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Candida albicans overgrowth disrupts the gut microbiota in mice bearing oral cancer

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ABSTRACT

Candida albicans is one of the most common opportunistic fungi in cancer patients. This study explored the influence of *C. albicans* on gut microbiota in oral tumour-bearing mice by means of 16S rRNA sequencing and ITS sequencing. It was found that *C. albicans* infection induced the decrease of alpha diversity of bacteria and fungi in the gut microbiome. For the bacteria, *C. albicans* caused the reduction of *Ralstonia*, *Alistipes*, *Clostridia UCG-014*, *Ruminococcus*, and *Lachnospiraceae NK4A136 group*. For the fungi, *C. albicans* inhibited the growth of other fungi including *Aspergillus*, *Cladosporium*, and *Bipolaris*. The neutralisation of γδT cells partly alleviated the out-of-balance of *Firmicutes*/*Bacteroidota* (F/B) ratio in the gut caused by *C. albicans* infection. However, γδT cell neutralisation boosted the overgrowth of *C. albicans*. Additionally, IL-17A neutralisation aggravated the microbial dysbiosis of bacteria and fungi caused by *C. albicans* infection. Further analysis indicated that *C. albicans* overgrowth might influence the correlations between fungal and bacterial kingdoms. In conclusion, *C. albicans* infection disturbed the gut microbiota of both bacteria and fungi in oral tumour-bearing mice, which may be associated with the intestinal immune components including γδT cells and IL-17A.

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1. Introduction

Candida albicans (*C. albicans*) is the most common opportunistic fungus inhabiting in the human oral cavity and intestinal tract, which shows close associations with various types of cancer including oral cancer (OC), oesophageal cancer, liver cancer, and colorectal cancer (Wang et al. 2023b). Among them, it was estimated that *C. albicans* infection could be detected in approximately 10%–68.2% of OC patients (Wang et al. 2023b).

OC is the malignant neoplasm of the lip and oral cavity. *Candida* presence is not only a risk factor for OC development but also associated with poor overall survival in OC patients (Mohamed et al. 2021). Both the cancer itself and cancer treatments including chemotherapy and radiotherapy are contributors to the overgrowth of *C. albicans* (Panghal et al. 2012). Unfortunately, *C. albicans* infection may induce mucosal bacterial dysbiosis (Bertolini et al. 2019; Zaongo et al. 2023), while gut microbiome influences immunotherapy against epithelial tumours (Routy et al. 2018b). However, little is known about the influence of *C. albicans* overgrowth during OC on the gut microbiota.

The human microbiota is a dynamic set of 40 trillion microbes, which is made up of more than 3,000 species containing bacteria, fungi, and viruses (Ting et al. 2022). The inter-kingdom interactions between bacteria and fungi are necessary for the development of the host immune system and homoeostasis of the mucosal barrier (Takiishi et al. 2017). The dyshomeostasis of microbiota is not only a reflection of pathological status, but may have a significant effect on host health and tumour therapeutic outcomes (Gopalakrishnan et al. 2018; Helmink et al. 2019).

Both the commensal microbiota and immune system contribute to the homoeostasis of the intestinal mucosal barrier. A previous study has revealed that *C. albicans* infection was associated with the loss of mucosal bacterial diversity in intestinal mucosa in mice receiving 5-fluorouracil (Bertolini et al. 2019). In turn, the commensal intestinal bacteria may protect the host from *C. albicans* challenge by altering their species diversity (Wang et al. 2021). However,

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whether immune factors play roles in the microbial disturbance caused by *C. albicans* infection is unclear.

Among the various types of immune cells and cytokines, γδT cells and IL-17A are important factors in response to *C. albicans* infection. γδT cells make up 10%–30% of CD3+ T cells in the human intestine, which play important roles in maintaining intestinal homoeostasis and resisting invasive pathogens (Shiromizu and Jancic 2018). During immune response, γδT cells usually act earlier than αβT cells and serve as a bridge between innate and adaptive immunity. In the early stage of the innate immune response, γδT cells recruit innate cells including neutrophils and macrophages; then in the middle stage, they regulate B cells to produce immunoglobulins, and present antigens to CD4+ T cells and CD8+ T cells; however, in the later stage, they can kill macrophages and αβT cells and promote tissue repair (Zhou et al. 2020). Additionally, IL-17A is an important cytokine during *C. albicans* infection, which is mainly produced by Th17 cells and γδT cells (Majumder and Mcgeachy 2021). The bidirectional interactions between γδT cells or IL-17A and microbiota have been noticed but not been fully understood (Majumder and Mcgeachy 2021; Papotto et al. 2021). Additionally, whether γδT cells and IL-17A play roles in influencing gut microbiota during *C. albicans* overgrowth has not been determined.

Considering the overgrowth of *C. albicans* in OC patients, the unrevealed interplay between *C. albicans* infection and gut microbes, as well as the undetermined roles of γδT cells and IL-17A in *C. albicans*-gut microbiota interplay, this study aimed to explore the changes of gut microbiome caused by *C. albicans* overgrowth in OC mice and the role of γδT cells and IL-17A during this process. As a result, this study exhibited the significant influence of *C. albicans* overgrowth on gut bacteria and fungi in oral tumour-bearing mice. Additionally, it was revealed that immune components (IL-17A and γδT cells) might play potential roles in modulating gut microbiota during *C. albicans* overgrowth.

2. Materials and methods

2.1. Oral tumour-bearing mouse model

C3H/HeN mice (6 to 8 weeks) were used in the study. All mice were maintained under specific pathogen-free conditions, fed with the same feed, and in the same facility and housing unit. Fresh faeces in the colon of oral tumour-bearing mice were collected with sterile instruments from our previous work (Wang et al. 2023a), and stored at −80 °C. Briefly, C3H/HeN mice were fed with 0.1% (wt/vol) tetracycline hydrochloride in drinking water for 1 week. Then, the mice in *C. albicans* infected group (CA group) were infected with *C. albicans* (SC5314 strain) by drinking water containing *C. albicans* for 2 weeks. After 2 weeks of pre-infection, all of the infected (CA group) and uninfected (CON group) mice were injected with SCC VII cells into the submucosa of the tongue dorsum to induce oral tumour. Two weeks later, the faeces were collected. The animal experiments were approved by the Biomedical Ethics Committee of Peking University.

