

Full Paper

Analysis of appendectomy samples identified dysbiosis in acute appendicitis

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Appendicitis is the most common cause of sudden-onset abdominal pain requiring surgery. Culture-independent techniques have revealed that the complex intestinal bacterial ecology is associated with various diseases. To evaluate differences in patient characteristics and gut microbiota distribution in patients with appendicitis, we enrolled 12 patients who underwent appendectomy for appendicitis (appendicitis group) and 13 patients who underwent ileocecal resection or right hemicolectomy for colon cancer (control group). Microbiota were analyzed using next-generation sequencing of surgical specimens from appendix swab samples collected postoperatively. Overall differences in the structure of the gut microbiota were evaluated using the α - and β -diversity indices, which were calculated using the weighted or unweighted UniFrac distance. Changes in the gut microbial distribution were taxonomically evaluated at the phylum and genus levels. The α -diversity of observed species was significantly different between patients with and without inflammation of the appendix. The appendiceal microbiome of patients with appendicitis exhibited the highest unweighted UniFrac distances. There were no significant differences at the phylum level. *Ruminococcus* ($p=0.02$) and *f_erysipelotrichaceae_g_clostridium* ($p=0.005$) were increased in the control group compared with the appendicitis group. This pilot study provides the first report of the correlation of the gut microbiota with the pathogenesis of appendicitis evaluated using mucus-origin sampling.

Key words: appendectomy, dysbiosis, appendicitis

INTRODUCTION

Acute appendicitis is the most common reason for acute surgical emergency worldwide, with an estimated lifetime risk of 7–8% [1]. Despite advances in diagnosis and treatment of the condition, little is known about the pathogenesis of acute appendicitis. Culture-independent techniques can identify bacteria on the basis of the nucleic acid sequences of 16S ribosomal RNA (rRNA) molecules and have recently revolutionized the understanding of the complex intestinal bacterial ecology associated with various diseases [2, 3]. Several studies on cancer have demonstrated an overabundance of *Fusobacterium*, *Alistipes*, *Porphyromonadaceae*, *Coriobacteridae*, *Staphylococcaceae*, *Akkermansia*, and *Methanobacteriales*

and a lack of *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, *Faecalibacterium*, *Roseburia*, and *Treponema* in patients with colorectal cancer [4]. However, the dysbiosis of inflammatory bowel disease is characterized by decreased bacterial diversity and reduced abundance of the phylum Firmicutes, with concomitant expansion of the phylum Proteobacteria [5].

Although the gut microbiota has often been suggested to be a pathogenetic element in appendicitis, surprisingly, there is little data available about this. Previous fluorescence in situ hybridization studies have reported that local invasion of *Fusobacterium* spp. underlies the majority of cases of suppurative appendicitis [6]. The presence of *Fusobacteria* in mucosal lesions is positively correlated with the severity of appendicitis, and the bacteria are completely absent in cecal biopsies from

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healthy patients and disease controls. The primary fecal bacteria, including *Bacteroides*, *Eubacterium rectale* (*Clostridium* cluster XIVa), *Faecalibacterium prausnitzii* groups, and *Akkermansia muciniphila*, are negatively correlated with the severity of appendicitis [7]. One study demonstrated that the prevalence of genera such as *Fusobacterium* could be linked to the severity of inflammation of the appendix [8]. The authors suggested that the microbial community of the appendix is subject to extreme variability and comprises diverse biota. However, analyses of biopsies and stool and fecal samples have led to questions regarding whether the microbial composition of appendices directly reflects the appendiceal microbiome because bacterial phyla differ among specific locations in the gut system, as reported by Peterson *et al.* [9]. Thus, we analyzed the microbial population of surgical specimens (appendix swab samples) via 16S rRNA gene sequencing using a next-generation sequencer. The present study was designed to investigate characteristics of the gut microbiota distribution in patients with appendicitis.

