REVIEW

Exploring the Potential of Breast Microbiota as Biomarker for Breast Cancer and Therapeutic Response

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Breast cancer tissue contains its own unique microbiota. Emerging preclinical data indicates that breast microbiota dysbiosis contributes to breast cancer initiation and progression. Furthermore, the breast microbiota may be a promising biomarker for treatment selection and prognosis. Differences in breast microbiota composition have been found between breast cancer subtypes and disease severities that may contribute to immunosuppression, enabling tumor cells to evade immune destruction. Interactions between breast microbiota, gut microbiota, and immune system are proposed, all forming potential targets to increase therapeutic efficacy. In addition, because the gut microbiota affects the host immune system and systemic availability of estrogen and bile acids known to influence tumor biology, gut microbiota modulation could be used to manipulate breast microbiota composition. Identifying breast and gut microbial compositions that respond positively to certain anticancer therapeutics could significantly reduce cancer burden. Additional research is needed to unravel the complexity of breast microbiota functioning and its interactions with the gut and the immune system. In this review, developments in the understanding of breast microbiota and its interaction with the immune system and the gut microbiota are discussed. Furthermore, the biomarker potential of breast microbiota is evaluated in conjunction with possible strategies to target microbiota in order to improve breast cancer treatment. *(Am J Pathol 2021, 191: 968−982; https://doi.org/10.1016/j.ajpath.2021.02.020)*

Breast cancer is a heterogeneous malignancy, accounting for 30% of all female cancers.[1,2] Molecular subtypes of breast cancer have been characterized, including luminal A, luminal B, *HER2*-enriched, and triple negative breast cancer (TNBC).[3] Luminal A subtype is enriched with the estrogen receptor (ER), and has the best clinical prognosis among all intrinsic subtypes, which is mostly due to the favorable response to endocrine therapy. Compared with luminal A cancers, luminal B cancers show lower expression of *ER*-related genes, but have higher expression of proliferation-related genes and variable expression of *HER2*-related genes.[4] The HER2 subtype comprises approximately 15% of all invasive breast cancers and are more aggressive in nature than luminal-like cancers.[4] This subtype is associated with the absence of ER and progesterone receptor (PR)

expression, and overexpression of *HER2/HER2* signaling-associated genes and genes located in *HER2* amplicon on chromosome *17q12*. TNBC lacks expression of ER, PR, and HER2.

Segregating breast tumors into subtypes is valuable for predicting prognosis and guiding clinical decision-making, in which an array of anticancer therapeutics, including chemotherapy, antiestrogen therapy, and anti−HER2-targeted agents can be selected.[5] Nevertheless, limitations in traditional subtyping exist, since it does not take interactions with the tumor microenvironment (TME) into account. Healthy tissue stroma normally acts as a physiological barrier against tumor formation.[6] During development of neoplastic

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cells, the adjacent tissue microenvironment is transformed by autocrine and paracrine mechanisms of cancer cells to maintain optimal conditions for tumor survival and progression. These alterations result in tumor-associated stroma, known as the TME. The TME consists of a heterogeneous collection of fibroblasts, myofibroblasts, neuroendocrine cells, adipocytes, extracellular matrix, blood and lymphatic vascular networks, and is enriched with many immune and inflammatory cells.[6]

Recent findings demonstrate the existence of microbiota, both in healthy stroma and in the TME of extraintestinal organs, including the lung, pancreas, and breast.[7–9] It is suggested that organ-specific microbiota play a role in tumor development and therapeutic resistance. In this review, microbial compositions in tumorous and healthy breast tissue are discussed. Moreover, microbiota interactions with the immune system, the gut microbiota and anticancer therapeutics are illustrated. Lastly, the biomarker potential of breast microbiota is evaluated in conjunction with possible strategies to target microbiota in order to improve breast cancer treatment.

Breast Microbiota in Health and Disease

Many extraintestinal tissues were traditionally considered sterile until the advancement of culture-independent DNA sequencing techniques, using next-generation sequencing technology.[10] The discovered link between colon cancer and certain gut bacteria led to novel investigations of organ-specific microbiota in the development of cancers in multiple tissues.[11] The existence of microbes in breast tissue was firstly demonstrated in 2014 by Xuan et al.[8] There is increasing evidence of the existence of a unique microbiota in breast tissue that is distinct from the overlying breast skin, and is unrelated to mastitis.[12] The breast microbiota is dominated by the phyla Proteobacteria and Firmicutes, which likely can be attributed to the fatty acid–rich environment in the breast. The discovery of microbiota in breast tissue has brought the attention to its potential role in the pathophysiological process of breast carcinogenesis.

Difference between Healthy and Tumorous Breast Tissue

Several studies have examined normal breast tissue adjacent to the breast tumor, approximately 5 cm away from the tumor margin. One study found a higher bacterial load and bacterial richness in breast tumor tissue, compared with adjacent normal breast tissue.[13] At the phylum level, highest presence of Proteobacteria was found in breast cancer tissue, compared with highest presence of Actinobacteria in adjacent normal breast tissue.[14] The families *Pseudomonadaceae, Sphingomonadaceae, Alcaligenaceae, Ruminococcaceae,* and *Clostridia* seemed to be decreased in adjacent breast tissue compared with breast cancer tissue.[8,15,16] At the class level, the absolute abundance in breast tumor tissue was highest for the classes Clostridia and Bacteroidia,[15] whereas at the genera level, *Ralstonia,*

followed by *Methylobacterium* and *Sphingomonas,* had the highest abundance.[15,17] In normal adjacent breast tissue, the family *Enterobacteriaceae*, of which *Escherichia coli* is a member, was increased compared with healthy controls.[18] *E. coli* isolates, cultured from normal adjacent tissue of breast cancer patients, induced DNA double-stranded breaks *in vitro* in HeLa cells.[18] Similarly, pks-positive *E. coli* bacteria induced DNA damage in human intestinal organoids by the production of the genotoxin colibactin.[19] This finding provides an explanation for a possible pathway by which bacteria, present in the breast, may contribute to breast carcinogenesis.

