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The presence of *Oxalobacter formigenes* in the microbiome of healthy young adults

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Abstract

Oxalobacter formigenes, a member of the human colonic microbiota that plays a major role in net colonic oxalate transport and secretion, is protective against formation of calcium oxalate kidney stones. We now describe the prevalence, relative abundance and stability of *O. formigenes* in healthy young adults in the United States, as revealed by Human Microbiome Project (HMP) data from fecal samples from 242 healthy young adults who had 1-3 study visits. Samples underwent whole-genomic shotgun (WGS) sequencing, and/or 16S rRNA sequencing. Three datasets available from the processed sequence data were studied: WGS metagenomic analysis by alignment to reference genomes (HMSCP) or using MetaPhlAn, or QIIME analysis of the V1-3 or V3-5 16S sequences. *O. formigenes* was detected in fecal samples using both the WGS and 16S rRNA data. Analysis of the WGS dataset, using HMSCP, showed that 29 (31%) of 94 subjects were *O. formigenes*-positive while the V1-3 and V3-5 analyses were less sensitive for *O. formigenes* detection. When present, *O. formigenes* relative abundance varied over 3 log₁₀, and was normally distributed. For 66 samples studied by all three methods, all assays agreed in 58 (88%). Of 14 subjects who were *O. formigenes*-positive at baseline, 13 (93%) were positive on follow-up visit, indicating the stability of colonization. *O. formigenes* appears to be stably present in fewer than half of healthy young USA adults and is most sensitively detected by WGS.

Keywords

kidney calculi; metagenome; microbiota; nephrolithiasis; oxalate; urolithiasis

INTRODUCTION

Oxalate is absorbed passively by the gut from dietary sources, and produced endogenously as an end-product of amino acid metabolism and excreted by the kidneys^{1, 2}. The urinary oxalate level, reflecting overall oxalate load, is a major determinant of the risk of calcium oxalate kidney stones, the most common type^{3, 4}. The increased prevalence of nephrolithiasis described in US adults⁵ and the dramatic rise in its incidence in children⁶

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suggest that oxalate stone prevalence is rising. The major factors contributing to this trend are unknown.

Oxalobacter formigenes is a gram-negative anaerobic bacterium that depends on oxalate metabolism for energy⁷, and that colonizes the human intestine⁸. *O. formigenes* plays a major role in regulating net intestinal transport of oxalate⁹. The lack of *O. formigenes* in the colon is believed to increase intestinal oxalate absorption, leading to an increase in urinary oxalate excretion¹⁰. Among oxalate-degrading bacteria in humans, *O. formigenes* appears to be the most efficient¹¹.

Human *O. formigenes* isolates are susceptible to many commonly used antibiotics¹²; thus, colonization status could reflect health status¹³, dietary habits¹⁴, or antibiotic exposure¹⁵. Because *O. formigenes* is difficult to culture, colonization status has been determined either indirectly using an oxalate-degradation assay or directly by polymerase chain reaction (PCR)^{16, 17}. In healthy American adults, colonization rates varied between 38 and 62% with geographic location, age, and method of testing accounting for much of the variation¹². In less developed countries, the prevalence of *O. formigenes* colonization is higher¹². Recent studies of an uncontacted tribal group detected *O. formigenes* in nearly all¹⁸. These observations suggest that *O. formigenes* may be disappearing as a consequence of modern lifestyles, including antibiotic use¹⁵.

As part of the NIH-sponsored Human Microbiome Program (HMP), the microbiota and metagenome of 242 healthy young adult volunteers were surveyed^{19, 20}. These studies provided the opportunity to address questions about the frequency of detecting *O. formigenes* in US subjects.

Materials and Methods

Population studied and samples interrogated

Institutional review board approval was not required since the study involved mining a publically available database consisting of microbial sequences from de-identified subjects. In our HMP data, 242 healthy subjects between the ages of 18 and 40 years old had 5300 samples taken at 15-18 body sites on up to three separate visits²⁰. During Phase I, the HMP investigators selected a subset of these samples for WGS and 16S rRNA sequencing analysis. The processed sequence data are available through the HMP Data Analysis and Coordination Center (DACC).

In the HMP shotgun metagenomic analysis, a subset of samples was selected for WGS sequencing and subsequent quality control tests^{19, 20}. The data available were from an initial phase of the HMP project. Although the entire data set included 690 samples (<http://hmpdacc.org/HMASM/>), and the number of subjects in the HMP is 242²⁰, the total number of subjects included in this database was 103 with only 94 of these subjects having at least 1 stool sample.

