

Human intestinal microbiota gene risk factors for antibiotic-associated diarrhea: perspectives for prevention. Risk factors for antibiotic-associated diarrhea.

Marie France de La Cochetière, Emmanuel Montassier, Jean-Benoît Hardouin, Thomas Carton, Françoise Le Vacon, Tony Durand, Valérie Lalande, Jean-Claude Petit, Gilles Potel, Laurent Beaugerie

▶ To cite this version:

Marie France de La Cochetière, Emmanuel Montassier, Jean-Benoît Hardouin, Thomas Carton, Françoise Le Vacon, et al.. Human intestinal microbiota gene risk factors for antibiotic-associated diarrhea: perspectives for prevention. Risk factors for antibiotic-associated diarrhea.: Diarrhea risk prediction from microbiota genes. Microbial ecology, 2010, 59 (4), pp.830-7. 10.1007/s00248-010-9637-2. inserm-00462416

HAL Id: inserm-00462416 https://inserm.hal.science/inserm-00462416

Submitted on 9 Mar 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1	Human intestinal microbiota gene risk factors for antibiotic-associated diarrhea:
2	perspectives for prevention
3	Marie-France de La Cochetière ^{1*} , Emmanuel Montassier ⁴ , Jean-Benoit Hardouin ⁵ , Thomas
4	Carton ⁴ , Françoise Le Vacon ³ , Tony Durand ⁴ , Valerie Lalande ² , Jean Claude Petit ² , Gilles
5	Potel ⁴ , Laurent Beaugerie ² .
6	
7	¹ INSERM, Université de Nantes, UFR Médecine, Thérapeutiques Cliniques et
8	Expérimentales des Infections, EA 3826, rue G. Veil, Nantes, F-44000 France.
9	² Service de Gastro-entérologie et Nutrition, Hôpital Saint-Antoine, 184 rue du faubourg
10	Saint-Antoine, 75012 Paris Cedex, France.
11	³ Atlangene [®] -Silliker, Bio Ouest, Ile de Nantes, 21 rue La Noue Bras de Fer, 44200 Nantes,
12	France
13	⁴ Université de Nantes, UFR Médecine, Thérapeutiques Cliniques et Expérimentales des
14	Infections, EA 3826, rue G. Veil, Nantes, F-44000 France.
15	⁵ Université de Nantes, UFR Médecine et Pharmacie, Biostatistics Clinical Research and
16	Subjective Measures in Health Sciences , EA 4275, rue G. Veil, Nantes, F-44000 France.
17	
18	Running title: Diarrhea risk prediction from microbiota genes
19	
20	*Corresponding author Dr. MF de La Cochetière Mailing address: UPRES EA 3826, UFR
21	de Médecine, 1 rue Gaston Veil, 44035 Nantes, cedex 01, France. Phone 33 (0)240412840,
22	Fax 33(0)240412854. E-mail marie-france.de-la-cochetiere@inserm.fr
23	
24	Financial support: Biocodex Inc
25 26	

28 Abstract

Antibiotic-associated diarrhea (AAD) is associated with altered intestinal microflora and other symptoms that may lead to possibly death. In critically ill patients, diarrhea increases rates of morbimortality. Assessing diarrhea risks is thus important for clinicians. For this reason, we conducted a hypothesis-generating study focused on antibiotic-associated diarrhea (AAD) to provide insight into methods of prevention. We evaluated the hypothesis of predisposing factors within the resident intestinal microbiota in a cohort of outpatients receiving antibiotherapy. Among the pool of tested variables, only those related to bacterial 16S rRNA genes were found to be relevant. Complex statistical analyses provided further information: amid the bacteria 16S rRNA genes, eight were determined to be essential for diarrhea predisposition and characterized from the most important to the least. Using these markers, AAD risk could be estimated with an error of 2%. This molecular analysis offers new perspectives for clinical applications at the level of prevention.

