Section A-Research paper

Molecular Study of *Lactobacillus* species in Patients with Breast Cancer: A Case-Control Study

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Abstract:

Background: The dysbiosis of the gut microbiota is a risk factor for breast cancer and could explain the difference in responses to therapy. **Aim:** To investigate the composition of different *Lactobacillus* species in patients with breast cancer compared to female control and in relation to different stages of breast cancer.

Methodology: The study included 100 female patients with breast cancer and cross-matched 100 healthy controls. Stool samples were freshly collected for a multiplex polymerase chain reaction (PCR). identification of different *Lactobacillus* species

Results**:** The patients were diagnosed mainly in stage II and 1V (34%, 37% respectively). In the patient's group, there was a significant decrease in most of *Lactobacillus* species compared to the control group with a detection frequency of *L. acidophilus* (49%, *P*=0.03), *L. delbrueckii* (44%, *P*=0.0001*), L. gasseri* (49%, *P*=0.032), *L. reuteri* (35%, *P*=0.001), *L. plantarum* (32%, *P*=0.0001) and *L. rhamnosus (*33%, *P*=0.0001).There was insignificant difference in the frequency of detection of *Lactobacillus* species between patients with different stages of breast cancer. **Conclusions:** The present study highlights the decreased prevalence of *Lactobacillus* species among patients with breast cancer compared to healthy control.

Keywords:

Breast cancer, *Lactobacillus*, gastrointestinal, microbiota, multiplex polymerase chain reaction

Statements and Declarations

Competing interests

There are no competing interests for any of the authors.

Introduction:

Breast cancer is a common malignancy that affects females worldwide. Based on the existence of estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2) and Ki67 protein, cancer breast is classified into Luminal A, Luminal B, HER2-enriched, and triple-negative subtypes [1]. There are various risk factors for the development of breast cancer including age, genetic factors, hormonal replacement therapy, lifestyle, and eating habits [2].

The composition of the microbiota in the gastrointestinal tract (GIT) may have an effect on the development of the different types of cancer. Under normal conditions, the microbiota interacts with the human host and affects different signaling pathways such as E-Cadherin/β-catenin [3] that lead to DNA double-strand breaks [4], enhance apoptosis, inhibit cell differentiation [5], and trigger an immune response and inflammatory pathway through interaction with the toll-like receptors leading to the maintenance of the hemostasis of the body [6]. The interplay between the human microbiome and cancer is termed "oncobiome" [7]. Based on recent studies, the dysbiosis of the gut microbiota is a risk factor for breast cancer and could explain the difference in responses to therapy [8].

The link between gut microbiota and breast cancer may be attributed to either disruption of the production of certain beneficial metabolites or affection of the estrogen metabolism [9]. In the experimental study, there was an alteration in the *Lachnospiraceae* and *Lactobacillus* in xenograft nude mice after challenging breast cancer cells [10].

Among the normal flora of the GIT, *lactobacillus* species is a common inhabitant. The most clinically important *Lactobacillus* species are *L. crispatus, L. gasseri, L. casei, L. acidophilus, L. delbrueckii, L. reuteri, L. plantarum, L. rhamnosus, L, jensenii*and *L. iners* [11-13]. Various species of *lactobacilli* such as *L. crispatus* and *L. acidophilus* have antiproliferative activity against breast cancer cells. Moreover, *lactobacilli* have inhibitory effects on the different cancer-testis antigens. The expression of the cancer-testis antigens is regulated by epigenetic mechanism. Thus, *lactobacilli* can lead to the down regulation of the epigenetic of this expression. There was a link between the expression of the cancer-testis antigens and the poor prognosis and high-grade tumors. Therefore, the use of *lactobacilli* in the downregulation may open up a new era of clinical research applications [14].

The detection of *Lactobacillus* species depends upon conventional culture techniques and biochemical identification. However, these methods are laborious and have limited accuracy [15, 16]. The development of molecular diagnostic laboratory methods has improved the identification of various organisms [17]. The successful molecular methods for *Lactobacilli* identification are based mainly on the use of 16S rDNA or 23S rDNA-targeted primers by PCR [18, 19]. The use of multiplex PCR has a good discrimination capacity for the detection of different *Lactobacillus* species by the use of the primers depending on the species-specific sequences of 16S rRNA or 16S–23S rRNA intergenic spacer region with its flanking 16S rRNA or 23S rRNA, respectively [20].

