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CHARACTERIZATION OF APPENDICEAL MICROBIOME AND APPENDICOLITHS IN ACUTE APPENDICITIS

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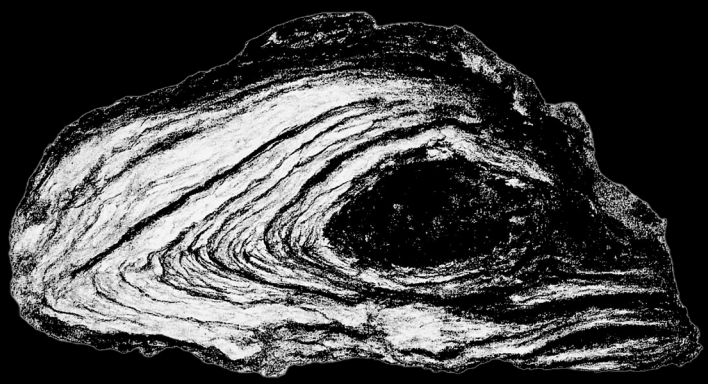

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To my family

UNIVERSITY OF TURKU
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ABSTRACT

Acute appendicitis presents as two different diseases: uncomplicated and complicated acute appendicitis. The majority are uncomplicated, which may be treated with antibiotics instead of surgery and possibly even with symptomatic therapy. Complicated acute appendicitis involves necrosis leading to perforation and either peritonitis or a periappendicular abscess. However, the microbiological differences and the role of appendicoliths, fecal concretions in the appendix, in appendicitis severity remain poorly understood.

The first part of the thesis was designing and conducting the Microbiology APPendicitis Acuta (MAPPAC) trial. The second aim was to identify differences in appendiceal microbiota between uncomplicated and complicated acute appendicitis. Third study aimed to revise an appendicolith classification from 1966 and to assess the structural and elemental composition of the appendicoliths. Fourth study explored the microbiome composition of appendicoliths to elucidate their formation mechanisms.

The prospective MAPPAC trial was conducted involving patients with CT confirmed acute appendicitis. The results showed differing appendiceal microbiomes associated with uncomplicated and complicated acute appendicitis. Appendicolith classification showed that even the softest appendicoliths (class 1) are visible by CT. Further results corroborate earlier observations of a concentrically layered inner structure built around distinguishable core, which was detected in most of the harder appendicoliths (class 2 and 3) and was associated with elemental composition. Appendicolith microbiome, enriched with specific *Actinobacteria*, suggest their potential roles in appendicolith formation.

In conclusion, this thesis bridges some knowledge gaps regarding the role of the appendiceal microbiome and appendicoliths in the severity of acute appendicitis, adding to the understanding of this very common surgical disease.

KEYWORDS: Acute appendicitis, complicated acute appendicitis, appendicolith, appendiceal microbiome

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TIIVISTELMÄ

Akuutti umpilisäketulehdus esiintyy kahtena eri taudin vaikeusasteena: komplisoitumaton eli lievä ja komplisoitunut eli vaikeampi akuutti umpilisäketulehdus. Valtaosa tapauksista on komplisoitumattomia ja voidaan hoitaa antibiooteilla leikkauksen sijaan, tai mahdollisesti jopa oireenmukaisella hoidolla. Komplisoituneeseen tautimuotoon liittyy nekroosi, perforaatio tai abskessi. Mikrobiologiset tekijät ja umpilisäkkeen ulostekiven eli appendikoliitin rooli eri tautimuotojen synnyssä ovat kuitenkin edelleen huonosti ymmärrettyjä.

Tämän tutkimuksen ensimmäinen osatyö oli MAPPAC-tutkimuksen suunnittelu ja toteutus. Toisen osatyön tavoitteena oli tunnistaa eroavaisuuksia umpilisäkkeen mikrobistossa komplisoitumattoman ja komplisoituneen tautimuodon välillä. Kolmas tutkimus pyrki luokittelemaan appendikoliitit päivittämällä aiempaa luokitusta vuodelta 1966 sekä arvioimaan niiden rakennetta ja alkuainekoostumusta. Neljäs tutkimus tarkasteli appendikoliittien mikrobiston koostumusta niiden muodostumismekanismien selvittämiseksi.

MAPPAC-tutkimus toteutettiin prospektiivisena kliinisenä tutkimuksena potilailla, joilla oli diagnosoitu akuutti umpilisäketulehdus. Tulokset osoittivat eroja umpilisäkkeen mikrobistossa eri tautimuotojen välillä. Appendikoliittien luokittelu osoitti, että jopa pehmeät (luokka 1) appendikoliitit ovat erotettavissa tietokonetomografiassa. Lisäksi tulokset vahvistivat aiempia havaintoja kerrostuneesta sisärakenteesta, joka rakentuu ytimen ympärille ja oli yleinen kovemmissa appendikoliiteissa (luokat 2 ja 3) sekä liittyi niiden alkuainekoostumukseen. Appendikoliittien mikrobistossa havaittiin rikastuneina tiettyjä Aktinobakteereita, mikä viittaa niiden mahdolliseen rooliin appendikoliittien muodostumisessa. Tämä väitöskirjatyö edistää ymmärrystä akuutin umpilisäketulehduksen tautimuotojen välisistä eroista liittyen umpilisäkkeen mikrobistokoostumukseen ja appendikoliitteihin.

AVAINSANAT: Akuutti umpilisäketulehdus, komplisoitunut akuutti umpilisäketulehdus, appendikoliitti, umpilisäkkeen mikrobistokoostumus

Table of Contents

Abbreviations

APPAC	APPendicitis ACuta
ASV	Amplicon sequence variant
BMI	Body mass index
CODA	Comparison of Outcomes of Antibiotic Drugs and Appendectomy
CRC	Colorectal carcinoma
CRP	C-reactive protein
CT	Computed tomography
dbRDA	Distance-based redundancy analysis
DNA	Deoxyribonucleic acid
GALT	Gut-associated lymphoid tissue
HU	Hounsfield units
MAPPAC	Microbiology APPendicitis ACuta
NGS	Next generation sequencing
OR	Odds ratio
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SD	Standard deviation
WBC	White blood cell
XRF	Micro-X-ray fluorescence spectroscopy

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Vanhatalo S, Munukka E, Sippola S, Jalkanen S, Grönroos J, Marttila H, Eerola E, Hurme S, Hakanen AJ, Salminen P, APPAC collaborative study group. Prospective multicentre cohort trial on acute appendicitis and microbiota, aetiology and effects of antimicrobial treatment: study protocol for the MAPPAC (Microbiology APPendicitis ACuta) trial. *BMJ Open,* 2019; 9: e031137.

II Vanhatalo S, Munukka E, Kallonen T, Sippola S, Grönroos J, Haijanen J, Hakanen AJ, Salminen P. Appendiceal microbiome in uncomplicated and complicated acute appendicitis: A prospective cohort study. *PLoS One*, 2022; 10: e0276007.

III Vanhatalo S, Mäkilä E, Hakanen AJ, Munukka E, Salonen J, Saarinen T, Grönroos J, Sippola S, Salminen P. Appendicolith classification: physical and chemical properties of appendicoliths in patients with CT diagnosed acute appendicitis - a prospective cohort study. *BMJ Open Gastroenterol,* 2024; 11: e001403.

IV Vanhatalo S*, Borman T*, Salminen P, Munukka E, Mäkilä E, Salonen J, Grönroos J, Sippola S, Pärnänen K, Lahti L, Hakanen A, Kallonen T. The microbial composition of appendicoliths in acute appendicitis. *Manuscript*. *Equal contribution

1 Introduction

Acute appendicitis is one of the most common surgical emergencies with an estimated lifetime risk of approximately 8% (Addiss et al., 1990). Epidemiological and clinical evidence indicates that acute appendicitis presents as two different forms of appendicitis severity: uncomplicated and complicated acute appendicitis (Andersson et al., 1994; Bhangu et al., 2015; Livingston et al., 2007, 2011). This preintervention differentiation between uncomplicated and complicated acute appendicitis is clinically of vital importance to allow assessment of all treatment options and this differential diagnosis is based on imaging and clinical findings. In addition to clinical practice, the accurate identification of appendicitis severity both preintervention and also postoperatively based on histopathological examination is central to appendicitis research (Di Saverio et al., 2020). Based on multiple clinical trials and meta-analyses, uncomplicated acute appendicitis can be safely treated with antibiotics (De Almeida Leite et al., 2022; Flum et al., 2020; Podda et al., 2019; Salminen et al., 2015; Scheijmans et al., 2025; Sippola et al., 2021) or even with symptomatic treatment (Park et al., 2017; Salminen et al., 2022). There are no standardized definitions for complicated acute appendicitis, but in general it is agreed to involve presence of gangrene, perforation, or periappendicular abscess (Bhangu et al., 2015). Complicated acute appendicitis is most often treated with emergency appendectomy; only the patients with periappendicular abscess should be treated with interval appendectomy (Mällinen et al., 2019).

Appendicolith, a fecal concretion in the appendix, has emerged as a clinically important factor in defining complicated acute appendicitis as the presence of an appendicolith has been clearly shown to be associated with a more complicated course of the disease (Kim et al., 2018; Scheijmans et al., 2025; Vons et al., 2011). However, the precise role of appendicoliths in appendicitis pathophysiology remains unclear.

Traditional culture-based approaches have provided insights on appendicitis microbiology. Molecular methods, such as 16S rRNA gene amplicon sequencing, have shed light on the complex and distinct microbiome of the appendix (Antonsen et al., 2023; Guinane et al., 2013). Studies indicate that appendiceal dysbiosis may contribute to inflammation, with certain bacterial taxa, such as *Fusobacterium*,

identified being more prevalent in complicated appendicitis (Blohs et al., 2023). The evolution of microbiological techniques has contributed to advancing the understanding of appendicitis etiology.

In this thesis a prospective Microbiology APPendicitis Acuta (MAPPAC) trial was designed and conducted. The thesis explored the appendiceal microbiome and specifically aimed to explore the microbiota differences between uncomplicated and complicated acute appendicitis. In addition, this thesis investigated structural and compositional characteristics of appendicoliths. By combining microbiological and compositional analysis of appendicoliths, this study aimed to provide insight on appendicolith formation. Through studies II, III and IV this thesis aimed to bridge the knowledge gaps of appendicitis severity and the factors potentially leading to complicated acute appendicitis.

2 Review of the Literature

2.1 Acute appendicitis

2.1.1 Anatomy and physiology of appendix

The vermiform appendix in humans is a narrow and approximately nine centimeters long, blind-ended tube-like structure that originates from the bottom of the cecum, below the junction of the ileum and colon (Schumpelick et al., 2000). This position corresponds to a point on the surface of the abdomen known as McBurney's point, a site where the pain is often focused during appendicitis (McBurney, 1891). Anatomically variation in the location within the cecum, shape of the appendix, and in the position is seen between the individuals (Schumpelick et al., 2000; Wakeley, 1933). Appendiceal like structures are known to exist also outside humans and higher class of primates. Human resembling appendix, defined as an extension of cecum with a significant decrease in the diameter, appears in specific species in lagomorphs such as rabbits and marsupials (Smith, 2022). For a long time, appendix was referred as a useless vestigial organ, but recent evolutionary studies show that appendix has evolved independently in various mammals indicating clear beneficial function to the host (Smith, 2022). In humans the appendix appears to have functions related to immune system and gut health that are further discussed in chapter 2.6. (Bollinger et al., 2007).

2.1.2 Incidence of appendicitis

Acute appendicitis is one of the most common causes of acute abdominal emergency in adults, often requiring emergency surgery (Stewart et al., 2014). The lifetime risk is about 8.6% for males and 6.7% for females (Addiss et al., 1990). In the United States, the incidence of appendicitis is approximately 9.4 cases per 10 000 people in a year (Buckius et al., 2012). In 2007, the incidence rate in Finland was 9.8 cases per 10 000 people (Ilves et al., 2014). The highest incidence of appendicitis appears in individuals between 10 and 19 years old (Buckius et al., 2012). There is also a male predominance in appendicitis cases with males being more frequently affected than females (Addiss et al., 1990; Buckius et al., 2012; Stein et al., 2012). The gender

disparity is most pronounced in young adults. However, despite the lower incidence in females, women have experienced a markedly higher rate of negative appendectomies meaning the surgical removal of a normal, non-inflamed appendix. The negative appendectomy is often due to diagnostic challenges in distinguishing gynecological conditions from appendicitis and the use of CT has significantly reduced its occurence (Stein et al., 2012).

2.1.3 Epidemiology, etiology and pathofysiology

In Western countries, the incidence of appendicitis has remained stable or decreased. In contrast, newly industrialized regions show upward trend in the incidence, suggesting that environment and lifestyle factors may have a role in the development of appendicitis (Ferris et al., 2017). Diet and especially fiber intake has been studied for their potential role in modulating the risk of appendicitis. The hypothesis that diet influences the development of appendicitis stems from epidemiological studies examining the incidence of acute appendicitis in populations with varying diets (Carr, 2000). Additionally, two observational case-control studies have linked diet, particularly low fiber intake, to an increased risk of appendicitis (Adamidis et al., 2000; Peeters et al., 2023). Seasonal fluctuations further underline the possible environmental influence, with multiple studies observing a higher incidence during the summer season and during warmer weather (Ilves et al., 2014; Rautava et al., 2018; Simmering et al., 2022). Genetics and family history may also play a role in the risk of developing appendicitis. The influence of genetic factors is supported by evidence showing that a positive family history increases the relative risk of appendicitis nearly three-fold (Ergul, 2007). Recent genome-wide association studies have further identified several loci associated with appendicitis, though the underlying mechanisms are not fully understood (Gaitanidis et al., 2021; Kristjansson et al., 2017; Orlova et al., 2019). Epidemiological studies suggest that uncomplicated (nonperforated) and complicated (perforated) acute appendicitis have distinct trends, reflecting different pathophysiological mechanisms (Andersson et al., 1994; Livingston et al., 2007).

Several etiological theories for appendicitis have been proposed. The variation in these theories might reflect differences in the pathophysiology and in the underlying causes of uncomplicated and complicated acute appendicitis. One suggested etiological theory is the possible obstruction of the appendiceal lumen by appendicolith or lymphoid hyperplasia. Obstruction would cause an increase in the intraluminal pressure followed by tissue damage and bacterial translocation through appendiceal wall (Wangensteen & Dennis, 1939). Obstruction theory has been questioned by for example Arnbjörsson, showing that only in minority of gangrenotic appendicitis cases obstruction of the lumen is present (Arnbjörnsson &

Bengmark, 1984). Yet, in cases of acute appendicitis associated with an appendicolith, which account for approximately 18% of all appendicitis cases, obstruction might be part of the pathophysiological mechanism (Singh & Mariadason, 2013). However, appendicoliths were also identified in 29% of negative appendectomy specimens, indicating that their presence alone is not a definitive marker of appendicitis (Singh & Mariadason, 2013).

Infection with bacterial or viral origin has been suggested to take part at least in part of appendicitis cases (Lamps, 2010). Bacterial species of wich some strains are known to be pathogenic such as *E. coli*, *B. fragilis* and *Fusobacterium* species have been identified from inflamed appendix but the significance in appendicitis etiology remains inconclusive as these species are found also in the normal appendix (Antonsen et al., 2023). It is hypothesized that mucosal ulceration often present in the histopathology would be the cause of viral or bacterial infection (Carr, 2000). The perception of normal appendiceal microbiota has evolved thorough sequencing-based research methods (Guinane et al., 2013). In addition to inflammation, a deleterious change in the appendiceal microbiota composition and a resulting inflammation has been suggested, and this is further discussed in chapter 2.5.3.

2.2 Classification of acute appendicitis

The core of appendicitis treatment and research lies in the differential diagnosis of uncomplicated and complicated acute appendicitis. The differentiation between appendicitis severity is reached through evaluation of clinical data and imaging findings for the clinical preintervention assessment and the histopathological findings are the reference standard for appendicitis research (Di Saverio et al., 2020). Uncomplicated appendicitis cases accounts for approximately 70-80%, depending on classification criteria used (Livingston et al., 2007; Yeh et al., 2021). This thesis is largely focused on appendicitis in adult patients, but similar pathology occurs in pediatric appendicitis, where disease classification is also used (Gil et al., 2023).