2.2. In vivo *neutralisation of γδT cells or IL-17A*

The *in vivo* neutralisation experiments were performed as previously (Wang et al. 2023a). As for the IL-17A neutralising experiment, the oral tumour-bearing mice were injected both intraperitoneally (50 μg, 2 times/week) and intratumorally (25 μg, 3 times/week) with anti-mouse IL-17A antibody (clone 17F3, BioXcell) for 2 weeks (AIL group: Mice with *C. albicans* infection plus IL-17A neutralisation). As for the γδT cell neutralising experiment, the tumour-bearing mice were also injected both intraperitoneally (50 μg, 2 times/week) and intratumorally (25 μg, 3 times/week) with anti-mouse TCRγ/δ antibody (clone UC7-13D5, BioXcell) for 2 weeks (ATG group: Mice with *C. albicans* infection plus TCRγ/δ neutralisation).

2.3. 16S rRNA and ITS sequencing

The 16S rRNA and ITS sequencings were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Briefly, DNA was extracted from faecal pellets using a Magnetic Soil and Stool DNA kit (TianGen, China, Catalog #: DP712). The DNA was amplified using 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) to target the V4 region of bacteria or using ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) to target the ITS region of fungi. Then the samples were barcoded

and pooled to construct the sequencing library, and sequenced by an Illumina Novaseq6000 to generate pair-ended 150 × 150 reads.

2.4. Bioinformatics analysis

Paired-end reads were merged using FLASH (V1.2.11) (Magoc and Salzberg 2021). Fastp (V0.23.1) software was used to perform quality filtering and obtain high-quality Clean Tags. The clean tags were compared with the reference database [Silva database (for 16S), https://www.arb-silva.de/; Unite Database (for ITS), https://unite.ut.ee/] using UCHIME Algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) to remove the chimera sequences (Edgar et al. 2011). Then, the effective tags were used to obtain ASVs (Amplicon Sequence Variants) with DADA2 or deblur module in the QIIME2 software (Version QIIME2–202006). Species annotation was performed using QIIME2 software (16S: Silva Database, ITS: Unite Database). Multiple sequencing alignment was performed using QIIME2 software. Then, the absolute abundance of ASVs was normalised using a standard sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on the output normalised data. The correlation analyses between 16S and ITS data was performed using Omicsmart, a dynamic real-time interactive online platform for data analysis (http://www.omicsmart.com).

2.5. Statistics

For the differential analysis of Chao1 richness, Observed OTUs, Shannon, and Pielou evenness index, a Student's *t*-test was used to determine the statistical relevance between two groups. Bray-Curtis distance was used to analyse the beta-diversity patterns. *T*-test was used to find out the differential species between groups. Wilcoxon test was used to analyse the Bray-Curtis distance between inter- and intra-group. Procrustes test was used to analyse the correlation between 16S and ITS in groups. Spearman correlation analysis was used to analyse the interactions between 16S and ITS data. Values of $P < 0.05$ were considered significant.

2.6. Data availability

Sequence data that support the findings of this study have been deposited in NCBI BioProject with the primary accession codes (PRJNA991314 for 16S, PRJNA991317 for ITS) and in NCBI Sequence Read Archive (SRA) with the primary accession codes (SRP447344 for 16S, SRP447374 for ITS).

3. Results

3.1. C. albicans infection disturbed the gut microbiota of oral tumour-bearing mice

In the 16S rRNA sequencing, a rarefaction curve based on the observed OTUs showed that the sequencing depth was sufficient to detect species in the samples (Figure 1a). Alpha diversity was estimated by Chao1, Observed OTUs, Pielou evenness, and Shannon index (Figure 1b). The results showed that the Observed OTUs ($P = 0.015$), Pielou evenness ($P = 0.005$), and Shannon index ($P = 0.002$) in the CON group were significantly higher than in the CA group (Figure 1b), which indicated that the *C. albicans* infection caused the reduction of community richness and diversity of gut bacteria in tumour-bearing mice. Beta diversity estimated by the PCoA plot showed a distinguishable microbial composition between the CON and CA groups (Figure 1c).

To further study the changes in the gut bacteria between the CON and CA groups, the compositional changes were evaluated. At the phylum level, it was noticed that the *Bacteroidota* was increased, but *Firmicutes* was reduced in the CA group compared to the CON group (Figure 1d). At the genus level, it was found that *Ralstonia*, *Alistipes*, *Clostridia UCG-014*, *Ruminococcus*, and *Lachnospiraceae NK4A136 group* were reduced, but *Muribaculum*, *Dubosiella*, *Parabacteroides*, *Lachnospiraceae UCG-001*, and *Erysipelatoclostridium* were increased in the CA group (Figure 1e). The Venn graph showed that there were 442 common ASVs between CON and CA groups, 488 unique ASVs in the CON group, and 230 unique ASVs in the CA group (Figure 1f). Further, the LEfSe (LDA Effect Size) result showed that *Firmicutes*, *Clostridia*, *Lactobacillus*, *et al*. were the main taxa enriched in the CON group, while *Bacteroidales*, *Muribaculum*, *Parabacteroides*, *et al*. were more abundant in the CA group (LDA score > 4, $P < 0.05$) (Figure 1g). The volcano plot showed the differentially