A standardized 16S rRNA sequencing protocol was used to profile 5177 samples, and two variable regions of the 16S rRNA gene, V1-3 and V3-5, important for taxonomy assignment

underwent sequence analysis¹⁹. The samples were amplified and sequenced using the Roche-454 FLX Titanium platform²⁰. The V1-3 variable region of the 16S rRNA gene was sequenced for 2,971 samples (including 193 stool samples) and the V3-5 variable region was sequenced for 4,879 samples (including 328 stool samples). By including only those samples with complete records and linkage across datasets, this study focuses on a subset of 187 stool samples from 144 subjects for the V1-3 analyses, and 319 stool samples from 210 subjects for the V3-5 analyses; samples were sequenced for either both the V1-3 and V3-5 16S rRNA regions, or only one (**Supplemental Figure 1**).

Metagenomic and 16S rRNA sequencing data analysis

Three datasets from the DACC were available for this analysis. Two contained the results of WGS metagenomic analysis using different approaches: shotgun community profiling by alignment to reference genomes (HMSCP) and through the MetaPhlAn algorithm²¹. In the first approach, WGS reads were mapped onto a reference genome database to determine the organisms present in the community. The results of the analysis included a bacterial strain abundance table for each HMP sample, available through www.hmpdacc.org/HMSCP/. The reference genome database includes organisms available in GenBank as of November 2009 and can be accessed at <http://www.hmpdacc.org/HMREFG/>. We used this dataset to examine prevalence of *O. formigenes* and to distinguish between *O. formigenes* Group I and Group II which are represented by strains OXCC13 and strain HOxBLS, respectively²². In the second approach, metagenomic data were taxonomically profiled using MetaPhlAn²¹, permitting estimation of relative abundances for the different taxa, with the output of the analysis at www.hmpdacc.org/HMSMCP.

The third dataset provides the output of QIIME-based analyses^{23,24} of 16S rRNA gene data. In QIIME analysis the 16S sequences are clustered into operational taxonomic units (OTUs), a representative sequence for each OTU is chosen, and then a taxonomy assigned using the RDP classifier. The complete output of QIIME analysis of the V1-3 and V3-5 16S data is available from the HMP DACC (<http://www.hmpdacc.org/HMQCP/>).

We constructed a MySQL database containing information from the publically available datasets above, through the HMP DACC. By restructuring the data using the MySQL database, we created a standardized pipeline for determining microbial prevalence, abundance, and diversity associations. Since particular *O. formigenes* 16S OTUs were identified from the HMP specimens, we sought to determine to which of the known *O. formigenes* Groups (I and II) they best matched. To accomplish this, we examined the HMP sequences with reference to the *O. formigenes* strains in the Greengenes database, and we identified the best match from each Group.

RESULTS

Prevalence of *Oxalobacter formigenes*. We sought to define the prevalence of *O. formigenes* in the intestinal tract of the healthy young adult HMP participants. *O. formigenes* was detected in fecal specimens in both the WGS and 16S rRNA analyses. In the WGS dataset, using the HMSCP-analytic tools, 29(31%) of 94 subjects were positive for *O. formigenes*; however, analysis of the V1-3 and V3-5 data, using QIIME, showed *O. formigenes*

prevalence of the studied subjects only at 15% and 11%, respectively (**Table 1**). In comparison, the prevalence of was 71, 12, and 40%, respectively, in the same three analyses. These disparate results highlight the difficulties intrinsic to interpreting the 16S and the WGS data,

Within species differences. The reference genome database used for the HMSCP WGS analysis contains strain OXCC13 that represents *O. formigenes* Group I and strain HOxBLS that represents Group II²⁵. In addition to genetic differences, the two Groups differ phenotypically in oxalate-degrading capacity, fatty acid content, oxygen tolerance and antigenic features⁸. In the WGS analysis, all 29 of the *O. formigenes*-positive subjects were colonized with sequences represented by Group I strains, and of these, 17 (59%) were simultaneously colonized with organisms with sequences represented by Group II strains. Thus, co-infection with both Group I and Group II was common, and colonization by Group II was only detected in the presence of Group I.

