











RESEARCH PAPER

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## Acute appendicitis manifests as two microbiome state types with oral pathogens influencing severity

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### ABSTRACT

Mounting evidence suggests that acute appendicitis (AA) is not one but two diseases: complicated appendicitis, which is associated with necrosis leading to perforation or periappendicular abscess, and uncomplicated appendicitis, which does not necessarily result in perforation. Even though AA is the most frequent cause of surgery from abdominal pain, little is known about the origins and etiopathogenesis of this disease, much less regarding the different disease types. In this study, we investigated the microbiome (inter-domain amplicon and metagenome sequencing) of samples from the appendix, rectum and peritoneum of 60 children and adolescents with AA to assess the composition and potential function of bacteria, archaea and fungi. The analysis of the appendix microbial community revealed a shift depending on the severity of the AA. This shift was reflected by two major community state types that represented the complicated and uncomplicated cases. We could demonstrate that complicated, but not uncomplicated, appendicitis is associated with a significant local expansion of oral, bacterial pathogens in the appendix, most strongly influenced by necrotizing *Fusobacterium* spp., *Porphyromonas* and *Parvimonas*. Uncomplicated appendicitis, however, was characterized by gut-associated microbiomes. Our findings support the hypothesis that two disease types exist in AA, which cannot be distinguished beyond doubt using standard clinical characterization methods or by analysis of the patient's rectal microbiome. An advanced microbiome diagnosis, however, could improve non-surgical treatment of uncomplicated AA.

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## Introduction

With approximately 100 cases per 100,000 person-years, acute appendicitis (AA) is the most common reason for emergency abdominal surgery in westernized countries. The lifetime risk of developing AA is estimated at between 6–17%, depending on a person's sex, life expectancy, region and socioeconomic status.<sup>1–4</sup> While a distinct morphological succession can be observed in the appendix during AA (Figure 1), the etiopathogenesis of this disease is still not fully understood. Historically, appendicitis was thought to result from (temporal) luminal obstruction, followed by distention, bacterial overgrowth and increased intraluminal pressure, eventually resulting in the disintegration of the vermiform appendix wall and thereby to gangrene or perforation.<sup>6</sup> However, this hypothesis has only limited support, as both obstruction by a fecalith and increased luminal

pressure are only found in about 20% and 25% of appendicitis patients, respectively.<sup>7,8</sup> Furthermore, researchers have argued that AA does not necessarily result in gangrene or perforation. In fact, appendicitis likely represent two different diseases: uncomplicated and complicated appendicitis,<sup>9</sup> each with a distinct epidemiology and pathophysiology,<sup>10–12</sup> that usually do not develop into the other.<sup>13,14</sup> Complicated appendicitis has been described as perforated appendicitis, periappendicular abscess, or peritonitis, which is defined as an acute inflammation of the peritoneum that occurs in addition to the infection of the appendix.<sup>15</sup>

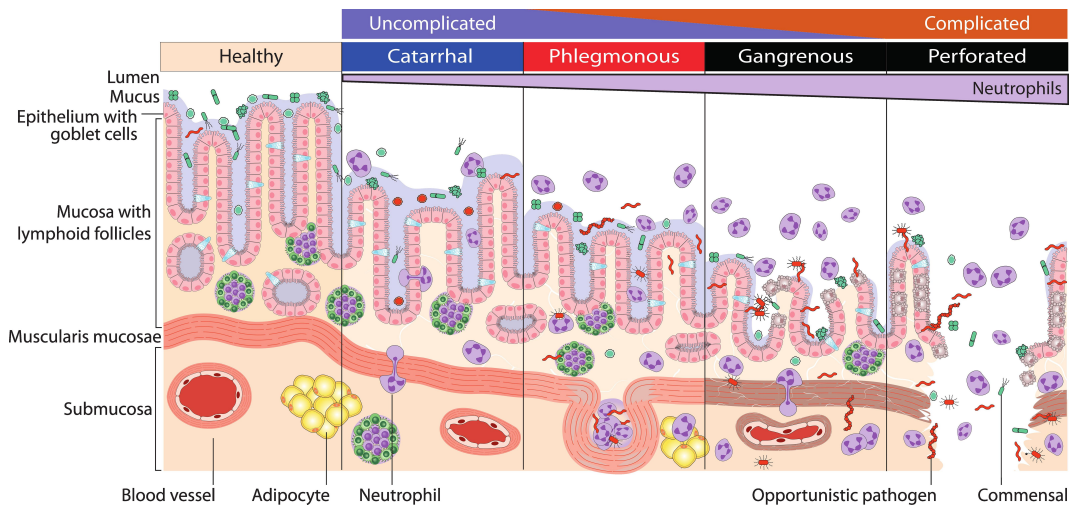
Recently, mounting evidence suggests that microbial dysbiosis together with an uncontrolled inflammatory response drive the development of the disease.<sup>16–22</sup> This claim is supported by the successful treatment of uncomplicated appendicitis with

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**Figure 1.** Disease stages of acute appendicitis. Catarrhal or early appendicitis is characterized by neutrophilic infiltration of the appendix wall, whereby neutrophils transmigrate into the lumen, caused by luminal obstruction or bacterial infection. Fluid accumulation and increased intraluminal pressure may lead to tissue distention, mucosal ulceration and bacterial passage through the epithelium. In phlegmonous appendicitis, the inflammation involves the entire appendix wall and leads to extensive ulceration, vascular thrombosis and frequently to intramural abscess formation. Increasing intraluminal pressure and thrombosis support bacterial tissue penetration and lead to gangrenous appendicitis with large areas of tissue necrosis. Terminally persisting tissue damage can result in perforation.<sup>5</sup>

broad-spectrum antibiotics as an alternative to surgery.<sup>23</sup> However, as in other gastrointestinal diseases, it is difficult to pinpoint the specific microorganisms that are responsible for the development of appendicitis. One problem is that each “healthy” microbiome is unique and may already include opportunistic pathogens that cause diseases. Another problem is the non-accessibility of healthy appendix microbiomes, as incidental appendectomies are not performed routinely.

Previous work using both culture-dependent and -independent methods suggested the existence of a tight connection between the occurrence and abundance of specific microbial taxa and AA. The latest studies based on amplicon sequencing and real-time quantitative polymerase chain reaction (RT-qPCR) identified a local expansion of oral cavity-associated microbes, including *Fusobacterium*, *Peptostreptococcus*, *Porphyromonas* and *Gemella*.<sup>17,19,24,25</sup> Other studies have connected the inflammation to food-borne pathogens such as *Campylobacter jejuni*.<sup>26</sup> Blood *et al.*<sup>25</sup> have even shown that some of the aforementioned oral pathogens can potentially survive the passage through the stomach, suggesting

that AA may be an infectious disease. Out of these oral microorganisms, only *Fusobacterium* spp. are recurring taxa commonly found in AA,<sup>16,17,19,24,27,28</sup> with *F. nucleatum* being capable of infiltrating the appendix lumen depending on the disease severity.<sup>27</sup> In contrast to their reportedly high impact in AA, Fusobacteria were only found in 62% of the appendicitis patients described by Swidsinsky *et al.*<sup>27</sup> and, likewise, other pathogens are also inconsistently found among AA patients, suggesting a different/individual etiopathogenesis for AA.

