



Exploring the Anti-Inflammatory and Antioxidative Potential of Selenium Nanoparticles Biosynthesized by *Lactobacillus casei* 393 on an Inflamed Caco-2 Cell Line

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Abstract

Selenium (Se) plays a crucial role in modulating inflammation and oxidative stress within the human system. Biogenic selenium nanoparticles (SeNPs) synthesized by *Lactobacillus casei* (*L. casei*) exhibit anti-inflammatory and anti-oxidative properties, positioning them as a promising alternative to traditional supplements characterized by limited bioavailability. With this context in mind, this study investigates the impact of selenium and *L. casei* in ameliorating inflammation and oxidative stress using a cell line model. The study is centered on the biosynthesis of selenium nanoparticles (SeNPs) by *L. casei* 393 under anaerobic conditions using a solution of sodium selenite (Na₂SeO₃) in the bacterial culture medium. The generation of SeNPs ensued from the interaction of *L. casei* bacteria with selenium ions, a process characterized via transmission electron microscopy (TEM) to confirm the synthesis of SeNPs. To induce inflammation, the human colonic adenocarcinoma cell line, Caco-2 was subjected to interleukin-1 beta (IL-1β) at concentrations of 0.5 and 25 ng/ml. Subsequent analyses encompass the evaluation of SeNPs derived from *L. casei*, its supernatant, commercial selenium, and *L. casei* probiotic on Caco2 cell line. Finally, we assessed the inflammatory and oxidative stress markers. The assessment of inflammation involved the quantification of NF-κB and TGF-β gene expression levels, while oxidative stress was evaluated through the measurement of Nrf2, Keap1, NOX1, and SOD2 gene levels. *L. casei* successfully produced SeNPs, as confirmed by the color change in the culture medium and TEM analysis showing their uniform distribution within the bacteria. In the inflamed Caco-2 cell line, the NF-κB gene was upregulated, but treatment with *L. casei*-SeNPs and selenium increased TGF-β expression. Moreover, *L. casei*-SeNPs upregulated SOD2 and Nrf2 genes, while downregulating NOX1, Keap1, and NF-κB genes. These results demonstrated the potential of *L. casei*-SeNPs for reducing inflammation and managing oxidative stress in the Caco-2 cell line. The study underscores the ability of *L. casei*-SeNPs to reduce oxidative stress and inflammation in inflamed Caco-2 cell lines, emphasizing the effectiveness of *L. casei* as a source of selenium. These insights hold significant promise for the development of SeNPs derived from *L. casei* as potent anti-inflammatory and anti-cancer agents, paving the way for novel therapeutic applications in the field.

Keywords Inflammation · Oxidative Stress · *Lactobacillus casei* · Selenium Nanoparticles

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Introduction

Selenium (Se) is critical in bolstering the immune system and functioning as an antioxidant. It is an indispensable component of selenoproteins, pivotal in regulating inflammation and oxidative stress [1]. Inadequate Se levels have been associated with heightened susceptibility to inflammatory conditions and compromised immune function [2]. While Se supplements, including inorganic forms such as sodium selenite or selenate and organic compounds like selenomethionine, are available, they may carry significant toxicity and limited absorption efficiency. It has become increasingly evident that dietary Se supplementation yields numerous beneficial effects on human health [3, 4]. Se has emerged as a promising agent in both cancer prevention and therapy [5].

Furthermore, clinical trials have indicated the anticancer properties of Se supplementation, particularly its notable effects against colorectal, lung, and prostate cancers [6]. It is crucial to recognize the narrow therapeutic window between the beneficial dose of selenium and its potentially toxic level [7]. Additionally, the nature and mechanism of Se toxicity appear closely linked to its chemical composition [8].

Selenium nanoparticles (SeNPs) exhibit distinctive physicochemical properties due to their nanoscale dimensions and reduced surface-area-to-volume ratio in comparison to other forms of Se. Notably, nano-Se demonstrates lower toxicity [9], superior bioavailability over Se salts [10], and improved performance relative to commercially available yeast-based selenium supplements [11]. Consequently, nano-Se is being positioned as a promising substitute for other forms of selenium in clinical practice [12–14].

These SeNPs demonstrate notable antioxidant and anti-inflammatory properties, positioning them as promising candidates in combating inflammation-associated conditions [15]. Numerous studies endeavors have delved into the anti-inflammatory characteristics of SeNPs. Through studies conducted on animal and cell models, it has been observed that SeNPs hold the capability to restrict inflammation by impeding the production of vital cytokines, including tumor necrosis factor-alpha (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6) in animal and cell models [16–18]. Furthermore, SeNPs can modulate signaling pathways crucial in inflammation, such as nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinases (MAPKs) [19, 20]. One of the fundamental roles of SeNPs lies in their capacity to shield against oxidative stress by augmenting the antioxidant defense mechanisms. This pivotal function of SeNPs is instrumental in preserving health by serving as a protective barrier against oxidative stress. Through their distinctive attribute of fortifying the body's antioxidant defense system, SeNPs play an essential

role in maintaining cells' health and optimal functioning [21]. Additionally, SeNPs demonstrate the capacity to augment the activity of critical antioxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) while concurrently reducing levels of lipid peroxidation and nitric oxide [22].

