Composition of the mucosa-associated microbiota along the entire gastrointestinal tract of human individuals

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Abstract

Background: Homeostasis of the gastrointestinal tract depends on a healthy bacterial microbiota, with alterations in microbiota composition suggested to contribute to diseases. To unravel bacterial contribution to disease pathology, a thorough understanding of the microbiota of the complete gastrointestinal tract is essential. To date, most microbial analyses have either focused on faecal samples, or on the microbial constitution of one gastrointestinal location instead of different locations within one individual.

Objective: We aimed to analyse the mucosal microbiome along the entire gastrointestinal tract within the same individuals.

Methods: Mucosal biopsies were taken from nine different sites in 14 individuals undergoing antegrade and subsequent retrograde double-balloon enteroscopy. The bacterial composition was characterised using 16S rRNA sequencing with Illumina Miseq.

Results: At double-balloon enteroscopy, one individual had a caecal adenocarcinoma and one individual had Peutz-Jeghers polyps. The composition of the microbiota distinctively changed along the gastrointestinal tract with larger bacterial load, diversity and abundance of Firmicutes and Bacteroidetes in the lower gastrointestinal tract than the upper gastrointestinal tract, which was predominated by Proteobacteria and Firmicutes.

Conclusions: We show that gastrointestinal location is a larger determinant of mucosal microbial diversity than inter-person differences. These data provide a baseline for further studies investigating gastrointestinal microbiota-related disease.

Keywords

Colonic microflora, gastrointestinal tract, intestinal microbiology, small bowel, colon

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What are the significant and/or new findings of this study?

- The bacterial load of mucosal samples decreased from oesophagus to proximal ileum, but drastically increased again in the lower GI tract.
- The composition of the microbiota markedly changes along the GI tract with larger diversity in the lower GI tract than the upper GI tract.

Introduction

In recent years, an increasing level of knowledge on the interaction between host and bacteria has made us come to regard the gut microbiota as a separate entity.\(^1\) The microbiota has important immunological, structural, metabolic and defence functions in the gut. Alterations in microbiota composition have been linked to intestinal disease, including colorectal cancer and inflammatory bowel disease (IBD). Unravelling the microbiota composition and its distribution along the gastrointestinal (GI) lining in healthy individuals is important to understand the role of the microbiota in disease.\(^2\)

Characterization of the microbiota in the entire GI tract is hampered by the fact that some locations are more difficult to access than others and most research has focused on the colonic faecal microbiota.\(^1\) The mucosal microbiome is arguably the more relevant compartment, as such mucosa-associated flora lives in close contact with the GI tract lining. The microbial composition of the colonic mucosa has been most often investigated. While it is clear that the composition and abundance of mucosal microbiota of the oesophagus and stomach in healthy individuals differ from that in the colon,\(^3\)–\(^5\) information about the microbial composition in the jejunum and ileum is scarce because of the inaccessibility of these sites.

Nevertheless, differences in the physiological functions of GI sites logically predict regional bacterial differences. The colonic microbiota for example, is driven by complex carbohydrates whereas simple carbohydrates fuel the microbiota in the small intestine.\(^2,6\) Furthermore, the composition of the mucus layer protecting the epithelial barrier from excessive bacterial contact differs along the intestinal tract.\(^7,8\)

Given the limited information about mucosal microbiota in the entire GI tract, we aimed to characterise the mucosal microbiota along the length of the entire GI tract within the same subjects.

Methods

Subject recruitment

Subjects, all inhabitants of The Netherlands, had abdominal symptoms of unknown cause requiring diagnostic antegrade and subsequent retrograde double-balloon enteroscopy (DBE). Exclusion criteria were: patients younger than 18 years, use of antibiotics three months before DBE, IBD, and failure to understand written Dutch. The study was conducted in accordance with the Declaration of Helsinki Principles and approved by the ethical committee of the Erasmus University Medical Center, Rotterdam (MEC-2017-151) on 3 April 2017.

