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Gut Microbiota and Estrogen Levels in Women Breast Cancer in Côte d'Ivoire

Gnahore Djeda Franck [a,b*], M'bengue Gbonon Valérie [a], Sekongo Yassongui Mamadou [c], Assohoun Stanislas [a,d], Ako Ako Aristide [a], Diplo Flore Bernadette [a], Coulibaly Safiatou [a], Osseni Akandji [e], Afran Sidje Arlette [b], N'guessan Jean David [b] and Dosso Mireille [a]

[a] Molecular Genetics Platform, Institut Pasteur de Côte d'Ivoire, Côte d'Ivoire.
[b] Laboratory of Biology and Health, UFR Biosciences, Félix Houphouët Boigny University, Côte d'Ivoire.
[c] National Blood Transfusion Center of Cote d'Ivoire, Côte d'Ivoire.
[d] Bioinformatics and Computational Biology Group, National Computing Center of Côte d'Ivoire, Félix Houphouët Boigny University, Côte d'Ivoire.
[e] Oncology Department, Treichville University Hospital, Côte d'Ivoire.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The gut microbiota is a complex ecosystem of microorganisms that maintains a symbiotic relationship with its host, contributing to digestion, metabolism, and immunity. Studies suggest that the microbiota may play a role in several non-communicable diseases, including certain cancers. It

**Corresponding author: E-mail: djedafranck@gmail.com;*

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may also influence circulating estrogen levels through enzymes like β-glucuronidase, which affects estrogen reabsorption and thus increases the risk of breast cancer, especially in postmenopausal women. This study aimed to characterize the microbiota of breast cancer patients in Côte d'Ivoire to identify bacterial markers potentially associated with increased plasma estradiol concentrations. A case-control study was conducted at the Oncology Department of the CHU of Treichville, the National Blood Transfusion Center, and the Institut Pasteur de Côte d'Ivoire, recruiting 85 participants, including 39 patients and 46 controls, both premenopausal and postmenopausal. Characterization of the gut microbiota revealed a significant difference in microbiota diversity between breast cancer patients and controls. Quantification of plasma hormones and the use of the LEfSe algorithm identified eight bacterial genera potentially associated with increased plasma estradiol concentrations. These results open research avenues on the gut microbiota and estrogen levels, which could have significant implications for the prevention, diagnosis, and targeted treatment of breast cancer.

Keywords: Gut microbiota; Estrogen levels; biomarkers; 16S metagenomic sequencing; breast cancer; LEfSe.

1. INTRODUCTION

The gut microbiota represents a complex ecosystem encompassing all unicellular microorganisms residing in the digestive tract, primarily bacteria, fungi, archaea, and even viruses [1]. Bacterial concentration is highest at the distal end of the digestive tract [2]. The relationship between the host and the microbiota is symbiotic; bacteria benefit from a stable environment (temperature, CO_2, pH, nutrients), while the host benefits from a multitude of capabilities in digestion, nutrition, metabolism, and immunity [1,3].

However, many studies suggest that the gut microbiota plays a crucial role in various non-communicable diseases, including obesity [4], chronic inflammatory bowel diseases, allergic and immune disorders [5,6], behavioral disorders [2,7], and even certain cancers [8,9]. Breast cancer is on the rise in developing countries, including Côte d'Ivoire, where it ranks first among cancers in women, followed by cervical cancer [10,11]. According to data from the Abidjan cancer registry, the age-standardized incidence rate was 44.7 per 100,000 women, with about 74% at late stages (III and IV), 1,785 deaths, and a mortality rate in 2020 of 25.3 per 100,000 women [12,13]. More than half of the diagnosed breast cancer cases in Côte d'Ivoire are hormone-dependent, meaning the cancer cells express hormone receptors to which estrogens and progesterone bind, promoting tumor growth [12,14,15]. Only 5 to 10% of breast cancers are attributable to a genetic predisposition (mutations in the *BRCA1* and *BRCA2* genes) [16], implying that the etiological mechanisms of this cancer are not yet fully understood [17-18].

