**Multi-cohort fecal metagenomic analysis reveals the altered fungal signatures in colorectal cancer and the carcinogenic potential of *Aspergillus rambellii***

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Introduction

Colorectal cancer (CRC) is the third most common cancer and has the second-highest mortality rate after lung cancer globally1,2. It is estimated that CRC incidence will increase by approximately 80% to over two million cases in the next two decades3. Sporadic CRC, which arises without known contribution from germline mutations or significant family history, accounted for about 75% of CRC, implying the importance of environmental factors in CRC pathogenesis4. The gut microbiome provides numerous essential metabolic and physiological functions for our bodies, including digestion, vitamins synthesis, immune system development, and more2. Recent studies have linked gut microbiota alteration to CRC occurence5. Dysbiosis such as reduced gut microbial diversity and enrichment of oncogenic microorganisms have been associated with CRC carcinogenesis. For instance, the notorious *Fusobacterium nucleatum* promoted glycolysis and oncogenesis of CRC by targeting IncRNA ENO1-IT15. A meta-analysis with approximately 1,000 individuals from five cohorts has revealed the microbial signatures of genes specific for CRC6 and the association between the gut microbiome and choline degradation7. Even though >90% of the gut microbiome are composed of bacteria, a perturbed gut fungal composition had also been described to be associated with inflammatory bowel disease8 and liver cirrhosis9. Fungi could influence the immunological responses of the host by dampening or promoting local inflammatory reactions 8–12. For instance, the mammalian intestinal fungal community interacts with the immune system through the innate immune receptor Dectin-111. The commensal fungi were shown to prevent hosts from colitis-associated colon cancer by prompting inflammasome activation and IL-18 maturation in murine model13. Therefore, it is apparent that fungi play a more significant role in CRC development than our previous anticipation. However, apart from our previous study14 aiming to discover potential fungal biomarkers for CRC detection, the exact role of fungi in CRC pathogenesis remains unexplored mainly due to their relatively low abundance and lack of well-characterized reference fungal genomes.

In this study, we performed a meta-analysis of eight available datasets. After rigorous and stringent data processing, a total of 1,329 samples from four continents, including 525 healthy individuals, 350 adenoma patients, and 454 CRC patients, were included in this analysis. After consistent data reprocessing, the specific fungal diversity and features associated with CRC were identified. Second, we trained the trans-kingdom CRC-screening models and compared them with pure bacterial or fungal classifiers. Moreover, we evaluated the inter-fungi and fungal-bacterial co-occurrence patterns in CRC and compared their correlations across the three groups: CRC, adenoma, and healthy controls. Finally, our abundant differential analysis targeting the fungi genome revealed the significant enrichment of Aspergillus rambellii in CRC patients. This was further validated by our in vitro experimental models. All these suggested that enteric fungi, especially Aspergillus rambellii might play a potential role in CRC carcinogenesis.

Methodology

Selection criteria and data retrieval

Fecal shotgun metagenomic sequencing data of CRC-related studies from 2014 to 2020 with a minimum of 2 subject categories (CRC patients and healthy controls) were retrieved from the NCBI. Eight published cohorts and our recently completed but unpublished cohort were included in this meta-analysis; five of o also had adenoma patients7,15–18 (table 1 and supplementary table 1). We downloaded seven public fecal shotgun metagenomic CRC datasets from European Nucleotide Archive (ENA) using the following ENA identifiers: ERP005534 for Zeller et al.15, ERP008729 for Feng et al.16, PRJEB10878 for Yu et al.19, PRJEB12449 for Vogtmann et al.20, PRJNA389927 for Hanningan et al.17, PRJEB27928 for Wirbel et al.6, and SRP136711 for Thomas et al.7. The cohort from Yachida et al. was downloaded from the DNA Data Bank of Japan (DDBJ) with the Accession numbers: DRA006684 and DRA00815618. For our cohort, fecal metagenomic sequencing data were used from samples collected in Hong Kong from 2009 to 2012. A subset of samples in this cohort was published previously14. These nine studies were organized from eight countries and various sampling procedures, sample storage, and DNA extraction protocols.

Hong Kong cohort with CRC, adenoma patients and healthy controls

Recruitment criteria included presentations of digestive symptoms to the outpatient gastroenterology clinics and asymptomatic individuals 50 years or older receiving colonoscopy screening from the Chinese University of Hong Kong Jockey Club Bowel Cancer Education Centre. Stool samples were collected by participants and stored at –20°C within 4 hours. For long-term storage, all samples were stored at –80°C within 24 hours of stool collection. Total DNA was extracted from stool samples by using the QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen, Germany). All subjects had intact colonic at the time of stool collection. An independent Chinese cohort with 112 healthy individuals, 111 patients with CRC, and 197 patients with colorectal adenoma were recruited. A subset of these samples has been published in a previous research21.