The microbiota composition of breast tissue adjacent to the tumor has also been compared with healthy breast tissue of women without breast cancer. Compared with healthy breast tissue, adjacent normal breast tissue contained a higher relative abundance of bacteria belonging to the phylum Bacteroidetes, the family *Comamondaceae*, and the genera *Bacillus* and *Staphylococcus*,[18] which could also suggest a gradual change in microbiota from healthy to cancerous states. However, not all studies identified differences between breast tumor tissue microbiota and adjacent normal breast tissue microbiota.[16–18] Similar microbiota in paired normal tissue and tumor tissue could suggest a predisposition of the entire breast tissue to carcinogenesis and thereby potentially predict breast cancer risk.

Microbiota Composition in Malignant versus Benign Breast Diseases

The breast microbiota composition of malignant disease, mainly invasive ductal carcinoma, can clearly be distinguished from that of benign breast disease, including fibroadenoma, intraductal papilloma, and atypical hyperplasia.[20] Phylum Proteobacteria, families *Micrococcaceae, Caulobacteraceae, Rhodobacteraceae, Nocordioidaceae,* and *Methylobacteriaceae,* and genus *Propionicimonas* were present in significantly higher levels in malignant breast cancer than in benign breast disease.[20] In another study investigating breast tissue of women with ER[+] breast cancer, increased abundance of specific genera, including *Fusobacterium, Atopobium, Hydrogenophaga, Gluconacetobacter,* and *Lactobaccilus*, was demonstrated in invasive breast cancer compared with benign disease.[12]

Different stages of breast cancer also coincide with a specific microbial profile. Higher stages were associated with reduced bacterial load.[8] In Stage I breast cancer, Proteobacteria, *Ruminococcaceae,* and *Hyphomicrobium* were most abundant, whereas Stage II breast cancer showed highest abundance in Euryarchaeota, Firmicutes, Spirochaetes, and the genus *Sporosarcina*.[15] Stage III and Stage IV breast cancer exhibited elevation in Thermi, Gemmatimonadetes, and Tenericutes, and higher abundance of *Bosea*. The genus *Agrococcus* was progressively enriched with increased malignancy.[20]

Table 1   Overview of Studies Analyzing Breast Microbiota Composition

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue, and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades, and stages
Xuan et al[8]	Analysis 1: 20 breast cancer patients; breast cancer tissue and adjacent breast tissue obtained from same patient Analysis 2: 39 breast cancer tissue samples, subdivided in breast cancer stages: Stage I: $n = 18$ Stage II: $n = 9$ Stage III: $n = 12$	FFPE and fresh frozen	ER$^+$	Comparing adjacent breast tissue with breast cancer tissue, higher absolute abundance of *Sphingomonas yanoikuyae* was found in adjacent tissue. Comparing breast cancer tissue with adjacent breast tissue, higher relative abundance of *Methylobacterium radiotolerans* was found in breast cancer tissue.	Decreased bacterial load was found in breast cancer tissue with more severe breast cancer stages.
Urbaniak et al[18]	58 adjacent breast tissue samples: 13 benign breast tumors; 45 malignant breast cancers 23 healthy breast tissue samples	Fresh tissue	*	Comparing healthy breast tissue with adjacent breast tissue, increased relative abundance of *Prevotella, Lactococcus, Streptococcus, Corynebacterium,* and *Micrococcus* was found in healthy breast tissue. Comparing adjacent breast tissue with healthy breast tissue, increased relative abundance of genera *Bacillus* and *Staphylococcus*, families *Enterobacteriaceae* and *Comamondaceae*, and phylum Bacteroidetes was found in adjacent breast tissue. Comparing adjacent breast tissue from patients with benign tumors with adjacent breast tissue from patients with malignant tumors and healthy tissue, microbial profiles of benign tumors were more similar to microbial profiles of malignant tumors than to microbial profiles of healthy breast tissue.	Comparing microbiota composition of adjacent breast tissue of women with different cancer stages, no differences were found.
Banerjee et al[21]	100 triple-negative breast cancer tissue samples 17 matched (adjacent breast tissue) and 20 nonmatched controls	FFPE	TNBC	Comparing TNBC tissue with healthy breast tissue, higher percentage of probes of the genera *Prevotella, Brevundimonas,*	In TNBC, highest prevalence of probes detecting *Arcanobacterium* (in 75% of samples) was found, followed by *Brevundimonas,*

(*table continues*)

Table 1 *(continued)*

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue, and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades, and stages
	(healthy breast tissue)			*Arcanobacterium, Escherichia, Sphingobacterium, Actinomyces,* and *Rothia* was found in TNBC tissue. Comparing TNBC tissue with healthy breast tissue, higher percentage of probes of the viruses MMTV, hepatitis C1, EBV1, BPSV, HCMV, KSHV, PCPV, HPV2, HTLV-2, HPV6B, MCPV, HTLV1, HPV18, hepatitis B, SV40, HPV16, HHV1, okra mosaic virus, FSV, hepatitis GB, viroids, and orf virus was found in TNBC tissue. Comparing TNBC tissue with healthy breast tissue, higher percentage of probes of the fungi *Pleistophora* and *Paecilomyces* was found in TNBC tissue.	*Sphingobacteria, Providencia, Prevotella, Brucela, Escherichia, Actinomyces, Mobiluncus, Propiniobacteria, Geobacillus, Rothia, Peptinophilus,* and *Capnocytophaga.* The highest hybridization signal in TNBC was found for the bacterial probe of *Prevotella,* for the viral probe of Herpesvirus, for the fungal probe of *Piedra,* and for the parasitic probe of *Trichuris.*
Hieken et al[12]	28 adjacent breast tissue samples: 13 benign breast tumors; 15 malignant breast cancer (67% Stage I, 33% Stage II, 13% positive lymph node)	Fresh frozen	All ER/PR$^+$, 29% HER2$^+$	Comparing malignant breast cancer tissue with benign breast tumors, higher relative abundance of *Fusobacterium, Atopobium, Hydrogenophaga, Gluconacetobacter,* and *Lactobaccilus* was found in malignant breast tissue.	*
Wang et al[16]	57 breast cancer tissue samples 21 healthy breast tissue samples Breast cancer stages: Stage I: $n = 22$ Stage II: $n = 30$ Stage III: $n = 5$	Fresh frozen	ER/PR$^+$ ($n = 50$) HER2$^+$ ($n = 9$)	Comparing breast cancer tissue with healthy breast tissue, decreased relative abundance of *Methylobacterium* and increased relative abundance of Alcaligenaceae were found in breast cancer tissue. Comparing breast cancer tissue with adjacent breast tissue, no significant differences were found.	Comparing HR$^+$ breast cancer tissue with HR$^-$ breast cancer tissue, increased Shannon diversity and decreased relative abundance of *Methylobacterium* was found in HR$^+$ breast cancer.
Thompson et al[14]	The Cancer Genome Atlas (TCGA) 668 breast cancer tissue	Fresh frozen	ER$^+$, HER2$^+$, TNBC (distribution is not given)	In breast cancer tissue, phylum Proteobacteria was most abundant,	*

