Dissertation

# The role of Archaea in the human microbiome

A journey towards “breathless” microbes that accompany us

submitted by

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**Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine**

under the supervision of

**Prof. Dr. Christine MOISSL-EICHINGER**

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# Declaration

“I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University’s regulations and procedures concerning plagiarism.

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_____

Graz, 07.08.2025 Marcus Blohs

“Smart people learn from everything and everyone, average people from their experiences, stupid people already have the answers.”

SOKRATES

Dear reader,

I am honored to present you the fruit of my last years of work. As you will see I decided for Archaea to be my primary focus of the thesis, a domain of life that has been misjudged as Bacteria first, later as sole extremophiles and still as rather meaningless for human health. But does this mean we do not need to study them just because they appear irrelevant in comparison to bacteria? Does it mean that they are not capable of colonizing the human microbiome just because they are known extremophiles? And does it mean that they have no effect on human health just because there are no known pathogens? As most of you will already guess, the answer to all these questions is: No. Thanks to a lot of smart people that Sokrates was referring to, science is not about having all the answers already but a process of uncovering the truth step by step - by learning from everyone and everything around us. During my thesis I tried to adopt this philosophy and got a glimpse into the archaeal impact on our everyday lives. I had the honor to dig a little bit deeper into the unknowns of the microbial cosmos surrounding us.

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All co-authors presented here gave their consent to re-use the data from the publications within this thesis. The following co-authors contributed to the data shown in the thesis:

Part 1: **The sanitary indoor environment—a potential source for intact human-associated anaerobes**

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- **Manuela-Raluca Pausan** and **Christine Moissl-Eichinger** initiated the project and were responsible for study design, performed the phylogenetic analysis and contributed to manuscript writing
- **Manuela-Raluca Pausan** performed sampling, biological samples processing, collection of metadata, microbiome data analysis

- **Alexander Mahnert** provided support for microbiome data and statistical analysis
- **Christine Moissl-Eichinger** performed genome mining

Part 2: **Acute appendicitis manifests as two microbiome state types with oral pathogens influencing severity**

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- **Georg Singer** and **Christine Moissl-Eichinger** initiated the project and were responsible for study design
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# Abbreviations and Definitions

| | |
|---|---|
| AA | acute appendicitis |
| ANOVA | analysis of variance |
| ASV | amplicon sequence variant |
| BE | built environment |
| BMI | body mass index |
| CRC | colorectal cancer |
| CST | community state type |
| FDR | false discovery rate |
| GIT | gastrointestinal tract |
| IBD | inflammatory bowel disease |
| IBS | irritable bowel syndrome |
| KEGG | Kyoto encyclopedia of genes and genomes |
| LED | light emitting diode |
| LefSE | linear discriminant analysis effect size |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PERMANOVA | permutational multivariate analysis of variance |
| PMA | propidium monoazide |
| ROS | reactive oxygen species |
| RT qPCR | real-time quantitative polymerase chain reaction |
| SCFA | short-chain fatty acid |
| SD | standard deviation |

# Zusammenfassung

Jeder Mensch ist Lebensraum für eine sehr individuelle Gemeinschaft von bis zu 600 verschiedenen mikrobiellen Arten: dem humanen Mikrobiom. Obwohl der Einfluss des Mikrobioms auf die menschliche Gesundheit und Krankheit unumstritten ist, wird in der wissenschaftlichen Literatur vorwiegend der Einfluss der Bakterien diskutiert. Archaeen werden oft aus der Diskussion um das Mikrobiom ausgeklammert. In dieser Dissertation wird die Rolle und Widerstandsfähigkeit von anaeroben Mikroorganismen - mit dem Schwerpunkt auf Archaeen - in zwei unterschiedlichen Kontexten untersucht: ihr Überleben in der bebauten Umwelt und ihr möglicher Beitrag zur mikrobiellen Entstehung der akuten Appendizitis (AA).

Im ersten Teil analysierten wir das Badezimmer-Mikrobiom von zehn Haushalten unter Verwendung von Propidiummonoazid (PMA) in Kombination mit 16S rRNA-Gen-Sequenzierung, um zwischen intakten Mikroben und freier DNA zu unterscheiden. Dabei wurden Archaeen, einschließlich methanogener Taxa wie *Methanobrevibacter smithii*, konsistent unter der pot. lebensfähigen Fraktion gefunden. Diese Ergebnisse stellen die traditionelle Annahme in Frage, dass strikte Anaerobier außerhalb des Wirts nicht überleben können, und legen die Möglichkeit einer Übertragung von archaeellen Taxa von der Umwelt auf den Wirt nahe.

Der zweite Teil des Projekts befasste sich mit der Untersuchung des Mikrobioms des Appendix vermiformis bei 60 jugendlichen AA-Patienten mittels Amplikonsequenzierung und Metagenomik. Wir beobachteten einen signifikanten mikrobiellen Unterschied mit zunehmender Krankheitsschwere, der durch eine Anreicherung von oral-assoziierten Pathogenen wie *Fusobacterium spp.* und *Porphyromonas* gekennzeichnet war. Dies ging einher mit einem sukzessiven Rückgang von kommensalen Darmbakterien wie *Bacteroides* und *Ruminococcaceae*. Die Analyse des Community-State-Typs (CST) stützte die Unterscheidung zwischen unkomplizierter und komplizierter Appendizitis. Die funktionelle Analyse wies auf einen erhöhten Aminosäurekatabolismus in schweren Fällen hin, was auf eine mögliche Rolle von *Fusobacterium* bei Gewebeschäden und Nährstoffabbau hindeutet. Archaeen wurden auch im Wurmfortsatz und in rektalen Proben nachgewiesen, jedoch mit geringer Prävalenz und ohne signifikanten Zusammenhang mit dem Schweregrad der AA.

Insgesamt wirft diese Arbeit ein Licht auf die Resilienz und ökologische Bedeutung von Archaeen. Die Arbeit plädiert für deren Einbeziehung in die künftige Mikrobiomforschung und wirft neue Fragen zu mikrobiellen Übertragungswegen, zur Dynamik von Gemeinschaften bei entzündlichen Erkrankungen und zur breiteren Rolle nichtbakterieller Domänen bei der Gestaltung der menschlichen Gesundheit auf.

# Abstract

Every person is habitat to a highly individual community of up to 600 different microbial species: the human microbiome. While the impact of the microbiome on human health and disease is indisputable, mainly the impact of bacteria is discussed in the scientific literature. Archaea are often excluded from microbiome discussions due to technical limitations in detection, underrepresentation in public databases, and a lack of characterized pathogenicity. This thesis investigates the role and resilience of anaerobic microorganisms—particularly archaea—in two distinct contexts: their survival in the built environment (BE) and their potential contribution to the microbial etiology of acute appendicitis (AA).

In the first part, we analyzed the bathroom microbiome from ten households using propidium monoazide (PMA) treatment combined with 16S rRNA gene sequencing to differentiate between intact microbes and free DNA. Several strictly anaerobic taxa, including methanogenic archaea such as *Methanobrevibacter smithii*, were consistently found among the intact microbial fraction. These findings challenge the dominant assumption that strict anaerobes cannot survive outside the host and suggest the possibility of environment-to-host transmission for archaeal taxa.

The second part focused on microbiome profiling of the vermiform appendix. The microbiome from 60 adolescent patients suffering from AA was analysied using amplicon and metagenomic sequencing. We observed a distinct microbial transition with increasing disease severity, marked by an enrichment of oral-associated pathogens including *Fusobacterium* and *Porphyromonas*, accompanied by a gradual decline in commensal gut bacteria such as *Bacteroides* and *Ruminococcaceae*. Community state type (CST) analysis supported a distinction between uncomplicated and complicated forms of appendicitis and functional analysis indicated increased amino acid catabolism in severe cases, suggesting a potential role for *Fusobacterium* in tissue damage and nutrient scavenging. Archaea were also detected in the vermiform appendix and rectal samples but at low prevalence and without significant association with AA severity.

In summary, this work sheds light on the resilience and ecological relevance of archaea within and beyond the host. It advocates for their inclusion in future microbiome research and raises new questions about microbial transmission pathways, community dynamics in inflammatory disease, and the broader role of non-bacterial domains in shaping human health.

# Introduction

The human body is host to a vast and diverse community of microorganisms, collectively known as the microbiome. With an estimated $3.8 \times 10^{13}$ prokaryotic cells [1] and a population-wide diversity of more than 45 million non-redundant eukaryotic genes [2], each person harbors a unique combination of microbes that is crucial in maintaining host´s health. However, we still lack a holistic understanding on how the human body interacts with a plethora of different bacteria, archaea, fungi and other protozoa. This complex relation between host and its resident microbes becomes quickly evident when studying the interactions within a multispecies gut microbiome. Laboratory experiments and computational models can only partially represent the dynamic interactions of microbes in these communities. This is due to several confounding factors: the vast genetic diversity among microbial species, heterogeneous sampling from different individuals and body sites, and the often-unknown microenvironmental conditions such as local pH, temperature fluctuations, or nutrient gradients. Additionally, the relationships between microbial and host genetics in combination with environmental influences are often non-linear and poorly defined, making it difficult to determine the specific role and function of individual taxa within a mixed microbial ecosystem. As such, assigning functional relevance to members of the human microbiome—especially in relation to health or disease—requires carefully controlled studies and advanced multi-omics approaches.

In this context, our research aimed to enhance the understanding of the human microbiome by studying two specific areas:

(1) The capability of strictly anaerobic commensals of survival in the build environment (BE) and
(2) the microbial contribution to acute appendicitis (AA) etiopathogenesis.

By focusing on these topics, our research seeks to contribute to a more comprehensive understanding of the human microbiome, particularly of strictly anaerobic human commensals, including human-associated archaea.

## Interplay between the human microbiome and health

The human microbiome has emerged as a critical factor in shaping health and disease, capturing both scientific and public interest in recent years. Discussions about its role often include oversimplified claims, such as "70% of the immune system is located in the gut" or "90% of diseases can be traced back to gut health." While these statements may seem plausible at first

glance, the multi-layered relationship between the microbiome and human health is far more complex. It is without a doubt a fact that the microbial composition is strongly correlated with various diseases, but correlation does not imply causation, and the gut microbiome is only one of many factors influencing health. However, scientific evidence for the microbial contribution in health and disease is in its infancy as compared to the idea that the gut is the primary origin of most diseases, which can be traced back as far as Hippocrates, who famously stated, "All diseases begin in the gut." While there is some truth to this notion, it is misleading to suggest that dysbiosis—a microbial imbalance in the gut—is directly linked to 90% of diseases. Instead, latest research suggests that many diseases lead to measurable shifts in microbial composition, rather than the microbiome itself being the initiating factor. For example, Gupta *et al.* [3] and Gacesa *et al.* [4] identified a common gut dysbiosis signature across various diseases, indicating that illness—regardless of its cause—triggers similar microbial changes in the gut. This means that while the microbiome may respond to disease states, its role in etiopathology remains elusive in many diseases.

A disease that is verifiably associated with microbial dysbiosis is inflammatory bowel disease (IBD). Numerous studies have linked IBD to microbial imbalances, with findings consistently showing reduced α-diversity (the variety of species within an individual) and specific shifts in β-diversity (differences in microbial composition between individuals) in affected patients [5,6]. For other diseases such as colorectal cancer (CRC), microbial diversity findings have been inconsistent, with studies reporting both increases and decreases in bacterial diversity in stool samples [7,8]. Such discrepancy indicates that microbiome changes alone are often insufficient to explain disease ethiopathology entirely. Especially, stool-based microbiome studies may not accurately represent microbial diversity within different regions of the gut, as distinct microbial communities exist in the mucosa and surrounding lumen [9].

Despite these complexities, the microbiome is undoubtedly a key player in immune system function and host defense. In fact, the gut microbiota acts as one among three major defense mechanisms that pathogens need to overcome in order to cause infection in the GI tract; complementing the intestinal epithelial layer, and the mucosal immune system [10]. Gut commensals achieve this through several strategies:

1. Colonization resistance: Beneficial microbes compete with pathogens for space and nutrients, limiting their ability to establish infections [11] .
2. Antimicrobial compound production: Many commensal bacteria produce bacteriocins and other antimicrobial substances that directly inhibit the growth of harmful bacteria [12].

3. Short-chain fatty acid (SCFA) production: SCFAs, such as acetate, propionate, and butyrate, play a crucial role in modulating immune responses and maintaining gut barrier integrity [13–15].

SCFAs are key metabolic products of gut microbial fermentation, primarily derived from dietary fiber breakdown by Firmicutes (which predominantly produce butyrate) and Bacteroidetes (which primarily generate acetate and propionate) [16]. These metabolites have far-reaching effects beyond the gut, influencing metabolic, immune, and neurological functions. SCFAs regulate histone acetyltransferases and histone deacetylases, which influence gene expression related to inflammation, including the inhibition of inflammatory responses mediated by polymorphonuclear cells, lymphocytes, and tissue phagocytes, thus, helping to maintain immune homeostasis [13–15].

Beyond their immunomodulatory effects, SCFAs also play a fundamental role in maintaining gut barrier integrity. Butyrate, in particular, supports the proliferation and function of colonocytes while reinforcing epithelial tight junctions, which helps reduce gut permeability and prevents the translocation of bacteria and microbial-derived molecules into the bloodstream [13]. By preserving epithelial integrity, SCFAs effectively limit exposure to bacterial endotoxins such as lipopolysaccharides, thereby mitigating systemic inflammation and maintaining a balanced host-microbe interaction.

The absorption of microbial metabolites by the gut epithelium extends far beyond localized effects within the gastrointestinal tract, influencing multiple physiological systems throughout the body. One intriguing concept that emerged from microbiome research is the gut-brain axis, which suggests that the microbiome plays a role in modulating neurological function and behavior [17]. This concept suggests that gut microbes can influence mood, cognition, and stress responses through several mechanisms such as Neurotransmitter production. Some gut bacteria produce neurotransmitters such as serotonin, γ-aminobutyric acid, and dopamine, which play critical roles in brain function [18,19]. It is further suggested that SCFAs alleviate brain inflammation and neuroplasticity, potentially impacting mood disorders such as depression and anxiety [20,21].

It is without a doubt that human microbiome is intricately linked to health and disease, serving as a protective barrier, metabolic regulator, and immune modulator. While it is true that many diseases coincide with microbial shifts, the extent to which these changes are causal or consequential remains an active area of research. As scientific understanding evolves, targeting the microbiome through diet, probiotics, and personalized medicine holds promise for future therapeutic strategies.

# Archaea and their role in human health

The human microbiome encompasses not just bacteria but a diverse range of microorganisms including viruses, fungi, protozoa, and archaea. Nevertheless, despite their notable prevalence and substantial functional potential, archaea have often been overlooked in human microbiome studies, which may be explained due to methodological challenges in their detection, cultivation, and characterization [22]. This underrepresentation, alongside a limited understanding of their ecological roles, has led to archaea being termed the "dark matter" of the microbiome, a category of organisms whose genomic and metabolic functions remain poorly annotated and understood [23].

"Life finds a way" is a particularly fitting phrase to describe the resilience and adaptability of archaea. These microorganisms have evolved extraordinary strategies to survive in some of the most extreme environments on Earth, ranging from the scorching hydrothermal vents of the deep sea to the hypersaline lakes and acidic hot springs to polar regions [24,25]. Their remarkable metabolic and structural capabilities allow them to thrive where only few to no other life forms can persist, utilizing alternative energy sources and biochemical pathways to sustain themselves even in the harshest conditions. Archaea are not only extremophiles but also capable of pushing the biochemical limits of life by demonstrating the ability to generate ATP from low-energy reactions that would be insufficient for most other organisms. Through their metabolic versatility, archaea significantly influence global biogeochemical cycles, notably by participating in ammonia and sulfur oxidation, methane cycling, and organic carbon scavenging [25,26]. Specifically, methanogenic archaea (methanogens) drive anaerobic carbon cycling by methane production, primarily through interspecies hydrogen transfer [27].

In the human microbiome, archaea are regarded as "keystone species", with *Methanobrevibacter smithii* being the predominant archaeal taxon found in approximately 96% of the adult population [28,29]. In fact, archaeal representatives have been identified from all conceivable body parts, including the skin, respiratory tract, oral cavity, and gastrointestinal tract [30,31]. In the gut, methanogens have been widely observed of forming syntrophic interactions with bacteria, where they consume fermentation products such as hydrogen gas, formate, and methylated amines – a process known as interspecies hydrogen transfer [32–35]. In coculture, *M. smithii* was shown to enhance growth and metabolic efficiency of bacterial strains such as *Bacteroides thetaiotaomicron* [36]. The removal of hydrogen and other inhibitory metabolic products is considered to facilitate a stable gut microbiome and appears beneficial for human health. It enables bacteria to sustain efficient fermentation, leading to elevated production of beneficial metabolites like SCFAs, particularly butyrate, ultimately supporting the stability of the gut

ecosystem by promoting the gut barrier integrity, modulation of inflammation, and maintenance of immune homeostasis (as described above).

While some archaea only seem to be transient residents of the human microbiome [37], several species have evolved unique strategies to colonize and thrive in the gut. These strategies include the expression of adhesins, bile salt hydrolases, and a diverse carbohydrate-active enzyme repertoire that closely resembles those found in gut bacteria, likely acquired through horizontal gene transfer events [38,39]. Intriguingly, *M. smithii* exhibits glycans on its cell surface that mimic human intestinal glycans, possibly aiding immune evasion or facilitating close adherence to the gut mucosal lining [40]. Moreover, the acquisition of bile acid metabolic genes, notably those encoding cholylglycine hydrolases, suggests an adaptive capacity to thrive within the bile-rich intestinal milieu, thereby enabling archaea to persist and proliferate alongside bacteria in the human gut [38,41].

Clinically, archaeal signatures have been associated with a range of health conditions and physiological states (*Table 1*). Most frequently, the presence and abundance of methanogens – especially *M. smithii* – has been linked to constipation [42], as methane appears to act as a neuromuscular modulator [43]. By inhibiting smooth muscle activity in the ileum and colon, methane slows intestinal transit, which may contribute to bloating and constipation or malignancies such as constipation-predominant irritable bowel syndrome (IBS) [42,43]. Supporting this, breath methane measurements correlate positively with *M. smithii* abundance and inversely with stool frequency [44]. On the other hand, *M. smithii* has also been associated with health benefits including improved cognitive outcomes. A recent study reported a positive correlation between *M. smithii* abundance and performance in domains such as executive function and cognitive flexibility, potentially mediated by its role in stabilizing gut fermentation processes and enhancing SCFA production [45]. This is contrasted by *Methanosphaera stadtmanae*, the second most prevalent methanogen in the human gut, which was detected in approximately 29.4% of healthy individuals in a European cohort [28]. Unlike *M. smithii*, *M. stadtmanae* is known to elicit significantly stronger pro-inflammatory responses upon immune cell stimulation, indicating species-specific interactions with the host immune system and suggesting a potentially greater immunogenicity [46].

Nevertheless, the involvement of archaea in diseases remains controversial. To date not a single archaeon was found which follows a pathogenic lifestyle or is causing disease directly [47,48]. While correlations have been reported in which the archaea were linked with conditions like multiple sclerosis, schizophrenia, Alzheimer's disease, and inflammatory bowel disease, these associations are not proven causative (*Table 1*). In many cases, archaeal populations appear to shift in response to overall microbial dysbiosis rather than directly contributing to disease

pathology (reviewed in [49]). However, archaeal populations can serve as indicators for gut health, and their reduced abundance may indicate dysbiotic conditions linked to disease states such as severe acute malnutrition [50].

Despite their critical roles, archaea remain challenging organisms to detect and characterize due to inherent methodological constraints [22]. Their cultivation requires specialized anaerobic techniques, which limits their routine analysis. Moreover, current sequence databases underrepresented archaeal genetic information, impeding accurate genomic annotations and functional predictions [51].

Taken together, archaea constitute essential, yet understudied, components of the human microbiome, contributing to key metabolic, immunological, and physiological processes. A thorough investigation into their diverse functions, genomic adaptations, ecological interactions, and roles in human health and disease is crucial. Such explorations promise not only to broaden our understanding of microbiome ecology but also to unveil novel therapeutic avenues for managing microbiome-associated health conditions.

**Table 1: Summary of studies that report a potential association between human-associated archaea and disease.** This table provides an overview of selected studies published since the year 2000, that investigated a potential correlation between the presence or abundance of archaeal taxa and human health conditions. Only investigations conducted on >10 human subjects or human-derived samples were included. For studies addressing irritable bowel syndrome (IBS), only those that specified subtypes—such as constipation-predominant (IBS-C) or diarrhoea-predominant (IBS-D)—were considered. It is important to note that none of the referenced studies provide evidence for a direct pathogenic role of any archaeal species. Rather, the data highlight associative trends, underscoring the need for further mechanistic research to clarify causality and function. Adapted from Mahnert *et al.*, 2018 [22] and Mohammadzadeh *et al., 2022* [52].

| Disease | Archaeal aspect | Methodology | References |
|---|---|---|---|
| **Anorexia nervosa** | abs. abundance *M. smitii* ↑ | qPCR | Armougom *et al.*, 2009 [53]<br>Gorwood *et al.,* 2016 [54] |
| **Appendectomy** | breath methane conc. ↓ | Breath test | Takakura *et al.*, 2020 [55] |
| **Archaemia / bacteremia** | Case reports of *M. smithii* presence in febrile patients;<br>codetection of methanogens with H2- or formate-producing bacteria | Microscopy, 16S rRNA gene PCR, cultivation | Drancourt *et al.*, 2021 [56] |
| **Atherosclerosis** | Absent or decreased *M. luminyensis* abundance | Proposed | Brugère *et al.*, 2014 [57] |
| **Crohn's disease** | Prevalence and abundance Methanobacteriales → | 16S rRNA gene qRT-PCR | Krawczyk *et al.*, 2021 [58,59]<br>Cisek *et al.*, 2024 [59] |
| **Colorectal cancer** | Controversial: methane excretion ↑ or → | Breath test | Roccarina *et al.*, 2010 [60] and references therein |
| | Prevalence Methanobacteriales → | mcrA gene PCR | Scanlan *et al.*, 2008 [61] |
| | rel. abundance of methanogens ↓ & of halophiles ↑ | Metagenomics | Coker *et al.*, 2020 [62]<br>Mathlouthi *et al.*, 2023 [63] |
| **Diverticulosis** | Prevalence methane-producer and breath methane conc. → | Breath test | Jang *et al.*, 2010 [64] |
| | Prevalence methane-producer and breath methane conc. ↑ | Breath test | Yazici *et al.*, 2016 [65] |
| **Diabetes type I** | Methane production 🔗 Glucose tolerance | Breath test; oral glucose tolerance test | Mathur *et al.*, 2014 [66] |
| **Diabetes Type II** | Slight increase of Methanobrevibacter and decrease of Methanosphaera | 16S seq. | Bhute *et al.*, 2017 [67] |
| **Encopresis** | Prevalence methane-producer ↑ | Breath test | Leiby *et al.*, 2010 [68] |
| **Inflammatory bowel disease** | Prevalence methane-producer ↓<br>Prevalence Methanobacteriales ↓ in ulcerative colitis | Breath test;<br>mcrA gene PCR | Pimentel *et al.*, 2003 [69]<br>Scanlan *et al.*, 2008 [61] |
| | Prevalence *M. stadtmanae* ↑<br>Msp.-specific IgG in patient serum ↑ | MtaB1 gene qPCR | Lecours *et al.*, 2014 [46] |

| | | | |
|---|---|---|---|
| **IBS: Constipation predominance** | Prevalence methane-producer ↑<br>*M. smithi* → | Breath test and qPCR | Pimentel *et al.*, 2003 [69]<br>Hwang *et al.*, 2010 [70]<br>Kim *et al.*, 2012 [71] |
| **IBS: Diarrhoea predominance** | methane-producer phenotype ↓ or Prevalence Archaea ↓ | Breath test or qPCR | Pimentel *et al.*, 2003 [69]<br>Orgler *et al.*, 2024 [72] |
| **Multiple sclerosis** | Controversial: rel. abundance Methanobrevibacter ↑ or →<br>🔗 pro-inflammatory protein expression ↑<br>Breath methane ↑ | 16S rRNA seq. and Breath test | Jangi *et al.*, 2016 [73]<br>Tremlett *et al.*, 2016 [74]<br>Yee Woh *et al.*, 2025 [75] |
| **Obesity** | Prevalence methane-producer ↑ | Breath test | Basseri *et al.*, 2012 [76]<br>Mathur *et al.*, 2013 [77] |
| | Breath methane conc. ↓ | Breath test | Wilder-Smith *et al.*, 2018 [78] |
| | Abs. abundance Methanobacteriales ↑ | qPCR | Zhang *et al.*, 2009 [79]<br>Patil *et al.*, 2012 [80] |
| | Abs. abundance *M. smithii* → | qPCR | Armougom *et al.*, 2009 [53] |
| | Abs. abundance Methanobrevibacter or *M. smithii* ↓ | qPCR | Schwiertz *et al.*, 2009 [81]<br>Million *et al.*, 2012 & 2013 [82,83] |
| **Parkinson's disease** | Rel. abundance of *Methanobrevibacter* and methanogenesis-associated genes ↑ | Meta-analysis from metagenomics studies | Romano *et al.*, 2021 [84] |
| **Periodontitis** | Prevalence & abundance *M. oralis* ↑<br>🔗 disease severity | qPCR or 16S rRNA gene seq. | Pérez-Nguyen-Hieu *et al.*, 2014 [85] and references therein<br>Horz *et al.*, 2015 [86]<br>Ramiro et al., 2018 [87]<br>Sogodogo *et al.*, 2019 [88] |
| **Sarcopenic cirrhosis** | rel. abundance of *Methanobrevibacter* ↓ | Metabolomics, metagenomics | Ponzani *et al.*, 2021 [89] |
| **Severe Malnutrition** | Abundance *M. smithii* ↓ | qPCR | Million *et al.*, 2016 [90]<br>Camara *et al.*, 2021 [50] |
| **Ulcerative colitis** | Prevalence Methanobacteriales ↓ | mcrA gene PCR or 16S rRNA gene qRT-PCR | Scanlan *et al.*, 2008 [61]<br>Cisek *et al.*, 2024 [59] |
| **Urinary tract infections** | Case reports: codetection of methanogens with H2-producing enterobacteria | Cultivation, 16S rRNA gene seq | Grine *et al.*, 2019 [91] |
| **Vaginosis** | 🔗 prevalence of *M. smithii* | FISH, PCR-seq, rtPCR, isolation and culture | Grine *et al.*, 2019 [92] |

