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Diseases & Research

Structural Modulation of Gut Bacterial and Fungal Community of C57BL/6 Mice by *Pseudobulbus Cremastrae Seu Pleionesaqueous* Extract

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Backgrounds: *Pseudobulbus Cremastrae Seu Pleiones* (PC), a traditional Chinese medicine (TCM) is used to treat various cancersin the modern pharmacological research. However, the potential mechanisms remain uncertain. The gut microbiome is involved in the pharmacological activities of many TCMs. Contemporary investigations into the interactions between gut microbiota and medicine have predominantly fixated on gut bacteria and routinely disregarded gut fungi. Present study firstly investigated the effects of PC on both gut bacteria and fungi and their interactions to explore its pharmacological mechanism.
Methods: Eighteen normal C57BL/6 mice were divided into PC and negative control groups, and orally administered an aqueous PC extract (1 g raw herb/kg) or an equivalent volume of distilled water, respectively. Fecal samples were gathered after four weeks and then under-went sequencing of the full-length 16S rRNA and ITS1/2 genes. Cecal samples were additionally obtained for determination of short-chain fatty acid levels.
Results: PC augmented the population of SCFA-producing bacteria like *Duncaniellamuris*, *Duncanielladubosii, Kineothrix alysoides* and *Faecalimonas umbilicata*, with a concomitant rise in cecal acetate and propionate amounts. In addition, PC significantly increased some potential beneficial fungi, such as *Cladosporium sp*., *Psathyrella candolleana*, *Nigrospora sphaerica*, and decreased some common fungi, like *Aspergillus spp*., *Penicillium spp*., *Cosmospora viridescens*. The interaction network demonstrated a complex relationship between gut bacteria and fungi after PC treatment.
Conclusion: These results suggest close links between an enriched abundance of SCFA-producing bacteria, gut fungi alterations, and anti-cancer effect of PC, highlighting the importance of gut fungi in mediating the in vivo effects of drugs.
Keywords: Traditional Chinese medicine; Gut bacteria; Gut fungi; Short-chain Fatty acid (SCFA).

Introduction

Trillions of microorganisms colonize the human intestine, jointly forming the gut microbiome. Emerging evidence suggests that gut bacteria are vital for host health and important targets for elucidating the mechanisms of many drugs[1–5]. Evidence suggests that some drugs, such as nuciferine[6] and berberine[7], could change the diversity and composition of gut bacteria. Recently, the causal relationship between drug efficacy and gut bacteria alterations has become more apparent. For example, drugs regulate the relative abundance of specific gut bacteria, change the levels of their metabolites, and activate or inactivate related molecular signaling pathways to alleviate disease symptoms[8]. Specifically, we found that the gut microbiota showed strong positive correlation with BBR's cholesterol-decreasing effect[9]. Remarkably, the initial abundances of *Alistipes* and *Blautia* were capable of precisely forecasting BBR's anti-hypercholesterolemic impact, and the lack of *Blautia* largely nullified it's cholesterol-reducing effectiveness[5]. Hence, modifications in gut bacteria might represent a novel approach for explicating drug mechanisms. Meanwhile, the changes in metabolites caused by the alteration in the abundance of gut bacteria may also have different regulatory effects on the body. SCFAs are metabolites produced by gut bacteria from indigestible carbohydrates, which provide energy for the host, and contribute anti-inflammatory and anticancer effects[10]. For instance, propionate upregulates Major Histocompatibility Complex-Class I Chain Related Proteins A and B (i.e., MICA/B) expression, a ligand of immune stimulatory Natural Killer Group 2D (i.e., NKG2D), on the surface of colon cancer cells, exerting its anticancer potential by immune response, related to mammalian target of rapamycin complex 2 (i.e., mTORC2) activity. Simultaneously, propionate inhibits breast cancer cell proliferation and induce apoptosis by regulating JAK2/STAT3/ROS/p38 MAPK signaling pathway[11]. Fungi and the fungal community are also indispensable components of the gut microbiome that have received little attention owing to their relatively small numbers in the gut compared to bacteria. Currently, the functions of many gut fungi remain unknown. Nevertheless, the crucial roles of gut fungi in various physiological and pathological conditions have been gradually recognized. For example, *Coker* et al. (2019) found that 14 fungal biomarkers could

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effectively differentiate colorectal cancer patients from healthy participants (area under the receiver-operating characteristic curve [AUC]: 0.93), emphasizing the crucial role of gut fungi in colorectal cancer[12]. Furthermore, a clinical study reported that the increased biodiversity and altered composition of the gut fungal community were present in patients with primary sclerosing cholangitis (PSC), suggesting that gut fungi participate in the pathogenesis of this disease and could be a novel treatment target[13]. Bacher et al. (2019) also reported that *Candida albicans* induced human T helper cell 17 (Th17) responses, contributing to *Aspergillus fumigatus*-related non-intestinal inflammation[14].

Moreover, many fungi have been identified as promising sources of anticancer and anti-inflammation drugs. For example, aspulvinone analogues isolated from *Cladosporium sp*. limited lactate dehydrogenase (LDH) secretion, interleukin-1β (IL-1β) production, and the activation of NLR Family Pyrin Domain Containing 3 (i.e., NLRP3) inflammasome; they also suppressed caspase-1 cleavage and pyroptosis to attenuate severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection-induced inflammatory reactions[15]. In addition, bioactive secondary metabolites, such as benzopyranone and xanthone derivatives, showed potent cytotoxicity against two human prostate cancer cell lines, C42B and 22RV1[16]. Furthermore, two novel tetracyclic diterpenoids (psathyrins A and B), extracted from *Psathyrella candolleana* were identified as the antibacterial agents that inhibit *Staphylococcus aureus* and *Salmonella enterica*[17]. Moreover, ergosterols isolated from cultures of the basidiomycete *Psathyrella candolleana* displayed cytotoxicity against several human cancer cell lines, such as HL-60 (myeloid leukemia), SMMC-7721 (hepatocellular carcinoma), A549 (lung cancer), MCF-7 (breast cancer), and SW-480 (colon cancer)[18]. Nigronapthaphenyl, a new compound extracted from the fungus *Nigrospora sphaerica*, also has potential anticancer and anti-inflammatory effects and inhibits α-glucosidase[19].