PATIENTS AND METHODS

In total, 12 patients who underwent surgery for appendicitis (appendicitis group) and 13 patients who underwent laparoscopic ileocecal resection or right hemicolectomy for colon cancer (control group) were included in the study. The study group included 8 males and 17 females, with a mean age of 28–86 years (median age, 73 years). Samples were obtained from 12 appendices removed via emergent laparoscopic appendectomy. Exclusion criteria were as follows: stage IV cancers and cancers located close to the appendiceal orifice. None of the patients received preoperative antibiotic treatment, and all patients underwent operation within 24 hr after the onset of symptoms of suspected acute appendicitis. The patients did not have a history of previous episodes, thus excluding chronic appendicitis, and did not use proton pump inhibitors or steroids.

We performed DNA extraction using DNeasy PowerSoil Kits (QIAGEN). Each library was prepared in accordance with the “Illumina 16S Metagenomic Sequencing Library Preparation Guide” with a primer set (27Fmod, 5'-AGR GTT TGA TCM TGG CTC AG -3', and 338R, 5'-TGC CTC CCG TAG GAG T -3') targeting the V1–V2 region of the 16S rRNA gene. The 251 bp paired-end sequencing of the amplicon was performed using a MiSeq. Obtained paired-end sequences were merged using PEAR (<http://sco.h-its.org/exelixis/web/software/pear/>). Subsequently, 30,000 reads per sample were randomly sampled using seqtk (<https://github.com/lh3/seqtk>) for taxonomic assignment. The sampled sequences were clustered into operational taxonomic units (OTUs), as defined by 97% similarity, using UCLUST version 1.2.22q. Representative sequences for each OTU were taxonomically classified using RDP Classifier version 2.2 with the Greengenes database (gg_13_8). The observed species, Chao1 value, and Shannon phylogenetic diversity index were calculated using the phyloseq package for R. The overall structure of the appendiceal microbiome was evaluated according to the presence or absence of inflammation using the β -diversity estimated using the UniFrac metric to calculate the unweighted UniFrac distances between samples, which were visualized using principal coordinate analysis (PCoA). The figures were generated using the QIIME version 1.9 software.

Statistical analysis

Statistical analyses were performed using JMP version 14 software (SAS Institute Inc., Cary, NC, USA). Categorical variables were compared using χ^2 or Fisher's exact tests, as appropriate. Continuous variables are presented as medians, and they were compared using the Mann-Whitney *U* test or analysis of variance. Observed species, Chao1 value, and Shannon phylogenetic diversity index were analyzed using the Wilcoxon test. Distances between samples were analyzed using analyses of similarity (ANOSIM).

Ethical approval

This study was performed in accordance with the ethical standards of the Committee on Human Experimentation of our institution.

RESULTS

Table 1 summarizes the patient clinical characteristics. Mean age was significantly different between the groups ($p=0.0002$). The pathology of appendicitis was catarrhalis in one patient, phlegmonous in five patients, and gangrenous in six patients. Appendicoliths were identified in four patients in the appendicitis group, but none were identified in the patients in the control group ($p=0.04$).

The α -diversity of the observed species was significantly different between patients with or without inflammation (Fig. 1). The appendiceal microbiome of patients with appendicitis demonstrated the highest unweighted UniFrac distances (Fig. 2).

Differences in the gut microbial structure taxonomically evaluated at the phylum level are illustrated in Fig. 3. The primary components of the appendiceal microbiome were *Bacteroidetes* (appendicitis group, 49.1%; control group, 33.7%), *Firmicute* (appendicitis group, 27.9%; control group, 31.6%), and *Proteobacteria* (appendicitis group, 15.3%; control group, 22.1%). The proportion of *Fusobacterium* was not significantly different between the groups (appendicitis group, 6.0%, control group, 8.5%). There were no significant differences with respect to *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Fusobacterium*, *Synergistetes*, and *Actinobacteria* at the phylum level.