Glossary:
↑/↓: significantly increased/decreased in patients as compared with the control group
→: no significant difference between patients and control
🔗: significantly connected to

# The Built Environment as a potential reservoir for microbial exchange

The human microbiome is shaped by a complex interplay of genetic, dietary, environmental, and social factors, with microbial transmission occurring through direct and indirect interactions with other individuals, food, water, and the surrounding environment [93]. Among these sources, the indoor microbiome [94–96] is considered to play a crucial role in shaping and maintaining a healthy microbiome [93]. In high-income, urbanized societies, individuals spend approximately 90% of their daily lives indoors [97], making the BE one of the primary reservoirs for microbial exposure. Given its constant interaction with human occupants, the BE can facilitate microbial colonization early in life [98,99] and likely contributes to microbiome recovery after disruptions, such as infections or antibiotic treatments [100,101]. Strikingly, cohabitation and environmental factors were found to be the predominant impact factors to shape the gut microbiome, rather than factors such as genetic relatedness [4].

The interactions between humans and the BE microbiome can have both positive and negative consequences. On one hand, certain microbial communities in indoor spaces have been linked to adverse health effects, particularly in healthcare settings where pathogen transmission and antibiotic resistance are significant concerns [102–104]. On the other hand, exposure to diverse microbial communities, particularly those resembling natural environments or agricultural settings, has been associated with reduced risks of allergies and asthma, highlighting the potential benefits of microbial diversity within indoor spaces [105–107].

For microbes to successfully transfer between hosts via the BE, they must survive outside their native habitat [93,108]. However, many human-associated microorganisms are susceptible to environmental stressors, including desiccation, UV exposure, and oxygen toxicity. This challenge is particularly relevant for gut commensals, as the vast majority of microbes residing in the distal colon are strict anaerobes that are described to be largely unable in tolerating oxygen exposure [109]. While some bacterial taxa have evolved mechanisms such as sporulation, allowing them to endure harsh conditions and later germinate within a new host, non-spore-forming anaerobes must rely on alternative survival strategies. These strategies remain largely uncharacterized, particularly for methanogenic archaea, which are common in the human gut but lack known spore-forming capabilities.

Methanogens—especially *M. smithii*—are among the most prevalent archaea in the human gut [28]. However, these organisms are highly susceptible to atmospheric oxygen and their presence is rare

in young children [110]. While methanogens have been detected in the oral cavity and GIT of infants just a few days after birth, they appear to be only transient inhabitants in infants, with their presence likely coupled to environmental input [111]. It is argued that stable archaeal colonization might occur not before one year of age, which raises fundamental questions regarding the mechanisms of methanogen transmission, as they lack known dormancy strategies such as spore formation, to reliably survive the ex-host environment.

Given that transmission routes of gut commensals remain largely unexplored, this study sought to investigate whether the BE could serve as a reservoir for anaerobic, commensal microbes, including archaea. To address this, we analyzed bathroom microbiomes from ten households, as bathrooms are considered a rich source of gut-derived microbes due to toilet-generated aerosols and frequent human contact [112–114]. A major limitation of previous BE studies has been their inability to distinguish between viable and non-viable microbes [93]. To address microbial viability, we combined propidium monoazide (PMA) treatment with high-throughput 16S rRNA gene sequencing.

## The role of microbial dysbiosis in acute appendicitis

AA is among the most frequent causes of emergency abdominal surgery in industrialized nations, with an incidence of approximately 100 cases per 100,000 person-years [115]. The likelihood of developing AA over a lifetime is estimated to range between 6% and 17%, influenced by factors such as sex, life expectancy, geographical region, and socioeconomic status [116–119]. Despite its high prevalence, the precise etiopathogenesis of AA remains incompletely understood. Traditionally, it was postulated that AA results from a temporary obstruction of the appendix lumen, leading to bacterial overgrowth, increased intraluminal pressure, and progressive ischemia, eventually culminating in gangrene or perforation (*Figure 1*) [120,121]. However, this classical model is only partially supported by clinical observations, as luminal obstruction by a fecalith is found in only about 20% of cases, and elevated intraluminal pressure is reported in roughly 25% of patients only [122,123]. Furthermore, perforation does not inevitably follow the course of AA, and not all cases progress from mild inflammation to severe complications [124]. Growing evidence suggests that AA may, in fact, encompass two distinct disease entities: uncomplicated appendicitis, characterized by localized inflammation, and complicated appendicitis, which involves perforation, periappendicular abscess, or peritonitis [124,125]. Both forms appear to have a unique epidemiology and pathophysiology [126–128], with most cases remaining within their respective categories rather than transitioning from one to the other [129,130]. While complicated appendicitis involves an aggressive inflammatory response that can extend beyond the appendix to the peritoneum,

uncomplicated appendicitis may be successfully treated with antibiotic therapy alone, further supporting the notion that different mechanisms underlie these two disease types [131].

Recent studies increasingly suggest that microbial dysbiosis, coupled with an uncontrolled inflammatory response, plays a crucial role in AA pathogenesis [132–138]. The efficacy of broad-spectrum antibiotics in treating uncomplicated appendicitis supports the hypothesis that shifts in microbial communities contribute to disease progression [131]. However, identifying the specific microorganisms responsible for AA remains challenging. The gut microbiome is highly individualized, and what constitutes a "healthy" microbiome varies widely between individuals. Moreover, many opportunistic pathogens exist as commensals in asymptomatic individuals, making it difficult to pinpoint causative agents. Another limitation in understanding AA microbiology arises from the fact that microbiome studies on healthy appendices are scarce, as incidental appendectomies are rarely performed.

So far, both culture-based and molecular approaches have provided insight into the microbial composition associated with AA. More recent high-throughput sequencing techniques, including amplicon sequencing and quantitative polymerase chain reaction (qPCR), have revealed a striking enrichment of oral cavity-associated microbes in AA [133,135,139,140]. Specifically, species belonging to the genera *Fusobacterium*, *Peptostreptococcus*, *Porphyromonas*, and *Gemella* have been frequently detected in inflamed appendices. Additionally, foodborne pathogens such as *Campylobacter jejuni* have also been linked to AA, suggesting that the disease may, at least in some cases, have an infectious etiology [140,141]. Among these microbial candidates, *Fusobacterium spp.* have emerged as particularly relevant, with multiple studies reporting a significant association to appendicitis severity [132,133,135,139,142]. *Fusobacterium nucleatum, for example, was show*n to infiltrate the appendix tissue in dependence of disease severity [142]. However, despite its potential pathogenic role, *Fusobacteria* are not universally present in AA cases. This study by Swidsinsky *et al.* [142] found *Fusobacteria* in only 62% of appendicitis patients, reinforcing the notion that AA may not have a single causative pathogen but rather a diverse and individualized microbial ethology.

While previous research has provided valuable insights into microbial shifts in AA, most studies have been limited in scope, with small sample sizes and a focus on amplicon sequencing [139,143,144]. Given that microbial composition and activity may vary with disease severity, there is a critical need for comprehensive, stage-specific microbiome analyses. To address this gap, we conducted a prospective study involving 60 patients with AA, categorizing them according to their post-surgical histopathological findings. In addition to sampling the appendix, we also collected peritoneal and rectal swabs to evaluate microbial transmission and potential systemic involvement. Our study employed 16S/23S rRNA gene amplicon sequencing for taxonomic

profiling across all sample types, and shotgun metagenomic sequencing of appendix samples to gain functional insights into microbial metabolic activity during AA progression. By integrating taxonomic and functional analyses, we aimed to determine the microbial features associated with different stages of AA and identify key bacterial, archaeal, and fungal taxa that may contribute to disease pathogenesis. Our findings provide deeper insights into the microbial shifts associated with AA, highlighting the complex interplay between host and microbiome in this highly prevalent gastrointestinal disease.

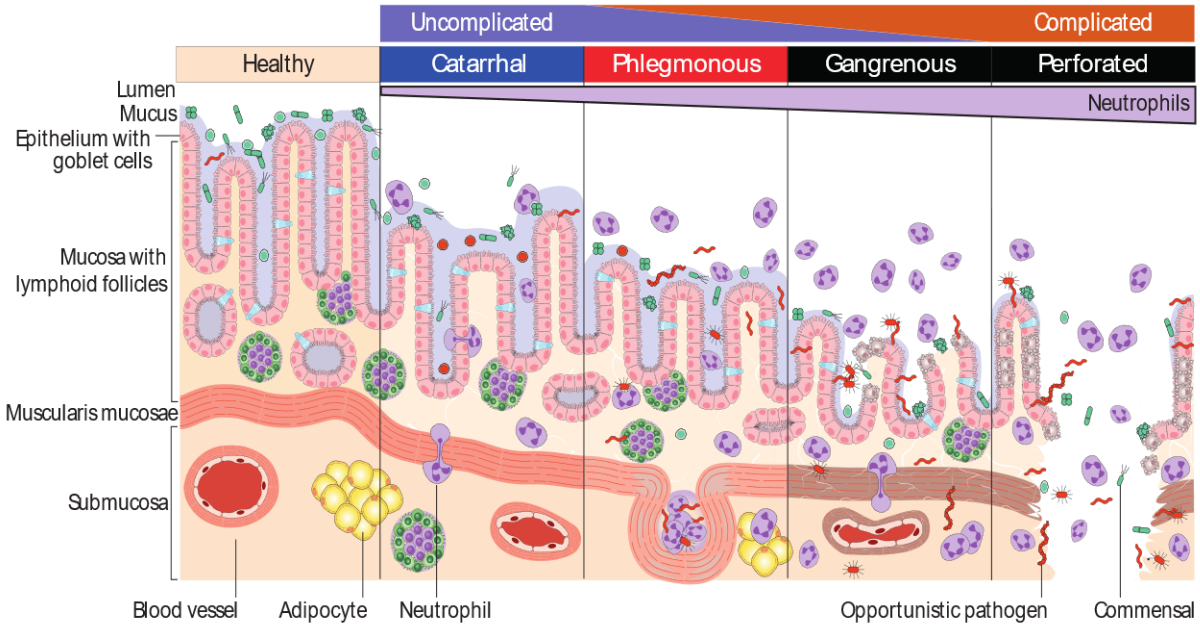



**Figure 1: Progression of Acute Appendicitis.** The schematic representation illustrates the sequential stages of acute appendicitis (AA), from a healthy appendix to advanced disease stages. *Catarrhal appendicitis* marks the early stage, characterized by neutrophilic infiltration into the appendix wall and lumen, often triggered by luminal obstruction or bacterial invasion. This stage involves mild tissue swelling, increased mucus production, and early immune activation. As the condition progresses to *phlegmonous appendicitis*, inflammation extends throughout the appendix wall, leading to mucosal ulceration, vascular thrombosis, and intramural abscess formation. The continued elevation of intraluminal pressure facilitates bacterial translocation across the epithelium, further exacerbating inflammation. In *gangrenous appendicitis*, widespread tissue necrosis results from severe vascular compromise, leading to loss of epithelial integrity and deeper bacterial invasion. This stage is frequently accompanied by extensive immune cell infiltration and breakdown of the appendix structure. Ultimately, *perforated appendicitis* represents the most severe stage, where persistent tissue damage leads to perforation, allowing bacterial spread into the peritoneal cavity and increasing the risk of peritonitis. Figure is taken from Blohs *et al.*, 2023 [145].

# Aim and scope of this thesis

As shown by Weiner et al. [146] on the example of the microbiome-gut-brain axis, Archaea are the least studied microorganisms in this field of research as compared to Fungi, Viruses, Parasites and especially Bacteria. Due to diverse shortcomings in archaeal detection and analysis, this basically applies to all areas of human microbiome research.

This thesis seeks to address these gaps by exploring two areas of human microbiome research in which Archaea may play an unrecognized but potentially important role: their survivability in the BE, and their involvement in the pathophysiology of AA. The first part of this work focused on the presence and viability of anaerobic microorganisms, especially human-associated methanogens, in indoor environments such as household bathrooms. Understanding the survival capabilities of obligate anaerobes under aerobic conditions is of substantial interest, given the increasing awareness of the BE as a microbial reservoir that shapes early-life microbiome development, supports recolonization after illness, and possibly mediates microbe–host transfer. We employed viability-PCR methods using PMA in conjunction with 16S rRNA gene amplicon sequencing to assess the intact fraction of microbial communities present in household environments, with special attention paid to archaeal taxa.

The second part of this thesis aimed for the taxonomic and functional microbial changes across different severity stages of acute appendicitis. The picture of the vermiform appendix, which was long regarded as vestigial, seems to have become repainted as a microbial reservoir and immunological organ. Its anatomical features, such as reduced peristalsis and a mucosa rich in immune tissue, make it a plausible niche for slow-growing, non-motile microorganisms such as methanogens. Using both 16S rRNA gene sequencing and shotgun metagenomics, we analyzed the microbial composition of appendix samples from 60 patients with varying grades of AA, from catarrhal to perforated. We explored whether the presence of archaeal taxa correlates with disease progression and evaluated potential shifts in microbial community structure, functional pathways, and virulence gene content. Additionally, rectal and peritoneal swabs were analyzed to determine whether changes in the appendix microbiota were mirrored in adjacent anatomical compartments.

# Material and Methods

## Indoor microbiome study design

The indoor microbiome study was designed by Manuela-Raluca Pausan. Sample collection, DNA extraction and PCR reactions have been performed by her. The detailed procedure is reported in Pausan & Blohs *et al.* [147] and summarized below.

Ten inhabited households within and around Graz, Austria, were sampled in March 2017. All metadata from the sampled sites are provided in Supplementary Table *1*. For sampling, two 30 cm² areas adjacent to the toilet were selected per household. Those locations were chosen due to a potentially high occurrence of GI-associated and anaerobic microorganisms. A representative sampling site is depicted in *Supplementary Figure 1*. Prior to sampling, each site was thoroughly cleaned with water and bleach and left uncovered for 7 days. Residents were instructed not to touch the cleaned surfaces during this period. After 7 days, the surfaces were sampled by applying sterile 0.9% NaCl solution to sterile nylon swabs (FLOQSwabsTM, Copan, Brescia, Italy) and swabbing the area three times, as illustrated in *Supplementary Figure 1b*. Negative controls were prepared by opening swabs and dipping them in sterile saline solution. One of the two samples from each household was treated with propidium monoazide (PMA; Biotium, Inc., Hayward, CA) according to the manufacturer's recommendations. This treatment aimed to mask free DNA by transferring swabs into sterile 50 µM PMA, 0.9% NaCl solution, followed by 5 min of incubation in the dark at RT and a final 15-minute exposure in the PMA-lite™ LED Photolysis device. These treated samples are referred to as "PMA" samples, while untreated samples are termed "non-PMA".

DNA extraction was performed using the FastDNA Spin Kit (MP Biomedicals, Germany) according to the manufacturer's instructions. Both bacterial and archaeal 16S rRNA gene amplification, as well as Illumina MiSeq v3-600, were carried out following the same procedures as described for the appendix samples below. DNA extraction controls and PCR controls were included.

# Appendectomy study design

A total of 60 children and adolescents diagnosed with acute appendicitis (AA) and undergoing appendectomy were recruited for this prospective study. Patients were recruited at the Department of Pediatric and Adolescent Surgery at the Medical University of Graz from April to June 2019.

Demographic data and serum parameters, including total leukocyte count and C-reactive protein, were collected preoperatively. The Alvarado Score and Pediatric Appendicitis Score were assessed. Rectal swabs (n = 60) and peritoneal swabs (n = 34) were taken using sterile nylon swabs (FLOQSwabsTM, Copan) prior to and during the surgery, respectively. After removing the vermiform appendix, approximately 1 cm of the proximal part was excised and stored at -80°C for further processing. The remaining tissue was used for standard histological examination. Appendicitis was diagnosed based on established criteria, as described in Carr et al. [121] into catarrhal (local inflammation with few intraepithelial neutrophils and reactive intraepithelial changes), phlegmonous (evidence of neutrophils invading the mucosa, submucosa and muscularis propria; intraluminal abscess and invasion of the surrounding tissue) or gangrenous appendicitis (additional intramural necrosis). Perforation was defined as microscopic or macroscopic perforation in the abdominal cavity. Sample information is provided in *Supplementary Table 2*.

# Ethical considerations

The AA study was approved by the ethics committee of the Medical University of Graz (EK31-004ex18/19) and conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants and/or their caregivers after providing them with written and oral information about the study. All data and samples were pseudo-anonymized to protect patient privacy.

# DNA extraction, 16S rRNA gene & ITS region amplification, and sequencing

Appendix samples were processed as follows: Two cross sections of about 2-3 mm were cut from the deep-frozen tissue using a sterile scalpel. The first slice was discarded, and approximately 25 mg of tissue was collected from the second slice, ensuring inclusion of the luminal part. DNA was extracted from tissue and rectal/peritoneal swabs using the DNeasy® PowerSoil® Kit

(Qiagen-Hilden) following the manufacturer's instructions with the addition of two cycles of bead beating using a MagNA Lyser (Roche Diagnostics GmbH) at 6500 rpm for 30s with intermediate cooling for lysis and homogenization of the samples. The DNA concentration was measured using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific).

All amplification reactions including the bacterial and archaeal V4 region as well as the fungal ITS2 region were performed as described elsewhere [31,148] with minor adjustments. In brief, the "universal" approach aimed for detection of a broad spectrum of prokaryotes applying the primers 515RF/806RB. The archaea-specific amplification was done with a nested PCR, using the primers 344F/1041R in the first, and primers 519F/806R (including sequencing adapters) in the second PCR under the conditions mentioned in *Table 2* and *Table 3*. For fungal barcoding the primer pair ITS86F/ITS4 including sequencing adapters was used to amplify the ITS2 region.

All PCR reactions were performed in a final volume of 25 µL containing: TAKARA Ex Taq® buffer with $MgCl_2$ (1X; Takara Bio Inc., Tokyo, Japan), primers 500 nM, dNTP mix 200 µM, TAKARA Ex Taq® Polymerase 0.5 U, water (Lichrosolv®; Merck, Darmstadt, Germany), and DNA template (1 to 2 µL of genomic DNA).

**Table 2: Primers used in this study.**

| Target | Primer name | Primer sequence (5'-3') | Reference |
|---|---|---|---|
| **Universal 16S rRNA gene (V4)** | 515FB | GTGYCAGCMGCCGCGGTAA | Walters *et al.,* 2016 [149] |
| | 806RB | GGACTACNVGGGTWTCTAAT | Walters *et al*., 2016 [149] |
| **Archaea 16S rRNA gene (V4** | 344F | ACGGGGYGCAGCAGGCGCGA | Klindworth *et al*., 2013 [150] |
| | 1041R | GGCCATGCACCWCCTCTC | Klindworth *et al*., 2013 [150] |
| | 519F | CAGCMGCCGCGGTAA | Klindworth *et al*., 2013 [150] |
| | 806R | GGACTACVSGGGTATCTAAT | Klindworth *et al*., 2013 [150] |
| **Fungi ITS2** | ITS86F | *GTGAATCATCGAATCTTTGAA* | Turenne *et al*., 1999 [151] |
| | ITS4 (R) | TCCTCCGCTTATTGATATGC | White *et al*., 1990 [152] |
| Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence] | | | |
| Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence] | | | |

**Table 3: PCR programs used in this study**

| Target gene | Primer pair | Initial denaturation | Denaturation | Annealing | Elongation | Final Elongation | No. of cycles |
|---|---|---|---|---|---|---|---|
| **Universal (16S rRNA gene)** | 515FB - 806RB | 3', 94°C | 45", 94°C | 1', 50°C | 1' 30", 72°C | 10', 72°C | 40 |
| **Archaea (16S rRNA gene)** | 344F-1041R | 5', 95°C | 30'', 94°C | 45'', 56°C | 1', 72°C | 10', 72°C | 25 |
| | 519F-806R | 5', 95°C | 40'', 95°C | 2', 63°C | 1',72°C | 10', 72°C | 30 |
| **Fungi (ITS2 region)** | ITS86F-ITS4 (R) | 1', 94°C | 30", 94°C | 30'', 56°C | 30", 72°C | 10', 72°C | 35 |

Library preparation and sequencing of the amplicons was performed at the Core Facility Molecular Biology at the Center for Medical Research at the Medical University of Graz, Austria. In brief, DNA concentrations were normalized using a SequalPrep™ normalization plate (Invitrogen), and each sample was indexed with a unique barcode sequence (8 cycles index PCR). The indexed samples were pooled and DNA fragments of the expected sizes were obtained via gel electrophoresis. Sequencing was performed using the Illumina MiSeq device and MS-102-3003 MiSeq® Reagent Kit v3-600cycles (2x251 cycles).

# Microbiota analysis and data collection

The data obtained from each of the three amplification approaches underwent individual processing following the standard operating procedures of QIIME2 v2019.7 [153], as previously described [154]. Briefly, the DADA2 algorithm [155] was utilized for raw read quality control, including demultiplexing and denoising of truncated reads as well as for the generation of amplicon sequence variants (ASVs). For fungal data, ITSxpress [156] was employed to preprocess raw reads, trimming sequences to the desired ITS2 region.

