

## Suppression of the malignancy of mammary tumor in mice model by inactivated preparation of *Mycobacterium obuense*

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### Abstract

Breast cancer (BC) is a significant cause of global mortality in women. This study was aimed to evaluate the immune-activation of malignant BC via the administration of attenuated *Mycobacterium obuense*. For this purpose, an *in vivo* model was developed with BALB/c mice. Mice were injected with  $2.00 \times 10^6$  4T1 cells with breast tumor cell line. Forty-two mice were equally divided into control as well as low dose ( $0.20 \text{ mg } 100 \mu\text{L}^{-1}$ ) and high dose ( $0.50 \text{ mg } 100 \mu\text{L}^{-1}$ ) groups of *M. obuense* to investigate gene expression in the antitumor effects of *M. obuense*. In one group, paclitaxel was administrated as a choice drug in BC treatment. Antitumor manners were characterized by cytotoxicity against tumor target cells, size of the tumor and the expression of some BC metastatic genes together with pathology. The MTT assay demonstrated that different concentrations of both low and a high dose of bacteria did present no cytotoxicity effect on 4T1 cells. According to our findings, *M. obuense* significantly repressed tumor growth. *M. obuense* downregulated the expression of collagen type I alpha 1 (COLIA1), cFos, alkaline phosphatase (ALP), claudin 3 (cldn3), and conversely, activated transcription factor 4 (ATF4) and Twist related protein-1 (Twist1). All these alternations induced a decrease in the migratory and invasive capabilities of BC. The result of pathology was indicative of tumor regression in the paclitaxel and HK- *M. obuense* -recipient group. Thus, it seems most likely that *M. obuense* might impinge upon cell growth and metastatic behavior of malignant cells exerting anti-tumor activity in BC.

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### Introduction

Breast cancer (BC) is the most commonly detected cancer and the second reason of cancer-related death in women worldwide both in developed and developing regions.<sup>1</sup> Of note, metastasis to the regional lymph nodes and other organs presents a considerable problem, usually resulting in higher mortality. In this regard, it has been proposed that the patients who present metastatic breast cancer occasionally have a 5-year survival rate of 21.00%.<sup>1</sup>

Despite significant advances in the treatment of breast cancer, effectiveness, drug resistance, and adverse side effects are important issue in selection of treatment tools. In this regard, the side effects of paclitaxel (PTX; as a commonly used anticancer drug) include hair loss, diarrhea, pain, vomiting, nausea and lowered white blood

cell counts as well as increased risk of anemia and infection in patients.<sup>2</sup> This obstacle caused to the focus for adjuvant therapy in the cancer with less toxic agents and the potential to boost anti-tumor activity and decrease adverse side effects that are proposed to be an alternative strategy for cancer treatment.<sup>3</sup>

Inactivated *Mycobacterium obuense* (as a bacteria-based immunomodulators) preparation has represented immunotherapeutic traits as well as potentials as a vaccine when examined in various disease settings.<sup>4,5</sup> The *M. obuense* was selected primarily due to its documented anti-tumor effect.<sup>4,6</sup> Previously, it was also demonstrated that *M. obuense* acts on cells of the innate immune system like  $\gamma\delta$  T-cells, antigen-presenting cells and granulocytes by interaction with several receptors.<sup>7</sup> It is believed that activation of these cells could have a cytotoxic effect

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against tumors. Also, it is proposed that *M. obuense* restores type-1 response, influences cytotoxic cell immune function and downregulates type 2 response.<sup>6</sup> In another study, Fowler *et al.*<sup>8</sup> indicated that BCG, *M. vaccae*, and *M. obuense* activated an anti-tumor program in peripheral blood T-cells that was characterized by T<sub>H</sub>1 cytokine secretion and increased cytotoxic responses against tumor. Reportedly, inactivated *M. obuense* was safe and well-tolerated in patients with melanoma.<sup>9</sup>

Gene expression profiles are the most informative profiles for cancer characterization, therefore, in our study this transcriptional feature was utilized as a preliminary profile for *M. obuense* response analysis in breast cancer. Hence, we hypothesized that *M. obuense* might have potential to further induce expression of genes related in breast cancer suppression. In addition, since breast cancer exhibits resistance to many drugs and there is an immediate need for finding new therapies, we evaluated the anti-tumor effects of *M. obuense* in breast cancer as a single agent. We also aimed to elucidate the mechanism of *M. obuense* in epithelial-to-mesenchymal transition which is a key malignant trait in carcinoma cells.