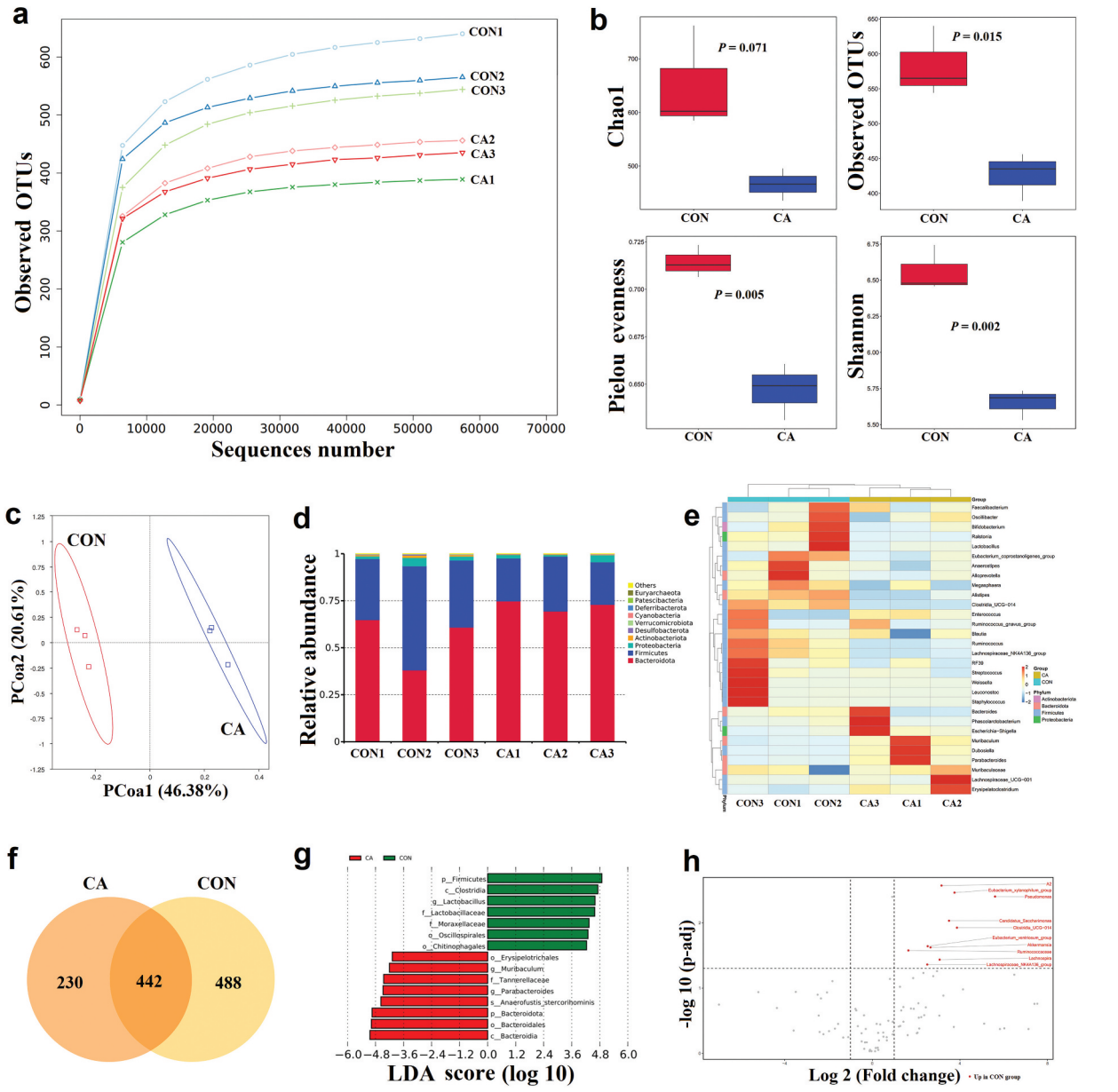

Figure 1. *Candida albicans* overgrowth induced the disorder of the gut microbiota of bacteria. (a) The rarefaction curve was based on the Observed OTUs. (b) Alpha diversity was evaluated by the Chao1, Observed OTUs, Pielou evenness, and Shannon index. (c) Beta diversity was measured by principal coordinate analysis (PCoA). (d) The distribution plot of relative abundance at the phylum level. (e) The heat map of relative abundance at the genus level. (f) The Venn diagram of the Amplicon Sequence Variants (ASVs) between groups. (g) The histogram of taxa with LDA scores more than 4 and *P* value less than 0.05. (h) The volcano plot of the dominant bacterial genus between groups.

altered species between groups, which indicated that *C. albicans* caused the significant reduction of various bacteria, such as *Clostridia UCG-014*, *Akkermansia*, and *Lachnospira* in the gut of mice ($P < 0.05$) (Figure 1h).

In the ITS gene sequencing, the sequencing depth was also sufficient to detect species in the samples (Figure 2a). The alpha diversity showed that the Pielou evenness ($P = 0.000779$) and Shannon ($P = 0.0168$) index in the CON group were significantly higher than in the CA group (Figure 2b). The PCoA plot showed a distinguishable fungal composition between the CON and CA groups (Figure 2c).

The compositional changes evaluated at the phylum level showed that *Ascomycota* was significantly increased in the CA group compared to the CON group (Figure 2d). At the genus level, it was found that *Candida* and *Malassezia* were increased, but others including *Aspergillus*, *Cladosporium*, and

