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Effect of antibiotic therapy on human fecal microbiota and the relation to the development of *Clostridium difficile*.

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Abstract

The gastrointestinal (GI) tract is a complex ecosystem. Recent studies have shown that the human fecal microbiota is composed of a consortium of microorganism. It is known that antibiotic treatment alters the microbiota, facilitating the proliferation of opportunists that may occupy ecological niches previously unavailable to them. It is therefore important to characterize resident microbiota to evaluate its latent ability to permit the development of pathogens such as *Clostridium difficile*. Using samples from 260 subjects enrolled in a previously published clinical study on antibiotic-associated diarrhea, we investigated the possible relationship between the fecal dominant resident microbiota and the subsequent development of *C. difficile*.

We used molecular profiling of bacterial 16S rDNA coupled with PLS regression analysis. Fecal samples were collected on day 0 (D₀) before antibiotic treatment and on day 14 (D₁₄) after the beginning of the treatment. Fecal DNA was isolated and V6-to-V8 regions of the 16S rDNA were amplified by PCR with general primers and analyzed by Temporal Temperature Gradient gel Electrophoresis (TTGE). Main bacteria profiles were compared on the basis of similarity (Pearson correlation coefficient). The characteristics of the microbiota were determined using Partial Least Square (PLS) discriminant analysis model.

Eighty seven TTGE profiles on D₀ have been analyzed. The banding pattern was complex in all cases. The subsequent onset of *C. difficile* was not revealed by any clustering of TTGE profiles, but was explained up to 46% by the corresponding PLS model. Furthermore 6 zones out of the 438 dispatched from the TTGE profiles by the software, happened to be specific for the group of patients who acquired *C. difficile*. The first approach in the molecular phylogenetic analysis showed related sequences to uncultured clones. As for the 87 TTGE profiles on D₁₄ no clustering could be found either, but the subsequent onset of *C. difficile* was explained up to 74.5% by the corresponding PLS model, thus corroborating the results found on D₀.

The non exhaustive data of the microbiota we found should be taken as the first step to assess the hypothesis of permissive microbiota. The PLS model was used successfully to predict *C. difficile* development. We found that important criteria in terms of main bacteria could be markedly considered as predisposing factors for *C. difficile* development. Yet the resident microbiota in case of Antibiotic-Associated Diarrhea (AAD) has still to be analyzed. Further more, these findings suggest that strategies reinforcing the ability of the fecal microbiota to resist to modifications would be of clinical relevance.

Introduction

The gastrointestinal (GI) tract is a complex ecosystem generated by the alliance of GI epithelium, immune cells and resident microbiota. Experimental systems such as cell culture, germ-free animal models and intestinal isografts have demonstrated that each member of the GI ecosystem can follow a predetermined developmental pathway, even if isolated from the other components of the ecosystem. However, the presence of all three components is required for full physiological function [19]. Genetic or functional alterations of any one component of this ecosystem can result in a broken alliance and subsequent GI pathology. In this work, we will focus only on resident microbiota.

In both health and disease, the colonic microbiota plays an important role in several areas of human physiology [15]. But this complex ecosystem is far from well known [29]. Culture-independent 16S rDNA analyses have previously been used to examine the microbial diversity of the human gut [25] and explorative multivariable analyses of 16S rDNA data to study specific microbial communities [23].

In a previous study Beaugerie *et al.* clarified the role of *C. difficile* in Antibiotic-Associated Diarrhea (AAD) in the community by prospectively studying a population of general-practice patients by means of routine screening for both *C. difficile* and the *C. difficile* toxin B. *C. difficile* was diagnosed by specific culture methods, and *C. difficile* toxin B was detected by its cytopathic effect. As for diarrhoea, it was scored with the help of validated visual support. Beaugerie's study was the first to demonstrate a high rate of acquisition (2,7%) of toxin-producing *C. difficile* during antimicrobial chemotherapy [2].

C. difficile produces two major toxins (toxins A and B). These are thought to be primarily responsible for the virulence of the bacterium and the major contributors to the pathogenesis of antibiotic-associated gastrointestinal disease [4]. Following most antibiotic treatment there will be a point at which the impact on the normal gut microbiota depresses colonization resistance to *C. difficile*. The composition of the pre-existing microbiota may have an important role as well.

Therefore, in view of literature data [7, 16, 21, 26], we judged as particularly promising to investigate the stool of the patients from Beaugerie's previous work. Thus the aim of the present study was to test the hypothesis of predisposing factors slot in the resident microbiota.

We used a genetic fingerprinting method. The characteristics of the microbiota were determined using Partial Least Square (PLS) discriminant analysis model.