There are in vitro studies about the role of some *lactobacillus* species such as *L. crispatus* and *L. acidophilus* in breast cancer with a

lack of studies about the *Lactobacillus* species composition in the gut in female patients with breast cancer and its difference from normal females

The aim of the present study was to investigate the composition of different *Lactobacillus* species in patients with breast cancer compared to female control and to study the composition of the *Lactobacillus* species in relation to the different stages of breast cancer.

Material and method

This study was a retrograde case- control study that included 100 female patients with breast cancer from January 2021 till March 2022 recruited from Mansoura University Hospital, Egypt. In addition, cross matched 100 healthy controls were recruited during the same period. The patients had inclusion criteria which were female patients above 18 years with a diagnosis of breast cancer by complete clinical examination, radiological investigations including ultrasound, mammogram, and bone scan and confirmed by pathological examination. Cases were staged in accordance with American Joint Committee on Cancer (AJCC) Tumor, Node, Metastasis (TNM) staging system for cancer breast, 8th edition (21). Patients were excluded if have a history of antibiotics intake in the previous month and patients with other malignancies were also excluded. The study was approved by the Mansoura Ethical Committee, Mansoura Faculty of Medicine, Egypt (**(R.21.07.1371)**), and written approval was obtained from each female.

Stool Specimens Collection

Stool specimens were freshly collected in clean leak-proof containers and then transported to the laboratory. The stool specimens were stored frozen at -80C until DNA extraction.

DNA extraction from stool specimens

The bacterial DNA was extracted from the obtained stool specimens by the aid of a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) in accordance to the manufacturer's instructions with prior handlings as previously discussed. Initially, one gram of stool was added to 100 ml of iced water with homogenization with glass rod and centrifuged at 1000 × g for 1 minute to eliminate the large fragments. After that, 300 milligrams of the supernatants were transported to another tube and 200 microliters of TE buffer (Tris-HCl [10mM], EDTA [10 mM], lysozyme [20mg/mL]; pH 8.0) was added. The tubes were vortexed for 1 minute and incubated at 37° C for 1 hour. Then the manufacturer protocol was completed with an increase of the lysis temperature to 95°C. The extracted DNA was stored frozen at −20 °C until further amplification procedures [22].

Multiplex PCR for *Lactobacillus* species

The primers utilized for multiplex PCR of *lactobacillus* species were enumerated in table 1. The reaction mixture was 25 microliters where Master Mix was supplemented from Qiagen (Qiagen, Hilden, Germany) with adding of 50 picomoles for each primer and 5 microliters of extracted DNA. PCR amplification was done by thermal cycler and the amplification process sequences as follows: initial heating at 94 °C for 2 minutes, followed by 35 cycles consisting of denaturation at 94 °C for 20 seconds, annealing at 51 °C for 40 seconds, extension at 68 °C for 30 seconds, and a 7 minutes final extension at 68 °C. Gel electrophoresis was done for the amplified products with the use of 1.5% agarose gel and stained with ethidium bromide [23, 24].

Table (1): The primers used to detect *lactobacillus* species.

Primer	Target bacteria	Primer sequence (5′→3′)	Product (bp)*	Target site **
IDL03R	*All Lactobacillus*	CAA CGC GAA GAA CCT TAC CAG		1178-1198
IDL04F	*All Lactobacillus*	CCA ACA TCT CAA CGA CAC GAG C		1499–1522
IDL11F	*L.casei*-group[d]	TGGTCGGCAGAGTAACTGTTGTCG	727	472–495
IDL22R	*L. acidophilus*	AACTATCGCTTACGCTACCACTTTGC	606	2079–2104
IDL31F	*L. delbrueckii*	CTGTGCTACACCTAGAGATAGGTGG	184	1015–1039
IDL42R	*L. gasseri*	ATTTCAAGTTGAGTCTCTCTCTC	272	1748–1770
IDL52F	*L. reuteri*	ACCTGATTGACGATGGATCACCAGT	1105	94–118
IDL62R	*L. plantarum*	CTAGTGGTAACAGTTGATTAAAACTGC	428	1900–1926
IDL73R	*L. rhamnosus*	GCCAACAAGCTATGTGTTCGCTTGC	448	1922–1946

*** Product** means the length of each PCR product derived from primer pair composed of species-specific primer and *Lactobacillus* conserved primer (IDL03R or IDL04F).