Key words: Data-Mining/Diarrhea/Microbiota genes/Prevention/Risk factors

63 Introduction

64 The collective effects of the intestinal microbiota are dictated by a complex network of interactions that span the cellular, immunological, and environmental levels. A primary 65 66 question is whether a simple system can be applied to predict and control the effects of this heterogeneous population composed of different subgroups. One approach in addressing this 67 68 question is to study representative genes that correlate with the health consequences of the bacteria mixture comprising the microbiota. The present study concerns specific bacterial 69 70 populations associated with antibiotic-associated diarrhea (AAD). This hypothesis-generating 71 experiment was conducted to discern whether an analysis of bacterial 16S rRNA genes from 72 pre-antibiotic resident faecal microbiota using with complex statistics could predict the 73 collective effects of the intestinal microbiota, thereby identifying individual risk factors for 74 diarrhea associated with antibiotic treatment. These specific gene sequences have been chosen 75 because they have been the far most common genetic marker used (16).

76 Antibiotic-associated diarrhea (AAD) is associated with altered intestinal microflora, 77 mucosal integrity, vitamin, mineral metabolism and crampy abdominal pain. If severe, AAD 78 may lead to electrolyte disturbances, dehydration, premature discontinuation of antibiotic 79 therapy, pseudomembranous colitis, toxic megacolon and possibly death. Reports in the 80 general population indicate that the incidence of AAD ranges from 25 to 62%, occurring at 81 any point from the initiation of therapy to two months after the end of treatment (2, 17). In 82 critically ill patients, diarrhea increases morbimortabilty. AAD leads also to longer hospital 83 stays and higher medical costs (1, 26, 27). The pathogenesis of AAD may be mediated 84 through the disruption of the normal microbiota and overgrowth of pathogens, or through metabolic imbalances (3, 31). The individual risk for AAD varies greatly, influenced by host 85 86 factors (age or diet), and type, dose, and duration of antibiotherapy (15, 22). Assessment of the diarrhea risk during antibiotic treatment is therefore worthwhile and may help controldiarrhea in defined high-risk individuals; however, few data exist on risk factors for AAD.

89 The role of the human intestinal microbiota in health and specific diseases is a 90 particularly important area of research (7, 12, 13). Substantial progress has recently been 91 made in characterizing the human intestinal microbiota, although its role in immune system 92 development and regulation, nutrition, and pathogenesis of the host are still not well 93 elucidated (30). Furthermore, the rapid rate of microbial evolution, combined with the global 94 rise of antimicrobial resistance and the low rate of novel antibiotic development underscores 95 the urgent need for innovative therapeutics (5, 10). Culture-based techniques have 96 traditionally been used to determine the faecal microbiota. However, molecular techniques 97 based on analysis of 16S rRNA genes directly amplified from bacterial DNA extracted from 98 feces have estimated that less than 25% of the faecal bacterial populations have been cultured 99 to date (11, 28). These approaches have provided considerable data about microbial 100 ecosystems, including that of the human gastrointestinal tract. Detailed phylogenetic 101 informations have been obtained by cloning and sequencing 16S rRNA genes. Further, 102 several studies with fingerprinting of 16S rRNA genes have reported its benefits in 103 monitoring community shifts (8, 19, 23, 29).

Hence, as a system model for the analysis of heterogeneous populations of bacteria, we analysed the 16S rRNA genes in the genomes of all bacteria using temporal temperature gradient gel electrophoresis (6), and multivariable data analysis (21). In this retrospective study, we hypothesized that the susceptibility to diarrhea may be linked to the resident intestinal microbiota. For this purpose we focused on a published clinical study (4), which included epidemiology forms, patient history, and laboratory reports; we analyzed database case records, specimen collection, and risk factors. We designed complex statistical analyses to determine the optimal procedures for providing the maximum relevant information withfingerprint data, and obtaining knowledge about the 16S rRNA gene system.