2.2.1 Uncomplicated acute appendicitis

Uncomplicated appendicitis also referred as simple, phlegmonous, or non-perforated appendicitis is histologically defined by the finding of transmural inflammation of the appendix without necrosis or perforation - the landmarks of the complicated acute appendicitis (Bhangu et al., 2015). The inflammation refers to the infiltration of neutrophils. Mucosal ulceration and vascular thrombosis are often seen in the histology (Bhangu et al., 2015; Carr, 2000). Microabscess formation can be also related to the histological findings of uncomplicated acute appendicitis. Macroscopically the appendix in uncomplicated acute appendicitis shows vascular

congestion, color changes and increased diameter from normal 6 mm or less (Bhangu et al., 2015).

In uncomplicated acute appendicitis, the entire appendiceal wall all the way to muscularis propria is inflamed according to the definition of appendicitis (Bhangu et al., 2015; Carr, 2000). In the histopathological evaluation the inflammation can be limited to the mucosa, referred to as catarrhal appendicitis, or extending to submucosa. When mucosal or submucosal inflammation occurs with slight macroscopic changes or small increase in the appendix diameter observed on CT imaging, it is sometimes described as borderline appendicitis or as an early appendicitis (Hoffmann et al., 2021). However, according to commonly accepted classification, inflammation extending to the submucosa but not to muscularis propria, is considered a normal finding (Bhangu et al., 2015). This highlights the ambiguity of the classification criteria. The pathologists can be inconsistent in their use of diagnostic criteria and the findings might be differently interpretated by surgeons (Riber et al., 1999). In uncomplicated cases, the classification is often based only on the clinical assessment and imaging without histopathological confirmation (Di Saverio et al., 2020). Further, diagnostic criteria may vary across clinical settings, influenced by factors such as available resources (Bass et al., 2023).

The distinction between mucosal and submucosal inflammation might have limited clinically relevance and it is not necessarily made by pathologist (Bhangu et al., 2015). Nevertheless, defining the degree of inflammation and understanding the progression might be important in elucidating the etiology or etiologies of appendicitis forms.

2.2.2 Complicated acute appendicitis

Complicated acute appendicitis includes appendicitis presenting with perforation, gangrene, abscess, and tumor (Di Saverio et al., 2020; Kim et al., 2018). Appendicolith appendicitis, where round or oval fecal concretions are present in the appendiceal lumen, with or without other above-mentioned complications, is currently most often regarded as complicated acute appendicitis (Di Saverio et al., 2020; Hoffmann et al., 2021; Scheijmans et al., 2025). Gangrenous acute appendicitis is histologically characterized by transmural inflammation with necrotic areas and extensive mucosal ulceration. Macroscopically appendicieal gangrene appears purple, green or black. Gangrenous appendicitis can progress to perforation (Bhangu et al., 2015; Carr, 2000). Perforation can present as sealed perforation with an abscess or as free perforation with content of the appendix including bacteria, air, and pus detected in abdominal cavity resulting in peritonitis (Hoffmann et al., 2021). According to a meta-analysis, appendiceal abscess was found in 3.8% (CI: 2.6–4.9) of all appendicitis cases (Andersson & Petzold, 2007). Appendiceal tumours are rare

and often incidental findings in the histopathological examination of a removed appendix, appearing in approximately 1% of patients treated with appendectomy (Teixeira et al., 2017). Appendiceal tumours are more prevalent in complicated appendicitis (3.2%) compared to uncomplicated appendicitis (0.9%), and the risk of tumor is especially high in patients with periappendiceal abscess (Lietzén et al., 2019; Mällinen et al., 2019).

2.3 Diagnosis and treatment of appendicitis

2.3.1 Diagnosis and differential diagnosis of appendicitis

Typical key clinical signs of appendicitis include migratory pain to the right lower quadrant, abdominal rigidity, and rebound tenderness at McBurney's point (Grover & Sternbach, 2012; McBurney, 1891). Inflammatory markers white blood cell (WBC) count and C-reactive protein (CRP), provide valuable diagnostic information in suspected acute appendicitis, but on their own they are not considered definitive. Signs of peritoneal inflammation (rebound and percussion tenderness, guarding) as well as pain migration combined with inflammatory markers provide valuable diagnostic information (Andersson, 2004).

CT is an essential diagnostic tool in distinguishing appendicitis from other conditions presenting with acute abdominal pain. CT criteria for appendicitis, in most studies, include appendiceal diameter larger than 6 mm, and the finding of periappendiceal inflammation or thickening of the caecal wall (Rud et al., 2019). The negative appendectomy rate represents the removed appendixes without histopathological signs of inflammation, and it is used to evaluate diagnostic performance and quality of treatment. In the era of modern CT imaging, the negative appendectomy rate should be very low, and the negative appendectomy rate is strongly affected by the availability of CT in the appendicitis diagnosis (Haijanen et al., 2021; Rao et al., 1999). Further, a low-dose CT, that is used to reduce the radiation exposure, has been shown to be noninferior to standard-dose CT (Kim et al., 2012; Sippola et al., 2018). In addition to CT, ultrasound and MRI can be used, especially for children, and pregnant women due to concerns about radiation exposure. MRI has demonstrated high accuracy in appendicitis diagnosis (D'Souza et al., 2021), whereas ultrasound shows lower diagnostic sensitivity, comparable to physical examination (Giljaca et al., 2017).

The differentiation between uncomplicated and complicated appendicitis seems to be most reliable when reached through evaluation of clinical data, laboratory markers, imaging findings (Scheijmans et al., 2024). CT imaging has been shown to produce the highest sensitivity in distinguishing the uncomplicated and complicated acute appendicitis (Atema et al., 2015; Lietzén et al., 2016). In a meta-analysis of

CT features in differentiating complicated appendicitis, ten CT findings were found to be informative of complicated appendicitis: Periappendiceal fat stranding, extraluminal or intraluminal appendicolith, extraluminal or intraluminal air, abscess, ileus, periappendiceal fluid collection, ascites, and a defect in the contrast enhancement of the appendiceal wall. Even though the specificity of these features was high the sensitivity was reported to be low (Kim et al., 2018). Appendicitis scoring systems that combine clinical, and imaging features have been developed to aid distinguishing complicated from uncomplicated appendicitis (Atema et al., 2015; Avanesov et al., 2018; Scheijmans et al., 2024).

2.3.2 Surgical treatment

In Finland, approximately 8000 appendectomies are performed annually (THL). In the United States, approximately 280 000 appendectomies are performed annually, making it one of the most common emergency surgeries (Livingston et al., 2007). Open appendectomy using a muscle-splitting McBurney incision described by Charles McBurney in 1894 was the standard approach for treating appendicitis before laparoscopy (McBurney, 1894; Semm, 1983). Laparoscopic appendectomy is the gold standard for surgical treatment of acute appendicitis. Laparoscopic appendectomy is associated with reduced postoperative pain, shorter hospital stays, and faster recovery (Jaschinski et al., 2018). Antibiotic prophylaxis administered according to guidelines is efficient in preventing the surgical site infection and intra-abdominal abscess formation (Bhangu et al., 2018; Di Saverio et al., 2020; Garcell et al., 2017). Guidelines for appendicitis treatment recommend administering single dose of broad-spectrum antibiotics within an hour before surgical incision (Di Saverio et al., 2020).

The classification of appendicitis plays a crucial role in evaluating the need for emergency appendectomy. Research indicates that delaying surgery for uncomplicated acute appendicitis by up to 24 hours does not increase the risk of complications (Jalava et al., 2023; van Dijk et al., 2018). However, delay in surgery for patients with complicated appendicitis, is linked to a higher risk of postoperative complications (Bolmers et al., 2022). According to current appendicitis management guidelines complicated acute appendicitis requires emergency appendectomy whereas uncomplicated appendicitis can be treated non-surgically with antibiotics (Di Saverio et al., 2020).

2.3.3 Non-operative treatment of uncomplicated appendicitis

Appendectomy as the gold standard for the treatment of acute appendicitis has been brought into question already 20-30 years ago by randomized controlled trials

(RCTs) comparing antibiotic treatment with emergency appendectomy (Eriksson & Granström, 1995; Styrud et al., 2006). In both of these trials, intravenous cefotaxime and tinidazole followed by oral tinidazole and ofloxacin was used and the antibiotics were shown to be feasibly treatment option with 88-95% treatment success (Eriksson & Granström, 1995; Styrud et al., 2006). Similar initial treatment success and recurrence rate of 14% was reported by Hansson et al comparing intravenous cefotaxime and metronidazole followed by oral ciprofloxacin and tinidazole to surgery (Hansson et al., 2009). A major limitation of these three studies was that they did not use CT to confirm appendicitis diagnosis and to exclude the complicated patients. In a French non-inferiority trial using CT to select only patients with uncomplicated appendicitis, antibiotic amoxicillin clavulanic acid was found not to be non-inferior to appendectomy (Vons et al., 2011). The treatment success was 88% and the recurrence rate was 25%, but the primary endpoint in this trial was postintervention peritonitis defined by repeat CT at 30 days, which was significantly higher in the antibiotic group (8%) compared to appendectomy group (2%). A large Finnish non-inferiority trial Appendicitis Acuta (APPAC), conducted by Salminen et al, compared intravenous ertapenem followed by oral levofloxacin and metronidazole with appendectomy (Salminen et al., 2015). While both Vons et al and Salminen et al used preintervention CT to differentiate uncomplicated and complicated appendicitis, only in the APPAC trial patients with appendicoliths were excluded (Salminen et al., 2015; Vons et al., 2011). Of the 256 patients treated with antibiotics 27% had had appendectomy at 1-year timepoint resulting to antibiotics not being non-inferior as the non-inferiority margin was at 24%, but the majority (73%) of the patients were successfully and safely treated with antibiotics (Salminen et al., 2015).

The most recent and largest study with 1552 CT confirmed patients with appendicitis is the Comparison of Outcomes of Antibiotic Drugs and Appendectomy (CODA) trial that has compared antibiotic treatment (various antibiotics) to surgery. The CODA trial included patients with appendicoliths and found that 29% of patients in antibiotic group underwent surgery at 90 days timepoint and if appendicolith patients were excluded, 25% underwent surgery by 90 days (Flum et al., 2020). At 1-year, 52% and 37% (without patients with appendicolith appendicitis) had undergone appendectomy (Davidson et al., 2021). In this trial the non-inferiority primary endpoint quality of life (QOL) measured based on the European Quality of Life–5 Dimensions (EQ-5D) questionnaire showed antibiotics to be non-inferior to surgery (Flum D. et al., 2020). In the long-term follow-up reports majority of the recurrent appendicitis cases appear within 1-year from the antibiotic treatment and after that the recurrence rate is lower (Davidson et al., 2021; Pátková et al., 2023; Salminen et al., 2018). In a meta-analysis of non-operative versus operative management of appendicitis the treatment success at 30-day follow-up and the

percentage of major adverse events was shown to be statistically nondifferent between the groups illustrating safety and efficacy of the antibiotic treatment of uncomplicated appendicitis (De Almeida Leite et al., 2022). Even though antibiotics are considered as a safe treatment option, another meta-analysis found antibiotics to be less effective than operative management at 1-year follow-up (Brucchi et al., 2024). In a recent individual patient data meta-analysis from six RCTs involving 2101 patients (1050 antibiotics, 1051 appendectomy) antibiotics were compared to surgery. In this meta-analysis the primary outcome was 1-year complication rate and secondary outcomes were rates of appendectomy, complications, and the impact of appendicoliths. At one year, 5.4% in the antibiotics group and 8.3% in the appendectomy group experienced complications demonstrating the safety of antibiotics. During the first year 33.9% of patients treated with antibiotics had appendectomy (Scheijmans et al., 2025). Results of the meta-analysis focusing on outcomes in patients presenting with appendicoliths are further discussed in chapter 2.4.3.

Today, antibiotics are considered to be safe treatment option for uncomplicated appendicitis (Di Saverio et al., 2020) and focus of research has moved into treatment optimization. In APPAC II non-inferiority randomized clinical trial oral moxifloxacin was compared with intravenous ertapenem followed by oral levofloxacin and metronidazole. Even though the noninferiority margin for oral antibiotics was not reached the success rate even in the oral antibiotics group at 1-year was 70% compared to the 73% in the intravenous followed by oral antibiotics group (Sippola et al., 2021). In APPAC II, 5% (29/583) of the patients were primary non-responders defined as patients undergoing surgery during the initial hospital stay and with the finding of complicated appendicitis. In the secondary analysis of this trial fever higher than 38°C on admission and appendiceal diameter 15 mm or wider on CT was found to be predictors of primary non-responsiveness (Haijanen et al., 2021). Wider appendiceal diameter was also associated with increased risk of treatment failure within 30 days after antibiotic treatment in CODA trial (Monsell et al., 2022). Based on information and treatment optimization the possibility of outpatient management is being considered due to shorter hospital stays and use of orally administered antibiotics (Talan et al., 2017, 2022).

Further, the possibility of only symptomatic treatment based on placebo-controlled trials has emerged (Iresjö et al., 2024; Park et al., 2017; Salminen et al., 2022). In the APPAC III superiority trial, the 10-day treatment success rate was 87% for placebo and 97% for antibiotics, demonstrating that antibiotics were not superior to placebo. A new multicenter Finnish RCT (APPAC IV) comparing oral antibiotics to placebo in uncomplicated appendicitis with an outpatient setting is currently recruiting patients (Lund et al., 2024).

2.4 Appendicoliths in patients with acute appendicitis

2.4.1 Appendicolith definition, identification and prevalence

Appendicoliths, also referred as faecoliths, coproliths or appendiceal calculi, do not have uniform and standardized criteria. It is therefore meaningful to examine appendicolith prevalence in conjunction with its definition.

Appendicoliths, even though not under that terminology, have been described already in 1886 as frequent findings from the appendix (Fitz, 1886). The preoperative finding of an appendicolith with x-ray became possible at the beginning of 20th century but findings were focused on the hardest and largest appendicoliths that could be detected in abdominal radiography (Forbes & Lloyd-Davies, 1966). In 1966, after many years of numerous case reports, appendicolith appendicitis and the appendiceal concretions were clinically and pathologically carefully characterized by Forbes and Lloyd-Davies (Forbes & Lloyd-Davies, 1966). In this work, 1800 appendiceal concretions with various appearances, were classified based on their physical characteristics and the degree of calcification. Three classes were defined as follows, (1) faecal pellet described as radiotranslucent faecal mass that is formed but soft, (2) calcified faecolith that is reasonably hard, partly radio-opaque mass, (3) calculus that is stony hard, calcified, and radio-opaque object with round or irregular shape. The hardest calculi that Forbes and Loyd-Davies refers as the true calculus, were relatively infrequent with an incidence of only 0.8%. In their classification, Forbes and Lloyd-Davies (1966) emphasized the level of calcification as a critical feature that correlates with hardness and radiodensity. (Forbes & Lloyd-Davies, 1966). Conversely, Shaw (1965), presenting his findings a year earlier, based his conclusions on a smaller yet comprehensive characterization of 117 appendiceal concretions. He asserted that, irrespective of the level of calcification—which he documented to range from 1% to 12% of calcium—all appendiceal concretions should be referred as appendix calculi (Shaw, 1965).

