Diploma Thesis

Changes of the Microbiome in Critically Ill Patients

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AFFIDAVIT

Hereby I, Lena Horvath, declare that I have written the present diploma thesis fully on my own and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of the thesis other than those indicated in the thesis itself.

Graz, 15th March 2018 Lena Horvath eh

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ABSTRACT

Background. The human microbiome of multiple body sites is supposed to be affected by critical illness and clinical interventions. When and how microbes change in intensive care unit (ICU) patients is not fully understood.

Methods. This pilot study investigated the short and long-term changes of microbiota composition in the pharynx, feces, tracheobronchial and gastric secretion of 6 ICU patients. The association of clinical factors (medication, infections, mechanical ventilation) was studied for each case. The microbiota of clinical samples was phylogenetically characterized by 16S rRNA sequencing. All samples were divided into 3 groups (early, mid, late), depending on sampling day after ICU admission.

Results and Discussion. Overall, the microbiome of both respiratory and gastrointestinal tract showed a clear loss of species-richness over the course of hospitalisation (seen by decreasing Chao1 indices). Low-diversity communities (one genus comprising over 75%) were detected in all patients, especially in pharyngeal, tracheobronchial and gastric samples, the majority after longer ICU stays. Microbial composition showed great interpatient differences, but *Staphylococcus* and *Enterococcus* were pathogens frequently observed in several patients. We found that patients with an infection showed the infecting pathogen in at least one sample before (3 of 5 patients) or after (all patients) the clinical diagnosis of the infection. In most of these cases, the same pathogen was detected simultaneously in multiple sample areas, suggesting colonization of different body habitats by dominant pathogens. Concerning other clinical factors, intense antibiotic therapies seem to have a big impact on changes in microbiota composition. Assessing the clinical influence of microbiota diversity-loss in relation to the abundance of nosocomial pathogens in critically ill patients is of great interest for the future.

ZUSAMMENFASSUNG

Hintergrund. Das menschliche Mikrobiom ist wahrscheinlich von dem Zustand kritischer Krankheit und den damit zusammenhängenden klinischen Interventionen betroffen. Wie und wann es zu Veränderungen der Mikrobiota-Zusammensetzung bei PatientInnen auf der Intensivstation (ICU) kommt, ist noch nicht ausreichend erforscht.

Methodik. Diese Pilotenstudie untersuchte die Kurz- und Langzeitveränderungen des Mikrobioms des Pharynx, Fäzes, der Magensaft- sowie tracheobronchialen Sekretion von 6 ICU- PatientInnen. Der Einfluss von klinischen Faktoren (Medikation, Infektionen, mechanische Ventilation) auf diese Veränderungen wurde für jeden Fall untersucht. Die Mikrobiota-Proben wurden mittels 16S rRNA Sequenzierung phylogenetisch charakterisiert. Alle Proben wurden in 3 Gruppen (früh, mittel, spät) aufgeteilt, abhängig vom Tag der Probenentnahme nach Aufnahme auf die ICU.

Ergebnisse und Diskussion. Insgesamt zeigte sich im Mikrobiom des Respirations- sowie Gastrointestinaltraktes eine Abnahme der Artendiversität im Verlauf der Hospitalisierung (erkennbar an abnehmenden Chao1 Indizes). Niedrig-diverse Populationen (ein Genus umfasste über 75%) wurden in allen PatientInnen gefunden, speziell in Proben aus dem Pharynx, der Tracheobronchial- und Magensaftsekretion, mehrheitlich nach längerem ICU-Aufenthalt. Die mikrobielle Zusammensetzung innerhalb der PatientInnenkohorte zeigte starke Variationen, jedoch wurden die Pathogene *Staphylokokkus* und *Enterokokkus* in den Proben einiger PatientInnen gefunden. In PatientInnen, welche an einer Infektion litten, wurde der betroffene Erreger in mindestens einer Probe vor (3 von 5 Patienten) oder nach (alle Patienten) der klinischen Diagnose der Infektion gefunden. In der Mehrheit dieser Fälle wurde der gleiche Pathogen gleichzeitig in Proben mehrere Körperregionen gefunden, was auf eine mögliche Kolonisierung dieser verschiedenen Regionen durch dominante Pathogene hinweist.
Von den anderen klinischen Faktoren zeigten intensive Antibiotikatherapien den größten Einfluss auf Mikrobiota-Veränderungen zu haben.
Es ist von großem Interesse den klinischen Einfluss des Diversitätsverlustes der Mikrobiota-Populationen sowie das Auftreten nosokomialer Erreger in kritisch kranken PatientInnen weiter zu untersuchen.

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ABBREVIATIONS

ARDS	Acute respiratory distress syndrome
BSI	Bloodstream infections
CAUTI	Catheter-associated UTIs
CRBSI	Catheter-related blood stream infections
CRIs	Catheter-related infections
DIC	Disseminated intravascular coagulation
ECMO	Extracorporeal membrane oxygenation
GALT	Gut-associated lymphoid tissues
GF mice	Germ-free mice
GIF	Gastrointestinal failure
GRV	Gastric residual volumes
HAI	Health care-associated infections
HAP	Health care-associated pneumonia
IAH	Intraabdominal hypertension
ICU	Intensive care unit
MCH II	Major histocompatibility molecules
MODS	Multiple organ dysfunction syndrome
MRSA	Methicillin-resistant S. aureus
NSTEMI	non-ST-elevation myocardial infarction
OTU	Operational Taxonomic Unit
PCoA	Principal Coordinate Analysis
PD	Phylogenetic distance
PPI	Proton pump inhibitors
ROSC	Return of spontaneous circulation
SCFA	Short chain fatty acids
SIRS	Systemic inflammatory response syndrome
SSTI	Skin and soft-tissue infections
TLR	Toll-like receptors
UTI	Urinary tract infections

VAP	Ventilator-associated pneumonia
16S rRNA	Small subunit (16S) ribosomal RNA gene sequencing
4MRGN	Multiresistant gram-negative bacteria (resistant to the 4 major groups of antibiotics)

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1 INTRODUCTION

1.1 The Healthy Microbiome

The human microbiome comprises all microorganisms, most of them bacteria, that live on and within a person's body.

Every human being, whether healthy or critically ill, is host to an enormous population of these. They make up about 10 times the number of human cells; their metagenome exceeds the human genome by a factor of 150 (1).

Every epithelial surface that stays in contact with the external world is covered with microbial organisms to a certain extent, including oropharynx, respiratory tract, genitourinary tract as well as the skin. The vast majority of these microbes, however, reside in the gastrointestinal tract (GI tract). As recently estimated, up to 10^{14} microbes are located in that region as well as numerous other microorganisms such as viruses, fungi and Archeae (1,2). As physiological conditions vary widely throughout the GI tract (e.g. pH, oxygen concentration, peristalsis), it provides various ecological niches for different specifically adapted bacterial populations to grow (3). These so-called microhabitats each maintain a unique environment optimal for the populating bacteria and therefore enable them to grow in symbiosis with the host (4).

1.1.1 Taxonomy of Bacteria

The term taxonomy includes the classification, identification and nomenclature of all bacteria. In the narrower sense, classification is the orderly categorization of bacteria into groups (taxa) based on similarities and relationships, which are nowadays expounded by 16S rRNA sequencing analysis as well as phenotypic data. Initially, all prokaryotes are assigned to the "domains" *Bacteria* or *Archeae*. This highest taxonomic rank is followed by the lower ranks *phylum, class, order, family, genus, species* and *subspecies.* As an explanation, a genus would be a group of related species and a family a group of related genera (5). For example, the classification of the species *Streptococcus pneumoniae* would be as follows: p_Firmicutes, c_Bacilli, o_Lactobacillales, f_Streptoccocaceae, g_Streptococcus.

Analyzing bacteria on the phylum level, as done in this study, helps understanding the overall relationship of the present bacteria. The genus (also done in this study) and species levels however have the most clinical relevance. A bacterial species represents the basic unit in taxonomy. It can be an organism with specific characteristics or a group of organisms that share the most important qualities of their organization (5). The number of new species-descriptions has increased dramatically in the last years, still many, if not most species are yet to be isolated (6).

1.1.2 The Microbiome of the Gastrointestinal Tract

Since the introduction of next-generation sequencing technologies, the knowledge on the composition of human microbial communities has expanded notably. Still, information on the exact composition, especially on the species level, is lacking (7).

Several studies have revealed the presence of a so-called "core" microbiome and a "variable" microbiome. The core microbiome is similar in all individuals and is composed of the most predominant species that grow at different sites of the body under healthy circumstances.

In contrast, the variable microbiome is unique for each individual as its composition depends on genotypic and phenotypic parameters (e.g. gender, ethnic group) as well as distinctive lifestyle and environmental factors (e.g. diet, medication, stress, smoking) (4,8).

Moreover, the existence of three robust clusters of the human microbiome (so-called enterotypes or enterogradients) that vary in species and functional composition is being debated controversially. What enterotype is present in each individual most likely depends on genetic host factors and environmental influences (7).

Although there are over 50 bacterial phyla known to exist on earth, human microbial communities are dominated by only four different phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. Nine other phyla (e.g. *Spirochaetes*, *Clamydiae*, *Fusobacteria*) are found in some sites and individuals. On the family and genera level these microorganisms are very much alike in most individuals, the respective species and strains however can be just as unique for each individual as a fingerprint (9). The variation of microbial communities over time is consistently lower intra-individually than between healthy individuals. This indicates that the uniqueness of a person's microbiome seems to stay relatively stable over time (10).

Throughout most time of life, the relationship between the human host and its microbiome is either symbiotic (beneficial to both) or commensal (beneficial to only one partner while not harming the other). Development of pathogenic interactions is comparatively rare (2).

The oral microbiome represents a particularly interesting part of the human microbiome, as the oral cavity is a major entrance point to the body. Hence, microbiota that grow within the oral cavity have a high chance of spreading to neighbouring sites of the body resulting in not only local infections but also systemic diseases such as bacterial endocarditis, aspiration pneumonia or cardiovascular diseases (11,12). Overall, more than 700 different species of bacteria have been detected to live inside the oral cavity (however, only less than 100 phylotypes per individual (13)), the majority of which belongs to the phylum of *Firmicutes* (12,14).
The stomach, the least colonized part of the GI tract, represents a very distinct ecological habitat for microbiota. Although the bacterial load of swallowed food is largely reduced by gastric acid, still numerous bacterial species managed to adapt to the extreme acidic conditions. At the phylum level *Firmicutes* and *Proteobacteria* dominate the gastric mucosa, whereas *Firmicutes*, *Bacteroidetes* and *Actinobacteria* are most abundant in the gastric fluid (3,15,16).

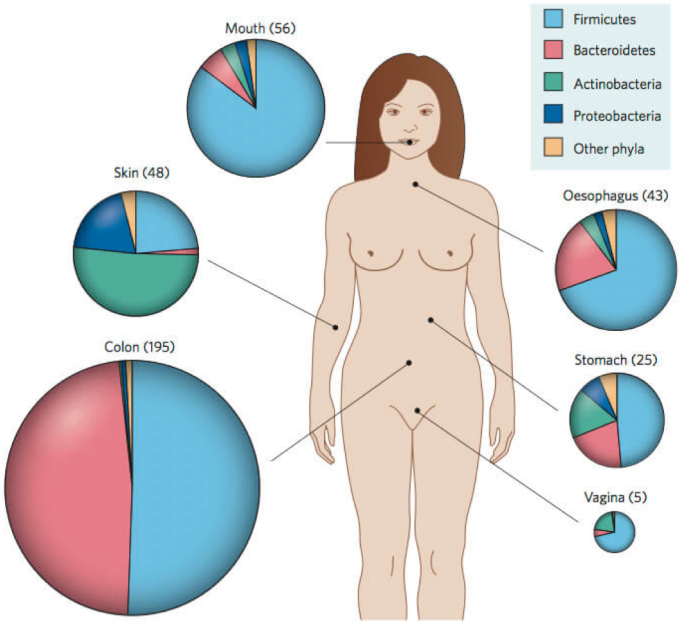

Figure 1. Bacterial composition on the phyla level at different body sites in healthy human beings (9).

The relatively low density of microbiota also characterizes the small intestine, encompassing duodenum, jejunum and ileum. This low colonization is due to strong peristaltic bowel movements, fast transit as well as bactericidal secretions (e.g. bile acid and pancreatic juice). It must be noted although that as the small intestine is poorly accessible in healthy individuals, information about the located microbiota is limited (3,17).

The most colonized part of the human body is the large intestine, consisting of the cecum, ascending-colon, transverse-colon, descending-colon, rectosigmoid-colon and the anal canal. As in the rest of the GI tract, all four major phyla can be found in the colon. However, more than 90% of all bacteria in this ecosystem belong to the phyla of *Firmicutes* and *Bacteroidetes*. *Firmicutes*, who are often dominating, are mainly divided into the two classes of Gram-positive bacteria: *Clostridia* (making up about 95%) and *Bacilli.* The *Bacteroidetes* phylum comprises only Gram-negative bacteria. On the genus level the three major groups *Bacteroides*, *Prevotella*, and *Porphyromonas* can be divided (3,18).

1.1.3 The Microbiome of the Respiratory Tract

The human respiratory tract, ranging from the nostrils to the alveoli, is inhabited by niche-specific diverse microbial communities. Similar to the GI tract, the 4 major phyla *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* are represented.

In the pharynx, proportions of *Bacteroidetes* are found to be greater than in other studied body sites (skin, vagina, intestines). On the genus level *Prevotella, Campylobacter, Veillonella, Streptococcus* and *Neisseria* are among the most prevalent bacteria (19).

Contrary to the long-lasting belief of the lungs being a sterile environment, microbial communities can be found from the trachea to alveoli. This is reasonable, since the lower respiratory tract is continuously connected to the external world as long as the larynx is open. The main immigration routes of bacteria to the lower respiratory tract are via microaspiration, inhalation of air and direct dispersion along mucosal surfaces. As a result, the microbiome of the lungs does not distinctly differ from that of the pharynx and oral cavity, however a relatively lower microbial biomass can be seen (2-4 times lower than in the pharynx). This could be due to the nutrient-poor environment of the lower respiratory tract and the selectively bacteriostatic surfactant produced by the alveoli. Although environmental conditions (e.g. oxygen concentration, pH, relative perfusion and ventilation, temperature) show a great

regional variability within the lungs of a single person, the overall microbial composition is relatively homogenous (19,20).

1.1.4 Functions of the Microbiome

Advances in next-generation sequencing have immensely improved the knowledge and understanding of the various functions of the microbiome. Increasing evidence indicates that most, probably all biological pathways connected to health, disease and aging are affected by the composition of these microbes and their activities in some way. More specifically, these functions comprise metabolic, protective and structural qualities, which are outlined closer in the following (3,21,22).

1.1.4.1 Development of Adaptive Immunity

One fundamental function of intestinal microbiota is their role in shaping the adaptive immune development.

It is known that germ-free mice (GF mice), born and raised in complete absence of microbes, have a vastly undeveloped immune system compared to mice with normal microbial populations. Changes found in these animals provide important information of how microbes influence the immune system: In GF mice the development of the gut-associated lymphoid tissues (GALT), representing the first line defence of the intestinal mucosa, is defective. Moreover, they show fewer and smaller Peyer's patches and mesenteric lymph nodes. Besides changes in tissue formation also molecular changes can be observed in the intestinal immune system. For instance, major histocompatibility molecules (MCH II) and toll-like receptors (TLRs), which are important for pathogen sensing and antigen presentation, are largely reduced in number in GF mice. Extra-intestinal changes, such as fewer CD4+ T-cell counts in the spleen as well as reduced antibody levels suggest that microbes even effect the maturation of systemic immunity (21,23,24).

1.1.4.2 Metabolism

The human microbes hold a wide range of metabolic functions that unquestionably influence the host's physiology and pathology. Again, findings in studies of germ-free mice can

underline the importance these microbes in human energy metabolism: GF animals need up to 30% less caloric intake compared to normal animals to uphold their body weight. These findings reasonably indicate that host and microbes may *compete* for the available nutrients (2).

Especially concerning carbohydrate digestion some very important functions of microbiota are known, but also protein and lipid metabolism may be influenced.

Complex plant-derived polysaccharides and unhydrolyzed starch reach the distal GI tract mostly undigested, since human enzymes cannot process these nutrients. However, anaerobic bacterial populations in the colon can hydrolyse complex carbohydrates to very important short chain fatty acids (SCFA). These SCFAs, acetate, propionate and butyrate are a major energy source to local bacteria and the intestinal mucosa on the one hand (mainly butyrate) and after subsequent absorption to different organs and tissues (mainly acetate and propionate) (2,25,26). In the liver for example, acetate and propionate serve as substrates for gluconeogenesis and lipogenesis (27). This arrangement promotes the symbiotic relationship between host and microbes, where the host can absorb valuable nutrients from otherwise undigested dietary carbohydrates, while bacteria are supplied with an abundance of fermentable carbon sources (28).

As to the impact of microbiota in protein metabolism, increasing evidence indicates that bacteria in the gut are able of *the novo* synthesis of essential amino acids as well as intestinal urea recycling. To determine the exact contributions of microbiota in protein metabolism, more work on this topic is needed (2,29,30).

The mechanisms by how gut microbiota effect lipid metabolism are not yet fully understood. However, by studying GF mice and human models some key aspects have been discovered in the last years of research. Firstly, energy storage in the body is regulated by the amount of lipogenic substrates (SCFAs, monosaccharides) that reach the liver. Several studies have demonstrated that levels of both SCFAs and monosaccharides are lower in GF mice, leading to a decrease in lipogenesis. Secondly, lower expression of the enzyme lipoprotein lipase (LPL) is seen in GF mice, which influences the release of fatty acids and triacylglycerol from circulating lipoproteins (31-35). Moreover, bile metabolism may be influenced by intestinal microbiota. Certain facultative and anaerobic bacteria have been shown to transform primary bile acids from the liver into secondary bile acids. A small part of these bacterially produced

bile acids gets absorbed in the distal ileum and can subsequently influence hepatic as well as systemic lipid and glucose metabolism via certain receptors, which mainly react to secondary bile acids (27,36,37).

1.1.4.3 Antimicrobial Protection

The tight colonization of the intestine with commensal microbiota plays an essential role in preventing an overgrowth of opportunistic pathogens, which, if present in sufficiently large number, can lead to disease (38).

Certain human and bacterial mechanisms allow the preservation of the healthy homeostasis between commensal and resident opportunistic bacteria in the gut.

Firstly, the two-tiered mucous layer of the large intestine plays an important role in antimicrobial protection, as it inhibits the contact of luminal bacteria with epithelial cells. More important in the small intestine, where the mucous layer is discontinuous (mucus production takes place in the top of crypts, the tips of the villi are not always covered (39)), is the production of antimicrobial proteins. Paneth cells under the influence of local microbiota produce these proteins, including cathelicidins, C-type lectins and defensins (24,40).

Secondly, the protective function of commensal bacteria plays a decisive part in fighting pathogens. One example is the production of lactic acid by *Lactobacillus* spp. Lactic acid can intensify the antimicrobial effect of human lysozyme, which destructs the outer membrane of bacterial cell walls (41).

Furthermore, microbiota, especially Gram-negative *Bacteroides* have generated methods to inhibit commensal bacteria from penetrating into the blood circulation. The underlying mechanism involves the activation of mucosal plasma cells to produce secretory IgA, which in further consequence leads to destruction of specific commensals without inducing general inflammatory responses (42).

Another mechanism of antimicrobial protection by commensals is the so-called colonization resistance. It is defined as the ability of indigenous microbes to inhibit the growth of foreign bacteria as well as the overgrowth of resident bacteria (commensal and pathogenic), which are normally only present at low levels. Stable and highly diverse bacterial communities as well as a lack of inflammation are necessary to obtain this condition.

The use of antibiotics or the presence of highly virulent bacteria can disrupt this harmonic intestinal ecosystem, paving the way for colonization by pathogens. Antibiotic-associated colitis due to *Clostridium difficile* infection is an example of impaired colonization resistance leading to disease (43,44).

1.1.4.4 Maintenance of the Structure and Function of the GI Tract

Increasing evidence supports the role of microbiota in maintaining the structure and function of the GI tract as well as the integrity of the gut barrier.

The observation of GF mice again highlights possible changes due to missing microbes. GF mice appear to have a smaller intestinal surface area (45), thinner villi due to reduced regeneration (46), a diminution of the villi capillary network (28) as well as perturbed peristalsis (47) – all factors that may lead to a decrease in nutrient digestion and absorption.

Some specific functions of particular bacteria have been discovered, for example the capability of *Bacteroides thetaiotaomicron* to express special proteins necessary for desmosome preservation (48). Furthermore, *Lactobacillus rhamnosus GG* strain can produce proteins that are able of inhibiting cytokine-induced apoptosis of intestinal epithelial cells (49,50).

1.2 The Microbiome in Critically Ill Patients

The condition of critical illness, commonly comprising sepsis, acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS), causes an enormous part of global mortality as well as an economic challenge. The physiological and pathological alterations of critical illness just as interventions of modern intensive care undoubtedly change the composition and function of intestinal microbiota (51,52), leading to so-called "dysbiosis", an overgrowth with low-diversity pathobiota (53).

1.2.1 Environmental Changes During Critical Illness Affect the Microbiome

Generally, the composition of a microbial community relies on the elimination rate of its members, the immigration rate of new members and the reproduction rate. All three factors are significantly influenced by the state of critical illness (51).

Under healthy circumstances gut bacteria can be rapidly eliminated via transit through the GI tract and lastly defecation. For example, a healthy human being excretes about 10^{14} bacterial cells each day (54). During critical illness, the transit time of bacteria through the GI tract is markedly reduced due to endogenous changes (e.g. electrolyte alterations, opioid production, cytokine production) on the one hand as well as medication (e.g. opiates, systemic catecholamines, sedatives, proton pump inhibitors) (51). Other ways of eliminating microbes from the GI tract, such as the production of bile salts (55) and antimicrobial peptides (56) as well as IgA excretion by plasma cells (57) can additionally be reduced as a consequence of critical illness. The result of the decrease in microbial elimination is an overgrowth with gram-negative bacteria, which are among the most prevalent ICU pathogens (58).

As to the growth conditions of intestinal microbiota during critical illness, there are multiple factors known to influence the environment in a way so that normal reproduction is impeded. It has been shown that intestinal inflammation can alter microbial communities, whereby the number and diversity of commensal bacteria is reduced while potentially pathogenic germs arise. Detailed explorations of these altered microbiota show a reduction of normally predominant *Firmicutes* (including *Clostridia* spp), whereas colitogenic species (able of

inducing colitis) such as *Proteus mirabilis* and *Klebsiella pneumonia* are increasingly presented (59). Moreover, it is known that intestinal epithelial hypoxia and splanchnic hypoperfusion, common consequences of surgical injury, can lead to perturbation of normal microbial growth. Instead, the overgrowth of more virulent pathogens such as *Pseudomonas aeruginosa*, one of the most frequent pathogens in critically ill patients and associated with the highest mortality rate, is promoted (60,61).

In order to survive the tough environmental changes occurring over the course of prolonged critical illness, some bacteria have evolved the capability of switching on virulence genes, shifting their behaviour from commensal to pathogenic (62). Several mediators released during host stress are known to activate those virulence genes. Endogenous opioids for example are among the first signals to be set free from different tissue sites. A number of studies could show that *P. aeruginosa* can intercept parts of these opioids, resulting in a state of higher virulence in which extremely harmful behaviours can be expressed (53,63). In mice models, similar effects have been shown for endogenous catecholamines: Norepinephrine, which is released under host stress, can increase certain binding proteins that in turn lead to significantly higher virulence of *P. aeruginosa* (60).