In recent years, traditional Chinese medicine (TCM) has become one of the primary strategies for treating diseases[20,21]. However, the mechanisms of most TCMs remain unclear; thus, identifying the pharmacological mechanisms of TCMs from multiple dimensions is necessary. Compelling evidence have demonstrated that the efficacy of TCMs is achieved partly through modulating gut microbiome. For example, astragaloside can improve the function of the intestinal mucosal barrier via increasing the abundances of beneficial bacteria (such as *Ruminococcaceae*), and reducing the abundances of harmful bacteria (such as *Escherichia* and *Shigella*)[22,23]. Besides having a regulatory effect on bacteria, natural products also have corresponding regulatory effects on fungi and their products. *Syzygium aromaticum* can inhibit the growth of *Aspergillus flavus* and *Aspergillus parasiticus*[24]. As the secondary metabolites of *Aspergillus*, *Penicillium* and *Fusarium,* mycotoxins are highly toxic to humans and animals. However, *Echinacea purpurea* and *Zingiber officinale* can increase resistance to mycotoxin poisoning and enhance immune responses[25]. The water-insoluble polysaccharides from *Wolfiporia cocos* can inhibit the overgrowth of intestinal fungi and *Proteobacteria*, and reduce liver steatosis caused by long-term dietary ethanol[26]. Our previous study demonstrated that *Coptidis Rhizoma* reduced the abundance of several *Aspergillus* (*A. chevalieri*, *A. luteovirescens*, *A. oryzae* and *A. sp. F51*) and *Penicillium* (*P. expansum*, *P. janthinellum*, *P. sp. BAB-5649* and *P. sp. GZU-BCECYN66-5*) species, but enriched the abundance of *Tilletia bornmuelleri* and *Tilletia bromip*[27]. Additionally, cachexia, a syndrome with multiple factors, is characterized by cancer-related muscle loss, which is a major comorbidity in oncology[28]. Several TCMs possess the potential in alleviating cachexia[29]. For example, polyphenol isolated from TCMs, such as quercetin[30] , ursolic acid[31], and theaflavin[32] can alleviate muscle atrophy via their anti-inflammatory and antioxidant effects. Additionally, the fungi species of Mucoromycota (*Rhyzopus oryzae*) can treat and/or prevent the development of cachexia[33]. Therefore, modulation of both gut bacteria and fungi may represent one of the pathway for TCMs to exert their functions.

Pseudobulbus Cremastrae Seu Pleiones (PC) is a common TCM derived from the dried pseudostem of the plants *Cremastra appendiculata* (D.Don) Makino, *Pleione bulbocodioides* (Franch.) Rolfe, and *Pleione yunnanensis* Rolfe. Based on the traditional property theory of TCM, PC is a cool-natured and sweet-flavored TCM that is used to clear heat-toxins, dissipate phlegm, and resolve masses. In modern clinical practice, PC is valuable for its anticancer effects in liver[34], lung[35], bladder[36], colorectal[37], gastric[38] and breast cancers[39]. Specifically, network pharmacology and molecular docking studies have determined that PC may prevent cancer by modulating several targets and pathways, such as Epidermal Growth Factor Receptor (i.e., EGFR), SRC (a proto-oncogene), Estrogen Receptor-1 (i.e., ESR1), Erb-B2 Receptor Tyrosine Kinase 2 (i.e., ERBB2), Mechanistic Target Of Rapamycin Kinase (i.e., MTOR), MCL1 (an apoptosis regulator), Matrix Metalloproteinase (MMP) 2 (i.e., MMP2), MMP9, Kinase Insert Domain Receptor (i.e., KDR), and Janus Kinase 2 (i.e., JAK2). However, the exact mechanisms controlling the pharmacological activities of PC are far from clear.

Elucidating how PC modulates the gut fungal and bacterial communities might cast new perspectives on the specific mechanisms of PC. Therefore, we delved into how PC influences the characteristics of bacteria and fungi and the associations between gut bacteria and gut fungi in mice, furnishing, for the first time, a comprehensive synopsis of the discrepant reactions of gut fungi and bacteria to the identical drug.

Methods

PC aqueous extract

Raw PC was purchased from Bohaotang Chinese Traditional Medicinal Crops (Bozhou, China) in February 2022. Professor Nian Kai Zeng authenticated the PC, which was deposited at Hainan Medical University with the numbers of FHMU6618. The water-based PC extract was prepared following the method described by Shi et al.[40]. Specifically, 200 g of PC was weighed and extracted twice with 2000 mL of distilled water using a decoction. The first time was decocted (100 °C) with 1000 mL of distilled water for 30 min, and the second time was decocted (100 °C) with 1000 mL of distilled water for 90 min. The high heat was used before boiling, and then switch to simmer after boiling. After extraction, the extracts were merged and filtered with gauze. The filtrate was finally concentrated to obtain 200 mL of PC extract with concentration of 1 g crude herb materials/mL water. The extract was prepared once and stored at 4 °C for animal experiment.

Animal experiments

Eighteen male C57BL/6 mice (weighing 22-25 g) at the age of 6-8 weeks were procured from GemPharmatech Co. LTD (situated in Haikou, China). Throughout the experiment, all the mice were housed in a specific pathogen-free (SPF) facility with regulated humidity (maintained at 40 ± 5%), temperature (kept at 23 ± 2 °C), and a 12 hour light/dark cycle. The mice had free access to food and water. The experimental operations in this study fully complied with the National Institutes of Health's guidelines on the care and use of laboratory animals, and approved by The Ethics Committee of Hainan Medical College (Haikou, China; No. HYLL-2023-467).

The animals were randomly divided into negative control (NC, N = 8) and PC (N = 10) groups after one week of feeding. Mice in the PC and NC groups were orally administered the aqueous PC extract (10 g/kg) or an equal volume of distilled water, respectively, by gavage daily for four weeks. Oral gavage was undertaken with 8-gauge gavage needles by professor Xiaopo Zhang. The PC dosage was determined in accordance with the dosages recommended for clinical use in the Pharmacopoeia of the People's Republic of China (2020 edition). Four weeks later, fresh fecal samples were obtained from each mouse, which had been positioned in a clean cage to defecate of its own accord. The fresh feces were immediately collected in a freezing tube, snap frozen in liquid nitrogen, and then transferred to a −80 °C freezer for storage until further gut bacteria and fungi analyses[41]. Finally, the mice were euthanized by cervical dislocation, and cecum was collected in cryogenic storage tube (Corning®), snap frozen in liquid nitrogen, and then transferred to a −80 °C freezer for short-chain fatty acid detection.

Full-length 16S ribosomal RNA (rRNA) and ITS1/2 (internal transcribed spacer 1 and 2) gene sequencing

Full-length 16S rRNA and ITS1/2 gene sequencing procedures were carried out as described previously[42]. In brief, the DNA of mouse fecal microbiota was isolated with a FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA, USA) by adhering to the producer's guidelines. The V1-V9 segments of the bacterial 16S rRNA gene and the ITS1/2 regions of the fungal gene were amplified via PCR (initially at 95 °C for 2 min, then 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with a final elongation at 72 °C for 5 min). PCR reactions were replicated thrice in a 20 µL mixture comprising 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were retrieved from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's protocol. PacBio libraries were constructed and sequenced on an Illumina HiSeq 2500 instrument (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions.

