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Multi-kingdom gut microbiome features associated with breast cancer and menopausal status

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ABSTRACT

Rising breast cancer incidences signal a growing need to improve understanding of its formation. This study explores the multi-kingdom link between the gut microbiome, short-chain fatty acids (SCFAs), and breast cancer risk, with a focus on menopausal status. Recently diagnosed breast cancer patients (n = 32) and age-matched controls (n = 32) were recruited for a case-control study. 16S rRNA and ITS1 amplicon sequencing, along with stool metabolome analysis, were conducted. Stratification by menopausal status revealed *Holdemanella* and unclassified Aureobasidiaceae were most prominent in postmenopausal cancer patients. Significant differences in beta-diversity were observed for both bacterial ($p = 0.007$) and fungal ($p = 0.004$) communities across groups. Within breast cancer patients, Spearman rank analysis identified *Holdemanella* as a central hub, significantly correlating with fungal genera *Rhizopus* ($p = 0.009$) and *Malassezia* ($p = 0.010$). Despite not reaching statistical significance ($p > 0.05$), the consistent pattern of increased propanoic acid and reduced butanoic acid suggests a meaningful shift in microbial metabolic activity rather than random variation. This is further supported by the predicted upregulation of β-galactosidase and β-glucosidase, indicating active functional changes within the microbiome pointing to estrobolome activities. This study identifies *Holdemanella*, *Aspergillus*, and unclassified Aureobasidiaceae as potential biomarkers and highlights the unique microbial signatures associated with breast cancer.

1. Introduction

Breast cancer stood as the foremost global cancer incidence in women and ranked as the fifth leading cause of worldwide mortality [1]. Developed countries exhibited higher incidence rates (>80 per 100,000), while developing nations showed lower rates (<40 per 100,000) [2]. Breast cancer is highly heterogeneous, displaying significant variability in onset, progression, and metastasis, even within specific subtypes of the disease. Despite extensive studies, the precise cause of breast cancer remains unclear, with up to 70% of sporadic cases lacking identifiable origins [3]. Lifestyle factors such as diet, alcohol consumption, and exposure to radiation may contribute to sporadic breast cancer aetiology. This prompts investigation into whether particular microbes such as gut bacteria and fungi, along with their associated metabolites, impact the development of breast cancer.

The gut microbiome, a community of bacteria residing in the host's gut, plays a crucial role. Dysbiosis, an imbalance within the microbiome, has been associated with an elevated risk of various diseases [4]. Studies on cancer risk factors suggest that microorganisms may be implicated in 15 to 20% of cases [5]. Elevated endogenous estrogen levels and alterations in estrogen metabolism have been implicated in increased breast cancer risk, particularly among postmenopausal women [6]. This idea is supported by the notion of the estrobolome, a group of bacteria which can regulate or change the levels of estrogen within the host; ultimately influencing the risk of breast cancer, especially within those whom have experienced menopause [1]. Mounting studies revealed disruptions to the estrobolome may lead to hormone-related illnesses which besides breast cancer can also include endometriosis, and polycystic oveary syndrome [7].

Despite growing clinical evidence, there remains a notable lack of

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comprehensive studies that systematically investigate the interplay between bacterial and fungal communities in breast cancer patients, particularly across pre- and postmenopausal states. We hypothesized that the composition of intestinal microbiota may vary in postmenopausal breast cancer patients, potentially revealing unique markers involving both bacteria and fungi. Although fungi have received less attention in research, they play a crucial role as either helpful inhabitants or harmful pathogens, affecting the immune system and posing a risk, particularly for individuals with weakened immunity like cancer patients [8]. This study addresses a critical gap by establishing baseline profiles of both fungal and bacterial gut microbiota while exploring their metabolic interactions in breast cancer. By identifying correlations between microbial signatures and disease status, it highlights their potential as biomarkers for prognosis and progression. At the same time, these microbes may offer protective roles through the production of anti-cancer metabolites. Collectively, the findings aim to inform more precise diagnostic and therapeutic strategies for breast cancer.

2. Methods

2.1. Patient recruitment and sample collection

A total of 64 participants were enrolled in this case-control study, comprising 32 recently diagnosed breast cancer patients and 32 age-matched individuals without cancer as controls. Recruitment took place at the Sultan Ahmad Shah Medical Centre @IIUM (SASMEC @IIUM) in Kuantan, Pahang. All participants received a detailed explanation of the study and provided signed consent forms. Female patients aged between 18 and 60 years were eligible for inclusion. Mammogram scans were conducted to assess the health status of each participant. Background information such as age, Body Mass Index (BMI), ethnicity, education level, fasting blood sugar levels, and cholesterol levels were collected for all participants. Specific details related to breast cancer patients, including cancer type (invasive carcinoma or lobular carcinoma), hormonal status (estrogen receptor, progesterone receptor, or HER2, and whether it was positive or negative), and cancer stage (I, II, III, or IV) were also documented. Breast cancer staging was determined according to the tumor, node, metastasis (TNM) system.

In this study, specific inclusion and exclusion criteria were established. Patients recently diagnosed with breast cancer, those currently undergoing pre-treatment for it, or individuals without breast cancer but with normal mammogram results were eligible for inclusion. However, women who were pregnant or lactating, had a history of inflammatory bowel disease (IBD) or known malignant diseases, were using steroids, reported recent antibiotic exposure within the past month, or had been on hormonal or estrogenic medications within the previous 12 months were excluded from the cohort.

Stool samples were obtained from study participants and stored properly using freezing elements during transportation and stored at −60 °C prior processing. The samples were then homogenized with distilled water at a 1:3 ratio and divided into multiple cryovial tubes for subsequent extraction, as outlined previously [9]. For metabolomics analysis, a 1:2 dilution ratio was employed during the process of preparation.

2.2. DNA extraction

Prior to DNA extraction, samples were centrifuged at 13,500 g to allow the separation of water from solid stool. The supernatant was removed, and 250 mg of solid stool pellets were utilized to recover the DNA. Samples were homogenized and processed according to the manufacturer's instructions for the QIAamp® PowerFecal® Pro DNA Kit with modification [10]. The quality of the DNA yields was assessed qualitatively using gel electrophoresis techniques and samples which meet the expected criteria were processed accordingly.

2.3. Library preparation and sequencing

The library for next-generation sequencing was prepared using the Nextera index Kit® from Illumina to prepare the DNA samples to the appropriate sequencer. Amplicons were prepared by enzymatically fragmenting DNA and attaching them with proper primers. Primers which targeted the 16S rRNA V3 [11] and the ITS1 region were utilized to amplify bacteria and fungi respectively. A 2-step polymerase chain reaction was used to amplify the adapter-ligated amplicons. Microbiome sequencing was conducted with the Illumina Novaseq sequencing systems for both V3 and ITS1 regions respectively. The amplicons with tags and adapters were pooled and purified before being sequenced.