Taxonomic changes in the microbial communities of patients with appendicitis evaluated at the genus level are illustrated in Fig. 4; no increase in the abundance of any genera was observed. *Ruminococcus* ($p=0.02$) and *f_erysipelotrichaceae_g_clostridium* ($p=0.005$) were increased in the control group compared with the appendicitis group.

Appendicolith-related appendiceal obstruction leading to appendicitis is a commonly encountered surgical emergency. A previous study demonstrated that patients with incidentally discovered appendicoliths did not develop appendicitis [10]. Preoperative computed tomography detected appendicitis with appendicolith. We examined the risk of appendicoliths using the same methods. There were no significant differences in the gut microbiome of patients with or without appendicoliths (data not shown).

DISCUSSION

Recently, it has been possible to successfully treat most patients with appendicitis with antibiotics alone. In the near

Table 1. Characteristics of the patients in the appendicitis and control appendix subgroups

	LpAppe (n=18)	LpAppe (interval) (n=6)	p value
Sex (M/F)	5/13	4/2	0.15
Age (median)	46	65	0.06
Stone	6	1	0.62
Catarrhalis/Phlegmonous/Gangrenous	2/7/9	6/0/0	0.0003

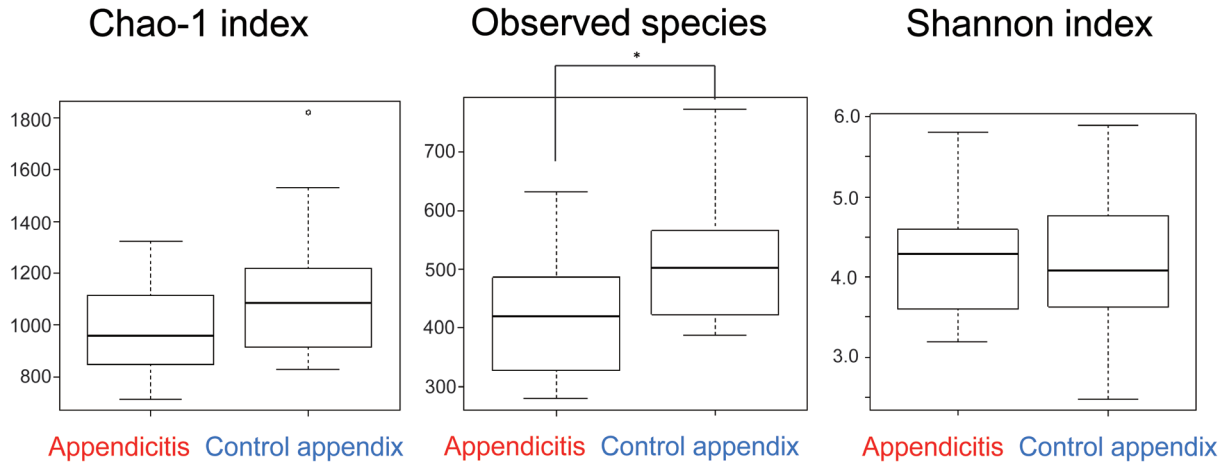


Fig. 1. Microbial communities of patients with or without inflammation of the appendix. The α -diversity indices are shown. Data are presented as the mean \pm standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