Bioinformatics analysis

Full-length 16S rRNA and ITS1/2 sequencing data were subjected to the bioinformatics data analysis including quality control, assembly and abundance quantification[43]. The vegan package (v2.7) in R version 4.0.2. (R Core Team, Vienna, Austria) was used to determine the alpha- and beta-diversity of the gut bacteria or gut fungi. Statistical differences in the Bray-Curtis distance of principal coordinates analysis (PCoA) and clustering analysis at the operational taxonomic unit (OTU) level were assessed by Adonis analysis. The interaction network was built based on Spearman's algorithm and visualized using Cytoscape 3.8.2 software. Differences among groups were analyzed using Student's *t*-test and *P*-value of < 0.05 was considered statistically significant.

Short-chain fatty acid (SCFA) quantification

SCFAs were analysed through a gas chromatography system (Agilent 7890, Agilent Inc., Santa Clara, CA, USA) in conjunction with a mass spectrometer (Agilent 5975 mass-selective detector and Agilent 7683B autosampler). The sample injection volume and helium flow rate were set as 1 µL and 1 mL/min, respectively. The temperature was 200 °C for injection and 230 °C for the ion source. The temperature program was as follows: 2 min at 70 °C, oven temperature ramp of 10 °C/min to 190 °C and 40 °C to 240 °C for the final 2 min. The mass spectrometry settings were as follows: positive electron impact mode (EI) at 69.9 eV, ionization energy in the 30–300 m/z scan range in the combined scan and selected ion monitoring (SIM) modes, and SIM targeted at 43, 45, 46, 60, 74 m/z. Mass Hunter Quantitative Analysis B.08.00 (Agilent Inc., United States) was used to evaluate the target peaks. The acetate, butyrate, and propionate concentrations are expressed as µmol/g of cecal materials.

Statistical analyses

Except for the statistical analyses used in microbiology experiments, the data are expressed as means ± standard errors of the mean (SEM). Student's *t*-tests were used to determine the statistical significance between the NC and PC groups using GraphPad Prism 9 (GraphPad Inc., San Diego, CA, USA). *P*-value of < 0.05 was considered statistically significant.

Results

PC increases gut bacteria diversity

Four weeks of administering PC or the NC slightly increased the food and water intake of the mice; thus, we evaluated body weight. The mice gained weight but the amount did not differ between the two groups (Fig. 1A-C), suggesting that PC did not influence appetite and body weight in normal animals.

Next, we evaluated the gut bacteria changes. The Chao1, ACE, Shannon and Simpson indices were notably higher in the PC group than in the NC group (Fig. 2A-D), suggesting that PC enhanced the alpha-diversity within the gut bacteria. PCoA based on the Bray-Curtis distance at the OTU level showed that the gut bacteria structure significantly differed between the PC and NC groups (Fig. 2E). Similarly, hierarchical cluster analysis demonstrated an obvious separation between the PC and NC groups (Fig. 2F).

PC alters gut bacteria composition

We also characterized the composition of the gut bacteria profile in each group at the species level to confirm PC-induced changes.

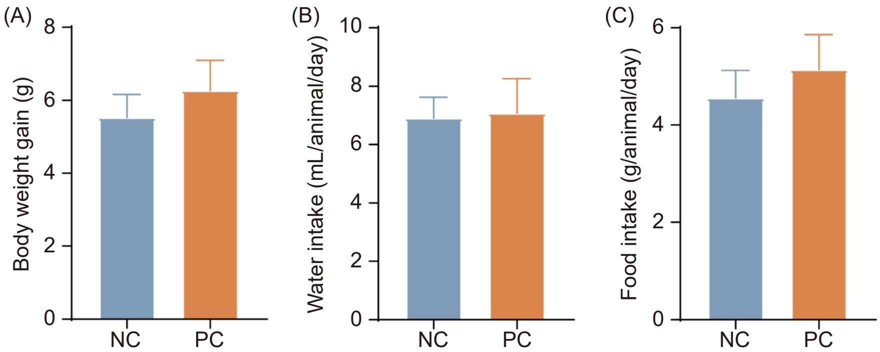

Fig. 1. The effect of PC on general parameters of mice.
A. Food intake.
B. Water intake.
C. Body weight.

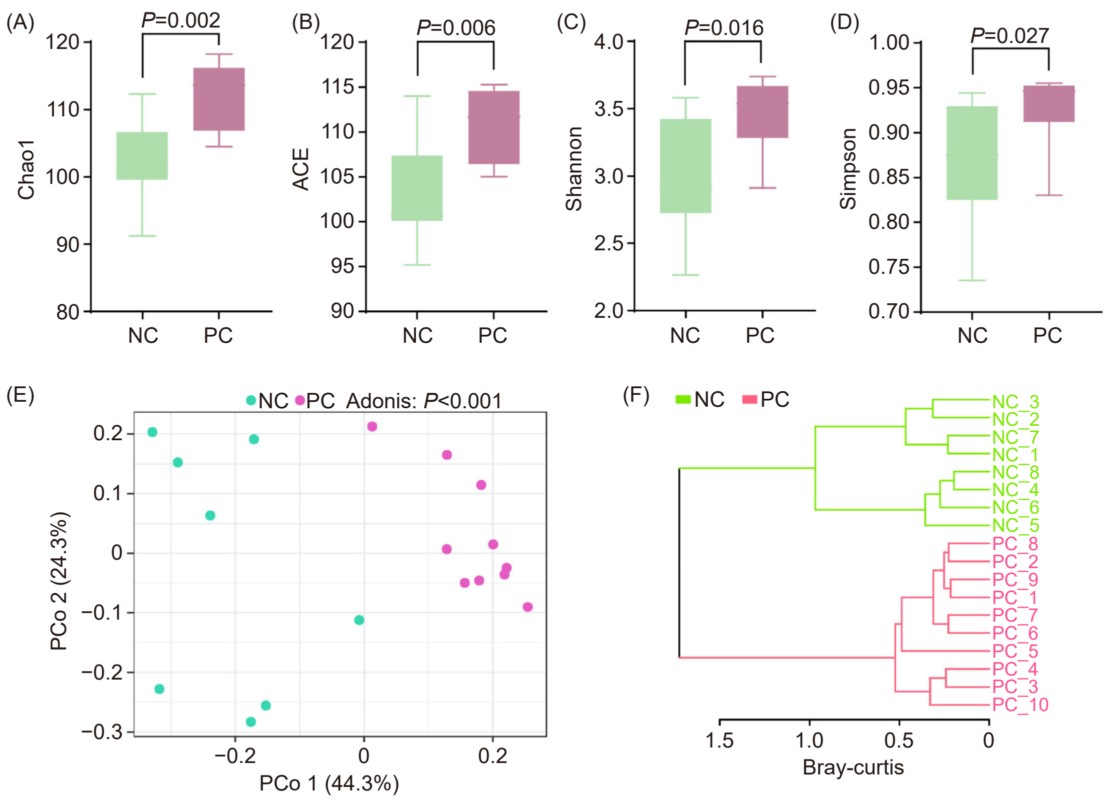

Fig. 2. PC increased the diversity of gut bacteria.
A. The alpha-diversity of gut microbiota was evaluated using Chao1.
B. The alpha-diversity of gut microbiota was evaluated using ACE.
C. The alpha-diversity of gut microbiota was evaluated using Shannon.
D. The alpha-diversity of gut microbiota was evaluated using Simpson indices.
E. The composition of gut bacteria was analysed by principal coordinate analysis (PCoA).
F. The composition of gut bacteria was analysed by hierarchical cluster analysis x.