Sample filtering

To ensure consistent and high-quality data, samples were subjected to filtering before analysis. Patients after surgery or with ambiguous conditions (CRC, adenoma or healthy controls) were discarded. We only included the PCR-free cohort because the PCR-free kits could reduce bias and cell spike-in controls for a more accurate quantification22. Finally, we excluded the samples with low-alignment reads (less than 1,000,000), which might be due to low sequencing depth and host reads contamination. In the second part, we removed the outliers or suspected contaminated cases. These include samples with high-fungi composition (the fungi composition is more than 1% of total gut microbiota), low-Fungi composition (the fungi composition is less than 0.01% of the gut microbiota), and bacterial or fungal contaminated samples (a particular species constitutes more than 50% of the gut microbiota). Finally, the samples with low-fungal sequence depth (fungi aligned read counts less than 10,000 reads) would be discarded, which was consistent with a previous study revealing that fungi could not be detected in at least 30% of individuals23.

Sequence pre-processing and taxonomic and functional profiling

We applied the KneadData default parameters for the quality control of all the metagenomic sequencing data. This separated microbial reads from the contaminated reads from the host or other user-defined sources using principled in silico methods. Next, taxonomic profiles were generated with the Kraken2 v2.0.9-beta across the custom database. Our custom library contained 9,543 bacterial and 909 fungal references from NCBI (https://www.ncbi.nlm.nih.gov/), FungiDB (https://fungidb.org/fungidb/), Ensemble (http://fungi.ensembl.org/index.html), and Broad Institute (<https://www.broadinstitute.org/>). The library was then established with the Jellyfish program by counting distinct 31-mer. We used the default parameters and discarded all reads with quality less than 20 and shorter than 50 nucleotides. Each query was classified to a taxon with the highest total hits of k-mer matched by pruning the general taxonomic trees affiliated with mapped genomes. The final metagenomic read counts were normalized by multiple methods, rarefaction, relative abundance (supplementary table 2 and supplementary table 8), and median normalization (supplementary table 3 and supplementary table 9) with the script (https://github.com/ifanlyn95/multi-CRC-fungi). To prevent the denominator from being zero, all zero values will be replaced by the normal distribution with a mean value of one-tenth of the non-zero minimum value and one-hundredth of the non-zero minimum value of the variance. The median normalization means dividing the relative abundance of each feature by the median of the control group:

: means the relative abundance of fungi or bacteria in sample , which belongs to cohort . In contrast, cohort has exactly sample to sample .

Differential abundance analysis

Three criteria were used to select the potential differentially abundant candidates between CRC and healthy individuals. First, we excluded the candidates with an average rarefied abundance of less than 0.1% fungal composition. We selected the same trend features (SSTF), required more than 3/4 cohorts (not less than six cohorts) to perform the same trends. And the log2 of Multiple Median Fold Change (log2MultMedFC) was the evaluation index of SSTF. We define the log2MultMedFC as:

means the counts of CRC/CTRL samples in an individual cohort.

means the fungal names.

means the relative abundance of species in sample .

The second measure was based on the Wilcoxon rank-sum test. We identified differentially abundant features between two groups on a per species basis using Wilcoxon rank-sum test and with p-values being adjusted using the conservative Bonferroni correction. For the last criteria, we discarded features with an absolute value of log2 of features' Fold Change less than 0.5. In addition, we ignored the unclassified strain of bacteria. The scripts were available on Github (https://github.com/ifanlyn95/multi-CRC-fungi).

The random forest based machine learning approach

Our machine learning analyses exploited the taxonomic species-level median normalized relative abundance by Kraken2 and its plugin Bracken. To obtain the test error estimates with lower bias, the LOSO (leave one set out) was used to perform the nested cross-validation. The feature selection and model training was conducted with the R package "random Forest". To choose the best model, and we utilized the max average AUC and best AUC in multi-features and single feature as the selected criteria, respectively. Only species appearing in the top three ranking features in at least one cohort were included in multi-features model characters selection. The code generating the analyses and the figures is available at https://github.com/ifanlyn95/multi-CRC-fungi.

