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LETTER TO THE EDITOR

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# Predicting and preventing intestinal dysbiosis on the basis of pharmacological gut microbiota metabolism

The possible relationship(s) between intestinal dysbiosis and various degenerative intestinal-based diseases, e.g. diabetes and obesity, is an ongoing focus of intense investigation.[1-4] As a consequence of the results of such research, the focus is now on therapeutic strategies for such diseases based on modification of the gut microbiota.[2,4] Fecal transplantation is one such strategy, which was suggested by Zhang and co-workers[2] as a treatment for diabetes mellitus and by Kang and co-workers[4] for treatment of obesity. Kang and co-workers also reported different strategies involving fecal gut microbiota transplantation to treat obesity. Other studies correlated other degenerative diseases to gut microbiota activities and suggested therapeutic strategies to alleviate those disease states.[1]

This interesting prospect, i.e., fecal gut microbiota transplantation to combat intestine-related diseases, has led to the development of methods by different research groups to evaluate the gut microbiota status in human subjects.[5-8] These approaches can be classified as:

a. Untargeted approaches that search for bacteria responsible for dysbiosis without any prior knowledge of which types of bacterium might be involved.[5,6]
b. Targeted approaches that search for specific pathogens and the correlation of these to gut microbiota-related dysbiosis.[7,8]

One example of an untargeted approach is that of Pinheiro de Oliveira and co-workers who developed a method that detects thousands of different species using DNA gene amplification.[5] For the aforementioned DNA approaches, it is difficult to determine a correlation between the presence of a bacterial species and its involvement in intestinal dysbiosis without knowledge of how its metabolism affects the intestinal environment.[5,6]

The current targeted approach incorporates the classical means by which pathogenic bacteria are detected, i.e., extraction of bacteria from fecal matter followed by culture on a specific medium that supports the viability of many different strains of bacteria.[7,8] Once the bacteria colonies have become visible (in ~2 days), they are identified usually by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[7,8] with their molecular pathways by database searching to confirm their identity.

Both untargeted and targeted approaches suffer from a lack of documentation of specific bacterial metabolic activities that can be used to evaluate the extent of gut microbiota-related dysbiosis. An approach to obtain this data has been the bacteria-toxin direct search.[9,10] Basically, this search is based on the detection of known bacteria toxins and metabolites. However, bacteria toxins are usually present at low level and, in particular, the development of gut microbiota-related dysbiosis[9] limits the applicability of this approach to that are highly sensitive, and thus costly, instruments.

All the current methods can be efficiently used in clinics; however, their main limitation is that they detect the presence of bacteria; whereas they do not consider complex microbiota metabolomic interactions (quorum sensing)[10] that can provide useful information on the pathogen causing dysbiosis, the developmental stage of dysbiosis, and potential therapeutic strategies. When a population of pathogen bacteria induces intestinal dysbiosis, the antagonist microorganism(s) produces quorum-sensing molecules that lead to specific control of the pathogen population (e.g., bacteria grown inhibitor).[11] Such antagonist quorum-sensing activity occurs even during the development of dysbiosis,[12,13] making it possible to re-equilibrate the environment to a healthy state before symptoms appear.[10]

Herein, we introduce a new method to increase the prediction efficiency of intestinal dysbiosis detection, based on the antagonist and pathogen bacteria pharmacological activity. Basically, feces molecular profiles are analyzed by liquid chromatography/surface-activated chemical-ionization/electrospray ionization (LC/SASI-MS) and tandem mass spectrometry (MS/MS)[14-16] in randomized data-dependent scan acquisition mode.[17] The antagonist and pathogen pharmacological active metabolites are correlated to latent dysbiosis on the basis of literature data. The molecular biomarker fingerprints are used to create a database to be routinely used for bacteria identification.

Feces from five apparently healthy subjects were subjected to metabolomic screening. Upon testing, one subject was found to be strongly positive for dysbiosis caused by *Clostridium* sp. (dysbiosis level degree = 80%). The description of our method and the data obtained from our study are shown and disclosed in detail below.

