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# **OPEN** Exploring the synergistic effects of vitamin D and synbiotics on cytokines profile, and treatment response in breast cancer: a pilot randomized clinical trial

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This study was designed to investigate the effect of vitamin D and/or synbiotics on the response to treatment, cytokines profile and hormonal biomarkers in breast cancer patients undergoing neoadjuvant therapy. A total of 76 patients were recruited and completed the course of the intervention between 2019 and 2021 in Kerman, Iran. breast cancer patients were randomly enrolled in this study. Patients divided into four groups to receive one of the following regimens: placebo, vitamin D, synbiotics and a combination of vitamin D and synbiotics. clinicopathologic parameters, inflammatory and anti-inflammatory biomarkers and hormonal levels were measured at the baseline and four months after intervention. The study results found no clear link between the interventions and achieving pathological complete response (pCR), and a similar trend was observed in Ki-67 index examination. After neoadjuvant therapy, TNF-α concentrations decreased, with vitamin D supplementation moderating this decline. Vitamin D supplemented groups showed a significant increase in serum IL-6 levels. While IL-10 levels decreased in the placebo group, all intervention groups were protected from this decline. Moreover, there was a notable increase in the anti-inflammatory index, particularly in the group receiving both vitamin D and synbiotic supplementation, suggesting potential synergistic anti-inflammatory effects from their combined administration. The outcomes suggest a potential anti-inflammatory function of this combination. Consequently, more extensive studies with prolonged follow-up periods and substantial sample sizes are warranted to thoroughly evaluate their potential benefits for breast cancer patients.

Keywords Breast cancer, Vitamin D, Synbiotics, Cytokine, Clinical trial, Prebiotics, Probiotics

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide. According to the American Cancer Society, there will be over 290,000 new cases of invasive breast cancer diagnosed in women in the United States this year, and more than 40,000 women will die from the disease<sup>1</sup>. The high incidence and mortality rates of breast cancer underscore the need for continued efforts to understand risk factors and find effective preventive and treatment strategies.

Previous research has identified certain risk factors for breast cancer, including older age, family history, mutations in the BRCA1 and BRCA2 genes, dense breast tissue, alcohol consumption, and postmenopausal

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hormone use. However, these factors do not account for all breast cancer cases, and lifestyle and environmental influences likely also play a role<sup>2</sup>. Identifying modifiable factors such as diet and nutrition that could help reduce breast cancer risk or enhance treatment response would have a significant impact on public health.

Inflammation and hormonal imbalances have been implicated in the initiation, promotion, and progression of breast cancer. Chronic inflammation in the breast microenvironment can lead to DNA damage, cellular proliferation, and angiogenesis, creating conditions favorable for cancer development. Inflammatory cytokines such as TNF-alpha, IL-1beta, and IL-6, and transcription factors like NF-kappa B and STAT3, which regulate expression of inflammatory genes, are often dysregulated in breast cancer<sup>3,4</sup>.

At normal physiological levels, these inflammatory cytokines help regulate immune responses and promote healing/tissue repair. However, in the context of breast cancer, they can contribute to tumor growth and progression. Increased or dysregulated levels of these cytokines in the breast tumor microenvironment can stimulate proliferation, survival, and spread of breast cancer cells. They also promote recruitment of immune cells that favor tumor growth instead of immune attack. This inflammation-driven tumor promotion effect highlights the detrimental role of inflammatory cytokines in breast cancer<sup>5</sup>.

Therefore, while inhibiting these cytokines could suppress breast cancer progression, completely blocking their effects may impair normal immune function. The key is to achieve regulation of these cytokines and immune pathways towards an anti-tumor balance. Lifestyle and natural interventions that can gently regulate inflammatory responses may be safer and more effective than complete cytokine inhibition<sup>6</sup>.

The effects of vitamin D in prevention, treatment and prognosis of breast cancer have been investigated in various studies<sup>7</sup>. The inverse correlation between higher serum concentrations of vitamin D and the risk of breast cancer incidence has been found throughout the mentioned studies, promoting the effective preventive role of vitamin D<sup>8</sup>.