Fecal colonization stability. To examine the stability of *O. formigenes* colonization in the WGS-tested subjects, we studied 42 subjects who had 2 visits at which samples were obtained; the remaining 52 subjects did not complete a second visit. Of the 42 subjects, 13(33%) were positive at baseline; 12 (93%) remained positive (**Figure 1**). Of the 29 subjects who were negative at the first visit, 27 (93%) remained negative, but in two subjects, the presence of the organism was detected on the second visit (and for one, on the third visit). The temporal stability of *O. formigenes* was mirrored at the strain level, for which most (93-100%) of the positive samples were Group I, with ~40-50% simultaneously positive for Group II, depending on the visit. Only 1 (7%) of the 14 subjects who were *O. formigenes*-positive at the first visit carried Group II with no evidence for Group I. However, at the second visit, both Groups were detected.

Abundance of *O. formigenes*: In 36 *O. formigenes*-positive stool samples from the WGS dataset analyzed by MetaPhlAn, the relative *O. formigenes* abundance was normally distributed around a median of 0.015% (**Figure 2A**). Abundance varied over ~3 log₁₀ while the abundance of *E. coli* (median 0.03%) and *Bacteroides fragilis* (median 0.036%) varied over 5 log₁₀ and 4 log₁₀, respectively (**Figure 2B**). The overall mean abundance was stable on follow-up visits across the population of those positive for all three species examined. For the nine subjects studied twice, the median values for *O. formigenes* abundance on the first visit (0.013%) and the second visit (0.017%) were similar (**Figure 2C,D**).

The *O. formigenes* and *E. coli* abundances also were compared in the 16S rRNA datasets. The V1-3 data showed *O. formigenes* abundance ranging between 0.01-0.41% (median 0.08%) and *E. coli* abundance ranging between 0.01-0.70%(median 0.48%) (**Figure 3A**). The V3-5 data showed lower *O. formigenes* (0.01-0.22%; median 0.03%) and slightly higher *E. coli* (0.01-11.67%; median 0.52%) abundance (**Figure 3B**). In the V1-3 and V3-5 analyses, the median abundance of *O. formigenes* was ~6-18-fold lower than that of *E. coli*, although *E. coli* varied over a larger range.

Relationship to other species. We applied the MetaPhlAn tool to compare *O. formigenes* prevalence to other gut microbiota to examine relationships between the different species in

the WGS dataset. This analysis, performed on 139 stool samples from 94 subjects, detected *O. formigenes* in 27 subjects (29%), *E. coli* in 67 (71%), and *B. fragilis* in 49 (52%). *O. formigenes* presence was independent of whether or not *E. coli* ($p=0.7$) or *B. fragilis* ($p=0.18$) were present. Using the V1-3 data, *O. formigenes* and *E. coli* were detected in 15 and 12%, respectively, but using the V3-5 data, *O. formigenes* prevalence was 11% and *E. coli* was 40% (**Table 1**). These differences in relative proportions illustrate the limitations of microbial census-taking using primers based on the 16S variable regions ²⁶.

O. formigenes diversity. We also examined *O. formigenes* diversity in 14 samples that were positive in both the V1-3 and V3-5 datasets. We detected three *O. formigenes* OTUs in each of the datasets (**Figure 4**). Two samples had OTU 11418 in the V1-3 dataset, and in both cases, they also showed OTU 15538 in the V3-5 dataset; this suggests that the two OTUs reflect the same strains. Similarly, all but one sample that had OTU 2496 in the V3-5 dataset had OTU 1285 in V1-3. OTUs 5310 and 22911 were similarly but not identically identified in the V1-3 and V3-5 dataset samples, respectively. These data suggest the presence of two major Groups and one minor Group of *O. formigenes* in the positive samples.

Comparing the 14 samples that underwent both V1-3 and V3-5 16S sequencing, 12 (86%) appear concordant between both analyses and two were discordant. All but one sample, positive for OTUs 1285 and 5310, showed only a single OTU in both analyses (**Figure 4**). When compared to the 16S rRNA genes of known Group I and Group II *O. formigenes*, the sequences of OTU 1285/2496 and OTU 5310/22911 were similar to Group II and Group I strains, respectively (**Supplemental Table 1**). OTU 11418/15538 could represent another less prevalent *O. formigenes* Group, which we now call “Presumptive Group III.” Further microbiological or molecular analysis are required to identify and characterize this group.