2.4. Short chain fatty acid extraction for targeted metabolomics analysis

Short-chain fatty acid (SCFA) evaluation based on targeted metabolomics analysis was conducted as previously described [12]. Briefly, SCFA targeted extraction was performed by mixing 0.5 ml of stool slurry samples with 1 ml of 0.3M oxalic acid in microcentrifuge tubes. An internal standard of 0.1 ml of 2 mM 2-ethylbutyrate was added to each tube. The samples were vortexed for 1 min to homogenize and then centrifuged at 12,000 g for 20 min at 20 °C using a refrigerated centrifuge. The supernatant was collected and filtered through a 25 mm disposable syringe filter (0.45 μm). Subsequently, the GC-MS analysis was then conducted on these filtered solutions as previously described.

2.5. Amplicon data analysis

Prior to clustering using NanoClust [13] the obtained raw sequences were filtered by quality (Oscore >8) and read length (between 500 bp and 1000 bp). An abundance table (OTU Table) was created using the NanoClust intermediate file. Racon and Medaka were used to error-correct the read clusters. After reads were extracted, they were treated to a BLAST-based taxonomic classification based on 16S rRNA V3 region using the qiime2 classify-consensus-blast tool. Next, to allow compatibility with MicrobiomeAnalyst webserver for secondary analysis, the sample metadata, OTU Table, and taxonomic assignments were manually formatted accordingly [9,11]. Additionally, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) analysis was conducted to predict the pathway expression found within the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved for the study.

2.6. Statistical analysis

Statistical analysis and microbial profiling were performed using the MicrobiomeAnalyst 2.0 webserver [14] using adjusted parameters [15]. Features were filtered using a minimum count of four with a 20% prevalence filter, and low-variance features (bottom 10% based on interquartile range) were removed to reduce noise. To address issues with feature sparsity, the analysis was conducted on rarefied data. Study yielded results for diversity analysis (Alpha- and Beta-diversity), taxonomic composition and biomarker identification via Discriminant Analysis Effect Size (LEfSe). Furthermore, pattern search analysis was conducted using Spearman Rank correlation to identify feature trends across study groups. Additionally. Inter-kingdom interactions between the gut bacteria and fungi were conducted in R (version 4.2.3) using the corrplot package and Spearman Rank within the RStudio environment (version 2030.03) to explore interactions. All statistical outputs were subjected to False Discovery Rate (FDR) adjustment to control for multiple testing, with significance set at $p < 0.05$.

3. Results

3.1. Demographic and clinical characteristics of the study participants

Information regarding the patients' socio-demographic distribution and breast cancer characteristics were presented in Supplementary Tables I and II, respectively. A metadata table encompassing multiple factors was compiled using information from both supplementary tables. The results and outcomes of testing these factors are documented in Supplementary Table III. Of the 12 factors tested across both datasets, only two were found to be significant to the study: direct comparisons between breast cancer patients and breast cancer-free individuals, as well as direct comparisons considering their respective menopausal statuses.

3.2. The bacterial and fungal composition in healthy and diseased individuals

The top 5 genera observed were *Bifidobacterium*, unclassified Lachnospiraceae, *Blautia_A*, *Entrerococcus_B*, *Collinsella* and *Candida*, *Saccharomyces*, *Aspergillus*, *Rhizopus*, *Trichosporon* for bacteria and fungi respectively. In terms of prevalence, about 60% of the total distribution for bacterial gut microbiome comprises of *Bifidobacterium*, unclassified Lachnospiriaceae, *Blautia_A*, *Enterococcus_B*, *Streptococcus*, and *Anaerobutyricum* between breast cancer patients and control group, respectively. Other noteworthy genera included *Holdemanella* and *Akkermansia* which were observed to be more abundant within breast cancer patients. Similarly, roughly 80% of the fungal gut mycobiome is made up of *Candida*, *Saccharomyces*, *Aspergillus*, *Rhizopus*, *Trichosporon*, and *Malassezia* between breast cancer patients and control group. Breast cancer patients were observed to have higher presence of *Candida*, unclassified Aureobasidiaceae, *Rhodoturula*, *Erythrobasidium*, and *Selenophoma* whereas control group were more abundant with the presence of *Saccharomyces*, *Exophilia*, *Caldosporium*, *Cystobasidium*, and *Megasporoporia* (Fig. 1(a)).

Based on Fig. 1(b), the alpha-diversity for the bacterial gut microbiome showed no significance ($p = 0.5714$) whereas there were significant findings for the fungal gut mycobiome ($p = 0.001$). Overall, the species diversity between breast cancer patients and the control group were recorded to be similar for gut bacteria but significantly different for gut fungi. The beta-diversity results from Fig. 1(c) depicted significant findings for both bacterial ($p = 0.002$) and fungal ($p = 0.005$) datasets. As such, the composition of gut bacteria and fungi were found to be different between breast cancer patients compared to those without cancer.

A pattern search analysis conducted using Spearman Rank correlation revealed several trends across groups in both bacterial and fungal datasets (Supplementary Fig. 1). Briefly, *Holdemanella* ($p < 0.005$), and unclassified Aureobasidiaceae ($p = 0.070$) were found be to strongly correlated with breast cancer patients. Conversely, *Blautia* ($p < 0.005$), *Fusicatenibacter* ($p = 0.006$), *Xeromyces* ($p < 0.005$), *Ceriporia* ($p < 0.005$), and *Perenniporia* ($p < 0.005$) were more strongly correlated with the control group. The detailed information of all the correlated features can be found in the supplementary files.

Microbial features associated to both study groups using Linear Discriminant Analysis Effect Size (LEfSe) analysis were summarized and recorded in Fig. 1(d). Detailed information of these significant findings can be found in Table 1. A total of four bacterial and three fungal features were identified from the analysis. It was found that the genera of *Holdemanella* and *Enterococcus_B* were potential bacterial biomarkers for breast cancer patients while *CAG_41*, and *Blautia_A*, were markers for the control group. In particular, *Holdemanella* (LDA = 2.79) can be considered as a reliable bacterial biomarker for breast cancer patients. For fungal features, several features were showcased by LEfSe analysis, it was found to that *Aspergillus*, *Xeromyces*, and *Ceriporia* were considered significant to the study; suggesting their use as biomarkers for control group. A more detailed report on all features listed in the analysis can be found in Supplementary Data 9.

