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ORIGINAL ARTICLE

Staphylococcus epidermidis modulates EMT-related gene expression and viability in MDA-MB-231 breast cancer cells

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ABSTRACT

Background and Objectives: Breast tissue microbiota differs between healthy and cancerous tissues, with some bacteria influencing tumor progression. *Staphylococcus epidermidis*, a common skin commensal found in breast tumors, may play a role in epithelial-mesenchymal transition (EMT), a key step in metastasis. This study evaluated the effects of *S. epidermidis* culture and cell-free supernatant (CFS) on MDA-MB-231 breast cancer cell survival and expression of EMT-related genes *Snail1*, fibronectin 1 (*FN1*), and N-cadherin (*CDH2*).

Materials and Methods: Different concentrations of *S. epidermidis* cultures and their CFS were applied to MDA-MB-231 cells. Cytotoxic effects were assessed by MTT assay at 2, 4, and 24 hours post-treatment. Real-time PCR analyzed gene expression after 24 hours of exposure to non-toxic concentrations (MOI 50 and 100 for cultures; 14% for CFS).

Results: Low concentrations did not affect viability, while higher doses (MOI 100 and 14% CFS) reduced viability by up to 60% and 90%, respectively, at 24 hours. MOI 50 did not significantly alter gene expression. At MOI 100, *Snail1* and *FN1* were significantly upregulated, but *CDH2* was unchanged. Treatment with 5% and 7% CFS significantly increased all three EMT gene expressions, indicating EMT induction.

Conclusion: *S. epidermidis* affects EMT gene expression and cell viability, indicating potential involvement in breast cancer progression.

Keywords: *Staphylococcus epidermidis*; Epithelial-mesenchymal transition; Breast neoplasms; Gene expression; Microbiota

INTRODUCTION

Globally, cancer is one of the primary causes of mortality, and among women, breast cancer is the most frequently diagnosed type. The number of breast cancer cases is steadily increasing, a trend attributed to factors such as an aging population and shifts in lifestyle (1). In contrast to benign tumors, malignant breast cancers are characterized by unchecked cell growth and can infiltrate surrounding

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tissues as well as spread to other parts of the body. Since nearly 90% of cancer fatalities are due to metastasis, it is crucial to develop treatments that address both the original tumor and the mechanisms driving metastatic spread (2).

Metastasis is largely driven by epithelial-mesenchymal transition (EMT), a biological program where epithelial cancer cells lose their polarity and adhesion properties while acquiring mesenchymal traits that enhance motility and invasiveness. EMT is a critical mechanism in cancer progression, making it a focal point for understanding tumor biology and developing targeted therapies (3).

Recent research increasingly demonstrates that the human microbiome can impact both the initiation and advancement of cancer. The microbial communities found within breast tissue are unique and show clear differences when comparing healthy individuals to those with breast malignancies (1, 4). Of particular interest, *Staphylococcus epidermidis*—a bacterium commonly present on the skin—has been repeatedly detected in breast tumor samples and is thought to facilitate tumor progression. Certain isolates of *S. epidermidis* from breast cancer patients have been shown to cause DNA double-strand breaks, thereby inducing genomic instability, which is recognized as a fundamental feature of cancer (5).

Additional research indicates that decreasing the abundance of *S. epidermidis* within the mammary tumor microbiome can strengthen antitumor immune responses and diminish tumor severity in mouse models. This suggests that *S. epidermidis* may promote tumor progression by modulating immune responses and altering the tumor microenvironment (6, 7). Additionally, *S. epidermidis* may influence the expression of EMT-related genes, thereby facilitating metastasis; however, the precise mechanisms remain to be fully elucidated (8).

Given these insights, understanding the interaction between *S. epidermidis* and breast cancer cells, particularly its effects on EMT-related gene expression is critical. This study investigates the impact of *S. epidermidis* culture and its cell-free supernatant on the viability of MDA-MB-231 breast cancer cells and the expression of key EMT markers: *Snail1* (Snail family transcriptional repressor 1), *FN1* (fibronectin 1), and *CDH2* (N-cadherin). Elucidating these interactions may uncover novel pathways by which *S. epidermidis* contributes to breast cancer progression and may identify potential therapeutic targets.

MATERIALS AND METHODS

Bacterial culture condition. *S. epidermidis* strain ATCC 12228 was sourced from the Pasteur Institute of Iran's bacterial collection. For routine cultivation, the bacteria were streaked onto brain heart infusion (BHI) agar plates (Quelab, Canada) and incubated under aerobic conditions at 37°C for one to two days to generate fresh bacterial colonies.