Methods

Patients. Our work is an explicative microbiological approach derived from a clinical study published elsewhere [2]. In short, 260 subjects enrolled in the latter study were adult out-patients living in the Paris area, who were prescribed a 5-10 day course of antimicrobial chemotherapy. Criteria for enrolment were prescription by a general practitioner of a 5-10 day course of antibiotics and age 18 years or older. Potential candidates were excluded if they were institutionalized subjects, had received antibiotic treatment during the previous 2 months, had been admitted to a hospital during the previous 6 months, had known human immunodeficiency virus infection, had any allergy, or had had a bout of diarrhea (more than 2 loose stools per day) the day before enrolment. All patients had given their written consent. Each patient was asked to store the last stool before the beginning of the antibiotherapy (D_0), and the stool 14 days after the beginning of the antibiotherapy (D_{14}), in double-thickness containers, and to keep them in a refrigerator or in a cool place until collection by the study monitor [2]. The antibiotics given were classified into 3 classes: class 1, amoxicillin/clavulanic acid; class 2, other beta-lactam agents; class 3, non beta-lactam agents. Among the 260 patients, 11 acquired *C. difficile*. Among the 249 remaining patients without *C. difficile*, 38 were chosen because they developed an AAD and paired with patients with no AAD according to age range (within 10-years) and class of antibiotic. Thus our study included a total of 87 patients. The 11 patients, 3 men and 8 women, with acquired *C. difficile* were from 28 to 73 years old, 5 had taken Pristinamycin, 3 of them Amoxicillin, and 3 of them Amoxicillin/clavulanic acid. Because a typical initial antibiotic dose has no effect on dominant fecal microbiota for at least 8 to 10 hours (data not shown) we considered the dominant microbiota profile on D_0 as the profile at equilibrium for each patient.

DNA isolation, 16S rDNA amplification. Stool samples were collected in sterile tubes and immediately stored at -80°C until analysis. Total DNA was isolated from fecal samples by using the bead beating method [28]: Immediately after collection, total DNA was extracted from a 125-mg fecal sample aliquot and purified as described by Godon et al.[10] The DNA concentration and its integrity (size, >21 kb) were estimated by agarose gel electrophoresis (with 1.5% [wt/vol] agarose- $1\times$ Tris-borate-EDTA-1 ng of ethidium bromide ml^{-1}). DNA was obtained from all samples (0.23 ± 0.1 $\mu\text{g}/\mu\text{l}$). DNA isolated was subsequently used as a template to amplify the V6 to V8 regions of the bacterial 16S rDNA with primers U968-GC (5' CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5' GCG TGT GTA CAA GAC CC). PCR was performed

using Hot Star Taq DNA polymerase (Qiagen, Courtaboeuf, France). PCR mixtures of 50 µL contained: 1X PCR Buffer, 1.5 mM Mg Cl₂, 0.1 mM of each dNTP, 0.5 µM of primers U968-GC and L1401, 2.5 U of Hot Star® Taq Polymerase, and approximately 1 ng of DNA. The samples were amplified in a Gene Amp PCR system 9700® (Perkin-Elmer, Nantes, France) by using the following program: 95°C for 15 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1.5 min, and finally 72°C for 15 min.

TTGE analysis of PCR amplicons Temporal Temperature Gradient gel Electrophoresis has been chosen (TTGE) as the culture independent method that allowed the main bacteria diversity to be compared among samples [27].

The Dcode universal mutation detection system (Bio-Rad, Paris, France) was used for sequence-specific separation of PCR products. Electrophoresis was performed through a 1 mm thick, 16 x 16 cm polyacrylamide gel (8% wt/vol acrylamide-bisacrylamide, 7 M urea, 1.25x % Tris-acetate EDTA (TAE), 55 µL and 550 µl of Temed and ammonium persulfate 10%, respectively) using 7 liters of 1.25x TAE as electrophoresis buffer. Electrophoresis was run at a fixed voltage of 65 V for 969 min with an initial temperature of 66°C and a ramp rate of 0.2°C/h. For better resolution, voltage was fixed at 20 V for 5 min at the beginning of electrophoresis. Each well was loaded with 100-200 ng of amplified DNA plus an equal volume of 2x gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, and 70% glycerol). As described earlier, a marker was used [7]. A temperature gradient from 66 to 70°C (ramp rate of 0.2°C/hour) was applied during electrophoresis. After completion of electrophoresis, the gel was stained in a 30 µg/mL Sybr Green solution (Sybr Green I, Sigma-Aldrich, St Quentin Fallavier, France), destained in 1.25x TAE, and analyzed using Quantity One® software of the Gel Doc 2000® system (Bio-Rad, Paris, France). Profiles were numerized and gray intensity recorded along a densitogram, each band giving rise to a peak.