Nowadays appendicoliths can be identified preoperatively with CT and verified postoperatively through pathological examination. In both CT and pathological examination, identification of appendicoliths is based on subjective interpretation of the ambiguous definition of the appendicolith (Weitzner et al., 2023). To clarify the definition, Weitzner et al. proposed including a threshold attenuation value measured in Hounsfield units (HU), suggesting it should be 180 HU greater than the attenuation of the appendiceal wall based on retrospective radiological evaluation of 88 patients with appendicitis. CT and postoperative identification were shown to be partly inconsistent. When both required positivity for confirmation, the number of detected appendicoliths decreased from 45 to 21 (Weitzner et al., 2023). Using CT

imaging, appendicoliths have been reported to be present in 38.7% to 44.0% of adult patients with acute appendicitis (Kaewlai et al., 2024; Ranieri et al., 2020). Ranieri et al., defined appendicoliths as “fecal calcific deposits within appendiceal lumen” and the attenuation range of appendicoliths was 104 to 1439 HU. This demonstrates that some appendiceal concretions considered appendicolith have relatively low attenuation values that do not meet the minimum threshold defined by Weitzner et al. In another study using CT together with pathological confirmation, a much lower prevalence of 13.7% (99/722) in adults and 29.9% (79/264) in pediatric patients was reported (Singh & Mariadason, 2013). The positive predictive value for CT for detecting appendicoliths was 48.8%, with a sensitivity of 53.1% for histopathologically confirmed appendicoliths (Singh & Mariadason, 2013). Later, in a larger cohort Mariadason et al reported slightly higher positive predictive value of 62% and sensitivity of 55% for CT detection. (Mariadason et al., 2022). Despite significant differences between studies, pediatric patients consistently show higher prevalence of appendicoliths compared to adult patients. (Lowe et al., 2000; Singh & Mariadason, 2013).

It appears that the accuracy of CT in appendicolith detection depends on definition and criteria used, weather pathological confirmation is required, and whether softer uncalcified concretions equivalent to faecal pellets are included. Additionally, appendicoliths with lower CT attenuation can be more easily left undetected leading to possible misdiagnosis (Kaewlai et al., 2024).

2.4.2 Appendicoliths and appendicitis severity

The presence of an appendicolith has been associated with complicated appendicitis in several studies showing higher appendicolith prevalence in complicated (gangrenous or perforated) appendicitis compared to uncomplicated (Flum et al., 2020; Ishiyama et al., 2013; Kaewlai et al., 2024; Kondo & Kohno, 2009; Ranieri et al., 2020; Singh & Mariadason, 2013; Sula et al., 2024; Yoon et al., 2018). In addition to complications, pediatric patients with appendicolith appendicitis show clinically more severe symptoms including higher CRP values, fever, and vomiting compared to no appendicolith group (Yoon et al., 2018). However, appendicoliths are also found without gangrenation or perforation (Flum et al., 2020; Hawkins et al., 2022; Ramdass et al., 2015). A study that compared the histopathological changes in uncomplicated appendicitis and appendicolith appendicitis without other complications, found that appendicolith appendicitis was associated with more frequent mucosal ulceration, micro-abscess formation, and a denser neutrophilic infiltration (Mällinen et al., 2019). Depth of inflammation did not differ and was less deep in appendicoliths appendicitis compared to uncomplicated appendicitis (Mällinen et al., 2019).

The association between the presence of an appendicolith and appendicitis severity may depend on appendicolith characteristics. In the 1966 classification of appendicoliths, faecal pellets were shown to have less clinical significance as they were more often detected from appendices with low grade inflammation or even without the inflammation compared to calcified faecoliths or calculi out of which majority were from appendices with acute inflammation. In addition, the stony calculus containing cases showed higher proportion of gangrenation and perforation (Forbes & Lloyd-Davies, 1966). In a risk factor analysis appendicolith diameter (≥5 mm) together with fever and prolonged abdominal pain predicted appendiceal perforation in children (Yoon et al., 2018). In adult patients, larger appendicolith size and location of the appendicolith at the proximal part (base) of the appendix have been associated to complicated appendicitis compared to uncomplicated appendicitis (Ishiyama et al., 2013; Kaewlai et al., 2024; Sula et al., 2024).

The significance of appendicoliths in the pathophysiology of appendicitis is complicated by finding of incidental appendicolith without signs of appendicitis on imaging (Jones et al., 1985; Ramdass et al., 2015). In a study following patients with incidentally discovered appendicoliths, no increased risk of developing appendicitis was observed compared to a matched control group over a four-year follow-up period (Khan et al., 2018). Interestingly, the size of the appendicolith, has been found to be larger in patients with appendicitis compared to normal appendices. In addition, the presence of multiple appendicoliths has been associated with appendicitis (Hawkins et al., 2022; Khan et al., 2019).

2.4.3 Appendicoliths in treatment options and outcomes

The differential diagnosis between uncomplicated and complicated appendicitis forms the foundation of assessing all treatment options and non-operative treatment strategy (Bom et al., 2021). In appendicitis scoring system used in differentiating uncomplicated acute appendicitis and complicated acute appendicitis, appendicolith as a CT feature scores for complicated appendicitis (Atema et al., 2015). Appendicitis presenting with an appendicolith is partly situated in the grey area between the different forms of appendicitis severity.

In one of the earliest studies comparing antibiotic treatment and surgery in CT confirmed acute appendicitis by Vons et al., patients with appendicoliths were included in the antibiotic group. This study failed to demonstrate the noninferiority of antibiotics compared to appendectomy, as the antibiotics group showed an increased incidence of postintervention peritonitis. Appendicoliths were identified as a factor associated with treatment failure. After excluding patients with appendicoliths from the analysis, there was no significant difference in peritonitis rates between these groups at 30-day follow-up (Vons et al., 2011).

The CODA trial also evaluated the impact of appendicoliths on short- and long-term treatment outcomes. Among the 1552 enrolled adults, 414 had an appendicolith. In the antibiotic group, patients with an appendicolith had a higher rate of appendectomy compared to those without an appendicolith (41% vs 29% at 90 days). Additionally, complications were more common in the antibiotic group for those with an appendicolith compared to those without (Flum David et al., 2020). In the long-term follow-up of CODA trial on appendicitis recurrence at 30 days to 1-year timepoint, the presence of appendicolith was more common in patients with recurrent appendicitis (27.8% vs 21.9%) but was not statistically significantly associated with appendectomy. Surprisingly, other signs of complicated appendicitis such as perforation, abscess or fat stranding at initial CT, were also not strongly linked to appendectomy within one year (Flum, 2023).

In recent meta-analysis the effect of appendicolith at pre-interventional imaging on treatment succes and complications were assessed (Scheijmans et al., 2025). At 1 year, 5.4% in the antibiotics group and 8.3% in the appendectomy group had complications while in patients with appendicoliths a higher risk of complications with antibiotics (15.0%) compared to surgery (6.3%) was observed. Nearly half (48.7%) of patients with appendicoliths initially treated with antibiotics required surgery within one year, compared to 30.6% without appendicoliths (Scheijmans et al., 2025). A subgroup analysis from a meta-analysis comparing the safety and efficacy of antibiotic treatment versus appendectomy in children showed that patients with appendicoliths had a higher risk of treatment failure compared to those without appendicoliths (Huang et al., 2017).

These studies highlight that antibiotic treatment for acute appendicitis may not be optimal for patients presenting with an appendicolith (Scheijmans et al., 2025). The increasing adoption of conservative treatment strategies has intensified the need for accurate preoperative diagnostics. If appendicoliths are considered a contraindication for antibiotic therapy, it becomes essential to have diagnostic methods that can reliably detect their presence. Alternative diagnostic methods, such as ultrasound or clinical examination, lack the sensitivity and specificity required to reliably detect appendicoliths. Thus, the use of CT in stratifying patients for surgical versus conservative treatment becomes more important as appendicolith cannot be reliable identified by other diagnostic methods (Lietzén et al., 2016).

2.4.4 Structure and chemical composition of appendicoliths

In one of the earliest studies on appendiceal concretions, Williams et al (1907) described white, laminated concretions to contain insoluble calcium soaps of saturated fatty acids, fats and inorganic salts like calcium and phosphates. Based on observed layered structure and the size larger than appendiceal opening Williams

hypothesizes that concretions would develop inside the appendix, likely driven by the appendix's fatty secretions from the intestinal mucosa (Williams et al., 1907). The silver nitrate staining method that Williams used and interpreted as calcium soap, was later disproved by Maver and Wells (1921) identifying the inorganic component as calcium phosphate rather than calcium soap (Maver & Wells, 1921). Their analysis of diverse concretions, including pigmented ones, showed that the organic portion primarily consisted of soaps formed from palmitic and stearic acids, koprosterol and to lesser extent cholesterol, while the inorganic fraction was mainly calcium phosphate. The heterogenicity of these concretions and the laminated structure, that was visible in radiographs as well, was corroborated by Shaw in 1965. He reported that the calcium percentage varied from 1% in softer and faintly radio opaque to 12% in densely radio opaque concretions, and that calcium was largely present as calcium phosphate. A significant variation was also observed in the proportion of organic material, ranging from 16% to 70% (Shaw, 1965).

Recently, over a century later, Prieto et al. analyzed five appendicoliths from pediatric patients, showing that appendicoliths are composed of inorganic and a complex array of organic compounds. Main elements were calcium ($11.0 \pm 6.0\%$ by weight) and phosphorus ($8.2 \pm 4.2\%$), followed by sodium, aluminium, magnesium, potassium, and iron. The organic proportion consisted of 32 fatty acids, and 249 human and bacterial proteins. In line with previous studies palmitic acid (29.7%) and stearic acid (21.3%) were the most common fatty acids, with some stearate present in crystalline form. Protein analysis revealed that proteins common to all samples, the most abundant being S100 calcium-binding protein A9, were linked to antioxidant activity and inflammation (Prieto et al., 2022).

2.5 The microbiology of appendix

In 2021, the National Center for Biotechnology Information (NCBI) updated the nomenclature of bacteria and archaea (Oren & Garrity, 2021). However, throughout this review of the literature, the microbiota is discussed using the previous taxonomic nomenclature, as the studies reviewed are mainly published before the adoption of the new nomenclature.

2.5.1 Functions and characteristics of a healthy appendix

The gastrointestinal tract is a habitat for an enormous amount of diverse microorganisms including bacteria, viruses, fungi, and archaea (Lynch & Pedersen, 2016). The balanced interaction of these gut microbes and the immune system is an important characteristic of a healthy gut (Atarashi et al., 2011; Geuking et al., 2011; Walker & Lawley, 2013). The gastrointestinal tract has gut associated lymphoid

tissue (GALT) which is responsible of the priming of lymphocytes and maintaining the homeostasis between the immune system and the gut microbiota. GALT consist of multi-follicular lymphoid tissues, such as Peyer's patches in the small intestine and of isolated lymphoid follicles (Mörbe et al., 2021). In the appendix, there is a dense GALT with a lymp node-like structure and the appendix is traditionally thought to act as an induction site during the development and function of the immune system (Gorgollón, 1978; Mörbe et al., 2021; Spencer et al., 1985). Further, the appendix has been suggested to serve as a safe house for bacteria from which beneficial bacteria would be inoculated back to the colon after disturbances such as diarrhea or an antibiotic course (Bollinger et al., 2007). This suggested function is explained by the exterior location of the elongated tube-like structure of the appendix that is unexposed to the normal fecal flow in the colon (Bollinger et al., 2007; Palestrant et al., 2004).

The possible role of the appendix in maintaining the gut homeostasis can be studied through the possible association of inflammatory bowel disease and appendectomy. A meta-analysis of observational studies has showed an increased risk of Crohn's disease in patients that have had appendectomy (Kaplan et al., 2008). However, the observed risk is higher during the first years after appendectomy, and is diminished thereafter which might rather reflect diagnostics challenges related to appendicitis in patients with Crohn's disease (Kaplan et al., 2007, 2008). The removal of appendix has also been suggested to protect from ulcerative colitis (Andersson et al., 2001; Russel et al., 1997) and that appendectomy would be positively associated with less severe form of ulcerative colitis (Cosnes et al., 2002; Radford-Smith et al., 2002). Further, in patients with a severe *Clostridioides difficile* infection (CDI), the rate of appendectomy is shown to be significantly higher than the general population's lifetime incidence and higher compared to patients with passing CDI, suggesting a potential association between appendectomy and severe CDI (Clanton et al., 2013; Yong et al., 2015). The appendix has also been suggested to protect from diarrhea according to retrospective collection of veterinary records of primates (Collard et al., 2023). Click or tap here to enter text.

Knowledge about the appendiceal microbiota composition and function in healthy uninflamed appendix has remained scarce due to the difficulty in obtaining samples. Same limitation has also restricted the understanding of the (normal) microbiota in other parts of the large intestine (cecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum) (Lawal et al., 2023). Different anatomical parts of the colon have different microbiota compositions that are affected by for example carbohydrate availability, pH, and transit time (Donaldson et al., 2015). Anatomically the appendix parts below the ileocecal valve in cecum (Schumpelick et al., 2000). The cecum microbiome is compartmentalized into luminal and mucosal communities and distinct microbial composition is observed in

the cecum compared to fecal microbiota (Eckburg et al., 2005; Marteau et al., 2001; Zaborin et al., 2020). A study examining microbiota across multiple gastrointestinal sites (oral cavity, esophagus, stomach, small intestine, appendix, and large intestine) in 33 deceased individuals revealed significant variation in microbial diversity and composition along the gastrointestinal tract (She et al., 2024). The appendiceal microbiome was similar to the small intestine in beta diversity. Within the large intestine, alpha diversity was lowest in the appendix and increased toward the rectum. The appendix was enriched with *Bacteroides* and *Parabacteroides*, which were also prominent in the small and large intestines, and with *Fusobacterium* which was also abundant in the oral cavity. Despite the anatomical proximity to the large intestine, the appendix showed unique microbial signatures (She et al., 2024). The appendix harbouring less diverse microbiome compared to rectum or fecal microbiome has been reported (Guinane et al., 2013; Peeters et al., 2019; Rogers et al., 2016). Several recent studies have been able to include a non-appendicitis control group in their analysis of appendiceal microbiome composition (Fonnes et al., 2024; Munakata et al., 2021; Oh et al., 2020). The normal microbiota as well as the microbiota composition during appendicitis is further discussed in the following chapters.

2.5.2 The methodological perspective on appendiceal microbiome

Appendicitis has long been attributed to various microbes, including bacterial, viral, fungal, and parasitic pathogens (Lamps, 2010). Case reports, often from immunocompromised patients, of for example *Aspergillus*-appendicitis have been reported (Yada et al., 2020), Blastocystic-associated appendicitis (Arredondo Montero et al., 2024) or Epstein-Barr virus appendicitis (AlMudaiheem et al., 2021) describe the broad spectrum of potential pathogens. Historically, culture-based methods have been used in identifying bacterial species associated to appendicitis and to evaluate differences between uncomplicated and complicated appendicitis. Bacteriological studies on appendix demonstrate mixed aerobic and anaerobic growth. The most commonly isolated bacterial species include *B. fragilis*, *E. coli*, *Streptococcus* sp. and *Pseudomonas aeruginosa*, (Baron et al., 1992; Bennion et al., 1990; García-Marín et al., 2018; Lau et al., 1984; Rautio et al., 2000; Song et al., 2018). The two most common species *B. fragilis* and *E. coli* are also the most common species isolated from blood culture positive samples in patients with appendicitis (Lau et al., 1984; Sula et al., 2022). Advantages of culture-based methods include accurate species and even strain level identification, and the ability to determine antimicrobial susceptibility and virulence factors of the isolates (García-Marín et al., 2018). For example, analysis of *E. coli* isolates from appendices

and feces of 146 patients with or without inflammation showed that specific fimbriated strains (hemolysin-producing, type 1C—fimbriated) and specific serotypes (025:K5:H1) are exclusively isolated from patients with appendicitis. This suggests that strain level identification of for example *E. coli* would be needed to interpret the microbiological pathophysiology (Saxén et al., 1996). As majority of bacteria in the appendix are anaerobes, a common limitation of culture-based studies is the reduced ability to grow fastidious or unculturable organisms. To overcome this limitation, next generation sequencing (NGS) has emerged in the detection of the microbiological factors in appendicitis etiology.