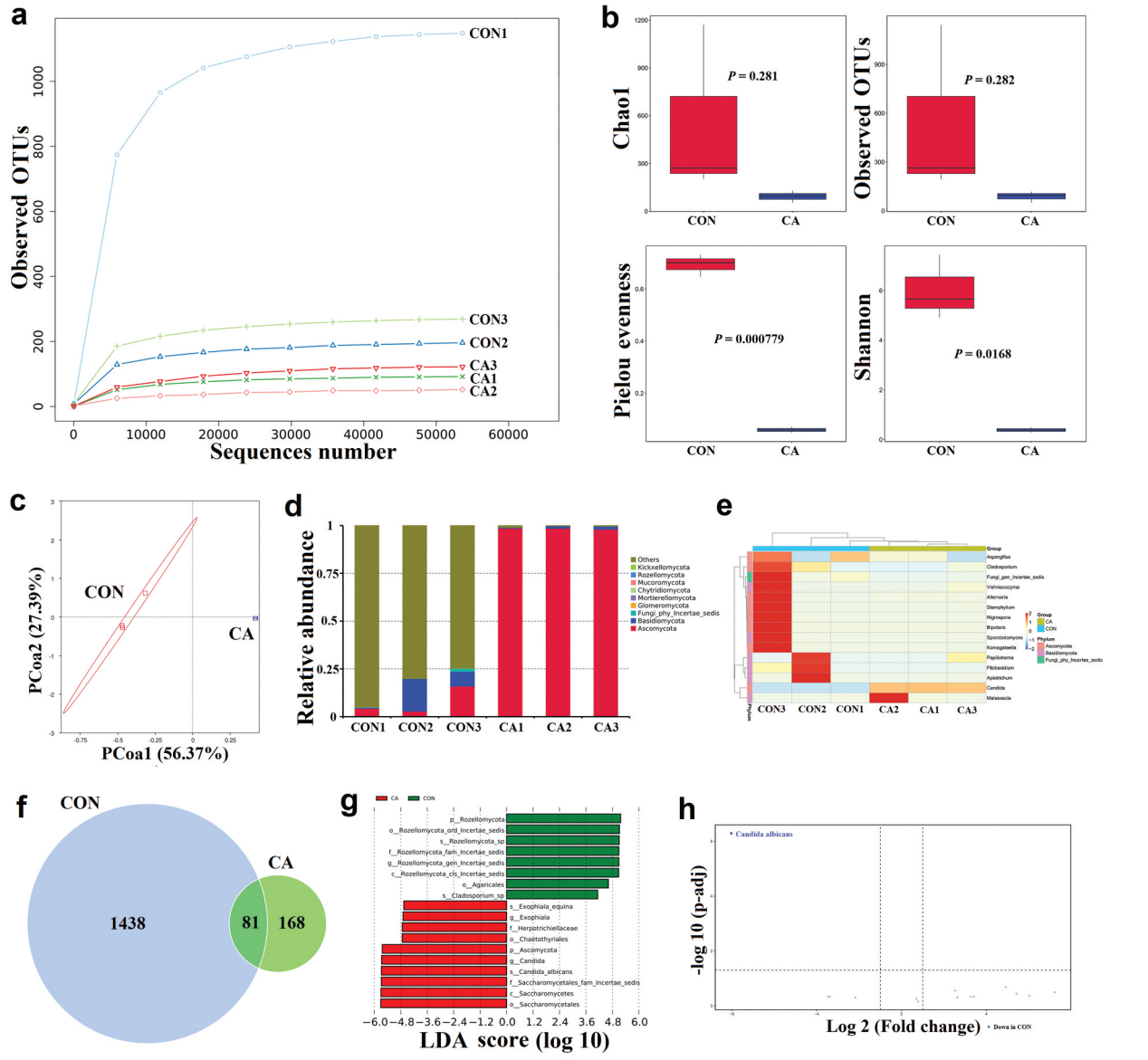

Figure 2. *Candida albicans* overgrowth induced the disorder of gut microbiota of fungi. (a) The rarefaction curve was based on the Observed OTUs. (b) Alpha diversity was evaluated by the Chao1, Observed OTUs, Pielou evenness, and Shannon index. (c) Beta diversity was measured by PCoA. (d) The distribution plot of relative abundance at the phylum level. (e) The heat map of relative abundance at the genus level. (f) The Venn diagram of the ASVs between groups. (g) The histogram of taxa with LDA scores more than 4 and *P*-value less than 0.05. (h) The volcano plot of the dominant fungal species between groups.

Bipolaris were reduced in the CA group (Figure 2e). The Venn graph showed that there were 81 common ASVs between the CON and CA groups, 1,438 unique ASVs in the CON group and only 168 unique ASVs in the CA group (Figure 2f). Further, the LEfSe result showed that *Rozellomycota*, *Agaricales*, and *Cladosporium* were the main taxa enriched in the CON group, while *Exophiala* and *Candida* were more abundant in the CA group (LDA score > 4, $P < 0.05$) (Figure 2g). The volcano plot demonstrated that *C. albicans* was the dominant fungus in the gut of mice with *C. albicans* infection (Figure 2h) ($P < 0.05$).

3.2. In vivo γδT cell neutralisation partly rescued the bacterial dysbiosis, but aggravated the fungal dysbiosis caused by C. albicans infection

γδT cells are a cluster of cells much more abundant as tissue-resident intraepithelial lymphocytes in the gastrointestinal tract (Ivanov et al. 2022), which is one of the important parts during *Candida* infection

(Mengesha and Conti 2017). To explore the role of γδT cells in the microecological disturbance caused by *C. albicans* infection, an *in vivo* γδT cell neutralisation experiment was performed.

As for 16S sequencing, the rarefaction curve showed that the sequencing depth was sufficient to detect species in the samples (Figure 3a). The alpha diversity showed that the Chao1, Observed OTUs, Pielou evenness, and Shannon index in the ATG group were higher than in the CA group, though the differences were not statistically significant ($P > 0.05$) (Figure 3b). The PCoA plot indicated that there was no significant difference in beta diversity between the CA and ATG groups (Figure 3c).

When compared to the CA group, the *Bacteroidota* was decreased, but *Firmicutes* was increased in the ATG group (Figure 3d), which indicated that the neutralisation of γδT cells partly alleviated the out-of-balance of *Firmicutes*/*Bacteroidota* (F/B) ratio in the gut caused by *C. albicans* infection. At the genus level, it was found that the reduced bacteria including *Alistipes*, *Clostridia UCG-014*, *Alloprevotella*, and