TTGE gel analysis. TTGE profiles were compared by using Gel Compare II software (Applied-Maths, Saint-Martens –Latem, Belgium). The analysis took into account the number of bands, their position on the gel, and their intensity. This software translates each TTGE profile into a densitometric curve, drawing a peak for each band (the area under the peak being proportional to the intensity of the band). A threshold area value was used to remove small peaks on the densitometric curves (these can be detected purely as a result of the amount of DNA applied to the gel). A marker consisting of a PCR amplicon mix of seven

cloned rDNAs from different bacterial species was used to normalize the profiles. During this step the gel strips were stretched or shrunk so that the assigned bands on the reference patterns matched their corresponding reference positions. Similarity coefficients (Pearson correlation method) were then calculated for each pair of profiles, yielding a similarity matrix. A dendrogram was constructed from this matrix by using a UPGMA algorithm (unweighted pair group method using arithmetic averages) [14].

Sequence analysis. Each electrophoregram consisted of a curve in which grey intensity = $f(\text{migration distance})$. The curves were digitized from the migration distances ranging from 1 to 438 at steps of 1 interval, using the Gel Compare II software. Thus TTGE profiles were dispatched into zones of interest from which dominant bands were selected and excised for PCR and sequence analysis. Gel fragments were washed once in 200 μL PCR water and kept in 100 μL PCR water overnight at 4°C for diffusion. Ribosomal DNA fragments were then amplified from the dialyzate. The PCR reaction was as described above. The size and concentration of the amplicons were evaluated on 1.5% agarose gel containing EtBr. PCR products were sequenced by Genome Express (Meylan, France). Newly determined sequences were compared with those in GenBank by BlastN search (NCBI) and using the Ribosomal Database Project RDP II sequence-match facility (Michigan State University, USA) in order to ascertain their closest relatives.

Multiple linear regression analysis. PLS-regression (PLSR) is a method for relating two data matrices, X and Y, by a linear multivariate model, but goes beyond traditional regression in that it models also the structure of X and Y. PLSR derives its usefulness from its ability to analyse data with many, noisy, collinear, and even incomplete variables in both X and Y. PLSR has the desirable property that the precision of the model parameters improves with the increasing number of relevant variables and observations. In the present study X variables are the TTGE profiles of dominant resident microbiota and variable Y is the presence or absence of *C. difficile*. Furthermore, this method allows the assumption that component X (dominant resident microbiota) is the component that is most relevant for predicting the variable Y (presence or absence of *C. difficile*) among others (sex, age, antibiotherapy). Relationships using PLS regression were established between dominant microbiota profiles and status of patients, using the SIMCA software, version 9.0 (UMETRI, Umeå, Sweden/www.umetrics.com). Each patient was given a code in which sex, stool sample, status, antibiotics and age were stated. First the TTGE profiles of each patient on D₀ and D₁₄

were established. Then they were analysed using the Gel Compare software. Finally, PLS regression was used to investigate the relations between TTGE profiles of patients (X variables) and the presence or absence of *C. difficile* (variable Y). The number of useful PLS components is determined by cross-validation (SIMCA-P 9.0, 2001). The X-loadings and the Y-loadings are noted w^* and c , respectively. Groups of patients are presented as situated on a plane defined by PLS components. The explanatory performance of the model is evaluated using the R^2 coefficient which corresponds to the part of the variance of variable Y explained by the X variables [18].

Results

Storage of fecal samples. In the previously published clinical study the interval between stool passage and laboratory processing was 26.9 ± 8.3 h (range, 3 to 71 h). In order to test the stability of the dominant fecal microbiota, fecal samples from 4 healthy volunteers were analysed in parallel. Those samples were kept simultaneously at -80°C , 4°C , and 20°C for 24, 48 and 72 hours and then analyzed by Temporal Temperature Gradient gel Electrophoresis (TTGE). Results are expressed as percentages of similarity of TTGE profiles after storage under the indicated conditions in comparison with an aliquot of the same sample stored at -80°C (as the gold standard). After 24 hours, the percentages of similarity of TTGE profiles were of $88.6\% \pm 5.2$ with storage at 4°C and $89.2\% \pm 1.8$ with storage at 20°C . After 48 hours they were $89.1\% \pm 4$ and $87.5\% \pm 1.9$ respectively and after 72 hours they were $82\% \pm 7.4$ and $86\% \pm 3.3$ respectively.