113

114 Methods

115 Patients. Subjects enrolled in this study were adults (age range, 20-60 years) living in 116 the Paris area. Subjects were prescribed a course of antimicrobial therapy for an ear, nose, or 117 throat infection. Criteria for enrollment included prescription by a general practitioner for a 118 5-to 10-day course of antibiotics and age of 18 years or older. Potential candidates were 119 excluded if they were institutionalized, had received antibiotic treatment during the previous 2 120 months, had been admitted to a hospital during the previous 6 months, had a known human 121 immunodeficiency virus infection, had any allergy, or had experienced a bout of diarrhea (>2 122 loose stools/day) the day before enrollment. All patients provided informed written consent. 123 Prescribed antibiotics were classified into one of the following groups: amoxicillin/clavulanic 124 acid, other beta-lactam agents, or non-beta-lactam agents. Diarrhea was defined as the 125 passage of at least three loose stools a day, AAD was defined as diarrhea associated with the 126 administration of antibiotics (in the absence of any other obvious reason) during the 14-day 127 study period. Each patient was asked to store the last stool before the beginning of the 128 antibiotherapy (D_0) in a double-thickness container. The dominant microbiota profile at D_0 129 was considered to be patient's profile at equilibrium (i.e., the resident microbiota). In 130 addition to the molecular study, bacteriological investigations were performed according to 131 standardized procedures.

132 **DNA isolation and 16S rRNA gene amplification.** Immediately after faecal sample 133 collection, total DNA was extracted from a 125-mg aliquot and purified as previously 134 described (9). DNA ($0.23 \pm 0.1 \mu g/\mu l$) was obtained from all samples. The DNA integrity 135 and concentration (size, >21kb) were determined by 1.5% agarose gel electrophoresis with

ethidium bromide. Isolated DNA was subsequently used as a template to amplify the V6 to
V8 regions of the bacterial 16S rRNA gene with primers U968-GC and L1401 (32).
Contamination and amplification controls were performed at each steps as previously
described (9).

TTGE analysis of PCR amplicons. The Dcode universal mutation detection system
(Bio-Rad, Paris, France) was used for sequence-specific separation of PCR products as
previously described. After electrophoresis, the gel was stained and analyzed using Quantity
One software of the Gel Doc 2000 gel documentation system (Bio-Rad, Paris, France).

144 TTGE gel analysis. Each grey band of the TTGE gels was considered an amplicon of 145 the 16S rRNA gene. To simplify analysis, we used zones of the electrophoretic gels to 146 describe the migration distances representing individual 16S rRNA genes. TTGE profiles 147 were compared by using Gel Compare II software (Applied-Maths, Sint-Martens-Latem, 148 Belgium). The analysis took into account the number of bands, their positions on the gel, and 149 their intensities. Gray intensities were then recorded along a densitogram, with each band 150 given rise to a distinct peak. Thus each electrophoresis pattern was represented by a curve 151 defined as grey intensity = function (normalized migration distance). A marker consisting of a 152 mixture of PCR amplicons (seven cloned 16S rRNA genes from different bacterial species) 153 was used to normalize the profiles as previously described (9). Similarity coefficients 154 (Pearson correlation method) were calculated for each profile, yielding a similarity matrix. A 155 dendrogram was constructed from this matrix using the UPGMA algorithm (unweighted pair 156 group method using arithmetic averages).

157 **Data collection.** The acquired data from all D_0 electrophoresis gels were collected as 158 147 retention times that resulted from digitizing each electrophoresis at equal distance 159 intervals. Each patient was also characterized by qualitative features related to age, sex,

therapy and the eventual presence of AAD. The ages were recoded into seven classes (from
ages 20-29 to 80-89). AAD was represented by two groups (absence or presence).

162

163 Multivariable data-analysis

164 Two analyses of the data has been carried out in order to detect retentions times predictive of 165 the AAD: ANOVA which compare the mean values at each retention times between the two 166 groups, and discriminant analysis which allows to detect the more important retention times to 167 predict AAD.

Analysis of variances. Each of the 147 retention times was explained by a four-way analysis of variance (ANOVA) including age, sex, therapy and AAD as independent variables. The aim of these analysis was to detect the retention times where there was significant differences between the two groups of patients (absence or presence of AAD), by adjusting the analysis on clinical variables (age, sex and therapy).