Only a few studies with a limited number of patients have been carried out to investigate differences in the microbial composition at different stages of AA, and the results were mostly based on amplicon-based analyses.<sup>24,28,29</sup> As microbial causes and/or responses may be specific to the grade of disease severity, we prospectively recruited 60 AA patients, grouped them according to postoperative pathological findings and analyzed the microbial composition in the appendix as well as in the peritoneum and rectum (swabs). For this purpose, we performed 16S/23S rRNA gene amplicon sequencing for all samples and shotgun metagenomic analysis of

appendix samples. Below, we describe the functional and compositional relevance of bacteria, archaea and fungi with respect to different stages of AA.

## Results

### Study population

In our study, we were able to monitor microbial differences in the appendix, rectum and peritoneum throughout three severity stages of acute appendicitis (AA). Tissue samples of the vermiform appendix and rectal swabs were obtained from 60 patients diagnosed with AA (median age: 12.0, range: 3 to 17 years), while peritoneal swabs were taken from 35 of the 60 recruited participants. A full list of samples and respective metadata can be found in Supplementary Table 1. The final evaluation of disease severity and sub-grouping of samples was performed based on a histopathological investigation as follows: subacute, catarrhal, phlegmonous, gangrenous and perforated (Table 1). In total, four patients without notable pathological findings (subacute samples) were excluded from further microbial analyses due to insufficient sample size. The defined patient groups were not significantly different regarding their age, sex, PAS and Alvarado score or the presence of a fecalith. However, the serum CRP concentration was significantly elevated in patients with gangrenous/perforated appendicitis as compared to those with phlegmonous appendicitis (Kruskal-Wallis test;  $P < .01$ ). Furthermore, the number of patients treated with antibiotics was significantly higher in gangrenous/perforated appendicitis (chi-squared test;  $P < .05$ ). A total of 20 patients (33%) were administered

antibiotics (cefuroxime 100 mg/kg/day intravenously three times daily, maximal dose 1.5 g; metronidazole 20 mg/kg/day intravenously three times daily, maximal dose 500 mg) intravenously before or during surgery, depending on the surgeon's assessment. We did not expect these antibiotics to affect the microbiome due to the short interval between administration and sampling. Even differences in bacterial beta diversity in relation to antibiotics treatment (amplicon data: PERMANOVA – Bray-Curtis,  $P = .048$ ; weighted UniFrac,  $P = .029$ ) are clear confounding factors of the disease severity (antibiotics are applied significantly more often in complicated cases; Table 1) and, therefore, were not considered to have a substantial effect on microbial diversity.

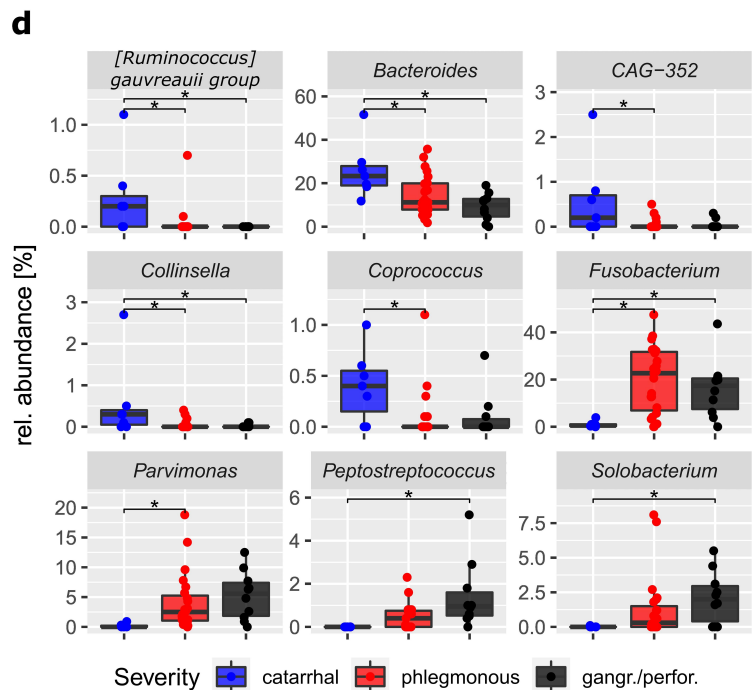
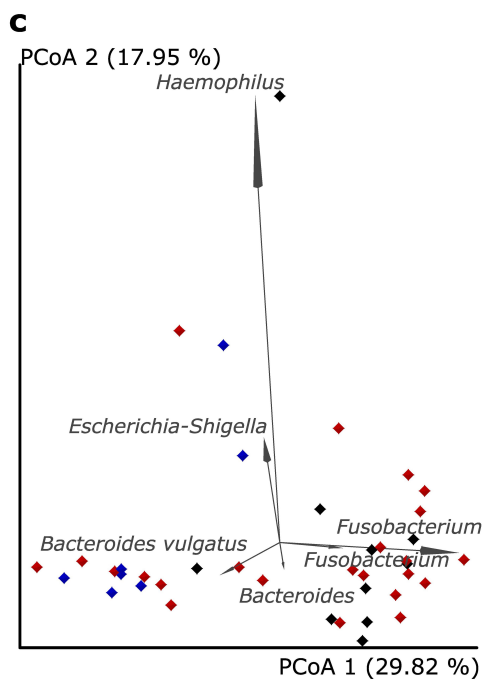
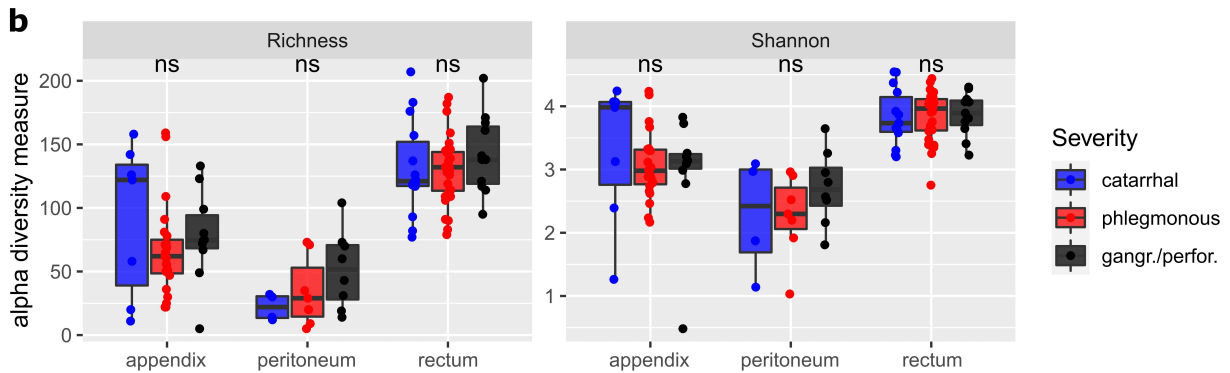
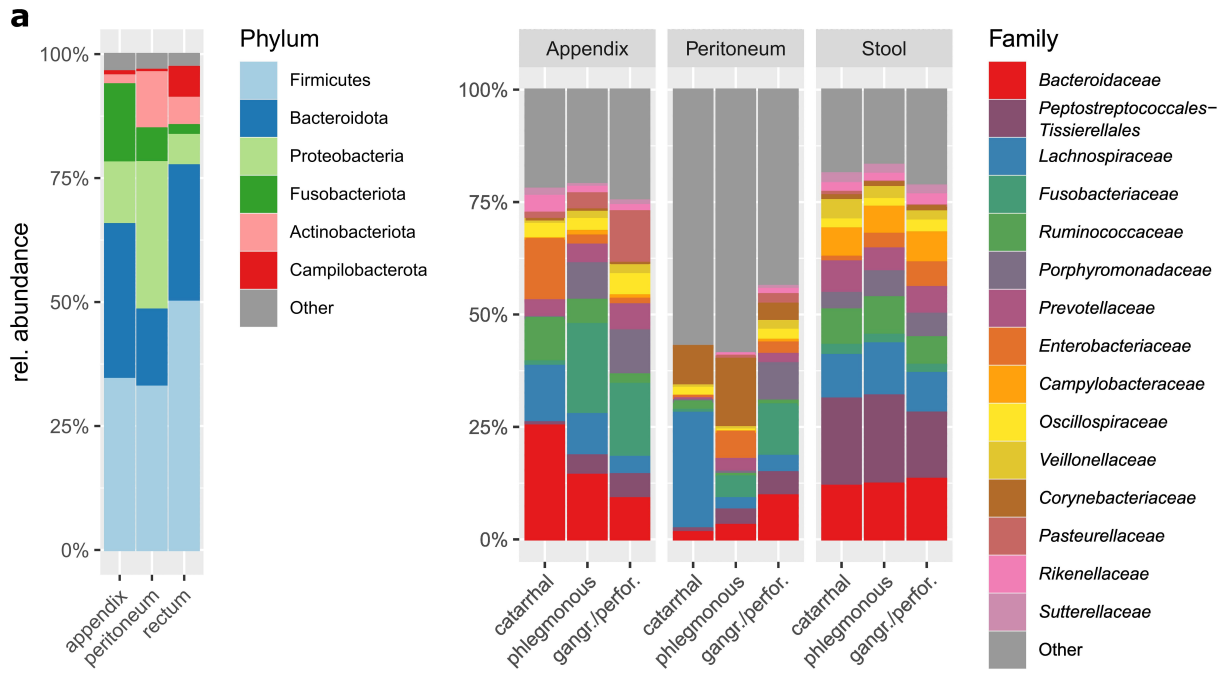
### Bacteriome in acute appendicitis patients