Intra-individual analysis. 174 TTGE profiles were analyzed, from D_0 and D_{14} for each of the 87 patients studied. The banding pattern was complex in all cases. The dendrogram analysis showed that the TTGE profiles did not cluster according to presence or absence of *C. difficile* (UPGMA dendrogram not shown). Similarity percentages between D_0 and D_{14} profiles for each of the 11 patients with acquired *C. difficile*, according to antibiotic classes, varied from i) 0% (patient with AAD) and 90.8% (patient without AAD) for amoxicillin – clavulanic acid, ii) 41% (patient with AAD) and 71.3% (patient without AAD) for other beta-lactam agents and iii) 60.4% (patient with AAD) and 84.4% (patient without AAD) for non beta-lactam antibiotics.

Inter-individual analysis and PLS model: Relationships between the profiles included in the PLS model and the results of “acquired *C. difficile*” are not easily established using a visual observation of the profiles. We calculated a PLS model that linked the 87 TTGE profiles of main bacteria (X variables) of patients and the subsequent onset of *C. difficile* (variable Y), first on D₀ then on D₁₄. The cross-validation led to R² coefficient which denotes the percentage of variation. On D₀ the corresponding PLS model explained 46% of the variation of the Y-matrix (development of *C. difficile*) (Fig. 1) and on D₁₄ 74.5% (Fig. 2).

Sequence analysis: D₀ TTGE profiles of the 11 patients with acquired *C. difficile* were compared on the same gel (Fig. 3). The analysis of the 438 zones dispatched from TTGE profiles by the software, showed that only 6 were specific for the group of patients with acquired *C. difficile*. From those 6 zones of interest, 7 main bands have been selected taking into account their optical density and distinctiveness. To gain insight into the phylogenetic positions of those amplicon DNA, they were extracted from the gel and sequenced. The sequences showed the highest similarity with sequences derived from different *Clostridium* clusters of the low guanine+cytosine (G+C) gram positive species [5]. The average determined length of the DNA sequences was 500 bases, and phylogenetic analysis was based on 400 to 450 aligned homologous nucleotides (corresponding to positions 900 to 1400 in *Escherichia coli* 16S rDNA). Using the same approach, 2 zones of interest were identified at D₁₄. Three bands were selected, extracted and sequenced. The characteristics of the 7 extracted sequences of D₀ and of the 3 extracted sequences of D₁₄ are shown in table 1 with the origin (fecal sample), sequence length (370-417), closest relative identification and percentage of identification. They all belong to Clostridiales order, Clostridiaceae, Eubacterium and Lachnospiraceae family.

Discussion

Our results support the concept of “permissive” microbiota. Using molecular profiling of bacterial 16S rDNA coupled with PLS regression analysis, we found that important criteria in terms of main bacteria of the fecal microbiota could be considered as predisposing factors for *C. difficile* development. We did not intend to sequence all implicate bacteria species. The studied patients were adult out-patients living in the Paris area. We assumed that all were exposed equally to *C. difficile* from the environment. The PLS regression analysis gave a predictive ability of 46% for the resident microbiota of those patients who developed a *C. difficile* after an antibiotherapy. These results explain the development of *C. difficile*

following antibiotic treatment and corroborate a metaproteomic approach to link biological functions to gene sequences. Furthermore, these findings suggest that strategies reinforcing the ability of the resident microbiota to resist to modifications would be of clinical relevance. Recent culture-independent molecular studies on healthy individuals have shown that the intestinal microbiota is specific to the host and resistant to modification over time [27]. Although, the difficulty to identify the exact profile at equilibrium has already been discussed [6], to take into account every patient's data including factors such as age, sex, clinical and/or antibiotherapy, is rather difficult. It needs the help of an abstract model that uses mathematical language to describe the behavior of the system by a set of variables and a set of equations that establish relationships between the variables. PLS-regression is a particular type of multivariate analysis which uses the two-block predictive PLS model to model the relationship between two matrices. PLS-regression derives its usefulness from its ability to analyze data with many, noisy, collinear, and even incomplete variables in both X and Y. PLS-regression has the advantageous property that the precision of the model parameters improves with the increasing number of relevant variables and observations [8]. Thus we chose PLS-regression for relating the resident microbiota to *C. difficile* development. PLS-regression has been used in various disciplines such as chemistry, economics, medicine, pharmaceutical science and microbiology [13, 18, 20]. The PLS-regression analysis of the resident microbiota on D₀ gives one significant component explaining 46% of the Y-variance. Our analysis shows that the data are clustered: The resident microbiota from patients with later *C. difficile* development deviates from the main cluster. In addition PLS-regression detected the variable that is highly linked to variable Y (acquired *C. difficile*) among a large number of X variables (TTGE profile, age, sex and class of antibiotic) as applied to a large number of observations. In our model the variable X (=TTGE profiles) had been detected based on its significance, other X variables (age, sex and class of antibiotic) were not relevant although a specific study with the different antibiotics would be warranted. Thus the present report provides evidence for predisposing factors in resident microbiota. Such scoring functions should aid in the identification of putative group of bacteria. Moreover the model could be used to predict the inclusion of new patients by incorporating their TTGE profiles into the model. Therefore, more information could be obtained from TTGE profiles than those given by densitometric analyses. This model enabled the parameters affecting the distribution of the microbiota to be examined. Nevertheless the molecular determinants and host specificity have yet to be identified.