As to the impact of clinical interventions commonly used in intensive care such as pharmaceutical agents (e.g. systemic antibiotics, proton pump inhibitors, opioids, vasopressors) or parenteral feeding, almost all of them can change the intestinal growth conditions for bacteria (51).

1.2.1.1 Antibiotics

Over the last decade, several studies could demonstrate significant effects of systemic antibiotic treatment on the intestinal microbiota. Acute side effects can range from self-limiting "functional" diarrhoea to life-threatening *Clostridium difficile* infection (pseudomembranous colitis) (64).

As investigations of mice and human guts could show, antibiotic therapy can decrease the total number, diversity, taxonomic richness as well as the evenness of the microbial community. While a complete sterilization of the intestine with antibiotics is hardly possible, some bacterial species react stronger to common antibiotics than others. The *Firmicutes* class for

example, seems to be most susceptible to many antibiotics. In contrast, other normally low-abundant species can become more dominant due to the environmental changes caused by antibiotics. Despite the pervasive antibiotic-induced perturbations, after ceasing treatment the total number of intestinal microbes recovers to the pre-therapeutically state within 1 to 4 weeks. However, the effects on specific microbial groups can persist for months or even years (43,64,65). New research was able to demonstrate that antibiotic treatment can result in the translocation of commensal bacteria across the colonic epithelium, leading to an inflammatory response and the possibility of worsened disease due to the simultaneous epithelial damage (66).

1.2.1.2 Proton Pump Inhibitors

Proton pump inhibitors (PPI) are among the ten most frequently used drugs worldwide. Their long-term usage is known to increase the risk for enteric infections, especially those with *C. difficile*, where the incidence is elevated by 65% compared to patients without PPI treatment (67). Also, the risk for infections with *Salmonella* spp, *Campylobacter* spp or *Shigella* spp is significantly increased (68). Different studies could show that PPIs lead to distinct changes in microbial composition as well as a reduced diversity (69-71). These changes are most possibly due to the reduced acidity of the stomach, resulting in an increased survival of swallowed bacteria, which would have normally perished. This hypothesis is underlined by the fact that species of the oropharyngeal microbiome, such as *Enterobacteriaceae, Micrococcaceae, Actinomycetaceae,* are more prevalent in the gut of PPI users (70).

1.2.1.3 Parenteral Nutrition

As to the ability of fulfilling the nutritional demands of a critically ill host, both enteral and parenteral feeding methods are equally effective. However, some disadvantages on the microbial level have been observed for parenteral nutrition. For one, the enteral uptake of nutrients seems to be an essential condition for the maintenance of mucosal immunologic functions. These functions include the production and secretion of secretory immunoglobulin A (sIgA), the primary defence molecule within the GI tract. It has been shown that under parenteral feeding IgA secretion is impaired, leading to mucosal inflammation (72,73).
Furthermore, there is evidence that the use of total parenteral nutrition or enteral nutrition with

processed liquid diets can provoke the translocation of intestinal bacteria to extraintestinal sites in the body, increasing the risk of infection (2).

Eventually all these ecologic alterations result in an unstable and often disrupted bacterial community- in extreme cases ultra-low diversity communities consisting of only 2 to 4 multidrug-resistant pathogens can remain. Frequently dominant species in critically ill are *S. aureus*, *Enterococcus* spp, *E. coli*, *Klebsiella* spp or *P. aeruginosa*, the latter one being very scarce under normal conditions. All these bacteria represent a threatening source for disseminating pathogens. In addition, some usually rare fungi can be found in abundance, especially *Candida albicans*, which is known to be a major cause of late-onset sepsis and mortality in critically ill (51,53).

1.3 Health Care-Associated Infections on the ICU

Health care-associated infections (HAI), formerly termed "nosocomial", are a major problem in intensive-care units. The CDC (Center for Disease Control and Prevention) defines HAI as "a localized or systemic condition resulting from an adverse reaction to the presence of an infectious agent(s) or its toxin(s). There must be no evidence that the infection was present or incubating at the time of admission to the acute care setting." These infectious agents can derive from endogenous (different sites of the patient's body) or exogenous sources (e.g. visitors, health care environment, medical devices).

In septic patients, the most frequent causes of infection are urosepsis, pneumonia, blood-stream infections, intra-abdominal infections, surgical wound infections and intravascular catheter-related sepsis (74,75).

1.3.1 Urosepsis

While urinary tract infections (UTI) among uncatheterised patients hardly play any role in causing systemic infections, catheter-associated UTIs (CAUTI) are the most frequent HAI within the USA (40% of all HAIs). The risk of developing an infection expectably depends on the duration of the catheter application; after 30 days, an infection is almost certainly. Especially important for ICUs is the fact that silent catheter-associated bacteriuria represents a relevant source of resistant germs. Similarly, the presence of isolated candiduria, mostly not needing treatment, can sometimes be the only sign of invasive candidiasis/candidemia.

Typical clinical symptoms of urosepsis include fever (over 38°C), urgency, frequency, dysuria, suprapubic tenderness or pyuria. Additionally, a positive urine culture is necessary for diagnosis (74,75).

1.3.2 Pneumonia

Health care-associated pneumonia (HAP) can either be defined as early onset or late onset pneumonia. Early onset pneumonia generally arises during the first 4 days of hospitalisation. The most frequently observed pathogens are *Moraxella catarrhalis*, *Haemophilus influenza* and *Streptococcus pneumoniae*. In contrast, late onset pathogens are mainly gram-negative

bacteria (especially *P. aeruginosa*) and *S. aureus*, methicillin-resistant S. aureus (MRSA) inclusive. Recently, the concept of early or late HAP has been questioned (76).

To confirm the clinical suspicion of HAP, an infiltration in chest-x-ray is required. To determine the chance of this infiltrate being of infectious origin, the factors fever, white blood cell count, sputum purulence, oxygenation and presence of pathogens in lower respiratory tract secretions should be measured. To assess the aetiology of pneumonia, microbiological samples need to be obtained and processed in the laboratory (74,75).

ICU patients requiring endotracheal intubation and mechanical ventilation are especially threatened of developing ventilator-associated pneumonia (VAP). The endotracheal tube can lead to iatrogenic lung injury as a result of improper setting and can impair the respiratory defences of the lungs, thus promoting the development of infection. The main pathophysiological mechanisms of VAP are the microscopic aspiration of colonized oropharyngeal secretions into the lower-respiratory tract, reduced bacterial clearance of the lung as a result of positive pressure ventilation and lastly the accumulation of secretions inside the bronchial system, which leads to subsequent overgrowth by pathogenic bacteria (77). Importantly, there is increasing evidence that pathogens overgrowing the stomach in critically ill patients can promote the development of VAP. These gastric microbiota, mainly enteric gram-negative bacteria, are the same that are frequently isolated from the sputum of patients suffering from VAP. To fully understand the importance of this so-called gastro-pulmonary route of infection, more research is needed (78-80).

VAP has been shown to increase the length of mechanical ventilation, ICU stay and hospital stay and to worsen a patient's outcome. Preventive measurements such as correct positioning of the mechanically ventilated patient, decontamination of the oropharyngeal microbiota or drainage of subglottic secretions can significantly reduce the risk of VAP (77,81).

1.3.3 Bloodstream Infections

Bloodstream infections (BSIs) are an important cause of severe sepsis and septic shock in ICU patients, accounting for up to 40% of all nosocomial infections. BSIs can be divided into a primary and secondary form: the primary form includes all BSIs that do not show a causative origin of infection. Secondary BSIs are caused by pathogens originating from an infection at

another body site. Frequent pathogens of BSI include *S. aureus*, *Enterococcus* spp, *Klebsiella* spp, *E. coli*, *Pseudomonas* spp, *Candida* and others. Common skin contaminants such as *Corynebacterium* spp, *Bacillu*s spp or coagulase-negative *Staphylococcus* can also cause BSI, however they require confirmation by at least two blood cultures drawn at different times. In patients with certain underlying conditions, e.g. prosthetic heart valves, stents in main arterial vessels, prosthetic joints or other foreign bodies, even a single positive blood culture with skin contaminants is suspicious of true bacteraemia, as these organisms form biofilms on foreign material (74,75).

1.3.4 Intra-Abdominal Infections

Intraabdominal infection can present a very heterogeneous appearance depending on their exact localisation. While retroperitoneal infections usually show a rather indolent development, intraperitoneal infections mostly appear with notable clinical symptoms. Peritoneal infections can be categorized by their aetiology: Primary peritonitis, also called spontaneous bacterial peritonitis, mainly occurs in patients with hepatic liver cirrhosis. There are no obvious visceral pathologies (e.g. abscess, perforation, localized infection, ischemic necrosis, penetrating injury, ileus) apparent, but pre-existing ascites is infected by translocation of intestinal pathogens or during bacteraemia. Secondary peritonitis develops as a result of the just mentioned visceral pathologies. The infectious agents are microorganisms from the intestinal microbiome. Depending on which part of the GI tract is affected, different germs will be present – anaerobic bacteria, for example, will be rare if the pathology is localized in the stomach, instead *Candida* are more likely. Tertiary peritonitis develops after appropriately treated primary or secondary peritonitis (≥48 hours) and is mainly caused by hospital-associated germs or fungi such as coagulase-negative *Staphylococcus*, *Pseudomonas*, *Enterococcus* or *Candida* (74,75).

1.3.5 Skin and Soft-Tissue Infections

The clinical spectrum of skin and soft-tissue infections (SSTI) is wide, ranging from focal cellulitis to life-threatening necrotizing fasciitis. These infections can develop spontaneously or as a result of local trauma or surgery, the latter being referred to as surgical site infection

(appearing within 30 days after the procedure and at the surgical site) (75). In most of the cases soft-tissue infections will present with local inflammatory changes, however septic complications are possible, mainly resulting from highly virulent pathogens, deep surgical site infections (affecting muscle of fascia) or tissue necrosis. Clinical examinations need to be done carefully as emerging necrotizing infections may initially present with only little cutaneous signs. Examples of non-surgical soft-tissue infections are erysipelas, folliculitis, pyoderma, abscess or necrotizing fasciitis (74,75).

1.3.6 Catheter-Related Infections

Catheter-related infections (CRIs), regarding central-venous catheters, appear as severe sepsis, shock, bacteraemia without other potential sources of infection being present or local soft tissue infections. As long as no appropriate culture results are available, CRIs are mostly diagnosis of exclusion (74). However, newer diagnostic measures, e.g. paired blood cultures with different time to positivity or Gram stain/Acridine orange Leukocyte cytospin test, allow fast and accurate diagnosis of catheter-related blood stream infections (CRBSI) (82).

The risk of developing a CRI increases when placing a catheter for more than 7 days and when placed under unsterile conditions. Clinical signs of localized CRI include erythema, cellulitis and especially pus in the catheter region, however most systemic infections do not show any local inflammatory changes (74).

1.4 GI Failure in ICU Patients

Gastrointestinal symptoms such as paralysis with absent or reduced bowel sounds, delayed gastric emptying or diarrhoea are very frequent within critically ill patients. According to a study from Reintam et al. around 59% of ICU patients suffer from at least one gastrointestinal symptom during their ICU stay (83). The so-called "gastrointestinal dysfunction", or in its most severe form named "gastrointestinal failure" is known to significantly impair the outcome of those affected. Although an exact definition of GI failure is still lacking, the term encompasses all changes of gut physiology that ultimately lead to intestinal barrier dysfunction, bacterial translocation and subsequent development of sepsis, cytokine-induced systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS), multiple organ dysfunction syndrome (MODS) and eventually death (84-86).

1.4.1 Multiple Organ Dysfunction Syndrome (MODS)

Although patients in intensive-care units are largely different concerning their gender, age, clinical history and actual disease, many of them suffer from MODS as a common premortal condition. The prevalence is especially high in patients with trauma, sepsis or shock. MODS is defined as "the development of potentially reversible physiologic derangement involving two or more organ systems not involved in the disorder that resulted in ICU admission, and arising in the wake of a potentially life threatening physiologic insult" (87,88).

The role of GI failure in the emergence and development of MODS has been known for years. The exact pathophysiological mechanisms of GI failure, however, are not yet fully discovered. It is most likely although that either direct organ injury or splanchnic hypoperfusion (due to an insult) can trigger the development. The result of both ways is the induction of immunoinflammatory responses, loss of mucosal barrier function and translocation of bacteria and toxins into the blood circulation and lymph system. Ultimately these events pave the way for the development of SIRS, ARDS and MODS (Figure 2) (84-86).

The translocation of intestinal bacteria across the mucosa to normally unsterile compartments such as mesenteric lymph nodes or other organs has been intensely studied over the last decade. This translocation process is thought to be physiologic, serving immunomodulatory

functions. Only when host defences are significantly impaired, just as it occurs in critical illness, the homeostatic condition gets lost and septic complications may arise.

Several factors can promote bacterial translocation: bacterial overgrowth, intestinal obstruction (leading to a breakdown of epithelial tight junctions) or ischemia/reperfusion injury as a result of hypoperfusion (84).

1.4.2 GI Failure Score

Evaluation of GI function in critically ill patients is difficult. On the one hand, many abdominal symptoms such as distension, discoloration of the abdominal skin or pain are often not solely related to the GI tract and on the other hand patient assessment, particularly pain evaluation, is difficult in sedated and mechanically ventilated patients (86).

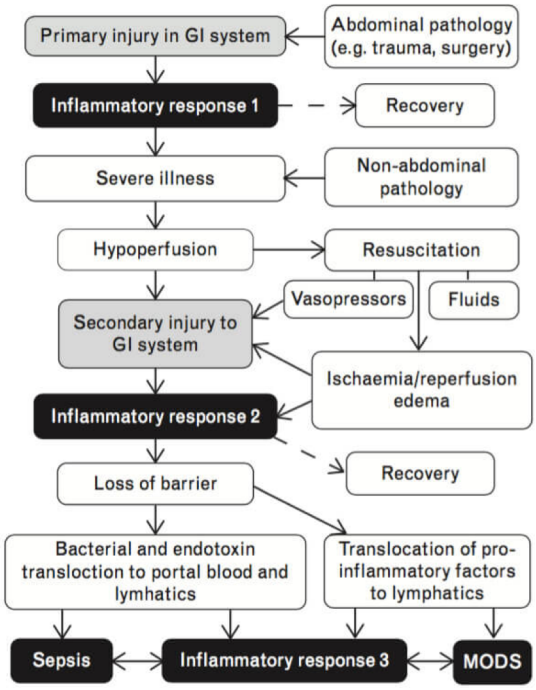

Figure 2. The pathophysiological events of acute gastrointestinal injury and their impact on the development of MODS (86).

To facilitate the evaluation of gastrointestinal symptoms in critically ill patients, Reintam et al. designed the Gastrointestinal Failure (GIF) score resting upon the presence of food intolerance

and intraabdominal hypertension (IAH) in patients (Table 1). The score defines GI failure to be the simultaneous presence of feeding intolerance and IAH (85).

Feeding intolerance is a common sign of GI dysfunction or failure, clinically identifiable as large gastric residual volumes (GRV) during enteral feeding. Scintigraphic investigations measured a GRV of at least 250ml to be predictive for delayed gastric emptying (86,89). However, some studies suggest that other factors such as diarrhoea, vomiting, ileus, bowel distension or abdominal pain would be more representative signs of feeding intolerance (85,90,91).

Intraabdominal hypertension is defined as an intraabdominal pressure persistently elevated above 12 mmHg and is a relatively common problem in ICU patients, showing a prevalence of 20-40%. It should be measured at least twice a day by intravesical catheters, even four times when measurements are pathological. The daily mean parameter should be used for calculating the GIF score. Abdominal compartment syndrome is the combination of an intraabdominal pressure above 20 mmHg and new or worsening organ failure, representing a potentially life-threatening condition (85).

GASTROINTESTINAL FAILURE SCORE

Points	Clinical symptomatology
0	Normal gastrointestinal function
1	Enteral feeding <50% of calculated needs or no feeding 3 days after abdominal surgery
2	Food intolerance (enteral feeding not applicable due to high gastric aspirate volume, vomiting, bowel distension, or severe diarrhoea) or IAH
3	Food intolerance and IAH
4	Abdominal compartment syndrome

Table 1. GIF score as designed by Reintam et al. (85)
IAH: intraabdominal hypertension, intraabdominal pressure above 12 mmHg; Abdominal compartment syndrome: intraabdominal pressure above 20 mmHg and new or worsening organ failure.

Although the exact pathophysiological correlation of IAH and GI failure is not yet completely understood, several studies could show an increased occurrence of GI symptom in patients with IAH as well as higher mortality rates. One fundamental problem could be the reduced blood flow to intraabdominal organs due to IAH, leading to functional impairment (85,86,92).

It was shown that a high mean GIF score during the first 3 days of a patient's stay on the ICU is significantly associated with higher rates of mortality. However, it must be noted that IAH does not specifically represent GI failure and therefore limits the use of the GIF score for evaluating GI function. Moreover, a more accurate definition of feeding intolerance would be needed to make the score comparable (84,85).

1.5 Analysis of the Human Microbiome

For over more than a century, scientists used conventional methods to study the microbial communities colonizing the human body, especially cultivation-based techniques. Concerning intestinal microbiota, these methods only revealed a minority of the present species, mainly because most intestinal bacteria are strictly anaerobic, only surviving a few minutes in the presence of oxygen. The development of cultivation-independent, molecular methods over the last decades has immensely broadened the understanding of composition and diversity of the human intestinal microbiota. These so-called metagenomics are based upon the analysis of the genomic content of microorganisms and comprise several next-generation sequencing methods, the most common one being small subunit (16S) ribosomal RNA gene sequencing (16S rRNA).

Genes that encode 16S rRNA are present in almost all bacteria, which is why they are chosen for analysis. Via 16S rRNA sequencing, bacteria can be taxonomically identified from the phylum level to approximately the genus level.

As to the procedure, by amplification and cloning sequencing of DNA segments using PCR, the 16S rRNA genes are generated. In further consequence, these can be compared with several database libraries to identify the exact organism (3,4,9,93).

The application of 16S rRNA sequencing is especially important for identifying rare bacteria or bacteria that are slow growing, uncultivable or all such showing unusual phenotypic profiles. Clear advantages are the rapid feasibility and accuracy. On the other hand, it must be noted that PCR may subject to bias and is sensitive to contamination. In practice, to detect the presence of already identified species, examiners can also use conventional microbiologic methods such as culture analysis, microscopy, enzyme analysis or immunoassays. Admittedly,

these methods are less advanced, yet they provide important information about the physical, chemical and metabolic characteristics of bacteria (4,94).

In order to identify the composition of intestinal microbiota, mainly faecal samples have been studied. However, also data from mucosal samples (biopsies, surgical specimens) are available. This is important, as mucosal-associated bacterial communities are known to considerably differ from faecal microbiota (1,95).

While 16S rRNA sequencing only offers taxonomic information helping to define bacterial diversity or dysbiosis, more complex technologies like so-called shotgun metagenomic sequencing can also provide functional information of the microbiome.

Shotgun sequencing examines the total DNA extracted from a microorganism, not only the 16S rRNA gene. By identifying functional genes, genes specific for certain diseases, antibiotic resistance genes or by discovering novel genes, this method provides numerous important insights into the bacteria- host relationship.

Unfortunately, there are still some factors limiting the widespread use of shotgun sequencing, especially the great costs and time, resulting from the high sequence coverage.

However, future improvement of these new technologies will immensely extend our knowledge on bacterial composition and function, inter-individual differences and on the role of microbes in the development of disease (4,96).

2 HYPOTHESES AND AIMS

2.1 Hypotheses

In this pilot study of 6 ICU patients, the following hypotheses were postulated:

1. Typical changes in microbiota composition can be observed in critically ill patients shortly after admission to the intensive care unit as well as over the longer course of hospitalisation.
2. These alterations can be observed not only in the intestinal microbiome, but also in microbiota communities of the pharynx, tracheobronchial secretion and gastric secretion – body regions not yet examined in critically ill patients.
3. Clinical interventions (e.g. medication, surgery, mechanical ventilation) as well as host factors (e.g. infections) might play an important role in changing microbiota composition.

2.2 Aims

To investigate the hypotheses listed above, six patients admitted to the intensive care unit were included into the study. Of each patient, multiple samples from the mentioned body areas were obtained at several time points during hospitalisation and analysed for their bacterial content. The clinical history of each patients was gathered retrospectively.

The aims of this study are as follows:

1. To investigate changes in microbiota composition in critically ill patients shortly after ICU admission as well as over the course of hospitalisation.
2. To investigate the microbiome of the pharynx, feces, tracheobronchial and gastric secretion over the course of time.
3. To explore short- and long-term changes of bacterial diversity and richness.
4. To examine possible influencing factors of these microbiota alterations, e.g. clinical interventions, medication, infections.

3 MATERIAL AND METHODS

This study was part of an ongoing prospective observational study investigating microbiota and mycobiota in various body locations of ICU patients related to gastrointestinal failure, nosocomial infections and death. Microbiota data from 6 patients were used for this thesis.
The human studies were approved by the Ethics Committee of the Medical University of Graz (protocol number: 19-322 ex 07/08).

3.1 Patient Selection

The study cohort comprised 6 patients (2 women, 4 men) who were admitted to the internal intensive care unit of the University Hospital Graz during between May 2015 and October 2015. The mean patient age was 66 years (age range 53-80 years).
The admitting diagnosis was an infection in 3 cases, cardiac arrest in 2 cases as well as respiratory insufficiency and delirium in one case.
The patients included where those showing a high chance of a prolonged ICU stay.

3.2 Clinical Data

Clinical and demographic variables of each patient were gathered retrospectively from the patient's charts and digital files and compiled in a Microsoft Excel® datasheet. For the individual patient analysis (described below in the results) parameters of interest were extracted from the datasheets.

CLINICAL AND DEMOGRAPHIC DATA	
General data	Age, duration of hospital stay, duration of stay until study inclusion, discharge from ICU and hospital
Actual data	Cause of admission to hospital or ICU, main and secondary diagnosis
Medication	Antibiotics (name, duration, dosage), PPI (name, duration), Analgosedation (name, duration), systemic and inhalative Glucocorticoids (name, dosage, duration), Catecholamines (name, duration)
Other therapies	Mechanical ventilation, diet (oral, tube feeding, parenteral)
Infections during ICU stay	Causative pathogen, onset, duration, diagnostic methods, therapy
Interventions	E.g. intubation, dialysis, gastroscopy, bronchoscopy, surgery
Scores	SAPS score III, GI Failure score
Stool	Daily assessment, categorized from 0 to 4: normal stool, no stool, diarrhoea, stool under medication, melena/haematochezia
Others	Fever (T >37.8°C), residual volume of gastric tube (ml), oxy-index, presence of foreign bodies (e.g. endoprosthetics)

Table 2. Clinical and demographic data.
Clinical and demographic data was gathered retrospectively from each patient.

3.3 Microbiota Analysis

Samples were obtained from the stool, gastric secretion, tracheobronchial secretion and from pharyngeal aspirate. These samples were collected at several time points from each patient; the number of samples taken differs between the patients depending on the length of their ICU stay.