Association calculation and comparison

Inter-fungal and fungal-bacterial co-occurrence and co-exclusion relationships were estimated using the DGCA algorithm24. DGCA is an algorithm for systematically assessing the difference in feature-feature regulatory relationships under different conditions. In our case, DGCA was used to assess the difference of inter-fungal and fungal-bacterial correlations between different groups (CRC vs Adenoma vs Healthy controls). P-values less than 0.05 were considered significant. When comparing the inter-fungal and fungal-bacterial correlation in different groups, DGCA leverages the permutation samples to calculate empirical p-values. The inclusion criterion for network plot features is the correlation index less than -0.2 or more than 0.5. Another important index used is the z-score, which represents the relative strength of differential correlation. We considered the empirical p-values less than 0.05 and the absolute values of the z-score larger than five as a significantly different correlation between different groups. When the inter-fungal or fungal-bacterial correlation in CRC is weaker than that of the healthy control, the z-score would be positive. Whereas, if the correlation is stronger in CRC, the z-score would be negative. Based on a threshold of correlation significance (p-value less than 0.05) and the direction of correlation changes in different conditions (i.e. the correlation is stronger or weaker in CRC compared to healthy controls), species-species correlations in each condition could be categorized into three classes: significant positive correlation, no significant correlation, and significant negative correlation. As we have two conditions (CRC vs Healthy controls), there were nine classes for differential correlation analysis, namely '+/+', '+/0', '+/-', '0/+', '0/0', '0/-', '-/+', '-/0', and '-/-'. The interactions between these selected features were clustered with affinity propagation clusters methodology25.

Additional validation experiments on cancer cell line

TBA

Results

Data preprocessing of metagenomic datasets for studying the associations between mycobiome and CRC

We collected shotgun metagenomic sequencing data from night cohorts. All the raw sequencing data were reprocessed using the KneadData, Kraken226, and Bracken27 for taxonomic profiling. Each sample has about 107.19 (median) high-quality paired reads that match the bacterial database. 104.31 (median) paired sequences were aligned to the fungal genome (figure 1a). The median ratio of fungi to bacteria was 10-2.80 (figure 1a), consistent with a previous study28 reporting that fungi make up about 0.1% of the total enteric microbes. The rarefaction curve (figure 1b) showed that all cohort samples reached a plateau at 10,000 sequencing reads. We applied strict sample filtering criteria to ensure rigorous outcomes and reduce the outlier effect (figure 1c). Collectively, we discarded 216 samples with unsatisfied sequencing quality, 211 outliers or contaminated samples. Notably, all samples from 2018\_HanniganGD were discarded because of their low sequencing depth and non-PCR-free processing. Additionally, 296 samples with low fungi sequencing depth were filtered. In the end, we included 1,329 samples with 454 CRC patients, 350 adenoma and 525 healthy controls. Altogether, this is consistent with a previous study23 that fungi could be detected in approximately 70% of individuals in the gut.

Alterations of enteric fungal and bacterial composition in CRC

Considering the overall fungal composition, *Ascomycota* was the most abundant fungal phylum among all the cohorts, while other dominating fungal phyla showed significant inter-cohort variations. For instance, unlike all other cohorts, the second-most abundant phylum in Yachida's Japanese cohort was *Mucoromycota* but not *Basidiomycota*. Other examples include *Microsporidia* taking up a smaller proportion in Asians than non-Asians (figure 2b).

For the altered microbial composition in CRC, we observed that bacterial phyla *Bacteroidetes* and *Fusobacteria* were enriched in the CRC group compared to the healthy control group. Conversely, *Firmicutes* and *Actinobacteria* were reduced in the CRC group (supplementary figure 1a). When we investigated the individual cohort, 6 of the total cohorts showed significant enrichment of *Fusobacteria* (p-value < 0.05) in the CRC group (supplementary figure 1b). Although no fungi were identified to show a stronger relationship with CRC than *Fusobacteria*, differentially abundant fungi were identified, which will be discussed in later sessions (figure 2c and supplementary figure 1).

In agreement with previous researches showing distorted microbial diversity in the diseased group29, we observed that alpha diversity was reduced in CRC patients compared to healthy individuals when considering all the cohorts together (figure 2d). When considering individual cohorts, the majority showed reduced alpha diversity (chao1 index). Despite the less apparent alteration of fungal composition compared to the bacterial composition in CRC and the heterogeneity in different cohorts, we could still observe remarkable differences in both fungi phyla composition and alpha diversity in the CRC group, which are not negligible.