The proportionate abundances of *Kineothrix alysoides*, *Paramuribaculum intestinale*, *Duncaniella muris*, *Duncaniella dubosii*, *Faecalimonas umbilicata*. *Enterocloster aldenensis* and *Enterocloster sp001517625* were upregulated, whereas *Muribaculum intestinale* and *Herbivorax saccincola* were downregulated in the PC group (Fig. 3A). Linear discriminant analysis effect-size (LEfSe) showed that *Duncaniella dubosii*, *Duncaniella muris*, *Kineothrix alysoides* and *Paramuribaculum intestinale* were the characteristic bacteria in the PC group, and *Muribaculum intestinale*, *Lactobacillus johnsonii* and *Mucispirillum schaedleri* were the characteristic bacteria in the NC group (Fig. 3B).

Next, we assessed the differences between the two groups. Seven species were significantly enriched in the PC group compared to the NC group: *Duncaniella dubosii* ($P < 0.01$), *Duncaniella muris* ($P < 0.05$), *Paramuribaculum intestinale* ($P < 0.01$),

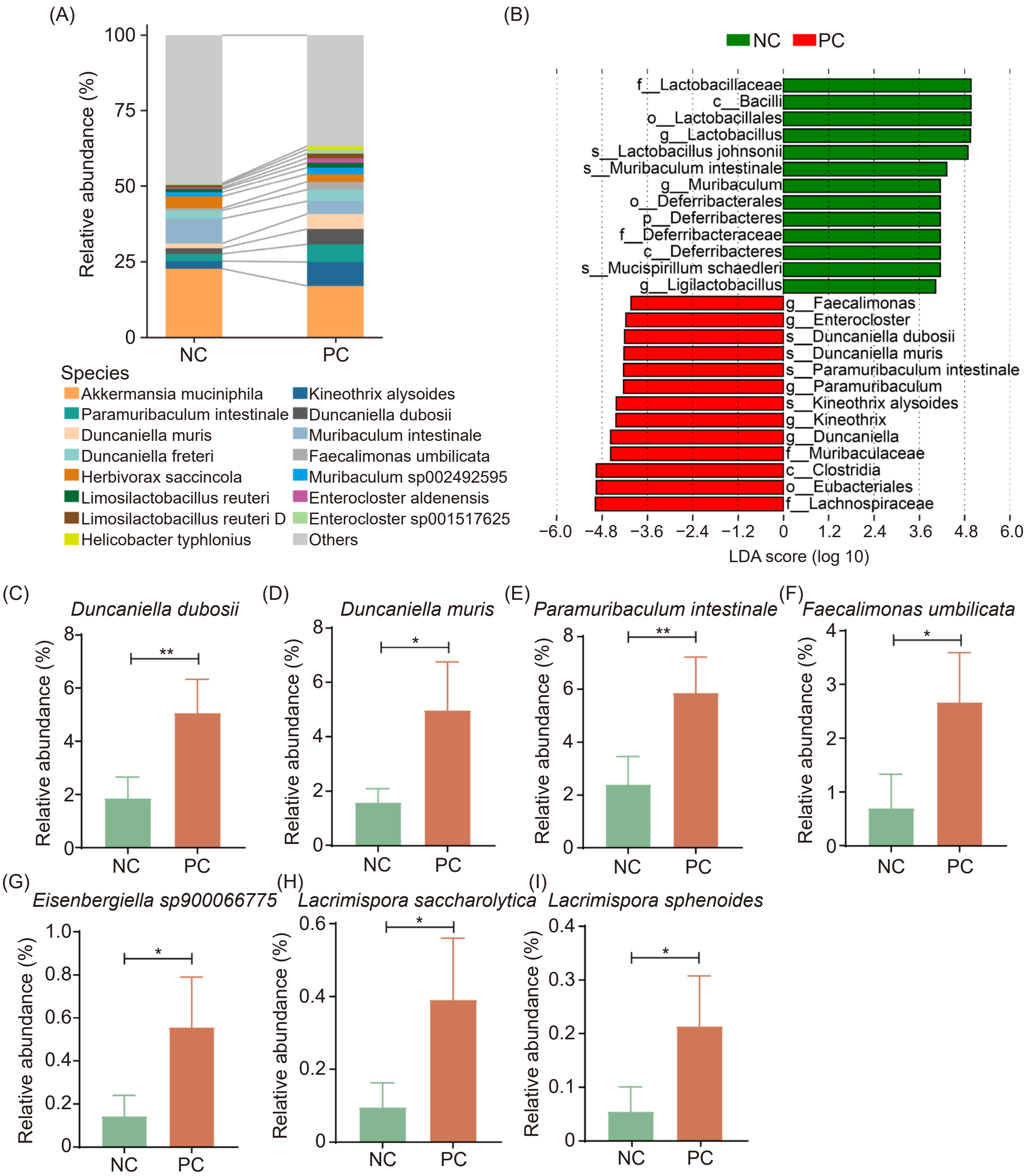

Fig. 3. PC altered the composition of gut bacteria.
A. The gut bacterial composition was assessed at species level.
B. Specific bacteria detected in PC and NC groups by LEfSe analysis.
C. The relative proportion of *Duncaniella dubosii*.
D. The relative proportion of *Duncaniella muris*,
E. The relative proportion of *Paramuribaculum intestinale*.
F. The relative proportion of *Faecalimonas umbilicate*.
G. The relative proportion of *Eisenbergiella sp900066775*.
H. The relative proportion of *Lacrimispora saccharolytica*.
I. The relative proportion of *Lacrimispora sphenoides*.
* $P < 0.05$, ** $P < 0.01$.

Faecalimonas umbilicata ($P < 0.05$), *Eisenbergiella sp900066775* ($P < 0.05$), *Lacrimispora saccharolytica* ($P < 0.05$) and *Lacrimispora sphenoides* ($P < 0.05$) (Fig. 3C-I). Therefore, *Duncaniella dubosii*, *Duncaniella muris*, *Paramuribaculum intestinale*, *Kineothrix alysoides* and *Faecalimonas umbilicata* are potential bacterial markers in PC-conditioned mice.