Currently, chemical reduction is the predominant method for synthesizing nano-Se [23]. Nevertheless, chemical synthesis entails high costs and necessitates specialized equipment and toxic chemicals. In recent years, there has been a notable surge in interest in harnessing biosynthetic pathways [24]. Realization of biosynthesis is possible in two ways - directly in living organisms or with the help of bioagents extracted from them. Sodium selenite/selenate, selenium acid, and selenium dioxide can be used as precursors for the synthesis of SeNPs added to a "bioreactor" - such as bacteria. The addition of precursors to the biological extract leads to the appearance of a red, red-orange, or orange color of the solution, which indicates the reduction of selenium compounds and the formation of colloidal SeNPs. In addition, the stabilization and capping processes of nanoparticles are carried out. The reduction of selenium salt to Se⁰ occurs due to various biopolymers that can fulfill this role. Enzymes of thioredoxin reductase, nitrite reductase, or other membrane reductases are responsible for SeNP synthesis in bacteria [25]. Specifically, various microorganisms have been documented to produce nano-Se through their detoxification mechanisms [26] or redox homeostasis [27]. Certain bacteria, which exhibit resistance to Se toxicity by biologically converting Se oxyanions to the less toxic Se(0), have been observed to synthesize elemental SeNPs during Se-anion reduction [28]. The resulting SeNPs from bacterial synthesis possess distinct arrangements of Se atoms, displaying significant variation among different species. These variances indicate the diversity of the enzymes that reduce Se-oxyanions in other microorganisms [29]. The array of nano-Se formulations derived from bacteria underscores the intricate nature of their physicochemical properties and, ultimately, the diversity of their biological effects [30].

In SeNPs production techniques, the biological method emerges as distinctive for its reliance on microorganisms as active participants [31]. Among the various microorganisms that can produce nano-Se are *Desulfovibrio desulfuricans* [32], *Stenotrophomonas maltophilia*, *Rhodobacter sphaeroides* [33], *Pseudomonas alcaliphila* [34], *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus plantarum* [35], the last three being probiotic strains that belong to the genus *Lactobacillus* [36].

Microorganisms serve as vital nano-factories, proficient in reducing harmful compounds and impeding their accumulation through a range of reductase enzymes. In recent years, substantial attention has been directed towards the

beneficial properties of probiotics, particularly the diverse species of the genus *Lactobacillus*, known for their immunomodulatory, anti-inflammatory, and anti-carcinogenic activities [37–39].

Probiotics, including *Lactobacillus casei* (*L.casei*), constitute a category of microorganisms essential for nanoparticle synthesis. Their widespread availability, rich diversity, and resilience in adverse conditions position probiotics as crucial facilitators in this process. *L.casei* first reported to accumulate intracellular Se in 1995, has been identified as one of the nano-Se-producing probiotic strains. Its potential application in nutritional supplementation studies was initially proposed by Calomme et al. [40], leading to subsequent confirmations of SeNPs synthesis by various probiotic strains [10].

Consequently, the field of nano-medicine, integrating probiotics, prebiotics, and postbiotics, significantly influences diverse facets of gastrointestinal physiology. Previous studies have underscored the substantial correlation between the beneficial attributes of probiotics and their physiological characteristics. The adhesive potency of a particular strain and its ability to endure gastrointestinal transit play a pivotal role in determining successful colonization within the digestive tract [31, 41]. Furthermore, probiotics such as *L. casei 393* have demonstrated beneficial effects in various inflammatory conditions, and their combination with selenium nanoparticles holds promise for a synergistic anti-inflammatory effect [42, 43]. *L. casei 393* showcases the ability to produce SeNPs that confer advantageous properties against oxidative stress [44].

This study aimed to investigate the synthesis of SeNPs by *L.casei 393* and evaluate their anti-inflammatory and anti-oxidative effects using an in vitro inflammatory model. The study delved into the evaluation of the characteristics and size of the nanoparticles as well as the assessment of inflammation through NF- κ B and transforming growth factor- β (TGF- β), as well as oxidative stress via nuclear respiratory factor 2 (Nrf2), kelch-like ECH-associated protein 1 (Keap1), NADPH oxidases 1 (NOX1), and SOD2 genes. These investigations are essential for elucidating the mechanisms underlying the anti-inflammatory and anti-oxidant effects of *L. casei*, *L. casei*-SeNPs, supernatant, and commercial selenium.

Method and Material

Probiotic Preparation and SeNPs Synthesis

Preparation of *L.casei 393*

The strain of *L.casei 393*, sourced from the Iranian Biological Resource Center (IBRC) with the accession number

IBRC-M 10711, was propagated on Man–Rogosa–Sharpe (MRS) (Sigma, Germany) agar plates and then cultivated at 37 °C for 48 h in an anaerobic setting. Subsequently, gram staining was performed to assess the morphology of the lactobacilli, confirm the purity of the culture, and visualize the bacteria under a microscope. Following a 24-h incubation period at 37 °C within an anaerobic jar, the bacterial culture was centrifuged at 12,000 $\times g$ for 10 min. The resulting supernatant was then discarded, and 1 ml of phosphate-buffered saline (PBS) was introduced to the bacterial pellet. Through meticulous pipetting, the pellet was thoroughly resuspended to ensure homogeneity, thereby aiding in eliminating the culture medium from the bacteria. The suspension underwent centrifugation at 12,000 $\times g$ for 5 min, with the process being repeated after discarding the supernatant. The concentration of the sample was determined by assessing its optical density (OD) using the McFarland 2 standard. Following the calculation of the total number of seeded cells and accounting for the total volume of the medium, the final bacterial concentration and level of contamination were evaluated based on a Multiplicity of Infection (MOI) of 10.