Sampling

Mucosal samples were obtained endoscopically using antegrade and subsequent retrograde double balloon enteroscopy (DBE) at the Erasmus Medical Center using Fujinon EN-450P5 and EN-450T5 (Fujinon Inc., Saitama, Japan) endoscopes. Endoscopes were disinfected before use. Mucosal biopsies using standard biopsy forceps were taken at nine different sites of the GI tract (Figure 1). Upper GI biopsies (oesophagus to proximal ileum) were collected using antegrade endoscopy and lower GI biopsies (distal ileum to rectum) with retrograde endoscopy. Between the antegrade and retrograde endoscopy the canal of the endoscope was cleaned with sterile water. All patients used bowel preparation before DBE consisting of macrogol and electrolytes (Klean-Prep (Norgine BV, Amsterdam, The Netherlands)).

Samples were stored in Eppendorf cups (0.2 ml) with a stabilising reagent Allprotect (Qiagen Gmbh, Hilden, Germany). The samples were homogenised using the MagNA Lyser machine (Roche Diagnostics, Mannheim, Germany), stored in Trizol tubes (Invitrogen, Groningen, The Netherlands) and immediately frozen and stored at −80°C for subsequent analyses. DNA was isolated from the samples using QIAamp DNA mini kit (Qiagen) with an initial bead beating step added to the protocol, as described previously.\(^9\)

Generation of 16 S rRNA gene amplicons

Sequencing libraries were prepared by amplifying the V3–V4 region of the 16 S rRNA gene using the 341f-805 r primers, as described earlier.\(^10\) After the initial amplification, PCR (Polymerase chain reaction) products were confirmed with gel electrophoresis and purified using Agencourt AMPure XP magnetic beads (Beckham Coulter Inc., Bromma, Sweden). A second PCR was performed to attach Illumina adapters and barcodes that allow for multiplexing and the products were purified as above, quantified and pooled into equimolar amounts. Samples were sequenced using the
Illumina MiSeq platform at Science for Life Laboratory, Solna, Sweden. From the generated sequence data, primer sequences were trimmed away and the paired-end reads produced by the sequencing instrument were merged using SeqPrep version 1.1 (https://github.com/jstjohn/SeqPrep) with default parameters and thereafter the merged sequences were processed with QIIME 1.8 pipeline (Quantitative Insights into Microbial Ecology). A de novo operational taxonomic unit (OTU) strategy was used to assign sequences to OTUs. Using the UCLUST algorithm built into the QIIME pipeline, sequences were clustered at 97% identity against the Greengenes reference database.

### PCR analysis

Conventional PCR was performed for the confirmation of bacterial and human DNA isolation of biopsies. While analysing the results of this study, we noticed that the family *Helicobacteraceae* were present not only in the antrum, but also in other parts of the GI tract. However, sequencing did not allow us identify this feature on species level. To improve our understanding, we performed additional analyses by PCR.

DNA amplification was executed with the Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, USA) using 16S (different from sequencing PCRs), *Helicobacter pylori* (HP) specific *UreA* and *VacA* S1/S1, and human *ACTB* primers (Supplementary Material Table S1). For HP genes, the reaction mixture contained GoTaq buffer (Promega, Madison, Wisconsin, USA), 1.25 mM MgCl₂ (Promega), 0.167 mM (each) deoxynucleotides (Roche Diagnostics), 2.5 U GoTaq polymerase (Promega), 333 nM of each primer (Sigma-Aldrich, St Louis, Missouri, USA) and 2 μl un-normalised stock DNA. PCR cycle consisted of four minutes 95°C, several...
cycles of 30 s denaturing at 95°C, 30 seconds annealing and one minute extension at 72°C, followed by the final extension for 10 min at 72°C. Annealing temperature was 60°C for 16S, *UreA* and *VacA* and 60.5°C for *ACTB*. Number of cycles was 40 for HP genes, and 35 for 16S and *ACTB*. Amplicons were analysed by gel electrophoresis using 2% agarose gel in 1X TBE (Tris-borate-EDTA) buffer and bacterial DNA load was quantified using Image J software.