Identification of fungal species associated with CRC by univariate meta-analysis

We next searched for the potential enteric fungal shifts in CRC patients as compared to healthy individuals. After filtering low abundant (< 0.1%) fungi from the 592 aligned species, 296 species were obtained for further analysis (figure 3a and supplementary table 2, 3). Using the Wilcoxon rank-sum test to compare data from all the cohorts together, 74 differentially abundant fungi were identified, which was named as the main set (FDR < 0.1). Among the 74 identified species, we further shortlisted 33 species that demonstrated significant alterations (FDR < 0.01) as the core set (figure 3a and supplementary table 4).

We then evaluated if these 74 fungi (main set) were consistently altered across all the eight cohorts using the SSTF and Wilcoxon rank-sum test (see Methods). We observed that the enrichment and depletion status of the 74 species were consistent in most of the cohorts except the 2019\_ThomasAM and 2019\_Yachida cohorts. Interestingly, most of the 74 species in the 2019\_ThomasAM cohorts either showed significant enrichment in CRC patients or no significant difference between CRC versus healthy individuals. Very few showed depletion in CRC patients. Whereas in the 2019\_Yachida group, most of the identified 74 fungi showed weak variance in CRC patients versus healthy individuals, unlike other cohorts. We also discovered that 3 of the 74 species showed consistent changes across all the cohorts with *Aspergillus* *rambellii* and *Erysiphe* *pulchra* being enriched while *Trichophyton* *mentagrophytes* being depleted in CRC (figure 3b and supplementary table 6). We further identified 15 species that were consistently altered in 7 out of the eight cohorts. Ten of them were enriched in CRC patients, while the remaining five were depleted (see supplementary table 6). Notably, only *Aspergillus* *rambellii* showed a significant difference (p-value < 0.05) in all the cohorts, except the 2019\_ThomasAM cohort (figure 3d and supplementary table 7).

For the 33 species in the core set, ten were enriched in CRC patients, and the remaining 23 were depleted (figure 3c). Notably, we identified that *Aspergillus* *rambellii* showed the most remarkable difference between the CRC patients and the healthy control groups (-log10FDR = 17.29). We observed that alterations of these 33 species in CRC patients versus healthy individuals were relatively consistent in most cohorts except the 2019\_ThomasAM and 2019\_Yachida cohorts. Moreover, most of the species showed consistent alterations in at least three cohorts.

In the meantime, we also compared the fungal community between CRC and adenoma patients (supplementary table 5 and supplementary figure 2). Seven of the identified species differed significantly (FDR < 0.01) in both CRC patients versus adenoma patients and CRC patients versus healthy individuals. These species include *Aspergillus rambellii*, *Moniliophthora perniciosa*, *Erysiphe pulchra*, *Sphaerulina musiva*, *Phytophthora capsici*, *Aspergillus kawachii*, and *Cordyceps sp. RAO-2017*. These species belong to the *Ascomycota* phylum except *Moniliophthora* *perniciosa* and *Phytophthora capsici* (supplementary table 12).

In summary, we identified 74 differentially abundant fungi between CRC patients and healthy individuals, of which 33 significant species were further selected as core-set for the downstream analysis. Despite cohort heterogeneity, we could still identify fungal features that were consistently altered in most of the cohorts.

*A. rambellii* is the most significant fungus associated with CRC

To identify the most crucial candidates associated with CRC, stringent criteria were used (see methods). We found that *A.* *rambellii* and *A.* *kawachii* were the only two significant fungi (figure 3c). *A. rambellii* was the only candidate with a significant difference among seven cohorts, excluding the 2019\_ThomasAM cohort (figure 3d). In contrast, *A. kawachii* was significantly different among 2014\_ZellerG, 2016\_VogtmannE, 2017\_JunY, and our unpublished dataset (figure 3d). Although they belong to the same genus, they were shown to have a different association with CRC, with *A. rambellii* being enriched in CRC, while *A. kawachii* being the opposite. Notably, they were also reported to have opposing actions in previous studies. *A. rambellii* has been demonstrated to accumulate aflatoxins (AF) and the aflatoxin precursor sterigmatocystin (ST)30. AF and ST are well known as the most carcinogenic natural products 31. In contrast, *A. kawachii* was reported to enhance anticancer effects of anticancer herbs, such as Korean mistletoe32 and fermented silkworm larvae33. All these previous literature supported our findings. Collectively, our meta-analysis revealed the essential fungi, *A.* *rambellii* and *A. kawachii*, that were significantly associated with CRC across multiple metagenomic studies.