3.3. Stratification of the bacterial and fungal composition based on menopausal status

For the second factor of the study, patients were divided into four groups: premenopausal control, postmenopausal control, premenopausal cancer, and postmenopausal cancer. It was found that the same top 5 genera for both bacteria and fungi from the previous analysis appeared in Fig. 2(a). Additionally, 60% of the bacterial distribution among the four study groups were also like the past analysis, being comprised of *Bifidobacterium*, unclassified Lachnospiraceae, *Blautia_A*, *Enterococcus_B*, *Collinsella*, and *Streptococcus* for the premenopausal control, postmenopausal control, premenopausal cancer, and postmenopausal cancer groups, respectively. Features such as *Holdemanella* and *Akkermansia* were also found, their presence being most prominent within the postmenopausal cancer group. Furthermore, it was observed that premenopausal cancer patients were the most abundant with *Ligilactobacillus* and *Clostridium* while postmenopausal cancer patients were more abundant with unclassified Enterococcaceae. It was also observed that regardless of the menopausal status, *Enterococcus_B* was the most abundant among breast cancer patients whereas *Anaerobutyricum* was more abundant within those without cancer.

As for the fungal makeup, 60% of the distribution is made up of the topmost prevailing features across the four groups; these are *Candida*, *Saccharomyces*, *Aspergillus*, *Rhizopus*, *Trichosporon*, and *Malassezia*. Other noteworthy features were also identified among the groups which were unclassified Aureobasidiaceae, *Rhodotorula*, *Erythrobasidium*, *Selenophoma*, *Megasporia*, and *Kurtzmaniella*. Overall, the diversity distribution is quite varied for the fungal gut mycobiome with premenopausal patients having the most skewed distribution compared to the menopaused counterparts.

Fig. 2(b) depicted non-significant alpha-diversity results for bacterial gut microbiome ($p = 0.875$) and significant findings for fungal gut mycobiome ($p = 0.001$). Similarly, species diversity was observed to be similar across the four study groups for gut bacteria whereas gut fungi had significantly different diversity between study groups. Beta-diversity results from Fig. 2(c) reported significant findings for both gut microbiome ($p = 0.007$) and mycobiome ($p = 0.004$). Overall, it was noted that the composition for both gut bacteria and fungi were vastly unique between each of the four study groups.

A multi-group pattern search analysis conducted using Spearman Rank correlation revealed several trends across both breast cancer and healthy individuals stratified by menopausal status (Supplementary Fig. 2). Briefly, *Eubacterium* ($p = 0.016$), *Holdemanella* ($p = 0.041$) and unclassified Aureobasidiaceae ($p = 0.004$) were found to be strongly correlated with breast cancer patients, particularly premenopausal cohorts. *Akkermansia* ($p = 0.018$) were more correlated with postmenopausal breast cancer patietns. Conversely, *Xeromyces* ($p = 0.028$), *Schizophyllum* ($p = 0.052$) were more strongly correlated with the healthy control groups, favoring premenopausal cohorts. Similarly, *CAG_41* ($p = 0.03$), and *Blautia* ($p = 0.032$) had strong correlation to healthy postmenopausal patients. The detailed information of all the correlated features across these stratified groups can be found in the supplementary files.

Fig. 2(d) recorded and summarized the microbial features found across the four study groups using LEfSe analysis. Detailed information on these findings was listed in Table 2. A total of one bacterium and eight fungal features were identified as significant through the analysis. It was found that *Holdemanella* was a potential bacterial biomarker for the postmenopausal cancer group.

In terms of potential fungal biomarkers, the analysis identified *Saccharomyces* and *Aspergillus* for the premenopausal control and premenopausal cancer groups, respectively Additionally, postmenopausal control group recorded features such as Unclassified Dothideales,

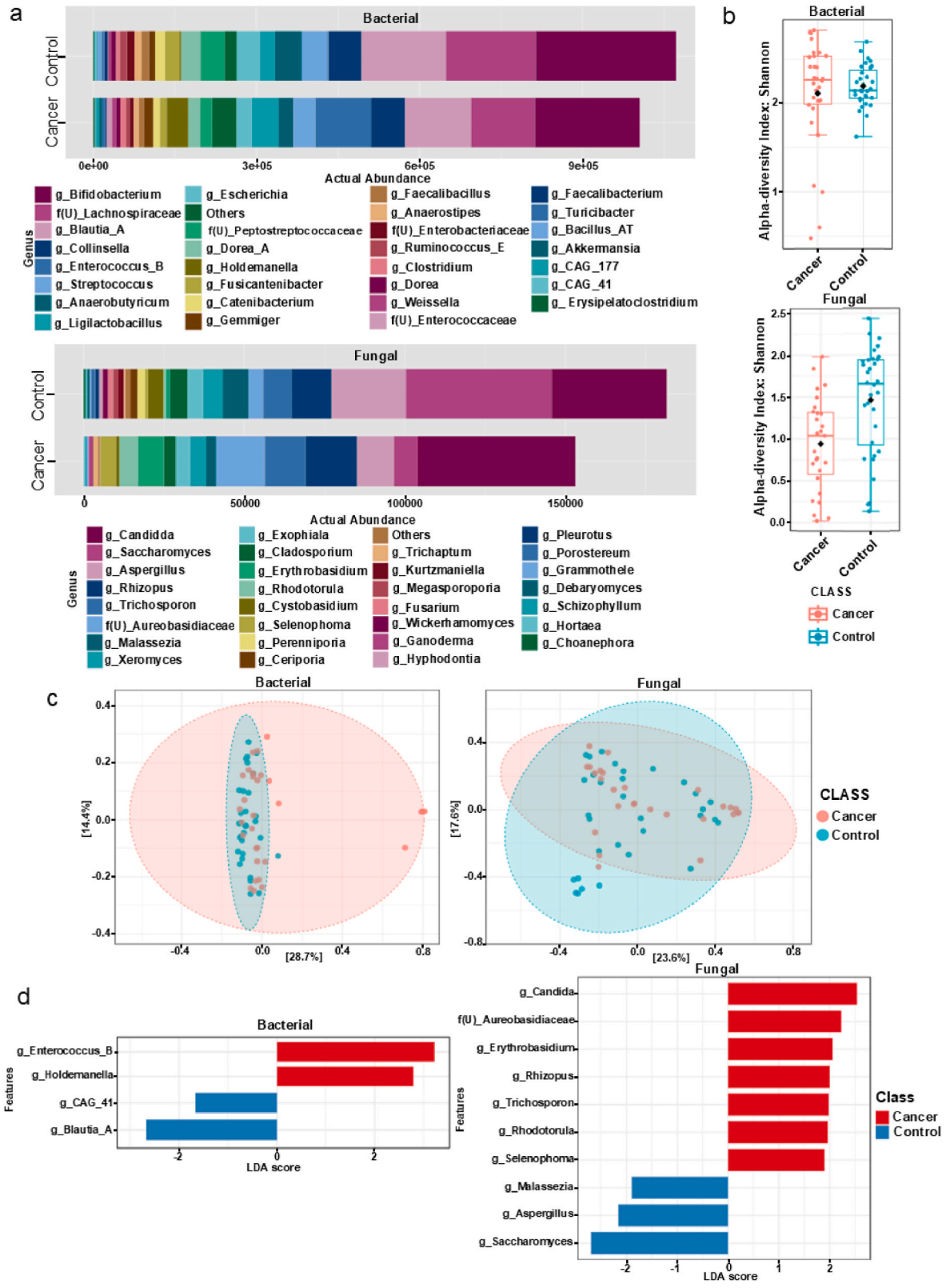

Fig. 1. The outcome of the analysis was based on direct comparison between breast cancer patients and control group for both bacterial and fungal datasets. **(a)** Top 30 features observed between cancer and control group at genus level. **(b)** Alpha-diversity based on rarefied dataset using Shannon diversity index and Mann Whitney method for bacteria and fungi ($p = 0.5714$, $p = 0.001$, respectively). **(c)** 2D PCoA plot of beta-diversity based on rarefied dataset using Bray-Curtis dissimilarity and PERMANOVA method for bacteria and fungi ($p = 0.002$, $p = 0.005$, respectively). **(d)** Top 4 bacterial and Top 10 fungal features associated with study groups based on LEfSe analysis.