### Statistical analysis

The similarity between two samples was calculated using weighted Unifrac distances. Biodiversity within a sample was measured using the Shannon index. All diversity calculations were also performed for a least detectable relative abundance of 0.1%, corresponding to 1000 sequences in a sample, but this did not alter the results (data not included). Principal coordinate analysis (PCoA) using Bray Curtis metrics based on abundance data from sequences classified to genus level was performed to determine clustering patterns among the subjects.

Differences in diversity and similarity indices were tested with Mann-Whitney or Kruskal-Wallis test using the IBM SPSS statistics 21 software (Chicago, Illinois, USA). For differences in relative abundance of specific bacterial taxa we used Wilcoxon tests and linear regressions using the *r* statistical framework, version 3.0.1.

### Results

#### Subject population

Fourteen subjects undergoing an antegrade and subsequent retrograde DBE were included. In 13 patients, the mucosal samples were also studied by histology. Twelve subjects had no relevant anomalies found with DBE and histology (Table 1). One patient had Peutz-Jeghers polyps in the distal jejunum and one patient had a caecum tumour in the distal ileum (Supplementary Material Table S1). Written informed consent was obtained from each patient included in the study.

#### Overview of sequencing data generated from the samples

A total of 118 mucosal samples were retrieved from nine locations of the GI tract in 14 individuals. Eight samples could not be sequenced due either to inability to analyse the retrieved samples or inability to reach the site.

First, we confirmed bacterial DNA isolation from all samples by conventional PCR. While human genomic

### Table 1. Baseline characteristics of subjects.

| Characteristics                  | Numbers |
|----------------------------------|---------|
| Mean age, mean (IQR) (years)     | 51 (42–60) |
| Sex, n (%)                       |         |
| Male                             | 7 (50%) |
| Race, n (%)                      |         |
| Caucasian                        | 10 (71%) |
| Other                            | 4 (29%) |
| BMI, mean (SD) (kg, m²)          | 22.9 (5.4) |
| Unknown, n                       | 5       |
| Current smoker, n (%)            |         |
| Yes                              | 8 (58%) |
| No                               | 3 (21%) |
| Unknown                          | 3 (21%) |
| Alcohol, n (%)                   |         |
| Yes                              | 6 (43%) |
| No                               | 5 (36%) |
| Unknown                          | 3 (21%) |
| Medication use, n (%)            |         |
| Yes                              | 11 (79%) |
| No                               | 3 (21%) |
| Medical history, n (%)           |         |
| Hypertension                     | 1 (7%)  |
| Diabetes                         | 2 (13%) |
| Cardiac disease                  | 1 (7%)  |
| Peripheral arterial disease      | 2 (13%) |
| Stroke                           | 1 (7%)  |
| Chronic pulmonary disease        | 1 (7%)  |
| Liver disease                    | 1 (7%)  |
| Resection part of GI tract       | 2 (13%) |
| Other                            | 2 (13%) |
| No medical history               | 2 (13%) |
| Presenting symptoms, n (%)       |         |
| Iron deficiency anaemia          | 5 (29%) |
| Diarrhoea                        | 4 (24%) |
| Abdominal complaints             | 4 (24%) |
| Weight loss                      | 3 (18%) |
| Rectal blood loss                | 1 (5%)  |
| Findings DBE, n (%)              |         |
| No abnormal findings             | 10 (71%) |
| Ulcerative lesions in small bowel| 1 (7%)  |
| Polyp in small bowel             | 1 (7%)  |
| Polyp in colon                   | 1 (7%)  |
| Pathology finding, n (%)         |         |
| No abnormal findings             | 10 (71%) |
| Reflux oesophagitis              | 1 (7%)  |
| Chronic inflammation antrum      | 1 (7%)  |
| Chronic inflammation SB          | 1 (7%)  |
| Peutz-Jeghers polyps             | 1 (7%)  |
| Ulcerative changes               | 1 (7%)  |

BMI: body mass index; DBE: double-balloon enteroscopy; GI: gastrointestinal; IQR: interquartile range; SB: small bowel; SD: standard deviation.
DNA content was similar in all samples (Supplementary Material Figure S1), the bacterial load decreased from oesophagus to proximal ileum, but increased again in the lower GI tract (Figure 2). Samples were subsequently subjected to 16S rRNA gene amplicon sequencing using a V3-V4 specific primer set, resulting in a total of 4,369,079 high-quality sequences, with 37,026 sequences per sample (range: 17,294–68,696).

