Investigating the Effects of Lactobacillus Casei on Gene Expression of CLR, TLR-4, and NF-κb in Oral Rat Cancer Induced by 4-Nitroquioline 1-Oxide

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the leading cause of mortality due to oral cancer. The anti-tumor effects of *Lactobacillus casei* (*L. casei*) on various tumors have been investigated, but the molecular mechanisms involved in these beneficial effects are not yet well understood. This study aimed to assess the anti-tumor effects of *L. casei* on the gene expression of receptors associated with the pathway pathogen recognition receptors, including tool-like receptor 4 (TLR4), and C-type lectin receptor (CLR) in the OSCC animal model induced by 4-Nitroquioline 1-Oxide (4NQO).

Methods: In this study, Twenty-eight male Wistar rats were divided into four groups of seven rats each: 1 (Control); 2 (4NQO-treated); 3 (live probiotic L. casei before and after treatment with 4NQO); 4 (live probiotic L. casei after treating with 4NQO). To induce the oral SCC model, the tongue of rats was treated with 4NQO solution using brush three times a week, and tongue carcinogenesis was followed for 16 consecutive weeks. Groups 3 and 4 received the 5×10^8 cfu/kg body weight live probiotic *L. casei* through oral gavage. The tissue sections were stained with hematoxylin and eosin (H&E) for pathological examination. Real-time polymerase chain reaction (RT-PCR) was used to assess the TLR4, DC-SIGN, Bcl-2, and NF-κB gene expression levels in the blood samples of rats.

Results: According to the pathological findings, a well-differentiated invasive SCC was developed in > 77% of rats after 16 weeks of 4NQO treatment. Rats gavaged with the probiotic *L. casei* showed potent anticancer effects. Analysis of real-time PCR data showed the highest NFκB gene expression in Group 4 compared to other groups (p-value < 0.001). Also, overexpression of the Bcl-2 gene was observed in Group 4 (p-value < 0.001). In addition, the expression of TLR4 and DC-SIGN genes in NQO-induced OSCC rat models that received *L. casei* was higher than in other groups (p-value < 0.05).

Conclusion: The present study's findings suggest that probiotic *L. casei* can be a potential strategy for treating OSCC by activating the NFκB signaling cascade through increased expression of TLR4 and Dc-SIGN. However, more studies are needed in this regard.
Keywords: oral squamous cell carcinoma; *Lactobacillus casei*; Probiotic; C-type lectin receptors; DC-SIGN; Toll-like receptor 4;

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Introduction

Malignant tumors characterize head and neck cancer from the epithelium covering the upper aerodigestive tract that can metastasize to other organs (1). Nowadays, it is evident that oral cavity cancer is a multifactorial disease like other cancers, which develops by various factors such as lifestyle, habits, and behaviors. Recently, dietary factors, such as iron deficiency and infectious agents like *human papillomavirus* (HPV), have been implicated as effective factors in developing oral diseases (4). Although few studies have assessed microbial compositions in subjects with squamous cell carcinoma (SCC), recent studies have shown that the microbial flora of patients and healthy individuals are different (5). Therefore, there is still a need to understand the biology better and uncover novel biomarkers/predictors of SCC to improve response to therapy (1).

