Investigating the human small intestinal microbiota

Microbiological characterization of jejunal and ileal samples collected during surgery

Heidi Cecilie Villmones

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2022

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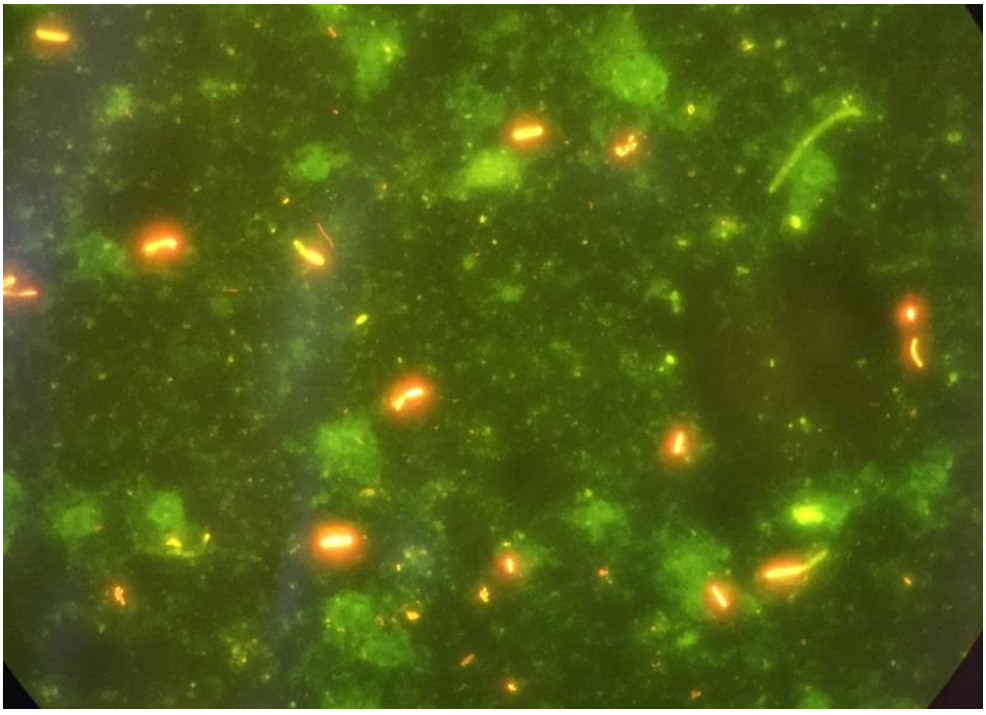

SAMMENDRAG

Kartlegging av tynntarmsmikrobiota hos mennesker

Mikrobiologisk beskrivelse av kirurgiske prøver fra jejunum og ileum

Beskrivelser av tynntarmsmikrobiota (tarmflora) i lærebøker og vitenskapelige artikler er lite konsistente. Nyere studier hevder at distale ileum har en mikrobiota som likner på den i tykktarmen, mens eldre studier i hovedsak rapporterer bakterier fra munn. Mikrobiota i jejunum beskrives også forskjellig i nyere litteratur og ingen vet sikkert om jejunum har en egen kjernemikrobiota.

Forskjellene mellom eldre og nyere studier kan skyldes at man har brukt ulike metoder for å påvise bakterier. Eldre artikler har brukt dyrkningsbaserte metoder mens nyere studier bruker sekvenseringsteknologi. I tillegg er det stor forskjell på å ta endoskopiske prøver via munn eller tykktarm og på å ta prøver direkte fra åpnet tynntarm under kirurgiske operasjoner.

Hovedmålet med dette PhD-prosjektet var å beskrive mikrobiota i jejunum og ileum på arts-nivå og definere en eventuell kjernemikrobiota, altså mikrober som antas å være viktige for funksjonen i tynntarmen vår, for begge segmenter.

Til dette formålet benyttet vi rene kirurgiske prøver fra tarmslimhinnen i proksimale og midtre del av jejunum på pasienter med sykelig overvekt under gastrisk bypass operasjon (n=60 x 2), og fra distale del av ileum på blærekreftpasienter under cystektomi med urinavledning (n=150). Alle prøvene ble dyrket i laboratoriet med standard metoder. I tillegg ble alle prøvene fra jejunum og 30 prøver fra ileum undersøkt med dypsekvensering av V3-V4-regionen av det bakterielle 16S rRNA genet.

Prøvene fra jejunum var dyrkningsnegative hos 51% av pasientene svarende til en mikrobetetthet på mindre enn 10^3 bakterier per milliliter. Hyppigste dyrkningsfunn var fra *Streptococcus salivarius-, S. sanguinis-* and *S. mitis*-gruppene. Dypsekvensering av 16S-rRNA-genet detekterte også lave nivåer av bakterielt DNA, primært fra

munnhulebakterier. De fleste artene ble bare sporadisk detektert og vi fant ikke holdepunkt for at det finnes en kjernemikrobiota i jejunum. De hyppigste artene detektert i jejunum ved dypsekvensering (tilstede i 40-48% av pasientene) tilhørte *Streptococcus mitis*-gruppen*, Streptococus sanguinis*-gruppen, *Granulicatella adiacens/para-adiacens*, *Schaalia odontolytica*-komplekset (tidligere *Actinomyces odontolyticus*) og *Gemella haemolysans/taiwanensis*. Hyppigste detekterte genera var *Corynebacterium, Streptococcus, Gemella, Granulicatella* og *Actinomyces.*

Sammenliknet med jejunum, var mikrobetettheten vesentlig høyere i ileum ned mot ileocøkal-klaffen. Vi fant mikrober ved dyrkning hos 93% av pasientene, men hos de fleste (79%) bare tilsvarende 1.6 x 10^4 bakterier per milliliter eller mindre. Hyppigste dyrkningsfunn var gjærsoppen *Candida albicans* sammen med bakteriearter fra *Streptococcus sanguinis*- og *S. mitis*-gruppene. I ileum fant vi også høyere nivåer av mikrobielt DNA (ca. 100-1000 ganger mer enn i jejunum) og det var mulig å definere en kjernemikrobiota. Hyppigste detekterte arter (tilstede i 89-100% av pasientene) var fra *Streptococcus mitis*- og *S. sanguinis*-gruppene, *Granulicatella adiacens, Schaalia odontolytica*-komplekset, *Solobacterium moorei*, *Gemella haemolysans/sanguinis* og *Rothia mucilaginosa.* Hyppigste identifikasjon på genus-nivå var *Streptococcus*, *Granulicatella, Actinomyces, Gemella, Rothia, Solobacterium*, *TM7(G-1)* og *Oribacterium.*

Vår studie viser at tynntarmsmikrobiotaen hos mennesker er sparsom og dominert av gram-positive bakterier assosiert med munnhulen. Mikroorganismene er hovedsakelig fakultative eller mikroaerofile, selv helt distalt i ileum. De hyppigste detekterte artene både i jejunum og ileum var fra *Streptococcus mitis*- og *S. sanguinis*-gruppene i tillegg til *Granulicatella adiacens.* Vi kunne definere en kjernemikrobiota i ileum, men finner ikke holdepunkt for at jejunum har en egen mikrobiota.

Front page figure: Smear from an ileal sample stained with acridine orange. DNA and RNA emit orange fluorescence allowing visualization of metabolically active bacteria (orange rods). The green background demarks debris from intestinal contents.

Photo: Heidi Cecilie Villmones

Contents

2022

Acknowledgements

The present work was initiated at the Department of Microbiology, Vestfold Hospital Trust and carried out from 2016 to 2022. The PhD and the scientific education was undertaken at the Department of Clinical Science, K2, University of Bergen.

Vestfold Hospital Trust is a medium sized hospital with closely connected departments, serving a population of about 230.000 individuals.

The study is a result of interdisciplinary collaborations between researchers and personnel from the Departments of Urology, Gastrointestinal Surgery, Infectious Medicine and Microbiology at Vestfold Hospital Trust and the Department of Microbiology, Haukeland University Hospital, Bergen.

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Summary

Results from previous characterizations of the small intestinal microbiota (*i.e.* the ecological community of resident microorganisms) are conflicting. Whereas modern investigations proclaim the presence of a colon-like microbiota in the distal ileum, older studies contradict these results and report bacteria characteristic of the oral cavity. Descriptions of the jejunal microbiota lack consistency and little is yet known as to whether the jejunum has a core microbiota of its own.

Such differences may be owing to different sensitivities of the most commonly used analytic methods – culturing and DNA sequencing, as well as to variations in sampling techniques – transluminal sampling, *e.g.* endoscopy, versus clean sampling of material from the lumen during surgery.

The main objective of this PhD-project was to perform a species-level description of the jejunal and distal ileal microbiota and to identify potential core-microbial species. Samples were collected surgically from the mucosa of the proximal and mid jejunum in a population with morbid obesity during gastric bypass surgical procedures (n=60 x 2), and from the distal part of the ileum in patients suffering from bladder cancer during cystectomy with urinary diversion (n=150). All samples were cultured using standard methods. In addition, all jejunal and 30 ileal samples were investigated using broad-range amplification and deep sequencing of the V3-V4-region of the bacterial 16S ribosomal ribonucleic acid (16S rRNA) gene.

Jejunal samples were culture-negative in 51% of the participants, corresponding to a bacterial density of less than 10^3 colony forming units (cfu)/ml. The species most frequently detected by culture belonged to the *Streptococcus salivarius group, S. sanguinis group* and *S. mitis* group. Deep sequencing and quantification of the bacterial 16S rRNA gene revealed low levels of typical oral bacteria. Most species were only sporadically detected, and we were not able to find evidence supporting the existence of a core resident jejunal microbiota. The most frequent species in the jejunum by deep sequencing (present in 40-48% of the patients) belonged to the *Streptococcus mitis group*, the *Streptococcus sanguinis group*, *Granulicatella adiacens/para-adiacens*, the *Schaalia odontolytica complex* (former *Actinomyces*

odontolyticus) and *Gemella haemolysans/taiwanensis*. The most frequently identified genera were *Corynebacterium, Streptococcus, Gemella, Granulicatella* and *Actinomyces.*

The density of microbial organisms was higher in ileum towards the ileocecal valve as compared to results from the jejunal samples. Ninety-three percent of ileal samples were culture-positive. Still, in 79% of the participants only 1.6 x 10^4 cfu/ml or less were detected. The most frequently cultured microbes in ileum were the yeast *Candida albicans* and the bacteria of the *Streptococcus sanguinis group* and the *S. mitis group*. In the distal ileum, we also found higher levels of microbial DNA (approximately hundred to thousandfold more than in jejunum) and were able to define a core microbiota. The most frequently detected species (present in 89-100% of the patients) were from the *Streptococcus mitis group*, the *S. sanguinis group*, *Granulicatella adiacens,* the *Schaalia odontolytica complex*, *Solobacterium moorei*, *Gemella haemolysans/sangui*nis and *Rothia mucilaginosa.* At the genus level *Streptococcus*, *Granulicatella, Actinomyces, Gemella, Rothia, Solobacterium*, *TM7(G-1)* and *Oribacterium* were most frequently detected.

Our data provide evidence that the human small intestine harbors a sparse microbiota dominated by gram-positive bacteria related to the oral cavity. Microorganisms are mostly facultative or microaerophilic even in the distal part of ileum. In both jejunal and ileal samples, the top three most frequent bacteria belong to the *Streptococcus mitis group,* the *S. sanguinis group* and *Granulicatella adiacens.* We were able to define a core microbiota in the ileum but our work does not support the presence of a resident jejunal core microbiota.

List of publications

Paper I (1)

Species level description of the human ileal bacterial microbiota.

Villmones HC, Haug ES, Ulvestad E, Grude N, Stenstad T, Halland A, Kommedal Ø. Scientific reports. 2018;8:4736.

Paper II (2)

The cultivable microbiota of the human distal ileum.

Villmones HC, Halland A, Stenstad T, Ulvestad E, Weedon-Fekjær H, Kommedal Ø. Clinical Microbiology and Infection. 2021; 27: 912.e7-912.e13.

Paper III (3)

Investigating the human jejunal microbiota.

Villmones HC, Svanevik M, Ulvestad E, Stenstad T, Anthonisen IL, Nygaard RM, Dyrhovden R, Kommedal Ø. Scientific reports. 2022;12:1682.

Preface

The gastrointestinal mucosa represents the greatest body-surface interacting with the body´s microbial surroundings, thus partly explaining the evolution of a large collection of lymphoid tissue located along the gastrointestinal system. Current investigations of luminal microbes and the human host are revealing intricate but still not fully understood mechanisms of interaction. Since these mechanisms may be relevant in both health and disease, huge resources are currently utilized to further investigate the causal webs of interaction.

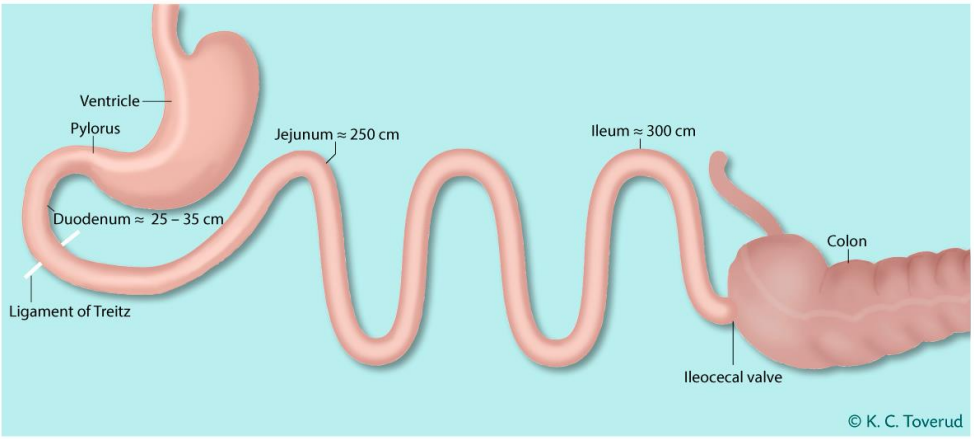
Most scientists working on host-microbe-interactions focus on the easily accessible fecal microbiota. This practice is partly justified by recent beliefs that feces reflects the microbiota of the distal part of the small intestine (4). However, there is little solid evidence to support this belief. Older investigations argue that feces is mostly "processed waist" that represent its own biological niche, that fecal microbiota should not be viewed as a proxy for the colon microbiota, and that the microbial contents of the small intestine cannot be extrapolated from the contents of the colon (5).

The literature describing the microbiota of the small intestine is still confusing and inconsistent, and the current investigations were set in motion to clarify the issues at hand.

Background

The small intestine –structure and function

Figure 1. Anatomy of the human gastrointestinal tract from the ventricle to colon.



The human small intestine constitutes the intestinal segment between the pylorus sphincter and the ileocecal valve. The total length varies from three to six meters, depending on a person's height, parasympathetic activity and genetics. The ligament of Treitz connects and supports the end of duodenum and the beginning of the jejunum. There is no distinct anatomical line to separate jejunum and ileum. Jejunum is by function more absorptive that ileum.

The surface area of the small intestinal mucosa is about fifteen times larger than the overall surface of the large intestine (6). Enterocytes are extruded every 4-5 days, making the epithelium the most rapidly renewing tissue in the adult mammal (7). Gastrointestinal luminal pH in normal healthy volunteers is highly variable, ranging from 1.7 to 4.7 in the stomach, 5.5 to 7.0 in the proximal small intestine and between 6.5 and 7.8 in the distal ileum (8, 9). Approximately 90% of the nutrients and minerals are absorbed along the small intestine (10). Sugars, amino acids and fatty acids are mainly absorbed in the jejunum (11), whereas the ileum absorbs primarily vitamin B12 and bile acids, but also remaining nutrients (12). The transit time of food varies considerably, and is estimated to be between 30 minutes and eight hours, with a median of approximately four hours (9, 13).

Enteroendocrine cells, which are scattered throughout the epithelium of the gastrointestinal tract, release more than 20 different neurotransmitters and hormones in response to components within the intestinal lumen. The enteroendocrine system is the largest endocrine system of in the body. It monitors the organism´s energy status and triggers appropriate physiological responses to reestablish metabolic homeostasis (14).

Host-microbe interactions

The mucosal surface of the small intestine regulates the host´s interactions with potentially harmful luminal substances. The epithelium is covered by mucus produced by goblet cells. The mucus serves as a physical barrier to infectious agents and as a repository for toxic glycoproteins. It may, however, also function as an energy source for microbes and as a substrate for bacterial attachment (15). Paneth cells, mainly located in the distal small intestine, secrete antibacterial peptides like defensins and lysozyme (7).

The intestine and its mesentery harbors the largest collection of lymphoid tissue in the body. About 70% of the peripheral lymphocytes and 40% of the body´s lymph nodes are located in close proximity to the intestine (16, 17). Between 70 and 80% of all Ig-producing cells in humans are located in the intestinal mucosa (18) and at least 70% of all immunoglobulins (Ig) produced by mammals is IgA produced by mucosal B-cells (19). Most bacteria in human feces are coated with specific IgA (20). In addition to its protective function, the massive IgA-secretion is also crucial for immunologic homeostasis within the lamina propria. Mucosal lymphocytes cells also play a role in educating the immune system to develop tolerance toward commensal microbes (21, 22).

Antigens derived from luminal microorganisms and diet are actively engulfed and presented to sub-epithelial immune cells by microfold cells (M-cells) (23, 24).

About 50% of Peyer´s patches – small groupings of lymphoid follicles in the intestinal membrane – are scattered along the distal 25 cm of ileum in humans (25). Follicle-associated epithelium with M-cells covers these (26) making Peyer's patches the major sites for initiation of adaptive immune responses to luminal antigens derived from bacteria and food (25, 27). Food proteins have been detected in the blood of humans after eating (28).

Lymph from the different segments of the intestine drains into lymph node aggregates in the mesentery; the gastric lymph nodes, the duodenopancreatic lymph nodes, the mesenteric lymph nodes and the caudal lymph nodes. These aggregations are the largest lymphoid aggregates in the body (17).

Lymphatic nodes in the mesentery collect bacterial and antigenic material derived from the adjacent intestine, and regulate migration of relevant immune cells to the associated intestinal mucosa (29).

A well-functioning small bowel peristaltic activity is crucial to hinder bacterial overgrowth in the small intestine (30). The small intestine undertakes two types of peristaltic activity: Postprandial motility and the fasting migrating motor complex (MMC) motility pattern (31, 32). After meals, the peristalsis shows irregular contractile activity and in a fasting status MMC performs migrating bands of more regular contractions (33). Food, stress and brain activation influences this parasympatic activity and possibly also certain microbial species (32, 34).

The human intestinal microbiota

The human intestine, which is sterile *in utero*, is seeded by a variety of microbes shortly after birth. Microbial diversity increases until about 2,5 years of age, after which it remains relatively stable until maturity (35). In the elderly, the gut microbiota

alters and seems to become less diverse. This may contribute to the physiological aging process and age-related comorbidities (36).

The composition of the intestinal microbiota, which is unique to each individual, is affected by host genetics, age and environmental factors such as mode of birth, diet, medications, diseases and infections.

Studies on the small intestinal microbiota

Scientific reviews of the small intestinal microbiota are conflicting and often vague on microbial compositions and abundancies in the various intestinal segments (37-51). This unfortunate state of affairs is partly owing to inadequacies related to methods for sample collection and to methods for microbiota identification. As presented in Table 1, surgical access assures samples to be uncontaminated by microbes from other intestinal segments, and therefore should be the preferred route of sampling. However, surgical access is restricted to the few conditions that are amenable for surgical interventions. Among the analytical methods, sequencing is preferred owing to its higher diagnostic sensitivity as compared to cultivation. However, since sequencing does not provide information as to whether the identified microbes are alive or not, cultivation retains a supplementary role when analyzing the intestinal microbiota. Cultivation is also traditionally the basis for density measures in colony forming units (cfu/ml). Density measures based on deep sequencing data are consistently missing (genomes/ml).

Table 1. Identification of the small intestinal microbiota: Advantages and disadvantages of the four methodic combinations of *access to samples* and *analytic method*

		Analytic method	
		Cultivation	Sequencing
		Uncontaminated	Uncontaminated

Access to samples	Surgical	Low diagnostic sensitivity	High diagnostic sensitivity
	Transluminal (endoscope, catheter, capsule, ileostomy effluent, autopsy)	Contaminated Low diagnostic sensitivity	Contaminated High diagnostic sensitivity

Studies on duodenum (52-65), jejunum (61, 63-75) and ileum (60, 62, 63, 65, 67-69, 76-92) from the past 20 years are mostly based on sampling techniques with a considerable risk of contamination from microbe-rich areas in the colon or mouth. The sampling techniques include endo/gastroscopies, nasoileal catheters/tubes, capsules and retrograde colonoscopy. Investigators have also studied intestinal samples collected from ileostomies and from autopsies. Only, Kleessen *et al.* 2002 (76), Ahmed *et al.* 2007 (82) and Pedamallu *et al.* 2016 (91) sampled material from the ileum during surgery.

Unfortunately, the three studies based on surgical sampling utilized analytic methods with a reduced diagnostic sensitivity. Kleessen *et al.* (76), who compared the ileal microbiota in patients with inflammatory bowel disease (IBD) with non-IBD controls, used fluorescent in situ hybridization (FISH) technique with probes for pre-selected species. The spectrum of probes was limited and did not include important genera like *Streptococcus, Actinomyces* and *Rothia.* More than 60% of the bacteria remained unidentified. Ahmed *et al.* (82) studied mucosal tissue from the terminal ileum of 26 patients in need of emergency surgery using FISH. Again, there were high numbers of unidentified bacteria in the terminal ileum, and *Streptococcus, Rothia* and *Actinomyces* were not included in the FISH probe panel. Pedamallu *et al.* (91) investigated 12 patients with Crohn´s disease and 12 patient controls with right sided cancer selected for surgery with Illumina whole genome sequencing. Although whole genome sequencing can provide very accurate identification, it can have a reduced sensitivity for low abundancy species. Their samples were paraffin-embedded tissues, which tend to increase significantly the problem of background contaminant DNA. Furthermore, their DNA extraction method was developed for the human host and not bacteria. Most

importantly, the results were reported mainly to compare the two groups of patients, and a description of the ileal microbiota was not attempted. Interestingly, viral, archaea and fungal sequences were not detected.

From the pre-sequencing era we identified four smaller microbiota-studies based on samples collected during surgery from patients with no reported gastrointestinal disorders: Elective gynecological surgery (jejunum and ileum) (Cregan *et al.* 1953 (93)), elective cholecystectomies (ileum) (Bentley *et al.* 1972 (94)), patients with morbid obesity selected for gastric bypass with no preoperative antibiotics (jejunum and ileum) (Corrodi et *al.* 1978 (95)), and exploratory laparotomy for gunshot/stab wounds (duodenum, jejunum and ileum) (Thadepalli *et al.* 1979 (96)). The sampling was performed during laparotomy by sterile needle aspiration of the luminal content and analyzed by culturing.

These four older studies (93-96) concluded that the duodenum is mostly sterile in 82% of the subjects, with sporadic findings of bacteria by culture. Jejunum was found to be sterile in 69 to 85% of the patients, and ileum was found sterile in 25% to 55% of subjects. Gram-positive microorganisms dominated in a scarce or absent microflora, with viridans streptococci, *Lactobacillus* and *Staphylococcus* most frequently reported. Gram-negative bacteria and strict anaerobic bacteria were only exceptionally detected.

Another three studies from 1953, 1958 and 1966 with sterile needle aspiration sampling were also tracked (97-99). The surgery was performed on patients with gastrointestinal disorders and thereby not relevant in the same manner as the above. The main findings were a missing or scanty microbiota with only exceptionally detected *E.coli* and strict anaerobic bacteria.

There was much debate during the pre-sequencing era as to whether the small intestine harbored a resident microbiota of its own (5, 99-101). The small intestine was thought to be normally sterile in nearly 50% of the population (95, 96) and in 1996 Berg *et al.* (102) postulated that bacteria cultured from the upper gastrointestinal tract represent passersby from the oral cavity and that they are not indigenous microorganisms.

The duodenal microbiota

The duodenal microbiota is estimated to harbor 10^1 – 10^4 colony forming units (cfu)/ml (10, 37-39, 42, 45, 46, 49). Studies report mainly *Lactobacillus* and *Streptococcus* (37, 39), *Lactobacillus/Lactobacillaceae, Escherichia coli/Enterobacteriales* (42, 45) and *Enterococcus faecalis* (42), or even *Bacteroides, Clostridium, Streptococcus, Candida* and *Saccharomyces* (51). The two most recent reviews list *Bifidobacteria, Prevotella, Streptococcus, Clostridium, Lactobacillus, Enterococcus, Veillonella* and *Neisseria* (47) and *Veillonellaceae, Lactobacillales, Pseudomonadales, Candida* and *Saccharomyces* (49).

The jejunal microbiota

The jejunal microbiota is estimated to contain 10^4 – 10^7 cfu/ml (10, 38, 41, 47-49). Some studies claim 10^3 – 10^4 cfu/ml (10, 42, 49), others 10^4 -10^7 cfu/ml (38, 47, 48) and even others 10^6 – 10^7 cells/g (41). Although the qualitative descriptions of the jejunal microbiota are conflicting, there seems to be a consensus that it is similar to the duodenum with a dominance of *Lactobacillus, Streptococcus, Staphylococcus* and *Bifidobacteria.* Some authors claim that *Enterococcus, Enterobacteriales* and/or strict anaerobes may be part of the normal jejunal microbiota (42, 45-48, 51, 103), while others do not report these genera (38, 49).

The ileal microbiota

Reviews have postulated that the ileal microbiota has a density of 10^5 -10^8 cfu/ml (10, 37-39, 41, 42, 45-47, 49, 50), most often 10^7-10^9 bacteria/cells/cfu/ml (37-39, 41, 42, 45, 46, 49, 50). Two reviews estimate the bacterial numbers to be 10^3-10^8 cfu/ml (47, 48). Some researchers claim that the distal ileal microbiota is mainly colon-like (4, 39) while others report a diverse spectrum of bacteria including strict anaerobic bacteria, Enterobacteriales and *Enterococcus* (38, 42, 45, 47-49). *Actinobacteria* other than *Bifidobacteria* like *Actinomyces* and *Rothia* are only exceptionally mentioned.

Bacterial microbiota proximally and distally to the small intestine

The oral microbiota

On average, humans ingest between 0.5 and 1.5 liters of saliva every day (104). The salivary microbiota, made up of around 10^9 cfu/ml (102, 105), varies with hygienic measures, eating, tooth brushing and dental status. The human oral microbiota is diverse and related to different niches in the oral cavity (106-113). The most recent information on the oral microbiome is registered in the Human Oral Microbiome Database (HOMD, http://www.homd.org/). The National Institute of Dental and Craniofacial Research launched the HOMD in 2010 for maintaining the information of orally-derived cultivable and non-cultivable microbial isolates (114, 115). The HOMD has been renamed as the expanded HOMD (eHOMD), and provides information of bacterial communities present in the oral cavity, pharynx, nasal passages, sinuses, and esophagus.

Frequently detected bacteria in the oral cavity include *Streptococcus, Prevotella, Veillonella, Neisseria, Haemophilus, Rothia, Porphyromonas, Fusobacterium* and many others (116). Some studies have demonstrated that the mouth harbors a microbial community that is less variable over time than that of the gut and skin, thus indicating that the oral bacterial environment is resilient and stable (117). A study by Zaura *et al.* from 2015 found the salivary microbiota to be significantly more robust to antibiotic exposure as compared to the fecal microbiota (118). A high variability of the oral microbiota has been observed amongst groups of individuals from different geographic locations. *Enterobacter* accounted for 28% of the sequences in individuals from Congo but was completely absent in samples from more developed, western countries. *Serratia* was particularly frequent in several individuals from Bolivia (119). Oral microorganisms have a role in dental caries (120), periodontal disease (121) and possibly in systemic diseases such as diabetes, obesity, cardiovascular disease and rheumatology (122, 123).

The esophageal microbiota

The esophagus is normally empty and collapsed. It is considered to harbor its own unique resident microbiota of about 10 cfu/ml of sample material (124). Bacteria from within the *Streptococcus* genus is recognized to represent the major components among other abundant taxa present in the oral cavity (124). *Prevotella, Fusobacterium* and *Veillonella* are also frequently reported.

The gastric microbiota

Until the discovery of *H. pylori* in 1982 by Robin Warren and Barry Marshall, the stomach was considered "sterile". A systematic review of current literature suggests a core microbiota dominated by *Prevotella, Streptococcus, Veillonella, Rothia* and *Haemophilus* (125). Typical concentrations of bacteria are estimated to be $10^3 – 10^4$ cfu/ml (102).

The colonic microbiota

The human colon harbors about 10^{11} to 10^{12} cfu/ml fecal material. The heavy colonization is probably the result of the slow transit time, up to 60 hours, and the low oxidation-reduction potentials. The majority of bacterial species belong to the phyla *Bacteroidetes* and *Firmicutes*, and to a lesser extent *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (4). The dominant genera are *Bacteroides* (126), *Prevotella*, *Ruminococcus* (51), *Faecalibacterium, Bifidobacterium (127), Eubacterium, Peptostreptococcus, Bacillus, Dorea, Alistipes, Clostridium, Lactobacillus* and *Enterococcus* (102, 128). Three basic enterotypes are suggested based on different relative enrichments of the genera: *Bacteroides, Prevotella* and *Ruminococcus* (Enterotypes 1, 2 and 3) (127). The colonic microbiota is 99.9% anaerobic, and obligate anaerobes are 100- to 1000-fold more numerous than facultative anaerobes. Around 60% of fecal solids consists of bacteria (129).

The non-bacterial intestinal microbiota

Bacteria make up the main volume of the intestinal microbiota, but there is also a diversity of microbes from other realms including fungi, viruses, parasites and archaea. We know less about these groups and their functioning than we do about the bacteria.