Diversity of the microbiota along the GI tract
To estimate the diversity of the microbial communities of the biopsies in the entire GI tract, analysis of alpha diversity, represented by Shannon’s index, was performed (Figure 1). The location of sampling had a significant influence on the alpha diversity of the microbiota, with samples taken from oesophagus to proximal ileum harbouring a lower level of microbial diversity than samples obtained from terminal ileum to rectum (p < 0.05). When comparing the average alpha-diversity of the individual locations from individual subjects, a wide spread in the mean Shannon index between individuals became apparent with, in particular, subject 12 showing a low diversity in all samples (Figure 3(a)). This patient was diagnosed with a caecum tumour. Nevertheless, all participants, except subject 10, showed a higher alpha-diversity in lower GI locations (Figure 3(b)) as compared to upper GI locations.

Differential microbial composition along the GI tract
We further searched for clustering patterns among samples according to their microbial population structure by PCoA based on Bray Curtis distance metrics. Again, a distinct separation of bacterial community structure was observed, with samples from the distal oesophagus to the proximal ileum clustering together, separately from distal ileum to rectum (Figure 4). Several samples clustered neither with the upper nor the lower GI locations, but belonged to the patient diagnosed with a caecum tumour. These samples from this patient appeared to be dominated by Enterobacteriaceae. (Supplementary Material Figure S2).

Cluster analysis using Euclidian distance at family level was used to visualise these data in a different way, which again demonstrates the separate clustering...
of this patient with a caecum tumour and the lower and upper GI tract samples (Supplementary Material Figure S3). Samples from individual patients appear to cluster more closely together in lower GI samples than upper GI samples (Supplementary Material Figures S3 and S4).

The similarity in microbiota composition between different sites in the GI tract was also visualised using weighted UniFrac distances, which showed that the microbial composition in the rectum was a good predictor for the microbial composition in the ascending and descending colon and – to a somewhat lesser extent – the distal ileum (Figure 5(a)). The composition of the microbiota in the distal oesophagus was also compared to the other locations in the GI tract. However, the microbiota in the distal oesophagus was not as good a predictor for the other locations in the upper GI tract as the rectum was for the lower GI tract (Figure 5(b)).

Characterization of mucosa-associated microbiota

All regions in the GI tract were dominated by three major bacterial phyla: Bacteroidetes, Firmicutes and Proteobacteria. Although ubiquitously dominant within the entire GI tract, each of the three phyla revealed distinct profiles along the length of the GI tract (Figure 1(d)). The mucosa-associated microbiota of the upper GI tract was dominated by Proteobacteria (mean abundance of 40 ± 2.1%) and Firmicutes (38 ± 2.3%). However, in the lower GI tract the level of Proteobacteria decreased consistently (distal colon (5.3 ± 0.4%)). Firmicutes, already highly abundant in the upper GI tract, dominated the large intestine with the highest level in the distal colon (mean abundance 64 ± 7%). Bacteroidetes was present at low levels in the upper GI tract (8 ± 1.6%), but became a dominant phylum in the lower GI tract (mean abundance in ascending colon 28 ± 1.6%).

The most prevalent bacterial families in the upper GI tract were Veillonellaceae, Pseudomonadaceae and Streptococcaceae (Figure 6). In contrast to other sites in the GI tract, Prevotellaceae (relative abundance of 8%) and Helicobacteraceae (relative abundance of 8%) were dominant in the antrum. Helicobacter species were detected in nine subjects, and predominated the antrum of one subject (S6) to the extent that other species were almost not found (Supplementary Material Figure S3). PCR analysis of the UreA and VacA gene confirmed that the Helicobacteraceae detected by sequencing were indeed Helicobacter pylori (Figure 7(a)). Helicobacter was present across the entire upper GI tract, and some lower GI tract locations in three subjects, which confirms data that this bacterium may spread beyond the stomach (Supplementary Material Figure S5). Interestingly, subject S14 showed high levels of Helicobacteraceae in the proximal duodenum, while not detected in the antrum (Figure 7(b)).