(table continues)

Table 1 (*continued*)

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue, and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades, and stages
	samples 72 adjacent breast tissue samples			followed by Actinobacteria and Firmicutes. In adjacent breast tissue, highest presence of phylum Actinobacteria was observed.	
Banerjee et al[22]	148 breast cancer tissue samples 20 healthy breast tissue samples	FFPE	ER/PR$^+$ ($n = 50$) HER2$^+$ ($n = 34$) ER/PR$^+$, HER2$^+$ ($n = 24$) TNBC ($n = 40$)	For all breast cancer subtypes, significant hybridization signals were found for *Actinomyces, Bartonella, Brevundimonas, Coxiella, Mobiluncus, Mycobacterium, Rickettsia,* and *Sphingomonas.* No bacterial signatures in healthy breast tissue were found of the following bacteria that were detected in breast cancer tissue: *Actinomyces, Aerococcus, Arcanobacterium, Bifigdobacterium, Bordetella, Cardiobacterium, Corynebacterium, Eikenella, Fusobacterium, Geobacillus, Helicobacter, Kingella, Orientia, Pasteurella, Peptinophilus, Prevotella, Rothia, Salmonella,* and *Treponema.*	In ER/PR$^+$ breast cancer, highest hybridization signals were found for probes of viruses Anelloviridae and Flaviviridae, and for fungal probes of *Filobasidiella, Mucor,* and *Trichophyton.* Comparing ER/PR$^+$ breast cancer tissue with healthy breast tissue, signals for *Arcanobacterium, Bifidobacterium, Cardiobacterium, Citrobacter,* and *Escherichia* were associated with ER/PR$^+$ breast cancer tissue. In HER2$^+$ breast cancer, highest hybridization signals were found for probes of the virus Togaviridae, and the fungi *Epidermophyton, Fonsecaea,* and *Pseudallescheria.* The virus Nodoviridae was only detected in HER2$^+$ breast cancer. In ER/PR$^+$, HER2$^+$ breast cancer, highest hybridization signals were found for probes of the virus Polyomaviridae and the fungus *Penicillum.* Comparing ER/PR$^+$, HER2$^+$ breast cancer tissue with healthy breast tissue, signals for *Bordetella, Campylobacter, Chlamydia, Chlamydophila, Legionella,* and *Pasteurella* were associated with ER/PR$^+$, HER2$^+$ breast cancer. In TNBC, highest hybridization signals were found for probes of viruses Picornaviridae and Anelloviridae, and fungi *Alternaria, Malassezia,*

(*table continues*)

Table 1 *(continued)*

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue, and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades, and stages
					Piedraia, and *Rhizomucor.* Comparing TNBC tissue with healthy breast tissue, signals for *Aerococcus, Arcobacter, Geobacillus, Orientia,* and *Rothia* were associated with TNBC tissue.
Meng et al[20]	94 breast tumor tissue samples: 22 benign breast tumors; 72 malignant breast cancer (grade I: $n = 7$, grade II: $n = 36$, grade III: $n = 13$, NO-grade: $n = 16$)	Fresh frozen	ER$^+$ ($n = 47$) ER- ($n = 25$)	Comparing malignant breast cancer tissue with benign breast tumor tissue, increased relative abundance of Proteobacteria, *Micrococcaceae, Caulobacteraceae, Rhodobacteraceae, Nocordioidaceae, Methylobacteriaceae,* and *Propionicimonas* was found in malignant breast cancer tissue.	Comparing grade III breast cancer tissue with grade I and II breast cancer tissue, higher alpha diversity was found in grade III breast cancer. With increasing grade, decreased relative abundance of *Bacteroidaceae* and increased relative abundance of *Agrococcus* were found.
Smith et al[15]	53 breast cancer tissue samples 11 adjacent breast tissue samples 8 healthy breast tissue samples Breast cancer stage: Stage I: $n = 13$ Stage II: $n = 24$ Stage III and IV: $n = 19$ Missing: $n = 8$ Non-Hispanic whites: $n = 51$ Non-Hispanic blacks: $n = 12$	Fresh frozen	Luminal A ($n = 22$) Luminal B ($n = 14$) HER2$^+$ ($n = 6$) TNBC ($n = 15$) Missing ($n = 7$)	Comparing healthy breast tissue with breast cancer tissue, higher alpha diversity, decreased relative abundance of *Pseudomonadaceae, Sphingomonadaceae,* and *Ruminococcaceae,* and increased relative abundance of *Actinomycetaceae* were found in healthy breast tissue. Comparing adjacent breast tissue to breast cancer tissue, lower relative abundance of *Ruminococcaceae* and *Clostridia* was found in adjacent breast tissue. Comparing adjacent breast tissue with healthy breast tissue, higher relative abundance of *Pseudomonadaceae* was found in adjacent breast tissue. In breast cancer tissue, class Clostridia, Bacteroidia, and family *Ruminococcaceae* were found to be most abundant. Family *Xanthomonadaceae* was	In luminal A breast cancer, order Xanthomonadales was most abundant. In luminal B breast cancer, genus *Clostridium* was most abundant. In the luminal subtypes, phyla Tenericutes, Proteobacteria, and Planctomycetes were most abundant. In HER2$^+$ breast cancer, genus *Akkermansia* and phyla Thermia and Verrucomicrobia were most abundant. In TNBC, genera *Streptococcaceae* and *Ruminococcus,* and phyla Euryarchaeota, Cyanobacteria, and Firmicutes were most abundant. In Stage I breast cancer, family *Ruminococcaceae,* and genus *Hyphomicrobium* were most abundant. In Stage II breast cancer, genus *Sporosarcina* was most abundant. In Stage III and IV breast cancer, genus *Bosea* was most abundant.