## Materials and Methods

**Bacterial preparation.** Here, an inactivated suspension of the whole-cell *M. obuense* (deposited as NCTC 13365), produced by good manufacturing practice (GMP) was used as 500 mg mL<sup>-1</sup> stock solution. The 2.00% and 5.00% solutions were prepared using 0.20 and 0.50 µg of bacteria from the original stock in 100 µL<sup>-1</sup> normal saline, respectively. The prepared solutions were stored at 4.00 °C for further experiments. To prepare inactivated *M. obuense*, suspensions were autoclaved in borate buffered saline at pH 8.00 at 121 °C for 15 min. Suspensions were diluted in sterile borate buffered saline pH 8.00.

**Cell line preparation.** For tumor induction 4T1 (ATCC= CRL-2539), one of the highly metastatic BC cell lines, was provided from Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. Briefly, the cells were cultured in the 95.00 mL cell culture flask routinely using high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, altogether with 10.00% fetal bovine serum (FBS; Gibco), 100 U mL<sup>-1</sup> and 100 µg mL<sup>-1</sup> penicillin/streptomycin (Gibco), respectively. All flasks containing the cultured cells were incubated at 37.00 °C with 5.00% CO<sub>2</sub>.

**Cytotoxicity assay.** The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Thermo Fisher Scientific, Bremen, Germany) assay was used to evaluate the possible toxicity effect of the bacteria. Briefly, 1.50 × 10<sup>3</sup> of 4T1 BC cells were seeded into 96-well plates which subsequently were incubated in a cell incubator at 37.00 °C for 24 hr. For this experiment, 6.00 µL of low dose (12.00 ng) and 15.00 µL of high dose (30.00 ng) bacteria

were independently mixed in 1.50 mL distilled water. Then, 17 ascending concentrations (including 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 7.00, 9.00, 11.00, 13.00, 15.00, 17.00, 19.00, 21.00 µL) of both low dose and high dose of bacteria were separately added to the wells. Moreover, 0.10 mL dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) was administered to the control group. Each concentration was performed triplicate and incubated in a cell incubator at 37.00 °C for 24 hr. Based on a previous study,<sup>10</sup> the MTT solution was utilized to incubate the cells at 37.00 °C for four hr in a cell incubator. After that, the cell culture medium was depleted from each well, and 200 µL of DMSO together with 25.00 µL of Sorenson's buffer was added to each well for solubilization of MTT formazan crystals and then incubated for 30 min at 37.00 °C. In the final step, the absorbance of each well was evaluated using a microplate reader at 490 - 570 nm (Sunrise, Tecan, Switzerland).

**Animals, breast cancer induction and sampling.** A total number of 42 inbred healthy adult female BALB/c mice ages and weights ranging between 7 - 9 weeks and 25.00 - 35.00 g body weight, were purchased from Pasteur Institute, Iran. All animal work was conducted by all institutional guidelines following approval by University of Tabriz (No. FVM.REC.1397.08). Upon arrival at the University of Tabriz were mice divided randomly into seven groups of six mice each. First, the animals were kept under 12 hr of dark and light cycles with free access to water and food. Animals were weighed daily, starting from the 17<sup>th</sup> day post tumor induction till the day of euthanasia. Breast cancer was induced in 24 mice using 4T1 cells which was previously described<sup>11</sup> with some modifications. Indeed, 2.00 × 10<sup>6</sup> cells (0.20 mL per animal) were administered for tumorigenicity via subcutaneous injection in the left flank of the mice under an intraperitoneal anesthesia by ketamine (80.00 mg kg<sup>-1</sup>; Vetoquinol, Buckingham, UK) and xylazine (10.00 mg kg<sup>-1</sup>; Agrar, Soest, The Netherlands). Prior to injection, the tip of the teat was snipped and the needle was inserted directly into the mammary duct through the teat opening. The size of tumor mass was measured in perpendicular direction using microcalipers three times a week (Mitutoyo, Sakado, Japan) and similarly the tumor mass was calculated using the following formula previously explicated in study<sup>12</sup> based on the largest diameter (*a*) and the shortest dimension (*b*):

$$Tumor\ mass = (a \times b^2) / 2$$

After 10 days, when the daily measurement of the tumor size reached 6.00 mg, the cancerous mice were divided into four equal groups. The experiment, fully summarized in Table 1, was performed. In brief, two groups of the cancerous mice were treated by intralésional injection by low dose (0.20 mg 100 µL<sup>-1</sup>) and high dose (0.50 mg 100 µL<sup>-1</sup>) bacteria, independently.

**Table 1.** Different treatments with 100 µL of *Mycobacterium obuense* were conducted twice a week for 38 days into seven groups of six BALB/c mice in each.

