# Acute cholecystitis associated with infection of Enterobacteriaceae from gut microbiota

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## Abstract

Acute cholecystitis (AC) is one of the most common surgical diseases. Bacterial infection accounts for 50% to 85% of the disease's onset. Since there is a close relationship between the biliary system and the gut, the aims of this study were to characterize and determine the influence of gut microbiota on AC, to detect the pathogenic microorganism in the biliary system, and to explore the relationship between the gut and bile microbiota of patients with AC. A total of 185 713 high-quality sequence reads were generated from the faecal samples of 15 patients and 13 healthy controls by 16S rRNA gene pyrosequencing. Patients' samples were significantly enriched in *Akkermansia, Enterobacter* and *Escherichia/Shigella* group. The healthy controls, however, showed significant enrichment of Clostridiales, *Coprococcus,* Coprobacillaceae, *Paraprevotella, Turicibacter* and *TM7-3* in their faecal samples. *Escherichia coli* was the main biliary pathogenic microorganism, among others such as *Klebsiella* spp., *Clostridium perfringens, Citrobacter freundii* and *Enterobacter cloacae* in the bile of the patients. Additionally, the amount of bile endotoxin significantly correlated with the number of Enterobacteriaceae, especially *E. coli.* Our data indicate that Enterobacteriaceae might play essential role in the pathogenesis and/or progress of AC. This was verified in an *in vivo* model using a pathogenic *E. coli* isolated from one of the patients in guinea pigs and observed marked gallbladder inflammation and morphologic changes. This study thus provides insight which could be useful for the prevention, diagnosis and treatment of AC and related diseases by controlling the growth of Enterobacteriaceae to alleviate the infection.



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## Introduction

Acute cholecystitis (AC), a sudden onset of inflammation of the gallbladder that causes severe abdominal pain, is increasingly becoming common, at a current prevalence of 5% to 10% in the elderly [1,2]. More than 90% of AC is caused by cholelithiasis [3], and the remaining acalculous cholecystitis is generally associated with trauma, parenteral nutrition or sepsis [4–6].

Recent studies suggested bacterial infection in the biliary system to play an important role in the aetiology of cholecystitis [7,8]. Diverse bacterial flora in the bile and on the walls of the gallbladder of patients with cholecystitis were observed, isolated and characterized through microorganism culture techniques [9–11]. The most frequent pathogens in biliary infection are Gram-negative anaerobes, dominated by *Escherichia coli, Klebsiella* spp., *Acinetobacter baumannii* complex and *Enterobacter* spp. [12,13].



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Intriguingly, a potential relationship between the gut flora and AC was also observed [13,14]. Gut microbes play regulating roles in bile acid metabolism as well as gallstone formation [15,16] and contributes to inflammatory responses. A hypothesis prevails that gut pathogens might enter the biliary tract by two paths: the ascending route through the gastrointestinal tract or the hematogenous route *via* the portal vein blood [17,18].

In this study, we investigated the gut and bile microbiota of a group of AC patients by using three techniques, pyrosequencing of 16S rRNA gene, denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) analysis. It was found that Enterobacteriaceae was the main organism in the patients of gut microbial dysbiosis and bile infection. Their bile endotoxin levels, as well as *in vivo E. coli* feeding experiment using guinea pigs, confirmed the observation. In addition, there was high consistency of Enterobacteriaceae counts between the gut and bile samples which suggested gut-originated pathogens to be responsible for the bile infection.

## Materials and methods

### Study subjects and sample collection

Subjects were recruited, and diagnosis by color Doppler ultrasonography for hepatobiliary diseases was conducted at the First and Second Affiliated Hospitals of Dalian Medical University of China. A total of 25 patients (including 20 cases of AC, four acute cholangitis cases, and one case of both diseases) and 13 healthy control subjects aged 38 to 79 years participated in this study. None of the subjects (including healthy controls) had any history of gastrointestinal tract disorder or took either antibiotics or probiotics within 8 weeks before sampling. However, most patients were diagnosed with common complications, including gallstones (cholecystolithiasis, choledocholith), obstructive jaundice and hepatic cyst. Detailed clinical information of all patients is presented in Supplementary Table S1. This study was approved by the Institutional Ethics Committee for Research of Dalian Medical University.

To study the effect of AC on gut microbiota, 28 faecal samples were collected from the AC patients ($n$ = 15) and healthy volunteers ($n$ = 13). Faecal samples were collected in sterile tubes at the hospitals and were immediately stored at −80°C until processing and use for high-throughput 16S rRNA gene pyrosequencing.