(table continues)

Table 1 (continued)

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue, and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades, and stages
				most abundant in breast cancer tissue of non-Hispanic white women. Genus *Ralstonia* was most abundant in breast cancer tissue of non-Hispanic black women. Families *Pseudomonadaceae, Sphingomonadaceae,* and *Caulobacteraceae* were most abundant in adjacent breast tissue.	
Costantini et al[17]	16 breast cancer tissue samples 16 adjacent breast tissue samples	Fresh tissue	ER/PR$^+$ ($n = 14$) ER/PR$^-$ ($n = 2$) HER2$^+$ ($n = 1$) HER2$^-$ ($n = 15$) TNBC ($n = 1$)	In breast cancer tissue, phylum *Proteobacteria* was most abundant, followed by *Firmicutes, Actinobacteria,* and *Bacteroidetes.* 50% to 75% of relative abundances belonged to genera *Ralstonia, Methylobacterium,* and *Sphingomonas.* 25% to 50% of relative abundances belonged to genera *Staphylococcus* and *Pseudomonas,* and to families *Bradyrhizobiaceae* and *Rhodocyclaceae.* Comparing breast cancer tissue with adjacent breast tissue, no significant differences were found.	*
Nejman et al[13]	355 breast cancer tissue samples 173 adjacent breast tissue samples 54 healthy breast tissue samples	Fresh frozen and FFPE (majority of samples)	ER$^+$, PR$^+$, HER2$^+$ (distribution is not given)	In breast cancer tissue, 9190 bacterial species were detected in total. Breast tumors had a richer and more diverse microbiome than other tumors (melanoma, lung, ovary, bone, and glioblastoma multiforme tumors). Comparing breast cancer tissue with adjacent breast tissue and healthy breast tissue, higher bacterial load and richness were found in breast cancer tissue.	Differences in microbiota composition were found between breast cancer subtypes, based on ER, PR, and HER2 expression. Most enriched pathways in bacteria within ER$^+$ breast cancer tissue were arsenate detoxification and mycothiol biosynthesis.

*Not described or investigated in the study.
FFPE, formalin-fixed paraffin-embedded; TNBC, triple-negative breast cancer.

Breast Microbiome in Breast Cancer Subtypes

Remarkably, the breast tumor subtypes luminal A, luminal B, TNBC, and HER2$^+$ were found to exhibit unique subtype-associated microbiota.[13,15,21,22] Luminal A tumor tissue exhibited highest abundance of order Xanthomonadales, and luminal B tumor tissue showed highest abundance of the genus *Clostridium*.[15] In most hormone-positive breast cancer tissues, the genus *Methylobacterium* had a decreased presence compared with that in healthy breast tissue.[16] In HER2 tumors, *Akkermansia* was most abundant.[15] TNBC tissue was shown to harbor *Streptococcaceae* and *Ruminococcus* at the highest level in one study.[15] In another study, using the PathoChip array, a higher percentage of *Prevotella, Brevundimonas, Arcanobacterium, Escherichia, Sphingobacterium, Actinomyces,* and *Rothia* was found in TNBC tissue compared with healthy breast tissue.[21]

All these studies show considerable heterogeneity in microbiota composition of healthy breast, normal adjacent, and tumorous breast microbiota. It seems clear that a breast microbiome exists and that it may change during the course of breast cancer development. However, a clear breast cancer microbiota profile has not been defined yet. This could be due to several reasons: i) the low microbial biomass in the breast organ, which increases the risk of contamination influencing the results; ii) small sample sizes; and iii) the variety of extraction and sequencing methods used. Understanding microbial differences and their role in carcinogenesis will be important to estimate breast cancer risk from a microbiome perspective. In Table 1, studies evaluating breast microbiota composition are chronologically listed in detail. In Table 2, the breast microbiota compositions of various breast cancer subtypes can be found.

Immune Involvement in Breast Carcinogenesis

The immune system plays a significant role in the initiation, progression, and control of cancer. This is illustrated by the process of cancer immunoediting, which describes the evolving interactions between host immunity and cancer cells, consisting of three distinct phases: elimination, equilibrium, and escape.[23] In the elimination phase, tumor cells are successfully recognized and eliminated by immune cells. During equilibrium, transformed cells escape elimination and are able to proliferate. However, this proliferation is still controlled by the immune system, in contrast to the escape phase, which is defined by uncontrolled proliferation. In a healthy situation, a balance exists between proinflammatory and anti-inflammatory signals, which is partly regulated by costimulation and coinhibition of T cells.[24] This enables sufficient clearance of foreign antigens, but concurrently prevents uncontrolled inflammation. Tumors are able to avoid immune destruction by several mechanisms, including loss of antigenicity and recruitment of immunosuppressive leukocytes.[25]