For preparation of the cell-free supernatant (CFS), an isolated colony of *S. epidermidis* was inoculated into 100 mL of BHI broth and incubated at 37°C with continuous shaking at 150 rpm. The incubation continued until it reached an optical density at 600 nm (OD600) of approximately 1.5, corresponding to the late exponential phase. Following this, the bacterial cells were then separated by centrifugation at 8,000 × g for 5 minutes at 4°C. The resulting supernatant was carefully collected, and its pH was adjusted to 7.4 using sterile phosphate-buffered saline (PBS) to maintain physiological conditions. To ensure sterility and removal of residual bacterial cells, the supernatant was filtered through a 0.22 μm pore-size membrane filter (Millipore). The sterile CFS was aliquoted and stored at -70°C until further use in cell culture experiments.

Cell culture and treatments. The MDA-MB-231 human breast cancer cell line was sourced from the National Cell Bank at the Pasteur Institute of Iran. Cells were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA) enriched with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 micrograms/mL streptomycin. Cultures were kept at 37°C in a humidified incubator containing 5% CO_2. For experimental procedures, cells were plated in 6-well plates at a density of 5×10^5 cells per well and cultured until they reached approximately 80% confluence. Before applying treatments, the culture medium was switched to DMEM with 10% FBS but without antibiotics to prevent any impact on bacterial survival.

Two treatment approaches were used: live *S. epidermidis* cultures were applied at MOI of 5, 10, 50, and 100, corresponding to 5 to 100 bacteria per cancer cell, while sterile CFS from *S. epidermidis* cultures was diluted to final concentrations of 5%, 7%, 10%, and 14% (v/v) in culture medium. Untreated cells cultured in antibiotic-free medium served as negative

controls. Cells were incubated with treatments for 2, 4, and 24 hours under standard incubation conditions (37°C, 5% CO_2, and humidity exceeding 90%). Following incubation, cell viability and the expression of EMT-related genes (*Snail1*, *FN1*, and *CDH2*) were assessed to evaluate the effects of *S. epidermidis* and its secreted factors on breast cancer cell behavior. The selected MOI values were based on a combination of previous literature investigating bacterial interactions with mammalian cells and preliminary dose-response experiments conducted in our laboratory. These MOIs were chosen to cover a broad range from low to high bacterial loads, allowing us to assess both sub-lethal and cytotoxic effects of *S. epidermidis* on breast cancer cells. This range ensures biological relevance by mimicking potential bacterial colonization levels in tumor (9, 10).

Investigation of cell viability. To determine the viability of MDA-MB-231 breast cancer cells after exposure to *S. epidermidis* culture and its cell-free supernatant (CFS), the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was utilized. This established colorimetric technique measures cellular metabolic activity, which reflects both cell survival and cytotoxicity (6). In summary, cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight to allow for proper adherence. Subsequently, cells received treatments with various concentrations of either *S. epidermidis* culture or CFS for durations of 2, 4, and 24 hours under standard environment (37°C, 5% CO_2). At each specified time point, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline) was introduced into each well. Plates were then incubated for 2 hours at 37°C, allowing viable cells to convert the yellow MTT into purple formazan crystals. After this incubation, the supernatant was carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) was added per well to dissolve the formazan. The plates were gently shaken for 10 minutes at 37°C to ensure complete dissolution. Absorbance readings were taken at 570 nm using a microplate reader. Cell viability was calculated as a percentage relative to untreated controls, using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD of 570 nm of treated cells}}{\text{OD of 570 nm of control cells}}$$

Each treatment was conducted in three separate wells, and each experiment was independently repeated in triplicate to confirm reproducibility. Cellular morphology was examined at 4 and 24 hours after treatment using an Olympus CK2 inverted phase-contrast microscope (olympus ck2, Japan).