Groups	Treatment twice a week for 38 days
1	Breast cancer induction treated with 2.00 mg <i>Mycobacterium obuense</i> (low dose bacteria); Intralesional
2	Breast cancer induction treated with 5.00 mg <i>Mycobacterium obuense</i> (high dose bacteria); Intralesional
3	Breast cancer induction treated with paclitaxel (15.00 mg kg <sup>-1</sup> ) (positive control); Intraperitoneal
4	Breast cancer induction treated with normal saline (negative control); Intralesional
5	Healthy mice treated with 2.00 mg <i>Mycobacterium obuense</i> (low dose bacteria); Intralesional
6	Healthy mice treated with 5.00 mg <i>Mycobacterium obuense</i> (high dose bacteria); Intralesional
7	Healthy mice treated with normal saline (healthy control); Intralesional

In one group as the positive control, paclitaxel (Taxol®; Sobhan Oncology, Rasht, Iran) was diluted in 0.90% sterile sodium chloride solution before injection. It was administrated as a choice drug in BC treatment. Moreover, the last cancerous group was treated with normal saline as a negative control. Eventually, three healthy groups were injected by saline, low dose, and high dose bacteria. The treatments were conducted twice a week continuously from day 10 to 38. All animals were euthanized- they are typically killed with carbon dioxide, and their necks are broken just to make sure- at the end of the experiment (day 38). Tumor tissue masses were extracted and stored at - 80.00 °C for further molecular analyses. Besides, the tumor masses and other organs comprising the liver and lung were given for histopathology, thus, were placed in a 10.00% formalin solution. Also, in low-dose and high-dose control groups, the liver, kidney and brain were isolated to evaluate the probable toxicity or other side effects of *M. obuense*.

**RNA extraction and molecular analysis.** To evaluate the expression levels of cancer-relevant genes including collagen type I alpha 1 (COLIA1), osteopontin (OPN), cFos, activating transcription factor 4 (ATF4), alkaline phosphatase (ALP), lymphotactin (XCL1), claudin 3 (cldn3), Twist related protein-1 (Twist1) and the total RNA of tumor tissues from all affected mice per group were extracted using FavorPrep Mini Kit (Favorgen, Ping-Tung, Taiwan). All of these genes possessed the invasive potential of breast cancer cells. RNA quantity and purity were evaluated using Bio-Rad spectrophotometer (Bio-Rad, Hercules, USA). Having used the cDNA Synthesis Kit (Yekta Tajhiz, Tehran, Iran), complementary DNA (cDNA) was amplified from 5.00 µg of total RNA to a final 20.00 µL reaction. Analysis of the target genes was performed by quantitative real-time reverse- transcription -polymerase chain (q-RT-PCR) reaction as well as SYBR® Premix Ex Taq™ II (Yekta Tajhiz). The PCR amplification of the target genes was prepared in 20.00 µL reaction total volume as follows: SYBR green supermix: 10.00 µL, each forward and reverse primer: 20.00 pmol of cDNA template: 1.00 µL. It should be emphasized that PCR amplification was run in triplicate to decrease the experimental error. The 2<sup>-ΔΔCT</sup> method was used to evaluate the fold change of mRNA expressions for the target genes. The β-actin gene was also used as an internal control (Table 2).

**Table 2.** Primers selected for the quantitative reverse transcription-polymerase chain reaction (q-RT-PCR) analysis in tumor mass of mice with experimental breast cancer.

Target gene	Nucleotide sequence (5'→3')
<b>Collagen type I alpha 1</b>	F: AGATTGAGAACATCCGCAGCC R: TCCAGTACTCTCCGCTTCCA
<b>Osteopontin</b>	F: CCAATGAAAGCCATGACCACA R: CGTCAGATTTCATCCGAGTCCAC
<b>cFos</b>	F: AATGGTGAAGACCGTGTCCAGGA R: CCCTTCGGATTCTCCGTTTCT
<b>Alkaline phosphatase</b>	F: TCCACGTTTTACATTTCGG R: CCCGTTACCATATAGGATGGCC
<b>ATF4</b>	F: GAGCTTCCTGAACAGCGAAGTG R: TGGCCACCTCCAGATAGTCATC
<b>Lymphotactin</b>	F: AGACTTCTCCTCCTGACTTTTCT R: GGACTTCAGTCCCCACACC
<b>Claudin 3</b>	F: GGCGGCTGCTCACCTTA R: CGTACAACCCAGCTCCCATC
<b>Twist related protein-1</b>	F: AGCGGGTATGGCTAACG R: GGACCTGGTACAGGAAGTCCA
<b>β-actin</b>	F: GATTACTGCTCTGGCTCCTAGC R: GACTCATCGTACTCCTGCTTGC

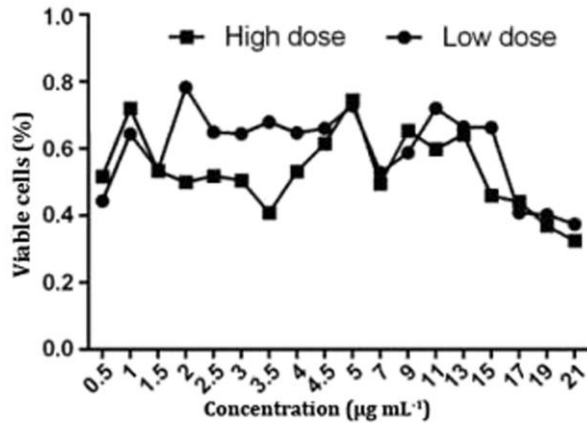
ATF4: activating transcription factor 4.