Machine learning models using fungal-bacterial markers to identify CRC patients from healthy populations

To identify the significant differentially abundant bacteria between CRC and healthy individuals, we performed Wilcoxon rank-sum test with stringent selection criteria (q-value < 0.01, , unclassified species removed) (supplementary table 10). Thirty-one differentially abundant bacteria were identified in CRC, which was more significant than fungi (supplementary table 10). At least half of the identified bacteria have been reported to be CRC-related, such as *Fusobacterium nucleatum*, *Parvimonas micra*, and *Gemella morbillorum*, or potential probiotics including *Roseburia* *intestinalis*, *Bifidobacterium* *bifidum*34–45, and *Streptococcus* *thermophilus*.46–51. The consistent results in differentially abundant bacteria analysis implied that our methods used in the previous differentially abundant fungi analysis were credible.

In previous researches6,7, the classifier distinguishing CRC patients from healthy individuals was trained using bacterial markers only. We, therefore, asked whether the fungal markers identified in this study could improve the accuracy of the classifier and further increase the potential of using fecal metagenomic markers to early identify CRC patients from the population. We trained the model with a single feature or multiple features to distinguish CRC from healthy individuals. Single feature refers to using only one fungus and bacteria in the core set as the predictor of the model. In contrast, multiple features refer to using a consortium of pure bacteria, pure fungi or a combination of fungi and bacteria in the core set as the predictor. Among the single-feature models, the average AUC value of the six features was greater than 60%. These include four bacteria (*Fusobacterium nucleatum*, *Parvimonas micra*, *Gemella morbillorum*, and *Porphyromonas asaccharolytica*) and two fungi (*Aspergillus rambellii* and *Aspergillus kawachii*) (table 2). Among these, *P. micra* showed the best performance, with an average AUC value of 67.79%, but it played a bad performance in 2016\_VogtmannE (AUC: 56.15%), in which *A. rambellii* performed the best (AUC: 67.57%). It revealed that the predictive values of fungi might supplement the bacteria in some situations. Next, we wanted to know whether the classifier model would be improved when using a combination of fungi and bacteria as predictors. We trained and compared the multi-features model with pure fungi, bacteria, and the fungal-bacterial combination, containing 17, 12, and 14 species, respectively (figure 4a, supplementary figure 3). Unexpectedly, the fungal classifier played more accurate role than the bacterial one in 2016\_VogtmannE (fungi: 77.27% vs bacteria: 70.63%) and 2019\_WirbelJ (fungi: 93.23% vs bacterial 89.39%). The AUC of the classifier with combined fungal and bacterial markers was 1.44% - 10.60% greater than the bacterial classifier in seven of eight cohorts (figure 4). Altogether, the classifier with combined fungal and bacterial markers was more accurate than the conventional pure fungal or bacterial classifiers.

Correlation between CRC-associated bacteria and CRC-associated fungi

Due to the complex and multifactorial nature of CRC, we asked whether the interactions among fungi were associated with CRC. We performed the correlation analysis with DGCA24 on the 33 differentially abundant fungi in the core set. We observed that the correlations within the core-set fungi were stronger in CRC than in healthy controls (figure 4 and supplementary figure 4). There were only four strong positive (correlation index ≥ 0.5) and three negative interactions (correlation index ≤ -0.15) in healthy individuals (figure 4a) as compared to the nine strong positive and four negative associations exhibited in CRC patients (figure 4b). Additionally, there were nine strong positives and one negative interaction in adenoma patients.

*Aspergillus* *rambellii*, *Rhizophagus* *irregularis*, *Rhizophagus* *clarus*, *Phytopythium* *vexans*, and *Edhazardia* *aedis* showed significant correlations in all three conditions (Healthy, Adenoma, CRC) (figure 4 and supplementary figure 3). However, no characteristic pattern was identified in the changes of their correlations across different conditions. A more exciting discovery was that 4 of the CRC-associated fungi, *Aspergillus* *rambellii*, *Erysiphe* *pulchra*, *Thielaviopsis* *punctulata*, and *Sphaerulina* *musiva,* were found to be positively correlated with one another in CRC patients. However, these correlations disappeared in the adenoma or healthy individuals (figure 4a and supplementary figure 3). In these two conditions, only *A. rambellii* still showed correlations with other fungi but not *T. punctulata*, *S. musiva*, and *E. pulchra*. Interestingly, CRC-depleted fungi such as *R. clarus*, *E. aedis*, *Naumovozyma dairenensis* and *R. irregularis* are correlated with one another in healthy individuals. Altogether, our results suggested that different sets of fungi might interact with one another in different conditions. While CRC-depleted fungi might be crucial for maintaining enteric homeostasis in a healthy gut, enrichment of CRC-associated fungi might break the homeostasis contributing to CRC carcinogenesis.