173 In order to take into account the multiplicity of the tests, the F statistics associated to 174 absence/presence of AAD of the ANOVA were compared to the value obtained with the 175 following process: 10000 simulated datasets were generated by using the observed retention 176 times and by simulating the factors age, sex, therapy and presence/absence of AAD with the 177 same distributions than these ones observed in the sample independently of the retention 178 times. The retained threshold for the F statistics (referenced as threshold F value) was the 179 value of the F where 5% of the simulated datasets were above. This threshold corresponds to 180 the 5% significant level obtained by chance. Only the retention times where the corresponding 181 F values are above this threshold are retained as significantly predictive of the AAD.

182 *Discriminant analysis*. A discriminant analysis was carried out in order to detect 183 retention times which allow predicting the AAD for the patients. Forward selection of the 184 retention times was realized: At each step, the retention time which allows improving the 185 more the clustering of the patients in the two groups (correct clustering between 186 absence/presence of AAD) is introduced in the analysis, until there is no more possible 187 improvement of the clustering.

188

189

190 **Results**

Among the 156 patients included in the study, 44 developed an AAD. None of the 44 patients with diarrhea had stool culture positive for the tested intestinal pathogens *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. and *Yersinia* spp.); however, six patients acquired *Clostridium difficile*.

195 TTGE gel analysis. From DNA extracted from D0 stool samples (before 196 antibiotherapy) 156 TTGE profiles were analyzed. The banding pattern was complex in all 197 cases (Figure 1), the number of bands ranged from 10 to 20. The dendrogram analysis 198 (unweighted pair group method with arithmetic mean (UPGMA) dendrogram not presented 199 here) showed that the TTGE profiles did not cluster according to the onset of AAD. These 200 results corroborate that, for each individual, microbial diversity and composition are specific 201 traits (20). Thus, the need for extra complex analyses is pointed out in order to find any 202 grouping within the microbiota profiles, before treatment.

203 *Multivariable data-analysis:* Using multivariate data analysis techniques to determine 204 potential causal factors for AAD, we found that only D_0 microbiota profiles were strongly 205 correlated with AAD. In this study, other factors (age, sex, and class of antibiotic) were not 206 relevant, thus we focused on the intestinal bacterial 16S rRNA genes: each TTGE profile was 207 converted into a curve, then digitized and further analysed to convert 16S rRNA genes to 208 numerical data (see complementary material). We applied advanced multivariate analysis to 209 confirm any predictive signature of the intestinal microbiota. 210 Variance analysis. Figure 2 shows the F- value of ANOVA carried out independently at each 211 data point of each electrophoresis at D₀. The studied factor was the presence/absence of AAD 212 after antibiotherapy. Six electrophoresis migration distance values were found exceed the 213 threshold F-value: (A) 58, (B) 70, (C) 174, (D) 321, (E) 358 and (F) 399. Figure 3 shows the 214 mean electrophoresis migration patterns associated with the absence and presence of AAD: 215 significant regions are emphasized using a grey color. Zones A and F are correlated to the 216 microbiota of patients with AAD, and zones B, C, D, and E are correlated to the microbiota of 217 patients without AAD.

Discriminant analysis. Six retention times explaining the absence/presence of AAD have
been selected by the forward selection of the variables for the discriminant analysis (FLDA).
These 6 variables allow clustering 142 among the 156 patients (91%) in the correct group.

221

Individual risk prediction of AAD from resident intestinal microbiota. Figure 4 demonstrates the degree of agreement between ANOVA and FLDA. Four of the first six variables selected by the discriminant analysis were also identified by ANOVA, producing distinct profiles for AAD and no AAD. The risk prediction for any new case of AAD could be calculated from its intestinal microbiota gene fingerprinting with an error of 2% in the case of AAD (1/44) and 11% (12/112) in the case of no AAD.