**Histopathological studies.** Formalin-fixed tissue samples were passaged routinely, embedded by paraffin, sectioned and stained using Hematoxylin and Eosin (H&E). The tissue sections including the liver, lung and tumor mass were studied under a light microscope (Olympus, Tokyo, Japan) for tumor proliferation, invasion and distance metastasis associated with the possible histopathological lesions comprising vascular congestion, hemorrhage, necrosis and inflammatory cell infiltration. Besides, the liver, kidney and brain were scrutinized for toxicity criteria in low-dose and high-dose control groups.

**Statistical analysis.** Statistical testing was carried out using SPSS Software (version 25.0; IBM Corp., Armonk, USA). Statistical differences were studied using one way ANOVA followed by LSD. Deviations from *p* values less than 0.05 were considered significant.

## Results

**Cytotoxicity evaluation using MTT cell viability assay.** According to MTT assay, different concentrations of both low dose and a high dose of bacteria did not show cytotoxicity effect on 4T1 BC cells (*p* > 0.05), (Fig. 1).



**Fig. 1.** Evaluation of the cytotoxicity effect of bacteria by MTT assay. There were no significant differences between high dose, low dose and control groups ( $p > 0.05$ ,  $n = 3$ ).

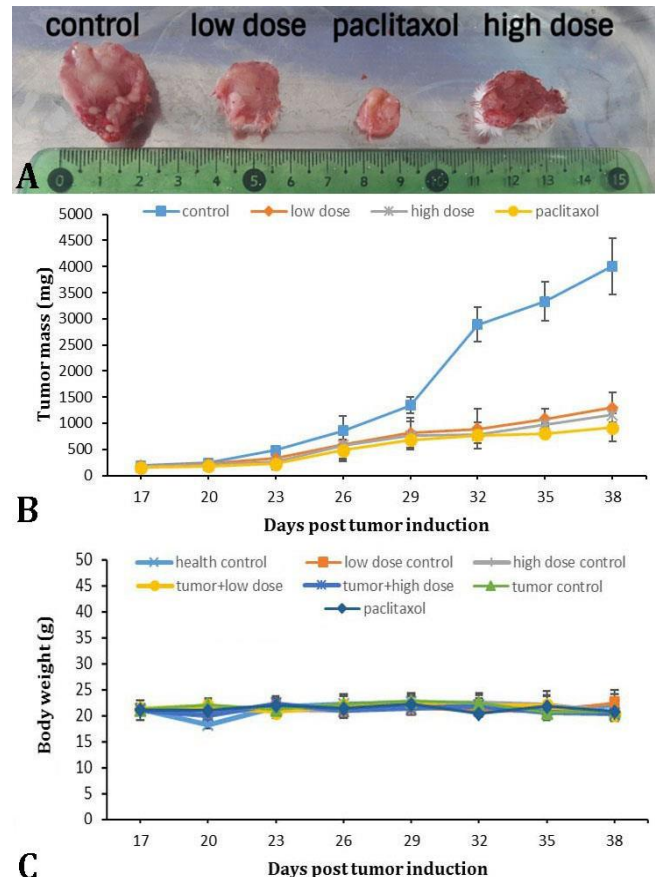
**Effect of *M. obuense* on animal weight.** To verify whether the administrated *M. obuense* induced symptoms of intoxication, the mice were constantly weighed during the experiment. Here, the body weight evaluation did not show difference between control and treated groups indicating that the present treatment did not have adverse side effects on the body weight (Fig. 2).

**Effect of *M. obuense* on tumor size.** The tumor size was measured during the experiment to confirm whether the *M. obuense* administration impacted the tumor tissue. A significant effect was observed in the treatment groups using one-way ANOVA upon the treatment time ended (Fig. 3). There were no significant differences until day 32, however, after day 32 there were significant differences in the tumor size from the control group and bacterial treated groups. There were a significant difference between taxol treated group and control group, also. There were no statistically significant differences between two doses of bacteria or between taxol treated group and *M. obuense* received groups.