Immunosuppression in Breast Tumor Tissue

Breast cancer is characterized by infiltrated immune cells in the tumor tissue, where immunosuppressive cells are dominant over proinflammatory cells. High magnitude of tumor-infiltrating lymphocytes in the breast is associated with better prognosis and therapeutic response in certain breast cancer subtypes.[26] Lymphocytes exhibiting antitumor activity include CD8$^+$ cytotoxic T lymphocytes, which eliminate cancer cells, and CD4$^+$ T helper 1 lymphocytes, which activate cytotoxic T lymphocytes.[27] In ER$^+$ HER2$^+$ tumors, presence of tumor-infiltrating cytotoxic (CD8$^+$) T cells was associated with a 27% reduction in the hazard of dying from breast cancer.[28]

It is hypothesized that tumors recruit immunosuppressive cells in order to evade immune destruction.[28] Repressors of T cells, such as myeloid-derived suppressor cells and T regulatory cells, are found in higher numbers in patients with breast cancer compared with healthy controls, and increase with tumor stage.[29,30] Tumor-associated macrophages, which resemble the anti-inflammatory M2-polarized macrophages, promote tumor growth by secretion of anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β.[31,32] High infiltration of tumor-associated macrophages in breast tissue is associated with malignancy, negative hormone receptor status, and poor disease-free and overall survival.[33]

Communication between the Immune System and Microbiota

The innate immune system is trained in recognizing microbes via pattern recognition receptors (PRRs) that bind to bacterial components, known as pathogen-associated molecular patterns.[34] Examples of PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and C-type lectin receptors, which are expressed by cells such as macrophages, dendritic cells, and natural killer (NK) cells.[34] TLRs induce an inflammatory response in reaction to binding with microbial structures such as lipopolysaccharide, peptidoglycan, flagella, or microbial DNA or RNA.[35] Activation of TLRs can either have tumor promoting or inhibiting effects, depending on the TLR subset, cancer type, and involved immune cells in the tumor.[36] In breast carcinomas of mice xenografts, TLR5 was found to be highly expressed.[37] *In vivo* administration of *Salmonella typhimurium* flagellin, a ligand of TLR5, stimulated secretion of proinflammatory cytokines and chemokines, mediating antitumor activity.[37] In another study, lipopolysaccharide/TLR4 signaling resulted in a protumorigenic effect by up-regulating production of IL-6 and IL-10.[38] These observations might explain the importance of breast microbiota in breast cancer, because certain microbial components recognized by PRRs in the breast can induce a tumor-inhibiting inflammatory response, contributing to recruitment of tumor-killing cells.

Table 2   Microbiota Composition of Breast Cancer Subtypes

Study	Breast cancer subtype definition	Microbiota composition of breast cancer subtype
Xuan et al,[8] Smith et al,[15] Banerjee et al[22]	HR+	Comparing HR+ breast cancer tissue to adjacent breast tissue, higher relative abundance of *Methylobacterium radiotolerans* was found in HR+ breast cancer tissue.
		Comparing HR+ breast cancer tissue with healthy breast tissue, signals for *Arcanobacterium, Bifidobacterium, Cardiobacterium, Citrobacter,* and *Escherichia* were associated with HR+ breast cancer tissue.
		Absolute abundance of order Xanthomonadales and phyla Tenericutes, Proteobacteria, and Planctomycetes was found highest in luminal A breast cancer.
		Absolute abundance of genus *Clostridium* and phyla Tenericutes, Proteobacteria, and Planctomycetes was found highest in luminal B breast cancer.
Banerjee et al[22]	HER2+	Comparing HR+/HER2+ breast cancer with healthy breast tissue, signals for *Bordetella, Campylobacter, Chlamydia, Chlamydophila, Legionella,* and *Pasteurella* were associated with HR+/HER2+ breast cancer.
		Absolute abundance of genus *Akkermansia* and phyla Thermia and Verrucomicrobia was highest in HER2+ breast cancer.
Smith et al,[15] Banerjee et al[21,22]	TNBC	Comparing TNBC tissue with healthy breast tissue, higher signals for *Prevotella, Brevundimonas, Arcanobacterium, Escherichia, Sphingobacterium, Actinomyces, Aerococcus, Arcobacter, Geobacillus, Orientia,* and *Rothia* were found in TNBC tissue.
		Absolute abundance of genera *Streptococcaceae* and *Ruminococcus*, and phyla Euryarchaeota, Cyanobacteria, and Firmicutes was highest in TNBC.

TNBC, triple-negative breast cancer.

Most of our understanding regarding immune cell and tissue-specific microbiota interactions, originates from studies investigating pancreatic and lung microbiota. In a study exploring microbiota in pancreatic cancer, microbiome diversity correlated with CD8+ T-cell infiltration.[39] Additionally, CD8+ immune infiltration was associated with three genera that were most abundant in long-term survivors, including *Saccharopolyspora, Pseudoxanthomonas,* and *Streptomyces.* Interestingly, in another pancreatic cancer study, TLR2 and TLR5 ligation was found to promote pancreatic cancer and induce immunosuppression.[40] Moreover, these immune-suppressive effects were absent when macrophages were deficient in TLR signaling, which suggests that immunosuppression is dependent upon TLR ligation between the tumor microbiome and immune cells. In mice, lung microbiota manipulation was demonstrated to reduce the local immunosuppressive environment. Treatment with antibiotic or probiotic aerosol, 2 weeks before melanoma cell injection, resulted in decreased bacterial load in the lung accompanied by enhanced activation of NK and T effector cells, promoting tumor immunity against lung metastases.[41]

In summary, correlations exist between intratumoral microbiota composition and immune cell infiltration, which indicates that an unfavorable microbiota composition may contribute to tumor immune evasion.

Is Therapeutic Efficacy and Cytotoxicity Governed by Intratumoral Microbiota?

Therapeutic resistance, which can be intrinsic or required, continues to be the limiting factor in achieving successful breast cancer treatment. Responsible determinants of drug resistance include alteration in expression or mutation of a drug target, tumor heterogeneity, reduced blood flow to the tumor, and prevention of immune evasion by the TME.[42] Biomarkers to predict therapeutic sensitivity of breast tumor cells are urgently required so that therapy and dose can be adjusted accordingly. In this context, gut microorganisms have been shown to mediate toxicity and related side effects of anticancer agents. For example, the inactive SN-38 G form of the prodrug irinotecan (CPT-11) is reactivated by intestinal bacterial β-glucuronidases into the active and toxic SN-38.[43] It is proposed that breast microbiota composition influences local availability and cytotoxicity of anticancer therapeutics as well, and thus could be used as a marker to determine drug efficacy and toxicity.