In the distal jejunum, Bradyrhizobiaceae (relative abundance of 6%) occurred more often compared to other parts of the GI tract. The same applies to Micrococcaceae (relative abundance of 4%) in the proximal ileum. The lower GI tract was dominated by Lachnospiraceae, Bacteroidaceae, Ruminococcaceae and Veillonellaceae. The highest abundance of the bacterial family Clostridiaceae (relative abundance of 1%) was seen in the distal ileum. Rikenellaceae was only seen with a higher relative abundance than 1% in the rectum.

Discussion

This study describes the composition of the microbiota along the entire GI tract in the same individuals.
Figure 4. Principal coordinate analysis (PCoA) plot illustrates a clear difference between gut location and composition of the microbiota. Different coloured dots represent different locations of the gastrointestinal (GI) tract. The green circle contains mainly oesophagus, antrum, proximal duodenum, distal jejunum and proximal ileum samples (upper GI tract), the yellow circle contains only distal ileum, ascending colon, descending colon and rectum samples (lower GI tract). The blue circle highlights samples dominated by *Enterobacteriaceae* which were all derived from one patient with a caecum tumour (S12).

Figure 5. Similarity between different sites in the gastrointestinal (GI) tract analysed using weighted UniFrac distances. (a) The microbiota in the rectum was compared to the eight other locations, and is a good proxy for other lower GI locations. (b) The microbiota of the distal oesophagus was compared to the eight other locations, and is a less efficient predictor for the microbiota of the other locations. Green: upper GI tract; Yellow: lower GI tract.
without significant pathology. In agreement with earlier reports, the bacterial load decreases from the oesophagus to the proximal ileum, but drastically increases again in the lower GI tract, starting from the distal ileum. The composition of the microbiota markedly changes along the GI tract, with the most prevalent bacterial families present in the upper GI tract Veillonellaceae, Pseudomonadaceae and Streptococcaceae, while the lower GI tract is dominated by Lachnospiraceae, Bacteroidaceae and Ruminococcaceae.

Our findings to a large extent reflect data obtained from other studies comparing only partly matched samples, but probing multiple locations within one patient may provide better accuracy. One report comparing only duodenal and rectal content from healthy individuals reported higher Shannon diversity values in both mucosa and luminal content from the duodenum, while others support our findings of a less complex luminal microbiota in the small intestine compared to the colonic content.

Arguably, the least studied GI sites in the current literature are the jejunum and distal ileum. In the jejunum, Proteobacteria and Firmicutes were the most dominant phyla, and at family level Veillonellaceae, Pseudomonadaceae and Streptococcaceae dominated. A previous study retrieving mucosal biopsies from the proximal jejunum of 19 healthy individuals also observed Proteobacteria, Bacteroidetes and Firmicutes as the predominant phyla, although family level classification indicated Brevibacteriaceae, Barnesiellaceae and Leuconostocaceae. Possible explanations for these discrepancies could be the difference in individual populations (Taiwanese versus Dutch population) as well as alternative methodologies used for sampling, preparation and analysis of the samples.

In terms of the proximal and distal ileum, our samples were found to have large differences in composition. In the proximal ileum, Proteobacteria and Firmicutes dominated, whereas Firmicutes and Bacteroidetes were the dominant phyla in the distal ileum.
ileum. It is conceivable that the distal ileum was contaminated from the colon, either due to sampling or through bowel movements. At present the only comparison that can be made in this context comes from animal studies. A study comparing 10 paired GI locations in mice showed that the largest difference between two locations in terms of bacterial diversity was seen between ileum and proximal cecum, with lower GI samples clustering away from upper GI samples. In pigs, a similar clear separation between the upper and lower GI could be seen, although in this case the dividing line appeared to lie between jejunum and ileum.

