Assessment of Fecal Microbiota and Fecal Metabolome in Symptomatic Uncomplicated Diverticular Disease of the Colon

Antonio Tursi, MD, Paola Mastromarino, MD,† Daniela Capobianco, BSci,†*
Walter Elisei, MD,‡ Alfredo Miccheli, MD,§ Giorgio Capuani, PhD,§
Alberta Tomassini, PhD,§ Giuseppe Campagna, MS, PhD,‖
Marcello Picchio, MD,¶ GianMarco Giorgetti, MD,#
*Federica Fabiocchi, MD,# and Giovanni Brandimarte, MD***

Goal: The aim of this study was to assess fecal microbiota and metabolome in a population with symptomatic uncomplicated diverticular disease (SUDD).

Background: Whether intestinal microbiota and metabolic profiling may be altered in patients with SUDD is unknown.

Patients and Methods: Stool samples from 44 consecutive women [15 patients with SUDD, 13 with asymptomatic diverticulosis (AD), and 16 healthy controls (HCs)] were analyzed. Real-time polymerase chain reaction was used to quantify targeted microorganisms. High-resolution proton nuclear magnetic resonance spectroscopy associated with multivariate analysis with partial least-square discriminant analysis (PLS-DA) was applied on the metabolite data set.

Results: The overall bacterial quantity did not differ among the 3 groups ($P = 0.449$), with no difference in *Bacteroides/Prevotella*, *Clostridium coccoides*, *Bifidobacterium*, *Lactobacillus*, and *Escherichia coli* subgroups. The amount of *Akkermansia muciniphila* species was significantly different between HC, AD, and SUDD subjects ($P = 0.017$). PLS-DA analysis of nuclear magnetic resonance -based metabolomics associated with microbiological data showed significant discrimination between HCs and AD patients ($R^2 = 0.733$; $Q^2 = 0.383$; $P < 0.05$, LV = 2). PLS analysis showed lower N-acetyl compound and isovalerate levels in AD, associated with higher levels of *A. municiphila*, as compared with the HC group. PLS-DA applied on AD and SUDD samples showed a good discrimination between these 2 groups ($R^2 = 0.69$; $Q^2 = 0.35$; LV = 2). SUDD patients were characterized by low levels of valerate, butyrate, and choline and by high levels of N-acetyl derivatives and U1.

Conclusions: SUDD and AD do not show colonic bacterial overgrowth, but a significant difference in the levels of fecal *A. muciniphila* was observed. Moreover, increasing expression of some metabolites as expression of different AD and SUDD metabolic activity was found.

From the *Gastroenterology Service, ASL BAT, Andria (BT); Departments of †Public Health and Infectious Diseases, Section of Microbiology; §Chemistry; ‖Experimental Medicine, Sapienza University; ‡Division of Gastroenterology, ASL RMH, Albano Laziale; ¶Division of Surgery, "P. Colombo" Hospital, ASL RMH, Velletri; #Digestive Endoscopy and Nutrition Unit, "S. Eugenio" Hospital; and **Division of Gastroenterology, "Cristo Re" Hospital, GIOMI Group, Rome, Italy.

The authors declare that they have nothing to disclose.

A.T. and P.M. contributed equally to this work.

Address correspondence to: Antonio Tursi, MD, Servizio di Gastroenterologia Territoriale, DSS no. 4, ASL BAT, Via Torino 49, Andria (BT) 76123, Italy (e-mail: antotursi@tiscali.it).

DOI: 10.1097/MCG.0000000000000626

Key Words: diverticular disease, fecal metabolome, fecal microbiota

(*J Clin Gastroenterol* 2016;50:S9–S12)

Symptomatic uncomplicated diverticular disease (SUDD) is a common condition that affects one fifth of patients harboring colonic diverticula.[1] Although its pathogenesis is not completely understood, it is generally thought that colonic bacterial overgrowth and microbial imbalance are 2 milestones in the occurrence of symptoms.[2] However, whether intestinal microbiota is really altered in those patients is unknown.