For further analysis, the samples were sent to the Core Facility Molecular Biology of the Center of Medical Research at the Medical University of Graz. Bacterial DNA isolation, PCR amplification and sequencing methods were implemented as described previously by Castellani et al. (97) and Spindelboeck et al. (98). The following descriptions are based on these publications.

3.3.1 DNA Isolation and PCR Amplification

DNA was extracted from stool, pharyngeal aspirate, tracheobronchial aspirate and gastric aspirate using the Magnapure Bacterial DNA Kit (Roche). DNA concentration was measured by picrogreen fluourescence. PCR was used to amplify the variable V1-V2 region of the bacterial 16S rRNA gene from 50ng DNA. Primers were 16s_515_S3_fwd: TGCCAGCAGCCGCGGTAA and 16s_806_S2_rev: GGACTACCAGGGTATCTAAT. Bacterial 16S rRNA was amplified with the Mastermix 16s Complete PCR Kit (Molzym, Bremen, Germany). The first PCR reaction product was subjected to a second round of PCR with primers fusing the 16s primer sequence to the A and P adapters necessary for Ion Torrent sequencing while additionally including a molecular barcode sequence to allow simultaneous multiplexing of up to 96 samples. PCR products were subjected to agarose gel electrophoresis and the band of the expected length (330nt) was excised from the gel and purified using the QiaQick (Qiagen, Hilden, Germany) gel extraction system. DNA concentration of the final PCR product was measured by picogreen fluorescence (97,98).

3.3.2 Sequencing

Amplicons from up to 60 samples were pooled equimolarly and subjected to emulsion PCR using the Ion Torrent One Touch 2.0 Kit according to manufacturer's protocols. After emulsion PCR the beads were purified on Ion ES station and loaded onto Ion Torrent 318 chips for sequencing. Sequencing reactions were performed on Ion Torrent PGM using the Ion 400BP Sequencing Kit running for 1082 flows (all reagents from Thermo Fisher Scientific, MA, USA). Sequences were split by barcode and transferred to the Torrent suite server. Unmapped bam files were used as input for bioinformatics (97,98).

3.3.3 Bioinformatics and Phylogenic Analysis

All sequences were initially trimmed by a sliding window quality filter with a width of 20nt and a cut-off of Q20. Reads shorter than 100 nucleotides and reads mapping to the human genome were removed using deconseq. The resulting reads were subjected to error correction using the Acacia tool leading to error correction of 10-20% of reads. Subsequently PCR

chimeras were removed by usearch algorithm in de-novo and reference based settings. The final sequence files were then analysed by QIIME 1.8 workflow scripts. OTU search was performed using the parallel_pick_open_reference_otus workflow script and the greengenes 13_8 reference database (97,98).

3.3.4 Statistical Analysis and Visualization

OTUs were visualized as OTU tables, bar charts, and PCoA plots using the QIIME core microbiome script. Groupings supplied in the mapping file were tested for statistical significance using the QIIME implementation of the Adonis test and significance of individual bacterial strains was determined by the Kruskal-Wallis test.
To determine the influence of the length of ICU stay, samples were assigned to three groups according to the sampling day related to the duration of ICU stay:

- *Early: sampling day 1 to day 5*
- *Mid: sampling day 6 to day 14*
- *Late: sampling day 15 and onwards*

Further analysis and visualisations were performed with custom R scripts (R 3.2.4). Number of observed species (depth of 8000 sequences per sample) was tested for significance by paired t-tests with FDR adjustment to account for multiple comparisons. Graphics were created using ggplot2 (version ggplot2_2.1.0) based on the relative abundance values at genus level calculated during bioinformatical microbiome analysis using QIIME (97,98).

3.4 Important Metrics Defined

OTU. Operational Taxonomic Unit (OTU) is a taxonomic unit referring to a cluster of 16S rRNA sequences with high similarity (usually 97%). Therefore, OTUs are proxies for microbial „species“ (99).

Diversity. Microbial diversity is defined as the number of different taxonomic units within a certain habitat. Diversity analysis can examine either alpha- or beta-diversity.

Alpha diversity is a measure of diversity within a sample or habitat. Factors such as *richness* (number of OTUs in one sample) and *evenness* (consistency of abundances of different OTUs in a sample) are taken into account when calculation alpha-diversity. The following metrics are commonly used to measure alpha-diversity:

- Observed species: unique OTUs in a sample
- Shannon's Index: measures richness and evenness. The greater the index number, the greater the diversity within the sample.
- Chao1: estimates the species richness. This index was mainly used in this study.
- PD (phylogenetic distance): based on the branch length of taxa.

Beta diversity instead describes how many taxa are shared among multiple samples by comparing the samples to each other. It measures the distance or dissimilarity between each sample pair (99). In this study UniFrac was used to evaluate beta diversity.

Rarefaction. To investigate all samples from a microbiome study at the same sampling depth (number of reads per sample), it is necessary to firstly specify the number of sequences. This can be done by so-called rarefaction analysis, where sequences from a sample are selected randomly to a certain sampling depth. The resulting rarefaction curves are then plotted against alpha diversity from 10% to 100% of all sequences. The sample has been sequenced to a sufficient depth, when the rarefaction curves starts to level off (99).

4 RESULTS

This cohort study examined the in-depth microbiota composition of 6 patients admitted to the intensive care unit of the Department of Internal Medicine at the University Hospital Graz. Microbial samples were collected from feces, pharynx, tracheobronchial and gastric secretions and evaluated via 16S rRNA sequencing methods. Clinical and demographic variables were gathered retrospectively. During the time of hospitalisation, every patient required mechanical ventilation, analgosedation and catecholamine therapy. All patients received proton pump inhibitors (on every day for the whole period), antibiotics (for the majority of days) as well as enteral nutrition (for the majority of days). In 5 out of 6 patients an infection was diagnosed during the time of hospitalisation. 3 patients died and 3 patients survived.
The results of each patient are described in detail on the following pages.

4.1 Patient 1

Patient 1, a 68-year-old man was admitted to the ICU with inspiratory stridor and hypercapnic coma due to pneumococcal sepsis and hypertrophic laryngotracheitis. On day 2 of the ICU stay a pneumococcal bacteraemia was diagnosed by positive blood cultures and treated with Moxifloxacin. On day 3 the patient received a tracheostoma, which was required for successful extubation. The patient recovered from the pneumococcal sepsis, but on day 13 of ICU stay he suffered from a urinary tract infection by *Proteus* spp, treated with Ciprofloxacin (delayed initiation of antibiotic therapy on normal ward on day 20).
As a result of successful mechanical ventilation and medication the weaning could start early on in the course, analgosedation was reduced to a minimum after one week and the patient was transferred to a normal ward after 17 days of ICU stay. The surgical ablation of the hypertrophic laryngeal mucosa was planned. After 23 days of hospital stay the patient was transferred to a nursing home.

Samples for this study were obtained from 5 areas: 4 pharyngeal samples, 4 tracheobronchial samples (one had to be excluded from analysis due to an insufficient number of sequence reads (<8000)), 1 fecal sample, 3 gastric samples (2 had to be excluded from analysis due to an insufficient number of sequence reads (<8000)) and one sample from the perianal skin region. The time points of sampling are explained below.

CLINICAL DATA OF PATIENT 1

Main diagnosis	Global respiratory insufficiency and pneumococcal sepsis
Comorbidities	COPD, Mb. Little, OSA
Medication	Antibiotics: Moxifloxacin (day 1-14), Ciprofloxacin (day 20-24); Corticosteroids (p.i. and i.v.), Analgosedation, Catecholamines, PPI
Infections	BSI: *Pneumococcus* spp (diagnosis day 2), UTI: *Proteus* spp (diagnosis day 13)
Mechanical ventilation	Invasive (endotracheal tube, tracheostoma)
Interventions	Tracheostomy
Other findings	Fever, tube feeding
Hospital stay	23 days
ICU stay	17 days
Outcome	Alive

Table 3. Patient 1 – Clinical data.
COPD: chronic obstructive pulmonary disease, OSA: obstructive sleep apnoea, BSI: blood stream infection, UTI: urinary tract infection.

4.1.1 Pharynx

Samples from the pharynx were collected on day 2, 4, 8 and 15 of the patient's ICU stay (Figure 3). On the phylum level a relatively constant microbial composition is seen over the course of the patient's illness, with *Firmicutes, Bacteroidetes* and *Fusobacteria* making up the majority of the 16S rRNA reads. A significant increase in the prevalence of *Firmicutes* and a decrease of *Bacteroidetes* is noticeable from day 8 to 15.

Analysis of the genus level shows a diverse microbiota population on the first 3 samples with a dominance of *Fusbacterium and Prevotella*.

Reads from the latest sample reveal a clear change in microbial composition – *Streptococcus*, already present on day 2 with around 10% emerged again on day 15 with a prevalence of 30% (possibly related to the blood stream infection with *Pneumococcus*/*Streptococcus pneumoniae,* clinically present from day 2 to 14). Furthermore, *Veillonella* (Firmicutes phylum) presented around 15% of all reads on this day.

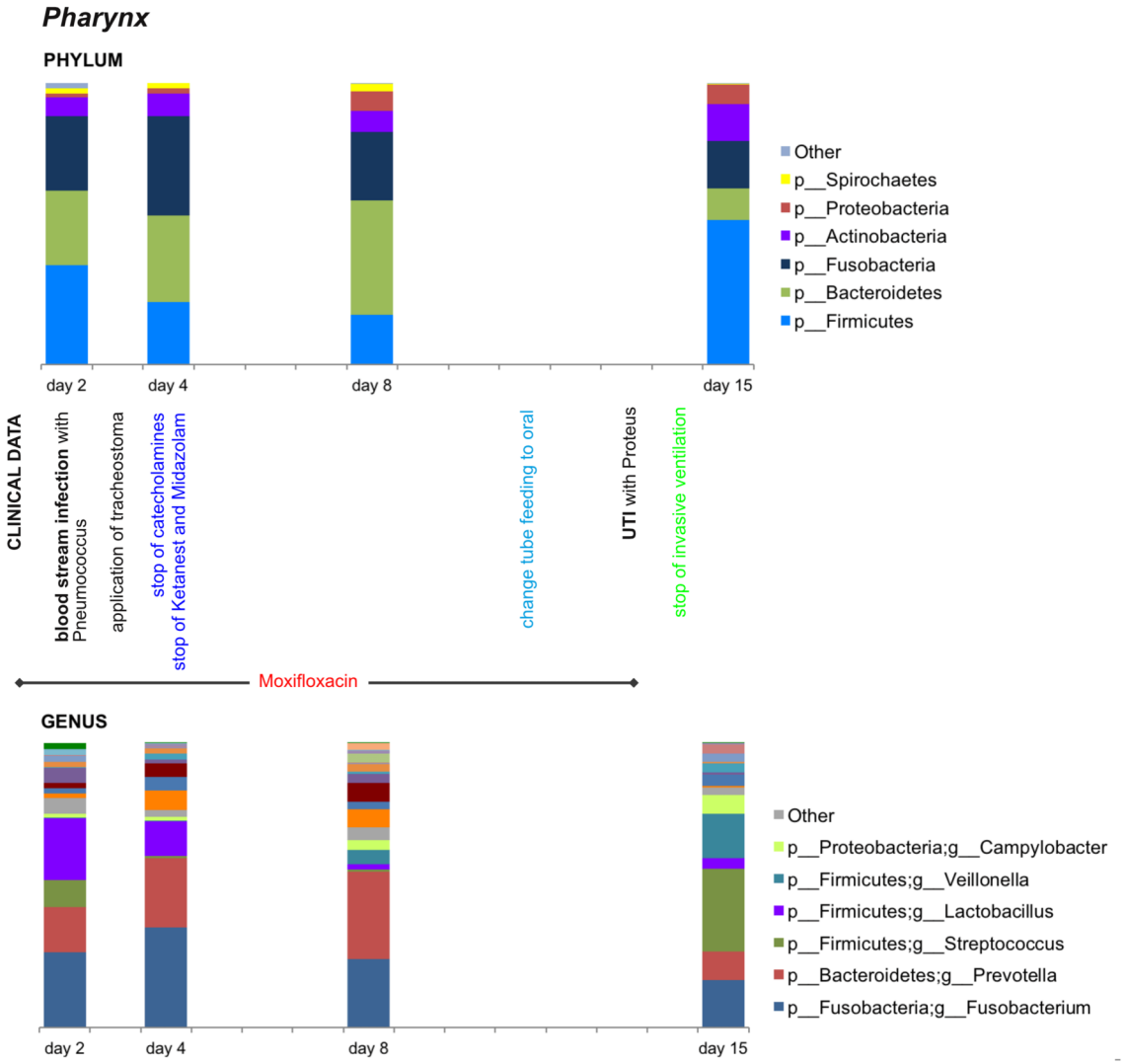

Figure 3. Patient 1 – Phylogenetic compositon of pharyngeal samples and analysis of clinical data.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. Note that not all illustrated taxa are labeled in the key."Other" includes all taxa that were represented below 2%. Above: important clinical factors are illustrated along the course of hospialisation; red: antibiotics; dark blue: catecholamines and analgosedation; light blue: feeding; green: ventilation: bold: infections (day of diagnosis).

4.1.2 Tracheobronchial Secretion

Samples of the tracheobronchial secretion were collected on day 1, 4 and 15 (Figure 4).

Overall, *Firmicutes* and *Proteobacteria* dominated on the phylum level, with the latter showing a marked increase from day 1 to day 4. *Firmicutes,* mainly accounting for *Streptococcus* on the genus level were present on all sample days. A marked decrease occurred from day 1 to day 4, however on day 15 *Streptococcus* increased to 61% of sequence reads.

Proteus accounted for 20% on day 15, however was not present at all in earlier samples. When comparing the clinical information, it is interesting that the patient was diagnosed with a urinary tract infection with *Proteus spp* on day 13 – e.g. these results could suggest simultaneous colonization of respiratory and urinary tract (discussed in the summary of patient 1).

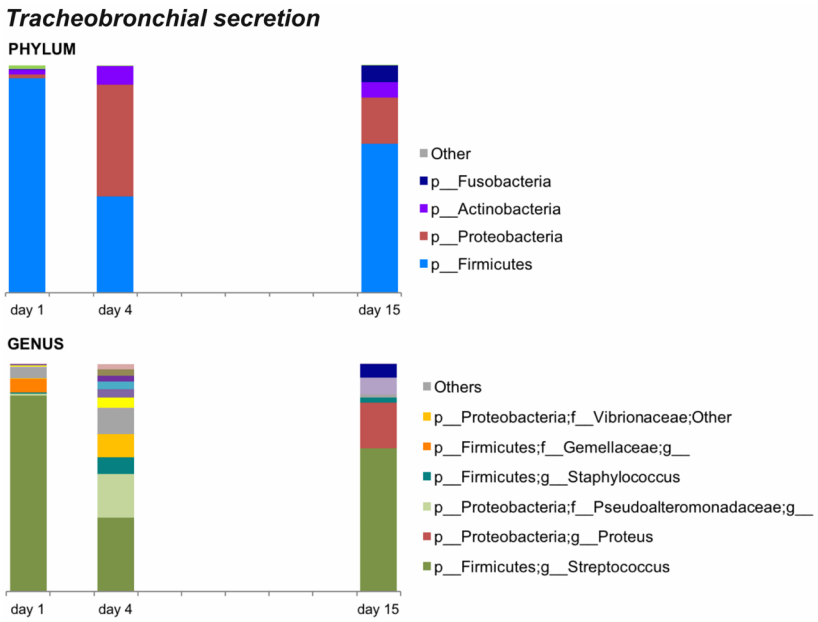

Figure 4. Patient 1- Phylogenetic composition of samples from the tracheobronchial secretion.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. Note that not all illustrated taxa are labeled in the key. ”Others” includes all taxa that were represented below 2%.The gaps between the samples represent 2 days each.

4.1.3 Gastric Secretion

The sample of the gastric secretion from day 8 was used for analysis (Figure 5-A). A low diversity 2-member community was identified, with 85% of the 16S rRNA reads indicating the presence *Pseudomonas* and 8% representing *Lactobacillus.* Clinically however, no *Pseudomonas spp* was detected in culture analysis of the blood.

4.1.4 Feces

Unfortunately, only one fecal sample (day 4) was obtained during the patient's ICU stay, which is why fecal microbial changes over the course of the illness cannot be interpreted (Figure 5-B).

On the phylum level *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (in descending order) represented the majority of sequence reads. Analysis of the genus level revealed a relatively high microbial diversity with *Bacteroides*, *Enterobacteriaceae* (family level), *Ruminococcaceae* and *Bifidobacterium* dominating.

4.1.5 Perianal Skin

One sample from the perianal skin region was obtained on day 3 (Figure 5-C). Similar to the fecal sample *Bacteroidetes, Firmicutes* and *Proteobacteria* dominate on the phylum level. *Prevotella* and *Porphyromonas* were the most prevalent genera, presenting 33% and 23%, respectively.

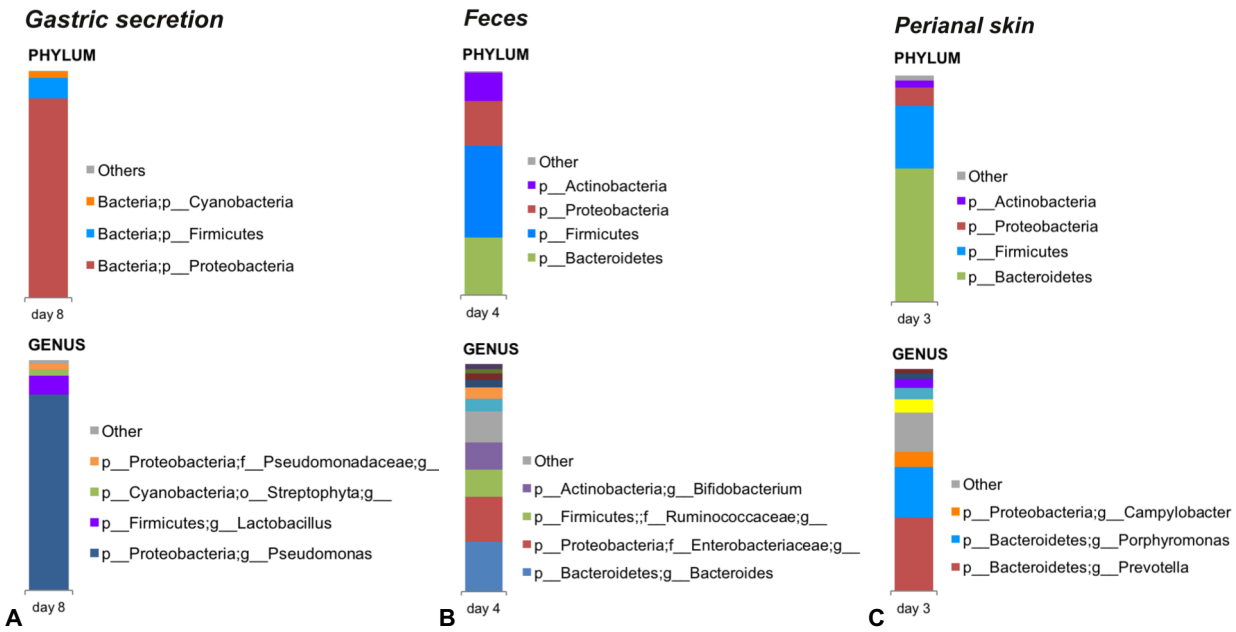

Figure 5. Patient 1 – Phylogenetic composition of samples from A) Gastric secretion B) Feces C) Perianal skin. The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. Note that not all illustrated taxa are labeled in the key. ”Others” includes all taxa that were represented below 2%.

4.1.6 Diversity

In this study, alpha diversity was calculated by the Chao1 index, a measure of bacterial richness. As illustrated in the rarefaction curves in Figure 6-A, there is a significantly greater microbial richness in samples from the early course of illness than at later time points. However, the fact that the only fecal sample belongs to the early time group (higher Chao1 index, compare Figure 6-C) is a bias in this analysis.

The difference in richness between early and late time points is especially visible samples from the pharynx and tracheobronchial secretion (day 1 versus day 15), as shown in the alpha rarefaction curves from the individual samples (Figure 6-B). Overall, the fecal sample showed a significantly higher richness than samples from the other sample areas (Figure 6-C) (note that there was only one early fecal sample). The same applies to rarefaction curves measuring the number of observed species and the phylogenetic distance curves (not illustrated).

Beta diversity, characterizing the similarities and differences between the individual samples, was evaluated with a Principal Coordinate Analysis (PCoA) of unweighted UniFrac distances. In this patient, the analysis showed a clustering of samples from the same sample area, but no clustering of samples from different areas (not illustrated).

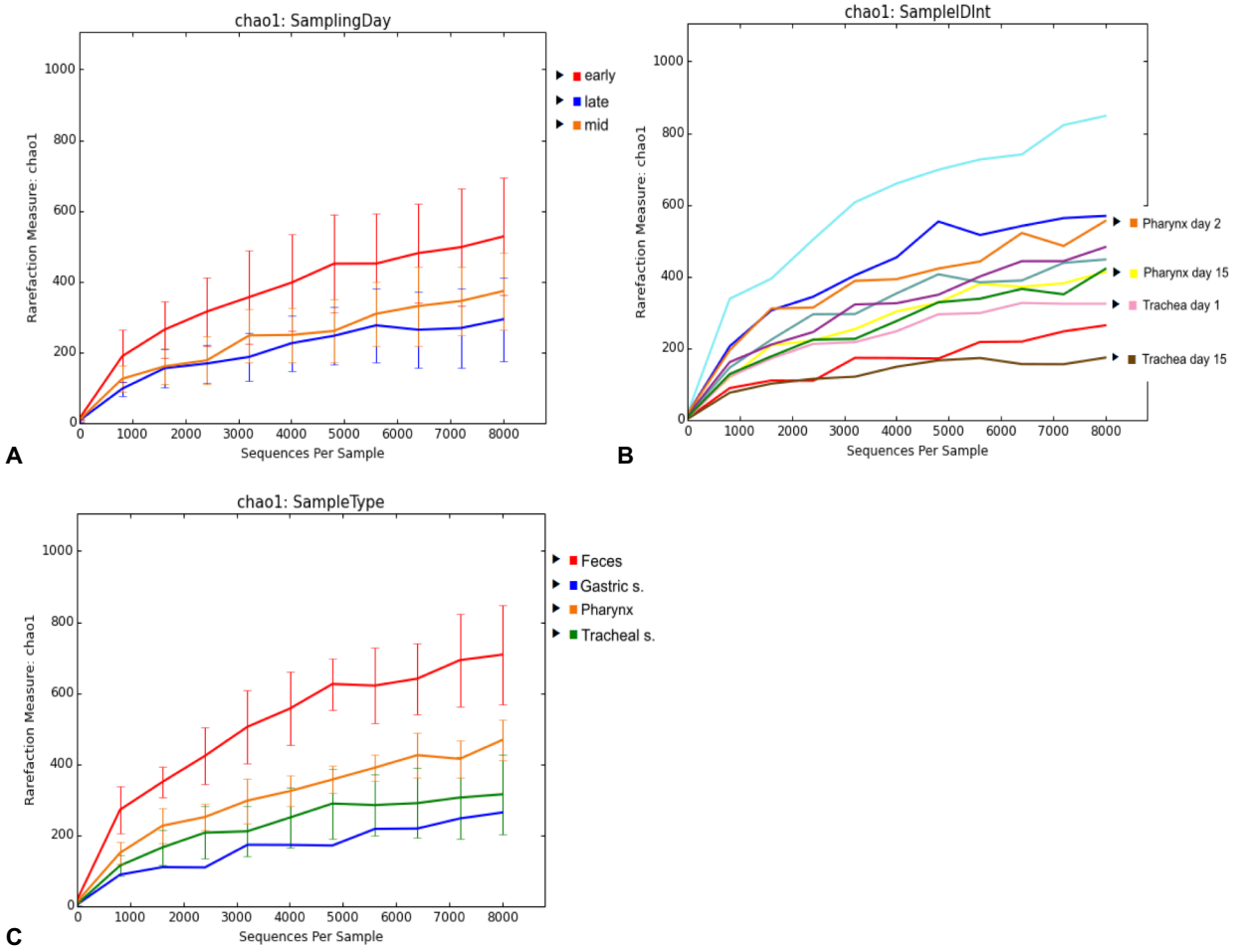

Figure 6. Patient 1 – Diversity analysis.
Alpha diversity rarefaction curves measuring Chao1 index (y-axis). A) Sampling time groups; early: sampling day 1-5, mid: sampling day 6-14, late: sampling day 15 onwards. B) Individual samples marked by sampling day. Only samples from the tracheobronchial secretion and pharynx are labeled, as a clear decrease in Chao1 index values over time can be observed in these samples; C) Sample type.