228

229 **Discussion**

Predicting and controlling the effects of a heterogeneous bacteria population is a highly challenging task with many biological and clinical applications. To study this question, we evaluated relationships between intestinal bacterial 16S rRNA genes and AAD, which is a growing health concern. Why some patients develop AAD while others do not is a recurrent and unresolved question. This paper describes for the first time a computational approach that accurately predicts the relationship between the resident microbiota and the riskfor developing AAD.

237

238 The important findings of this study are as follows:

i.) Among the studied patient variables (antibiotherapy, age, sex, bacterial 16S rRNA
genes), only the pre-antibiotic resident faecal bacterial 16S rRNA genes were found to be
correlated to AAD.

ii.) Among the bacterial 16S rRNA genes studied, one group was found to be crucial for
the predisposition for AAD. This group is composed of eight specific electrophoretic
distances, thus eight specific set of genes, classified from most important to least important:
all eight were required for the development of diarrhea.

iii.) Risk factors could be calculated; AAD could be predicted from the resident intestinal
bacterial 16S rRNA gene analysis with an error of 2%, and no AAD with an error of 11%.

248

These observations are based on the investigation of the dominant faecal bacterial populations before any antibiotherapy, by fingerprinting techniques applied to samples from a cohort of outpatients treated by antibiotherapy for ear, nose and throat infections (non invasive sampling). Those conclusions are valid within the context of the study, but do not account for variation outside the dataset.

The strengths of this study include the uniqueness of this type of analysis on bacterial genes from the resident faecal microbiota. It is based on a comprehensive causal model that describes the relationships among numerous risk factors for AAD. The weakness of this study is the lack of phylogenetic analysis; the study does not provide information regarding the specific microorganisms involved in AAD risk. Our aim was to determine the presence of risk factors for AAD among the 16S rRNA genes. The 16S rRNA gene is an ~1500 base pair

260 gene that codes for a portion of the 30S ribosome. Partial (500-base pair) 16S rRNA gene 261 sequencing has emerged as an accurate method to identify a wide variety of bacteria and has 262 been successfully implemented in clinical laboratories (24, 25). A major limitation of the 16S 263 rRNA gene sequencing, though, is its inability to discriminate among all bacterial taxa. In that 264 case alternative gene targets can provide better separation of closely related species (e.g. rpoB 265 gene) (18). However, in this work, the aim was not to identify bacteria, although it looks 266 rather frustrating not to. Ultimately, the goal is to associate differences in communities with 267 differences in metabolic function and/or disease (AAD). Thus these work and the results 268 explain here, stand as one first step toward it. Therefore, the findings of this study are being 269 used for further work on the impact of the resident microbiota. Then, in-depth phylogenetic 270 analysis of the microbiota will be needed and preventing strategies developed.

We started with the assumption of a predictive signature of the microbiota. The statistical analysis shows significant differences in the migration patterns between the two groups (absence/presence of AAD). Moreover, this analysis shows that the AAD can be correctly predicted with data based exclusively on migration distances. As a consequence, it can be conclude that the nature of the microbiota before antibiotherapy may play a role in AAD.

277 The variance analysis identified six significant zones (A to F); the discriminant 278 analysis also identified six significant regions (1 to 6) in order of decreasing importance, all of 279 which are required for prediction. Interestingly, only four zones were common between the 280 two analyses (B to 4, C to 2, E to 6 and F to 1). Further, if an intestinal microbiota 16S rRNA 281 gene profile shows the six zones indicating no AAD and does not show the two zones 282 indicating AAD, it can be assumed that the patient is not likely to develop AAD. One 283 potentially confusing aspect of this study is that we speak of numerical data, so we describe 284 risk in terms of "zones", that is to say, electrophoretic distances representing the16S rRNA genes. The next area of study will be to determine to which extend they are associated tophylogenetic species.