Metabolic profiling is a powerful exploratory tool for understanding the interactions between nutrients, the intestinal metabolism, and the microbiota composition in health and disease and to gain more insight into metabolic pathways. Currently, metabolomics technologies are being increasingly used for discovery of gastrointestinal disease signatures and have been applied for the screening of different pathologic conditions that are linked to a metabolic imbalance.[3] These host-microbiota metabolic interactions complicate the interpretation of metabolite profiles, and the outcome of metabolome analyses clearly depends on the biomatrix chosen. The contribution of the microbial metabolism is more likely reflected in the fecal metabolome than in urinary or serum of breath profiles.

We therefore assessed fecal microbiota and fecal metabolome in SUDD patients, comparing it with those of healthy controls (HCs) and people with asymptomatic diverticulosis (AD).

PATIENTS AND METHODS

Setting and Participants

From September 2012 to June 2014, 44 consecutive female patients fulfilling the following characteristics were enrolled: middle aged; having given birth by vaginal delivery, with exclusive breastfeeding; living in the same geographical area; having the same body mass index; no intake of antibiotics in the 3 months before enrollment; incidence of ongoing or past acute complicated and uncomplicated diverticulitis; and absence of bacterial and/or parasitic intestinal diseases (by stool cultures) and of lactose malabsorption (by lactose H_2 breath test).

We enrolled 15 SUDD patients, 13 AD patients, and 16 HCs. SUDD was defined as the presence of symptoms in

patients with diverticulosis, with presence of abdominal pain recorded in the lower left quadrant for > 24 hours and absence of any complications (stenosis, abscesses, fistulas).[4,5]

Fecal Samples Collection

Stool samples were collected at least 4 weeks after colonoscopy. They were collected early morning at home, in sterile plastic tubes, transported on ice to the laboratory within 2 hours, and stored at −80°C until analysis.

Fecal Microbiota Assessment

Bacterial DNA from fecal samples was extracted using the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany), as previously reported.[6] Real-time polymerase chain reaction (PCR) was used to quantify total bacteria, *Bifidobacterium* and *Lactobacillus* genera, *Clostridium coccoides* and *Bacteroides-Prevotella* groups, *Escherichia coli* subgroup, and *Akkermansia muciniphila*, using the primers and conditions reported.[6–11] PCR amplification and detection were performed on optical-grade 96-well plates with SensiMix SYBR PCR Master Mix (Bioline) using the Applied Biosystems 7500 Real-Time PCR instrument. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from *Lactobacillus brevis*, *Bacteroides fragilis*, *Bifidobacterium breve*, *E. coli*, *C. coccoides,* and *A. muciniphila*. All samples were analyzed in duplicate in 2 independent real-time PCR assays.

Fecal Metabolome Assessment

Fecal samples (analysis conducted on μmol/g) were analyzed in HC individuals, SUDD patients, and AD patients by means of ^{1}High-resolution Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy. Twenty-five fecal metabolites, of which 3 were not assigned, were assessed. All the ^{1}H NMR spectra were acquired at 298 K using a Bruker AVANCE 400 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) equipped with a magnet operating at 9.4 T and at 400.13 MHz for ^{1}H frequency, by applying a standard solvent presaturation sequence. Signal assignments were achieved by standard 2-dimensional (2D) ^{1}H Homonuclear Total Correlation Spectroscopy, ^{1}H ^{13}C Heteronuclear Single Quantum Correlation, and Heteronuclear Multiple Bond Correlation on selected samples and confirmed by comparison with home data sets and HMDB.[12,13] One-dimensional NMR spectra were processed and quantified using the ACD Lab 1D-NMR Manager version 12.0 software (Advanced Chemistry Development Inc., Toronto, ON, Canada), whereas 2D-NMR spectra were processed by using Bruker TopSpin version 3.1. The NMR spectra were manually phased, baseline corrected, and referenced to the chemical shift of the trimethylsilylpropionate methyl resonance at $\delta = 0.00$. The quantification of metabolites was performed by comparing the specific signal integrals to the trimethylsilylpropionate integral.