High concentrations of estradiol and progesterone would increase the risk of breast cancer by about 10%, especially in postmenopausal women where this rate is normally low [19,20]. Recent studies have shown that the gut microbiota can influence circulating estrogen levels in postmenopausal women through bacterial genes (estrobolome) capable of producing enzymes like β-glucuronidase, which affect the enterohepatic circulation of estrogens and their reabsorption. Disruptions in the microbiota could therefore lead to high levels of circulating estrogens and their metabolites, thus increasing the risk of breast cancer. Many studies suggest a crucial role of gut microbiota imbalance (dysbiosis) in various pathologies, including breast cancer.

Characterizing the microbiota allows us to assess potential differences between a normal and an imbalanced microbiota. Moreover, bioinformatics analyses could highlight potential bacterial biomarkers that could serve as targets in approaches using prebiotics or probiotics, allowing for the rebalancing of the gut microbiota. The objective of our study was to characterize the microbiota of breast cancer patients in Côte d'Ivoire to identify potential bacterial markers associated with increased plasma Estradiol 2 concentrations.

2. MATERIALS AND METHODS

2.1 Recruitment of Women

This case-control study was conducted from May 2020 to September 2023 at the Oncology Department of the University Hospital Center of Treichville for case recruitment, at the blood

donation service of the National Blood Transfusion Center of Treichville, and at the Reception, Welcome, and Sampling Unit of the Institut Pasteur de Côte d'Ivoire for control recruitment. Two groups of women were included in this study: the first group consisted of premenopausal and postmenopausal women with breast cancer (the cases), while the second group consisted of premenopausal and postmenopausal women without the disease (the controls). For the cases, women of all ages diagnosed with breast cancer at any stage were included, and for the controls, women of all ages with a normal mammogram/breast ultrasound less than a year old were included. Pregnant women, those who had used hormones in the 6 months preceding their inclusion, and those who had started chemotherapy were excluded from the study.

2.2 Biological Material

The biological material consisted of venous blood samples and fresh stool samples.

2.3 Methods

2.3.1 Interview and data collection

Sociodemographic information, clinical status, and Body Mass Index (BMI) were collected using a questionnaire and by consulting medical records.

2.3.2 Collection, transport, and preservation of samples

Blood samples were collected in red tubes with clot activator on the same day of inclusion for postmenopausal women and during the follicular phase for premenopausal women (between the 4th and 7th day of the menstrual cycle). Stool samples were collected using a specially designed collection kit. Stool samples were transported to the laboratory within less than 2 hours after emission and stored at a temperature of -80 degrees Celsius at the Biological Resources Center of the Institut Pasteur de Côte d'Ivoire.

2.3.3 Hormone quantification

Estradiol 2 (E2) and progesterone hormones were quantified using Elecsys® Estradiol III and Elecsys® Progesterone III kits on the Cobas® e411 Analyzer according to the manufacturer's protocol (Roche Ltd, Switzerland).

2.3.4 16S rRNA metagenomic analysis

For metagenomic analysis, ten (10) stool samples from cases and ten (10) from controls were randomly selected by multi-stage sampling. Total bacterial DNA extraction was performed using the Quick DNA™ Fecal/Soil Microbe Microprep kit (ZYMO RESEARCH) and quantification of the extracts was performed on the Nanodrop UV/Vis spectrophotometer. Sequencing libraries were prepared using the Quick-16S™ Plus NGS Library Prep Kit (V4) (ZYMO RESEARCH) targeting the hypervariable V4 region of the 16S rRNA gene (515f: GTGYCAGCMGCCGCGGTAA; 806r: GGACTACNVGGGTWTCTAAT). Libraries were sequenced on the Illumina® Miseq platform.

2.3.5 Bioinformatics analysis

Bioinformatics analyses were conducted using the mothur pipeline version 1.48 [21,22]. A reference sequence Silva seed version 132 [23] truncated to the V4 region of the 16S rRNA gene was used for alignment, and RDP version 18 reference taxonomy files were used for taxonomy.

2.3.6 Identification of bacterial biomarkers associated with plasma E2 concentration

Linear Discriminant Analysis (LDA) coupled with effect size (LEfSe) was performed using the LEfSe program [24] to determine differentially abundant genera in each group, with a logarithmic LDA score > 2 considered significant. E2 concentration was categorized relative to normal reference values for premenopausal (follicular phase) and postmenopausal women [25].

