Unveiling the Gut Microbiota Landscape in Bangladeshi Female Breast Cancer Patients through Next-Generation Sequencing

Sabekun Nahar[1*] Maruf Ibne Monowar[2] Tanvir Faysal[3] Laila Anjuman Banu[4]

Abstract

Background: Scientific evidence indicates that imbalances in the composition of gut microbiota are closely linked to the onset of many diseases. This study aimed to employ Next Generation Sequencing (NGS) to investigate gut microbial imbalances in Bangladeshi female breast cancer patients, enabling simultaneous analysis of numerous bacterial DNA fragments.

Materials and methods: In this case-control study, stool sample was collected from 30 cases of Bangladeshi female breast cancer patients and 30 female controls. Microbial genomic DNA was isolated to amplify targeted (V3-V4) region of the 16S ribosomal RNA (rRNA) gene. After indexing, NGS was done in Illumina sequencer using the MiSeq platform. Data were analyzed using QIIME and MiSeq Reporter to identify microbiome.

Results: Microbial differences were noted in Bangladeshi female breast cancer patients compared to the control group. At the phylum level (L2) 15 Operational Taxonomic Units (OTUs) were identified, fourteen phyla belonging to domain Bacteria and other to the domain Archaea. In the control group, bacteria belonging to phylum Firmicutes (44%) were dominant, were as in cases phylum Bacteroidetes were dominant. At the order level (L4) 53 OTUs were identified. In breast cancer and control groups, bacteria belonging to order Bacteroidales were dominant. In this research, gut microbial variation in Bangladeshi female breast cancer patients from that of controls was observed.

Conclusion: The emerging connection between the gut microbiome and female breast cancer could serve as a valuable lead for future research. Potential impact of gut microbiome would be further investigated for better management of female breast cancer patients.

1. Assistant Professor of Anatomy
Sheikh Hasina National Institute of Burn and Plastic Surgery, Dhaka.
2. Associate Professor of Anatomy
Enam Medical College, Savar, Manikgonj.
3. Lecturer of Anatomy
Dhaka Medical College, Dhaka.
4. Professor of Genetics & Molecular Biology and Chairman
Department of Anatomy
Bangabandhu Sheikh Mujib Medical University (BSMMU) Dhaka.

***Correspondence: Dr. Sabekun Nahar**
Cell : 01716 06 96 91
E-mail: snigdha.snigdho@gmail.com

Submitted on : 24.03.2024
Accepted on : 15.04.2024

Key words: Breast cancer; Gut microbiome; Next Generation Sequencing (NGS); 16S rRNA.

Introduction

Recent advancements in medical research suggest that humans host billions of bacteria, known as microbiota, which play a significant role in their genetic makeup.[1] The collection of genomes of microbiota is referred as microbiome.[2] The differences in the microbiome among individuals, as revealed by the Human Microbiome Project (2008 to 2013) suggest its potential role as an influencing factor for disease development.

Metabolites released by gastrointestinal microbiota, may play essential roles in influencing host immunity and overall health.[3] Germ-free models, which are raised in sterile environments without microorganisms, effectively highlight the essential role of gut microbiota in developing immunity.[4] This suggests a potential connection between gut microbiota and various human diseases. In the past decade, numerous intriguing links between gut microbiota and risks of obesity, metabolic disorders, and inflammation have been documented. Epidemiological data, animal studies and in vitro research have demonstrated that reproductive hormones, especially estrogen, are crucial in the development of breast cancer.[5] Gut microbiota affects estrogen balance through enterohepatic circulation, with bacterial species producing β-glucuronidases and β-glucuronides playing a key role in estrogen metabolism by enabling its deconjugation and conjugation.[6] This process reactivates unbound estrogens, allowing them to re-enter the bloodstream, which may influence hormonal disorders such as ER/PR+ breast cancer.[6] In this context, intestinal microflora can be regarded as an environmental factor contributing to the development and progression of breast cancer.[7] The review of Thu M S et al. clarifies the intricate interactions between the microbiome, breast cancer and treatment options, aiming to establish connections that could enhance research efforts and advance personalized medicine to improve patient quality

of life.[8] Studies show that breast cancer patients often have less diverse gut microbiota than healthy individuals, with higher levels of Firmicutes and lower levels of Bacteroidetes.[9]
Previously, staining and microbial cultures identified only a small fraction of microbes due to the difficulty in culturing 20% to 60% of human bacteria.[10] Metagenomics uses advanced genomics to study microbial communities directly in their natural settings, bypassing the need for isolation or cultivation.[4] When Next Generation Sequencing (NGS) is used as a high-throughput approach for molecular research, a huge number of bacterial DNA fragments can be thoroughly analyzed at the same time. The 16S rRNA gene is highly conserved across many bacteria, but its hypervariable regions, which vary between bacteria.[11]

