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RESEARCH ARTICLE

# Whole Genome Sequencing of *Enterococcus faecalis* Isolated from Stool Sample of a Postmenopausal Women with Breast Cancer Patient in Malaysia

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**ABSTRACT -** Breast cancer stands as a formidable and prevalent cause of mortality among women in Malaysia, prompting rigorous research into potential contributors to its development. Given the well-established association between gut microbiota and various forms of cancer, a particular focus has been placed on exploring the role of *Enterococcus* in breast cancer pathogenesis. Several studies have identified *Enterococcus* as a significant component of the estrobolome, the collection of gut microbiota involved in the metabolism of estrogens. This association has garnered attention due to its potential link to breast cancer. The estrobolome's role in modulating estrogen levels in the body suggests that Enterococcus could influence breast cancer risk by affecting estrogen homeostasis. Thus, this study endeavours to unravel the potential implications of *Enterococcus faecalis* in breast cancer by delving into its genome and decoding gene functions through bioinformatics analysis. Whole genome sequencing emerged as the methodological linchpin, revealing a distinctive gene expression profile within *Enterococcus faecalis*. The size of draft genome sequenced was found to be 2.8Mb, with a GC content of 37.7%, and 4430 protein coding sequences were detected within the genome. Notably, the bacterium sequence subjected to gene annotation exhibited an expression of β-glucosidase, an enzyme intricately involved in the deconjugation of estrogen. While these findings underscore the plausible contribution of *Enterococcus faecalis* to breast cancer, a considerable knowledge gap persists regarding the genetic variations within specific strains of this bacterium. As such, a more nuanced and comprehensive exploration is warranted to bridge this existing gap in our understanding.

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## 1. INTRODUCTION

Breast cancer, a well-known and life-threatening variant, manifests as a complex and multifactorial ailment, represents a formidable global health challenge, ranking among the primary causes of mortality worldwide [1]. Despite extensive research endeavours, the precise etiology of breast cancer in the majority of cases remains elusive. Although genetic and epigenetic interactions have been acknowledged, their comprehensiveness falls short of fully elucidating the root causes of breast cancer especially in sporadic cases [2]. Consequently, the integration of advanced sequencing technologies and collaborative research approaches has brought forth insights into the potential influence of gut microbiota on the severity and mortality of breast cancer [3]. Several studies have suggested a correlation between gut dysbiosis and the development of breast cancer. A pilot study conducted by Goedert et al. that analyzed faecal samples indicated that postmenopausal women diagnosed with breast cancer exhibited less diverse and compositionally distinct faecal microbiota compared to postmenopausal women without breast cancer [4]. Moreover, a research conducted by Khalid et al. (2022) identified a higher relative abundance of the genera *Holdemanella* and *Akkermansia* in the gut microbiota of breast cancer patients when compared to healthy women [5]. These findings highlight the potential role of gut microbiota in the pathogenesis of breast cancer and underscore the importance of further research in this area.

Scientific investigations have also delineated that specific microorganisms within the gastrointestinal tract, collectively known as the estrobolome, can exert an impact on estrogen metabolism [1]. Estrogen is a hormone that plays a significant role in breast cancer development. While estrogen is essential for normal breast development and function, prolonged exposure to high levels of estrogen can contribute to the growth and progression of breast cancer, particularly hormone receptor-positive breast cancer. Hence, the regulation of estrogen metabolism emerges as a pivotal factor in the modulation of breast cancer pathogenesis [6]. Estrogen metabolism involves the process of conjugation and deconjugation of estrogen that predominantly occur in the liver and gut microbiota. Estrogens, such as estradiol, estrone, and estriol, undergo conjugation in the liver, where they are converted into water-soluble forms by attaching to various molecules,

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such as glucuronic acid or sulfate. This conjugation renders estrogen molecules more hydrophilic, facilitating their excretion from the body through urine or bile.

Here, we leveraged a cultivation-based approach and advanced high-throughput sequencing via Oxford Nanopore Technologies (ONT) to sequence the genome of Enterococcus faecalis isolated from stool sample of a postmenopausal woman with breast cancer. The genome sequence analysis was conducted to find out the potential genes that could contribute to the development of breast cancer. Through detecting bacteria carrying specific genes correlated with estrogen metabolism, we can utilize these microbes as biomarkers for breast cancer diagnosis. This approach could significantly improve the diagnostic process, allowing for early detection and timely intervention. Such early predictions would enable preventive measures, such as lifestyle and dietary modifications to be implemented sooner, potentially curbing the progression of the disease at an earlier stage. Understanding these genomic characteristics could provide critical insights into the microbial factors influencing breast cancer, thereby enhancing early detection and enabling timely preventive strategies.

## 2. METHODS AND MATERIAL

### 2.1 Sample Preparation, Bacteria Cultivation and Identification of Bacterial Cell Morphology

Faecal sample from a breast cancer patient was collected at Sultan Ahmad Shah Medical Centre at International Islamic University Malaysia (SASMEC IIUM), Kuantan, Pahang, Malaysia and homogenized with autoclaved distilled water in the ratio of 1:2 for faeces : water as previously described [7]–[9]. A ten-fold serial dilution of the suspended sample was conducted before culturing on Brain Heart Infusion agar plates. The colonies formed were then isolated through streak plate technique [10]. Gram staining was performed to identify the isolated bacterial cell morphology. A single bacterial colony was aseptically transferred to a clean microscopic slide. Crystal violet, iodine, decolourizer, and safranin were added to the slide sequentially before rinsing off and examining under light microscope with oil immersion as previously described [11]–[13].