We first evaluated the impact of the bacterial microbiome upon disease progression for the three sample types (appendix, peritoneal and rectal samples). Based on 16S rRNA gene amplicon sequencing of the V4 region, we identified a total of 2,158 ASVs in the dataset after quality control, filtering and SRS normalization (Supplementary Table 5a-c). All three sample types were dominated by the phyla Firmicutes (see Figure 2a; appendix: 34.4%, peritoneum: 32.9%, rectal: 50.0%) and Bacteroidota (A: 31.1%, P: 15.5%, R: 27.5%). Proteobacteria were identified as the second most abundant phylum in peritoneum samples (29.7%) but were less abundant in the other two sample types (A: 12.4%, R: 6.1%). Fusobacteriota were solely represented by the genus *Fusobacterium* in our dataset and constituted 15.7% of the microbes

**Table 1.** Clinical and demographic data for the study cohort grouped by pathological findings of acute appendicitis.

Study population	subacute (n = 4)	catarrhal (n = 14)	phlegmonous (n = 31)	gangrenous/ perforated (n = 11)	P-value
Age (yrs) <sup>a</sup> ± SD	10.0 ± 4.2	10.3 ± 3.0	10.9 ± 3.2	10.3 ± 4.7	.63
Male sex, n (%)	2 (50)	9 (64)	26 (84)	5 (45)	.12
PAS score (0–10) <sup>a</sup> ± SD	6.5 ± 1.9	6.6 ± 2.3	6.7 ± 2.3	8.7 ± 1.5	.19
Alvarado score (0–12) <sup>a</sup> ± SD	6.8 ± 1.9	6.6 ± 2.6	6.6 ± 2.4	8.2 ± 2.7	.20
Leukocyte count (10 <sup>9</sup> /L) <sup>a</sup> ± SD	15.4 ± 8.4	13.2 ± 4.6	14.1 ± 5.6	14.5 ± 4.6	.88
C-reactive protein (mg/l) <sup>a</sup> ± SD	68.5 ± 63.2	37.9 ± 43.2	*23.5 ± 35.7	117.2 ± 101.3	<b>.002</b>
Fecalith detected, n (%)	0 (0)	1 (7)	5 (16)	1 (9)	.69
Antibiotic treatment <sup>§</sup> , n (%)	*0 (0)	*2 (14)	*10 (32)	8 (73)	<b>.007</b>

<sup>a</sup>, mean values; \*, sign. different from gangrenous/perforated; SD, standard deviation; <sup>§</sup>, antibiotic treatment prior to surgical removal of the appendix (cefuroxime 100 mg/kg/day intravenously three times daily, maximal dose 1.5 g; metronidazole 20 mg/kg/day intravenously three times daily, maximal dose 500 mg)



found in the inflamed appendices (P: 6.8%, R: 2.0%), making it the genus with the highest relative abundance in the appendix samples.

As expected, rectal samples showed the highest alpha diversity among the three sample types with an average richness of  $132 \pm 30$  ASVs, followed by the appendix and peritoneum samples with  $75 \pm 41$  and  $37 \pm 28$  ASVs per sample, respectively. While the alpha diversity was significantly different between the three sample types (richness and Shannon diversity; ANOVA:  $P < .001$ ), we did not observe significant changes in relation to disease severity (Figure 2b). In terms of beta diversity, a significant difference from catarrhal to phlegmonous and from catarrhal to gangrenous/perforated appendicitis was observed in appendix samples (but not in rectal and peritoneal samples; weighted UniFrac and Bray-Curtis PERMANOVA:  $P_{\text{adj}} < .05$  and  $P_{\text{adj}} < .01$ , respectively; Figure 2c and Suppl. Figure 1). This indicates that disease progression in AA is defined by a local microbial shift toward a dysbiotic and inflammation-promoting microbiota.

The results of the biplot analysis suggest that *Bacteroidetes* spp. and *Fusobacterium* spp. serve as indicators for disease severity in AA, as they tend to reflect catarrhal and gangrenous/perforated severity, respectively (Figure 2c). Interestingly, two *Fusobacterium* ASVs were found among the six highest-impact features in the biplot analysis for both appendix and peritoneum samples. *Fusobacterium* has been repeatedly reported to be associated with disease severity in AA,<sup>16,17,19,24,27,28</sup> and the results of differential abundance analysis confirm this finding in our dataset (Figure 2d). By applying MaAsLin2 (Multivariable Association Discovery in Population-scale Meta-omics Studies), we identified a total of nine differentially abundant genera, in which the abundances significantly increased or decreased upon disease progression in appendix samples. In addition to observing a significant increase in the relative abundance of *Fusobacterium* signatures, we also observed an increased abundance of other typically oral cavity-

associated microbes including *Parvimonas*, *Peptostreptococcus* and *Solobacterium*. Strikingly, all these (potentially) opportunistic pathogens show a very low abundance at catarrhal severity ( $< 1\%$  rel. abundance) and display a successive local expansion upon disease progression. In contrast, the respective abundance of the genera *Bacteroides*, *Ruminococcus gauvreauii* group, *Collinsella*, *Coprococcus* and CAG-352 (family Ruminococcaceae) was found to be significantly increased in catarrhal appendicitis as compared with higher severity (Figure 2d).