Via endogenous enzymes, bacteria have the ability to transform organic compounds.[44] Administration of bacteria found in breast cancer tissue, namely Gram-negative *E. coli* and Gram-positive *Listeria welshimeri*, are able to either enhance or reduce efficacy and cytotoxicity of different
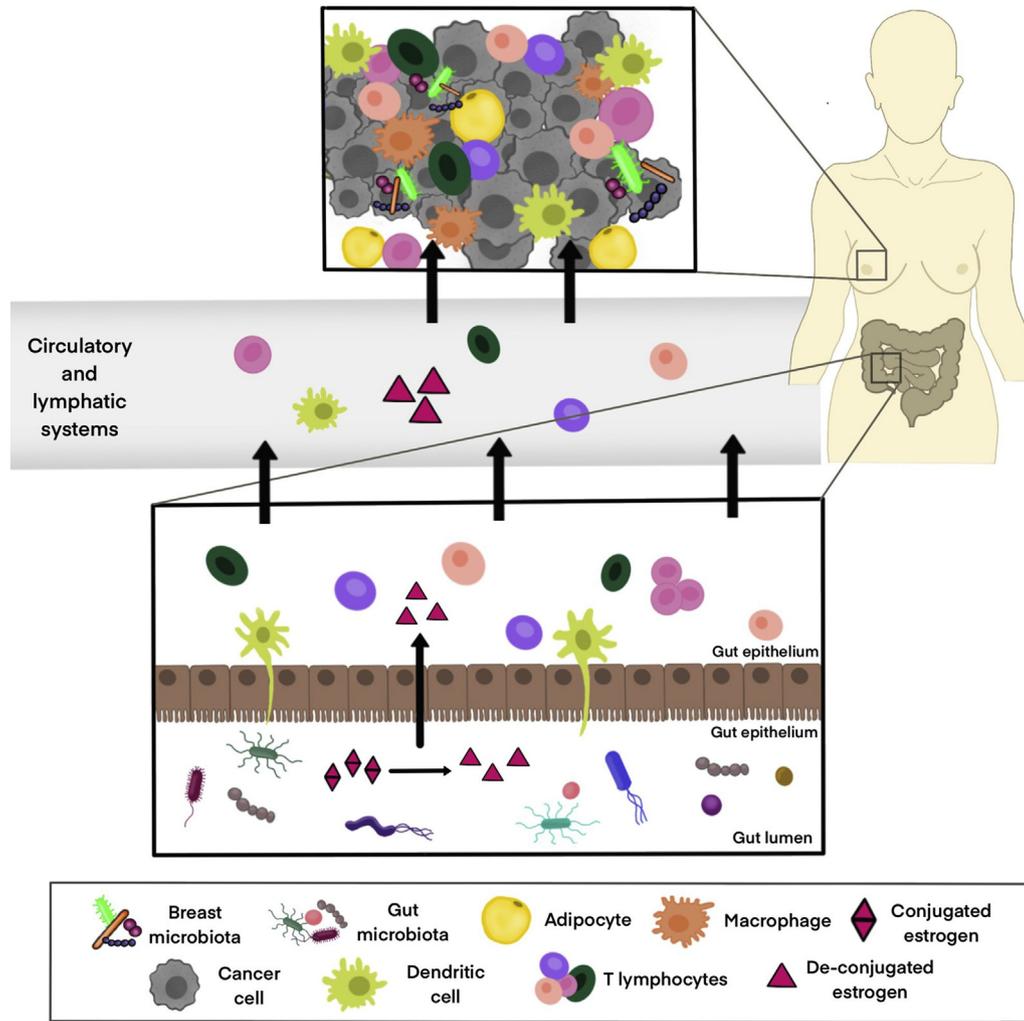
Figure 1 Interaction mechanisms between the gut and the breast. A connection between the gut and the breast has been demonstrated in human and mouse studies. The gut microbiota composition interacts with the host immune system and subsequently influences systemic immunity and the local immune environment in the breast. Furthermore, the gut microbiota has the capacity to influence systemic availability of estrogen through enterohepatic recycling of estrogen, because certain bacteria contain the enzyme beta-glucuronidase.

chemotherapeutics through biotransformation in both *in vitro* and *in vivo* cancer models.[44] In this study, *E. coli* increased *in vitro* cytotoxicity of tegafur, fludarabine de phosphate, 5-fluorcytosine, 6-mercaptopurine-2′-deoxyriboside, AQ4N, and CB1954, and decreased cytotoxicity of cladribine, vidarabine, gemicitabine, doxorubicin, daunorubicin, etoposide phosphate, mitoxantrone, β-lapachone, and menadione. *In vitro* findings were confirmed in a CT26 murine colon carcinoma model. Intratumoral growth of *E. coli,* together with gemicitabine administration, resulted in increased tumor volume and reduced survival compared with a control group with only gemicitabine administered.[44]

To confirm that bacteria determine effectiveness of anticancer therapy, a pancreatic cancer study was sued to demonstrate reduction in gemcitabine concentration due to the presence of intratumoral Gammaproteobacteria, and increased chemotherapeutic sensitivity when treated with antibiotics.[45] Proteobacteria are abundant in pancreatic cancer tissue, and when transferred to a colon cancer mouse model, these bacteria were shown to mediate gemcitabine resistance by metabolizing and inactivating the drug via the long form of bacterial enzyme cytidine deaminase (CDD$_L$).[45] Chemotherapeutics can also induce changes in intratumoral microbiota composition, because neoadjuvant chemotherapy reduced bacterial diversity in breast tumor tissue.[46]