Recent research has investigated the potential for natural compounds and lifestyle interventions to regulate inflammation and hormones, and thereby suppress tumor growth or enhance medical therapies<sup>9,10</sup>. For instance, vitamin D, omega-3 fatty acids, and curcumin have anti-inflammatory effects and may reduce levels of inflammatory cytokines and mediators in breast cancer<sup>11</sup>. Among these, the role of the human microbiome, particularly the gut and breast microbiota, has emerged as a critical area of interest<sup>12,13</sup>. Probiotics, live microorganisms that confer health benefits to the host, are increasingly recognized for their potential in modulating cancer risk, influencing immune responses, and affecting treatment outcomes<sup>14,15</sup>. As such, studies on combinations of diet, supplements, and exercise have promising implications for reducing inflammation and hormones that fuel breast cancer and dampening their detrimental effects. Therefore, there is a need for further research in this area to better understand the potential benefits and limitations of vitamin D and synbiotics supplementation in breast cancer patients. Given the current absence of studies evaluating the co-supplementation of vitamin D and synbiotics supplementation for synergistic effects between these two interventions, the present study has been designed to address this research gap.

# Method and material Study design

The protocol of this research has been registered at Iranian Registry of Clinical Trials (IRCT registration code: 20200313046756N1, date: 28–3-2020). This study is a double-blinded (physician and patients), randomized trial. 88 patients who referred to Javad Al-A'meh Clinic and Bahonar Hospital, Kerman, Iran was evaluated for eligibility criteria during 2019–2021. Informed consent was obtained from all participants before enrollment in the study.

Inclusion criteria:

Age  $\geq$  18 years

Histologically confirmed T1-3, N0-2, non-metastatic primary invasive breast cancer (ductal or lobular carcinoma)

Candidates for neoadjuvant therapy No prior treatment for the current breast cancer diagnosis Provision of written informed consent

Exclusion criteria:

History of vitamin D or synbiotics-containing products consumption in the past three months Previous medical history related to hormonal therapy Antibiotic usage within the past three months Presence of distant metastases Any non-invasive or non-ductal/lobular breast cancer histology

All pathological analyses were conducted by a singular pathologist. Estrogen and progesterone receptor levels and HER2 status were defined as per American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines<sup>16</sup>. Also, Subsequent to neoadjuvant therapy, assessments were made regarding residual cancer burden (RCB) and the Miller-Payne score<sup>17</sup>.

# Randomization

Patients were randomly assigned to one of four treatment groups (factorial) using a computer-generated random permuted block. Treatment allocation was not masked for researchers. Each participant will receive a unique identifier according to the random number table that will appear on the patient's drug box to ensure anonymity. Medicines did not differ in appearance, taste and odor. The patients consumed two capsules (synbiotic and Vitamin D) on a daily basis.

# Interventions

The synbiotic supplements (Familact 2Plus, Zist-Takhmir, Tehran, Iran) consisted of eight strains of beneficial bacteria: *Lactobacillus casei, L. acidophilus, L. rhamnosus, L. salivarius, L. reuteri, Bifidobacterium lactis, B. longum, and B. bifidum* (each at  $1 \times 109$  CFU/g), plus 38.5 mg fructo-oligosaccharide. Vitamin D was administered as 1000 IU/day pearls (Arvand Pharmed, Tehran, Iran). Patients in all groups took two capsules daily—one vitamin D (or placebo) and one synbiotic (or placebo)—for 18 weeks. The synbiotic capsules and placebos were indistinguishable in appearance, fragrance, color, shape, and size. Patients were instructed to store the capsules in a refrigerated environment (2–7 °C) for the duration of the study.

Patients were randomly assigned to one of four treatment groups which is shown in Fig. 1:

Placebo group: Received placebo capsules for both vitamin D and synbiotics Vitamin D group (VitD): Received vitamin D 1000 IU/day and placebo for synbiotics Synbiotic group (Pro): Received synbiotic capsule and placebo for vitamin D Vitamin D + synbiotic group (VitD + Pro): Received both vitamin D 1000 IU/day and synbiotic capsule



Fig. 1. CONSORT diagram illustrating the flow of participants through each stage of the clinical trial.