Methanol, acetonitrile, doubly distilled water, and formic acid were purchased from Sigma Aldrich (Milan, Italy).

Feces from three apparently healthy males and two apparently healthy females were used in the study and were collected in plastic tubes. 1 g of feces from each volunteer was extracted in 50 mL of methanol. Each mixture was then centrifuged, and 95 µL of each supernatant was treated with 5 µL of Agilent Tuning Mixture (Agilent Technologies, Santa Clara, CA, USA). Then, 100 µL of $H_2O$ was

  

added into each mixture. The solutions were subjected to LC/SASI-MS and MS/MS conditions as described below.

An Ultimate 3000 ultra-performance liquid chromatography (UPLC) system (Thermo Fisher, San Jose, CA, USA) was employed to obtain analyte separation of each sample before mass spectrometric analysis. A C-18 reversed-phase chromatographic column (50 × 2.1 mm; particle size, 5 µm; pore size, 100 Å; Phenomenex, San Jose, CA, USA) was used for the separations. The mobile phases were: (A) 0.2% (v/v) HCOOH and (B) $CH_3CN$. The composition of the elution gradient was 2% (v/v) B between 0 and 2 min; 2 to 30% B between 2 and 7 min; 30 to 80% between 7 and 9 min; 80% B between 9 and 12 min; 80 to 2% B between 12 and 12.1 min; and the column was equilibrated with 2% B between 12.1 and 17 min. The eluent flow rate was 0.25 mL/min and the injection volume was 15 µL.

Mass spectrometry was performed using a HCT ion trap spectrometer (Bruker Daltonics, Bremen, Germany) coupled to a SACI/ESI source (ISB, Milan, Italy) and operated in positive ion mode. Full-scan mass spectra were acquired between *m/z* 50 and 1000. The ion source parameters were: ESI capillary voltage, 2750 V; SACI surface voltage, 47 V; drying gas (nitrogen) flow rate, 12 L/min; nebulizer gas (nitrogen) pressure, 60 psi; and drying gas temperature,