The 16S rRNA gene or the whole DNA in shotgun metagenomic sequencing is nowadays a common method in studying the microbiota composition of the gastrointestinal tract (Wensel et al., 2022). Appendiceal microbiota characterisations have started with the use of 16S rRNA gene amplicon sequencing of more than 10 years ago (Guinane et al., 2013) followed by numerous other studies that are outlined in Table 1. In the most recent studies shotgun metagenomic sequencing was used (Blohs et al., 2023; Fonnes et al., 2024; Yuan et al., 2021). Both in adults and children in non-inflamed appendiceal microbiota three most abundant phyla detected are *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* followed by smaller proportion of *Actinobacteria* and *Fusobacteria.* Minor proportions of *Synergistetes*, and in some studies *Tenericutes*, *Lentisphaera,* and *Verrucomicrobia* have been detected (Arlt et al., 2015; Fonnes et al., 2024; Munakata et al., 2021; Oh et al., 2020; Zhong et al., 2014). Common genera found in the normal appendix were *Bacteroides*, *Escherichia*, *Fusobacterium*, *Parabacteroides*, *Collinsella Odoribacter*, *Prevotella*, *Lachnospiraceae*, and *Ruminococcus.* Majority of appendiceal samples across the different studies exhibited rich and diverse appendiceal microbiome. Additionally, the variation in the microbiota composition between individuals was high (Fonnes et al., 2024; Oh et al., 2020; Rogers et al., 2016; She et al., 2024; Zhong et al., 2014).

Table 1. Summary of NGS based studies on appendiceal microbiota composition.

Study (year)	Age range	Total N	Uncomplicated /Complicated	Control N	Appendix sample	Sequencing and bioinformatics	Appendicolith
Guinane (2013)	5–25	7	4/3	0	Lumen swab	16S rRNA v4, Roche 454, SILVA database	No mention
Zhong (2014)	<18	22	10/5, 2 recurrent	5 non-inflamed	Lumen swab	16S rRNA v2v4, Roche 454 sequencing, QIIME, database not mentioned	No mention
Jackson (2014)	<18	21	6/9	6 non-inflamed	Lumen swab	16S rRNA v1v3, Roche 454, CLoVR-16S, database not mentioned	No mention
Arlt (2015)	18–63	8	4 without specification	4 non-inflamed, CRC	Biopsy	16S rRNA v1v2, Roche 454 sequencing, SILVA	No mention
Rogers (2015)	<18	70	37/15	18 non-inflamed	Lumen swab	16S rRNA v2v4/v4, Roche 454 and Illumina, QIIME with SILVA	No mention
Schulin (2017)	<18	16	12/4	0	Lumen swab	16S rRNA v1v3, Illumina, QIIME	No mention
Salö (2017)	<18	22	11/8	3	Biopsy	16S rRNA v1v3, Illumina, QIIME	In both groups
Oh (2020)	18–53, female only	85	50 (non-perforate	35 non-inflamed	FFPE biopsy	16S rRNA v3v4, Illumina, CLC Genomics Workbench with Greengenes	No mention
Munakata (2020)	28–86	25	6*/6	13 non-inflamed, CRC	Lumen swab	16S v1v2, Illumina, RDP Classfier with Greengenes	In appendicitis group
Blohs (2023)	<18	56	45* /11	0	Biopsy	16S v4 and 23S ITS2 rRNA, Illumina, QIIME2 with SILVA and metagenomic seq.	In both groups
Yuan (2021)	7–64	19	9/10	0	Biopsy	Metagenomic seq. (BGI-SEQ-500)	No mention
Aiyoshi (2023)	<18	50	15/14	17 (no appendix sample)	Lumen swab	16S rRNA v1v2, Illumina, GLSEARCH with Refseq	In both groups
Fonnes (2024)	18–85	53	26/16	11	Lumen swab	Metagenomic seq., Metaphlan4	No mention

*catarrhal and/or phlegmonous, CRC= colorectal carcinoma, FFPE=formalin-fixed paraffin-embedded

2.5.2.1 Common limitations of studies on appendiceal microbiome

A common limitation in several NGS studies on appendiceal microbiome seems to be a relative high rate of negative samples (30 to 40%), meaning samples that have either failed at the library preparation or have been excluded from the analysis due to very low number of reads (Fonnes et al., 2024; Salo et al., 2017). Both 16S rRNA gene and shotgun metagenomic sequencing face experimental and computational challenges, with sequencing depth being a critical factor for accurate taxonomical analysis in both approaches (Bharti & Grimm, 2021). This might be a common difficulty related to both appendiceal tissue and lumen samples that have been used in determining the appendiceal microbiome. The proportion of host cells is high in tissue samples and can even be higher in samples from infected sites due to the infiltration of immune cells (Nelson et al., 2019). The overpower of host reads in the shotgun metagenomic sequencing data results to a poor sequencing dept measured in the number of microbial reads which has an impact on the taxonomical profiling (McArdle & Kaforou, 2020; Pereira-Marques et al., 2019). Therefore, the challenges related to microbiome analysis of the appendix are even more evident as they are in the gut microbiome studies, highlighting the importance of carefully considering the methods when interpreting research findings. Further, the concept of a healthy gut microbiota in comparison to dysbiotic, according to current knowledge, includes not only the composition of the microbiota but also its functional characteristics (Lloyd-Price et al., 2016; Turnbaugh et al., 2009). To date, only one study has reached the level of functional prediction analysis of the appendiceal microbiota, due to methodological challenges discussed above (Blohs et al., 2023). Yet, by harnessing the NGS methods it has become possible to understand the diversity of the appendiceal normal microbiota in ways that was not possible by culturing methods.

Studies on appendiceal microbiome have significant heterogeneity both in sequencing and analysis methods (Table 1) limiting the comparability of results. Studies vary in the sample collection (lumen vs biopsy), DNA extraction, target variable (V) region of the 16S rRNA gene, sequencing system and library preparation (Roche vs Illumina), read amount and quality, taxonomical classification (QIIME vs others, OTU vs ASV, taxonomical level used), reference database used (Greengenes vs SILVA), and the plethora of downstream statistics and reporting.

In addition to methodological differences, variability in the clinical and pathological classification appeared (Table 1). In addition, many studies lacked comrehensive reporting of clinically and methodologically relevant aspects of the study design. A common limiting factor was the small sample size, which is particularly important given the substantial inter-individual variation observed in microbiome composition. Additionally, other patient-related factors, such as comorbidities, medications, and diet, may have influenced the microbiome composition, further contributing to variability of results and to the risk of bias. In

most studies (8/12) there is no mention about appendicoliths that according to current understanding form a relevant factor in differentiating uncomplicated and complicated appendicitis (Atema et al., 2015; Kim et al., 2018).

Even though the control group without appendicitis in these studies would be a strength, its representativeness can be considered. In several studies the control group consists of patients whose appendix at the time of surgery has not shown any macroscopic or microscopic marks of inflammation, but they have had appendicitis-like symptoms (Fonnes et al., 2024; Jackson et al., 2014; Rogers et al., 2016; Zhong et al., 2014). In two of the studies the control group consisted of patients with colorectal carcinoma (CRC) participating in hemicolectomy (Arlt et al., 2015; Munakata et al., 2021), one pediatric study included patients with inguinal hernia undergoing surgery (Aiyoshi et al., 2023), and in one study controls were women undergoing incidental appendectomy during gynecological surgery for indications such as endometriosis and leiomyoma (Oh et al., 2020). Control group characteristics as well as differences in the classification of appendicitis may influence the conflicting results seen in comparative analyses.

2.5.3 Dysbiosis and inflammation of appendix

In a heathy gut a large diversity of microbes that are neutral or beneficial for host health form the majority of microbiota profile. Healthy gut microbiota may include opportunistic pathogens but the competition of resources and the crosstalk with host immune system maintain a homeostatic balance (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Lozupone et al., 2012; Walker & Lawley, 2013). Dysbiosis characterized by an imbalance in gut microbial composition or function, has been implicated in the pathophysiology of numerous chronic and acute diseases, including inflammatory bowel disease (Manichanh et al., 2012).

A growing number of studies have investigated potential imbalances in the appendiceal microbial community that may be linked to appendicitis or may help explain differences between uncomplicated and complicated acute appendicitis. These appendiceal microbiota differences compared to the non-inflamed appendiceal microbiota has been investigated in four studies including adult patients and in four studies including pediatric patients (Table 1). Consistent result according to majority of these studies was that in alpha diversity indexes or in species richness there was no significant change between noninflamed and inflamed appendiceal microbiota (Fonnes et al., 2024; Munakata et al., 2021; Oh et al., 2020; Rogers et al., 2016; Salo et al., 2017; Zhong et al., 2014). Several studies have shown differences in the beta diversity and have identified various taxa were differentially abundant between the groups. However, only few genera were consistently observed across these studies. In pediatric studies, an increased abundance of *Porphyromonas* and

Parvimonas was reported (Jackson et al., 2014; Zhong et al., 2014). Additionally, two studies noted a reduced abundance of *Bacteroides* (Rogers et al., 2016; Zhong et al., 2014) while another two reported a reduction in *Faecalibacterium* abundance (Fonnes et al., 2024; Jackson et al., 2014). Particularly a decrease in *Faecalibaterium*, could indicate a dysbiotic appendiceal microbiota. The most well-known member of the *Faecalibacterium* genus, *F. prausnitzii*, is recognized for its contribution to a healthy human gut through its metabolic and anti-inflammatory properties (Martín et al., 2014).

The most consistently identified taxa positively associated with appendicitis is *Fusobacterium* (species *F. nucleatum* and *F. necrophorum*) (Aiyoshi et al., 2023; Arlt et al., 2015; Jackson et al., 2014; Rogers et al., 2016; Schülin et al., 2017; Zhong et al., 2014). While fusobacteria are present in the normal appendiceal microbiome, their enrichment suggest a potential role in the etiology of appendicitis (Aiyoshi et al., 2023; Arlt et al., 2015; Jackson et al., 2014; Rogers et al., 2016; Schülin et al., 2017; Zhong et al., 2014). This is further supported by studies comparing the appendiceal microbiome in uncomplicated and complicated appendictis, where fusobacteria are more strongly associated with complicated cases (Arlt et al., 2015; Blohs et al., 2023; Guinane et al., 2013; Salo et al., 2017). Interestingly, Aiyoshi et al. demonstrated that at genus level *Fusobacterium* was enriched in saliva and feces of pediatric appendicitis patients (Aiyoshi et al., 2023). Pathogenic potential of fusobacteria, particularly *F. nucleatum* and *F. necrophorum*, was demonstrated in study that investigated appendices using rRNA-based fluorescence in situ hybridization (Swidsinski et al., 2011). This study found that fusobacteria, were present in the mucosal and submucosal lesions of acute appendicitis in 62% of cases, correlating with disease severity. Fusobacteria were absent in caecal biopsies, suggesting a localized infection of fusobacteria (Swidsinski et al., 2011). The study was repeated in different populations in Germany, China, and Russia with similar findings (Loening-Baucke et al., 2012). This invasiveness is consistent with their known virulence factors, including adhesins and immune-modulatory molecules, which facilitate tissue colonization and inflammation (Engevik et al., 2021; Rubinstein et al., 2013). In adult patients, however, the evidence is less conclusive. Two studies in adult patients showed similar abundance of *Fusobacterium* between appendicitis and control groups (Fonnes et al., 2024; Munakata et al., 2021). These findings may be partly explained by control group selection in Munakata's study, as fusobacteria are strongly associated with CRC etiology (Bullman et al., 2017), which may confound results.

Like inflamed versus noninflamed, the comparison of uncomplicated and complicated appendiceal microbiomes demonstrates differences in the beta diversity but no consistently differentiating taxa across studies is found (Arlt et al., 2015; Blohs et al., 2023; Fonnes et al., 2024; Jackson et al., 2014; Munakata et al., 2021;

Salo et al., 2017), despite the fusobacteria discussed above. In two most recent studies based on shotgun metagenomic sequencing and including adult patients no significant difference was observed between uncomplicated (non-perforated) and complicated groups (Fonnes et al., 2024; Yuan et al., 2021). In the subgroup analysis of Fonnes's study patients with complicated appendicitis (16), uncomplicated appendicitis (26), and without appendicitis (11) the beta diversity at species level was dissimilar between subgroups. However, the dissimilarity was significant only between inflamed and noninflamed groups, illustrating the importance of including a control group (Fonnes et al., 2024). A study by Oh et al, is the largest to date with 50 adult patients and with a control group of 35 subjects without appendicitis. They showed several differences including decrease in unidentified genus from *Burkholderiaceae* and *Enterobacteriaceae* in appendicitis samples, while 14 genera, including *Neisseria*, *Acinetobacter*, and *Campylobacter*, were increased. They studied further the *Campylobacter* levels with quantitative PCR showing significantly higher levels of *C. jejuni* in appendicitis samples compared to controls, with 40% of cases testing positive versus 6% of controls (Oh et al., 2020). This is interesting finding; however, the study design is much different from other studies in including only women and that formalin-fixed paraffin-embedded appendiceal tissue biopsy is used. The appendicitis cases included appear to be non-perforated, although clinical data description is limited.

A notable presence of genera that are frequently detected in the oral cavity were observed in the appendix, for instance *Fusobacterium*, *Porphyromonas*, and *Parvimonas*, suggesting that the oral bacteria may translocate to the appendix and contribute to infection (Aiyoshi et al., 2023; Blohs et al., 2023). Blohs et al. describes the increase in *Fusobacterium*, *Porphyromonas*, and *Parvimonas* species particularly in complicated appendicitis with simultaneous decrease in *Bacteroides* (Blohs et al., 2023). In addition, in the event of the taxa showing over presented growth in their abundance in the appendix, they might be also detected in rectal or fecal samples (Aiyoshi et al., 2023).

In addition to the microbial changes in appendiceal microbiome, host immunological response to changes or to the enrichment of pathogenic bacteria in the appendix, have been suggested to explain the development of complicated appendicitis. In a recent study, a specific immune profile with disrupted T cell response and increased Th17 response was observed in children with complicated appendicitis. The increased IL-17A-production was associated with *Proteobacteri*a, but significant species level association was not detected (The et al., 2023). An increased Th17 response has also been observed in adult patients with gangrenotic appendicitis compared to those with phlegmonous appendicitis (Rubér et al., 2010). Another study indicated that the underlying weaknesses in the immune defence caused by polymorphism in the IL-6 gene might increase the risk of developing

gangrenous or perforated appendicitis (Rivera-Chavez et al., 2004). Additionally, the polymorphism patterns in IL-17 and CD44 have been associated with an increased risk of severe appendicitis, further supporting the idea that etiological differences between uncomplicated and complicated appendicitis may be mediated by genetic factors related to immune response (Dimberg et al., 2020).

These findings highlight the unique characteristic of complex appendiceal microbiome and its potential role in appendicitis, warranting further investigation into microbial composition and function in reference to gut microbiome as well as microbiome-host interactions in the appendix. The etiological factors reviewed above including appendiceal microbiota dysbiosis in uncomplicated and complicated acute appendicitis are illustrated in Figure 1.

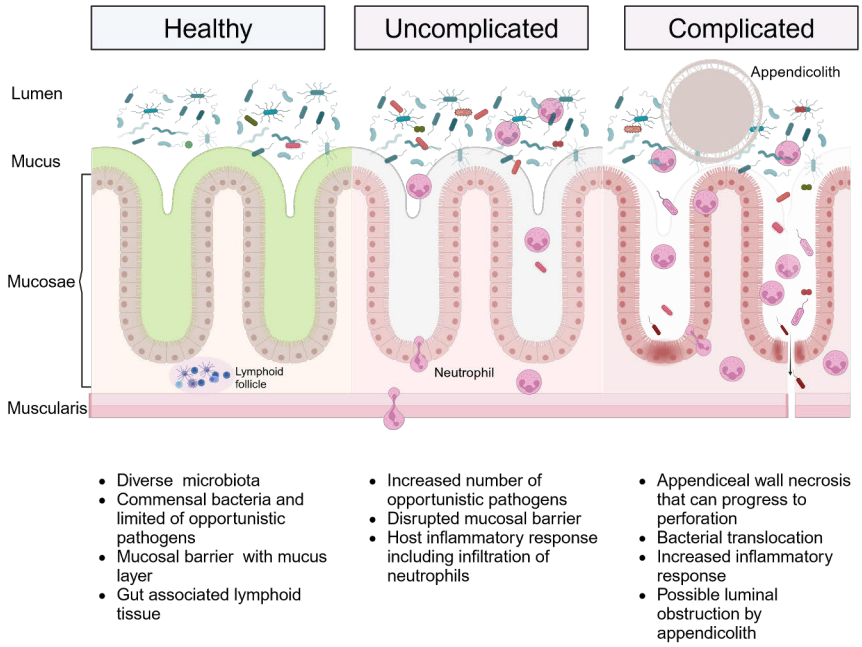

Figure 1. In heatlhy appendix the microbiota composition is diverse and balanced crosstalk with bacteria and host immune system is taking place. In acute appendicitis microbiota changes characterized by enrichment of opportunictic pathogens initiate host inflammatory response including infiltration of neutrophils. Mucosal barrier disruption might allow bacterial translocation. In complicated acute appendicitis inflammatory response is exacerbated and the appendiceal wall shows necrosis and/or perforation. Obstruction of appendiceal lumen by an appendicolith is often present in complicated appendicitis. Figure is adapted from Blohs et al. 2023 and Walker et al. 2013.