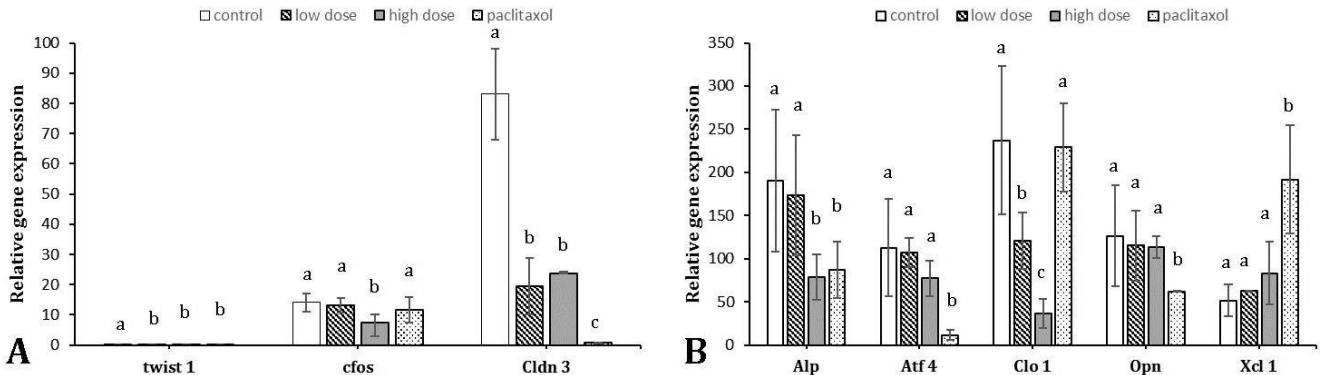
**Effects of *M. obuense* on COLIA1, OPN, cFos, ALP, ATF4, XCL1, cldn3, and Twist1 genes expression.** During this phase of investigation, the expression of COLIA1, OPN, cFos, ALP, ATF4, XCL1, cldn3 and Twist1 genes in tumor samples following *M. obuense* treatment was investigated. The obtained *M. obuense* concentrations were used to analyze the impact exerted on expression: 0.20 and 0.50 mg. In the present findings, no significant impact of the '*M. obuense* concentration' factor was detected on ATF4, XCL1, and OPN expression (Fig. 3). A remarkable effect of *M. obuense* on the gene expression level for COLIA1, Twist1, cldn3, ALP and cFos genes were observed. In brief, the expression levels of genes were mostly affected by a high dose of bacteria.

**Histopathological findings.** The histopathological examination showed severe malignancy in the control group due to displaying a high nuclear/cytoplasmic ratio, presence of bizarre neoplastic cells, cell degeneration, and

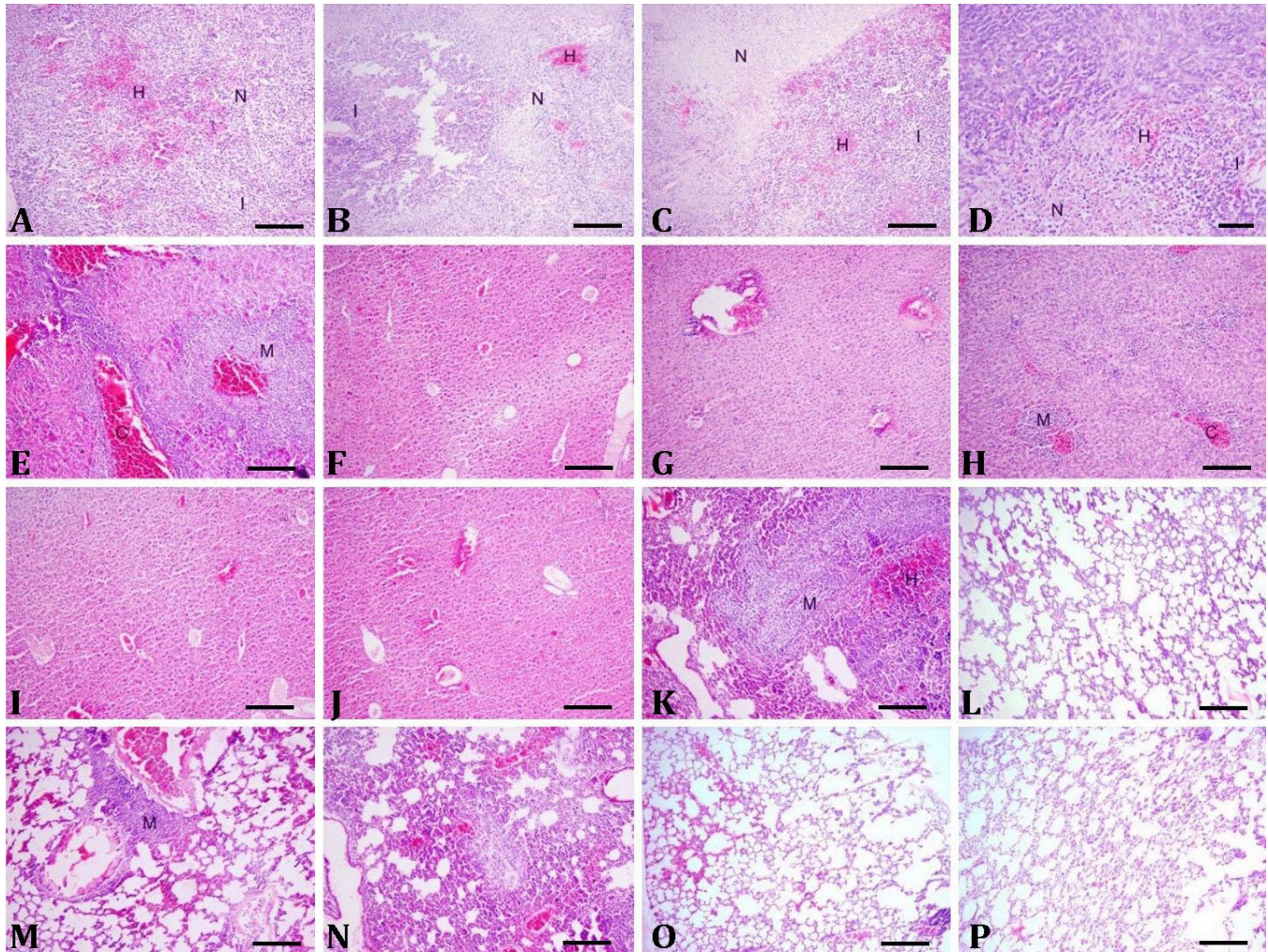
necrosis associated with severe hemorrhage (Fig. 4). The pathological lesions were remarkably decreased after using paclitaxel ( $p < 0.05$ ). Similarly, the histological changes and tumor malignancy were considerably attenuated in the low dose and particularly in high dose in *M. obuense*-recipient groups ( $p < 0.05$ ). In both bacteria-treated groups, higher cell degeneration and necrosis together with mild hemorrhage and negligible bizarre neoplastic cells were observed. Extensive metastases associated with severe coagulative necrosis, vascular congestion and hemorrhage were pinpointed in the liver and lung tissue sections of the tumor group treated by saline. Microscopic metastases to the liver and lung as well as other related histopathological lesions were significantly reduced ( $p < 0.05$ ) in the tumor + paclitaxel group and also in tumor + low dose and tumor + high dose groups. Microscopically, there were no toxic effects such as hemorrhage, cellular degeneration and necrosis on the important organs including the brain, liver and kidney in the control-low dose and control-high dose groups.