Surprisingly, we did not observe significant microbial differences in rectal and peritoneum samples that were associated with disease severity or any other clinical parameter. In rectal samples, we only observed certain tendencies, such as a successive increase in the genus *Escherichia-Shigella* from acute to gangrenous/perforated severity, based on the results of a pairwise Kruskal-Wallis test ( $P = .026$ ); however, none of these results remained significant after FDR correction. Thus, we hypothesize that appendectomy has only limited impact on rectal samples. In contrast, peritoneum samples show a high heterogeneity between severity grades, displaying a high abundance of skin-associated microbes (e.g. *Staphylococcus*: 7.9%, *Streptococcus*: 10.3%, *Pseudomonas* 5.9%) in cases of catarrhal severity and shifting toward a microbial community that resembled the appendiceal microbiome in cases of phlegmonous and gangrenous/perforated severity. The apparent lack of significance of these results may be explained by the low number of catarrhal and phlegmonous samples with detectable bacterial signals ( $n = 4$  and  $7$ , respectively).

### Impact of archaea and fungi in acute appendicitis

In all sample types, archaeal taxa were dominated by the genera *Methanobrevibacter* (A: 90.6%, P: 78.4%, R: 69.5%) and *Methanosphaera* (A: 8.7%, P: 4.4%, R: 22.7%). Few samples yielded signatures

**Figure 2.** Bacterial diversity in children and adolescents with acute appendicitis ( $n = 60$ ). (a) Microbial composition on phylum and family levels. (b) Alpha diversity measures (left, ASV richness; right, Shannon diversity) are not significantly different within the three sample types (pairwise Wilcoxon signed-rank test, all within-sample type comparisons  $P_{\text{adj}} > .26$ ). For appendix samples (c), the biplot analysis of the six most important ASVs (weighted UniFrac PCoA) and (d) the nine genera that are significantly different between severity grades are shown, respectively (MaAsLin2; \*,  $P_{\text{adj}} < .05$ ). The catarrhal severity grade was used as a reference for the differential abundance analysis.

of Methanomethylphilaceae or the thaumarchaeotal families Nitrososphaeraceae and *Candidatus Nitrosotenuis*, which are believed to represent skin contaminants. We did not observe significant differences between the archaeal composition and disease severity (or any other clinical parameter). Interestingly, *Methanosphaera* abundance and prevalence increased with AA severity, but not significantly (Kruskal-Wallis test for catarrhal and gangrenous/perforated severity:  $P = .08$ ).

In ITS2 gene amplicon sequencing, only 12 appendix samples yielded detectable amounts of fungal signatures after quality control. *Malassezia restricta* represented the most prevalent fungal species that was predominantly found in the non-inflamed and catarrhal appendicitis samples. However, again no significant association between fungal taxa and disease severity or other clinical parameters was observed.

### Community state type analysis

As mentioned earlier in this communication, AA is hypothesized to comprise two different diseases: complicated appendicitis, which reaches the stage of perforation eventually, and uncomplicated appendicitis, which does not. In many cases, however, it is very difficult or even impossible to assess whether an unperforated appendix would have developed a perforation later on. As we observed a marked shift in the abundance of several bacterial taxa based on disease severity, we further investigated whether the microbial composition could be specific for complicated and uncomplicated appendicitis, respectively. In order to test this hypothesis, we performed a *de novo* community state type (CST) clustering based on the abundances of normalized ASV in the bacterial-dominated universal dataset.

Notably, we obtained a total of three different CSTs, two of which correlate well with the proposed disease types (Figure 2). Within CST 2, most of the gangrenous/perforated and, in fact, all of the perforated cases cluster together. This CST is postulated to represent cases of complicated appendicitis, with the key taxa including oral-cavity-associated *Fusobacterium*, *Porphyromonas* and *Parvimonas* species (Figure 2c) which, as we and others show,<sup>16,17,19,27,28</sup> correlate closely with

disease severity. CST 3, on the other hand, is believed to represent cases of uncomplicated appendicitis, as this group contains almost all cases of catarrhal appendicitis, with the gut-associated *Bacteroides* and *Faecalibacterium* as the dominant taxa. The major discriminating factor between these clusters is the disease severity, which is significantly higher in CST 2 as compared to CST 3 (chi-squared test,  $P = .004$ ). This is also reflected in the number of patients with elevated blood leukocytes: 86.3% of CST 2 patients were diagnosed with leucocytosis but only 57.1% of CST 3 patients ( $P = .048$ ). However, other diagnostic parameters did not show significant associations with both CSTs, including CRP (Wilcoxon rank-sum test;  $P = .470$ ), PAS ( $P = .077$ ) and Alvarado score ( $P = .083$ ). Interestingly, we also observed another cluster of four samples in CST 1. This group is unique as we identified very high abundances of either *Haemophilus* (rel. abundance of 98.9% and 40.7%) or *Escherichia-Shigella* (rel. abundance of 28.9% and 53.5%) in the corresponding samples. CST 1 thus represents the “bacterial overgrowth” cluster, in which a single genus or species is hypothesized as being responsible for the AA.

### Metagenomic analysis of inflamed appendices

16S rRNA gene amplicon sequencing provides data about the microbial composition but cannot reliably be used to resolve taxa beyond the genus level or infer the genetic functionality of the microbiome. For this reason, we performed shotgun metagenomic sequencing of all 60 appendix samples and strove to identify key species/subspecies and virulence factors involved in AA pathogenesis. The results of the 16S rRNA gene analysis (Supplementary Table 6a) revealed the species *Fusobacterium necrophorum* (pairwise Kruskal-Wallis;  $P = .029$ ), *Peptostreptococcus stromatis* ( $P = .042$ ) and *Solobacterium moorei* ( $P = .042$ ) to be enriched in gangrenous/perforated compared to catarrhal appendicitis. Furthermore, two *Porphyromonas* species (*P. uenonis*,  $P = .006$  and *P. asaccharolytica*,  $P = .01$ ) were also enriched in gangrenous/perforated samples. However, none of the enriched taxa were found to be significantly differently abundant after FDR correction. Such

a lack of significant differences was also reported recently by Yuan et al.<sup>29</sup> who used a similar classification for AA severity.