Taxonomic assignment for prokaryotes was based on the SILVA database v132 for the indoor dataset and v138 for the AA dataset, on 99% ASVs [157]. Fungal taxonomic assignment utilized the UNITE v8.0_dynamic database [158]. In the archaeal dataset, sequences assigned to bacterial taxa were removed. By employing the R package decontam (version 1.2.1), contaminants in the archaeal and fungal datasets were excluded using the prevalence method with thresholds of 0.5 for the indoor datasets and 0.25 for the AA dataset [159]. The used R version was 3.5.1+ [160] . All datasets underwent rarefaction using the scaling with ranked subsampling (SRS) method [161]. The bacterial ("universal") datasets were rarefied to a depth of 1000, archaeal datasets to a depth of 100, and fungal datasets to a depth of 50 reads per sample.

To assess the impact of the human microbiome on the indoor microbiome, we compared the indoor data with datasets from various human body sites, obtained from studies conducted in-house. Samples were collected from the nasal cavity, saliva, skin, stool, and vagina. The methodology for DNA extraction involved a combination of enzymatic and mechanical lysis. 16S rRNA gene amplification for bacteria ("universal" approach) was either performed with the primers according to Caporaso *et al.* [162] or with the adopted primers by Walter *et al.* [149] as described in *Table 2*. For the archaeal approach the same primer sets were used as described in *Table 2*. Library preparation and sequencing were performed by the Core Facility Molecular Biology at the Medical University of Graz, Austria. Only raw reads obtained from healthy patients were included for subsequent analysis, with data processing conducted identically and in parallel to the indoor microbiome dataset.

**Table 4: Description and origin of included datasets from the human microbiome used in this study.**

| | Body part | Number of samples | Mean age of subjects (SD) | Male : Female ratio | Medical University of Graz Ethical approval number | ENA accession number | Reference |
|---|---|---|---|---|---|---|---|
| **Bacterial datasets** | Nasal cavity, adult | 10 | 41.3 (14.3) | 08:02 | 1573/2017 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| | Nasal cavity, child | 30 | 5.0 (2.1) | 19:11 | 1573/2017 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| | Gastrointestinal tract (stool) | 100 | 24.1 (3.5) | 49:51 | GZ. 39/44/63 ex 2017/18 | PRJEB41867 | Kumpitsch *et al.*, 2021 [163] |
| | Saliva | 11 | 46.7 (15.2) | 10:1 | 1325/2016 | PRJEB37299 | Wolf *et al.*, 2017 [164] |
| | Skin, forearm | 38 | 47.3 (13.6) | 21:17 | 31-110 ex 18/19 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| | Oral cavity | 31 | 31.93 (5.63) | 0:31 | 28-524 ex15/16 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| | Vagina | 31 | 31.93 (5.63) | 0:31 | 28-524 ex15/16 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| | Urinary tract (urine) | 31 | 31.93 (5.63) | 0:31 | 28-524 ex15/16 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| **Archaeal datasets** | Nasal cavity | 30 | 25.9 (5.33) | 11:19 | 39/80/63 ex 2014/15 | PRJEB41618 | Koskinen *et al.*, 2018 [165] |
| | Gastrointestinal tract (stool) | 38 | 29.5 (5.35) | 17:21 | 28-524 ex 15/16 and 27-289 ex 14/15 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| | Skin, forearm | 7 | 28.71 (4.75) | 6:1 | 27-289 ex 14/15 | PRJEB41618 | Blohs *et al.*, 2023 [145] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Oral cavity | 26 | 28 (4.81) | 9:17 | 28-524 ex15/16 and 27-289 ex 14/15 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| | Vagina | 16 | 31.25 (4.31) | 0:16 | 28-524 ex15/16 | PRJEB41618 | Blohs *et al.*, 2023 [145] |
| | Urinary tract (urine) | 43 | 29.62 (4.86) | 19:24 | 28-524 ex15/16 and 27-289 ex 14/15 | PRJEB41618 | Blohs *et al.*, 2023 [145] |

# Whole genome sequencing of vermiform appendix samples

To achieve higher taxonomic resolution and identify functional changes in the microbiome associated with AA, shotgun metagenome sequencing was conducted on all 60 vermiform appendix samples. DNA extraction from the samples followed the procedure described in the section "DNA extraction, 16S rRNA gene & ITS region amplification, and sequencing." For six samples (no. 25 - 30), an additional enrichment of prokaryotic DNA was performed using the NEBNext® Microbiome DNA Enrichment Kit according to manufacturer's recommendations, aiming to reduce the eukaryotic signal.

A total of 200 ng DNA from each sample was sent to Macrogen Europe (Amsterdam, Netherlands) for library preparation and sequencing. Library preparation utilized the TruSeq DNA PCR-Free kit (Illumina), and sequencing was conducted on the Illumina NovaSeq 6000 platform (150 bp paired end) without depletion of ribosomal DNA. All samples yielded >20 million raw reads for both forward and reverse reads.

## Processing of WGS data

Raw reads were subjected to quality control using Fastqc (v0.11.8), followed by trimming and filtering with trimmomatic (v0.38) accordingly [166,167]. Reads with a Phred33 quality score of <20 in a sliding window of 5 bp and a length below 50 bp were discarded. Accepted reads were mapped against the human chromosome hg19 using Bowtie2 (v2.3.5), and only unmapped reads were retained using SAMtools (v1.9) [168,169]. Settings applied for SAMtools were -b -f 12 -F 256. Non-host forward and reverse sequences were extracted using Bedtools (v2.29.0) [170].

For draft genome generation, host-depleted reads were co-assembled in MEGAHIT (v1.1.3) and binned with MaxBin (v.2.2.4) [171,172]. However, independent of the settings applied, we were incapable of yielding any genomes with this gene-centric approach; neither for the unprocessed samples nor for the Microbiome DNA Enrichment Kit-treated samples. In all cases, about 99% of the reads were mapped to the human genome and thus, were excluded. For this reason, a gene-

centric approach was performed instead. With DIAMOND (v2.0.8), blastx was used to annotate the reads against the NCBI nr database (release Sep. 2020) [173]. By using Megan (6.18.0), eukaryotic taxa classified as Metazoa were removed due to a high likelihood of them being miss-annotated or contaminations [174]. Subsequently counts were normalized, taxonomic and functional annotation was performed based on the SEED database, and data was exported for statistical analysis [175].

## Mapping WGS data for virulence factors and prominent taxa

Quality-controlled contigs were screened for the presence of virulence and antimicrobial resistance genes using ABRicate with all available databases: ARG-ANNOT (Jul 2019), CARD (Jul 2019), EcOH (Jul 2019), Ecoli_VF (Jul 2019), MEGARES 2.00 (Feb 2020), NCBI AMRFinderPlus (Jul 2019), PlasmidFinder (Jul 2019), Resfinder (Jul 2019) and VFDB (Jul 2019) [176]. ABRicate was run on default settings.

Contigs were also mapped against genomes from species of interest, including the facultative pathogen *Fusobacterium nucleatum* (NCBI Reference Sequence: NZ_LN831027.1) as well as archaeal species that were commonly found in the human GIT such as *Methanobrevibacter smithii* (DSM 2374 and strain WWM1085), *Methanosphaera stadtmanae* (DSM 3091), *Methanomassiliicoccus luminyensis* (B10), *Methanocorpusculum*, *Methanobacterium*, *Halorubrum lipolyticum* (DSM 21995) and *Haloferax sp. Arc-Hr* [38]. For this purpose, the trimmed sequences were mapped against the target species using Bowtie2 (v2.3.5) and matching sequences were extracted using SAMtools (v1.9) and Bedtools (v2.29.0) as described above.

# Statistical analysis

## Clinical Parameter Analysis

Statistical analysis of clinical parameters and utilized the IBM® SPSS® Statistics 26 software package. Gaussian normal distribution was assessed using the Shapiro-Wilk test. Nonparametric data underwent Kruskal-Wallis tests, while parametric data comparison utilized one-way analysis of variance (ANOVA) and a Tukey post-hoc test. Categorical data were analyzed with the chi-square test.

## Microbial Data Analysis

Microbial data analysis and visualization were conducted using QIIME2 (V2019.11+) and R (V4.1.1+). Phylogenetic distances for amplicon sequencing data were calculated in QIIME2 using the fasttree plugin. Biplots were generated based on the weighted UniFrac distance matrix using the biplot plugin in QIIME2. The Emperor plugin in QIIME2 was employed to illustrate the results of the biplot analysis.

## Differential Abundance and Alpha Diversity Testing:

Normalized data was used for differential abundance in R for alpha diversity testing and visualization, utilizing packages such as phyloseq, ggplot2, Maaslin2, and DESeq2 (for a complete list of R packages used in this thesis see *Supplementary Table 3*). Alpha diversity was calculated based on the filtered and normalized datasets, with mean values ± standard deviation (SD) reported. Pairwise Wilcoxon signed-rank tests were performed after testing for normality, with resulting P-values corrected for false discovery rates (FDR) according to Benjamini and Hochberg. For MaAsLin2, default settings were applied, except for max_significance = .05 and min_prevalence = .001. DESeq2 analysis was considered significant if $P_{adj} < .05$ and Log2 Fold Change ≥ .58 (corresponding to a 1.5-fold change).

Comparative analysis between indoor and human microbiomes (see *Microbiota analysis and data collection*) was conducted based on the mixed dataset. Bar charts, box plots, and bubble plots were constructed for both bacterial and archaeal communities at various taxonomic levels using primarily the phyloseq and ggplot2 packages in R.

## BugBase and SourceTracker2 Analysis

BugBase [177] was utilized to predict potential phenotypes present in the PMA and non-PMA treated indoor samples. SourceTracker2 analysis was done on indoor samples in comparison with the human microbiome dataset (see *Data availability*) and was performed with default settings at a sampling depth of 1000 reads for both source and sink.

## Community state type analysis

Using the rarefied 16S rRNA data from the AA dataset obtained with "universal" primers, a *de novo* community state type (CST) analysis was conducted. CSTs were determined based on the weighted UniFrac distance matrix for the bacterial dataset, following the methodology outlined by

DiGiulio *et al.* [178]. The provided R script was adapted to accommodate the specifics of the current dataset and analytical requirements. Gap statistics were utilized to determine the optimal number of clusters for the dataset, revealing an ideal number of clusters of k = 3.

## Data availability

The datasets supporting the conclusions of this thesis are available in the European Nucleotide Archive repository, Primary Accession: PRJEB41618 (indoor data) and PRJEB49215 (AA data) in https://www.ebi.ac.uk/.

## Cultivation of methanogenic strains and oxygen-tolerance test

This dissertation involved the propagation of three established cultures of human-associated methanogenic archaea, namely *Methanobrevibacter smithii* DSM no. 2375, *Methanosphaera stadtmanae* DSM no. 3091, and *Methanomassiliicoccus luminyensis* DSM no. 25720, obtained from DSMZ. Additionally, a new strain, designated *Methanobrevibacter smithii GRAZ-2*, was isolated from human feces (as described in Weinberger et al. [179]). All cultures were cultivated in 100 ml flasks that were sealed with a rubber stopper and a headspace of $H_2/CO_2$ gas mixture (80:20, vol/vol) applied. 20 ml medium were inoculated with 0.5 ml of densely grown culture and incubated at 37°C and 30 rpm. *Methanobrevibacter spp.* were grown in recommended ATCC 1340 MS medium for methanogens (https://www.atcc.org/), while *M. stadtmanae* and *M. luminyensis* were cultured in adapted MpT1 medium [180,181]. The adapted MpT1 medium contained the following components (L): 1 g NaCl, 0.5 g KCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.3 g $NH_4Cl$, 0.2 g $KH_2PO_4$, 0.15 g $Na_2SO_4$, 2 g casamino acids, 2 g yeast extract, 1 ml trace element solution SL 10 (Widdel 1983), 20 µl selenite-tungstate solution [182], and 0.082 g Na-acetate. The suspension was deoxygenated with $N_2$ and subsequently, 0.24 g L-cysteine and 2.52 g $NaHCO_3$ were added. After autoclaving, the medium was supplemented with 1 ml seven-vitamins solution [182], 2 ml methanol, 0.154 g dithiothreitol, 0.24 g L-cysteine, 0.034 g Na-formate and 0.01 g Na-coenzyme M.

For the oxygen-tolerance test, cultures were allowed to grow densely for 3 to 7 days, depending on the strain. The subsequent steps were performed under anoxic conditions (in the anaerobic chamber and with all media used being deoxygenated) or under ambient air and with standard media in a sterile bench (aerobic conditions). Cells were pelleted, washed with phosphate-buffered saline PBS and resuspended in PBS before being exposed to normoxic or anoxic conditions for up to 7 days. At predetermined time points, cell suspensions were transferred to fresh culture medium and allowed to grow for up to 3 weeks. Microscopic screening, optical density measurement at 600 nm, and methane production measurement using a spectrometer and methane sensor were

performed on all samples. Controls were included for each strain individually, including positive, negative/contamination, and medium-only controls. Furthermore, it was tested whether the medium remained oxygenated in the applied set-up. The oxygen saturation of the PBS was monitored using the FireStingO2 optical oxygen meter and oxygen probe OXROB10. The oxygen saturation in the medium was confirmed to be >80% at all time points and for all samples using identical methodology as described above. Each of the four strains was monitored in triplicates for a period of 24 h.

# Results

## The indoor environment as potential source for obligate anaerobes

As mentioned earlier, the establishment of facultative and obligate anaerobic microbes is essential for the proper functioning of the gastrointestinal tract (GIT). Throughout one's lifespan, the balance of microbial communities in the GIT is frequently influenced by internal and external factors, leading to changes in population size and the acquisition or loss of commensal microorganisms. Particularly intriguing is the process by which humans acquire obligate anaerobic commensals. Aside from the initial colonization during birth and within the first month of life, it remains largely unknown how obligate anaerobic species become part of the GIT's microbial community. The transmission of these microbes would subject them to harsh environmental conditions, including exposure to oxygen, radiation, and stomach acidity, which are considered lethal to most gut commensals. Methanogens, a group of highly oxygen-sensitive species, are present in the majority of the human population but are rarely detected in newborns and infants. Furthermore, species of the genus *Methanomassiliicoccus* are primarily found in elderly individuals [183,184]. The means by which obligate anaerobic microbes are transmitted remains elusive. One intriguing possibility is that the indoor environment may contribute to the spread and transmission of gut commensals. Humans may shed as many as $6x10^6$ microbes each day, which can be dispersed through dust and aerosols, forming what has been termed the "microbial cloud" [185].

To investigate whether commensal obligate anaerobes, including methanogens, can be acquired from the indoor environment, we sampled ten bathroom floors. Bathrooms, known to be a potentially rich source of GIT-associated microorganisms, served as a proxy for assessing the capability of the built environment to harbor and spread obligate anaerobes. We collected samples from two surfaces adjacent to the toilet, each measuring approximately 30 $cm^2$. One of these samples was subsequently treated with propidium monoazide (PMA). PMA has the ability to mask cell-free DNA in downstream analyses, enabling us to compare the overall microbiome in the untreated "non-PMA" samples with the intact and potentially viable microbiome in the "PMA" samples. The microbial composition was determined using amplicon sequencing of the 16S rRNA gene V4 region, employing both a "universal" primer set designed to encompass all prokaryotes as well as an archaea-specific nested-PCR approach.

## The human microbiome is the predominant source of BE microbes

The built environment (BE) has traditionally been characterized as a "microbial wasteland" due to its limited access to nutrients and liquid water [186]. This challenging environment restricts the ability of most microbes to propagate, leaving them to be considered dormant or in a state of decline. However, counterintuitively, our analysis has unveiled the BE as a habitat teeming with unique microbial patterns and remarkable diversity, surpassing even that found in human stool samples.

In our amplicon sequencing approach, we acquired a dataset of over 380,000 high-quality 16S rRNA gene sequences using universal primers (Bacteria: 99.86%, Archaea: 0.14%). These sequences were then assigned to 3684 amplicon sequence variants (ASVs), with an average of 473 ± 110 ASVs per household. Intriguingly, the majority of these ASVs (in total 3333 ASVs or 90.5%), were unique to a specific household, highlighting the distinctiveness of each environment and reflecting the unique microbiome of the residents. Only a limited subset of 351 (9.5%) ASVs was shared between at least two households, and a mere 26 (0.7%) ASVs were common to all bathroom samples. Within these 26 shared ASVs, we predominantly identified typical skin-associated taxa, including *Staphylococcus* (11 ASVs), *Corynebacterium* (8 ASVs), and *Finegoldia** (6 ASVs). Notably, a single shared ASV was attributed to the genus *Peptoniphilus**, a microorganism commonly found in the human vaginal and GIT microbiome.

A total of 32 different phyla were detected (*Figure 2a*), reflecting the wide array of microbial life thriving in this seemingly inhospitable environment. Notably, the distribution of sequences classified under these phyla remained largely stable, with only minimal variations observed upon PMA treatment. Statistical analyses, employing the Linear Discriminant Analysis Effect Size (LEfSe) method and the Wilcoxon signed-rank test, revealed significant differences primarily within four low-abundance phyla. Specifically, we noted an increase in Actinobacteria and a decrease in Tenericutes, Fusobacteria, and Epsilonbacteraeota upon PMA treatment ($P < .05$).

Unsurprisingly, many of the most abundant genera are closely associated with human health and anatomy. These genera include those linked to the human skin, such as *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Neisseria*, *Micrococcus*, and *Cutibacterium*, as well as those commonly found in the gastrointestinal and genitourinary tracts, such as *Lactobacillus*, *Finegoldia**, *Bacteroides**, *Anaerococcus**, *Peptoniphilus**, and *Lachnospiraceae** (*Figure 2c*). Thus, we performed a comparative analysis with the human microbiome, using data from prior in-house studies that applied an equivalent methodology. This analysis revealed distinct patterns regarding the abundance commensals in BE samples as compared to samples retrieved from the human nasal and oral cavity, skin, stool, and vaginal regions (*Figure 2d*). Particularly, signatures of typical human commensals like *Corynebacterium*, *Anaerococcus**, *Dolosigranulum*, *Finegoldia**,

*Gardnerella*, and *Staphylococcus* were significantly enriched in non-PMA bathroom samples compared to human samples ($P_{adj} < .001$; Mann–Whitney U test). This enrichment strongly suggests an efficient transfer of these microorganisms onto indoor surfaces. A reliable transfer of microbes was also observed for genera, such as *Bifidobacterium**, *Rothia*, *Bacteroides**, *Blautia**, *Dialister**, *Faecalibacterium**, *Gemella*, *Subdoligranulum** and *Haemophilus*, which are found in every single household. However, their presence in PMA samples was not as uniform, frequently even entirely eradicated, indicating a possible vulnerability to environmental stressors.

The continuous accumulation of both human and non-human associated microbes is strongly reflected in the high alpha diversity measures. In comparison to our reference samples from diverse human body sites, non-PMA samples were equal to stool sample regarding the Shannon diversity and even exceeded stool samples in terms of richness by more than twofold (mean richness: 282 ± 65.3 for non-PMA indoor samples and 136 ± 30.6 for stool samples; see *Figure 3b*). Intriguingly, even PMA-treated BE samples matched stool samples in richness and showed a Shannon index comparable to oral samples.

It is tempting to speculate that skin, being the most exposed body site, may exert the most significant influence on the BE microbiome. To explore this notion, we conducted a principal coordinate analysis using Bray-Curtis dissimilarity on all human and BE samples. With the exception of urine and vaginal samples, which could not be differentiated ($P_{adj} = .81$; PERMANOVA), all human body parts formed separate and significantly distinct clusters ($P_{adj} < .01$; *Figure 3a*). Additionally, PMA and non-PMA indoor samples showed no significant difference from each other. Nevertheless, indoor samples remained significantly distinct from all the analyzed body sites.

To gauge the extent of human influence on the microbial composition of the BE, we next employed SourceTracing 2 analysis, a tool designed to estimate the proportion of a novel community (here: the BE) originating from a predefined set of source environments (such as human body sites). Regardless of the PMA treatment, on average ~75% of the indoor microbiome could be traced back to human sources. These findings underscore the human body as the predominant source of the microbes detected in bathroom samples (*Figure 3a, b*). However, it's noteworthy that, depending on the household, we observed significant fluctuations in the proportion of human-associated taxa. Particularly in PMA-treated samples, this proportion ranged from 41% to 89%. This variability suggests differing frequencies and durations of surface exposure to human occupants in the tested bathrooms. The strongest impact of the PMA treatment was observed on taxa associated with the human vaginal, stool, and oral microbiomes, all of which exhibited a significant decline, while the proportion of microbes originating from the human skin significantly

increased ($P < .05$, Wilcoxon signed-rank test; see *Figure 3f*). These findings substantiate the prominent role of skin-associated taxa in shaping the BE microbiome. Notably, with an estimated proportion of 40.2% in non-PMA samples and 62.9% in PMA samples, skin-associated microbes not only account for the largest share of microbes in our indoor samples but also demonstrate a remarkable ability to withstand the challenging indoor conditions.

Subsequently, we explored the possibility of the BE serving as a source of microbes for the human body (*Figure 3e*). For microbes to successfully colonize the human body from the BE, their ability to survive outside their host is a crucial trait. Interestingly, skin not only proved to be the most abundant source of microbes in the examined bathroom samples but also appears to be most influenced by the BE, sharing an average of 29% and 32% of microbial signatures with non-PMA and PMA-treated bathroom samples, respectively. In contrast, the impact of the BE on other body sites appears to be considerably lower, particularly in terms of potentially viable microbes. PMA-treated samples shared on average just about 6% of taxa with urine, 5% with the nasal cavity, 4% with the vaginal region, and less than 0.01% with oral and stool samples. This discrepancy can be possibly attributed to the notably lower amount of biological material shed into the BE in comparison to that from the skin as well as the oxygen sensitivity of the corresponding microbes. Overall, the influence of the bathroom microbiome on various human body sites, except for the skin, appears to be minimal, if not negligible.