287 Fingerprinting techniques such as TTGE are powerful analysis tools for detecting 288 biomedically relevant markers such as nucleic acids and proteins and ultimately diseases or 289 disease progression that can alter the structure of biological systems like intestinal microbiota. 290 Multivariate data-analysis techniques are essential to manipulate and interpret these enormous 291 amounts of data, and appropriately address the inherent complexity of data derived from 292 In addition, different multivariate algorithms must be tested to biomedical samples. 293 determine the most suitable method(s) for establishing reliable, robust, and accurate 294 classification or regression models, while minimizing false-positive and false-negative results. 295 Nonetheless, multivariate data-analysis techniques should be used cautiously, as a 296 complement to optimized diagnostic techniques that already provide relevant information. 297 Specifically, useful information obtained by fingerprinting techniques like TTGE (i.e., 298 bacterial diversity) increases with a priori knowledge of the samples and the individual (age 299 group, treatment), which enhances the accuracy and reliability of classification and regression 300 techniques based on pattern recognition. It is noteworthy that our study, neither age groups 301 nor antibiotic treatments were important indicators of AAD.

302 In conclusion, we are aware of the limitations of relatively small number of patients 303 used in this study, the complex structure of the data, and the need for verification of our 304 findings. The broad application spectrum of sequence-dependent fingerprinting techniques in 305 the field of intestinal microbiology has been largely examined. It ranges from primary 306 assessments of the bacterial complexity and diversity of intestinal community structures to the 307 monitoring of compositional changes at different population levels upon dietary or therapeutic 308 intervention (14). In this model, causal modeling was based on current TTGE gel analysis 309 and thus has the same limitations of any genetic analysis using biomolecular engineering (e.

310	g. DNA extraction, amplification). Therefore, it is possible that not all possible confounders
311	are represented in the models, and some factors that are designated as no confounders might
312	actually be so. Additional population-based studies with multivariable analyses structured on
313	causal models are required to confirm the findings of this study. In addition, this study was
314	primarily a hypothesis-generating study of resident microbiota genes, which utilized ever-
315	improving molecular techniques and analyses, and demonstrates that important part of risk
316	factors for AAD can be found within the individual microbiome. As such, it offers new
317	perspectives for clinical applications at the level of prevention.
318	
319	
320	
321	
322	
323	
324	Acknowledgments:
325	We thank Dr Bertrand D. from INRA, Unité de Sensometrie-Chimiometrie (Nantes, France)
326	for his assistance in this investigation.
327	Financial support: Biocodex, Inc.
328	Potential conflicts of interest: The authors declare no conflict of interest
329	
330	
331	
332	
333	
334	