Statistical Methods

Statistical analyses were performed using SAS version 9.4 and JMP 12 (SAS Institute, Cary, NC). Normally continuous variables were presented as mean ± SD and non-normally distributed variables as median (interquartile range). Normality was tested with the Shapiro-Wilk test. The differences between the 3 groups (HC, AD, and SUDD) were analyzed using analysis of variance for normally distributed variables and by the Kruskal-Wallis test for skewed variables. Homoscedasticity was tested with the Levene and Brown-Forsythe test. For post hoc analysis Dunnett (HC vs. AD and HC vs. SUDD) and Tukey (AD vs. SUDD) tests were performed. Pearson (normal distribution) and Spearman (non-normal distribution) correlations were used to assess the correlations between variables. When data were not normally distributed, logarithmic transformation was performed. A P-value < 0.05 was considered significant. Multivariate analysis on fecal metabolome was carried out using Unscrambler X 10.3 software (CAMO, Oslo, Norway). Data were mean-centered and scaled before analysis. Principal component analysis was performed to verify the presence of outlier data, followed by partial least squares-discriminant analysis (PLS-DA) to check the existence of a model explaining the differences between pairs of classes based on the metabolic profiles of their subjects. For this reason the uncertainty test, based on cross-validation, jack-knifing, and stability plots, was applied to test the significance of the models.

Ethics Approval

The protocol was approved by the Ethics Committee. This study is registered with www.ClinicalTrials.gov, number NCT01831323.

RESULTS

Characteristics of the Studied Populations

The median age of the HCs, AD patients, and SUDD patients was 59.5 (range, 55 to 69) years, 62.0 (range, 56 to 68) years, and 64.5 (range, 52 to 70) years, respectively (P = NS). The median body mass index of the HCs, AD patients, and SUDD patients was 25.5 (range, 23.0 to 29.0), 26.5 (range, 24.3 to 32.0), and 26.5 (range, 21.6 to 33.7), respectively. Symptoms other than left lower-quadrant pain > 24 hours in SUDD patients were as follows: constipation in 6 patients (35.29%), diarrhea in 9 patients (52.94%), bloating in 2 patients (11.76%), and mucus with feces in 2 patients (11.76%). All of them showed overexpression of fecal calprotectin: it was between 15 and 60 μg in 10 patients (64.70%) and ≥ 60 μg in 5 patients (35.30%).

Analysis of the Fecal Microbiota

The total number of bacteria in the fecal samples was similar between the HCs, AD patients, and SUDD patients, and no significant differences were observed ($P = 0.449$). Thus, a colonic bacterial overgrowth was absent in SUDD patients. Average normalized abundance for each bacterial group of HCs, AD patients, and SUDD patients is presented in Figure 1. There was no difference in fecal abundance of dominant groups, defined as those found to represent 1-10% (between −2.0 and −1.0 log no. of bacteria) or more of the fecal bacterial population (*Bacteroides*/*Prevotella*, *C. coccoides*, and *Bifidobacterium*). Also the levels of less abundant microorganisms such as *Lactobacillus* and *E. coli* subgroups were not significantly different in participants of the 3 study groups.

Interestingly, the amount of *A. muciniphila* species was significantly different between HCs, AD patients, and SUDD patients ($P = 0.017$). Their counts in the HC microbiota (−4.57 ± 1.05) were significantly lower when compared with those of the AD group (−3.41 ± 1.13, $P = 0.019$) and SUDD patients (−3.56 ± 1.27, $P = 0.044$).