### 2.2 DNA Extraction and Sanger Sequencing

The bacterial colony was isolated and cultured in Brain Heart Infusion broth to enrich the bacterial culture where the enrichment process was carried out at 37°C for 72 hours under anaerobic conditions. The enriched bacterial culture was subjected to DNA extraction, which was carried out using QIAamp PowerFecal Pro DNA Kit following manufacturer's protocol with modification. The DNA extracted was then amplified using polymerase chain reaction. The amplification process targeted the V4 region of 16S rRNA with the use of 515F (5'-GTGCCAGCMGCCGCGGTAA-3') forward primers and 806R (5'-GGACTACHVGGGTWTCTAAAT-3') reverse primers. Both the quality of DNA extracts and PCR products were assessed using gel electrophoresis. The concentration of DNA was measured using Qubit 4 Fluorometer.

The amplified PCR products was outsourced to external laboratory for Sanger sequencing. The raw reads generated was filtered and compared with the reference sequences in the database of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) to identify the bacterial isolate.

### 2.3 Library Preparation and Whole Genome Sequencing

The identified bacterial isolate was then subjected to DNA library preparation prior to whole genome sequencing performed by Oxford Nanopore Technologies using ONT MinION sequencer. Nanopore Ligation Sequencing Kit SQK-LSK 110 was used to prepare DNA library with some modifications made [14]–[15]. Firstly, the DNA sample was subjected to end repair and A-tailing. The fragmented DNA was repaired to create blunt ends and an adenine (A) overhang was added to the 3' ends using end repair and A-tailing enzymes, preparing the DNA fragments for adapter ligation. Next, sequencing adapters were ligated to the A-tailed DNA fragments, which contained platform-specific sequences, motor protein required for binding to the sequencing flow cell. After DNA library preparation, the sample was loaded onto the ONT MinION flow cell before sequencing process was conducted. The MinION flow cell was thawed to room temperature before use. The cover of flow cell priming port was opened and checked for any small air bubble under the cover. 800µL of Priming Mix that consisted of Flush Buffer and Flush Tether was loaded into the flow cell via priming port and incubated for 5 minutes at room temperature. The SpotON sample port cover was gently lifted to make the SpotON sample port accessible. 200µL of the Priming Mix was then loaded into the flow cell priming port. Following that, 75µL of the sample was loaded to the flow cell via SpotON sample port using dropwise method. The cover of SpotOn sample port was replaced, and the priming port was closed. The ONT device lid was then replaced and connected to the computer for sequencing run.

### 2.4 Data Analysis

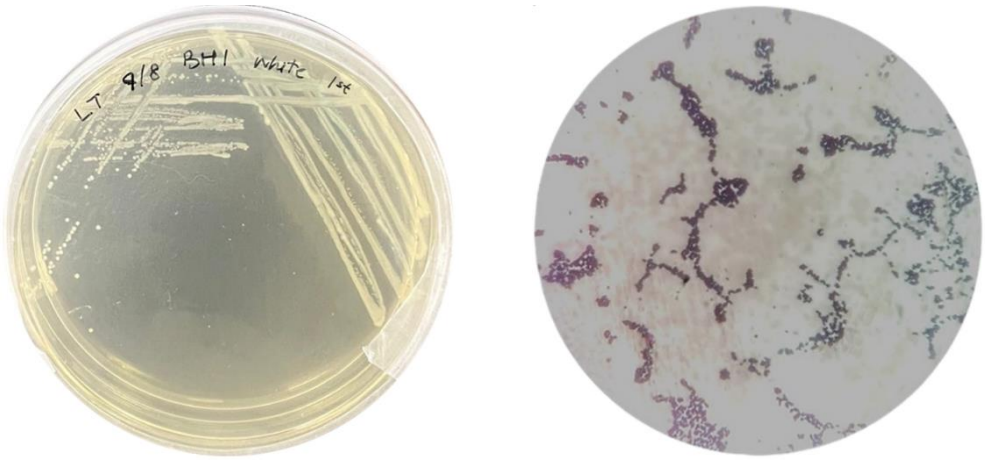
The raw reads obtained from sequencing was base-called using Guppy v6.5.7. The raw reads were filtered and subjected to de novo assembly using Flye v2.8.1 and polished using medaka v1.7.2 to generate the final consensus assembly. QUAST was employed to generate statistics on genome assembly, while BUSCO5 assessed genome completeness based on the enterococcus_obd10 lineage. Annotation of the sequenced genome utilized Proksee, incorporating features such as CARD RGI for antibiotic gene assessment. Additionally, Rapid Annotation using

Subsystem Technology (RAST) predicted protein-encoding genes and their functions within subsystems. Finally, the genome sequence of the bacterial isolate was deposited in the NCBI public database [16].