335 **References**

- 336 1. Arbuckle, R. B., S. L. Huber, and C. Zacker. 2000. The consequences 337 of diarrhea occurring during chemotherapy for colorectal cancer: a 338 retrospective study. Oncologist 5:250-9.
- 339 2. Bartlett, J. G. 2002. Clinical practice. Antibiotic-associated 340 diarrhea. N Engl J Med 346:334-9.
- 341 3. Bartlett, J. G. 2002. Clostridium difficile-associated Enteric 342 Disease. Curr Infect Dis Rep 4:477-483.
- 343 4. Beaugerie, L., A. Flahault, F. Barbut, P. Atlan, V. Lalande, P.
 344 Cousin, M. Cadilhac, and J. C. Petit. 2003. Antibiotic-associated
 345 diarrhoea and Clostridium difficile in the community. Aliment
 346 Pharmacol Ther 17:905-12.
- 347 5. Blakelock, R. T., and S. W. Beasley. 2003. Infection and the gut. 348 Semin Pediatr Surg 12:265-74.
- 349 6. Bosshard, P. P., S. Abels, R. Zbinden, E. C. Bottger, and M. Altwegg. 350 2003. Ribosomal DNA sequencing for identification of aerobic grampositive rods in the clinical laboratory (an 18-month evaluation). J 352 Clin Microbiol 41:4134-40.
- 353 7. Collier, C. T., C. L. Hofacre, A. M. Payne, D. B. Anderson, P. 354 Kaiser, R. I. Mackie, and H. R. Gaskins. 2008. Coccidia-induced 355 mucogenesis promotes the onset of necrotic enteritis by supporting 356 Clostridium perfringens growth. Vet Immunol Immunopathol 122:104-15.
- 357 8. De La Cochetiere, M. F., T. Durand, V. Lalande, J. C. Petit, G. 358 Potel, and L. Beaugerie. 2008. Effect of Antibiotic Therapy on Human 359 Fecal Microbiota and the Relation to the Development of Clostridium 360 difficile. Microb Ecol.
- 361 9. De La Cochetiere, M. F., H. Piloquet, C. des Robert, D. Darmaun, J. 362 P. Galmiche, and J. C. Roze. 2004. Early intestinal bacterial 363 colonization and necrotizing enterocolitis in premature infants: the 364 putative role of Clostridium. Pediatr Res 56:366-70.
- putative role of Clostridium. Pediatr Res 56:366-70.
 Dethlefsen, L., S. Huse, M. L. Sogin, and D. A. Relman. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6:e280.
- 36811.Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen,369M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman. 2005.370Diversity of the human intestinal microbial flora. Science 308:1635-3718.
- Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J.
 Gordon. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science 291:881-4.
- 375 13. Hrncir, T., R. Stepankova, H. Kozakova, T. Hudcovic, and H.
 376 Tlaskalova-Hogenova. 2008. Gut microbiota and lipopolysaccharide 377 content of the diet influence development of regulatory T cells: 378 studies in germ-free mice. BMC Immunol 9:65.
- 379 14. Huys, G., T. Vanhoutte, and P. Vandamme. 2008. Application of 380 sequence-dependent electrophoresis fingerprinting in exploring 381 biodiversity and population dynamics of human intestinal microbiota: 382 what can be revealed? Interdiscip Perspect Infect Dis 2008:597603.
- 383 15. Iapichino, G., M. L. Callegari, S. Marzorati, M. Cigada, D. Corbella, 384 S. Ferrari, and L. Morelli. 2008. Impact of antibiotics on the gut microbiota of critically ill patients. J Med Microbiol 57:1007-14.
- 386 16. Janda, J. M., and S. L. Abbott. 2007. 16S rRNA gene sequencing for 387 bacterial identification in the diagnostic laboratory: pluses, 388 perils, and pitfalls. J Clin Microbiol 45:2761-4.
- 389 17. Johnston, B. C., A. L. Supina, M. Ospina, and S. Vohra. 2007. 390 Probiotics for the prevention of pediatric antibiotic-associated 391 diarrhea. Cochrane Database Syst Rev:CD004827.
- 392 18. La Scola, B., V. A. Gundi, A. Khamis, and D. Raoult. 2006. Sequencing 393 of the rpoB gene and flanking spacers for molecular identification of 394 Acinetobacter species. J Clin Microbiol 44:827-32.