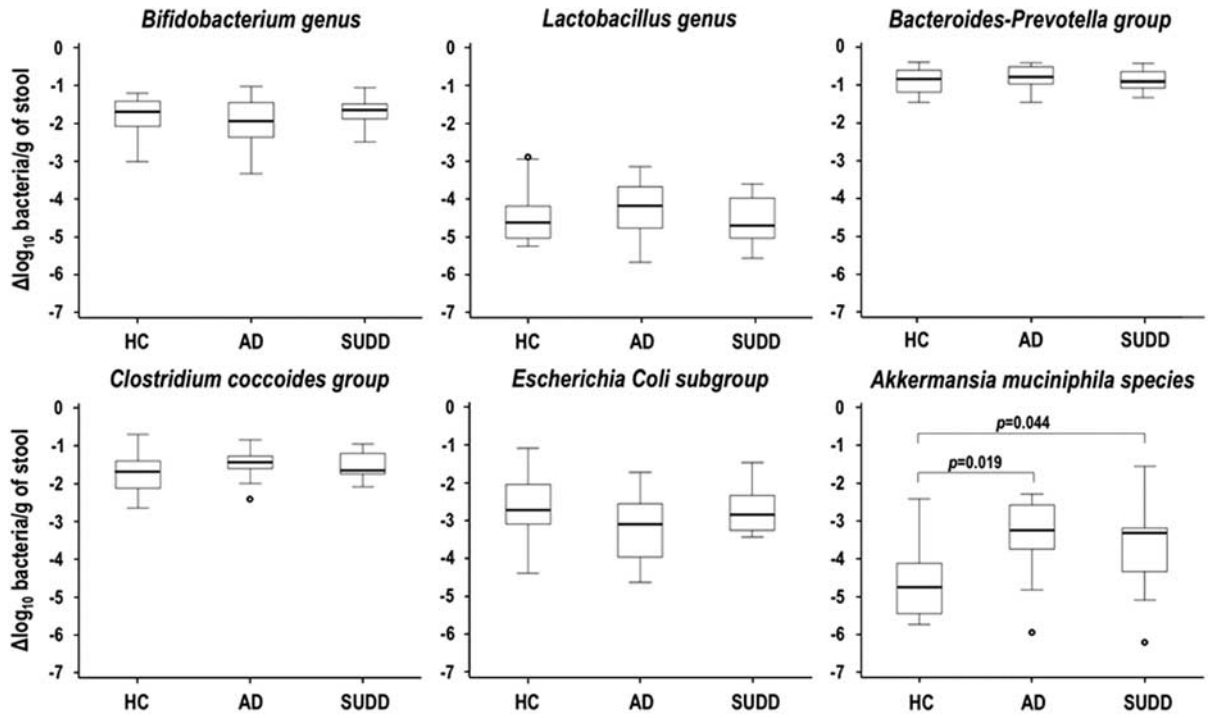

FIGURE 1. Quantification of fecal microbiota in healthy controls (HCs), asymptomatic diverticulosis (AD) patients, and symptomatic uncomplicated diverticular disease (SUDD) patients. The qPCR results were plotted as boxes and whiskers graph. The boxes (containing 50% of all values) show the median (horizontal line across the middle of the box) and interquartile range. The amount of each bacterial group was normalized for each subject and calculated as the log number of targeted bacteria minus the log number of total bacteria.

Analysis of the Fecal Metaboloma

Twenty-two fecal metabolites were identified and quantified: short-chain fatty acids (SCFA) (butyrate, valerate, acetate, and isovalerate), organic acids (3-hydroxy-phenylpropionate, 3-phenylacetate, formate, and fumarate), alcohols (ethanol and methanol), amino acids (alanine, glutamate, valine, glycine, and methionine), trimethylamine derivatives (choline, methylamine, and trimethylamine), glucose and N-acetyl-glucosamine (also including other glycans), niacinamide, uracil, and finally 3 not assigned resonances at 2.11, 6.38, and 6.87 ppm (indicated as U1, U2, and U3). The principal component analysis was applied on mean-centered and autoscaled data from NMR spectra of HC, AD, and SUDD patients as an explorative technique. As shown in Figure 2A, the principal component scores plot did not show a clustering of the participants belonging to different groups. Further supervised analysis, such as PLS-DA, did not show any significant statistical model for HC and SUDD comparison. Interestingly, a good separation between HC and AD participants was shown by PLS-DA applied on the respective metabolomic and microbiological data set ($R^2 = 0.733$; $Q^2 = 0.383$; latent variable = 2). Although the cross-validation of the model is not high enough, the model showed that the variable changes for the separation of the 2 classes were represented by a significant increase in *A. municiphila* abundance ($P = 0.003$) associated with a significant decrease in N-acetyl-compounds (such as N-acetyl-glucosamine; $P = 0.002$), isovalerate ($P = 0.033$), and U1 levels ($P = 0.019$) in AD patients. These results were also confirmed by the univariate analysis (1-way analysis of variance and Dunnett test, or Kruskal-Wallis associated with Tukey test for the whole data set). The Dunnet test for comparison between HC and AD showed significant decrease in N-acetyl-glucosamine in AD patients (0.27 ± 0.11 and 0.15 ± 0.05 μmol/fresh weight, respectively; $P = 0.012$).