Fungi

The mycobiota, particularly yeast, make up 0.001% to 0.1% of the microbes in the human gastrointestinal tract (130, 131). Fungi are most often detected in the colon (132, 133). The gut mycobiome is especially complex to understand, since fungi are ubiquitous in the environment including fermented food products like bread, cheese and beverages. The degree to which detected fungi are intestinal residents or simply passers-by, is thus a difficult but important issue to elucidate.

Recent data, based on internal transcribed spacer 2 (ITS2) deep sequencing of feces from 307 subjects, led to the conclusion that "Compared to bacterial communities, the human gut mycobiome is low in diversity and dominated by yeast including *Saccharomyces cereviciae, Malassezia restricta* and *Candida albicans*." (134). These three genera were present in at least one sample from nearly every volunteer. The abundance of *Candida* could be associated with recent consumption of carbohydrates (135).

Only a few studies have applied culture-based methods to investigate whether fungi could be true viable residents of the gut ecosystem. A study from 1969 collected and cultured samples from oropharynx, jejunum, ileum and feces in 27 patients. They reported *Candida albicans* to be the most frequent fungus present in 30% of samples from oropharynx, 54% from jejunum, 55% from ileum and 65% from feces (136).

Viruses

Viruses, especially bacteriophages, are part of the microbial ecosystem (137). Investigations with transmission electron microscopy (TEM) have shown dense bacteriophage communities in mucosal biopsies (138) of which Caudovirales, Myoviridae-, Podoviridae-, Ackermannviridae- and Herelleviridae bacteriophage families are most frequently detected. Viruses of eukaryotic cells are found at very low levels, most often Anelloviridae (49). Most research has been conducted on feces (139-141). An old study based on traditional cell culturing from 1966 did not detect any viruses (142). The cell-lines used (HEp2, Monkey kidney cell and suckling mice) cover most of the enteroviruses (polio-, coxsackie A- and B-, enteric cytopathic human orphan (ECHO)- and Entero 68-71). Adenoviruses are difficult to culture and rota-, noro-, sapo- and astroviruses would not be detected in these cell-lines (Halvor Rollag, personal communication, December 2018).

Parasites

Parasites are not regarded as members of the human microbiota in Western countries, but a variety of non-pathogenic commensal intestinal parasites/protists such as *Entamoeba coli, Blastocystis spp.,* and *Endolimax nana* are regularly found in individuals from low income countries with water supply deficits (143).

Archaea

There is limited information on the abundance and diversity of archaea in the human gut. Theoretically, some 16S rRNA gene probes will also detect some archaea (144), but sensitivity data of the different variable regions are scarce. In the human gut, methane-producing archaea (methanogens) are predominant. Important species are *Methanobrevibacter smithii*, *Methanosphaera stadtmanae* and *Methanomassiliicoccales* (145). Methanogenes can only use substrates from anaerobic degradation of organic matter effectuated by hydrolytic or fermentative bacteria (146).

Aims of the thesis

We aimed to give a comprehensive description of the human small intestinal microbiota by investigating per-operative samples collected from intestinally healthy patients suffering from morbid obesity and patients diagnosed with muscle invasive bladder cancer.

The aims were:

- To characterize the jejunal microbiota with sampling from both proximal and mid-segmental parts of jejunum during gastric bypass by both culture and deep sequencing of the 16S rRNA gene.

- To characterize the ileal microbiota with sampling from the distal ileum during cystectomy with urinary diversion both by culture and deep sequencing of the 16S rRNA gene.

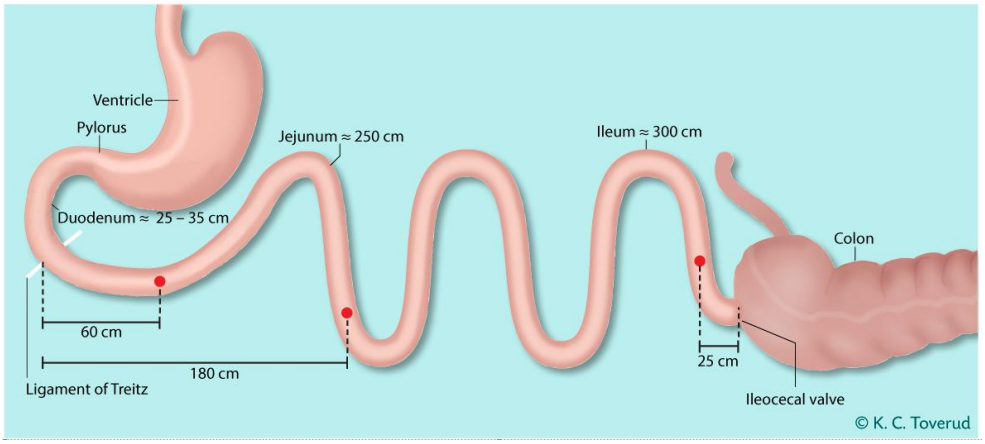
- To provide reliable bacterial density measures for all three sampling sites.

Materials and methods

Summary of study population, study design and sample collection

Surgical access to three different segments of the small intestine was obtained during elective surgical procedures. Samples were collected by rubbing a swab against the luminal mucosa and thereafter transported to the laboratory in a Transwab medium (MWE, Medical Wire, England). For the investigations of the ileal microbiota we collected samples 25 cm proximal to the ileocecal valve from 150 patients undergoing radical cystectomy with urinary diversion. All ileal samples were investigated using standard culturing techniques (Paper II). Due to cost considerations at the time, only the first 27 ileal samples were analyzed using 16S rRNA gene deep sequencing (Paper I). For the investigation of the jejunal microbiota, we collected samples 60 and 180 cm distally to the ligament of Treitz from 60 patients undergoing gastric bypass surgery. All 120 jejunal samples were described using both culture, 16S rRNA gene deep sequencing and a universal quantitative PCR.

Figure 2. Sampling sites for the studies (red dots).



Collaborators at the Departments of Urology and Gastrointestinal Surgery collected clinical data from the journal system according to the study protocol. We registered age, gender, body mass index (BMI), medications, American Society of Anesthesiologists (ASA) score, smoking, neoadjuvant chemotherapy, antibiotic prophylaxis, tumor stadium and comorbidities like diabetes, hypertension, dyslipidemia, obstructive sleep apnea. Characteristics of the 150 patients with bladder cancer are described in Table 2, Paper II: *Demographic and clinical data*. Characteristics of the 60 patients undergoing bariatric surgery are provided in Table 1, Paper III: *Patient characteristics*.

Methods

Cultivation and MALDI-TOF MS

Vortexed content from the Transwab medium was cultured on blood, chocolate, MacConckey and Sabouraud agar plates and incubated in a 5% CO_2 enriched atmosphere at 37°C for 5 days. The samples were also cultured in an anaerobe atmosphere on blood agar plates, Menadione agar plates and in a Thio broth.

We used Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany) to identify microbial colonies. Analysis was performed using the Biotyper versions 4.1.70.0 – 4.1.90.0 software available between 2016 and 2019. Scores between 2000 and 3000 with consistent naming (category A) were accepted for identification at the species level and scores between 1700 and 1999 at the genus level.

DNA extraction

In Paper I, we used the "Pathogen complex kit" (Qiagen, Hilden, Germany) on a QIAsymphony (Qiagen) platform for DNA extraction and purification. Negative controls from the relevant batches of Transwab media were processed in parallel. In

Paper III we included an additional pre-treatment with bead-beating in Matrix E tubes (MP Biomedicals, United States) on a Fastprep 24 (M.P. Biomedicals, India) instrument for 2 X 45 seconds to assure effective lysis of all bacteria prior to extraction and purification using the "DSP DNA Mini kit" (Qiagen, Hilden, Germany) on the QIAsymphony platform. In addition to the negative processing controls we included weak-positive controls spiked with *Legionella pneumophila*.

Quantification by microbial culture; cfu/ml

Quantification by culture was performed by seeding a standardized amount of sample material on agar plates and counting colonies as described in Paper II and III. The detection limit by culture was estimated to be around 160 cfu/ml and the upper limit for quantification was $>1.6 \times 10^4$ cfu/ml.

Quantification by real-time 16S rRNA gene-PCR; genomes/ml

In Paper III, we measured the number of bacterial genomes per milliliter of sample material using a quantitative universal 16S rRNA real-time PCR. Standardization was based on dilution series of *Streptococcus pneumonia* that has a 16S rRNA gene copy number close to the mean number of 16S rRNA genes in organisms within the kingdom of bacteria. The lower limit of detection was estimated to be 2816 bacterial genomes/ml.

16S rRNA-gene deep sequencing

In both Papers I and III, deep sequencing was based on amplicons containing the 16S rRNA gene variable areas V3 and V4. However, deep sequencing was performed on different platforms at different laboratories for the two papers. There were also slight differences in primer designs, but we do not believe this to have had any significant impact on the results.

In Paper I, the 16S rRNA gene amplification and sequencing were outsourced to the Public Health Agency of Sweden. They used an IonTorrent sequencing instrument (Thermofisher, Foster City, CA).

In Paper III, amplification and sequencing was done at the Department of Microbiology, Haukeland University Hospital. Here we used a MiSeq instrument (Illumina, San Diego, CA) with MiSeq reagent kit V3 (2x300 basepair reads).

Bioinformatics

We used the RipSeq next generation sequencing (NGS) software (Pathogenomix, Santa Cruz, CA) for sequence data analysis. Reads were clustered *de novo* into operational taxonomic units (OTUs) using a 99% similarity threshold. The most representative sequence from each OTU was assigned using a BLAST search against the Pathogenomix Prime database. This is a semi-curated database constructed from more than 2500 manually curated references, all references from the Human oral microbiome project, NR references from GenBank, 16S rRNA genes extracted from GenBank complete genomes and GenBank type-strain references. We required ≥99.0% homology with a high-quality reference sequence and a minimum distance of more than 0.8% to the next alternative species for a species level identification. Homologies between 97% and 99.0% were reported at the genus-level.

In Paper I, we also used QIIME (Quantitative Insights into Microbial Ecology), an open-source bioinformatics pipeline for microbiome analysis to perform comparative sample analyses. Alfa-diversities (diversity within a sample/species richness) were reported as Shannon index and number of bacterial identifications, while beta-diversities (diversity between samples) were calculated using weighted and unweighted UniFrac analyses (147).

Elimination of chimera and filtration of contaminant background DNA

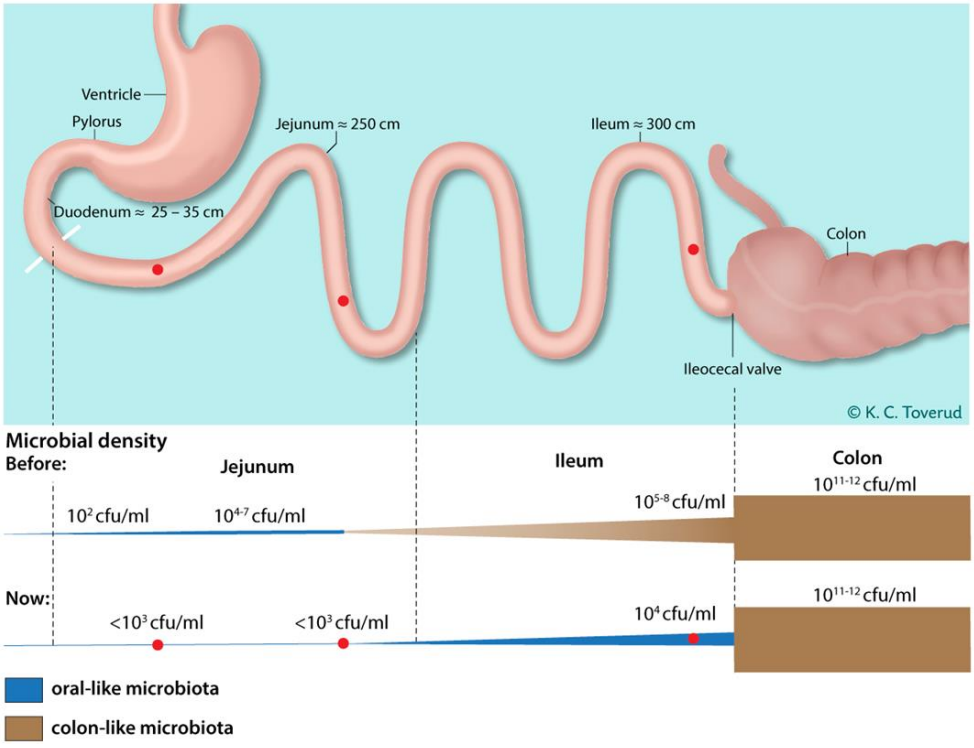
We used the RipSeq NGS chimera check to remove chimeric OTUs from all samples. In Paper I, we filtered contaminant background DNA by simply removing any species also detected in the negative controls. In Paper III we adopted a more sophisticated approach and defined a sample specific cutoff for each sample based on the most abundant contaminants as described by Dyrhovden *et al.* (148). All species identified from negative controls were rejected from the samples unless they appeared in higher concentrations than the five most abundant contaminants. In addition, we filtered species detected only once among our 120 samples (singletons) and finally any remaining species considered biologically unexpected.

Statistics

In Paper II, we applied descriptive statistics and univariate statistical analyses with 95% confidence intervals. P-values were calculated with Pearson´s Chi-squared test or Fisher´s exact test for tables with few cases. Potential trends were tested with binary logistic regression. Multivariate logistic regression was utilized to adjust for age and sex. SPSS Statistic 25 package, Stata 16 and R 3.5 package were the preferred software.

Summary of results

Figure 3. Bacterial densities of the small intestine. Our results compared to current literature.



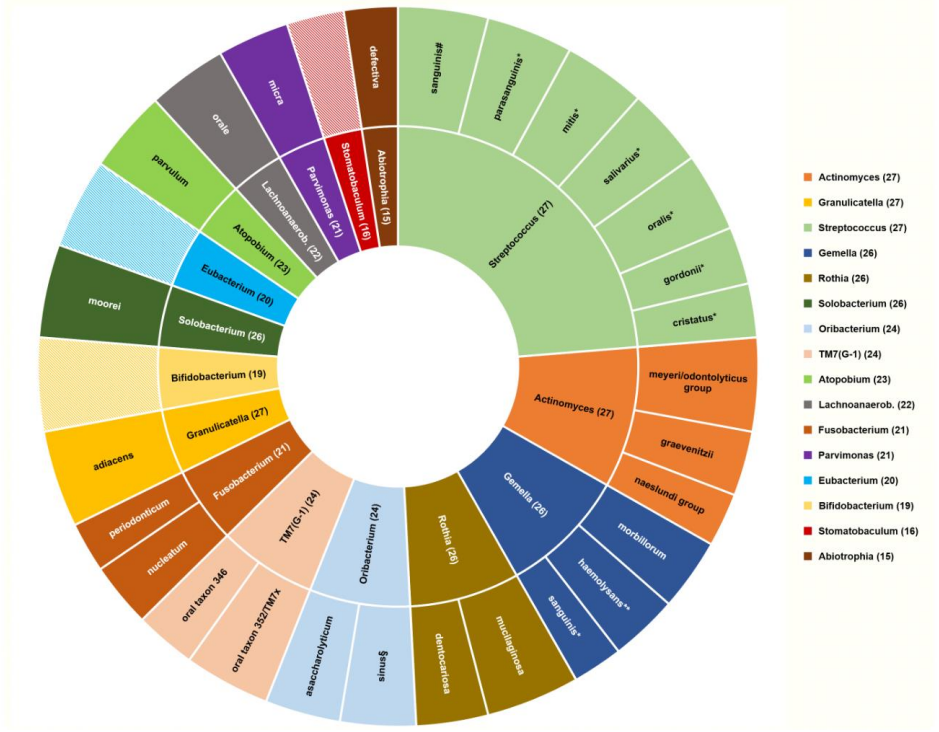
Paper I –Species level description of the human ileal bacterial microbiota

Sampled material from the distal ileal mucosa of 27 patients undergoing elective cystectomy were characterized using deep sequencing of the 16S rRNA gene. In the ileum, we were able to define a core microbiota at both the genus level (Figure 3 in Paper I) and the species level. The most frequently detected species (present in 96 – 100% of the patients) belonged to the *Streptococcus mitis group*, the *S. sanguinis group*, *Granulicatella adiacens,* the *Schaalia odontolytica complex*, *Solobacterium moorei*, *Gemella haemolysans/sangui*nis and *Rothia mucilaginosa.* At the genus level *Streptococcus* (100%), *Granulicatella* (100%)*, Actinomyces* (100%)*, Gemella* (96%)*, Rothia* (96%), *Solobacterium* (96%), *TM7(G-1)* (89%), *Oribacterium* (89%),

Atopobium (85%), *Lachnoanaerobaculum* (81%), *Fusobacterium* (78%), *Parvimonas* (78%), *Eubacterium* (74%) *Bifidobacterium* (70%), *Stomatobaculum* (59%) and *Abiotrophia* (56%) were most frequently detected. Proteobacteria and strict anaerobes were only exceptionally detected.

Figure 4 (Figure 3 from Paper I)

Core microbiota of the ileum. The inner circle represents the genus-level core microbiota defined as genera present in >50% of samples. The outer circle represents the species-level core microbiota defined as species present in >50% of samples. For the outer circle, the width of a segment is proportional to the observed incidence for that species. *Species level identification obtained with targeted *gdh* or *rpoB* Sanger sequencing. **Species level identification obtained with *rpoB* sequencing. Includes *Gemella haemolysans* sensu strictu (n = 7) and the newly proposed species *Gemella para-haemolysans* (n = 9) and *Gemella taiwanensis* (n = 6). #Only 0.7% distance to *Streptococcus sinensis*. Formally *S. sanguinis* (*S. sinensis*). §Only 0.7% distance to *Oribacterium parvum*. Formally *O. sinus* (*O. parvum*).



Paper II – The cultivable microbiota of the human distal ileum

Material from the distal ileal mucosa of 150 patients with bladder cancer were characterized using traditional culturing and density measures. Only 7% of the samples were culture-negative. Among the culture-positive samples, 79% contained $\leq 1.6 \times 10^4$ cfu/ml. The three most frequently cultured species from ileum were *Candida albicans* (48% of patients), *Streptococcus sanguinis group* (45%) and *S. mitis group* (42%). Other viridans streptococci, *Actinomyces, Rothia* and *Lactobacillus* were also relatively frequent findings. Constipation was associated with increased recovery of colon related bacteria. High age was significantly associated with increased fungal growth. Proton pump inhibitors seemed to be associated with increased levels of both bacteria and fungi. We did not find evidence that antibiotic treatment prior to the surgical procedure affected microbial density.

Paper III – Investigating the human jejunal microbiota

Jejunal samples from both the proximal and mid-jejunal segments were collected from 60 patients undergoing gastric bypass. Cultivation and 16S rRNA gene deep sequencing were performed in all samples and quantitation was undertaken using both culture and a universal quantitative 16S rRNA real-time PCR. In the jejunum, 51% of the samples were culture-negative – corresponding to less than 10^3 cfu/ml. Deep sequencing and quantification of the bacterial 16S rRNA gene revealed low levels of typical oral bacteria. 76,7% had $\leq 10^4$ bacterial genomes/ml. Most species were only sporadically detected, and we were not able to find evidence supporting the existence of a core resident jejunal microbiota. The most frequent species in the jejunum detected by deep sequencing (present in 40 - 48% of the patients) were *Streptococcus mitis group*, *Streptococus sanguinis group*, *Granulicatella adiacens/para-adiacens*, the *Schaalia odontolytica complex* (former *Actinomyces odontolyticus*) and *Gemella*

haemolysans/taiwanensis. At the genus level *Corynebacterium* (83%), *Streptococcus* (67%), *Gemella* (53%), *Granulicatella* (47%) and *Actinomyces* (30%) were most frequently identified. The three most frequently cultured groups of species were the *Streptococcus salivarius group* (25% of patients), the *S. sanguinis group* (17%) and the *S. mitis group* (12%).

Discussion

Microbiota - Viable or dead microbes?

Establishing whether the bacteria identified are viable or not, is of importance when considering the presence of a core microbiota. This task is, however, methodologically arduous. Culture, which is the gold standard technique to confirm the existence of viable microbial cells, has a low sensitivity for anaerobic and fastidious organisms within the intestines. A large proportion of the intestinal microbiota will thus go undetected by culturing procedures. DNA-based methods like PCR and deep sequencing will principally detect all microbes in the sample. These techniques can, however, not be used to discriminate between viable and dead bacterial cells. For instance in this project, *Corynebacterium vitaeruminis* detected by deep sequencing in seven jejunal samples and *Enterococcus cecorum* detected by sequencing in seven other jejunal samples are generally considered to be part of the normal flora in domestic animals and not humans (149, 150). Whether or not the two bacterial species are members of the human gastrointestinal flora, is therefore still not clarified.

Although the living/dead-issue is an important one for research on the microbiota, the biological interactions between the host and the microbes could be unhindered by this distinction. Dead bacteria are sometimes as efficient immune modulators as live bacteria (151). Dead lactobacilli with intact cell walls will release the microbial beta-galactosidase (lactase) in the intestinal lumen (152). Heat-killed *Enterococcus faecalis* have been demonstrated to stimulate the immune system in chicks and increase neutrophil phagocytes in dogs (151). Likewise, killed *Saccharomyces cerevisiae*, *Candida utilis* and *Kluyveromyces marxianus* show beneficial effects on the immunological and intestinal health in fish industry (149, 153).

Microbiota - Resident or transient microbes?

Most studies do not problematize the demarcation between resident (indigenous or autochthonous) and transient (allochthonous) microbiota (5). A resident microbe is per definition ever-present in a niche, whereas transient microbes will only exceptionally colonize a habitat in perturbed situations like ileus or stasis. Transient microbes contribute little to the local ecology and may be present only in dormant forms.

Neither culture nor sequencing can be used to discriminate between resident and transient microbes derived from upstream intestinal locations.

There is no simple solution to these methodological matters, but it seems reasonable to assume that DNA from non-resident bacterial species will be inconsistently present and only at low levels. In our investigations, we have thus considered the more abundant species consistently detected in a large proportion of patients to represent live members of the local microbiota.

Clinical and scientific implications

Although contemporary reviews and textbooks take the existence of a human jejunal microbiota for granted, with reported bacterial concentrations between 10^4 and 10^7 cfu/ml, evidence from older investigations contradict this conclusion by stating that the human jejunum lacks a resident microbiota (5). Our results, which correlate well with those of the older studies and with theories in comparative anatomy and physiology, support a conclusion stating that the jejunum lacks a resident microbiota. From this, we infer that microbes do not play an important part in digestion and uptake of nutritive substances in this part of the gastrointestinal system.

Predominantly herbivorous animals are dependent on the microbial ability to access nutrients from the food they eat. In such animals, the intestinal tract slows down the passage of food to provide sufficient time for microbial food degradation. In contrast, the small intestinal canal of meat-eaters and omnivores like humans lack spaces for

food accumulation and fermentation. They therefore have to digest food by enzymes largely of their own making. A resident microbiota in the small bowel would not be useful to such animals, as it would compete with the host for nutritive substances (154). There is even evidence to suggest that the human small intestine regularly performs a peristaltic cleaning procedure to prevent the buildup of a microbial flora, the fasting migrating motor complex (MMC).

Whereas we found the jejunum to lack a microbiota of its own, our results support the presence of a resident microbiota in the distal part of ileum. The resident microbiota was more similar to the oral than to the colonic microbiota, but considerably less dense with microbial concentration around 10^4 cfu/ml. This contradicts previous coloscopy-based studies of the ileum, reporting a colon-like flora with up to 10^8 cfu/ml. Our results should thus put an end to the assumption that the colonic microbiota is a relevant proxy for the overall intestinal microbiota (155, 156).

Our results from both the jejunum and ileum raise essential questions regarding current sample collection procedures from the small intestine in both research and diagnostics. Non-surgical sampling via endoscopes is prone to significant contamination from the colonic or oral microbiota. In research, fine needle aspiration from the small intestine during other types of abdominal surgery could represent an alternative to obtain uncontaminated samples from intestinally healthy people if deemed ethically acceptable. However, even this is not optimal since as we have discussed in our papers, patients will be in a fasting state and normally receive some sort of antimicrobial prophylaxis. Therefore, we need to develop new sample collection techniques in order to advance this research field further and in particular if mapping and monitoring of the small intestinal microbiota is to be used for diagnostic and therapeutic purposes. More advanced capsule technologies, improved sterile brushing during endoscopic procedures or sterile needle aspiration during laparotomy could secure clean uncontaminated mucosal sampling.

Bacterial dysbiosis is among suggested causes for “functional” gastrointestinal disorders like irritable bowel syndrome (IBS), small intestinal bacterial overgrowth (SIBO) and unexplained food intolerance syndromes. Bacterial overgrowth in the

small intestine is also among the proposed causes for tropical sprue, a malabsorption disease with flattening of villi and inflammation of the small intestine observed in tropical regions (154). Microbes have almost unlimited possibilities in metabolic pathways. Some convert dietary precarcinogens and carcinogens to noncarcinogens (157) and the other way around, some synthesize beneficial substances like vitamins (158), while others produce toxins or harmful endogenous substances. Bariatric surgery like gastric bypass and gastric sleeve procedures, could result in higher microbial density because of disturbed intestinal motility and a decreased antibiotic acidic treatment of food in the ventricle (42). This might contribute to the postoperative weight loss observed for this group of patients. It could be of importance to map and even monitor the small intestinal microbial content in several disorders. However, as discussed above, novel non-invasive sample collection procedures will be necessary to obtain this.

An unexpected and very interesting finding in our ileal samples was the frequent detection (89% of samples) of bacteria from the Candidatus Saccharibacteria-phylum, in some samples constituting almost a quarter of the present bacteria. The provisional species TM7(G-1) HMT-352 was the most frequently detected Saccharibacterium followed by TM7(G-1) HMT-346. We also relatively frequently detected Saccharibacteria at lower concentrations in the jejunal samples (30% of patients) predominantly TM7(G-1) HMT-352. Representatives from this phylum have not previously been reported in the small intestine.

TM7(G-1) HMT-352 is a bacterial species living on the surface of its bacterial host. This species has been co-isolated from human saliva together with its obligate bacterial host, *Schaalia odontolytica* (159). Increased abundancies of OTUs from TM7(G-1) HMT-346 is associated with periodontitis in elderly individuals (160).

The ultra-small gram-positive coccoid bacteria from the TM7 genera are known to be ubiquitous members of the human oral microbiome (161). Owing to difficulties with cultivation of this genus, knowledge about physiology and relevance in microbial ecological systems and in human health and disease are scarce or missing. Genomic analysis reveals that bacteria from these species are unable to synthesize nucleotides,

lipids and amino acids required for their own growth, and TM7 is classified as auxotrophic - not able to synthesize particular organic compounds and thus dependent on other organisms (162). The parasitic bacteria are known to affect their bacterial host with increased stress responses, reduced cellular growth rate and cell lysis under nutrient starvation (163, 164). In research performed on dental microbiota, it has been hypothesized that TM7 can be beneficial for the bacterial host by promoting biofilm formation (165), thereby protecting it from salivary flow, toxic substances in the surroundings and the human immune system. Such effects could be even more important to the bacterial host in the ileum with massive concentrations of lymphoid cells and immunoglobulins, and mechanical rubbing caused by the combination of intestinal content and peristaltic movements. Frequent and abundant identifications in our project implicate intact living cells present. The phyla represent almost a quarter of the reads in some of the ileal samples. To this date, research on this mysterious phylum is limited but next generation sequencing technologies and novel approaches for culturing are promising tools to accelerate the field.

The existence of fungi as part of the small intestinal microbiota is debated. A study from 2018 (132) hypothesized on the basis of serial investigations of feces from four volunteers, that fungi are not true colonizers of the human healthy gastrointestinal system. They report that only 0.01 % to 0.1 % of deep sequencing reads from adult stool samples are mapped to fungal species and postulate that these reads represent remnant DNA and non-viable *Candida* cells originating from the oral cavity. Our high culture recovery rate (48% of ileal samples) contradict this conclusion. In order to effectively colonize the small intestine, microbes should probably possess adherence factors. Biofilm formation is a well-known phenomenon of *Candida albicans* and these fungi often exist as part of surface-attached microbial communities that could interact with other members in the ileal microbiota (166). More studies on biofilm formation in the small intestine are needed.

The scarce microbiota in jejunum and ileum challenge the relevance of the currently recommended antibiotic prophylaxis regimens prior to surgery on the small intestine. These recommendations seem to focus on the gram-negative enteric rods and strict

anaerobic bacteria of the colonic microbiota. Future clinical studies will decide whether antimicrobial prophylaxis prior to gastric bypass should be modified.

Methodological considerations

Study population and design

Our populations consisted of patients with morbid obesity and patients with muscle invasive bladder cancer, most without gastrointestinal disease. Although such patients are not representatives of a standard healthy population, they do represent individuals without overt gastrointestinal disease.

The patients with muscle invasive bladder cancer were treated for a condition unrelated to the intestines and can therefore be considered intestinally healthy. Most of them were old with a median age of 73 years (range 48-89), and most had American Society of Anesthesiologists (ASA) score 2 or 3. In addition, surgery necessitates anesthesia that influences intestinal motility. These factors could influence the generality of our results.

The patients with morbid obesity received surgery for a condition that could be associated with an altered gut microbial composition. However, since we were not able to find a resident microbiota in jejunum and since this conclusion correlates well with older studies on other populations (93, 96) our results seem generalizable.

It is important to acknowledge that we have investigated a homogenous population from a high-income country and that our results might not be completely representative of other populations in different parts of the world. It has, for example, been demonstrated that South Indians and Guatemalans have greater numbers of coliforms and *Bacteroides* in the small bowel, maybe due to malnutrition and contaminated food (167).