Table 1
Detailed information of significant features provided from LEfSe analysis at the genus level by direct comparison of groups.

Name	P-value	FDR	LDA Score	Dataset Origin
Holdemanella	1.73E-08	1.21E-06	2.79	Bacteria
Enterococcus_B	0.0022485	0.039349	3.22	Bacteria
CAG_41	1.49E-05	0.00048463	−1.67	Bacteria
Blautia_A	0.19416	0.4465	−2.67	Bacteria
Aspergillus	0.00043905	0.0085109	−2.16	Fungi
Xeromyces	0.00048634	0.0085109	−1.02	Fungi
Ceriporia	0.0010635	0.010577	−1.53	Fungi

Ceriporia, *Parasympodiella*, and *Cystobasidium*. Furthermore, *Xeromyces* and unclassified Aureobasidiaceae were found for the postmenopausal cancer group. A more detailed report can be found in Supplementary Data 9.

3.4. Correlation between bacterial and fungal composition

A correlation network analysis was conducted to study the possible interaction effects between the gut bacteria and fungi found within the participants of the study. Using R language, the analysis was visualized as a Principal Component Analysis (PCA) plot. The outcome of this correlation analysis for both factors was summarized in Fig. 3. To explore the relationships between bacterial and fungal communities, a Spearman rank correlation analysis was also conducted (Fig. 3(c); Supplementary Data 11). A robust network centered on the breast cancer biomarker *Holdemanella* was identified. *Holdemanella* abundance showed significant negative correlations with several fungal genera, including *Rhizopus* ($p = 0.41$, $p = 0.009$), *Malassezia* ($p = 0.40$, $p = 0.010$), and *Aspergillus* ($p = 0.34$, $p = 0.032$). Additionally, *Aspergillus* was positively associated with members of the Lachnospiraceae family ($p = 0.009$) and *Akkermansia* ($p = 0.014$). Additionally, Table 3 showcases the top 10 most significant findings from the Inter-Kingdom correlation analysis. These results indicate a coordinated shift in both bacterial and fungal populations in the breast cancer group.

3.5. The link between functional gene predictions and metabolomics analysis

Based on Fig. 4(a), after filtering the enzyme expression predictions, it was found that the expression of both β-galactosidase and β-glucosidase, as well as butyrate kinase expression were considered significant to the study. In particular, the two prior enzymes were observed to be expressed more within the control group compared to breast cancer patients. Additionally, predicted pathway expressions that were significant to the study were also found as shown in Fig. 4(b). These were 'superpathway of UDP-N-acetylglucosamine-derived O-antigen building blocks biosynthesis', 'photorespiration', and 'gallate degradation I'; the latter being more relevant to the study. Moreover, the biosynthesis pathway for galloylated catechin can be found in Supplementary Fig. 6. Furthermore, based on Fig. 4(c) butyrate kinase expression was significant to study ($p = 0.025$); it was observed to be less prevalent among breast cancer patients compared to the control group. Moreover, analysis of short-chain fatty acids (SCFAs) using Mann-Whitney *U* test showed no significant differences between groups (Propanoic acid, p = 0.222; Butanoic acid, p = 0.857; Supplementary Fig. 3). A non-significant trend of higher propanoic acid and lower butanoic acid was observed in a limited subset of samples, though these results should be interpreted with caution due to small size (n = 7 for controls, n = 9 for BC patients) resulting from many samples falling below the limit of detection. Acetic acid also remained below the limit of detection in both groups. The full expression of predicted enzymes and PICRUSt analysis for menopausal states can be found in Supplementary Figs. 4–7.

4. Discussion

The bacterial and fungal diversity are notably lower among diseased individuals, particularly breast cancer patients. Studies consistently show reduced microbial diversity in cancer patients compared to healthy controls, with the most significant disparity observed in fungal diversity among premenopausal breast cancer patients. This reduction in diversity is linked to the instability and dysbiosis caused by cancer, compounded by the less stable fungal gut mycobiome in younger, premenopausal individuals [16]. The significant alpha-diversity results for fungi and beta-diversity results for both bacteria and fungi across datasets indicate a distinct microbial composition in breast cancer patients, highlighting dysbiosis. While bacterial dysbiosis in disease progression has been widely studied [1], the role of fungal mycobiomes remains less understood but appears crucial in disease prognosis.

The significant presence of *Holdemanella* and *Blautia* in breast cancer groups, contrasted with the prominence of *Anaerobutyricum* and *CAG_41* in control groups, underscores this point. Notably, *Holdemanella* emerges as a potential breast cancer biomarker, given its consistent prevalence in both taxonomic composition and pattern search results across datasets. In contrast, *Anaerobutyricum,* a natural commensal gut bacterium known for producing beneficial metabolites such as butyrate and propionate, highlights its protective role in gut health [17]. These metabolites are crucial for reducing inflammation and preventing leaky gut syndrome, both conditions that can increase cancer risk. The absence of *Anaerobutyricum* in cancer patients suggests a higher susceptibility to inflammation and subsequent cancer development, reinforcing its significance in maintaining a healthy gut microbiome [18].

The role of *Holdemanella* in health and disease is highly complex and contentious, highlighting the need for further research to fully understand its impact. On one hand, past studies have reported the positive aspects of *Holdemanella* demonstrating its capabilities in inflammation protection [19], maintaining health stability and potential inhibition of human tumor cell proliferation through the production SCFAs [20]. Conversely, other studies paint a more concerning picture. Despite its reported anti-cancer properties, *Holdemanella* has been implicated in tumor formation. Moreover, the presence of *Holdemanella* in patients with melasma, suggests its involvement in hormonal regulation [21]. The bacteria's ability to produce GUSs enzymes, which deconjugate estrogens and potentially increase their reabsorption, ties it to the estrobolome and possibly elevates circulating estrogen levels [6]. The evidence points to *Holdemanella* potentially playing an active role in breast cancer development, but whether its influence is harmful or beneficial remains uncertain. This dichotomy necessitates further research to unravel the complex mechanisms at play. Understanding these mechanisms could be crucial for determining whether *Holdemanella* should be targeted or harnessed in therapeutic strategies for breast cancer and other conditions.