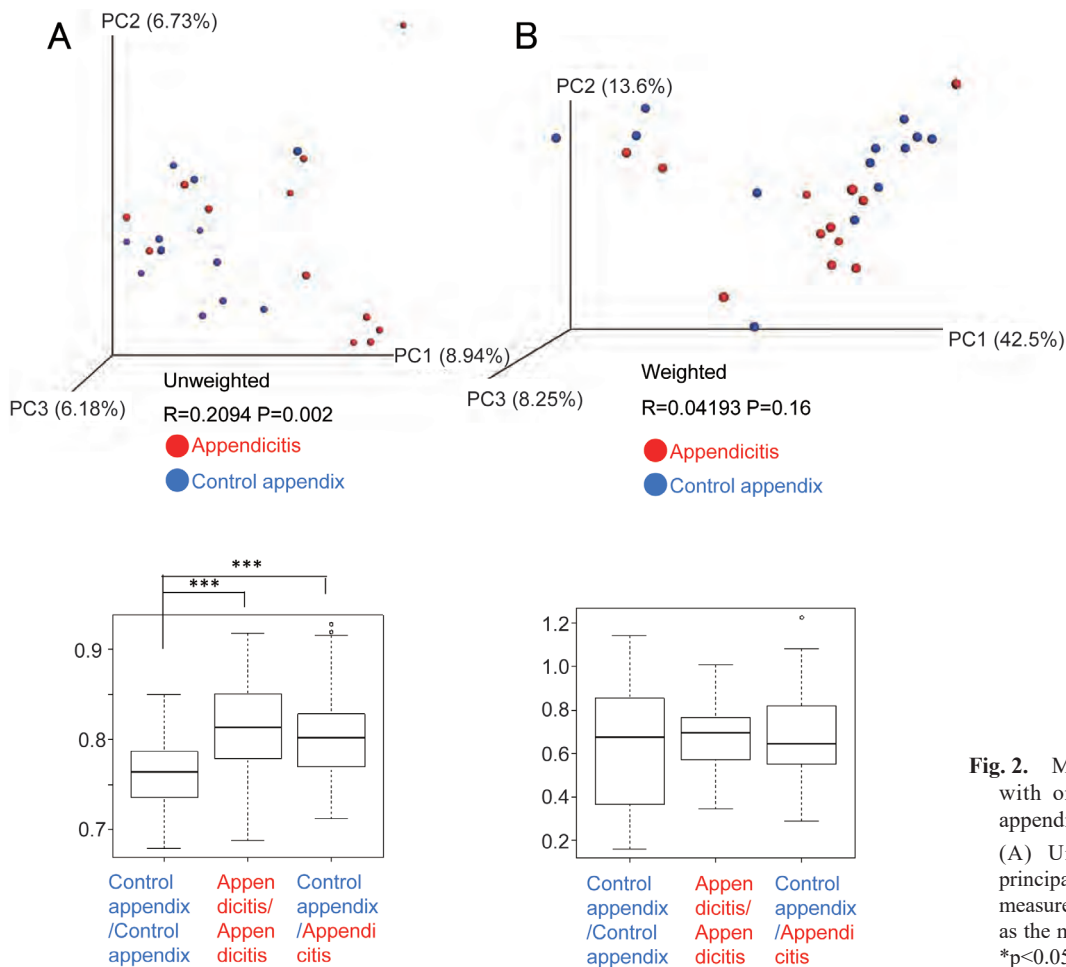


Fig. 2. Microbial communities of patients with or without inflammation of the appendix.

(A) Unweighted and (B) weighted principal coordinate analysis of β -diversity measures of all samples. Data are presented as the mean \pm standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