** **Target site** indicates the start and end point of the complimentary sequences annealing the forward and reverse primer, respectively.

Statistical analysis

Data were statistically analyzed using SPSS22 (SPSS Inc., Chicago, IL, USA). The numerical data was formulated as mean and standard deviation (SD) and the qualitative data was formulated as numbers and percentages. The comparison between numerical data was done by T-test and between qualitative data by chi-square. *P* value was interpreted as significant if it was < 0.05.

Results

Demographic and clinical characteristics of the studied patients

The study included 100 patients with breast cancer with mean age ± SD is 43.64± 15.73 years and 100 healthy controls with mean age ± SD is 40.78 ± 15.6 years with insignificant difference between both groups (P=0.2), data not shown. The clinical data of the patients revealed that the breast cancer stages were mainly stage IV (37%) and stage II (34%) followed by stage III (21%) and stage I (8%). The most common presenting symptom was breast mass (78%), while pain, axillary mass, and nipple discharge were less recorded (12%, 8%, and 5%, respectively), table 2.

Table (2): Clinical and demographic data of the patients with breast cancer (n=100)

Clinical and demographic data	**No. of Patients (%)**
Age (years) Mean ±SD	 43.64 ± 15.73
Tumor grade Stage I (No. - %) Stage II (No. - %) Stage III (No. - %) Stage IV (No. - %)	 8 8% 34 34% 21 21% 37 37%
Pain (No. - %)	12 12%
Breast mass (No. - %)	78 78%
Discharge from the nipple (No. - %)	5 5%
Axillary mass (No. - %)	8 8%

The *Lactobacillus s*pecies detected in the stool samples

The commonest detected *Lactobacillus* species by multiplex PCR in the control subjects were *L. delbrueckii (74%), L. rhamnosus (64%)* and*L. gasseri (63%)*. In the patients group, there was significant decrease in the most *Lactobacillus* species with detection frequency of *L. acidophilus* (49%, OR 1.78- 95%CI 1.01-3.14-*P*=0.03), *L. delbrueckii* (44%, OR 0.276- 95%CI 0.15-0.5-*P*=0.0001*),L. gasseri* (49%, OR 0.564- 95%CI 0.32-0.99-*P*=0.032), *L. reuteri* (35%, OR 0.41- 95%CI 0.32-0.72-*P*=0.001), *L. plantarum*(32%, OR 0.29- 95%CI 0.161-0.52-*P*=0.0001)and *L. rhamnosus (*33%, OR 0.28- 95%CI 0.155-0.49-*P*=0.0001), table 3.

Table (3): The *Lactobacillus s*pecies detected by multiplex PCR in patients with breast cancer compared to the control subjects

Lactobacillus species	Patients subjects (n=100) No. %	Control subjects (n=100) No. %	Odds Ratio (OR)	95% Confidence Interval (CI)	*P* value
L. casei-group[d]	50 50%	61 61%	0.63	0.365-1.12	0.08
L. acidophilus	49 49%	35 35%	1.78	1.01-3.14	0.03
L. delbrueckii	44 44%	74 74%	0.276	0.15-0.5	0.0001
L. gasseri	49 49%	63 63%	0.564	0.32-0.99	0.032

L. reuteri	35	35%	57	57%	0.41	0.32-0.72	0.001
L. plantarum	32	32%	62	62%	0.29	0.161-0.52	0.0001
L. rhamnosus	33	33%	64	64%	0.28	0.155-0.49	0.0001

Lactobacillus species detected in relation to the different stages of the breast cancer

There was insignificant difference in the frequency of the detection of *Lactobacillus* species between patients with different stages of breast cancer, *L. casei*-group[d] (*P*=0.341), *L. acidophilus* (*P*=0.41), *L. delbrueckii* (*P*=0.461), *L. gasseri* (*P*=0.341), *L. reuteri* (*P*=0.261), *L. plantarum*(*P*=0.615) and *L. rhamnosus* (*P*=0.201), table 4.