Besides, *CAG_41* is a common gut bacterium, but its role is not well understood. It is often found in higher amounts in diet intervention studies [22], and is reduced in people with neurodegenerative diseases or mild cognitive impairment, suggesting it may have health benefits [23]. There might be a link between low dietary fibre intake and increased *CAG_41* level, though this needs more research [24]. In breast cancer studies, the higher presence of *CAG_41* in postmenopausal control patients might be due to low fiber diets, a common issue in Malaysia [25]. Understanding CAG_41 's role could lead to better dietary guidelines and treatments. On contrary, *Blautia* is often linked to obesity studies [26], and is known for its antibacterial and anti-inflammatory properties [27]. In breast cancer research, *Blautia* is found in abundance among patients with severe clinical stages [26]. However, some species show anti-cancer effects by transforming carcinogenic substances or converting lignans into beneficial compounds. These conflicting roles suggest that *Blautia*'s impact on disease may vary by species, indicating the need for more detailed research at the species level [27]. More research is needed to fully understand *Blautia's* role in

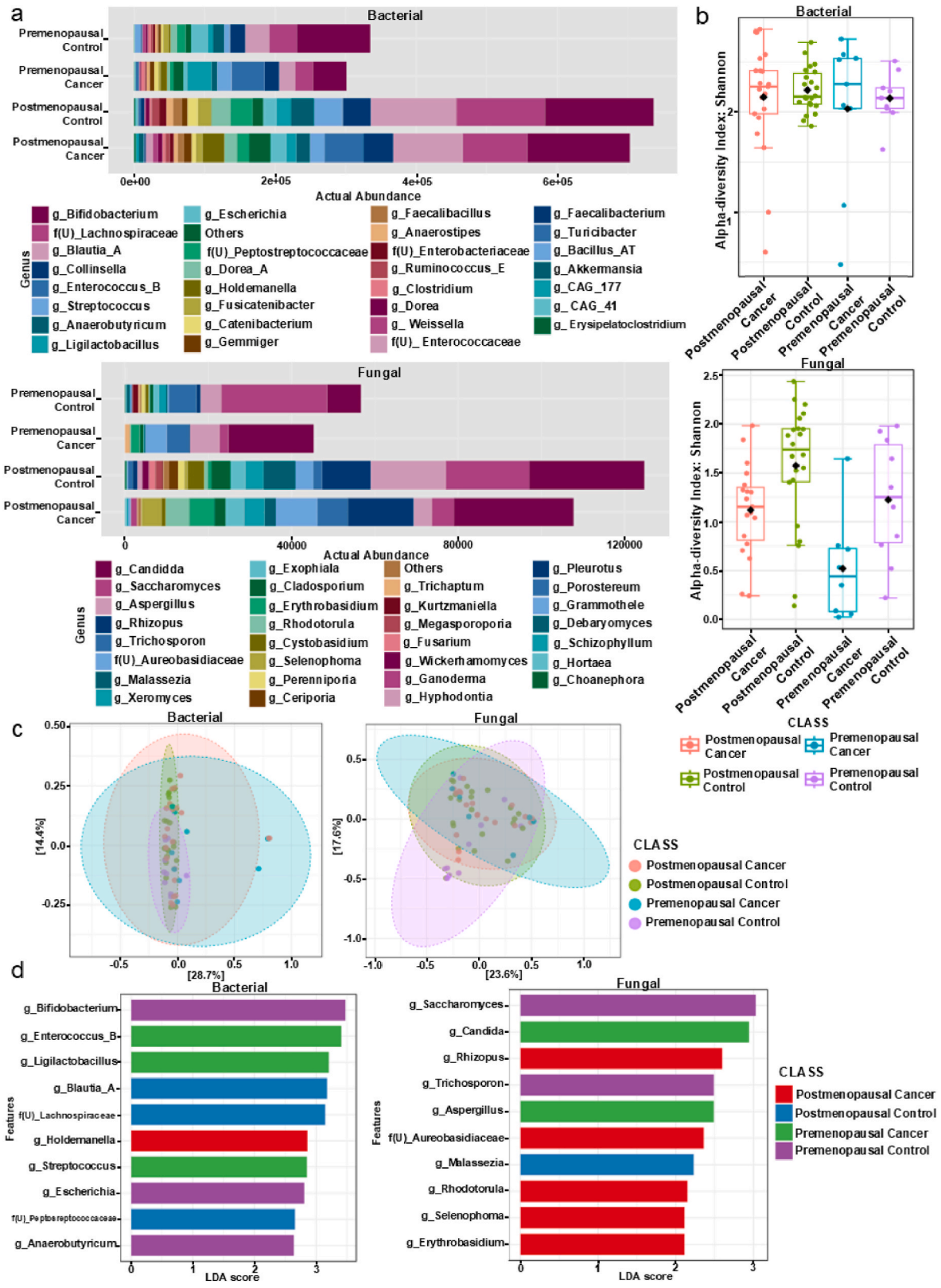

Fig. 2. The outcome of the analysis was based on direct comparison between study groups paired with their respective menopausal status for both bacterial and fungal datasets. **(a)** Top 30 features observed between the four groups at genus level. **(b)** Alpha-diversity based on rarefied dataset using Shannon diversity index and Kruskal Wallis method for bacteria and fungi ($p = 0.8752$, $p = 0.001$, respectively). **(c)** 2D PCoA plot of beta-diversity based on rarefied dataset using Bray-Curtis dissimilarity and PERMANOVA method for bacteria and fungi ($p = 0.007$, $p = 0.004$, respectively). **(d)** Top 10 bacterial and Top 10 fungal features associated with study groups based on LEfSe analysis.

Table 2
Detailed information of significant features provided from LEfSe analysis at the genus level found in menopausal dataset across the four study groups.

Name	P-value	FDR	LDA Score	Dataset Origin
Holdemanella	3.65E-07	2.56E-05	2.86	Bacteria
Unclassified Dothideales	0.0005047	0.017664	1.11	Fungi
Ceriporia	0.002329	0.038042	1.68	Fungi
Aspergillus	0.0036456	0.038042	2.49	Fungi
Sacharomyces	0.0049412	0.038042	3.03	Fungi
Xeromyces	0.0056252	0.038042	2.08	Fungi
Parasympodiella	0.0078641	0.038042	1.42	Fungi
Cystobasidium	0.0081054	0.038042	1.91	Fungi
Unclassified Aureobasidiaceae	0.0086954	0.038042	2.36	Fungi

breast cancer development.