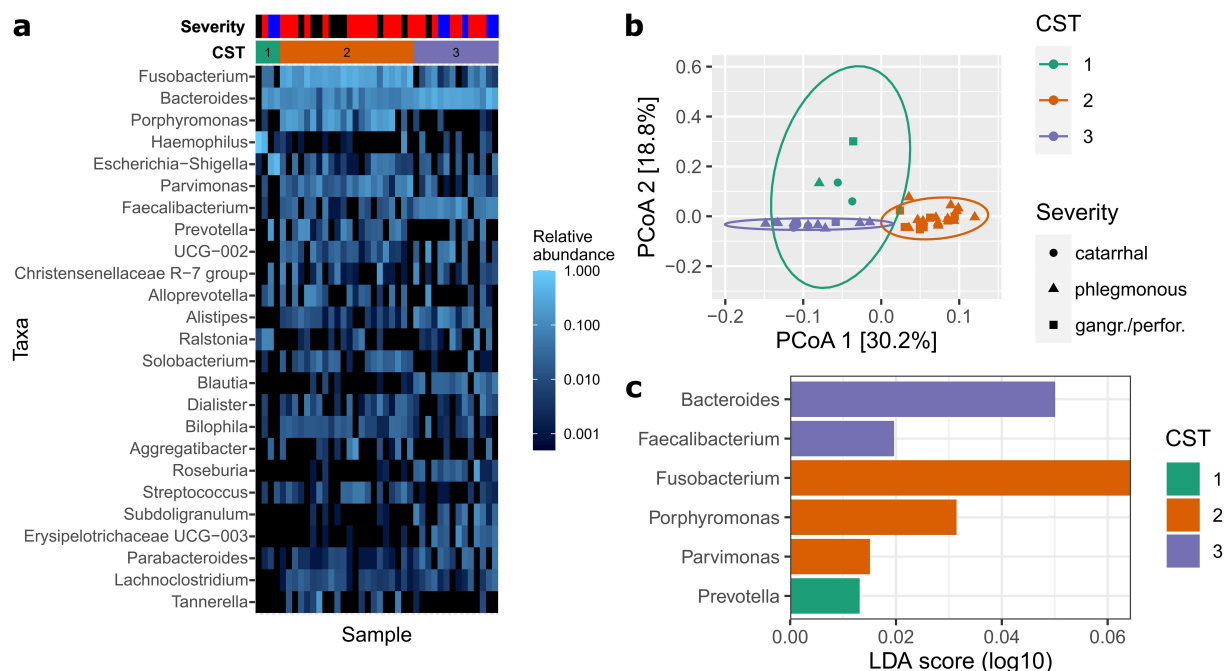
Again, it is likely that pathological categorization is not necessarily linked to the microbial composition alone, especially for phlegmonous cases. Therefore, we applied the 16S rRNA gene amplicon sequencing-based CST clustering method to our metagenomic data and performed a differential abundance analysis on both the taxonomic and functional levels. Based on MaAsLin2, only a significant enrichment of *Fusobacterium necrophorum* ( $P_{\text{adj}} = .035$ ) was determined in CST 2 as compared to CST 3, further highlighting the importance of Fusobacteria with respect to disease severity. *Porphyromonas asaccharolytica* was also found to be enriched in CST 2 but not significantly after FDR correction ( $P_{\text{adj}} = .14$ ). This microbial shift was accompanied by an altered abundance of functional genes in the community (Supplementary Table 6b). In particular, we observed a higher abundance of catabolism pathways in CST 2 and especially for amino acids, including lysine fermentation to crotonyl-CoA ( $P_{\text{adj}} = .035$ ), histidine degradation ( $P_{\text{adj}} = .051$ ), glutamate fermentation ( $P_{\text{adj}} = .066$ ) and the associated Na-driven 2-hydroxyglutarate pathway ( $P_{\text{adj}} = .051$ ), as well as the bacterial proteasome pathway ( $P_{\text{adj}} = .051$ ).

To further validate and test the robustness of the results reported above, DESeq2 was performed (Figure 3). On the taxonomic level, *F. necrophorum* was confirmed as being significantly enriched in more complicated cases (CST 2) but also *F. nucleatum*, two *Porphyromonas* species (*P. endodontalis* and *P. uenonis*) and two unspecified species of the genera *Prevotella* and *Alloprevotella* were significantly enriched in CST 2. Despite the marked expansion of those species, no significant change was observed at the functional level. As noted in the MaAsLin2 analysis, an enrichment of catabolic pathways was apparent in CST 2, indicating a potentially increased release of nutrients by, e.g. apoptotic or necrotic host cells. However, this hypothesis is highly speculative and needs to be verified via physiological characterization of the corresponding species.

Neither the differential abundance nor ABRicate analysis yielded any indications that antimicrobial resistance or virulence genes were enriched among the disease severities.

## Discussion

In this study, we identify microbial signatures that are characteristic and potentially responsible for the different severity stages of AA. We observed a shift in the microbial community in the appendix



**Figure 3.** Community state type (CST) analysis for appendix samples. (a) shows a heatmap of the 25 most abundant genera in the appendix samples, sorted by the three defined CSTs. Appendicitis severity grades are as follows: blue, catarrhal; red, phlegmonous; black, gangrenous/perforated. (b) CST clustering is shown and based on Principal Coordinates Analysis (PCoA). (c) Indicates the most important genera that contribute to the corresponding CSTs as defined by Linear discriminant analysis Effect Size (LEfSe) analysis.

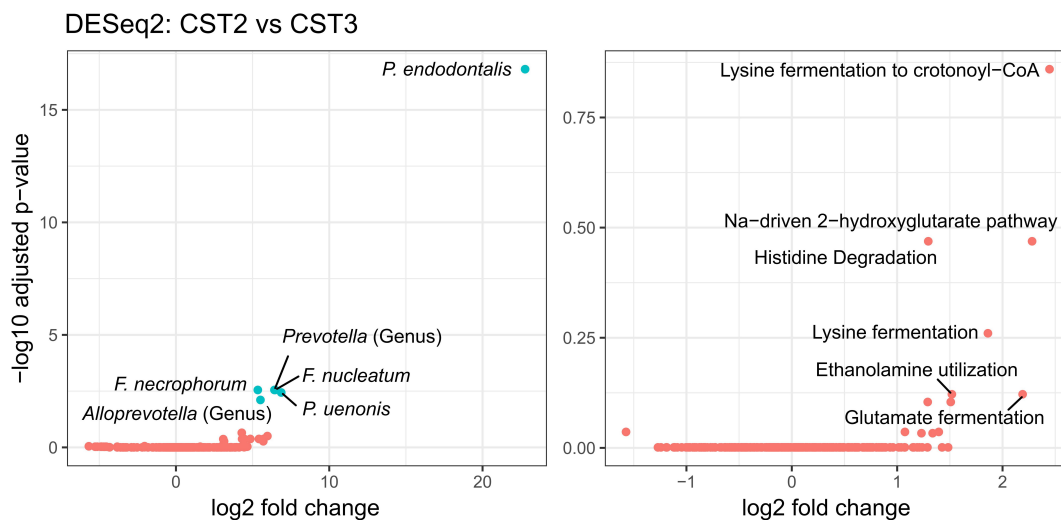
(Figure 2), depending on the severity of the disease, that was primarily attributed to a change in the bacterial beta diversity, but not to an increased richness, as previously suggested.<sup>24</sup> This observed shift in diversity is almost exclusively due to changes in bacterial taxa without any significant contribution from fungi or archaea. Both 16S rRNA gene amplicon and metagenomic shotgun sequencing of the appendix microbiome clearly indicate a local expansion of mainly oral cavity-associated microbes in complicated appendicitis, including *Fusobacterium*, *Porphyromonas*, *Parvimonas*, *Peptostreptococcus* and *Solobacterium*. Likewise, we could provide support for the previous observation of a stepwise decline in gut-associated *Bacteroides* as the disease severity increased.<sup>17,24,27,28</sup> This decline is accompanied by a substantial reduction in the relative abundance of *Ruminococcaceae*, *Collinsella* and *Coprococcus* from catarrhal to phlegmonous and/or gangrenous/perforated appendicitis (Figure 2d).