Real-time PCR analysis of EMT-related genes (*FN1*, *Snail1*, and *CDH2*). Total RNA was isolated from both treated and control MDA-MB-231 cells using TRIzol reagent (DNA Biotech) in accordance with the manufacturer's guidelines. The integrity of the extracted RNA was checked by running samples on an agarose gel, while RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm with a spectrophotometer. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using the Easy cDNA Synthesis Kit (ParsTous) according to the supplier's protocol. Quantitative real-time PCR (qRT-PCR) was carried out with SYBR Green chemistry in a final volume of 20 µL, comprising 1 µL of cDNA (about 50 ng), 10 µL of 2X SYBR Green Master Mix (Thermo Fisher Scientific), 0.7 µL of each primer (forward and reverse, final concentration 350 nM), and nuclease-free water. The PCR program included an initial denaturation at 95°C for 2 minutes, followed by 20 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 15 seconds. Primer sequences for the target genes were designed using Primer3 software to have uniform melting temperatures (~60°C) and are as follows: *Snail1* forward 5'-CACATCCGAAGCCACACG-3' and reverse 5'-ACTGGTACTTCTTGACATCTGAG-3'; *FN1* forward 5'-GTGTCAGATACCAGTGCTAC-3' and reverse 5'-GAAATGTGAGATGGCTGTGG-3'; *CDH2* forward 5'-TCGAAGGATGTGCATGAAGG-3' and reverse 5'-TTCTCACGGCATACACCATG-3'. *GAPDH* was used as the internal reference gene with primers forward 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse 5'-ACCACCCTGTTGCTGTAGCCAA-3'. Primer specificity was confirmed by melting curve analysis, ensuring a single specific amplification product.

To verify reproducibility, every qPCR assay was carried out in triplicate. Primer specificity for each target gene was confirmed by melt curve analysis, which demonstrated single, sharp peaks, indicating specific amplification without primer-dimer formation. Amplification efficiency was validated using standard curves, which was within the acceptable range (90-110%) for all target genes. Due to the rou-

tine use and well-established specificity of *GAPDH* as a reference gene, representative plots for *GAPDH* are not shown; however, specificity was confirmed in all runs. These data collectively demonstrate single, specific amplification products and efficient qPCR performance for each gene analyzed. Relative expression of target genes was determined by the comparative Ct (ΔΔCt) approach, with normalization to *GAPDH* and comparison between treated and control groups.

Data analysis. All statistical analyses were conducted with SPSS software version 24 (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard deviation (SD) from at least three independent experiments. Comparisons among multiple groups were made using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant. All graphs and figures were generated using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).

Ethics statement. Not applicable since only commercially available cell lines were applied in current research.

RESULTS

Cytotoxicity of *S. epidermidis* culture and cell-free supernatant (CFS). The cytotoxic effects of *S. epidermidis* culture and its CFS on MDA-MB-231 breast cancer cells were evaluated using the MTT assay at 2, 4, and 24 hours (Figs. 1 and 2). Treatment with increasing MOI of *S. epidermidis* culture (MOI 5-100) resulted in a modest, dose- and time-dependent reduction in cell viability. At 24 hours, cell viability decreased slightly with increasing MOI, reaching approximately 80% at the highest MOI (100). However, these reductions were not statistically significant at any time point or MOI tested, as indicated by the "ns" (not significant) annotations in Fig. 1. In contrast, exposure to increasing concentrations of CFS (S1: 5%, S2: 7%, S3: 10%, S4: 14%) produced a much more pronounced and significant decrease in cell viability, particularly at higher concentrations and longer incubation times. At 24 hours, 10% CFS (S3) reduced cell viability to approximately 30%, and 14% CFS (S4) reduced viability to below 10% compared to the controls. This effect was both dose- and time-dependent and was substantially greater than the reduction observed with live bacterial cultures at any MOI (Fig. 2). These results demonstrate that the cell-free supernatant of *S. epidermidis* exerts a markedly stronger cytotoxic effect on MDA-MB-231 cells than the live bacterial culture itself. The pronounced reduction in viability at higher CFS concentrations highlights the significant role of secreted bacterial factors in mediating anti-proliferative effects.

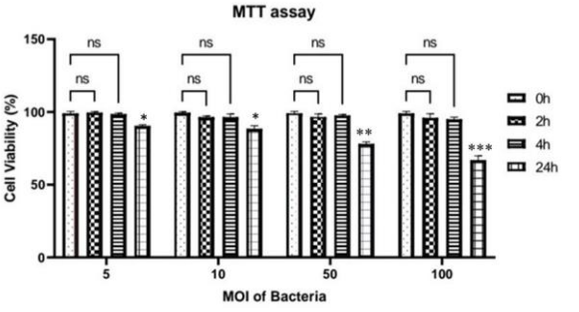

Fig. 1. Viability of MDA-MB-231 cells following exposure to different MOIs of *Staphylococcus epidermidis* culture at 2, 4, and 24 hours. Results are presented as mean ± standard deviation (SD) from a minimum of three independent experiments. Statistical differences among groups were assessed using one-way ANOVA followed by Tukey's post hoc test, with comparisons made against the untreated control group. *p < 0.05, **p < 0.01, ***p < .001; ns indicates no statistically significant difference.