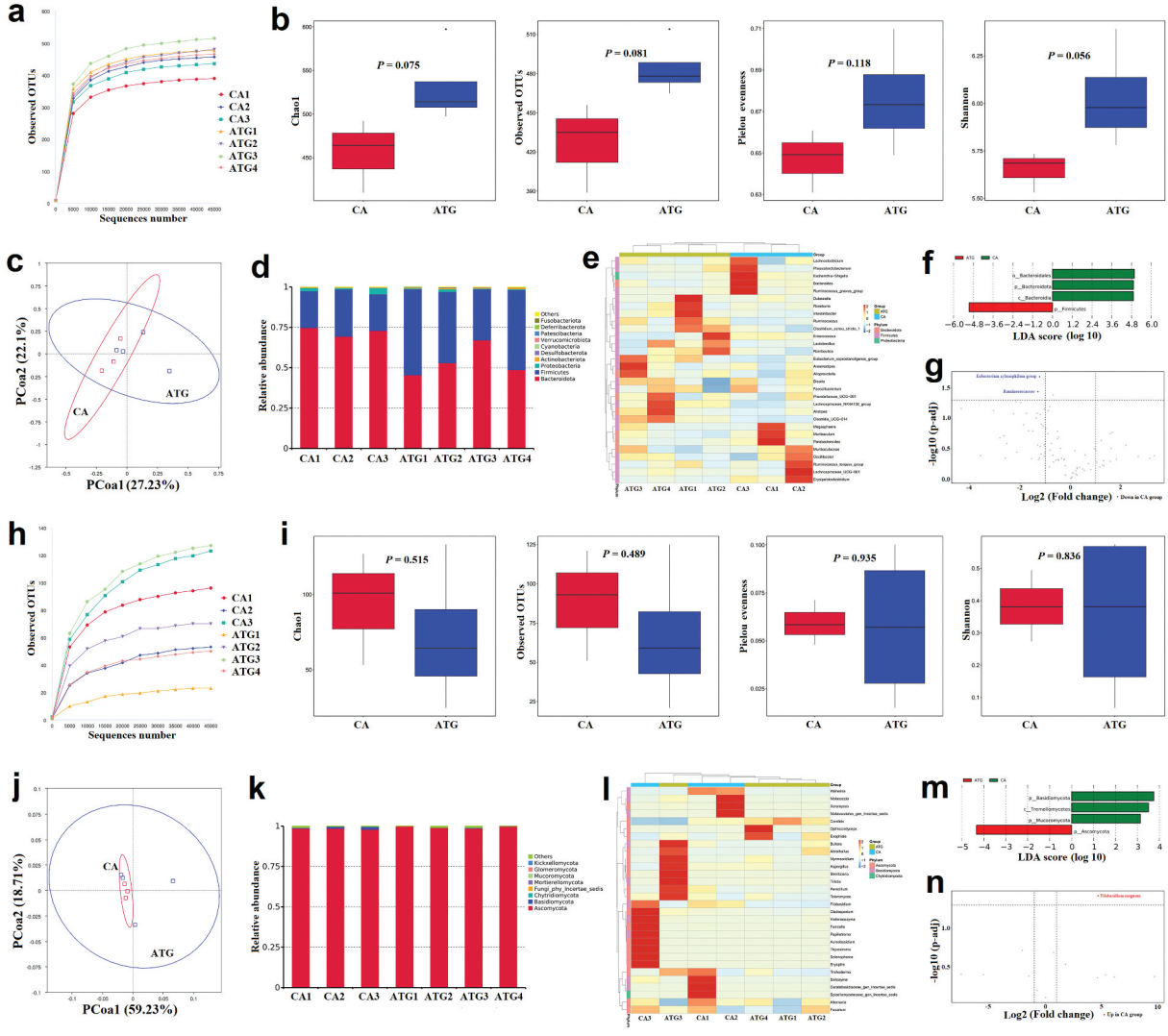

Figure 3. *In vivo* γδT cell neutralisation partly rescued the bacterial dysbiosis but aggravated the fungal dysbiosis caused by *Candida albicans* overgrowth. (a) The rarefaction curve for 16S sequencing was based on the Observed OTUs. (b) Alpha diversity was evaluated by the Chao1, Observed OTUs, Pielou evenness, and Shannon index. (c) Beta diversity was measured by PCoA. (d) The distribution plot of relative abundance at the phylum level. (e) The heat map of relative abundance at the genus level. (f) The histogram of taxa with LDA scores more than 4 and *P* value less than 0.05. (g) The volcano plot of the dominant bacterial genus between the CA and ATG groups. (h) The rarefaction curve for ITS sequencing was based on the Observed OTUs. (i) Alpha diversity was evaluated by the Chao1, Observed OTUs, Pielou evenness, and Shannon index. (j) Beta diversity was measured by PCoA. (k) The distribution plot of relative abundance at the phylum level. (l) The heat map of relative abundance at the genus level. (m) The histogram of taxa with LDA scores more than 3 and *P* value less than 0.05. (n) The boxplot of the differential fungal species between the CA and ATG groups.

Lachnospiraceae NK4A136 group caused by *C. albicans* infection were partly rescued after the neutralisation of γδT cells (Figure 3e). The LEfSe result showed that *Firmicutes* was the main taxa enriched in the ATG group, while *Bacteroidota* was more abundant in the CA group (LDA score > 3, $P < 0.05$) (Figure 3f). Additionally, it was shown that *Eubacterium xylanophilum group* and *Ruminococcaceae* increased in the ATG group when compared to the CA group ($P < 0.05$) (Figure 3g).

As for the ITS sequencing, the sequencing depth was also sufficient to detect species in the samples (Figure 3h). The alpha diversity showed that the Chao1 and Observed OTUs index in the ATG group were lower than in the CA group, though there were no statistically significant differences ($P > 0.05$) (Figure 3i). The PCoA plot indicated that there was no difference in beta diversity between the CA and ATG groups (Figure 3j).

The *Basidiomycota* in the ATG group was lower than in the CA group (Figure 3k). At the genus level, it was found that *Wallemia* and *Filobasidium* were decreased but *Candida* and *Exophiala* were increased in the ATG group compared to the CA group (Figure 3l). The LEfSe result showed that *Ascomycota* was more abundant in the ATG group compared to the CA group (LDA score > 4, $P < 0.05$) (Figure 3m). Additionally, it was shown that *Filobasidium magnum* was significantly decreased in the ATG group ($P < 0.05$) (Figure 3n).

3.3. In vivo *IL-17A neutralisation aggravated the microbial dysbiosis caused by* C. albicans *infection*

IL-17A is an important cytokine for antifungal immunity, which mediates the antimicrobial immune response and contributes to wound healing of injured epithelium (Akuzum and Lee 2022). Thus, we further explored the influence of IL-17A on gut microbiota during *C. albicans* infection.

The sequencing depth was sufficient to detect species in the samples (Figure 4a and h). The alpha diversity of bacteria showed that the Chao1 and Observed OTUs index in the AIL group were lower than in the CA group, though the differences were not statistically significant ($P > 0.05$) (Figure 4b). The PCoA plot indicated that there was no significant difference in beta diversity between the CA and AIL groups (Figure 4c).

When compared to the CA group, the *Bacteroidota* was increased, but *Firmicutes* was decreased in the AIL group (Figure 4d), which indicated that the neutralisation of IL-17A aggravated the out-of-balance of F/B in the gut caused by *C. albicans* infection. At the genus level, it was found that the neutralisation of IL-17A reduced *Clostridia UCG-014*, *Alistipes*, *Muribaculum*, *Straphylococcus*, *Enterococcus*, and *Lactobacillus*, but increased *Alloprevotella*, *GCA-900066575*, and *Lachnoclostridium* in mice gut with *C. albicans* infection (Figure 4e). Additionally, it was found that *Muribaculaceae* was the main taxa enriched in the CA group (Figure 4f). The volcano plot demonstrated that the neutralisation of IL-17A reduced the *UCG-010*, but increased the *Alloprevotella* and *Streptococcus* in the gut of mice with *C. albicans* infection ($P < 0.05$) (Figure 4g).

As for the mycobiome, it was found that the Chao1, Observed OTUs, Pielou evenness, and Shannon index in the AIL group were lower than in the CA group, though there were no significant differences ($P > 0.05$) (Figure 4i). The PCoA plot showed a difference in beta diversity between the CA and AIL groups (Figure 4j). When compared to the CA group, the *Ascomycota* was increased, but other fungi were decreased in the AIL group (Figure 4k), which indicated that the neutralisation of IL-17A aggravated the fungal disorder caused by *C. albicans* infection. It was found that the neutralisation of IL-17A boosted the *C. albicans* overgrowth ($P < 0.05$) but inhibited most of the other fungi in the gut (Figures 4l, m and n).