2.4 Statistical Analyses

Data were collected using EPI info™ (CDC) software version 7.2.4.0 and exported to Excel. Statistical analyses were performed using R Studio version 2022. Statistical differences between cases and controls were studied using the Student's t-test for normally distributed variables and the Wilcoxon-Mann-Whitney test for non-normally distributed variables. For binary variables, the significance of differences between cases and controls was studied using the Chi-squared test with a significance threshold $\alpha = 0.05$. Alpha diversity was calculated using the Sobs and Chao1 diversity indices to estimate

community richness. Beta diversity was studied using non-metric multidimensional scaling (NMDS) and molecular variance analysis based on the Bray-Curtis dissimilarity matrix.

3. RESULTS

3.1 Characteristics of the Women Included

In total, we recruited 85 women, including 39 cases and 46 controls, both premenopausal and postmenopausal. The average age of the patients was 53.7 years ± 12 years. Analyzing the average age of menarche and menopause, it appears that postmenopausal cases had an early menarche (~13 years) and late menopause (~52 years) compared to the controls in the same group ($p<0.05$). Postmenopausal cases were therefore under hormonal influence longer than the controls in the same group.

3.2 Plasma Levels of Estradiol 2 and Progesterone

3.2.1 Hormones in premenopausal women

In the cases, the average value of estradiol 2 was 50.58 pg/mL, with a minimum of 29.6 pg/mL and a maximum of 86.84 pg/mL, while in the controls it ranged from 32.73 pg/mL to 247 pg/mL with an average of 110.47 pg/mL. In these women, the average plasma level of estradiol 2 was higher in the controls than in the cases ($p<0.05$), whereas there were no differences between the two groups in progesterone levels (Table 2).

3.2.2 Hormones in postmenopausal women

The estradiol 2 level ranged from below 5 pg/mL to 38.07 pg/mL with an average of 13.54 pg/mL in the cases and from below 5 pg/mL to 16.5 pg/mL with an average of 9.45 pg/mL in the controls. For progesterone measurements, the average was 0.186 ng/mL in the cases, with variations ranging from 0.073 ng/mL to 0.720 ng/mL. Postmenopausal cases had higher plasma levels of estradiol 2 and progesterone than the controls ($p<0.05$) (Table 2).

3.3 Metagenomics analysis

3.3.1 α Diversity

Calculating the alpha diversity indices revealed that there was a significant difference between the microbiota of cases and controls for both the Sobs and Chao1 diversity indices (Sobs index $p = 0.005$; Chao1 index $p = 0.004$) (Fig. 1).

3.3.2 β Diversity

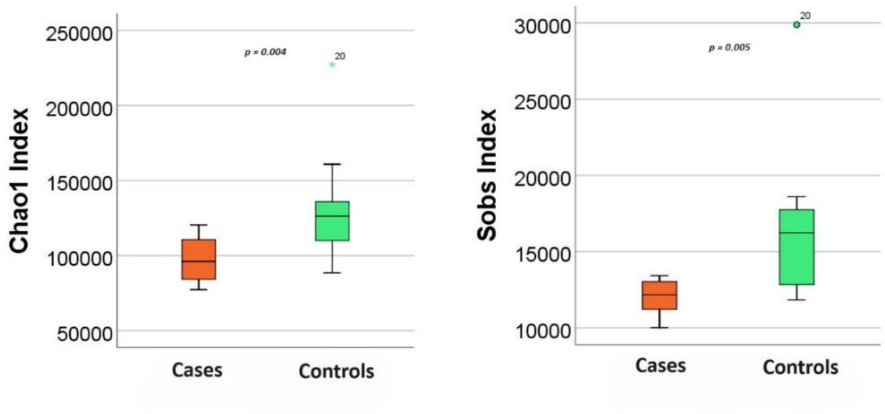
Non-metric multidimensional scaling (NMDS) analysis using the Bray-Curtis dissimilarity matrix revealed a difference in the distribution distance of the microbiota of cases compared to controls. The gut microbiota samples from the women were distributed in space, representing two