PC increases the cecal contents of SCFAs

Taxonomic analysis indicated that PC-enriched bacteria, such as *Duncaniella dubosii*, *Duncaniella muris*, *Kineothrix alysoides*, and *Faecalimonas umbilicata* are SCFA-producing microbes. Therefore, we analyzed the cecal contents of the main SCFAs, including acetate, propionate and butyrate, to establish a link between PC-modulated gut bacteria and SCFA production. Four weeks of oral PC administration significantly increased the cecal acetate and propionate contents (Fig. 4A, B). The PC group also had moderate and non-significantly enhanced butyrate levels (Fig. 4C). These data align with the gut microbial results, suggesting that PC-induced SCFA production may contribute to the beneficial effects of PC.

PC minimally affects gut fungal diversity

Given its crucial functions in human health and physiology and therapeutic potential[44], we assessed alterations in the gut fungal community after PC treatment. The richness and evenness of the fungal diversity increased but insignificantly (Fig. 5A-D). Furthermore, the gut fungi structure differed between the PC and NC groups (PCoA diagram; Fig. 5E), as did the hierarchical cluster

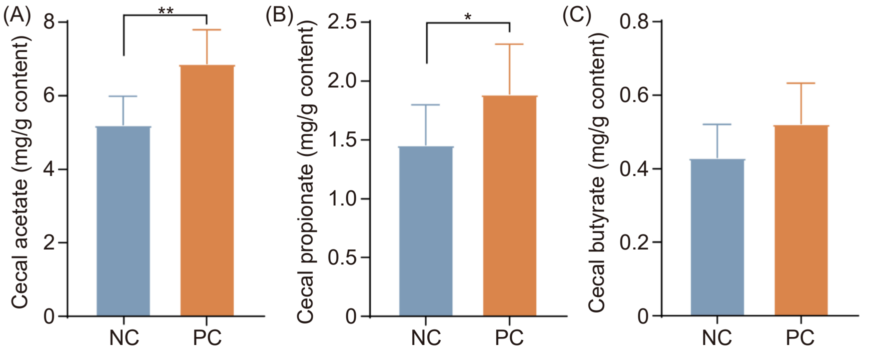

Fig. 4. PC increased cecal contents of SCFAs.
A. The cecal levels of acetate.
B. The cecal levels of propionate.
C. The cecal levels of butyrate.
* $P < 0.05$, ** $P < 0.01$.

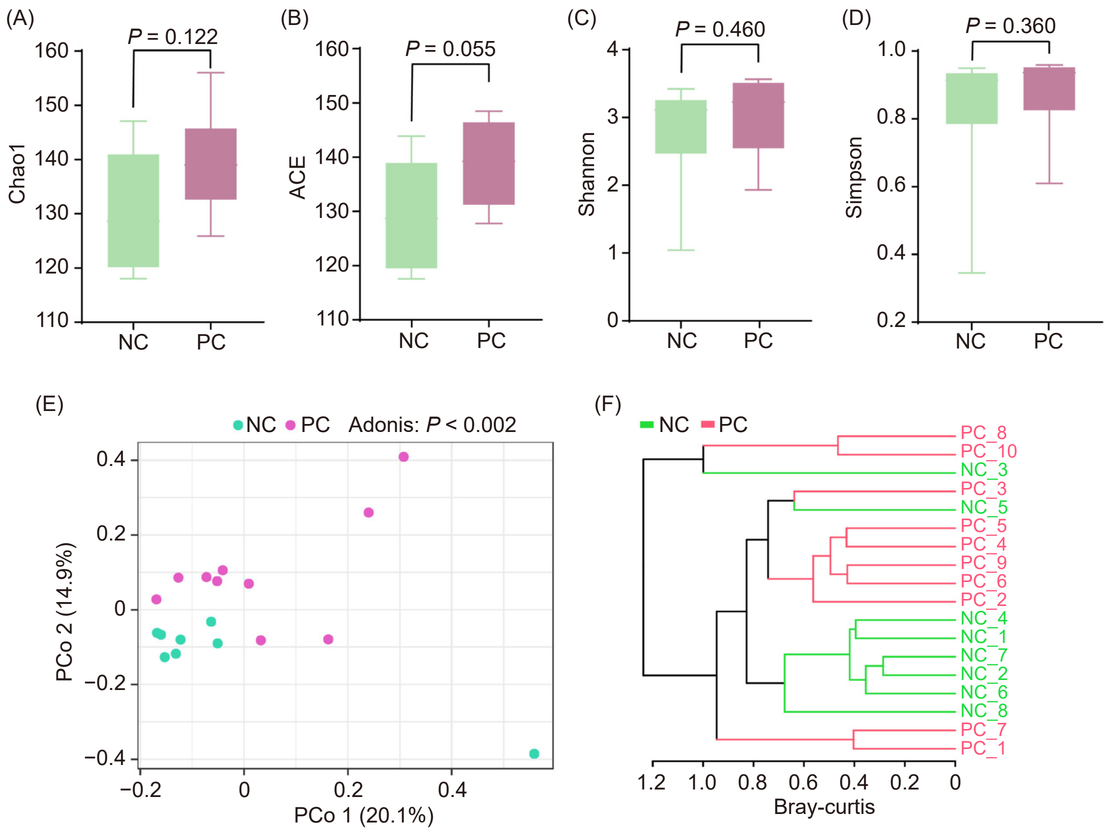

Fig. 5. PC exerted relatively little impact on gut fungal diversity.
A. The alpha-diversity of gut fungi was evaluated with Chao1.
B. The alpha-diversity of gut fungi was evaluated with ACE.
C. The alpha-diversity of gut fungi was evaluated with Shannon.
D. The alpha-diversity of gut fungi was evaluated with Simpson indices.
E. The structure of gut fungi was analysed via principal coordinate analysis (PCoA).
F. The structure of gut fungi was analysed via hierarchical cluster analysis.

analysis results (Fig. 5F). Therefore, PC minimally affected gut fungal diversity compared to its effect on gut bacteria.

PC alters the gut fungi composition

Besides the diversity of gut fungi, the composition of gut fungi in both groups was evaluated at the phylum, genus and species levels. Ascomycota, Basidiomycota and Mucoromycota were the dominant phyla in both groups. However, Ascomycota decreased, and Basidiomycota and Mucoromycota increased in the PC group compared to the NC group (Fig. 6A). At the genus level, the relative abundances of *Nigrospora*, *Neosartorya* and *Cladosporium* were upregulated, and *Aspergillus* and *Penicillium* were downregulated in the PC group compared to the NC group (Fig. 6B). At the species level, *Nigrospora sphaerica*, *Neosartorya sp*., *Cladosporium sp*., *Aspergillus ruber* and *Mortierella kuhlmanii* were enriched, and *Aspergillus proliferans*, *Aspergillus penicillioides*, *Aspergillus spiculosus*, *Aspergillus flavus*, *Penicillium oxalicum* and *Penicillium janthinellum* were reduced in the PC group compared to the NC group (Fig. 6C).