Next, we asked whether the correlations between the differentially abundant fungi and bacteria are associated with CRC. DGCA24 was performed to calculate the correlation between fungi and bacteria. We discovered that the fungal-bacterial correlations were weaker than inter-fungal correlations across all three conditions (Healthy, Adenoma, CRC) (supplementary figure 5 and supplementary table 11). However, the fungal-bacterial correlation was still more vigorous in CRC when compared to healthy individuals, which showed the same pattern as in inter-fungal correlations (figure 4, supplementary figure 5 and supplementary table 11). This suggested that although the fungal-bacterial interactions are weaker than inter-fungal interactions, they might still be associated with CRC tumorigenesis.

Differential inter-fungal and fungal-bacterial correlation analysis in CRC versus Healthy controls

After determining the inter-bacteria, inter-fungal and fungal-bacterial correlations in CRC and healthy conditions, we next asked whether these correlations are significantly different between these two conditions. DGCA was used to perform the differential correlation analysis. Interestingly, inter-bacterial correlations were stronger in CRC patients than in healthy individuals, while inter-fungal correlations were stronger in healthy individuals. (figure 5a). When assessing fungal-bacterial correlations, two peaks at -5 and +5 were observed in the density graph with Z-score, indicating the strength of fungal-bacterial correlations do not show simple unidirectional changes across two conditions. While a group of fungal-bacterial interactions became stronger in CRC patients, another group of fungal-bacterial interactions became weaker. Collectively, our differential correlation analysis demonstrated distinct differences in the correlation changes among inter-fungal, inter-bacterial and fungal-bacterial interactions.

We also defined the nine cases in the pair correlation comparison (figure 5b left panel and see methods). Our results showed that the most significant correlations were '+/+', '+/0', and '0/+', indicating that the most meaningful comparisons (*pm Val* < 0.05) were based on the positive correlations; in other words, negative correlations were rare (figure 5c). Notably, only the intra-fungi had six '-/+' cases, which means the feature pair correlation in CRC was negative, while its association in healthy control was positive (figure 5c). It might reveal some potential markers or changes in the stage alteration.

Sixty-four microbes (31 bacteria and 33 fungi) were separated into six clusters with affinity propagation clusters (figure 5d). Among these, two clusters contained most of the candidates identified. We named the biggest one the Fun\_cluster because 18 of 22 microbes were fungi. We named the second biggest one the Bac-cluster because 17 of the 21 microbes were bacteria. As the clustering results were based on the z-score, we can observe that the alteration of inter-bacteria and inter-fungal correlation have distinct differences. Notably, some bacteria were present in the Fun\_cluster, while some fungi were present in the Bac\_cluster. This implies that these might be the special species that have more trans-kingdom interactions and might be important in CRC pathogenesis.

In the Fun\_cluster, the correlations between the enriched fungi were enhanced in CRC compared to healthy individuals. We identified that *E. pulchra* and *A. rambellii* were the core fungi in the eEuk\_cluster. Three CRC enriched bacteria, including *F. nucleatum*, *F. periodonticum*, and *P. micra,* had strong correlations with these fungi (figure 5d). The most significant fungi, *A. rambellii*, and the cancer-related pathogens, *F. nucleatum*, were in the same cluster. Its z-score was -5.95, and it belonged to the '+/+' case (see supplementary table 11), indicating that the correlation between fungi *A. rambellii* and bacteria *F. nucleatum* was positive in both conditions, and the correlation is even stronger in CRC. Also, *A. rambellii* showed a strong correlation with another CRC-enriched bacteria *P. micra*, with a z-score -5.07, belonged' +/0' (see supplementary table 11). This means that their correlation is much stronger in CRC when compared to healthy control. In contrast, there was no direct correlation between another critical fungus, *E. pulchra*, and these three CRC-enriched bacteria. It revealed that *A. rambellii* might play a role in the CRC carcinogenesis contributed by *F. nucleatum*.