The present research aimed to determine the gut microbiome of the Bangladeshi female breast cancer patients through NGS. This microbiome profiles may help in the development of gut microbial genome data bank biomarkers for female breast cancer patients of Bangladesh. The results of this study could also assist clinicians in diagnosing breast cancer by analyzing changes in the composition of gut microbiota. Emerging treatment strategies for breast cancer that target the gut microbiome, including prebiotics, probiotics and dietary changes, are still in early stages and will be further investigated in future research.

Materials and methods

Following formal approval from the Institutional Review Board (IRB) of Bangabandhu Sheikh Mujib Medical University (BSMMU) this case-control study was conducted in the Department of Anatomy, BSMMU, Dhaka, Bangladesh from March 2018 to January 2020 where the cases were female having breast cancer and the controls were females without that. The patients were recruited from the Department of Surgery of Bangladesh Medical College Hospital and the Department of Surgery and the Department of Oncology, BSMMU. All the females were informed about the ethical aspects relevant to the present study and 'informed written consent' was taken from each participant. Approximately 90 female breast cancer patients were interviewed, most of them could not be included as the sample because of their co-morbidities. The participants who was smoker, had respiratory and digestive tract infection, thyroid disease, coronary artery disease, stroke, use antibiotics, pregnant or breast feeder was excluded.[5] To complete the study in time and make its cost affordable, thirty cases and thirty controls were selected initially. Out of sixty samples, genetic sequencing of only 11 cases and 10 controls were done considering the quality of DNA.
The participants were provided a stool collection kit named "OMNIgene.GUT Feces Collection Kit (DNA Genotek, Canada)" as well as explained the procedure so that their queries were answered by the investigator. A leaflet describing the collection procedure was also provided, which increased their confidence regarding this procedure.
Genomic DNA of the microbiota was isolated from feces samples by means of QIAamp DNA Stool Mini Kit (Qiagen, Germany) and FavorPrep Stool DNA Isolation Kit (Favorgen, Taiwan). After isolation, the quantity and DNA purity was assessed using the NanoDrop spectrophotometer. The absorbance at 260
nanometers and 280 nanometers and the A_{260}/A_{280} ratio was recorded. The DNA concentration was found at a time from NanoDrop 2000 Spectrophotometer.
Primer set for the amplification of the targeted region of the gut microbiome was selected. The 16S V3-V4 region was the target of the primers employed in this technique, which were selected from the Klindworth et al. article as the most efficient bacterial primer pair.[12] Illumina adapter overhang nucleotide sequences were supplemented to the gene specific sequences.
Polymerase Chain Reaction was performed using one µL of genomic DNA, one µL of prepared forward and reverse primers, 12.5 µL KAPA HiFi master mix and rest filled with 9.5 µL nuclease-free water in a PCR tube. The reaction mixture was briefly (10-20 seconds) centrifuged and then placed in a thermal cycler. Gel electrophoresis of amplicons produced by PCR was done to check whether the desired DNA segment was amplified or not. After checking, amplicons were cleaned up using AMPure XP beeds (Beckman Coulter, USA). The Qubit Fluorometer was used to measure each purified amplicon (Thermo Fisher Scientific, USA).

The library of the targeted genomic DNA was prepared for Next Generation Sequencing. The V3-V4 region of the selected 16S rRNA genes was prepared in accordance with "16S Metagenomic Sequencing Library Preparation" module provided by Illumina. Following the module, the target amplicon was attached to dual index barcodes and Illumina sequencing adapters. For sequencing desired libraries were pooled together employing the complement of Nextera XT indices.

The quality and quantity of the NGS Library quality were checked. The hypervariable V3-V4 regions of the 16S rRNA were sequenced using Illumina MiSeq paired-end sequencing at Apical Scientific Sdn Bhd in Selangor, Malaysia. Paired 300 bp reads and MiSeq Reagents Kit v3 were used and sequencing was completed in Illumina Miseq Sequencer (Illumina, San Diego, USA).