A further notable finding in our study was that a patient who had a caecum tumour showed a significant dysbiosis predominated by Enterobacteriaceae in all other GI sites tested. A role for Enterobacteriaceae in carcinogenesis has been suggested before, as several enterobacterial strains are known to produce DNA-damaging genotoxins and may therefore cause mutations. The major strength of this study is that we collected nine mucosal samples along the entire GI tract of 14 different individuals allowing us to study the composition of the microbiota along the length of the gut. Since all individuals underwent an antegrade DBE followed directly by a retrograde DBE, no bias could have occurred based on the timeframe.

There are also a number of limitations. Firstly, the same endoscope was used for anterograde and retrograde DBE. Although the canal of the endoscope was cleaned with sterile water between the antegrade and retrograde DBE, it is impossible to exclude contamination from the upper GI tract to the lower GI tract using this methodology. However, the low level of similarity of the microbial composition in the upper and lower GI tract suggests that this is not a major issue in our study. Secondly, the subjects in our study underwent DBE for unexplained symptoms and therefore may not fully represent healthy individuals. However, ethical considerations preclude performing DBE in individuals without clinical indication and thus we consider our study the best that can be achieved with current technical approaches. Third, neither DBE nor histopathology of

Figure 7. *Helicobacter pylori* predominates in the antrum from one patient, and extends beyond the stomach. (a) Relative abundance of *Helicobacter* species across the nine different gastrointestinal (GI) sites in subject S6 as determined by sequencing. Identity of *Helicobacter pylori* at species level was confirmed by PCR in the high Helicobacter abundant samples by UreA and VacA. The antrum was dominated by *H. pylori*, resulting in a low diversity in this sample (see Figure 2 and Supplementary Material S4). 16S PCRs, similar to Figure 2, are shown here to allow comparison of total bacterial abundance in these samples. (b) Relative abundance of *Helicobacter* species across the different GI sites in subject S14 as determined by sequencing. Identity of *H. pylori* at species level was confirmed by PCR of UreA. Numbers are as described above, X represents a missing sample. While *H. pylori* was not detected in the antrum, high levels were present in the proximal duodenum. 1: distal oesophagus; 2: antrum; 3: proximal duodenum; 4: distal jejunum, 5: proximal ileum; 6: distal ileum; 7: ascending colon; 8: descending colon; 9: rectum; +: positive control of pure *H. pylori* culture strain ATCCÂ®43504 (American Type Culture Collection, Rockville, Maryland, USA); −: negative control (water).
the retrieved biopsies showed clinical abnormalities except for one patient with a caecum tumour and one patient with Peutz-Jeghers polyps. Fourth, patients were treated with colonic lavages prior to DBE, which could potentially have diminished the diversity of the mucosa-associated microbiota. Unfortunately, a DBE cannot be performed without bowel preparation. Finally, stool samples were not collected of these patients and therefore the faecal microbiota could not be analysed. Whether stool and mucosal microbiome correlate well is somewhat debated in literature, and having stool samples would have been of value. With the exception of the patient with a caecum tumour, the data represented here could be conceived as representing the ‘normal’ mucosal microbiome. While it is already well described that education of the immune system depends on the intestinal microbiome, to what extent local mucosal differences affect local immunological responses is less well elucidated. Diseases like IBD are largely driven by an altered immunological response towards intestinal microbes. Thus a comparison of disease-location specific mucosal microbial changes to normal microbiome signatures at these sites may be of use. The use of faecal microbiota transplantation for IBD has been advocated, and it is thought that optimal donor selection is important for clinical efficacy, although more research is needed to identify which components of the gut microbiome constitute key members.

In conclusion, we have generated a first overview of the composition of the microbiota along the entire GI tract. This study is of particular importance in helping us to understand the interactions between bacterial communities and human cells and takes us to the next step in describing the impact of the microbiota on health and its involvement in diseases.

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Ethics approval
The study was conducted in accordance with the Declaration of Helsinki and approved by the ethical committee of the Erasmus Medical Center, Rotterdam (MEC-2017-151) on 3 April 2017.

Informed consent
Written informed consent was obtained from each patient in the study.

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