Table 1. Characteristics of women included in the metagenomic study

	Cases (n)	Controls (n)	p- value
Premenopausal women	11	18	
Average age in years**,** (sd)	36.09 (5.30)	37.5 (7.45)	***0.58***
Average age of menarche in years, (sd)	12.63 (1.74)	12.72(1.17)	***0.87***
Average age of first pregnancy years (sd)	25.18 (7.05)	23.13 (4.50)	***0.37***
Parity (n), mean, (sd)	2.18 (1.47)	2.11 (1.99)	***0.71***
Average BMI (kg/m ^{2})(sd)	27.15 (6.41)	26.15 (2.87)	***0.9***
Family history of CS (%)	9.09	5.55	***1***
Hormonal contraceptive use (%)	90.90	72.22	***0.228***
Postmenopausal women	28	28	
Average age, (sd)	60.71 (6.88)	59.42 (6.63)	***0.48***
Average age of menopause, (sd)	52.46 (3.52)	49.85 (2.90)	***0.01***
Average age of menarche (sd)	13 (1.15)	14.10 (1.61)	***<0.0001***
Average age at first pregnancy (sd)	22.14 (5.89)	19.60 (2.74)	***0.14***
Parity (n), mean, (sd)	4.89 (3.2)	3.71 (1.6)	***0.186***
Average BMI (kg/m ^{2}) (sd)	28.54 (4.29)	27.58 (4.43)	***0.41***
Family history of CS (%)	32.14	28.57	***0.77***
Hormonal contraceptive use (%)	53.57	53.57	***1***

Table 2. Plasma concentration of E2 and progesterone

	Patients (n)	Controls (n)	p- value
Premenopausal women	11	18	
Estradiol 2 (pg / mL) mean(sd)	50.58(22.24)	110.47 (19.98)	**0.001**
Progesterone (ng / mL) mean(sd)	0.211 (0.082)	0.188 (0.096)	**0.509**
Menopausal women	28	28	
Estradiol 2 (pg / mL) mean(sd)	13.54 (7.95)	9.45 (3.68)	**0.04**
Progesterone (ng / mL) mean(sd)	0.186 (0.127)	0.130 (0.059)	**0.017**



a **b**

Fig. 1. Comparison of alpha diversity between cases and controls

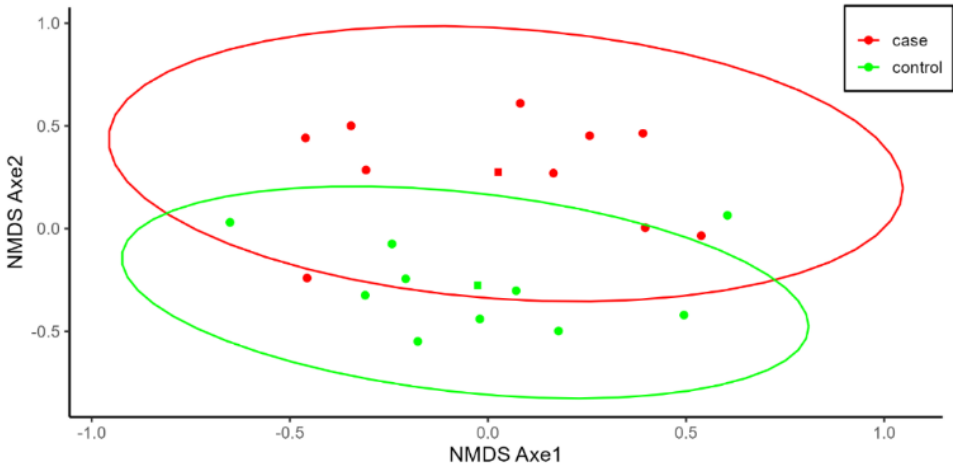


Fig. 2. Non-metric Multidimensional Scaling (NMDS) of samples

distinct groups. One group consisted predominantly of cases samples, while the other group consisted predominantly of control samples (Fig. 2). Additionally, molecular variance analysis (AMOVA) showed a significant difference between the two groups (p = 0.001).

3.3.3 Taxonomic composition analysis

The analysis of taxonomic composition highlighted five major phyla (>3%) in the microbiota of both population groups: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. The other phyla and unclassified genera in this study represent the remaining 3%.

In women with breast cancer, we observed a reduction in the relative abundance of the phyla *Firmicutes* (47.97%) and *Bacteroidetes* (6.68%) compared to the controls, which had respective abundances of 56.83% and 19.42%. Conversely,

we observed an increase in the phyla *Actinobacteria* (28.75%) and *Verrucomicrobia* (6.49%) compared to the controls, which had abundances of 12.56% and 2.87%, respectively. Regarding the phylum *Proteobacteria,* there were no significant variations between cases (7.29%) and controls (6.07%) (Fig. 3).