3 Aims

The differences between uncomplicated and complicated acute appendicitis are not well understood and there are major knowledge gaps in the role and etiology of appendicoliths. The aim of this study was to assess differences between uncomplicated and complicated appendicitis focusing on the microbiological factors and the structure and role of appendicoliths in acute appendicitis.

The specific research objectives were:

1. To design a prospective clinical cohort study to assess the microbiological factors and appendicolith characteristics in the etiology of appendicitis severity.
2. To study the possible differences in the appendiceal microbiome composition in patients with uncomplicated and complicated acute appendicitis.
3. To define, characterize, and categorize appendicoliths and their structure in acute appendicitis by revisiting an appendicolith classification from 1966.
4. To study the chemical and microbiome composition of appendicoliths and their association with appendicolith formation.

4 Materials and Methods

4.1 MAPPAC study design and participants

All studies (II-IV) are based on Microbiology APPendicitis ACuta (MAPPAC) trial. MAPPAC trial was conducted in close synergy with APPendicitis ACuta II (APPAC II) and APPAC III randomised clinical trials. APPAC II was an open-label, noninferiority trial comparing oral moxifloxacin with intravenous ertapenem followed by oral levofloxacin and metronidazole (Haijanen et al., 2018; Selänne et al., 2024; Sippola et al., 2021). APPAC III was a double-blind, placebo-controlled, superiority study comparing antibiotic therapy (intravenous ertapenem followed by oral levofloxacin and metronidazole) with placebo in the treatment of uncomplicated appendicitis (Sippola et al., 2018). The MAPPAC trial was an observational prospective cohort study with a single-center and a multicenter arm. The single-center study arm at Turku University Hospital focused on exploring potential differences in the etiology of complicated and uncomplicated acute appendicitis regarding the role of microbiota and appendicoliths. In all studies (II-IV), the single-center patient cohort of MAPPAC study was used, which included patients with uncomplicated and complicated acute appendicitis undergoing appendectomy. Patients with recurrent appendicitis were excluded from the analysis included in this thesis as the focus was on primary appendicitis and to avoid confounding factors by recent treatment with broad spectrum antibiotics.

The multicenter longitudinal arm of the MAPPAC trial was conducted in five university hospitals Turku, Helsinki, Tampere, Oulu, and Kuopio and in five central hospitals Jyväskylä, Pori, Mikkeli, Seinäjoki, and Rovaniemi. In the multicenter arm MAPPAC study aimed to assess the impact of antibiotic and placebo treatments on the gut microbiota and to the potential development of antimicrobial resistance reservoir within the gut microbiota. Additionally, the multicenter study aimed at exploring immunological and microbiological factors that may contribute to initial non-responsiveness to treatment or recurrence of appendicitis followed by successful initial antibiotic therapy during long-term follow-up. These longitudinal multicenter arm results are not reported in this thesis.

Patients aged 18 to 60 years with suspicion of acute appendicitis underwent standard contrast enhanced or low dose protocol CT imaging of the abdomen. Patients with a body mass index (BMI) under 30 kg/m² were imaged using a low-dose CT protocol, while those with a BMI over 30 kg/m² underwent standard CT imaging. Patients with CT-confirmed acute appendicitis were included in the study. Acute appendicitis was confirmed by histopathology and appendicitis was defined by evidence of intramural neutrophil invasion in the histopathological examination of the removed appendix.

For study III, patients with both a CT-visible appendicolith and a retrieved appendicolith sample were included. Further selection of 15 patients was done for appendicolith elemental and microbiome analysis (study III and IV). For this subgroup, patients with BMI under 30 were included to mitigate potential bias related to the CT imaging as these patients underwent low-dose CT. Patient sex was also considered in the representative sample selection by including both males and females.

4.1.1 Appendicitis classification and severity

Appendicitis was defined according to CT criteria as an appendiceal diameter larger than six millimeters with a thickened contrast enhancement of appendiceal wall and periappendiceal edema and/or minor fluid collection. Acute appendicitis was classified as uncomplicated if no features of complicated appendicitis were present. Complicated appendicitis was defined by the presence of an appendicolith, gangrene, perforation, abscess, suspicion of a tumor, or a combination of these. To ensure the accuracy of distinguishing between uncomplicated and complicated acute appendicitis, two investigators independently evaluated clinical data, CT findings, surgical observations, and histopathological results without knowledge of each other's assessments. In study III, the severity of appendicitis was evaluated by categorizing patients into two subgroups: patients with an, but no signs of other complications (appendicolith appendicitis) and those with an appendicolith accompanied by other indicators of complicated appendicitis (appendicolith appendicitis with complications).

4.1.2 Ethics

MAPPAC study was approved by the ethics committee of the Hospital district of Southwest Finland (approval number ATMK:142/1800/2016) and by the Finnish Medicines Agency. All participants gave an informed written consent for the collection and analysis of clinical data and microbiological samples.

4.2 Sample collection and processing

Microbiological samples, i.e., appendiceal swab, biopsy, and appendicoliths were collected from uncomplicated and complicated appendicitis patients immediately after appendectomy. Given the emergency surgery nature of appendicitis, samples were collected by the surgeons on call and during office hours by researchers and technicians. In both cases, sample collection was performed in similar manner. To collect the microbiological samples, the appendix was longitudinally opened using a sterile scalpel. In case of an appendicolith, it was first collected with sterile tweezers into a sterile container and stored in +4℃ up to 48 hours until transferred for -75℃ freezer. Appendiceal lumen swabs were collected with Puritan DNA/RNA Shield tube (Puritan DNA/RNA swab, Puritan Medical products, Guilford, Maine) containing a sterile swab. The Shield fluid (Zymo Research, Irvine, California, USA) in the tube preserves the sample in room temperature (RT) until DNA extraction. In practise samples were stored in RT for 3 to 5 days before DNA extraction and to uniform the storing time, none of the samples were extracted before 3 days. Appendiceal biopsies for DNA extraction were taken approximately 2 cm from the base of the appendix or directly from observable inflammation site. Biopsy, approximately the size of 0.5 x 0.5 cm, was cut with a sterile scalpel and was immersed in RNAlater solution (Thermo Fischer Scientific, Waltham, MA, USA) and was stored in +4℃ for 24-72 hours before the DNA extraction.

4.2.1 DNA extraction (II, IV)

DNA from appendiceal lumen swabs and appendicoliths was extracted with a semiautomated GXT Stool Extraction Kit (Hain Lifescience GmbH, Nehren, Germany) together with NorDiag Arrow extraction instrument (Diasorin, Saluggia, Italy). For biopsies an equivalent semiautomated GXT NA Extraction Kit (Hain Lifescience, GmbH, Nehren, Germany) was used. DNA extraction included a pre-treatment adjusted to each sample type as follows: For lumen samples 500 ul of the shield fluid containing the sample was transferred to a 1.4 mm Ceramic Powerbead Tube (Qiagen, Hilden, Germany) with a lysis buffer, and homogenized with MOBIO Powerlyzer 24 Bench Top Homogenizer (MO BIO Laboratories, Inc., USA) at 1000 rpm for 3 minutes. For appendicolith DNA extraction, a piece of appendicolith was sectioned with a sterile scalpel and weighed. Bead beating with lysis buffer and 1.4 mm Ceramid Powerbead Tubes was performed in similar manner although with repeated homogenization; first at 1000 rpm for 3 minutes followed by second at 2500 rpm for 3 minutes. After homogenization and centrifugation at 5000 x g for 4 minutes, the DNA was extracted from the supernatant according to the manufacturer's instructions. For appendiceal biopsy extraction, the biopsy was first removed from the RNAlater solution which was then centrifuged at 16 000 x g for 6

minutes. The pellet was resuspended into ultrapure water. Subsequently, the resuspension containing also the added biopsy was treated with proteinase K in thermomixer at 56℃ for 30 minutes. From the resulting solution, DNA extraction was carried out according to manufacturer's instructions. Concentration of eluted DNA was quantified with Qubit dsDNA HS and BR assay kits (Thermo Fisher Scientific) using Qubit fluorometer (Life Technologies, Carlsbad, California, USA). DNA was stored in two separate aliquots at −75℃ until further analysis.

4.3 Microbiome analysis (II, IV)

4.3.1 16S rRNA amplicon sequencing (II)

The microbiome compositions of appendiceal lumen samples in study II were determined with targeted NGS of V3-V4 hypervariable region of the bacterial 16S rRNA gene. Sequencing libraries were prepared using the KAPA HiFi HotStart ReadyMix kit (KAPA biosystems, Roche, Basel, Switzerland) and by following the Illumina protocol (Illumina) that was modified by increasing DNA template amount in the amplicon PCR from standard 12.5 ng to 75 ng. The total volume of the PCR was also increased from 25 ul to 33 ul in order to have a sufficient amount for library quality monitoring. Control samples including negative DNA extraction control, negative PCR control and positive mock community (ZymoBiomics microbial community DNA standard, Zymo Research) were included in each sequencing run. Sequences of the V3-V4 gene specific full length forward and reverse primers were 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3', respectively (Klindworth et al., 2013). Sample specific Nextera XT indices were added in the second PCR using Nextera XT Kit (Illumina, San Diego, California, USA) resulting in PCR product of approximately 630 bp in size.

The PCR products were purified with AMPure XP Magnetic beads (Beckman Coulter, Inc., Brea, CA, USA) together with DynaMagTM-96 magnetic plate (Life Technologies). The DNA concentrations of the final libraries were quantified with Qubit fluorometer (Life Technologies) and dsDNA HS Assay Kit (Thermo Fisher Scientific). This was followed by equimolar pooling (4nM), denaturation and dilution to 4 pM concentration. For sequencing control and to increase the nucleotide diversity a 10% PhiX (Illumina) was added to the pool. Sequencing was performed using MiSeq Reagent Kit v3 and 2 x 300 bp paired-end run on Miseq System (Illumina).

4.3.2 16S rRNA gene amplicon sequencing analysis (II)

The analysis of 16S rRNA gene amplicon sequencing data was performed in collaboration with Aluke Oy (Helsinkin, Finland). The quality of the raw data was checked with FastQC program (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Primers were removed from amplicon sequences using Cutadapt (version 2.7.) (Martin, 2011). Amplicon sequence variants (ASVs) of the 16S rRNA gene sequences were generated using dada2 package (v. 1.18.0) with the following pre-processing steps. Amplicon sequences were quality filtered and trimmed using filterAndTrim by truncating the forward reads after 250 bp and reverse reads after 225 bp. Sequencing errors were detected and modelled with the function learnErrors separately for the two sequencing batches. Sequences from separate batches were pooled and sequences were inferred using the function dada. ASV table was created with the function makeSequenceTable after which chimeric sequences were removed by the function removeBimeraDenovo (Callahan et al., 2016). ASV annotation to taxonomy was based on SILVA rRNA gene reference database (v. 138)(McLaren, 2020).

Decontamination was performed using R package decontam with the method "Prevalence" and a threshold of 0.225 which compares prevalence of each ASV in true samples to the prevalence in negative controls (Davis et al., 2017). Results of the decontamination were manually inspected and corrected. For the final analysis a cut-off value of 1800 reads was determined for a successfully sequenced sample that was included in the analysis. Raw sequence counts were transformed to relative abundances of ASVs which were used in following downstream analyses of the sequencing data.

Alpha diversity measures (Chao1, Shannon entropy and the number of observed species) describing microbial diversity and richness were calculated with R package vegan (2.5.6) using functions estimateR and diversity (Oksanen et al., 2007). The association of diversity and appendicitis severity was assessed with standard linear regression model. In addition to the appendicitis severity, the age, sex and BMI of patients were tested separately. The differences in the overall bacterial diversity across the appendiceal samples i.e., beta diversity based on Bray-Curtis dissimilarities were calculated in Principal Coordinate Analysis (PCoA) with vegan (v. 2.5.6) and the permutational multivariate analysis of variance (PERMANOVA) with R package BiodiversityR (Kindt & Coe, 2005). PERMANOVA was controlled for age, sex and BMI of the patients. The microbiota compositions, differentially abundant species and the alpha diversities between the uncomplicated and complicated groups were visualized using GraphPad Prism (v. 9).

The differences in microbiota composition between uncomplicated and complicated appendicitis groups were compared with DESeq2 method (Love et al.,

2014) using raw counts. Differential abundance of ASVs, species, genera and phylum were tested. The p-values were adjusted using Benjamini-Hochberg method and adjusted p-values <0.01 were considered as statistically significant.

4.3.3 Shotgun metagenomic sequencing and analysis (IV)

Shotgun metagenomic sequencing of appendicolith-, appendiceal swab- and appendiceal biopsy samples was performed by CosmosID (Germantown, Maryland, USA) as follows: Sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and Nextera Index Kit (Illumina) with total DNA input of 1ng. Genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. Sample specific indices were added followed by 12 cycles of PCR to construct libraries. DNA libraries were purified using AMPure Magnetic Beads (Beckman Coulter). DNA libraries were quantified using Qubit fluorometer and Qubit dsDNA HS Assay Kit. Libraries were sequenced with 150 bp paired-end sequencing on Illumina HiSeq platform.

Host removal was performed using Qiagen CLC Microbial Genomics Module v23.0 (QIAGEN Digital Insights, Aarhus, Denmark) by mapping raw reads to the human genome (GRCh38). Reads mapped to the human genome were discarded, while unmapped and microbiome-mapped reads were retained for analysis. Quality was assessed with FastQC and MultiQC (v1.19), and adapters were removed using Cutadapt (v4.6). For each sample, the forward and reverse reads were merged. Taxonomic profiling was performed using HUMAnN (v3.8) with default parameters. The sequences were aligned against the CHOCOPhlAn pangenome database (v. October 2022) (Beghini et al., 2021).

Statistical analysis was conducted using R (4.4.2). Microbiome data were processed with the mia R package (1.15.6). Abundance plots were generated using miaViz (1.15.3). Alpha diversity comparisons were made using mia, while a linear mixed model (lme4 package) assessed the association between appendicitis severity, appendicolith hardness, and sample type. Sample type, diagnosis, and appendicolith hardness were modeled as fixed effects, while patient identity was modeled as a random effect allowing the model to capture variance explained by sample type. Beta diversity analysis employed Bray-Curtis dissimilarity PCoA in scater R package (1.33.4). Species-level community composition was evaluated using dbRDA (mia), modelling patients as confounding factor. Differential abundances between appendicitis severity class were analyzed with Maaslin2 (1.19.0) using linear model and CLR-transformed data, with appendicolith hardness and sample type as fixed effects and patient information as a random effect so that the effect of sample type is correctly captured.

4.4 Appendicolith analysis

For study III, MAPPAC patients (41/308) with both a CT-visible appendicolith and an available appendicolith sample were included. Further selection of representative patients (15/41) was performed for appendicolith elemental and microbiome analysis (study III and IV). For this subgroup, patients with BMI under 30 were included to mitigate potential bias related to the CT imaging as these patients underwent low-dose CT. Patient sex was also considered in the representative sample selection by including both males and females.