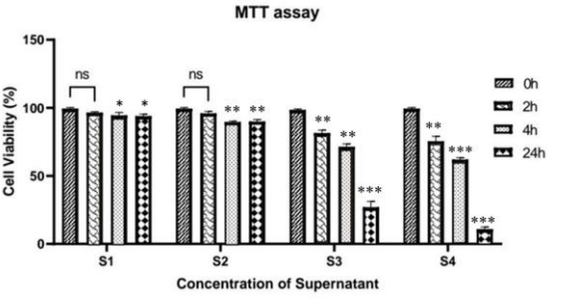

Fig. 2. Viability of MDA-MB-231 cells following treatment with escalating concentrations of *Staphylococcus epidermidis* cell-free supernatant (CFS) at 2, 4, and 24 hours. Data represent the mean ± standard deviation (SD) from three independent experiments. Statistical significance compared to the untreated control was evaluated using one-way ANOVA followed by Tukey's post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001; ns denotes no significant difference.

Morphological changes induced by bacterial suspension. Given the stronger impact of the bacterial suspension on cell viability, we further evaluated morphological changes in MDA-MB-231 cells treat-

ed with serial dilutions (1:1, 1:10, 1:100, and 1:1000) of the *S. epidermidis* suspension at 4 and 24 hours post-treatment (Fig. 3). In this qualitative analysis, serial dilutions were used to broadly assess the effects of increasing bacterial concentrations on cell morphology and apparent proliferation, while defined MOIs were reserved for quantitative assays as described in the Methods.

Microscopic observations revealed a clear, concentration- and time-dependent increase in cell density and confluency. At both 4 and 24 hours, higher concentrations of the bacterial suspension (particularly the 1:1 and 1:10 dilutions) resulted in visibly greater cell density and more extensive cell spreading compared to the lower concentrations (1:100 and 1:1000) and the untreated controls. This effect was most pronounced at the 1:1 dilution after 24 hours, where the monolayer appeared denser and cells exhibited more pronounced morphological changes, such as increased elongation and cell-to-cell contact. In contrast, the lowest concentration (1:1000) showed only a modest increase in cell number and less pronounced morphological alterations. Taken together, these qualitative observations suggest that exposure to *S. epidermidis* suspension enhances both the proliferation and morphological alterations of MDA-MB-231 cells in a manner dependent on both concentration and duration. These effects align with the trends observed in the MTT cell viability assay.

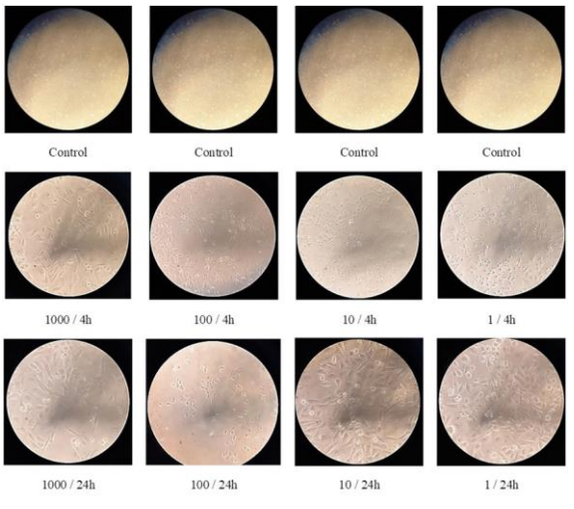

Fig. 3. Microscopic images of MDA-MB-231 cells following treatment with serial dilutions (1:1, 1:10, 1:100, and 1:1000) of *Staphylococcus epidermidis* suspension at 4 and 24 hours. Untreated controls are shown in the top row. A concentration- and time-dependent increase in cell density and confluency is observed, with the most pronounced effect at the 1:1 dilution after 24 hours. Images are representative of three independent experiments.