3.4. *Significant correlations between fungal and bacterial kingdoms were found in the gut microbiome during* C. albicans *infection*

Pairwise comparisons of Bray-Curtis dissimilarity values (Nash et al. 2017) based on OTUs between samples from the same groups (within the CON group or the CA group) and between samples from different groups (between CON and CA group) for the 16S rRNA and ITS sequencing data were performed. The results showed that the Bray-Curtis distance for both 16S and ITS was significantly higher in inter-group (between) than in intra-group (within) ($P < 0.01$) (Figure 5a), which indicated that the *C. albicans* infection influenced both the bacterial and fungal microbiome. Procrustes correlation testing (Longa et al. 2017) for PCoA analysis showed a correlation of 0.714 at Class level ($M^2 = 0.4896$, $P < 0.05$) (Figure 5b),

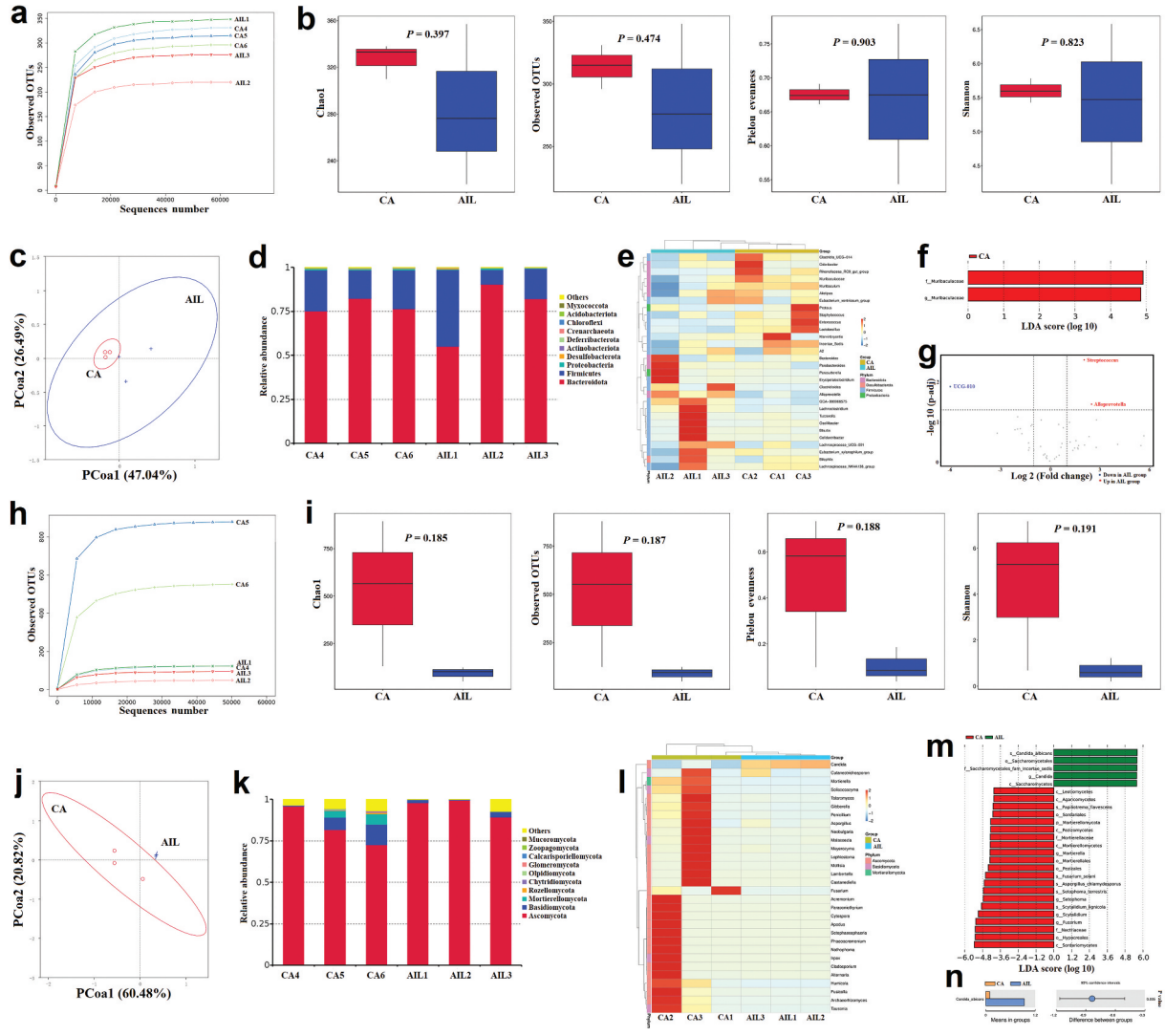

Figure 4. *In vivo* IL-17A neutralisation aggravated the microbial dysbiosis caused by *Candida albicans* overgrowth. (a) The rarefaction curve for 16S sequencing was based on the Observed OTUs. (b) Alpha diversity for 16S sequencing was evaluated by the Chao1, Observed OTUs, Pielou evenness, and Shannon index. (c) Beta diversity for 16S sequencing was measured by PCoA. (d) The distribution plot of relative bacterial abundance at the phylum level. (e) The heat map of relative bacterial abundance at the genus level. (f) The histogram of bacterial taxa with LDA scores more than 4 and *P* value less than 0.05 between the CA and AIL groups. (g) The volcano plot of the dominant bacterial genus between the CA and AIL groups. (h) The rarefaction curve for ITS sequencing was based on the Observed OTUs. (i) Alpha diversity for ITS sequencing was evaluated by the Chao1, Observed OTUs, Pielou evenness, and Shannon index. (j) Beta diversity for ITS sequencing was measured by PCoA. (k) The distribution plot of relative fungal abundance at the phylum level. (l) The heat map of relative fungal abundance at the genus level. (m) The histogram of fungal taxa with LDA scores more than 4 and *P* value less than 0.05 between the CA and AIL groups. (n) The histogram of the differential fungal species between the CA and AIL groups.

meaning that bacterial and fungal diversity reacted in a similar way to *C. albicans* challenge. Then, the correlations between fungal and bacterial kingdoms were analysed. In the family level, the proportion of inter-kingdom similarity values with an absolute value > 0.5 ($P < 0.05$) that were positive was 46.2% (6 positives, 7 negatives) (Figure 5c). In the genus level, the proportion of positive intra-kingdom bacterial and fungal similarity values was 67.1% (108 positives, 53 negatives) and 73.3% (22 positives, 8 negatives), respectively, both of which were higher than the proportion of inter-kingdom positive correlations ($P = 0.037$ and 0.065 respectively) (Figure 5d). Comparing abundances of fungal and bacterial genera revealed that there were strong positive correlations between *Candida* and *Muribaculum*, *GCA-900066575*, and *Erysipelatoclostridium*, but negative correlations between *Candida* and *Ruminococcaceae UCG-014*, *Akkermansia*, *Anaerostipes*, *Eubacterium ventriosum group*, *A2*, and *Bilophila* (Figure 5e).