The LEfSe analysis showed that the species *Nigrospora sphaerica*, *Neosartorya sp*., *Aspergillus ruber* and *Mortierella kuhlmanii* were the characteristic fungi in the PC group, and *Purpureocillium lilacinum*, *Aspergillus proliferans* and *Cephaliophora tropica* were the characteristic bacteria in the NC group (Fig. 6D). In the PC group, compared to the NC group, the relative abundance significantly decreased in four species: *Aspergillus oryzae* ($P < 0.01$), *Cosmospora viridescens* ($P < 0.05$), *Penicillium oxalicum* ($P < 0.05$) and *Aspergillus proliferans* ($P < 0.05$) (Fig. 6E); the relative abundance significantly increased in fourteen species: *Sporobolomyces roseus* ($P < 0.01$), *Tilletia bromi* ($P < 0.05$), *Cladosporium sp*. ($P < 0.05$), *Aspergillus ruber* ($P < 0.05$), *Nigrospora sp. TA26-9* ($P < 0.05$), *Fusarium polyphialidicum* ($P < 0.05$), *Tilletia bornmuelleri* ($P < 0.05$), *Psathyrella candolleana* ($P < 0.05$), *Radulotubus resupinatus* ($P < 0.05$), *Talaromyces sp. MG-2016* ($P < 0.05$), *Leptosporomyces aff. fuscostratus UC2022884* ($P < 0.05$), *Aspergillus turcosus* ($P < 0.05$), *Loweporus sp. 2-LS-1-C-12-B.2* ($P < 0.05$) and *Cladosporium halotolerans* ($P < 0.05$) (Fig. 6F). Therefore, PC decreases *Aspergillus* (*A. oryzae*; *A. proliferans*) and *Penicillium oxalicum* levels and enriches *Cladosporium sp*., *Cladosporium halotolerans, Psathyrella candolleana*, *Nigrospora sphaerica*, *Aspergillus ruber*, *Tilletia* (*T. bornmuelleri*; *T. bromi*) levels.

The associations between the gut bacteria and fungi in PC-conditioned mice are complex.

Finally, we constructed a bacteria-fungi interaction network by means of the Spearman algorithm to further explore the inherent connection between gut bacteria and fungi following PC treatment. Fig. 7 presents the intricate associations between gut bacteria and gut fungi in PC-conditioned mice. Specifically, *Ligilactobacillus animalis* bacteria were negatively associated with *Auxarthron filamentosum*, *Cystofilobasidium infirmominiatum*, *Penicillium dimorphosporum*, *Eutypella sp. ES1* and *Podospora glutinans* fungi. Simultaneously, *Candida sp. (in: Saccharomycetales)* positively correlated with *Lactobacillus*, such as *Lactobacillus taiwanensis*, *Lactobacillus gasseri*, *Lactobacillus paragasseri* and *Ligilactobacillus animalis*. Also, *Septoria orchidearum* was negatively associated with bacterial *Duncaniella dubosii* and *Duncaniella muris*. Other associations between gut bacteria and fungi demonstrating the complexity of the gut microecological environment also exist.

Discussion

This study explored, for the first time, PC-induced gut bacteria and fungi alterations in normal mice, and established a correlation network between gut bacteria and fungi, expanding the body of evidence on the modulation of gut fungi by TCMs. Intriguingly, we found that PC significantly enriched SCFA-producing bacteria, such as *Duncaniella muris*, *Duncaniella dubosii*, *Kineothrix alysoides* and *Faecalimonas umbilicata*. PC also increased the relative abundance of some beneficial fungi, such as *Cladosporium sp*., *Psathyrella candolleana* and *Nigrospora sphaerica,* and decreased *Aspergillus* (*A. proliferans*; *A. penicillioides*; *A. spiculosus*; *A. flavus*; *A. oryzae*), *Penicillium* (*P. oxalicum*; *P. janthinellum*; *P. oxalicum*) and *Cosmospora viridescens* levels.

We found that SCFA-producing bacteria, such as *Duncaniella dubosii*, *Duncaniella muris*, *Kineothrix alysoides*, and *Faecalimonas umbilicate*, were notably enriched after PC treatment. Therefore, we speculate that PC's anticancer activity might be due to enriched SCFA-producing bacteria. Correspondingly, PC administration significantly increased the cecal contents of acetate and propionate contents and moderately increased the butyrate level. Therefore, enhancing the SCFA concentration could be a new research avenue for uncovering the detailed pharmacological mechanisms of PC. Culturomics and mono-colonialization experiments have also demonstrated that gut microbiota from the genus *Duncaniella* exerted protective effects against dextran sulfate sodium (DSS)-induced inflammatory injury[45]. Therefore, PC-altered gut bacteria may benefit the host's health.

Intriguingly, the relative abundances of *Cladosporium sp*., *Psathyrella candolleana,* and *Nigrospora sphaerica*,which can produce active products with anti-cancer effects, were also notably increased in the PC group in our study, implying that PC-induced gut fungi alterations may contribute to the anticancer effects of PC. Although our study was conducted in normal mice, it provides experimental data support for further research. Primary sclerosing cholangitis (PSC) is a chronic liver disease tightly related with cholangiocarcinoma. Current research shows that the occurrence of PSC is closely related to gut microbiota dysbiosis[46]. For example, in patients with PSC-IBD, the abundance of *Bacteroides*, while the abundances of *Enterococcus*, *Fusobacterium* and *Lactobacillus* were increased, leading to the decline of butyric acid[47,48]. Unfortunately, there is currently relatively little research on the link between PSC and gut fungi. However, existing results found Th17 is involved in the immune response and usually has a pro-inflammatory effect, and some gut fungi serve as the immune inducers of Th17[49,50]. Based on this, we can reasonably hypothesise that gut fungi may have an impact on the development of PSC through mechanisms such as modulating inflammation and immunity in the host.