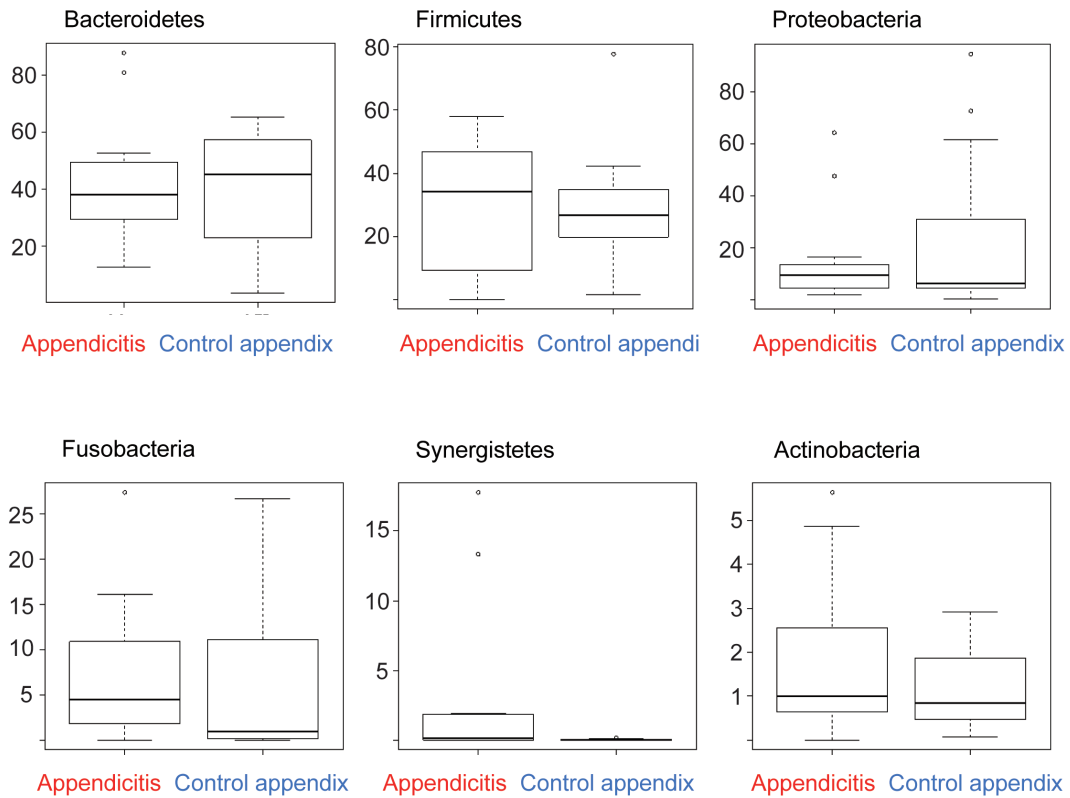


Fig. 3. Comparative analyses of the taxonomic composition of microbial communities at the phylum level. Data are presented as the mean ± standard error of the mean. Numbers in bars are percentages.

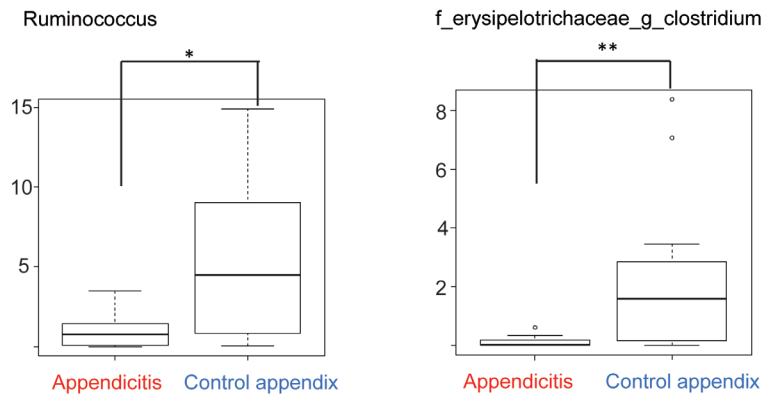


Fig. 4. Comparative analyses of the taxonomic composition of microbial communities at the genus level. Data are presented as the mean ± standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Numbers in bars are percentages.

future, it is possible that resection of the appendix may no longer be necessary for appendicitis. However, patients should be made aware of the failure rate of treatment with antibiotics alone, which is approximately 25–30% at 1 year with a requirement of readmission or surgery [11]. Furthermore, the etiology of the appendiceal microbiome cannot be revealed using antibiotics. Compared with previous studies, this study, for the first time, provided comparative data on regions of the appendix that are not the sites of inflammation.