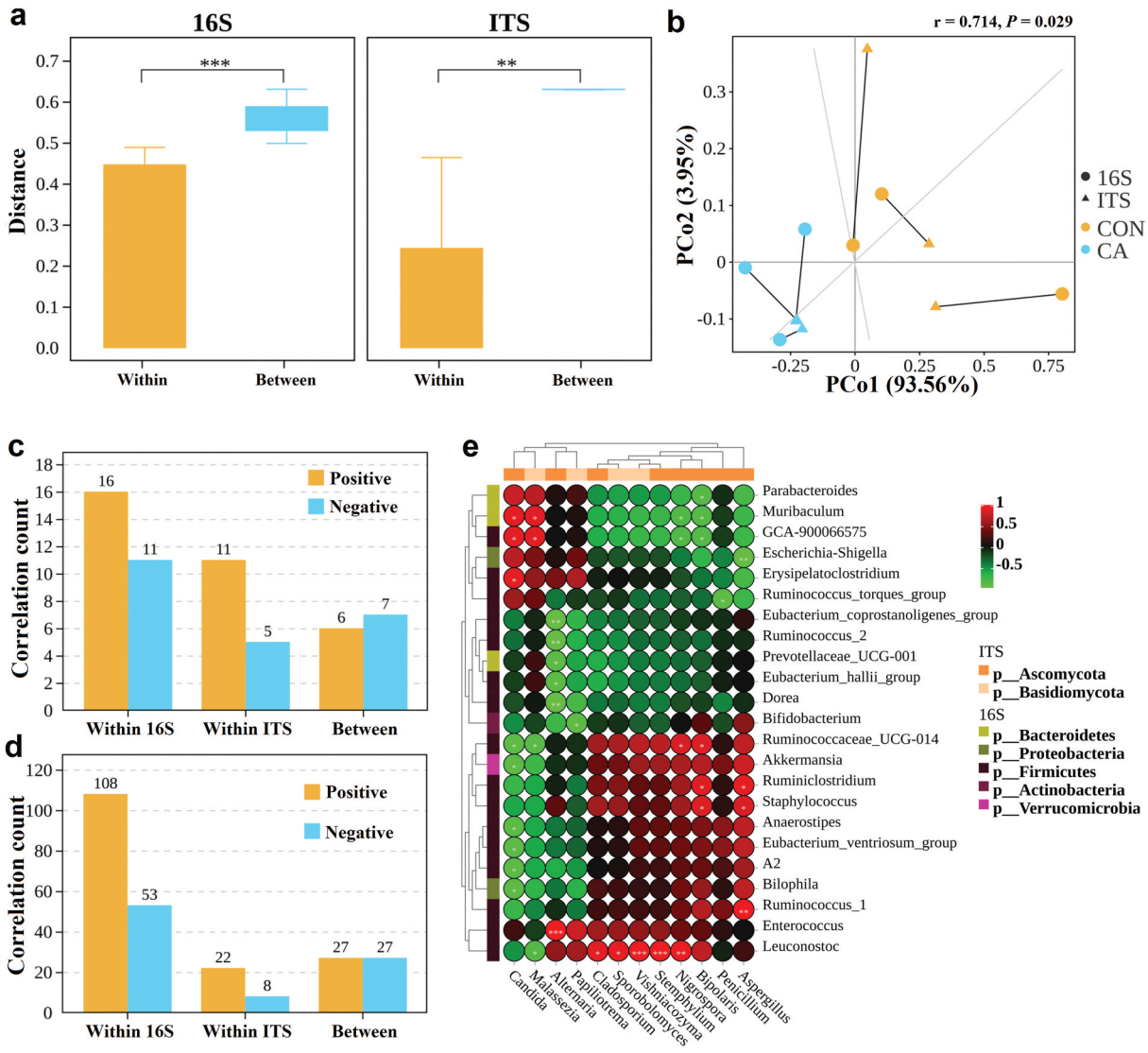

Figure 5. Significant correlations between fungal and bacterial kingdoms were found in the gut microbiome during *Candida albicans* overgrowth. (a) Box plot of the Bray-Curtis distance between samples in the same group (within the CON group or the CA group) and between samples in different groups (between the CON and CA groups) for 16S and ITS sequencing data. Bray-Curtis dissimilarity values range from 0 to 1, with 0 being the least dissimilar and 1 being the most dissimilar. Wilcoxon test, $^{**}P < 0.001$, $^{***}P < 0.0001$. (b) Procrustes test depicting the significant correlation between gut bacterial community and fungal community based on Bray-Curtis dissimilarity metrics (Class level, sum of squares $M^2 = 0.4896$, $r = 0.714$, $P = 0.029$, 999 permutations). (c and d) Bar graph of the significant correlation count for intra-bacteria (within 16S), intra-fungi (within ITS), and inter-kingdom (between) in family level (c) and genus level (d) based on Spearman correlations (with an absolute correlation value > 0.5 and $P < 0.05$). (e) Heatmap of the correlations between bacteria and fungi in genus level based on Spearman correlations. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

4. Discussions

C. albicans is the main pathogen to oropharyngeal and gastrointestinal candidiasis in cancer patients receiving chemotherapy and radiotherapy (Umazume et al. 1995; Bertolini et al. 2019; Kumari et al. 2021). In this study, we investigated the influence of *C. albicans* infection on the composition of intestinal bacteria and fungi in the context of oral cancer. It was demonstrated that *C. albicans* overgrowth led to a profound taxonomic imbalance on the gut microbiota. It was also discovered that the neutralisation of γδT cells partly rescued the bacterial dysbiosis caused by *C. albicans* infection, while the neutralisation of IL-17A aggravated the microbial dysbiosis caused by *C. albicans* infection. Additionally, the inter-kingdom relationships between bacteria and fungi seemed to be influenced during *C. albicans* overgrowth. Thus, the results indicated that the overgrowth of *C. albicans* in oral cancer

patients could induce the dysbiosis of gut microbiota, which was partly associated with the intestinal immune components including γδT cells and IL-17A.