Although Paper I could arguably have included a larger number of patients (n=27), the number is still large compared to other deep sequencing microbiota-studies on the small intestine. This study was of descriptive nature and the low number of patients did not allow for further statistical analyses.

In Paper II we included a robust number of subjects (n=150) for culture, making it possible to search for statistical associations between microbiological density and acknowledged clinical parameters.

In Paper III we present results from the jejunal microbiota from an obese population (n=60) both by deep sequencing and culture. The number made it possible to also run some bivariate analyzes.

Antibiotic prophylaxis given prior to both cystectomy and gastric bypass surgery, could affect microbial findings. Intravenous metronidazole is detectable in feces shortly after administration as demonstrated in patients suffering from *Clostridioides difficile* infection (168). However, we believe that the epithelial mucus layer will protect the microbiota against antibiotic effects at least temporarily as has been demonstrated for the salivary microbiota (118). In paper II we looked for potential effects of antibiotic treatment administered the previous 6 months and during the week ahead of hospital admission on microbial culture, without finding any evidence of statistical significance (Table 5 and 6, Paper II). DNA-based analyses are generally less vulnerable to antibiotic effects, in particular to recent brief exposures like surgical prophylaxis.

Unfortunately, we did not have access to clinical information related to oral health and dental status. A study from 2018 found less *Candida* in fecal samples from patients with increased frequency of teeth brushing (132). Oral health and teeth cleaning routines should thus be investigated in future studies.

In addition to oral status, diet, eating habits and medications could all affect the composition and relative abundancies of the small intestinal microbiota. Recent food intake could possibly also serve as a bias. An apple will for example harbor its own microbiota (169) and a recent study demonstrated that microbial findings in duodenal

aspirates correlated better with diet than with symptoms like diarrhea, abdominal pain and bloating (170). Most published studies abstain to inform the reader about participants fasting status. In our studies, all our included subjects were fasting.

Microbial specimen collection

A major strength of this project is the clean surgical sampling procedures. To our knowledge, the only elective procedures that provide access to the small intestine of intestinally healthy patients are gastric bypass surgery and cystectomy with urinary deviation.

Other abdominal surgery procedures are performed on patients suffering from gastrointestinal disorders like inflammatory bowel disease, cancer, ileus, traumas etc. Non-surgical procedures, including sampling during e.g. endoscopy techniques through the oral cavity or the colon, come with a great risk of contamination due to the extremely high bacterial concentrations in the mouth and in the colon as compared to the small intestine. The salivary microbiota contains about 10^9 cfu/ml and the colonic $10^{11\text{-}12}$ cfu/ml and therefore even the slightest introduction of oral or colonic content will significantly influence the results from the small intestines containing several orders of magnitude fewer bacteria.

For paper III, we also consider it a strength that we sampled from two different sections of the jejunum in all patients. This enabled us to investigate differences between the two jejunal sections and also doubled the number of jejunal samples. The overall lack of microbial consistency not only between patients but also between the two sections in the same patients strengthen our conclusion that the jejunum does not have a microbiota of its own, and that our findings instead represent low levels of transient microbes.

Since microorganisms close to the epithelium may be of higher biological relevance than microbes in the lumen, we further believe that sample collection by rubbing the mucosal surface is important.

Alternative methods for sampling the small intestinal microbiota:

Upper endoscopy/gastroscopy only reaches the proximal part of jejunum and introduces a great risk for contamination from the oral cavity. However, for inspection of the upper parts of the small intestine, a protected sterile brush technique (30) might be adequate.

Nasojejunal or ileal catheters often demands swallowing fluids to evoke peristalsis. This technique would be highly susceptible to contamination from the oral cavity. It can often take up to 24 hours for the tube to reach the distal part of ileum. It is also difficult to determine the exact position of the tube, the tube could provoke alterations in intestinal motility and there is an unknown dilution factor for measurements of densities.

Sampling by mechanical capsules designed to open at electronic signals represents a very attractive alternative. It is however difficult to follow and determine the position of the capsule. Further, the capsules are currently not capable of collecting mucosal samples, only free luminal content. The capsule follows the natural flow in the intestine and is recovered from feces. The resulting delay between sample collection and cultivation allows for both bacterial multiplication and death inside the capsule that could distort the findings.

Retrograde coloscopy is often used in studies to get samples from the distal parts of ileum. Colon contains approximately 1-10 million times more cfu/ml than the distal parts of ileum and even the slightest trace of contamination from colon will bias the results. A randomized trial from 2011 (85) compared sheathed versus standard forceps for obtaining biopsy specimens from terminal ileum during coloscopy. They found no significant differences between the methods and concluded that sheeting was not necessary. However, they failed to consider the possibility that both methods were

insufficient to avoid contamination. Unfortunately, this study might have contributed to the generally held belief that current methods for sampling from the ileum by coloscopy are adequate, and thus cemented the misconception that the distal ileal microbiota is colon-like.

Autopsies are not ideal because of an obligate delay after death, allowing profound alterations of the intestinal contents. The temperature fall together with the physiological leakage postmortem across all cell membranes in the dead body will completely change the microbial growth conditions. In addition, cessation of peristalsis and disruptions of other antibacterial mechanisms in the small intestine will contribute to rapid and large changes in the microbiota.

Ileostomies present effluent material from ileum in an artificial opening in the abdominal wall exposed to air. Ileostomy effluent is by no means representative of the physiological state of the intact small intestinal lumen. A study from 1967 (171) demonstrated significant differences in the microbiota from the normal terminal ileal content sampled through peroral intubation compared with effluent from ileostomy. Especially coliforms and anaerobes were more numerous in effluent samples.

Fine needle aspiration: In studies from the 1950s, 1960s and 1970s (93-96), a syringe needle was inserted obliquely into the antimesenteric border of the small bowel. 2 ml of sterile Ringer solution was injected into the lumen while constricting the bowel with fingers above and below the site of injection. Thereafter, the surgeon washed the bowel with the fluid by sucking it back and forth with the syringe before sampling. For future studies, given the potential importance of detailed and correct knowledge of the microbiota in the small intestine, sterile needle aspiration during surgery might be considered ethically acceptable also today. Using this approach, younger healthier patients could be included for microbiota investigations.

Microbial investigations

Cultivation versus 16S rRNA gene deep sequencing

The establishment of DNA sequencing technologies in the late 1970s led to revolutionary understandings of the human microbiota. From around 2009, novel "next generation" sequencing technologies like Roche´s 454-sequencing instrument provided new possibilities for affordable and accurate massive parallel sequencing or "deep sequencing" of pre-amplified targets like e.g. partial 16S rRNA genes. Our understanding of this methodology has evolved over the years and has, together with improved bioinformatics tools and recommendations for the inclusion of control samples, made results more refined and reliable. Many of the earlier studies in this field provided identifications only at high taxonomic levels. In addition, they either utilized insufficient methods for handling contaminant DNA or failed to provide a description on how this was done. Therefore, the results from many of these early studies should be interpreted with care.

Cultivation offers information about viable microorganisms, but fail to detect many slow-growing, fastidious or anaerobic microorganisms. The reported complementarity between culture-dependent and culture-independent studies varies; some have found that about 15% of species are detected concomitantly in gut microbiota by the two techniques (172). Furthermore, 16S rRNA gene deep sequencing studies have a depth bias, just like cultivation. In feces, with approximately 10^{12} cfu per ml, large-scale molecular studies have been found to underreport bacteria at concentrations below 10^{5} cfu/ml (173). Culture-independent techniques on human feces also report a significant amount of microorganisms considered remnant DNA from dead microbes or DNA originating from food (169). In our projects, the median number of detected species in ileum by cultivation was three compared to 50 by deep sequencing (Paper I). In jejunum, the median numbers were zero and six, respectively. In jejunum the bacterial concentrations were at the limit of or below the lower level or detection for both culture and 16S rRNA gene sequencing, probably explaining why detected species are randomly reported from the two segments and also between patients.

The 16S rRNA gene is a bacterial gene not present in eukaryotic cells. Deep sequencing of alternative fungal targets like ITS2 or 18S was not a part of this work. However, cultivation allowed us to look into the presence of yeast.

Cultivation

Cultivation is the basis for identification of living microorganisms. The Transwab medium used for sample transportation in our studies is according to the manufacturer designed for aerobic, anaerobic and fastidious bacteria.

The major limitation of our culture based approach and of culture in general is the low sensitivity. The cultivation methods used were the standard procedures in daily diagnostic use at our laboratory. We know that many bacteria from the human intestine fail to grow under such conditions. In addition, in polymicrobial samples like ours, bacteria better fitted to the conditions in the laboratory could suppress or overgrow other more fastidious or slow-growing species. Ideally, more selective plates to separate species in a polymicrobial environment and mimicking the environment in the small intestinal lumen in terms of acidity, presence of mucus, antimicrobial substances, pH, nutrients and atmospheres should be included (173). However, these are highly specialized non-standardized approaches not easily implemented in a hospital laboratory.

Experienced lab technicians scrutinized the plates and picked colonies with different morphologies for identifications by MALDI-TOF MS. Multiple species were detected. However, we know that colonies from many species look very similar and therefore can be difficult to distinguish even for an experienced eye (173).

Despite these limitations, representatives from all the most abundant and frequent genera detected by 16S rRNA deep sequencing, were also detected by cultivation from some samples except from the fastidious genera *Gemella, Granulicatella, TM7* and *Solobacterium.* However, the overall recovery rate by culture was low compared to that of deep sequencing.

Our cultivation methods were suitable for the recovery of yeast. We found *Candida spp.,* mostly *Candida albicans,* in particular in the ileal samples (56%) but also in one jejunal sample (1.7% of patients). Previous deep sequencing studies have reported the presence of *Saccharomyces cerevicia* ("bakers yeast") in the small intestine, but this yeast did not grow in any of our samples. One possible explanation is that the detections by deep sequencing in literature represent dead or inactivated remnants from ingested food, and that this yeast should not be considered part of the human gut flora (134).

We did not cultivate in order to detect molds like *Aspergillus*. Molds demand lower temperature, typically 25 degrees, and longer incubation times.

Quantification by microbial culture; cfu/ml

The referred microbial densities in the literature are based on microbial culture measured as number colony forming units visible to the human eye on plates per ml or milligram of aspirate. The obvious weakness of quantification by culture is all the fastidious or non-cultivable genera, like *Gemella, Granulicatella and Helicobacter*.

We calculated microbial densities from an input volume that was not possible to accurately standardize. We rubbed the swab against the mucosa and assumed it to hold 150 microliters of sample material (174) that would necessarily be a mixture of rubbings from the mucosa and luminal juices. Our calculations therefore must be viewed as approximations, but the mucosal material is difficult to measure in any other way, and there are no standard procedures available. Our results are in congruence with older studies on surgically collected samples.

16S rRNA gene deep sequencing

DNA extraction

Bead-beating for mechanical disruption of the bacterial cell wall was not included in the extraction procedure in the first article, and the results in Paper I could therefore potentially be under-estimating the amounts of typically gram-positive microorganisms with more robust cell walls. In the third article, the extraction was optimized with bead-beating.

16S rRNA gene amplicon deep sequencing

The 16S rRNA gene is composed of alternating variable and conserved areas. The conserved areas are used as primer-targets for universal amplification of selected regions whereas the variable regions in between are the basis for identification. Among the nine distinct hypervariable regions V1-V9, there might be differences in the resolution i.e. the possibility to reliably discriminate between certain species (123). We amplified a region of about 430-460 basepairs (bp) that includes the variable regions V3 to V4 in both Paper I and Paper III. This region is one of the most commonly used regions from Sanger-based identification of bacteria (alongside the regions V1-V3) and is the region used in the standard Illumina protocol (175). All the 16S variable regions have limitations when it comes to species resolution within some genera. It is a strength of these studies that we adhered to the CLSI criteria for species and genus level identifications, and reported species level identification whenever possible.

In Paper I, the sequencing was outsourced to Folkhalsomyndigheten in Sweden, which left us with somewhat less control of the wetlab part of the experiment. However, the sequencing depth was good (median valid 97,047, range 15,079 to 376,370) and we performed the bioinformatics analyzes ourselves. The investigation profited from the inclusion of additional dedicated PCRs and Sanger-sequencing reactions for discrimination to the species level among the most frequent species of *Streptococcus* and *Gemella*: The gene encoding glutamate dehydrogenase (gdh) was selected to distinguish between streptococci according to Jensen *et al.* (176) and RNA dependent polymerase B (rpoB) to discriminate within the Gemella cluster (177). Species level description of the microbiota is of importance because different species within a

genus, could possess different metabolic properties and effects in the micro ecology. The approach with amplification and sequencing of additional genes was not applied in our third article because of the considerable workload and the fact that we could not find evidence for a core microbiota that needed an accurate description.

In Paper III we used a MiSeq sequencing instrument at Haukeland University Hospital, thus enabling better control of the sequencing process itself. In addition, we developed and used a quantitative universal 16S rRNA gene PCR that enabled us to calculate microbial densities more accurately. Contrary to the investigations on ileum, all patient samples were both cultured and analyzed with deep-sequencing. The sequencing depth was deeper and more consistent than in the ileum study although the final number of valid reads varied extensively due to low and varying microbial concentrations (median valid reads 19,568, range 2700 to 282,249). As expected, more reads were rejected in the weak positive samples.

Shotgun sequencing could in theory have secured species level identification for all bacterial findings. Shotgun sequencing is also a hypothesis free method able to detect fungi, bacteriophages, virus, parasites and archaea. However, shotgun sequencing is more expensive, complex and laborious. In 2016, when we started this project, the methodology and interpretation was also less mature, and the availability of complete genome references much lower. Shotgun sequencing is dependent on the availability of the correct whole genome sequences in the database. In a material like this, containing a substantial number of unusual and unnamed species, whole genome references will be lacking for a number of the present organisms (e.g. the TM7 phylum). In shotgun sequencing it is also difficult and very costly to assure a sufficient sequencing depth to fully describe low-abundant parts of a complex population. However, the methodology offers power to differentiate even different strains within a species.

Deep sequencing of the 16S rRNA gene deep sequencing has proven its immense value through many years and remains a practical tool for microbiota studies.

Bioinformatics

We used the Pathogenomix RipSeq NGS software that has been commercially available since 2014 (178). This software was developed specifically for diagnostic microbiology, since at that time available pipelines like QIIME did not meet the level of accuracy needed. In particular, it was problematic that QIIME back then only allowed for OTU-clustering at 97%. The Pathogenomix NGS software has been used in a number of publications. In these publications, the software has been evaluated against culture and Sanger sequencing (2, 178-181), species specific PCRs (1) and shotgun sequencing (182). Recently, the software was thoroughly evaluated in a publication in mBio (148) with focus on the ability to distinguish between relevant identifications and background contaminant DNA. In this latter publication, it was also benchmarked against commonly used free software on a commercial staggered mock-community.

The Pathogenomic databases are composed entirely of sequences selected from GenBank based on certain quality criteria for more robust identification. "The Prime database" available in the program, contains 2500 manually verified 16S rRNA gene references from GenBank, all NR_sequences (i.e. sequences curated by GenBank staff), all type strain references, all references from the Human oral microbiome project database and finally extracted 16S rRNA sequences from all GenBank complete bacterial genomes. In ileum 21 of 27 samples contained one or more unidentified OTUs below cutoff (97% similarity for genus), most of them far below 1% of the total amount of reads. In jejunum 59 out of 60 patients had unidentified OTUs from one of the two or both segments, averaging 22% of total number of the reads. We believe the increased levels of unassigned reads in jejunum reflects the low microbial levels and a relative much higher proportion of reads representing environmental contaminant species, unspecific amplification of non-bacterial targets and sequencing artifacts.

The Pathogenomic NGS software is based on reference-free OTU clustering, so called *de novo* clustering. All relevant sequences were clustered using a 99% homology cutoff. After clustering, the most dominant sequence type from each OTU is matched

against the databases mentioned in the text using a standard Basic Local Alignment Search Tool (BLAST)-search. The software provides a complete scoring list for each OTU and all alignments are directly available for visual inspection. This is a highly transparent process, comparable to analyzing sanger-sequences. We believe the species assignment is of high quality in our studies. OTU-clustering was performed after removal of short reads <250 bp.

Amplicon sequence variants (ASV) is now generally considered better than the approach with operational taxonomic units (OTU). As for the OTU vs. ASV debate, for diagnostic purposes the main problem with OTUs was the previously used clustering at 97%. When clustering is performed at 99% homology, a level that corresponds better with the observed intra-species variation of the 16S rRNA, the performance of OTU is comparable to that of ASV (Microbiome Informatics: OTU vs. ASV (zymoresearch.com).

For both ASV and OTU-approaches, it is important to acknowledge that the main problem is actually the inherent limitations linked to the resolution of the 16S rRNA gene within a range of genera. In diagnostic microbiology, single nucleotide differences are generally not considered sufficient to distinguish between species since intra-species sequence variation tend to be larger than that. This is well handled in the RipSeq software where you can also set criteria for genetic distance to next possible ID and will obtain a slash-result when this is not reached (we used 0.8% or higher as recommended by CLSI (183)).

One of the reviewers from Scientific Report for Paper I was concerned about the fact that 2/3 of the sequencing reads were removed during the filtration for short reads (below 250 bp), small clusters (<10 reads), chimera and contaminants from the negative controls. A likely explanation for this is that the Thermo-Fisher IonTorrent ION 5S sequencer used in paper I, have more premature read truncations compared to the Illumina technology, as shown by Salipante *et al.* (184). In Paper I, removal of sequences <250 accounted for the largest loss, typically about 50% of the reads. We expected the amplicon length to be 430-460 bp, and applied a cutoff of 250 bp for Paper I. With the pair-end sequencing of Illumina in Paper III we expected most

relevant reads to have expected length and therefore only removed reads shorter than 300 bp.

Species level assignment remains tricky when using partial 16S rRNA gene sequences and several strategies have been developed to make the best-possible tentative taxonomic assignments. We have analyzed our data using identity cut-off of ≥99% (>99.3% in Paper I) and distance of >0.8 to the next alternative species according to the widely accepted Clinical and Laboratory Standard Institute (CLSI) (183). Between Paper I in 2018 and Paper III in 2022, we observe inconsistent naming for some species due to the taxonomic evolution. For instance, *Actinomyces odontolyticus* is now classified as *Schaalia odontolytica*.

Elimination of chimera and contaminant background DNA

In both deep sequencing papers I and III, we included negative controls from the relevant Transwab-batches in the protocols. We used a rather basic method for filtering contaminants in the ileal samples (Paper I) and simply removed any OTU also present in the negative controls. Filtering of contaminants is generally both more critical and more challenging in weak negative samples, like those from jejunum (Paper III), where they constitute a much larger proportion of the results. In the ileal samples, that generally contained much higher concentrations of microbial DNA, this is less critical and we still believe the applied filtering approach was adequate for these samples. In Paper III we used a more sophisticated approach as described above and also included a *Legionella* spiked weak positive control to ensure control of low levels of DNA.

We detected a wide range of *Corynebacterium* species in jejunum and *Corynebacterium* was the most frequently detected genus in jejunum. This genus is a frequent contaminant in clinical samples but all corynebacteria detected in the negative sequencing controls (Paper III, Supplementary Table S2) were appropriately removed from the sequencing results. The most common among these were *C. tuberculostearicum* (50% of negative controls). However, many species were only

detected in the clinical samples (Supplementary Table S4), some of them rather frequently like *C. kroppenstedtii* and *C. aurimucosum* (both 25% of patients) and *C. durum* (18% of patients). These three species were are all first described in human samples. The other corynebacteria deemed relevant in this study are also associated with the human microbiota and/or human infections

Quantification by a real-time 16S rRNA gene PCR; genomes/ml

For Paper III we developed a bacterial genomic quantification based on universal amplification and quantification of the bacterial 16S rRNA gene. Such approaches will also be approximate since the copy number of the 16S rRNA gene among species can vary from one to fifteen (185). Most bacteria detected in this project have between three and seven copies.

For the standard curve in our quantitative PCR we therefore used a serial dilution of *Streptococcus pneumoniae*. It is similar to the other bacteria in the mitis group that constitutes an important part of the small intestinal flora and also harbors four copies of the 16S rRNA gene.

Measures of densities as cfu/ml by culture versus genomes/ml by quantitative PCR is displayed in Supplementary Table S3 in Paper III. Most of the 76 culture negative samples had high CT (cycle threshold, i.e. the number of cycles required for detection of the target sequence in a PCR reaction) values corresponding to $<10^3$ genomes/ml. Only 28% had CT values corresponding to concentrations between 10^3 and 10^4 genomes/ml. Among the three samples with growth of more than 1.6×10^4 cfu/ml, the CT values corresponded to $<10^6$, $<10^5$ and $<10^3$ bacterial genomes/ml respectively. The discrepancy for the last sample could be explained by the use of separate swabs used for culture and sequencing.

In Paper I, although all samples were analyzed using a universal 16S rRNA gene real-time PCR, thus providing a semi-quantitative indication on the levels of microbial DNA, we did not perform a more accurate quantification using a standardized quantitative PCR. This was unfortunate since it prohibited a later direct comparison

with the quantitative results from jejunum. 16S Ct-values from ileum, however, indicated bacterial concentrations at least hundred fold higher than in jejunum.

Statistical associations

Paper I and III are descriptive studies. In Paper II we did investigate a few clinical correlations. Due to the relatively small sample size of the dataset, our statistician strongly advised us to carefully select the parameters included in the multivariate analysis. A limited set of parameters is important to avoid over-parameterization or random false positive results. We mostly used the Fisher´s exact test for p-value calculations due to low case numbers. We used simple bivariate analyses (cross tables) to investigate relevant factors mentioned in the Patient characteristics Table 1 (age, proton pump inhibitors (PPIs), antibiotics, diabetes mellitus type 2 or status after cholecystectomy).

The use of proton pump inhibitors was associated with increased growth of both bacteria and *Candida*. To keep microbial numbers low, gastric acid and low pH are important. This finding is coincident with other studies (186). High age was also found to be associated with increased growth of *Candida*.

Constipation was associated with increased recovery of colon related bacteria. The peristaltic activity in the gut is important to keep the small intestine free from a harmful anaerobic colonization. Various bacterial endotoxin lipopolysaccharides have been demonstrated to delay gastric emptying and induce intestinal dysmotility and sphincter dysfunction by affecting the enteric nervous system (187, 188). *E. coli* and *Micrococcus luteus* are shown to be suppressive (34, 189). The association between constipation and colon-related bacteria in distal parts of ileum could be a relevant

finding but need supporting studies. In addition, eventual physiological consequences of this phenomenon should be further investigated.

Increased growth of *Candida*, not bacteria, was associated with increased age. To verify the association between age and *Candida* and to clarify the possible physiological effect on the human organism more studies are needed.

We did not observe any difference in the ileal microbiota in patients that had received antibiotics during the last six months or during the last week prior to surgery as compared to those that had not. Regarding an eventual effect of antibiotics distributed as prophylaxis just prior to surgery this is not possible to measure reliably due to the lack of a control group that has not been given prophylaxis.

In Paper III, no significant correlations with bacterial growth were found of substantial or non-substantial growth (for proximal and mid jejunal samples merged) like the associations we showed for age and PPI in paper II.

Ethical considerations

The study was approved by The Regional Committee for Medical and Health Research Ethics in the South-Eastern Norway Regional Health Region (2017/106 and 2016/926). All investigations were performed in accordance with this approval and in accordance with the relevant guidelines and regulations. Informed written consent was obtained from all participants. Participants were already selected for elective surgery and no inclusion or exclusion criteria were set. The sampling procedure prolonged the time of surgery by a couple of minutes, but was not considered to constitute any risk for the patients.

In accordance with the ethical approval, none of the results were reported back to the patients. Some patients (three in Paper I and five in Paper III) had 16S rRNA genes from *Helicobacter pylori* detected. *Helicobacter pylori* could potentially cause gastric cancer, but national guidelines do not support asymptomatic screening. The bacterium

is considered to be an ancient bacterium coexicting with humans for at least 100.000 years (190). *Tropheryma whipplei* were found in three patients in Paper I and two patients in Paper III. It can cause Whipples disease, a rare opportunistic infection in susceptible patients with unknown predisposing factors (191). However, in most carriers it causes no known health problems. In conclusion, we do not believe these findings to be ethical problematic, and the detection of both *H. pylori* and *T. whipplei* only reflects the expected background prevalence in the human gut microbiota in a western country.

For Paper I and Paper III we reported one conflict of interest. My main supervisor Øyvind Kommedal is a co-developer of the RipSeq NGS software and a stockholder in Pathogenomix. Neither other co-authors nor I reported any conflicts of interests.

Conclusions and future perspectives

The small intestine utilizes a variety of mechanisms to gauge and control microbial proliferation. Our results provide evidence that these mechanisms are utilized to a greater extent than reported in current reviews and textbooks, thus contradicting current opinion on the microbial composition of the small intestine. The microbiological descriptions of the jejunal and ileal segments of the human gastrointestinal tract presented in this work expand our understanding of the present microbiology in this physiologically important part of the gut.

We believe that some of the present contradictions in the literature is owing to methodological issues. Non-surgical sampling of the small intestinal microbiota through the oral cavity or colon will inevitably lead to contamination. More advanced capsule technologies, improved sterile needle brushing during laparoscopic procedures or sterile needle aspiration during laparotomy could secure clean uncontaminated mucosal sampling in the future. In addition, deep sequencing technologies have introduced new pitfalls related to low-resolution identifications and a considerable risk of reporting background DNA and contaminants as relevant clinical results. Density measurements by quantitative PCR and culture secure a more stringent interpretation of the identifications.

Some of the inconsistencies in the literature may also be owing to the clinical and therapeutic status of the patients investigated. The intestinal microbiota is responsive to a wide variety of external and internal perturbations, including eating habits, oral hygiene, medications and factors affecting the peristaltic activity. To assure consistency and for relevant comparisons between studies, the research community should elaborate standardized clinical guidelines for reporting on the small intestinal microbiota. Such standardization might as well increase the understanding of pathophysiological processes in the intestines and maybe even for systemic diseases not yet fully understood.

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Errata

Appendix

Paper I-III

The editorial services from the papers *Scientific Reports* and *Clinical Microbiology and Infection* have approved the reprints below.

Supplementary material

Paper I

Paper II

Paper III

I

SCIENTIFIC REPORTS

OPEN

Species Level Description of the Human Ileal Bacterial Microbiota

Heidi Cecilie Villmones[1], Erik Skaaheim Haug[2], Elling Ulvestad[3,4], Nils Grude[1], Tore Stenstad[5], Adrian Halland[2] & Øyvind Kommedal[3]

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The small bowel is responsible for most of the body's nutritional uptake and for the development of intestinal and systemic tolerance towards microbes. Nevertheless, the human small bowel microbiota has remained poorly characterized, mainly owing to sampling difficulties. Sample collection directly from the distal ileum was performed during radical cystectomy with urinary diversion. Material from the ileal mucosa were analysed using massive parallel sequencing of the 16S rRNA gene. Samples from 27 Caucasian patients were included. In total 280 unique Operational Taxonomic Units were identified, whereof 229 could be assigned to a species or a species group. The most frequently detected bacteria belonged to the genera *Streptococcus*, *Granulicatella*, *Actinomyces*, *Solobacterium*, *Rothia*, *Gemella* and *TM7(G-1)*. Among these, the most abundant species were typically streptococci within the *mitis* and *sanguinis* groups, *Streptococcus salivarius*, *Rothia mucilaginosa* and Actinomyces from the *A. meyeri/odontolyticus group*. The amounts of Proteobacteria and strict anaerobes were low. The microbiota of the distal part of the human ileum is oral-like and strikingly different from the colonic microbiota. Although our patient population is elderly and hospitalized with a high prevalence of chronic conditions, our results provide new and valuable insights into a lesser explored part of the human intestinal ecosystem.

The human gut microbiota has been extensively investigated in recent years owing to its impacts on human health and disease[1–3]. Most research on host-microbe interactions are based on studies of the faecal microbiota. The feasibility of this practice is questionable[4–6]. The small bowel is responsible for most of the body's nutritional uptake and for the development of intestinal and systemic tolerance towards microbes. It has protruding villi, making its surface area approximately fifteen times greater than that of the colon[7]. The small intestines also have a thinner, more vulnerable mucin barrier than the colonic epithelium, offering closer contact between the luminal content and mucosal enteroendocrine and immunological cells[8,9]. Peyer's patches are present mainly in the distal jejunum and ileum and increase to a maximum together with the Paneth cells in the distal ileum[10–12].

A recent study on rats have concluded that faecal sampling misrepresents the microbiota at different intestinal locations[13]. Similar studies have been difficult to perform in humans, due to major challenges in the procurement of representative and uncontaminated samples from intestinally healthy individuals. Sampling techniques have included naso-ileal catheters[14], capsules[15,16] and retrograde colonoscopy[16,17]. Investigations have also been performed on ileostomy effluent samples[12,14,18,19], autopsies[20] and samples from patients suffering from inflammatory bowel disease or in need of emergency surgery[21–23].

Patients undergoing radical cystectomy, a treatment where the bladder is removed, have their urinary diversion created using a segment of ileum. Most of these patients have no known intestinal diseases, despite their high median age and bladder condition. In this study, by using uncontaminated samples collected directly from the distal ileal mucosal surface during this procedure, we aimed to characterize the microbiota of the ileum using massive parallel sequencing of the bacterial gene encoding 16S ribosomal RNA (16S rRNA gene). When the 16S rRNA gene provided insufficient species-level discrimination, we supplemented the characterization with targeted sequencing of alternative genes providing higher taxonomic resolution. Species-level identification is necessary to increase our understanding of the microbial ecosystem and host-microbe interactions, but also the microbiota's role in infections through leaking, translocation and haematogenous spread of potentially pathogenic organisms.