Operational Taxonomic Unit or molecular species is defined as a set of sequences with less than 2% divergence in 400-450 aligned homologous nucleotides [25]. Thus most of the sequences identified in this work were related to uncultured bacterium clones (99-98%) from *Clostridiales* order. Among the anaerobes the *Clostridiales* order are known to have a strong catalytic activity.

The non exhaustive data of the microbiota we found show species only from *Clostridiales* order, *Clostridiaceae*, *Eubacterium* and *Lachnospiraceae* family. This is not surprising since novel or yet uncultured species are most often identified upon characterization of fecal microbiota using cloned 16S rDNA genes libraries [11]. Recent culture-independent studies have shown that approximately 70% of the dominant human gut microorganisms have not been isolated and described [3].

Interestingly, after subtractive densitometric analyses on D₀ for the selected band 1, we found that it was common in 10 out of the 11 TTGE profiles of the selected patients and only 2 out of the 76 others. Furthermore, among the 11 patients with acquired *C. difficile*, 5 developed AAD. They clustered separately from the 6 patients without AAD on D₀ as well as on D₁₄, suggesting two different “sub-groups” of dominant microbiota. These results’ analyses are consistent with our hypothesis and merit confirmation. This will be tested with patients who developed AAD.

Within D₁₄ of antibiotherapy, the human fecal microbiota of patients was markedly modulated. The alterations observed here (0% patient with AAD and 90.8% patient without AAD) in the structure of the microbiota upon amoxicillin – clavulanic acid treatment are important enough to suggest two groups of patients. It had been determined that upon natural oscillations of dominant fecal microbiota TTGE profiles would remain within 90% of similarity with the equilibrium state over a period of two years in one volunteer [24]. Thus, these observations suggest the occurrence of a specific resistant microbiota to amoxicillin – clavulanic acid and would warrant confirmation.

From the 5 patients with AAD one was found neither toxinogenic nor with toxin. Thus, in this case, *C. difficile* could not be considered as cause of diarrhea, although a negative search for toxin and/or toxinogenesis obviously does not constitute final proof for the absolute lack of *C. difficile* spore in the microbiota.

The dendrogram analysis showed that the TTGE profiles did not cluster according to presence or absence of *C. difficile*. But the PLS –regression model explained 46% of the variation of the Y-matrix (development of *C.difficile*). This study assessed for each individual, the significance of resident microbiota but did not intend to determine the composition of the

dominant fecal microbiota in terms of bacterial genera or species. The new sequences found in the genus *Clostridium* indicate the importance of this genus inside the microbiota and its putative role in development of pathogens [7]. Molecular analyses of the bacterial microbiota based on 16S rDNA have attracted attention as reliable methods for detection and identification of bacterial species [1, 12, 17]. Techniques such as temporal temperature gradient gel electrophoresis are attractive because they are conducive to high throughput studies. TTGE successfully differentiates bacterial gene fragments of the same size but different thermal stability. The uses and limits of TTGE in microbial ecology have already been explored [7, 22]. The ability to apply statistical methods makes denaturing gel electrophoresis fingerprinting techniques such as TTGE tools with great potential [7, 9]. Only the dominant fraction of the fecal microbiota is assessed using the PCR-TTGE technique, as applied here, with universal primers. The complexity of the profiles observed by TTGE will represent the most prevalent species.

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Table 1: Data of the sequences: origin, sequence length, closest relative identification (accession number), % identity.