SeNPs synthesis by *L. casei 393*

In the process of synthesizing SeNPs utilizing the probiotic strain *L. casei 393*, a solution containing sodium selenite (Na₂SeO₃) was prepared by dissolving 200 μ g of sodium selenite in 1 ml of the bacterial culture medium. The resultant mixture was incubated for 24-h period at 37 °C within an anaerobic culture jar. Over this incubation duration, the bacteria engaged with selenium ions, metabolizing them to reduce and facilitate the generation of SeNPs.

Measurement of SeNPs synthesized by *L.casei 393* probiotic bacteria

To confirm the synthesis of SeNPs and to determine their dimensions by utilizing *L.casei* strain 393 bacteria, transmission electron microscopy (TEM) was employed. After 24 h of treating the aforementioned bacteria with sodium selenite, the observations were made. This approach was undertaken to validate the formation of nanoparticles and assess their size accurately, which was executed to ensure the precision of the results.

Preparation of SeNPs supernatant from *L.casei 393* probiotic bacteria

Following a 24-h incubation period, a liquid culture containing SeNPs derived from *L.casei 393* bacteria was extracted from the microtube. The ensuing supernatant was

removed, and the pellet was resuspended in fresh TE buffer and washed through centrifugation at $12,000 \times g$ for 10 min. This process was repeated to ensure the homogeneity of the mixture. The culture's absorbance was measured at a wavelength of 600 nm until a value of McFarland 2 was obtained, indicating about 6×10^8 CFU/ mL bacterial density.

To lyse the bacterial cells, 2 mg/ml of lysozyme was added to the resuspended culture, and the mixture was thoroughly vortexed. The lysed mixture was then incubated in a thermomixer at 37°C for 1 h. After incubation, the mixture was boiled for 20 min and promptly transferred to a freezer set at -21°C for 2–3 min. Finally, the mixture was centrifuged at $10,000 \times g$ for 10 min to separate the supernatant containing the desired SeNPs from the cellular debris.

Cell Culture Conditions and Treatments

Culturing Caco-2 cell line

The Caco-2 human colon cancer cell line was obtained from the IBRC. Cells were cultured in T25 flasks using High glucose Dulbecco's Modified Eagle Medium (DMEM) (Biosera, France), supplemented with 10% fetal bovine serum (FBS) (Gibco, Germany) and 1% penicillin-streptomycin antibiotics (Biosera, France) at 37°C with a CO_2 concentration of 5%. After five days, the cells were transferred to T75 flasks, and incubated in a cell culture incubator at the same conditions.

Upon reaching the optimal growth phase, cells were distributed into 12-well plates, each well containing approximately 1 million cells. Following an initial 48-h incubation period, the cells underwent treatment with interleukin-1 beta (IL-1 β) at concentrations of 0.5 and 25 ng/ml for 24 h.

Optimizing IL-1 β levels to induce inflammation in Caco-2 cells

Inflammation in the Caco-2 cell line was triggered using IL-1 β concentrations of 0.5 and 25 ng/ml [45–47]. The inflammatory response was evaluated by quantifying *NF- κ B* gene expression using quantitative reverse transcription polymerase chain reaction (RT-qPCR) via specific primers after 24 h.

Evaluating the impact of *L.casei* 393 probiotic and SeNPs on inflamed Caco-2 cells

Cells were exposed to predetermined IL-1 β concentrations and incubated for 24 h. Cell morphology was microscopically inspected for uniformity, and each well's culture

medium was changed to match the specific treatment conditions. For selenium supplementation, 200 μg of selenium was added to the appropriate well. In the case of *L. casei* treatments, cells were added to an antibiotic-free culture medium in designated wells and infected with bacteria at an MOI of 10. Similarly, SeNPs in the supernatant were diluted with an antibiotic-free culture medium and added to cells at an MOI of 10. The plate was gently agitated and then incubated for 48 h. All treatments were conducted in duplicate. Following incubation, the cell supernatant was aspirated, and the cell pellet was collected for RNA extraction.

RNA extraction and RT-qPCR

Following a 24-h incubation period, cells were collected, and RNA extraction was performed using the EZ-10 Total RNA Mini-Preps Kit (BIO BASIC, Canada) according to the manufacturer's instructions. The quality of the isolated RNAs was evaluated using a NanoDrop[®] ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, complementary DNAs (cDNAs) were synthesized from each RNA sample utilizing the AddScript cDNA synthesis kit (addbio, Korea).

For inflammation assessment, the NF- κ B and TGF- β genes were selected, while the Nrf2, Keap1, NOX1, and SOD2 genes were chosen for measuring oxidative stress. Primer sequences were validated using Primer-BLAST (NCBI), Oligo 7, and Gene Runner software, and were synthesized by Gen Fanavaran in Iran. The primer sequences, product sizes, and the thermal cycling program can be found in Table 1.

The qPCR reaction mixture had a total volume of 10 μl , comprising 5 μl of SYBR Green Master mix 2X (Amplicon, Denmark), 0.25 μl each of the forward and reverse primers, and 1 μl of cDNA. The thermal cycling program included an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at the temperature specified in Table 1 for 25 s, and extension at 72°C for 25 s. A subsequent melting curve analysis was conducted in the temperature range of 70 to 95°C . The samples were run on the LightCycler[®] 96 instrument from Roche, with each sample tested in duplicate for precision.

Statistical Analysis

The data is shown as mean \pm standard deviation (\pm SD). Statistical analysis was performed using GraphPad Prism version 6.07 (GraphPad Software, San Diego, CA). Student's *t* test was analyzed to evaluate the differences between groups. Results with a *P*-value of ≤ 0.05 were considered statistically significant.