[1]Department of Microbiology, Vestfold Hospital Trust, 3103, Tønsberg, Norway. [2]Department of Urology, Vestfold Hospital Trust, 3103, Tønsberg, Norway. [3]Department of Microbiology, Haukeland University Hospital, 5021, Bergen, Norway. [4]Department of Clinical Science, University of Bergen, 5021, Bergen, Norway. [5]Department of Infectious Medicine, Vestfold Hospital Trust, 3103, Tønsberg, Norway. Correspondence and requests for materials should be addressed to H.C.V. (email: heivil@siv.no)

Population characteristics	Years/ kg/m2	(range)	Patient identification, sample number
Age, median years (min-max)	71	(53–85)	
BMI, median kg/m^2 (min-max)	27	(21–40)	
BMI $\geq$ 30	5		4, 5, 10, 21, 29
BMI $\geq$ 40	1		29
Population characteristics	**Number of patients**	**(%)**	**Patient identification, sample number**
Sex, male	21	(78)	
Sex, female	6	(22)	9, 13, 14, 15, 16, 20
Neoadjuvant chemotherapy	8	(29)	
MVAC	5	(19)	3, 9, 14, 20, 28
GC	3	(11)	10,11,16
Antibiotic prophylaxis	27	(100)	
quinolone + metronidazole	25	(93)	
trimethoprim sulfa + metronidazole	1	(4)	13
furadantin + metronidazole	1	(4)	18
Indication, bladder cancer	24	(88)	
Indication, prostate cancer	2	(7)	2, 27
Indication, chronic cystitis	1	(4)	15
Chronic diseases none	5	(19)	8, 11, 14, 18, 30
Chronic diseases yes	22	(81)	
Diabetes mellitus 2	2	(7)	10, 22
Cardiovascular disease*	15	(56)	1, 2, 3, 4, 5, 9, 10, 13, 16, 19, 20, 22, 23, 24, 28
COPD/Asthma	5	(19)	5, 6, 12, 28, 29
Irritable bowel disease	1	(4)	20
Constipation	1	(4)	28
Cancer coli operata	1	(4)	6
Regular medications, none	5	(19)	8, 11, 14, 18, 30
Regular medications, yes	22	(81)	
Statins	15	(56)	1, 2, 4, 5, 10, 13, 16, 19, 21, 22, 24, 26, 27,28, 29
PPI	3	(11)	3, 26, 27
Antidiabetics	2	(7)	10, 22
Antibiotics prior to admission (last week)	5	(19)	11, 13, 15, 16, 19
Antibiotics prior to admission (last six months)	15	(56)	1, 2, 9, 11, 12, 13, 14, 16, 18, 19, 20, 26, 28, 29, 30

Table 1. Demographic and clinical data. BMI: body mass index, MVAC: Methotrexate, vinblastine, neomycin (Adriamycin), cisplatin. GC: Gemcitabine, cisplatin. COPD: Chronic Obstruction Pulmonary Disease, *including hypercholesterolemia and hypertension

Results

The mean and median age of the 27 patients was 71 years (range 54–86). Indications for cystectomy were bladder cancer for 24 patients, complications following prostate cancer for two patients and chronic cystitis for one. Nine patients received neoadjuvant chemotherapy prior to surgery but none had received radiotherapy or immunotherapy. Most suffered from additional chronic diseases and 22 out of 27 used medications on a regular basis. Fifteen out of 27 had at least one antibiotic treatment during the last six months prior to admission, mainly for urinary tract infections. Further demographic and clinical data are displayed in Table 1.

The mean number of reads was 318,742 per sample (Supplementary Table S1). After removal of short reads (<250 base pairs), small clusters (<10 reads) and chimeras, a mean of 100,276 valid reads remained (range 15,079 to 376,370, median 97,047) for each sample. The main reason for this substantial loss was short reads, probably due to premature truncation in the ion semiconductor sequencing technology[24]. About 50% of the reads in all samples had a length below 250 base pairs.

By 16S rRNA sequencing, a total of 280 unique Operational Taxonomic Units (OTU's) were accepted, whereof 191 could be identified to the species level, 38 to a species group level, and 51 to the genus level (Supplementary Table S2). The detailed results from the targeted Sanger-sequencing of gdh and rpoB-genes, providing species level identification for some of the bacteria that could only be assigned to the group level by 16S, are provided in Supplementary Table S3. Whenever species level identifications obtained by these supplementary genes are used in the text, the gene is indicated in parenthesis after the species name. The mean species richness was 51 (range 15 to 124, median 50) with an average Shannon index of 2.84 (range 0.83 to 3.72, median 2.56). Most samples had species richness between 30 and 79 and a Shannon-index between 2.36 and 3.72 (Fig. 1a,b and Supplementary Table S4).

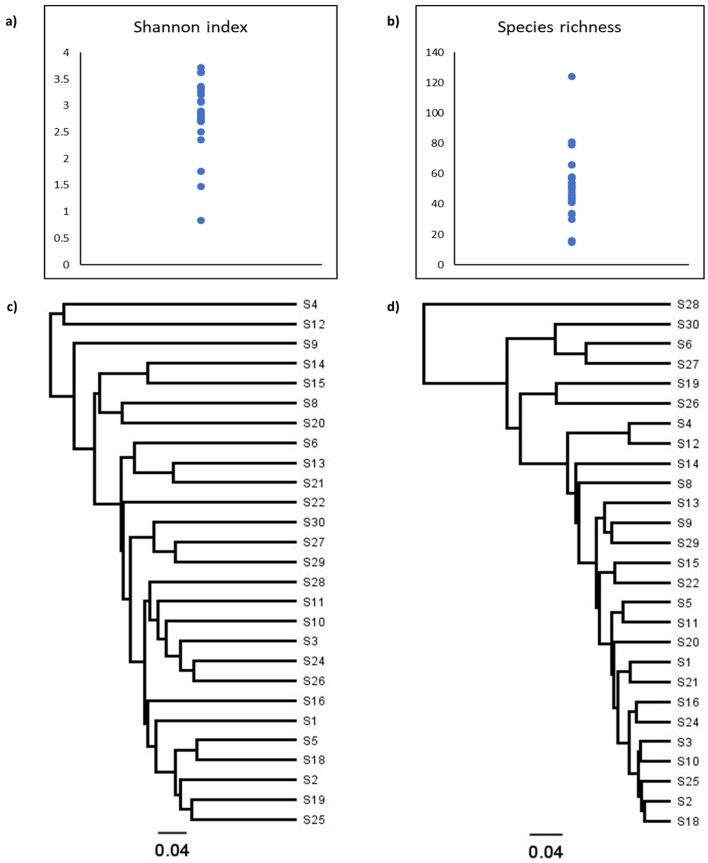

Figure 1. Alpha- and beta diversities. (**a**) Shannon index distribution. (**b**) Species richness distribution. (**c**) Unweighted (qualitative) UniFrac analyses. (**d**) Weighted (quantitative) UniFrac analyses.

Bacterial structure of the ileal samples. The most abundant phylum in the distal ileum was Firmicutes followed by Actinobacteria. Most samples also contained Candidate division TM7 (24/27), Proteobacteria (24/27) and Fusobacteria (23/27) (Fig. 2a). In four samples species from the latter three phyla flourished at the expense of both Firmicutes and Actinobacteria. These were *Fusobacterium periodonticum* in sample 30, *TM7(G-1) sp. oral taxon 352* in samples 6 and 27 and *Escherichia coli* in sample 28. Although outliers in the weighted UniFrac analysis, they remained within the main cluster in the unweighted UniFrac analysis (Fig. 1c,d), reflecting that the qualitative species compositions were not atypical. The most extreme outlier, sample 28 with 77% reads from *E. coli*, was from a patient with long term constipation that had been using a combination of high-osmotic and contact-laxative medications on a regular basis. We observed no congruence between clusters in the UniFrac analyses and BMI category, gender, use of statins, antibiotic treatment or neoadjuvant chemotherapy. Bacteroidetes were found in 11 out of 27 samples whereas species within the phyla Syngergistetes, Tenericutes and Spirochetes were detected at low levels in only one sample each.

The distal ileum core microbiota at genus and species level, defined as genera and species that occurred in more than 50% of the individuals, are displayed in Fig. 3. The typical microbiota is dominated by the facultative anaerobic genera *Streptococcus*, *Actinomyces*, *Gemella*, *Granulicatella* and *Rothia* and in most cases also contain the genera *Atopobium*, *Lachnoanaerobaculum*, *Oribacterium*, *Solobacterium* and *TM7(G-1)* (Fig. 2b). On average, strict anaerobic bacteria constituted only 10% of the reads (range 0.5% to 29%, median 7%), whereof the most significant were *Atopobium parvulum*, *Oribacterium asaccharolyticum*, *Oribacterium sinus*, *Solobacterium moorei*, *Fusobacterium nucleatum*, *Fusobacterium periodonticum*, *Parvimonas micra* and *Bifidobacterium longum*. *Clostridium* species were detected only sporadically and at low levels, except from *Clostridium celatum*, which was found in seven samples. *Clostridium celatum* was also the dominant anaerobe in one individual. The newly described species *Romboutsia timonensis* (Clostridiales order), was recovered from six samples and among the dominant anaerobes in three. *Prevotella* and *Bacteroides* species were found in only six and four samples

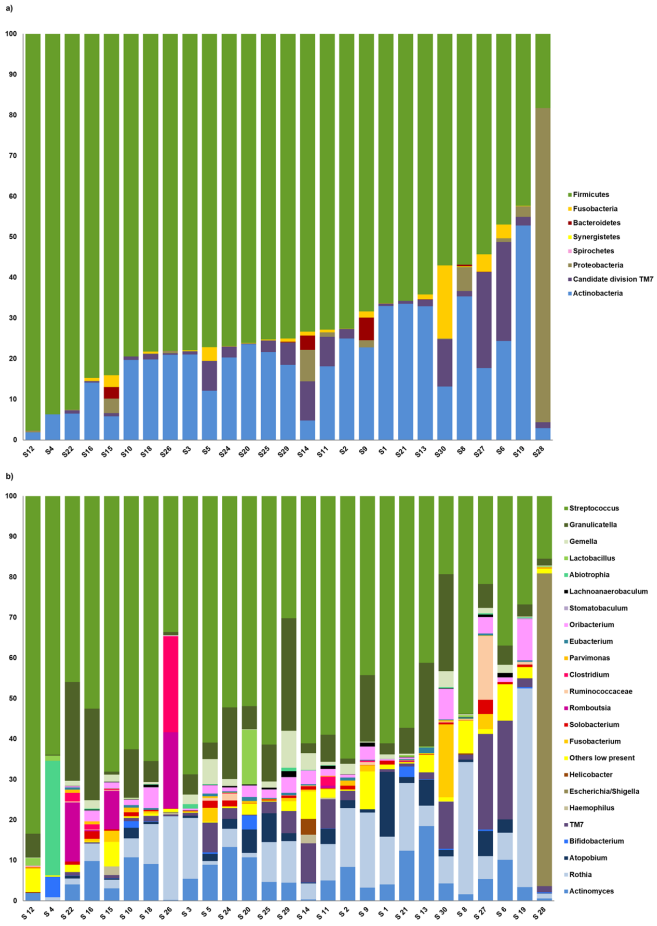

Figure 2. Phylum and genus distribution. (**a**) Phylum distribution. Relative abundances based on number of reads (%). (**b**) Distribution of most abundant genera. Relative abundances based on number of reads (%).

respectively. *Bacteroides fragilis* was only detected in one patient with 0.01% of the total reads. *Faecalibacterium prausnitzii*, considered a dominant and ubiquitous member of the human intestinal flora[25], was detected in only two patients with <0.1% of the total reads. Microaerophilic bacteria were represented by *Helicobacter pylori* and *Campylobacter* species, mainly *Campylobacter concisus*. Strict aerobic bacteria were present in small fractions in seven samples except for sample 19 with 2.0% of reads representing *Stenotrophomonas maltophila*.

Discussion

In this study we have identified a group of patients that provides surgical access to the ileal lumen and that potentially represents an attractive population for future research on host-microbiota interactions. To obtain species level identification we chose a bioinformatic approach specifically developed for use in diagnostic microbiology.

The study reveals fundamental differences between the microbiota of the distal ileum and the colon. This contradicts previous studies based on samples collected by retrograde colonoscopy which indicate that the small bowel microbiota at the level of the distal ileum is similar to the colonic microbiota[16,17]. Our findings also diverge significantly from the other small and inconsistent studies on samples from ileostomy patients or collected from healthy individuals using naso-ileal catheters[12,14,15,18,19]. The colonic content is dominated by the Firmicutes and Bacteroidetes phyla[4,26–28]. Although Firmicutes is the major phylum also in ileum, it is here represented mainly by facultative anaerobic species within the Bacilli class (Streptococcaceae, Lactobacillacae, Aerococcaceae, Carnobacteriaceae) and not the strict anaerobic Firmicutes from the Clostridia class (Peptostreptococcaceae, Eubacteriaceae, Clostridiaceae, Lachnospiraceae, Ruminococcacea) found in colon (Fig. 2b). In our samples, Clostridia account for less than 5% of the sequences whenever present. The second most dominant phylum in

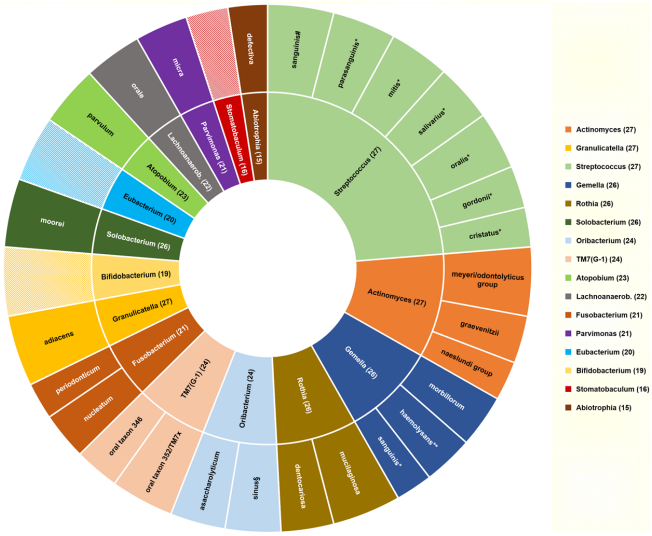

Figure 3. Core microbiota of the ileum. The inner circle represents the genus-level core microbiome defined as genera present in >50% of samples. The outer circle represents the species-level core microbiome defined as species present in >50% of samples. For the outer circle, the width of a segment is proportional to the observed incidence for that species. *Species level identification obtained with targeted gdh or rpoB Sanger sequencing. **Species level identification obtained with rpoB sequencing. Includes *Gemella haemolysans* sensu strictu (n = 7) and the newly proposed species *Gemella para-haemolysans* (n = 9) and Gemella taiwanensis (n = 6). #Only 0.7% distance to *Streptococcus sinensis*. Formally *S. sanguinis (S. sinensis)*. §Only 0.7% distance to *Oribacterium parvum*. Formally *O. sinus (O. parvum)*.

ileum was Actinobacteria whereas species from the Bacteroidetes phylum were detected only sporadically and at low levels. The microbiota of the distal ileum has a higher resemblance to the oral microbiota with dominance of viridans streptococci and high contributions of *Rothia*, *Gemella*, *Granulicatel metagenomic sequencing was performed at the la* and *Actinomyces* species. The major differences are higher abundances of *Neisseria*, *Haemophilus*, *Prevotella* and *Veillonella* in the oral cavity and higher levels of the TM7 phylum in the ileum[28–32]. A study using 16S metagenomics to analyse capsule biopsies from jejunum[15] also found similarity with the oral microbiota. In jejunum even the contributions of *Haemophilus*, *Prevotella* and *Veillonella* were preserved. The same study demonstrated an inverse relationship between *Streptococcus* and *Prevotella* abundances, supporting the almost complete absence of *Prevotella* in our *Streptococcus*-dominated ileal specimens.

Twenty-four individuals harboured TM7 genera (3–11 species each), representing almost a quarter of the reads in some samples. Although previously detected in the oral cavity and colon, the TM7-phylum has not previously been reported from the small intestine. The most likely explanation for this discrepancy is methodological. Microarrays designed for the gut-microbiome like the HITChip[33] or HuGChip[34] do not target the TM7 phylum. In sequence based studies, the lack of TM7-references in databases might have rendered TM7-derived reads unassigned or even erroneously assigned to other phyla. Only two reports exist on the successful cultivation of TM7-species from human samples. In the first study[35], several TM7-species were identified in mixed bacterial cultures together with *Actinomyces naeslundi* group, *Fusobacterium nucleatum*, *Parvimonas micra*, *Shuttleworthia satelles*, *Streptococcus gordonii* and *Veillonella parvula*, all frequently detected in this study as well. Dual-species biofilm experiments demonstrated synergistic biofilm formation with *F. nucleatum*, *P. micra* and *S. gordonii*. (gdh) Interestingly, the cellular shape of the TM7-species shifted between coccoid and filamentous depending on which bacterium it was co-cultivated with. In the second study[36], a TM7-species was recovered as an epibiont of a specific clone of *Actinomyces odontolyticus*. Together, this creates the impression of a phylum with potential for close interactions with its neighbours. Establishing the human ileum as a major TM7-reservoir represents an intriguing novel discovery that should stimulate research on this enigmatic phylum.

An interesting finding was the presence of *Helicobacter pylori* in three ileal samples, representing as much as 3.9% of the 16S sequences in sample 14. It has until now been thought to colonize only the gastric mucosa. Additional studies, including fluorescence *in situ* hybridization of biopsies, need to be undertaken to further illuminate *H. pylori* colonization of the ileum.

Colonic and ileal microbiota are reported to change with diet[14,37,38]. A strength of our study is therefore that the collection of mucosa-near samples under standardized preoperative diet restrictions assured that microbiotas were compared under similar nutritional conditions. However, we only obtain a snapshot of the ileal microbiota under these specific conditions. Theoretically, other species could dominate after the intake of more protein-rich or fat-rich food.

Despite the almost complete absence of known intestinal illnesses in our patient population, the samples obtained are not necessarily representative of a normal ileal flora. The median age was high and most patients suffered from chronic conditions including COPD and cardiovascular diseases. These conditions themselves as well as some of the medications prescribed to treat them could affect the intestinal microbiota. Another concern is the impact of the preoperative antibiotic prophylaxis. Antibiotics are documented to impact gut microbiota[39,40]. It can be argued that the luminal surface of the ileum is covered with a mucus layer[21] likely to protect bacteria against both direct and systemic effects of antibiotics at least in the short-term. It can also be argued that results from DNA-based analyses are less vulnerable to short-term effects of antibiotics due to detection of DNA from non-viable bacteria and even persisting free DNA from lysed bacterial cells. However, the exact impact of the antimicrobial exposure in our population remains unknown. In the UniFrac analysis, patients that had received antibiotics during the last six months or during the last week prior to surgery did not cluster separately from the remaining population.

Patients undergoing cystectomy are currently the closest we might get to a "normal" population. Obtaining clean surgical samples for unbiased metagenomic characterization from this group, presents no major ethical dilemmas. However, high median age, underlying cancer, chronic illnesses and antibiotic prophylaxis could all impact microbiota composition. Some of the differences observed between this and previous studies could arise from these factors.

In conclusion, the distal part of ileum harbours a distinctive niche of the human gut ecosystem that differs more from the colonic than the salivary flora. These findings oppose the relevance of the bacterial flora in colon as proxy for the overall intestinal microbiota. In future studies of host-microbe interactions, it will be necessary to pay greater attention to the ileal microbiota.

Materials and Methods

Population and sample collection. Patients undergoing cystectomy with urinary diversion in Vestfold Hospital Trust (Tønsberg, Norway) are enrolled in a local quality registry based on broad informed consent. Informed consent was obtained from all subjects. Thirty patients were consecutively included in the current survey that was approved by the Regional Ethical Committee of Southern and Eastern Norway Regional Health Authority (2016/926 REK Sør-Øst D) and performed in accordance with European Association of Urology (EAU) and local guidelines and regulations.

All included patients were Caucasian. None of the patients had current gastrointestinal diseases except from patient number 20 with irritable bowel syndrome and patient number 28 with chronic obstipation (Table 1). Due to low output of reads after sequencing, three of the patients (number 7, 17 and 23) were later excluded (Supplementary Table S1). Patients were routinely fasting for solid food for 20 hours prior to surgery. They were given standardized carbohydrate drinks (PreOp Nutricia, Danone, The Netherlands) the evening before and the morning prior to surgery, and otherwise followed the Enhanced Recovery After Surgery (ERAS)-regimen[41]. Two patients with diabetes (patient 10 and 22) did not receive PreOp drinks. All patients received peroral ofloxacin 400 mg (25 patients) prophylaxis two hours before surgery or according to findings in preoperative urine-culture (Table 1). Metronidazole 1000 mg along with tranexamic acid 1000 mg was administered intravenously from the start of surgery as standard.

The surgeon collected the sample about 25 cm proximal to the ileocecal valve by rubbing the swab against the luminal wall, and then inserted the swab in a standardized Transwab medium (MWE, Medical Wire, England). Samples were frozen immediately at minus 70 °C for later DNA extraction.

Pre-PCR treatment of samples. The collected sample-suspension was diluted 1:2 with Qiagen ATL buffer (Qiagen, Hilden, Germany) and extracted using the "Pathogen complex kit" (Qiagen) according to the manufacturer's instruction on a QIAsymphony platform (Qiagen). Negative controls from the relevant batches of Transwab media were extracted in the same manner.

16S metagenomic analysis. 16S metagenomic sequencing was performed at the Public Health Agency of Sweden that offers this as a commercial service for the Nordic countries. Briefly, a fragment containing the variable areas V3 and V4 was amplified using forward-primer 5′-CGG-CCC-AGA-CTC-CTA-CGG-GAG-GCA-GCA -3′ and reverse-primer 5′-GCG-TGG-ACT-ACC-AGG-GTA-TCT-AAT-CC-3′[42]. An Ion Chef instrument (Thermofisher, Foster City, California) was used for automated library preparation and bidirectional sequencing was done using the IonS5 sequencer (Thermofisher). Barcode separated FASTQ-files were processed individually using the RipSeq NGS software[43] (Pathogenomix, Santa Cruz, California). Reads shorter than 250 base pairs were removed before de-novo clustering into OTU's using a similarity threshold of 99%. OTUs containing less than 10 sequences were rejected. For each of the remaining OTUs the most representative sequence variant was used for a BLAST-search against the curated "RipSeq. 16S human pathogen"-database (Pathogenomix) that currently contains more than 2500 non-redundant references for clinically relevant bacteria and commensals. OTU's that did not obtain a species-level match were re-analysed against GenBank (nr/nt-database) and the "Human oral microbiome" database (www.homd.org) assuring the highest possible level of identification for all clusters. RipSeq NGS flags the quality of a BLAST-result based on adjustable interpretation criteria. For unambiguous species-level identification, the Clinical and Laboratory Standard Institute (CLSI) guidelines for 16S sequence interpretation recommends $\geq$99% homology with a high-quality reference combined with a minimum distance of $>$0.8% to the next alternative species[44]. These recommendations are based on the V1-V3 segment. Due to the lower overall variability in the V3-V4 segment we adopted a more stringent cut off of $\geq$99.3% while retaining the minimum distance to the next species at $>$0.8%. OTU's with a similarity $>$99,3% but with a distance to next species of $\leq$0,8% was assigned to a group of species (group-level identification) whereas OTU's with a similarity of 97–99.2% was assigned to the genus level. When appropriate, the provisional taxonomy developed by the

Human oral microbiome project (www.homd.org) was used for hitherto unnamed species[45]. Chimeras and any species/OTU found in the negative controls were rejected. A comparison between results obtained by QIIME, the software package typically used in 16S rRNA metagenomic studies[46] and RipSeq NGS on a commercial Mock-up community is provided in supplementary table S5. This comparison demonstrates the better performance of the RipSeq NGS software in obtaining species level identification.

Comparative analyses were performed using the QIIME. Alfa-diversities were measured using the Shannon-index and weighted and unweighted UniFrac analyses were used to calculate beta-diversities[47,48].

Sanger sequencing of alternative genes. For discrimination among bacteria with too similar 16S-amplicons, we designed species-specific PCRs for genes with higher mutations rates (Supplementary Table S6). Due to limited volumes of DNA-eluate we prioritized species that consistently came out among the top-scoring organisms in frequently encountered ambiguous 16S clusters. For streptococci, the gene for glutamate dehydrogenase (gdh) was selected and we applied the taxonomical modifications proposed by Jensen *et al.*[49]. Within the mitis group, we targeted *Streptococcus oralis* (including ssp. *dentisani*, *oralis* and *tigurinus*) and *Streptococcus mitis*. For *Streptococcus infantis* a single gdh-PCR could not be designed due to varying sequence patterns. Recent whole genome-based comparisons of strains within the *S. infantis* clade expose significant unresolved taxonomical matters[49]. Within the parasanguinis group, PCR's were designed for *Streptococcus cristatus* (synonym: *S. oligofermentans*), *S. parasanguinis* and *S. gordonii*. *Streptococcus salivarius* and *S. vestibularis* were targeted by a shared PCR and discriminated by the nucleotide sequence. *S. sanguinis* was unambiguously identified based on the 16S rRNA-gene.

For discrimination within the *Gemella haemolysans/sanguinis* cluster the gene for RNA-dependent polymerase beta (rpoB) was used. In addition to specific PCRs for *G. haemolysans* and *G. sanguinis* we designed a shared PCR for *G. haemolysans* and the newly proposed species *Gemella parahaemolysans* and *Gemella taiwanensis*[50].

All primers were aligned against available references in GenBank (nr/nt and wgs databases) to assure coverage of all known sequence variants. The PCR-products from positive reactions were sequenced using Sanger-sequencing and aligned against the GenBank nr/nt and wgs databases for confirmation. Due to lack of established cut-offs for a valid species level identification all species level assignments were supported by a pairwise comparison of the alignment table in GenBank using the "distance tree of results" function.

Primer sequences, primer concentrations and PCR conditions are listed in Supplementary Table S6. SYBR-green real-time PCRs were performed in 25 µl reaction tubes on a SmartCycler real-time apparatus (Cepheid, Sunnyvale, California). PCR mixtures consisted of 12.5 µl ExTaq SYBR master mix (TaKaRa, Otsu, Japan), 0.4 or 0.6 µM of each primer (corresponding to 1.0 or 1.5 µl from a 10 µl stock solution), 8.0 or 8.5 µl PCR-grade water (depending on primer-volume) and 2 µl extracted DNA. Thermal profiles included an initial polymerase activation step at 95 °C for 30 seconds, followed by 45 cycles of 95 °C for 10 seconds, PCR-specific annealing temperature for 10 seconds and 72 °C for 20 seconds.

The products from positive PCR reactions were spun out of the SmartCycler reaction tubes into a 1.5-ml Eppendorf tube and cleaned up using ExoSAP-IT enzymatic degradation (Affymetrix, Santa Clara, California). Cycle sequencing was run for 28 cycles with annealing temperature 50 °C for all amplicons. Sanger sequencing was performed using an ABI prism 1.1 Big Dye sequencing kit and an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California 16S).

Data availability. Source data of this study are available from the corresponding author upon reasonable request. Not all patient data are publicly available due to restrictions from the Regional Ethical Committee (REK Sør-Øst D).

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Author Contributions

H.C.V., E.S.H., T.S. and N.G. initiated the study. H.C.V. and Ø.K. were responsible for the study design and E.S.H. and A.H. enrolled patients and collected samples. H.C.V. and Ø.K. performed the 16S metagenomic data analysis. Ø.K. designed and performed the Sanger-sequencing experiments. H.C.V., Ø.K. and E.U. wrote the manuscript with inputs from all the authors.

Additional Information

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Original article

The cultivable microbiota of the human distal ileum

Heidi Cecilie Villmones [1, *], Adrian Halland [2], Tore Stenstad [3], Elling Ulvestad [4, 5], Harald Weedon-Fekjær [6], Øyvind Kommedal [4]

[1] Department of Microbiology, Vestfold Hospital Trust, Tønsberg, Norway
[2] Department of Urology, Vestfold Hospital Trust, 3103, Tønsberg, Norway
[3] Department of Infectious Medicine, Vestfold Hospital Trust, 3103, Tønsberg, Norway
[4] Department of Microbiology, Haukeland University Hospital, 5021, Bergen, Norway
[5] Department of Clinical Science, University of Bergen, 5021, Bergen, Norway
[6] Oslo Center for Biostatistics and Epidemiology, Research Support Services, Oslo University Hospital, Oslo, Norway

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ABSTRACT

Objectives: The existing literature on the microbiota of the ileum is inconsistent. To further characterize the microbiota, we analysed samples obtained directly from resected ileums used for urinary diversion after radical cystectomy.

Methods: We included 150 patients with bladder cancer operated on from March 2016 to March 2019. Samples obtained by rubbing a swab against the ileal mucosa 25 cm from the ileocecal valve were cultivated at the local laboratory. Microbial colonies were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF).

Results: The microbial density of the distal ileum was low. Among our samples, 79% (95% confidence interval (CI) 71%, 84%) harboured less than 1.6×10^4 cfu/mL, whereas 36% (95% CI 28%, 44%) harboured less than 1.6×10^3 cfu/mL. The flora was dominated by viridans streptococci, *Candida, Actinomyces*, *Rothia* and *Lactobacillus* species. Colon-related bacteria i.e. strict anaerobic bacteria, *Enterobacteriales* and enterococci, were recovered from 14% of the samples. Constipation was associated with increased recovery of colon-related bacteria. Antibiotic treatment prior to surgical procedures did not affect culture results. Increased age was significantly associated with more substantial fungal growth and use of proton pump inhibitors seemed to increase both bacterial and fungal growth.