3.3.4 LefSe analysis

The Linear Discriminant Analysis Effect Size (LEfSe) analysis revealed that the serum level of E2 was significantly positively correlated with the abundances of the bacterial genera *Bifidobacterium*, *Erysipelatoclostridium*, *Eggerthella*, *Dorea*, *Blautia*, *Slackia*, *Anaerobutyricum*, and *Collinsella*, with LDA discriminant values greater than 2 and p-values < 0.05 (Table 3).

4. DISCUSSION

In our study, the average age of patients at the time of diagnosis was 52.7 years. This result aligns with an African meta-analysis showing an average age at diagnosis ranging between 46 and 60 years [26]. However, this result differs from those in industrialized countries where the average age of breast cancer onset has been advancing in recent years [27–29] and is around 67 years, for instance, in France. This difference could be explained by the fact that in developing countries, populations are increasingly adopting a Westernized lifestyle with an increase in risk factors, while populations in developed countries are returning to a much healthier lifestyle, resulting from awareness policies within at-risk populations.

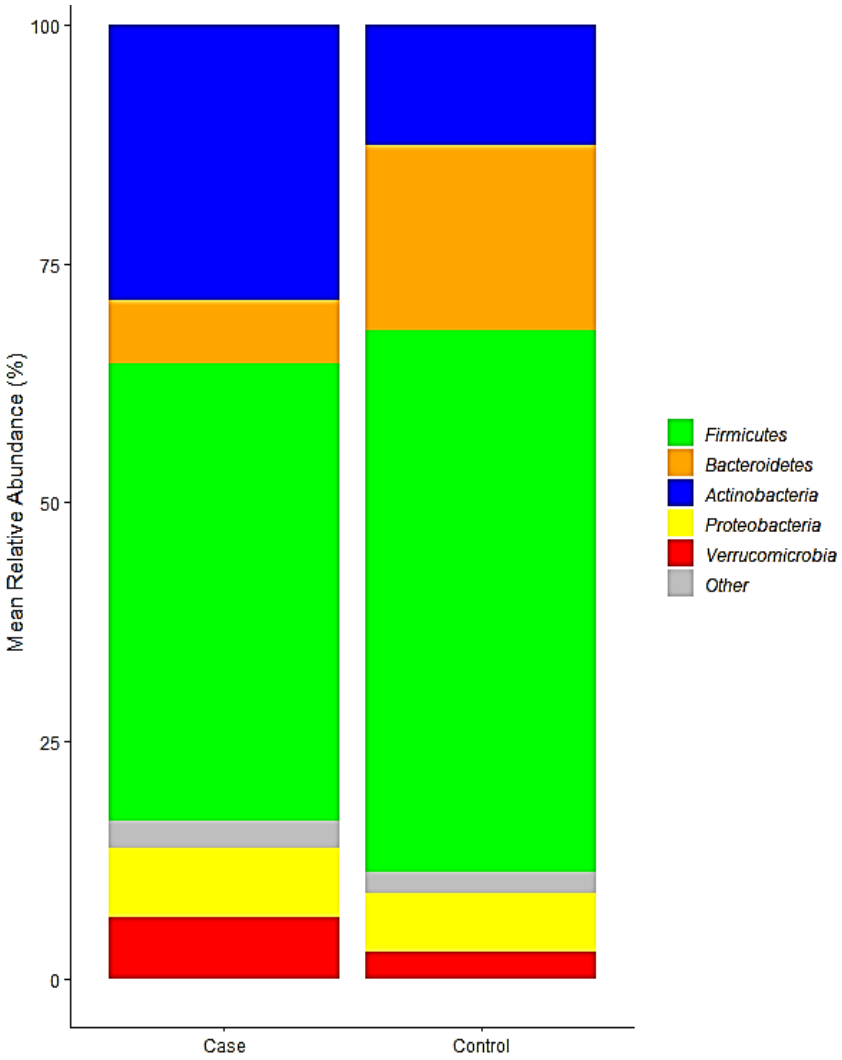


Fig. 3. Comparison of the relative abundance of major phyla between cases and controls

Table 3. Linear Discriminant Analysis (LDA) Coupled with Effect Size (LEfSe) on Plasma Concentration of E2.