Table 1 Oligonucleotide primers used for qPCR in this study

Gene	Primer sequences	Annealing temperature (°C)	Product size (bp)
<i>NF-κB</i>	F: 5'-TCACTCGGCAGATCTTGAGC-3' R: 3'-CCAGACCAACAACAACCCCT-5'	57	197
<i>TGF-β</i>	F: 5'-CAATTCCTGGCGATACCTCAG-3' R: 3'-GCACAACCTCCGGTGACATCAA-5'	55	86
<i>SOD2</i>	F: 5'-TGGACAAACCTCAGCCCTA-3' R: 3'-CTGATTGGACAAGCAGCAA-5'	60	198
<i>NOX1</i>	F: 5'-GTTTTACCGCTCCCAGCAGA-3' R: 3'-GGATGCCATTCCAGGAGAGA-5'	60	156
<i>NRF2</i>	F: 5'-CAGCGACGGAAAGAGTATGA-3' R: 3'-TGGGCAACCTGGGAGTAG-5'	60	200
<i>KEAP1</i>	F: 5'-GGCTGTCTCAATCGTCTCC-3' R: 3'-TCTGTTTCCACATCGTAGCG-5'	60	231
<i>GAPDH</i>	F: 5'-CTCAAGATCATCAGCAATGCCT-3' R: 3'-ACAGTCTTCTGGGTGGCAGT-5'	57	134

Result

Synthesis of SeNPs by *L.casei* Probiotic

Following a 24-h anaerobic incubation at 37 °C, the cultural medium of the probiotic *L. casei* exhibited a noticeable color change when sodium selenite was added. The color of the MRS medium shifted to red after the introduction of sodium selenite, as a visual indicator of successful production of SeNPs by *L. casei* probiotic.

Examination of TEM Imaging Results

TEM images, as shown in Fig. 1, were utilized to analyze the distribution of SeNPs within *L. casei* bacteria. The results revealed a predominant distribution of SeNPs, ranging from 150 to 200 nm, within the *L. casei* bacteria. The cytoplasm of *L. casei* exhibited uniformity, showing no presence of dark particles within the bacterial cell by the TEM images. These findings indicate that *L. casei* bacteria have the potential to be utilized as an effective delivery vehicle for SeNPs.

Confirmation of Inflammation Induction in Caco-2 Cell Line

To confirm the presence of inflammation in the Caco-2 cell line, we assessed the expression level of the *NF-κB* gene, a pivotal regulator in inflammatory processes that becomes transcriptionally activated upon initiation of the *NF-κB* pathway [48]. The gene expression was quantified following treatments with two distinct concentrations (0.5 and 25 ng) for 24 h.

The qPCR analysis revealed a noteworthy upregulation of the *NF-κB* gene in the Caco-2 cell line, with a LogFC

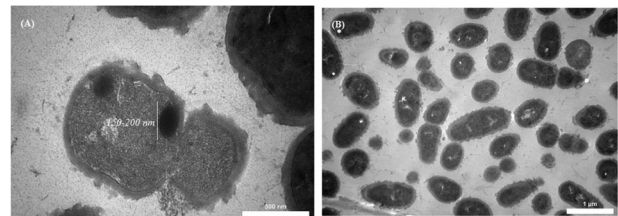


Fig. 1 Transmission electron microscopy (TEM) imaging. **A** The image of nanoparticles made with a size of 150–200 nm. **B** The image of homogeneous cytoplasm without black particles of *Lactobacilli*

18.29 increase (p -value = 0.0065) at the 0.5 ng concentration and a LogFC 13.81 increase (p -value = 0.0085) at the 25 ng concentration after 24 h compared to the uninflamed control group. Inducing an inflammatory response in the Caco-2 cell line is more effective with 0.5 ng of IL-1 β . Furthermore, the expression level of *TGF-β*, *SOD2*, *Nrf2*, *NOX1*, and *Keap1* genes was determined in the inflamed Caco-2 cell line (Fig. 2).

Expression of *TGF-β* and *NF-κB* in Treated Caco-2 Cell Line

The comparison of the inflamed Caco-2 cell line showed different degrees of response to different treatments. Treatment with commercial selenium with LogFC 2.033 (p -value = 0.0444), *L.casei* with LogFC 2.27 (p -value = 0.0937), and *L.casei* containing SeNPs with LogFC 3.57 (p -value = 0.0132) showed to increase the expression level of *TGF-β*. In addition, supernatant treatment with a LogFC of 5.91 (p -value = 0.0063) caused a significant increase in *TGF-β* expression in inflamed cells compared to the control well (Fig. 3A).

Furthermore, analysis of the qPCR data indicates a reduction in the *NF-κB* gene expression in the inflamed