Sensitivity of *O. formigenes* detection in the V1-3, V3-5, and WGS dataset analyses. We compared *O. formigenes* detection in 66 stool specimens for which samples were analyzed using all three HTS methods. By all three testing methods, *O. formigenes* was undetectable in 51 and identified in 7; thus 58 (88%) of 66 were completely concordant in all three analyses. For the non-concordant eight, their mean relative abundance (0.037%) in the WGS dataset was similar to the seven concordant positives (0.039%) (Student’s T-test, $p=0.92$) All samples positive for *O. formigenes* in either the V1-3 or V3-5 datasets also were positive by WGS; however, *O. formigenes* was detected in two samples in the WGS analysis, but was negative in the V1-3 and V3-5 analyses.

O. formigenes at non-fecal sites. Sequences representative of *O. formigenes* also were detected in non-stool samples, ~2.2% and 1.3% of the samples subjected to V1-3 and V3-5 analyses, respectively; most were from the cutaneous sites. The positive samples in the V1-3 analysis were collected from four subjects, two of whom also had *O. formigenes* detected in non-stool samples by V3-5 testing (**Table 2**).

DISCUSSION

The HMP is of a similar scope to the Human Genome Project in that it sought to more fully characterize the micro-organisms coexisting with humans^{19, 20}. Selection of young healthy

subjects was intended to represent adults prior to the development of comorbidities. To our knowledge, study of the HMP has not yet appeared in the urology literature nor applied to the study of urolithiasis. To eventually more precisely characterize the longitudinal risk of kidney stones, studies that determine *O. formigenes* prevalence in unaffected, healthy adults will be essential. Developing appropriate methodologies will also be critical.

The protective role of *O. formigenes* against hyperoxaluria is well-established in animal models²⁷ and also may be applicable to humans¹⁰. The absence of *O. formigenes* in humans has been associated with higher urinary stone prevalence²⁸. In the rat colon, *O. formigenes* induces oxalate secretion into the lumen, and decreases the oxalate gradient that drives oxalate absorption across the colonic epithelium⁹.

O. formigenes is detected in the gut based on the ability of anaerobic cultures to metabolize oxalate^{7, 16}, a method that is time-consuming, limited by variation in culturability and metabolism, and non-specific, since other species may metabolize oxalate²⁹. To improve accuracy, molecular assays have been designed to detect *O. formigenes oxc* which encodes Oxc (oxalyl coenzyme-A decarboxylase)³⁰, but the heterogeneous distribution and the range in *O. formigenes* abundance in the human gut indicated by the present study adds to the complexity of detection strategies.

With the increasing availability of open source 16S rRNA and WGS studies of the microbiome, understanding the sensitivity, specificity, and utility of each of those methods for *O. formigenes* detection is needed for future investigations of its relationship to kidney stone prevalence. The HMP, with V1-3 and V3-5 16S rRNA and WGS data from the same individuals, represents a valuable resource for comparing these methods. This study serves as a model for analysis of such databases for specific bacterial species and strains to relate to pathologic conditions.

No prior studies showed that *O. formigenes* can be detected by WGS and HTS genomic techniques. Among the HMP methods, WGS was the most sensitive. The relatively low *O. formigenes* prevalence (31%) detected by WGS is consistent with prior US studies using different methodologies^{15, 28}. When *O. formigenes* is present at low abundance, it can be induced by high oxalate diet¹⁴. Giving an oxalate load may be one way to increase assay sensitivity. With the present study design, using de-identified data, we cannot determine whether the absence of colonization is associated with higher risk of urolithiasis. We also do not have 24 hour urine data to correlate *O. formigenes* colonization with lower urinary oxalate excretion.

The large inter-individual variation in *O. formigenes* abundance could partially explain the discrepant sensitivities of the WGS and 16S rRNA methods used; variation in dietary oxalate intake also could contribute to the variation in abundance. Stable *O. formigenes* colonization was detected in subjects tested on multiple occasions, at both the strain and OTU levels. Our results resemble those of our prior study showing that 91.7% of patients with *O. formigenes* at baseline remained positive when retested one and six months later¹⁸. This relative stability is notable since neither of these studies controlled dietary oxalate content. The 16S rRNA “universal” PCR primers differ in their sensitivity for particular

taxa²⁶, for detecting both *E. coli* and *O. formigenes*, the PCRs based on the V1-3 region were less sensitive than for V3-5.