N° extract. bande	Accession Number	Origin	Sequence length (letter)	Closest relative	Phylum/class of the closest relative	% Iden tity
1	EU196222	Fecal sample Individual	395	Uncultured bacterium	Genus Ruminococcus, (DQ802748)	99
2	EU196223	Fecal sample Individual	394	Uncultured bacterium	Unclassified_Clostridiale s (AY984391)	99
3	EU196224	Fecal sample Individual	395	Clostridium	Clostridium sp. (AJ582080)	98
4	EU196225	Fecal sample Individual	391	Uncultured bacterium	Genus Ruminococcus (DQ905852)	99
5	EU196226	Fecal sample Individual	370	Uncultured bacterium	Genus Ruminococcus (AM277309)	99
6	EU196227	Fecal sample Individual	386	Uncultured bacterium	Genus Dorea/ family Clostridiaceae (DQ802652)	99
7	EU196228	Fecal sample Individual	394	Uncultured bacterium	Genus Eubacterium (AM275432)	91
8	EU196229	Fecal sample Individual	405	Uncultured bacterium	unclassified_Lachnospir aceae (AY235653)	100
9	EU196230	Fecal sample Individual	417	Uncultured bacterium	Genus Ruminococcus	
10	EU196231	Fecal sample Individual	389	Uncultured bacterium	Genus Ruminococcus Uncultured Firmicutes (EF071261)	94

Legends to figures

Figure 1: 3D representation of the PLS regression model showing relationship between TTGE profiles of dominant species before any antibiotic treatment (D_0) and the subsequent onset of *C. difficile*. The corresponding model explained 46% of the estimated modification. Positions of the D_0 TTGE profiles of patients who acquired *C. difficile* are indicated in this 3D representation, by rectangles, position of the D_0 TTGE profiles of others are indicated by triangles.

Figure 2: 3D representation of the PLS regression model showing relationship between TTGE profiles of dominant species 14 days after the antibiotic treatment (D_{14}) and the subsequent onset of *C. difficile*. The corresponding model explained 74.5% of the estimated modification. Positions of the D_{14} TTGE profiles of patients who acquired *C. difficile* are indicated in this 3D representation, by rectangles, position of the D_{14} TTGE profiles of others are indicated by triangles.

Figure 3: Temporal temperature gradient gel electrophoresis of 16S rDNA amplicons (amplified with universal primers for the V6-V8 region of the gene) of fecal samples obtained at day 0, before any antibiotherapy, from the 11 patients who acquired a *C. difficile* after the antibiotherapy.

Figure 4: Example of Temporal temperature gradient gel electrophoresis of 16S rDNA amplicons (amplified with universal primers for the V6-V8 region of the gene) of fecal samples from patients 6 and 9, who acquired *C. difficile* after the antibiotherapy, at Day 0 and Day 14.

Figure 1:

