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Original Article

Culture-based bacterial evaluation of the appendix lumen in patients with and without acute appendicitis☆

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ABSTRACT

Purpose: Controversy exists over whether bacterial flora within the appendix differs between patients with and without appendicitis. To examine these potential differences, we cultured the appendiceal luminal microbiota of patients with and without acute appendicitis, and identified the bacterial species therein.

Methods: Fifty-seven patients with acute appendicitis and 37 patients without acute appendicitis who underwent curative resection of colorectal cancer and prophylactic appendectomies (control group) were included. Appendicitis patients were classified into the phlegmonous group or the gangrenous appendicitis group histopathologically. There was no patient with perforated appendicitis. Aerobic isolates were identified using standard identification schemata, and anaerobic isolates were identified according to the Japanese guidelines.

Results: There were no significant differences among the three groups in the median number aerobe species present per patient. However, the median number anaerobe species in the gangrenous appendicitis group was significantly higher than that of the control group and the phlegmonous appendicitis group. In addition, the incidence of patients with *Bacillus* species, *Fusobacterium nucleatum*, and *Bilophila wadsworthia* increased as the disease progressed from phlegmonous to gangrenous appendicitis.

Conclusion: The present results suggest that increased diversity of anaerobes and the translocation of *Bacillus* species, *F. nucleatum,* and *B. wadsworthia* are associated with the progression of acute appendicitis.

1. Introduction

Acute appendicitis is a common disease often requiring emergency surgery. The pathogenesis of appendicitis is classically described as the mechanical obstruction of the appendiceal lumen by a fecalith or lymphatic hyperplasia, followed by secondary stasis, an accumulation of undrained secretions, alteration and overgrowth of microbes, and epithelial damage [1,2]. Meanwhile, the mechanism may be identified in only around one-third of patients with appendicitis [3], and the luminal obstruction theory cannot explain all cases of appendicitis. There is growing evidence that mechanical obstruction is unlikely to be the primary cause of appendicitis [4] and that a specific appendiceal microbiota plays a key role in the pathogenesis of acute appendicitis [5,6].

Several studies have provided compelling evidence that the appendiceal microbiota differs between patients with and without appendicitis [5,7,8]. Baron et al. identified *Bilophila* species from cultures obtained from appendicitis tissue in 1989 [7]. Additionally, Swidsinski et al. identified *Fusobacterium*

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species as the major microbe responsible for acute appendicitis using rRNA-based fluorescence *in situ* hybridization [5]. In contrast, studies by Roberts and Salo et al. indicated no differences in appendiceal bacteria between patients with and without appendicitis [9,10].

The aim of the present study was to investigate whether the appendiceal microbiota differs between patients with and without acute appendicitis using bacterial culture methods. The specific methods used in our study include the following: (a) the appendiceal luminal content was examined in patients excluding those with perforated appendicitis; (b) not only aerobes but also anaerobes were cultured; and (c) the microbiome was compared according to histopathological perspective, preoperative antibiotic administration, and computed tomography (CT) findings of the appendix.

2. Patients and methods

2.1. Patients

This study was approved by our institutional review board (No. 2011-921), and informed consent was obtained from all included patients for prophylactic appendectomy and microbiological examinations of the appendiceal lumen.

Fifty-seven patients (34 men and 23 women) who were clinically diagnosed with acute appendicitis and underwent appendectomies at Japanese Red Cross Nagoya First Hospital (Nagoya, Japan) from July 2011 to April 2013 were included in the present study following their informed consent. The mean patient age was 35.2 years (range, 3–87). All the patients had histopathological findings consistent with acute appendicitis, and were classified into two groups according to the inflammation severity: (1) phlegmonous appendicitis, in which inflammatory cells invaded through the appendix wall without destruction of its architecture (Fig. 1A); (2) gangrenous appendicitis, in which inflammatory cells invaded the appendix with destruction of the wall architecture but without perforation (Fig. 1B). Consequently, sixteen patients had phlegmonous appendicitis, and 41 had gangrenous appendicitis. There was no patient with perforated appendicitis. Thirty-seven patients with colorectal cancer (CRC) (20 men and 17 women) in which prophylactic appendectomies were performed during the curative resection of CRC to reduce the subsequent risk of appendicitis served as a control group (mean age, 64.8 years; range, 31–84). Histologically, there was no inflammation in their appendixes. In addition, we recorded the histories of preoperative antibiotic administration and investigated hyperdense material in the appendiceal lumen suggesting a fecalith visualized by preoperative CT.