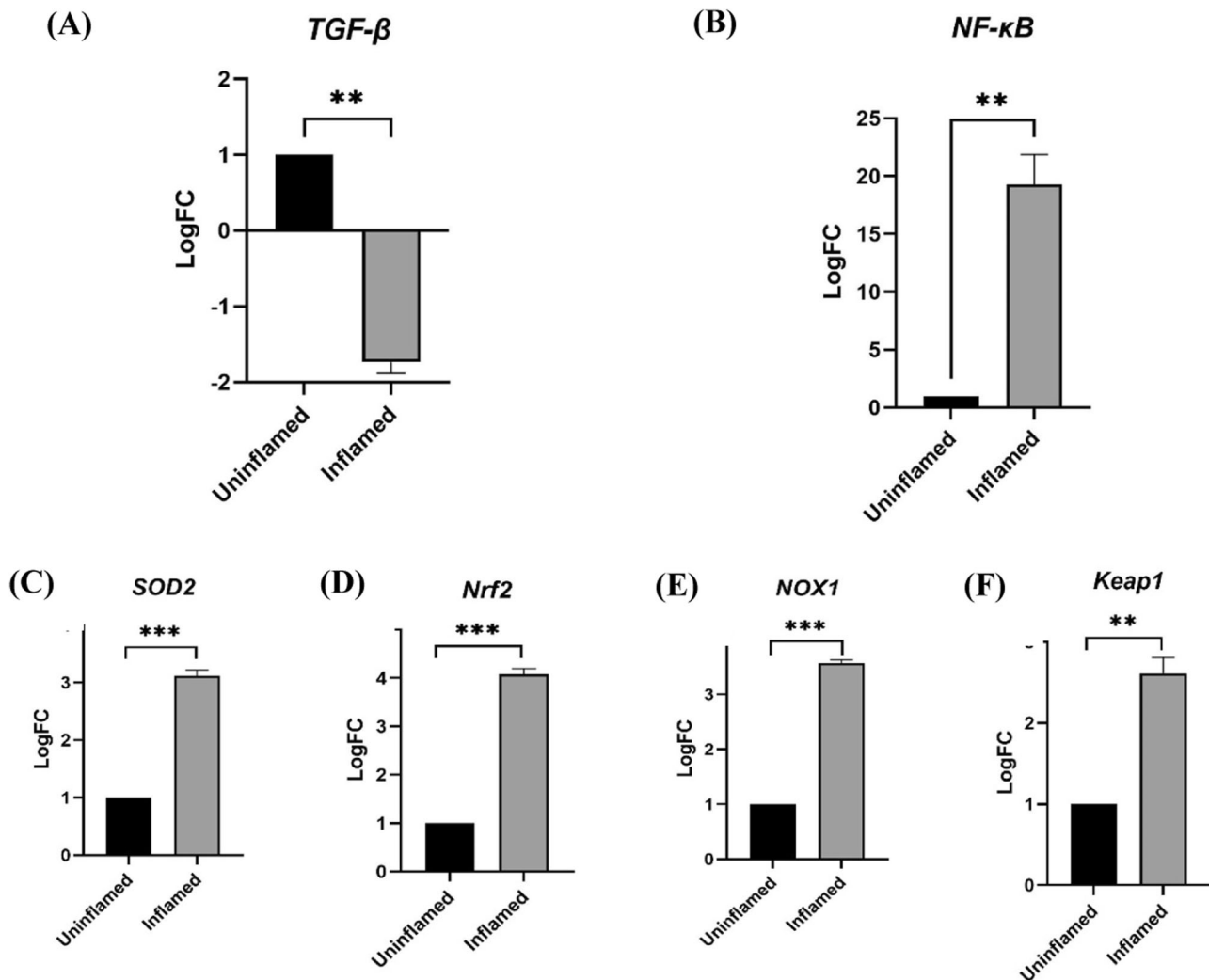


Fig. 2 Expression of the *TGF-β* (A), *NF-κB* (B), *SOD2* (C), *Nrf2* (D), *NOX1* (E), and *Keap1* (F) genes in the Caco-2 cell line after 24-h treatment with *IL-1β* at concentrations of 0.5 ng. The foldchange

results are Log2 transformed (LogFC) and the statistical analysis is denoted by *, **, ***, and ****, which represent *p*-values of <0.05, <0.001, <0.0001, and <0.00001, respectively

Caco-2 cell line after treatment with various substances. Notably, the treatments included commercial selenium with a LogFC of -0.72 (p -value = 0.0046), *L.casei* LogFC -4.67 (p -value = 0.0214), *L.casei* containing SeNPs LogFC -2.31 (p -value = 0.0095), and supernatant LogFC -2.93 (p -value = 0.1172), decreased when compared to the control well (Fig. 3B).

The treatment of inflamed Caco-2 cells with various substances resulted in diverse degrees of response. Specifically, treatment with commercial selenium, *L.casei*, and *L.casei* containing SeNPs led to increased expression levels of *TGF-β*. Furthermore, the application of supernatant significantly enhanced *TGF-β* expression in inflamed cells compared to the control well.

In contrast, analysis of qPCR data revealed a reduction in *NF-κB* gene expression in the inflamed Caco-2 cell line following treatment with different substances. Notably,

treatment with commercial selenium, *L.casei*, *L.casei* containing SeNPs, and supernatant decreased *NF-κB* expression compared to the control well.

Expression of Anti-oxidative and Oxidative Genes in Inflamed Caco-2 Cell Line

The results from qPCR analysis of antioxidant genes showed an increase in *SOD2* with LogFC 4.323 (p -value = 0.0017) and *Nrf2* with LogFC 2.73 (p -value = 0.0003) expressions, following treatment with *L. casei*-SeNPs compared to the inflamed Caco-2 cell line.