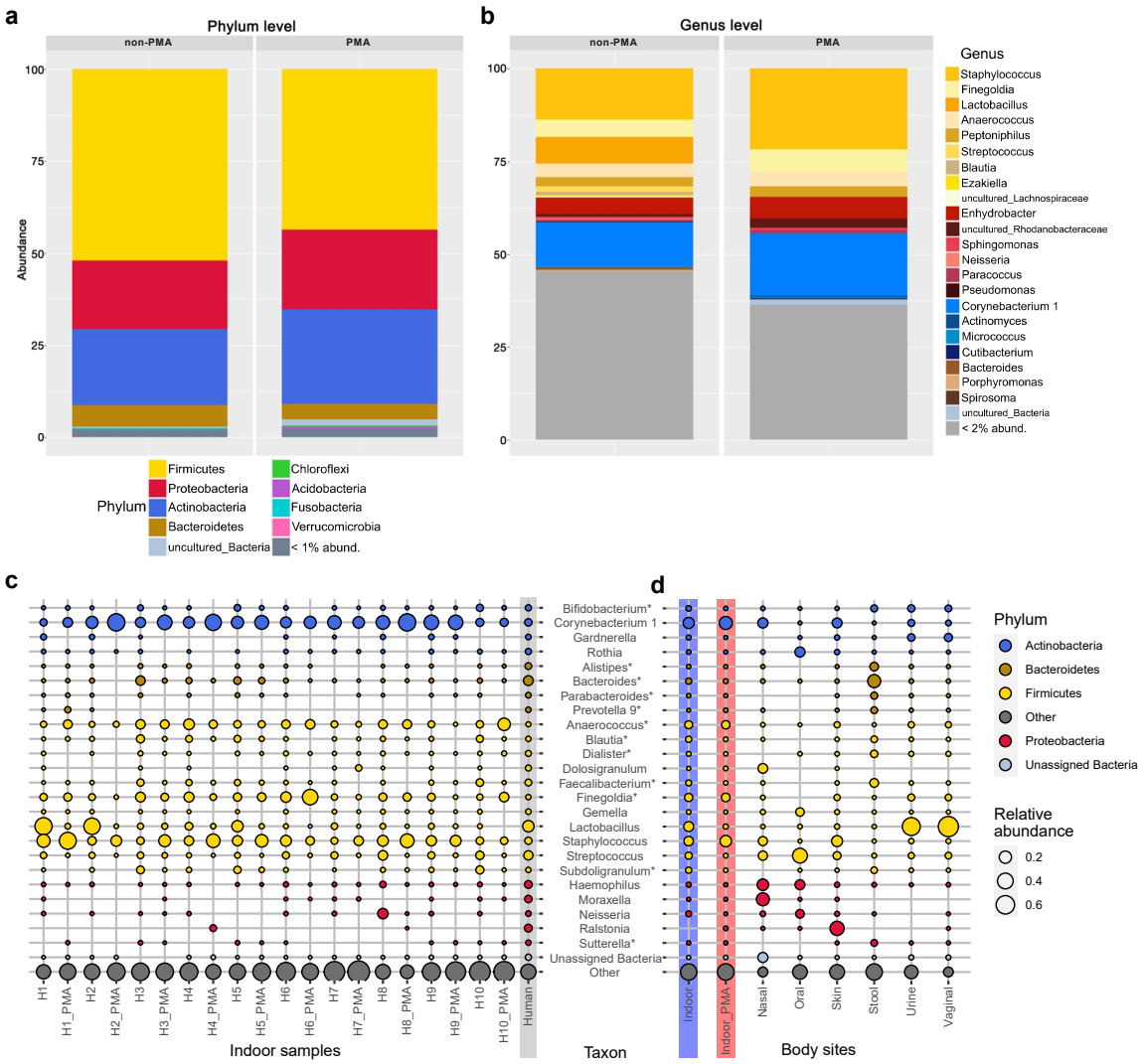
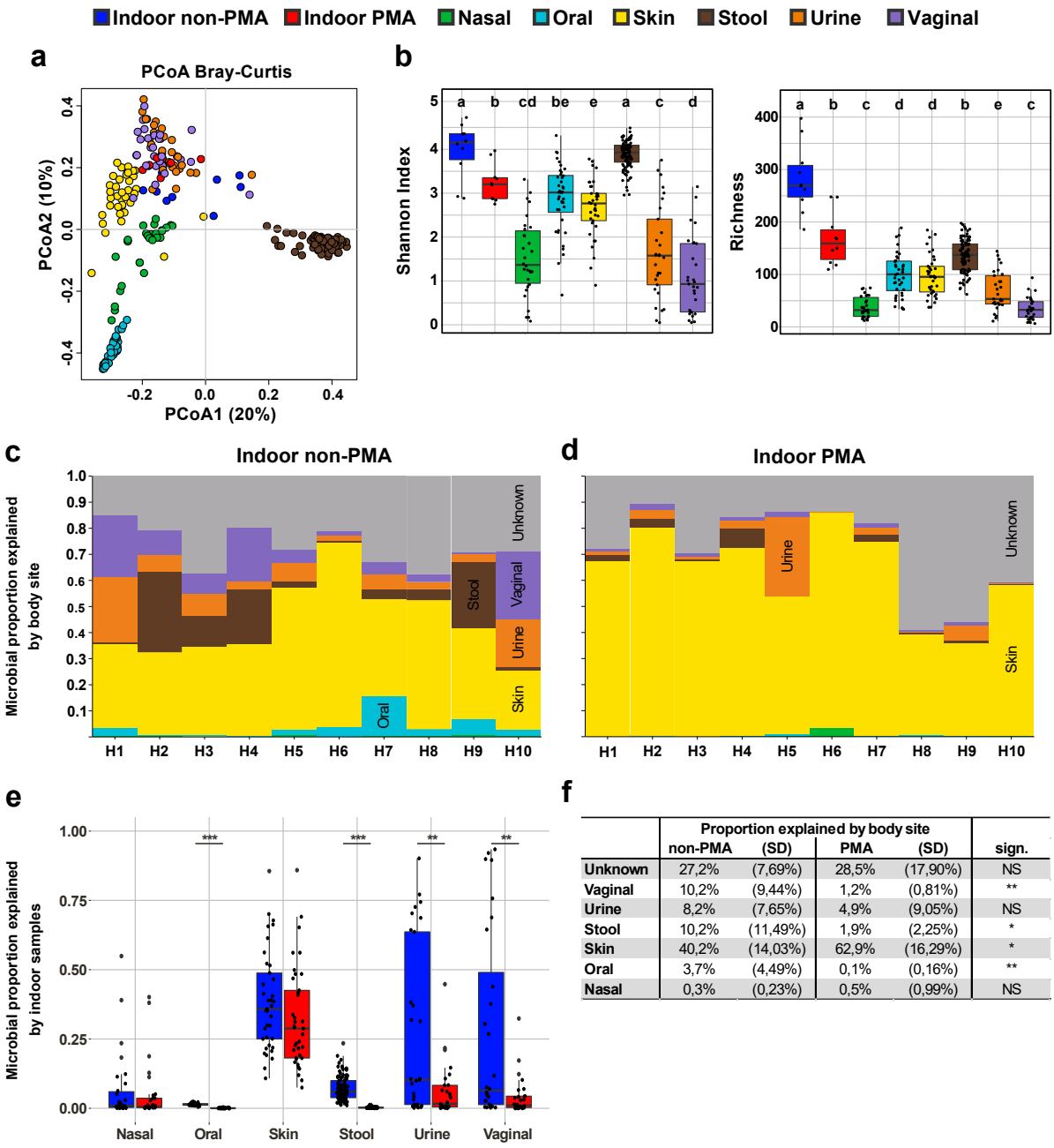



**Figure 2: Bacterial Taxa Distribution and Abundance in Bathroom Samples.** Distribution and relative abundance of bacterial taxa in bathroom floor samples collected from various households. Bar charts depict the microbial composition of both non-PMA and PMA-treated samples at the **(a)** phylum and **(b)** genus levels. Genera with less than 2% relative abundance are summarized in gray. **(c)** In the bubble plot, individual household samples (H1–H10) are presented for both non-PMA and PMA treatment alongside human samples (gray background) from different body sites, including the nasal cavity, skin, vagina, urine, stool, and oral samples. Panel **(d)** compares human samples to non-PMA (blue background) and PMA (red background) bathroom samples. Genera are color-coded based on their taxonomic phylum, with taxa predominantly containing strict anaerobes marked by an asterisk. Figure is taken from Pausan, Blohs et al., 2022 [147].




| | Proportion explained by body site | | | | |
|---|---|---|---|---|---|
| | non-PMA | (SD) | PMA | (SD) | sign. |
| Unknown | 27,2% | (7,69%) | 28,5% | (17,90%) | NS |
| Vaginal | 10,2% | (9,44%) | 1,2% | (0,81%) | ** |
| Urine | 8,2% | (7,65%) | 4,9% | (9,05%) | NS |
| Stool | 10,2% | (11,49%) | 1,9% | (2,25%) | * |
| Skin | 40,2% | (14,03%) | 62,9% | (16,29%) | * |
| Oral | 3,7% | (4,49%) | 0,1% | (0,16%) | ** |
| Nasal | 0,3% | (0,23%) | 0,5% | (0,99%) | NS |

**Figure 3: Comparative Analysis of Indoor and Human Microbiomes. (a)** Principal coordinates analysis plots based on Bray-Curtis dissimilarity and **(b)** alpha diversity indices showing Shannon diversity (left) and richness (right) are depicted for all indoor samples (non-PMA in blue, PMA-treated in red), alongside representative samples from various human body sites (green for nasal cavity, light blue for oral, yellow for skin, brown for stool, pink for urine, and purple for vaginal). Statistically significant differences are denoted by distinct letters above the bars, as determined by the Mann–Whitney U test (P < .05, FDR-adjusted). Samples sharing the same letter do not exhibit significant differences. The proportion of microbes in ten different bathrooms (H1–H10) are depicted for **(c)** non-PMA and **(d)** for PMA samples, which can be attributed to human body sites or remain of unknown origin (grey). Each household is represented by a vertical bar, and the values have been scaled to 100%. **(e)** Demonstrates the proportion of microbes found in or on various body sites that are potentially influenced by the bathroom microbiome. Significant differences between treatments were identified using a Kruskal–Wallis test, with **P < .01 and ***P < .001. **(f)** The table provides mean values and standard deviations for (c) and (d), as well as significant differences upon PMA treatment as defined by Wilcoxon signed-rank test, with NS indicating non-significance, *P < .05, and **P < .01. Figure is taken from Pausan, Blohs *et al.*, 2022 [147].

## Microbial indoor survival is primarily dictated by oxygen tolerance

Human commensals exposed to the BE encounter various stress factors, raising questions about their resilience in this environment over extended periods. To address this, we employed PMA treatment to selectively mask DNA from disrupted cells, which led to a significant reduction in the absolute abundance of microbial 16S rRNA gene copies (*Figure 4a*). Specifically, we observed a remarkable 4.2-fold decrease in gene copy numbers following PMA treatment, indicating that less than 25% of all microbial signatures can be attributed to intact, presumably living cells. This reduction is further reflected in a stark decrease of alpha diversity parameters in PMA-treated samples compared to untreated indoor samples (Shannon: $P_{adj}$ = .007, richness: $P_{adj}$ = .0006; Wilcoxon rank-sum tests, *Figure 3b*). Principal coordinates analysis (PCoA) plots based on Bray-Curtis distances also reveal a significant separation attributed to the PMA treatment ($P_{adj}$ = .018), even though indoor samples exhibit greater similarity to one another than to any human body site (*Figure 2a*).

Subsequently, we conducted LEfSe analysis on PMA and non-PMA samples to identify taxa indicative of PMA-treated samples, which represent more resilient microbes, likely to endure in the indoor environment for an extended period of time. Representative taxa for PMA-treated samples were primarily environmental and aerobic (or aerotolerant) groups, including *Acidobacteria*, *Micromonosporales*, *Solirubrobacterales*, *Rhizobiales*, *Nocardiaceae*, *Bacillus*, *Sphingomonas*, as well as human skin commensals such as *Corynebacterium_1*, *Staphylococcus*, and *Rombustia**. In contrast, representative taxa for non-PMA samples exhibited greater diversity in terms of physiological traits and origins. They were predominantly (facultatively) anaerobic, non-spore forming, and human-associated groups, encompassing *Bacteroidales**, *Lactobacillaceae*, multiple genera within the *Lachnospiraceae** and *Ruminococcaceae** families, *Bifidobacteriaceae**, *Christensenellaceae**, *Veillonellaceae**, *Haemophilus*, *Neisseria*, and the *Eubacterium coprostanoligenes group**. Intriguingly, some opportunistic pathogens, such as *Atopobium*, *Rothia*, and *Campylobacter*, were notably characteristic of and significantly enriched in non-PMA samples, suggesting their inability to persist on BE surfaces over prolonged periods.

Following PMA treatment, we observed a significant alteration in the relative abundance of 53 taxa within the most abundant bacterial genera and families ($P < .05$, $n_{genus} = 100$, $n_{family} = 50$, Wilcoxon signed-rank test). However, only 10 taxa remained significant after multiple testing adjustments ($P_{adj} < .05$, *Figure 4c, d*). In line with the findings from our LEfSe analysis, a substantial reduction in signals was noted for non-spore forming, (facultatively) anaerobic human commensals, specifically *Lactobacillus*, *Streptococcus*, *Gemella*, and the *Eubacterium coprostanoligenes group**. Members of the *Ruminococcaceae* family and several of its genera also exhibited a substantial

decrease in relative abundance following PMA treatment, implying susceptibility to BE-associated stress factors. Conversely, taxa within the *Acetobacteraceae* family and other commensals like *Bacillus* and *Staphylococcus* appeared exceptionally well-suited for survival under BE conditions.

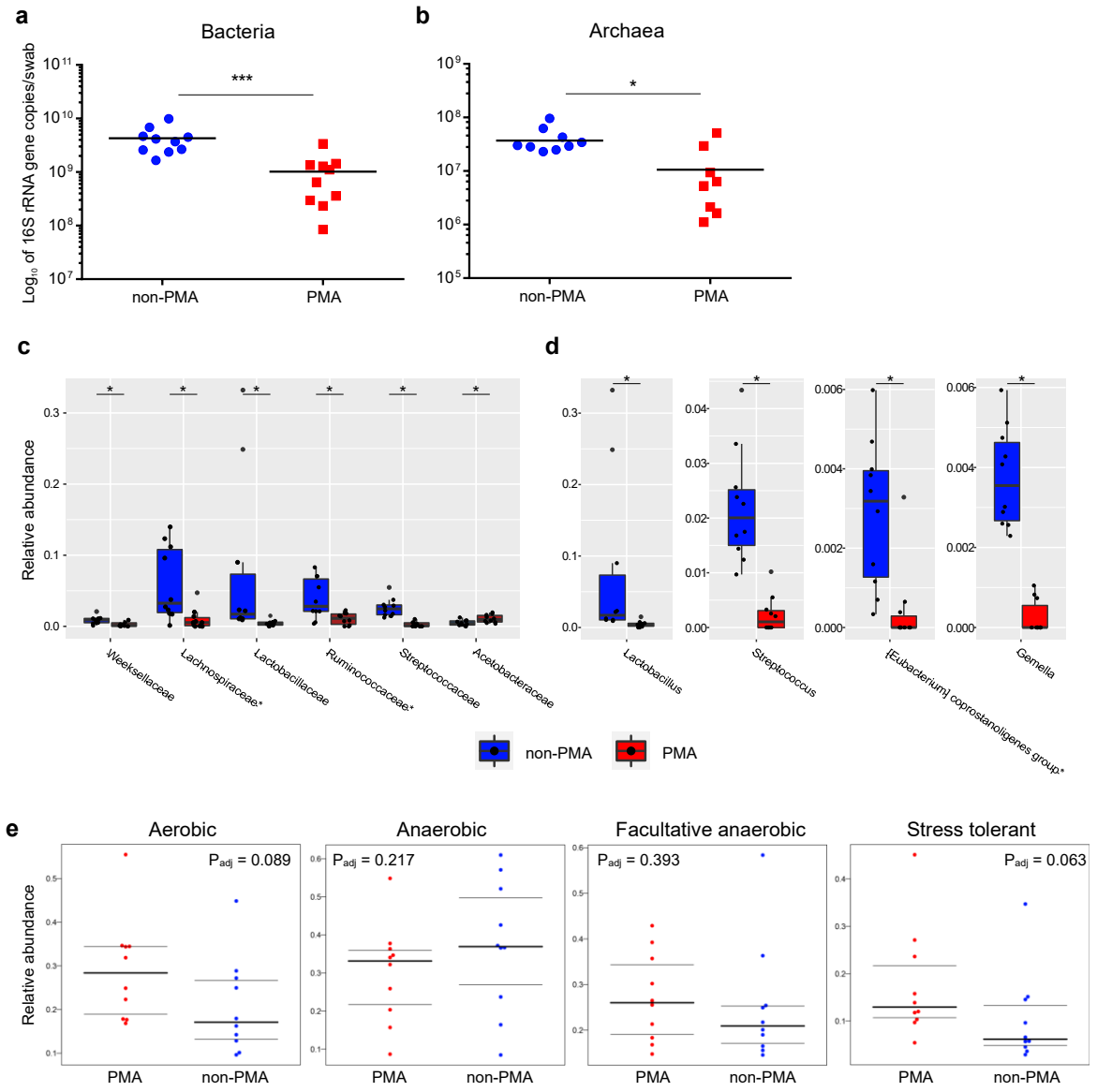



**Figure 4: Influence of PMA treatment on the indoor prokaryotic microbiome. (a)** Quantitative PCR analysis of bacterial and **(b)** archaeal abundance in the surveyed households, comparing untreated (non-PMA, blue) and PMA-treated (red) samples. Significant alterations among the **(c)** 50 most-abundant bacterial families and **(d)** 100 most-abundant bacterial genera are shown (FDR-adjusted Wilcoxon signed-rank test; *$P_{adj}$ < 0.05; ***$P_{adj}$ < 0.01). **(e)** Impact of PMA on microbial composition based on phenotypes determined through BugBase analysis (P-values determined by Mann–Whitney U test in BugBase). Figure is taken from Pausan, Blohs *et al.*, 2022 [147].

To further corroborate these observed traits in non-PMA and PMA samples, we utilized BugBase. As compared to non-PMA samples, PMA-treated samples demonstrated an increased relative abundance of aerobic ($P_{adj}$ = .089) and facultative anaerobic communities ($P_{adj}$ = .393) and a slight decrease in anaerobic communities ($P_{adj}$ = .217; *Figure 4e*). Intriguingly, the abundance of taxa exhibiting stress-tolerant phenotypes expanded in the PMA samples ($P_{adj}$ = .063), suggesting that microorganisms capable of surviving in the indoor environment may possess greater adaptations to cope with BE-related stress factors.

## Archaea as integral part of the BE

To date, Archaea are recognized colonizers of various mesophilic ecosystems like soil, aquatic environments and host-associated habitats. For this reason, we expected archaeal signatures to be present in the BE as a result of passive shedding and dormancy from external sources in a seemingly "extreme" setting. In fact, we observed Archaea to be a stable component within the investigated households, even with surprising capabilities to endure the BE-stressors for highly these oxygen susceptible taxa.

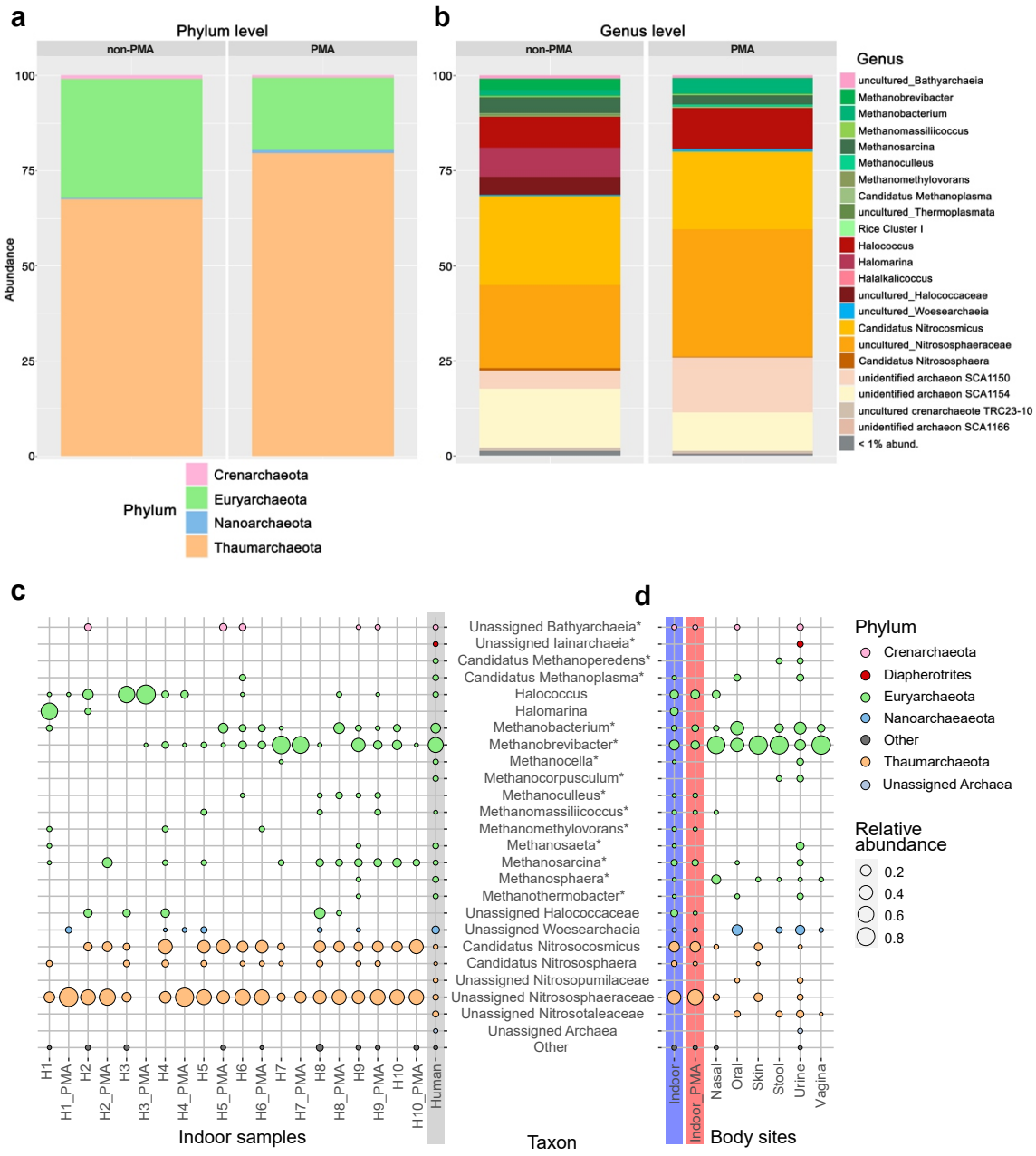
By employing nested-PCR for specific archaeal signature detection, signal amplification was facilitated against a vast bacterial presence. This method revealed a total of 376,000 high-quality archaeal reads across the sampled households, corresponding to 373 ASVs. On average, each household exhibited 70.2 ± 22.8 ASVs (53.6 ± 16.8 for non-PMA and 35.9 ± 22.1 for PMA samples), with 79% of ASVs unique to a single household.

The archaeal community in the investigated households was predominantly composed of Euryarchaeota (relative abundance in non-PMA samples: 36.6%, PMA: 35.0%) and Thaumarchaeota (now Thermoproteota; non-PMA: 62.2%, PMA: 64.1%). Crenarcheota (non-PMA: 0.8%, PMA: 0.5%) and Nanoarchaeota (non-PMA: 0.4%, PMA: 0.5%) both accounted for less than 1% of the relative abundance each (*Figure 5a*). Thaumarchaeota signatures were primarily classified as Nitrososphaeraceae (non-PMA: 62.1%, PMA: 64.1%), the sole archaeal family identified in every investigated household (Fig. 5b; with 9 Nitrososphaeraceae ASVs found in 9 of the 10 households respectively). *Cand. Nitrosocosmicus* (non-PMA: 22.5%, PMA: 15.9%) and *Cand. Nitrososphaera* (non-PMA: 0.9%, PMA: 0.2%) were the only identified genera within this ubiquitous found family (*Figure 5b*). Euryarchaeota, in contrast, exhibited greater diversity in the BE. Its most prevalent family, *Halococcaceae* (non-PMA: 19.7%, PMA: 28.7%), contained the highly PMA-stable genus *Halococcus* (non-PMA: 13.7%, PMA: 28.6%) and the notably susceptible genus *Halomarina* (non-PMA: 3.4%, PMA: 0%). Particularly intriguing was the presence of various methanogenic signatures consistently detected in the investigated households. Among the most abundant

methanogenic genera identified were: *Methanobacteria** (non-PMA: 3.7%, PMA: 3.5%), *Methanomicrobia** (non-PMA: 4.0%, PMA: 2.6%), *Methanosarcina** (non-PMA: 3.2%, PMA: 1.8%), *Methanobrevibacter** (non-PMA: 2.4%, PMA: 0.2%), *Methanobacterium** (non-PMA: 1.3%, PMA: 3.3%), and *Methanomassiliicoccus** (non-PMA: 0.4%, PMA: 0.2%) — all of which are considered to be susceptible to oxygen exposure [187].

The observed archaeal composition in the BE suggests a substantial fraction to be of human origin, notably involving skin-associated Thaumarchaeota, Haloarchaea, and GI-associated methanogens present in the sampled bathrooms. To explore this, the BE data was compared to archaeal profiles from various human body sites, encompassing nasal (n = 30), oral (n = 26), skin (n = 7), stool (n = 38), urine (n = 43), and vaginal (n = 16) samples that have been previously analyzed in our lab (*Figure 5d*).