OTU	Class	LDA	pValue	Bacterial Genus
Otu000003	up	4.47723	0.00699598	*Bifidobacterium*
Otu000030	up	3.62793	0.0109411	*Erysipelatoclostridium*
Otu000040	up	3.56318	0.00852749	*Eggerthella*
Otu000035	up	3.36341	0.016706	*Dorea*
Otu000036	up	3.02947	0.0333367	*Blautia*
Otu000220	up	2.30609	0.0429313	*Anaerobutyricum*
Otu000238	up	2.25746	0.0230033	*Collinsella*
Otu000316	up	2.08458	0.0165425	*Slackia*

Postmenopausal patients had an early menarche (13 years) and a late menopause (52.46 years) compared to controls in the same group. This observation has been made by several authors [30,31]. Indeed, early menarche and late menopause can influence the risk of breast cancer through long-term effects on sex hormone levels [32,33]. These factors cause prolonged exposure to endogenous estrogens throughout a woman's life. In a meta-analysis of 13 case-control studies in postmenopausal women, circulating estradiol levels were 6% lower in women who had their periods at 14 years or older compared to women who had their periods before 12 years [19].

Regarding parity and age at first full-term pregnancy, there was no significant difference between cases and controls. Several studies have shown that nulliparous women have a 20 to 40% higher risk of developing postmenopausal breast cancer than women who had their first childbirth before age 25 [34,35]. However, further studies should be conducted to deepen the potential links between these risk factors and breast cancer in Ivorian women.

In this study, no association could be established between BMI, family history of breast cancer, and the use of hormonal contraceptive methods in the different groups. This corroborates the work of Akoko et al. [36] on Tanzanian women [36]. However, more in-depth studies on these risk factors need to be conducted because some of these risk factors have been mentioned among African American women [37,38].

In our study, among premenopausal women, the average estradiol 2 level measured in patients was (50.58 pg/mL). Some authors obtained similar results (48 pg/mL) [39] in a similar population. Moreover, in the premenopausal controls of our study, we recorded an average level of 110.47 pg/mL, which was higher than in the patients. Sturgeon et al. [40] reported similar results in a case-control study among premenopausal women during the late follicular phase [40]. Indeed, interpreting hormone quantification results in premenopausal women can be complex due to intra-subject variations within the cycle, involving considerable variations during the follicular phase [39]. This differentiation during the follicular phase was not performed in our study.

Postmenopausal patients had higher plasma levels of estradiol (13.54 pg/mL) and progesterone (0.186 ng/mL) than the controls. These results are comparable to those of Zhang et al. [41], who showed that higher levels of estradiol 2 and progesterone were associated with an increased risk of ER+/PgR+ tumors [41]. This phenomenon was observed in a meta-analysis of 9 prospective studies on hormonal risk factors for breast cancer in postmenopausal women [42].

The involvement of the gut microbiota composition has already been demonstrated in several digestive cancers such as stomach cancer [43], liver cancer [44], and colon cancer [45,46]. In breast cancer, some studies have suggested that the microbiota composition could modulate the reabsorption of estrogens at the level of enterohepatic circulation [47,48]. In this regard, Goedert et al. [49] showed a difference in microbiota composition between breast cancer patients and healthy women [49], suggesting that the composition and stability of the gut microbiota are crucial for maintaining good biological activities in the body.

In this study, the alpha diversity analysis revealed a significant difference in the Sobs and Chao1 indices ($p < 0.05$). Women with breast cancer had lower alpha diversity than healthy women. This same observation was also made by several authors in studies on similar

populations in Ghana, the United States, and China [50–52]. Moreover, a study in China presented opposite results, indicating that postmenopausal cases had a higher Sobs diversity index than postmenopausal controls, and the Shannon index was higher in premenopausal cases. However, in this study, microbiota characterization was performed by shotgun metagenomics, and the presented results were not adjusted for the case-control groups [53]. The gut microbiota has been repeatedly implicated in estrogen regulation. For example, in postmenopausal women, previous studies suggested a negative correlation between gut microbiota alpha diversity and estrogen concentrations in stools, while a positive correlation was observed in urine [54].