**Fig. 2.** A) Image of breast tumor harvested from mice carrying 4T1 tumors 38 days after injection in four treated groups. B) Inactivated *M. obuense* treated breast cancer grew slower than control group *in vivo*. C) Mouse weight. Data are expressed as a mean  $\pm$  SD ( $n = 6$ ). Significant difference in tumor size was observed between control (tumor- induced, no *M. obuense* or paclitaxel) and other groups after adjustment for time.



**Fig. 3.** Analysis of breast cancer tumor genes after the *M. obuense* treatment. **A)** *cfos*, *cldn3*, and *Twist1* genes, and **B)** *ATF4*, *XCL1*, *Alp*, *COLIA1* and *OPN* genes. <sup>abc</sup> Different superscripts indicate a significant difference ( $p < 0.05$ ).



**Fig. 4.** Malignant breast carcinoma induced by 4T1 cell line, mice, H&E (A-C: bars = 200  $\mu$ m; D: bar = 60  $\mu$ m). **A)** Control group treated with normal saline (bar = 200  $\mu$ m). **B)** Breast carcinoma treated with paclitaxel 15.00 mg  $kg^{-1}$ . **C)** Breast carcinoma treated with 5.00% *M. obuense* (high dose bacteria). **D)** Breast carcinoma treated with 2.00% *M. obuense* (low dose bacteria). Liver in malignant breast carcinoma induced by 4T1 cell line, mice, H&E (E-J, bars = 200  $\mu$ m). **E)** Tumor-control group treated with normal saline. **F)** Breast carcinoma treated with paclitaxel 15.00 mg  $kg^{-1}$ . **G)** Breast carcinoma treated with 5.00% *M. obuense* (high dose bacteria). **H)** Breast carcinoma treated with 2.00% *M. obuense* (low dose bacteria). **I)** Low-dose control group with normal structure. **J)** High-dose control group with normal structure. Lung in malignant breast carcinoma induced by 4T1 cell line, mice, H&E (K-P, bars = 200  $\mu$ m). **K)** Tumor-control group treated with normal saline. **L)** Breast carcinoma treated with paclitaxel 15.00 mg  $kg^{-1}$ . **M)** Breast carcinoma treated with 5.00% *M. obuense* (high dose bacteria). **N)** Breast carcinoma treated with 2.00% *M. obuense* (low dose bacteria). **O)** low-dose control group with normal structure. **P)** High-dose control group with normal structure. H: hemorrhage, N: necrosis, I: inflammatory cells, C: congestion, and M: metastatic cells.