## 3. RESULTS AND DISCUSSION

### 3.1 Observation of Bacterial Cultivation and Isolation

The morphological characteristics observed from the isolated colonies were small, spherical, smooth and white in colour. The colonies appeared as pinpoint colonies as they grew individually across the streak plate. Under the observation of light microscope, the isolated colony was stained purple during Gram staining, indicating that it was a Gram-positive bacterium (Figure 1). Besides that, the colony was observed to be cocci and spherical in shape, and appeared to be either single, in pairs, or in chains.

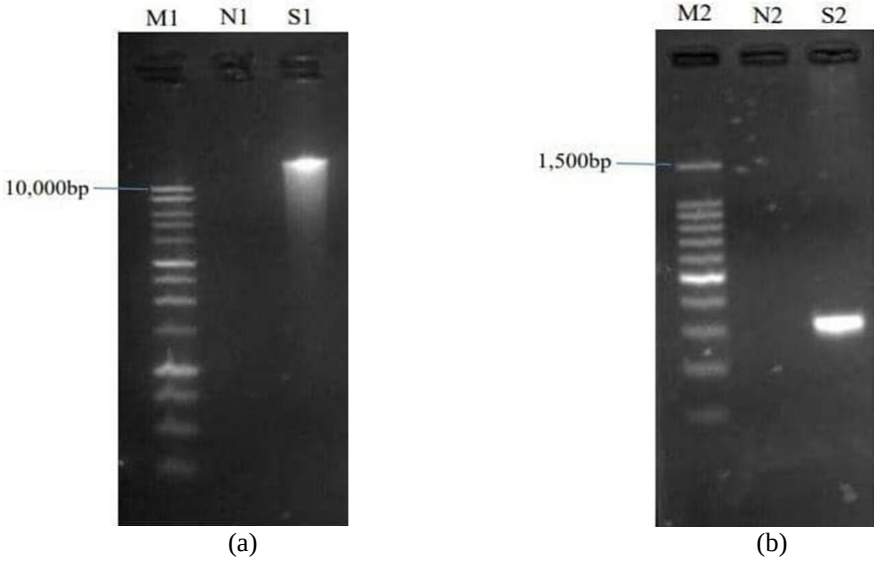



(a) (b)

Figure 1. Bacteria culture (a) Observation of isolated colony on Brain Heart Infusion agar plate, and (b) Morphological observation of isolated colony after gram staining at 100X magnification

### 3.2 Bacterial Identification by Sanger Sequencing

The size and quality of DNA extract were assessed using 1% agarose gel electrophoresis. The DNA band observed was intact, indicating that the DNA extracted is of good quality. The size of the DNA extract was observed to be more than 10,000bp, and this result was validated by the absence of band in the negative control. The concentration of DNA measured was 65.8 ng/µL, which provides sufficient amount of DNA for sequencing.




(a) (b)

Figure 2. DNA band of (a) gDNA and (b) PCR product on agarose gel visualized under ultraviolet light; (a) M1: 1kb DNA ladder, N1: negative control, S1: gDNA (b) M2: 1,500 bp DNA ladder, N2: negative control, S2: PCR product

The DNA extract was also PCR amplified using 515F (5'-GTGCCAGCMGCCGCGGTAA-3') forward primers and 806R (5'-GGACTACHVGGGTWTCTAAAT-3') reverse primers that target the V4 hypervariable region of 16S rRNA gene, and the DNA band observed on agarose gel displayed a size of approximately 300bp, showing an intact band with prominent brightness, which indicates that the DNA has been successfully amplified. The V4 region was chosen as it can amplify the DNA of a diverse spectrum of bacteria and archaea and determine the specific genera or species of an

organism at a relatively low cost [17]. The raw reads after filtered were compared to reference sequence in NCBI database using BLAST. The top 5 hits from BLAST where majority of the hits were shown to be uncultured *Enterococcus* sp. However, the bacterial identity could only be confirmed until genus level as most of the hits were not specified to species level.

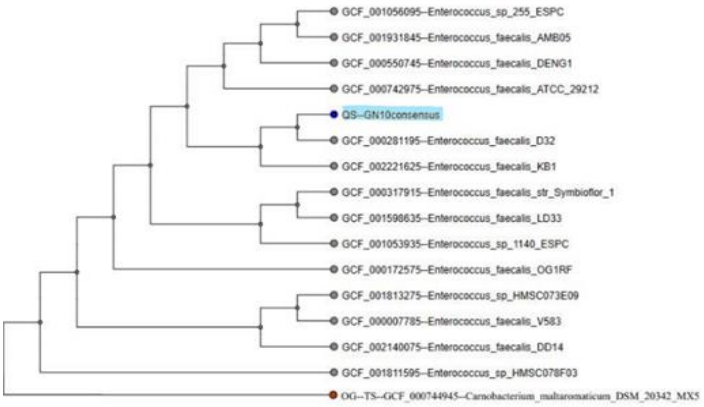
### 3.3 Whole Genome Sequencing Data Analysis

The assembled genome sequence was annotated using the Rapid Annotations using Subsystem Technology (RAST) platform to predict protein-coding sequences and their functions. According to the data generated by RAST, the genome annotation of *Enterococcus faecalis* revealed a total of 4,430 coding sequences and 71 RNA-encoding genes.