Several archaeal ASVs, including genera such as *Halococcus*, *Methanobacterium**, *Methanobrevibacter**, *Methanosarcina**, unassigned Nitrososphaeraceae, and Woesearchaeia, were identified in both bathroom and human-associated samples, suggesting a potential exchange of microbes between these habitats. Nevertheless, both the human and BE microbiome revealed features that were characteristic to the corresponding habitat. Thaumarchaeota exhibited significant enrichment in the BE ($P_{adj}$ = 3.5 × $10^{-11}$, Mann–Whitney U test), while Euryarchaeota were significantly enriched in human-associated samples ($P_{adj}$ = 1.3 × $10^{-5}$). Key discriminators included *Cand.* Nitrososphaera ($P_{adj}$ = 2.9 × $10^{-26}$), *Cand.* Nitrosocosmicus (Padj = 1.1 × $10^{-24}$), and unassigned Nitrososphaeraceae ($P_{adj}$ = 1.0 × $10^{-20}$), which showed low abundances in human-associated samples but significant enrichment in the bathroom samples. Notably, these taxa are predominantly found on human skin and appear to be steadily shed and accumulated in the BE. In fact, Skin shared the highest number of ASVs with the BE (four *Cand. Nitrosocosmicus* ASVs and 16 ASVs uncultured *Nitrososphaeraceae*; see *Figure 5e, f*), supporting the continuous exchange of skin microbes, both bacteria and archaea, into the BE. Interestingly, nine ASVs, all classified under the *Methanobrevibacter** genus, were shared among body sites and the investigated indoor samples.


a
Phylum level
non-PMA
PMA
Abundance
100
75
50
25
0
Phylum
Crenarchaeota
Euryarchaeota
Nanoarchaeota
Thaumarchaeota
b
Genus level
non-PMA
PMA
Genus
uncultured_Bathyarchaeia
Methanobrevibacter
Methanobacterium
Methanomassiliicoccus
Methanosarcina
Methanoculleus
Methanomethylovorans
Candidatus Methanoplasma
uncultured_Thermoplasmata
Rice Cluster I
Halococcus
Halomarina
Halalkalicoccus
uncultured_Halococcaceae
uncultured_Woesearchaeia
Candidatus Nitrocosmicus
uncultured_Nitrososphaeraceae
Candidatus Nitrososphaera
unidentified archaeon SCA1150
unidentified archaeon SCA1154
uncultured crenarchaeote TRC23-10
unidentified archaeon SCA1166
< 1% abund.
c
Unassigned Bathyarchaeia*
Unassigned Iainarchaeia*
Candidatus Methanoperedens*
Candidatus Methanoplasma*
Halococcus
Halomarina
Methanobacterium*
Methanobrevibacter*
Methanocella*
Methanocorpusculum*
Methanoculleus*
Methanomassiliicoccus*
Methanomethylovorans*
Methanosaeta*
Methanosarcina*
Methanosphaera*
Methanothermobacter*
Unassigned Halococcaceae
Unassigned Woesearchaeia
Candidatus Nitrosocosmicus
Candidatus Nitrososphaera
Unassigned Nitrosopumilaceae
Unassigned Nitrososphaeraceae
Unassigned Nitrosotaleaceae
Unassigned Archaea
Other
H1
H1_PMA
H2
H2_PMA
H3
H3_PMA
H4
H4_PMA
H5
H5_PMA
H6
H6_PMA
H7
H7_PMA
H8
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H9
H9_PMA
H10
H10_PMA
Human
Indoor samples
Taxon
d
Indoor
Indoor_PMA
Nasal
Oral
Skin
Stool
Urine
Vagina
Body sites
Phylum
Crenarchaeota
Diapherotrites
Euryarchaeota
Nanoarchaeaeota
Other
Thaumarchaeota
Unassigned Archaea
Relative abundance
0.2
0.4
0.6
0.8
e
Nasal
Indoor
Oral
Stool
Skin
489
267
41
24
5
f
Nasal
Indoor_PMA
Oral
Stool
Skin
491
199
44
24
5

**Figure 5: Composition of native and PMA-treated archaeal communities in the built environment (BE). (a)** Relative abundance of archaeal 16S rRNA gene reads on phylum and **(b)** genus levels, detected in ten bathroom surfaces using archaea-specific nested PCR, with and without prior PMA application (non-PMA and PMA samples, respectively). Bubble plots illustrate the 25 most-abundant archaeal genera found in human and bathroom samples. **(c)** Individual representation of all sampled bathrooms, with and without PMA treatment, in comparison to human samples (gray background), aggregating reads from various body sites: nasal and oral cavity, skin, stool, urine, and vagina. **(d)** Archaeal composition in specific human body sites compared to native (blue) and PMA-treated (red) indoor samples. The number of shared ASVs among selected human body sites and **(e)** non-PMA, as well as **(f)** PMA indoor samples, is presented. The body sites were chosen based on the highest number of shared ASVs with the BE among the six investigated body sites. Figure is taken from Pausan, Blohs *et al*., 2022 [147].

## Demonstrating oxygen tolerance in methanogens using human isolates

Energy generation in methanogenic archaea necessitates strict anaerobic conditions within a low redox potential environment. Due to their vulnerability to oxygen, which can disrupt their metabolic pathways, methanogens are traditionally considered highly oxygen-sensitive. Nevertheless, the widespread presence of *Methanobrevibacter** signatures across all examined human body sites, as well as in PMA and non-PMA indoor samples, suggests a certain degree of oxygen tolerance.

To test this hypothesis, we used four methanogens previously isolated from human samples as proxies to assess the ability of strictly anaerobic archaea to survive in oxygenated conditions. The strains used include three well-characterized species: *Methanobrevibacter smithii** (DSM no. 2375), *Methanosphaera stadtmanae** (DSM no. 3091), *Methanomassiliicoccus luminyensis** (DSM no. 25720), along with a new isolate of *Methanobrevibacter* from human feces: *M. smithii GRAZ-2* (Weinberger et al., 2025). All cultures were either exposed to normoxic conditions or kept in an anoxic environment over a period of up to 7 days (0, 6, 24, 48 and 168 h) and subsequently transferred to fresh anoxic culture media. Afterwards, the cells were allowed to grow for 2–3 weeks, with growth recorded through $OD_{600}$ and methane measurements.

Remarkably, all four strains demonstrated survival in aerobic conditions for at least 6 hours (*Figure 6*). *M. luminyensis** exhibited the highest resilience among the tested strains, enduring exposure to aerobic conditions for over 48 hours. However, following 7 days of dormancy, no regrowth was observed for *M. luminyensis**, neither from oxygenated nor anoxic conditions. This suggests that additional factors, such as starvation, potentially hindered normal outgrowth. In contrast, both *Methanobrevibacter sp.** and *M. stadtmanae** were capable of surviving under anaerobic conditions for at least 7 days.

Collectively, these findings indicate a certain degree of oxygen tolerance among the studied methanogenic archaea, albeit within a limited timeframe. This discovery strengthens our hypothesis of methanogens to be transmitted and potentially reacquired through the built environment.

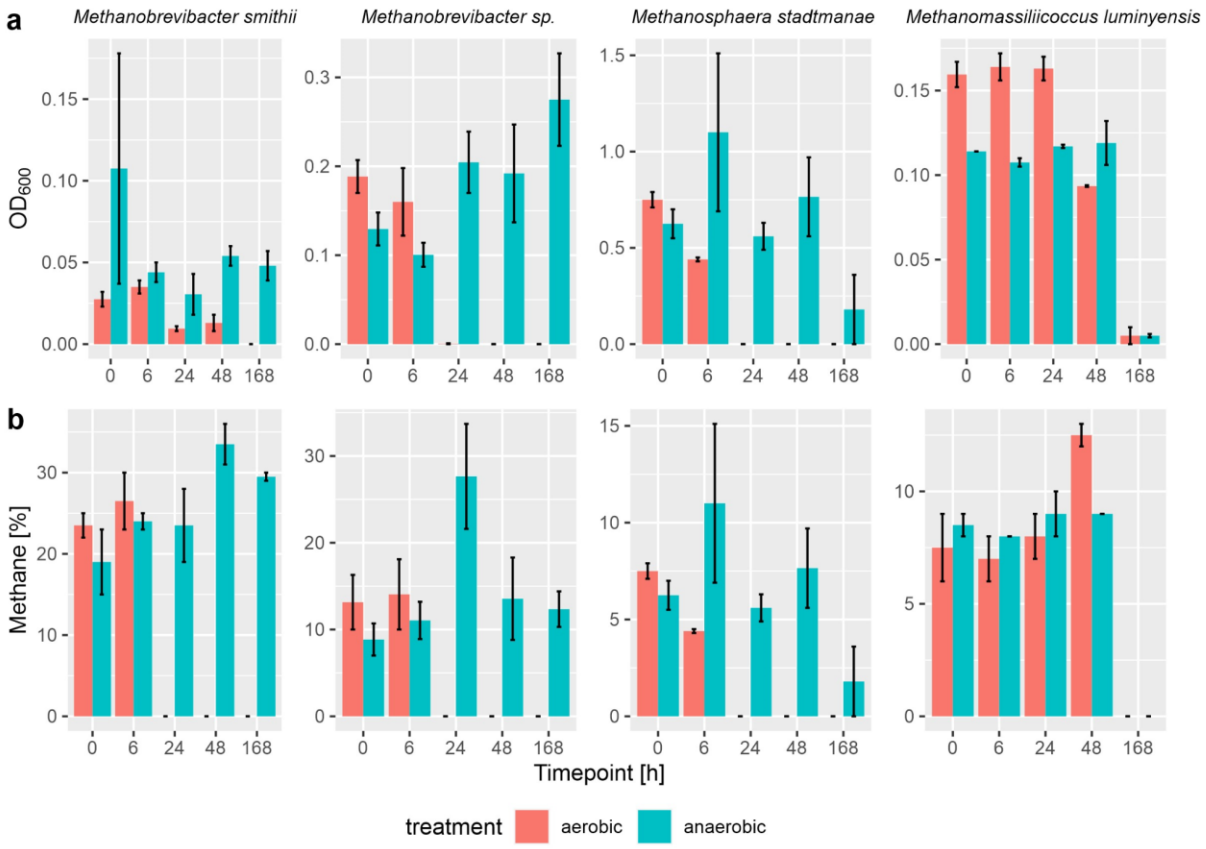



**Figure 6: Methanogenic strain growth and metabolic activity post-exposure to ambient air.** Four isolated methanogenic strains from human feces were subjected to either oxygenated (red) or anoxic (blue) conditions for a duration of up to 7 days. Survival was assessed through **(a)** $OD_{600}$, measuring culture growth, and **(b)** methane production, indicating metabolic activity (n = 2). Error bars represent standard deviations. Figure is taken from Pausan, Blohs *et al.*, 2022 [147].

# Microbial impact in acute appendicitis etiopathogenesis

Considering the continual acquisition and alterations in archaeal dynamics throughout an individual's lifespan, it becomes crucial to explore the potential health implications of Archaea on the human body. As of now, no archaeal species has been identified as a pathogen, and virulence factors within Archaea remain undiscovered as well [188]. However, changes in the abundance or acquisition of archaea could contribute to a shift in the overall microbial composition, possibly fostering the growth of commensal pathogens. This section aims to investigate the role of archaea in the etiopathogenesis of acute appendicitis (AA), a condition with an unclear origin and unknown impact of its archaeome.

## Study cohort and design

To investigate factors contributing to the development and progression of AA, we enrolled 60 children and adolescents who underwent appendectomy (median age: 12.0, range: 3 to 17 years). Tissue from the proximal part of the appendix and rectal swabs were collected from all participants, while peritoneal swabs were obtained from 35 out of the 60 participants. Disease severity and sample grouping were determined through histopathological assessments, categorizing cases as acute, catarrhal, phlegmonous, gangrenous, or perforated (*Table 5*). Following this assessment, four patients without notable pathological findings (subacute samples) were excluded due to insufficient sample size. Patient groups, stratified by severity, did not exhibit significant differences in regard to patient age, sex, Alvarado score and Pediatric Appendicitis Score, nor in the presence of a fecalith. However, serum C-reactive protein concentration was notably higher in patients with gangrenous/perforated appendicitis as compared to those with phlegmonous appendicitis (Kruskal-Wallis test; $P < .01$). Additionally, the number of patients treated with antibiotics was significantly greater in cases of gangrenous/perforated appendicitis (chi-squared test; $P < .05$). Twenty patients (33%) received 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) before or during surgery, based on the surgeon’s assessment. These antibiotics were not anticipated to substantially alter microbial composition, given the short interval between administration and sampling. The observed differences in bacterial beta diversity upon antibiotics treatment (amplicon data: PERMANOVA – Bray-Curtis, $P = .048$; weighted UniFrac, $P = .029$) were regarded as clear confounding factor of disease severity (as antibiotics were more frequently applied in complicated cases; *Table 5*) and were therefore considered not to have a substantial effect on microbial diversity.

**Table 5: Clinical and demographic data for the study cohort of patients 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)[a] ± 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)[a] ± SD | 6.5 ± 1.9 | 6.6 ± 2.3 | 6.7 ± 2.3 | 8.7 ± 1.5 | .19 |
| Alvarado score (0-12)[a] ± SD | 6.8 ± 1.9 | 6.6 ± 2.6 | 6.6 ± 2.4 | 8.2 ± 2.7 | .20 |
| Leukocyte count ($10^9$/L)[a] ± SD | 15.4 ± 8.4 | 13.2 ± 4.6 | 14.1 ± 5.6 | 14.5 ± 4.6 | .88 |
| C-reactive protein (mg/l)[a] ± SD | 68.5 ± 63.2 | 37.9 ± 43.2 | ***23.5 ± 35.7** | 117.2 ± 101.3 | **.002** |
| Faecalith detected, n (%) | 0 (0) | 1 (7) | 5 (16) | 1 (9) | .69 |
| Antibiotic treatment[§], n (%) | ***0 (0)** | ***2 (14)** | ***10 (32)** | 8 (73) | **.007** |

[a], mean values; *, sign. different from gangrenous/perforated; SD, standard deviation; [§],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)

## The bacteriome as driving factor of acute appendicitis progression

At first, we investigated the entire microbial composition across all three sampling sites (appendix, peritoneum and rectum). Given the assumption that GIT-associated archaea might not be the primary drivers of pathogenesis but rather contribute to the accumulation and growth of bacterial (opportunistic) pathogens, we conducted 16S rRNA gene amplicon sequencing, specifically targeting the V4 region. After meticulous quality control, filtering, and SRS normalization, we identified a total of 1,796 ASVs among all samples and sample types (samples that passed the quality control for each sample type: $n_{appendix} = 42$, $n_{peritoneum} = 21$, $n_{rectal} = 60$).

The bacteriome across all three sample types was dominated by the two phyla Firmicutes (Appendix: relative abundance 34.4%, Peritoneum: 32.9%, Rectal: 50.0%) and Bacteroidota (A: 31.1%, P: 15.5%, R: 27.5%; *Figure 7a*). Proteobacteria were notably prevalent in the peritoneum of acute appendicitis (AA) patients (29.7%), comparatively less in the appendix (12.4%), and the least in the rectum (6.1%). Noteworthy was the substantial prevalence of the genus *Fusobacterium* – the sole taxon within the Fusobacteria phylum – constituting 15.7% of bacterial taxa in the

investigated appendices (peritoneum: 6.8%, rectum: 2.0%), making *Fusobacterium* the genus with the highest relative abundance.

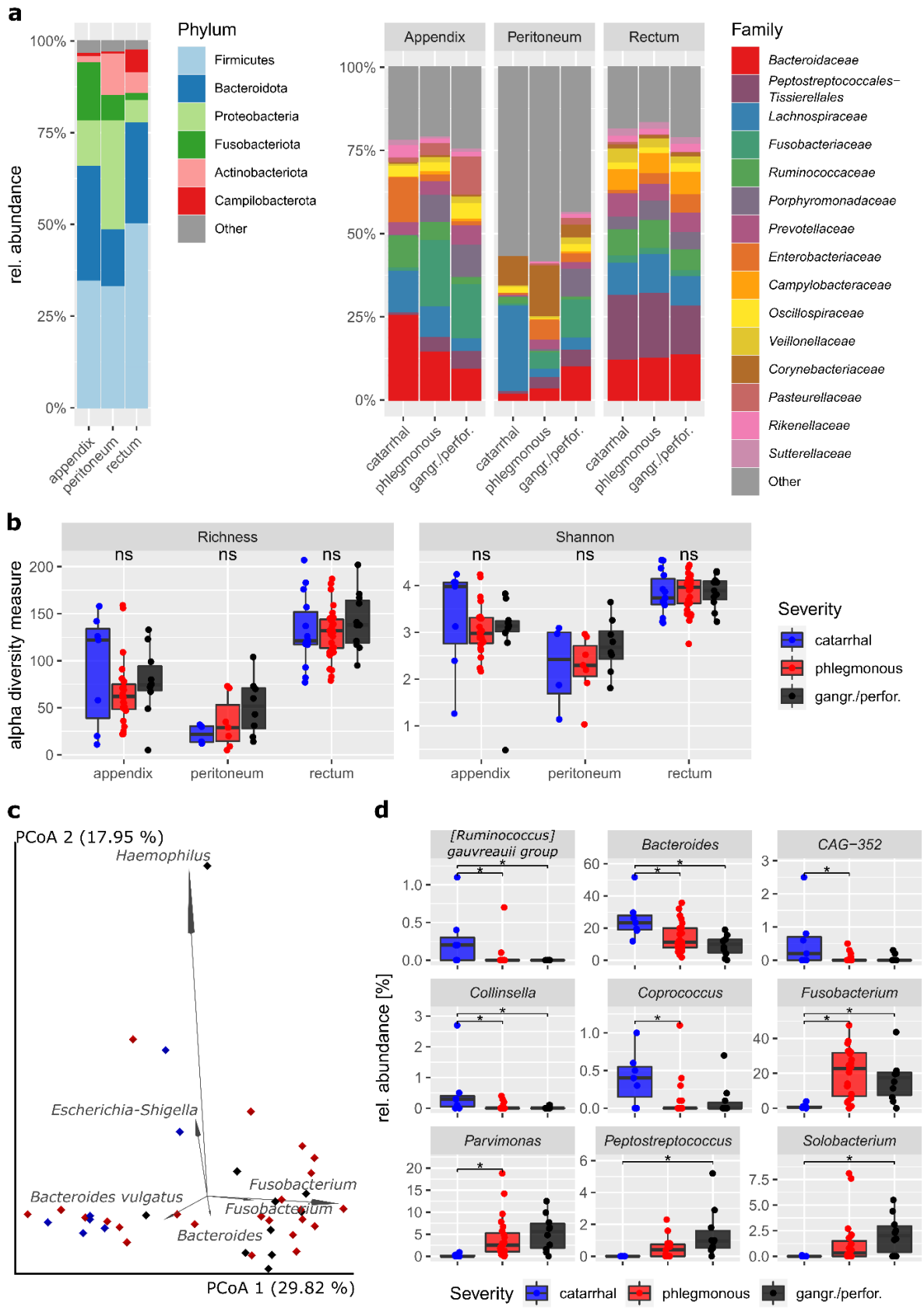
In terms of alpha diversity, the observed richness revealed an expected pattern among thee sample types, with peritoneal samples exhibiting the lowest richness with 37 ± 28 ASVs on average, followed by appendix samples displaying 75 ± 41 ASVs, and the rectum topping the scale with 132 ± 30 ASVs. The alpha diversity differences were significantly different between the three sample types (appendix, peritoneal and fecal samples) in terms of richness and Shannon diversity (ANOVA: $P < .001$), while both metrics remained fairly stable between disease states (*Figure 7b*). In contrast, a noteworthy shift in beta diversity was observed in the appendix samples regarding disease states (*Figure 7c* and *Supplementary Figure 2*). A distinct shift was evident from catarrhal to phlegmonous states (weighted UniFrac and Bray-Curtis PERMANOVA: $P_{adj} < .05$) and further from catarrhal to gangrenous/perforated appendicitis ($P_{adj} < .01$). Such significant differences were not identified in peritoneal or rectal samples. Hence, one might speculate that the progression of AA involves a local microbial transition towards an inflammation-promoting, dysbiotic state.

We further analyzed the key features of dysbiosis in AA via biplot analysis and differential abundance testing. In biplot analysis, species of two genera were striking indicators for disease severity in AA. While *Bacteroidetes spp.* rather indicated a less severe AA with catarrhal cases, *Fusobacterium spp.* tend to indicate more severe cases of gangrenous/perforated outcome (*Figure 7c*). Interestingly, both *Fusobacterium* ASVs were found to be among the six highest-impact features in the biplot analysis for both appendix and peritoneum samples. The importance of *Fusobacterium* for AA disease severity has been widely discussed previously [132,133,135,139,142,143] and is further confirmed by differential abundance testing in our dataset (*Figure 7d*). In the appendix, a total of nine genera were found to be significantly different depending on the disease state, as confirmed by MaAsLin2. A statistically significant increase of typically oral-associated microbes was found to be associated with severe disease and included the genera *Fusobacterium*, *Parvimonas*, *Peptostreptococcus* and *Solobacterium*. These four genera were found at very low abundance in catarrhal severity (< 1% relative abundance) but displayed a substantial increase at higher disease stages. Conversely, five genera — *Bacteroides*, CAG-352 (family *Ruminococcaceae*), *Collinsella, Coprococcus, and Ruminococcus gauvreauii* group — exhibited a significant decrease in abundance at higher disease stages compared to catarrhal appendicitis.

This expansion of oral-associated microbes and the reduction of commensals in AA disease was specifically localized to the appendix and not extended to rectal samples. Remarkably, we did not identify any significant microbial differences in rectal samples associated with disease severity or

any investigated clinical parameter. Only subtle trends, such as a gradual increase in the genus *Escherichia-Shigella* with disease progression (pairwise Kruskal-Wallis test, P = .026), were observed, yet no feature retained significance after false discovery rate (FDR) correction. This indicates a microbial shift in appendicitis that is confined to the appendix and is not linked to overall microbial changes in the (terminal) gut. Surprisingly, the microbial analysis of peritoneal samples revealed no significant differences either. In instances of catarrhal severity, we noted a high abundance of typically skin-associated microbes, such as *Staphylococcus* (relative abundance 7.9%), *Streptococcus* (10.3%), or *Pseudomonas* (5.9%), likely acquired through skin contact. In contrast, higher severity samples more closely resembled the appendix microbiome, potentially linked to a gradual disruption of the gut barrier with advancing disease. Despite the heterogeneity observed in the peritoneal swab microbiome, the lack of significance may be attributed to the limited number of samples from catarrhal and phlegmonous patients that provided sufficient bacterial signals (n = 4 and 7, respectively).

**Figure 7: Bacterial diversity dynamics in relation to acute appendicitis severity in children and adolescents (n = 60). (a)** Relative abundance across all sampled sites (appendix, peritoneum & rectum) presented at phylum (left) and family level (right). **(b)** Alpha diversity within each site (left, ASV richness; right, Shannon diversity) remained significantly unchanged with disease state (pairwise Wilcoxon signed-rank test, all within-sample type comparisons $P_{adj} > .26$). Microbial shifts in the appendix, illustrated by **(c)** biplot analysis, highlighting the six most driving ASVs (weighted UniFrac PCoA), and **(d)** nine genera exhibiting significant changes in relative abundance between severity grades (MaAsLin2; *, $P_{adj} < .05$). The catarrhal severity grade served as the reference for the differential abundance analysis. Figure is taken from Blohs *et al.*, 2023 [145] and shown on the following page.


a
Phylum
Firmicutes
Bacteroidota
Proteobacteria
Fusobacteriota
Actinobacteriota
Campilobacterota
Other
rel. abundance
100%
75%
50%
25%
0%
appendix
peritoneum
rectum
Appendix
Peritoneum
Rectum
catarrhal
phlegmonous
gangr./perfor.
Family
Bacteroidaceae
Peptostreptococcales-Tissierellales
Lachnospiraceae
Fusobacteriaceae
Ruminococcaceae
Porphyromonadaceae
Prevotellaceae
Enterobacteriaceae
Campylobacteraceae
Oscillospiraceae
Veillonellaceae
Corynebacteriaceae
Pasteurellaceae
Rikenellaceae
Sutterellaceae
Other
b
Richness
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alpha diversity measure
ns
Severity
catarrhal
phlegmonous
gangr./perfor.
c
PCoA 2 (17.95 %)
PCoA 1 (29.82 %)
Haemophilus
Escherichia-Shigella
Bacteroides vulgatus
Fusobacterium
Bacteroides
d
[Ruminococcus] gauvreauii group
Bacteroides
CAG-352
Collinsella
Coprococcus
Fusobacterium
Parvimonas
Peptostreptococcus
Solobacterium
rel. abundance [%]
Severity
catarrhal
phlegmonous
gangr./perfor.