Conversely, oxidant genes *NOX1* and *Keap1* exhibited a significant downregulation: decrease in *NOX1* with LogFC -5.45 (p -value = 0.0017) and decrease in *Keap1* expression LogFC -6.77 (p -value < 0.0001) after *L. casei* treatment, as well as reduction in *NOX1* with LogFC -3.673 (p -value

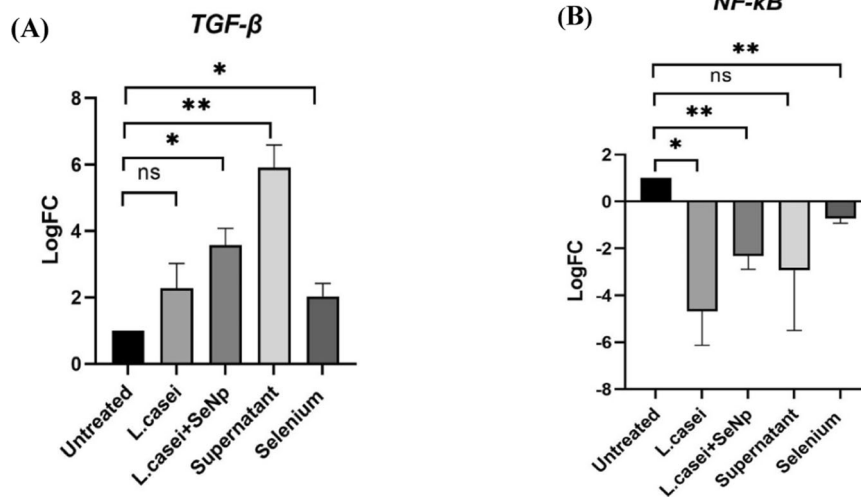


Fig. 3 The anti-inflammatory factor TGF- β and inflammatory factor NF- κ B expression level in various treatments of inflamed Caco-2 cell line. **A** The expression level of TGF- β elevated in response to the supernatant of SeNPs, *L.casei* containing SeNPs, *L.casei*, and commercial selenium, compared to the inflamed Caco-2 cell line. **B** Treatment with *L. casei* led to a significant decrease in NF- κ B

expression. Additionally, a significant reduction in NF- κ B expression was induced by supernatant containing SeNPs, *L. casei* with SeNPs, and commercial selenium. All the cells were treated with 0.5 ng IL-1 β and the foldchange results are Log₂ transformed (LogFC). The statistical analysis are denoted by *, **, ***, and ****, which represent *p*-values of <0.05, <0.001, <0.0001, and <0.00001, respectively

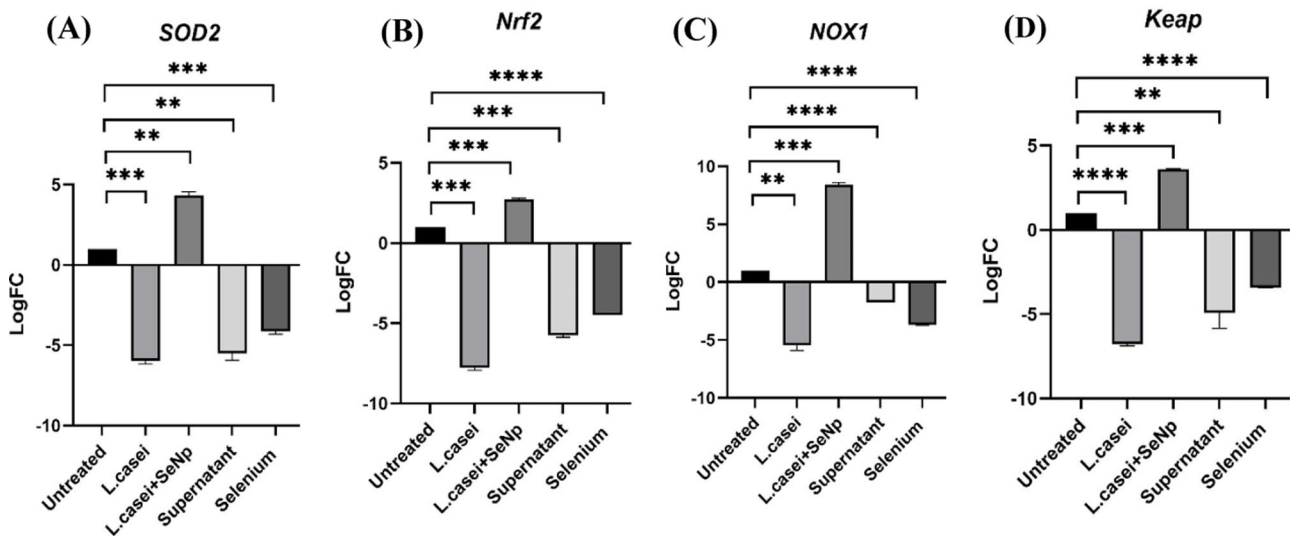


Fig. 4 Expression of antioxidant and oxidative genes in inflamed Caco-2 cell line. **A** *SOD2* is significantly upregulated in *L.casei*-SeNP treated cells and down-regulated in *L.casei*, supernatant, and selenium treated cells compared to untreated control. **B** *Nrf2* is significantly upregulated in *L.casei*-SeNP treated cells and down-regulated in *L.casei*, supernatant, and selenium-treated cells compared to untreated control. **C** *NOX1* is significantly up-regulated in *L.casei*-SeNP and

supernatant-treated cells and down-regulated in *L.casei* and selenium, treated cells compared to untreated control. **D** *Keap1* was significantly increased in cells treated with *L.casei*-SeNP and decreased in cells treated with *L.casei*, supernatant, and selenium compared to untreated control. All the cells were treated with 0.5 ng IL-1 β and the foldchange results are Log₂ transformed (*p*-value: *<0.05, **<0.001, ***<0.0001, ****<0.00001)

<0.0001) and *Keap1* LogFC -3.43 (*p*-value <0.0001) following treatment with commercial selenium. Notably, *Keap1* expression decreased with LogFC -4.9 (*p*-value = 0.0081) after treatment with the supernatant (Fig. 4).

The qPCR analysis revealed significant changes in the expression levels of antioxidant and oxidant genes

following treatment with *L. casei*-SeNPs compared to the inflamed Caco-2 cell line.