## Discussion

Administration of anti-tumor therapeutic agents through intralesional (IL) injection has been used for the treatment of BC. In this regard, it was previously suggested that IL treatment strategy can modulate local disease and reduce potential complications of surgical therapy and systemic toxicity.<sup>13</sup> While many of these agents result in suppression of injected lesions, systemic responses are rarely presented, thus, limiting this approach for patients with the metastatic tumors. Immunotherapeutic tools associated with IL approach to promote anti-tumor immune responses are being proposed as a potential strategy to lead both local and systemic tumor regressions. IL injection of agents that can enhance the expression of tumor-specific antigens, promote presence and activity of immune cells or increase ongoing immunity may leading to the induction of strong systemic anti-tumor immunity. In this regard, injection of adjuvants such as Bacillus Calmette-Guérin (BCG) has been shown to increase tumor-specific immunity in human and mice.<sup>13,14</sup> Moreover, IL injection of rose Bengal in murine models of melanoma and BC in mice has previously been reported.<sup>15,16</sup>

Alterations in body weight are commonly related to the adverse effects of drugs and chemicals and it will be regarded significant if the weight loss exceeds 10.00% from the initial body weight.<sup>17</sup> In this research, the body weight evaluation did not show a notable disparity between control and treated groups implying that the present treatment did not have any detrimental side effects on the body weight.

The MTT test is frequently recommended and used for the evaluation of the cytotoxic impact of various agents in the laboratory condition. In the current study, it was used for the scrutiny of the probable toxicity effect of the bacteria. As expected, there was no evidence of cytotoxicity effect following treatment with different doses of bacteria in BC cells *in vitro*. Additionally, the data were confirmed in further *in vivo* histopathological evaluations of the tissue samples in which tissue structures were found normal after administration of the low dose and high dose of bacteria to healthy animals.

The findings of our study showed that *M. obuense* down-regulated CLDN3 in breast cancer when used in a low dose. CLDN3 belongs to a family of proteins essential in tight junction formation and function. Recently, it is reported that CLDN3 gene expression contributes to the invasive potential of breast cancer cells. Zhang *et al.* pointed out that silencing the expression of CLDN3 in adenocarcinoma cells also inhibited tumor growth *in vivo*.<sup>18</sup> Similarly, *M. obuense* downregulated CLDN3 and hampered tumor growth in this research.

The XCL1, lymphoactin, as the sole member of the C chemokine family, plays a pivotal role in chemotaxis of natural killer (NK) and CD4+ and CD8+ T cell responses.

Although the functional role of XCL1 has not yet been verified in BC, XCL1 has been used in combination with interleukin-2 (IL-2) in patients with neuroblastoma.<sup>19</sup> XCL1 has been presented to reduce tumor growth and increase survival in mouse melanoma through the induction of IL-2 and production of interferon- gamma from T cells.<sup>20</sup> In our research, XCL1 was increased in both treated groups although the differences were not significant compared to the control group.

There are several experimental data presenting evidence of aberrant Twist1 reactivation in human BC associated with tumorigenesis and lung metastasis formation.<sup>21,22</sup> Some studies propose the role of Twist1 in carcinogenesis depends on various tissue environments and/or oncogenic factors.<sup>23,24</sup> Twist1 is expressed at early stages of tumorigenesis which is essential for the initiation of skin tumors. The knockdown of Twist1 in established BC cell lines inhibits their metastases in immune-defective host mice.<sup>24,25</sup> In our study, *M. obuense* reduced the Twist1 level substantially. The size of tumors shrank markedly in *M. obuense*-recipient groups, too.

Angiogenesis is an essential limiting factor in tumor growth and progression. Growing evidence reports that ATF4 relates to tumor angiogenesis. It has been previously demonstrated that tumor-associated macrophages (TAMs) in the tumor microenvironment (TME) are proangiogenic and the infiltration of macrophages in tumor tissues is influenced by ATF4 overexpression.<sup>26</sup> Our results showed a fall in ATF4 gene expression in *M. obuense*-treated groups. The previous study showed that ATF4 enhanced the proliferation of breast cancer *in vivo*.<sup>27</sup> In our research, the size of tumors was decreased in groups with low expression of ATF4, also.

Here, OPN expression was downregulated by *M. obuense*, even though moderately. OPN is expressed at higher levels in different transformed cell lines than in their non-tumorigenic cell counterparts.<sup>28</sup> It has been importantly associated with higher malignancy in BC, however, its functional role in this process is poorly known. The OPN transcripts can be detected in both invasive and *in situ* carcinoma components of human BC. Overexpression of OPN has occurred in early tumor metastasis and been modulated in tumor growth, angiogenesis and metastasis.<sup>29</sup>

In BC, increased cFOS expression was related to weak prognosis.<sup>30</sup> The knockdown of cFos expression could increase patient survival and decrease the proliferation and invasiveness of BC xenografts.<sup>31</sup> The cFos protein contributes in normal development, cellular growth and apoptotic cell death in the proliferative conditions or in response to cellular injury.<sup>32</sup> Our study demonstrated that *M. obuense* in high dose decreased c-Fos gene expression. According to Lu *et al.*<sup>31</sup> cFos is an essential activator of genes involved in tumor growth and our research was in agreement with this finding, as well.