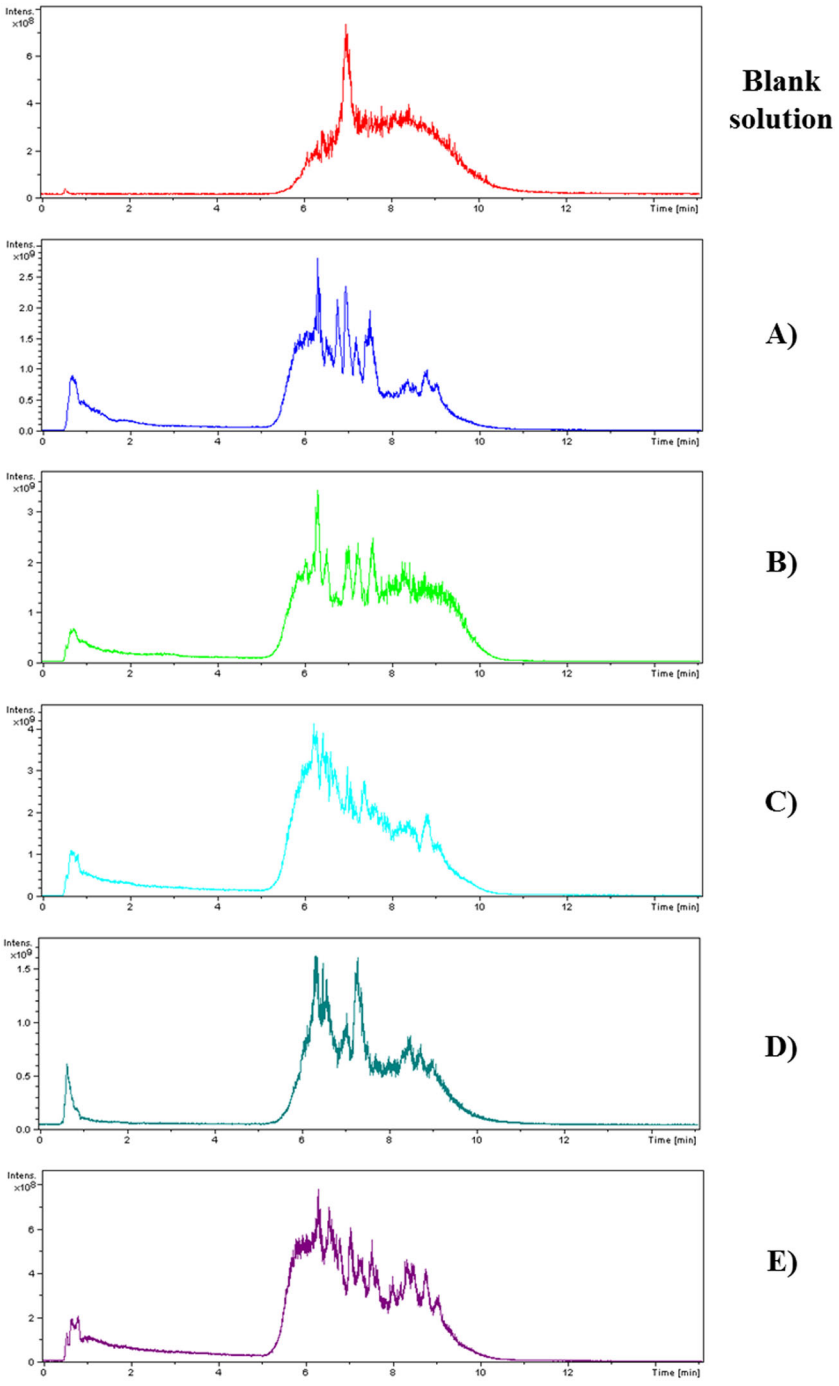


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**FIGURE 1** Total ion current mass chromatographs of: A, water sample (blank solution; top chromatogram) and the extracts of the feces from our five subjects (A–E) [Color figure can be viewed at wileyonlinelibrary.com]

**TABLE 1** Molecular fingerprint potentially produced by gut microbiota antagonist vs *Clostridium* sp. and by the pathogen

| ID | Compound | Detected full scan ion | Pathogen bacteria | Origin | Biological activity |
|---|---|---|---|---|---|
| 1 | 2-methoxyestradiol | $[M+H]^+$ | *Clostridium difficile* | Different antagonist intestinal bacteria | Selective activity vs *Clostridium difficile* superoxide dismutase |
| 2 | Deoxycholic acid | $[M+H]^+$ | *Clostridium bifermentans* | Produced by the pathogen | Bile acid transformation |
| 3 | Ursodeoxycholic acid | $[M+H]^+$ | *Clostridium sordellii* | Produced by the pathogen | Bile acid transformation |
| 4 | Dexamethasone | $[M+H]^+$ | *Clostridium difficile* | Cladosporium sp antagonist | Toxin B inhibitor |
| 5 | Pinolenic acid | $[M+H]^+$ | *Clostridium perfringens* | Eubacteria Lactobaccilus antagonist | Antibacterial activity |

300°C. The ion trap vacuum pressure was $2.5 \times 10^{-5}$ Torr. MS/MS data were acquired in data-dependent scan mode[17] with dynamic exclusion enabled. For each isolated chromatographic peak, its most abundant *m/z* peak was isolated twice and then added into a dynamic exclusion list for 60 s. During this time, the peak was not fragmented. The MS/MS isolation width was 1.5 *m/z* units The collision-induced dissociation (CID) activation voltage was 80% of its normalized value (1 V peak to peak).