4.4.1 Appendicolith characterization and classification (III)

In case of multiple appendicoliths, the largest one was selected for characterisation and analysis. All appendicoliths were classified into three categories based on the degree of hardness of the outer surface. This classification was adapted from the classification described in 1966 by Forbes and Lloyd-Davies (Forbes & Lloyd-Davies, 1966). This revised classification included only appendicoliths that were visible on CT imaging, and the classification was based exclusively on the hardness of their outer surface. Similarly, to the original classification, soft, class 1 appendicoliths were defined by the ability to be squeezed by tweezers in contrast to class 2 and 3 appendicoliths. Class 2 appendicoliths were characterize by a firm to hard surface and stony class 3 appendicolith, unlike others, made a distinct clinking sound when dropped on metal surface. Appendicoliths were intended to be classified before freezing; however, this was not feasible for nine appendicoliths due to the acute care surgery context of the study.

Appendicoliths were examined for their morphology, measured and weighed Photographs from both their surfaces and cross-sections were obtained using a Canon EOS 60D digital camera with a Canon EF-S 60 mm macro lens. They were weighed and measured immediately after being removed from the −80°C freezer to prevent drying before analysis.

4.4.3 Initial computed tomography and micro-computed tomography (III)

The initial CT images for all patients taken at the emergency department were evaluated in retrospect by two abdominal radiologists to assess the radiographical characteristics of the appendicoliths. Appendicoliths were defined as highly attenuated round or oval concretions with a size of 3 mm or larger. The radiographic hardness of the appendicoliths was assessed by measuring their density in Hounsfield units (HU). The regions of interest were measured with an oval shape to cover 80-

90% of the appendicoliths surface area at the largest diameter using Philips PACS, Philips Medical Systems, version 12.

Representative appendicoliths from six patients were selected, with two from each hardness class, for Micro-CT, i.e., X-ray microtomography (XMT) measurements. Micro-CT measurements were performed using a high-resolution XMT scanner Phoenix Nanotom 180 NF (GE Measurement and Control Solutions GmbH, Germany). Scanning was performed with a divergent cone beam configuration using a source voltage of 60 kV and a current of 150 µA. The imaging parameters were selected to provide a voxel size of 15 µm. A total of 720 transmission radiographs were taken from each sample. The tomographic reconstruction of the transmission images to create to create three-dimensional (3D) images was done with Datos|x software supplied by the manufacturer. The image processing of the reconstructions was performed with Dragonfly software (v. 2020.2 and 2022.2) (Object Research systems (ORS) Inc., Montreal, Canada).

4.4.4 Appendicolith elemental analysis (III)

To investigate the elemental composition and spatial distribution of the elements within the appendicoliths, high-resolution micro-X-ray fluorescence spectroscopy (micro-XRF) was employed. To prepare a flat cross-sectional surface for micro-XRF analysis, appendicoliths were sectioned, and their surfaces were refined using a microtome. For microtome trimming, half of each appendicolith was embedded in paraffin, providing structural support during the process. The analysis was conducted using a Bruker Tornado M4 system (Bruker Nano GmbH, Germany). X-rays were produced with a rhodium (Rh) tube operating at 50 kV and a source current of 600 µA. Elemental mapping was achieved with a 20 µm spot size, generated through polycapillary optics, a 50 µm step size, and a dwell time of 20 ms. Calibration of the instrument was performed using a reference sample block provided by the manufacturer. Quantitative elemental analysis was derived directly from the acquired micro-XRF maps.

The carbon, hydrogen, and nitrogen (CHN) content of the appendicoliths were analysed with Flash 2000 CHNS organic elemental analyser (Thermo Fisher Scientific Inc., USA). Sulphanilamide standard was used to calibration of the instrument.

4.5 Statistical analysis

In Study II, group differences in numerical (age, BMI) and categorical (sex) baseline characteristics were tested with Wilcoxon rank sum test and Pearson´s chi-squared test, respectively.

In Study III, appendicolith classification on the degree of hardness (class 1 to 3) as categorical variable was expressed as frequency with percentages, and Fisher's test was used for the analysis as the number of samples in class 3 was low (<5). Radiographic density in HU units as continuous variable with non-normal distribution was presented as median (range) and the correlation between the three classes and HU units was evaluated with Spearman's correlation test. Kruskal-Wallis test was used to compare the diameter and HU units from collected and analysed to uncollected appendicolith samples. Appendicolith maximum length, width, and weight as continuous variables were analysed using univariate logistic regression expressed as odds ratio (OR) and 95% confidence interval (CI). A two-sided P value < 0.05 was considered statistically significant. Statistical analyses were performed with SAS System for Windows, Version 9.4 (SAS Institute Inc, Cary, NC, USA).

5 Results

5.1 Appendiceal microbiome in uncomplicated and complicated acute appendicitis (II)

5.1.1 Clinical data and sample characterization

The MAPPAC study enrolled 308 patients with acute appendicitis between April 2017 and March 2019. Patients with histopathologically confirmed uncomplicated or complicated acute appendicitis undergoing appendectomy were included (n=169). Due to incomplete sample collection (n=21), failed library preparation (n=26), and low library size (n=4), a total of 118 (70%) samples were included in the final analysis. Patient characteristics are presented in Table 2.

The groups were similar in age and BMI, but the proportion of women was higher in the uncomplicated acute appendicitis group compared to the complicated acute appendicitis group (66% vs. 45%, p=0.0346).

Table 2. Patient baseline characteristics and subtypes of complicated appendicitis. Modified from original publication II.

CHARACTERISTIC	UNCOMPLICATED (N=41)	COMPLICATED (N=77)
Sex, Female/Male, n (%)	27 (66)/ 15 (34)	35 (45)/ 42 (55)
Age (years), median (range)	37 (16-75)	43 (18-69)
BMI (kg/m^2), median (range)	25.8 (20.4-39.5)	26.6 (18.4-42.7)
Complications, n (%)		
Appendicolith		30 (39.0)
Gangrenous/perforation		14 (18.2)
Gangrenous/perforation and appendicolith		21 (27.3)
Periappendicular abscess*		6 (7.8)
Gangrenous/perforation/abscess and appendicolith		3 (3.9)
Abscess and appendicolith		1 (1.3)
Tumor and appendicolith		1 (1.3)
Gangrenous/perforation/tumor and appendicolith		1 (1.3)

* Perforated acute appendicitis limited by nearby tissues and organs resulting in periappendicular abscess

5.1.2 Overall appendiceal microbiome composition in appendicitis

The appendiceal lumen microbiota in both uncomplicated and complicated groups primarily (>95%) consisted of the phyla *Bacteroidota*, *Firmicutes*, *Proteobacteria*, and *Fusobacteriota*. Less abundant phyla were *Verrucomicrobiota*, *Desulfobacterota*, *Campilobacterota*, *Actinobacteriota*, *Synergistota*. The microbial profiles of the appendiceal lumen at genus level are illustrated in Figure 2**.** Altogether, 419 distinct genera and 599 different species were identified, underscoring the complexity of the appendiceal microbiome.

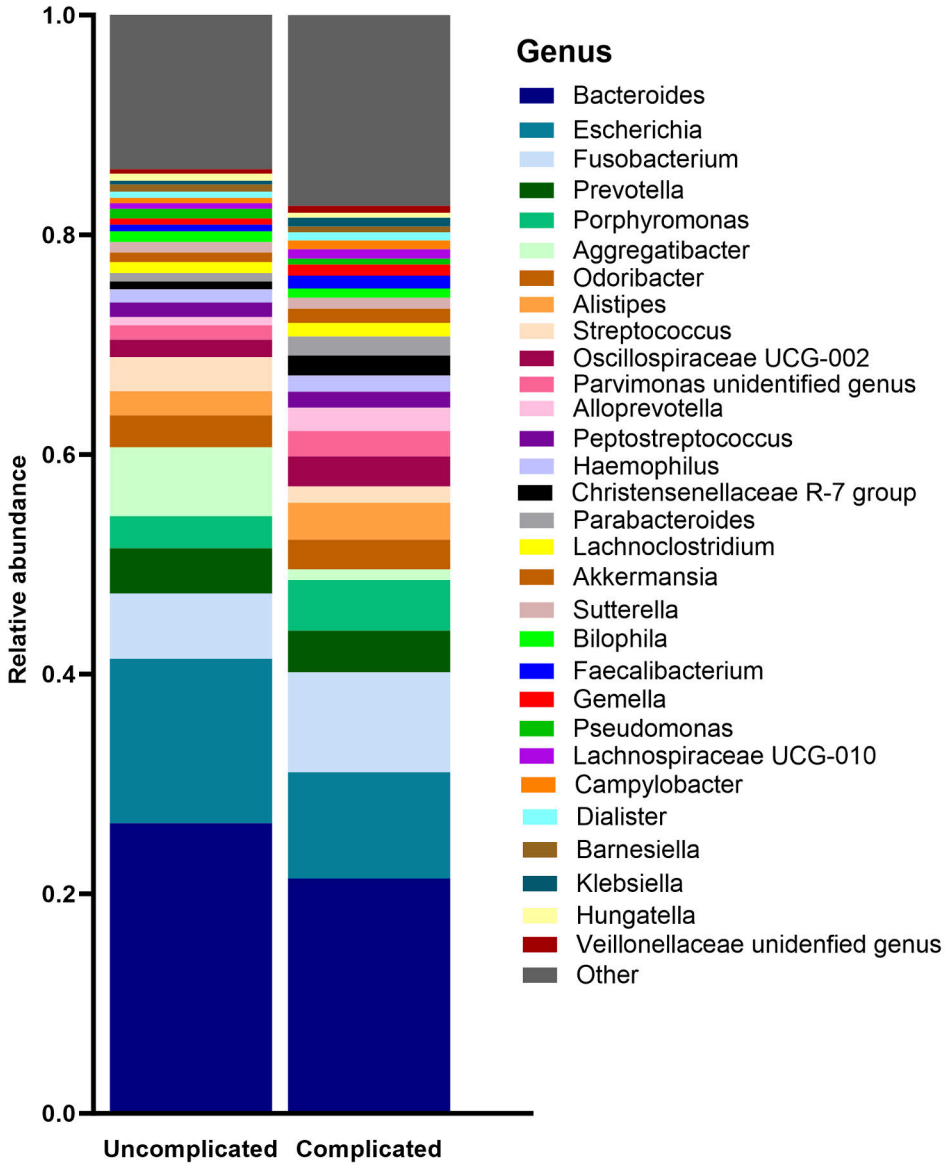

Figure 2. Appendiceal microbiome composition at genus level in uncomplicated and complicated acute appendicitis. Modified from original publication II. Stacked barplots indicate the mean relative abundance of top 30 genera grouped by disease form.

5.1.3 Differences in the appendiceal microbiome according to appendicitis forms

The composition of the appendiceal microbiome differed significantly between uncomplicated and complicated appendicitis, as reflected in both alpha and beta diversities. Alpha diversity indices, Shannon and Chao1, were lower in uncomplicated cases compared to complicated ones ($p=0.011$ and $p=0.006$, respectively). Similarly, beta diversity analysis using Bray-Curtis dissimilarity indicated distinct microbial compositions between the two groups, with pairwise comparisons showing significant differences ($p=0.002$).

The relative abundances of bacteria between uncomplicated and complicated groups were compared in phylum, genus, and species level. At phylum level, there were no significant differences. Statistically significantly (adjusted $p< 0.01$) differentially abundant bacterial species and genera (with mean relative abundance >0.1%) are summarized in Table 3.

Table 3. Species and genera with significant differences between study groups.

SPECIES	BASE-MEAN	LOG2 FOLD CHANGE	ADJUSTED P-VALUE	UNCOMPLI-CATED RELATIVE ABUNDANCE %	COMPLI-CATED RELATIVE ABUNDANCE %
Veillonella parvula	31.4	6.4	5.6E-11	0.52	0.03
Haemophilus UNK	34.6	5.7	3.2E-06	0.45	0.01
Veillonella UNK	12,3	5.3	1.4E-03	0.48	0.03
Aggregatibacter aphrophilus	406.4	4.6	5.0E-03	3.80	0.18
Enterobacteriaceae UNK	44.5	4.6	4.8E-03	0.92	0.05
Streptococcus UNK	272.7	2.7	2.8E-03	2.39	0.72
Bacteroides fragilis	8268.5	2.4	5.0E-03	12.28	7.42
UCG-005 UNK	202.2	-2.4	5.0E-03	0.15	0.76
Clostridia UCG-014 UNK	43.4	-2.5	9.2E-03	0.03	0.21
Christensenellaceae UNK	30.3	-3.6	2.6E-05	0.09	0.37
Dialister UNK	38.9	-4.0	5.1E-03	0.03	0.29
Porphyromonas endodontalis	146.5	-4.8	4.8E-03	1.11	1.41
Bacteroides faecis	681.8	-5.4	4.0E-06	0.92	2.15
Phocaeicola abscessus	22.9	-7.1	2.8E-03	0.00	1.04
GENUS					
Aggregatibacter	2849.8	5.3	1.6E-04	6.27	0.97
Veillonella	35.0	5.1	4.5E-10	0.98	0.06
Enterobacteriaceae UNK	56.8	4.9	4.0E-03	0.90	0.05
Oscillibacter	60.3	-2.2	2.1E-03	0.08	0.19
Subdoligranulum	25.1	-2.6	1.8E-03	0.01	0.15
Christensenellaceae UNK	22.3	-3.2	3.0E-04	0.12	0.37
Phocaeicola	22.7	-7.0	4.0E-03	0.00	1.04

UNK= unidentified species/genus, positive values in Log2fold change indicate higher abundance in uncomplicated appendicitis compared to complicated appendicitis

5.1.4 Species poor appendiceal microbiota profiles with specific predominant bacteria was observed across appendicitis forms

At species level, an unidentified species from *Escherichia* and *Bacteroides fragilis* were the most abundant and prevalent in both groups. Beside this, samples showed high interindividual variation in composition and in the number of observed species. In some samples, the appendiceal microbiome comprised of hundreds of species, while in others, only a few predominant species were detected at significant levels, resulting in low alpha diversity values. The appendiceal microbiome was dominated by a single bacterial species with a relative abundance exceeding 50% in 17% (7/41) of uncomplicated cases and 12% (9/77) of complicated acute appendicitis cases. Regardless of disease severity, *B. fragilis* and an unidentified *Escherichia* species were the most commonly predominant species. *Aggregatibacter aphrophilus, A. segnis*, and unidentified *Streptococcus* species dominated exclusively in uncomplicated appendicitis. Only complicated appendicitis microbiome contained an unidentified *Fusobacterium* species, *Haemophilus parainfluenzae*, *B. faecis*, *B. dorei*, and an unidentified *Bacteroides* species.

5.2 Features of appendicolith appendicitis

5.2.1 Patients with appendicolith appendicitis

Out of 308 patients, 78 MAPPAC patients had acute appendicitis with appendicolith observed in CT. Out of 78 patients, appendicoliths were available for analysis from 41 (52.6 %) patients. Studies III and IV were based on this subpopulation with an appendicolith appendicitis. Figure 3 shows the patient flow and study design. Of the 41 patients with CT-visible and harvested appendicolith, 21 (51.2%) were classified as having appendicolith appendicitis with additional complications such as gangrene, perforation, or abscess. In 20 patients (48.8%), the appendicolith was the sole finding of complicated appendicitis. The mean age was 44.1 years, with 23 (56.1%) being male and 18 (43.9%) female. The mean BMI was 27.3 kg/m^2. Almost half (48.8%) of the patients presented with multiple appendicoliths, with median of 2, and the highest number being 6 appendicoliths from single individual.