The sequenced data generated after Next Generation Sequencing was available in BaseSpace and analyzed in the MiSeqReporter software. QIIME software was used for taxonomy categorization and quality reads filtering.[16] The samples' taxonomic makeup was assessed, and a reference-based method based on the database of 16S rRNA gene sequences was used to identify the microbiome up to the order level. Greengenes v. 13.5.

Statistical Package for Social Science (SPSS) version 23.0 (IBM Corporation) was used to analyze the socio-demographic data.

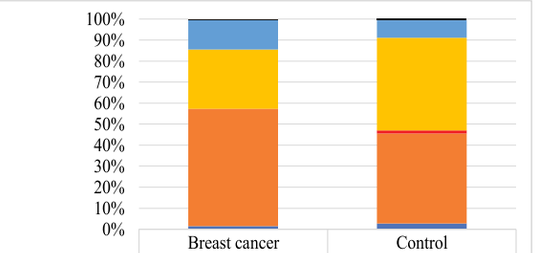
Results

A case-control study was conducted involving 60 participants with an average age spanning from 30 to 70 years while maximum belonged to the age group of 41 to 50 years. About 70% of the breast cancer cases developed the disease before age 50, and 56.67% experienced menarche before age 12, while only 26.6% of the controls had menarche before age 12. Most of the cases had their first live pregnancy before age 21 and the use of hormonal contraceptives was higher among them, at 56.7%. The demographic data was shown in Table I.

Table I Demographic and reproductive characteristics of the controls and cases

Parameter	Breast cancer patient mean (± SD)	Control mean (± SD)	p-value
Age in years	46.7 (± 8.2)	37.3 (± 12.5)	0.001*
Age at menarche	11.7(±1.6)	12.4(±1.4)	0.096
Parity	2.1(±0.2)	1.5(±1.3)	0.047*
Age at first childbirth	18.6(±3.4)	24(±3.8)	0.004*
Age at menopause	42.3(±3.6)	43.3(±2.6)	0.447

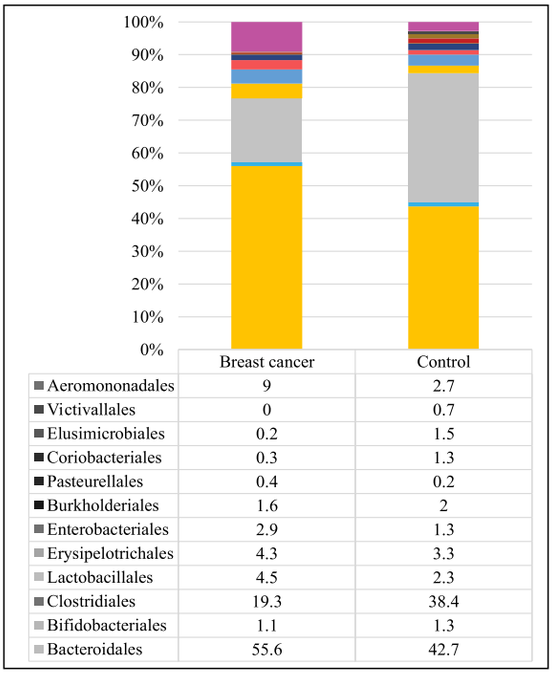
Paired-end sequencing on Illumina MiSeq of fecal samples showed that all 21 samples were positive for the presence of bacterial DNA. On an average 1421950.615 paired reads per sample were yielded. At L2 (phylum), the Bacteroidetes, Firmicutes, and Proteobacteria constituted the majority of the microbiota in stool samples in both of the groups and the remaining phyla constituted only a fraction of the gut microbiota composition in the samples. Relative percentage distribution of microbiota at the phylum level in case and controls are presented in Figure 1.



	Breast cancer	Control
Cyanobacteria	0.4	0
Lentisphaerae	0	0.7
Proteobacteria	14	8.1
Firmicutes	28.2	44
Elusimicrobia	0.2	1.5
Bacteroidetes	55.6	42.7
Actinobacteria	1.4	2.7

Figure 1 Relative percentage distribution of microbiota at the phylum level (L2) in two groups

At the order level (L4) 53 OTUs were identified. Among which, 51 orders were from the domain Bacteria and the rest two were from the domain Archaea. In both breast cancer and control females, bacteria belonging to order Bacteroidales were dominant and constituted the following percentage of bacterial composition: 55.6% in the cases and 42.7% in the controls. Relative percentange distribution of microbiota at the order level (L4) in case and controls are presented in Figure 2.