## The dark microbial matter in acute appendicitis: Archaea and Fungi

To our knowledge, the impact of fungal taxa and Archaea on AA has not been investigated previously. Utilizing archaea-specific amplicon sequencing, we detected archaeal signatures in 34 appendix and 25 rectal samples out of a total of 60 samples, respectively. In the peritoneum, only 12 samples contained detectable amounts of archaea out of 34 samples. Among the identified signatures, the majority were classified under the genera *Methanobrevibacter* (appendix: 90.6%, peritoneum: 78.4%, rectum: 69.5%) and *Methanosphaera* (appendix: 8.7%, peritoneum: 4.4%, rectum: 22.7%). A few samples contained sequences classified under the families *Methanomethylophilaceae*, *Nitrososphaeraceae*, and *Ca. Nitrosotenuis*. However, the latter two families belong to the Thermoproteota phylum, commonly found on human skin, suggesting potential contamination. The presence and abundance of Archaea in our AA samples did not show a significant association with disease severity or any other investigated clinical parameter. While the abundance and prevalence of *Methanosphaera* tended to increase with AA severity, this trend was not statistically significant (P = .08 for catarrhal compared to gangrenous/perforated severity; Kruskal-Wallis test).

Similarly, the observation of fungal taxa in the appendix revealed an unremarkable pattern. Following amplicon sequencing targeting the ITS2 region, only 12 samples provided sufficient fungal features after quality control. The most prevalent feature detected was *Malassezia restricta*, predominantly found in sub-acute and catarrhal severity samples. None of the features displayed a significant association with disease severity or any other clinical parameter investigated.

## Community State Type analysis: a potential tool for distinguishing complicated from uncomplicated appendicitis

As previously discussed, the literature suggests that AA may encompass two distinct diseases: complicated appendicitis, which ultimately results in tissue perforation, and uncomplicated appendicitis, which did not. Distinguishing between these types may become crucial in the endeavor to reduce surgical treatment, yet current diagnostic tools fall short in this regard.

To investigate the hypothesis of two distinct disease types, we conducted de novo community state type (CST) clustering using the universal dataset dominated by bacteria, as differential abundance testing identified bacteria as the primary determinant of disease severity. Notably, the most optimal model derived from our data comprised three distinct CSTs, with two CSTs potentially representing complicated and uncomplicated appendicitis (*Figure 8*). CST 2, was associated with high-severity cases and predominantly clustered gangrenous/perforated cases

(indeed, all perforated cases fell within this CST group). Key taxa characterizing CST 2 included *Fusobacterium*, *Porphyromonas*, and *Parvimonas* species, which have been closely linked to disease severity in our study and by others [132,133,135,142,143]. Therefore, we speculate that CST 2 corresponds to cases of complicated appendicitis. In contrast, CST 3 encompassed almost all catarrhal cases and was characterized by typical gut commensals such as *Bacteroidetes* and *Fecalibacterium*, suggesting a representation of uncomplicated AA cases.

The most significant discriminating factor between CST 2 and CST 3 was found to be disease severity, markedly elevated in CST 2 as compared to CST 3 (P = .004, chi-squared test). Additionally, the proportion of patients with elevated blood leukocytes was higher in CST 2 as compared to CST 3 (86.3% versus 57.1%; P = .048), while other clinical parameters showed no significant differences between both clusters. Unexpectedly, an additional cluster was identified - CST 1. In contrast to both other clusters, CST 1 comprised samples that were dominated by single genera, namely Haemophilus (rel. abundances of 98.9% and 40.7%) or *Escherichia-Shigella* (rel. abundances of 28.9% and 53.5%) in the respective samples. We hypothesize that bacterial overgrowth from one of these genera may have contributed to AA in these cases.

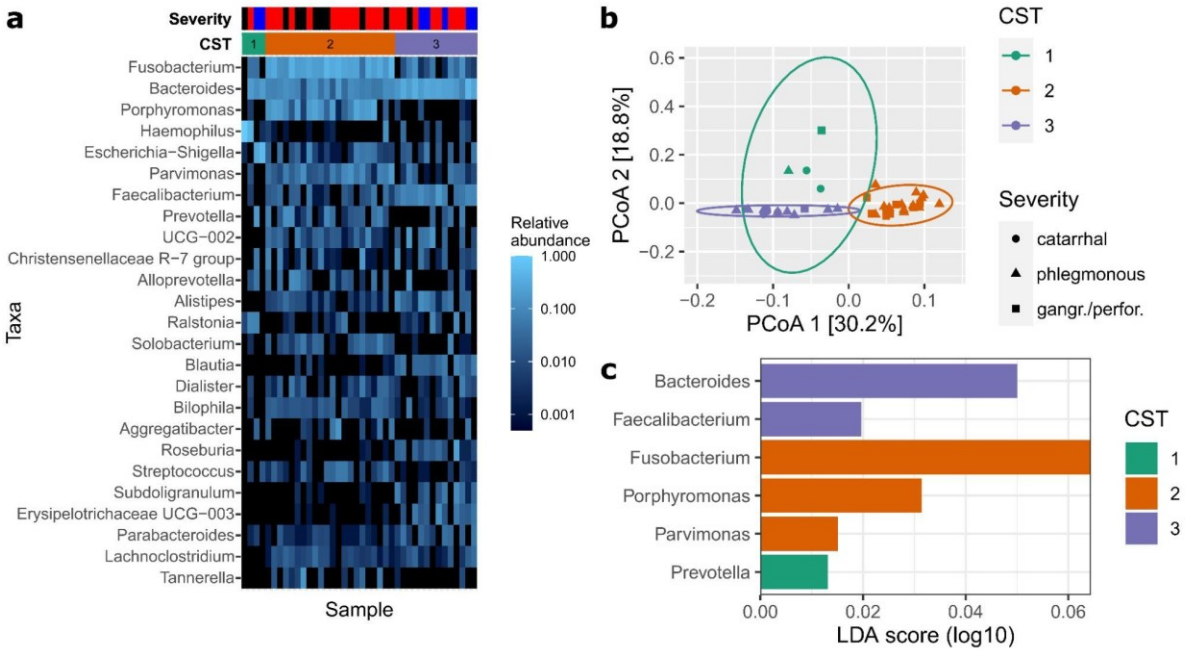


**Figure 8: The appendix microbiome can be characterized by three community state types (CST). (a)** Heatmap displaying the 25 most abundant genera in the appendix samples, sorted by the three defined CSTs. Appendicitis severity grades are indicated by color: blue for catarrhal, red for phlegmonous, and black for gangrenous/perforated. **(b)** CST clustering based on Principal Coordinates Analysis, with circles indicating a 95% confidence interval for the respective CST. **(c)** Bar charts highlighting the key genera contributing to the corresponding CSTs, identified through Linear Discriminant Analysis Effect Size (LEfSe) analysis. Figure is taken from Blohs *et al.*, 2023 [145].

## Metagenomic analysis of the vermiform appendix microbiome in AA

Up to this point, our analysis has primarily relied on amplicon sequencing of the 16S rRNA gene (and ITS region), limited in taxonomic resolution and prone to primer-associated bias. To delve deeper into the taxonomic and functional alterations underlying disease progression and severity, we conducted metagenomic sequencing on all 60 vermiform appendix samples. Analysis of the 16S rRNA genes identified through this approach revealed a disease stage-specific shift toward facultatively pathogenic species. Relative to catarrhal appendicitis, *Fusobacterium necrophorum* (P = .029; pairwise Kruskal-Wallis), *Peptostreptococcus stromatis* (P = .042), *Solobacterium moorei*, and *Porphyromonas* species including *P. uenonis* (P = .006) and *P. asaccharolytica* (P = .01) were enriched in gangrenous/perforated severity. However, no significant differences were observed in microbial composition across disease severity grades following FDR correction, consistent with previous metagenomic analyses [144].

It's worth considering that the histological classification may not adequately reflect the microbial phylotype responsible for disease, particularly in cases of phlegmonous appendicitis where both etiopathologies may overlap, thus complicating analysis. Hence, we applied the described CST clustering to the metagenomic data in order to explore microbial shifts at taxonomic and functional levels. MaAsLin2 analysis revealed a significant enrichment of *Fusobacterium necrophorum* ($P_{adj}$ = .035) in the "complicated disease" cluster CST 2 compared to the "uncomplicated disease" cluster CST 3, underscoring the pivotal role of *Fusobacteria* species in AA severity. Additionally, CST 2 showed enrichment in *Porphyromonas asaccharolytica* ($P_{adj}$ = .14), although not significant after FDR correction.

On the metabolic level (*Figure 9*), CST 2 displayed an intriguing increase in catabolic pathways relative to CST 3, including lysine fermentation to crotonyl-CoA ($P_{adj}$ = .035), histidine degradation ($P_{adj}$ = .051), and pathways for bacterial proteasome ($P_{adj}$ = .051), glutamate fermentation ($P_{adj}$ = .066), and associated Na-driven 2-hydroxyglutarate pathway ($P_{adj}$ = .051).

To validate these findings, we employed DESeq2 analysis, confirming the significant enrichment of *F. necrophorum* in the presumed complicated AA cluster CST 2. Additionally, DESeq2 revealed enrichment of *F. nucleatum*, *P. endodontalis*, *P. uenonis*, as well as two species of the genera *Prevotella* and *Alloprevotella* in CST 2 cases. Even though no significant functional changes were observed, both DESeq2 and MaAsLin2 hinted at an increase in catabolic pathways in CST 2, possibly indicative of heightened nutrient availability from apoptotic or necrotic host cells.

Finally, we explored whether AA severity correlated with increased availability of antimicrobial resistance or virulence genes. However, neither ABRicate nor differential abundance testing yielded any significant associations in this regard.

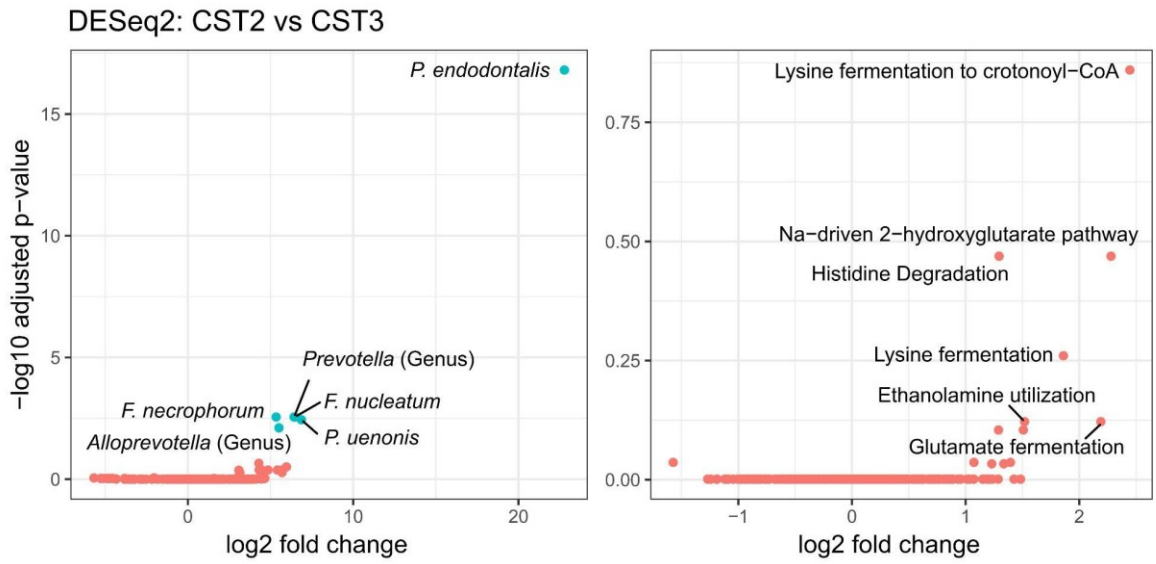


**Figure 9: Differences in both taxonomic and functional profiles between community state types (CST) 2 and 3.** DESeq2 analysis results are presented for the taxonomic (left) and functional level (right), with the six most crucial features labeled. Significantly differentially abundant features are highlighted in blue ($P_{adj}$ < .05 and fold change > 1.5). Species abbreviations are: *Fusobacterium necrophorum, Fusobacterium nucleatum, Porphyromonas endodontalis,* and *Porphyromonas uenonis*. Figure is taken from Blohs *et al.*, 2023 [145].

# Discussion

To gain a comprehensive understanding of the dynamics of the human microbiome, it is crucial to identify all the participants within the system, elucidate their roles in the microbial community, and explore their interconnections with one another and with the host. This thesis aims to contribute a small piece to this complex puzzle by examining the roles of archaea and other anaerobic microorganisms within the human microbiome. Archaea are recognized as keystone species in the human gastrointestinal tract (GIT) and are believed to play an important role in maintaining gastrointestinal health and microbial balance. Despite their potential significance, the impact of these microorganisms is often underrepresented in existing literature, primarily due to methodological challenges, their relatively low abundance in human-associated microbiomes, and the absence of known archaeal pathogens or diseases directly linked to them [22].

## Microbial transmission via the build environment

Human-associated archaea, such as methanogens, are highly adapted to their host [38–41] and are considered to have co-evolved with humankind [189]. A reliable transmission of methanogens between hosts is thus imperative for their evolutionary fitness and survival of these archaea. Nevertheless, the exact transmission route for methanogens is not yet resolved. In this context, we explored the capability of host-associated microbes to survive the hostile built environment (BE), while focusing on strictly anaerobic taxa. These microbes play a substantial role in maintaining human health and constant microbial exchange of viable anaerobes is arguably of great importance for training of the immune system [190] and the (re-)colonization of the human microbiome following disease or antibiotic treatment [100,101]. In urban settings, where individuals often spend the majority of their time indoors, the potential for anaerobic microbes to interact with humans through various reservoirs in the BE thus requires further exploration.

In the first step, we analyzed which microbes accumulated in the ten investigated bathrooms within one week. The results, described above clearly support the notion of normal households to harbor a unique microbial community, dominated by human-associated microbes (*Figure 2*) [191]. This high richness and diversity in human-associated microbes supports the notion of a personalized “microbial cloud” that is leading to a shedding and accumulation on indoor surfaces [185]. We observed only about a quarter of the microbes from other environmental sources, contrasting previous studies that report up to 75% to be of environmental origin [95,192–194]. However, this effect is very much dependent on several factors, mainly defined by the room type investigated and “low traffic” vs. “high human traffic” areas and restroom surfaces typically show the highest

abundance of human-associated microbes [195]. The authors further speculate that especially kitchens and restrooms reveal more consistent microbial community patterns due to the higher likelihood of moisture exposure and periodic inoculation from microbe-rich sources such as food or the human body [195]. With about 40% contribution, skin-associated microbes constituted the highest fraction to the investigated bathroom microbiome, which is in concordance with previous reports [113,114]. A much higher variability was observed among the ten households for microbes that presumably originated from fecal, vaginal, and urinary sources. Approximately 10% of the total microbial community on bathroom floors comprised those microorganisms, with their presence likely linked to aerosols generated during toilet flushing [196]. Interestingly, actions such as closing the toilet lid before flushing, as practiced in households H4 and H10, did not markedly change the proportions of fecal, vaginal, and urinary-associated microbes, although the small sample size limits the ability to draw definitive conclusions. This finding may draw further exploration into alternative reservoirs, including showers [197] or sinks [112].

While the presence of molecular markers can provide valuable insights into the BE microbiome, the sole analysis of available DNA offers limited information regarding the ability of the microbes to be transmitted from the BE to a new host. In general, molecular surveys of the BE microbiome do not discriminate between viable and dead microbes and DNA is very stable in the environment. Signatures of microbes remain detectable for a long time and the presence of DNA signatures alone does not serve as a suitable indicator for viability and metabolic activity *per se*. Once dispersed, DNA from human-associated taxa can still be tracked on public restroom floors and walls after several weeks [113]. Thus, the proportion of relevant microorganisms in the BE might be overestimated in conventional microbiome approaches. On the other hand, cultivation-based assays are hindered by their technological limitations and might reflect only a small, non-representative proportion of living indoor microbiota [198]. To address this limitation, we utilized PMA treatment to specifically investigate the intact and potentially viable microbial community. Although this approach has been rarely applied to built environment samples in the past (e.g., Emerson et al., [199]), it has been instrumental in assessing living microorganisms in spacecraft environments to evaluate the risk of contamination for extraterrestrial research missions as addressed by planetary protection [103,200,201]. Notably, under stringent cleanroom conditions, a proportion of 1-45% of the detected microbial community was deemed to be alive under cleanroom conditions [201,202].

Our experiments confirmed the hypothesis that the majority of microbial signatures originated from free DNA or disintegrated cells. Specifically, we observed a more than three- and four-fold reduction in the absolute numbers of archaea and bacteria, respectively, following PMA treatment, as indicated by RT-qPCR (

*Figure **4**a*). This suggests that over 25% of the identified prokaryotic signatures—and notably, more than 30% of the archaeal signatures—derive from intact and potentially viable cells that may have the capability to colonize the human body. As to be expected, these potentially viable cells were associated with traits that enable them to withstand the challenging conditions of the BE, including an aerobic lifestyle and enhanced stress tolerance (

*Figure **4**e*). The PMA-treated household samples predominantly contained typical aerotolerant taxa, including those associated with humans, environmental sources, and spore-forming organisms. This aligns with the proposition made by Gibbons *et al.* [186] that microbes in the BE do not proliferate but instead persist in indoor environments through mechanisms of dormancy and accumulation. A notion that is further supported by our findings.

Although aerotolerant taxa were predominant in our PMA-treated samples, strictly anaerobic taxa were still present in all investigated bathrooms. Notably, archaea were identified as stable components of the BE microbiome. Although their abundance is approximately 100 times lower than that of bacteria, archaea were detected in every household, both in PMA and non-PMA samples. Specifically, members of the *Nitrososphaeraceae* family demonstrated substantial resilience within the BE. They were among the most prevalent features in PMA samples and accounted for the most abundant family in non-PMA samples (*Figure 5*), which highlighted their stability in indoor environments. In contrast, Euryarchaeota appeared to be more transient. Most genera of this phylum were detected in only a few households and their signals frequently disappeared in PMA-treated samples. Most members of Euryarchaeota are strict anaerobes, suggesting that they may struggle to survive in the challenging conditions of indoor environments.

An exception among the apparently sensitive members of the Euryarchaeota phylum is the taxon *Methanobrevibacter** [taxa with predominantly strict anaerobes are marked with an asterisk for convenience], which is recognized as the most abundant and widespread archaeal taxon in the GIT [47]. Contrary to the prevailing notion that methanogens are highly susceptible to oxygen [187,203–205], our findings revealed *Methanobrevibacter** signatures not only in non-PMA samples but also in PMA samples, which indicates the presence of intact and potentially viable methanogens in the investigated bathroom samples. In fact, the oxidative susceptibility of methanogens has been challenged by metagenomic analyses conducted by Lyu and Lu [206], which provided in-depth insights into the genetic mechanisms of methanogens to counteract ROS. Although *Methanobrevibacter** species have been classified as "Class I methanogens", indicating limited genetic capacity to manage ROS, both our experimental data and that of others confirm the ability of representative methanogens to survive in fully oxygenated environments for several hours or even days [207–209]. Our experiments demonstrated that methanogens isolated from the human

gastrointestinal tract could be regrown after exposure to oxygen for several hours. Both tested *M. smithii** strains as well as *M. stadtmanae**, classified as Class I methanogens, remained viable after six hours of oxygen treatment, while *Methanomassiliicoccus luminyensis** — more closely related to Class II methanogens, which possess greater genetic resilience to ROS — was capable of regrowth even after 48 hours (*Figure 6*).

Building on the previous findings by Lyu and Lu [206], we explored the genomic capabilities of methanogens to identify mechanisms enabling them to withstand oxidative stress, as detailed in Pausan, Blohs *et al.* [147]. Our focus was on pinpointing key genes linked to oxidative stress resistance within the representative genomes of *M. stadtmanae**, *M. smithii**, and *M. luminyensis**. Utilizing the Microbial Genome Annotation and Analysis Platform (MaGe) [210] facilitated a comprehensive comparative genomic assessment of these methanogens. Our analysis revealed that all examined methanogens, with the exception of *M. stadtmanae** strain DEW79, contained essential genes associated with surviving oxygen exposure, including rubrerythrin, ferritin, thioredoxin, and rubredoxin. Notably, *Methanomassiliicoccus spp.** exhibited enhanced resilience to oxygen exposure, supported by the presence of catalase and peroxiredoxin, while *M. luminyensis** even contained superoxide dismutase.

Overall, our experimental results, combined with genomic data, indicate that human-associated archaea have the potential to survive in oxygenated environments for at least six hours under the conditions tested. This resilience highlights the possibility of an environment-to-host transfer of these methanogens. Nevertheless, it remains plausible that the *Methanobrevibacter** signatures identified in our bathroom samples originate from other sources as the human body site, as the loss of genes to mitigate oxidative stress is common to host-associated strains [38]. Our objective was to ascertain whether the methanogens detected in the indoor samples originated from human hosts or were linked to archaeal signatures from natural environments. For this purpose, we performed phylogenetic reconstruction with the 16S rRNA gene sequences of methanogenic archaea from our bathroom datasets (both PMA and non-PMA) and reference data from various environments available in public databases (Figure 5 in Pausan, Blohs *et al.* [147]). The majority of the *Methanobrevibacter* sequences identified in our indoor samples clustered closely with sequences known to be host-associated, including *M. smithii*, *M. oralis* and *M. millerae*, which are typically found in humans and other mammals like cattle and sheep. This clustering suggests that the detected *Methanobrevibacter* signatures are host-associated and likely originate from human microbiota, given the strong association of this taxon with host organisms and the sample origin.

In the context of the *Methanomassiliicoccales* order, our findings from phylogenetic reconstruction concurred those of Borrel *et al.* [211], which indicated that nearly all human-associated

*Methanomassiliicoccales* sequences are found within a host-associated clade, with a few exceptions such as Candidatus *M. intestinalis* and *M. luminyensis*. These two taxa, while isolated from human samples, were found to cluster with sequences from microorganisms primarily associated with soil and sediment, suggesting that environmental *Methanomassiliicoccales* taxa can transfer from their natural habitats to the human gut and establish colonization. In our analysis, the *Methanomassiliicoccales* sequences identified in the bathroom samples were predominantly grouped within the environmental clade, aligning closely with sequences obtained from soils, sediments, and anaerobic digesters. This supports the notion that the source of the *Methanomassiliicoccales* sequences in our samples were likely environmental. In contrast, the sequences belonging to the genus *Methanobacterium* demonstrated a stronger association with human origins. Most *Methanobacterium* sequences from our human dataset formed a distinct cluster, with only one ASV showing close association with *Methanobacterium ferruginis* and another identified in the non-PMA samples. Notably, seven ASVs from the PMA samples clustered with *Methanobacterium oryzae*, sharing a node with the phylogenetic group composed of human-associated ASVs. Taken together, our phylogenetic and abundance-based analyses collectively indicate that the majority of methanogenic sequences identified in the bathroom floor samples, particularly those from *Methanobrevibacter* and *Methanobacterium*, are likely of human origin, emphasizing the potential role of these archaea in an indoor-host transmission route.