Table (4): The *Lactobacillus* species detected by multiplex PCR in relation to different stages of the breast cancer

Lactobacillus species	Stage I (n=8) No. %	Stage II (n=34) No. %	Stage III (n=21) No. %	Stage IV (n=37) No. %	*P* value
L. casei-group[d]	6 75%	14 41.2%	10 47.6%	20 54.1%	0.341
L. acidophilus	2 16%	19 55.9%	10 47.6%	18 48.6%	0.471
L. delbrueckii	2 16%	18 52.9%	8 38.1%	16 43.2%	0.461

L. gasseri	2 16%	16 47.1%	13 61.9%	18 54.1%	0.348
L. reuteri	3 24%	9 26.5%	11 52.4%	12 32.4%	0.261
L. plantarum	2 16%	9 26.5%	9 42.9%	12 32.4%	0.615
L. rhamnosus	2 16%	9 26.5%	11 52.4%	11 29.7%	0.201

Discussion

Lactobacilli are part of the normal human microbiota that colonizes the mouth, GIT, and female genitourinary tract [25]. Many species of *Lactobacilli* are considered probiotic bacteria which have a protective role and even a therapeutic approach to many types of cancer. The anti-cancer role of probiotics includes the inhibition of the growth of pathogenic bacteria which is involved in mutagens production, modification in carcinogen metabolism, and DNA protection from oxide injury as well as regulation of the immune system [26]. Moreover, it is implicated in the change of different gene expressions leading to death and apoptosis[27], invasion and metastasis [28], cancer stem cell preservation [29] as well as cell cycle control [30]. Further studies have demonstrated their regulatory effects on the cancer-related signaling pathways in a cell type-specific method [31-33].

The patients with breast cancer in the present study were diagnosed mainly in stages 1V and II (37%, and 34% respectively). It is previously reported by *Bray et al.* that the incidence of breast cancer is more elevated in high-income countries than in low and middle-income countries [34]. However, in high-income countries, the diagnosis is reported to be mainly in stages I and II in around 70% of the patients

compared to 50% in low and middle-income countries which is close to our findings (35). This delay in the diagnosis of breast cancer leads to poor prognosis with higher mortality rates [36, 37].

In this study, the mean age ± SD of the patients with breast cancer was43.64± 15.73 years. In agreement, previous study from Mansoura cancer registry reported that the median age at diagnosis for females with different cancers was 52 years with increased incidence in the different types of cancer diagnosis above 45 years and decreasing incidence after the age group of 65 years [38].

Among breast cancer patients in the current study, the common presenting symptom was breast mass (78%), while pain, axillary mass and nipple discharge were less recorded (12%, 8% and 5%, respectively). Similarly, a study conducted by *Koo et al*. reported the presence of breast lump in about 83% of patients. The other reported symptoms were nipple abnormalities (7%), breast pain (6%), and axillary lump (1.2%) [39].

There are more than fifty *Lactobacillus* species that were detected in stool samples [40], suggesting that a persistent population of *lactobacilli* could inhabit the GIT of the normal population.

In the present study, the commonest detected *Lactobacillus* species by multiplex PCR in the control subjects were *L. delbrueckii (74%), L. rhamnosus (64%)* and *L. gasseri (63%)*. It has been recorded that *L.gasseri* [41] and *L. rhamnosus* [25] are normal inhabitants of GIT while *L. delbrueckii* may be introduced through food as it is transient *lactobacillus* species. A previous study indicated the survival of the food *lactobacilli* during gastrointestinal passage and it is detected by various laboratory methods such as the culture of stool samples [42]. The probiotic activity of *Lactobacillus* species is associated with its ability to survive in low PH values and its ability to adhere to the mucus layer by pill and cell wall protein [43].