Conclusions: The microbiota of the human distal ileum is sparse and differs significantly from the colonic microbiota both quantitatively and by composition. These findings contradict recent metagenomics studies based on samples collected by retrograde colonoscopy and emphasize the crucial importance of adequate sampling techniques. **Heidi Cecilie Villmones, Clin Microbiol Infect 2021;27:912.e7–912.e13**

Introduction

The microbiota of the distal part of the healthy human ileum has been variously described. While most contemporary authors conclude that the microbiota is colon-like with a dominance of anaerobic bacteria and with an estimated cultivable bacterial load of approximately 10^6–10^8 cfu/mL [1–10], some older studies [11–13] postulate that the microbiota is more scanty and oral-like. These discrepancies may be owing to sample collection procedures or methods of detection. The older investigations referred to above used cultivation techniques on samples collected directly from the ileal lumen during surgery whereas more recent studies applied molecular techniques to analyse samples collected by retrograde transcolonic procedures [14–25], but also from ileostomies [26–29] or from autopsies [30]. These later sampling approaches are all afflicted with uncertainty considering representativeness. Contemporary studies on more proximal parts of the small intestine, conducted using samples collected by antegrade enteroscopy [23,24,31], capsules [32] or indwelling luminal catheters [33], find the jejunal microbiota to have a composition more similar to the oral microbiota.

* Corresponding author: H.C. Villmones, Department of Microbiology, Vestfold Hospital Trust, Postbox 2168, 3103, Tønsberg, Norway. Tel.: +47 3334568.
E-mail address: heivil@siv.no (H.C. Villmones).

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In a recent investigation [34], we utilized 16S deep sequencing to characterize 27 samples collected directly from the ileal mucosa of patients undergoing radical cystectomy, and our results strongly support that also the distal ileal microbiota are more similar to the oral microbiota. Although the general validity of our findings has been questioned as most patients were of high age, exhibited comorbidities and had received antibiotics [35], we believe that representativeness is maintained because the patients mainly had no acknowledged intestinal diseases. We therefore suggest that the substantial discrepancy in the literature is more likely a classic example of the importance of adequate sample collection.

In the present article we aimed to extend our previous study, by describing the cultivable ileal micro-organisms of an expanded cohort of 150 patients. The cohort includes the 27 patients from our recent deep-sequencing study for whom we could directly compare culture and sequencing results.

Methods

Patient and public involvement

Patients undergoing cystectomy at Vestfold Hospital Trust (SiV HF) are enrolled in a local quality registry. All 150 patients operated for bladder cancer from March 2016 to March 2019 were consecutively included in this study. Informed consent was obtained according to concession of the local registry. The study was approved by the Regional ethical committee (2016/926 REK sør-øst D).

No exclusion criteria were set. All patients were fasting for solid food during the last 20 h prior to surgery. They were given standardized carbohydrate drinks (PreOpR Nutricia, Danone, The Netherlands) the evening before and the morning prior to surgery, and otherwise followed the Enhanced Recovery After Surgery (ERAS) regimen [36]. All patients received peroral antibiotic prophylaxis, either ofloxacin as standard or adjusted according to findings in patients with a positive preoperative urine culture. Antibiotic tablets were given together with 1 g paracetamol 2 h before surgery and 1000 mg of metronidazole together with 1000 mg tranexamic acid was administered intravenously from the start of surgery.

Surgical sample collection

The surgeon collected microbiological samples about 25 cm from the ileocecal valve by rubbing a swab against the luminal wall to absorb ileum mucosal secrete. The swab was transported to the laboratory in a standardized Transwab medium (MWE, Medical Wire, UK).

Cultivation and identification

For cultivation, 50 μL of vortexed content from the Transwab medium was spread on blood, chocolate, MacConckey and Sabouraud Dextrose agars. Another 50 μL were inoculated in a Thio broth and together with the agar-plates incubated in 5% CO_2 enriched air at 37°C for 5 days. An additional blood agar and a Menadione agar plate were incubated anaerobically for 5 days. Bacterial growth was carefully inspected by experienced lab technicians, and all colony variants were submitted for matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany) identification according to the manufacturer's instructions. Spectra was performed using FlexControl microflex, analysis was performed using the Biotyper version 4.1.70.0–4.1.90.0 software available between 2016 and 2019. Scores between 2000 and 3000 with consistent naming (category A) were accepted for identification at the species level and scores between 1700 and 1999 at the genus level. Scores above 2000 and consistent category B were accepted for viridans streptococci at the group level. Due to well-known difficulties of categorization within the viridans streptococci by both MALDI-TOF and 16S sequencing [37,38], we used slash-names (*S. mitis/oralis/peroris/pseudopneumoniae* and *S. vestibularis/salivarius)* for these species. Each bacterial species, as determined by colony appearance, was quantified on the plate where it had the most abundant growth.

Quantification of cultivated colonies

The swab, which absorbs 150 μL of fluid, was inserted into the Transwab tube containing 1050 μL of solution, giving a total volume of 1200 μL and a 1:8 dilution of the sample. Fifty microlitres of the suspension were distributed on to each of the agar plates. A single colony on a plate therefore corresponded to $1 \times 8 \times 20 = 160$ cfu/mL in the original sample, which consequently was our lower limit of detection. It was possible to reliably count 100 colonies per relevant plate, giving us a quantitative range from 160 to 1.6×10^4 cfu/mL. More than 100 colonies was reported as $>1.6 \times 10^4$ cfu/mL.

Statistics

For the statistical analysis, growth was categorized as substantial or non-substantial using $>1.6 \times 10^3$ cfu/mL as a cut-off (Table 1). Univariate statistical analyses of group data was analysed giving 95% confidence intervals (CIs) based on Wilcox confidence intervals for proportions, and score test for comparison of proportions. p-Values were calculated using Pearson's Chi-squared test or Fisher's exact tests for tables with few cases (as indicated in the text). Binary logistic regression was used for analysing potential trends in fungal growth by age and use of proton pump inhibitors (PPIs) and analysis adjusted for age and sex was performed using multivariate logistic regression. Data was analysed using the SPSS Statistics 25 package, Stata 16 and R 3.5 package with the 'binom', 'PropCIs' and 'epitools' add-on libraries.

Results

Description of the population

A total of 150 patients were recruited. All had urothelial cancer, predominantly staged T1–T4. Twenty percent had regional lymph node involvement and only 1% had known metastasis. Almost all patients (98%) had American Society of Anesthesiologists (ASA) score 2 or 3 (57% and 41%, respectively). Further clinical and demographic details are provided in Table 2.

Microbiological findings in ileum

Eleven of 150 samples (7%) were culture negative. For 43 samples (29%) growth was only obtained in broth culture or as single colonies on plates. Thus 36% (95% CI 29%, 44%) of samples were categorized as having non-substantial growth. Substantial growth was observed in 96 samples (64%) and only 32 (21%) exceeded the upper limit of quantification (Table 3).

All bacterial and fungal species cultured in this material are listed in Supplementary Table S1. *Candida albicans* was the most frequently detected microbial species (48% of samples) followed by *S. mitis/oralis/peroris/pseudopneumoniae* (40%), *Streptococcus parasanguinis* (41%), *S. salivarius/vestibularis* (38%), *Actinomyces odontolyticus* (31%), *Rothia mucilaginosa* (22%), *Rothia dentocariosa* (15%), *Actinomyces oris* (11%), *Lactobacillus gasseri* (5%) and *Lactobacillus fermentum* (5%).

Table 1
Quantification of cultivated colonies

Growth	Colonies on plate	cfu/mL	Growth
No growth = 'lower limit of detection'	0	<160	Non-substantial
Single colonies/growth from broth	1–9	160 to <1.600	
Sparse growth	10–49	1.600 to <8.000	Substantial
Moderate growth	50–100	$0.8–1.6 \times 10^4$	
Abundant growth	>100	$>1.6 \times 10^4$	

Viridans streptococci were the most dominant group of microbes in 118 samples (79%), most frequently represented by species within the sanguinis-group (45%), mitis-group (42%) and salivarius-group (41%).

In total, 84 samples (56%) grew *Candida spp*., of which eight were in monoculture. *Candida albicans* ($n = 72$) was the most frequently detected species, but also *C. dubliniensis* ($n = 7$), *C. glabrata* ($n = 5$), *C. tropicalis* ($n = 4$), *C. parapsilosis* ($n = 4$), *C. inconspicua* ($n = 1$) and *C. kefyr* ($n = 1$) grew in our samples, sometimes in combinations.

Samples growing bacteria typically associated with the colonic microbiota, i.e. Gram-negative enteric rods, strict anaerobic bacteria and enterococci, were categorized as colon-like, whereas samples missing the colon-related bacteria above, were categorized as oral-like (Table 4). Only 21 samples (14%) harboured colon-like bacteria: 10 samples (7%) contained Gram-negative rods, including four with *Klebsiella pneumonia*, three with *Escherichia coli*, one with *Enterobacter cloacae complex*, one with *Serratia marcesens* and one with *Pseudomonas aeruginosa*. Nine samples (6%) had growth of strict anaerobic bacteria. Only four samples contained *Bacteroides spp.* and *Bacteroides fragilis* was not detected. Four samples (3%) contained enterococci; two with *Enterococcus faecalis* and two with *E. faecium*.

For the 27 samples that had previously been investigated using 16S sequencing, a comparison between culturing and sequencing results is provided in Supplementary Table S2. As expected, and as demonstrated by others [24], sequencing identified far more anaerobes and fastidious bacteria than culture. Corresponding to the higher number of detected species, the Ct-values in the universal 16S PCR also indicated somewhat higher levels of bacteria than estimated by culture, correlating to an approximate median microbial density around 10^5 bacteria/mL. Notably, three samples

Table 2
Demographic and clinical data

Population characteristics	Number of patients $N = 150$	Percent
Age, median years (min–max)	73.00 (range 48–89)	
BMI, median kg/m^2 (min–max)	25.4 (range 14.5–45.0)	
Sex, male	113	75%
Sex, female	37	25%
ASA score 1 (American Society of Anesthesiologists)	2	1%
ASA score 2	86	57%
ASA score 3	61	41%
ASA score 4	1	1%
Current smoker	35	23%
Neoadjuvant chemotherapy	25	17%
T a	1	1%
Tis	5	3%
T1	45	30%
T2	38	25%
T3	45	30%
T4	16	11%
N+ stage	30	20%
M+ stage	1	1%
Diabetes any type	14	9%
Per oral antidiabetics	11	7%
Antibiotics ongoing prior to surgery (within 7 days)	21	14%
Antibiotics ongoing and/or recent 6 months prior to surgery	110	73%
Any use of medication	127	85%
Proton pump inhibitor	20	13%
Inflammatory bowel disease	2	1%
Resected ileocaecal valve	4	3%
Constipation	19	13%
Reduced mobility	8	5%

Table 3
Colony quantities in cultivated ileal samples (fungi and bacteria)

Growth	cfu/mL	Number of patients ($n = 150$)	Percent	Cumulative percent with 95% confidence interval
No growth	<160	11	7%	7% (4%, 13%)
1–9 colonies/broth	160 to <1.600	43	29%	36% (29%, 44%)
10–49 colonies	1.600 to <8.000	21	14%	50% (42%, 58%)
49–100 colonies	$0.8–1.6 \times 10^4$	43	29%	79% (71%, 84%)
>100 colonies	$>1.6 \times 10^4$	32	21%	100%

Table 4
Distribution of microbiological findings in cultivated ileal samples.

Growth		*n*	Percent with 95% confidence interval	Percent in subcategory
No growth		11	7% (4%, 13%)	
Only Candida		8	5% (3%, 10%)	
Oral-like		110	73% (66%, 80%)	
	Viridans streptococci	110		100%
	Actinomyces	57		52%
	Rothia	44		49%
	Lactobacillus[*]	23		21%
	Candida[*]	71		65%
Colon-like		21	14% (9%, 20%)	
	Gram negative rods	10		48%
	Strict anaerobic bacteria	9		43%
	Enterococcus	4		19%
	Viridans streptococci[Ω]	14		67%
	Actinomyces[Ω]	5		24%
	Rothia[Ω]	6		29%
	Lactobacillus[*]	3		14%
	Candida[*]	13		62%
Total		150	100%	

[Ω] Classified as oral-related, listed due to their high frequencies. *n* = number of samples. Many samples contained more than one microbe.
[*] Classified as neither colonic nor oral, but are listed due to their high frequencies in both categories.

were negative by bacterial culture, but still contained abundant bacterial DNA as indicted by low 16S PCR Ct-values.

Effect of antibiotic treatment

There was no observed difference in bacterial growth between the group of patients that were antibiotic naïve before submission ($n = 40$) and the patients that had received antibiotics within 6 months prior to submission ($n = 110$) ($p > 0.99$, 95% CI for difference 18% to 17%) (Table 5).

There was also no appreciable difference in fungal growth between the antibiotic naïve group and the patients that had received antibiotics within 6 months prior to submission ($p = 0.99$, 95% CI for difference -19% to 21%) (Table 6). Odds ratios (ORs) were not considerably changed when adjusted for of age and sex (Supplementary Tables S3 and S5).

Associations between ileal microbiota and other clinical parameters

The number of patients in this study is somewhat low for performing multivariate analyses, thus only cross tables were used to look for indications of varying bacterial and fungal growth by the key clinical factors: body mass index, constipation, diabetes mellitus, gender, neoadjuvant chemotherapy and smoking.

High age was found to be associated with increased growth of yeast (Fig. 1 and Supplementary Tables S3 and S4). Of patients in their 80s, 36% had substantial growth of fungi, compared with only 8% of patients aged less than 60 years. When changing cut-off to any fungal growth versus no growth compared with age, the trend was not significant (Supplementary Fig. S1). There was no clear association between age and substantial growth of bacteria (Supplementary Table S5).

PPI-treatment could favour bacterial growth in the ileum. Sixteen patients (80%) in the PPI-treatment group had substantial bacterial growth compared with 74 (67%) in the group without PPIs (Supplementary Table S6). A stronger association was observed for growth of fungi (Supplementary Table S7): 11 (55%) of 20 patients had substantial fungal growth in the PPI treatment group, while 34 (26%) of 130 patients not taking PPIs had substantial growth. The observed correlation between PPI and substantial fungal growth was not significantly reduced when adjusted for age and sex in multivariate analysis (Supplementary Table S3), whereas the possible correlation between PPI-treatment and bacterial growth was weakened (Supplementary Table S5).

Table 5
Preoperative antibiotic treatment vs bacterial growth

Preoperative antibiotic treatment		Non-substantial bacterial growth <1.600 cfu/mL	Substantial bacterial growth >1.600 cfu/mL	Odds ratio (with 95% CI)	p [*]
Ongoing prior to surgery (last 7 days)	No	50 (39%)	79 (61%)		
	Yes	10 (48%)	11 (52%)	0.7 (0.3, 1.7)	0.57
Recent 6 months or/and ongoing	No	16 (40%)	24 (60%)		
	Yes	44 (40%)	66 (60%)	1.0 (0.5, 2.1)	>0.99

[*] Fisher's exact test.

Table 6
Preoperative antibiotic treatment vs fungal growth

Preoperative antibiotic treatment		Non-substantial fungal growth <1.600 cfu/mL	Substantial fungal growth ≥1.600 cfu/mL	Odds ratio (with 95% CI)	p [*]
Ongoing prior to surgery (last 7 days)	No	90 (70%)	39 (30%)		
	Yes	15 (71%)	6 (29%)	0.9 (0.3, 3.3)	>0.99
Recent 6 months or/and ongoing	No	30 (75%)	10 (25%)		
	Yes	75 (68%)	35 (32%)	1.4 (0.6, 3.2)	0.60

[*] Fisher's exact test.

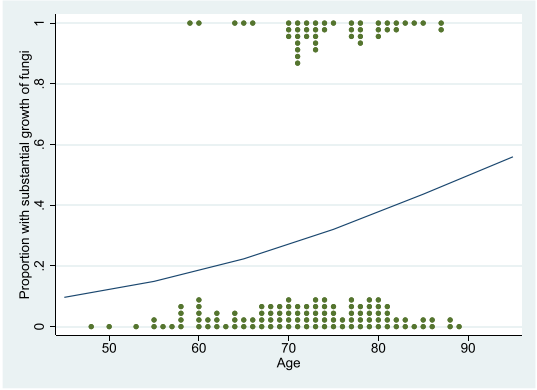

Fig. 1. Estimated probability of substantial (≥1.600 cfu/mL) fungal growth by age. Solide line shows the estimated probability based on a binary logistic regression model, while dots indicate indivdual observations of substantial growth (1) or non-substantial growth (0).

Nineteen patients (13%) reported problems with constipation. For this group we did not find any considerable trends towards increased growth of bacteria or fungi overall (Supplementary Tables S6 and S7). However, samples from patients with constipational complaints yielded colon-related bacteria more often compared with those without (Fisher's exact test; $p = 0.01$, 95% CI for difference from 7% to 49%). A possible association is detected, however numbers are low (Supplementary Table S8).

For the remaining clinical factors listed above, no statistically significant correlations were seen.

Discussion

In the present study, we found that the distal ileal microbiota differs radically from the colonic microbiota and by composition instead appears more related to the microbiota of the proximal parts of the small intestine [23,24,31–33]. Most samples (79%) were estimated to harbour 1.6×10^4 or less cfu/mL, with a dominance of microbes associated with the oral cavity including *Candida spp*. These findings support our recent study based on 16S deep sequencing and oppose contemporary medical textbooks and recent reviews advocating that the ileal microbiota becomes gradually more similar to the colonic microbiota by decreasing distance to the ileocecal valve. The latter hypothesis is built on microbiological studies on ileal biopsies collected mainly through transcolonic procedures [14–22]. Using a low-resolution method (restriction fragment length polymorphism) for microbial profiling, Dave et al. [15] concluded that a sheathed forceps did not significantly alter the microbial results compared with a non-sheathed forceps across the ileocecal valve. This has been interpreted in support of the idea that the colonic and ileal floras are similar and that retrograde transcolonic biopsies collected with or without a sheathed forceps represent adequate sample material for characterizing the ileal microbiota. However, the authors and others fail to address the possibility that the massive bacterial load in the colon might introduce an overriding contamination bias regardless of sheathing. Given a cultivable bacterial density of around 10^4 cfu/mL in the ileum compared with 10^{11}–10^{12} in the colon [2,39], we believe that transcolonic sample collection procedures will unavoidably lead to sample contamination.

Three older publications from Cregan et al. in 1953 [11], Bentley et al. in 1973 [13] and Corrodi et al. in 1978 [12] support the findings of the current study. These investigators collected samples by sterile needle aspiration directly from the distal ileal lumen during elective gynaecological surgery, elective cholecystectomy and elective gastric bypass, respectively. To our knowledge, these are the only previous studies on the ileum microbiota based on direct surgical sampling from intestinally healthy patients apart from our recent 16S metagenomics study [34]. Cregan et al. demonstrated sparse or even no growth. Bacteria were "chiefly those Gram-positive species that are usually found in the mouth and throat". Bentley et al. concluded: "The terminal ileum harboured a relatively sparse flora, contrasting with the large concentration of microorganisms on the other side of the ileocecal valve". Although contemporary investigators have access to advanced molecular techniques, these advancements cannot compensate for inappropriate sample collection.

We cultured one or more *Candida* species from 56% of our samples. Two culture-based studies from 1967 [40] and 1969 [41] support our findings. Additionally, neither they nor we detected any *Saccharomyces* or *Malassezia species*, thought by some to be commensals of the human gastrointestinal microbiota [42]. Others have suggested that their presence in sequencing-based studies might represent non-viable remnant DNA from ingested food or the oral cavity [43,44]. One of these [44] questions whether fungi colonize the gastrointestinal tract of healthy adults at all. The frequent findings of viable *Candida spp.* in the present study contradict the latter hypothesis.

The representativeness of our patient population has been questioned [35]. Antibiotics are known to impact the intestinal ecosystem [45,46] and all patients received ofloxacin and metronidazole prophylaxis or antibiotics as guided by preoperative findings in urine. Ofloxacin is rapidly absorbed in the upper gastrointestinal tract with a bioavailability of 95%, and a direct luminal effect in the ileum at the time of surgery is less likely. Metronidazole is given intravenously at the start of surgery and could possibly impact mucosa-associated anaerobes. Studies have shown high concentrations of unchanged or inactivated metronidazole in the small intestine, but in caecum reduced, activated metronidazole has been detected [47,48]. In this material, we did not find any correlation between growth and antibiotic exposure 6 months before surgery. Importantly, these observations do not exclude a potential effect on the non-cultivated population of anaerobe and fastidious organisms detected by deep sequencing only. However, in our previous 16S deep-sequencing study on the subset of 27 patients, we did not observe any differences between exposed and non-exposed patients either. An eventual effect of the antibiotic prophylaxis given at the start of surgery is difficult to measure regardless of methods, due to the lack of a control group that has not been given prophylaxis. That is a weakness of this study. Both high age and treatment with PPIs were associated with increased microbial growth. A younger and healthier population could have a stronger physiological barrier between the ileal and colonic flora and thereby possibly present even lower microbiological counts than reported in this study.

The predominance of viridans streptococci, *Actinomyces* and *Rothia* correlates well with our previous 16S deep sequencing study [34] where these species were detected in all or almost all patients and constituted a high proportion of the sequencing reads. The present study further confirmed the existence of viable bacteria from the genera *Gemella, Lachnoanaerobaculum* and *Abiotrophia,* which were also defined as members of the ileal core microbiota in the 16S study. Genera defined as part of the core ileum microbiota by 16S deep sequencing that were not recovered by culture in this study include *Oribacterium, TM7, Fusobacterium, Granulicatella,*

Solobacterium, Eubacterium, Atopobium, Parvimonas and *Stomatobaculum*. All these are fastidious, anaerobic or non-cultureable species for which culture has a reduced sensitivity. Thus, our main findings with traditional culturing represent a significant extension of our previous investigation, especially as the findings are unbiased by remnant DNA from by-passing dead bacteria that could interfere with results from sequencing-based methods. In Supplementary Table S2 we show a comparison of the two methods, indicating that most bacterial species except from Staphylococcus and Pseudomonas, were identified by metagenomics.

Conclusion

The cultivable microbiota in the distal ileum is sparse and considerably less diverse than previously reported and distinctly different from the colonic microbiota both quantitatively and by composition. It is dominated by viridans group Streptococci whereas *Enterobacteriales* and strict anaerobes were cultured from only 7% and 6% of samples, respectively. Interestingly, *Candida albicans* was the most prevalent species, found in 48% of the samples. Our results emphasize the importance of adequate sample collection and continue to challenge the use of faecal material as a relevant representative for the overall gut microbiota.

Author contributions

H.C.V. designed the study. E.U. and Ø.K. obtained funding together with H.C.V. H.C.V., Ø.K., E.U., T.S. and H.W.F. wrote the manuscript. A.H. collected samples and obtained clinical characteristics from journals. H.C.V. did the main laboratory work and the description analyses. H.W.F. was responsible for the statistical analyses. All authors contributed to interpretation of the data and read and approved the final version of the manuscript.

Transparency declaration

The authors declare no competing interests. This work was supported and funded by Department of Microbiology and Department of Research and Innovation, Vestfold Hospital Trust, and Department of Microbiology, Haukeland University Hospital, both Norway.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.08.021.

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Investigating the human jejunal microbiota

Heidi Cecilie Villmones[1✉], Marius Svanevik[2,3], Elling Ulvestad[4,6], Tore Stenstad[5], Inger Lill Anthonisen[1], Randi Monsen Nygaard[6], Ruben Dyrhovden[6] & Øyvind Kommedal[4,6]

Descriptions of the small intestinal microbiota are deficient and conflicting. We aimed to get a reliable description of the jejunal bacterial microbiota by investigating samples from two separate jejunal segments collected from the luminal mucosa during surgery. Sixty patients with morbid obesity selected for elective gastric bypass surgery were included in this survey. Samples collected by rubbing a swab against the mucosa of proximal and mid jejunal segments were characterized both quantitatively and qualitatively using a combination of microbial culture, a universal quantitative PCR and 16S deep sequencing. Within the inherent limitations of partial 16S sequencing, bacteria were assigned to the species level. By microbial culture, 53 patients (88.3%) had an estimated bacterial density of < 1600 cfu/ml in both segments whereof 31 (51.7%) were culture negative in both segments corresponding to a bacterial density below 160 cfu/ml. By quantitative PCR, 46 patients (76.7%) had less than 10^4 bacterial genomes/ml in both segments. The most abundant and frequently identified species by 16S deep sequencing were associated with the oral cavity, most often from the *Streptococcus mitis group*, the *Streptococcus sanguinis group*, *Granulicatella adiacens/para-adiacens*, the *Schaalia odontolytica complex* and *Gemella haemolysans/taiwanensis*. In general, few bacterial species were identified per sample and there was a low consistency both between the two investigated segments in each patient and between patients. The jejunal mucosa of fasting obese patients contains relatively few microorganisms and a core microbiota could not be established. The identified microbes are likely representatives of a transient microbiota and there is a high degree of overlap between the most frequently identified species in the jejunum and the recently described ileum core microbiota.

The longstanding debate as to whether antibacterial mechanisms of the intestinal epithelium along with peristalsis prevent the formation of a resident jejunal microbiota, is still not resolved[1–4]. Descriptions of the jejunal microbiota remains vague and there is little consistency both among microbial quantifications and described microbial compositions. Contemporary textbooks[5] and reviews[6–12] report bacterial concentrations of 10^4 to 10^7 cfu/ml, dominated by lactobacilli, streptococci, enterococci and *Veillonella* spp. *Enterobacteriales* are also considered to be prominent participants[8–10,13,14].

Deep sequencing approaches[13–21] have failed to define a consistent core microbiota. *Streptococcus*, *Prevotella, Veillonella* and *Fusobacterium* are frequently detected genera along with a range of Proteobacteria including *Enterobacteriales, Haemophilus spp*. and *Neisseria spp*. These studies are typically based on indirect sample collection procedures like endoscopies[13,14,16,17,19,20,22], nasoileal catheters[18,23], capsules[15] or from autopsies[21]. Despite the use of indirect sampling, the possibility for sample contamination from more abundantly colonized parts of the gastrointestinal tract has rarely been addressed.

We have identified four older studies on samples collected directly from the jejunal lumen during surgery[3,24–26]. These studies, published between 1953 and 1979 were based on culture-dependent techniques. They consistently report a high proportion of jejunal samples to be sterile, 71%, 20%, 63% and 69% respectively. The sporadic species detected, typically gram-positive facultative bacteria like viridans streptococci, were related to the oral cavity and generally considered transient microorganisms. Strict anaerobes, *Enterobacteriales* and enterococci were rarely detected.

In an attempt to provide a comprehensive and methodically sound description of the jejunal microbiota, we collected samples from two separate jejunal segments in a cohort of 60 patients during scheduled gastric bypass

[1]Department of Microbiology, Vestfold Hospital Trust, Postbox 2168, 3103 Tønsberg, Norway. [2]Department of Gastrointestinal Surgery, Vestfold Hospital Trust, 3103 Tønsberg, Norway. [3]Morbid Obesity Center, Vestfold Hospital Trust, 3103 Tønsberg, Norway. [4]Department of Clinical Science, University of Bergen, 5021 Bergen, Norway. [5]Department of Infectious Medicine, Vestfold Hospital Trust, 3103 Tønsberg, Norway. [6]Department of Microbiology, Haukeland University Hospital, 5021 Bergen, Norway. ✉email: heivil@siv.no

Population characteristics	Number of patients (n = 60)	Percent
Age years, median (range)	45 (19–65)	
BMI kg/m^2, median (range)	41 (34–57)	
Gender, male	18	30
Gender, female	42	70
ASA score 1	0	0
ASA score 2	3	5
ASA score 3	56	93
ASA score 4	1	2
Current smoker	0	0
Systolic BP, mean (95%CI)	136 (132–140)	
Diastolic BP, mean (95%CI)	85 (83–87)	
Comorbidities	49	82
Diabetes, any type	10	17
Peroral antidiabetics	7	12
Insulin dependant	1	2
Hypertension	21	35
Dyslipidemia	12	20
Obstructive sleep apnea (OSA)	17	28
OSA with CPAP	15	25
Previous cholecystectomy	10	17
Proton pump inhibitor	14	23
	Median (range)	
Operative time, min	56 (31–101)	
Postoperative stay, days	1 (1–7)	

Table 1. Patient characteristics. *BMI* Body Mass Index, *ASA* American Society of Anesthesiologists, *BP* blood pressure, *CPAP* continuous positive airway pressure.

Growth	cfu/ml	Proximal jejunum (*n*)	Mid jejunum (*n*)	Patient level (*n*) (Both segments combined)*	
No growth	< 160	34	43	51.7% (31)	88.3%
Single colony/broth only	160– < 1600	21	13	36.7% (22)	
Sparse growth	1600– < 8000	3	0	3.3% (2)	11.6%
Moderate growth	$0.8–1.6 \times 10^4$	1	2	3.3% (2)	
Abundant growth	$> 1.6 \times 10^4$	1	2	5% (3)	
In total		60	60	100%	100%

Table 2. Microbiological densities estimated in jejunum by aerobic and anaerobic culture. *Counted by the most bacteria rich segment.

surgery. The samples were characterized qualitatively and quantitatively using a combination of microbial culture, a universal quantitative PCR and 16S deep sequencing. The study population was a selected group of patients with morbid obesity otherwise considered intestinally healthy. Although some components of their microbiota might differ from that of a normal weight population, we believe the overall findings will be representative and can contribute to our understanding of the normal human jejunal microbiota.

Results

Patient characteristics. A total of 60 patients were included with a median age of 45 years and a preponderance of females (70%). All patients were intestinally healthy, but due to morbid obesity and other comorbid conditions most are classified with an ASA risk score 3 (Table 1).