2.2. Data analysis

The number of aerobic and anaerobic bacterial species per patient and type of bacteria were compared among the control, phlegmonous appendicitis, and gangrenous appendicitis groups. In addition, they were compared according to preoperative antibiotics administration and hyperdense material in the appendiceal lumen visualized by preoperative CT.

2.3. Microbiological methods

After opening the appendiceal lumen, a Seed Swab (Eiken Chemical Co., Ltd., Tokyo, Japan) was used to obtain the luminal contents. The swab was promptly stored in an anaerobic transporter (Eiken Chemical) and delivered to the microbiological laboratory. The swab was cultured directly onto the following agar plates: sheep blood agar M58 (Eiken Chemical), DHL agar (Eiken Chemical), Brucella HK agar (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan), *Bacteroides* bile-esculin (BBE) agar (Kyokuto Pharmaceutical Industrial), and modified FM agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan). Sheep blood agar M58 and DHL agar were incubated at 35 °C overnight in a 5% carbon dioxide incubator and in normal air without carbon dioxide, respectively. Aerobic isolates were identified via standard identification schemata [11]. Brucella HK agar, BBE agar, and modified FM agar were incubated at 35 °C for 2 days with an AnaeroPack-Anaero Gas Generator (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) to produce an anaerobic atmosphere. The anaerobic isolates were identified according to the Guidelines for Examination of Anaerobic Bacteria 2012 by the Japanese Society for Clinical Microbiology [12]. The microorganisms not described in these guidelines were classified into four categories according to their Gram-staining characteristics (i.e., other anaerobic Gram-negative bacilli, other anaerobic Gram-positive bacilli, other anaerobic Gram-negative cocci, and other anaerobic Gram-positive cocci).

2.4. Statistical analysis

Continuous variables were expressed as medians (interquartile range: IQR). For multigroup comparisons, a Kruskal–Wallis nonparametric analysis of variance was performed, and the Mann–Whitney *U*-test with Bonferroni correction was used for post hoc comparisons. Categorical variables were analyzed using Chi-square and Fisher's exact tests as appropriate. All the tests were two-tailed, and $p < 0.05$ was considered statistically significant, except when the Mann–Whitney *U*-test with Bonferroni correction was used, in which case $p < 0.01$ was considered statistically

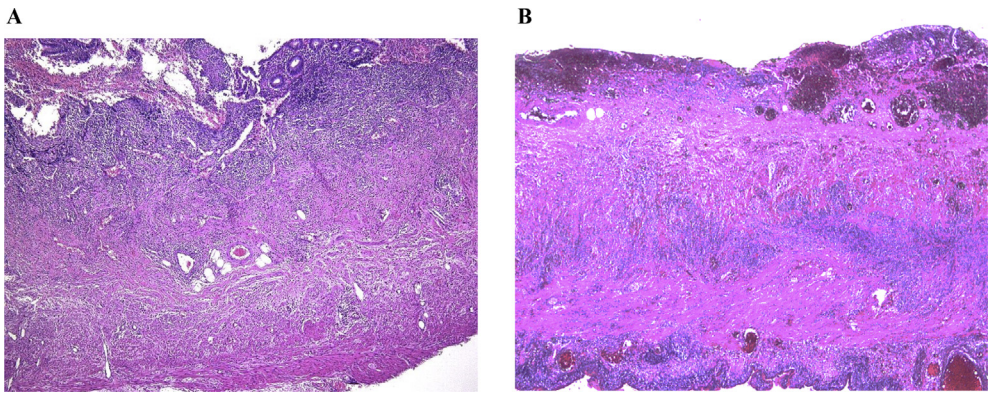

Fig. 1. Representative histopathological pictures of appendixes from patients with phlegmonous (A) and gangrenous appendicitis (B).

significant. StatView 4.5 software (Abacus Concepts, Berkeley, CA) was used to perform the statistical analyses.