The Bac\_cluster contained most of the reported probiotics or potential probiotics identified in this study, including *S. thermophilus*, *S. salivarius*, *A. hadrus*, and *E. eligens* (figure 5d). Some cancer-enriched bacteria such as *D. pneumosintes*, *S. anginosus*, and *P. intermedia* were also included in this clustering (figure 5d). These results were consistent with many previous studies reporting the correlations among bacteria. Interestingly, *P. kudriavzevii* was the only one correlated with three of the four probiotics. In previous research52, colon cancer cell proliferation, inhibition of endogenous and exogenous apoptosis would be inhibited by metabolites from *P. kudriavzevii*. Collectively, these findings revealed that the inter-fungal, fungal-bacterial and bacterial-bacterial correlations were significantly different in CRC. The specific relationships between *A. rambellii* with CRC-carcinogen and *P. kudriavzevii* with CRC-probiotics revealed that fungi might play an important role in CRC development or be potential predictive biomarkers.

*Aspergillus rambellii* and its conditioned medium promote the viability of colon cancer cells.

[TBA]

Discussion

Our study performed the first comprehensive multi-cohort meta-analysis of enteric fungi shotgun metagenomics in CRC. Samples from eight publicly available cohorts and one unpublished dataset were assembled in this study. We were able to demonstrate the distinct mycobiota alteration in CRC patients versus healthy individuals. Using robust statistical methods, we identified differentially abundant fungi present in CRC patients, which could be used together with bacterial markers to improve the accuracy of distinguishing CRC patients from tumor-free healthy individuals based on fecal metagenomes. Our DAGC analysis further inferred the correlations among fungal markers and bacterial markers identified. We further calculated the correlation differences in CRC patients versus healthy controls, which revealed the potential fungi-bacterial interactions associated with CRC pathogenesis. **[TBA]**

Previously, researchers mainly focused on the relationship between gut bacteria and host pathology. Mycobiota is usually overlooked because of their low proportion in the enteric microbiome53. From our alpha diversity rarefaction curve, a sequencing depth of at least ten thousand reads is required to study enteric fungi. To improve credibility and accuracy, we adopted strict criteria to obtain 1,329 from 2,052 by filtering low-quality samples23,54.

Cohort heterogeneity of the enteric mycobiota was observed across different studies. From our principal component analysis, there were significant p-values for the fungal compositional differences across cohorts. This is consistent with previous studies showing the crucial roles of genetic background, age, dietary habits, lifestyle, and local environments in microbiota composition across different populations55.

Similar to previous research studying gut bacterial composition, we also observed the altered mycobiota composition in CRC versus healthy controls. We found that the fungal chao1 index in CRC was lower than that of healthy controls, as in the case of gut bacteria56,57. This phenomenon was also observed in other intestinal diseases such as inflammatory bowel diseases (IBD)8. In IBD, the enrichment of *Saccharomyces cerevisiae* and *Candida albicans* was found to be the culprit of low alpha diversity58. However, we could not observe the enrichment of these two fungi in CRC patients. Although a low alpha diversity was observed to be associated with various diseases, the mechanisms causing a low alpha diversity and subsequently how is a low alpha diversity related to the pathogenesis process vary across different diseases.

The meta-analysis approach has been used to evaluate and combine results from comparable studies59 with significant advantages of reducing the influence by cohort-specific bias and increasing statistical power. Using the rank-sum test and SSTF in our meta-analysis, we identified 33 fungi and 31 bacteria that were associated with CRC across eight cohorts. Our results suggested that *A. rambellii* was the most significant CRC-enriched fungus, which showed universal associations with CRC in seven of eight cohorts. This finding was supported by previous studies showing the ability of *A. rambellii* in synthesizing carcinogenic products, aflatoxin and aflatoxin precursor sterigmatocystin30,31. Interestingly, the most significant CRC-depleted fungi were *A. kawachii*, also from the genus *Aspergillus*. Even though both fungi were from the same genus, they play opposite roles in CRC. The crude enzyme extract derived from *A. kawachii* could enhance the antioxidative activities of Viscum album var. coloratum32 (Korean mistletoe), a promising agent for immunomodulation, treating colon cancer60 and hepatoma. Another study also reported that the fermented silkworm produced by *A. kawachii* solid-state fermentation could inhibit the human hepatocellular carcinoma cells33. *R.* *irregularis* was the second most significant CRC-depleted fungi. Supporting evidence from previous studies showed that *A. Officinalis*-*R. irregularis* symbiosis can lead to the production of salvianolic acid, which has anticancer effects61,62. Furthermore, other beneficial effects of *A. Officinalis*-*R. irregularis* symbiosis included the production of rosmarinic acid, ferulic acid and caffeic acid, presenting several health-related properties, such as antioxidant, anti-inflammatory, and antimicrobial effects63,64. These observations support their roles in the promotion or inhibition of colorectal carcinogenesis. Since only very few studies have been done to investigate the CRC associated fungi, we have to ensure the statistical methods we used to identify differentially abundant fungi in CRC were robust and accurate. Therefore, the same statistical methods were used to identify the CRC-associated bacteria from the metagenomics sequencing data. More than half of the CRC-related bacteria we identified were reported previously to be cancer-related or commonly used probiotics. Four out of the top five were well-known CRC-associated microbes, namely, *F. nucleatum*65, *P. micra*19, *G. morbillorum*66,67, and *A. hadrus*68. This proved that the statistical methods used in our study are reliable and that the CRC-associated fungi we identified might be potential pathogenic fungi or probiotics.