Strain-level analysis of fecal samples has been limited, with conflicting results on the dominant *O. formigenes* strains in the US population. Although no prior studies have detected colonization with more than one *O. formigenes* strain^{8, 14}, our WGS analysis demonstrates that healthy adults can simultaneously harbor both Group I and Group II strains. The lack of detected colonization with Group II strains alone suggests either obligate dependence on Group I or that the methods used are more sensitive for Group I. With both sets of 16S rRNA primers, three *O. formigenes* OTUs were detected in the study samples, remaining stable over time. In contrast to the WGS data, Group II appears more common than Group I, and a new “Presumptive Group III” was identified. Since the relative effects of Group I and Group II strains on oxalate balance are not established, the importance of our findings to the epidemiology of stone disease remains to be determined.

That *O. formigenes* was present in some specimens obtained from non-fecal sites raises the possibility that it is a normal constituent of that site, or more likely represents contamination from the GI tract. Several subjects who had non-fecal positivity also had stool-positivity, favoring the hypothesis of contamination from fecal sources.

A limitation of our study is that it involves mining an existing database that was not designed to study *O. formigenes*. The non-uniform testing of samples/subjects adds to the limitations on the interpretability of the results. Nevertheless, using these imperfect data sets, we discovered novel information regarding *O. formigenes* colonization in humans, including presence of a newly identified “Presumptive Group III”.

CONCLUSIONS

In conclusion, all *O. formigenes*-detection methods appear insensitive, possibly due to the extensive variation in its relative abundance. Yet most (88%) of the samples examined by all three-test methods yielded completely consistent results. Although the optimal method for *O. formigenes* detection remains to be established, WGS was most sensitive in this study. The relatively lower rate of *O. formigenes* detection in relation to other non-USA populations¹², provides further evidence to support a general trend of the disappearance of this organism with socioeconomic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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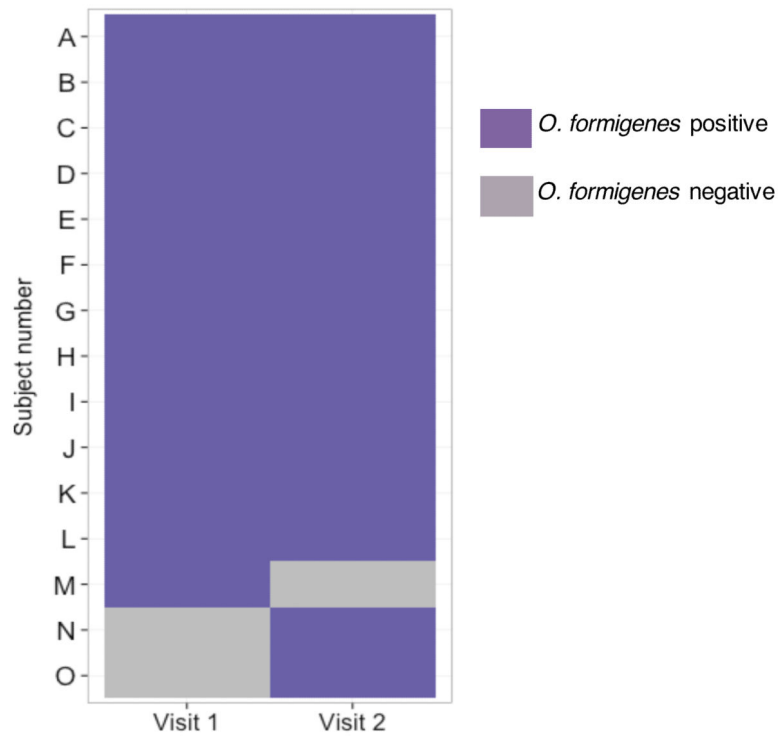
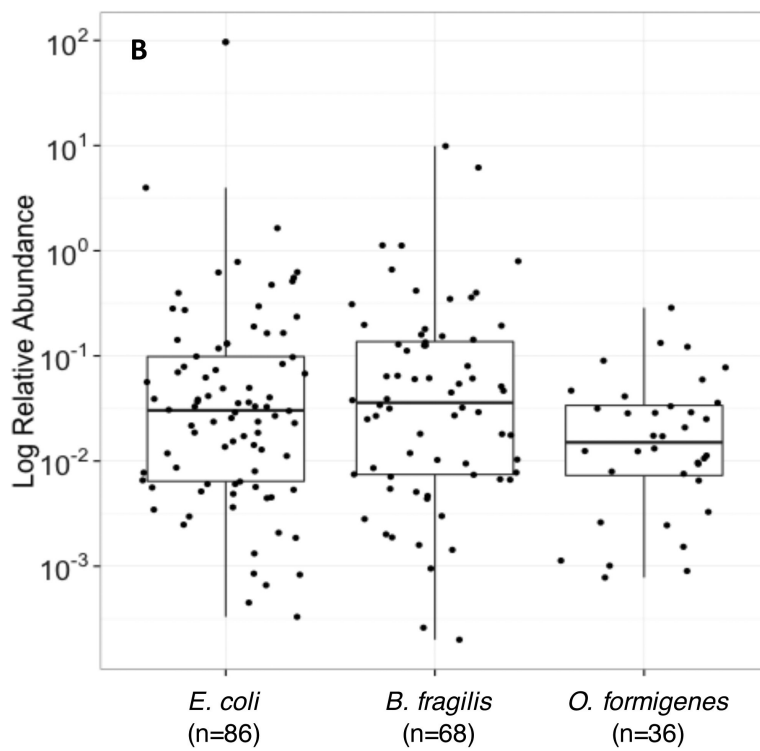
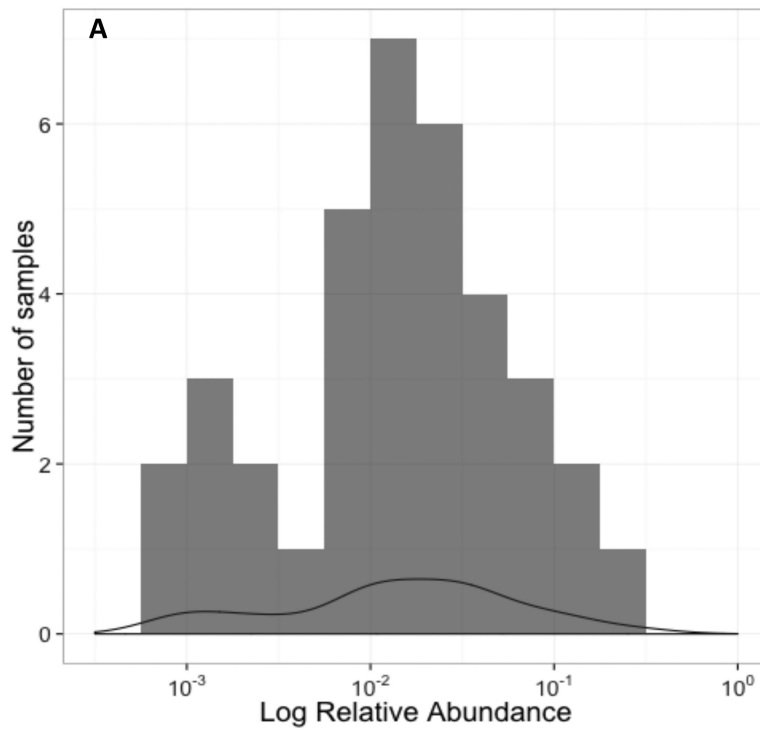