3. Results

Fig. 2 shows the median number of aerobic and anaerobic bacterial species per patient isolated from the control ($n = 37$), phlegmonous appendicitis ($n = 16$) and gangrenous appendicitis groups ($n = 41$). There were no significant differences among the three groups in the median number of aerobe species per patient (control group, 1.0 (1.0–2.0) species; phlegmonous appendicitis group, 1.5 (1.0–2.0) species; gangrenous appendicitis group, 2.0 (1.0–3.0) species), while the median number of anaerobe species in the gangrenous appendicitis group (5.0 (4.0–6.0) species) was significantly higher than those of the control group (3.0 (1.0–4.0) species) and the phlegmonous appendicitis group (3.5 (1.3–4.8) species). The diversity of anaerobic species was significantly different between the three groups ($p < 0.001$).

Table 1 summarizes the microorganisms that were cultured from the three patient groups. For the aerobes, *Streptococcus* species and *Bacillus* species were frequently isolated from the gangrenous appendicitis group (29% and 20%, respectively). For the anaerobes, *Fusobacterium nucleatum* and *Bilophila wadsworthia* were isolated very frequently from the gangrenous appendicitis group (46% and 44%, respectively). The incidence of patients with *Bacillus* species, *F. nucleatum*, and *B. wadsworthia* increased with disease progression from phlegmonous to gangrenous appendicitis. The incidences of patients without these bacteria, namely, *Bacillus* species, *F. nucleatum*, and *B. wadsworthia*, were 77% (30/37), 62% (10/16), and 29% (12/41) in the control, phlegmonous appendicitis, and gangrenous appendicitis groups, respectively. On the other hand, *Escherichia coli* (57%, 69%, and 71%, respectively) and isolates of the *Bacteroides fragilis* group (70%, 69%, and 83%, respectively) were frequently observed in each group; that is, the incidence of these bacteria was not significantly different among the three groups ($p = 0.462$ and 0.333, respectively).

The proportions of patients with preoperative antibiotic administration in the control, phlegmonous appendicitis, and gangrenous appendicitis groups were 8% (3/37), 19% (3/16), and 24% (10/41), respectively. Notably, there were no significant differences in terms of the median numbers of aerobic and anaerobic bacterial species per patient in accordance with the preoperative antibiotic administration (Fig. 3). With the preoperative antibiotic administration, the detection rate of *B. wadsworthia* was found to be low in the gangrenous appendicitis group (10% vs. 55%, $p = 0.03$).

The incidences of patients with hyperdense material in the appendiceal lumen visualized on CT were 0% (0/37), 38% (6/16), and 59% (24/41) in the control, phlegmonous appendicitis, and gangrenous appendicitis groups, respectively. Further, the number of aerobic and anaerobic bacterial species per patient was significantly higher in patients with CT-hyperdense material than that in patients without CT-hyperdense material (Fig. 4). In the presence of CT-hyperdense material, the detection rate of *Bacillus* species was high in the acute appendicitis patients (27% vs. 4%, $p = 0.03$).

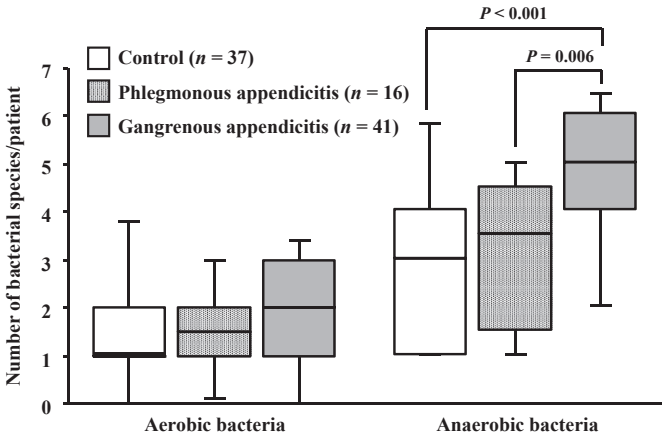

Fig. 2. The number of bacterial species per patient in the control, phlegmonous, and gangrenous appendicitis groups. Boxes represent the 25th–75th percentiles, with horizontal lines and whiskers indicating the median value and range, respectively.

4. Discussion

The present study demonstrated that the diversity of anaerobic species isolated from patients, as well as the incidence of *Bacillus* species, *F. nucleatum*, and *B. wadsworthia*, increased with disease progression. These results suggest that the growth of specific microorganisms, including *Bacillus* species, *F. nucleatum,* and *B. wadsworthia*, are associated with the progression of acute appendicitis. To the best of our knowledge, this is the first study to investigate the bacterial species including anaerobes in the appendiceal luminal content according to the progression of acute appendicitis.