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# **Clinical data**

Demographic and clinical information was obtained through personal interviews and medical records, documented in a checklist. Anthropometric measurements, including height and weight, were taken for all participants at the beginning of the study. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m<sup>2</sup>). Tumor size, grade, stage, RCB, and Miller-Payne score were retrieved from the patient medical records.

#### Enzyme linked immunosorbent assay (ELISA)

At the baseline and end of the intervention peripheral blood samples were collected from patients and stored at -60 C refrigerator. Serum vitamin D and Estradiol levels were measured using ELISA kits (Monobind, Lake Forest, USA; and Kit Cat. No DCM003-10; DiaMetra, Segrate, Italy, respectively). Serum levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IL-10 were assessed using ELISA kits as per the manufacturer's instructions (Invitrogen, Termofisher scientific, Cleveland, OH, USA).

#### Immunohistochemistry

Estrogen receptor (ER), progesterone receptor (PR), primary Ki67 expression (from the core needle sample), and HER2 status were collected from the patient medical documents. Tumor tissue blocks following surgery, reviewed by an expert pathologist for immunohistochemistry (IHC), are sliced into 4- $\mu$ m sections. To evaluate secondary Ki67 expression, slides undergo recutting, xylene immersion, distilled water washing, and boiling in Tris buffer (pH = 9). After cooling, slides are immersed in a 1:9 ratio of oxygenated water to distilled water for 10 min, followed by EDTA buffer washing. A marker outlines the tissue, and a 5-min protein block application precedes washing and the addition of the primary antibody. Incubation occurs for half an hour at 37 degrees Celsius, followed by a final wash.

Following the primary antibody, two secondary antibodies are applied to the slides for 20-min intervals, each followed by a washing buffer rinse. Subsequently, a chromogen DAB step is implemented on the slides for 1–3 min, succeeded by a distilled water wash and immersion in hematoxylin. After water washing and water extraction, the slides are mounted. The same collaborative pathologist assesses all slides for Ki67 expression. Randomly selecting 10 high-magnification fields (400×), the pathologist counts over 100 cells in each field, reporting Ki67 expression as a percentage of the total cells enumerated.

#### **Outcome measures**

The primary outcomes were the pCR vs. residual tumor, providing an assessment of treatment success. Additionally, secondary measures involved the analysis of inflammatory biomarkers, offering valuable insights into the overall impact of the intervention on inflammatory processes within the subjects.

#### Statistical analysis

Descriptive statistics in tables and plots were mean (standard deviation), median (interquartile range), and frequencies (%). The Shapiro–Wilk test assessed data distribution. Between-group comparisons at baseline and study end employed ANOVA, Kruskal–Wallis, and Chi-square/Fisher exact tests. mixed effect model was used for within-group univariate analysis. Significance level was 0.05, with post hoc adjustments for multiple testing. Repeated measures multivariate analysis was done using generalized estimating equations (GEE) in R program (geepack). GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was utilized for graph plotting in this study. The study followed intention to treat analysis, considering all randomized subjects, irrespective of non-compliance, deviations, withdrawals, or post-randomization events.

#### Results

# Study population and adherence

Between 2019 and 2021, a cohort of 88 eligible women participated in the study. Ten patients were excluded due to non-adherence, while one patient succumbed during the study. Additionally, one patient was excluded as they exhibited positive distant metastasis. the remaining number of patients reduced to 76.

The more detailed data is available in Fig. 1 of the CONSORT diagram. Table 1 provides an overview of the baseline characteristics of the participants. No significant differences were observed between groups concerning age, weight, body mass index (BMI), tumor histologic type, ER and PR status, as well as tumor grade. For ease of presentation, subsequent sections detailing the results are organized into baseline, after 4 months, and pairwise comparisons, where applicable.

# Vitamin D and estradiol profile

#### *Baseline and after 4 months*

Estradiol and vitamin D levels were not significantly different in four groups at baseline, and the data depicted in section A in Fig S1, and S2, respectively. no significant between group variation was seen in serum estradiol level following intervention. Succeeding the intervention, serum vitamin D level was significantly up-regulated in all interventional groups compared to the placebo group (p < 0.0001 for Vitamin D, p < 0.0001 for Synbiotics, p < 0.0001 for VitD + Pro). Also, it became apparent that vitamin D level in VitD + Pro group is meaningfully higher than Pro group (41.81 ng/ml ± 5.83 vs 35.24 ng/ml ± 10.54, p = 0.0212) but no significant difference was seen between VitD, and Pro groups in terms of serum vitamin D.