	Breast cancer	Control
Aeromononadales	9	2.7
Victivallales	0	0.7
Elusimicrobiales	0.2	1.5
Coriobacteriales	0.3	1.3
Pasteurellales	0.4	0.2
Burkholderiales	1.6	2
Enterobacteriales	2.9	1.3
Erysipelotrichales	4.3	3.3
Lactobacillales	4.5	2.3
Clostridiales	19.3	38.4
Bifidobacteriales	1.1	1.3
Bacteroidales	55.6	42.7

Figure 2 Relative percentage distribution of microbiota at the order level (L4) in the two groups

Orders with a representation of 0.5% and higher relative abundance are presented in Figure 3.

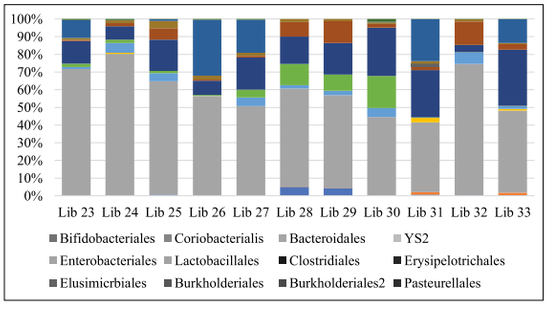


Figure 3 Relative abundance of microbiota among the cases and control at the order level (L4)

Discussion

The comparative bacterial percentages at the phylum level (L2) observed in this study are consistent with those reported by Luu et al.[13] Their study found that in female breast cancer patients, Firmicutes and Bacteroidetes were the two most prevalent phyla, representing 39.4% and 13.0% of all bacteria respectively.

In this study, the relative percentages of Firmicutes and Bacteroidetes among the controls were 44% and 42.7% respectively. Lin et al. found that over 80% of the gut microbiome in healthy adults from both Bangladesh and the United States is made up of Firmicutes and Bacteroidetes.[14] Additionally, a study of the Western Indian population reported a higher relative percentage of Bacteroidetes in the control group (71.5%) and a Bacteroidetes/Firmicutes (B/F) ratio of 1.9 in cases versus 0.031 in controls.[15]

At the order level (L4), both groups were primarily dominated by Bacteroidales, with the phylum Bacteroidetes mainly represented by this order under the class Bacteroidia. Goedert et al. found that Clostridiales and Bacteroidales were more prevalent in postmenopausal female breast cancer patients, similar to the current study.[16] A study from China found a higher percentage of Enterobacteriales in female breast cancer patients, which contrasts with the present study's findings.[14] In the current study, Bacteroidales accounted for 42.7% in the controls, while Clostridiales was less prevalent in the case group (19.3%) compared to the controls (38.4%). This result is consistent with the findings of Goedert et al.[16]

Limitations

Considering the cost and amount of data generated, 30 female breast cancer patients and 30 females without breast cancer were selected, and this was mostly due to the limitations with the time. Out of sixty samples, genetic sequencing of only 11 cases and 10 controls were done due to financial restraints.

Conclusion

As this study is one of the first 16S metagenomics study on female breast cancer patients in Bangladesh to date. With this sample size, it was observed that the fecal microbiota of women with breast cancer was compositionally different from that of similar women without breast cancer. The findings imply that the gut microbiota may affect breast cancer risk. This study may serve as a baseline data for further investigation of female breast cancer based on metagenomics study.

Recommendation

Multicenter study with larger sample size is recommended to comprehend the link between the gut microbiome and breast cancer to develop tailored therapies aimed at enhancing patient outcomes.

Acknowledgement
Authors express their gratitude to honorable co-guide Professor of Genetic Engineering and Biotechnology, University of Dhaka, Bangladesh for precious guidance.

Contribution of authors

SN-Concept, data collection, data analysis, manuscript writing & final approval.

MIM-Data collection, interpretation of data, critical revision & final approval.

TF-Data collection, data analysis, manuscript writing & final approval.