Saccharomyces are best known for their capability to contribute to fermentation processes, and bioethanol production, and are widely used in the food and beverage industry. In breast cancer studies, these fungi are used as models to study cancer development and have shown anti-cancer properties by inducing apoptosis in cancer cells [28]. Some species also suppress gene expression and induce apoptosis in MCF-7 breast cancer cells [29]. Similarly, the genus *Debaryomyces* also used in various industries for metabolizing lactic acid and increasing pH levels, are generally non-pathogenic, with only minor infections reported [30]. These fungi are known to enhance host immunity through beta-glucan production [31]. While not extensively studied in breast cancer, some homologous genes for breast cancer resistance protein have been identified in *Debaryomyces* [32], but more research is needed to confirm any connection. The *Malassezia* often associated with skin conditions [33], and are commensal members of the gut mycobiome, likely due to interactions between the skin and gut. They are usually harmless but can infect immunocompromised individuals and have been linked to IBD and skin cancer. There's no strong evidence linking *Malassezia* to breast cancer, but it's speculated that the fungi may become pathogenic in cancer patients, exacerbating the condition. This hypothesis requires more scientific testing for confirmation.

The fungus *Aspergillus* is often associated with food spoilage and can produce carcinogens. It can also cause infections in immunocompromised individuals and has been linked to liver tumor formation [34]. However, some species of Aspergillus have shown apoptotic effects in breast cancer cells [35]. Similarly, *Rhizopus* can affect plant growth and causes mucormycosis infections in humans [36], particularly in immunocompromised individuals [8]. Despite this, they have also demonstrated apoptotic effects in breast cancer cells [37]. The Aureobasidiaceae fungi are often plant pathogens, but some species can produce commercially valuable biodegradable substances [38]. The more distinct member, the genus *Aureobasidium* are commercially wanted for its ability to produce pullulan with biodegradable properties [38]. The presence of the fungi is mostly commensal in nature. However, the genus is considered to be an opportunistic pathogen with its ability to produce potential carcinogenic metabolites [39]. The role of these unclassified Aurebasidiaceae fungi in breast cancer development is still unclear and requires more detailed research to understand their functions in this context. These fungi are opportunistic pathogens in immunocompromised individuals, which is relevant to breast cancer as cancer patients are often immunocompromised as well [8]. However, further research is needed to confirm their role in breast cancer development.

Spearman rank correlation network analysis revealed a complex inter-kingdom interplay, with the bacterial genus *Holdemanella* emerging as a central hub. *Holdemanella* exhibited significant correlations with several fungal taxa, most notably *Rhizopus* ($p = 0.009$), *Malassezia* ($p = 0.010$), and *Aspergillus* ($p = 0.032$). These findings are particularly significant given that *Holdemanella* species are known producers of short-chain fatty acids (SCFAs), specifically butyrate, which plays a critical role in maintaining intestinal barrier integrity and modulating systemic inflammation [20]. In the context of breast cancer, the prominence of *Holdemanella* and its fungal partners suggests a state of multi-kingdom dysbiosis. While some studies suggest *Holdemanella* may have anti-tumorigenic properties in colorectal models [20], its strong association with fungal biomarkers like *Aspergillus* and *Rhizopus* in this study's BC cohort may point to its role within the estrobolome—the aggregate of enteric bacterial genes whose products are capable of metabolizing estrogens. Bacteria including *Holdemanella*, *Weissella*, *Clostridium*, and *Streptococcus* possess enzymes such as beta-glucuronidase that deconjugate estrogens, leading to their reabsorption into the circulation and potentially increasing breast cancer risk [1]. Furthermore, while our metabolomic analysis of SCFAs showed only non-significant trends (Propanoic acid, $p = 0.222$; Butanoic acid, $p = 0.857$), the strong taxonomic correlations involving known butyrate-producers like *Holdemanella* and *Faecalibacterium* suggest that the microbial community's functional potential is altered in BC patients. These inter-kingdom associations likely contribute to a pro-carcinogenic environment, influenced by the patient's menopausal and hormonal status, necessitating further longitudinal research to establish causality.

Metabolic pathway predictions using PICRUSt2 to forecast functions based on microbial genomes revealed intriguing results, including the predicted expression of β-galactosidase and β-glucosidase. β-galactosidase is among the GUSs, enzymes known to deconjugate estrogens, potentially increasing their reabsorption into the system instead of their natural elimination from the body, leading to elevated estrogen levels [6]. Elevated estrogen levels pose risks as they may bind to estrogen receptors, initiating cell cycle progression and promoting cell proliferation, thereby increasing the risk of cancer formation. While GUSs are typically associated with β-glucuronidase and β-galactosidase, some studies suggest that β-glucosidase may also influence the estrogen pool to some extent [40]. Although most research emphasizes the role of β-glucosidase in bacterial growth by breaking down complex carbohydrates [41], the analysis results showed reduced expression of these enzymes in breast cancer patients, contrary to the expected pattern. We speculated that β-glucosidase inhibition significantly sensitized breast cancer cells to chemotherapy *in vitro* and *in vivo*, suggesting that inhibiting β-glucosidase effectively targeted breast cancer cells resistant to elimination by chemotherapy alone [42] indicating readiness for chemotherapy as a treatment option. Additionally, the reduced expression of the 'gallate degradation I' pathway in breast cancer patients compared to the control group may indirectly affect cancer formation. Gallic acid, which can be converted into anti-cancer compound EGCG, might be more available in breast cancer patients, potentially aiding in combating tumor cells [43] although further research is needed to confirm this.

Lastly, we conducted a metabolomic study to measure SCFAs in stool samples to investigate gut microbial activity in healthy and diseased individuals. Although butanoic acid levels appeared lower in the limited number of BC samples analysed, this was not statistically significant, the lack of significance is likely due to limited sample size, as many stool samples had SCFA concentrations below the detection threshold. The current metabolomic data findings remain preliminary. Butanoic acid has been linked to anti-cancer properties, such as inducing apoptosis in cancer cells [44] or reducing cell viability in breast cancer cells [45] suggesting its importance in cancer development. While the showed non-significant difference, these observations, while preliminary, aligns with documented biological role of SCFA in tumor suppression and underscores the need for further validation in larger cohorts. Furthermore, breast cancer patients showed predicted expression of butyrate kinase, which catalyzes the conversion of butyric acid to butanoic acid. This might explain the absence of *Anaerobutyricum* among breast cancer patients.