Before patients were readmitted to hospital with recurrence of appendicitis, we recently recommended that this be handled as

chronic appendicitis and that the patients undergo appendectomy. To examine recurrence factors, appendix samples collected from patients with chronic appendicitis or recurrence of appendicitis should be analyzed to identify the causative microbiome of recurrence.

We compared the overall microbial diversity between groups using the unweighted and weighted UniFrac distance matrices. The unweighted UniFrac identified that there was a significant difference due to the large number of minor species found; however, the number of reads was small. On the other hand, because there was no significant difference in weighted UniFrac

distance, there was no difference in the composition of major bacterial species. Therefore, the appendicitis group had fewer individual differences in minor species than the control group.

Interestingly, we identified small differences among the sites in the appendix. *Ruminococcus* and *f_erysipelotrichaceae_g_clostridium* were increased in the control group compared with the appendicitis group. *Ruminococcus* appear to be host-associated with physiological and metabolic adaptations that allow them to survive within the gut environment. They can deconstruct and utilize a wide diversity of plant polysaccharides, with implications for both host health and potential exploitation for biotechnology [12]. *f_erysipelotrichaceae_g_clostridium* have been associated with high-fat diets, consistent with the strong positive correlation with dietary fat content and negative correlation with fat digestibility. Moreover, subsequent research showed that they were significantly reduced in inflammatory bowel disease and increased in colorectal cancer patients [13]. However, future work will be needed to further elucidate their versatile biology and intricate roles as key members of the gut microbial community.

Several members of *Bacteroides/Prevotella* can degrade host mucus, and increased abundance of them has been reported in inflammatory bowel diseases [14]. Guinane *et al.* reported that *Bacteroides* represent a small population in the context of appendicitis and suggested that *Fusobacterium* was linked to the severity of appendicitis in their study involving seven patients [8]. Senghor *et al.* reported that differences in the composition and diversity of the gut microbiota can be attributed to food products, dietary habits, geographical origin, and use of antibiotics [15, 16].

Appendicoliths were thought to be involved in the pathogenesis of acute appendicitis. The earliest description of incidentally discovered appendicoliths was published by Forbes and Lloyd-Davies in their study on appendicoliths that were associated with appendicitis. They recommended that prophylactic appendectomy be performed in cases where appendicoliths are incidentally found [17]. Thus, we speculated that appendicoliths have a specific appendiceal microbiome community. However, we did not identify any significant differences in terms of the appendiceal microbiome between patients with and without appendicoliths.

The present study utilized appendectomy samples obtained in the operating room. One advantage of this approach, as opposed to the use of stool samples, is that it prevents contamination. Endoscopic brush and biopsy samples do not reflect the microbiome of the appendix because endoscopy does not enable easy collection of appendix tissue.

This study has some limitations, which include its small sample size; limited investigation of the influence of diet; and the impact of potential biases, including the uneven distribution of gender, previous antibiotic use, dietary habits, and geographical origin within the study population. As more data are required to elucidate the mechanism underlying the occurrence of acute appendicitis, further studies are warranted. In addition, the control appendices were collected from cancer patients. There are possibly differences between appendices from normal patients and those from cancer patients (control). appendix. However, we have never been able to obtain normal appendix flora from healthy volunteers. Considering the importance of the data provided here and for identification of potential biomarkers, further investigation with purposely designed, large, confirmatory studies is warranted.

CONCLUSION

This pilot study provides the first report of the correlation of the gut microbiota with the pathogenesis of appendicitis.

DATA AVAILABILITY

Raw data were generated at Osaka University. Derived data supporting the findings of this study are available from the corresponding author on request.

AUTHOR CONTRIBUTIONS

Data acquisition (SM, HM, YT, KA, TH); analysis and interpretation of data (SM, MT, DM, SN); drafting of the manuscript (SM); critical revision of the manuscript (SM, KS, SW).

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CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest regarding the publication of this paper.

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