A phylogenetic tree was generated for the sequenced genome by establishing the maximum likelihood alignment between the query genome sequence and the reference genome sequence, subsequently categorizing them. Based on the tree, the genome sequence was classified within the order of *Lactobacillales*, genus of *Enteroccocus*, and the closest strain to the sequenced genome was *Enterococcus faecalis D32*, with an estimated Average Nucleotide Identity (ANI) of 99.1% (Figure 3). *Enterococcus faecalis* is the most common species of *Enterococcus* residing in the gastrointestinal tract of human. It has been strongly associated with breast cancer, as studies have reported a significantly higher abundance of *Enterococcus* in the gut microbiota of breast cancer patients compared to healthy female individuals [5]. Nevertheless, there is currently no specific literature directly linking the *E. faecalis D32* strain to breast cancer cells.

| Attribute | Description |
|---|---|
| Genome size (bp) | 2,819,447 |
| Genome GC Content | 37.7% |
| N50 | 1597140 |
| Number of Contigs | 3 |
| Number of Protein Coding Sequences | 4430 |
| Number of RNAs | 71 |

(a)




(b)

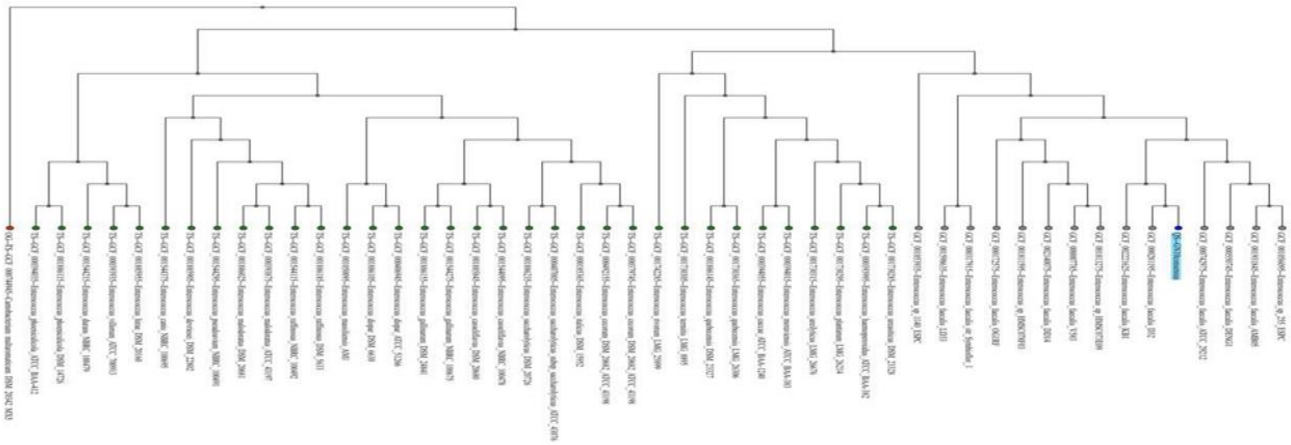


Figure 3. Genomic feature of Enterococcus faecalis; (a) Details of the genome, (b) Simplified autoMLST-generated phylogenetic tree using sequenced genome of isolated *Enterococcus faecalis*, and (c) autoMLST-generated phylogenetic tree using sequenced genome of isolated *Enterococcus faecalis*

### 3.4 Gene Annotation and Functional Prediction

The human gut microbiome is often referred to as the hidden "organ" due to its vital role in human health [18]. Distinct changes in the gut microbiome also known as "dysbiosis" is often associated with a wide range of diseases, including gastrointestinal diseases, central nervous system related diseases, cardiovascular diseases, etc. [19]. Dysbiosis influences the development and progression of cancer through various mechanisms by triggering immune response and increasing inflammatory markers that can promote tumor growth. There is a dynamic balance between the human gut microbiota and the immune system whereby a set of innate and adaptive immune responses is established to maintain homeostasis [20]. For example, *Helicobacter hepaticus* infection in mouse models was shown to foster breast cancer progression through increased neutrophil infiltration. On the other hand, neutrophil depletion was shown to delay tumor formation instead [21]. Human gut microbiota can influence estrogen metabolism, particularly hormone receptor-positive (HR+)