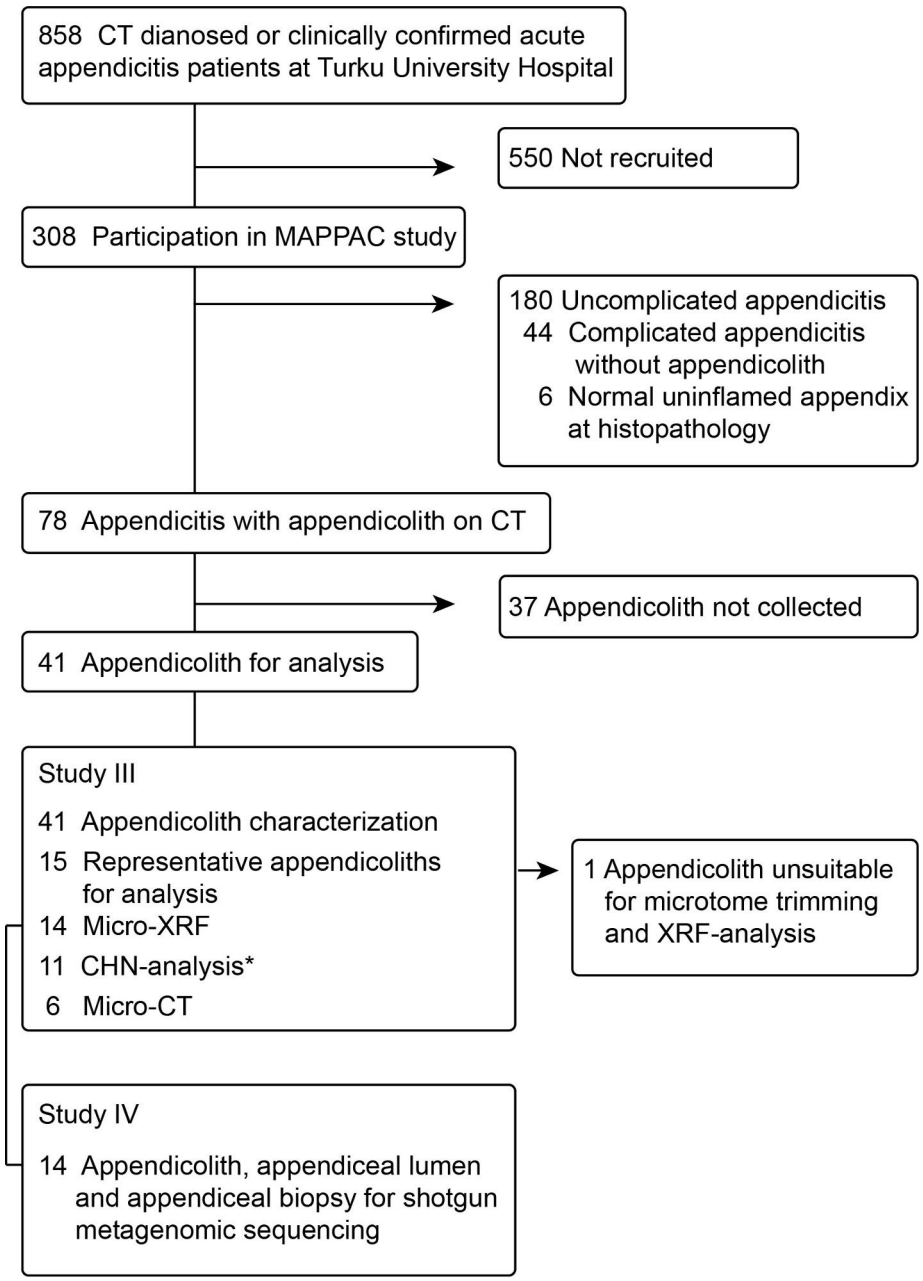

Figure 3. Flow of patients in study III and IV. Modified from original publication III. *Insufficient amount of appendicolith left for CHN-analysis (N=3).

5.2.2 Appendicolith classification and morphological characterization

Appendicoliths were classified based on the degree of hardness into three classes. The classification including appendicolith characteristics is presented in Table 4.

Table 4. Appendicolith classification and physical characteristics. Table is from Vanhatalo et al., 2024 (original publicaton III).

CLASS	DESCRIPTION	OCCURRENCE N/41 (%)	WEIGHT (MG)	MAXIMUM LENGTH (MM)	MAXIMUM DIAMETER (MM)
CLASS 1	Clear form, but soft faecal- like structure. Cross-sectionally amorphous. No visible core.	17 (41.5)	243 (11-816)	9.9 (4-20)	6.3 (2-9)
CLASS 2	Firm to hard surface. Cross-sectionally concentrically layered structure. Visible core.	16 (39.0)	589 (19-1600)	12.6 (4-22)	8.0 (3-13)
CLASS 3	Stony hard outer surface. Cross-sectionally concentrically layered structure. Visible core.	8 (19.5)	646 (15-2985)	10.9 (6-19)	7.2 (3-14)

Appendicolith weight, maximum length and diameter are expressed as mean (range). Maximum diameter of the appendicolith is the vertical measure of the maximum length.

The classification was compared with radiographical density of appendicoliths expressed as Hounsfield units (HU). In Class 1, appendicoliths exhibited a median radiographic density of 174.0 HU (37-337 HU), Class 2 appendicoliths displayed a higher median density of 201.0 HU (138-371 HU), and Class 3 appendicoliths demonstrated the highest radiographic density with a median attenuation of 486.0 HU (263–730 HU). This classification, based on appendicolith hardness, correlated with radiographic density values observed in CT images (Spearman coefficient 0.74, $p < 0.001$). For comparison, we also measured HU values and diameters in patients whose appendicoliths were not collected for analysis. The median attenuation of collected appendicoliths was 187.0 HU (37–730 HU), while uncollected appendicoliths exhibited a median attenuation of 222.0 HU (75–1293 HU). The

difference in attenuation between the two groups was not statistically significant (p=0.52). Collected appendicoliths were significantly larger in size compared to the uncollected appendicoliths (p = 0.0026, median 11.0 mm vs. 8.0 mm).

In addition to the morphological characterization, the three-dimensional architecture of six representative appendicoliths (two from each class) was analyzed using micro-CT imaging. Both the visible structure on cross-sectional surfaces and the X-ray attenuation patterns observed in the micro-CT slices showed that appendicoliths in classes 2 and 3 exhibit a concentrically layered structure surrounding a central core. In contrast, the majority (71%) of class 1 appendicoliths (12/17) lacked both a distinct core and the layered structure. Micro-CT imaging further demonstrated that the dense, outer surface of class 3 appendicoliths displayed high X-ray attenuation, resulting in a continuously bright appearance, while the softer, class 1 appendicoliths did not exhibit an apparent outer layer. The cores of most appendicoliths were softer, as indicated by lower attenuation, which appeared darker in the cross-sectional micro-CT images.

The relationship between appendicolith classification and the severity of appendicitis was assessed. Among patients with an appendicolith and other findings of complicated appendicitis, 23.8% (5/21) had a class 3 appendicolith. By comparison, class 3 appendicoliths were identified in 15.0% of patients with appendicitis presenting only with an appendicolith, without other findings of complication (p = 0.85). Likewise, there was no statistically significant association between appendicitis severity and appendicolith size ((maximum length (OR 1.03, 95% CI 0.91–1.17, p = 0.60), and diameter (OR 1.05, 95% CI 0.84–1.30, p = 0.68) or weight (OR 1.00, 95% CI 0.99–1.00, p = 0.45)).

5.2.3 Elemental composition of appendicoliths

Elemental distribution on the appendicolith dissection surfaces showed of calcium and phosphorus as the primary components. The mean weight percentage of these elements increased with appendicolith hardness, while the calcium-to-phosphorus atomic ratio ranged from 1.9 to 4.3. Trace amounts of titanium, iron, manganese, and copper were detected in all samples. Spatial variations in calcium, phosphorus, and potassium distributions were observed within most appendicoliths. Detailed analysis of core and shell regions of appendicoliths showed compositional differences between these areas. CHN analysis of appendicoliths showed that the estimated average percentages by weight of organic proportion was 44.4% for class 1, 33.8% for class 2, and 36.4% for class 3.

5.2.4 The microbiome of appendicoliths in comparison to appendiceal microbiome (IV)

Study IV examined 14 appendicoliths from adult patients with CT-confirmed and pathologically diagnosed appendicitis described in chapter 5.2.1 (Figure 3). Microbiome profiles of the appendicolith, appendiceal lumen (swab), and wall (biopsy) were analyzed with a total of 41 samples (one biopsy was unavailable). Associations between microbiome composition, appendicitis severity, and appendicolith hardness were evaluated.

The metagenomic analysis resulted to an average of 42 million reads for appendicolith samples, 72 million reads for biopsy and 63 million reads for lumen samples, though human DNA presence reduced microbial reads to 19M, 10M, and 9M, respectively. ZymoBiomics standards confirmed accuracy, detecting all expected species except *Bacillus subtilis*. The appendicolith microbiome comprised 17 phyla, 436 genera, and 712 species, with Firmicutes (59.7%), Bacteroidetes (22.0%), Actinobacteria (15.4%), and Proteobacteria (1.7%). Key genera included *Clostridiaceae* (unclassified), *Alistipes, Eggerthella, Lawsonella*, genus GGB40351 from phylum Firmicutes, Enterocloster, and Bididobacterium. Appendicoliths exhibited higher species richness than lumen ($p=1.49 \times 10^{-5}$) and biopsy ($p=2.33 \times 10^{-6}$), likely due to greater microbial reads. Of 702 identified species, 441 were unique to appendicoliths.

The microbiome profile showed significant variation based on sample type, appendicolith hardness, and severity, as analyzed using dbRDA at the species level. The composition of the appendicolith microbiome was distinct from that of the appendiceal lumen and biopsy, with sample type accounting for 20.0% of the observed variance. Certain bacterial species, including *Clostridiaceae bacterium Marseille-Q4149*, *SGB15090* (family *Clostridiaceae*), *Eggerthella lenta*, GGB40351_SGB14301 (phylum *Firmicutes*), and *Lawsonella clevelandensis*, were more abundant in appendicolith samples. Conversely, appendicoliths had a lower relative abundance of species such as *Prevotella nigrescens*, *Paraburkholderia fungorum*, *E. coli*, *Porphyromonas endodontalis*, and *B. fragilis* compared to lumen or biopsy.

Differential abundance analysis with Maaslin2 confirmed findings from dbRDA. *E. lenta* was enriched in appendicoliths (4.9% vs. 0.8% and 1.1% in lumen and biopsy samples, p=0.0289). Additionally, *Collinsella aerofaciens* was also higher in appendicoliths (p=0.0289), while *E. coli* was more abundant in lumen (p=0.0289) and GGB9713_SGB15249 (*Oscillospiraceae*) in biopsies (p=0.0289).

6 Discussion

6.1 Microbial differences between uncomplicated and complicated appendicitis

Appendix has a distinct microbiome composition (She et al., 2024). NGS based studies within last ten years have shown that the non-inflamed appendiceal microbiome is predominantly composed of three major phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, and with smaller proportions of *Actinobacteria* and *Fusobacteria.* These NGS studies consistently demonstrate that the appendiceal microbiome is rich, diverse, and highly variable between individuals (Arlt et al., 2015; Fonnes et al., 2024; Guinane et al., 2013; Jackson et al., 2014; Munakata et al., 2021; Oh et al., 2020; Salo et al., 2017; Schülin et al., 2017; Yuan et al., 2021; Zhong et al., 2014). During appendicitis several changes in the appendiceal microbiome have been observed, particularly in beta diversity and in the abundance of bacterial species. However, the specific taxa associated with appendicitis differ between these studies. To date, only two studies focusing on adult patients have directly compared appendiceal microbiome in uncomplicated and complicated appendicitis, both utilizing metagenomic sequencing. The first study compared 9 uncomplicated and 10 complicated cases, with the analysis primarily emphasizing comparisons between culture and sequencing results (Yuan et al., 2021). The second study analyzed 26 uncomplicated and 16 complicated cases (Fonnes et al., 2024). Additionally, two earlier studies, each involving fewer than 10 adult patients, included both uncomplicated and complicated cases but did not systematically compare these groups (Arlt et al., 2015; Guinane et al., 2013).

In study II 118 patients with confirmed acute appendicitis microbial composition of the appendiceal lumen between uncomplicated (n=41) and complicated (n=77) acute appendicitis were studied. The overall appendiceal microbiome composition in this study was relatively well in line with other studies on appendiceal microbiota composition at phylum, genus and species level. Further, we corroborated findings showing high variability in appendiceal microbiome composition across different

forms of appendicitis (Fonnes et al., 2024; Guinane et al., 2013; Munakata et al., 2021; Oh et al., 2020; Yuan et al., 2021). Alpha diversity (e.g., Shannon and Chao1 indices) was higher in complicated acute appendicitis compared to uncomplicated acute appendicitis. Beta diversity further revealed distinct microbial profiles between the groups, indicating divergent microbial ecosystems potentially driving different disease severities. In uncomplicated acute appendicitis, gram-negative *Aggregatibacter* species, particularly *A. segnis* and *A. aphrophilus*, were enriched. These bacteria are members of the HACEK group known for causing infective endocarditis and other infections (Nørskov-Lauritsen, 2014). In addition, uncomplicated acute appendicitis showed higher abundance of *Veillonella parvula* accompanied with an unidentified *Streptococcus* species, both linked to gastrointestinal and oral microbiota (Said et al., 2014; van den Bogert et al., 2013). Previous culture-based studies report *Streptococcus* species as one the most frequent findings in appendicitis along with *E. coli (Jeon et al., 2014; Rautio et al., 2000)*. Conversely, complicated appendicitis exhibited a higher abundance of oral pathogens such as *Porphyromonas endodontalis*, *Dialister* species, and *Phocaeicola abscessus*. These species have been previously associated with complicated acute appendicitis in pediatric populations (Blohs et al., 2023; Jackson et al., 2014; Schülin et al., 2017; Zhong et al., 2014). *P. endodontalis* has been implicated in polymicrobial infections in the oral cavity and is known for its capacity to produce virulence factors that may exacerbate inflammation (Bedran et al., 2012; van Winkelhoff et al., 1992). These characteristics suggest a potential role for *Porphyromonas* in the pathogenesis of complicated acute appendicitis, where the microbiome undergoes a dysbiotic shift that may promote infection.

Individual samples from both groups showed very low diversity dominated by a single bacterial species, often exceeding 50% of relative abundance. Such predominance may play a significant etiological role, potentially indicating infection by the dominant species. On the other hand, the enrichment and dominance can be a consequence rather than a cause of inflammation. Species that can thrive in infection due to their metabolic traits are likely to dominate. In the appendix, bacterial species that dominate during infection likely possess specific metabolic traits that enable them to outcompete other members of the microbiome (Zeng et al., 2017). One critical factor is the ability of these bacteria to exploit the appendix's mucus layer as a nutrient source. This, in turn, may exacerbate the infection by depleting the protective mucus layer on the intestinal surface (Johansson et al., 2011). Both uncomplicated and complicated acute appendicitis were dominated by *B. fragilis* or *Escherichia*. *A. aphrophilus*, *A. segnis*, and unidentified species from genus *Streptococcus* were predominant only in uncomplicated acute appendicitis. While unidentified *Fusobacterium* species emerged as the predominant species exclusively in complicated acute appendicitis.

Fusobacterium species are considered to be noteworthy members of the appendiceal microbiome in appendicitis etiology. Some studies have shown that fusobacteria would be associated with the more complicated course of appendicitis (Arlt et al., 2015; Blohs et al., 2023; Guinane et al., 2013; Salo et al., 2017). However, conflicting results have shown especially in studies on adults that *Fusobacterium* is equally abundant in normal vs. inflamed and in uncomplicated vs. complicated acute appendicitis (Fonnes et al., 2024; Munakata et al., 2021; Oh et al., 2020). In this study, *Fusobacterium* genus was prevalent in both study groups and there was no statistically significant difference in the abundance of *Fusobacterium* species. This suggest that fusobacteria might have different relevance in adult and pediatric appendicitis.

One possibly important factor in appendiceal microbiome changes and in the onset of appendicitis might be the presence of an appendicolith. In majority of cases, appendicolith causes mechanical obstruction of the appendiceal lumen (Ranieri et al., 2020) preventing normal blood flow or nutrients to the appendiceal lumen. Thus, appendicoliths may play a significant role in the pathophysiology of appendicitis and in the development of microbial changes. As discussed in the literature review, appendicoliths are not necessarily even mentioned or included in the appendicitis classification in studies on appendiceal microbiome. Future research on the appendiceal microbiome should consider incorporating the presence of appendicoliths into their analyses.