Figure 1. Stability of *O. formigenes* over time in subjects studied twice and analyzed by shotgun community profiling

In 42 subjects tested on two consecutive visits analyzed using the HMSCP dataset, 27 were negative on both visits and are not shown. Shown are the 15 subjects with at least one positive result.



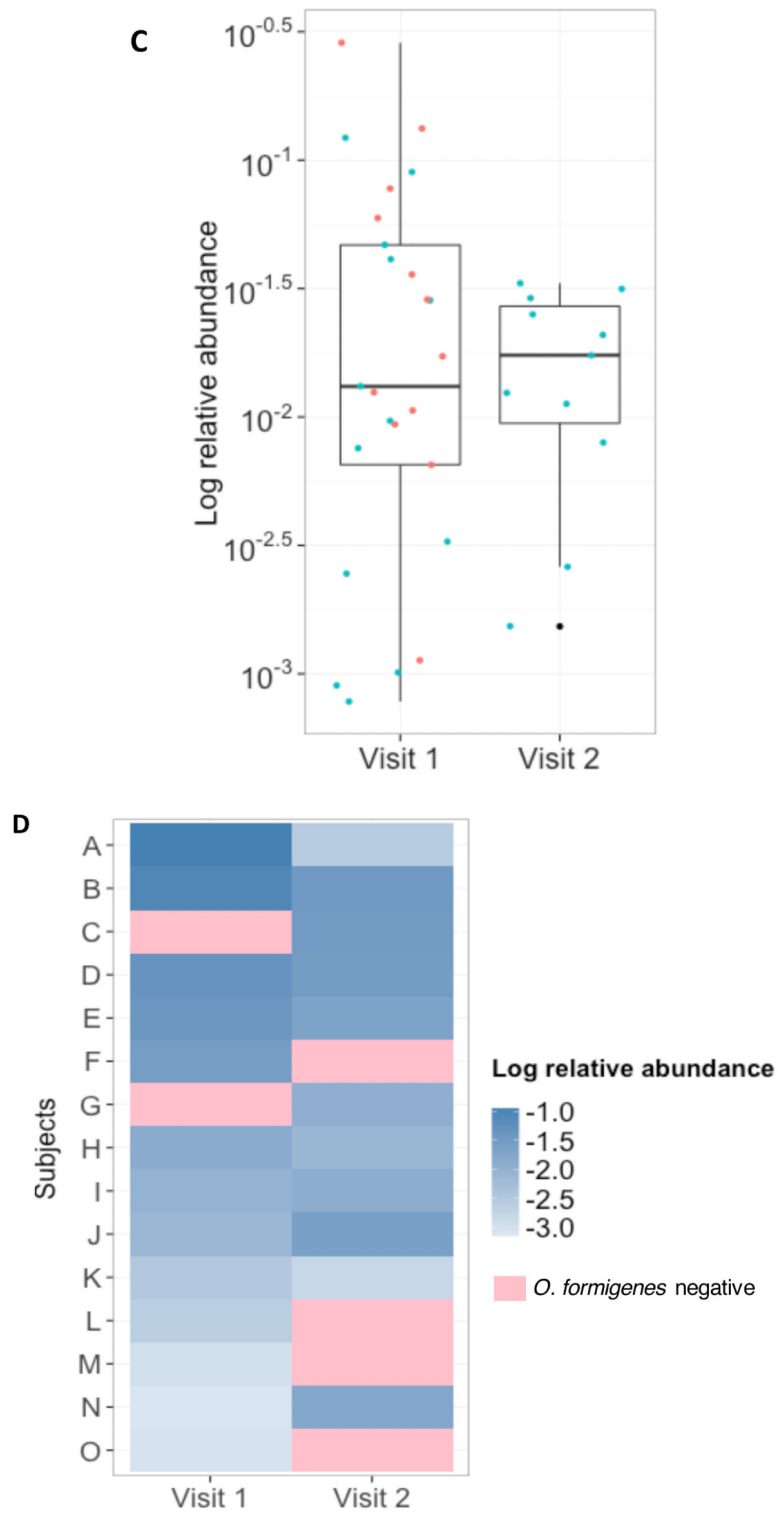


Figure 2. Relative abundance of *O. formigenes* in the 36 fecal samples in the MetaPhlAn WGS dataset

Panel A: Histogram of number of samples by log relative abundance of *O. formigenes*.

Panel B: Log relative abundance of *O. formigenes* (n=36) compared to *E. coli* (n=86) and *B.*

fragilis (n=68). Box indicates median and inter-quartile range, and lines indicate 95% confidence interval. **Panel C:** Stability of *O. formigenes* log relative abundance in HMP subjects examined once, (red circles; n=51) or twice (blue circles; n=43), and found to be positive on at least one sample. **Panel D:** *O. formigenes* relative abundance by visit for the 15 subjects studied twice.