For over a decade now, researchers have reported that uncomplicated and complicated appendicitis do not share the same etiopathogenesis. This claim is supported by the observation – among others – that not all cases of AA eventually lead to perforation. While the time span from pain onset to surgery positively correlates with perforation, some cases remain phlegmonous even after a long duration of pain.<sup>30–32</sup> Our data on the microbial composition support this difference in etiopathogenesis for AA, as we observed a decisive microbial shift from catarrhal to gangrenous/perforated appendicitis, indicated by the results of the differential abundance and beta diversity analyses as well as by *de novo* CST analysis (Figure 3). We identified three CST clusters with different characteristics. The first cluster is associated with bacterial overgrowth typified by either *Haemophilus* or *Escherichia-Shigella* (CST 1). The second cluster was enriched with Fusobacteria and other oral cavity-associated microbes (such as *Porphyromonas* and *Parvimonas*), a characteristic which appears to be a hallmark of complicated appendicitis (CST 2). In the third cluster, cases are defined by higher relative abundance of typical gut-associated bacteria such as *Bacteroides* and *Faecalibacterium* without an apparent enrichment of oral microbes. These cases might be unlikely to develop

perforation and can be attributed to uncomplicated AA. However, while CST 3 and CST 2 clearly separate catarrhal and perforated appendicitis, the phlegmonous and (to a minor extent) gangrenous cases are distributed in both clusters. It is plausible that the microbial community is only partially responsible for AA severity and that some patients may develop complicated appendectomy with a CST-3-like microbial composition. AA is a multifactorial disease that depends on multiple aspects including lifestyle, diet and genetic predisposition.<sup>1,2,4,20</sup> It is apparent that proper tissue function and microbial homeostasis require a delicate balance between the immune system, microbiome and host epithelial cells. Previous work by Rivera-Chavez *et al.*<sup>20</sup> for example, indicated that single nucleotide polymorphisms in the IL-6 gene can partially explain the development of complicated appendicitis. Thus, an analysis of the microbiome alone does not allow us to explain the disease etiopathogenesis and an uncomplicated microbial state (CST 3) may still (rarely) lead to gangrene. Due to a lack of longitudinal data, we also cannot exclude the possibility that CSTs can shift from one state to another as the disease progresses. For example, uncomplicated (CST 3) may shift to complicated appendicitis (CST 2) upon the further growth of Fusobacteria and colonization by *Porphyromonas* and *Parvimonas* species.

The latest and current findings indicate that special attention should be paid to the presence of Fusobacteria in appendicitis patients, especially when other oral pathogens such as *Porphyromonas* or *Parvimonas* are present. Fusobacteria are robustly and frequently associated with an increased disease severity and appear to be hallmark taxa in the development of complicated appendicitis. While species of this taxon were also found to be part of the normal appendix microbiota,<sup>16,28</sup> their abundance, prevalence and tissue invasion are greatly increased in complicated appendicitis (Figures 2 and 3).<sup>27</sup> The pathogenic expansion and tissue invasion in AA is mainly attributed to the species *F. nucleatum* and *F. necrophorum* (Figure 4),<sup>27,33</sup> two well-known, opportunistic pathogens that are also associated with other gastrointestinal diseases such as inflammatory bowel disease, primary sclerosing cholangitis and colorectal cancer.<sup>34</sup> *Fusobacterium nucleatum* is a mutualistic microorganism that interacts with human tissue in ways that





**Figure 4.** DESeq2 analysis comparing community state types (CST) 2 and 3 on taxonomic (left) and functional level (right). In both plots, the six most important features were labeled and the significantly differentially abundant features are highlighted in blue ( $P_{adj} < .05$  and fold change  $> 1.5$ ). Species abbreviations: *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Porphyromonas endodontalis*, *Porphyromonas uenonis*.

range from neutral to pathogenic. Several disease-promoting mechanisms have been described, ranging from immunomodulatory effects to tissue and cell invasion and on to recruitment and virulence enhancement of other, potentially pathogenic microbes (nicely reviewed in Brennan and Garrett<sup>35</sup>). As such, *F. nucleatum* has been shown to induce antimicrobial and pro-inflammatory host responses (such as  $\beta$ -defensin, IL-6 and IL-8 expression) and to be capable of actively penetrating and surviving in human tissue and immune cells. Furthermore, Fusobacteria are important biofilm-forming bacteria. Due to the high variety of adhesins they produce, *F. nucleatum* can bind to host cells, enabling it to act as a docking hub for other microorganisms due to its elongated shape. In colorectal cancer, Fusobacteria frequently co-occur with other oral microbes such as *Peptostreptococcus* spp. and *Leptotrichia* spp. and it is feasible that a *Fusobacterium*-mediated colonization of secondary oral pathogens such as *Porphyromonas*, *Parvimonas*, *Solobacterium* or *Peptostreptococcus* also occurs in complicated appendicitis.<sup>36,37</sup> All of the mechanisms employed by Fusobacteria are common virulence factors that aid bacteria in establishing new niches, acquiring nutrients and evasion of the immune system.<sup>38</sup> As such, the increase in the relative abundance of amino acid metabolizing pathways that was observed in the metagenomic data may be directly linked to the growth of Fusobacteria. Free lysine,

histidine, glutamate and serine are required for *F. nucleatum* growth,<sup>39</sup> and the relative abundance of the catabolic pathways for the production of the former three amino acids were enhanced in CST 2. Especially lysine fermentation to crotonyl-CoA was predominantly found to be enriched in more severe cases (CST 2), and this pathway has been described in only few microbes, including *F. nucleatum* and *Porphyromonas gingivalis*.<sup>40</sup> It is tempting to speculate that Fusobacteria actively trigger apoptosis in intestinal epithelial cells during AA to release peptides and amino acids.

Considering all of these aspects, Fusobacteria appear to play a causative role in several diseases. However, other researchers have also suggested that Fusobacteria only play a passenger role in disease,<sup>41</sup> especially since not enough evidence is available to satisfy Koch's postulates. The same holds true in case of AA. Fusobacteria are certainly associated with disease severity, and particularly with complicated appendicitis, but we do not yet know whether they play a role as a driver or passenger in the disease.

In recent decades, major improvements in AA diagnosis have been achieved, e.g. by implementing and evaluating standardized clinical scoring systems, such as PAS and Alvarado, or by applying routine imaging techniques to the lower abdomen, including ultrasound or computed tomography.<sup>42</sup> These tools help physicians choose the appropriate therapy and can help to prevent unnecessary

surgery. Reliable and easily accessible diagnostic and microbial markers could help to further improve AA diagnosis and even might indicate whether antibiotic treatment is suitable.