Current immunotherapeutic strategies aim to block coinhibitory molecules in the TME, in order to reduce immunosuppression found in cancer. Immune checkpoint inhibitors, such as programed cell death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) blocking antibodies, function by preventing T-cell inhibition.[47] However, the clinical benefit of immune checkpoint inhibitors in breast cancer is not as effective as in other cancer types.[48] To enhance immunotherapeutic efficacy, it is needed to better understand the mechanisms underlying immunotherapy resistance. The TME is a promising target to improve responsiveness to immunotherapy, which can be achieved by modulating protumor inflammation.[25] The influence of gut microbiota on immunotherapeutic efficacy has been
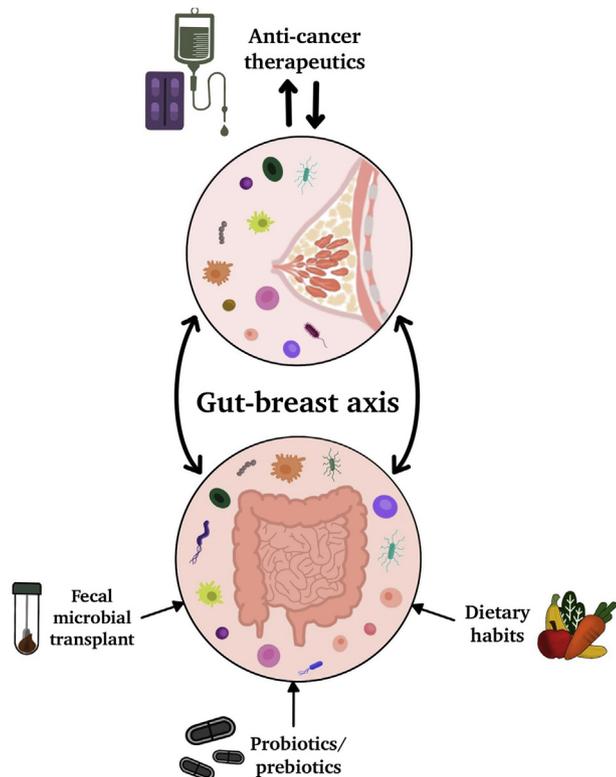
Figure 2   Proposed strategies to influence the gut—breast axis. Breast cancer is characterized by a unique microbiota and immune composition. Though little is known about manipulating these compositions in the breast, alteration of the gut microbiota composition might influence the breast tumor microenvironment as well. Strategies to positively alter gut microbiota comprise healthy dietary habits, pre- or probiotics, or more rigorously, fecal microbiota transplantation. Moreover, certain bacterial species potentially have the capacity to modulate anticancer agents by bacterial enzymatic activity.

described previously.[48–50] However, it remains to be established whether intratumoral breast microbiota also influence the functioning of immunotherapeutics. This is a reasonable assumption, because activation of TLRs by bacterial products stimulate maturation and priming of immune cells.

Gut—Breast Microbiota Axis

Other than the direct effects of organ-specific microbiota on local tissue, the gut microbiota possibly affect breast cancer development through several mechanisms.[11] Disruption of gut microbiota homeostasis, characterized by low gut microbial diversity and less beneficial bacteria, is associated with breast cancer.[51] In addition, antibiotic treatment, which is known to disrupt gut microbiota homeostasis, is associated with increased breast cancer risk.[52,53] Furthermore, established risk factors for breast cancer, such as obesity and alcohol consumption, are associated with dysbiosis of the intestinal microbiota.[54,55]

The breast parenchyma can be influenced by the gut microbiota through different mechanisms, including via enterohepatic recycling of estrogens and bile acids, and microbial interaction with the innate and adaptive immune system.

Estrogen and Bile Acids

Estrogens are normally conjugated in the liver and delivered into the gut via bile excretion.[56] In the gut reside bacteria with beta-glucuronidase enzymatic activity, capable of deconjugating conjugated estrogen. Deconjugated estrogen is reabsorbed into the circulation, resulting in systemically increased estrogen exposure and thereby increasing breast cancer risk.[57]

A main metabolic feature of intestinal microbes is bile acid conversion. Bile acids are soluble amphipathic molecules derived from cholesterol in the liver.[58,59] Microbiota that reside in the intestinal lumen have the capacity to convert primary bile acids into secondary bile acids by the process of deconjugation and 7α-dehydroxylation.[58] Certain specific bile acids are functionally similar to hormones, due to their capacity to alter metabolic pathways in distant organ tissues by activating several receptors, including farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D receptor (VDR), and Takeda G-protein-coupled receptor 5 (TGR5).[58] The farnesoid X receptor has been detected in invasive breast carcinoma.[60] Bile acids were found to be accumulated in breast tumors and were associated with antiproliferative effects and improved patient prognosis.[61] Another study evaluated the effects in breast cancer of a separate bile acid. Lithocholic acid decreased breast cancer cell proliferation by oxidative stress induction and improved antitumor immunity by increasing tumor-infiltrating lymphocyte count in the breast.[62]

Shaping Systemic Immunity

The intestinal microbiota shapes the immune system. Gut dysbiosis modulates immune functioning, which might contribute to carcinogenesis.[63,64] Various experimental mouse models exist, demonstrating carcinogenic regulation by gut microbes via interaction with the immune system. In mice, commensal dysbiosis of the gut, induced by oral antibiotics, significantly increases the number of myeloid cells present in normal adjacent breast gland tissue at both early and advanced stages of breast tumor progression.[65] Moreover, these infiltrated myeloid cells exhibit high expressions of inflammatory mediators arginase-1 and IL-6. Myeloid recruitment into breast tissue is explained by corresponding increases in myeloid chemoattractants, namely CXCL10 and CCL2, which were up-regulated in dysbiotic mice compared with nondysbiotic mice.[65]

Neutrophils, members of the innate immune system, have been identified to mediate between the gut and extraintestinal organs. A specific bacterium, *Helicobacter hepaticus*, residing in the gut was shown to affect distant neoplastic progressions in breast tissue, in which neutrophils played a tumorigenic role.[66] Mice with a predisposition for breast

cancer underwent accelerated cancer progression when injected with *H. hepaticus*, compared with noninfected mice. However, mice systemically depleted of neutrophils showed only preneoplastic and early neoplastic lesions in the breast tissue, thus inhibiting carcinogenesis.