Many studies have reported that *E. coli* and Gram-negative anaerobes were the primary organisms in acute appendicitis [13,14]. Furthermore, several studies have indicated that *Bacteroides* species play an important role in the progression of acute appendicitis [15,16]. In agreement with other studies [17,18], the present study showed that *E. coli* and *Bacteroides* species were frequently isolated from the appendiceal lumen; however, their incidence was similar in patients with or without acute appendicitis. The disparity in the results between the previous studies and our current study may be due to differences in the patient population, collected samples, and sampling methods. We have collected materials from appendiceal lumina without perforation. This is one of the specific features of the present study, in which the appendix lumina were not contaminated with materials from the abdominal cavity.

The present study showed that *F. nucleatum* was isolated more frequently from the appendix lumen as appendicitis progressed. *Fusobacterium nucleatum* is indigenous to the human oral cavity, as well as an invasive, adherent, and pro-inflammatory anaerobic bacterium [19]. Several studies have reported that the presence of *Fusobacteria* in mucosal lesions is positively correlated with the severity of acute appendicitis, and suggested that *Fusobacteria* plays a role in the invasive microbial process underlying acute appendicitis [5,6,8]. *F. nucleatum* can adhere to epithelial cells and invade them by exploiting the cell signaling and the cytoskeletal elements of the host cells [20,21]. Although the pathogenic mechanisms of *Fusobacteria* are complex, several toxins or secreted products, such as leucotoxin, endotoxin, hemolysin, hemagglutinin, and adhesion have been implicated as virulence factors.

B. wadsworthia has been reported as the third most common anaerobic organism in acute and advanced appendicitis [17,18]. In the present study, *B. wadsworthia* was isolated more frequently from the appendix lumen as appendicitis progressed. In addition, our study showed that the detection rate of *B. wadsworthia* was low in the gangrenous appendicitis group after antibiotic treatment, suggesting that *B. wadsworthia* can be controlled in the appendix by empiric antibiotic treatment. Since *B. wadsworthia* was initially isolated in 1989 from patients with appendicitis and related clinical conditions [7,17,18], the natural habitat of *B. wadsworthia* was assumed to be the gastrointestinal tract. *B. wadsworthia* can release lipopolysaccharide as an endotoxin, though its endotoxic activity is

Table 1
Microorganisms cultured from control, phlegmonous appendicitis and gangrenous appendicitis patients.

	Control (n = 37)	Phlegmonous appendicitis (n = 16)	Gangrenous appendicitis (n = 41)	*P* value
Aerobic microorganisms				
Escherichia coli	21 (57)	11 (69)	29 (71)	0.462
Klebsiella pneumoniae	4 (11)	2 (13)	9 (22)	0.395
Klebsiella oxytoca	2 (5)	2 (13)	2 (5)	0.551
Enterobacter species	2 (5)	0 (0)	1 (2)	0.774
Citrobacter species	1 (3)	1 (6)	3 (7)	0.701
Proteus species	2 (5)	1 (6)	0 (0)	0.211
Pseudomonas aeruginosa	4 (11)	4 (25)	8 (20)	0.358
Nonfermentative Gram-negative bacilli	3 (8)	0 (0)	1 (2)	0.389
Enterococcus faecalis	4 (11)	1 (6)	1 (2)	0.272
Enterococcus faecium	6 (16)	3 (19)	6 (15)	0.933
Enterococcus avium	2 (5)	0 (0)	0 (0)	0.47
Streptococcus species	5 (14)	0 (0)	12 (29)	0.023
Bacillus species	0 (0)	1 (6)	8 (20)	0.009
Anaerobic microorganisms				
Fusobacterium nucleatum	2 (5)	4 (25)	19 (46)	<0.001
Fusobacterium necrophorum	0 (0)	0 (0)	1 (2)	NA
Fusobacterium varium	5 (14)	3 (19)	7 (17)	0.81
Fusobacterium mortiferum	0 (0)	0 (0)	4 (10)	0.128
Bacteroides fragilis group	26 (70)	11 (69)	34 (83)	0.333
Bacteroides vulgatus	14 (38)	3 (19)	16 (39)	0.346
Bilophila wadsworthia	6 (16)	3 (19)	18 (44)	0.019
Pigmented *Prevotella/Porphyromonas* species	2 (5)	4 (25)	9 (22)	0.056
Non-Pigmented *Prevotella/Porphyromonas* species	5 (14)	0 (0)	8 (20)	0.151
Peptococcus species	2 (5)	1 (6)	1 (2)	0.662
Peptostreptococcus species	4 (11)	4 (25)	11 (27)	0.173
Veillonella species	1 (3)	0 (0)	1 (2)	NA
Clostridium species	1 (3)	0 (0)	1 (2)	NA
Other anaerobic Gram-negative bacilli	9 (24)	5 (31)	21 (51)	0.041
Other anaerobic Gram-positive bacilli	16 (43)	3 (19)	20 (49)	0.113
Other anaerobic Gram-negative cocci	0 (0)	1 (6)	4 (10)	0.144
Other anaerobic Gram-positive cocci	0 (0)	0 (0)	1 (2)	NA