In the patient's group, there was a significant decrease in most *Lactobacillus* species with a detection frequency of *L. acidophilus* (49%, *P*=0.03), *L. delbrueckii* (44%, *P*=0.0001*), L. gasseri* (49%, *P*=0.032), *L. reuteri* (35%, *P*=0.001), *L. plantarum* (32%, *P*=0.0001) and *L. rhamnosus (*33%, *P*=0.0001). The composition of *lactobacilli* in GIT is dynamic and it is affected by various factors whether internal or external such as age, race, diet, infections and drugs intake [42].

Furthermore, *L. acidophilus* has shown to increase the survival time in experimental animal study if administered before breast cancer tumor transplantation and this finding was attributed by the stimulation of the immune responses with the increase of pro-inflammatory cytokines such as IFN-γ and suppression of anti-inflammatory cytokines such as IL-4 and IL-10 [44]. Another study, confirmed the anticancer activity of *L. acidophilus* in mice bearing breast tumors [45].

Another experimental study confirmed that the use of *L. casei* decreased the growth rate of transplanted breast adenocarcinoma to mice and prolonged the survival time due to the stimulation of T helper 1 cell cytokine production and enhancement of the cytotoxicity of natural killer cells [46]. The use of selenium nanoparticles enriched with *L. plantarum* in mice model revealed an increase in IFN-γ and IL-2 levels and improved natural killer cell activity [47]. In a human study, *L. gasseri* has an immunomodulation effect on both innate and adaptive immune responses [41]. Though all these findings were experimental findings, the findings in the present study with significant reduction of the detection frequency of probiotics species of *Lactobacilli* in patients with cancer breast compared to healthy control may indicate their potential protective role toward breast cancer.

Currently, there was an insignificant difference in the frequency of the detection of *Lactobacillus* species between patients with different

stages of cancer breast. This finding may be due to the beneficial effect of the quantification of different species of *lactobacilli* on the breast cancer stages rather than simply its presence. There is a clinical trial (NCT03358511)held by *Mendoza*, investigating the role of therapeutic administration of 15 billion colony-forming units of 13 species of beneficial bacteria to patients with breast cancer, who will be given 2-4 weeks of probiotics before surgery in operable stage I-III breast adenocarcinoma tumors ≥1.0 cm with follow up after surgery to determine the effect of this therapy on the survival [48].

Conclusion

The current study highlights the prevalence of *Lactobacillus* species among patients with breast cancer compared to healthy control. There was significant reduction of the prevalence of *L. acidophilus*, *L. delbrueckii*, *L. gasseri*, *L. reuteri*, *L. plantarum* and *L. rhamnosus* species among patients with breast cancer compared to healthy control. This finding has to be validated in large-scale studies.

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Author contributions

Noha Mostafa Mahmoud had shared in the laboratory study, the draft preparation and data analysis of the study. Maha Saif, shared in the clinical data collection and data analysis of the study. Tamer Akl shared in the clinical data collection and draft preparation of the article Maysaa El Sayed Zaki designed the study, shared in the laboratory study and draft preparation of the article. Mohamed Mostafa Mahmoud shared the clinical data collection and draft preparation of the article. Ehab M.

Fahmy shared in the laboratory study and draft preparation of the article. All authors read and approved the final manuscript.

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Availability of data and materials

The data of the present study is available at https://data.mendeley.com/datasets/3yz3jnbjmb

Ethics approval and consent to participate

The study was approved by Mansoura Faculty of Medicine Ethical Committee (R.21.07.1371) and informed written consent was obtained from each participant. The study was performed according to the declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

There are no competing interests for any of the authors.

Author Contribution

Maysaa El Sayed Zaki shared in the laboratory study, the draft preparation of the article and data analysis of the study. in the laboratory study draft preparation of the article. designed the study, collecting clinical data of the studied children and writing the article. collected clinical data from the studied children and wrote the article. collected clinical data of the studied children and wrote the article. ar shared the laboratory study and draft preparation of the article. All authors read and approved the final manuscript.

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