Real-time PCR analysis of EMT-related genes in MDA-MB-231 cells. The expression of EMT markers—*Snail1, FN1,* and *CDH2*—was analyzed in MDA-MB-231 cells exposed to *S. epidermidis* culture and its CFS, using SYBR Green-based real-time PCR. *GAPDH* was utilized as the internal control gene, and results were compared to those from untreated control cells. Treatments were administered at concentrations previously identified as non-cytotoxic in MTT assays. As illustrated in Fig. 4, exposure to an MOI of 50 did not significantly alter the expression of *Snail1, FN1,* or *CDH2* compared to the controls. However, at an MOI of 100, there was a notable upregulation of *Snail1* and *FN1*, while the increase in *CDH2* expression was not statistically significant. In contrast, treatment with 5% (S1) and 7% (S2) CFS resulted in a marked upregulation of all three EMT-related genes. Lower CFS concentrations and shorter incubation periods did not produce significant changes in gene expression.

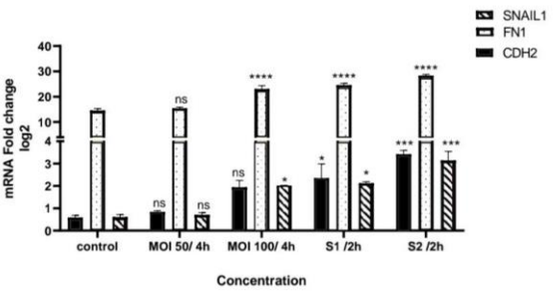

Fig. 4. Relative mRNA levels of *Snail1, FN1,* and *CDH2* in MDA-MB-231 cells after treatment with *Staphylococcus epidermidis* culture at MOIs of 50 and 100, as well as cell-free supernatant (CFS) at 5% (S1) and 7% (S2) concentrations for 2 hours. Data are presented as mean ± standard deviation (SD) from three independent experiments. Statistical analysis: ns, not significant; *P < 0.05; **P < 0.001; ***P < 0.0001.

DISCUSSION

Recent research has identified distinct bacterial communities within breast tumor tissues, notably including *Staphylococcus epidermidis*, which ap-

pears to influence breast cancer development and progression through various mechanisms. These include modulation of the immune microenvironment, induction of DNA damage, and alteration of cellular pathways associated with proliferation and metastasis (5, 11). Understanding these interactions is critical for elucidating the complex function of the tumor microbiota in breast cancer biology. Our study reveals important insights into the intricate relationship between *S. epidermidis* and breast cancer cells, highlighting the differential effects of the live bacterial cultures versus their CFS on MDA-MB-231 cell viability and EMT-related gene expression.

Notably, while live *S. epidermidis* cultures induced only modest and statistically non-significant decreases in the cell viability—approximately 20% at the highest MOI (MOI 100) after 24 hours—treatment with CFS resulted in a pronounced, dose- and time-dependent cytotoxic effect, with the highest concentration (14%) reducing viability by over 90%. This stark contrast suggests that secreted bacterial factors, rather than the presence of live bacteria per se, are the primary mediators of anti-proliferative activity. This may be explained by the concentration and bioavailability of bacterial metabolites, toxins, and extracellular vesicles within the CFS, which can more effectively interact with cancer cells than live bacteria that may be limited by host immune clearance or bacterial viability constraints (1, 5).

Our qualitative comparison indicates that CFS is approximately eight times more potent than live bacteria in reducing cancer cell viability, consistent with prior studies demonstrating that bacterial secreted products—such as phenol-soluble modulins—can induce apoptosis and disrupt cancer cell signaling more efficiently than whole bacterial cells (1, 8). These secreted factors have been shown to modulate mitochondrial function, oxidative stress pathways, and caspase activation, thereby promoting tumor cell death (12). The observed dose- and time-dependent cytotoxicity further supports the notion that bacterial metabolites accumulate to biologically effective concentrations with prolonged exposure, emphasizing the importance of secreted molecules in the tumor microenvironment (13, 14). Of note, this study did not determine exact IC_{50} values due to the restricted number of concentrations examined. While our data clearly demonstrate dose- and time-dependent cytotoxic effects, future studies employing a broader range of concentrations will be essential to accurately quantify the potency of these bacterial factors and to facilitate more rigorous comparisons between treatments.