4.1.7 Summary of Patient 1

When analysing microbial changes over time, samples from pharynx and tracheobronchial secretion were useful in this patient. In these samples, a loss of diversity was shown when comparing early to late samples. Tracheobronchial samples revealed the lowest diversity of all

sample areas, with a dominance of *Streptococcus* and *Proteus.* Both these bacteria could be associated to the diagnosed blood stream infection with *S. pneumoniae* and the urinary tract infection with *Proteus spp*. If so, the increase in *Streptococcus* in late pharyngeal and tracheobronchial samples is surprising, as the infection was actually treated with Moxifloxacin from day 1 to day 13. Also, a connection between a pathogen from the urine and the tracheobronchial microbiome would be interesting, but more exact analysis would be necessary to clarify this relation.

Analysis of the gastric sample obtained on day 8 interestingly showed the presence of a low diversity mainly 2-member community consisting of *Pseudomonas* and *Lactobacillus* (85% and 8%, respectively).

Concerning the clinical factors in this patient, no clear relation to the observed microbial changes was evaluable. However, the detection of both infecting bacteria (on the genus level) in sample areas other than the actual site of infection after the clinical diagnosis of the infection is definitely an interesting finding. A generalized colonization by these bacteria, an external source of infection or else the displacement of pathogens (e.g. iatrogenic) from one body area to the other could be taken into consideration as possible causes.

4.2 Patient 2

Patient 2 was an 80-year-old woman who suffered from ventricular fibrillation. She was admitted to the ICU with ROSC (return of spontaneous circulation) due to successful preclinical cardiopulmonary resuscitation. As the cause of the ventricular fibrillation a non-obstructive hypertrophic cardiomyopathy was diagnosed. The patient underwent external cooling over the first two days of the ICU stay. Hemodynamic stability and a reduction of mechanical ventilation were achieved soon. Despite ending analgosedation, the patient remained comatose as a result of significant hypoxic encephalopathy. The patient eventually died under best supportive therapy on day 11.

Samples were obtained from the pharynx, feces, perianal skin, stomach and tracheobronchial secretion. Unfortunately, the tracheobronchial samples had to be excluded from analysis due to an insufficient number of sequence reads (<8000). To evaluate microbiota changes in the early course of an ICU stay, samples from the first days of hospitalisation were collected in this patient.

CLINICAL DATA OF PATIENT 2

Main diagnosis	Cardiac arrest due to ventricular fibrillation
Comorbidities	Hypertrophic non-obstructive cardiomyopathy, coronary heart disease I, diabetes mellitus II, arterial hypertension
Medication	Antibiotics: Amoxicillin/Clavulanic acid (day 2-11); Analgosedation, Catecholamines, PPI
Infections	No infections
Mechanical ventilation	Invasive (endotracheal tube)
Interventions	Cooling, coronary angiography
Other findings	Fever, tube feeding
Hospital stay	11 days
ICU stay	11 days
Outcome	Dead

Table 4. Patient 2 – Clinical data

4.2.1 Pharynx

Samples from the pharynx were obtained on day 2 and 3 of the patient's ICU stay. As illustrated in Figure 7, most clinical interventions were started in the first days of hospitalisation and were continued over the course of the patient's illness. On the phylum level *Bacteroidetes* and *Firmicutes* dominated in both samples; *Fusobacteria* accounted for 25% of sequence reads in the first sample but decreased to only 1% in the second sample. On the genus level, the increase of *Porphyromonas* to around 30% on day 3 was noticeable, the prevalence of *Prevotella* and *Streptococcus* in contrast stayed relatively stable.

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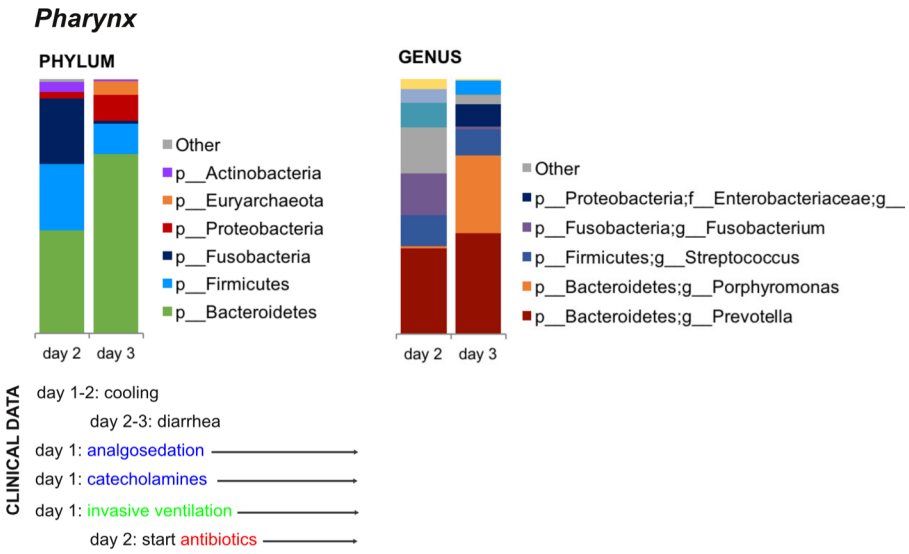


Figure 7. Patient 2 – Phylogenetic composition of the pharynx and analysis of clinical data.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (left: phylum level, right: genus level) for each sample. The x-axis shows the samples sorted over time. Note that not all illustrated taxa are labeled in the key. "Other" includes all taxa that were represented below 2%. Left: important clinical factors are illustrated along the course of hospialisation; red: antibiotics; dark blue: catecholamines and analgosedation; light blue: feeding; green: ventilation.

4.2.2 Gastric Secretion

Samples of the gastric secretion were collected on days 1, 2 and 3 of the patient's ICU stay (Figure 8-A).

As to the microbial composition, *Firmicutes* represented a majority of sequence reads in each of the gastric samples. *Bacteroidetes* were the second largest phylum on samples of day 1 and 2, however were replaced by *Cyanobacteria* on day 3. Analysis of the genus level revealed a relatively high diversity in all gastric samples; samples of day 1 and 2 showed similar results with *Streptococcus* and *Prevotella* accounting for around half of all sequence reads. A change in microbial composition was seen on day 3: *Streptophyta* (genus unknown) (38%) and *Enterobacteroaceae* (genus unknown) (12%) were the most prevalent genera. As these bacteria were not present in the earlier two samples, an external source of bacteria (e.g. food) or else a contamination of the sample should be considered

4.2.3 Feces

Fecal samples were collected on day 3 and 4 of the patient's ICU stay (Figure 8-B).

As seen in samples from the other areas, *Firmicutes* and *Bacteroidetes* accounted for the majority of sequence reads. Contrary is the presence of *Euryarchaeota* (*Methanobrevibacter* on the genus level), which presented 21% and 15% on days 3 and 4, respectively.

Generally, analysis of the genus level demonstrated a relatively great number of observed genera (5 genera comprised around 60% of all reads).

Commensal bacteria such as *Faecalibacterium, Ruminococcus* or *Blautia*, representing around 5% in healthy individuals (100) averagely accountet for 2%, 1,5% and 6% of reads, respectively.

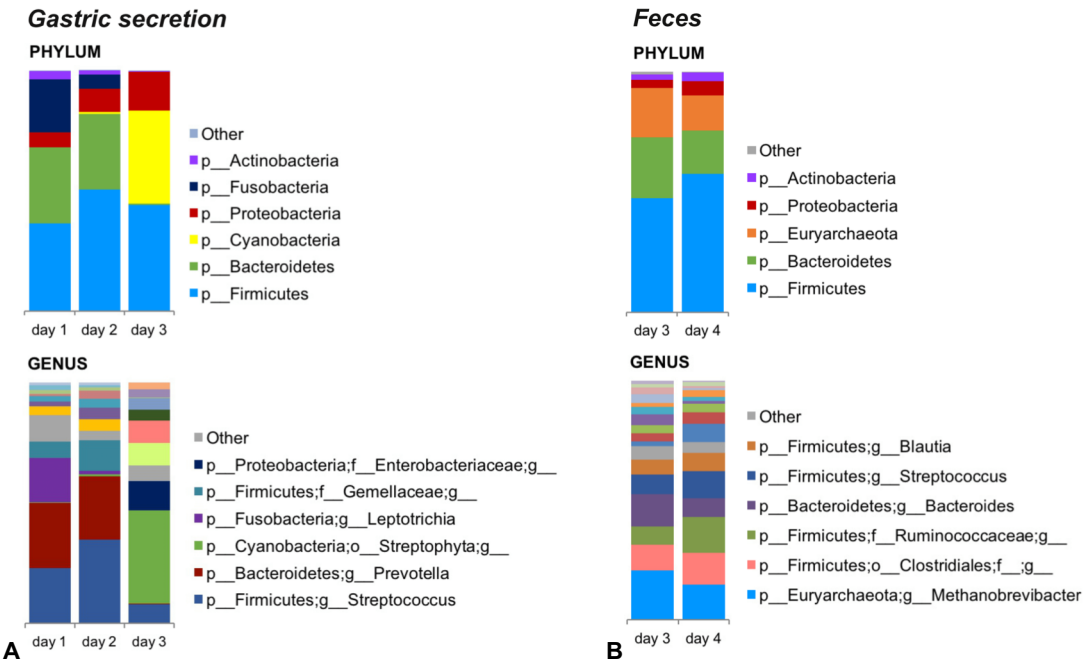


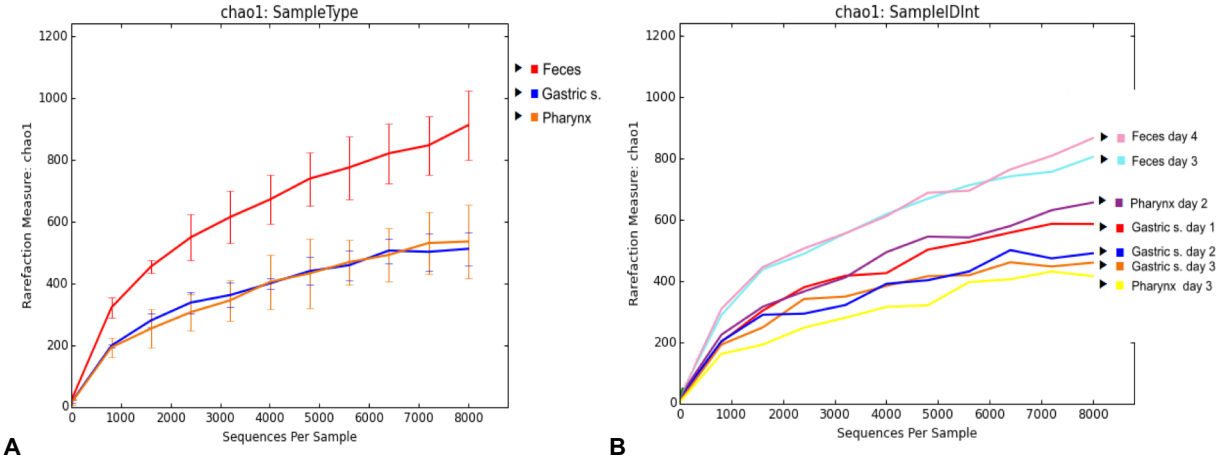
Figure 8. Patient 2 – Phylogenetic composition of samples from A) Gastric secretion and B) Feces.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. Note that not all illustrated taxa are labeled in the key. "Other" includes all taxa that were represented below 2%.

4.2.4 Diversity

Diversity analysis of patient 2 showed a relatively high microbial richness in fecal samples in comparison to other patients from this study as seen by a Chao1 index value of around 900

(Figure 25-A). Moreover, richness was markedly greater in fecal samples than in pharyngeal or gastric samples (Figure 9-A).

As there were only early samples in this patient, diversity changes could not be observed over a longer course. However, already a slight loss of Chao1 index numbers over the first days of hospitalisation were seen in fecal, gastric and especially pharyngeal samples (Figure 9-B).



A B

Figure 9. Patient 2 – Diversity analysis.
Alpha diversity rarefaction curves measuring Chao1 index (y-axis); A) Sample type; B) Individual samples, marked by sampling day.

4.2.5 Summary of Patient 2

In this patient, *Firmicutes* and *Bacteroidetes* dominated the microbial communities. On the genus level, fecal samples showed the highest diversity.

In contrast to other patients from this study, there was no typical pathogen present in any of the samples. This could be associated to the fact that the patient did not suffer from an infection throughout the course of the illness (the only patient of this cohort).

Some shifts in bacterial composition were observed from day 2 to day 3 in pharyngeal and gastric samples. Importantly, analysis of the Chao1 index revealed a loss of bacterial richness (in pharyngeal and gastric samples) already in these early days of ICU stay. This is a first indicator that microbial changes might already happen very early in the course of hospitalisation.

It was not possible to interpret the influence of clinical interventions on the microbiome in this case, as samples of later time points were not collected.

4.3 Patient 3

Patient 3, a 60-year-old woman was admitted to the hospital with pancytopenia and agranulocytosis, primarily as a result of a Methotrexate and Metamizole therapy for the underlying rheumatoid arthritis. She was transferred to the ICU on day 2 due to increasing cardiorespiratory instability (especially hypotonia, tachycardia, tachypnea). *Staphylococcus aureus* was detected in her blood culture as well as in a puncture of the left thumb (diagnosed as septic *Staphylococcus aureus* arthritis). Consequently, the initial antibiosis with Piperacillin/Tazobactam was changed to Cefazolin and Fosfomycin. Additionally, on day 7 a fungaemia by *Candida albicans* was diagnosed and treated with Anidulafungin. In the course of the ICU stay the patient developed progressive kidney and liver failure, a ventilator-associated pneumonia and suffered from a paralytic ileus. Despite high-dose catecholamine therapy and blood transfusions, the patient died on day 17 of the ICU stay.

Three samples were collected from each feces, pharynx, tracheobronchial and gastric secretion on the days 9, 12 and 16 of the patient's ICU stay.

CLINICAL DATA OF PATIENT 3

Main diagnosis	*Staph. aureus* and *Candida* sepsis, MODS, agranulocytosis
Comorbidities	Rheumatoid arthritis
Medication	Antibiotics: Piperacillin/Tazobactam (day 1-3), Doxycyclin (day 1-2), Cefazolin (day 3-17), Fosfomycin (day 3-17), Meropenem (day 6-15); Antimycotics (Anidulafungin), Analgosedation, Catecholamines, Steroids, PPI
Infections	BSI: *Staph. aureus* (on admission), *Candida albicans* (diagnosis day 7); joint infection: *Staph. aureus* (diagnosis day 3)
Mechanical ventilation	Invasive (endotracheal tube)
Other findings	GI failure, fever, tube feeding
Interventions	Ci-Ca-Dialysis, bronchoscopy, biopsy of thumb and hip joint, TEE
Hospital stay	18 days
ICU stay	12 days
Outcome	Dead

Table 5. Patient 3 – Clinical data.
MODS: multiorgan dysfunction syndrome, BSI: blood stream infection, TEE: transesophageal echocardiography.

4.3.1 Pharynx

When analysing the pharyngeal samples of patient 3, relatively great microbial changes were observable. Firstly, an increasing prevalence of *Bacteroidetes* from day 9 to day 16 (mainly corresponding to *Porphyromonas* on the genus level) was prominent, with a dominance of 54% on day 16. Concomitantly, *Fusobacteria (*corresponding to *f_Leptotrichiaceae,* genus unknown*)* decreased from 50% on day 9 to only 2% on day 16. Similarly, the prevalence of *Firmicutes* clearly decreased over time (mostly due to a loss of *Pediococcus* on the genus level). In the sample of day 12, around 30% of sequence reads indicated the presence of *Enterobacteriaceae* which however were not present by day 16 (Figure 10).

4.3.2 Tracheobronchial Secretion

Similar to samples from the gastric secretion, tracheobronchial samples showed a dominance of *Firmicutes* and *Proteobacteria* on the phylum level. The latter showed a noticeable increase from 17% on day 12 to 73% on day 16 (similarly in numbers is the decrease of *Firmicutes*).
In the samples (Figure 11-A) of day 9 and 12, 58% and 79% of sequence reads demonstrated the presence of *Staphylococcus,* which surprisingly were not present in any other sample area of this patient (considering the blood stream infection with S. aureus). By day 16 *Staphylococcus* had decreased to around 16% of sequence reads. Clinically, no factor occurred between day 12 and day 16 that could explain the dramatic decrease of *Staphylococcus* (no new initiation of antibiotic). *Cupriavidus (*p_*Proteobacteria)* was prevalent in all tracheobronchial samples, however showed a clear increase from around 10% on day 12 to 42% on day 16.

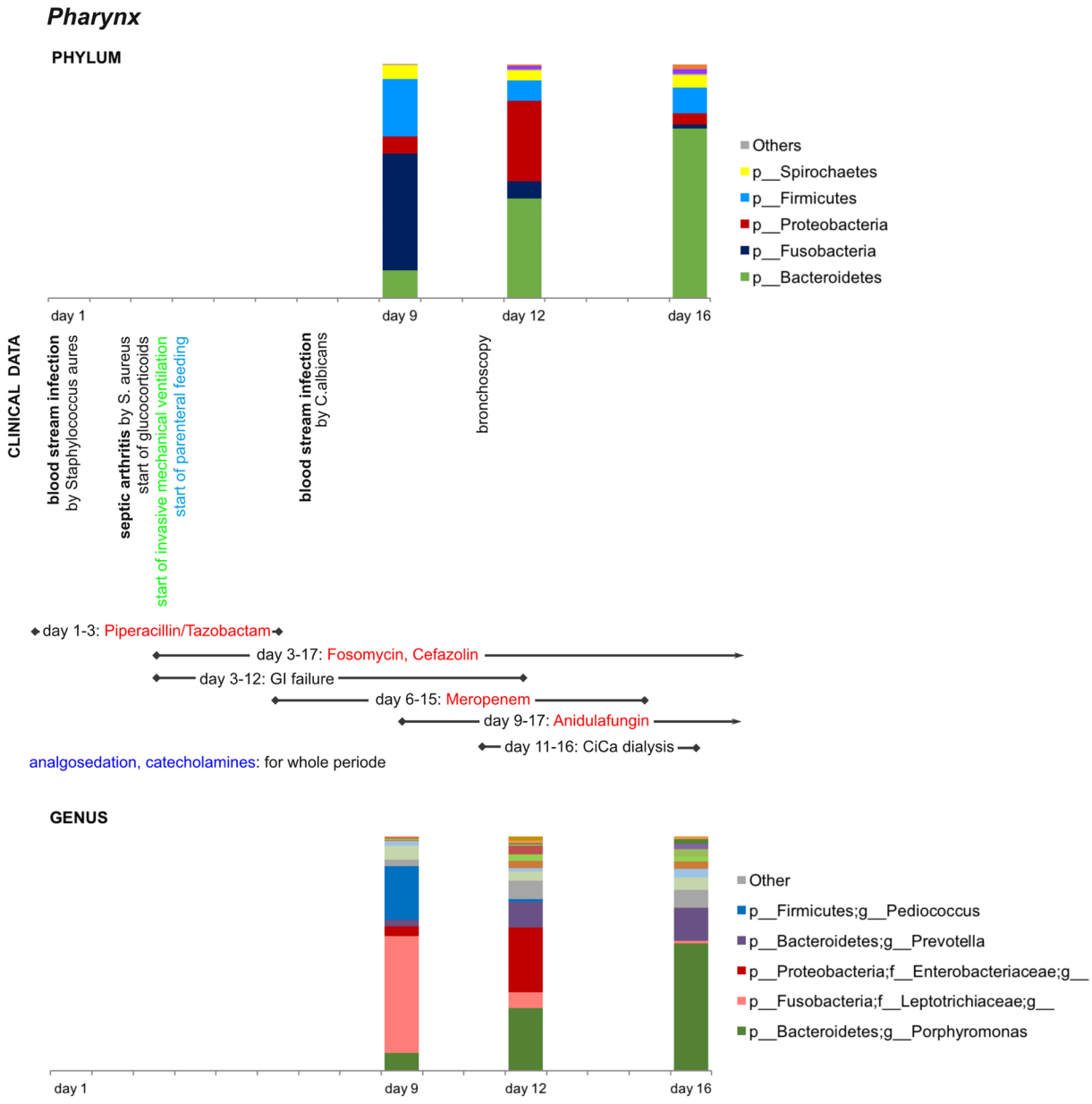


Figure 10. Patient 3 – Phylogenetic composition of pharyngeal samples and analysis of clinical data.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each fecal sample. The x-axis shows the samples sorted over time. Note that not all illustrated taxa are labeled in the key. ”Others” includes all taxa that were represented below 2%. Above: important clinical factors are illustrated along the course of hospialisation; red: antibiotics; dark blue: catecholamines

4.3.3 Gastric Secretion

Compared to samples from feces and pharynx of the same patient, the samples of the gastric secretion show a remarkable lower number of different bacteria resulting in a low-diversity microbiota community (also seen by low Chao1 index numbers, Figure 12-B). On the phylum level *Firmicutes* dominated with 97%, 93% and 83% of sequence reads on days 9, 12 and 16, respectively. The rest accorded to the phylum of *Proteobacteria.* The corresponding genera were identified as *Pediococcus*, comprising the majority of reads, as well as *Lactobacillus* (on days 9 and 12 with around 15%) and *Streptococcus* (on day 16 with 12%) (Figure 11-B).

4.3.4 Feces

On the phylum level fecal samples showed the presence of *Bacteroidetes*, *Firmicutes* and *Actinobacteria* (Figure 11-C). The prevalence of *Bacteroidetes* represented the majority of sequence reads with an increase from 44% to 64% over the course of time, mainly corresponding to the genera *Bacteroides* and *Parabacteroides*.

From day 9 to day 16 the number of identified genera decreased notably, resulting in a low diversity 3-member community on day 16, consisting of *Bacteroides*, *Corynebacterium* and *Enterobacteriaceae* (genus unknown).

Commensal bacteria such as *Faecalibacterium* and *Ruminococcus* only presented less than 1% and were not detectable on day 16 at all.