	Placebo	Vitamin D + Placebo	Synbiotics + Placebo	Vitamin D + Synbiotics	
Characteristic	Group A (n = 22)	Group B (n=15)	Group C (n = 21)	Group D (n = 18)	Significance
Age, years (mean)	50	49.6	46.5	46.7	0.61
Weight (kilogram)	72.4	72.7	71	72.8	0.92
BMI (kg/m <sup>2</sup> )	28.2	28.3	28.9	29.1	0.58
Tumor grade					
Low	3 (13.6)	2 (13.3)	3 (14.3)	0	0.251
Intermediate	16 (72.2)	6 (40.0)	11 (52.4)	12 (66.6)	
High	3 (13.6)	6 (40.0)	7 (33.3)	5 (27.7)	
Not known	0	1 (6.6)	0	1 (5.5)	
Histologic Type					
Ductal	20 (90.9)	14 (93.3)	19 (90.5)	18 (100)	0.709
Lobular	2 (9.1)	1 (6.6)	2 (9.5)	0	
ER status					
Positive	16 (72.7)	9 (60)	11 (52.4)	11 (61.1)	0.701
Negative	6 (27.3)	5 (33.3)	9 (42.8)	7 (38.9)	
PR status					
Positive	13 (59.1)	7 (46.6)	9 (42.8)	9 (50)	0.842
Negative	9 (40.9)	7 (46.6)	11 (52.4)	9 (50)	
Not known	0	1 (6.6)	1 (4.7)	0	
HER2 status					
0	13 (59.1)	7 (50)	11 (55)	9 (50)	0.933
1	1 (4.5)	1 (7.1)	3 (15)	2 (11.1)	
2	2 (9.1)	1 (7.1)	0	2 (11.1)	
3	6 (27.3)	5 (35.8)	6 (30)	5 (27.8)	

Table 1. Baseline demographic and clinicopathological characteristics by randomized treatment groups.

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#### Pairwise comparison

Pairwise analyses showed that a universal significant down-sloping pattern were happened in all group regarding the serum level of estradiol following neoadjuvant therapy (p = 0.0113 for placebo, p < 0.0001 for VitD, p < 0.0001 for Pro, p = 0.0008 for VitD + Pro) (Fig S1, sections B, and C). The serum vitamin D level was increased following treatment (p < 0.0001 for all groups) (Fig S2, sections B, and C).

# Inflammatory profile

TNF-α

<u>Baseline and after 4 months.</u> There were no significant changes in TNF-alpha levels between different groups at both the baseline and after 4 months (Fig. 2, section A).

Pairwise comparison. There was a significant decrease in TNF-α levels in the placebo group (2.041 ng/ ml ± 0.009 vs. 2.032 ng/ml ± 0.007, p = 0.004) (Fig. 2, sections B, and C). Although there was a decrease in TNF-α levels in the VitD group, the reduction was not statistically significant (2.046 ng/ml ± 0.010 vs. 2.037 ng/ml ± 0.01, p = 0.0937). A significant decrease in TNF- α levels was observed in the Pro group (2.042 ng/ml ± 0.009 vs. 2.034 ng/ml ± 0.006, p = 0.0285). The TNF- α level remained unaltered in VitD + Pro group (2.038 ng/ml ± 0.010 vs. 2.039 ng/ml ± 0.005, p = 0.9754), indicating that the combination of vitamin D and synbiotics did not lead to the expected reduction in TNF-α observed in the other groups.



Fig. 2. Serum TNF-alpha levels at baseline and after 4 months of intervention.

# IL-1 $\beta$

Baseline and after 4 months. There is no significant difference between groups (Fig S3, section A).

Pairwise comparison. There were no statistically significant changes noted (Fig S3, sections B, and C).