Treatment with *L. casei*-SeNPs resulted in a substantial upregulation of the antioxidant gene *SOD2*, indicating an enhanced antioxidant response compared to the inflamed cells. Similarly, treatment with *L. casei*-SeNPs led to an

upregulation of Nrf2 gene expression, suggesting activation of the Nrf2-mediated antioxidant pathway.

In contrast, the oxidant genes NOX1 and Keap1 exhibited significant downregulation following treatment with *L. casei* or commercial selenium. Treatment with *L. casei* resulted in a substantial decrease in NOX1 expression, indicating a potential reduction in oxidative stress. Additionally, Keap1 expression was downregulated after *L. casei* treatment. Treatment with commercial selenium also significantly decreased NOX1 expression and downregulated Keap1 expression. Notably, treatment with the supernatant led to a decrease in Keap1 expression.

Discussion

Se is a vital trace element crucial for the physiological functions of various organisms, including both humans and animals [49]. While Se supplements are available in both organic and inorganic forms, their bioavailability is limited, and they carry the risk of toxicity. Biogenic SeNPs have attracted interest due to their safety profile and biological activities. SeNPs biogenic synthesis using probiotic bacteria emerging as a promising strategy [50–53]. Our study revealed an interesting phenomenon of intracellular synthesis of small red selenium nanoparticles in *L. casei* 393 cells. Observation of these nanoparticles through TEM confirms their synthesis through a reduction mechanism. In addition, the results of TEM showed that the size of produced selenium nanoparticles is 150–200 nm.

Additionally, *Lactobacillus* species are recognized for their anti-inflammatory properties and their significant role in regulating various diseases [54]. These microorganisms have shown promise as a primary therapeutic intervention for conditions associated with oxidative stress [55].

Oxidative stress results from an imbalance between reactive oxygen species (ROS) production and antioxidant availability [56]. Excessive ROS can oxidize biomolecules and alter protein and gene expression, triggering signaling cascades that contribute to inflammation [57–59]. Inflammatory cells release soluble mediators that attract more inflammatory cells to the injury site, increasing active species' presence. These mediators initiate signal transduction cascades and affect transcription factors like NF- κ B and Nrf2 [60], leading to immediate cellular stress responses. Prolonged exposure to inflammation and oxidative stress harms nearby healthy cells, potentially leading to carcinogenesis [61]. In this paper, we investigated the complex dynamics of inflammation in the Caco-2 cell line. To induce inflammation, a carefully selected approach was used, including the administration of IL-1 β , a potent proinflammatory cytokine known for its pivotal role in the initiation and perpetuation of inflammatory responses. After

interacting with its specific receptors embedded on the cell surface, IL-1 β triggered a cascade of events, activating not only the NF- κ B inflammatory pathway but also the Nrf2 oxidative stress pathway. This simultaneous activation elucidates the remarkable interplay and interrelation between these two critical pathways and emphasizes their concerted participation in the complex coordination of cellular responses to inflammation.

The I κ B/NF- κ B signaling network integrates signals from Toll-like receptors (TLR) and interleukin-1R (IL-1R), regulating inflammatory responses through transcriptional responses and proinflammatory cytokine production [62, 63]. Selenium deficiency is associated with NF- κ B pathway activation, reduced anti-inflammatory cytokines, oxidative stress, and inflammation. Studies suggest that selenium supplementation can increase TGF- β expression, an anti-inflammatory cytokine often diminished in individuals with low selenium levels [64, 65]. Additionally, certain probiotics, particularly from *Lactobacillus* species, have been found to enhance TGF- β signaling and mitigate NF- κ B-induced inflammatory responses [66]. The results of this study provided evidence demonstrating the remarkable effectiveness of all the substances investigated in significantly downregulating the expression of the inflammatory factor, NF- κ B. Moreover, our research sheds light on a noteworthy observation: the supernatant, bacteria containing SeNPs, *L. casei*, and commercial selenium, led to a remarkable increase in the expression of the anti-inflammatory factor, TGF- β . These findings highlight these substances' potential therapeutic value and promising implications in modulating inflammatory responses and establishing a balanced immune system.

The Nrf2/ARE pathway is crucial for cellular balance, detoxification, and response to oxidative stress. NRF2, a vital transcription factor, regulates this pathway, while Keap1, a repressor protein, controls NRF2 activity through degradation [67]. The interplay between NF- κ B and Nrf2 is vital in oxidative stress, where the absence of Nrf2 can increase NF- κ B activity and inflammation. NF- κ B acts as an antioxidant and pro-oxidant during oxidative stress [68]. Activating Nrf2 can enhance antioxidant expression without oxidative stress, improving resilience and reducing inflammation orchestrated by NF- κ B [69]. *L. casei* influenced the Nrf2/Keap1 pathway and suppressed NF- κ B. This effect reduced p65 phosphorylation and enhanced GPx2 activity, highlighting *Lactobacilli*'s ability to reduce ROS accumulation and maintain membrane integrity [54]. Se supplementation upregulates Nrf2 expression and inhibits pyrin-3 inflammasome domain (NLRP3) activation, which is crucial for inflammation regulation. Selenium offers protective benefits against oxidative stress [70]. Additionally, selenium modulates the Keap1/NRF2 pathway to protect hepatocytes from oxidative stress [71].