LAB-Conception, design, critical revision & final approval.

Disclosure
All the authors declared no competing interests.

References

1. Tandon D, Haque MM, R S, Shaikh S, P S, Dubey AK, S S, Mande. A snapshot of gut microbiota of an adult urban population from western region of india. PLoS ONE. 2018; 13(4):1-20.
doi:10.1371/journal.pone.0195643.

2. Sirisinha S. The potential impact of gut microbiota on your health: current status and future challenges. Asian Pac J Allergy Immunol. 2016; 34:249-264.
doi: 10.12932/AP0803.

3. Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA et al. The role of intestinal microbiota and the immune system. Eur.Rev.Med. Pharmacol Sci. 2013; 17:323–333. doi: 10.1002/cbdv.201100359 .

4. Smith, K, McCoy, KD & Macpherson, AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Seminars in Immunology. 2006; 18(1): 43-51.
doi: 10.1016/j.smim.2006.10.002.

5. Althuis, MD, Fergenbaum, JH, Garcia-Closas, M, Brinton, LA, Madigan, MP & Sherman, ME. Etiology of hormone receptor–defined breast cancer: A systematic review of the literature. Cancer Epidemiology, Biomarkers & Prevention. 2004; 13(10): 1558-1568.
doi: 10.1158/1055-9965.1558.13.10.

6. Jotshi A, Sukla KK, Haque MM, Bose C, Varma B, Koppiker CB et al. Exploring the human microbiome : A step forward for precision medicine in breast cancer. Cancer Reports (Hoboken). 2003; 6(11): 1877.
doi: 10.1002/cnr2.1877.

7. Xuan C, Shamonki JM, Chung A, DiNome ML, Chung M, Sieling PA, Lee DJ. Microbial dysbiosis is associated with human breast cancer. PLoS ONE. 2014; 9(1):1-7.
doi:10.1371/journal.pone.0083744 .

8. Thu, MS, Chotirosniramit, K, Nopsopon, T, Hirankarn, N & Pongpirul, K. Human gut, breast and oral microbiome in breast cancer: A systematic review and meta-analysis. Frontiers in Oncology. 2023; 13: 1144021.
doi: 10.3389/fonc.2023.1144021.

9. Nandi, D, Parida, S & Sharma, D. The gut microbiota in breast cancer development and treatment: The good, the bad, and the useful! Critical Reviews in Oncology/ Hematology. 2023; 176: 2221452.
doi: 10.1080/19490976.2023.2221452.

10. Sharma S, Rana S, Singh R. A short note-metagenomics. International Journal of Biomedical Research. 2012; 3(4):181-186.
doi: 10.7439/ijbr.v3i4.396 .

11. Coenye T, Vandamme P. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. FEMS Microbiology Letters. 2003; 228(1): 45–49.
doi: 10.1016/S0378-1097(03)00717-1.

12. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glockner FO. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research. 2012; 41(1): 1-11.
doi: 10.1093/nar/gks808 .

13. Luu TH, Michel C, Bard JM, Dravet F, Nazih H, Bobin-Dubigeon C. Intestinal Proportion of Blautia spp. is associated with clinical stage and histoprognostic grade in patients with early-stage breast cancer. Nutrition and cancer. 2017; 69:267–275.
doi: 10.1080/01635581.2017.1263750.

14. Lin A, Bik EM, Costello EK, Dethlefsen L, Haque R, Relman DA, Singh U. Distinct distal gut microbiome diversity and composition in healthy children from Bangladesh and the United States. PLoS ONE. 2013; 8(1):53838.
doi: 10.1371/journal.pone.0053838.

15. Vaiserman A, Romanenko M, Piven L, Moseiko V, Lushchak O, Kryzhanovska N. Differences in the gut Firmicutes to Bacteroidetes ratio across age groups in healthy Ukrainian population. BMC Microbiology. 2020; 20: 221.
doi.org/10.1186/s12866-020-01903-7.

16. Goedert JJ, Jones G, Hua X, Xu X, Yu G, Flores R, Falk RT, Gail MH, Shi J, Ravel J, Feigelson HS. Investigation of the association between the fecal microbiota and breast cancer in postmenopausal women: a population-based case-control pilot study. JNCI: Journal of the National Cancer Institute.2015; 107(8):1-5.
doi: 10.1093/jnci/djv147.