Findings by microbial culture. Bacterial concentrations as estimated by microbial culture are presented in Table 2. No growth in either segment was observed for 31 patients (51.7%) and only three patients (5%) had growth that exceeded the upper limit of quantification ($> 1.6 \times 10^4$). Cultivated bacteria are listed in Supplementary Table S1. When combining results from both jejunal segments, the most frequent bacteria at the patient level were: *Streptococcus salivarius/vestibularis* (25% of patients), *S. parasanguinis* (16%), *S. mitis/oralis* (12%), *Rothia mucilaginosa* (10%), *Actinomyces odontolyticus (8%), Haemophilus parainfluenzae (8%), Neisseria flave-*

scens/subflava (5%) and *Neisseria parahaemolyticus (5%). Enterobacteriales* were only detected in one patient (a *Klebsiella pneumoniae*). Fungi, a *Candida albicans,* grew in only one sample collected from a mid-segment.

Deep sequencing technical results. The median number of reads per sample was 445,263 (range 264,689 to 911,244). After removal of chimera, small OTUs (< 50) and contaminants, the median number of remaining reads was 19,568 (range 2700 to 282,249). The median percentage of retained reads was 9.9% (range 0.5% to 97.4%) with only 16 of 120 samples having more than half of the reads left.

Microbial findings by 16S deep sequencing. In total, after filtering of possible contaminants, we identified 218 different species (Supplementary Table S2). A per sample description at the genus level is provided in Fig. 1 and at the phylum level in Supplementary Fig. S1. Actinobacteria, especially *Corynebacterium spp*., seems to be more abundant in samples with low bacterial loads whereas the presence of Firmicutes, in particular *Streptococcus spp*., *Gemella spp*. and *Granulicatella spp*. increases in samples with higher bacterial loads.

An overall species-level comparison of results from the upper and middle part of jejunum revealed no noticeable differences (Fig. 2). In addition, most species were found at low concentrations close to the limit of detection and consequently prone to random detection. Therefore, for the remaining part of this manuscript, results from the two segments were merged and reported per patient. No species was detected in more than 50% of the population and only six species/groups of species were found in more than 30% of participants: *Enterobacteriales* were only exceptionally detected; *Escherichia coli* in 3 patients (5%), *Serratia grimesii/proteamaculans/liquefaciens* in 3 patients (5%) and *Klebsiella pneumonia complex* in 2 patients (3%).

Quantification by PCR. Bacterial genome concentrations as determined by the quantitative 16S rRNA PCR are presented in Table 3. The median density was found to be below our limit of genomic quantification i.e. $< 2.9 \times 10^3$ bacterial genomes per ml.

Intra-patient concentration differences between proximal and mid-jejunal samples were generally small, and there was no overall tendency towards a higher bacterial load in neither of the segments. In four patients (9, 49, 53 and 55) we observed a more than tenfold concentration difference between the two samples, two of them with the highest load in the proximal sample and two with the highest load in the more distal sample.

Results from microbial culture and 16S deep sequencing compared. A comparison of findings by microbial culture versus by deep sequencing is provided in Supplementary Table S3. As expected, deep sequencing identified far more species than culture. Out of 120 samples, only 43 were culture positive. Still, culture made 22 identifications not reproduced by sequencing. These identifications were most often from the species *Haemophilus parainfluenzae, Actinomyces odontolyticus, Micrococcus luteus, Streptococcus mitis group* and *Streptococcus salivarius/vestibularis.*

Comparison of sequencing results from this study with previously reported results from the ileum. In a recent study on peroperative ileal samples from patients undergoing radical cystectomy[27] we defined an ileum core microbiota consisting of 22 species, each present in more than half of the patients. In Fig. 3a, we show that except from the provisional species *TM7(G-1) oral taxon 346,* all 22 ileum core microbiota species were also detected in the jejunal samples although much more sporadically. In Fig. 3b we compare the most frequent species in jejunum with their corresponding frequencies in ileum. Except from *Corynebacterium kroppenstedtii, C. aurimucosum/minutissimum/singulare* and *Acinetobacter junii*, all these were also part of the ileum core microbiota. Again, most of them were much more frequently detected in ileum. *Granulicatella adiacens, Streptococcus mitis group* and *Streptococcus sanguinis group* were the three most frequent identifications in both ileum and jejunum.

Discussion

Our results indicate that the jejunum is more scarcely populated by bacteria than specified by contemporary reviews and textbooks. Using a combination of cultivation and deep sequencing with a limit of detection in the range 10^2 to 10^3 cfu/genomes per milliliter of sample material, we were unable to recover a resident core microbiota in the proximal and middle parts of the human jejunum.

Keystone species are thought to be important for shaping the organization and diversity of an ecological community, and a common core microbiota has been variably defined as microbial taxa present in between 30 and 95% of a population[28]. In our material, only five identifications were made in more than 30% of the patients: *Streptococcus mitis group* (48%), *Streptococcus sanguinis group* (47%), *Granulicatella adiacens/paraadiacens* (45%), *Schaalia odontolytica complex* (42%) and *Gemella haemolysans/taiwanensis* (40%). All of these are groups of species not possible to differentiate by 16S rRNA sequencing, and therefore most likely, as previously demonstrated in ileum[27], represent more than one species each. Further, microbial resemblance between samples was low both between the upper and middle segments in each patient and between patients.

We detected a wide range of *Corynebacterium* species in this study, and they also dominated in some of the weak positive samples. Among these, *Corynebacterium kroppenstedtii, Corynebacterium aurimucosum* and *Corynebacterium durum* were detected rather frequently (18–25% of patients). *Corynebacterium kroppenstedtii* was first isolated from a sputum sample, *C. durum* from respiratory samples and *C. aurimucosum* from human clinical samples. Also the other Corynebacteria deemed relevant in this study are associated with the human microbiota and/or human infections with the exception of *C. vitaeruminis*, a vitamin-B producing microbe

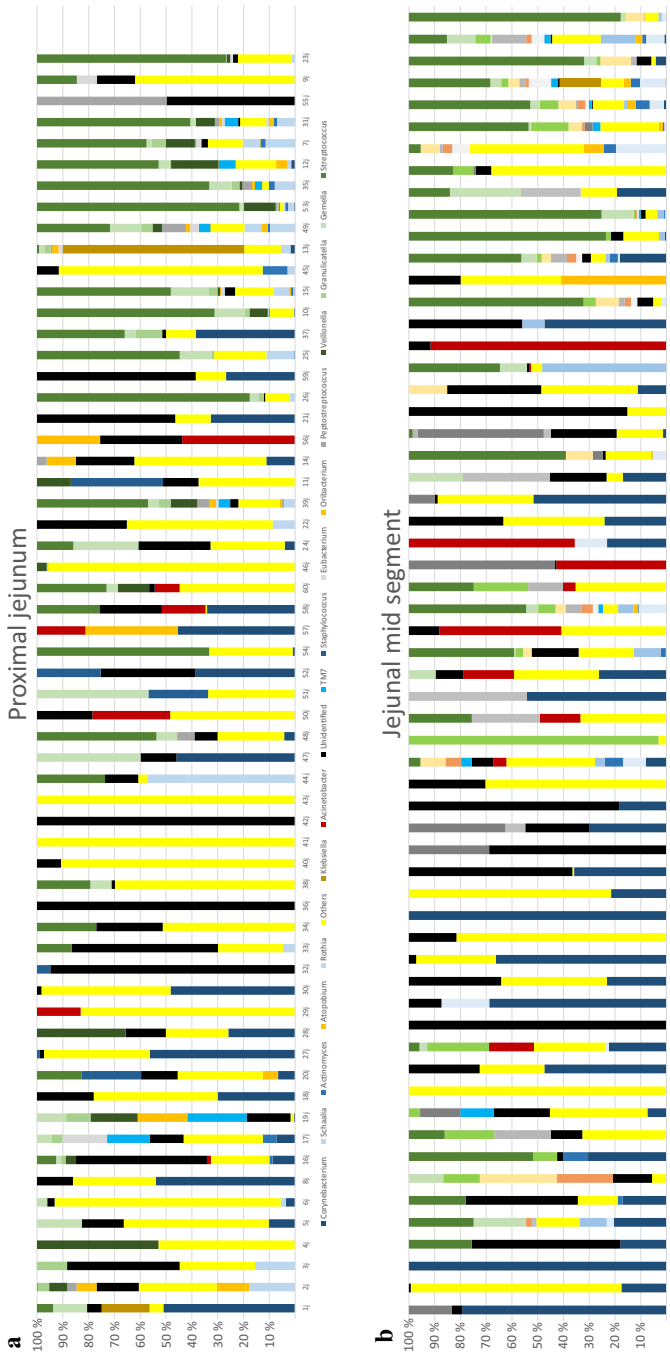

Figure 1. Relative distribution of most abundant genera in (**a**) proximal part of jejunum and (**b**) jejunal mid-segment. All 60 patients included. Samples are sorted by increasing bacterial concentration. Samples with concentration below the level of quantification (Ct-value ≥ 34.17) are sorted by name on the left side (35 proximal samples 1j-60j and 38 mid-segment samples, 1i-60i).

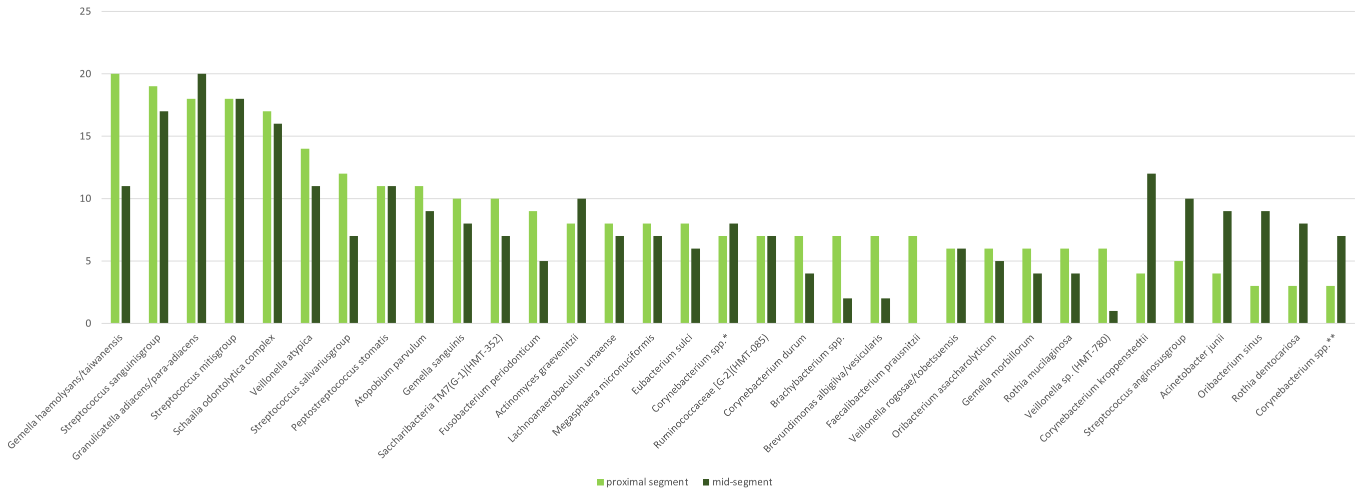

Figure 2. Comparison of most frequent species in upper and middle jejunum. (y axis number of patients). Based on all 60 patients.

Genome copies/ml	Proximal jejunum (*n*)	Mid jejunum (*n*)	Patient level (n) (Both segments combined)*
10^5– < 10^6	5% (3)	5% (3)	5% (3)
10^4– < 10^5	16.7% (10)	6.7% (4)	18.3% (11)
2.9×10^3– < 10^4	20% (12)	25% (15)	36.7% (22)
< 2.9×10^3	58.3% (35)	63.3% (38)	40% (24)

Table 3. Microbial densities estimated by quantitative 16S rRNA PCR. *Counted by the most microbial genome-rich segment.

isolated from the rumen of cows. It could be argued that *C. vitaeruminis* should be removed as biologically unexpected, but is was detected in seven patient samples.

The jejunal samples also generally revealed low bacterial densities, both by cultivation and by 16S rRNA quantification. The median density was < 1600 cfu/ml or < 2.9×10^3 bacterial genomes/ml. Only 23.3% of the samples had an estimated bacterial concentration above 10^4 genome copies per ml. In contrast to cultivation, 16S deep sequencing was positive in all samples, although sometimes only with a single species.

Despite being performed on two different patient populations, the overall spectrum of bacteria identified from the jejunal samples in the present study, bears a striking resemblance to the ileal core microbiota as defined by our recent studies on surgically collected ileal samples (Fig. 3a,b)[27,29]. 16S Ct-values indicate that the bacterial concentration in ileum is at least 100 fold higher than in jejunum. The overall impression is that bacterial cells are capable of multiplying and forming a stable microbiota with a definable core microbiota only more distally in the ileum. Consequently, our results revitalize discussions as to whether the human jejunum harbors only transient microbes and no resident microbiota.

The higher bacterial concentrations in jejunum reported by previous 16S deep sequencing studies might reflect the use of indirect sample collection methods by endoscopies, nasoileal catheters and capsules with an inherent risk of contamination from the more abundantly colonized upper gastrointestinal tract. The human oral cavity has a rich and dense microbiota[30] and saliva is estimated to contain 10^9 cfu/ml[31]. Provided a bacterial density of maximum 10^3–10^4 in jejunum, this represents a gradient of at least 1:10^5 suggesting that both endoscopies and nasoileal tubes introduce a considerable risk for contamination[32]. This can also affect microbial composition. In a study using endoscopy to collect samples[33] they found *Bacteroides, Prevotella* and *Helicobacter* to be among the most frequent and dominant genera. Although we also found multiple species from these genera in our samples they were mainly represented as only minor constituents. To the best of our knowledge, the present study is the first to combine 16S deep sequencing with samples collected directly from the jejunal mucosa during surgery. Our results are more in line with older culture-based studies on samples collected by needle aspiration from the jejunum of intestinally healthy individuals during abdominal surgery.

This investigation has some notable limitations. There might be differences in the microbiota of obese patients versus a normal weight population. Obesity has earlier been associated with increased risk of small intestinal bacterial overgrowth[34] in which case the bacterial load in jejunum from non-obese might be even lower than observed in this study. On the other hand, our patients were subjected to a 3 week low calorie diet with an unknown impact on the jejunal microbiota. Further, the patients were in a fasting state. Earlier publications have found the small intestine to harbor more bacteria after meals[35] and that environmental and food-related bacteria then make up a considerable part of the findings[36]. Therefore, as the goal of this study was to describe a normal physiological core microbiota, data from a fasting state might be preferable.

The antibiotic prophylaxis in this study, trimethoprim/sulfamethoxazole (TMP/SMX), is active against both gram negative and gram positive bacteria, but less effective against anaerobic bacteria. The antibiotic prophylaxis could interfere with results from cultivation. However, in 1978, Corrodi et al.[25] collected jejunal content by sterile needle aspiration in eight obese patients during a gastric bypass procedure without antibiotic prophylaxis. They found 63% of samples to be cultivable sterile, even more than in our material (51.7%). The 16S deep sequencing is less likely to be noticeably affected by the antibiotics given only 2 h before surgery. Finally, although the surgeons were instructed to rub the sample collection swab firmly against the intestinal mucosa it could be that some mucosa-associated bacteria were not effectively sampled.

The strengths of this study are a high number of subjects compared to previous studies, the investigation of two separate jejunal segments from each patient and the use of surgically collected mucosal samples free of contamination from other parts of the gastrointestinal tract. Future studies on the small intestine should attempt to reduce the sampling biases by also using surgically collected samples. Unfortunately, it is a significant challenge to obtain these samples from patients not a priori in need of surgery on the small intestines. Sterile needle sampling during other types of elective abdominal surgery as used in the older culture-based studies, could represent an alternative. Although ethical and patient safety aspects need to be re-evaluated by contemporary experts in both ethics and clinical medicine, this might represent an acceptable approach in order to provide reliable data from the understudied segment of the gastrointestinal tract most essential for nutrient uptake and probably also host-microbe interactions.

Conclusion

Proper sample collection methods is crucial for studies on the small intestine. To the best of our knowledge, this is the largest study of the jejunal bacterial microbiota collected surgically on intestinally healthy patients. Our data fail to demonstrate a jejunal resident core microbiota. Most species identified by both cultivation and deep

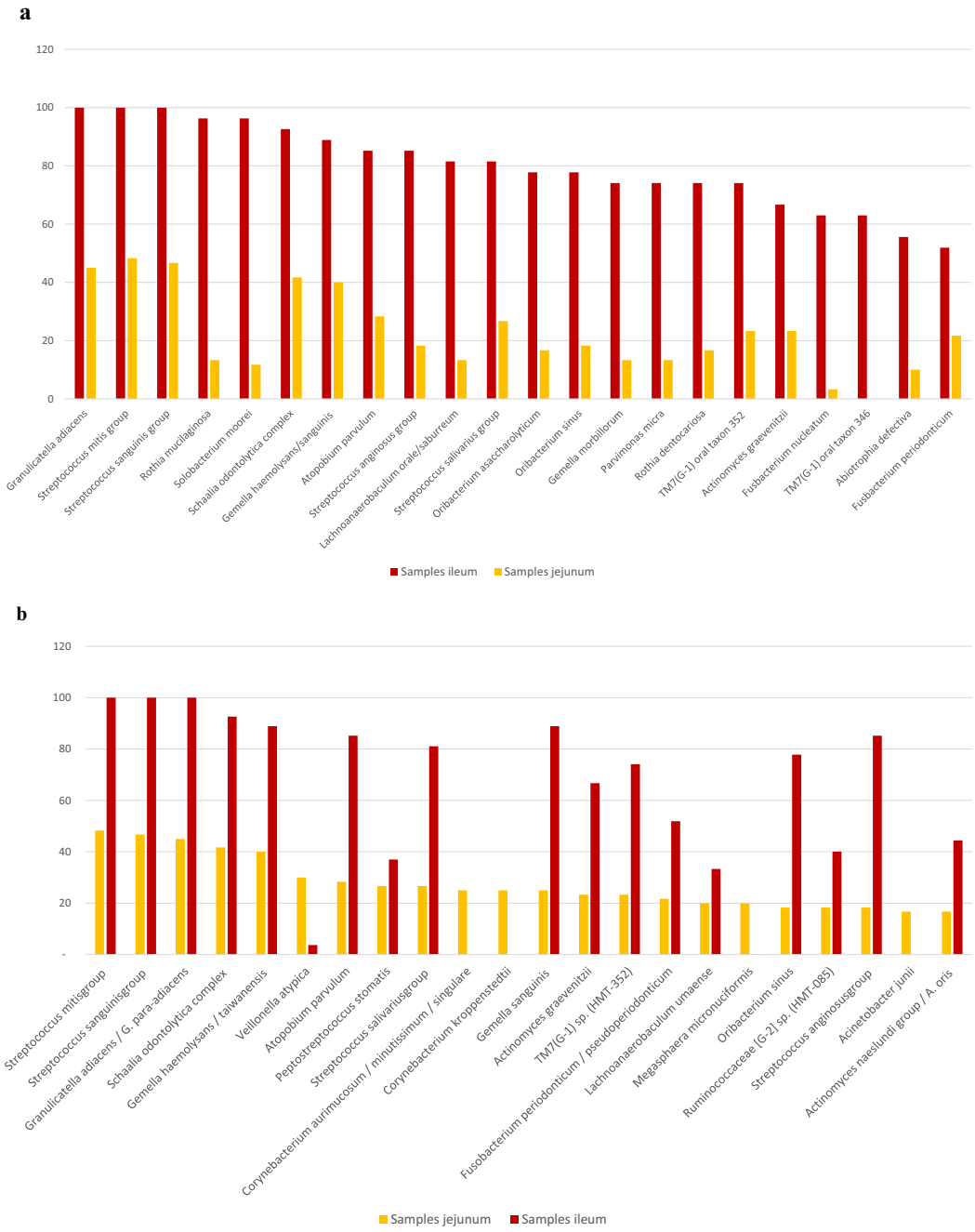

Figure 3. (**a**) Previously reported ileum core microbiota (based on 27 patients) sorted by frequency (%) compared to observed frequency in jejunum (upper and middle segment combined for all 60 patients) and (**b**). Most frequently detected species in jejunum (upper and middle segment combined) sorted by frequency (%) compared to previously reported frequency in ileum. Based on all 60 patients.

Growth	Colonies on plate	Cfu/ ml	Growth
No growth (lower limit of detection)	0	< 160	Non-substantial < 1600 cfu/ml
Single colonies/ broth only	1–9	160– < 1600	
Sparsely growth	10–49	1600– < 8000	Substantial ≥ 1600 cfu/ml
Moderate growth	50–100	$0.8–1.6 \times 10^4$	
Abundant growth (upper limit of detection)	> 100	$> 1.6 \times 10^4$	

Table 4. Quantification by microbial culture.

sequencing appear only sporadically with high intra-individual differences and also considerable differences between the upper and mid segments in each patients.

Patients and methods

Population. Sixty patients scheduled for gastric bypass surgery at Vestfold Hospital Trust (SiV HF) were consecutively enrolled from December 2017 to September 2018. The study was approved by The Regional Committee for Medical and Health Research Ethics in the South-Eastern Norway Regional Health Region (2017/106 REK sør-øst D). All methods were performed in accordance with this approval and in accordance with the relevant guidelines and regulations. Informed consent was obtained from all participants. There were no predefined exclusion criteria.

All patients were prescribed a preoperative low-calorie diet (< 1000 kcal/day) 3 weeks before surgery, and recommended a preoperative weight loss of approximately 5%. Patients underwent fasting for solid foods a minimum of 6 h before surgery, and fluids were withheld 2 h before the procedure. Standard per oral preoperative antibiotic prophylaxis was given in the form of TMP/SMX (160 mg/800 mg) 2 h prior to surgery. All patients were examined preoperatively by a surgeon and there were no evidence of intestinal disease.

Surgical sample collection. A standard laparoscopic gastric bypass was performed in all patients with an antegastric antecolic Roux-en-Y configuration using linear staplers. Four microbiological samples were collected from the openings of the small bowel prior to forming the two intracorporal anastomoses. Two samples were taken 60 cm from the ligament of Treitz prior the gastrojejunostomy, and two 120 cm further along the jejunum (180 cm from the ligament of Treitz) before creating the jejunojejunostomy. The swab from a standard Transwab medium (MWE, Medical Wire, UK), was introduced through a clean laparoscopy trocar and rubbed against the luminal wall to absorb the jejunum mucosal secretion. One sample from each site was cultivated within 2 h. The other pair of samples were frozen at − 70° for later DNA extraction.

Sample cultivation and identification of bacterial colonies. 50 µl of vortexed content from the Transwab medium was distributed on blood, chocolate, MacConckey and Sabouraud Dextrose agars respectively for incubation in 5% CO_2 enriched air at 37 °C for 5 days. The same amount was spread on blood and Menadione agar plates and inoculated to a Thio broth and incubated anaerobically for 5 days. Growth was evaluated by experienced lab technicians and all colony variants were submitted for matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) (Bruker Daltonics, Bremen, Germany) identification using the Biotyper version 4.1.70.0–4.1.90.0 software. Scores above 2000 with consistent naming (category A) were accepted for identification at the species level. Scores between 1700 and 1999 were accepted for a genus level identification. For each species, colonies were quantified on the plate with the most abundant growth.

Quantification by microbial culture. The swab in the Transwab kit absorbs 150 µl of sample material. The Transwab tube contains 1050 µl of solution, giving a 1:8 dilution of the sample. Provided 50 µl are spread on an agar plate, one colony (or growth in broth exclusively) corresponds to $1 \times 8 \times 20 = 160$ cfu/ml in the original sample which was our lower limit of detection. Quantification by bacterial growth was performed according to Table 4.

Quantification by real-time PCR. The quantitative 16S rRNA gene-PCR was based on the dual priming oligonucleotide (DPO) principle to avoid interference from human DNA[37]. The 5-end of the primers were modified according to Dyrhovden et. al.[38] (16S_DPO_Short-F: 5'-AGAGTTTGATCMTGGCTCAIIIIIAACGCT-3' and 16S_DPO_Short-R: 5'-CGGCTGCTGGCAIIIAITTRGC-3'). The universal anti-sense probe was designed for this study and placed in a highly conserved region of the 16S rRNA gene (Escherichia coli 16S rRNA position 360 to 341)[39] (16S-Pb: FAM-CCYACTGCTGCCTCCCGTAG-BBQ). The PCR reaction mixture consisted of 12.5 µL Premix Ex Taq Mastermix (TaKaRa Bio, Kusatsu, Japan), 1.5 µL of each primer (from a 10 µM solution), 0.5 µL probe (from a 10 µM solution), 7 µL PCR grade water and 2 µL of template giving a total reaction volume of 25 µL. The PCR was run on a QuantStudio5 real-time PCR instrument (ThermoFisher) using a two-step thermal profile: (1) Enzyme activation at 95 °C for 30 s (2) melting at 95 °C for 10 s (3) annealing/extension at 60 °C for 20 s. Step (2) and (3) were repeated 40 times.

Streptococcus pneumoniae was selected as a quantitative standard due to its similarity to the other bacteria in the mitis group constituting an important part of the small intestinal microbiota. It also possesses four copies of the 16S rRNA gene which is close the estimated average number of 16S copies in bacterial genomes[40,41]. We

extracted total nucleic acids from a heavy suspension of *Streptococcus pneumoniae* ATCC 49619 in PCR grade water using a MagNaPure Compact automated extractor (Roche, Mannheim, Germany). The DNA concentration in two individual samplings of the eluate was measured on a Qubit Fluorometer (Qiagen). Based on these measurements (37.3 and 39.3 ng/µl; average 38.3 ng/µl) and a genome size of 2,096,423 basepairs (ATCC 49619/GenBank accession GCA_003966485.1) we calculated the concentration of *S. pneumoniae* in our eluate to be 1.69×10^7 genomes/µl. From this eluate we made a ten-fold dilution series from 1.69×10^7 to 1.69×10^{-2}. Each of the nine dilution steps was run in triplet in the quantitative real-time 16S PCR. The PCR was found to be linear down to dilution step 7, i.e. 1.69×10^0 genomes/µl with an average Ct-value of **34.17** (Supplementary Fig. S2). Provided 2 µl of template and 4 copies of the 16S rRNA gene per genome this corresponds to approximately 14 target copies per PCR reaction. Taking into account the 1:833 dilution of our clinical samples during sample collection and DNA extraction, it further corresponds to 2816 bacterial genomes/ml of jejunal content which was therefore our lower limit of molecular quantification.

The *S. pneumoniae* 1.69×10^6 dilution step was included as a standard in the subsequent analysis of the jejunal samples. The standard was run in triplet and the average Ct-value used to adjust the quantitative estimates for the jejunal samples. The observed inter-run variation for the standard was small (average Ct-values 31.25, 31.11 and 30.93 respectively). The estimated genome copy number for the standard was $2 \times 1.69 \times 10^1$ i.e. 33.8 genomes which corresponds to $\sim 2.9 \times 10^3$ bacterial genomes/ml in a jejunal sample.

Sample preparation and DNA extraction for 16S deep sequencing. Two-hundred µl of nuclease-free water (Ambion, Thermo Fisher Scienfic) and 450 µl sample solution from the Transwab media (MWE, Medical Wire, England) were transferred to Matrix E glasses (mpbio, MP Biomedicals, United States) and run on a FastPrep 24 instrument for 2×45 s. After bead-beating, the samples were centrifuged for 2 min at 13,000 rpm. Thereafter 200 µl of supernatant from each sample was used for DNA extraction and purification on a QIAsymphony automated extractor using the "DSP DNA Mini kit" (Qiagen, Hilden, Germany).

Negative controls. Unused Transwab sample collection tubes from the two batches used in this study were included as negative controls. In addition, one Transwab tube from each batch was spiked with 1 µl of a 0.5 McFarland suspension of *Legionella pneumophila* (corresponding to 1.5×10^5 bacterial cells) and included as weak positive extraction controls. Each extraction set-up therefore included two negative and two weak positive controls. Five extraction set-ups were necessary to process all samples, resulting in a total of ten negative and ten weak positive controls. Air-swabs were not included as negative controls in this study.

16S deep sequencing. Deep sequencing of the 16S rRNA gene was based on the Illumina V3-V4 16S metagenomics protocol with some modifications as described previously[38]: PCR amplification of the V3-V4 region was done as a real time PCR reaction on a LightCycler 480 PCR instrument (Roche) using the TBGreen Premix Ex Taq (TaKaRa, Shiga, Japan) mastermix instead of the KAPA HiFi HotStart ReadyMix. The PCR mixture consisted of 12.5 µl mastermix, 8.5 µl PCR-grade water, 1 µl of each primer (from a 10 µM solution, giving a final concentration of 0.4 µM in the PCR) and 2 µl template. After an initial polymerase activation step of 30 s at 95 °C the thermal profile included 45 cycles of 20 s at 95 °C (melting), 30 s at 60 °C (annealing), and 30 s at 72 °C (extension). The PCR products from the real time TBGreen reaction were used directly in downstream steps. The rest of the procedure was performed according to the Illumina protocol without further modifications. Sequencing was done on a MiSeq instrument (Illumina, San Diego, CA) using the Miseq reagent kit V3 (2×300 bp reads).

Sequence data analysis. After sequencing, FASTQ-files were analyzed using the RipSeq NGS software (Pathogenomix, Santa Cruz, CA). After merging of R1 and R2 files, sequences shorter than 300 base pairs were removed before de novo clustering into operational taxonomic units (OTUs) using a 99% similarity threshold. OTUs with fewer than 50 sequences were rejected. Remaining OTUs were annotated using a blast search against the Pathogenomix Prime database. For an unambiguous species-level identification, we required $\geq 99.0\%$ homology with a high-quality reference sequence combined with a minimum distance of $> 0.8\%$ to the next alternative species. For hits above 99% but with less than 0.8% distance to the next alternative species the alternative species is presented in parenthesis. Slashed results were used for OTUs that obtained identical scores against more than one species. Homologies between 97 and 99% qualified for genus-level identification.