When PLS-DA was applied on AD and SUDD samples, the model showed a good discrimination between these 2 groups ($R^2 = 0.69$; $Q^2 = 0.35$; latent variable = 2). The SUDD patients were characterized by low levels of valerate ($P = 0.009$), butyrate ($P = 0.047$), and choline ($P = 0.009$) and by high levels of N-acetyl -glucosamine ($P < 0.001$) and U1 ($P = 0.003$). In post hoc analysis SUDD patients showed significant increase in N-acetyl-glucosamine ($P = 0.0015$) and U1 ($P = 0.017$), compared with AD patients.

We observed significant relationships between the amount of *A. muciniphila* and several SCFA. *A. muciniphila* abundance was correlated with Valerate ($r = 0.33$, $P = 0.037$) and Formate ($r = 0.37$, $P = 0.02$) levels when all groups were examined together. Both in AD and SUDD groups, *A. muciniphila* was positively correlated with Isovalerate ($r = 0.63$, $P = 0.037$ and $r = 0.57$, $P = 0.04$, respectively), whereas in the AD group *A. muciniphila* was also associated with Valerate ($r = 0.66$, $P = 0.028$) and Formate ($r = 0.63$, $P = 0.037$).

DISCUSSION

Despite the small sample size of the study population, our results revealed for the first time changes in the fecal microbiota and metabolome composition in individuals with diverticula in comparison with HCs. The relative abundance of *A. muciniphila* was significantly higher in fecal samples of both AD and SUDD in comparison with HC. From the present study we cannot conclude whether

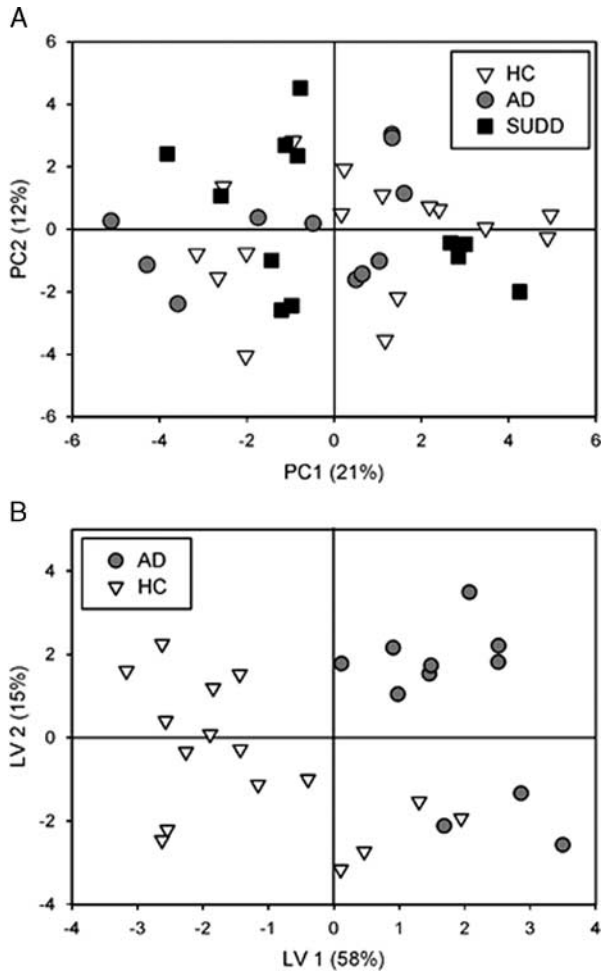

FIGURE 2. A, The explorative PC scores plot carried out from metabolomic and microbiological data of healthy controls (HCs), asymptomatic diverticulosis (AD), and symptomatic uncomplicated diverticular disease (SUDD) patients. The LV score plot (B) carried out from the whole data set (metabolomic and microbiological sets) showed a significant separation between HC and AD groups.