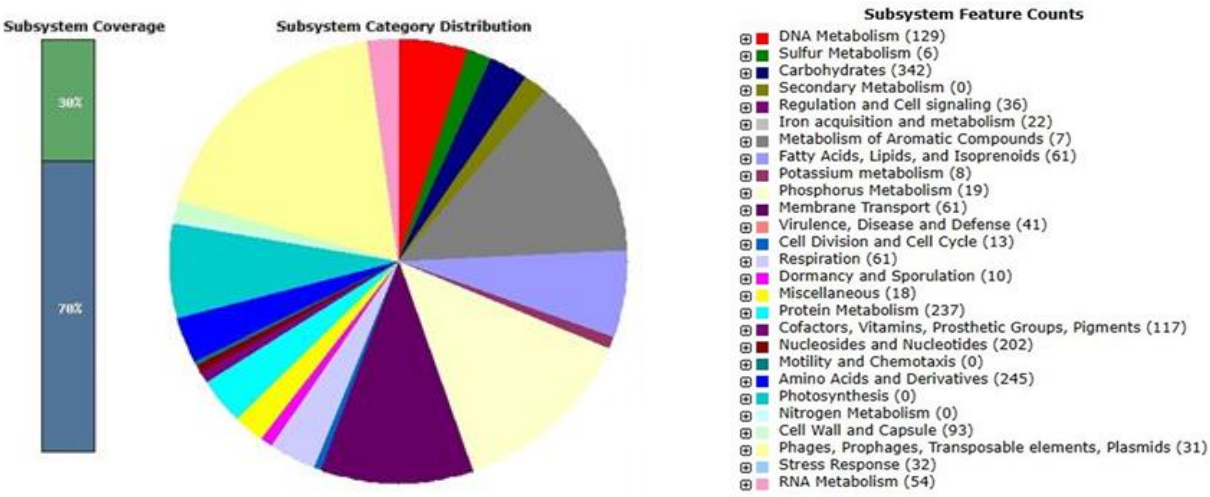
breast cancer by bacterial β-glucuronidase which can deconjugate estrogens, resulting in the release of free estrogens which are reabsorbed and enter the bloodstream where they can bind to estrogen receptors on breast cancer cells, promoting proliferation. Bacterial communities like *Clostridia* and *Ruminococcaceae* are particularly associated with this process, and dysbiosis from factors like antibiotics, diet, or alcohol can raise the risk of cancer progression [20]–[24]. Moreover, metabolites synthesised by the human gut microbiota may either contribute to cancer progression or exert anti-tumor effects even though at present, no pro-tumorigenic metabolites have been identified for breast cancer. Short chain fatty acids (SCFAs) like butyrate often exhibit anti-tumor properties [25]–[29]. Overall, the gut microbiome can influence breast cancer through immune cell modulation, estrogen regulation, and the production of metabolites that can either promote or inhibit tumor growth.

The analysis of the sequenced *Enterococcus faecalis* genome using RAST revealed a coverage in 30% of subsystems, encompassing a total of 1314 genes. Among these, 1258 genes were identified as functional, while 56 genes were classified as hypothetical proteins with uncharacterized functions. These genes were systematically categorized based on their functional associations within the specified subsystems. The subsystem features predicted by RAST included carbohydrates (342 genes), amino acid and derivatives (245 genes), protein metabolism (237 genes), nucleosides and nucleotides (202 genes), DNA metabolism (129 genes), cofactors, vitamins, prosthetic groups, pigments (117 genes), cell wall and capsule (93 genes), fatty acids, lipids, and isoprenoids (61 genes), membrane transport (61 genes), respiration (61 genes), RNA metabolism (54 genes), virulence, disease and defense (41 genes), regulation and cell signaling (36 genes), stress response (32 genes), phages, prophages, transposable elements, plasmids (31 genes), iron acquisition and metabolism (22 genes), phosphorus metabolism (19 genes), miscellaneous (18 genes), cell division and cell cycle (13 genes), dormancy and 81 sporulation (10 genes), potassium metabolism (8 genes), metabolism of aromatic compounds (7 genes), and sulfur metabolism (6 genes).