Alkaline phosphatases are a family of metalloenzymes that catalyze the hydrolysis of organic phosphate esters at an alkaline environment with low substrate specificity.<sup>33</sup> Previous studies reported that cultured human osteosarcoma cell lines<sup>34</sup> and an animal osteosarcoma cell line produced a large amount of ALP.<sup>35</sup> By the way, another study has found that an elevated ALP was prominently associated with lymph nodes in resected esophageal cancer patients;<sup>36</sup> thus, ALP level may be a sensitive marker of tumor proliferation. Kim *et al.*<sup>37</sup> showed that ALP predicts liver metastases.

Collagen 1A1 (COL1A1) is a significant component of the tumor-stromal environment playing vital functions in cancerous cells.<sup>38</sup> COL1A1 is an individual extracellular matrix gene and has been reported to be associated with tumor invasive and metastatic behaviors. With increased extracellular levels of COL1A1, tumor cell invasiveness and metastasis are increased in animal models.<sup>39</sup> Expression of COL1A1 in our study was significantly lower in *M. obuense*-recipient groups which was in agreement with the findings of pathologic investigations.

Despite the tremendous advances in cancer research, the potentials of histopathology have not been fully exploited for the evaluation of microscopic cancer characteristics. Here, histopathological examination was conducted for microscopic analyses of the tumor mass. As previously described, histopathological data indicated a remarkable attenuation in the tumor malignancy features with an extensive cell degeneration and necrosis after the course of treatment by paclitaxel and inactivated *M. obuense* compared to the control group which was consistent with the results of tumor weight and size. Briefly, the obtained data were indicative of tumor regression in the paclitaxel and inactivated *M. obuense* - recipient groups, particularly in high dose.

Epithelial-to-mesenchymal transition (EMT) is a key malignant trait in carcinoma cells which is induced by reprogramming in their differentiation state.<sup>40</sup> This trans-differentiation process is phenotypically characterized by repression of epithelial markers, overexpression of mesenchymal markers, and alterations in morphology associated with cell migration. EMT instills invasive and drug resistant attributes into cancer cells, enabling them to induce primary metastatic tumor seeding which is hard to be differentiated from tumor-initiating or stem cells in terms of function.<sup>41</sup> Inhibition of ATF4 signaling prevents EMT at the cellular and molecular levels which stands in contrast to the Twist1 approach.<sup>42</sup> On the other hand, COL1A1 is occasionally found in most connective tissues and is abundant in bone, cornea, dermis, and tendon. Besides, its expression level is closely related to EMT.<sup>43</sup> Lin *et al.*<sup>44</sup> research on the human carcinoma cell lines 2008 and HEY revealed that knockdown of CLDN3 was accompanied by an increase in the expression of EMT markers and concurrently, the upregulation of Twist. Still,

in our experiment, we noticed the up-regulation of both CLDN3 and Twist following the treatment of mice with *M. obuense*. Furthermore, Muhammad *et al.*<sup>45</sup> showed that c-Fos overexpression in the non-tumorigenic cell line made the cells tumorigenic and enhanced the EMT marker genes. In our experiment, c-Fos gene expression was declined in a high dose receiving group which was consistent with pathologic findings. As EMT can affect the level of ALP, knockdown of the key EMT transcription factor results in diminished ALP activity implicating a mechanism by which ALP may be regulated.<sup>46</sup> Our study revealed a remarkable decrease of ALP in the high dose group that might be attributed to low metastasis in the liver in the high dose bacteria-recipient group.

Previous studies had explored the determining role of *M. obuense* for patients with melanoma. They concluded that inactivated *M. obuense* was safe and tolerable for these patients.<sup>6</sup> Our results demonstrated the safety of this reagent by MTT and pathology tests as well. Consistent with our hypothesis, in our study, the size of the tumor in the groups treated with *M. obuense* was smaller than that of the control group. However, the examination of Fowler *et al.*<sup>4</sup> on colorectal and melanoma tumors revealed no significant effect on tumor growth.

In summary, this was the first report that showed the IL injection of *M. obuense* suppressed tumor growth and cancer-related gene expression. We do acknowledge the need to investigate *M. obuense* in combination with standard of care in treatment of BC alone and in combination with paclitaxel.

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## Conflict of interest

The authors report no conflicts of interest associated with this work.

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