In the present study, *C. albicans* infection disrupted the gut microbiota including bacteria and fungi in oral tumour-bearing mice. As for bacteria, the alpha diversity and F/B ratio were significantly reduced after *C. albicans* infection. *Firmicutes* and *Bacteroidota* are two dominant phyla occupying together up to 90% of the total gut microbiota (Human Microbiome Project Consortium 2012). The F/B ratio is suggested to be an important index of the health of gut microbiota (Vaiserman et al. 2020). The reduced gut F/B ratio is associated with many pathological conditions including breast cancer (An et al. 2023), Hepatitis C virus (Aly et al. 2016), and inflammatory bowel disease (Stojanov et al. 2020). Thus, the reduced F/B ratio caused by *C. albicans* infection indicated an abnormal intestinal homoeostasis. Similarly, *C. albicans* also induced the reduction of alpha diversity in fungi and caused the decrease of some other fungi levels, which indicated that *C. albicans* overgrowth might inhibit the growth of some fungi in the gut.

Microbiota is reported to affect the development and homoeostasis of γδT cells, and vice versa, γδT cells play important roles in the selection and maintenance of commensal microbiota (Papotto et al. 2021). Our study demonstrated that the *in vivo* γδT cell neutralisation partly rescued the bacterial dysbiosis caused by *C. albicans* infection, which indicated that γδT cells might be potential henchmen for *C. albicans* inducing gut bacterial disorders. However, the neutralisation of γδT cells further boosted the overgrowth of *Ascomycota* in the gut. It is worth mentioning that microbiota-related IL-17A producing γδT cells were reported to promote OC and colorectal cancers (Reis et al. 2022; Wei et al. 2022). That's to say, γδT cells may be important parts in maintaining microbial homoeostasis, the abnormality of which may promote cancer development.

IL-17A, an important cytokine produced at low levels in response to the resident microbiota, contributes to maintaining a healthy bacterial and fungal population on mucosa and skin (Majumder and Mcgeachy 2021). However, the unrestrained IL-17 signal is proposed to be associated with cancer progression (Mcgeachy et al. 2019). Though IL-17A is important in fighting against *C. albicans* (Douzandeh-Mobarrez and Kariminik 2019), the role of IL-17A in regulating gut microbiota during *C. albicans* infection is unclear. Interestingly, our results showed that the neutralisation of IL-17A during *C. albicans* infection further reduced the F/B ratio and boosted the overgrowth of *Candida* in the gut of mice with oral cancer. Thus, it is indicated that IL-17A is an important cytokine in inhibiting *Candida* overgrowth and maintaining intestinal homoeostasis.

C. albicans infection influenced the abundance of some bacterial species, which are closely associated with intestinal diseases and prognosis. It was shown that *C. albicans* infection caused the decrease of *Alistipes*, *Clostridia UCG-014*, *Akkermansia*, and *Lachnospiraceae NK4A136 group*. Correlation analysis further supported the negative relationships between *C. albicans* and *Akkermansia*. Among them, *Alistipes* is a controversial genus of bacteria possessing protective effects against colitis or pathogenic effects on colorectal cancer (Parker et al. 2020). *Clostridia UCG-014* is reported to be decreased in patients with Crohn's disease and may be associated with gut barrier function (Leibovitzh et al. 2022). *Akkermansia muciniphila* is accepted to be a promising probiotic and has important roles in regulating host functions that are disrupted in various diseases including ulcerative colitis (Derrien et al. 2017). *Lachnospiraceae NK4A136 group* is also a short chain fatty acids (SCFA)-producing bacterium, which enhances gut barrier function (Ma et al. 2020). Thus, the decrease of potential beneficial bacteria caused by *C. albicans* overgrowth may further affect the host's immune system and intestinal mucosal barrier.

Accumulating evidence has demonstrated the importance of gut microbiota in the prognosis of various types of cancers (Helmink et al. 2019; Sepich-Poore et al. 2021). Whether *C. albicans* influences the efficacy of tumour treatment is rarely reported. From our results, it is speculated that *C. albicans* infection may influence the efficacy of tumour treatment by disrupting intestinal homoeostasis. *Akkermansia muciniphila* was reported to be associated with the clinical benefit of PD-1 blockade in patients with non-small-cell lung cancer or kidney cancer (Derosa et al. 2022). Additionally, *Bifidobacterium* spp. and *Faecalibacterium* spp. were demonstrated to be associated with favourable anticancer immune responses (Routy et al. 2018a). Thus, the reduced abundance of

Akkermansia, *Bifidobacterium*, and *Faecalibacterium* during *C. albicans* overgrowth may indicate an impaired anticancer immune efficacy.

Though many studies have demonstrated the role of bacteria in cancer treatment, relatively few studies explored fungi. Some recent studies provided evidence supporting fungal roles in cancer treatment. It was reported that the depletion of fungi enhanced the responsiveness of breast cancer and melanoma to radiotherapy (Shiao et al. 2021). Additionally, the enriched presence of *Filobasidium* spp. in donor faeces was demonstrated to be associated with the beneficial response to faecal microbiota transplantation (FMT) for patients with ulcerative colitis (van Thiel et al. 2022), which fungus was reduced during *C. albicans* overgrowth in our study. Thus, it would make sense to further explore the role of fungi including *C. albicans* in cancer treatment.

The limitations of this study should be noticed. Firstly, this study only focused on the roles of γδT cells and IL-17A in *C. albicans* influencing gut microbiota, but did not explore the other immune factors, such as macrophages, Th17 cells, and B cells. Secondly, the interplay between fungi and bacteria in the host is complex, further studies should explore this matter from multi-dimensional time and space. Lastly, different levels and statuses of *C. albicans* infection may contribute differently to intestinal immunity and intestinal flora, which should be considered.

5. Conclusions

In conclusion, this study presented the disturbed intestinal flora caused by *C. albicans* infection in the context of oral cancer. *C. albicans* overgrowth inhibited the growth of some beneficial bacteria, such as *Lachnospiraceae NK4A136 group* and *Akkermansia*, which indicated that *C. albicans* overgrowth during cancer development and treatment might damage the intestinal mucosal barrier and even impair the efficacy of cancer treatment. Additionally, it was speculated that immune components such as γδT cells and IL-17A might participate in the maintenance of intestinal homoeostasis. Thus, it is suggested to control *C. albicans* infection during the period of cancer treatment.

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