Data are number of patients (%); NA, not applicable because of zero value.

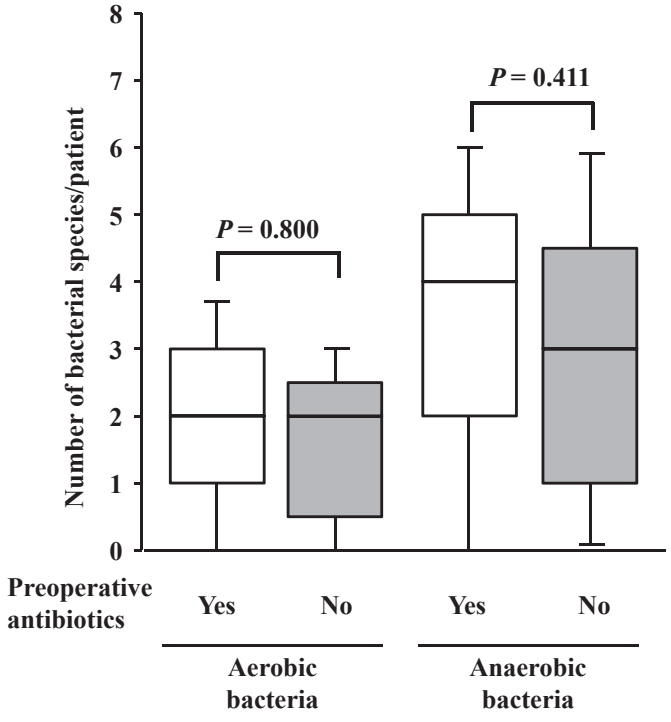

Fig. 3. The number of bacterial species per patient according to preoperative antibiotic administration. Boxes represent the 25th–75th percentiles, with the horizontal lines and whiskers indicating the median values and ranges, respectively.

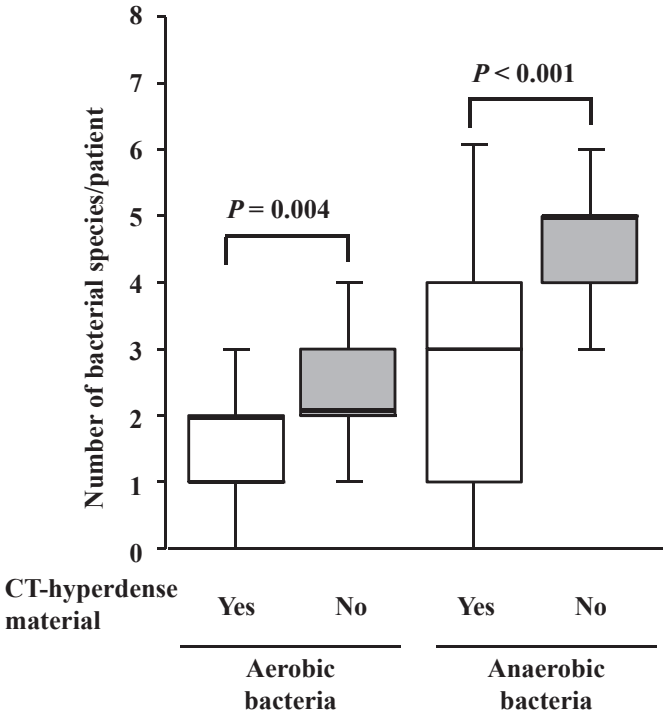

Fig. 4. The number of bacterial species per patient according to hyperdense material in the appendiceal lumen visualized by preoperative computed tomography. Boxes represent the 25th–75th percentiles, with the horizontal lines and whiskers indicating the median values and ranges, respectively.