#### IL-6

Baseline and after 4 months. There were no significant differences observed at the baseline and the end of the study (Fig. 3, section A).

<u>Pairwise comparison</u>. After 4 months of treatment, a statistically significant increase was observed in the VitD (2.044 ng/ml±0.012 vs. 2.059 ng/ml±0.011, p=0.0432) and the VitD + Pro group (2.045 ng/ml±0.013 vs. 2.061 ng/ml±0.019, p=0.0220) (Fig. 3, sections B, and C).

#### Anti-inflammatory profile

#### Baseline and after 4 months

In terms of IL-10 levels, significant differences were observed at baseline (Fig. 4, section A), with the placebo group displaying significantly higher IL-10 levels ( $3.518 \text{ ng/ml} \pm 1.144$ ) compared to other study arms (p < 0.01 for all groups). By the end of the study, the serum levels of IL-10 were comparable across all groups.

#### Pairwise comparison

The placebo group showed a substantial decrease in IL-10 levels (3.320 ng/ml  $\pm$  0.964 vs. 2.475 ng/ml  $\pm$  0.816, p = 0.0068) (Fig. 4, sections B, and C). In contrast, all interventional groups were protected from a decrease in IL-10 levels, and this indicates a stable IL-10 profile in these groups over the study period. To assess the simultaneous changes of pro and anti-inflammatory cytokines, anti-inflammatory index (IL-10/TNF- $\alpha$  ratio) was calculated and it showed that the anti-inflammatory index is solely increased in VitD + Pro group (1.014  $\pm$  0.056 vs. 1.291  $\pm$  0.664, p = 0.0403) following intervention (Fig. 5, sections B, and C).



Fig. 3. Serum IL-6 levels at baseline and after 4 months of intervention.



Fig. 4. Serum IL-10 levels at baseline and after 4 months of intervention.



Fig. 5. Anti-inflammatory index ratio at baseline and after 4 months of intervention.

# Multivariate longitudinal analysis

TNF-  $\alpha$  has a statistically meaningful positive relationship with the vitD group (p = 0.016). None of the serum vitamin D, estradiol and the interventional groups were associated with IL-1 $\beta$ . IL-6 has a positive significant relationship with serum vitamin D level (p = 0.0052). all interventional groups, including vitD (p = 0.0395), Pro (p = 0.0011), and vitD + Pro (p = 0.0275) groups were positively associated with serum IL-10 which indicates the anti-inflammatory role of the interventions. However, this relationship was not observed with serum vitamin D (p = 0.4286) and estradiol level (p = 0.2924).

# **Pathologic features**

All study arms, except for the VitD group, had a higher relative number of patients with residual tumors compared to those achieving pCR (Fig. 6). A difference was found between the placebo group and the VitD group, with a nonsignificant contrast in these outcomes (p=0.05). To measure the tumor growth tendency, Ki-67 marker has been compared in patients with residuals tumor after neoadjuvant therapy. no differences were observed in between-group comparisons at both baseline and the end of the study (Fig S4, section A). However, in pairwise comparisons, a decreasing trend was noted in all study groups (Fig S4, sections B, and C). Only the Placebo group exhibited a significant reduction in Ki67 percentage ( $30.20 \pm 18.85$  vs.  $10.33 \pm 8.06$ , p=0.0042).

Estimated tumor size at baseline and following intervention is statistically not significant in between groups and within groups comparisons (Fig S5). In terms of RCB, and Miller-Payne score no significant difference was observed (data were not shown).

### Discussion

The present study aimed to explore the effect of synbiotics, vitamin D, and their combination on breast cancer patient pathologic response and inflammatory profiles. Studies showed that neoadjuvant chemotherapy can increase the prevalence of vitamin D insufficiency<sup>18</sup>. On the other hand, the results from a meta-analysis in 2021 concluded that lower levels of serum vitamin D was associated with reduced survival in breast cancer<sup>19</sup>. However, the findings did not indicate a clear link between the interventions and achieving pCR. A similar pattern was observed in Ki-67 index.