The potential for the transmission of obligate anaerobic human commensals between individuals through the BE remains unverified to date, even though some publications do support this notion [99,212–214]. It is known that microbial taxa such as clostridia are unlikely to be directly passed from mother to child, despite being the most abundant bacterial group in the maternal gut [212]. It is suggested that clostridia depend on transmission through relatives or indirect pathways, such as environmental reservoirs [212]. This idea is further supported by our findings, which indicate a higher survival rate for spore-forming bacteria, including clostridia, capable of enduring oxygen exposure and potentially surviving the acidic conditions of the stomach. The likelihood of such horizontal, indirect transmission occurring in healthy adults may be low, as several factors influence the success of transmission. These factors include the specific routes of transmission, the efficiency of dispersal, the ability of microbes to survive outside a host, human colonization resistance, and the capacity of gut commensals to withstand gastric and bile acids [93]. While these factors have not been thoroughly explored for commensals, it can be inferred that both pathogens and commensals may utilize similar mechanisms for transmission. For instance, well-established transmission routes for many pathogens include direct inhalation of aerosols and dust (e.g., *Bacillus anthracis*, *Mycobacterium tuberculosis*) and surface contact transmission (e.g., *Clostridioides difficile*, *Staphylococcus aureus*, *Enterococcus faecalis*) [198]. It is likely that analogous mechanisms are also applicable for the spread of human commensals. However, further research

is needed to evaluate this hypothesis, and microbial genomic sequence variation analysis could provide valuable insights to substantiate the transfer of commensals between individuals via the BE. Intriguingly, a later publication by Gacesa *et al*. [4] underscored the importance of the (indoor) environment and cohabitation on the human microbiome. Analyzing data from over 8,200 participants, the authors found that nearly 50% of the gastrointestinal taxa were significantly influenced by cohabitation, with 9% of the taxa attributable to the history of shared living conditions. In contrast, only 6.6% of the taxa were found to be heritable, suggesting that cohabitation has a more substantial impact on microbial composition than genetic inheritance or early life exposures. Regarding archaea, the only taxon Gacesa *et al*. [4] reported was *Methanobrevibacter* spp. Other archaeal taxa were likely cut by restrictive quality control and / or methodological constraints as mentioned in the introduction. The study revealed that the prevalence and abundance of *Methanobrevibacter* spp. were predominantly linked to anthropogenic factors, including BMI, sex, and age, rather than early-life exposures, geographical factors, or socioeconomic status. This aligns with previous observations related to *Methanomassiliicoccales*, which tend to predominantly colonize the gut in older adults [183,184]. It appears that the colonization of methanogens is more contingent on finding suitable conditions within the human gut, rather than on opportunistic transmission routes. Therefore, widespread transmission through food sources or the built environment seems plausible and together these findings emphasize the necessity of exploring the BE as a potential transmission route for human commensals.

While our research provides insights into the presence of archaea and other strict anaerobes in the BE, it is crucial to view those results in the light of inherent strengths and limitations of our methodology. Although PMA treatment effectively masks free DNA, its efficacy can be influenced by various factors, including the physicochemical properties of the samples and the structural characteristics of the microbial community. Especially the optical density, turbidity and pH of the sample as well as cellular properties such as cell wall structures, natural intake and efflux mechanisms of the microbes may significantly affect PMA treatment results [199,215]. As such, various bacteria are susceptible to PMA treatment as PMA was found to exert antimicrobial effects to *Listeria* and *Legionella* species [216,217]. Furthermore, PMA was shown to penetrate intact cells of several Gram-negative bacteria, including *Escherichia*, *Staphylococcus*, *Streptococcus* and *Mycobacterium* species [218]. In our analysis of relatively low-biomass samples, PMA appears to have an ambiguous impact on the microbial community. On the one hand, it is likely that we may have underestimated the diversity of viable microorganisms as suggested by Wang *et al*. [219]. On the other hand, PMA may counteract the susceptibility to contamination that especially impact samples of low microbial load and/or diversity. Masking of free DNA is an efficient tool to minimize contaminating DNA, achieving a much more stable alpha- and beta diversity [220]. As demonstrated

in human saliva samples, PMA treatment was also shown to effectively cover human DNA, resulting in a reduction of host derived sequencing reads from about 90% in untreated samples to about 9% post-treatment [221]. Taken together, we are confident that the taxa identified in the PMA dataset predominantly originate from intact cells, even though biases affecting certain genera, such as *Corynebacterium* and *Staphylococcus* which have shown "PMA-resilience", cannot be excluded [219]. Furthermore, the limited sample size and the heterogeneity among households may introduce biases, which should be considered when interpreting the data. Our study focused exclusively on bathroom surfaces, leaving out other potential reservoirs of microorganisms, such as showers, sinks, and door handles, which could provide a more comprehensive understanding of the indoor microbiome and should be considered in future studies. Additionally, monitoring environmental factors such as humidity, temperature, and ventilation frequency would help elucidate the conditions that favor microbial survival [222,223]. Finally, our functional analyses were limited by the constraints of amplicon sequencing, which restricted our ability to assess the metabolic capacities of the microbiome comprehensively. The functional analysis conducted using BugBase [177] relies solely on predicting phenotypes based on amplicon sequence variants of the microbiome. While this approach can offer a functional overview of the microbial communities within the investigated households, it is inadequate for making claims about specific taxa. Furthermore, the methodology we employed does not allow for discrimination at the species or strain level, which hampers our understanding of microbial diversity. Although newer bioinformatics tools and updated annotation libraries, such as ASVmaker [224], Unassigner [225] and Greengenes2 [226], may help address some of these issues, other limitations inherent to targeted sequencing methods — including the introduction of significant detection biases in microbiome richness and Shannon diversity based on primer selection (e.g., V1-V2 vs. V3-V4) — cannot be fully overcome [227]. To effectively verify the transfer of specific human commensals through the BE, it is essential to achieve resolution at the strain level or conduct assessments using techniques like random amplified polymorphic DNA analysis. Therefore, future research should adopt untargeted methodologies, such as shotgun metagenomics or long-read real-time sequencing with MinION technology, that offer enhanced taxonomic classification, diversity estimation, and resolution on rare taxa [228]. These approaches offer enhanced taxonomic resolution and more comprehensive functional profiling of the indoor environment microbiomes.

Collectively, our results support the hypothesis that methanogens can survive aerobic conditions for a limited duration, suggesting a potential transmission pathway that may be particularly relevant for infants. This introduces the possibility that methanogens, such as *Methanobrevibacter*, could colonize the gastrointestinal tract via alternative sources, such as the built environment or dietary products like yogurt and organic vegetables [229].

## Role of microbial dysbiosis in acute appendicitis

With Archaea being an integral part of the human microbiome, the assessment of their impact in human health is underrepresented so far. To date, Archaea are not known to possess virulence factors or to be pathogenic [47,48]. While Archaea are not currently recognized as direct pathogens or possessing virulence factors, their potential involvement in microbial dysbiosis is a topic of discussion nevertheless (*Table 1*). Although not inherently harmful, changes in the abundance or diversity of archaeal species could contribute to disruptions in the balance of the microbiome, potentially influencing health outcomes (as reviewed in Cisek *et al.* [49]). To investigate the role of archaea in disease, we focused on microbial community changes in acute appendicitis (AA), one of the most common inflammatory conditions of the GIT. The vermiform appendix, which is largely isolated from bowel movement, presents an ideal site for hosting non-motile and rather slow-growing methanogens, making it a compelling candidate for this study.

In our study, we identified microbial signatures that were distinctively associated with the different severity stages of AA and revealed a shift in the microbial community composition within the appendix as the disease progressed (*Figure 7*). In fact, major changes were observed in bacterial beta diversity rather than an increase in microbial richness, which is in contrast to previous reports [139]. This shift in diversity was driven almost entirely by changes within bacterial taxa, with fungi and archaea showing no significant contributions. Both 16S rRNA gene amplicon sequencing and metagenomic shotgun sequencing results revealed a distinct localized increase in opportunistic pathogens in cases of complicated appendicitis. In those cases, the appendix microbiome showed a significant increase in oral cavity-associated microbes, such as *Fusobacterium*, *Porphyromonas*, *Parvimonas*, *Peptostreptococcus*, and *Solobacterium*. Concurrently, a stepwise reduction in gut-associated *Bacteroides* was observed upon increasing disease severity, which is in line with previous reports [133,139,142,143]. This decline was accompanied by a significant decrease in the relative abundance of other commensals like *Ruminococcaceae*, *Collinsella*, and *Coprococcus* as AA progressed from catarrhal to phlegmonous and/or gangrenous/perforated stages (*Figure 7d*).

For over a decade, researchers have suggested that uncomplicated and complicated appendicitis follow distinct etiopathogenic pathways. This notion is supported by evidence showing that not all cases of appendicitis progress to perforation [230–232]. While the duration between the onset of pain and surgical intervention often correlates with the likelihood of perforation, some cases remain phlegmonous even after an extended period of symptoms. Our findings align with this hypothesis, demonstrating a clear microbial shift as the disease progresses from catarrhal to

gangrenous/perforated appendicitis. This shift is evident from both differential abundance and beta diversity analyses, as well as through a de novo community state type (CST) analysis. The CST analysis revealed three distinct clusters with unique microbial compositions. The first cluster (CST 1) was characterized by bacterial overgrowth dominated by either *Haemophilus* or *Escherichia-Shigella*. The second cluster (CST 2), which we strongly associated with complicated appendicitis, exhibited an enrichment of *Fusobacterium* and other oral-cavity associated microbes such as *Porphyromonas* and *Parvimonas*. The third cluster (CST 3), potentially indicative of uncomplicated cases, featured a higher relative abundance of gut-associated bacteria like *Bacteroides* and *Faecalibacterium*, with minimal presence of oral pathogens. Interestingly, while CST 2 and CST 3 clearly differentiated catarrhal from perforated appendicitis, phlegmonous and some gangrenous cases appeared in both clusters, suggesting that microbial composition is only one factor influencing disease severity. Appendicitis is a multifactorial condition, impacted by a combination of microbiome dynamics, immune responses, genetic predisposition, and lifestyle factors [116,117,119,136]. For instance, prior research by Rivera-Chavez *et al.* [136] highlighted the role of single nucleotide polymorphisms in the *IL-6* gene for the progression of complicated appendicitis.

The presence of *Fusobacterium*, particularly *F. nucleatum* and *F. necrophorum*, emerged as a consistent hallmark of complicated appendicitis. These species, known for their pathogenic potential in gastrointestinal diseases such as primary sclerosing cholangitis, inflammatory bowel disease and colorectal cancer [233], were significantly enriched in CST 2. *F. nucleatum* is particularly notable for its ability to interact with host tissue, modulate immune responses, and enhance the virulence of other pathogens. Its capacity to form biofilms and to act as a docking hub for secondary colonizers like *Porphyromonas*, *Parvimonas*, and *Peptostreptococcus* [234] further underscores its role in disease progression. Even though both *Fusobacterium* species were found to be part of the healthy appendix microbiota [132,143], we and others show that their abundance and prevalence are significantly enriched in complicated appendicitis (*Figure 7d* & *Figure 8*) [142]. Moreover, the observed increase in amino acid metabolizing pathways in our metagenomic data suggests a potential connection to the proliferation of fusobacteria. Growth of *F. nucleatum* relies on free lysine, histidine, glutamate, and serine [235], and our analysis revealed an enrichment of catabolic pathways for the production of lysine, histidine, and glutamate specifically in CST 2. Notably, lysine fermentation to crotonyl-CoA, a pathway documented in only a limited number of microbes—including *F. nucleatum* and *Porphyromonas gingivalis* [236]—was significantly elevated in more severe cases (CST 2). This finding raises the possibility that fusobacteria may actively induce apoptosis in intestinal epithelial cells during AA, potentially releasing peptides and amino acids to support their growth and survival.

Despite its strong association with complicated appendicitis, the role of fusobacteria remains debated. While some researchers propose it as a direct causative agent [133,142], others consider it a secondary opportunist that merely thrives in dysbiotic conditions without initiating the disease itself [237]. Addressing this uncertainty requires further studies to satisfy Koch's postulates and confirm its pathogenic role in AA. Furthermore, it would be intriguing to explore whether certain species, such as *F. nucleatum*, have the capacity to exploit the nutrient release resulting from host cell lysis. This nutrient surplus may not only support bacterial proliferation but could also induce the expression of virulence factors, thereby promoting further host cell damage and establishing a self-reinforcing cycle of inflammation and tissue destruction. Recent findings provide evidence for this concept: Franklin *et al*. [238] demonstrated that elevated levels of free ethanolamine, a breakdown product of phosphatidylethanolamine and a major component of eukaryotic and prokaryotic cell membranes, enhanced the virulence of *F. nucleatum*. This suggests that cell lysis during inflammation may trigger an adaptive metabolic and pathogenic response in *Fusobacterium*, allowing it to actively shape its environment to its own advantage and potentially accelerate disease progression.

Advancements in diagnostic tools, such as clinical scoring systems and imaging techniques, have significantly improved the management of appendicitis and contributed to the prevention of unnecessary surgery [239]. Incorporating microbial markers into diagnostic protocols could further enhance the accuracy of disease classification and guide treatment strategies. For instance, the presence of *Fusobacterium* and its co-occurring oral pathogens might serve as indicators for complicated cases, potentially informing decisions on antibiotic therapy. However, our study did not reveal significant differences in the microbiome of rectal samples within the analyzed cohort. The localized expansion of opportunistic pathogens in the appendix did not appear to influence the rectal microbiome. This finding could be attributed to the substantial microbial diversity present in the large intestine, which likely dilutes any microbial signatures originating from the appendix as they transit through the gut. Consequently, rectal samples are likely unsuitable for distinguishing between complicated and uncomplicated appendicitis. However, they might still hold potential for differentiating AA from healthy states or other gastrointestinal conditions that present with abdominal pain, such as colitis. Supporting this notion, previous studies have demonstrated an increased microbial richness and higher abundance of genera such as *Bulleidia*, *Dialister*, and *Porphyromonas* in rectal samples from AA patients compared to healthy controls [139]. Longitudinal studies conducted both before and after appendectomy are needed to determine whether microbial changes in the distal colon are causally linked to AA.

In peritoneal samples, we noted a gradual, though not statistically significant, shift from skin-associated taxa dominating in catarrhal cases to intestinal-associated taxa becoming more

prominent in cases of complicated appendicitis. These samples may act as a proxy for AA severity, as invasive pathogens like fusobacteria were detected in patients with perforated appendicitis and, in some instances, even in gangrenous or phlegmonous appendicitis (*Figure 7* & *Figure 8*). While the diagnostic value of peritoneal samples is limited due to the invasive nature of sampling, our findings confirmed the presence of pathogen signatures in the peritoneal cavity even in non-perforated appendicitis. The clinical significance of this observation remains unclear but emphasizes the importance of prompt therapeutic intervention, such as surgery or antibiotic treatment, in cases of AA. Additionally, we found a significantly higher occurrence of leukocytosis in complicated appendicitis compared to uncomplicated cases (*Supplementary Table 2*). Although the diagnostic utility of white blood cell and lymphocyte counts in AA remains debated [240], the presence of uncommon microbes in the gastrointestinal tract, particularly oral-associated pathogens, might contribute to inflammation and may be reflected by elevated leukocyte levels.

Finally, it needs to be discussed whether archaea may influence the etiopathogenesis of AA. Within our dataset, only *Methanosphaera* showed a weak tendency to correlate with AA severity. However, the direct involvement of *Methanosphaera* in the disease's etiopathology seems unlikely, particularly given its low prevalence in adolescents. Studies have shown that only 8.3% of children aged 6 to 10 harbor *Methanosphaera* in their fecal samples [229] as compared to 29.4% in the general population [28]. Consistent with these findings, our 16S rRNA gene analysis revealed a similarly low prevalence among diseased adolescents, with 10% of rectal and 5% of appendix samples that were positive for *M. stadtmanae* signatures. With such low prevalence in the diseased vermiform appendix, its etiopathological significance appears negligible. While it is tempting to hypothesize that the presence of *Methanosphaera* may exacerbate disease progression due to its stronger inflammatory response compared to *Methanobrevibacter* [46,241], the limited sample size in our study precludes definitive conclusions. As opposed to previous reports, we detected reliable signals of *Methanobrevibacter* in only 28.3% (17/60) of rectal samples, markedly lower than the 78.2% prevalence observed in the stool of a healthy adolescent cohort [229]. In the vermiform appendix, *Methanobrevibacter* signatures were present in 43.3% (26/60) of participants.

Given the limited data on archaea, their impact on AA remains speculative. Methanogens are mutualistic organisms that can influence the broader gut microbiota and even appear to indicate colonic homeostasis [72]. *Methanobrevibacter* spp., in particular, support the growth of adjacent microbes by scavenging hydrogen produced during fermentation, thereby relieving inhibition on fermentative processes [32–35]. It is conceivable that such growth promotion by archaea could increase the production of beneficial metabolites, such as SCFAs, and enhance colonization resistance, thus, reducing susceptibility to pathogens. However, the same mechanisms may also support the growth of opportunistic pathogens, potentially contributing to dysbiosis and disease.

Thus, methanogens presumably play a "catalytic" role in the gut microbiota, fostering mutualistic interactions and enabling the growth of taxa that are typically suppressed by high hydrogen partial pressure. However, whether this catalytic effect skews toward health promotion or disease facilitation depends on the specific microbial context. It is tempting to speculate that the low prevalence of methanogens in our cohort might indicate a reduced colonization resistance rather than direct support for opportunistic pathogens. Future investigations should aim to elucidate this relationship. For instance, co-culture experiments testing the growth dynamics of *F. nucleatum* or *F. necrophorum* alongside gut-associated methanogens such as *M. smithii* or *M. stadtmanae,* and other gut commensals like *Bacteroides thetaiotaomicron*, could provide valuable insights. Such studies could clarify whether archaea influence the etiopathogenesis of AA by fostering conditions that either stabilize the gut microbiota or promote pathogen overgrowth.

Several questions remain unanswered regarding the origins and progression of appendicitis. The hypothesis that appendicitis is an infectious disease driven by specific pathogens warrants further exploration, especially in light of recent observations during the COVID-19 pandemic. Intriguingly, during this time a significant reduction in paediatric appendicitis cases was observed, which was potentially linked to improved hygiene and reduced microbial exposure [242,243]. The role of oral health and microbial translocation from the oral cavity to the appendix is another area of interest, given the increasing evidence of oral pathogens' involvement in gastrointestinal inflammation [140,233,244,245]. In conclusion, while this study provides valuable insights into the microbial dynamics of appendicitis, further longitudinal and mechanistic studies are needed to unravel the complex interplay between the microbiome, host immune responses, and genetic factors in the disease's etiopathogenesis.

Our findings on the microbial landscape in AA must also be critically assessed in light of methodological limitations inherent in our study. As with any 16S rRNA gene analysis, previously described biases—such as amplification efficiency, primer specificity, and database annotation—likely influenced our results. Another major challenge was the overwhelming presence of host DNA in our samples, which severely impeded the detection of bacterial and archaeal reads, particularly in metagenomic sequencing. This issue was exacerbated by factors associated with inflammation and infection, including immune cell infiltration, tissue damage, and necrosis, which further elevated the human-to-microbe DNA ratio in our dataset [246]. Several strategies have been proposed in the literature to improve the recovery of microbial DNA from low-biomass samples dominated by host DNA. One approach involves depleting methylated host DNA, using kits such as the NEBNext® Microbiome DNA Enrichment Kit [247]. However, in our study, this method did not significantly enhance microbial read recovery. To improve future analyses of the vermiform appendix microbiome, it will be essential to refine sampling strategies that minimize disruption to

host cells while maximizing microbial recovery. Techniques that preserve microbial integrity and selectively target bacterial and archaeal DNA without amplifying host DNA will be pivotal.

The detection and interpretation of archaeal signatures was further hindered by analytical challenges. It is estimated that 30–80% of archaeal genes remain uncharacterized, referred to as archaeal "dark matter" [23,51]. As a result, KEGG annotations may provide limited or inaccurate functional insights for archaeal proteins. Future studies should incorporate structure-based annotation methods to better predict the function of uncharacterized archaeal genes [51].

Lastly, stool samples may not provide a comprehensive representation of intestinal microbial diversity. Some archaeal species, including *M. smithii* and *M. stadtmanae*, form biofilms and adhere to the intestinal epithelium, rendering them largely inaccessible in stool-based analyses [248]. Therefore, complementary sampling methods, including targeted biopsies and mucosal scrapings, should be considered in future studies to capture the full spectrum of archaeal diversity and their functional roles in the gastrointestinal tract. These methodological advancements will be critical in unravelling the complex interactions between the microbiome and host during AA.

# Conclusion and outlook

Through a combination of PMA treatment and high-throughput sequencing, we were able to demonstrate that archaea are not only present in the BE but may persist there intact and in a potentially viable state. Their detection in all investigated households, even after oxygen exposure, is in contrast to the prevailing assumption that human-associated archaea cannot survive outside their host environment. The repeated identification of viable *Methanobrevibacter* species, including *M. smithii*, suggests that environmental survival may allow for indirect transmission routes and that the BE may serve as a microbial reservoir—not just for bacteria but also for archaea. This finding opens new perspectives on the role of the BE in shaping the human microbiome and calls for its consideration in microbiome-focused public health strategies, particularly in early life colonization and microbial resilience after disease or antibiotic treatment.

In the second part of this thesis, we explored the microbial ecology of AA across gradual increasing stages of disease severity. We confirmed a stepwise compositional shift within the appendix microbiome, particularly the proliferation of oral cavity-associated taxa such as *Fusobacterium*, *Parvimonas*, and *Porphyromonas* in gangrenous and perforated cases, supporting the idea of microbial dysbiosis as a driving factor in complicated appendicitis. The decline of beneficial gut commensals alongside functional enrichment of amino acid catabolism pathways further supports the hypothesis that certain microbial communities may contribute to disease progression by modulating host–microbe interactions, nutrient availability, or tissue integrity. While the presence of archaea in AA samples was confirmed, particularly *Methanobrevibacter* and *Methanosphaera*, their prevalence was relatively low and not significantly associated with disease severity in this adolescent cohort.

Our experimental and comparative genomic analyses revealed that human-associated methanogens possess a range of stress-response genes enabling survival in oxygenated conditions, an adaptation that may enhance their resilience in fluctuating environments such as the gut or BE. This survival capacity, together with their syntrophic interactions with fermentative bacteria and role in hydrogen scavenging, underscores their catalytic influence within microbial networks, capable of supporting either health-promoting or potentially dysbiotic communities depending on the context.

In conclusion, this thesis provides new insights into the archaeal domain within the human microbiome. Our findings aim to encourage broader inclusion of archaeal taxa in microbiome research and highlight the value of integrating functional, compositional, and viability assessments when evaluating microbial ecosystems in human health and disease. In the future,

understanding the ecological roles of archaea, their survival strategies, and their interdependencies with bacterial partners will be crucial in developing a truly comprehensive view of the human microbiome and its implications for personalized medicine and public health.