6.2 Appendicolith appendicitis

6.2.1 Appendicolith classification and appendicitis (III)

Appendicoliths have been recognized since 1886 (Fitz, 1886), though their definition and clinical significance was not truly identified and still remains debated. Early studies linked appendicoliths to more severe appendicitis (Carras & Friedenberg, 1960; Forbes & Lloyd-Davies, 1966). While studies on incidental appendicoliths found on imaging do not seem to increase appendicitis risk (Khan et al., 2018), their presence in acute appendicitis is associated with worse outcomes, including perforation, gangrene, and treatment failure in non-operative management (Flum et al., 2020; Vons et al., 2011; Yoon et al., 2018). According to appendicitis treatment guidelines, surgery is the recommended treatment option for patients with uncomplicated appendicitis and appendicoliths (Di Saverio et al., 2020). Advances in imaging, particularly CT, have improved preoperative identification of complicated appendicitis (Kim et al., 2018). Variability in appendicolith definition and criteria complicates accurate detection (Weitzner et al., 2023). In 1966, Forbes

and Lloyd-Davies provided a detailed description of the various types of fecal concretions observed in the appendix and classified them into three categories based on physical properties and hardness. These categories were: (1) faecal pellet, (2) calcified faecolith, and (3) calculus (Forbes & Lloyd-Davies, 1966).

The aim of study III was to revisit and update the 1966 appendicolith classification and characterization. In addition, the characterization and updated classification of appendicolith was complemented with modern structural and elemental analysis using micro-CT and micro-XRF. This appendicolith characterization and classification was performed in a clinically well-defined patient cohort with CT diagnosed and histopathologically confirmed acute appendicitis. All appendicoliths in our classification, including the soft appendicoliths (class 1), corresponding to faecal pellets in the original 1966 classification, were visible by CT. This highlights that the whole spectrum of appendicoliths from soft to stony are CT visible with potential clinical relevance in the treatment options for uncomplicated acute appendicitis. The classification of appendicoliths based on the hardness of the outer surface correlated with the radiographical density of appendicoliths expressed as Hounsfield units (HU). The CT attenuation range detected was 37 HU to 730 HU. To uniform the nomenclature and to make appendicolith definition cleared Weitzner et al. proposed defining a CT-visible appendicolith as having a maximum HU value at least 180 HU above the appendiceal lumen or wall (Weitzner et al., 2023). However, in our study with CT-visible appendicoliths and the smallest HU value being 37, the suggestion of detection threshold based on HU values seems impractical. Taken together, appendicolith characteristics provide valuable insights into their classification and clinical detection. Appendicolith could be used as a term for all appendiceal concretions, regardless of hardness, calcification or HU values. This would in future enable better comparison of different studies assessing patients with appendicolith appendicitis.

In this study III, there was no statistically significant association with appendicolith characteristics (hardness, diameter, weight) with the severity of appendicitis. Among the 41 patients approximately half (48.8%) had uncomplicated appendicitis without gangrene or perforation, while the other half (51.2%) presented with appendicolith appendicitis with complications. Our analysis of appendicitis severity across different appendicolith classes showed that while class 3 appendicoliths were slightly more prevalent in group of appendicolith appendicitis with complications, no statistically significant relationship was found between appendicitis severity and appendicolith hardness. Similarly, the size of the appendicolith (longest diameter, width, or weight) showed no association with disease severity. Increasing number of other studies have examined how appendicolith characteristics influence appendicitis severity. Contrary to our findings, Ishiyama et al. identified an appendicolith size exceeding 5 mm and its

location at the base of the appendix as factors associated with gangrenous appendicitis (Ishiyama et al., 2013). Similarly, a pediatric study reported that an appendicolith with a maximum diameter of 5 mm or more was a significant risk factor for perforated acute appendicitis (Yoon et al., 2018). The conflicting result may be due to limited sample size in our study. In the most recent studies by our group and others with larger sample size, larger appendicolith size has been associated with complicated (perforation, gangrene, an abscess) appendicitis (Kaewlai et al., 2024; Sula et al., 2024).

6.2.2 Insights into appendicolith composition and formation

Study by Williams et al. more than hundred years ago, described laminated concretions in the appendix, hypothesizing that their formation was driven by fatty secretions from the intestinal mucosa. These concretions were composed of insoluble calcium soaps of saturated fatty acids, fats, and inorganic salts like calcium and phosphate (Williams et al., 1907). Subsequent studies refined this understanding by identifying calcium phosphate, rather than calcium soap, as the primary inorganic component, organic fraction consisting of palmitic and stearic acid, koprosterol, and smaller amounts of cholesterol (Maver & Wells, 1921). The heterogeneity of these concretions, including their layered structure and variability in calcium and organic content, was later corroborated (Shaw, 1965). After paucity, Prieto et al. analyzed pediatric appendicoliths and provided a comprehensive profile of their composition. Findings confirmed the presence of both inorganic elements—primarily calcium and phosphorus—and a diverse array of organic compounds, including fatty acids palmitic and stearic acid, consistent with earlier studies (Prieto et al., 2022),

In study III, we observed that most class 2 and class 3 appendicoliths exhibit a distinct layered structure, visible both in cross-sectional imaging and micro-CT. This concentrically layered morphology shown implies that appendicoliths form layer by layer inside the appendix, supporting the idea proposed as early as 1907 that appendicoliths might not be merely faecal concretions but instead would be the result of a more intricate biological and chemical process. By using micro-XRF imaging, we confirmed that calcium and phosphorus were the predominant inorganic elements across all classes, aligning with previous studies (Maver & Wells, 1921; Prieto et al., 2022). In addition, we showed that the proportion of calcium increased with appendicolith hardness. Notably, the elemental maps revealed distinct core and outer layer regions in many appendicoliths, reinforcing the notion of layered growth. The absence of layering in many class 1 appendicoliths, along with their smaller size and lower weight, suggests that these softer concretions may represent an earlier stage of appendicolith development. Interestingly, previous studies have observed that appendicoliths tend to be larger in patients with appendicitis compared to those in

normal appendices (Hawkins et al., 2022; Khan et al., 2019). These findings provide intriguing further insights into appendicolith formation, even though definitive conclusions about the mechanisms of appendicolith formation cannot be drawn from this study. A considerable heterogeneity in external appearance, cross-sectional structure, and elemental composition was observed, even within each class. This variability may indicate that appendicolith formation may follow multiple pathways. Beyond our study design remains the knowledge gap of actual time of appendicolith formation and/or association of symptoms. When thinking about the progression of inflammation and histopathological changes as well as the concept of consecutiveness of the complicated acute appendicitis, it would be intriguing to know the effect of time.

6.2.3 Appendicolith microbiome composition

Appendicoliths are not only critical in the clinical characterization of appendicitis but may also hold insights into the pathophysiology and microbial dynamics of this common surgical condition. Despite their clinical relevance, the understanding of the appendicolith formation and the precise role of appendicoliths in the onset and progression of appendicitis remains poorly understood. A prevailing theory suggests that appendicolith obstructs the appendiceal lumen, causing increased intraluminal pressure, tissue ischemia, and subsequent bacterial invasion (Wangensteen & Dennis, 1939). However, the primary processes underlying appendicolith formation might involve bacteria and their interaction with the appendiceal microbiome. For example, in gall stone formation, bacteria have an active role in the stone development (Kose et al., 2018). The traditional obstruction model for appendicolith pathogenesis might therefore be complemented by a microbial-related view. This involves an idea that appendicolith formation is affected by bacterial activity. Similarly, bacteria within the appendix could influence appendicolith formation through their metabolic activities and biofilm production.

Using metagenomic sequencing, we characterized the bacterial composition of appendicoliths in comparison to the appendiceal lumen and wall microbiota. Our findings revealed distinct microbial signatures, with specific taxa potentially contributing to appendicolith formation. The enrichment of *E. lenta* in appendicolith samples is particularly noteworthy. As a metabolically versatile anaerobe, *E. lenta* is capable of adapting to diverse gut environments, including conditions imposed by inflammation. Its known associations with Th17-mediated inflammation and its ability to process bile acids and host-derived compounds suggest it may actively contribute to the inflammation and structural formation of appendicoliths (Noecker et al., 2023; The et al., 2023). Other taxa from phylum *Actinobacteria*, such as *Lawsonella clevelandensis* and *Collinsella aerofaciens*, were also associated with

appendicoliths, implicating the phylum *Actinobacteria* in the microbiological dynamics of this condition.

Interestingly, while *E. coli* and *B. fragilis*, common residents of the appendiceal microbiome, were abundant in lumen and biopsy samples, they showed a negative association with appendicoliths. This distinction highlights potential differences in bacterial ecology and pathogenesis between appendicolith-associated and non-appendicolith appendicitis. Additionally, species such as *Alistipes onderdonkii* and *P. endodontalis*, previously linked to appendicitis (Blohs et al., 2023; Jackson et al., 2014; Rautio et al., 2003; Schülin et al., 2017), were prevalent across sample types and warrant further investigation to clarify their roles in disease progression.

Appendicoliths exhibited the highest bacterial species richness among sample types, possibly reflecting their layered structure and the ability to trap past and present microbiota. This observation aligns with previous findings of distinct elemental and morphological layers in harder appendicoliths discussed in chapter 6.2.2. Future studies should focus on spatially resolved sampling to differentiate microbiota composition across appendicolith layers, which may shed light on their formation process. Culturing appendicolith samples could also help to determine whether bacteria within these structures are viable and actively contribute to their development or merely become incidentally entrapped.

In conclusion, this first comprehensive characterization of the appendicolith microbiome highlights distinct bacterial profiles, particularly the role of *E. lenta*, that may influence appendicolith formation and appendicitis pathogenesis. These findings warrant further research into the microbiome associated with appendicoliths.

6.3 Limitations

Our study has several limitations. Like many other studies on the appendiceal microbiome, our research lacks a healthy control group as appendectomy on healthy controls would not naturally be ethical. As a result, we could only compare uncomplicated and complicated acute appendicitis without knowing the baseline composition of the normal microbiota in the appendiceal lumen or wall. In study II a significant portion of samples had to be excluded due to inability to create amplicon PCR libraries or due to low sequencing read counts. This reduced the sample size and may have affected the comprehensiveness of the microbiome analysis. In study IV the variability in sequencing depth across sample types may have influenced the detection of less abundant taxa. Whole-genome amplification in some cases, while necessary to maximize DNA yield, could also introduce bias. The proportion of host reads in appendiceal lumen sample was almost as high as in appendiceal biopsies which might be because of the infiltration of immune cells (Nelson et al., 2019).

In study IV, the number of samples included in the microbiome analysis in limited. The small sample size combined with the inherent "spikiness" of appendiceal microbiome data (where a single dominant microbial profile can skew results) pose challenges for beta diversity and differential abundance analyses. This can result in overrepresentation of certain taxa, limiting the robustness of the conclusions. Microbiome studies in appendicitis research, in general, have faced challenges related to sample size due to the difficulty in obtaining microbiological samples in the acute care surgery setting. The high variability in microbiome data influences the number of samples needed to achieve more reliable and certain results. Additionally, diet and other possible important important confounders in microbiome studies, was not sufficiently controlled in study II and IV which may have influenced the microbiome results

In study III the number of patients included in the study, particularly within subgroups according to the severity, is relatively modest. In study IV the small sample size (14 patients) is a notable constraint. Small sample size limits the statistical power and generalizability of the findings to larger and more diverse populations. A common limitation with appendicolith analysis in study III and IV include the lack of prospectiveness, which may introduce bias and inconsistencies in data collection.

In study III, 41 patients out of 78 patients had appendicolith available for characterization and analysis. The inability to retrieve a significant number of the eligible appendicoliths constitutes a major limitation of the study leading to small sample size. This was due to the acute care surgery setting of the study creating major challenges for patient enrolment and sample collection.

In study III and study IV only a subpopulation of appendicoliths were analyzed in depth. Even though appendicoliths were selected to be representative of the specific class, this might have caused selection bias especially due to the heterogeneity of the appendicoliths.

In study IV functional characteristics of microbial communities, such as their metabolic capabilities or interactions with host tissues, were not comprehensively examined. Study also lacks the spatial resolution in microbiome analysis as we aimed to maximize the DNA yield and wanted to capture the entire appendicolith microbiome. Despite this we had to use whole genome amplification for four samples, which can introduce bias to the microbiome analysis.

6.4 Future perspectives

This study provides basis for future investigations into the microbiological underpinnings of uncomplicated and complicated acute appendicitis including appendicoliths and their role in appendicitis. In the development of appendicitis treatment and especially in considering symptomatic treatment, increased understanding of microbiological factors, inflammation, and infection in appendicitis etiology are needed. Future studies would need to overcome limitations related to sequencing depth to reach strain level resolution in the microbiome analysis and to be able to estimate the functional properties of appendiceal microbiome.

The observed spatial variability in elemental distribution and structural properties, alongside with the distinct appendicolith microbiome, underscores the intricate and multifactorial nature of appendicolith formation. This complexity highlights the need for additional studies to explore appendicolith formation. A crucial direction for future work lies in elucidating patient-related risk factors that predispose individuals to appendicolith formation. Factors such as diet, genetics, immune function, and alterations in gut microbiota composition may play a significant role.

Cumulative antibiotic exposure is associated with an increased risk of new-onset IBD, particularly UC and CD, with the strongest associations linked to broad-spectrum antibiotic use (Nguyen et al., 2020). These findings provide an example of the impact of antibiotic-induced microbiome alterations on chronic disease pathogenesis. Moreover, antibiotic use contributes to antimicrobial resistance, which is of major health concern at both individual and societal levels (Naghavi et al., 2024). Thus, the safety and efficacy of symptomatic treatment with the potential to reduce antibiotic use when taken into clinical practice, should be studied further. The Appendix has been proposed to be a useful organ for the gut microbiome in restoring it after crisis like antibiotic use and protecting from severe *C. difficile* infection (Clanton et al., 2013; Yong et al., 2015). Further, the appendix is recognized as an immune organ contributing to the development of the immune system (Gebbers & Laissue, 2004; Gorgollón, 1978; Spencer et al., 1985). These functions are at least temporarily impacted by the antibiotic treatment of appendicitis, but also and irreversibly by the appendectomy. Consequently, the surgical and nonsurgical treatment of the appendix might have lifelong consequences that might be partly mediated through the gut microbiota. Recent advances in appendicitis research indicate that uncomplicated cases can be safely treated with antibiotics (Flum David et al., 2020; Salminen, Paajanen, Rautio, Nordström, et al., 2015; Sippola et al., 2021). However, the short- and long-term effects of both surgical and nonsurgical treatments on the gut and appendiceal microbiome remain largely unexplored. Given the high incidence of appendicitis, understanding the long-term health effects

of appendicitis treatment, particularly those mediated by gut microbiota, should be addressed in future research. The ongoing studies of MAPPAC trial evaluate antibiotic and placebo effects on gut microbiota composition and antimicrobial resistance.

7 Conclusions

1. The MAPPAC trial protocol was successfully designed and patient recruitment resulted in a large number of subjects (308 in total) compared to the previous studies, allowing a comprehensive research of appendicitis etiology. Studies II-IV were based on MAPPAC trial.
2. Uncomplicated and complicated acute appendicitis were associated with distinct appendiceal microbiome profiles, supporting the hypothesis that uncomplicated and complicated appendicitis represent distinct clinical entities rather than a single disease spectrum. However, in both disease forms dysbiotic appendiceal microbiome was observed and characterized by samples with low diversity and dominance of opportunistic pathogens.
3. The 1966 appendicolith classification was described before modern CT era. This study confirmed the categorization of CT-visible appendicoliths into three distinct classes. This study demonstrated the clinical relevance of even the softest appendicoliths, which were detectable on CT. Harder appendicoliths (class 2 and 3) exhibited layered inner structure, but this structure was absent in soft appendicoliths.
4. This study confirmed calcium and phosphorus as the main inorganic elements in all appendicolith classes, with calcium proportion increasing with hardness. Elemental maps highlighted distinct core and outer layers. This study identified several bacterial species, including *E. lenta* that were associated with appendicoliths. This study expands our understanding on appendicolith microbiome and the possible mechanisms of appendicolith formation.

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