesicles have been associated with the infection (**CDI**), since they can induce the expression of pro-inflammatory genes and epithelial cells cytotoxicity.

PURPOSE

The aim of this study was to evaluate **the microbial diversity of feces and secreted EMV** in **healthy patients**, **diarrheic patients** and **patients with CDI.** The link between microbiota composition and their derived EMV could reveal newt insights into the **microbial activities in the host**. Furthermore, the identification of these changes opens up new possibilities of **disease diagnostic and assessment**.

METHODS

C. DIFFICILE DETECTION

Fresh faecal samples of all patients were tested for the presence of ***C. difficile*** using:

- ✓ chromatographic immunoassay rapid test
- ✓ rapid PCR
- ✓ classical microbiological culture

Further confirmation and characterisation of the isolates was performed by classical PCR with the detection of the *tpi* gene, and the toxin genes *tcdA*, *tcdB* and *cdtA*.

EMV ISOLATION

Vesicles isolation was performed by

- ✓ **faecal dilution and centrifugations** (5000-5600 rpm 30-40 min 4ºC x 3 times)
- ✓ **supernatant filtrations** (filters of 0.45 µm and 0.22 µm)
- ✓ **ultracentrifugations** (130.000g 180 min 4ºC)

The vesicles concentrations and sizes were analysed using the Nanosight technique, with the technology of nanoparticle tracking analysis (NAT).

METAGENETIC ANALYSIS

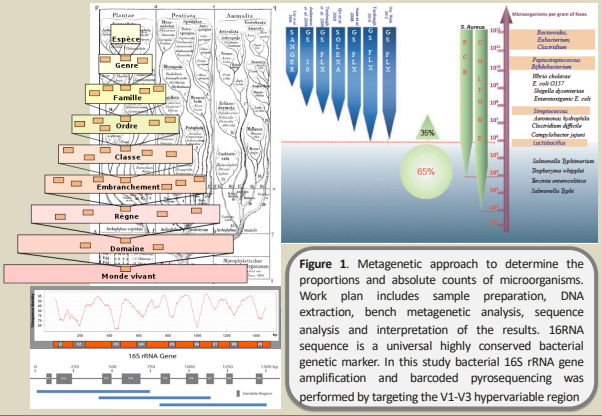


Figure 1. Metagenetic approach to determine the proportions and absolute counts of microorganisms. Work plan includes sample preparation, DNA extraction, bench metagenetic analysis, sequence analysis and interpretation of the results. 16RNA sequence is a universal highly conserved bacterial genetic marker. In this study bacterial 16S rRNA gene amplification and barcoded pyrosequencing was performed by targeting the V1-V3 hypervariable region

RESULTS

Figure 2. **(a, b)** Vesicles (V) composition versus faecal microbiota (F) composition at genus level in a group of 10 patients with diarrhea **(c)** Vesicles (V) composition versus faecal microbiota (F) composition at genus level in a group of 10 healthy patients and 10 patients with diarrhea **(d)** Statistical differences using 2-way ANOVA and Sidak multiple comparisons test at genus level between faecal microbiota composition and vesicles composition of diarrheic patients **(e)** Multiple comparisons between the different groups at genus level (i) feces healthy (ii) feces diarrhea (iii) vesicles diarrhea (iv) vesicles healthy. Statistical differences were found in taxa *Alistipes*, *Bacteroides*, *Holdemia*, *Barnesiella*, and *Veillonellaceae* (difference in mean proportions %).

High throughput sequencing results: diarrhea vs *C. difficile* diarrhea

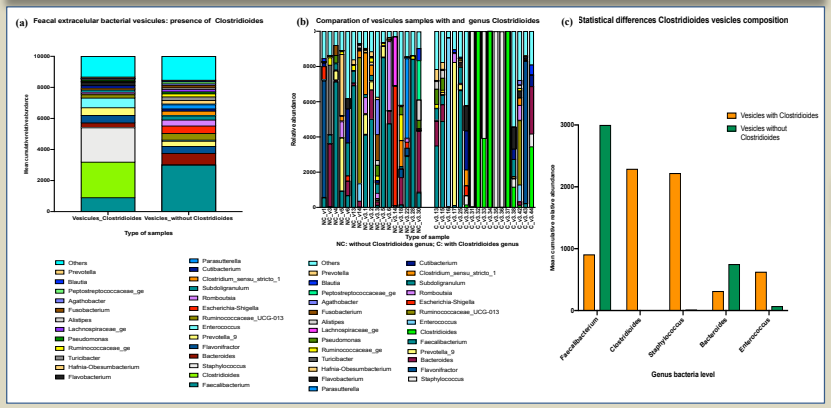
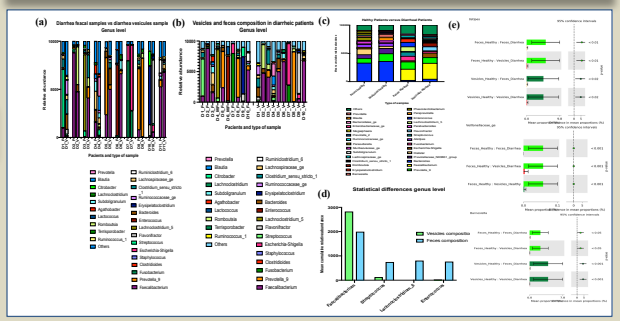


Figure 3. (a) Mean cumulative relative abundance at genus level. Vesicles with Clostridioides versus Vesicles without Clostridioides (n=17) **(b)** Microbiota composition at genus level in a group of 17 patients. Vesicles with *Clostridioides* versus vesicles without *Clostridioides* **(c)** Statistical differences using 2-way ANOVA and multiple T test comparisons were found at genus level when we compare vesicles composition with and without *Clostridioides* from diarrheic patients. *Faecalibacterium,* and *Bacteroides* are reduced when proportions of *Clostridioides, Staphylococcus and Enterococcus* are high

High throughput sequencing results: healthy vs diarrhea



Characterization of the isolated EMV

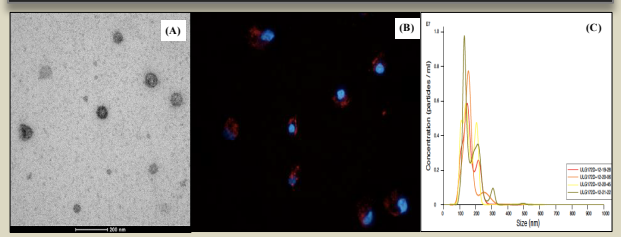


Figure 4. A) Electron microscope image of EMVs **B)** Fluorescence microscope photography. Cell nucleus (blue) and bacterial vesicles (red) **C)** Characterization and quantification of EMVs by Nanosight technology

CONCLUSIONS

EMV were enriched in the 3 groups of patients, but their composition differed significantly between them. Regarding global differences between feces and EMV, *Lachnoclostridium* and *Streptococcus* were more abundant in feces, but their vesicles production was limited and dominated by *Faecalibacterium*. At genus level, proportions of *Clostridioides*, *Staphylococcus* and *Enterococcus* were significantly higher in vesicles from CDI patients than in the other groups. These findings suggest that the increased production of EMV by these taxa could be associated with the dysbiosis establishment, and therefore with the development of the infectious disease. More extensive research to investigate the specific role of the identified EMV in the CDI is now warranted.

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