To characterize the biliary tract microbiota, 16 bile samples were obtained from the AC patients during either percutaneous transhepatic cholangial drainage or percutaneous transhepatic gallbladder drainage operation, and stored at −80°C. For subsequent analysis, each sample was divided into four parts for qPCR, DGGE, endotoxin analysis and bacterial culturing. Faecal samples of these patients were also collected for qPCR analysis to reveal the relationship between gut and bile flora.

### DNA extraction, 16S rRNA gene amplification and pyrosequencing

Total bacterial DNA was extracted according to the manufacturer's instructions of QIAamp DNA Blood Mini Kit (Qiagen, Germany). The quality of the nucleic acid was visually determined by electrophoresis on 1% agarose gel containing ethidium bromide. The DNA concentration was measured spectrophotometrically using BioPhotometer plus (NanoVue, USA).

For 16S rRNA gene analysis, the V3 variable region was amplified using the following primers, primer-F: 5′-GC clamp (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG)-CCTACGGGAGGCAGCAG-3′; primer-R: 5′-ATTACCGCGGCTGCTGG-3′. The GC clamp was attached to the 5′ end of the forward primer to allow detection of the corresponding PCR products with DGGE [19]. PCR amplification was performed in an automated thermocycler (Thermo, USA). After the reaction, the size of the obtained amplicon was verified *via* electrophoresis. Pyrosequencing was performed on a 454 CS FLX titanium sequencer (Roche 454 Life Science, USA).

### DGGE and sequence analysis

DGGE and sequence analysis were used to characterize the dominant pathogens in bile. DGGE was performed by a Universal Mutation Detection System (Bio-Rad, USA); the method was similar to what we previously reported [20] with slight modifications. The electrophoresis with gradient of urea as well as formamide ranged from 35% to 55% was run at 180 V for 10 minutes at 60°C, followed by fixed voltage at 65 V for 7 hours. The gels were stained in 0.5 μg/mL ethidium bromide solution for 60 minutes, washed with deionized water, and viewed in a Gel Documentation System (Bio-Rad, USA).

After this, DNA sequences of DGGE bands were cloned and analysed. Specific bands were excised from polyacrylamide gel under UV illumination, washed, and kept in 50 μL sterile water at −20°C overnight. The extracted gel mix was incubated in a 90°C water bath for 10 minutes, and 4 μL of the solution was taken and used as the DNA template for reamplification by PCR using the original primers with GC clamp. After purification, the amplicons were ligated with the pMD18-T vector (TaKaRa, Japan). The *E. coli* NovaBlue competent cells were transformed by ligation reaction, and the positive colonies carrying the plasmids with insertions were screened according to blue–white selection. The recombinant plasmids were

isolated from white colonies and sequenced by ABI PRISM 3730XL DNA Analyzer (TaKaRa, Japan). For taxonomy assignment, the obtained sequences were aligned to the non-redundant nucleotides of the National Center for Biotechnology Information (NCBI-nt) by BLAST search [21].

### Quantitative PCR

Real-time qPCR with group-specific primers was used to quantify three important bacterial groups: Enterobacteriaceae, *Escherichia coli* and *Clostridium perfringens* (Supplementary Table S2, [22–24]). The qPCR reactions were performed in 25 μL volume according to the instruction of SYBRPremix Ex Taq II (TliRNaseH Plus) (TaKaRa, Japan). Each reaction mixture of 25 μL contained 12.5 μL of 2× SYBR Green PCR Mix, 9.1 μL sterile deionized water, 1 μL of each primer (20 μM), 1 μL of genomic DNA of samples and 0.4 μL ROX Reference Dye II. The qPCR reactions were detected by Real-Time QPCR System (Agilent Technologies Stratagene Mx3005P, USA). For Enterobacteriaceae, the amplification parameters were set as following: one cycle of 95°C for 1 minute, 40 cycles of 95°C for 5 seconds, 55°C for 30 seconds and 72°C for 30 seconds. For *E. coli,* the parameters were as follows: one cycle of 95°C for 2 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 20 seconds and 72°C for 30 seconds. For *C. perfringens,* the parameters were as follows: one cycle of 95°C for 5 minutes, 35 cycles at 95°C for 15 seconds, 55°C for 20 seconds and 72°C for 30 seconds. To obtain the melting curve, an extra cycle of 95°C for 1 minute, 55°C for 30 seconds and 95°C for 30 seconds was carried out. A tenfold dilution series of recombinant plasmids containing the insert of interest was used in each real-time qPCR assay to generate standard curves for absolute quantification of target DNA in test samples. The correlation coefficient values of the standard curves were limited from 0.99 to 1.0, and the amplification efficiency was confined between 90% and 110%.