When analysing the clinical factors, it is surprising that no *Staphylococcus* was present in any of the fecal samples, considering that the patient suffered from a blood stream infection with *Staphylococcus aureus* for the first two weeks of stay (treated with 4 different antibiotics).

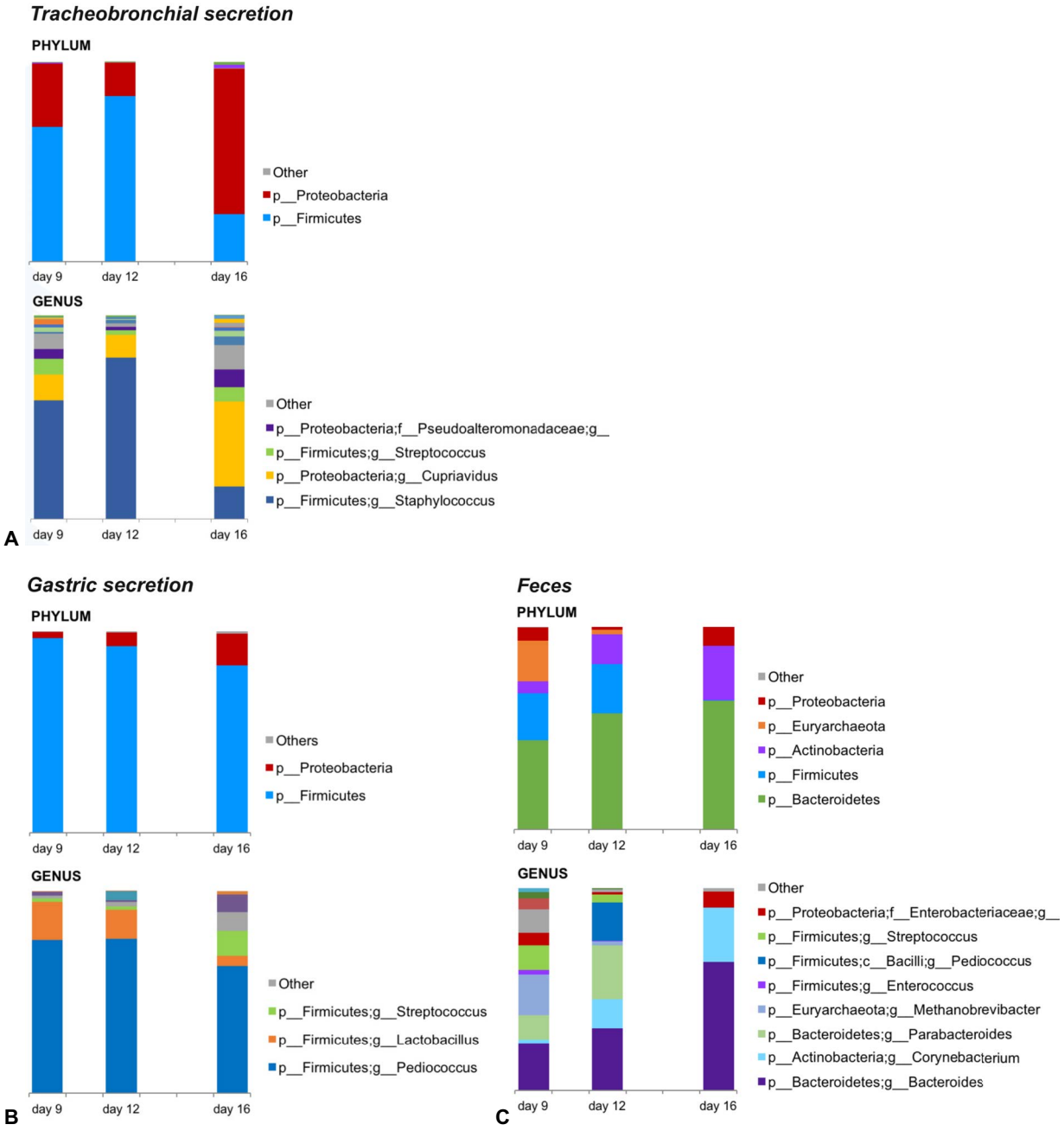


Figure 11. Patient 3 – Phylogenetic composition of samples from A) Tracheobonchial secretion; B) Gastric secretion; C) Feces.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time.
Note that not all illustrated taxa are labeled in the key. ”Other” includes all taxa that were represented below 2%.

4.3.5 Diversity

Alpha diversity rarefaction curves (Figure 12-A/B) demonstrated a relatively low microbial richness for all sample types with index numbers around 300 to 400. When analysing microbial richness over the course of hospitalisation (Figure 12-C), a significant reduction of diversity was seen in fecal samples, a moderate reduction in gastric samples. There was an increase in richness seen from mid to late tracheobronchial samples.

In this patient, analysis of the phylogenetic distance showed interesting results and are therefore illustrated additionally (Figure 12-D). A significant difference in microbial diversity between the individual sample types can be seen: pharyngeal samples showed by far the greatest phylogenetic diversity while samples from the gastric secretion showed the lowest.

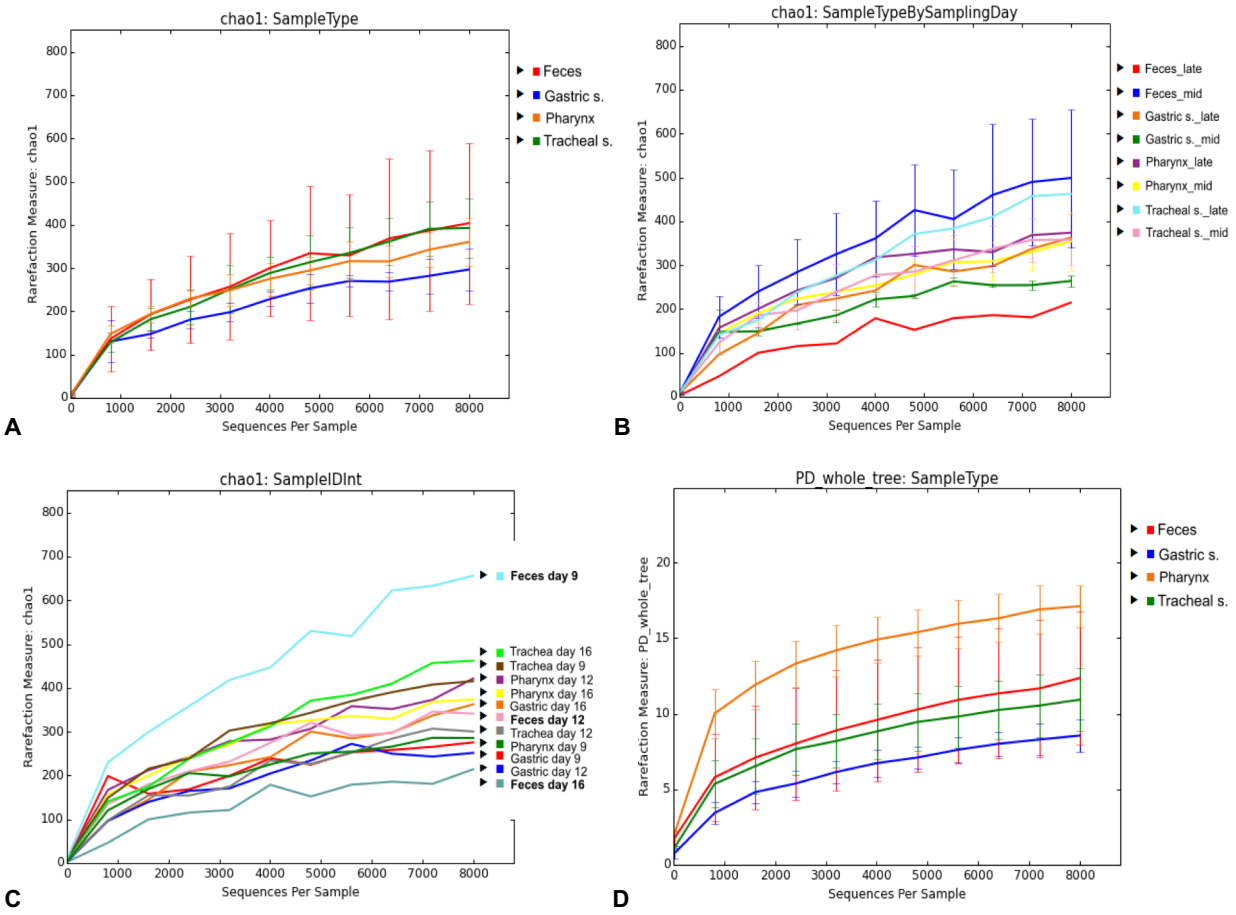


Figure 12. Patient 3 – Alpha diversity rarefaction curves.
A) Calculation of Chao1 Index (y-axis) for sample type; B) Calculation of Chao1 index for sample types by sampling time groups; early: sampling day 1-5, mid: sampling day 6-14, late: sampling day 15 onwards. C) Calculation of Chao1 index for the individual samples, interesting results were found for fecal samples (marked in bold), where a decrease in bacterial richness over the time can be seen. D) Phylogenetic distance analysis of all sample types.

4.3.6 Summary of Patient 3

Overall, microbial richness was low in all sample areas in this patient.

Interestingly, there was one genus comprising over 50% of reads in samples from all areas, however the dominating genera were differing between areas: *Bacteroides* in fecal samples, *Porphyromonas* in pharyngeal samples, *Pediococcus* in gastric samples and *Staphylococcus* in tracheobronchial samples.

Another interesting point is the relatively high prevalence of *Cupriavidus* in all tracheobronchial samples with a clear increase over time – there are some case reports of pneumonia caused by *Cupriavidus pauculus* (101-103).

As for the influence of clinical factors on microbial community changes, it is possible that the summation of therapeutic interventions (Figure 10) during the first week of stay (multiple antibiotics, invasive ventilation, parenteral feeding) could play a role in the loss of microbial richness and the emergence of dominating genera. A specific clinical factor however was not identifiable with the methods used in this study.

Although patient number 3 suffered from a severe blood stream infection by *S. aureus* for the first two weeks of ICU stay (treated with 4 different antibiotics), *Staphylococcus* was not generally present (as seen in other patients from this study).

4.4 Patient 4

Patient 4, a 73-year-old man was transferred from an outside hospital for further treatment after cardiac arrest, ventricular fibrillation and NSTEMI (non-ST-elevation myocardial infarction). On admission, the patient showed cardiorespiratory instability under high-dose catecholamine therapy, then suffered another cardiac arrest but could be stabilized over the course of the first days on the ICU. Additionally, the patient was treated for a pneumothorax, acute cholecystitis and DIC (disseminated intravascular coagulation). Due to impaired kidney function a Ci-Ca-dialysis was started. On day 10 *coagulase-negative Staphylococcus* was detected in the blood culture, consequently the antibiotic therapy was changed from Levofloxacin and Clindamycin to Meropenem. Despite cardiorespiratory stability the patient suffered another cardiac arrest, however he could be stabilized soon afterwards. After 20 days

of ICU stay, the patient was transferred to a normal ward in good condition and was released from the hospital after 32 days of stay.

Samples were collected from the feces (6 samples, but one had to be excluded from analysis), pharynx (5 samples) and from gastric and tracheobronchial secretion (2 samples each).

CLINICAL DATA OF PATIENT 4

Main diagnosis	Cardiac arrest due to ventricular fibrillation and NSTEMI
Comorbidities	Pneumothorax, DIC, beginning coronary artery disease, acute kidney failure, aortic stenosis I
Medication	Antibiotics: Ampicillin/Sulbactam (day 1-5), Levofloxacin (days 4-10), Clindamycin (day 4-10), Meropenem (day 10-23); Analgosedation, Catecholamines, PPI
Infections	Blood stream infection: *coagulase-negative Staphylococcus* (diagnosis day 10)
Mechanical ventilation	Invasive (endotracheal tube), non-invasive
Other findings	Fever, tube feeding
Interventions	Ci-Ca-Dialysis, bronchoscopy, CT-Angiography, puncture of V. jugularis interna (non poss.)
Hospital stay	32 days
ICU stay	20 days
Outcome	Alive

Table 6. Patient 4 - Clincial data.
NSTEMI: non-ST-elevcation myocardial infarction, DIC: disseminated intravascular coagulation.

4.4.1 Pharynx

Samples from the pharynx were obtained on day 5, 8, 12, 16 and 18 of the patient's ICU stay (Figure 13). In the sample of day 5 there were 6 different phyla present at more than 5%- in descending order *Bacteroidetes*, *Fusobacteria*, *Tenericutes*, *Firmicutes*, *Spirochaetes* and *Proteobacteria*. On the genus level a relatively high diversity of different genera was shown.

Reads from day 8 and 12 presented a similar microbial composition on the phylum level with a marked increase of *Proteobacteria* compared to day 5.

On these two days members of the *Enterobacteriaceae* family presented 52% and 20%, respectively (this family was not present on day 5). Furthermore, reads from day 12 showed the presence of *Tanerella* (of the Bacteroidetes phylum) at 20%.

Importantly, a dramatic shift was visible between day 12 and later samples: samples of day 16 and 18 were dominated by *Staphylococcus*, comprising 92% and 78% of the 16S rRNA sequences, respectively (≤1% in the earlier samples). Additionally, *Streptococcus* presented around 6% of the reads on day 18.

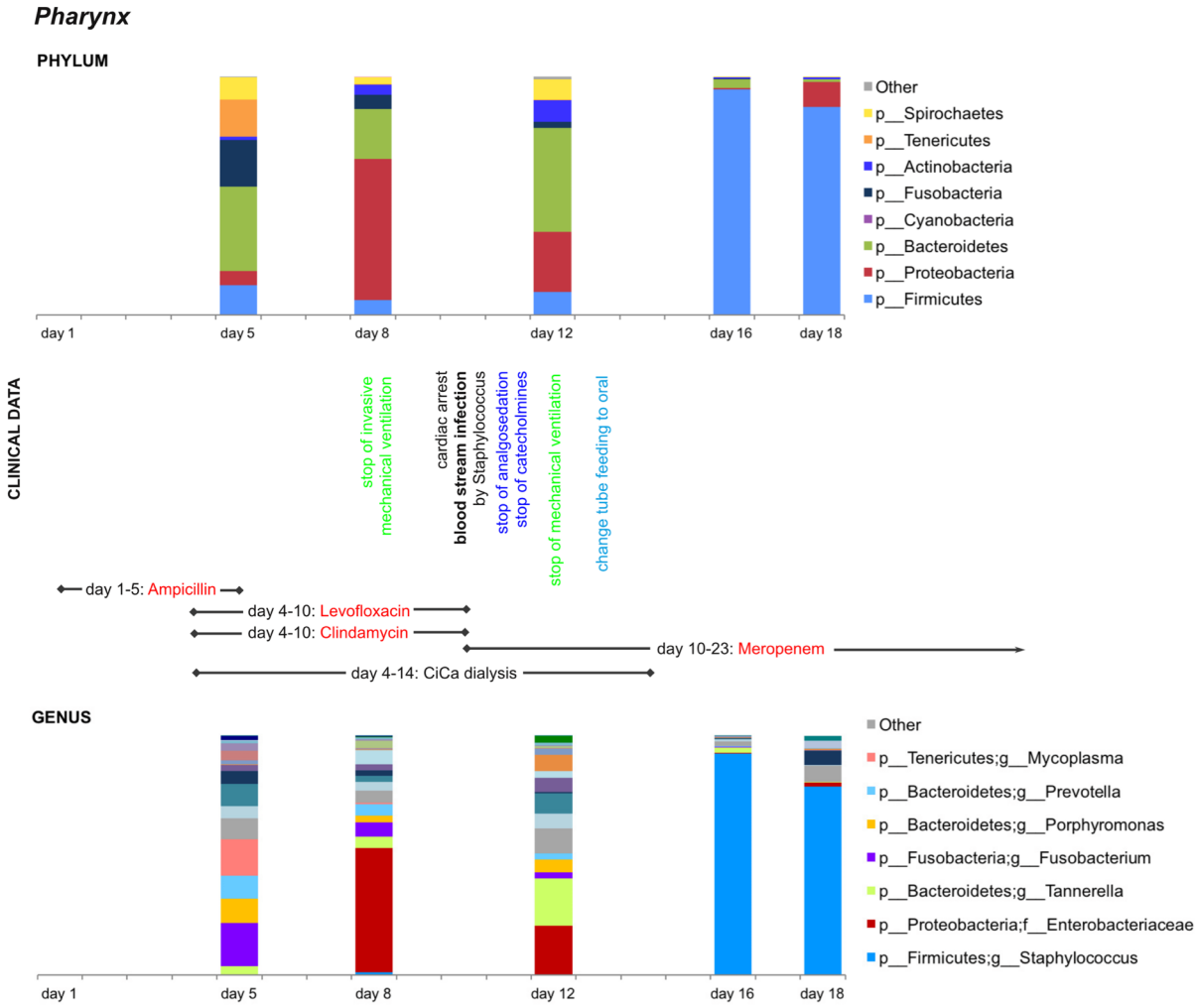


Figure 13. Patient 4 – Phylogenetic composition of pharyngeal samples and analysis of clinical data.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. Note that not all illustrated taxa are labeled in the key."Other" includes all taxa that were represented below 2%. Above: important clinical factors are illustrated along the course of hospialisation; red: antibiotics; dark blue: catecholamines and analgosedation; light blue: feeding; green: ventilation: bold: infections (day of diagnosis).

4.4.2 Tracheobronchial Secretion

Samples from the tracheobronchial secretion were collected on day 5 and 8 of the patient's ICU stay (Figure 14-A). Sequence reads from the first sample showed a dominance of *Mycoplasma*, *Streptococcus* and *Fusobacterium* (28%, 26%, 24%).
In the later sample, 98% of the reads comprised the family *Xanthomonadaceae* (p_*Proteobacteria*). In this case, contamination should be considered (*Xanthomonadaceae* are typically a part of the oral microbiome (104)).

4.4.3 Gastric Secretion

Samples from gastric secretion were taken on day 5 and day 8 of the patient's ICU stay (Figure 14-B). On the phylum level *Firmicutes*, *Proteobacteria* and *Cyanobacteria* dominated in both samples, with *Firmicutes* decreasing and *Cyanobacteria* increasing from day 5 to day 8. On the genus level the first sample showed a diverse microbial population, however within a few days the majority of genera was lost with only two genera dominating in the sample of day 8; *Streptophyta* (genus unknown) and *Enterobacteriaceae* (47% and 36%, respectively) (genus unknown), the latter not having been present on day 5.

4.4.4 Feces

Fecal samples were collected on day 4, 5, 8, 16 and 18 of the patient's ICU stay (Figure 14-C). On the phylum level samples of day 4 and 5 showed a dominance of *Firmicutes* (57% and 77%), mainly constituted of *Streptococcus* and *Clostridia* (genus unknown). Noticeable is the absence of *Bacteroidetes* in all samples. Moreover, *Euryarchaeota (g_ Methanobrevibacter)* presented 30% on day 4, but decreased to only 2% on day 5. Only 3 days later a marked microbial change could be observed, with an emergence of f_*Enterobacteriaceae* (genus unknown) to 26% (2% on day 4). Interestingly, *Enterobacteriaceae* also presented *52%* in the pharyngeal sample of day 8.
Similar to the pharyngeal samples, a dramatic loss of genera was seen on days 16 and 18, with *Firmicutes* making up 87% and 95%, respectively.

On the genus level the microbiome was dominated by a 4- member community with *Streptococcus*, *Staphylococcus, Lactobacillus* and *Enterobacteriaceae* (genus unknown).

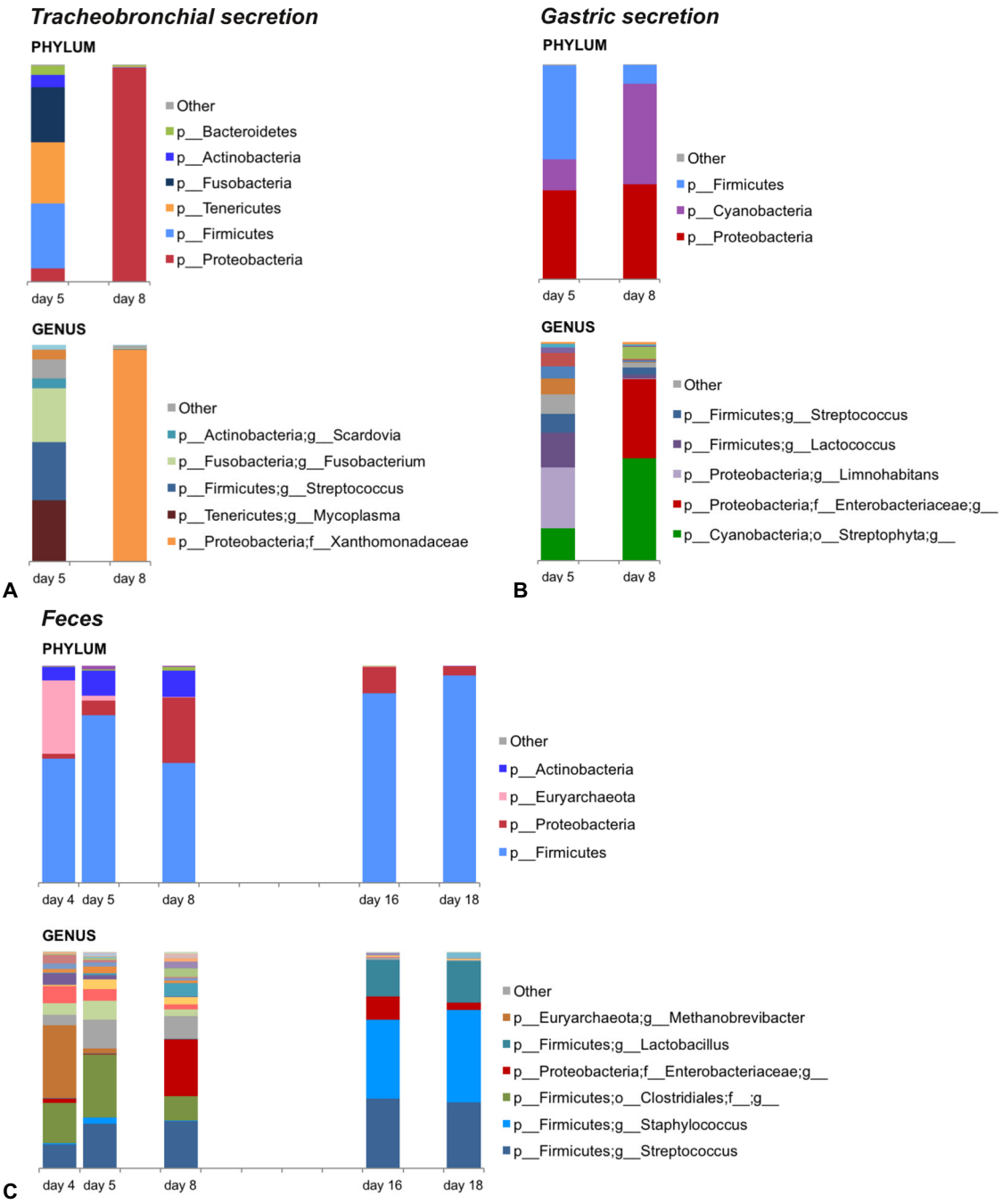


Figure 14. Patient 4 – Phylogenetic composition of samples from A) Tracheobronchial secretion, B) Gastric secretion, C) Feces.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. One gap between the samples represents two days. Note that not all illustrated taxa are labeled in the key. ”Other” includes all taxa that were represented below 2%.