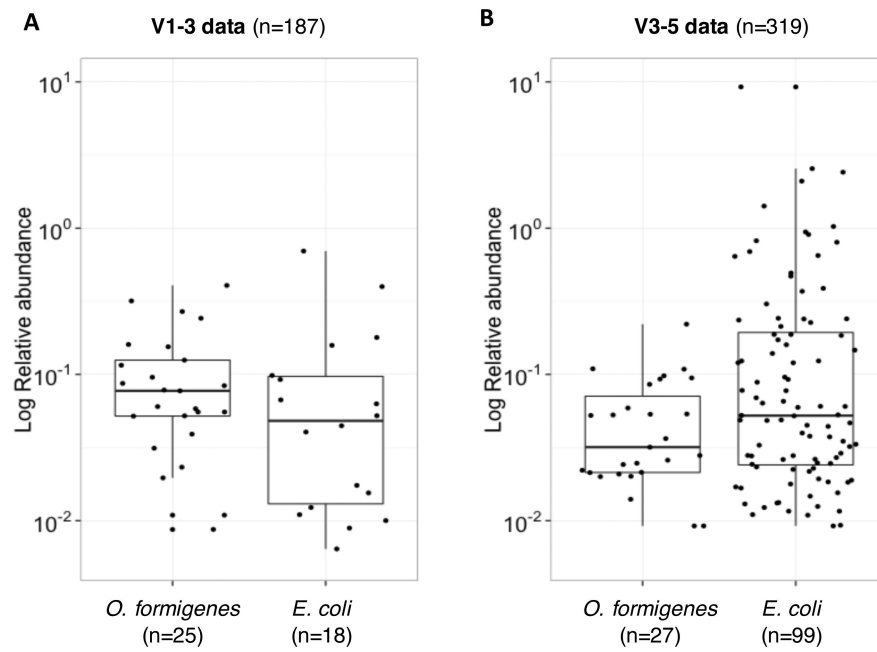


Figure 3. Abundance of *O. formigenes* and *E. coli* in HMP fecal samples, using QIIME 16S rRNA data

Panel A: *O. formigenes* and *E. coli* relative abundances in 25 and 18 positive subjects, respectively, using the V1-3 dataset. **Panel B:** *O. formigenes* and *E. coli* relative abundance in 27 and 99 positive subjects, respectively, using the V3-5 dataset.

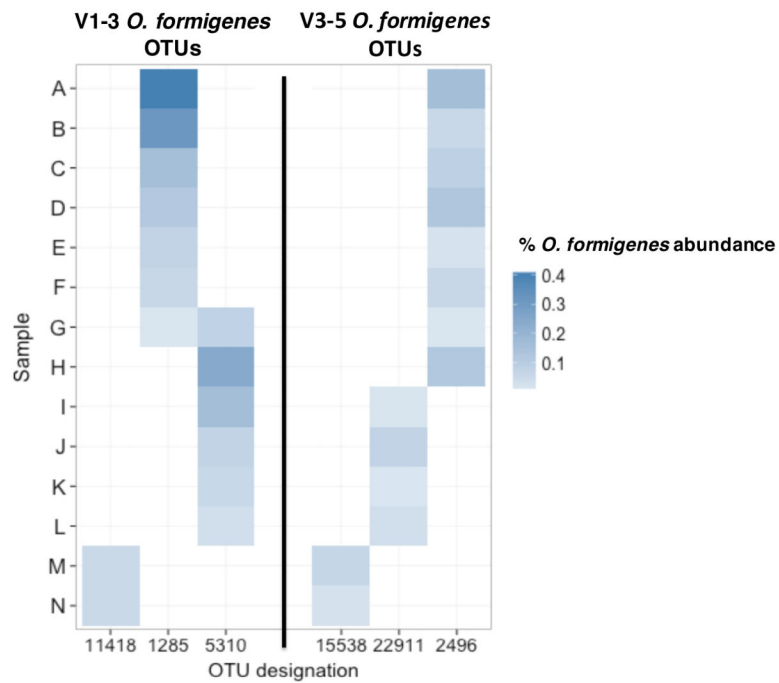


Figure 4. *O. formigenes* OTU abundance for the 14 stool samples that were positive in both the V1-3 and V3-5 datasets

The heatmap shows the percent relative abundance of the three different OTUs that each were detected in the V1-3 or V3-5 datasets, respectively.

Table 1

Prevalence of *O. formigenes* and *E. coli* in HMP subjects, based on separate analyses.

Microbe status	Metagenomic WGS		V1-3 16S rRNA		V3-5 16S rRNA	
	Subjects (n=94)	Samples (n=139)	Subjects (n=144)	Samples (n=187)	Subjects (n=210)	Samples (n=319)
<i>O. formigenes</i> +						
HMSCP	29 (31%)	42 (30%)	22 (15%)	25 (13%)	23 (11%)	27 (9%)
MetaPhlAn	27 (29%)	36 (26%)				
<i>E. coli</i> +	67 (71%)	86 (62%)	17 (12%)	18 (10%)	84 (40%)	99 (31%)

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Table 2

O. formigenes status by body site in V1-3 and V3-5 16S HMP data.

	V1-3 samples (n=2898)		V3-5 samples (n=4743)	
	Total number	<i>O. formigenes</i> +	Total number	<i>O. formigenes</i> +
Stool	187	25	319	27
Skin	664	3	990	4
Airways	161	2	269	0
Oral cavity	1622	1	2774	0
Vagina	264	0	391	0

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