Unfortunately, we did not detect significant differences in the microbiome of rectal samples within our study cohort. The local expansion of opportunistic pathogens within the appendix had no apparent effect on the rectal microbiome. This result may arguably be due to the high diversity of microbes in the large intestine along with a “dilution” of signatures or microbes from the appendix as they pass through the large intestine. Rectal samples are not suitable for distinguishing complicated and uncomplicated appendicitis but may be useful for discriminating between AA and healthy patients or patients with other gastrointestinal diseases that cause abdominal pain, such as colitis. In fact, previous studies have suggested that rectal samples from AA patients show an elevated richness and increase in the abundance of *Bulleidia*, *Dialister* and *Porphyromonas* as compared to healthy controls.<sup>24</sup> Longitudinal analyses prior to as well as after appendectomy are required to show whether microbial changes in the distal colon are causally linked with AA.

In peritoneal samples, we observed a stepwise but not significant shift from skin-associated taxa dominating in catarrhal cases to intestinal-associated taxa dominating in complicated appendicitis cases. In fact, peritoneal samples may serve as a proxy for AA severity, as invasive pathogens such as *Fusobacteria* can be detected both in patients with perforated appendicitis and even in some cases with phlegmonous and gangrenous appendicitis. While the diagnostic value of peritoneal samples is rather low since sampling requires invasive interventions, we could confirm the presence of pathogen signatures in the peritoneal area even in non-perforated appendicitis. The health implications of this finding are uncertain but underline the importance of quick therapeutic intervention in case of AA via surgery or antibiotics.

Furthermore, we observed a significantly higher incidence of leucocytosis in complicated as compared to uncomplicated appendicitis cases. While the diagnostic value of white blood cell and leukocyte counts in AA is controversial,<sup>43</sup> the presence of “unknown” microbes in the GIT, such as oral cavity-associated pathogens, may be indicated by increased leukocytes counts.

Several questions regarding the etiopathogenesis of AA are still open. First of all, the origins of appendicitis are still unknown. One possibility was proposed by A. Swidsinsky and his team,<sup>27</sup> who postulated that appendicitis is a pathogen-induced infectious disease. This idea was discussed recently in the context of the COVID-19 crisis, as the number of pediatric AA cases substantially dropped (~40%) in 2020 as compared to those in previous years.<sup>44,45</sup> Even though the reasons for this development are unknown, it is possible that social distancing and improved hygiene could have contributed to lower incidence rates by reducing exposure to specific microbes. Furthermore, a recent analysis of stimulated peripheral blood mononuclear cells did not show evidence for innate immune dysfunction in patients with a history in AA, but suggested a possible connection regarding responses between complicated and uncomplicated appendicitis.<sup>22</sup> These findings further strengthen the idea that bacterial infection and/or dysbiosis causes AA. In this regard, it is also of crucial importance to trace the origin of the potential pathogens. Recently, the impact of oral microbes in several inflammatory gastrointestinal diseases was highlighted and received an increased amount of attention.<sup>25,34,46,47</sup> The history of dental health and oral cavity integrity in AA patients should be investigated in further studies as well, since both factors may show a putative link to appendicitis severity.

## Limitations

AA is a multifactorial disease defined by several host- and microbiome-based factors. Subsequent studies should not be restricted to a singular aspect of this disease, but include holistic analyses of both the host (genetics, immunological parameters) and microbiome (composition, metabolomics, transcriptomics). Considering the potentially negative effects of oral pathogens involved in AA, saliva and subgingival sampling should also be considered in future studies. Whether the tendencies and borderline-significant results we show are truly associated with AA could be further resolved in studies with larger cohorts, ideally including a suitable number of incidental appendectomies that could serve as controls.

## Conclusion

We show that uncomplicated AA was characterized rather by the increased relative abundance of typical gastrointestinal microorganisms, whereas complicated AA was associated with misplaced oral microorganisms. *Fusobacterium* and its associated fatal group are obviously involved in necrotizing activities, which could substantially lead to barrier breaks and perforation. As these microorganisms originate from the oral cavity, their transmission into the gastrointestinal tract needs to be resolved further, including an examination of the function of the stomach barrier or dental issues. Although a reliable microbiome-based biomarker for distinguishing uncomplicated and complicated AA could not be identified in easily-accessible samples in this study, other (clinical) parameters could fulfil this requirement. As a consequence, further studies are required to delineate the clinical phenotype of *Fusobacterium* infection in appendicitis.

## Materials and methods

### Study design

A total of 60 children and adolescents with acute appendicitis (AA) undergoing appendectomy at the Department of Pediatric and Adolescent Surgery at the Medical University of Graz from April to June 2019 were prospectively recruited. The study was approved by the Ethics committee of the Medical University in Graz (31-004 ex 18/19) and performed according to the Helsinki Declaration. Written informed consent was given by the participants and/or the caregivers after they were provided with written and oral information about the study. All data and samples were pseudo-anonymized.

### Sample and data collection

Preoperatively, demographic data and serum parameters were obtained, including total leukocyte count and C-reactive protein (CRP). Additionally, the Alvarado score and Pediatric Appendicitis Score (PAS) were assessed.<sup>48,49</sup>

Rectal ( $n = 60$ ) and in cases of fluid collection in the abdominal cavity peritoneal swabs ( $n = 34$ ) were taken with sterile nylon swabs (FLOQSwabs™, Copan) prior to and during the

operation, respectively. After the removal of the vermiform appendix, about 1 cm of the proximal part was excised and stored at  $-80^{\circ}\text{C}$  together with the other samples until further processing. The remaining tissue was used for standard histological examination. According to Carr,<sup>5</sup> catarrhal appendicitis was defined as a local inflammation with few intraepithelial neutrophils and reactive intraepithelial changes. Phlegmonous appendicitis was diagnosed in cases with evidence of neutrophils invading the mucosa, submucosa and muscularis propria, intraluminal abscess and invasion of the surrounding tissue. Gangrenous appendicitis additionally showed intramural necrosis. Perforation was defined as microscopic or macroscopic perforation in the abdominal cavity. Sample information can be accessed in the Supplementary Table 1.

### Sample processing

Appendix samples were processed as follows: While keeping the tissue deep-frozen, two cross sections of about 2–3 mm were cut with a sterile scalpel. The first slice was discarded, and about 25 mg tissue were collected from the second, making sure to include luminal content. DNA was extracted from tissue and rectal/peritoneal swabs using the DNeasy® PowerSoil® Kit (Qiagen-Hilden) according to manufacturer's instructions with the following exception: Lysis and homogenization of the samples was performed in two cycles of bead beating using a MagNA Lyser (Roche Diagnostics GmbH) at 6500 rpm for 30s with intermediate cooling. The DNA concentration was measured using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific).

### PCR and amplicon sequencing

To identify the microbial communities from the appendix, rectum and peritoneum, we amplified the V4 region of the 16S rRNA gene to detect bacteria and archaea and the internal transcribed spacer 2 (ITS2) region of the 23S rRNA gene to detect fungi. Sequences from prokaryotic specimens were amplified with the universal primer pair 515 F and 806 R containing Illumina adapter sequences.<sup>50</sup> We performed a nested PCR to specifically amplify archaea DNA by using the primer combination 344 F-1041 R/Illu519F-Illu806R as described previously.<sup>51,52</sup> PCR reactions

were carried out in a final volume of 25  $\mu$ L containing: TAKARA Ex Taq<sup>®</sup> buffer with MgCl<sub>2</sub> (10 X; Takara Bio Inc.), primers (200 nM of each), dNTP mix (200  $\mu$ M of each), TAKARA Ex Taq<sup>®</sup> Polymerase 0.5 U, water (Lichrosolv<sup>®</sup>; Merck) and DNA template (1–2  $\mu$ L of genomic DNA). To amplify DNA from fungal specimens, we used the primer pair ITS86F-ITS4 containing Illumina adapter sequences.<sup>53</sup> The PCR reaction was carried out with the same setup as described above, but 400 nM of each primer were used instead. All primers and PCR conditions used are listed in Supplementary Tables 2 and 3.