The analysis conducted in our study demonstrates the significant enhancement in the expression of the crucial antioxidant gene, Nrf2, attributed to the application of *L. casei* supplemented with SeNPs (*L. casei*-SeNPs). This notable upregulation of Nrf2 expression signifies the capacity of *L. casei*-SeNPs treatments to effectively reinforce cellular antioxidant defense mechanisms. Furthermore, our investigation strengthens the efficacy of treatments involving *L. casei* alone and commercial selenium supplementation. These treatments have exhibited a substantial reduction in the expression of the oxidant gene Keap1, thereby underlining their potential to mitigate the burden of oxidative stress within the cellular environment effectively.

These findings emphasize the intricate interplay between *L. casei*, selenium, and the delicate balance of antioxidant and oxidant gene expression. By augmenting Nrf2 and attenuating Keap1 expression, these treatments offer promising avenues for modulating cellular redox balance and combating oxidative stress-induced damage.

The NOX1 gene is vital for the internal antioxidant defense system, encoding the NADPH oxidase enzyme responsible for generating ROS in different cell types. It has a dual role, balancing the signaling effects of superoxide with its potential for oxidative damage [72, 73]. Lactobacillus strains have been shown to influence NOX activity and regulate the expression of NOX-1 and NOX-4 genes [74]. However, it is important to note that Lactobacillus can also stimulate NOX1-dependent ROS production [75]. Selenium complexes have been reported to suppress the expression of pro-oxidant genes like NOX1 and NOX2 while increasing the levels of genes encoding ROS-neutralizing proteins such as SOD1 and SOD2 [76, 77]. Various lactobacilli have been identified as agents that enhance the activity of antioxidant enzymes like catalase (CAT), SOD, and GPx [78, 79]. This study, which included the inflamed cell line Caco-2, showed that *L. casei*, together with SeNPs, led to a significant increase in the expression of the antioxidant SOD2 gene. This finding emphasizes the potential of *L. casei*-SeNp treatments to enhance cellular antioxidant defense mechanisms, mainly through increasing SOD2 expression.

Furthermore, our study has practical implications as it shows the effectiveness of *L. casei* isolates and commercial selenium treatments in reducing NOX1 oxidant gene expression. This reduction in NOX1 expression further proves the viability of *L. casei* and selenium treatments as prospective strategies to reduce inflammation and oxidative stress by reducing the burden of oxidant gene expression.

Our research outcomes significantly contribute to expanding the scientific evidence, endorsing the potential benefits of probiotics like *L. casei* and selenium in ameliorating inflammation and combating oxidative stress. These

findings propose promising therapeutic pathways through the utilization of probiotics and Se as synergistic agents to mitigate the detrimental effects of inflammation and oxidative stress on cellular health.

Conclusion

The probiotic bacterium *L. casei* 393, which has anti-inflammatory properties, reduced toxic selenium to non-toxic red SeNPs. The evidence presented in this paper demonstrates the highest anti-inflammatory and antioxidant effects of probiotic *L. casei* 393 bacteria enriched with SeNp in cellular conditions. We compared these effects to those of supernatant or probiotics alone, highlighting the necessity of relative evaluation in understanding the biological impacts of enriched probiotics.

In summary, the findings of the study demonstrate the remarkable potential of *L. casei*-SeNPs in mitigating inflammation, enhancing anti-inflammatory mechanisms, improving antioxidant levels, reducing oxidative stress, and paving the way for innovative avenues in future research. The remarkable capability of *L. casei* to produce SeNPs opens up exciting possibilities for leveraging this probiotic strain as a therapeutic nanoparticle delivery system with potent anti-inflammatory and antioxidant properties. The results of this study will be further investigated and validated in an animal model, a promising and impactful direction for future research efforts.

Author Contribution A.A.S.: Investigation, Data Curation, Writing — original draft, Writing — review & editing, Visualization. M.F.: Methodology, Validation, Software, Formal analysis, Writing — review & editing. K.J.: Writing — original draft, Writing — review & editing, Resources. N.K.: Methodology, Validation, Software, Formal analysis, Writing — review & editing. S.H.B.: Conceptualization, Resources, Project administration, Supervision. H.H.: Methodology, Supervision. F.A.: Resources. A.S.: Resources, Supervision.

Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

Abbreviation

Se	Selenium
SeNPs	Selenium Nanoparticles
<i>L. casei</i>	<i>Lactobacillus casei</i>
Nrf2	Nuclear factor erythroid 2-related factor 2
SOD2	Superoxide Dismutase 2
TGF- β	Transforming Growth Factor- β
Keap1	Kelch-like ECH-associated protein 1
NOX1	NADPH Oxidase 1
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B
TNF-a)	Tumor Necrosis Factor-alpha
IL-1	Interleukin-1

IL-6	Interleukin-6
MAPKs	Mitogen-activated Protein Kinases
GPx	Glutathione Peroxidase
SOD	Superoxide Dismutase
IBRC	Iranian Biological Resource Center
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
IL-1 β	Interleukin-1 beta
PCR	Polymerase Chain Reaction
PBS	Phosphate-Buffered Saline
OD	Optical Density
MOI	Multiplicity Of Infection
TEM	Transmission Electron Microscopy
CFU	Colony-Forming Unit
cDNA	complementary DNA
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
MRS	Man-Rogosa-Sharpe agar
HIF1- α	Hypoxia-Inducible Factor 1 α
STAT3	Signal Transducer and Activator of Transcription 3
AP-1	Activator Protein 1
NFAT	Nuclear Factor of Activated T cells
TLR	Toll-like receptor
IL-1R	Interleukin 1 Receptor
ARE	Antioxidant Responsive Element
NLRP3	Nucleotide-binding domain Leucine-Rich family Pyrin-3 inflammasome domain
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ROS	Reactive Oxygen Species
CAT	Catalase.

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