Elimination of chimera and contaminant background DNA. Chimeric OTUs were filtered from all samples using the RipSeq NGS chimera check. Sequencing results from all twenty negative/weak positive controls were pooled. The most abundant contaminant species (*Cutibacterium acnes, Ralstonia pickettii* and *Staphylococcus capitis/caprae/epidermidis, Aquabacterium* and *Hydrotalea flava)* were highly consistent across all controls and used to define sample-specific cutoffs for valid identifications[42]. All species/sequence-types detected in any of the negative controls were removed from the sample sequencing results unless when they appeared in higher concentrations than all the most abundant contaminants listed above. All cultured bacteria were accepted as valid findings. Some of the cultured species were also represented in the negative sequencing controls, and therefore could not be included based on the sequencing data. This illustrates the value of combining two independent detection principles. A complete list of bacteria identified in our negative/weak positive sequencing controls is provided in Supplementary Table S4. Finally, species appearing only once among all the 120 samples, and species considered clearly biologically unexpected were removed. A complete list of rejected identifications is presented in Supplementary Table S5.

Data availability

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB46597 (https://www.ebi.ac.uk/ena/browser/view/PRJEB46597).

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Author contributions

H.C.V., T.S. and M.S. designed the study. E.U. and Ø.K. structured the manuscript together with H.V. M.S. collected samples and obtained clinical characteristics from journals. Ø.K., I.L.A. and R.M.N. did the main laboratory work together with H.C.V. H.C.V. did the description analyzes with support from Ø.K. All authors contributed to interpretation of the data and read and approved the final version of the manuscript.

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Competing interests

ØK has contributed to the development of the RipSeq NGS software and is a shareholder in Pathogenomix. The other authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to H.C.V.

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SUPPLEMENTARY INFORMATION

Title:

SPECIES LEVEL DESCRIPTION OF THE HUMAN ILEAL BACTERIAL MICROBIOTA

AUTHOR INFORMATION

Villmones, Heidi Cecilie[1]; Haug, Erik Skaaheim[2]; Ulvestad, Elling[3,4]; Grude, Nils[1]; Stenstad, Tore[5], Halland, Adrian[2] and Kommedal, Øyvind[3]

1. Department of Microbiology, Vestfold Hospital Trust, 3103 Tønsberg, Norway.
2. Department of Urology, Vestfold Hospital Trust, 3103 Tønsberg, Norway.
3. Department of Microbiology, Haukeland University Hospital, 5021 Bergen Norway.
4. Department of Clinical Science, University of Bergen, 5021 Bergen, Norway.
5. Department of Infectious Medicine, Vestfold Hospital Trust, 3103 Tønsberg, Norway.

Corresponding author:

Heidi Cecilie Villmones, Department of Microbiology,

Vestfold Hospital Trust, Postbox 2168, 3103 Tønsberg, Norway

E-mail: heivil@siv.no

Telephone number 0047 92214631 or 0047 33346598

Supplementary Table S1. CT-values from the universal 16S rRNA-PCR and technical data from the metagenomic analysis

Sample/patient number	CT 16S	Cluster 10c1p	Used reads	Total reads
1	24,2	183	157,399	447,090
2	23	159	81,872	242,510
3	21,4	362	376,370	1,060,039
4	25,1	59	45,722	127,220
5	21,7	135	59,851	181,708
6	31	61	15,079	55,545
7	**31**	**22**	**2,233**	**10,692**
8	28,7	131	99,875	279,138
9	26	126	72,423	230,495
10	17,5	196	119,260	380,629
11	25,7	126	97,047	335,058
12	25,7	122	14,938	79,404
13	27,7	118	53,053	194,219
14	25	138	58,427	224,234
15	19,4	284	185,680	705,976
16	24,4	59	59,991	187,406
17	**31,1**	**19**	**2,532**	**10,750**
18	19,8	136	59,929	228,237
19	27,2	65	46,223	149,186
20	24,2	137	99,054	296,087
21	24,6	107	101,140	278,633
22	24,5	221	152,484	498,152
23	**30,4**	**83**	**24,256**	**80,232**
24	21,1	153	102,794	338,886
25	21,1	105	89,416	283,769
26	18,1	256	129,408	463,484
27	21,5	153	103,337	376,853
28	24,1	103	102,493	259,576
29	24,9	112	67,555	23,1127
30	24,9	201	156,623	47,1374
Neg ctr 0B	32,3	11	4,958	16,279
Neg ctr 0A	33,1	20	3,661	12,709
FOHMnegCtr		14	3,964	15,293

CT 16S: Cycle Threshold in the universal 16S PCR; Cluster 10c1p: Number of clusters with above 10 reads and less than one percent variation; Used reads: The final number of reads after removal of short reads, small clusters and chimeras; Total reads: The total sequence output for each sample

Supplementary Table 2: Alphabetical list of OTU´s and species obtained by 16S sequencing

OTUs / SPECIES	Samples of total 27	Mean	Median	Min	Max
Abiotrophia defectiva	15	1,18	0,17	0,01	28,15
Abiotrophia (genus only)	2	0,01	0,09	0,03	0,15
Achromobacter (genus only)	3	0,02	0,03	0,02	0,41
Acinetobacter baumannii	1	NA	NA	0,05	0,05
Actinobaculum sp. oral taxon 183	2	NA	NA	0,01	0,08
Actinomyces cardiffensis	2	NA	NA	0,01	0,01
Actinomyces dentalis	1	NA	NA	0,01	0,01
Actinomyces (genus only)	17	0,29	0,16	0,01	1,82
Actinomyces gerencseriae	3	0	0,01	0,01	0,02
Actinomyces graevenitzii	18	0,43	0,06	0	3,79
Actinomyces naeslundi group	15	0.66	0,11	0,02	15,69
Actinomyces meyeri/odontolyticus group	26	4,32	3,67	0,03	12,84
Actinomyces oral taxon 178	9	0,06	0,02	0,01	1,31
Actinomyces oral taxon 448	8	0,04	0,04	0,01	0,5
Actinomyces oral taxon 848	2	NA	NA	0,01	0,03
Actinomyces massiliensis	3	0	0,01	0,01	0,04
Actinomyces sp. (sp4-iso1_H03x4)	10	0,26	0,41	0,02	2,79
Actinomyces turicensis	1	NA	NA	0,01	0,01
Aerococcus christensenii	1	NA	NA	0,03	0,03
Afipia (genus only)	2	NA	NA	0,07	0,15
Aggregatibacter segnis	2	NA	NA	0,01	0,04
Aggregatibacter aphrophilus	1	NA	NA	0,03	0,03
Aggregatibacter (genus only)	1	NA	NA	0,04	0,04
Aggregatibacter oral taxon 458	2	NA	NA	0,02	0,1
Aggregatibacter oral taxon 513	1	NA	NA	0,06	0,06
Alistipes finegoldii	2	NA	NA	0,13	0,52
Alistipes putredinis	1	NA	NA	0,69	0,69
Alloprevotella tannerae	1	NA	NA	0,02	0,02
Alloprevotella oral taxon 308	1	NA	NA	0,11	0,11
Alloprevotella oral taxon 473	1	NA	NA	0,01	0,01
Alloscardovia omnicolens	12	0,15	0,16	0,01	1,58
Atopobium deltae (A. minutum)	2	NA	NA	0,01	0,08
Atopobium (genus only)	12	0,04	0,02	0	0,39
Atopobium parvulum	23	2,35	1,53	0,04	15,88
Atopobium rimae	12	0,05	0,02	0,01	1,12
Atopobium vaginae	2	NA	NA	0,03	0,04
Bacterioidales(G-2) oral taxon 274	2	NA	NA	0,01	0,05
Bacteroides caccae	2	NA	NA	0,01	0,45
Bacteroides dorei (B. vulgatus)	2	NA	NA	0,03	1,78
Bacteroides faecis	1	NA	NA	0,33	0,33
Bacteroides fragilis	1	NA	NA	0,01	0,01
Bacteroides massiliensis	1	NA	NA	0,76	0,76
Bacteroides stercoris	1	NA	NA	0,5	0,5

Bacteroides uniformis	2	NA	NA	0,01	0,08
Bacteroides xylanisolvens	1	NA	NA	0,06	0,06
Barnesiella (genus only)	2	NA	NA	0,09	0,27
Below cutoff	21	0,6	0,09	0,01	6,39
Bifidobacteraceae(G-2) oral taxon 407	1	NA	NA	0,03	0,03
Bifidobacterium adolescentis	1	NA	NA	0,01	0,01
Bifidobacterium dentium	13	0,13	0,03	0,01	2,96
Bifidobacterium longum	13	0,42	0,3	0,01	5,01
Bifidobacterium (genus only)	1	NA	NA	0,02	0,02
Blautia faecis	2	NA	NA	0,02	0,17
Blautia (genus only)	1	NA	NA	0,01	0,01
Blautia wexlerae	1	NA	NA	0,68	0,68
Brevundimonas vesicularis	1	NA	NA	0,02	0,02
Bulleidia extructa	5	0	0,02	0,01	0,06
Butyrivibrio oral taxon 455	4	0	0,02	0,01	0,06
Campylobacter concisus (C. mucosalis)	9	0,06	0,06	0,01	0,6
Campylobacter oral taxon 044	1	NA	NA	0,02	0,02
Campyobacter rectus/showae	3	0	0,03	0,02	0,04
Capnocytophaga gingivalis (C. granulosa)	2	NA	NA	0,01	0,15
Campylobacter gracilis	2	NA	NA	0,03	0,17
Capnocytophaga (genus only)	1	NA	NA	0,05	0,05
Capnocytophaga granulosa (C. gingivalis)	2	NA	NA	0,01	0,22
Capnocytophaga leadbetteri	2	NA	NA	0,03	0,15
Capnocytophaga oral taxon 336	1	NA	NA	0,19	0,19
Capnocytophaga oral taxon 338	1	NA	NA	0,01	0,01
Capnocytophaga sputigena	1	NA	NA	0,01	0,01
Cardiobacterium hominis	2	NA	NA	0	0,06
Cardiobacterium valvarum	1	NA	NA	0,03	0,03
Catonella morbi	5	0,01	0,03	0,01	0,08
Clostridium bartlettii	4	0,03	0,14	0,01	0,4
Clostridium celatum/disporicum	7	0,83	1,28	0,01	16,66
Clostridium glycolicum	1	NA	NA	0,05	0,05
Clostridium (genus only)	3	0,26	0,27	0,15	6,47
Clostridium citroniae/boltae/clostridioforme	1	NA	NA	0,09	0,09
Clostridium paraputrificum	2	NA	NA	0,04	0,12
Clostridium perfringens	1	NA	NA	0,01	0,01
Collinsella aerofaciens	1	NA	NA	0,06	0,06
Corynebacterium accolens (C. fastidiosum/mcginley/segmentosum/tuberculostearicum)	1	NA	NA	1,61	1,61
Corynebacterium durum	8	0,03	0,03	0,01	0,29
Corynebacterium fastidiosum	1	NA	NA	0,01	0,01
Corynebacterium (genus only)	1	NA	NA	0,04	0,04
Corynebacterium matruchotii	1	NA	NA	0,02	0,02
Corynebacterium proinquum/pseudodiphteriticum	2	NA	NA	0,01	0,42
Corynebacterium vitaeruminis (pseudotuberculosis/ulcerans/argentoratense)	1	NA	NA	0,04	0,04
Cryptobacterium curtum	5	0,01	0,03	0,02	0,06

Dialister invisus	3	0	0,02	0,01	0,07
Dialister micraerophilus	1	NA	NA	0,01	0,01
Dialister oral taxon 119	1	NA	NA	0,01	0,01
Dialister pneumosintes	1	NA	NA	0,01	0,01
Dolosigranulum pigrum	2	NA	NA	0,03	0,04
Dorea formicigenerans	1	NA	NA	0	0
Eikenella sp. (NML130454) (Kingella denitrificans)	1	NA	NA	0,14	0,14
Erysipelotrichaceae(G-1) sp. oral taxon 905	1	NA	NA	0,06	0,06
Erysipelotrichaceae(G-1) sp. oral taxon 904	9	0,04	0,06	0,01	0,29
Erysipelotrichaceae (genus only)	5	0,02	0,02	0,01	0,41
Escherichia coli/Shigella boydii/S. dysenteriae/ S flexneri	8	2,72	0,01	0,01	73,24
Escherichia/Shigella (genus only)	1	NA	NA	4,05	4,05
Eubacterium brachy	13	0,18	0,23	0,04	1,35
Eubacterium hallii	1	NA	NA	0,05	0,05
Eubacterium infirmum	7	0,01	0,03	0,02	0,1
Eubacterium nodulatum	1	NA	NA	0,02	0,02
Eubacterium ramulus	1	NA	NA	0,04	0,04
Eubacterium rectale	1	NA	NA	0,16	0,16
Eubacterium saphenum	2	NA	NA	0,01	0,15
Eubacterium sulci (infirmum)	12	0,12	0,29	0,03	0,65
Faecalibacterium prausnitzii	2	NA	NA	0,08	0,09
Filifactor alocis	3	0	0,03	0,01	0,08
Flavobacteriaceae genomosp. C1	2	NA	NA	0,01	0,03
Fretibacterium fastidiosum	1	NA	NA	0,17	0,17
Fusobacterium necrophorum	1	NA	NA	0,03	0,03
Fusbacterium nucleatum	18	0,16	0,08	0,01	1,88
Fusbacterium periodonticum	14	1,03	0,32	0,01	17,21
Fusbacterium (genus only)	2	0,01	0,15	0,06	0,25
Gardnerella vaginalis	1	NA	NA	0,3	0,3
Gemella bergeriae	5	0,01	0,03	0,01	0,08
Gemella haemolysans/sanguinis	24	1,11	0,83	0,03	6,11
Gemella morbillorum	20	0,38	0,07	0,01	2,23
Gemella (genus only)	5	0,03	0,02	0,01	0,63
Gemella haemolysans/morbillorum/sanguinis	9	0,11	0,32	0,01	1,02
Gemmiger formicilis	3	0,03	0,34	0,16	0,52
Gemmiger (genus only)	1	NA	NA	0,01	0,01
Granulicatella adiacens	27	8,44	5,18	0,13	23,91
Granulicatella elegans	10	0,33	0,11	0,02	6,36
Granulicatella (genus only)	9	0,03	0,03	0,01	0,48
Haemophilus haemolyticus	2	0,02	0,28	0,03	0,53
Haemophilus parainfluenzae	11	0,15	0,03	0,01	1,66
Haemophilus parahaemolyticus (paraphrohaemolyticus)	1	NA	NA	0,37	0,37
Haemophilus sp. oral taxon 035	1	NA	NA	0	0
Haemophilus (genus only)	1	NA	NA	0,02	0,02
Haemophilus sputorum	1	NA	NA	0,1	0,1
Helicobacter pylori	3	0,17	0,28	0,25	3,93

Holdemania (genus only)	1	NA	NA	0,02	0,02
Kingella oralis	1	NA	NA	0,03	0,03
Lachnoanaerobaculum (genus only)	2	NA	NA	0,02	0,02
Lachnoanaerobaculum oral taxon 089	1	NA	NA	0,01	0,01
Lachnoanaerobaculum orale/saburreum	22	0,17	0,08	0,01	1,09
Lachnoanaerobaculum umaense	9	0,1	0,1	0,03	1,45
Lachnospiraceae(G-2) oral taxon 096	4	0	0,03	0,02	0,03
Lachnospiraceae (G-3 oral taxon 097)	1	NA	NA	0,01	0,01
Lachnospiraceae(G-3) oral taxon 100	3	0,02	0,21	0,02	0,36
Lachnospiraceae(G-7) oral taxon 163	4	0,01	0,08	0,01	0,12
Lactobacillus casei/paracasei/rhamnosus	2	NA	NA	0,01	0,43
Lactobacillus crispatus/gallinarum (S. acidophilus/ultunensis)	1	NA	NA	0,33	0,33
Lactobacillus (genus only)	4	0	0,03	0,01	0,07
Lactobacillus gasseri	6	0,58	0,51	0,04	13,38
Lactobacilus fermentum	4	0,04	0,24	0,06	0,43
Lactobacillus reuteri	2	NA	NA	0,02	0,05
Lactobacillus salivarius	2	NA	NA	0,01	0,36
Lautrophia mirabilis	3	0,01	0,08	0,04	0,09
Leptotrichia (genus only)	5	0,05	0,07	0,03	0,77
Leptotrichia goodfellowii	1	NA	NA	0,01	0,01
Leptotrichia hofstadii	1	NA	NA	0,04	0,04
Leptotrichia oral taxon 215	2	NA	NA	0,02	0,06
Leptotrichia oral taxon 218	1	NA	NA	0,11	0,11
Leptotrichia oral taxon 221	2	NA	NA	0,04	0,06
Leptotrichia oral taxon 225 (L. buccalis)	1	NA	NA	0,01	0,01
Leptotrichia sp. oral taxon 417	5	0,09	0,32	0,02	1,73
Leptotrichia sp. oral taxon 462	3	0,04	0,08	0,02	0,89
Leptotrichia oral taxon 498	3	0	0,01	0,01	0,02
Leptotrichia hongkongensis	2	NA	NA	0,01	1,09
Leptotrichia wadei	2	NA	NA	0,03	0,1
Megasphaera micronuciformis	2	NA	NA	0,02	0,13
Mesorhizobium (genus only)	1	NA	NA	0,22	0,22
Microbacterium (genus only)	1	NA	NA	0,22	0,22
Micrococcus luteus	2	NA	NA	0,03	0,62
Mobiluncus (genus only)	2	NA	NA	0,02	0,13
Mycoplasma salivarium	1	NA	NA	0,01	0,01
Neisseria bacilliformis	1	NA	NA	0,02	0,02
Neisseria elongata	3	0,01	0,02	0,01	0,17
Neisseria perflava/subflava (cinerea/flavenscens)	6	0,05	0,04	0,01	0,94
Neisseria (genus only)	1	NA	NA	0,09	0,09
Neisseria mucosa/sicca (pharyngis)	3	0,01	0,1	0,01	0,1
Odoribacterium splanchnicus	1	NA	NA	0,05	0,05
Olsenella sp. oral taxon 807	1	NA	NA	0,02	0,02
Oribacterium asaccharolyticum	21	0,76	0,56	0,01	7,5
Oribacterium oral taxon 078	6	0,01	0,02	0,01	0,03
Oribacterium (genus only)	12	0,12	0,04	0,01	2,03

Oribacterium parvum (sinus)	1	NA	NA	0,78	0,78
Oribacterium sinus (parvum)	21	1,16	0,63	0,03	7,33
Oribacterium (genus only)	2	NA	NA	0,02	0,08
Oscillibacter sp. (Marseille-P2778)	1	NA	NA	0,28	0,28
Parabacteroides (genus only)	1	NA	NA	0,03	0,03
Parascardovia denticolens	4	0	0,03	0,02	0,06
Parasutterella excrementihominis	2	NA	NA	0,1	0,71
Parvimonas micra	20	0,25	0,19	0,01	1,26
Parvimonas (genus only)	1	NA	NA	0,07	0,07
Peptococcus oral taxon 167	1	NA	NA	0,01	0,01
Peptoniphilus lacrimalis	1	NA	NA	0,02	0,02
Peptostreptococcaceae(XI)(G-4) oral taxon 369	3	0	0,02	0,02	0,02
Peptostreptococcus stomatis	10	0,02	0,03	0,01	0,1
Peptostreptococcus (genus only)	1	NA	NA	0,02	0,02
Porphyromonas endodontalis	2	NA	NA	0,06	0,64
Porphyromonas (genus only)	1	NA	NA	0,65	0,65
Porphyromonas oral taxon 279	1	NA	NA	0,16	0,16
Prevotella denticola	1	NA	NA	0,02	0,02
Prevotella (genus only)	1	NA	NA	0,09	0,09
Prevotella histicola	4	0,03	0,08	0,02	0,52
Prevotella melaninogenica	4	0,08	0,56	0,03	1,14
Prevotella nanceiensis	1	NA	NA	0,1	0,1
Prevotella nigrescens	1	NA	NA	0,03	0,03
Prevotella oral taxon 304	1	NA	NA	0,06	0,06
Prevotella oral taxon 306	2	NA	NA	0,02	0,14
Prevotella oral taxon 317 (P. conceptionensis)	2	NA	NA	0,01	0,02
Prevotella oris	1	NA	NA	0,03	0,03
Prevotella pallens	1	NA	NA	0,47	0,47
Prevotella pleuritidis	1	NA	NA	0,02	0,02
Prevotella salivae	2	NA	NA	0,17	0,18
Propionibacterium acidifaciens	1	NA	NA	0,04	0,04
Pseudoflavonifractor (genus only)	1	NA	NA	0,01	0,01
Pseudomonas (genus only)	4	0,01	0,02	0,01	0,11
Pseudomonas putida	1	NA	NA	0,01	0,01
Pseudomonas stutzeri	1	NA	NA	0,65	0,65
Reyranella (genus only)	3	0,01	0,02	0,02	0,26
Reyranella soli	1	NA	NA	0,03	0,03
Romboutsia timonensis	6	1,53	4,91	0,26	18
Romboutsia (genus only)	3	0,11	0,85	0,3	1,75
Roseburia intestinalis (R. hominis)	1	NA	NA	0,06	0,06
Rosebruria hominis (R. intestinalis)	1	NA	NA	0,02	0,02
Roseburia faecis	1	NA	NA	0,02	0,02
Roseburia inulinivorans	3	0	0,02	0,01	0,03
Rothia aeria	5	0,04	0,05	0,02	0,71
Rothia dentocariosa	20	0,74	0,36	0,04	4,71
Rothia (genus only)	11	0,05	0,04	0,02	0,76

Rothia mucilaginosa	26	9,07	6,55	0,33	48,96
Ruminococcaceae(G-1) (genus only)	1	NA	NA	0,09	0,09
Ruminococcaceae(G-1) sp. oral taxon 075	6	0,55	0,1	0	14,03
Ruminococcaceae(G-2) sp. oral taxon 085	11	0,11	0,09	0,01	1,67
Ruminococcus faecis	1	NA	NA	0,18	0,18
Ruminococcus gnavus	2	NA	NA	0,06	1,68
Ruminococcus lactaris	1	NA	NA	0,51	0,51
Ruminococcus bromii	2	NA	NA	0,03	0,2
Ruminococcus torques	1	NA	NA	0,28	0,28
Ruminococus (genus only)	1	NA	NA	0,02	0,02
Scardovia (genus only)	1	NA	NA	0,59	0,59
Scardovia inopinata	1	NA	NA	0,01	0,01
Scardovia wiggsiae	12	0,23	0,2	0,01	1,73
Shuttleworthia satelles	5	0,01	0,02	0,01	0,06
Slackia exugia	1	NA	NA	0,01	0,01
Solobacterium moorei	26	0,64	0,45	0,04	3,51
Solobacterium (genus only)	6	0	0,02	0,01	0,02
Sp.hingobium (genus only)	1	NA	NA	0,04	0,04
Staphylococcus (genus only)	1	NA	NA	0,03	0,03
Stenotrophomonas maltophila	2	NA	NA	0,05	1,96
Stomatobaculum sp. oral taxon 097	8	0,01	0,02	0,01	0,1
Stomatobaculum sp. oral taxon 373	1	NA	NA	0,02	0,02
Stomatobaculum longum	10	0,03	0,03	0,02	0,29
Streptococcus anginosus group	23	0,81	0,16	0,01	8,05
Streptococcus (genus only)	26	0,87	0,5	0,01	6,12
Streptococcus mitis group	27	26,7	20,74	0,36	77,15
Streptococcus mutans group	8	0,07	0,05	0,01	1,21
Streptococcus salivarius group	22	4,13	2,72	0,1	14,49
Streptococcus sanguinis group	27	17,89	17,47	0,15	41,27
Tannerella oral taxon 286	3	0	0,01	0,01	0,05
Tannerella forsythia	1	NA	NA	0,01	0,01
Tetragenococcus halophilus	1	NA	NA	0,02	0,02
TM7(G-1) (genus only)	21	0,38	0,24	0,02	3,91
TM7(G-1) oral taxon 346	17	0,29	0,05	0,01	4,6
TM7(G-1) oral taxon 347	6	0,04	0,09	0,01	0,72
TM7(G-1) oral taxon 348	2	NA	NA	0,04	0,12
TM7(G-1) oral taxon 349	2	NA	NA	0,01	0,02
TM7(G-1) oral taxon 352/TM7x	24	2,73	0,62	0,02	23,32
TM7(G-1) oral taxon 488	2	NA	NA	0,01	0,03
TM7(G-2) oral taxon 350	3	0	0,02	0,01	0,02
TM7 (G-3) oral taxon 351	8	0,02	0,04	0,01	0,26
TM7 (G-3) (genus only)	1	NA	NA	0,01	0,01
TM7(G-4) oral taxon 355	2	NA	NA	0,02	0,07
TM7(G-5) sp. oral taxon 356	7	0,01	0,01	0,01	0,16
TM7(G-6) sp. oral taxon 870	8	0,25	0,69	0,26	2,14
TM7(G-6) (genus only)	5	0,34	0,22	0,08	5,6

Treponema medium/vincentii	1	NA	NA	0,01	0,01
Tropheryma whipplei	3	0,04	0,18	0,12	0,84
Turicibacter (genus only)	2	NA	NA	0,01	0,02
Turicibacter sanguinis	4	0,07	0,54	0,04	0,71
Veillonella atypica	1	NA	NA	0,01	0,01
Veillonella dispar (V. parvula)	9	0,01	0,03	0	0,09
Veillonella (genus only)	8	0,01	0,02	0,01	0,03
Veillonella parvula (V. tobetsuensis/dentocariosa)	1	NA	NA	0,01	0,01

Supplementary Table S3: Specific amplification and Sanger sequencing of selected gdh and rpoB -gene targets. Results per sample and per target

Sample ID	*S. mitis* (gdh)	*S. oralis* (gdh)	*S. cristatus* (gdh)	*S. gordonii* (gdh)	*S. salivarius* and *S. vestibularis* (gdh)	*S. parasanguinis* (gdh)	*G. haemolysans* (rpoB)	*G. haemolysans group* (rpoB)	*G. sanguinis* (rpoB)
1	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	-	parahaemolysans	sanguinis
2	mitis	oralis	-	-	salivarius	parasanguinis	-	-	sanguinis
3	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	haemolysans	taiwanensis	sanguinis
4	mitis	oralis		gordonii	-	-	-	-	-
5	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	-	parahaemolysans	sanguinis
6	-	oralis	-	-	salivarius	-	-	-	-
8	-	-	-	-	salivarius	parasanguinis	-	-	-
9	mitis	oralis	cristatus	-	salivarius	parasanguinis	-	-	sanguinis
10	mitis	oralis	-	gordonii	salivarius	parasanguinis	haemolysans	taiwanensis	sanguinis
11	mitis	oralis	cristatus	-	salivarius	parasanguinis	-	taiwanensis	-
12	mitis	oralis	-	-	-	-	-	parahaemolysans	-
13	-	oralis	-	gordonii	-	parasanguinis	-	-	-
14	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	-	taiwanensis	-
15	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	haemolysans	-	sanguinis
16	mitis	-	-	gordonii	-	parasanguinis	haemolysans	-	-
18	mitis	oralis	-	gordonii	salivarius	parasanguinis	-	parahaemolysans	sanguinis
19	mitis	-	-	-	-	parasanguinis	haemolysans	-	-
20	-	-	cristatus	-	salivarius	parasanguinis	-	-	-
21	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	-	parahaemolysans	-
22	mitis	oralis	cristatus	-	salivarius	parasanguinis	haemolysans	-	sanguinis
23	mitis	-	-	-	-	-	-	-	-
24	mitis	oralis	-	gordonii	salivarius	parasanguinis	-	parahaemolysans	sanguinis
25	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	-	-	sanguinis
26	mitis	oralis	cristatus	-	salivarius	parasanguinis	-	taiwanensis	sanguinis
27	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	-	parahaemolysans	sanguinis
28	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	-	parahaemolysans	-
29	mitis	oralis	cristatus	gordonii	salivarius	parasanguinis	-	parahaemolysans	sanguinis
30	mitis	-	-	-	salivarius	parasanguinis	haemolysans	taiwanensis	-
Total positive	24	22	15	16	22	24	7	15	14
Homology (%)	97.5-100	94.3-100	94.1-100	97.9-100	100	97.5-100	98.2-99.1	98.5-100	99.1-100

Dash-sign (-): target not detected.

Homology (%): Range of observed % homologies with closest reference for all samples. Due to lack of established cutoff-values for a valid species-level assignment, all identifications were supported by a pairwise comparison of the alignment table in GenBank using the "distance tree of results" function.

Supplementary table S4. Alpha diversities

SAMPLE	Shannon index Average 2,84 Mean 20,86	Species richness Average 51 Mean 50
1	2,70	46
2	2,75	42
3	2,78	54
4	1,76	15
5	3,26	48
6	3,34	30
8	2,36	66
9	3,33	54
10	2,89	51
11	2,85	58
12	0,83	16
13	3,34	46
14	3,07	79
15	2,71	124
16	2,79	33
18	2,86	57
19	2,50	34
20	3,20	58
21	2,86	41
22	3,09	57
24	3,36	50
25	2,77	41
26	2,89	44
27	3,72	66
28	1,48	44
29	3,62	52
30	3,62	81

Supplementary Table S5: A comparison between RipSeq NGS (5b) and QIIME (5c) for the analysis of a commercial bacterial mock community (5a)

Summary: RipSeq NGS identifies all the mock community species to the best possible level within the limitations defined by the resolution of the 16S rRNA gene itself. QIIME identifies most of the sequences to the genus, family or order-level only. It also provides several erroneous or inaccurate species level designations. Both pipelines find fewer *Rhodobacter sphaeroides* sequences than expected, most likely reflecting inefficient amplification or sequencing of this target. The RipSeq NGS pipeline underestimates the number of *Staphylococcus aureus* sequences when OUT-clustering is performed with a 99 % similarity threshold. This is because *Staphylococcus aureus* shares 99.3 % homology with *Staphylococcus epidermidis* leading to most of the reads being erroneously assigned to the *S. epidermidis* cluster. This is a well-known issue when using de-novo OTU clustering for very similar species. As demonstrated in table S6b the problem can be diminished by using a higher similarity threshold of 99.5 %. For the study we nevertheless used a OTU-clustering of 99 % since using a higher threshold also increased the number of small groups representing out-layer sequences and consequently increased the workload in the quality assuring of the data-analysis.