Compounds were identified using a first NIST database search[18] screening followed by European Union database match mode (EU directive 2002/657/EC).[15] The dysbiosis level expressed as a percentage is calculated using Equation 1:

$$Ci = ((Fi-1)/\Sigma j\ (Nt-1))^*100 \quad (1)$$

where Ci is the dysbiosis percentage contribution of the i-th pathogen bacterium, Fi is the frequency associated with antagonist and pathogen metabolic biomarkers and Nt is the total number of detected metabolites associated to all the detected bacteria.

The identities of the bacteria causing the dysbiosis were confirmed in the MultiMedica Research and Cure Scientific Institute (IRCCS, Milan, Italy) Laboratory, by means of culture growth.

The first step in the method is to obtain fecal metabolites by means of a methanol extraction.[19] After that data acquisition takes place. Figure 1 shows the total ion current mass chromatographs (LC/SASI-MS and MS/MS) of a 1:1 $H_2O/CH_3OH$ blank solution (top chromatogram) and those of the methanol-extracted feces metabolites from our subjects (Figures 1A–1E). As can be seen, the LC/SASI-MS and MS/MS profiles are quite similar, because some of the components found in the feces, e.g., mercaptans, are present in relatively large amounts and, therefore, exhibited strong signals (counts/s, $>1 \times 10^8$). Consequently, MS/MS data were acquired in the dynamic exclusion mode to maximize the number of characterized compounds. In this mode, the most abundant species were fragmented twice. Next, the *m/z* value of each precursor ion was added into a dynamic exclusion list, and the precursor ion was not fragmented for the following 60 s (which was twice the elution-time width of a standard HPLC peak). For compound identification the raw binary MS/MS data were converted into Mascot generic format (Ascii files) using an automated script based on the data acquisition software Dynamic Link Library (DLL) under the Windows operative system. Analytes were identified using a similarity approach and the NIST algorithm.[18] The identified molecules were confirmed using analytical standards of the compounds and the European Union low recognized database match mode (EU directive 2002/657/EC).[15] The dysbiosis contribution percentage is calculated for each identified bacterium using Equation 1. A metabolic molecular fingerprint-pattern strategy[9] was, therefore, used to identify pathogens that potentially could cause dysbiosis in both apparently healthy and sick individuals.

A strong dysbiosis was detected in one of the subjects under study. In this case, the NIST database search showed the presence of the molecular fingerprint reported in Table 1. All the precursor ions were detected as $[M+H]^+$ species. These molecules could be potentially produced by the antagonist and pathogen (*Clostridium* sp.) bacteria. As an example, two interesting compounds detected are dexamethasone and 2-methoxyestradiol. Dexamethasone is

**TABLE 2** Example of a report output obtained by determining the dysbiosis status of an apparently healthy subject

| ID | Bacteria | Detection frequency | Dysbiosis contribution % |
|---|---|---|---|
| 1 | *Clostridium*, *clostridium* genus (species related to *C. bifermentans*; *C. sordellii*; *C. leptum*), *Clostridium perfringens*, *Clostridium difficile* | 5 | 80 |
| 2 | Bacteroides, Bacteroides genus | 2 | 20 |
| 3 | Intestinal anaerobic bacteria | 1 | - |
| 4 | Intestinal bacteria | 1 | - |
| 5 | *Eubacterium* sp. | 1 | - |
| 6 | *Xanthomonas* | 1 | - |
| 7 | *Collinsella* | 1 | - |
| 8 | *Ruminococcus* | 1 | - |
| 9 | *Eggerthella* | 1 | - |
| 10 | Non-selectively isolated strains (HD-17, b-8 e c-25) | 1 | - |