The non-metric multidimensional scaling (NMDS) analysis using Bray-Curtis dissimilarity indices revealed a difference in the distribution distance of the microbiomes of the sick women compared to the microbiomes of the healthy women. Indeed, the gut microbiota samples from the women are visually distributed in space, representing two distinct groups. One group was predominantly formed by the patients' samples, and another group was predominantly formed by the controls' samples. The separation of the samples into two groups could be explained by the observed and estimated richness differences, represented respectively by the Sobs and Chao1 indices. In He et al. [55] study, a similar clustering of case and control samples in premenopausal women was shown [55]. However, the dissimilarity data representation was performed by redundancy analysis (RDA). Similarly, Byrd et al. [51] showed a significant difference in the distribution of samples from sick women and healthy women, using principal coordinates analysis with the Bray-Curtis matrix [51].

Several hypotheses suggest that changes in the composition (dysbiosis) and functions of several bacterial genera in the intestine can contribute to the development and progression of breast cancer through various pathways [56].

This study revealed a difference in composition between the microbiomes of the two subject groups (cases-controls). Specifically, there was a reduction in the relative abundance of the phyla *Firmicutes* and *Bacteroidetes*, and an increase in the phyla *Actinobacteria* and *Verrucomicrobia* in breast cancer patients. Two other studies had already observed a difference in composition within the gut microbiota of patients. However, comparing our Ivorian study to these, the relative abundance of major phyla differs. Indeed, in Ma et al. [52] study in China, the relative abundances of *Firmicutes* and *Proteobacteria* were reduced, while that of *Bacteroides* increased [52]. Also, in Bobin-Dubigeon et al. [57] study in France, the relative abundance of *Bacteroidetes* was reduced, while that of *Firmicutes* increased [57]. Moreover, the Molecular Analysis of Variance (AMOVA) revealed a significant difference between the microbiota of cases and controls ($p = 0.001$). These differences between the study results could be explained by factors such as diet and geographical distance between the studied populations [58,59], implying that data from multiple continents and various populations are necessary to better understand the role of gut microbiota in breast cancer.

The Linear Discriminant Analysis Effect Size (LEfSe) revealed that serum E2 level was significantly positively correlated with the abundances of the bacterial genera *Bifidobacterium*, *Erysipelatoclostridium*, *Eggerthella*, *Dorea*, *Blautia*, *Slackia*, *Anaerobutyricum*, and *Collinsella*, with LDA discriminant values greater than 2 and p-values < 0.05. The bacterial genera *Blautia*, *Dorea*, and *Bifidobacterium* have been associated with elevated serum E2 levels [60–62]. However, for the other genera, this is the first report of this association. Moreover, *Bifidobacterium*, *Dorea*, and *Blautia* possess the *GUS* gene and are capable of synthesizing the beta-glucuronidase enzyme, responsible for deconjugating conjugated estrogens in the gastrointestinal tract [63]. These deconjugated estrogens can be reabsorbed into the bloodstream and maintain their effects on the body.

5. CONCLUSION

The characterization of the intestinal microbiota in this study highlighted that the patients' microbiota is less diverse, with lower abundance and representation of species. The LEfSe analysis identified eight bacterial genera potentially associated with increased plasma E2 concentration. Some of these genera, such as *Bifidobacterium*, *Dorea*, and *Blautia*, possess the GUS gene, involved in the deconjugation of estrogens in the gastrointestinal tract. These results show an association between the gut microbiota and estrogen levels in women with

breast cancer, opening up research perspectives on new strategies for breast cancer prevention, diagnosis, and treatment, taking into account female hormone levels and modulation of the gut microbiota.

6. LIMITATIONS OF THE STUDY

Although we used rigorous sampling methods to select the sample, we are aware that the small sample size and the non-distribution of non-menopausal women by early and late follicular phase may limit the statistical power of the results.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

CONSENT AND ETHICAL APPROVAL

Before proceeding with the interview and sampling, each participant gave informed consent. The study protocol was approved by the National Ethics Committee for Life Sciences and Health (Côte d'Ivoire) under number IRB000111917.

DATA AVAILABILITY

Data are available at Institut Pasteur de Côte d'Ivoire and with authors. Authors are ready to share on demand at any moment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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