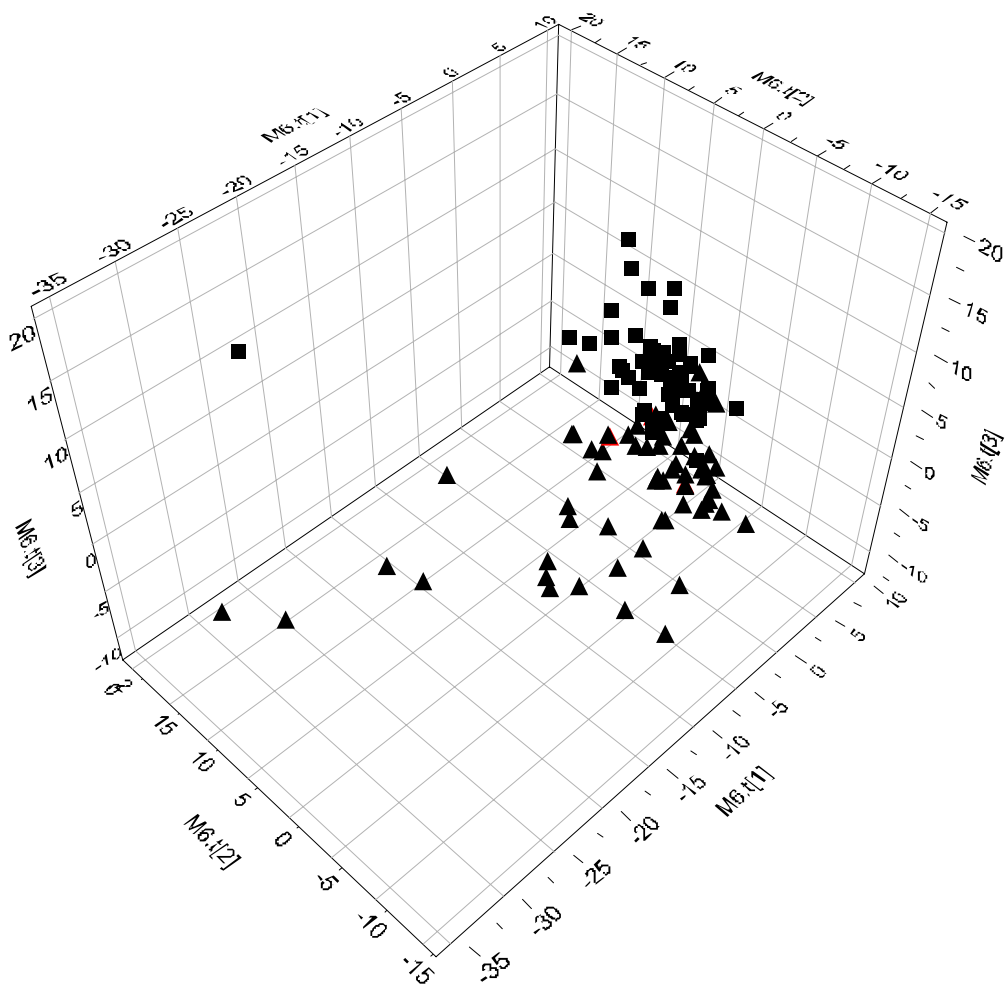
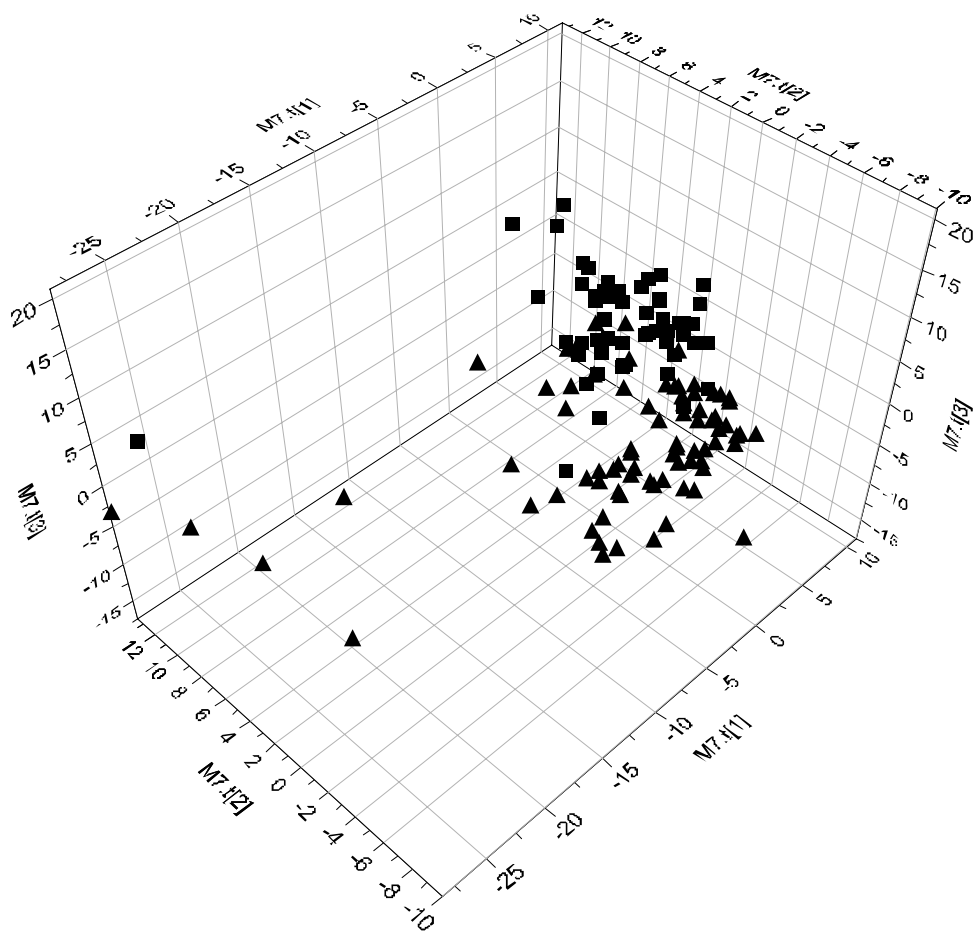