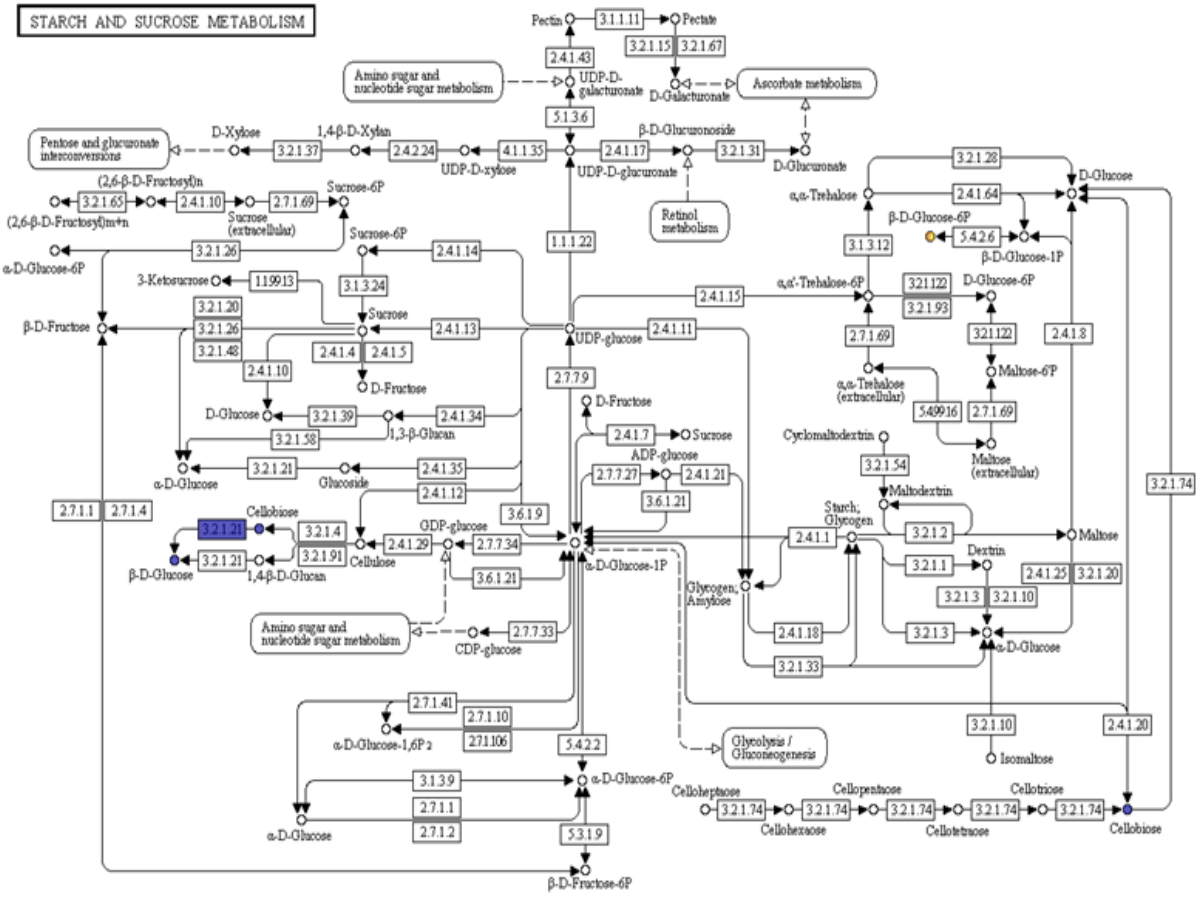
One of the notable subsystems to be highlighted is carbohydrate subsystem. There were several metabolisms categorized under the carbohydrate subsystem. *β-glucosidase* coding sequence, which encodes for β-glucosidase, was discovered to be one of the key genes involved in Beta-Glucoside Metabolism. This enzyme contributes to estrogen level regulation through its involvement in the deconjugation of estrogen glucuronides in the gut. Estrogen conjugates, such as estrogen glucuronides, are initially rendered inactive and less readily absorbed by the body. However, beta-glucosidase, which is produced by gut bacteria, catalyzes the hydrolysis of these conjugates. This enzymatic activity releases free, biologically active estrogen molecules, consequently leading to an increase in free estrogen levels. Subsequently, this heightened estrogen can be reabsorbed into the bloodstream, potentially concentrating in breast tissue and contributing to the development of breast cancer [8],[11]–[12]. Notably, breast cancer patients exhibited elevated β-glucosidase levels, indicating robust enzyme activation in breast cancer contexts [13]. This direct patient-derived evidence strongly suggests that overexpression of β-glucosidase may play a role in breast cancer development (Figure 4).

After conjugated estrogens are excreted into the gastrointestinal tract via bile, they can be deconjugated by bacterial enzymes produced by the gut microbiota. The community of estrobolome could produce an enzyme known as beta-glucuronidases (GUS), which contributes to the conversion of estrogens into their active forms, elevating the levels of intestinal estrogen available for absorption into the bloodstream [9]. This pathway facilitates the dissemination of free estrogens to various distant organs, including the breast, where they play a pivotal role in promoting breast tumour progression. This influence is exerted by orchestrating breast dysbiosis, leading to chronic inflammation and subsequent alterations in DNA breaks, proliferation, angiogenesis, metastasis, and invasion [3]. In addition to beta-glucuronidases, another enzyme secreted by the estrobolome, namely beta-glucosidases, is implicated in the deconjugation of estrogen [8]. These enzymes are predicted to contribute to the escalation of breast cancer development.

According to the research conducted by Mahno [6], a compelling association was unveiled between several genera exhibiting glucuronidase (GUS) activity and an increased likelihood of breast cancer. Reported genera with positive correlations to breast cancer include *Weissella*, *Turicibacter*, *Ruminococcus*, *Prevotella*, *Porphyromonas*, *Megamonas*, *Marvinbryantia*, *Klebsiella*, *Fusobacterium*, *Faecalibacterium*, *Eubacterium*, *Escherichia*, *Enterococcus*, *Enterobacter*, *Coprobacillus*, *Clostridium*, *Butyricicoccus*, *Blautia*, *Bacteroides*, *Anaerostipes*, *Actinomyces*, and the order *Clostridiales*. Among these, the genus *Enterococcus* was notably supported by other studies as having a significant association with an elevated predisposition to breast cancer. One of the researchers supporting this finding was conducted by Zhu et al.'s [10] which found a positive association between *Enterococcus sp.* and breast cancer cases, lends support to the findings of Mahno [6]. Furthermore, these findings were corroborated by Khalid's [5] study, which showed that the gut microbiota of breast cancer patients included comparatively more *Enterococcus sp.* and other intestinal microbes than that of healthy women. Hence, in the endeavor to elucidate the intricate relationship between gut microbiota and breast cancer, the gastrointestinal bacterium *Enterococcus faecalis* underwent comprehensive whole genome sequencing to unravel its contributory role in the development of breast cancer.




(a)




(b)

Figure 4. (a) Functional classification of sequenced *Enterococcus faecalis* using RAST subsystems, and (b) KEGG Pathway that reveals genes involved in starch and sucrose metabolism.