Our study contributes unique insights into the gut microbiome of BC patients within the Malaysian context, our finding of non-significant

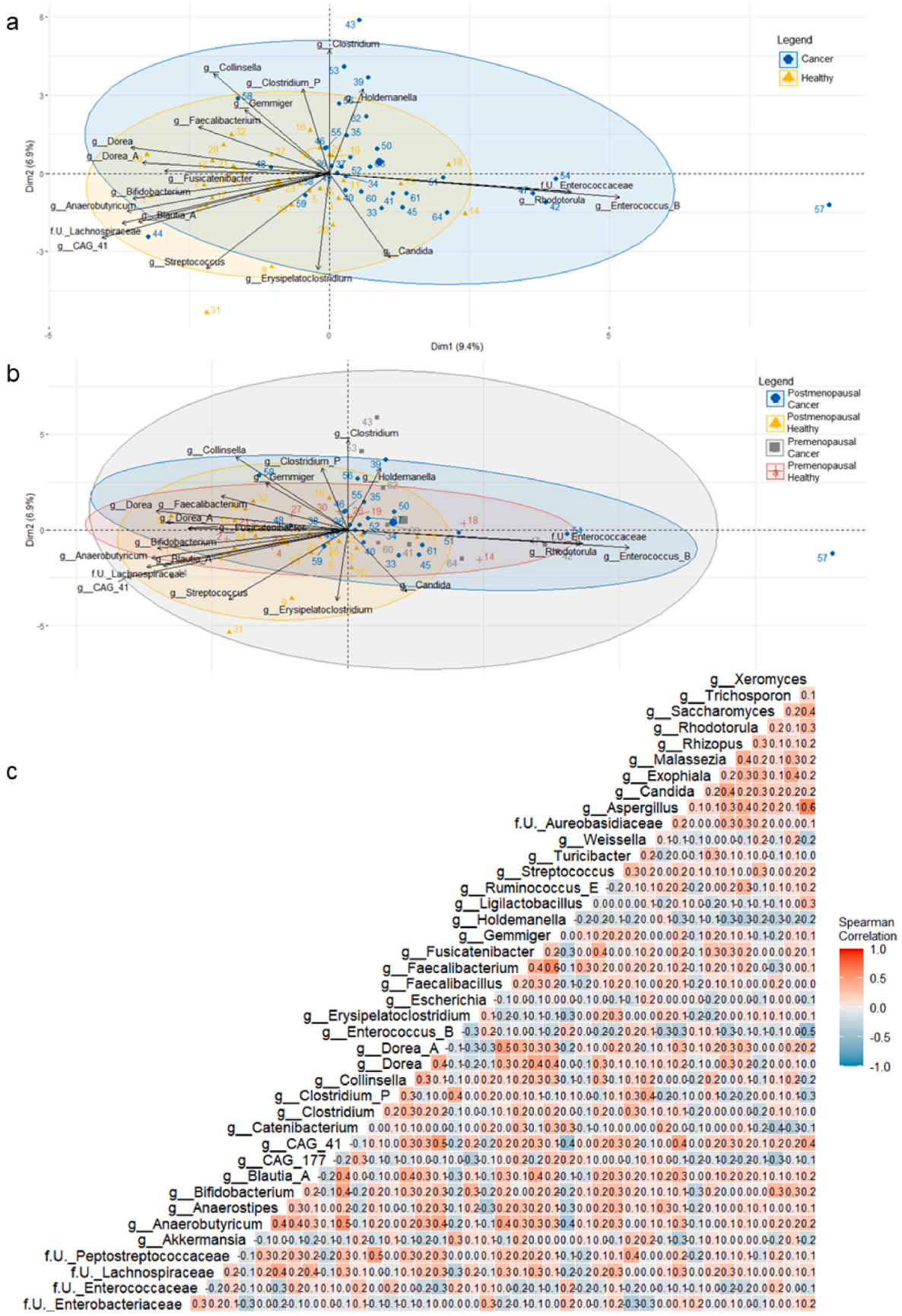

Fig. 3. shows the outcome of the correlation network analysis between bacterial gut features and fungal gut features found among the study participants. **(a)** PCA correlation plot at the genus level based on direct comparisons. **(b)** PCA correlation plot at the genus level based on direct comparison and respective menopausal states. **(c)** Correlation Matrix scores of the bacteria-fungi pairings.

Table 3
Inter-kingdom spearman rank correlation analysis of gut microbiota.

Bacteria	Fungi	Spearman Correlation (ρ)	P-Value (Spearman)	Significance
Enterococcus_B	*Xeromyces*	−0.5	0.0001	***
CAG_41	*Aspergillus*	0.4	0.0002	***
Holdemanella	***Rhizopus***	−0.3	**0.0090**	**
Ruminococcus_E	*Rhizopus*	0.3	0.0074	**
Lachnospiraceae	*Aspergillus*	0.3	0.0097	**
Holdemanella	***Malassezia***	−0.3	**0.0104**	**
Bifidobacterium	*Saccharomyces*	0.3	0.0059	**
Akkermansia	*Aspergillus*	−0.3	0.0143	*
Holdemanella	***Saccharomyces***	−0.3	**0.0246**	*
Holdemanella	***Aspergillus***	−0.3	**0.0327**	*

Note: Significance levels are represented as * ($p< 0.05$), ** ($p< 0.01$), *** ($p < 0.001$).

differences in bacterial alpha-diversity between BC patients and controls stands in contrast to the broader consensus reported in several international cohorts. For instance, studies conducted in Ghana, France, and the United States (Colorado) typically observed significantly lower alpha-diversity in breast cancer cases [46–49]. However, our results align with findings from specific Chinese cohorts, such as those in Southern Anhui and Guangxi, which similarly reported no significant statistical differences in alpha-diversity metrics like the Shannon index or Chao1 [50,51]. These geographical variations extend to specific microbial taxa; while we identified *Holdemanella* as a reliable bacterial biomarker for Malaysian BC patients, other studies have highlighted different genera. For example, *Faecalibacterium* was found to be positively correlated with BC in France and the USA [46,48,49] but showed a negative correlation in Taiwan [52]. Interestingly, our results mirrored the Taiwan trend, with *Faecalibacterium* showing a positive correlation with our healthy control groups ($p = 0.097$). Furthermore, our study is one of the few to explore the gut mycobiome, finding significant differences in fungal alpha-diversity where previous global studies have primarily focused on the bacteriome. Another significant finding of this study is the identification of an inter-kingdom correlation network that characterizes the breast cancer gut microbiome. It was revealed that the bacterial biomarker *Holdemanella* acts as a central hub, correlating with multiple fungal taxa such as *Rhizopus* and *Aspergillus*. Although these correlation coefficients are moderate, their statistical significance suggests a shared ecological niche within the dysbiotic gut of cancer patients. This co-occurrence may suggest a synergistic relationship where certain bacterial taxa and opportunistic fungi thrive together under the inflammatory or metabolic conditions associated with breast cancer. By identifying these multi-kingdom signatures, our study provides a more holistic view of the microbial landscape in Malaysian breast cancer patients compared to traditional bacterial-only analyses. Notably, our predictive functional analysis also revealed a reduced expression of beta-galactosidase and beta-glucosidase in Malaysian cancer patients. This is an intriguing departure from the typical estrobolome model, often supported by Western studies which suggests that an increase in these enzymes leads to higher levels of circulating estrogen and elevated cancer risk [3,41,53]. These discrepancies likely reflect the impact of distinct geographic, ethnic, and dietary factors inherent to the Malaysian population, underscoring the importance of region-specific microbiome profiling in understanding breast cancer pathogenesis [1].