We developed machine learning models to distinguish CRC patients from healthy controls using stool bacterial and fungal markers. The AUC obtained when we used pure bacterial and fungal markers were 0.81 and 0.73, respectively. However, when we combined both bacterial and fungal markers, an average AUC of 0.83 was obtained. In 7 out of the 8 cohorts, the combined classifier showed an improvement of 1.44% - 10.60% compared with the traditional bacterial classifier. Moreover, the performance of the fungal classifier in 2016\_VogtmannE and 2019\_WirbelJ was better than the bacterial one implying that bacteria may not be the critical CRC-associated factor in all situations. Notably, with the limitation of the metagenomic DNA extraction kit and the low sequencing depth of current studies, we could only detect limited numbers of fungi in the stool of each patient. Given the current challenge, we could still identify several important CRC-associated fungi. This implies that future studies targeting enteric fungi with higher sequencing depth may be able to pick up more important pathogenic fungi and elucidate their roles in CRC pathogenesis.

The fungal-fungal interaction in CRC was significantly different between the healthy controls and CRC patients. Most previous studies have focused on the role of a single key microorganism or metabolite in CRC development69–71. However, microbes in our gut interact with one another. From our results, multiple strong positive or negative correlations in CRC disappeared or weakened in adenoma or healthy control groups. It may be the interaction of multiple species that causes carcinogenesis. Therefore, we cannot only focus on the abundance changes of fungi in two groups but also the alteration of inter-fungal correlation. The most inter-fungal interactions in these three groups were significantly different, but *Aspergillus* *rambellii*, *Rhizophagus* *irregularis*, *Rhizophagus* *clarus*, *Phytopythium* *vexans*, and *Edhazardia* *aedis* appeared in all groups. It suggested that they might play a vital role in the stability of the entire intestinal ecology.

From our differential correlation analysis, we obtained two main clusters, the Bac\_Cluster and Fun\_Cluster. Our results showed that the inter-fungal correlations were weakened in CRC, while inter-bacterial correlations were enhanced. The disruption of the inter-fungal correlation may break the healthy intestinal environment and induce colorectal carcinogenesis. On the other hand, the increased bacterial correlations in CRC may potentially contribute to colorectal carcinogenesis. Interesting results were observed when comparing the fungal-bacterial interactions in two conditions (CRC vs Healthy controls). Our results showed that the fungal-bacterial correlations with smaller changes across two conditions (|z-score| < 2) contained low proportions. The CRC strengthen and weaken correlations performed the primary and secondary ratios in fungal-bacterial correlation comparisons, respectively. It revealed that the internal-kingdom associations and external-kingdom correlations were significantly different. This suggested that bacterial kingdom dysbiosis may cause the fungi to tremble rapidly, which was not similar to the warm alteration of internal-kingdom relationships as previously described14,72.

We discovered that most reported or potential probiotics were separated in the Bac\_Cluster, and *P. kudriavzevii* had multiple correlations with probiotics in this study. Supporting evidence from previous studies showed that *P. kudriavzevii* derived metabolites possess anticancer effects by inhibiting cell proliferation and inducing intrinsic and extrinsic apoptosis in colon cancer cells52. There were also strong correlations among *A. rambellii*, *F. nucleatum*34,65,73, and *P. micra*19, from which the latter two were the famous CRC-related pathogens. A previous study revealed that the altered trans-kingdom association between bacteria and virus are associated with CRC21. We proposed that the trans-kingdom interactions between bacteria and fungi are also important colorectal carcinogenesis. However, this discovery was explored only in a metagenomic sequencing study. More experiments are needed to verify and prove this hypothesis.

TBA (in vitro)

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