## 4. CONCLUSION

Based on the findings, this research offered predictions of *Enterococcus faecalis* as a gut bacteria in the contribution of breast cancer development. The identification of β-glucosidase gene in the draft genome of *Enterococcus faecalis* points to a likely mechanism by which the bacterium may cause breast cancer. Notable is the role of β-glucosidase in estrogen deconjugation, which raises the free active form of estrogen. After being reabsorbed into the bloodstream, this active estrogen may make its way to the breast tissue, where it may cause persistent inflammation and accelerate the development of breast cancer. Nevertheless, despite the encouraging findings of this study, much remains unknown about the particular genetic variants, virulence factors, and metabolic pathways found in Enterococcus faecalis strains that may contribute to the development of breast cancer. As a result, there is a strong need for further in-depth research on the gut microbiome to identify any possible roles that gut bacteria may have in the emergence of breast cancer.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS CONTRIBUTION

Jasmine Teoh Wen Yi and Lim Li Tong (Investigation; Validation; Writing – Original Draft, Formal analysis)

Hajar Fauzan Ahmad (Conceptualization; Supervision; Methodology; Writing - Review & Editing)

All authors critically revised all versions of the manuscript and approved the final version.

## REFERENCES

[1] N. E. Mahno, D. D. Tay, N. S. Khalid, A. S. M. Yassim, N. S. Alias, S. A. Termizi, et al., "The relationship between gut microbiome estrobolome and breast cancer: A systematic review of current evidences," *Indian Journal of Microbiology*, vol. 64, no. 1, pp. 1-19, 2023.

[2] N. X. Thang, S. Yoo, H. La, H. Lee, C. Park, K. S. Park, et al., "Epigenetic factors as etiological agents, diagnostic markers, and therapeutic targets for luminal breast cancer," *Biomedicines*, vol. 10, no. 4, p. 748, 2022.

[3] S. Viswanathan, S. Parida, B. T. Lingipilli, R. Krishnan, D. R. Podipireddy, and N. Muniraj, "Role of gut microbiota in breast cancer and drug resistance," *Pathogens*, vol. 12, no. 3, p. 468, 2023.

[4] J. J. Goedert, G. Jones, X. Hua, X. Xu, G. Yu, R. Flores, et al., "Investigation of the association between the fecal microbiota and breast cancer in postmenopausal women: A population-based case-control pilot study," *Journal of the National Cancer Institute*, vol. 107, no. 8, p. djv147, 2015.

[5] N. S. Abd Khalid, N. E. Mahno, D. D. Tay, H. F. Ahmad, J. Kasian, A. F. Othman, et al., "IDDF2022-ABS-0263 Gut microbiome of women diagnosed with breast cancer within Pahang, Malaysia," *Gut*, vol. 71, p. A175 LP-A177, 2022.

[6] M. Kwa, C. S. Plottel, M. J. Blaser, and S. Adams, "The intestinal microbiome and estrogen receptor-positive female breast cancer," *Journal of the National Cancer Institute*, vol. 108, no. 8, p. djw029, 2016.

[7] R. L. Bechshøft, S. Reitelseder, G. Højfeldt, J. L. Castro-Mejía, B. Khakimov, H. F. B. Ahmad, et al., "Counteracting age-related loss of skeletal muscle mass: A clinical and ethnological trial on the role of protein supplementation and training load (CALM Intervention Study): Study protocol for a randomized controlled trial," *Trials*, vol. 17, no. 1, p. 397, 2016.

[8] T. Jensen, R. L. Bechshoeft, D. Giacalone, M. H. Otto, J. Castro-Mejía, H. F. B. Ahmad, et al., "Whey protein stories – An experiment in writing a multidisciplinary biography," *Appetite*, vol. 107, pp. 285–294, 2016.

[9] D. D. Tay, S. W. Siew, S. Shamzir Kamal, M. N. Razali, and H. F. Ahmad, "ITS1 amplicon sequencing of feline gut mycobiome of Malaysian local breeds using Nanopore Flongle," *Archives of Microbiology*, vol. 204, no. 6, p. 314, 2022.

[10] Z. U. Afeera, N. Mohamad Zain, J. Arumugam, N. Kamarudin, M. A. Zainal Abidin, et al., "Near-complete whole-genome sequencing of two burkholderia pseudomallei strains harbouring novel molecular class D beta-lactamase genes, isolated from Malaysia," *Microbiology Resource Announcements*, vol. 11, no. 10, p. e00468-22, 2022.

[11] M. Y. Choo, S. W. Siew, N. S.-A. Abdullah, M. A. N. Muhammad, and H. F. Ahmad, "Complete whole genome sequencing of Vibrio parahaemolyticus strain UMP001VA, isolated from sea cucumber Holothuria leucospilota from Malaysia harbouring blaCARB-33 gene," *Materials Today: Proceedings*, vol. 75, pp. 68–73, 2023.

[12] S. N. Soffian, M. I. H. Nasharudin, R. A. Ruzaidi, A. N. F. Mohd Anera, W. A. W. Hashim, M. S. Ismail, et al., "Whole genome sequencing of bovine Pasteurella multocida type B isolated from haemorrhagic septicaemia during 2020 major outbreak in East Coast, Malaysia," In *AIP Conference Proceedings*, vol. 2682, no. 1, p. 050011, 2023.