Staphylococcus and *Lactobacillus* were present in much lower levels in the earlier samples; *Streptococcus* in contrast was abound in all samples taken. The commensal *Ruminococcus* markedly decreased from 5,5% on day 4 to 0% on day 18, indicating a damaged intestinal microbiome. As seen in Figure 13, antibiotic therapy was changed on day 10, which could be a possible explanation for the dramatic change in microbiota composition.

4.4.5 Diversity

As shown in the alpha-diversity rarefaction curves in Figure 15-A, the early samples showed a significantly greater microbial richness than the late samples in feces, pharynx and tracheobronchial secretion (mid samples did not fit into this pattern; gastric samples did not change a lot). When looking at the alpha diversity by sample type (Figure 15-B), a

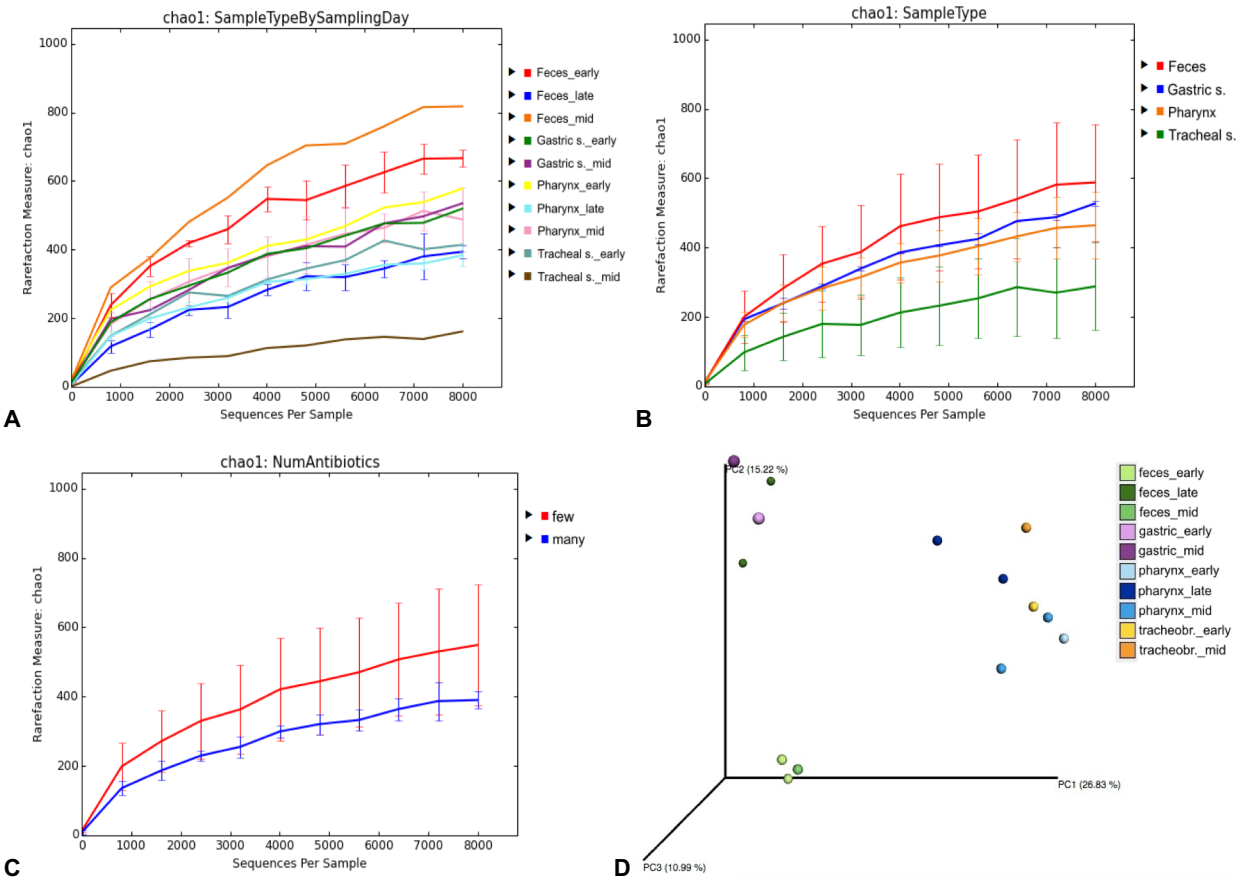


Figure 15. Patient 4 – Diversity analysis.
A) Alpha diversity rarefaction curves measured by Chao1 index for sample type by sampling day; B) Chao 1 index measured by sample type; C) Chao1 index measured for number of antibiotics, few: 2-3 antibiotics, many: 4 or more antibiotics; D) Beta diversity; Principal Coordinate Analysis (PCoA) of unweighted UniFrac distances.

significantly lower diversity was seen in samples from the tracheobronchial secretion as compared to samples from the other areas. Figure 15-C shows that microbial richness decreased with a greater number of antibiotics. It must be noted that these curves mainly represent the shift from early and mid to late time points (the change in the number of antibiotics was on day 10). Therefore, the curves for mechanical ventilation (stop on day 12) appear similarly. Still, it indicates a significant change in microbial diversity which was likely associated to therapeutic interventions.

Interestingly, beta diversity analysis revealed clustering of late fecal samples with samples of the gastric secretion, indicating greater similarities between those samples (Figure 15-D). This pattern was not found in any other patient.

4.4.6 Summary of Patient 4

In this patient, the fecal and pharyngeal samples are especially useful for analysing the microbial shift over time due to the quantity of samples collected. In both sample areas a dramatic loss of microbial diversity was shown in the late samples (day 16 and 18) compared to earlier samples. Overall, a dominance of *Firmicutes* and *Proteobacteria* in late samples with little to no presence of *Bacteroidetes* was revealed.

Clinically, several factors occurred over the course of day 8 to day 13 (Figure 13), namely a change in antibiotic therapy, stop of mechanical ventilation, stop of analgosedation and catecholamine therapy, diagnosis of a blood stream infection and stop of tube feeding. All these changes could result in a reduction of microbial diversity; nevertheless, a specific clinical factor is not definable with our analysing methods.

Especially interesting is the dominance of *Staphylococcus* in the late pharyngeal and fecal samples, which could be associated to the patient's blood stream infection with coagulase negative *Staphylococcus* from day 10 to 23 (further sequencing analysis would be necessary to verify whether these were of the same species).

Lastly, the relatively high prevalence of *Enterobacteriaceae* (genus unknown) in mid samples of feces, pharynx and gastric secretion is notable.

4.5 Patient 5

Patient 5, a 59-year old man, was admitted to the ICU due to acute delirium tremens. On admission, the patient was comatose and in bad general condition, requiring mechanical ventilation, catecholamine therapy and analgosedation. On the second day, aspiration pneumonia with oxacillin-sensitive *Staphylococcus aureus* was diagnosed via culture analysis from the tracheobronchial secretion. Later, the patient also suffered from a blood stream infection with *Enterococcus*, which was treated with Vancomycin. On day 16 weaning was initiated, however failed due to respiratory insufficiency. Subsequently, Herpes simplex virus 1 (HSV1) was detected in the bronchial lavage and treated with Aciclovir. Also, Meropenem was added to the antibiosis. Despite intensive care therapy, the patient recovered slowly, but could be transferred to a normal ward after 31 days on the ICU. He was discharged to a rehabilitation centre after a total hospital stay of 62 days.

Samples were collected from the pharynx (3 samples), tracheobronchial secretion (2 samples), gastric secretion (4 samples, but 2 had to be excluded from analysis) and feces (3 samples).

CLINICAL DATA OF PATIENT 5

Main diagnosis	Acute delirium tremens, *S. aureus* and HSV1 pneumonia, *Enterococcus* blood stream infection, liver cirrhosis Child C
Comorbidities	Epilepsy, anemia, thrombocytopenia, caput medusae, critical illness neuropathy, coarse tremor
Medication	Antibiotics: Piperacillin/Tazobactam (day 2-19), Vancomycin (day 13-32), Rifaximin (day 13-41), Meropenem (day 19-32), Aciclovir (day 21-28); Analgosedation, Catecholamines, PPI
Infections	Pneumonia: *S. aureus* (diagnosis day 2), HSV1 (diagnosis day 20); BSI: *Enterococcus* (diagnosis day 11)
Mechanical ventilation	Invasive (endotracheal tube)
Interventions	Ascites puncture (3x), bronchoscopy (5x), TEE
Other findings	Fever, tube feeding
Hospital stay	62 days
ICU stay	32 days
Outcome	Alive

Table 7. Patient 5 – Clinical data.
HSV1: Herpes simplex virus 1, BSI: blood stream infection, TEE: transesophageal echocardiography.

4.5.1 Pharynx

Samples from the pharynx were obtained on days 10, 15 and 22 of the patient's ICU stay (Figure 16). Already on the phylum level, a clear change in microbial composition over time was visible. In the first sample, *Firmicutes* dominated with around 70% of 16S rRNA sequence reads, mainly corresponding to *Enterococcus* on the genus level. By day 15 *Enterococcus* reads had decreased to only 5%. This change could possibly be associated to the blood stream infection with *Enterococcus*, diagnosed on day 11 and the subsequent antibiotic therapy with Vancomycin.

Mycoplasma was modestly represented in the first two samples (16% and 13%, respectively). On day 15, *Enterobacteriaceae* emerged to around 40% of reads and subsequently increased further to around 95% on day 22, resulting in a low diversity mainly 2-member community (2 genera of the *Enterobacteriaceae* family (genus unknown).

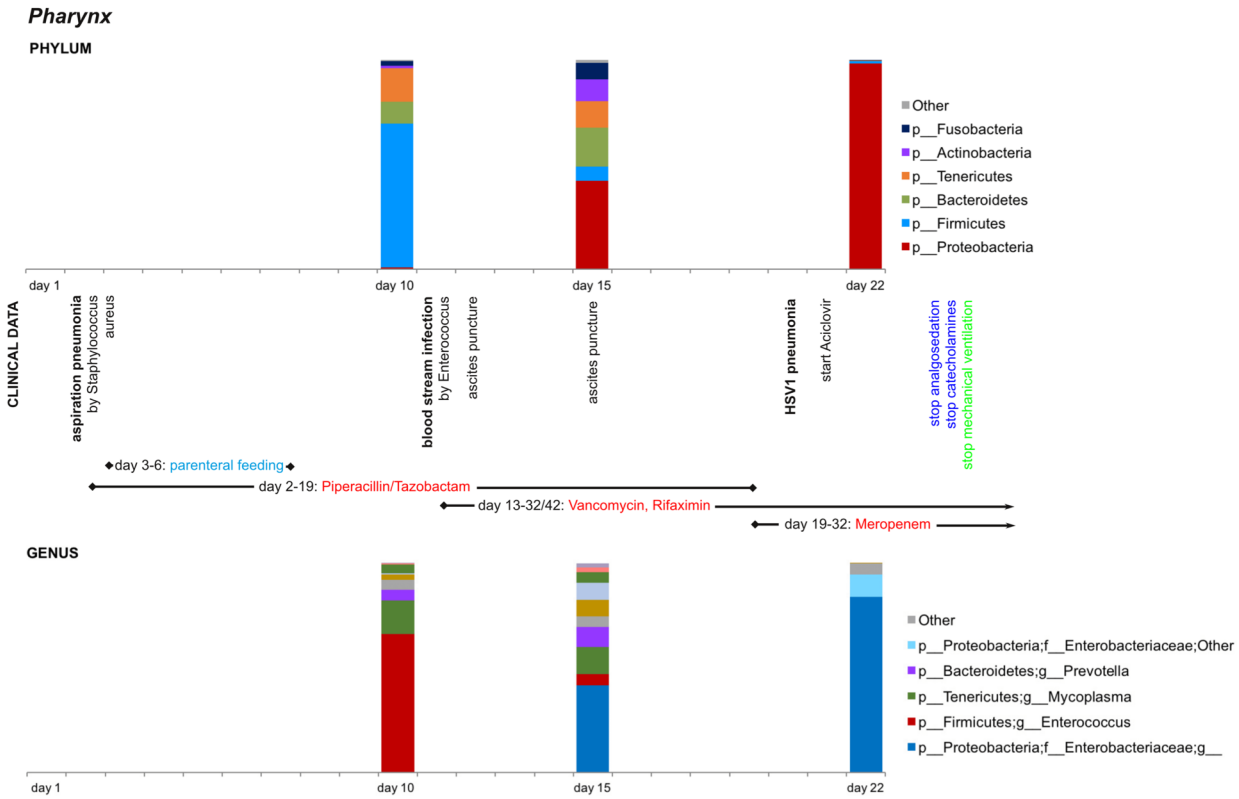


Figure 16. Patient 5 – Phylogenetic composition of pharyngeal samples and analysis of clinical data.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. "Other" includes all taxa that were represented below 2%. Note that not all illustrated taxa are labeled in the key. Above: important clinical factors are illustrated along the course of hospialisation; red: antibiotics; dark blue: catecholamines and analgosedation; light blue: feeding; green: ventilation: bold: infections (day of diagnosis).

4.5.2 Tracheobronchial Secretion

Samples from the tracheobronchial secretion were collected on days 10, 15 and 22 (Figure 17-A). The first two samples were dominated by *Proteobacteria* on the phylum level; *Cupriavidus* decreased notably over the course, from 52% on day 10 to only 2% on day 22; *Enterobacteriaceae* (genus unknown) appeared on day 15 with 48%, but decreased to only 5% by day 22 (not present on day 10 at all). Moreover, *Streptococcus* was modestly present on both fist samples. By day 22 the microbial composition had changed distinctly with the most prominent genera being *Mycoplasma* (38%) and *Fusobacterium* (14%). In comparison to day 15 however, the number of prevalent genera had increased again. Interestingly at this point is that Meropenem was added to the antibiotic therapy on day 19.

4.5.3 Gastric Secretion

Samples from the gastric secretion were collected on days 11 and 15 (two later samples had to be excluded due to an insufficient number of reads (<8000)) (Figure 17-B). The sample of day 11 almost exclusively presented *Enterococcus*, comprising 95% of sequence reads. By day 15 *Enterococcus* had decreased to 43%, instead *Enterobacteriaceae* (genus unknown) emerged to 44% of sequence reads (not present on day 11 at all). These changes in microbial composition were similar in pharyngeal samples, where *Enterococcus* dominated on day 10 and *Enterobacteriaceae* arose on day 15, possibly associated to clinical factors (e.g. *Enterococcus* infection and antibiotic therapy).

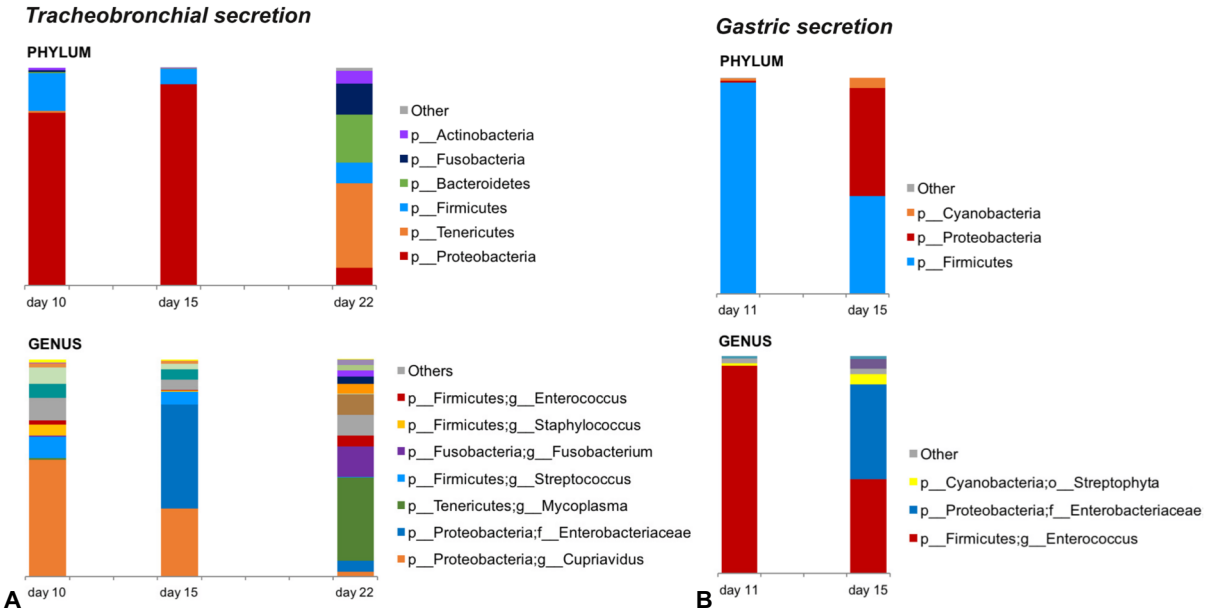


Figure 17. Patient 5 – Phylogenetic composition of samples from the A) Tracheobronchial secretion; B) Gastric secretion.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. One gap between the samples represents two days. Note that not all illustrated taxa are labeled in the key.

4.5.4 Feces

Fecal samples were obtained on days 10, 19 and 30 of the patient's ICU stay (Figure 18). On the phylum level *Firmicutes* and *Bacteroidetes* clearly dominated. Analysis of the genus level showed a high number of prevalent genera in the first two samples, but a significant loss of diversity in the last sample of day 30.

Interestingly, the community composition changed clearly from day 10 to day 19 with no genus being prominent in both samples (note that both were collected from the perianal skin; the sample from day 10 also stands out in alpha diversity analysis, Figure 19-A). In the sample of day 30, *Bacteroides* reads accounted for 74% and *Enterococcus* for 11%, representing a low diversity community compared to the earlier samples.

Typical commensals such as *Faecalibacterium, Ruminococcus* and *Blautia* only represented around 1,5% of reads on day 10 and were not detected anymore later on, indicating a damaged intestinal microbiome.

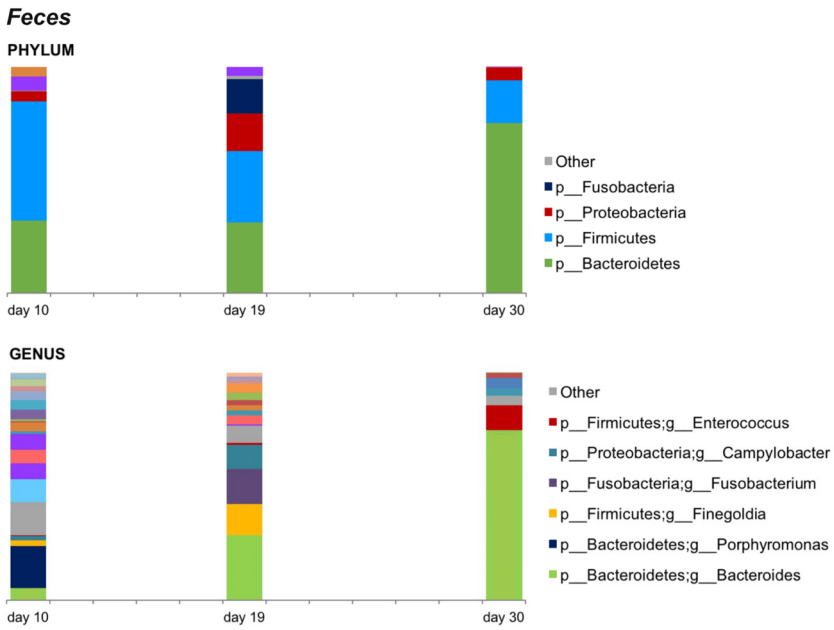


Figure 18. Patient 5 – Phylogenetic composition of fecal samples.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. One gap between the samples represents two days. Note that not all illustrated taxa are labeled in the key.

4.5.5 Diversity

When analysing changes in alpha diversity over the course of time (Figure 19-A), no significant loss of diversity was found from day 10 to later time points, as seen in other patients from this study. Only one fecal sample (day 10) showed a significantly higher Chao1 index number, however this sample was obtained from the perianal skin region, which could possibly explain the difference (note: the fecal sample from day 19 was also obtained from the perianal skin, but fitted into the cluster of other samples).

Alpha diversity analysis by sample type (Figure 19-B) revealed the highest microbial richness in fecal samples, the lowest in samples from the gastric secretion.

As shown in Figure 19-C, a greater diversity was observed before the emergence of the nosocomial infection (in this patient summarizing all samples from day 10) than during/after the occurrence of the infection.

Interestingly, beta diversity plots (Figure 19-D) demonstrated a clustering of pharyngeal and tracheobronchial samples, which has not been detected in any other patient from this study.

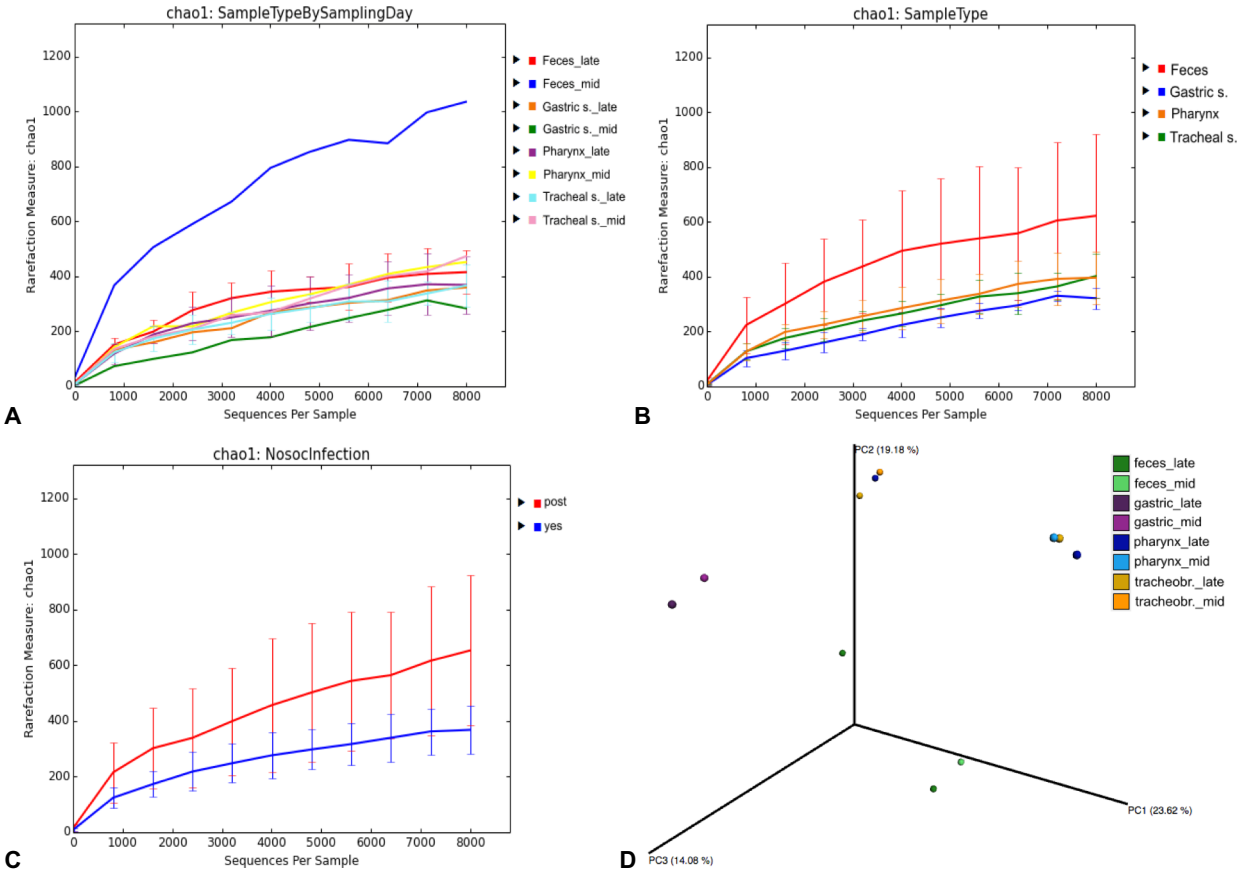


Figure 19. Patient 5 – Diversity analysis.
A) Alpha diversity rarefaction curves measured by Chao1 index for sample type by sampling day; B) Chao1 index by sample type; C) Chao1 index measured in samples before and after nosocomial infection; post: no infection present at the time of sampling, but occurance of infection at later time points of hospitalisation; yes: infection present at the time of sampling. D) Beta diversity analysis of unweighted UniFrac distances.