Advanced cancer often presents with cachexia, a serious condition that results in numerous fatalities. Fungi are identified as one of the factors of cachexia. It is reported that the proportion of *Mucoromycota* in cachectic mice was reduced, while the chitosan

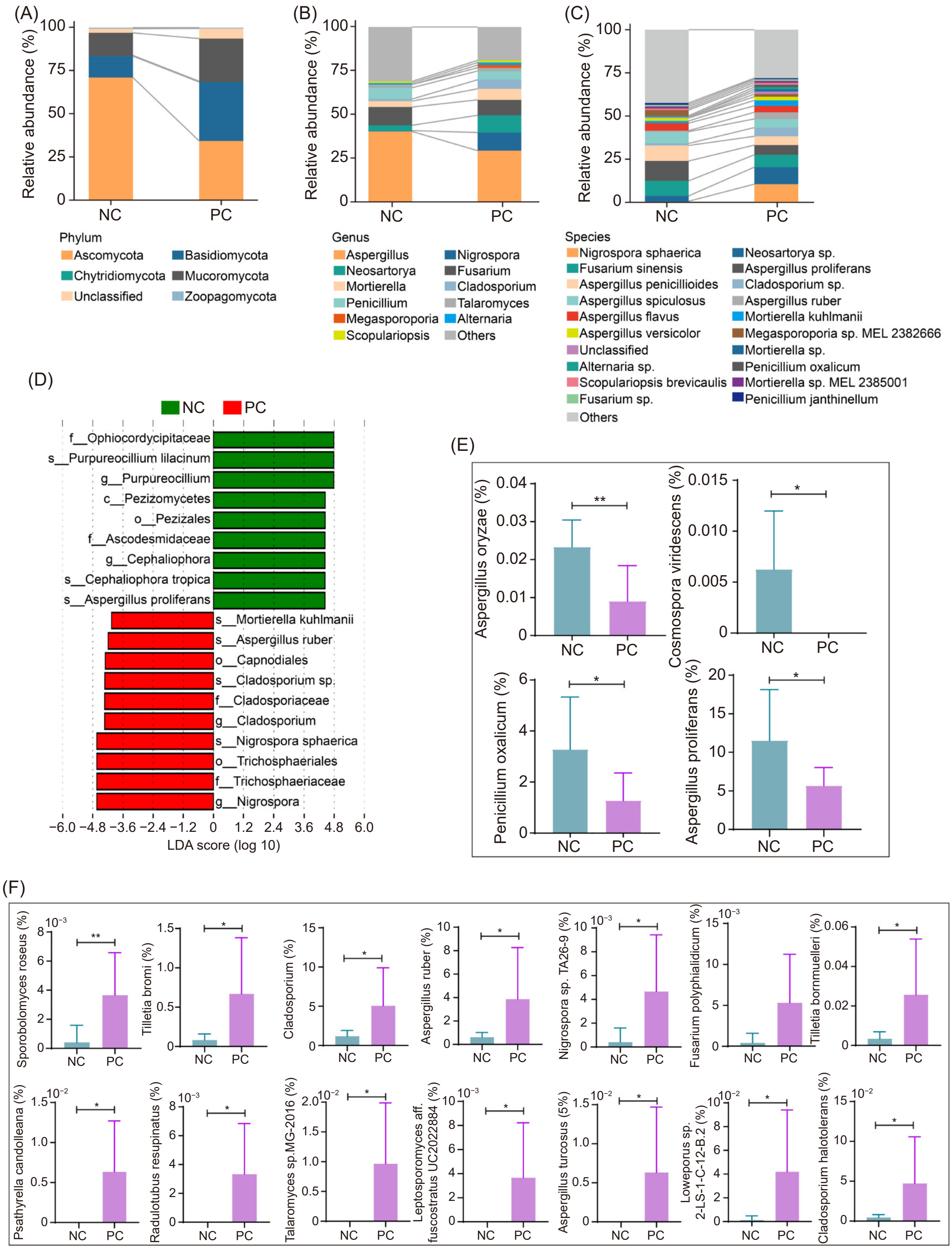

Fig. 6. PC altered the composition of gut fungi.
A. The gut fungal composition was assessed at phylum.
B. The structure of gut fungi was analysed via genus.
C. The structure of gut fungi was analysed via species level.
D. Distinct fungi identified in PC and NC group via LEfSe analysis.
E. The relative abundance of four down-regulated fungi by PC.
F. The relative abundance of fourteen up-regulated fungi by PC.
*$P < 0.05$, **$P < 0.01$.

in the cell walls of *Mucoromycota* can regulate energy metabolism and serve as a therapeutic approach to treat or prevent cachexia[33]. In our study, the abundance of *Mucoromycota* increased under the intervention of PC, which indicates that PC might alle-

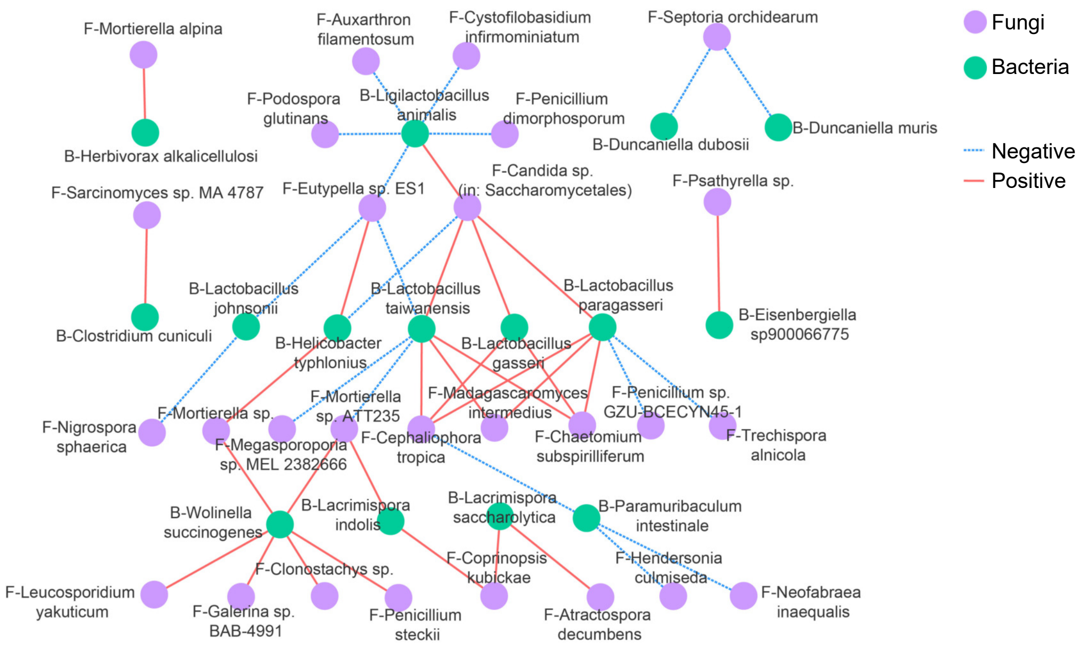

Fig. 7. The interaction between intestinal bacteria and fungi.
Green dots: bacteria; purple dots: fungi; red solid line: positive correlation; blue dotted line: negative correlation.

viate cachexia in cancer development. Moreover, SCFAs have also been reported to improve the function of muscle fibres[51]. For example, butyrate can induce the differentiation of type I fibres in skeletal muscle, significantly increase the contents of ADP and AMP in the muscle, improve the metabolism of glucose in muscle fibres and reduce the accumulation of fat[52]. After PC intervention, the abundance of SCFAs-producing bacteria is enhanced, and consequently rises the amount of SCFAs. Those alterations may potentially relieve cachexia-induced sarcopenia, contributing the anti-cancer effect of PC.