These findings indicate that the gut microbiota has the capacity to affect immune cell expression in breast tissue as well. In breast cancer patients, higher gut microbiota diversity is associated with increased tumor-infiltrating lymphocyte expression in breast tissue.[67] A summary of breast microbiota interactions is illustrated in Figure 1.

Translational Gaps—the Future of Using Microbiota to Fight Cancer

Microbiota comprise a promising field of research, in which the breast microbiota can be used as biomarker to establish disease characteristics and subsequently used as a therapeutic target. Accumulated evidence confirms the presence of a unique microbial community in the breast. However, small sample sizes, interindividual heterogeneity of the microbiota, variances in methodological approaches (DNA extraction kit, target hypervariable region selection for sequencing, tissue extraction and storage), and patient characteristics (demography, dietary habits, menopausal status, breast cancer subtypes) limit comparability of results. Furthermore, the observational design of most studies only provides associative, but not causal, evidence for the relationship between microbial dysbiosis and breast carcinogenesis. Moreover, because tumor samples contain a low bacterial biomass, contamination can contribute to inaccurate findings. A major challenge in this field will be making the shift from descriptive analysis to functional metagenomics and metabolomics, in order to outline the functional roles of bacteria present in the breast and its interaction with the internal environment. Furthermore, rather than holding a single pathogen responsible for cancer progression, the accumulative effects of an entire microbial community are more likely to determine disease processes.

The Breast Microbiota as a Biomarker for Cancer

The breast microbiota could be used as a biomarker in several ways: i) pretreatment, to determine the molecular characteristics of the cancer, so that response or resistance to therapeutics can be predicted; ii) during treatment, enabling the adjustment of the therapy when unresponsive; and iii) during tumor progression, to elucidate the achieved resistance to therapeutics.[68] Using microbiota as a pretreatment biomarker would provide most value to oncologists, because microbiota analysis would offer additional information on tumor aggressiveness and tumor sensitivity to anticancer therapeutics, after which, treatment type and regimen could be adjusted accordingly. Moreover, as per current protocols in breast cancer diagnosis, tissue specimens have already been obtained prior to treatment, placing no additional burden on patients.

To bring a biomarker to the clinic, five phases have been outlined.[69] These include preclinical studies, clinical assay development, retrospective studies, prospective studies, and control studies. Currently, research regarding the breast microbiota is still in the preclinical, exploratory phase, because most studies have focused on identifying characteristics that are unique to breast tumor tissue, by comparing the microbiota of breast tumor tissue to healthy tissue.

Even though the breast microbiome exhibits great potential as a prognostic and predictive biomarker, further investigations are still required to consider the breast microbiome as a biomarker for prognosis or therapeutic response. First, more participants should be included in future studies, taking clinicopathological variability into account. Furthermore, by performing prospective studies, the prognostic value of pretreatment breast microbiota composition can be evaluated. Second, confounding factors should be taken into consideration, including menopausal status, age, ethnicity, body mass index, and lifestyle-related factors, such as alcohol consumption and dietary habits. Lastly, microbiota compositions should be identified that are able to predict efficacy and resistance to anticancer therapeutics, by comparing microbial composition of patients with differences in tumor progression and between responders and nonresponders. In order to obtain in-depth mechanistic insight into the relationship between breast cancer microbiota and anticancer agents, the use of animal models will be required.

Modulating Breast Microbiota

No studies have been conducted yet on how the breast microbiota composition could be manipulated. However, because the gut microbiota is able to affect the breast through several mechanisms, discussed above, the gut microbiota could be targeted. Beta-glucuronidase activity and gut-derived metabolites can be modulated by diet.[56] Diets high in fat were found to increase beta-glucuronidase activity in gut bacteria and diets high in fiber reduced this activity. Other factors that are known to elevate estrogen levels in the circulation are alcohol ingestion and adiposity.[70] A more rigorous approach to manipulate gut microbiota composition is by the method of fecal microbiota transplantation.[71] Another strategy encompasses the intake of pro- or prebiotics.[72] However, more research is needed to understand how gut microbiota interacts with microbiota residing in the breast, and other breast microbial targeting possibilities need to be explored. In Figure 2, the proposed strategies to influence the gut-breast axis are summarized.

Microbiota Investigation Techniques

It remains to be investigated which intratumoral bacteria in the breast modulate anticancer agents, in order to identify

possible targets for breast cancer therapeutics. Alongside this, its interactions with the immune system deserve attention as well. This can be best done by combining metagenomics and metabolomics sequencing with preclinical models, such as gnotobiotic mice and patient-derived tumor xenografts (PDTXs).[73] Gnotobiotic mice refers to animals with known microbiota composition, either germ-free mice or ex—germ-free animals.[74] PDTXs exist of immunodeficient mice to which patient-derived material is transferred.[75] Another possible preclinical model that can be combined with sequencing is the *in vitro* three-dimensional model termed organoid.[75] Transferring breast cancer microbiota to these models will contribute to the understanding of patient-specific tumorigenic and therapeutic modulatory potential of intratumoral bacteria. Identifying which microbial composition favors a positive response to anticancer therapy is important to increase therapeutic effectiveness.[73]

Summary

Breast microbiota holds many possibilities to reveal more insight into the pathophysiology of breast cancer. Using the breast microbiome as a marker to predict breast cancer prognosis and therapeutic response holds promise. Targeting microbiota could be used to improve therapeutic efficiency and reduce related toxicity. Before breast microbiota analysis can be implemented, major knowledge gaps need to be considered. There is limited understanding of the contribution of breast microbiota to cancer development and treatment response. Because indications exist that breast microbiota components interact with the gut microbiota and the immune system, these need to be explored in parallel.

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