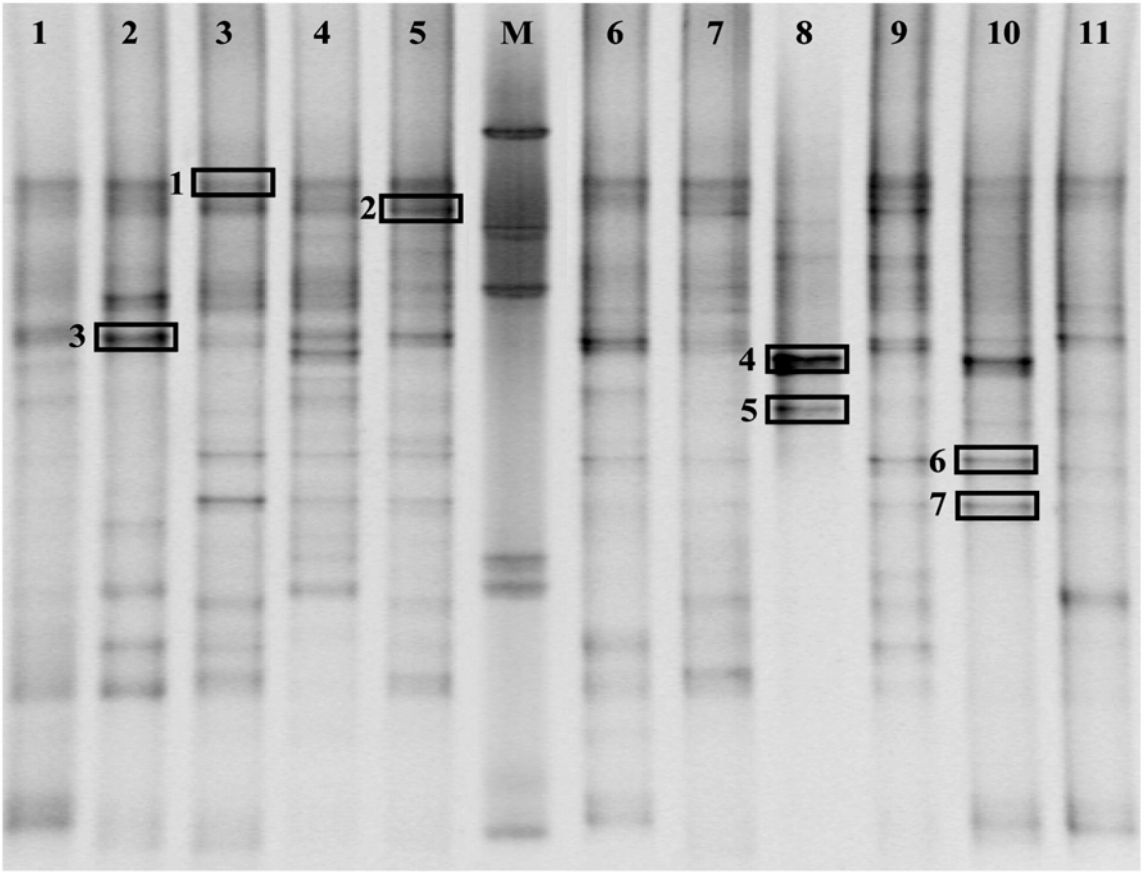
Figure 2:



561 **Figure 3:**

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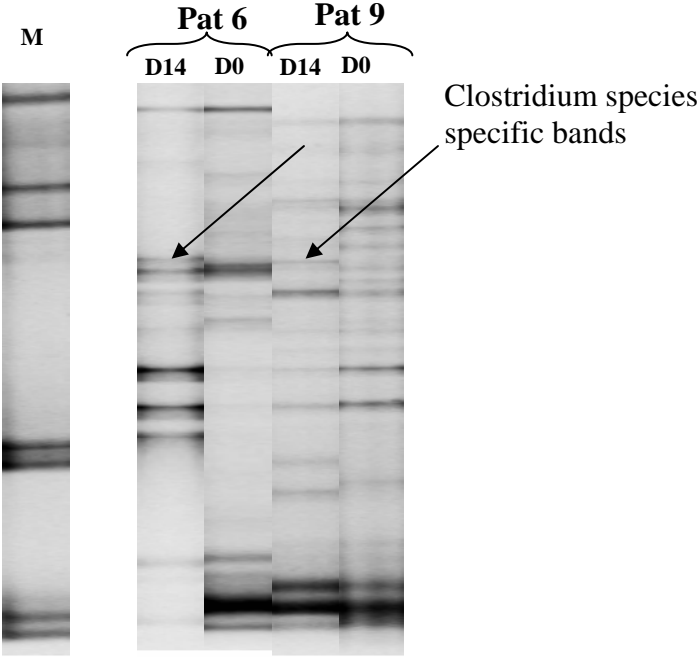
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566 **Figure 4:**
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