relatively low compared with that of *E. coli* [22]. *B. wadsworthia* can also induce procoagulant activity, and its ability to adhere to human colonic epithelial cells is considered to be the first step of infection [22,23]. In addition, the metabolic end-product of dissimilatory sulfate and sulfite reduction—hydrogen sulfide—is produced by *B. wadsworthia*, and is well documented as pro-inflammatory and toxic to mucosal tissue at higher physiological doses [24]. These features of *B. wadsworthia* may contribute to its pathogenicity in acute appendicitis.

In the present study, *Bacillus* species were frequently found in patients with gangrenous appendicitis. In addition, the detection rate of *Bacillus* species was remarkably high in patients with CT-hyperdense material, suggesting a role of *Bacillus* species in obstructive appendicitis. *Bacillus* species are generally perceived to be inconsequential, and to have little clinical significance [25]. However, isolates of the *B. cereus sensu lato* group are known to produce a number of toxins involved in their ability to cause gastrointestinal diseases, as well as serious systemic diseases such as septicemia, endocarditis, peritonitis, liver failure, and meningitis [26]. The virulence factors produced by *Bacillus* species include necrotizing exotoxin-like hemolysins, phospholipases, delta endotoxins, collagenases, and proteases [25,27]. Furthermore, these bacteria are able to tolerate adverse conditions because of their endospore-forming abilities, and may proliferate in a wide range of environments, including the appendiceal lumen.

Given that the number of aerobes and anaerobes increased in patients with CT-hyperdense material in the appendiceal lumen, the increased number of bacterial species may have been induced by the appendiceal luminal obstruction. Regarding this, we hypothesized two underlying mechanisms: (a) a causative role of bacteria in the development of acute appendicitis and (b) bacterial growth caused by environmental alteration due to luminal obstruction, elevated intraluminal pressure, and circulatory disturbance.

We acknowledge that our study has some limitations. First, we could not quantify the amounts of bacteria present in the appendixes of the patients. Although the quantities of microorganisms in the appendixes of the patients may have been highly variable, this could not be evaluated in our laboratory. Further study is needed to reveal the contribution of the amount of bacteria present to disease progression. Second, we evaluated appendiceal microbiota using culture-based methods. Because difficult-to-culture bacteria may have been easily overlooked, the negative result does not eliminate the possibility of the presence of these species. Third, the control group did not comprise healthy subjects, and the appendices of the control group patients were simultaneously surgically resected for the treatment of CRC. Indeed, an association between CRC and gut microbiota has been suspected for decades [28]. Given that it is ethically impossible to prepare healthy controls for such a study, to our knowledge, this is the largest study so far to investigate the appendiceal bacterial flora in patients with and without appendicitis. Fourth, we could not identify *Bacillus* species to the species level. Therefore, it was not possible to distinguish pathogens related to disease progression from contaminating organisms. Further study is required to investigate which *Bacillus* species may play a role in appendicitis. Lastly, antimicrobial susceptibility testing of isolates was not performed. Information about the microbial antibiotic resistance profiles of these bacteria is important for the treatment of patients with appendicitis.

The frequent observation of *F. nucleatum* and *B. wasworthia* in gangrenous appendicitis indicates that these anaerobic microorganisms had translocated into the appendiceal lumen, and suggests their role in the progression of acute appendicitis. Because antibiotic administration has emerged as a reasonable first-line treatment strategy for patients with uncomplicated appendicitis [29], an understanding of the role of patient microbiome in the early phase of acute appendicitis is of substantial importance. *F. nucleatum* and *B. wadsworthia* produce β-lactamases and are susceptible to β-lactam-β-lactamase inhibitors, carbapenems, and clindamycin [30–32]; therefore, these antibiotics are indicated in patients with acute appendicitis.

Declarations of interest

None.

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Conflicts of interest

None.

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