4.5.6 Summary of Patient 5

In this patient, the presence of *Enterococcus* and *Enterobacteriaceae* in many samples from different sample areas was prominent. *Enterococcus* dominated in samples from pharynx and

gastric secretion on days 10 and 11, with 66% and 95%, respectively. This could be associated to the blood stream infection with *Enterococcus*, diagnosed on day 11 and treated with Vancomycin. *Enterobacteriaceae* (genus unknown) were highly prevalent in samples from day 15, comprising 40% in the pharynx (95% on day 22), 48% in tracheobronchial secretion and 44% in gastric secretion. Importantly, *Enterobacteriaceae* were not present in any sample before day 15, which could possibly indicate an external source of the bacterium. In contrast to *Enterococcus*, there was no infection with any *Enterobacteriaceae* spp identified clinically in this patient. However, the generalized prominence is noteworthy.
As in patient 3, samples of the tracheobronchial secretion included a great proportion of *Cupriavidus* (52% and 31% on days 10 and 15, respectively).

4.6 Patient 6

Patient 6, a 53-years old man was transferred to the ICU from another hospital with septic conditions, acute anuric kidney failure and thrombocytopenia. Serology was positive for Hantaan virus; more specific analysis identified Dobrava-Belgrad virus. Already on admission the patient showed progressive respiratory insufficiency and ARDS (an *Aspergillus fumigatus* pneumonia was diagnosed), subsequently extracorporeal membrane oxygenation (ECMO) had to be started. In consequence of a failed arterial puncture and the resulting surgical correction, the patient developed a compartment syndrome of the left leg. Due to avitality of the muscle and septic conditions an amputation of the lower leg and later on of the whole extremity had to be performed. In the course, the patient suffered from a wound infection with *Enterococcus*, coagulase negative *Staphylococcus* and MRSA, which all were treated with Daptomycin. Coagulase negative *Staphylococcus* was also detected in the blood culture. After an intermittent clinical improvement, multiresistant bacteria (4MRGN; *Pseudomonas* spp and *Klebsiella pneumoniae)* were detected in smear material (anus, groin, wound) and treated with Gentamicin and Colistin. The permanent comatose status, cardiorespiratory instability as well as the multiresistant infections led to the decision of best supportive care. The patient died after 54 days of ICU stay.

Samples were collected from the pharynx (5 samples), from gastric secretion (5 samples, one had to be excluded due to an insufficient number of sequence reads (<8000)), feces (3 samples) and tracheobronchial secretion (4 samples, 3 had to be excluded due to an insufficient number of sequence reads (<8000)).

CLINICAL DATA OF PATIENT 6

Main diagnosis	MODS due to Dobrava virus-infection (acute kidney failure, ARDS), cSSTI
Comorbidities	Compartment syndrome, rhabdomyolysis, paralytic ileus
Medication	Antibiotics: Piperacillin/Tazobactam (day 1-10), Meropenem (day 11-54), Daptomycin (day 19-36), Tigecyclin (day 20-31), Gentamicin (day 38-41), Colistin (day 42-53), Erythromycin (intermitted prokinetic use); Antimycotics: Voriconazol (day 3-15), Anidulafungin (day 20-39); Steroids, Analgosedation, Catecholamines, PPI
Infections	Dobrava virus infection (on admission), Pneumonia: *Aspergillus fumigatus* (diagnosis day 2), BSI: *coa. -neg. Staphylococcus* (diagnosis day 18), cSSTI: *Enterococcus* (diagnosis day 16), MRSA (diagnosis day 20), 4MRGN *(Klebsiella pneumoniae, Pseudomonas* spp, diagnosis of both on day 16)
Mechanical ventilation	Invasive (endotracheal tube)
Other findings	Fever, tube feeding, GI failure, diarrhoea
Interventions	Ci-Ca dialysis, bronchoscopy (4x), ECMO, TEE, fasciotomy/necrectomy, amputation (2x), discontinuous dialysis, gastroscopy, tracheostoma application
Hospital stay	54 days
ICU stay	54 days
Outcome	Dead

Table 8. Patient 6 – Clincial data.
MODS: multiorgan dysfunction syndrome, ARDS: acute respiratory distress syndrome, cSSTI: complicated skin and soft tissue infection, BSI: blood stream infection, MRSA: multi resistant Staphylococcus aureus, 4MRGN: multiresistant gram-negative bacteria (resistant to the 4 major groups of antibiotics). ECMO: extracorporeal membrane oxygenation, TEE: transesophageal echocardiography.

4.6.1 Pharynx

Samples from the pharynx were obtained on days 3, 5, 15, 20 and 23 of the patient's ICU stay (Figure 20). On the phylum level a high prevalence of *Firmicutes* was seen on the first 4 sample days; in the sample of day 23 *Tenericutes* represented the highest proportion.

Especially interesting is the change of *Staphylococcus* over the longer course, which comprised around 60% on day 3, decreased to 15% on day 5 and subsequently increased again to around 60% on day 15. On day 20 and 23 it had diminished below 5%. Instead, *Enterococcus* emerged on day 20 with 71% of reads (not detected on days 3, 5 and 15), but got reduced to 16% on day 23. Clinically, both pathogens were associated to wound and blood stream infections from day 16 onwards, which were treated with Meropenem and later also Daptomycin and Tigecyclin. *Mycoplasma*, already modestly present on days 15 and 20, was the most prevalent genus in the last sample with 37% of sequence reads.

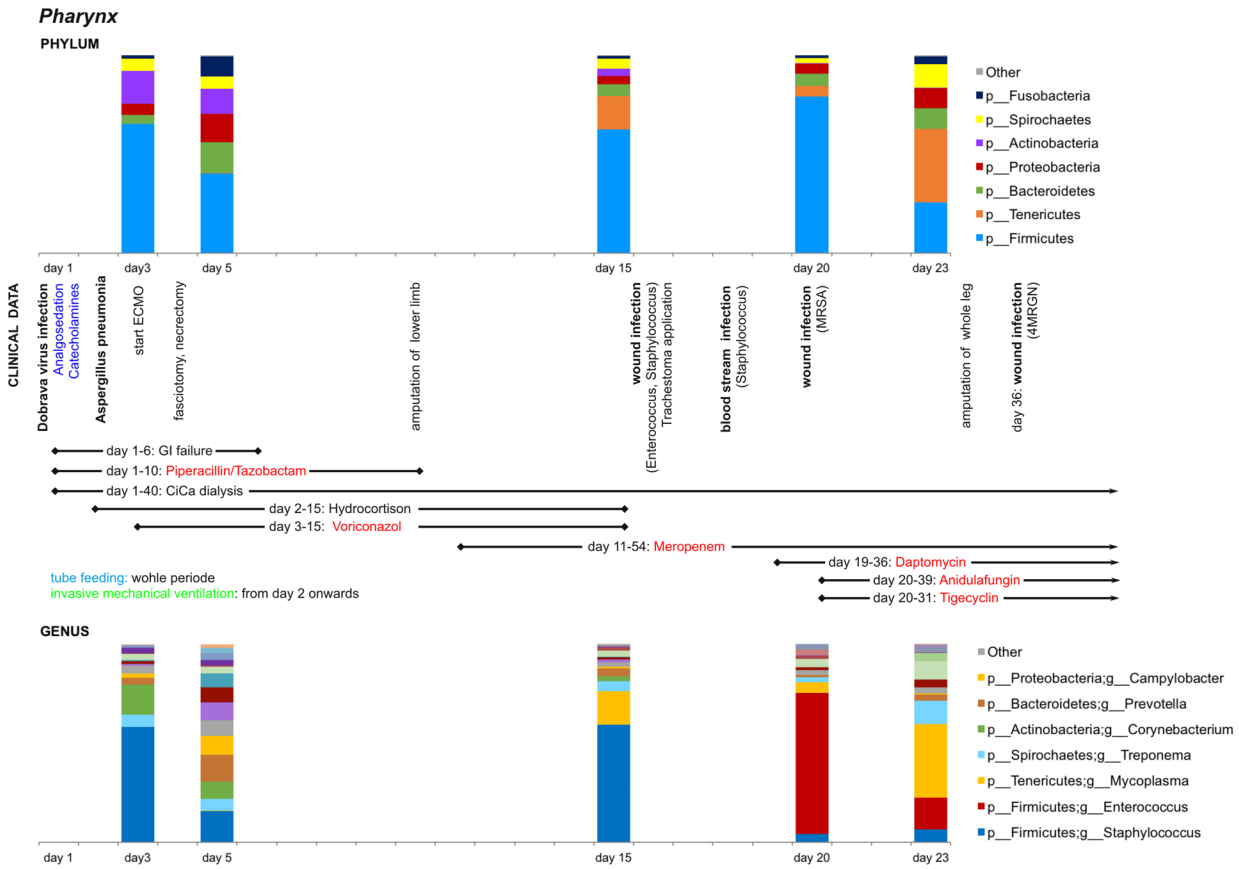


Figure 20. Patient 6 – Phylogenetic composition of pharyngeal samples and analysis of clinical data.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. Note that not all illustrated taxa are labeled in the key."Others" includes all taxa that were represented below 2%. Above: important clinical factors are illustrated along the course of hospialisation; red: antibiotics; dark blue: catecholamines and analgosedation; light blue: feeding; green: ventilation: bold: infections (day of diagnosis). MRSA: multiresistant Staphylococcus aures, ECMO: extracorporal membrane oxygenation. 4MRGN: multiresistant gram-negativ bacteria (resistant to the 4 major groups of antibiotics).

4.6.2 Tracheobronchial Secretion

The sample of day 23 collected from the tracheobronchial secretion could be used for analysis (Figure 21-A). *Enterococcus* reads dominated with around 80%.

4.6.3 Gastric Secretion

Samples from the gastric secretion were collected on days 5, 15, 20 and 23 of the patient's ICU stay (Figure 21-B).

Overall, gastric samples showed low-member microbial communities (mainly consisting of one or two bacterial members), however the dominating genera differed between samples. *Corynebacterium* reads comprised around 78% on day 5. Sample of day 15 showed a 2-member community with *Staphylococcus* (87%) and *Corynebacterium* (11%). Samples from days 20 and 23 showed a completely different composition than earlier samples, with *Enterococcus* dominating with around 92% on both days.

4.6.4 Feces

Samples from the feces were obtained on days 15, 20 and 23 of the patient's ICU stay (Figure 21-C). On the phylum level *Firmicutes* and *Bacteroidetes* represented the most dominant phyla. In contrast to other sample areas from this patient, fecal samples showed a relatively stable composition on the genus level. *Staphylococcus* was present in all samples, comprising 31%, 11% and 29% on days 15, 20 and 23, respectively. Additionally, *Corynebacterium* reads comprised 15% on day 15 (also seen in gastric samples of day 5 and 15). *Bacteroides* markedly increased on days 20 and 23 with 39% and 32%, respectively. *Enterococcus*, highly present in pharynx and gastric secretion, corresponded to 12% and 9% of reads on days 20 and 23, respectively. Typical commensals were not significantly represented in any of the fecal samples.

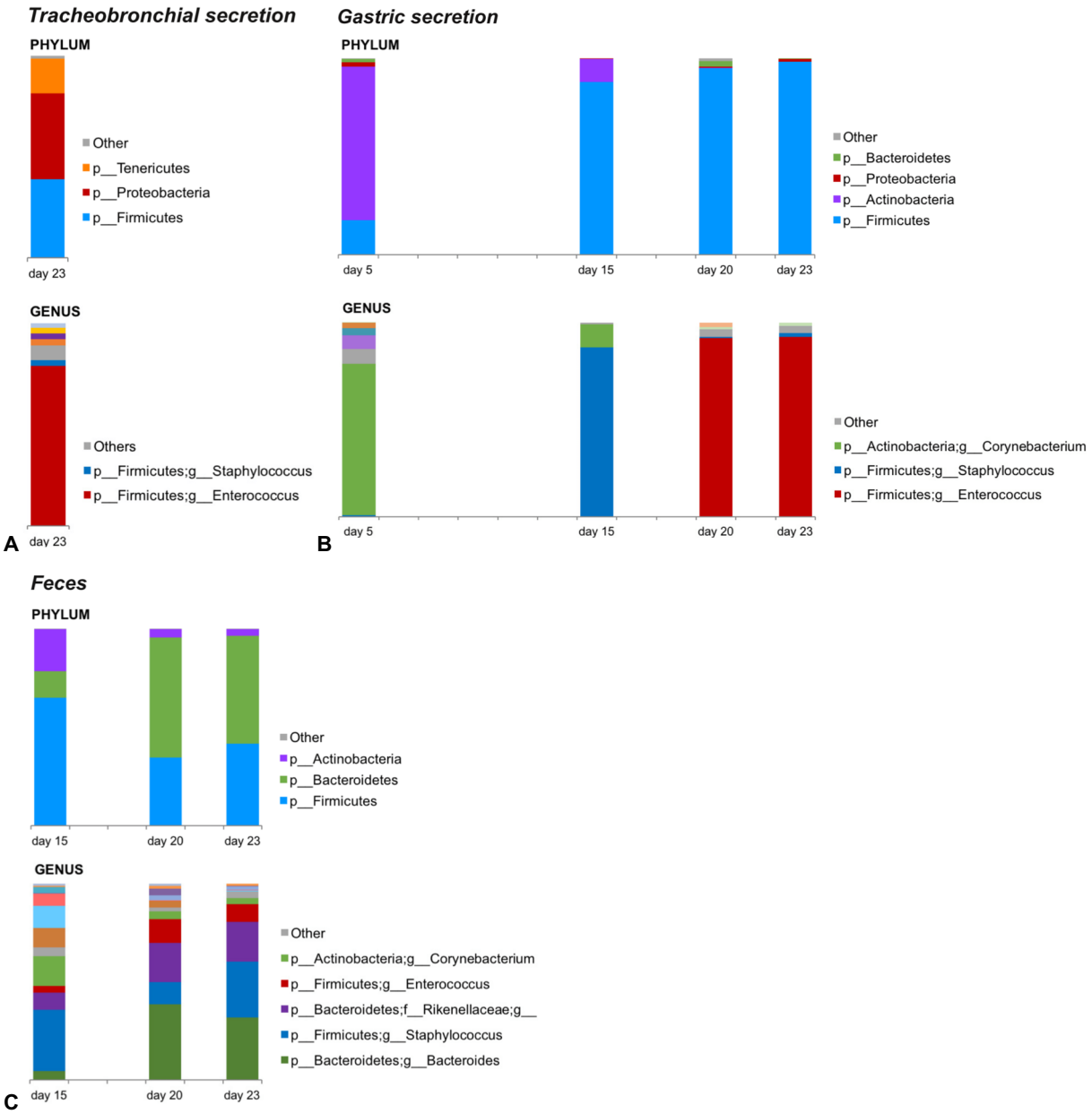


Figure 21. Patient 6 – Phylogenetic composition of samples from A) Gastric secretion; B) Tracheobronchial secretion; C) Feces.
The y-axis shows the relative distribution of the represented bacterial taxa on two phylogenetic levels (above: phylum level, below: genus level) for each sample. The x-axis shows the samples sorted over time. One gap between the samples represents two days. Note that not all illustrated taxa are labeled in the key.

4.6.5 Diversity

Analysis of alpha diversity rarefaction curves showed a markedly higher microbial richness in fecal and pharyngeal samples in comparison to gastric and tracheobronchial samples (Figure 22-A). When comparing sample types by sample day (possible in gastric and pharyngeal samples), a significantly greater diversity was seen in mid samples (collected day 6 -14) than in late samples (collected day 15 and afterwards) within each area (Figure 22-B) (early samples could not be included into this analysis).

Interestingly, a lower richness (number of observed species) was seen at the time after GI failure than during GI failure (day 1-6) (Figure 22-C). This change was also moderately visible in patient 3 (the only two patients who had suffered from GI failure in this cohort).

When looking at the beta diversity analysis of unweighted UniFrac distances (Figure 22-D), a clearly parted clustering of pharyngeal samples and fecal samples was shown. Samples from the gastric secretion did not cluster among themselves, however they clustered with pharyngeal (sample of day 20) and with tracheobronchial (sample of day 23) samples.

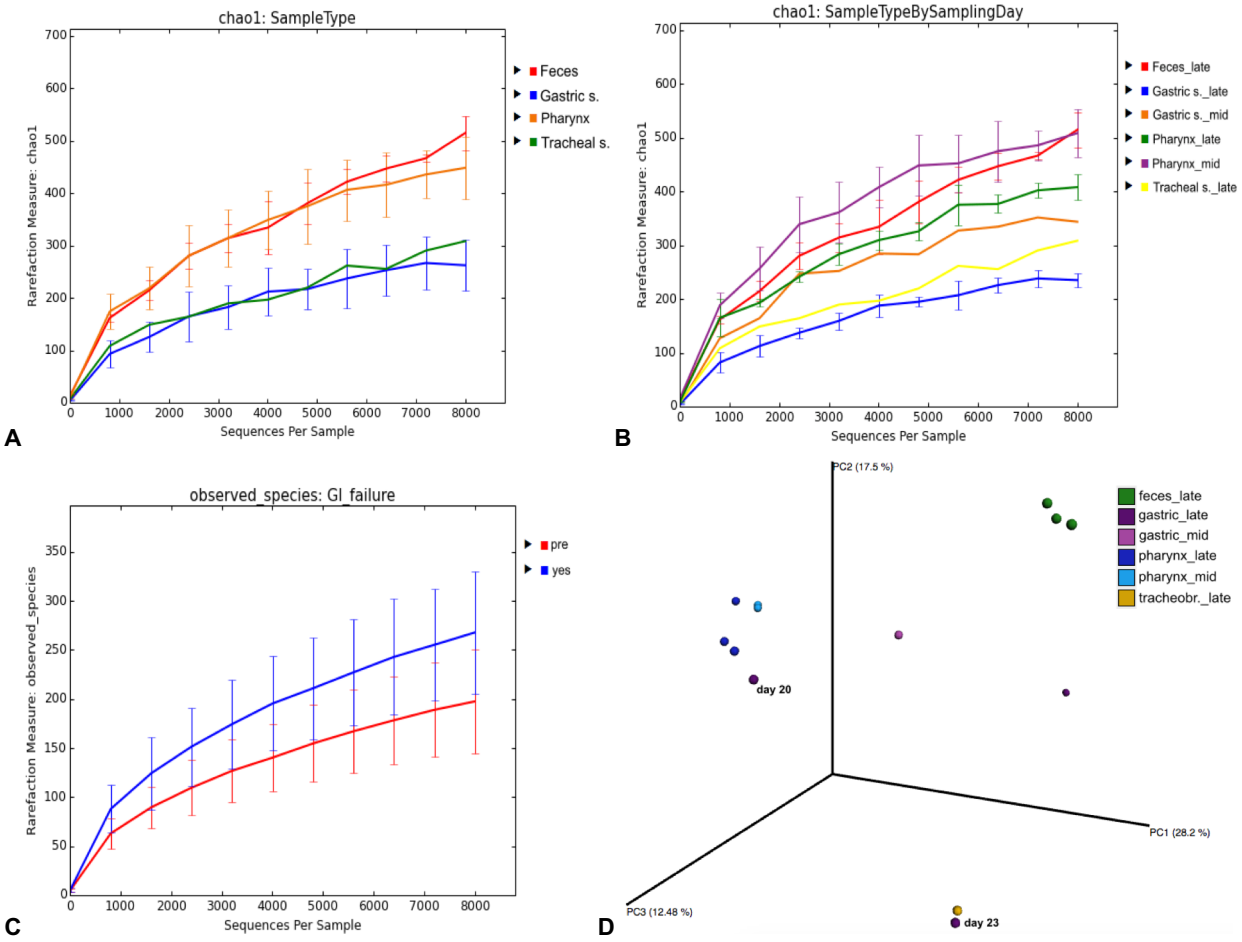


Figure 22. Patient 6 – Diversity analysis.
All graphs are discussed closer in the text. A) Alpha diversity rarefaction curves measuring Chao1 index for sample types. B) Alpha diversity rarefaction curves measuring Chao1 index for sample type by sampling time groups; mid: sampling days 6-14, late: sampling days 15 onwards. C)) Alpha diversity rarefaction curves measuring Chao1 index for samples before and during GI failure, pre: patient had suffered from GI failure in the past, but not at the time point of sample collection; yes: patient suffered from GI failure at time point of sample collection. D) Beta diversity analysis of unweighted UniFrac distances. A clustering of fecal samples and pharyngeal samples among themselves is seen. Late gastric samples (day 20 and 23) show clustering with other sample areas.

4.6.6 Summary of Patient 6

In patient 6, marked microbial changes could be observed over the longer course (especially in pharynx and gastric secretion).

The most interesting finding was a generalized presence of pathogens: *Staphylococcus* reads dominated in **all** samples of day 15, comprising 60% in pharyngeal samples, 87% in gastric samples and 31% in fecal samples. The clinical history revealed infections with coagulase

negative *Staphylococcus* on the one hand (a wound infection diagnosed on day 16 and a blood stream infection diagnosed on day 18) and with *multi-resistant Staphylococcus aureus* (wound infection diagnosed on day 20) on the other hand. Due to the temporal proximity, an association between the dominating bacterium and the infecting pathogen seem likely. As this evaluation shows, microbial changes in the pharynx, feces and gastric secretion occurred several days before the infection got detected in the clinical course of infection.

A similar pattern was shown for *Enterococcus,* which was present on days 20 and 23 in samples from all sample areas; 70% in the pharynx (day 20), 92% in gastric secretion (both days), 80% in tracheobronchial secretion (day 23) and around 10% in feces (both days). This finding could be associated to the wound infection with *Enterococcus,* diagnosed on day 16.

Overall microbial diversity decreased over the course of time in this patient (seen in pharyngeal and gastric samples). As seen in other patients from this study, alpha diversity was the lowest in samples from the gastric secretion and highest in feces and pharynx.

Finally, an overall loss of diversity (all sample areas summarized) after GI failure was demonstrated (also seen in patient 3). However, a greater number of patients with GI failure would be necessary to interpret a possible significance of this observation.