Concurrently, our gene expression analyses demonstrated that significant upregulation of EMT markers *Snail1* and *FN1* occurred only at the higher bacterial concentration (MOI 100), while *CDH2* expression did not increase significantly at this MOI. In contrast, treatment with cell-free supernatant (CFS) at 5% (S1) and 7% (S2) led to a notable elevation in the expression of all three genes—*Snail1, FN1*, and *CDH2*. These findings indicate that the induction of EMT-related genes by *S. epidermidis* is both concentration- and treatment-dependent. *Snail1* functions as a transcriptional repressor of epithelial markers, *FN1* (fibronectin) contributes to extracellular matrix remodeling, and *CDH2* (N-cadherin) enhances cell motility and invasive capabilities, all of which are key components of the EMT process facilitating cancer cell detachment, migration, and invasion (4, 8, 15).

The simultaneous occurrence of cytotoxicity and EMT gene upregulation presents a complex biological scenario. One plausible interpretation is that EMT activation may represent a cellular stress response to bacterial challenge, potentially enabling a subset of cancer cells to survive under adverse conditions by adopting a more invasive phenotype. Alternatively, EMT induction could reflect a pro-metastatic shift directly stimulated by bacterial components or inflammatory mediators present in the CFS. These hypotheses align with reports that bacterial lipoteichoic acid and other pathogen-associated molecular patterns can activate EMT via MAPK and NF-κB signaling pathways, linking microbial infection to tumor progression and metastasis (15).

Comparing our findings with the existing literature reveals both consistencies and novel contributions. While previous studies have documented the presence of *S. epidermidis* in breast tumors and its association with inflammation and immune modulation (5, 6), our work is among the first to demonstrate its direct influence on EMT-related gene expression in breast cancer cells. Furthermore, the differential cytotoxicity between live bacteria and CFS underscores the need to focus on secreted bacterial metabolites as key effectors in tumor-microbiota interactions. In contrast, certain probiotic bacteria, such as *Lactobacillus* species—have demonstrated anti-tumor properties by promoting cancer cell apoptosis

and modulating critical signaling pathways including AKT/PTEN. This emphasizes the species-specific and context-dependent nature of bacterial influences on cancer biology (16).

Despite these advances, our study has limitations that warrant consideration. We did not assess the cytotoxic or EMT-modulating effects of *S. epidermidis* and its secreted factors on normal, non-cancerous breast epithelial cells. Such analyses are crucial to determine the selectivity and safety of potential microbiota-targeted therapies and to define therapeutic windows that minimize off-target toxicity. Additionally, while gene expression changes provide valuable insights, protein-level validation and functional assays—such as migration, invasion, and in vivo metastasis models—are necessary to confirm EMT activation and its biological consequences. Further studies should focus on fractionating the cell-free supernatant to identify and characterize the individual metabolites responsible for the observed biological activities. This approach could facilitate the development of targeted therapeutic strategies.

Clinically, our findings suggest that *S. epidermidis* and its secreted products may contribute to breast cancer progression by promoting EMT and altering tumor cell viability, positioning bacterial metabolites as potential targets for novel interventions. Modulating the tumor microbiota or neutralizing pro-metastatic bacterial factors could complement existing therapies and improve patient outcomes. However, comprehensive evaluation of safety, efficacy, and mechanisms in physiologically relevant models remains essential before clinical translation.

In summary, this study advances the comprehension of the dualistic function of *S. epidermidis* in breast cancer, demonstrating that its secreted factors exert potent cytotoxic effects while simultaneously inducing EMT-related gene expression. These findings highlight the intricate interplay between tumor cells and their associated microbiota, emphasizing the need for integrated mechanistic and translational research to harness microbial influences for cancer therapy.

CONCLUSION

The microbial composition of breast tissue shows significant differences between healthy and cancerous environments, with resident bacteria playing varied roles in tumor initiation and progression. In this study, we investigated the impact of live *S. epidermidis* cultures and their cell-free supernatant on breast cancer cell viability and the expression of key genes involved in EMT. Our findings indicate that while lower concentrations of bacterial cultures and CFS allow continued proliferation of cancer cells, higher doses markedly reduce cell viability. Notably, both forms of *S. epidermidis* exposure led to increased expression of EMT-associated genes, suggesting a potential role for this bacterium in promoting metastatic behavior in breast cancer. To our knowledge, this is the first report to demonstrate that *S. epidermidis* can influence EMT-related gene expression in breast cancer cells. These results highlight the significance of the tumor-associated microbiota in cancer progression and suggest that targeting *S. epidermidis* or its secreted factors could offer a novel therapeutic approach. Future studies should aim to elucidate the underlying molecular mechanisms and evaluate the clinical implications of manipulating the tumor microbiome in breast cancer therapy.

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