Table S5a: The content of the commercial mock community

Mock community species	operons	% dist.
Rhodobacter sphaeroides	1000000	21.91 %
Staphylococcus epidermidis	1000000	21.91 %
Streptococcus mutans	1000000	21.91 %
Escherichia coli	1000000	21.91 %
Bacillus cereus	100000	2.19 %
Clostridium beijerinckii	100000	2.19 %
Pseudomonas aeruginosa	100000	2.19 %
Staphylococcus aureus	100000	2.19 %
Streptococcus agalactiae	100000	2.19 %
Acinetobacter baumannii	10000	0.22 %
Helicobacter pylori	10000	0.22 %
Lactobacillus gasseri	10000	0.22 %
Listeria monocytogenes	10000	0.22 %
Neisseria meningitidis	10000	0.22 %
Propionibacterium acnes	10000	0.22 %
Acitinomyces odontolyticus	1000	0.02 %
Bacteroides vulgatus	1000	0.02 %
Deinococcus radiodurans	1000	0.02 %
Enterococcus faecalis	1000	0.02 %
Streptococcus pneumoniae	1000	0.02 %
	4565000	100.00 %

Mock community: HM-783D BEI Resources (Virginia, US)

% dist = relative distribution of reads in percent

Table S5b: Results obtained using RipSeq NGS

Mock community species	RipSeq NGS result	Reads 99 %	% dist. 99 %	% dist. 99.5 %
Rhodobacter sphaeroides	*R. johrii /megalophilus /sphaeroides*	7793	6.01%(L)	5.96%(L)
Staphylococcus epidermidis	*S. capitis/caprae/epidermidis (aureus/cohnii/hominis/lugdunensis/ saccharolyticus)*	38075	29.38%	27.52%
Streptococcus mutans	*S. mutans*	33725	26.02%	25.97%
Escherichia coli	*E. coli/Shigella spp.*	35979	27.76%	27.85%
Bacillus cereus	*B. anthracis/cereus/thuringiensis*	4003	3.09%	3.06%
Clostridium beijerinckii	*C. beijerinckii (puniceum)*	2703	2.09%	2.11%
Pseudomonas aeruginosa	*P. aeruginosa*	1469	1.13%	1.14%
Staphylococcus aureus	*S. aureus (croceolyticum/petrasii)*	102	0.08%(L)	2.01%
Streptococcus agalactiae	*S. agalactiae*	4119	3.18%	3.11%
Acinetobacter baumannii	*A. baumannii*	305	0.24%	0.24%
Helicobacter pylori	*H. pylori*	358	0.28%	0.27%
Lactobacillus gasseri	*L. gasseri (johnsonii)*	216	0.17%	0.16%
Listeria monocytogenes	*L. innocua/ivanovii/marthii/ monocytogenes /seeligeri/welshimeri*	333	0.26%	0.26%
Neisseria meningitidis	*N. meningitidis (polysaccharea)*	221	0.17%	0.17%
Propionibacterium acnes	*P. acnes*	145	0.11%	0.11%
Acitinomyces odontolyticus	*A. meyeri/odontolyticus group*	9	0.01%	0.01%
Bacteroides vulgatus	*B. vulgatus*	11	0.01%	0.01%
Deinococcus radiodurans	*D. radiodurans*	20	0.02%	0.02%
Enterococcus faecalis	*E. faecalis*	19	0.01%	0.01%
Streptococcus pneumoniae	*S. pneumoniae/pseudopneumoniae (mitis/oralis/infantis)*	8	0.01%	0.01%
	Total	129613	100.00%	100.00%

Results obtained using the RipSeq NGS software and a cluster similarity of 99 %. The % distribution of reads is also showed for an analysis using a cluster similarity of 99.5 %.

% dist = relative distribution of reads in percent

Slash-results: Species with identical 16S rRNA-genes in the sequenced area. No discrimination possible.

Results in parenthesis: species within 0.8 % from the top-scoring sequence. Robust discrimination not possible.

L = significantly lower than expected

Table S5c: Results obtained using QIIME

Mock community species	QIIME-result	reads	% dist.
Rhodobacter sphaeroides	*R. sphaeroides*^	9780	6.29%(L)
Staphylococcus epidermidis	*S. epidermidis*^	302	26.30%
	Staphylococcus (g)	40608	
Streptococcus mutans	*Streptococcus* (g)*	40596*	26.10%*
Escherichia coli	*E. coli*^	18	27.69%
	Enterobacteriaceae (f)	43051	
Bacillus cereus	*B. cereus*§	163	4.07%
	B. alkalitolerans (E)	20	
	B. anthracis§	10	
	Bacillus (g)	4847	
	Bacillales (f)	1295	
Clostridium beijerinckii	*C. butyricum* (E)	40	2.28%
	Clostridiales (o)	3510	
Pseudomonas aeruginosa	*P. alcaligenes* (E)	1712	1.44%
	Pseudomonas (g)	532	
Staphylococcus aureus	*S. aureus*	5030	3.23%
Streptococcus agalactiae	*Streptococcus* (g)	*	*
Acinetobacter baumannii	*Acinetobacter* (g)	486	0.31%
Helicobacter pylori	*H. pylori*	510	0.36%
	H. pullorum (E)	41	
Lactobacillus gasseri	*Lactobacillus* (g)	318	0.20%
Listeria monocytogenes	*L. seelingeri*#	447	0.29%
Neisseria meningitidis	*N. cincerae* (E)	324	0.21%
Propionibacterium acnes	*P. acnes*	211	0.14%
Acitinomyces odontolyticus	*Actinomyces* (g)	13	0.01%
Bacteroides vulgatus	*Bacteroides* (g)	27	0.02%
Deinococcus radiodurans	*Deinococcus* (g)	25	0.02%
Enterococcus faecalis	*Enterococcus* (g)	42	0.03%
Streptococcus pneumoniae	*Streptococcus* (g)	*	*
	Gemellales (o) (E)	184	0.12%
	Planococcaceae (f) (E)	1418	0.91%
Total		155560	100.00%

Results obtained using the QIIME bioinformatics pipeline version 1.9.1 with command "pick_closed_reference_otus.py" and default 97 % cutoff for species identification.

% dist = relative distribution of reads in percent

E = Erroneous identification or species assignment, L = significantly lower than expected f = family-level, g = genus-level, o = order-level

*No discrimination between S. agalactiae, S. mutans and S. pneumoniae. All merged into single genus-level identification

^Unambiguous identification is not possible, since this species is identical to other species in the sequenced area. It should have been a slash-result. Please refer to table S6b.

[§]B. anthracis and B. cereus are identical in the sequenced area. Should have been a slash-result B. cereus/anthracis

[#]L. seelingeri is identical to L. monocytogenes in the sequenced area. Should have been a slash-result L. seelingeri/monocytogenes.

Supplementary Table S6. Primer sequences and PCR conditions for targeted Sanger-sequencing of the rpoB and gdh genes

Target species	Gene	Primers	Conc. (µM)	Anneal. (ºC)
G. haemolysans	rpoB	Forward: 5'-AGGAATCATTCGTATTGG-3' Reverse: 5'-AACATCTTCATCAGTAGC-3'	0.4 0.4	56
G. haemolysans *G. parahaemolysans* *G. taiwanensis*	rpoB	Forward: 5'-TAAAGTTACACCGAAAGG-3' Reverse: 5'-CATCAAATACTGGTGTTG-3'	0.4 0.4	56
G. sanguinis	rpoB	Forward: 5'-GTGGTATTATTCGTATAGGT-3' Reverse: 5'-CATCAAATACTGGTGTTG-3'	0.4 0.4	56
S. cristatus	gdh	Forward: 5'-CTAATCTGCTGTTTGAAA-3' Reverse: 5'-GATATAGATGGTTAGGACA-3'	0.4 0.4	53
S. gordonii	gdh	Forward: 5'-AGCYAATAATTCTGCTGAAG-3' Reverse: 5'-CAGAATCTATGACTGAAACTT-3'	0.4 0.4	57
S. mitis	gdh	Forward: 5'-ATGAAGARCTTAAAGAAYACT-3' Reverse: 5'-TGGCTAAAGTTAGTTGAGT-3'	0.6 0.4	53
S. oralis	gdh	Forward: 5'-CTGAGGAAGAAYTGAAAGAA-3' Reverse: 5'-ATTGGTWGARTTGTTRTTCA-3'	0.4 0.6	53
S. parasanguinis	gdh	Forward: 5'-CTTTACYAAGGATGCRATTC-3' Reverse: 5'-GATCCAGACTTGTATTCATAGA-3'	0.6 0.4	55
S. salivarius *S. vestibularis*	gdh	Forward: 5'-TTGGGAGCTATTGATGTC-3' Reverse: 5'-GCRTCTGCAACTTTAAGG-3'	0.4 0.4	57

Conc.: Final concentration in the 25 µl PCR-reaction tube, Anneal.: PCR-specific annealing temperature

SUPPLEMENTARY INFORMATION

THE CULTIVABLE MICROBIOTA OF THE HUMAN DISTAL ILEUM

Corresponding author Heidi Cecilie Villmones[1], Department of Microbiology,

Vestfold Hospital Trust, Postbox 2168, 3103 Tønsberg, Norway

E-mail: heivil@siv.no, Telephone number 0047 92214631 or 0047 3334568

Supplementary Table 1: Bacteria identified by culturing of ileum content from 150 patients

Microorganism		**Number of samples**	**Percent of samples**
Viridans streptococci		**118**	**78.7**
	Streptococcus sanguinis group	68	45.3
	Streptococcus mitis group	63	42
	Streptococcus salivarius group	60	40.7
	Streptococcus anginosus group	2	1.3
	Streptococcus mutans group	6	4.0
	Streptococcus bovis group	2	1.3
Streptococcus	*Streptococcus agalactiae*	1	0.7
Enterococci		**4**	**2.7**
	Enterococcus faecalis	2	1.3
	Enterococcus faecium	2	1.3
Actinomyces		**61**	**40.7**
	Aactinomyces odontolyticus	46	30.7
	Actinomyces oris	16	10.7
	Actinomyces naeslundi	4	2.7
	Actinomyces graevenitzii	1	0.7
	Actinomyces lignae #*	1	0.7
Rothia		**49**	**32.7**
	Rothia mucilaginosa	33	22.0
	Rothia dentocariosa	22	14.7
	Rothia aeria	2	1.3
Lactobacillus		**26**	**17.3**
	Lactobacillus gasseri	8	5.3
	Lactobacillus fermentum	7	4.7
	Lactobacillus plantarum	3	2.0
	Lactobacillus rhamnosus	2	1.3
	Lactobacillus paracasei	2	1.3
	Lactobacillus salivarius	1	0.7
	Lactobacillus vaginalis	1	0.7
	Lactobacillus reuteri	1	0.7
	Lactobacillus oris	1	0.7
	Lactobacillus johnsonii	1	0.7
Other *lactobacillaceae*			
	Bifidobacterium longum	2	1.3

	Lactococcus lactis	1	0.7
	Weissella confusa #	1	0.7
	Leuconstoc lactis	1	0.7
Enterobacteriacea		**10**	**6.7**
	Escherichia coli	3	2.0
	Klebsiella pneumonia	4	2.7
	Enterobacter cloacae complex	1	0.7
	Serratia marcesens	1	0.7
Pseudomonas		**1**	**0.7**
	Pseudomonas aeruginosa	1	0.7
Anaerobic bacteria		**9**	**6.0**
	Bacteroides ovatus	2	1.3
	Bacteroides vulgatus	2	1.3
	Bacteroides faecis/thetaiotaomicron	1	0.7
	Parabacteroides merdae	2	1.3
	Clostridium paraputrificum	2	1.3
	Clostridium perfringens	2	1.3
	Lachnoanaerobaculum umense #	1	0.7
	Veillonella parvula	1	0.7
Haemophilus		**2**	**1.3**
	Haemophilus parahaemolyticus	2	1.3
Gemella		**2**	**1.3**
	Gemella sanguinis	1	0.7
	Gemella haemolysans	1	0.7
	Gemella sp.	**1**	**0.7**
Staphylococcus		**8**	**5.3**
	Staphylococcus epidermidis	6	4.0
	Staphylococcus aureus	1	0.7
	Staphylococcus haemolyticus	1	0.7
	Staphylococcus warneri	1	0.7
Micrococcus		**1**	**0.7**
	Micrococcus luteus	1	0.7
Alloscardovia		**1**	**0.7**

	Alloscardovia omnicolens	1	0.7
Abiotrophia		**1**	**0.7**
	Abiotrophia defectiva	1	0.7
Candida		**84**	**56**
	Candida albicans	72	48.0
	Candida dubliniensis	7	4.7
	Candida glabrata	5	3.3
	Candida tropicalis	4	2.7
	Candida parapsilosis	4	2.7
	Candida kefyr	1	0.7
	Candida inconspicua	1	0.7

*A. lignae = A. meyeri/odontolyticus group

Identified by 16S-gene analysis, V3-V4

Viridans streptococci group	**No of samples**	**Detected species with MaldiTOF score > 2, category > A**	**No of samples**	**Percent of samples**
S. mitis group	63	*Streptococcus mitis/oralis/peroris/pseudopneumoniae*	60	40.0
		Streptococcus cristatus	4	2.7
		Streptococcus infantis	1	0.7
		Streptococcus massiliensis	1	0.7
S. sanguinis group	68	*Streptococcus sanguinis*	6	4.0
		Streptococcus parasanguinis	62	41.3
S. salivarius group	60	*Streptococcus salivarius/vestibularis*	57	38.0
S. anginosus group	2	*Streptococcus anginosus*	1	0.7
		Streptococcus intermedius	1	0.7
S. mutans group	6	*Streptococcus mutans*	3	2.0
		Streptococcus sobrinus	3	2.0
S. bovis group	2	*Streptococcus lutetiensis*	1	0.7

Supplementary Table 2. Comparison of bacterial culturing and metagenomics of a subset of 27 samples

Sample	Culture number	16S number	Detected both methods	Only detected by culture	CFU/ml bacteria	16S PCR CT value
1	3	46	3		0.8-1.6 x 10^4	24.2
2	3	42	3		>1.6 x 10^4	23
3	2	52	2		>1.6 x 10^4	21.4
4	1	15	1		160-<1.600	0.8-1.6 x 10^4
5	5	47	5		0.8-1.6 x 10^4	21.7
6	0	30	0		0	31
8	1	66	1		160-<1.600	28.7
9	3	53	3		>1.6 x 10^4	26
10	6	51	6		0.8-1.6 x 10^4	17.5
11	3	58	3		160-<1.600	25.7
12	2	16	1	*Staphylococcus epidermidis*		27.7
13	3	46	3		160-<1.600	25
14	0	79	0			19.4
15	4	121	4		0.8-1.6 x 10^4	24.4
16	0	33	0		0	19.8
18	1	56	1	*Pseudomonas aeruginosa*	>1.6 x 10^4	19.8
19	2	33	2		1.600->8.000	27.2
20	1	59	1		1.600->8.000	24.2
21	4	40	4		1.600->8.000	24.6
22	2	57	2		0.8-1.6 x 10^4	24.5
24	2	50	2		160-<1.600	21.1
25	3	41	3		>1.6 x 10^4	21.1
26	3	44	3		>1.6 x 10^4	18.1
27	0	68	0		0	21.5
28	3	44	3		>1.6 x 10^4	24.1
29	4	53	3	*Staphylococcus lugdunensis*	160-<1.600	24.9
30	4	81	4		160-<1.600	24.9
NegKtr1						32.3
NegKtr2						33.1

Supplementary Table 3: Univariate and multivariate analysis of substantial growth of **fungi**

Variable	Odds ratios (with 95% CI)	
	Univariate	Adjusted for age and sex
PPI	3.45 (1.32, 9.05)	3.30 (1.23, 8.91)
Age (per 10 year)	1.64 (1.05, 2.57)	1.61 (1.03, 2.54)*
Antibiotic treatment, recent 6 months or/and ongoing	1.40 (0.62, 3.18)	1.13 (0.48, 2.65)

* Adjusted only for sex

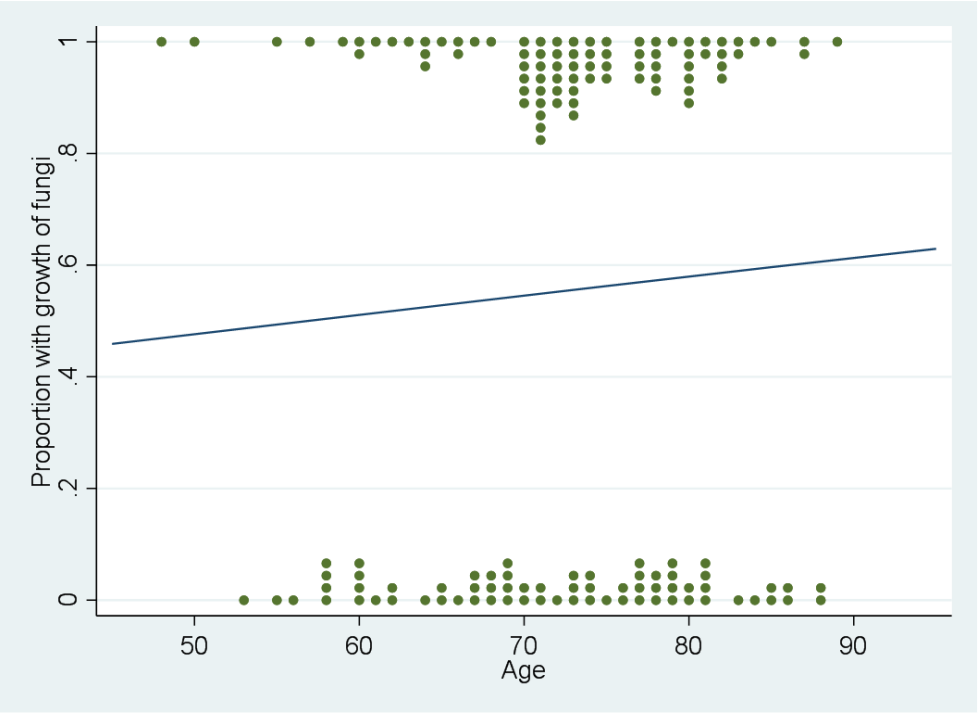
Supplementary Table 4: **Fungal** growth by age (p-value = 0.03* between fungal growth and age)

Age, years	**Non-Substantial fungal growth <1.600 CFU/mL**	**Substantial fungal growth ≥1.600 CFU/mL**	**Percent with substantial fungal growth (with 95% CI)**	**Odds ratio (OR) (with 95% CI)**
<60	11	1	8% (1% - 35%)	OR= 0.15 (0.02, 1.24)
60s	28	5	15% (7% - 31%)	OR= 0.30 (0.10, 0.86)
70s	45	27	38% (27% - 49%)	OR= 1.0 (reference)
80s	21	12	36% (22% - 53%)	OR= 0.95 (0.41, 2.24)

*Fishers exact test

Supplementary Figure 1: Estimated probability of any amount of fungal growth by age

Solid line shows the estimated probability based on binary logistic regression model, while dots indicate individual observations of any growth (1) or no growth (0) of fungi.



Supplementary Table 5: Univariate and multivariate analysis of substantial growth of **bacteria**

Variable	Odds ratios (with 95% CI)	
	Univariate	Adjusted for age and sex
PPI	3.02 (0.96, 9.55)	3.05 (0.96, 9.69)
Age (per 10 year)	0.89 (0.61, 1.32)	0.89 (0.60, 1.31)*
Antibiotic treatment, recent 6 months or/and ongoing	1.00 (0.48, 2.09)	1.01 (0.47, 2.15)

* Adjusted only for sex

Supplementary Table 6: **Bacterial** growth and microbiota category vs proton pump inhibitor-treatment and constipation

Clinical variable		Non-Substantial bacterial growth <1.600 CFU/mL	Substantial bacterial growth ≥1.600 CFU/mL	Percent with substantial bacterial growth (with 95% CI)	Difference (with 95% CI) p-value	Odds ratio
Use of PPI-treatment	no	56	74	57% (48%, 65%)	23% (0%, 39%) p=0.05*	3.02
	yes	4	16	80% (58%, 92%)		
Patient reported constipation	no	54	77	59% (50%, 67%)	10% (-14%, 28%) p=0.46*	1.52
	yes	6	13	68% (46%, 85%)		

*Fishers exact test

Supplementary Table 7: **Fungal** growth and microbiota category vs proton pump inhibitor-treatment and constipation

Clinical variable		Non-Substantial fungal growth <1.600 CFU/mL	Substantial fungal growth ≥1.600 CFU/mL	Percent with substantial fungal growth (with 95% CI)	Difference (with 95% CI) p-value	Odds ratio
Use of PPI-treatment	no	96	34	26% (19%, 34%)	29% (6%, 50%) p=0.02*	3.45
	yes	9	11	55% (34%, 74%)		
Patient reported constipation	no	90	41	31% (24%, 40%)	-10% (-26%, 13%) p=0.43*	0.58
	yes	15	4	21% (9%, 43%)		

*Fishers exact test

Supplementary Table 8: Growth of colon-related bacteria vs proton pump inhibitor-treatment and constipation

Clinical variable		Non-colon-like flora*	Colon-related bacteria#	Percent with growth of colon-related bacteria (with 95% CI)	Difference (with 95% CI) p-value	Odds ratio
Use of PPI-treatment	no	114	16	12% (8%, 19%)	13% (-3%, 35%) p=0.16*	2.38
	yes	15	5	25% (11%, 47%)		
Patient reported constipation	no	117	14	11% (6%, 17%)	26% (7%, 49%) p=0.01*	4.76
	yes	12	7	37% (19%, 59%)		

Gram negative rods, strict anaerobic bacteria or enterococci

*Fishers exact test

SUPPLEMENTARY INFORMATION

Investigating the human jejunal microbiota

Heidi Cecilie Villmones [1], Marius Svanevik[2,3], Elling Ulvestad[4,6], Tore Stenstad[5], Inger Lill Anthonisen[1], Randi Monsen Nygaard[6], Ruben Dyrhovden[6], Øyvind Kommedal[4,6]

[1] Department of Microbiology, Vestfold Hospital Trust, 3103 Tønsberg, Norway

[2] Department of Gastrointestinal Surgery, Vestfold Hospital Trust, 3103 Tønsberg, Norway

[3] Morbid Obesity Center, Vestfold Hospital Trust, 3103 Tønsberg, Norway

[4] Department of Clinical Science, University of Bergen, 5021 Bergen, Norway

[5] Department of Infectious Medicine, Vestfold Hospital Trust, 3103 Tønsberg Norway

[6] Department of Microbiology, Haukeland University Hospital, 5021 Bergen, Norway

Corresponding author Heidi Cecilie Villmones, Department of Microbiology, Vestfold Hospital Trust, Postbox 2168, 3103, Tønsberg, Norway. Tel.: +47 3334598. E-mail: heivil@siv.no

Supplementary Table S1. Cultivated bacterial species from jejunum by frequency

Growth (genus)	Growth (species)	Proximal segment (*n*)	Mid segment (*n*)	Patient level (*n*)*
Streptococcus	*S. salivarius/vestibularis*	**13**	**6**	**15**
	S. parasanguinis	**9**	**3**	**10**
	S. mitis/oralis	**7**	**1**	**7**
	S. sanguinis	1	1	1
	S. australis	1		1
	S. sobrinus		1	1
Rothia	*R. mucilaginosa*	6	2	6
	R. dentocariosa	1	1	1
Actinomyces	*A. odontolyticus*	4	3	5
	A. graevenitzii	1		1
Haemophilus	*H. parainfluenzae*	3	2	5
	H. haemolyticus	1	1	2
Neisseria	*N. parahaemolyticus*	3		3
	N. flavescens/subflava	2	1	3
	N. flava	1		1
	N. elongata		1	1
Micrococcus	*M. luteus*	3	1	4
Cutibacterium	*C. acnes*	3		3
	C. avidum		1	1
Staphylococcus	*S. aureus*	1	1	2
	S. epidermidis	1	1	1
	S. pasteurianus	1		1
	S. warneri		1	1
	S. hominis		1	1
	S. capitis		1	1
Gemella	*G. sanguinis*	1		1
	G. haemolysans		1	1
Lactobacillus	*L. gasseri*	1		1

	L. fermentum		1	1
	L. salivarius		1	1
Aggregatibacter	*A. segnis*	1		1
Veillonella	*V. parvula*	1		1
	Veillonella sp.		1	1
Klebsiella	*K. pneumoniae*	1	1	1
Enterococcus	*E. faecalis*		1	1
Corynebacterium	*C. tuberculostearicum*		1	1
	C. aurimucosum		1	1
Brachybacterium	*Brachybacterium sp.*		1	1
Dietzia	*Dietzia sp.*		1	1
Ponticoccus	*P. gilvus*		1	1
Candida	*C. albicans*		1	1

* Both segments combined

Supplementary Table S2. List of species in jejunum alphabetical and by abundance

Separate EXCEL-table

Supplementary Table S3. A comparison of findings by microbial culture versus by deep sequencing

Separate EXCEL file

Supplementary Table S4. List of identifications from negative and positive negative controls

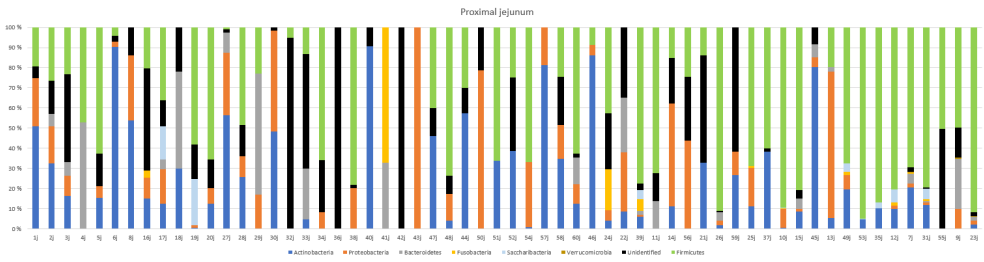
Separate EXCEL file

Supplementary Table S5. Rejected identifications not present in the controls

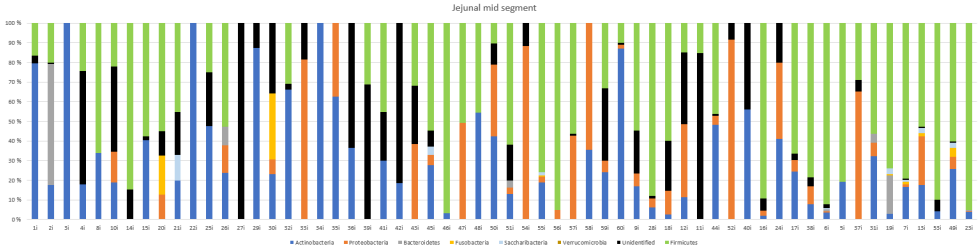
Separate EXCEL file

Supplementary Figure S1. Relative distribution of most abundant phyla in (a) proximal part of jejunum and (b) jejunal mid-segment. Samples are sorted by increasing bacterial concentration. Samples with concentrations below the level of quantification (Ct-value ≥34.17) are sorted by name on the left side (35 proximal samples 1j-60j and 38 mid-segment samples 1i-60i)

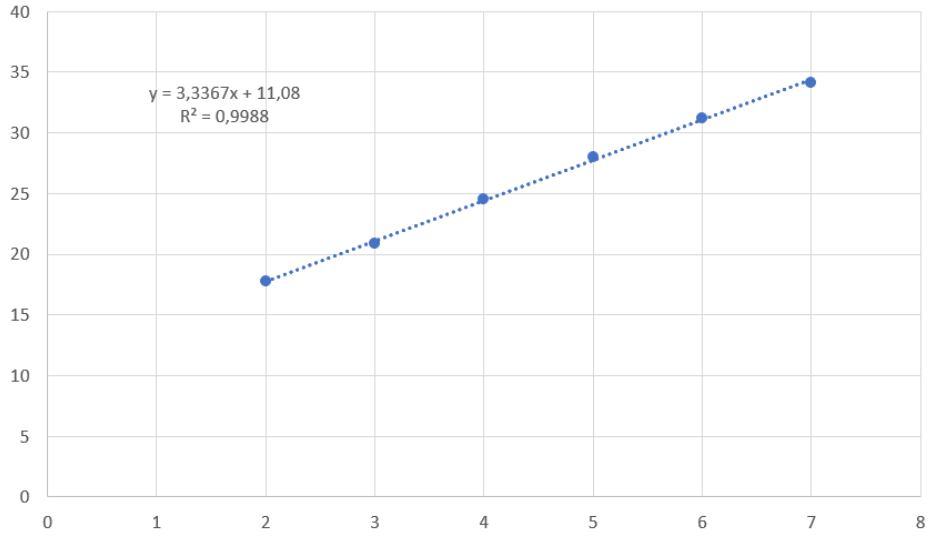
(a)



(b)



Supplementary Figure S2. 10-fold dilution series of *S. pneumonia*



Y-axis: PCR Ct-values. X-axis: 10-fold dilutions from 10^2 (x=2) to 10^7 (x=7).

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