This is in contrast with the findings of a cohort study in 2018 involving 144 breast cancer patients undergoing neoadjuvant therapy, that showed the presence of vitamin D deficiency was associated with a lower rate of achieving pCR<sup>20</sup>. Data obtained from the NEOZOTAC trial study (2016) indicated that post neoadjuvant therapy, there was a reduction in serum vitamin D levels; however, no significant association with pCR was observed<sup>21</sup>. Similar outcomes were observed in another investigation (2018) involving 374 breast cancer patients undergoing neoadjuvant chemotherapy. This study revealed that the status of vitamin D was not linked with either pCR or survival rates<sup>22</sup>. This could be attributed to either the brief period between intervention and result analysis or the limited size of the sample. Additionally, adjusting for molecular subtype may help control for this variability and provide a more robust assessment of the relationship between interventions and achieving pCR.

Concerning Ki-67 expression, a study conducted in 2014 involving 33 postmenopausal breast cancer patients who received vitamin D supplementation (0.50  $\mu$ g/day PO, for 30 days) compared to 23 control subjects revealed a moderate decrease in Ki-67 levels (15.7% vs. 10.2%, p = 0.03) following the intervention<sup>23</sup>. This inverse correlation between serum vitamin D level and Ki-67 was also corroborated in another study conducted in 2022 on 145 patients from Iran<sup>24</sup>. A randomized phase two clinical trial conducted in 2019 investigated the effect of high-dose vitamin D supplementation (40,000 IU/day for 2–6 weeks before surgery) on Ki-67 and apoptosis but found no significant differences<sup>25</sup>. In conclusion, vitamin D supplementation alone or in combination with synbiotics, did not seem to impact tumor mitotic index in pre-surgical period.



Fig. 6. Pathologic response following interventions.

Following neoadjuvant therapy, the levels of TNF- $\alpha$  were reduced. In cohorts receiving vitamin D supplementation, this reduction in TNF- $\alpha$  levels was attenuated. In groups receiving vitamin D supplementation, there was a notable increase in serum IL-6 levels. This is in contrast with the findings of a case–control study in 2022, in which a negative correlation were observed between vitamin D deficiency and IL-6 (r2 = 0.537, p < 0.0001), and TNF- $\alpha$  (r2 = 0.484, p < 0.0001) levels<sup>26</sup>. A randomized, triple-blind, placebo-controlled trial in 2021 assessed the relationship of symbiotic supplementation with a low-calorie diet in breast cancer survivors, and showed that the intervention could decrease TNF- $\alpha$  level significantly<sup>27</sup>. In research conducted by Vafa et al.<sup>28</sup>, breast cancer patients with unilateral lymphedema were supplemented with Lacto Care (Zist Takhmir Co., Tehran, Iran) over a 10-week period, coupled with a weight reduction regimen. Their findings indicated a decrease in IL-1, TNF- $\alpha$ , and leptin levels, a result that contradicts our own study outcomes.

Studies showed that TNF- $\alpha$  and might has contradictory roles in breast cancer and this effect might be stage specific<sup>29,30</sup>. The cross-talk between vitamin D and TNF- $\alpha$  can be affected by the vitamin D receptor gene polymorphism<sup>31</sup>. Also, it is necessary to consider the dysregulation of vitamin D metabolism in tumor tissues that may abolish the anti-tumor benefits of this essential nutrient<sup>32</sup>.

A notable reduction in IL-10 levels was observed in the placebo group, while all interventional groups were shielded from this decrease. Additionally, the anti-inflammatory index showed a significant increase essentially in the VitD + Pro group, suggesting potential synergistic anti-inflammatory effects of co-supplementation with synbiotics and vitamin D. The observations also agree with the results reported by<sup>33</sup>. the concurrent administration of vitamin D and synbiotics could potentially result in an enhanced anti-inflammatory response.

This study has several strengths and limitations that should be acknowledged. One of the key strengths of our work is its novelty; this is one of the first studies to investigate the combined effects of vitamin D and synbiotics on breast cancer treatment response and cytokines profile, providing novel insights into potential synergistic anti-inflammatory effects. The study utilized a double-blinded, randomized controlled trial design, which helps minimize bias and adds robustness to our findings. Additionally, we conducted comprehensive biomarker analyses, measuring a broad range of inflammatory and anti-inflammatory biomarkers, which allowed us to gain a detailed understanding of the immunomodulatory effects of the interventions.