Bacteria, fungi, archaea and viruses inhabit the host's intestine, forming intricate interactions with the host[53]. For example, treatments with antibiotics or colistin may lead to changes in the composition of various fungi, including *Candida*[54]. Similarly, antifungal drugs can also have an impact on the composition of the gut bacteria[55]. A disruption in this balance elicits ill effects on the host[56]. A significant increase in the abundance of *Escherichia coli* may raise the risk of hepatocellular carcinoma[57]. *Candida albicans* is an immune inducer of human helper Th17, and the dysregulation of *Candida albicans* may lead to inflammatory bowel disease[14]. The richness and diversity of fungal species in patients with alcoholism are relatively low. Inhibiting the increase in alcohol-related gut fungi can alleviate the characteristics of ethanol-induced liver disease in mice[58]. Hence, investigating the effects of PC on the interaction between gut bacteria and fungi is instrumental for analyzing the beneficial or unfavorable effects on the host. In this study, the gut fungus *Septoria orchidearum*, a common plant-pathogenic fungus[59], was significantly and negatively associated with *Duncaniella dubosii* and *Duncaniella muris* bacteria. Furthermore, the OTU data indicated that the relative abundance of *Septoria orchidearum* decreased from 0.23% to 0%, suggesting that the inhibition of pathogenic fungi may have resulted from the enrichment of SCFA-producing bacteria and, thus, an increased SCFA concentration. Moreover, we observed a positive correlation between *Candida sp. (in: Saccharomycetales)* and *Lactobacillus taiwanensis*, *Lactobacillus gasseri*, *Lactobacillus paragasseri* and *Ligilactobacillus animalis* bacteria, which decreased after PC treatment. Trans-kingdom interactions between *Candida* and bacteria, either synergistic or antagonistic, may influence the virulence of both *Candida* and bacteria, further affecting the pathogen-host immune response[56].

The interactions between gut fungi and bacteria are complex. In colitis models, antifungal treatment will exacerbate disease, as well as decrease the relative abundance of *Bacteroides*, *Allobaculum*, *Clostridium*, *Desulfovibrio*, and *Lactobacillus spp.*, and increase the relative abundance of *Anaerostipes*, *Coprococcus*, and *Streptococcus*[55]. Another study also found that in dextran sulfate sodium (DSS)-induced colitis, vancomycin treatment could significantly decrease the alpha diversity of gut fungi and alter their composition. Meanwhile, colistin treatment led to the decrease in the bacteria-fungi correlation[54]. The harmful effects of *Candida albicans* and the beneficial effects of *Saccharomyces boulardii* on colitis are most likely dependent on the presence of colistin-sensitive bacteria of the Enterobacteriaceae family in the gut microbiota. Moreover, in the absence of colitis, bacteria of the Enterobacteriaceae family cooperate with fungi to promote their gut colonization[54]. Therefore, it is reasonable to assume that the interaction between gut bacteria and gut fungi plays a fairly important role in their survival and function exertion. Unfortunately, our understanding of this cooperation within competition is rather limited. According to our research, bacteria and fungi might be connected through short-chain fatty acids (SCFAs). Secondary metabolites could be one of the ways they cooperate. The interpretation of relationship between gut bacteria and fungi could provide new ideas for the treatment of gut inflammation and other diseases caused by fungal or bacterial dysbiosis.

This study is still in the preliminary stages; therefore, several limitations exist. First, the experiments were performed in normal animals, resulting in a lack of indicators for assessing the pharmacological effects of TCM. Therefore, we did not establish a correlation between pharmacological activity and the alterations of gut bacterial and gut fungal species, leading to insufficient evidence to support the theory that gut bacteria and fungi changes contribute to PC efficacy. Nevertheless, we observed alterations in the gut microbiome after PC treatment, and identified a correlation between gut bacteria and gut fungi in PC-conditioned mice. Future studies should establish corresponding pathological models, set up more groups, and employ deeper analysis methods to investigate the mechanism of TCMs from a gut microbiome perspective.

Conclusions

This is the first study to evaluate the regulatory impacts of PC regarding the gut microbiome and the inherent association between gut bacteria and fungi. Specifically, PC enriched the SCFA-producing bacteria, such as *Duncaniella muris*, *Duncaniella dubosii*, *Kineothrix alysoides* and *Faecalimonas umbilicata*, and some potential beneficial fungi, like *Cladosporium sp.*, *Psathyrella candolleana*, and *Nigrospora sphaerica*. These alterations appear to be closely linked with PC's anticancer effects, highlighting the importance of gut fungi in mediating the in vivo effects of drugs.

Abbreviations

DSS, dextran sulfate sodium; EGFR, Epidermal Growth Factor Receptor; ERBB2, Erb-B2 Receptor Tyrosine Kinase 2; ESR1, Estrogen Receptor-1; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; ITS1/2, internal transcribed spacer 1 and 2; JAK2, Janus Kinase 2; KDR, Kinase Insert Domain Receptor; LDH, lactate dehydrogenase; LEfSe, Linear discriminant analysis effect-size; MICA/B, Major Histocompatibility Complex-Class I Chain Related Proteins A and B; MMP2, Matrix Metalloproteinase (MMP) 2; MTOR, Mechanistic Target Of Rapamycin Kinase; mTORC2, mammalian target of rapamycin complex 2; NC, negative control; NKG2D, Natural Killer Group 2D; NLRP3, NLR Family Pyrin Domain Containing 3; OUT, operational taxonomic unit; PC, Pseudobulbus Cremastrae Seu Pleiones; PCoA, principal coordinates analysis; PSC, primary sclerosing cholangitis; Rrna, ribosomal RNA; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2SCFA, short-chain fatty acid; SEM, standard errors of the mean; SIM, selected ion monitoring; SPF, specific pathogen-free; TCM, Traditional Chinese medicine; Th17, T helper cell 17.

Ethics approval and consent to participate

The animal study was approved by the Ethics Committee of Hainan Medical University (ethical code: HYLL-2023-467).

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

BC: Formal analysis, Visualization, Writing - review & editing. YF: Writing - review & editing. YNY: Data curation, Formal analysis, Visualization, Writing - original draft. JLC: Writing - review & editing. WYL: Writing - review & editing, XPZ: Methodology, Writing-review & editing. CMW: Conceptualization, Data curation, Project administration, Validation, Writing - original draft, Writing - review & editing.

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