4.7 Results Summarized

4.7.1 Microbial Composition

4.7.1.1 Phylum Level

Overall, *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria* and *Fusobacteria* represented the most abundant phyla. *Tenericutes* and *Cyanobacteria* were abundant in lower numbers in some body habitats (Figure 23).

Fecal samples of all 6 ICU patients summarised show a dominance of *Firmicutes* and *Bacteroidetes* in all 3 time groups (early, mid and late samples) (Figure 23). The changes between each time period of hospitalisation were more pronounced in the other investigated body habitats.

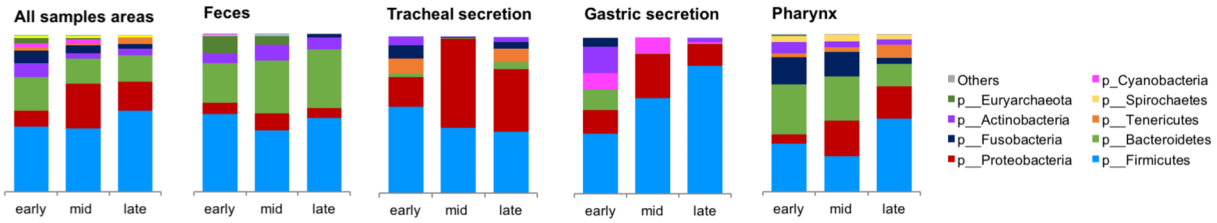


Figure 23. Relative proportions of the bacterial composition in samples of all patients on the phylum level. Samples were divided into the 3 groups early (day 1-5), mid (day 6-14) and late (day 15 onwards).

When analysing the trends of lower intestinal microbiota individually for each patient (Figure 24) dynamic changes in composition were observed in all patients. An increase of *Bacteroidetes* over the course of time was seen in 3/6 patients; in each case *Bacteroidetes* dominated over *Firmicutes* in these later samples. One sample did not include any Firmicutes. In contrast, patient 4 presented a total dominance of *Firmicutes* in late fecal samples.

Inconstant results concerning the changes of *Firmicutes* and *Bacteroidetes* in the feces of critically ill patients have been found in the few studies available – our results accord with findings from Ojima et al. (105), who also detected an increase of *Bacteroidetes* in the majority of cases. In contrast, a study by Zaborin et al. (53) reported a dominance of *Firmicutes* and *Proteobacteria* as well as a concomitant reduction of *Bacteroidetes* in fecal samples of critically ill patients.

Overall, a loss of phyla over the course of time was visible in fecal samples of patients 3, 4, 5 and 6. Importantly, there was no clear difference in the prevalent phyla between deceased and discharged patients.

Concerning the other sample areas, a total dominance of either *Proteobacteria* or *Firmicutes* in at least one sample was found in pharyngeal samples of 3/6 patients, in gastric samples of 4/6 patients and in tracheobronchial samples of 3/6 patients. In all these cases (except one gastric sample), the total abundances were found in samples from the mid and late time points. In contrast to fecal samples, *Bacteroidetes* showed a clear decrease in the pharynx and were only minimally to not detectable in tracheal and gastric samples.

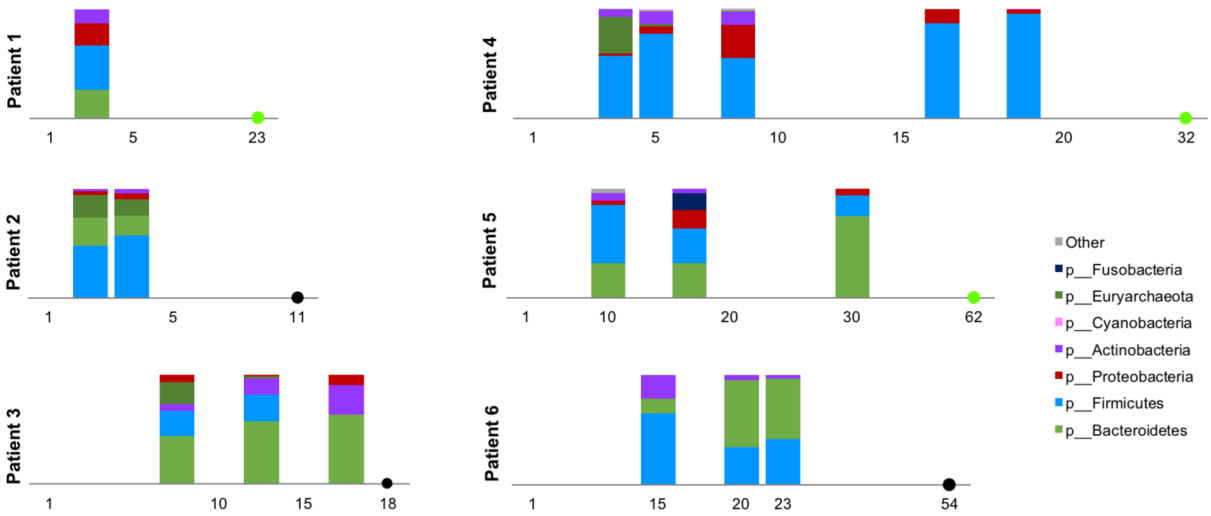


Figure 24. 16S rRNA analysis of microbial composition in fecal samples of all patients on the phylum level. The clinical outcome is illustrated by circles on the time line; black: death, green: alive. The x-axis is measured in days on the ICU.

4.7.1.2 Genus Level

To summarize the results of all patients, we found bacterial communities consisting of a small number of different genera in samples from all habitats; especially in samples from later time points of hospitalisation.

Low diversity communities with a single bacterial genus accounting for over 75% of sequence reads were found in 5 of the 6 patients. Interestingly, this mainly concerned samples from pharynx, tracheobronchial and gastric secretion; only one fecal sample showed a mainly one-member community (p_*Bacteroidetes;* g_*Bacteroides* at 74%).

Extremely low diversity communities, where one member comprised over 90% of sequence reads were found in patients 4, 5 and 6 from gastric and pharyngeal samples. In these low and

extremely low diversity communities *Staphylococcus* and *Enterococcus* were the most frequently seen genera (dominating in around 56% of these samples), which is consistent with result from other ICU studies (53,100,106). Other dominating taxa were *Streptococcus, Bacteroides, Pseudomonas, Pediococcus, Corynebacterium, Porphyromonas* and *f_Enterobacteriaceae*, indicating a large variation in dominating genera among the studied patients.

Regarding the time points of collection, low diversity communities were mainly observed in samples from mid and late groups (only two samples from the early time group showed a low diversity community, compared to 12 from mid and late groups). This suggests a loss of bacterial community members in the later course of hospitalisation, which is also seen at low Chao1 indices (described below).

As representation of important commensal fecal microbiota, representing around 5% in healthy individuals (100) the proportions of *Faecalibacterium, Ruminococcus* and *Blautia* were evaluated. In all patients, these genera were almost completely lost over the course of hospitalisation.

In all those patients who had suffered from a bacterial infection during the course of hospitalisation (5/6 patients) the infecting pathogen (genus level) was detected by 16S rRNA sequence analysis in at least one sample. The pathogens were detected in samples from before and/or after the clinical diagnosis of the infection. A correlation between the type of infection and the sample area positive for the pathogen was not found. However, in most cases the sample area positive for the pathogen differed from the original site of infection – e.g. patient 6 suffered from a BSI with *Staphylococcus*, and *Staphylococcus* was detected in the pharyngeal aspirate before the clinical diagnosis of infection.

Generally, pharyngeal samples were the sample type most frequently positive for infecting pathogens; fecal samples the least often.

4.7.1.3 Species Level

With the 16S rRNA data of this study it was not possible to exactly assess the species level of the present genera.

However, in those cases where a clinically detected pathogen (detected via culture analysis, e.g. *Streptococcus pneumoniae*) was found in our study samples on the genus level (e.g. *Streptococcus*), the chances are high of those bacteria to be the same species. We also included this assumption into the discussion of the results.

4.7.2 Diversity

4.7.2.1 Alpha Diversity

In all patients, fecal samples showed the highest number of observes species, while gastric samples showed the lowest number of observed species in 5 out of 6 patients. In phylogenetic distance analysis, diversity was the greatest in pharyngeal samples (in 3/6 patients) and fecal samples (in 3/6 patients); lowest phylogenetic diversity was seen in gastric samples (in 4/6 patients).

Measurements of the Chao1 diversity index, an estimator for microbial richness, are summarised in Figure 25 of all 6 patients; gastric samples showed the lowest index numbers with Chao1 ≤ 300 in 4 out of 6 patients (Figure 25-A). Generally, Chao1 indices of fecal and gastric samples varied widely between the patients (feces: median 600, range 490; gastric secretion: median 300, range 300), while indices of the tracheobronchial secretion and pharynx were relatively similar in all patients (tracheobronchial secretion: median 300, range 110; pharynx: median 475, range 160). Measurements by sample day (Figure 25-B) revealed consistently decreasing Chao1 index numbers from early to late samples. This indicates a clear loss of microbial richness over the course of hospitalisation in all patients (not evaluable in patient 2). Interestingly, the fecal samples of patient 2 had a distinctly higher Chao1 index (900) than those of other patients, noting that this patient was the only not suffering from an infection. What is more, in this patient only early samples were obtained from the feces, possibly resulting in a higher index number.

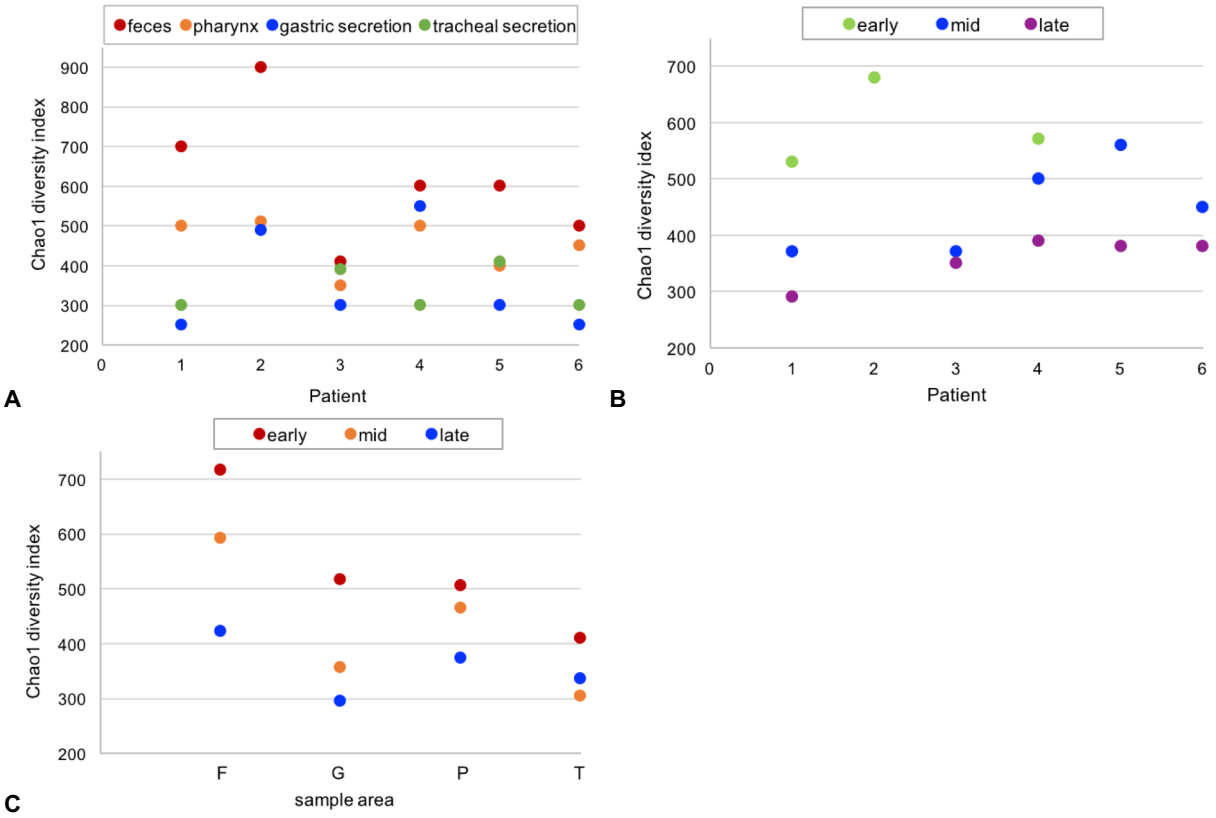


Figure 25. Chao1 diversity index of bacterial composition of all samples.
A) Chao1 index of all sample areas for each patient; fecal samples had the highest index numbers of all areas in all patients; gastric samples had the lowest numbers in 4/6 patients. Mean Chao1 index values and standart deviation (SD): feces 618 (SD 155), pharynx 452 (SD 95), gastric s.: 357 (SD 119), tracheobronchial s.: 340 (SD 49). B) Chao1 index of all sample time groups for each patient; early: sampling days 1-5, mid: sampling days 6-14, late: sampling days 15 onwards. The index number decreased from early to mid and later sample time points in all patients. Mean Chao1 index values: early 593 (SD 63), mid 450 (SD 74), late 358 (SD 37). Some patients did not have samples from every group. C) Chao1 index of all sample time groups for each sample area; F:feces, G: gastric secretion, P: pharynx, T: tracheobronchial secretion. In all sample areas a decrease in Chao1 indices can be seen over time. Mean Chao1 index values and standart deviation (SD): early 538 (SD 111), mid 430 (SD 110), late 357 (SD 47).

4.7.2.2 Beta Diversity

Beta diversity analysis of unweighted UniFrac distances area illustrated in Figure 26. Analysis by sample type (Figure 26-A) revealed a rough clustering of samples from the same sample area. Especially pharyngeal samples showed a very tight grouping which indicates strong similarities in the pharyngeal microbiome across the patient population. Tracheal samples did not cluster as clearly, thus showing microbial similarities with samples from the gastric

secretion and pharynx, however not with fecal samples. A possible reason would be the anatomic proximity of those areas. In Figure 26-B, the outliers are marked by patient name and sampling day. As an example, the pharyngeal sample of patient 4 (day 18) was quite separated from other pharyngeal samples – here, *Staphylococcus* dominated with 78%. Interestingly, the pharyngeal sample of the same patient from two days earlier was very similar on the taxonomic level (*Staphylococcus* comprised 92%), however this sample clustered with the other pharyngeal samples. This example suggests that, at least in this study, beta diversity analysis might not only cluster by the taxonomic composition.

Beta diversity analysis by sample day (Figure 26-C) showed a separation of early and late time points for the majority of samples, which is in accordance with the changes found in alpha diversity analysis. In Figure 26-D, fecal samples are specifically marked, as here a clear separation of late samples to early and mid samples can be seen.

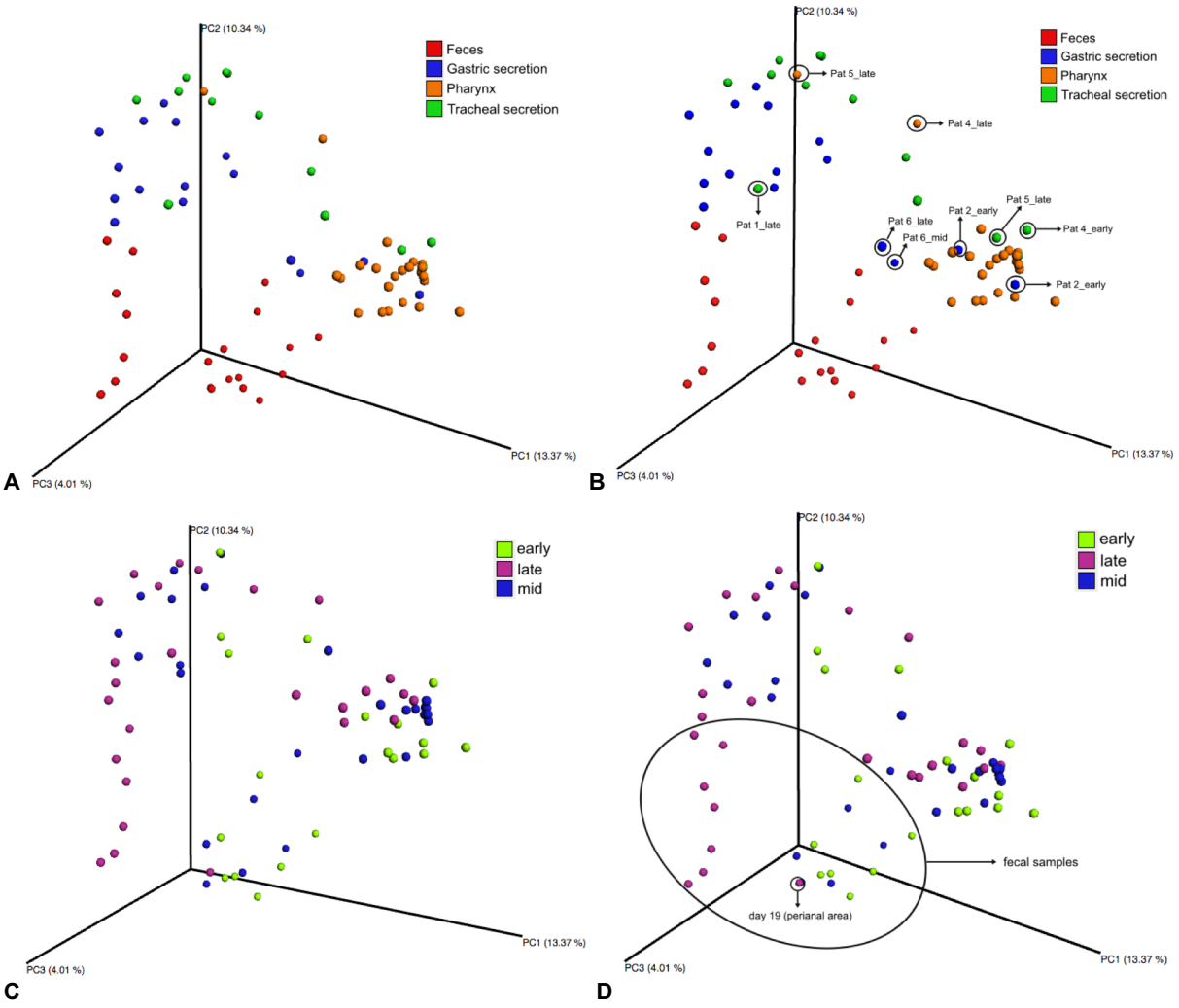


Figure 26. Beta diversity of all samples evaluated with Principal Coordinate Analysis (PCoA) of unweighted UniFrac distances.

A) Analysis by sampling day; a clustering of samples from the same area is visible - especially tight in pharyngeal samples (orange). B) Analysis by sampling day; the outliers are marked by patient number and sampling day. Some outlier-samples are discussed in the results. C) Analysis by sample type; a separation of early and late samples can be seen in the majority of cases - especially visible in fecal samples. D) Analysis by sample type; the circle encompasses all fecal samples - here a clear separation of early and late samples is visible. One late fecal sample (day 19) does not cluster with the fecal samples, noting that it was obtained from the perianal area.

5 DISCUSSION AND CONCLUSIONS

The interpretation of the results, especially for the individual patients, is already included in the result section for better understanding. Therefore, substantial parts of the discussion can be found in the summaries of each patient in the result section.

In this pilot study, high throughput 16S rRNA sequence analysis was performed in six critically ill patients to evaluate changes of the microbiome in feces, pharynx, gastric and tracheobronchial secretion over the course of hospitalisation. To our knowledge, this is the first study evaluating temporal changes in microbial composition in the respiratory, upper and lower gastrointestinal tract in adult ICU patients.

In this small cohort, clear shifts in microbiota composition over time have been found in samples from all sample areas. These changes were detected on the phylum level as well as on the genus level. Overall, a different microbial composition was found in each sample area in beta diversity analysis, however with a loss of site specificity seen in samples from gastric and tracheobronchial secretion. Our data confirmed the expected loss of bacterial diversity over the course of hospitalisation, especially in terms of richness, which was seen in samples from all patients and all sample areas. Measurements of bacterial richness of the respiratory tract showed similar results in all patient (smaller range of Chao1 indices) in contrast to the gastrointestinal tract, where a bigger range was found.

Moreover, low diversity one- to two-member-communities were detected in samples from all patients, especially in those from pharynx, gastric and tracheobronchial secretion. The majority was observed at mid and late time points. The typical nosocomial pathogens *Enterococcus* and *Staphylococcus* were most frequently detected in these communities. This is in accordance with results from other reports, which investigated only the stool microbiota of critically ill patients (53,100,106).

Importantly, in those patients who suffered from a bacterial infection during the course of hospitalisation we found the clinically detected pathogen (on the genus level) in at least one sample from before or after the clinical diagnosis of the infection. In most cases, the same pathogen was found simultaneously in samples from different sample areas and in areas other than the original site of infection. These results suggest a colonization of different body habitats by single bacterial strains, thus underlining the theory of the microbiome displaying a

source of disseminating pathogens in critically ill, as already proposed by Zaborin et al. (53). Crucially, our investigations showed that not only the intestinal microbiome might serve as the reservoir for nosocomial infections, and that microbiota from different body habitats are potentially involved in development of infectious complication. Subsequently, this raises the question of whether infections or the risk for nosocomial infections could be diagnosed earlier in the course of an illness via microbiome analysis. Consequent benefits would be an earlier initiation of therapeutic interventions or even one step ahead, i.e. the prevention of pathogens from systemic spreading. However, to investigate these questions, more research in this field will be needed.

As discussed in other microbiome studies (100,106) an almost complete loss of commensal, "health-promoting" genera such as *Faecalibacterium*, *Ruminococcus* or *Blautia* was observed in fecal samples of all patients from this study.

The dramatic loss of microbial diversity and the simultaneous emergence of pathobiota at multiple body sites in ICU patients may one the one hand develop as a result of the immense host stress during the state of critical illness. On the other hand, therapeutic interventions including multiple antibiotics, vasopressors, mechanical ventilation, proton pump inhibitors, to name but a few, as well as bacterial infections may all play an important part in the development of dysbiosis. In our results, we identified intense antibiotic treatment as the most influencing clinical factor on microbiota changes, which was especially visible in the analysis of patients 3 to 6. There, changes in microbial composition were visible after initiation of new antibiotics or after a change in the antibiotic regime. However, to ascertain the influence of individual clinical factors, bigger patient cohorts will be needed.

This pilot study has a several limitations. First of all, the patient cohort was too small and the interpatient heterogeneity too great to assess specific causes of the observed microbial shift. Moreover, the number of samples per time group (early, mid, late) was irregular among the patients and not all patients had samples from every single time group (in many cases due to the exclusion of samples with insufficient number of sequence reads). This especially impeded the comparison of sample areas within each patient, as not every sample area had samples from all time groups. Lastly, this study did not include healthy individuals to serve as controls. However, previous investigation showed that admission to ICU rapidly (during the first 3 days) shifted lower respiratory tract microbiota and heavily differed from healthy controls.

In conclusion, this study showed a marked loss of bacterial diversity and an abundance of nosocomial pathogens in samples from feces, pharynx, gastric and tracheobronchial secretion in critically ill patients. As seen by longitudinal analysis, this so-called dysbiosis increases with the duration of hospitalisation. Pathogens associated to a patient's infection can be detected in samples from the gastrointestinal AND respiratory tract before or after the clinical diagnosis of the infection. Therefore, the microbiome from multiple body sites might serve as source of disseminating pathogens in critically ill.

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