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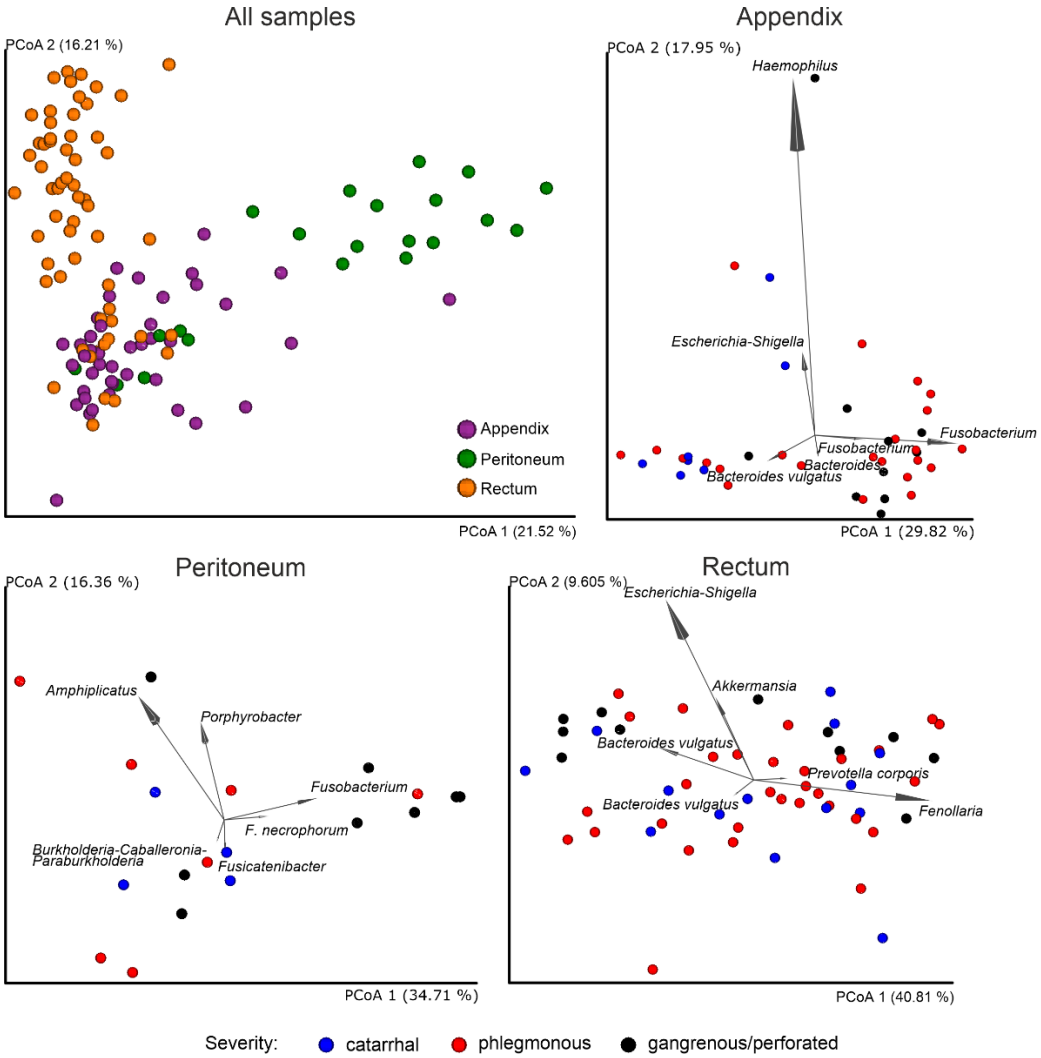
# Supplementary information

**Supplementary Figure 1: Sampling areas of a representative bathroom floor.**




**Supplementary Figure 2: Weighted UniFrac PCoA Biplots of the six most important features for the corresponding sample sites.**

**Supplementary Table 1: Sample data and metadata from all sampled households.**

| | | | | | | | gender distribution | | Age distribution | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Samples** | **gDNA (ng/ul)** | **PMA-treated samples** | **gDNA (ng/ul)** | **Sampling date** | **Number of residents** | M | F | under 3y | 3-10y | 10-20y | 20-40y | 40-60y | over 60y |
| Household 1 | **H1_1** | 0.564 | **H1_2** | 0.126 | 28.03.2017 | 1 | 0 | 1 | | | | 1 | | |
| Household 2 | **H2_1** | 0.372 | **H2_2** | 0.384 | 28.03.2017 | 3 | 1 | 2 | | | | 3 | | |
| Household 3 | **H3_1** | 0.242 | **H3_2** | n.d. | 29.03.2017 | 2 | 1 | 1 | | | | 2 | | |
| Household 4 | **H4_1** | 0.608 | **H4_2** | 0.1 | 30.03.2017 | 2 | 1 | 1 | | | | 2 | | |
| Household 5 | **H5_1** | 1.23 | **H5_2** | 0.17 | 30.03.2017 | 4 | 1 | 3 | | 2 | | | 2 | |
| Household 6 | **H6_1** | 1 | **H6_2** | 1.07 | 31.03.2017 | 3 | 2 | 1 | 1 | | | 2 | | |
| Household 7 | **H7_1** | 0.742 | **H7_2** | 0.208 | 29.03.2017 | 4 | 1 | 3 | 1 | 1 | | 1 | 1 | |
| Household 8 | **H8_1** | 0.66 | **H8_2** | n.d. | 29.03.2017 | 6 | 2 | 4 | 1 | 1 | | 2 | | 2 |
| Household 9 | **H9_1** | 0.876 | **H9_2** | 0.208 | 27.03.2017 | 4 | 3 | 1 | | 2 | | 1 | 1 | |
| Household 10 | **H10_1** | 0.782 | **H10_2** | 0.464 | 30.03.2017 | 1 | 1 | 0 | | | | 1 | | |

| **ID** | **Separated toilet*** | **Sink next to toilet** | **Window in the same room as the toilet** | **Window close to toilet** | **Flushed with toilet seat close** | **Use of daily toilet products (e.g. toilet rim block)** | **Cleaning the toilet with bleach solution** | **Number of guests in between samplings** |
|---|---|---|---|---|---|---|---|---|
| Household 1 | yes | no | no | no | no | yes | no | 0 |
| Household 2 | yes | no | no | no | no | yes | no | 4 |
| Household 3 | yes | yes | no | no | no | no | no | 0 |
| Household 4 | yes | no | no | maybe? | yes | no | no | 0 |
| Household 5 | no | yes | no | no | no | no | no | 0 |
| Household 6 | no | no | yes | yes | no | no | yes | 0 |
| Household 7 | yes | yes | no | no | no | no | no | 0 |
| Household 8 | no | yes | yes | yes | no | no | no | 0 |
| Household 9 | no | yes | yes | yes | no | yes | no | 2 |
| Household 10 | no | yes | yes | yes | yes | no | no | 1 |

* when the shower/bathtub is in a different room than the toilet

**Supplementary Table 2: Patient data of the acute appendicitis cohort.**

| Patient | Gender | Patient age | PAS score | Alvorado score | Leukocytes | CRP | Antibiotic treatment | Severity score | CRP rating | BB rating | Alvorado rating | severity rating | Empyem | Faecalith | Perforated |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P1 | Female | 16 | 5 | 5 | 12.45 | 31.7 | no | 4 | high | leukocytosis | intermediate | complicated | yes | no | no |
| P2 | Male | 8 | 7 | 5 | 13.22 | 11.3 | no | 3 | increased | leukocytosis | intermediate | phlegmonous | yes | no | no |
| P3 | Female | 9 | 4 | 3 | 16.96 | 4.1 | no | 2 | normal | leukocytosis | low | acute | no | no | no |
| P4 | Male | 7 | 9 | 8 | 21.8 | 9.2 | no | 2 | increased | leukocytosis | high | acute | no | yes | no |
| P5 | Male | 6 | 6 | 5 | 17.54 | 17 | no | 3 | high | leukocytosis | intermediate | phlegmonous | yes | yes | no |
| P6 | Female | 12 | 7 | 7 | 18.65 | 2.3 | no | 3 | normal | leukocytosis | high | phlegmonous | yes | no | no |
| P7 | Male | 9 | 7 | 7 | 12.48 | 35.2 | yes | 3 | high | leukocytosis | high | phlegmonous | yes | no | no |
| P8 | Male | 11 | 9 | 9 | 19.26 | 66.1 | no | 3 | high | leukocytosis | high | phlegmonous | yes | yes | no |
| P9 | Male | 14 | 6 | 6 | 11.48 | 7.4 | no | 3 | increased | leukocytosis | intermediate | phlegmonous | yes | no | no |
| P10 | Female | 5 | 8 | 6 | 6.19 | 138.7 | yes | 5 | high | normal | intermediate | complicated | yes | no | yes |
| P11 | Male | 14 | 7 | 6 | 10.93 | 13.2 | no | 3 | increased | normal | intermediate | phlegmonous | no | no | no |
| P12 | Male | 16 | 4 | 4 | 5.17 | 24.4 | no | 3 | high | normal | low | phlegmonous | no | no | no |
| P13 | Male | 10 | 3 | 3 | 7.69 | 14 | yes | 3 | increased | normal | low | phlegmonous | yes | no | no |
| P14 | Female | 14 | 9 | 9 | 19.28 | 36.7 | yes | 5 | high | leukocytosis | high | complicated | yes | yes | yes |
| P15 | Male | 16 | 6 | 5 | 9.4 | 22.7 | no | 3 | high | normal | intermediate | phlegmonous | no | no | no |
| P16 | Female | 16 | 8 | 9 | 19.16 | 63.7 | yes | 5 | high | leukocytosis | high | complicated | no | no | yes |
| P17 | Female | 11 | 6 | 6 | 9.45 | 116.7 | no | 4 | high | normal | intermediate | complicated | yes | no | no |
| P18 | Female | 12 | 4 | 3 | 9.39 | 0.6 | no | 3 | normal | normal | low | phlegmonous | yes | no | no |
| P19 | Male | 9 | 7 | 7 | 15.02 | 14.3 | no | 2 | increased | leukocytosis | high | acute | no | no | no |
| P20 | Male | 13 | 6 | 6 | 18.21 | 21.6 | no | 3 | high | leukocytosis | intermediate | phlegmonous | yes | yes | no |
| P21 | Male | 10 | 7 | 7 | 17.79 | 2.9 | no | 3 | normal | leukocytosis | high | phlegmonous | yes | no | no |
| P22 | Male | 8 | 9 | 9 | 13.66 | 79.6 | no | 2 | high | leukocytosis | high | acute | no | no | no |
| P23 | Male | 6 | 10 | 9 | 11.37 | 226.25 | yes | 5 | high | leukocytosis | high | complicated | no | no | yes |
| P24 | Male | 13 | 8 | 7 | 8.06 | 5.1 | no | 3 | increased | normal | high | phlegmonous | no | no | no |
| P25 | Female | 8 | 5 | 7 | 10.44 | 136 | no | 1 | high | normal | high | none | no | no | no |
| P26 | Male | 12 | 0 | 0 | 6.39 | 0.7 | no | 3 | normal | normal | low | phlegmonous | no | no | no |
| P27 | Male | 13 | 8 | 9 | 19.22 | 3.7 | yes | 5 | normal | leukocytosis | high | complicated | no | no | yes |
| P28 | Male | 10 | 7 | 8 | 16.26 | 137.8 | no | 2 | high | leukocytosis | high | acute | no | no | no |
| P29 | Male | 3 | 9 | 9 | 19.03 | 55.3 | yes | 5 | high | leukocytosis | high | complicated | no | no | yes |
| P30 | Male | 6 | 10 | 10 | 20.07 | 13.3 | yes | 3 | increased | leukocytosis | high | phlegmonous | no | no | no |
| P31 | Male | 7 | 8 | 7 | 8.59 | 59.5 | yes | 3 | high | normal | high | phlegmonous | yes | no | no |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P32 | Male | 7 | 7 | 6 | 8.56 | 10.4 | no | 3 | increased | normal | intermediate | phlegmonous | yes | no | no |
| P33 | Male | 9 | 7 | 7 | 15.83 | 1.4 | no | 3 | normal | leukocytosis | high | phlegmonous | yes | yes | no |
| P34 | Male | 14 | 9 | 8 | 13.41 | 108.7 | no | 1 | high | leukocytosis | high | none | no | no | no |
| P35 | Female | 15 | 5 | 4 | 7.07 | 0.6 | no | 2 | normal | normal | low | acute | no | no | no |
| P36 | Male | 14 | 9 | 9 | 13.18 | 116.9 | no | 5 | high | leukocytosis | high | complicated | yes | no | yes |
| P37 | Male | 14 | 3 | 4 | 5.37 | 27.7 | yes | 3 | high | normal | low | phlegmonous | yes | no | no |
| P38 | Male | 10 | 9 | 9 | 20.68 | 3.4 | no | 3 | normal | leukocytosis | high | phlegmonous | yes | no | no |
| P39 | Male | 8 | 7 | 7 | 15.8 | 55.8 | no | 3 | high | leukocytosis | high | phlegmonous | yes | no | no |
| P40 | Male | 12 | 8 | 8 | 18.24 | 73.2 | yes | 3 | high | leukocytosis | high | phlegmonous | yes | no | no |
| P41 | Male | 13 | 5 | 5 | 9.02 | 5.08 | no | 3 | increased | normal | intermediate | phlegmonous | no | no | no |
| P42 | Female | 4 | 10 | 10 | 21.58 | 183.3 | yes | 3 | high | leukocytosis | high | phlegmonous | no | no | no |
| P43 | Male | 10 | 9 | 10 | 12.44 | 1.4 | no | 3 | normal | leukocytosis | high | phlegmonous | yes | no | no |
| P44 | Female | 10 | 9 | 9 | 12.91 | 354.2 | yes | 4 | high | leukocytosis | high | complicated | yes | no | no |
| P45 | Female | 13 | 9 | 10 | 15.43 | 7.2 | no | 3 | increased | leukocytosis | high | phlegmonous | yes | no | no |
| P46 | Female | 13 | 9 | 9 | 19.28 | 4 | yes | 3 | normal | leukocytosis | high | phlegmonous | yes | no | no |
| P47 | Male | 10 | 3 | 5 | 15.63 | 14.8 | yes | 3 | increased | leukocytosis | intermediate | phlegmonous | no | yes | no |
| P48 | Male | 8 | 8 | 8 | 28.82 | 15.7 | yes | 3 | high | leukocytosis | high | phlegmonous | yes | no | no |
| P49 | Male | 13 | 5 | 4 | 9.87 | 13.2 | no | 1 | increased | normal | low | none | no | no | no |
| P50 | Male | 12 | 8 | 9 | 13.39 | 27.1 | no | 2 | high | leukocytosis | high | acute | no | no | no |
| P51 | Male | 13 | 8 | 8 | 11.2 | 0 | yes | 2 | normal | leukocytosis | high | acute | no | no | no |
| P52 | Male | 6 | 10 | 10 | 17.43 | 145.2 | yes | 5 | high | leukocytosis | high | complicated | yes | no | yes |
| P53 | Female | 14 | 10 | 10 | 9.49 | 31.6 | no | 2 | high | normal | high | acute | no | no | no |
| P54 | Male | 16 | 7 | 8 | 19.87 | 0.1 | no | 2 | normal | leukocytosis | high | acute | no | no | no |
| P55 | Male | 17 | 8 | 9 | 14.96 | 8.4 | no | 3 | increased | leukocytosis | high | phlegmonous | no | no | no |
| P56 | Male | 14 | 2 | 2 | 8.06 | 15.9 | no | 2 | high | normal | low | acute | no | no | no |
| P57 | Female | 16 | 7 | 7 | 14 | 56.4 | yes | 2 | high | leukocytosis | high | acute | no | no | no |
| P58 | Female | 5 | 7 | 8 | 27.74 | 16.1 | no | 1 | high | leukocytosis | high | none | no | no | no |
| P59 | Male | 12 | 7 | 7 | 10.52 | 47 | no | 2 | high | normal | high | acute | no | no | no |
| P60 | Female | 14 | 3 | 3 | 7.29 | 106.8 | no | 2 | high | normal | low | acute | no | no | no |

**Supplementary Table 3: R plugins and versions used.**

| Plugin | Version | Reference |
|---|---|---|
| ape | 5.5 | Paradis E. & Schliep K. 2019. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35: 526-528. |
| Biobase | 2.52.0 | W. Huber, V.J. Carey, R. Gentleman, ..., M. Morgan (2015) Orchestrating high-throughput genomic analysis with Bioconductor. Nature Methods, 2015:12, 115. |
| BiocGenerics | 0.38.0 | W. Huber, V.J. Carey, R. Gentleman, ..., M. Morgan (2015) Orchestrating high-throughput genomic analysis with Bioconductor. Nature Methods, 2015:12, 115. |
| cluster | 2.1.2 | Maechler, M., Rousseeuw, P., Struyf, A., Hubert, M., Hornik, K.(2021). cluster: Cluster Analysis Basics and Extensions. R package version 2.1.2. |
| cowplot | 1.1.1 | Claus O. Wilke (2020). cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'. R package version 1.1.1. https://CRAN.R-project.org/package=cowplot |
| DESeq2 | 1.32.0 | Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2 Genome Biology 15(12):550 (2014) |
| dplyr | 1.0.7 | Hadley Wickham, Romain François, Lionel Henry and Kirill Müller (2021). dplyr: A Grammar of Data Manipulation. R package version 1.0.7. https://CRAN.R-project.org/package=dplyr |
| forcats | 0.5.1 | Hadley Wickham (2021). forcats: Tools for Working with Categorical Variables (Factors). R package version 0.5.1. https://CRAN.R-project.org/package=forcats |
| GenomeInfoDb | 1.28.4 | Sonali Arora, Martin Morgan, Marc Carlson and H. Pagès (2021). GenomeInfoDb: Utilities for manipulating chromosome names, including modifying them to follow a particular naming style. R package version 1.28.4. https://bioconductor.org/packages/GenomeInfoDb |
| GenomicRanges | 1.44.0 | Lawrence M, Huber W, Pages H, Aboyoun P, Carlson M, et al. (2013) Software for Computing and Annotating Genomic Ranges. PLoS Comput Biol 9(8): e1003118. doi:10.1371/journal.pcbi.1003118 |
| ggdist | 3.0.0 | Kay M (2021). _ggdist: Visualizations of Distributions and Uncertainty_. doi: 10.5281/zenodo.3879620 (URL: https://doi.org/10.5281/zenodo.3879620), R package version 3.0.0, <URL: https://mjskay.github.io/ggdist/>. |
| ggplot2 | 3.3.5 | H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016. |
| ggpubr | 0.4.0 | Alboukadel Kassambara (2020). ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0. https://CRAN.R-project.org/package=ggpubr |
| ggrepel | 0.9.1 | Kamil Slowikowski (2021). ggrepel: Automatically Position Non-Overlapping Text Labels with 'ggplot2'. R package version 0.9.1. https://CRAN.R-project.org/package=ggrepel |
| ggthemes | 4.2.4 | Jeffrey B. Arnold (2021). ggthemes: Extra Themes, Scales and Geoms for 'ggplot2'. R package version 4.2.4. https://github.com/jrnold/ggthemes |
| gridExtra | 2,3 | Baptiste Auguie (2017). gridExtra: Miscellaneous Functions for "Grid" Graphics. R package version 2.3. https://CRAN.R-project.org/package=gridExtra |
| gtable | 0.3.0 | Hadley Wickham and Thomas Lin Pedersen (2019). gtable: Arrange 'Grobs' in Tables. R package version 0.3.0. https://CRAN.R-project.org/package=gtable |
| igraph | 1.2.6 | Csardi G, Nepusz T: The igraph software package for complex network research, InterJournal, Complex Systems 1695. 2006. https://igraph.org |
| IRanges | 2.26.0 | Lawrence M, Huber W, Pag\`es H, Aboyoun P, Carlson M, et al. (2013) Software for Computing and Annotating Genomic Ranges. PLoS Comput Biol 9(8): e1003118. doi:10.1371/journal.pcbi.1003118 |
| Maaslin2 | 1.6.0 | Mallick H, Rahnavard A, McIver LJ (2020). MaAsLin 2: Multivariable Association in Population-scale Meta-omics Studies. R/Bioconductor package, http://huttenhower.sph.harvard.edu/maaslin2. |
| maps | 3.4.0 | Original S code by Richard A. Becker, Allan R. Wilks. R version by Ray Brownrigg. Enhancements by Thomas P Minka and Alex Deckmyn. (2021). maps: Draw Geographical Maps. R package version 3.4.0. https://CRAN.R-project.org/package=maps |
| markovchain | 0.8.6 | G.A. Spedicato (2017). Discrete Time Markov Chains with R. R package version 0.6.9.7. https://journal.r-project.org/archive/2017/RJ-2017-036/index.html |
| MatrixGenerics | 1.4.3 | Constantin Ahlmann-Eltze, Peter Hickey and Hervé Pagès (2021). MatrixGenerics: S4 Generic Summary Statistic Functions that Operate on Matrix-Like Objects. R package version 1.4.3. https://bioconductor.org/packages/MatrixGenerics |
| matrixStats | 0.61.0 | Henrik Bengtsson (2021). matrixStats: Functions that Apply to Rows and Columns of Matrices (and to Vectors). R package version 0.61.0. https://CRAN.R-project.org/package=matrixStats |

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|---|---|---|
| microbiome | 1.14.0 | Leo Lahti et al. microbiome R package. URL: http://microbiome.github.io |
| microbiomeMarker | 0.0.1.9000 | Yang Cao (2021). microbiomeMarker: microbiome biomarker analysis. R package version 0.0.1.9000. https://github.com/yiluheihei/microbiomeMarker |
| microViz | 0.7.10.9040 | Barnett et al., (2021). microViz: an R package for microbiome data visualization and statistics. Journal of Open Source Software, 6(63), 3201, https://doi.org/10.21105/joss.03201 |
| phyloseq | 1.36.0 | phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. Paul J. McMurdie and Susan Holmes (2013) PLoS ONE 8(4):e61217. |
| phytools | 0.7-90 | Revell, L. J. (2012) phytools: An R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3 217-223. doi:10.1111/j.2041-210X.2011.00169.x |
| plyr | 1.8.6 | Hadley Wickham (2011). The Split-Apply-Combine Strategy for Data Analysis. Journal of Statistical Software, 40(1), 1-29. URL http://www.jstatsoft.org/v40/i01/. |
| psych | 2.1.9 | Revelle, W. (2021) psych: Procedures for Personality and Psychological Research, Northwestern University, Evanston, Illinois, USA, https://CRAN.R-project.org/package=psych Version = 2.1.9,. |
| purrr | 0.3.4 | Lionel Henry and Hadley Wickham (2020). purrr: Functional Programming Tools. R package version 0.3.4. https://CRAN.R-project.org/package=purrr |
| RColorBrewer | 1.1-2 | Erich Neuwirth (2014). RColorBrewer: ColorBrewer Palettes. R package version 1.1-2. https://CRAN.R-project.org/package=RColorBrewer |
| readr | 2.1.0 | Hadley Wickham and Jim Hester (2021). readr: Read Rectangular Text Data. R package version 2.1.0. https://CRAN.R-project.org/package=readr |
| reshape2 | 1.4.4 | Hadley Wickham (2007). Reshaping Data with the reshape Package. Journal of Statistical Software, 21(12), 1-20. URL http://www.jstatsoft.org/v21/i12/. |
| rstatix | 0.7.0 | Alboukadel Kassambara (2021). rstatix: Pipe-Friendly Framework for Basic Statistical Tests. R package version 0.7.0. https://CRAN.R-project.org/package=rstatix |
| S4Vectors | 0.30.0 | H. Pagès, M. Lawrence and P. Aboyoun (2021). S4Vectors: Foundation of vector-like and list-like containers in Bioconductor. R package version 0.30.0. https://bioconductor.org/packages/S4Vectors |
| stringr | 1.4.0 | Hadley Wickham (2019). stringr: Simple, Consistent Wrappers for Common String Operations. R package version 1.4.0. https://CRAN.R-project.org/package=stringr |
| SummarizedExperiment | 1.22.0 | Martin Morgan, Valerie Obenchain, Jim Hester and Hervé Pagès (2021). SummarizedExperiment: SummarizedExperiment container. R package version 1.22.0. https://bioconductor.org/packages/SummarizedExperiment |
| tibble | 3.1.5 | Kirill Müller and Hadley Wickham (2021). tibble: Simple Data Frames. R package version 3.1.5. https://CRAN.R-project.org/package=tibble |
| tidyr | 1.1.4 | Hadley Wickham (2021). tidyr: Tidy Messy Data. R package version 1.1.4. https://CRAN.R-project.org/package=tidyr |
| tidyverse | 1.3.1 | Wickham et al., (2019). Welcome to the tidyverse. Journal of Open Source Software, 4(43), 1686, https://doi.org/10.21105/joss.01686 |