### Endotoxins analysis

The Limulus Amebocyte Lysate (LAL) kit was used to quantify the bacterial endotoxin, which refers to the lipopolysaccharide level in the bile of AC patients. Briefly, 50 μL bile samples and 50 μL LAL solutions were added in pyrogen-free microplate, mixed and incubated for 10 minutes at 37°C; the chromogenic substrate solution was added and incubated for 6 minutes at 37°C. The reaction was carried out according to the manufacturer's protocol of pyrochrome LAL kit (Associates of Cape Cod, USA) [25]. The reactants were checked under 545 nm wavelength by a multimode plate reader (Perkin-Elmer, USA). For endotoxin analysis, the odds ratio was calculated between the Enterobacteriaceae or *E. coli* counts and the endotoxin levels based on the logistic regression model.

### Isolation and identification of *E. coli* strain in bile

To identify Enterobacteriaceae in AC, we attempted to isolate the bacteria strains from the bile samples of these patients. For bacteria proliferation, bile of 500 μL was added into 50 mL of sterile Luria-Bertani liquid medium, and the culture was incubated for 14 to 18 hours at 37°C. The culture was then spread on Luria-Bertani solid agar to separate single colonies. A pathogenic *E. coli* strain was isolated from one of the patients (patient 9) which had a great quantity of Enterobacteriaceae. To validate this strain, the bacterial genomic DNA was prepared by EZNA Bacterial DNA Kit (Omega, USA), and the full-length 16S rRNA gene was sequenced by Sangon Biotech Ltd. The obtained sequence was identified by aligning against the NCBI-nt database using BLAST, which returned the best hit as *E. coli* strain BW25113 genome (Supplementary Fig. S1a). The bacterial sample was also analysed by mass spectrometry (Ultraflextreme MALDI-TOF/TOF MS), and the result showed the bacterial sample was *E. coli* (Supplementary Fig. S1b). Twenty-two kinds of *E. coli* single factor serum, Serotest *E. coli* (O&K) Antisera (S&A Reagents Lab, Thailand), were used to implement the serotype identification. The result showed that the serotype of the pathogenic *E. coli* strain corresponded to O124:K72, which belonged to enteroinvasive *E. coli* (EIEC).

### Guinea pigs *E. coli* feeding experiment

Five 6-week-old male guinea pigs were obtained from the specific-pathogen-free animal center at Dalian Medical University. They were randomly divided into two groups with free access to normal food and water in a temperature-controlled room with a 12:12 hour light–dark cycle and acclimatized for a week before experimentation.

For the bacteria-treated groups, one guinea pig was orally provided with the isolated *E. coli* strain at $1 \times 10^8$ colony-forming units (CFU)/mL physiologic saline daily, and the other two were given rectal suppositories containing $1 \times 10^8$ CFU of the *E. coli* in 1 mL cocoa butter as the ground substance, according to a previously described method [26]. After 30 days, all the animals were dissected to collect organs, fix tissue samples and perform morphologic and histologic observation. Hematoxylin and eosin (HE) staining was undertaken for organs including gallbladder, liver, spleen, duodenum and colon. The tissue preparations and examinations were carried out in the pathology department of Dalian Medical University and read by a pathologist who was blinded to the experimental design.

### Statistical methods

Statistical analysis was implemented using the R platform (v3.1.0). The relationship between two groups of samples was characterized by the Pearson correlation coefficient and was visualized by the ggplot2 package [27]. Statistical significance

 

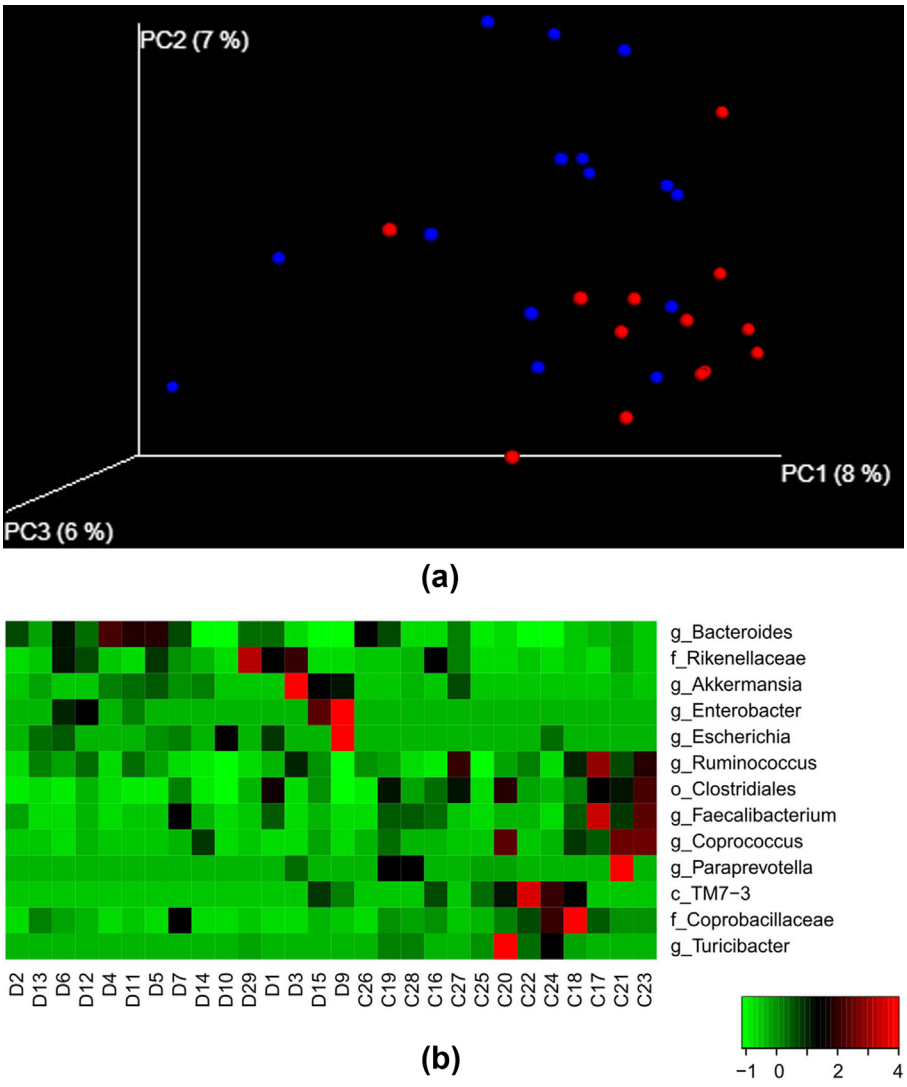


**FIG. 1.** Gut microbiota composition differs between AC patients and healthy controls. (a) Principal coordinate analysis of unweighted Unifrac distances. (b) Heat map constructed using amount of significantly different genera. Colors represent increase or decrease of each genus relative to average level.

was set at $p < 0.05$. For endotoxin level analysis, the odds ratio was calculated by the logistic regression model.

## Results

### Comparison of gut microbiota from AC patients with healthy controls

To characterize the gut microbiota composition during AC, the faecal samples of 15 patients and 13 healthy controls were examined using 16S rRNA gene pyrosequencing. A total of 185 713 high-quality sequence reads were generated, with an average of 6633 (±2453 SD) reads for each subject. All sequences were clustered into 9013 operational taxonomic units based on 97% nucleotide similarity cutoff for species-level bacterial phylotypes [28].

Biodiversity indexes were calculated to estimate the richness and evenness of the microbial communities. The results showed that the patients had significantly lower Shannon index (p 0.03, Student *t* test), Chao1 index (p 0.02), phylogenetic diversity (p 0.02) and observed number of species ($p < 0.01$), indicating gut microbial dysbiosis in AC. Unifrac distance was then used to measure similarity between subjects [29]. The principal coordinates analysis of the unweighted Unifrac distances showed apparent separation between the patient and control groups (Fig. 1a).

For 16S rRNA-based microbiome studies, it is appropriate to analyse the taxonomic difference across samples at the genus level [30]. The differential genera between patients and healthy controls were identified using a two-side Wilcoxon rank sum test and were displayed through a heat map (Fig. 1b). Patients' samples were significantly enriched in *Akkermansia, Enterobacter*

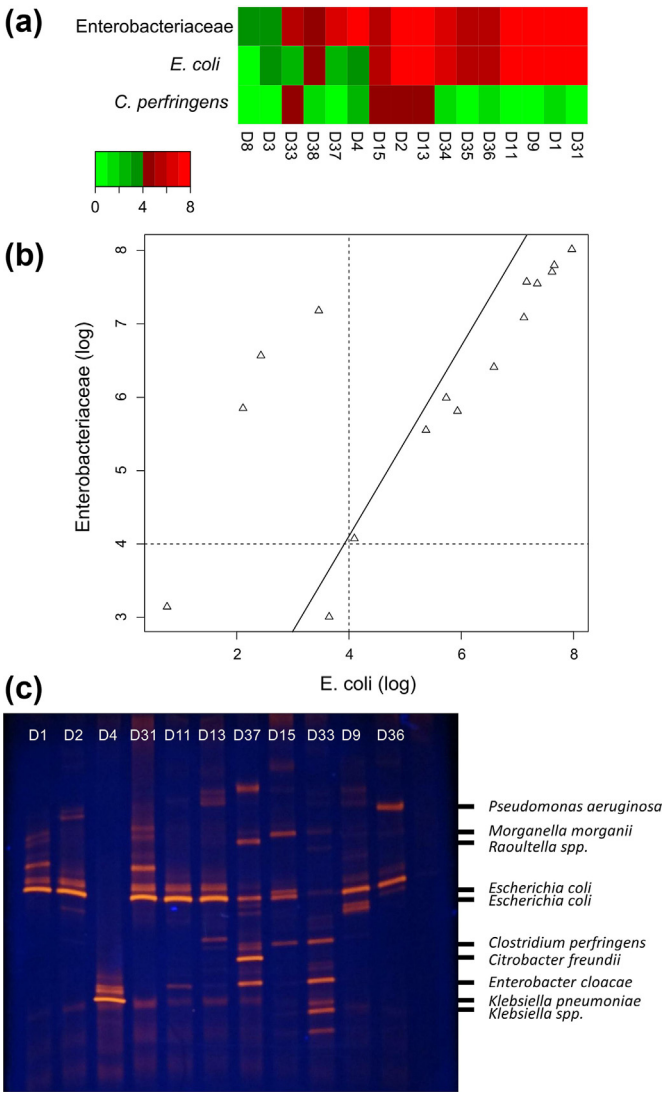


**FIG. 2.** Content of bile bacteria in AC patients. (a) Copy numbers of three bacteria revealed by qPCR. (b) Positive correlation of Enterobacteriaceae and *E. coli* content. Hollow triangles represent subjects. (c) DGGE profiles. Bacteria of main lanes were identified by DNA sequence analysis (see Materials and Methods).

and *Escherichia/Shigella* group. *Bacteroides* and Rikenellaceae were also increased, but not significantly (p 0.09). One study demonstrated that Enterobacteriaceae can be highly enriched in gut microbiota of gallbladder stones patients [13], which was also observed in our AC subjects ($p < 0.01$). For the healthy controls, Clostridiales, *Coprococcus,* Coprobacillaceae, *Parabacteroides, Turicibacter* and *TM7-3* were significantly enriched, as were *Faecalibacterium* (p 0.08) and *Ruminococcus* (p 0.08).

### Enterobacteriaceae are enriched in bile microbiota of AC patients

To quantify the bacterial content of the bile samples from AC patients, qPCR analyses was performed using Enterobacteriaceae universal primers and the specific primers of two species, *Escherichia coli* and *Clostridium perfringens*. The results showed that Enterobacteriaceae was observed in most (14/16) samples at a threshold of 10 000 bacteria copies per milliliter of bile, with copy numbers ranging from $1.06 \times 10^4$ to $1.04 \times 10^8$ (average $2.33 \times 10^7$) (Fig. 2a). The widely distribution and high enrichment of Enterobacteriaceae in the bile samples were consistent with those from the gut samples in this study and with a gallbladder stones study [13]. We further observed that 11 of the 14 Enterobacteriaceae-enriched samples consisted mainly of *E. coli* and had close correlation of bacteria copies (Fig. 2b). On the other hand, *C. perfringens* was found distributed in only four samples, and with very low copy numbers ranging from $1.18 \times 10^4$ to $5.68 \times 10^4$ (average $3.37 \times 10^4$).

DGGE investigations for further precise species information validated *E. coli* as the major component of the Enterobacteriaceae-enriched bile samples. Three other non–*E. coli* components—*Klebsiella* spp., *Citrobacter freundii* and *Enterobacter cloacae*—were also identified (Fig. 2c). The other

Enterobacteriaceae species that frequently occurred in the bile might be *Morganella* and *Raoultella,* as well as *Salmonella,* suggested in other studies [31].

An interesting question was whether the Enterobacteriaceae of the gut microbiota and the bile flora could be somehow connected. To address this, we used qPCR to quantity Enterobacteriaceae and *E. coli* in the faecal and bile samples of these patients. There were significant positive correlations between the gut and bile for both Enterobacteriaceae ($r = 0.91$, $p\ 0.001$) and *E. coli* ($r = 0.95$, $p < 0.001$) (Fig. 3). This finding provided reliable evidence that the bile pathogens might have originated from the gut, and it was in agreement with the previous hypothesis [32].

### Endotoxin level of AC patients

Endotoxins act as determinants of virulence in Gram-negative bacteria [33,34] and function to incite systemic inflammation in the host organism [35]. For the 16 bile samples from AC patients, appreciable endotoxin levels were detected and were significantly associated with the copy numbers of both Enterobacteriaceae (odds ratio 4.99, 95% confidence interval 1.21–20.7, p 0.006) and *E. coli* (odds ratio 2.34, 95% confidence interval 0.90–6.08, p 0.022). The high/medium endotoxin levels occurred mostly in the higher Enterobacteriaceae samples (Fig. 4), leading to 14-fold higher Enterobacteriaceae count in high/medium endotoxin level samples than the low endotoxin level samples. The consistency of endotoxin level and insight from the qPCR data indicated that Enterobacteriaceae might play a major role in producing endotoxin in the biliary tract of AC patients.

### Guinea pigs infected with *E. coli* develop cholecystitis

To determine if Enterobacteriaceae is sufficient to promote cholecystitis, a pathogenic *E. coli* strain (serotype O124:K72, pathogenic type EIEC) was isolated from an AC patient and inoculated into guinea pigs by intragastric ($n = 1$) or rectal suppository ($n = 2$) administration. After 30 days of feeding and daily inoculation (see Material and Methods), the experimental guinea pigs became inactive, drank less and ate more compared to the control group ($n = 2$) (data not show). Anatomic images and histomorphologic changes are shown in Fig. 5. Anatomy revealed that animals in the experimental group had dark yellow opaque bile, deformed gallbladder and stones compared with clear and bright yellow bile, and normal gallbladder in the control group (Fig. 5a). This changes were similar to the gallbladder of guinea pigs fed a high-cholesterol diet in a previous study by Fan *et al.* [36]. HE staining showed mucosa epithelial hyperplasia in the gallbladder and higher inflammatory cells

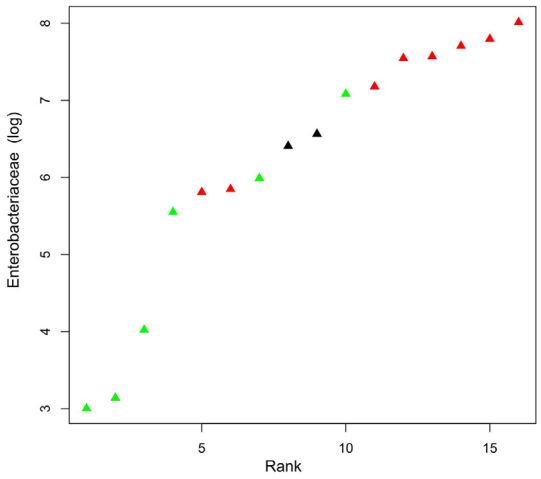


**FIG. 4.** Endotoxin level is associated with Enterobacteriaceae content. Subjects are represented by solid triangles with color codes: red, high endotoxin level; black, medium endotoxin level; green, low endotoxin level.

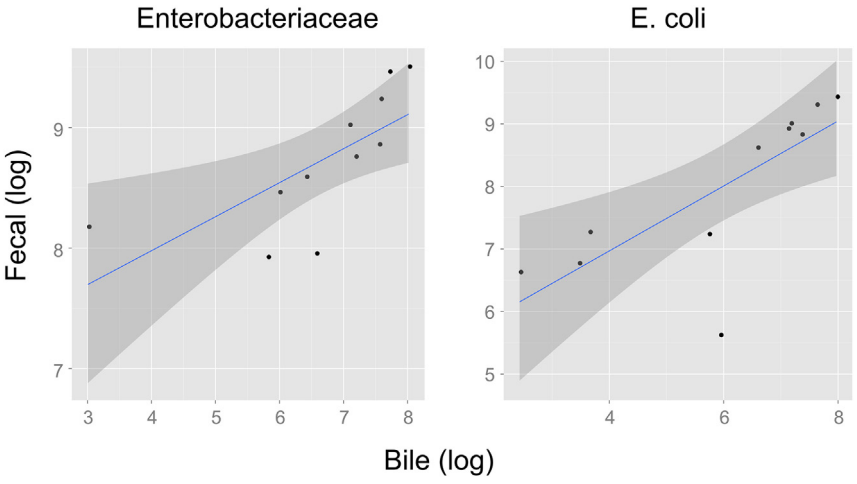


**FIG. 3.** Enterobacteriaceae content in faecal and bile samples. Subjects are represented by solid points. Line of best fit is shown flanked by shadows for 95% confidence interval on both sides.

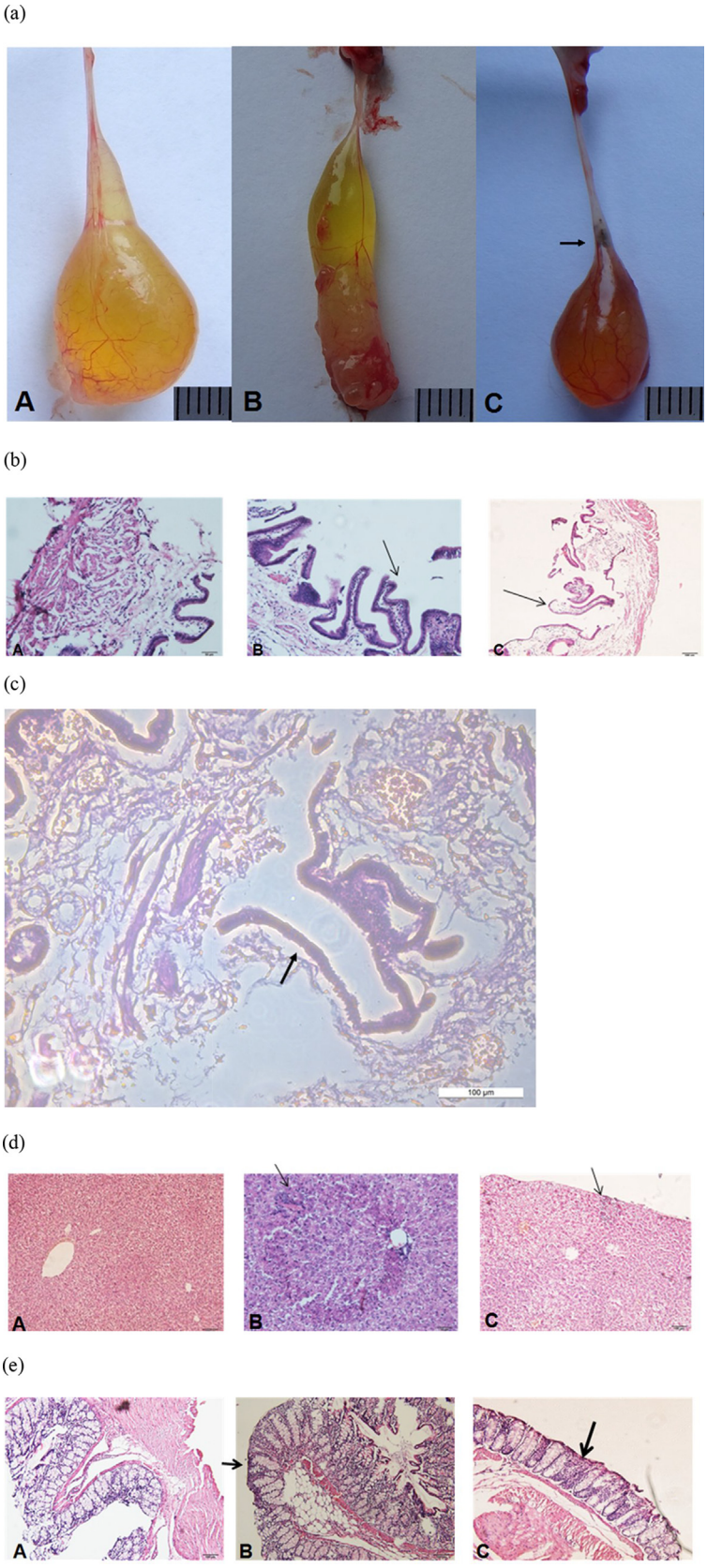


**FIG. 5.** Anatomic and histomorphologic images of experimental guinea pigs. (a) Image of morphologic and anatomic changes of gallbladder of guinea pigs. (A) Normal gallbladder from a negative control group. (B, C) Experimental group showing dark yellow opaque bile as well as deformed gallbladder and stones (arrow). Minimum division value = 1 mm. (b) Representative images of gallbladder wall stained with HE; original magnification ×20. Gallbladder of experimental group showed mucosa epithelial hyperplasia with obvious elongation of mucosal fold into cystic cavity. Mild inflammation was apparent (arrow). (c) Representative sign of gallbladder epithelial hyperplasia with typical HE staining. Rokitansky-Aschoff sinus (RAS) (arrow) appeared in one gallbladder sample of experimental group, which was treated with rectal suppository containing *E. coli* (original magnification ×20). (d) Normal liver tissue from negative control group (original magnification ×10) (A) and experimental group showing mild edema in liver cells at (B) original magnification ×20 and (C) original magnification ×10. There were tiny foci of phlogocyte infiltration in certain parts of liver (arrow). (e) Representative images of colon wall stained with HE. (A) Control group, original magnification ×10. Experimental group at (B) original magnification ×20 and (C) original magnification ×10 show experimental group to have more inflammatory cell infiltrations in stratum mucosum (arrow).

infiltrations of the liver and colon of the experimental group animals (Fig. 5b–e), an indication that *E. coli* inoculation led to gallbladder and colon inflammation. These data suggested that *E. coli* infection may have the propensity to cause cholecystitis in guinea pigs and lead to gut microbiota imbalance.

## Discussion

Enterobacteriaceae present as the major harmful members in human symbiotic microbial community [37] and cause a large amount of physical illnesses, including acute pelvic inflammatory disease, necrotizing enterocolitis and urinary tract infection [38–41]. Enterobacteriaceae infections are commonly observed in the gastrointestinal or urinary tract. However, some of these strains can cause diseases if they contaminate a normally sterile site such as the body cavities [42]. In the current study, noticeable enrichment of Enterobacteriaceae in both the gut and bile microbiota of AC patients was found by using multiple technologies. We found that, firstly, the quantities of Enterobacteriaceae significantly correlated with the bile endotoxin levels, and secondly, a pathogenic *E. coli* strain isolated from a patient and inoculated into guinea pigs could promote inflammation and induce morphologic changes in the gallbladder of the animals. These findings indicated that Enterobacteriaceae might be the primary causative organisms of AC. In agreement with our findings, other studies have implicated several genera of Enterobacteriaceae, including *E. coli, Klebsiella pneumonia* and *Salmonella,* to be the main pathogenic bacteria in bile flora [8,43,44].

A strong correlation between the quantities of Enterobacteriaceae existing in the gut and bile flora was also found. According to the data from Almeida *et al.* [45], *E. coli* infection can degrade intestinal barrier and thereby compromise its integrity. This may permit gut flora to transmit to otherwise sterile sites and/or systems of the host such as the blood and biliary circuits. Wu *et al.* [13] verified this and reported that 70% of gut bacterial operational taxonomic units from gallstone patients were detectable in the biliary tract, and injection of *E. coli* into the portal vein in rabbits also led to chronic inflammation and stone formation in the gallbladder [46]. Therefore, a hypothesis has arisen from these and other similar studies that bacteria can be transferred from the gut into the bile in some way.

A larger amount of *Akkermansia* was observed in the gut microbiota of AC patients. *A. muciniphila* is a mucin degrader which exists in the human intestinal tract [47] and which is associated with protecting the mucus lining of healthy intestines [48]. Nevertheless, the effect of *A. muciniphila* on patients remains unknown. For the healthy controls, Clostridiales, *Coprococcus, Paraprevotella, Faecalibacterium* and *Ruminococcus,* which are normal gut microflora, were significantly enriched. *Coprococcus* and *Faecalibacterium* especially can produce butyrate, which is essential for the energy metabolism and normal development of colonic epithelial cells and therefore play a protective role in relation to colonic disease [49]. *Faecalibacterium* has also been suggested to protect colonic mucosa against the development of inflammation and could be crucial to gut homeostasis [50].

This study therefore provides additional insight essential for the prevention, diagnosis and treatment strategies against AC and related diseases by controlling the growth of Enterobacteriaceae to alleviate the infection. Probiotics and prebiotics might be utilized to regulate the intestinal flora to reduce the incidence of cholecystitis. However, further studies are needed to test which selected prebiotics or probiotics could lower the risk of AC.

## Transparency declaration

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.05.017.

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