- 395 19. Lepage, P., P. Seksik, M. Sutren, M. F. de la Cochetiere, R. Jian, P. 396 Marteau, and J. Dore. 2005. Biodiversity of the mucosa-associated 397 microbiota is stable along the distal digestive tract in healthy 398 individuals and patients with IBD. Inflamm Bowel Dis 11:473-80.
- 39920.Manichanh, C., E. Varela, C. Martinez, M. Antolin, M. Llopis, J.400Dore, J. Giralt, F. Guarner, and J. R. Malagelada. 2008. The gut401microbiota predispose to the pathophysiology of acute402postradiotherapy diarrhea. Am J Gastroenterol 103:1754-61.
- 40321.Martens, H., S. R. Veflingstad, E. Plahte, M. Martens, D. Bertrand,404and S. W. Omholt. 2009. The genotype-phenotype relationship in405multicellular pattern-generating models the neglected role of406pattern descriptors. BMC Syst Biol 3:87.
- 407 22. McFarland, L. V. 2008. Antibiotic-associated diarrhea: epidemiology, 408 trends and treatment. Future Microbiol 3:563-78.
- 409 23. Seksik, P., P. Lepage, M. F. de la Cochetiere, A. Bourreille, M. 410 Sutren, J. P. Galmiche, J. Dore, and P. Marteau. 2005. Search for 411 localized dysbiosis in Crohn's disease ulcerations by temporal 412 temperature gradient gel electrophoresis of 16S rRNA. J Clin 413 Microbiol 43:4654-8.
- 414 24. Simmon, K. E., A. C. Croft, and C. A. Petti. 2006. Application of 415 SmartGene IDNS software to partial 16S rRNA gene sequences for a 416 diverse group of bacteria in a clinical laboratory. J Clin Microbiol 417 44:4400-6.
- 41825.Simmon, K. E., S. Mirrett, L. B. Reller, and C. A. Petti. 2008.419Genotypic diversity of anaerobic isolates from bloodstream420infections. J Clin Microbiol 46:1596-601.
- 421 26. Stringer, A. M., R. J. Gibson, J. M. Bowen, D. M. Keefe, R. M. Logan,
 422 A. S. Yeoh, and J. Hamilton. 2009. Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change
- 424 Gastrointestinal microflora and mucins may play a critical role in the 425 development of 5-Fluorouracil-induced gastrointestinal mucositis. 426 Curr Drug Metab 10:79-83.
- 427 27.
 428 A. M., R. J. Gibson, J. M. Bowen, R. M. Logan, A. S. Yeoh,
 428 and D. M. Keefe. 2007. Chemotherapy-induced mucositis: the role of
 429 gastrointestinal microflora and mucins in the luminal environment. J
 430 Support Oncol 5:259-67.
- 431 28. Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D.
 432 Collins, and J. Dore. 1999. Direct analysis of genes encoding 16S
 433 rRNA from complex communities reveals many novel molecular species
 434 within the human gut. Appl Environ Microbiol 65:4799-807.
- 435 29. Tannock, G. W. 2001. Molecular assessment of intestinal microflora. 436 Am J Clin Nutr 73:410S-414S.
- 437 30. Tannock, G. W. 2007. What immunologists should know about bacterial
 438 communities of the human bowel. Semin Immunol 19:94-105.
- 43931.Wistrom, J., S. R. Norrby, E. B. Myhre, S. Eriksson, G. Granstrom, L.440Lagergren, G. Englund, C. E. Nord, and B. Svenungsson. 2001.441Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-442treated hospitalized patients: a prospective study. J Antimicrob443Chemother 47:43-50.
- 444 32. Zoetendal, E. G., A. D. Akkermans, and W. M. De Vos. 1998. 445 Temperature gradient gel electrophoresis analysis of 16S rRNA from 446 human fecal samples reveals stable and host-specific communities of 447 active bacteria. Appl Environ Microbiol 64:3854-9.
- 448 449
- 450
- 451

Figure legends

453 Figure.1. Representative TTGE gel image of faecal microbiota DNA before antibiotic454 treatment. M control marker DNA.

Figure.2. Analysis of Variance (ANOVA). Fisher *F*-values (AAD) are plotted against the
migration distance. The Fisher *F* limit (threshold value) was obtained using randomisation
procedures (see Methods). Six electrophoresis distances were found to exceed this threshold,
and were thus considered significant.

Figure.3. Mean TTGE electrophoresis at D_0 . The grey bars emphasize the regions detected by ANOVA tests. Solid line, pattern from patients with AAD; Dotted line, pattern from patients without ADD.

462 Figure.4. Comparison of ANOVA and discriminant analysis. *Top*: regions detected by
463 ANOVA (grey bars) *Bottom:* first six variables introduced in forward discriminant analysis
464 (vertical lines). Vertical lines are numbered in order of introduction of the corresponding
465 variable. Solid line, pattern from patients with AAD; Dotted line, pattern from patients
466 without AAD.

Pat 1 Pat 2 Pat 3 M Pat 4 Pat 5 M Pat 6 Pat 7 Pat 8 Pat 9



Figure 1





Figure 3



Figure 4