Both the library preparation and sequencing of the amplicons were performed at the Core Facility Molecular Biology, Center for Medical Research at the Medical University Graz, Austria. In brief, SequalPrep<sup>™</sup> normalization plates (Invitrogen) were used to normalize the DNA concentration, and each sample was subsequently indexed with a unique barcode sequence (8 cycles index PCR). All indexed samples were pooled, and the products of the indexing PCR were purified with gel electrophoresis. Sequencing was performed using an Illumina MiSeq device and the MS-102-3003 MiSeq<sup>®</sup> Reagent Kit v3-600 cycles (2x251 cycles).

The MiSeq data for all three approaches (universal, archaeal, fungal) were analyzed individually using QIIME2 V2019.11 as described previously.<sup>54,55</sup> Briefly, the DADA2 algorithm was used to demultiplex and de-noise truncated reads as well as to generate amplicon sequence variants (ASVs).<sup>56</sup> Taxonomic assignment was based on the SILVA v138 database for prokaryotic specimens and on the UNITE v8.3 database for fungi.<sup>57,58</sup> Fungal raw reads were also pre-processed with ITSxpress,<sup>59</sup> trimming reads to the desired ITS2 region. The datasets were filtered as follows: Potential contaminants were identified and removed with the R software package decontam by providing negative controls (DNA extraction and PCR negative controls) and applying a threshold of 0.25.<sup>60</sup> Control samples were subsequently removed from the dataset. Unassigned ASVs, those classified as chloroplast and mitochondria, and ASVs with fewer than 10 total reads were also removed. Rarefaction of the datasets was performed by scaling with ranked subsampling (SRS) using rarefaction depths of 1000, 100 and 50 for bacteria, fungi and archaea, respectively.<sup>61</sup>

### Whole genome sequencing (WGS)

Shotgun metagenome sequencing was performed for all 60 appendix samples. A total of 200 ng of extracted DNA from each sample was sent to Macrogen Europe (Amsterdam, Netherlands). Library extraction was performed with the TruSeq DNA PCR-Free kit (Illumina) and sequenced with the Illumina NovaSeq 6000 platform (Illumina).

Raw reads were processed as described previously.<sup>54</sup> In brief, quality control and filtering were performed with fastqc (v0.11.8) and trimmomatic (v0.38).<sup>62,63</sup> Accepted reads were mapped to the human chromosome hg19 with bowtie2 (v2.3.5),<sup>64</sup> and unmapped reads were retained using samtools (v1.9).<sup>65</sup> Bedtools (v2.29.0) was subsequently used to extract host-removed files.<sup>66</sup> Unfortunately, a genome-centric approach was not possible for this dataset, as the assembly with Megahit (v1.1.3) and the subsequent binning using MaxBin (v2.2.4) did not yield any draft genomes.<sup>67,68</sup> Even though all samples had ~20 Mil. raw reads, about 99% of all reads mapped to the human genome. This high abundance of host reads could not be mitigated by using the NEBNext<sup>®</sup> Microbiome DNA Enrichment Kit in our approach. Instead, we performed a gene-centric analysis, performing blastx with diamond (v2.0.8) to annotate the host-removed, quality-filtered reads against the NCBI nr database (release Sep. 2020).<sup>69</sup> Megan (6.18.0) was used to remove reads that were classified as Metazoa and to compare samples based on normalized counts.<sup>70</sup> Subsequently, the taxonomic and functional annotation based on the SEED database was exported and used for statistical analysis.<sup>71</sup>

In addition, contigs were screened for antimicrobial resistance and virulence genes with ABRicate using all available databases: ARG-ANNOT, CARD, EcoOH, Ecoli\_VF, MEGARES 2.00, NCBI AMRFinderPlus, PlasmidFinder, Resfinder and VFDB.<sup>72</sup> Furthermore, we mapped the quality filtered contigs against the genome of *Fusobacterium nucleatum* (NCBI Reference Sequence: NZ\_LN831027.1) and several archaeal genomes, using species that were repeatedly reported to be associated to the human gut, including *Methanobrevibacter smithii* (DSM 2374 and strain

WWM1085), *Methanosphaera stadtmanae* (DSM 3091), *Methanomassiliicoccus luminyensis* (B10), *Methanocorpusculum*, *Methanobacterium*, *Halo rubrum lipolyticum* (DSM 21995) and *Haloferax* sp. Arc-Hr.<sup>73</sup>

### Statistical analysis

Statistical analysis of clinical parameters was performed using the IBM® SPSS® Statistics 26 software package. After testing for Gaussian normal distribution using the Shapiro-Wilk test, Kruskal-Wallis tests were applied to nonparametric data, while the comparison of parametric data was conducted with a one-way analysis of variance (ANOVA) and a Tukey post-hoc test. Comparisons of categorical data were performed with the chi-square test.

Microbial data analysis and visualization were done with QIIME2 (V2019.11) and R (V4.1.1). Phylogenetic distances for the amplicon sequencing data were calculated in QIIME2 using the fasttree plugin and subsequently analyzed following the core-metrics-phylogenetic command without further subsampling.<sup>74</sup> Biplots were performed based on the weighted UniFrac distance matrix and were calculated using the biplot plugin in QIIME2. The Emperor plugin was used to illustrate the results of the biplot analysis.<sup>75,76</sup>

Differential abundance and alpha diversity testing as well as visualization were performed using normalized data within R and the packages phyloseq,<sup>77</sup> ggplot2,<sup>78</sup> Maaslin2,<sup>79</sup> DESeq2.<sup>80</sup> A full list of all packages and versions used can be found in Supplementary Table 4. Alpha diversity was calculated based on the filtered and normalized dataset, and mean values  $\pm$  standard deviation (SD) are reported. After testing for normality, pairwise Wilcoxon signed-rank tests were performed, and the resulting *P*-values were corrected for false discovery rates (FDR) according to Benjamini and Hochberg.<sup>81</sup> For MaAsLin2, the default settings were applied except for max\_significance = 0.05 and min\_prevalence = 0.001. DESeq2 analysis was considered significant if  $P_{\text{adj}} < 0.05$  and  $\log_2\text{FoldChange} \geq 0.58$  (corresponds to a 1.5-fold change).

### Community state type (CST) analysis

We performed *de novo* community state type (CST) clustering based on rarefied 16S rRNA gene data (universal primers) and the corresponding weighted UniFrac distance matrix. Calculation of the CSTs was applied using the methodology of DiGiulio *et al.*<sup>82</sup> Based on gap statistics, the number of clusters was determined ( $k = 3$ ).

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## Data availability statement

The datasets supporting the conclusions of this article are available from the European Nucleotide Archive (ENA) repository, Primary Accession: PRJEB49215 in <https://www.ebi.ac.uk/>

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