However, the study also has several limitations. Firstly, the relatively small sample size limits the generalizability of our findings, and larger studies are needed to confirm our results and provide more statistically robust conclusions. Secondly, the four-month follow-up period may have been insufficient to observe long-term effects of the interventions, suggesting that future studies should consider longer follow-up periods to evaluate the sustained impact of vitamin D and synbiotics supplementation. Additionally, we did not collect detailed information on the specific chemotherapy regimens used by participants, frequency and severity of chemotherapy-induced adverse effects in groups which limits our ability to analyze the potential interactions between the interventions and different chemotherapy drugs. Future research should include this data to better understand the modulation of supplementation protocols based on chemotherapy treatments. Another limitation of our study was the limited scope of anthropometric measurements collected. We only measured height and weight to calculate BMI, but did not assess other potentially relevant anthropometric variables such as waist circumference, hip circumference, or waist-to-hip ratio. These additional measurements could have provided more comprehensive data on body composition changes and their potential impacts on inflammatory markers and treatment response. Moreover, the COVID-19 pandemic caused delays in follow-up and chemotherapy for some patients, which may have affected the study outcomes. Lastly, there is a possibility that some participants may have used other supplements during the study, which could have influenced the results. These limitations should be anticipated and addressed in future studies to provide a more comprehensive understanding of the impact of vitamin D and synbiotics supplementation in breast cancer patients.

#### Conclusion

In summary, our study explored the potential anti-inflammatory and therapeutic benefits of vitamin D and synbiotics supplementation in breast cancer patients undergoing neoadjuvant therapy. While our findings did not establish a clear link between the interventions and achieving pathological complete response (pCR) or significant changes in the Ki-67 index, there were notable observations in the inflammatory profiles. Specifically, vitamin D supplementation moderated the decline in TNF- $\alpha$  levels, increased serum IL-6 levels, and, along with synbiotics, protected against a decrease in IL-10 levels. The combination of vitamin D and synbiotics notably increased the anti-inflammatory index, suggesting potential synergistic anti-inflammatory effects. These outcomes suggest a potential anti-inflammatory function of this combination, which may contribute to the supportive care of breast cancer patients. However, the limitations of our study, including the small sample size and short follow-up period, highlight the need for more extensive studies. Future research should incorporate larger sample sizes, longer follow-up periods, and detailed chemotherapy regimen data to thoroughly evaluate the potential benefits and establish appropriate dosage and duration of supplementation with vitamin D and synbiotics in breast cancer patients. By adhering to such rigorous methodological designs, researchers may contribute significantly to the advancement of clinical practice knowledge in this important area.

#### Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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# Author contributions

A.T., and M.R. conceptualized the study; M.K.H., V.M., B.K.K., A.T., and E.J., designed the research methodology and supervised the research; A.T., M.H.E., and Z.S. performed the laboratory research; E.J. performed the histological study; A.R. prepared the initial drafts; N.N., and M.R. analyzed the data; M.K.H. achieved the funding; All the authors read, revised, and approved the final manuscript.

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# **Competing interests**

The probiotic supplements were graciously supplied by Zist Takhmir Pharmaceutical Company to Dr. Vahid Moazed, with a transparent affirmation that the company did not participate in the formulation of the study hypothesis, design, implementation, analysis, or interpretation thereof. The other authors declare no competing interests. The authors of this study gratefully acknowledge the financial support provided by the Kerman University of Medical Sciences.

# **Ethical approval**

The research study's protocol has been officially registered in the Iranian Registry of Clinical Trials (IRCT) under the registration number IRCT 20200313046756N1 (date: 28–3-2020). The study was conducted in accordance with the Declaration of Helsinki, and informed consent was obtained from all participants before enrollment. The experimental protocols involving human participants and/or the utilization of human tissue samples received review and authorization from the Ethics Committee of Kerman University of Medical Sciences. The project underwent ethical scrutiny and was approved, adhering to the ethical standards observed by the committee. The ethical approval identifier for this study is IR.KMU.REC.1398.709.

# Additional information

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