**TABLE 3** Results achieved by accessing the dysbiosis status of five subjects

| Subject | Bacteria | Dysbiosis contribution % | Risk factor based on skatole and indole urinary level (low – medium – high) |
|---|---|---|---|
| 1 | *Clostridium*, *Clostridium* genus (species related to *C. bifermentans*; *C. sordellii*; *C. leptum*), *Clostridium perfringens*, *Clostridium difficile* | 80.0 | High |
| | Bacteroides, Bacteroides genus | 20.0 | |
| 2 | *Staphylococcus aureus* | 35.8 | Low |
| | Intestinal microflora, intestinal microbiome, colonic bacteria | 35.8 | |
| | *Shigella* spp., *Shigella flexneri* | 7.1 | |
| | *Vibrio cholerae*, *Vibrio parahemolyticus* | 7.1 | |
| | *Bacillus* spp., *Bacillus cereus* | 7.1 | |
| | *Pseudomonas aeruginosa* | 7.1 | |
| 3 | *Bifidobacterium adolescentis*, *Bifidobacterium breve*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium longum* | 45.5 | Low |
| | Intestinal microflora, intestinal bacteria, intestinal anaerobic bacteria, fecal bacterial flora | 27.3 | |
| | *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus acidophilus* | 18.2 | |
| | *Staphylococcus aureus* | 9.0 | |
| 4 | *Pseudomonas* (*Pseudomonas citronellolis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas miyamizu*) | 33.3 | Low |
| | *Staphylococcus* (*Staphylococcus sa-prophyticus*, *Staphylococcus aureus*) | 25.0 | |
| | *Escherichia* (*Escherichia fergusonii*, *Escherichia coli*) | 25.0 | |
| | Lactic acid bacteria (*Lactobacillus plantarum*) | 16.7 | |
| 5 | *Staphylococcus aureus* | 66.6 | |
| | *Pseudomonas* – *Pseudomonas aeruginosa* | 16.7 | Low |
| | *Lactobacillaceae lactobacillus* | 16.7 | |

usually classified as a steroid-derived metabolic active drug. The metabolic production/conversion of this compound class can be catalyzed by gut microbiota bacteria[20,21] and it exhibits an action vs *Clostridium difficile*.[22] Basically, it inhibits the biological activity of toxin B of *Clostridium difficile*. The second compound, 2-methoxyestradiol, can be produced by intestinal gut microbiota[23] and it exhibits a specific activity vs *Clostridium difficile* through the inhibition of superoxide dismutase and providing valuable benefits in curing *Clostridium difficile*.[24] This strongly suggests that these compounds could be produced by the antagonist bacteria. To confirm the presence of *Clostridium* sp., a list of compounds derived from the direct metabolism of the pathogen was added to the biomarker list (Table 1). These substances are all derived by bile acid trasformation.[25] An example of a report obtained by determining the dysbiosis status of an apparently healthy subject is shown in Table 2. As can be seen, in this case the presence of potential dysbiosis due to *Clostridium* sp. is obtained with a confidence of 80%. Validation tests performed in the hospital confirmed the presence of the detected pathogens only after 3 months from the first detection when the infection symptoms became evident.

The shown procedures were applied to all the five subjects involved in the study. The dysbiosis prediction results confirmed by the official hospital institution are shown in Table 3. The obtained dysbiosis data were integrated with the classical clinical determination based on skatole and indican urinary quantitation (Table 3).[26] Basically, this is a nonspecific test that indicates the dysbiosis degree, on the basis of toxins produced by the intestinal tryptophan metabolism. Subject 1, who was confirmed to be positive to *Clostridium difficile* after 3 months by means of culture grown, exhibits both *Clostridium* sp. prediction and high clinical dysbiosis risk obtained by the classical approach.

Concluding, the new approach based on pharmacological gut microbiota metabolism correlated to antagonist and pathogen bacteria was able to predict human subjects affected by intestinal dysbiosis. The detection efficiency of pathogen bacteria in our subjects that cause intestinal dysbiosis was clinically confirmed using a classical bacterial culture approach. The ideal method application is for an initial screen for the diagnosis of intestinal dysbiosis that is then confirmed by the classical bacterial culture or with DNA-based approaches usually employed in clinics.[7,8] The obtained results suggests that the method could be used for early detection and prevention of dysbiosis.

Future studies will be focused on applying the developed method on a wide sample number with the collaboration of a network of research and clinical institutes. Finally, a specific metabolite fingerprint database, to be used for bacterial dysbiosis identification, will be compiled.

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