fecal bacterial abundance is directly proportional to abundance in the gut. Microbiota in the mucus layer differs from that of the intestinal lumen, and *A. muciniphila* is closely associated with the gut mucosal layer.[14] The observed differences in abundance of *A. muciniphila* into feces may be due to actual changes in bacterial numbers, or alterations of the mucosal layer and gut architecture. *A. muciniphila* produces a variety of fermentation products, including SCFAs, through mucin degradation.[15] We observed in both AD and SUDD patients a correlation between *Akkermansia* levels and several SCFAs, indicating that microbiota component changes co-occurred with metabolic changes associated with diverticula.

Our results indicate a fecal metabolic and microbiological signature in AD, characterized by a high *A. municiphila* abundance associated with a decrease in N-acetyl-glucosamine, isovalerate, and an unidentified compound (U1). However, the lack of significant correlations among *A. municiphila* abundance and N-acetyl-carbohydrates, when all groups were examined together, suggests that these metabolite changes are covariations not depending on the specific metabolic activity of *Akkermansia* spp. The decrease in N-acetyl-carbohydrates could be the result of a complex change in the microbiota ecosystem associated with the presence of diverticula. The significant difference in some metabolites in SUDD patients as compared with AD also indicates the presence of different metabolic activities that need further investigation.

In conclusion, the study shows significant fecal metabolome and microbiota differences between AD patients and healthy individuals, as well as between AD and SUDD patients. However, it needs to be determined whether changes in the gut microbiome and metabolome indeed are a cause or just a consequence of diverticular disease.

REFERENCES

1. Strate LL, Modi R, Cohen E, et al. Diverticular disease as a chronic illness: evolving epidemiologic and clinical insights. *Am J Gastroenterol*. 2012;107:1486–1493.
2. Tursi A. New physiopathological and therapeutic approaches to diverticular disease: an update. *Expert Opin Pharmacother*. 2014;15:1005–1017.
3. De Preter V, Verbeke K. Metabolomics as a diagnostic tool in gastroenterology. *World J Gastrointest Pharmacol Ther*. 2013;4:97–107.
4. Tursi A, Elisei W, Picchio M, et al. Moderate to severe and prolonged left lower-abdominal pain is the best symptom characterizing symptomatic uncomplicated diverticular disease of the colon: a comparison with fecal calprotectin in clinical setting. *J Clin Gastroenterol*. 2015;49:218–221.
5. Tursi A, Papa A, Danese S. Review article: the pathophysiology and medical management of diverticulosis and diverticular disease of the colon. *Aliment Pharmacol Ther*. 2015;42:664–684.
6. Mastromarino P, Capobianco D, Campagna G, et al. Correlation between lactoferrin and beneficial microbiota in breast milk and infant's feces. *Biometals*. 2014;27:1077–1086.
7. Nadkarni MA, Martin FE, Jacques NA, et al. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*. 2002;148:257–266.
8. Matsuki T, Watanabe K, Fujimoto J, et al. Use of 16 S rRNA gene targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol*. 2004;70:7220–7228.
9. Bartosch S, Fite A, Macfarlane GT, et al. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl Environ Microbiol*. 2004;70:3575–3581.
10. Malinen E, Kassinen A, Rinttilä T, et al. Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology*. 2003;149:269–277.
11. Collado MC, Derrien M, Isolauri E, et al. Intestinal integrity and *Akkermansia muciniphila,* a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. *Appl Environ Microbiol*. 2007;73:7767–7770.
12. Wishart DS, Jewison T, Guo AC, et al. HMDB 3.0 — The Human Metabolome Database in 2013. *Nucleic Acids Res*. 2013;41(D1):D801–D807.
13. Del Chierico F, Vernocchi P, Petrucca A, et al. Phylogenetic and metabolic tracking of gut microbiota during perinatal development. *PLoS One*. 2015;10:e0137347.
14. Dao MC, Everard A, Aron-Wisnewsky J, et al. *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut*. 2016;65:426–436.
15. Derrien M, Vaughan EE, Plugge CM, et al. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol*. 2004;54:1469–1476.

Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved.