Molecular characterization of the microbiota in patients with ulcerative colitis in the Kingdom of Saudi Arabia

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Background
Ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract. Herein, we report a comparative analysis of intestinal microbiota in Saudi patients with UC and healthy individuals using a culture-independent approach.
Materials and methods
Intestinal biopsies of the five Saudi patients with UC and five healthy citizens were collected, homogenized, and DNA extracted. Genomic libraries of 16S rDNA were constructed using these biopsies.
Results and discussion
Among the 96 clones analyzed, 39 distinct bacterial strains were found to belong to two main genera: Bacteroides (46%) and clostridium (26%). Levels of uncultured bacteria and uncultured Bacteroidetes were higher in patients with UC than in healthy individuals, and there was a marked decrease in bacterial diversity and evenness in patients with UC relative to healthy individuals. A group of bacteria in healthy individuals was absent in the microbiome of patients with UC, including *Bacteroides fragilis*, *Bacteroides vulgatus*, *Prevotella* spp., *Bacteroides coprocola*, *Escherichia coli*, and *Streptococcus thermophiles*, whereas another group of bacteria found in Saudi patients with UC was not detected in healthy individuals, including *Staphylococcus warneri*, Bacteruim LF48, *Weissella confusa*, and enterococci. The results confirm that UC is a multifactorial disease in origin, and some specific bacteria act as etiological agents of UC.
Conclusion
UC is a multifactorial illness, expressed not only by the dysbiosis of the intestinal microbial flora but also is referred to other causes like the type of diet of each patient, his/her immunity, and genetics.

Keywords:
genomic library, microbiota, molecular characterization, phylogenetic, 16S rDNA, ulcerative colitis

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Introduction

All surfaces of the human body, including mucosal surfaces, skin, genitals, and gastrointestinal tracts are occupied by habitat-specific microbes whose cell numbers are more than the human body cells by about 10 times [1]. The human gut microbiota involves one of the most complex microbial communities, in which the extremely high diversity undergoes a functional homogenization, playing essential roles in nutrients processing, energy production, and synthesis of a variety of cellular components [2,3]. Furthermore, the presence of more than 9×10^6 unique microbial genes in the human gut has been reported [4].

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease; both are chronic inflammatory disorders of the gastrointestinal tract that were previously considered uncommon but now are emerging with an alarming frequency over the past decades [5]. Patients with UC show symptoms ranging from diarrhea and weight loss to mucosal inflammation in colon, starting in the rectum and extending proximally in a continuous manner, leading to ulceration, perforation, and obstruction of the gastrointestinal tract [6,7]. The causes and etiology of UC disease syndromes are still under investigation and uncertain as of yet. However, it is generally accepted that several factors are involved in the development of the disease such as abnormal immune responses, host genetics, environmental factors, nutrition, and intestinal dysbiosis [8,9]. Furthermore, most studies suggest that the gut microbiota is an important factor in the pathogenesis of UC [10]. UC disease is obviously

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associated with gut dysbiosis which is an imbalance in the numbers and/or functions of gut microbiota that disrupts host-microbe and immune homeostasis [5,11,12]. Thus, it is believed that members of the gut microbiota may play a significant role in the etiopathogenesis of UC disease. However, no specific bacterium has been proven to be the etiological agent of UC disease or colon disorders [7,13]. In addition, it should be emphasized that UC disease is defined as a complex syndrome that results from the interaction between the host's genetic background, immune response, and the commensal microbiota inhabitants [14]. Previously, intestinal bowl disorder (IBD) was considered as an illness of the western world with geographic variations. However, there is a continuous increase in the prevalence of IBD in the countries previously known to have low incidence [15,16]. This increase of IBD prevalence is apparently owing to the westernized lifestyle accompanied by an increase in the fast food consumption, environmental changes, and the improvement in hygienic standards [12]. The studies are not enough on the nature of microbiota in patients with UC syndromes in Kingdom Saudi Arabia. Therefore, the main objective of this work was the comparative analysis of intestinal microbiota in Kingdom Saudi Arabia patients with ulcerative colitis and healthy individuals to determine the microbes that may play a role in the pathogenesis of UC disease among the Saudi population.

Patients and methods

Patient and biopsy sample collection

Human intestinal flush samples were collected from 10 Saudi individuals, including five colonoscopy-negative adult volunteers, without any dietary restrictions or antibiotic treatment, as well as five patients with UC from local hospitals. Consent was obtained from all patients. UC diagnosis was based on standard consensus criteria, including clinical, endoscopic, radiological, and histological findings [17]. Intestinal flushes were performed at four different locations of the intestine in each of the 10 individuals and/or patients using 40 ml normal saline solution (0.9%); 10 ml of each of the four samples was pooled and mixed together in new sterile Falcon tubes and stored at −80°C. The Ethics Committee for Human Medical Research of King Saud University approved this study.

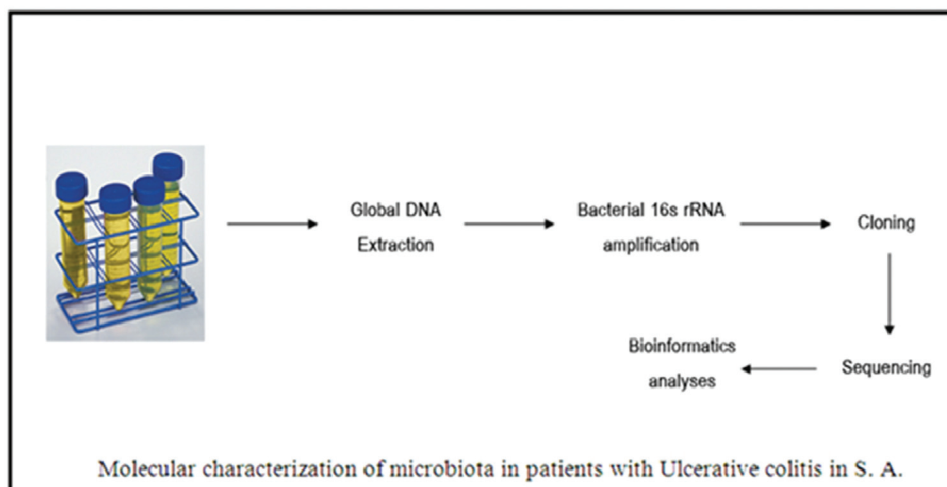
XXXX16s rDNA library construction

A clone library of 16S rDNA genes was constructed from the intestinal biopsy samples according to standard protocols, including genomic DNA isolation from the collected intestinal flush samples, 16S rDNA amplification and purification, gene cloning in the pGEM-T vector and transformation into *Escherichia coli*, plasmid purification from transformed cells, and 16S rDNA gene sequencing and analysis [18,19]. The scheme applied for the experiments is shown Fig. 1.

Genomic DNA isolation and purification

The collected intestinal sample suspensions were centrifuged at 5000*g* for 15 min in a refrigerated centrifuge. The supernatant was discarded, and the pellets were re-suspended in 10 ml of cell lysis buffer (10 mmol/l Tris-HCl buffer, pH 8; 10 mmol/l NaCl; 1 mmol/l EDTA and 0.5% SDS). The suspensions were vortexed for 5 min and homogenized by shaking for 10 min, and then another 10 ml of the lysis buffer was added followed by further homogenized for 10 min. The

Figure 1



A scheme of the molecular characterization of the microbiota of patients with ulcerative colitis [18].

particulate materials were removed by centrifugation at 5000*g* for 15 min. Total DNA was precipitated by adding 5 ml of 7.5 mol/l ammonium acetate and 25 ml of cold ethanol (95–100%) and placed at −20°C for 20–30 min. The DNA was collected by centrifugation at 7000*g* for 15 min; in this step, the obtained precipitated DNA was not colourless and contained bile salts. Therefore, the DNA pellets were re-suspended in 600 μl TE buffer (pH: 8) and incubated at 65°C for 15 min. The DNA was further purified using a conventional single-step phenol/chloroform/isoamyl-alcohol (25 : 24 : 1) protocol [18].

XXXX16S rDNA amplification

PCR amplification of the 16S rDNA gene was performed using universal eubacteria-specific forward and reverse primers (lane, 1991): 8F (5′-GCGGATCCGCGGCCGCTGCAGAGTTTGA TCCTGGCTCAG-3′) and 806 R (5′-GCGGATCCGCGGCCGCGGACTACCAGGG TATCTAAT-3′). PCR amplification was carried out using the Master Mix kit (Qiagen, NY, USA) in a final reaction volume of 25 μl containing 12.5 μl Master Mix, 1 μl forward primer (10 μmol/l), 1 μl reverse primer (10 μmol/l), 5 μl DNA template (200 ng), and 5.5 μl nuclease-free water. Amplification was carried out in a DNA thermal cycler under the following thermal profile: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, primers annealing at 52°C for 1 min and extension at 72°C for 1.5 min, with a final step for 10 min at 72°C to ensure full extension of the products. Thereafter, the PCR products were mixed with 3 μl of loading dye solution and separated by 1% (w/v) agarose gel electrophoresis in 1× TAE buffer, pH 8.0 (40 mmol/l Tris-acetate, 1 mmol/l EDTA). The gel was placed in ethidium bromide solution (1 μg/ml) for 30 min and then placed on an ultraviolet transilluminator to visualize the products. The amplified 16S rDNA amplicons were excised from the gel using a sterile razor blade, and the DNA was purified using a DNA gel extraction kit (Qiagen) following the manufacturer's instructions.

DNA cloning and transformation

The purified 16S rDNA fragments were cloned into the pGEM-T vector (Promega), a linear plasmid with T-overhang at both ends. The 10-μl ligation mixture contained 5 μl of 2× rapid ligation buffer, 1 μl (50 ng) of pGEM-T vector, 2 μl of PCR products (120 ng), 1 μl (3 U/μl) of T4 DNA ligase and buffer (Promega) and 10 μl of nuclease-free water. Positive and negative controls were included, and the mixtures were incubated for 1 h at room temperature and then kept overnight at 4°C. Transformation of *E. coli* JM109 competent cells was carried out by mixing 2 μl of each of the ligation-reaction mixtures with 50 μl of JM109 competent cells in sterile 1.5 ml Eppendorf tubes on ice. The mixtures were subjected to heat shock for 1.5 min at 42°C; 800 μl Luria–Bertani (LB) broth was added, and the mixtures were kept for 1 h at 37°C in a shaking water bath. The efficiency of the transformation process was tested by blue-white screening on LB agar medium containing 100 μg/ml ampicillin, 1.2 mg isopropyl-β, d-thiogalactopyranoside, and 1.0 mg 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-Gal) dissolved in *N*,*N*-dimethylformamide [18]. The appearance of white colonies was an indication of the successful ligation and transformation. White colonies (n=96) were selected, inoculated into 5 ml LB broth containing ampicillin (50 mg/ml), and incubated at 37°C overnight. 16S rDNA-containing plasmids were extracted using QIAprep Miniprep (Qiagen) following the manufacturer's instructions.

DNA sequencing and bioinformatics

After plasmid extraction and purification, the 16S rDNA inserts were sequenced using pGEM-T (Promega, NY, USA) plasmid-specific primers (USP and RSP) and universal eubacteria primers for 16S rDNA. DNA sequencing was performed using the Cycle Reader DNA sequencing kit (MBI Fermentas, Hamburg, Germany) according to the manufacturer's instructions and the LI-COR automated DNA sequencing machine (MWG-Biotech). The eubacterial primers used included forward primers 16F530 (5′-GTG CCA GCC GCG G-3′) and 16F926 (5′-AAA CTC AAA KGA ATT GAC GG-3′) and reverse primers 16R519 (5′-GAA TTA CCG CGG CTG-3′) and 16R907 (5′-CCG TCA ATT CAT TTA AGT TTT-3′) [20]. The obtained 16S rDNA gene sequences of the selected clones (n=96) were aligned with the reference 16S rDNA sequences available at NCBI and the Ribosomal Database Project. Sequence alignment was achieved using CLUSTAL W.

Results and discussion

Human intestinal flush samples were collected from 10 Saudi individuals, including five healthy adults and five patients with UC, at local hospitals (Saudi Arabia). Total bacterial DNA was successfully extracted from the intestinal flushes with good yield and quality. 16S rDNA genes were amplified from total DNA by conventional PCR using universal eubacterial primers, and the products were analyzed by agarose gel electrophoresis, revealing successful amplification of 16S rDNA genes with the expected size of 1500 bp.

Figure 2

Escherichia coli clones on Luria–Bertani AMP plates with blue-white screening using the pGEM-T Easy vector. This approach is based on detection of the vector that has been ligated to the target gene in the presence of an X-Gal analogue and IPTG. White colonies indicate successful ligation of the insert with the vector; blue colonies indicate no ligation.

Table 1 Summary of the total number of bacteria isolated from intestinal samples from healthy individuals (*n*=5)

Bacterial identification	Number of clones
Uncultured bacterium	27
Clostridium perfringens	6
Bacteroides fragilis	4
Bacteroides vulgatus	4
Bacteroides thetaiotaomicron	2
Prevotella spp.	1
Escherichia coli	1
Bacteroides coprocola	1
Parabacteroides merdae	1
Streptococcus thermophiles	1
Faecalibacterium prausnitzii	1
Uncultured Bacteroidetes	1

Table 2 Summary of the total number of bacteria detected in the intestinal samples of patients with ulcerative colitis (*n*=5)

Names of bacteria	Number of clones
Uncultured bacterium	30
Clostridium perfringens	4
Bacteroides thetaiotaomicron	2
Uncultured Bacteroidetes	2
Enterococcus faecium	1
Staphylococcus warneri	1
Bacterium LF48	1
Enterococcus faecalis	1
Weissella confuse	1
Enterococcus hirae	1
Parabacteroides merdae	1
Faecalibacterium prausnitzii	1

The amplified 16S rDNA products were purified, and the purity and concentration were assessed. A clone library of the 16S rDNA genes was then construct.

Clone library of 16S rDNA

The purified amplified 16S rDNA amplicons were cloned into pGEM-T Easy vector, followed by transformation into *E. coli* JM109 and selection. The results showed the appearance of several white colonies (Fig. 2), indicating successful cloning and transformation of JM109 cells with plasmids containing the 16S rDNA genes. A number of individual white colonies (*n*=96) were selected, and the plasmids were purified, confirming the presence of 16S rDNA genes through PCR amplification and analysis.

XXXX16S rDNA gene sequencing and alignment

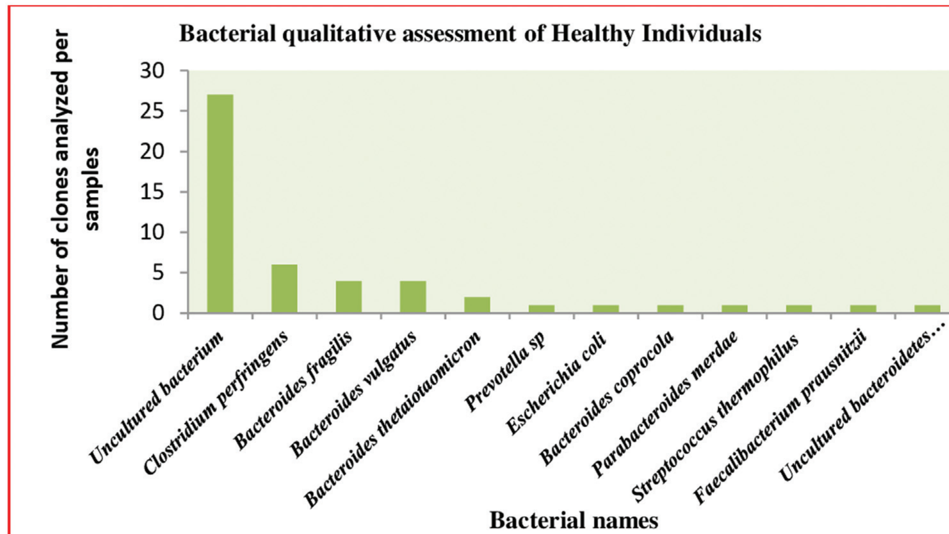
After sequencing of selected clones (*n*=96), the 16S rDNA genes obtained were aligned and compared with known sequences of other bacteria available in the GenBank database. The results of alignments and bacterial identification of intestinal samples collected from patients with UC and healthy individuals are shown in Tables 1 and 2 and Figs 3 and 4, respectively. Identity was high and reached 98–100% for most of the clones.

Phylogenetic analysis

Phylogenetic trees were constructed to investigate the taxonomic placement of the clones obtained from the healthy and patients with UC, and the results are shown in Figs 5 and 6, respectively. The average length of the obtained DNA sequences was 800 bp, and the phylogenetic analysis was based on multiple alignment of 16S rRNA sequencing using CLUSTAL W. As the identified groups were not identical, the evolutionary relationships represented by nodes and lines differ according to their ancestor within the tree.

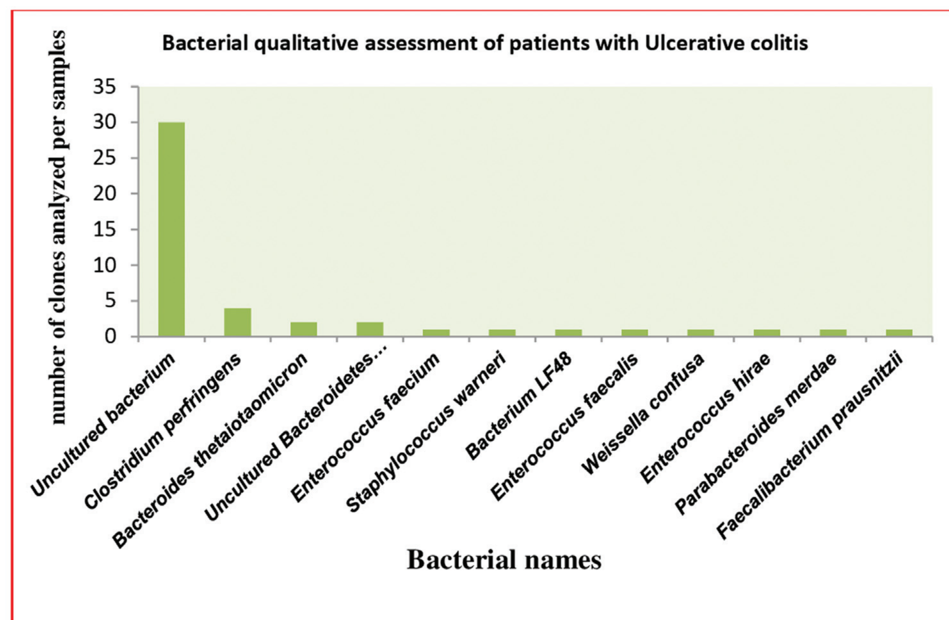
IBD, including UC, is a chronic disease with the highest prevalence and distribution in western countries. However, owing to more industrialization and adoption of the western lifestyle, IBD is expanding globally, affecting populations in Asia, North Africa, the Middle East, and South America [5]. Previous

Figure 3



Summary of the total number of bacteria detected in the intestinal samples of healthy individuals (n=5).

Figure 4

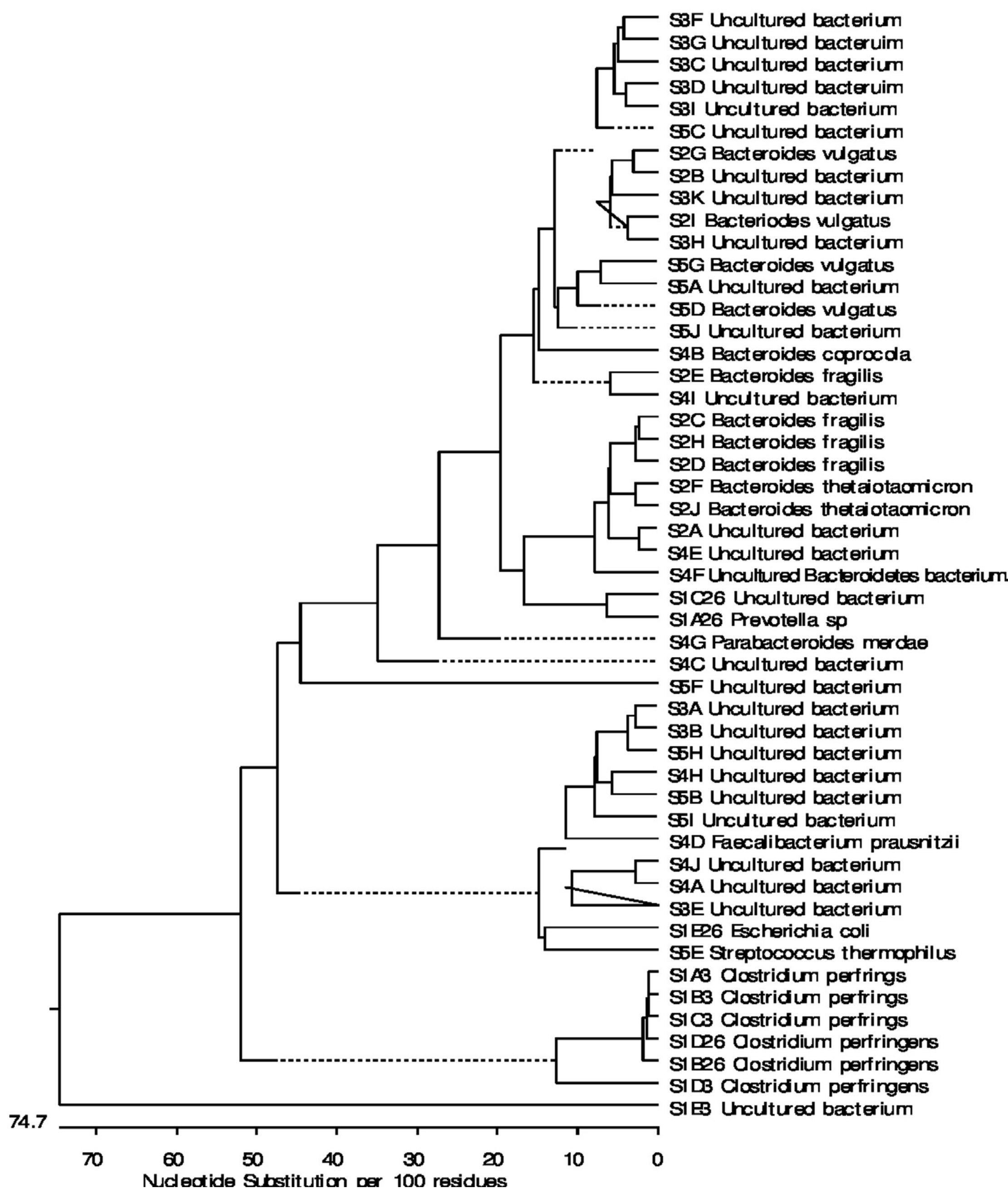


Summary of the total number of bacteria detected in the intestinal samples of patients with ulcerative colitis (n=5).

studies have suggested that abnormalities of the colonic microbiota occur in both UC and Crohn's disease [8,21]. The gut contains a large number of microbes that play a significant role in human nutrition, metabolism, and immunity; some members of the gut microbiota induce inflammatory reactions, others suppress the microbial population immune and inflammatory responses by amplification of regulatory immune cells [22]. Hence, it has been proposed that UC is caused by a breakdown in the balance between putative species of beneficial versus harmful intestinal bacteria, a phenomenon termed dysbiosis [7,23]. Regardless, culture-dependent studies have failed to identify particular pathogens or bacterial groups as aetiological agents of UC when compared with healthy patients. However, the application of molecular techniques has allowed for better characterization of unculturable bacteria and thus can provide more details about the gut microbiota and dysbiosis in patients with UC [24].

In this study, we employed culture-independent phylogenetic analysis to characterize gut microbiota dysbiosis in Saudi patients with UC relative to healthy

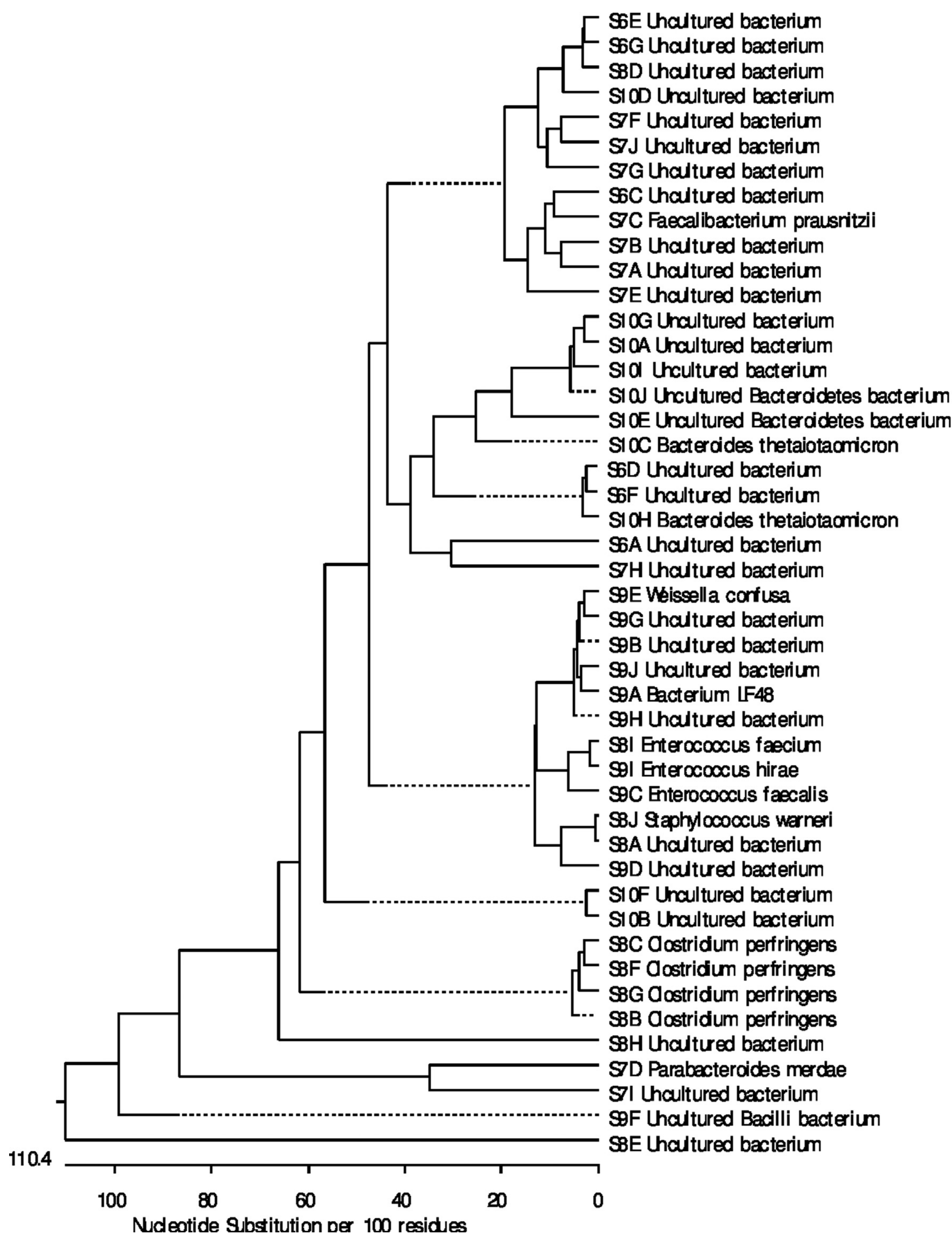
Figure 5



Phylogenetic tree of bacterial clones isolated from healthy individuals, constructed based on partial DNA sequence of the 16S rRNA gene.

controls. We used colonic mucosal biopsies rather than fecal samples to examine the gut wall-associated microbiota, which may play a more critical role than fecal microbes in UC pathogenesis [14]. By applying this approach, we confirmed the existence of significant abnormalities in the predominant gut bacteria of patients with UC relative to healthy individuals. A genomic library of 16S rDNA obtained from healthy individuals and patients with UC was constructed, and among the 96 clones analyzed, there were 39 distinct bacterial strains: 28 (72%) major strains belonging to two main genera, bacteroides (46%) and clostridium (26%). The percentage of uncultured bacteria among the tested 96 clones was 60%, which provides an estimation of the biodiversity within the original sample under the applied PCR conditions. The species diversity in this study was in accordance with that of the established indigenous human gut microbiota, including *Bacteroides* spp., *Enterococcus* spp., *Prevotella* spp., *Streptococcus thermophiles*, *Clostridium perfringens*, and *Faecalibacterium prausnitzii* [7,14,25–27]. However, some other reported gut microbiota species were not detected in this study, such as *Bifidobacterium* spp. [27], *Collinsella aerofaciens* [28], and *Eubacterium biforme* [29]. The results generally showed that a group of bacteria in

Figure 6



Phylogenetic tree of bacterial clones isolated from patients with ulcerative colitis, constructed based on partial DNA sequence of the 16S rRNA gene.

healthy individuals was absent in the microbiome of patients with UC, including *Bacteroides fragilis*, *Bacteroides vulgatus*, *Prevotella* spp., *Bacteroides coprocola*, *E. coli*, and *S. thermophiles*. Another group of bacteria not detected in healthy individuals was present in Saudi patients with UC, including *Enterococcus facium*, *Staphylococcus warneri*, Bacterium LF48, *Enterococcus faecalis*, *Weissella confuse*, and *Enterococcus hirae*. In addition, the results clearly demonstrated a decrease in bacterial diversity and evenness in the patients with UC relative to the healthy individuals. Recently, Mirsepasi-Lauridsen *et al.* [30] reported that patients with UC with and without active disease had significantly decreased

intestinal bacterial diversity and evenness in comparison with healthy persons.

In this study, *E. coli* was detected in healthy individuals (1 clone) but not in patients with UC, whereas *Clostridium perfringens* was detected in healthy individuals more frequently than in patients with UC (6 and 4 clones, respectively). Uncultured bacteria and uncultured Bacteroidetes were found more often in the patients with UC (30 and 2, respectively) than in the healthy individuals (27 and 1, respectively). However, another recent study demonstrated a greater prevalence of virulence factors from *E. coli* in patients with UC; in contrast, virulence factors from *Clostridium perfringens* were only present in patients with UC but were undetectable in healthy controls [31]. Furthermore, some species were identified in the Saudi patients with UC in this study but not in the healthy individuals (n=4), such as a number of enterococci. Indeed, Moustafa *et al*. [31] reported that the most prevalent virulence factors among patients with IBD were enterotoxins produced by enterococci.

Nonetheless, comparison between the microbiota of the Saudi patients with UC and healthy individuals did not lead to a precise identification of a specific sequence or group of sequences exclusively in the samples from the patients with UC. In contrast to our findings, a few previous studies have reported that *B. fragilis*, which has been associated with active disease and relapse, might be responsible for the aetiology of UC [21,26,32]. Prorok-Hamon *et al*. [24] reported the detection in IBD of *E. coli* that expresses genes relevant to pathogenic processes, including M-cell translocation, angiogenesis, and genotoxicity. However, in our study, both *B. fragilis* and *E. coli* were detected in healthy individuals and absent in Saudi patients with UC. This finding was in accordance with the accepted hypothesis that UC is associated with gut dysbiosis that disrupts host-microbe interactions and immune homeostasis rather than with the presence of a specific bacterium acting as an aetiological agent of IBD, including UC. Therefore, it is expected that UC is multifactorial in origin and is a complex syndrome that results from the interaction among the host's genetic background, environmental factors, unregulated immune responses, and the gut microbiota [7,13,14,32,33].

Conclusion

A comparative analysis of the intestinal microbiota in Saudi patients with UC and healthy individuals was carried out using a culture-independent approach via construction of genomic libraries of 16S rDNA amplified from intestinal biopsy samples. In general, a group of bacteria was detected in healthy individuals, such as *B. fragilis*, *B. vulgatus*, *Prevotella* spp., *B. coprocola*, *E. coli*, and *S. thermophiles*, which were not present in the microbiome of Saudi patients with UC. Conversely, another group of bacteria was found in patients with UC but not detected in healthy individuals, including *E. facium*, *S. warneri*, Bacterium LF48, *E. faecalis*, *W. confuse*, and *E. hirae*. In addition, there was a significant decrease in bacterial diversity and evenness in patients with UC in comparison with healthy individuals. The results confirmed the generally proposed hypothesis that UC disease is multifactorial in origin rather than occurring owing to the presence of specific bacteria acting as aetiological agents.

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Conflicts of interest

There are no conflicts of interest.

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