The present research primarily involves an association study and predictive analysis based on the available data. The identified microbial biomarkers and SCFA profiles are of particular interest, as they could potentially play a significant role in influencing the development and progression of breast cancer. This study highlights the correlation between these microbial factors and breast cancer, suggesting that alterations in the microbiome and its metabolic outputs may be linked to tumor biology. The findings, while preliminary, establish a foundational baseline that paves the way for more in-depth exploration. Future research should aim to delve deeper into these associations, utilizing larger and more diverse cohorts, as well as experimental models to elucidate the mechanistic pathways involved. Such studies could lead to the development of novel diagnostic tools or therapeutic strategies that target the microbiome to manage or even prevent breast cancer. Therefore, the current research not only contributes to our understanding of the microbiome's role in cancer but also underscores the need for comprehensive investigations to confirm these initial findings and explore their clinical implications.

5. Conclusion

The study concluded that both bacterial and fungal aspects of the gut microbiome are linked to breast cancer. Significant results were observed when comparing study groups based on menopausal states. The study found a link between the gut microbiome, including both bacterial and fungal aspects, and breast cancer. Breast cancer patients showed associations with certain bacteria like *Holdemanella* and fungi like unclassified Aureobasidiaceae, and *Aspergillus*, suggesting these could be biomarkers for breast cancer detection. Additionally, through inter-kingdom correlation analysis, it was revealed that *Holdemanella* acted as a central hub to many interactions with various fungal features such as *Rhizopus* and *Aspergillus*. Metabolomics analysis revealed lower levels of butyrate and enzymes like β-galactosidase and β-glucosidase in cancer patients, indicating potential disruptions in gut metabolites. The study highlights the complex relationship between gut microbiota and breast cancer, suggesting that gut microbiome analysis could enhance understanding and treatment of the disease.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Ethical approval

This study was performed in line with the principles of the Declaration of Helsinki. Ethical approval for human research was obtained from the International Islamic University Malaysia under reference number IIUM/504/14/11/2/IREC 2020-008.

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CRediT authorship contribution statement

Noor Ezmas Mahno: Conceptualization, Data curation, Funding acquisition, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Darren Dean Tay:** Data curation, Formal analysis, Investigation, Software, Visualization, Writing – original draft, Writing – review & editing. **Nurul Syazwani Khalid:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Sahrol Azmi Termizi:** Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **Hajar Fauzan Ahmad:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial

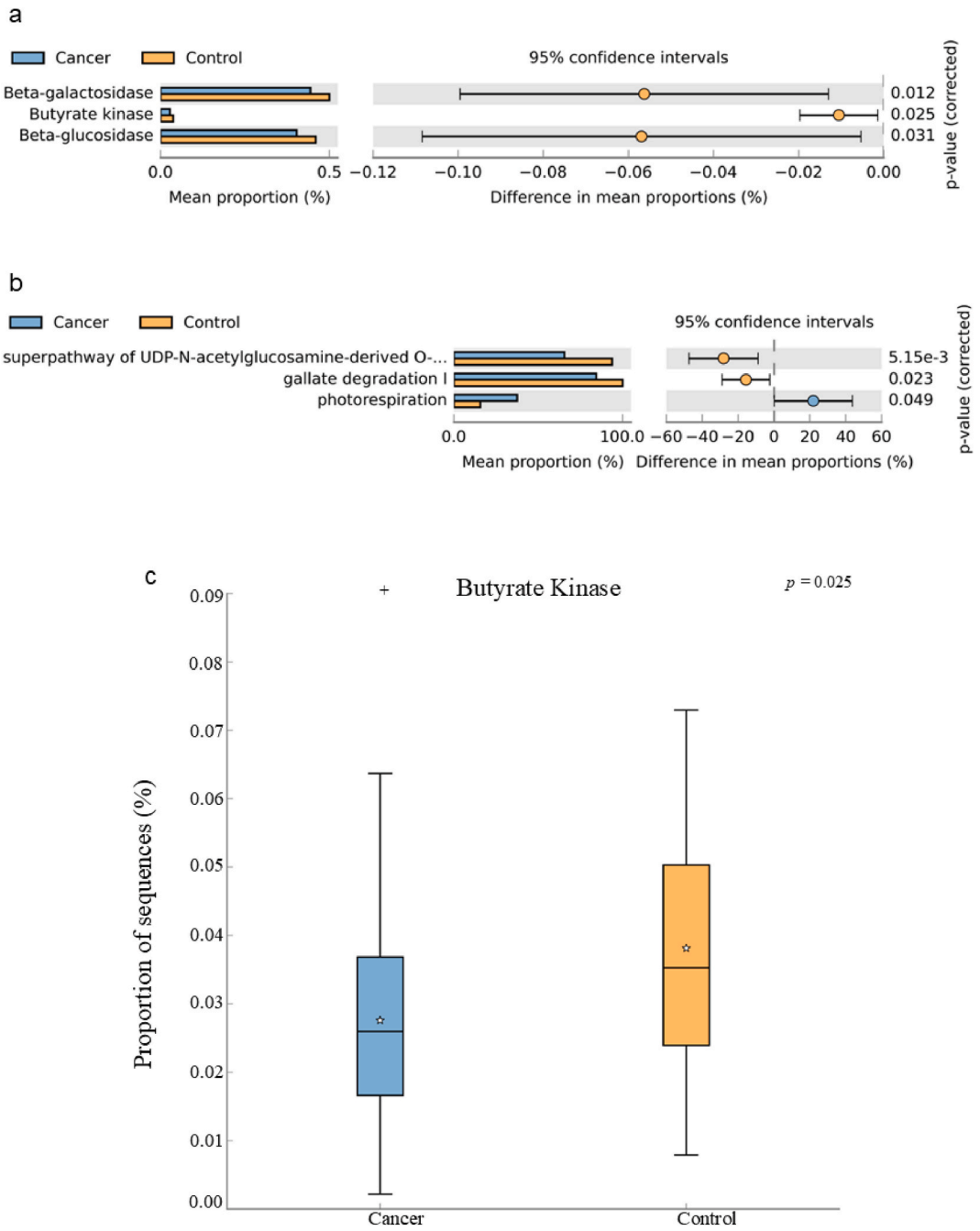

Fig. 4. The outcomes from the metabolomics analysis. **(a)** Filtered expression of predicted enzymes found between participants of the study. **(b)** Expression of predicted pathways found between participants of the study. **(c)** Predicted expression of butyrate kinase among participants.

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gutmic.2026.100009.

Data availability

Data was uploaded in NCBI.

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