[13] M. S. Munirah, S. S. Wei, T. D. Dean, and A. H. Fauzan, "Near-complete whole-genome sequence of paenibacillus sp. nov. Strain J5C2022, a Sucretolerant and endospore-forming bacterium isolated from highly concentrated sugar brine," *Microbiol. Resour. Announc.*, vol. 12, no. 4, p. e01055-22, 2023.

[14] S. W. Siew, C. M. Yen, I. P. G. Marshall, H. Abd Hamid, S. S. Kamal, D. S. Nielsen, et al., "Gut microbiome and metabolome of sea cucumber (Stichopus ocellatus) as putative markers for monitoring the marine sediment pollution in Pahang, Malaysia," *Marine Pollution Bulletin*, vol. 182, p. 114022, 2022.

[15] S. W. Siew, S. M. Musa, N. 'Azyyati Sabri, M. F. Farida Asras, and H. F. Ahmad, "Evaluation of pre-treated healthcare wastes during COVID-19 pandemic reveals pathogenic microbiota, antibiotics residues, and antibiotic resistance genes against beta-lactams," *Environmental Research*, vol. 219, p. 115139, 2023.

[16] Z. U. Afeera, S. S. Wei, M. S. Munirah, S. S. Nellisa, P. Petrick, and A. H. Fauzan, "Whole-genome sequence of a Stenotrophomonas maltophilia isolate from tap water in an intensive care unit," *Microbiology Resource Announcements*, vol. 12, no. 2, p. e00995-22, 2023.

[17] W. Walters, E. R. Hyde, D. Berg-Lyons, G. Ackermann, G. Humphrey, A. Parada, et al., "Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys," *Msystems*, vol. 1, no. 1, p. 10-1128, 2015.

[18] S. Filardo, M. D. Pietro, and R. Sessa, "Current progresses and challenges for microbiome research in human health: a perspective," *Frontiers in Cellular and Infection Microbiology*, vol. 14, p. 1377012, 2024.

[19] Barkha Madhogaria, P. Bhowmik, and A. Kundu, "Correlation between human gut microbiome and diseases," *Infectious Medicine*, vol. 1, no. 3, pp. 180–191, 2022.

[20] G. Bernardo, V. Le Noci, M. Di Modica, E. Montanari, T. Triulzi, S. M. Pupa, et al., "The emerging role of the microbiota in breast cancer progression," *Cells*, vol. 12, no. 15, pp. 1945–1945, 2023.

[21] J. R. Lakritz, T. Poutahidis, S. Mirabal, B. J. Varian, T. Levkovich, Y. M. Ibrahim, et al., "Gut bacteria require neutrophils to promote mammary tumorigenesis," *Oncotarget*, vol. 6, no. 11, pp. 9387–9396, 2015.

[22] J. Russo and I. H. Russo, "The role of estrogen in the initiation of breast cancer," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 102, no. 1–5, pp. 89–96, 2006.

[23] J. Russo, R. Moral, G. A. Balogh, D. Mailo, and I. H. Russo, "The protective role of pregnancy in breast cancer," *Breast Cancer Research*, vol. 7, no. 3, pp. 1-12, 2005.

[24] X. Li, J. Yang, L. Peng, A. A. Sahin, L. Huo, K. C. Ward, et al., "Triple-negative breast cancer has worse overall survival and cause-specific survival than non-triple-negative breast cancer," *Breast Cancer Research and Treatment*, vol. 161, no. 2, pp. 279–287, 2016.

[25] N. I. Hanafi, A. S. Mohamed, A. Kadir, and M. H. D. Othman, "Overview of bile acids signaling and perspective on the signal of ursodeoxycholic acid, the most hydrophilic bile acid, in the heart," *Biomolecules*, vol. 8, no. 4, pp. 159–159, 2018.

[26] E. Mikó, A. Vida, T. Kovács, G. Ujlaki, G. Trencsényi, J. Márton, et al., "Lithocholic acid, a bacterial metabolite reduces breast cancer cell proliferation and aggressiveness," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1859, no. 9, pp. 958–974, 2018.

[27] N. Garmpis, C. Damaskos, A. Garmpi, E. Kalampokas, T. Kalampokas, E. Spartalis, et al., "Histone deacetylases as new therapeutic targets in triple-negative breast cancer: Progress and promises," *Cancer Genomics & Proteomics*, vol. 14, no. 5, 2017.

[28] V. Salimi, M. Shabani, Mitra Nourbakhsh, and Masoumeh Tavakoli-Yaraki, "Involvement of 15-lipoxygenase-1 in the regulation of breast cancer cell death induced by sodium butyrate," *Cytotechnology*, vol. 68, no. 6, pp. 2519–2528, 2016.

[29] J. Semaan, S. El-Hakim, J.-N. Ibrahim, R. Safi, A. A. Elnar, and C. El Boustany, "Comparative effect of sodium butyrate and sodium propionate on proliferation, cell cycle and apoptosis in human breast cancer cells MCF-7," *Breast Cancer*, vol. 27, no. 4, pp. 696–705, 2020.