A rising body of evidence suggests that microbiota plays a crucial role in developing and treating cancers. Probiotics, whether in food or pills, are a simple and effective technique to change the composition of the microbiota. These findings will likely pave the way for regular probiotics use to be considered an adjuvant technique in cancer prevention and treatment (Panebianco *et al.*, 2020). The oral microbiota is crucial to the human microbiome and overall health. Oral microorganisms and oral squamous cell carcinoma (OSCC) may be linked. In a study by Zhang et al., the microbiota compositions of 50 individuals with OSCC were examined between tumor locations and opposite normal tissues. There was a significant variation in oral bacterial profiles between cancer locations and normal tissue (Zhang *et al.*, 2020). Probiotics are suggested as an adjunctive and complementary strategy to increase the efficiency of chemotherapy and immunotherapy. Several *Lactobacillus casei* (*L. casei*) have been used in the food industry as probiotics. Recently, *L. casei* has been reported to have anticancer activity, directly inhibiting cancer cell growth and metastasis in vitro and in vivo. The mechanism through which the bacterium induces cell death in cancer microenvironments remains unclear, particularly at the transcription level, where the metabolic products of *L. casei* can signal a cell to undergo apoptosis (12). *L. casei* Shirota (LcS), a microbe with health-promoting benefits, primarily by enhancing the human immune system, is one of the most well-known and researched probiotic strains (Panebianco *et al.*, 2020). However, several immunological factors could contribute to inducing *L. casei*-associated cancer cell death. Accordingly, innate immunity might be triggered due to the bacterial pathogen-associated molecular patterns (PAMPs) through pathogen recognition receptors (PRRs).

The toll-like receptor (TLR) family is critical in aiding pathogen recognition and the subsequent activation of innate immunity. TLR4 is the central receptor of innate immunity and functions to identify pathogens. As a crucial regulator of inflammation and immune responses, the NF-κB transcription factor regulates the transcription of target genes closely related to cell survival, cell proliferation, apoptosis, invasion, and metastasis. However, the significance of the expressions of both NF-κB and TLR-4 in the occurrence, development, and prognosis of oral SCC remains unclear(11).

Multifunctional C-type lectin receptors (CLRs) are vital in recognizing pathogens and regulating innate and adaptive immune responses. Specific regulation of CLR signaling by modulating tumor microenvironments such as glycoligands and immune cells should lead to the best application of CLRs biology (13). DC-SIGN is a CLR-type PRR that recognizes high-mannose N-glycans on viruses, bacteria, and fungi. Recent research has shown that, in addition to acting as an adhesion molecule, DC-SIGN can start innate immunity via regulating toll-like receptors (Den Dunnen *et al.*, 2009).

Some of the studies also focused on how treatment may induce the apoptosis of tumor cells, and it is known that various cells have different apoptosis thresholds, making their responses to treatment differ. As the induction and regulation of apoptosis are very complex, the mechanisms of inducing apoptosis by various tumor drugs are completely different in many aspects [15]. To elucidate the anti-tumor effects of *L. casei* on OSCC development, we developed a rat OSCC model using the carcinogen 4NQO. We investigated the carcinogenesis process and the gene expression analysis of receptors associated with the NFκB signaling pathway, including pathogen recognition receptors, tool-like receptor 4 (TLR4), and DC-SIGN. The results of the present study demonstrated a significant effect of *L. casei* on inducing apoptosis and enhanced expressions of TLR-4, NF-κB, and DC-SIGN genes in cancer cells, and it was found that the related processes, which help provide evidence for the application of *L. casei* in experimental practices.

Materials and methods

Microbial strains and culture conditions

L. casei (ATTC:1608) was provided by Persian Type Culture Collection (PTCC) and was used to treat oral cancer in rats. MRS agar (HiMedia, Mumbai) was used for routine cultivating *L. casei*. Approximately 10^8 CFU/ml of *L. casei* was suspended in 0.85% saline and used for the treatment study. Lactobacillus grown on MRS agar was tested with the microscopic examination (Gram staining), biochemical characteristics, including catalase activity, growth at different NaCl concentrations, and fermentation of carbohydrates. Moreover, bile, acid resistance, and antibiotic sensitivities were evaluated.

Animals homing and treatment groups

twenty-eight male Wistar rats with an average weight of 150 gr were purchased from (Pasteur Institute of Amol, Mazandaran, Iran). The animals were held in quarantine for a minimum of one week before the initiation of the study. Additionally, rats underwent a hand-held physical examination to ensure their suitability as test subjects. Animals were housed on hardwood bedding in polycarbonate shoebox cages (seven per cage) in a windowless room with a 12/12 hr light/dark cycle at 22±1 °C and 30%-70% humidity. Rats were permitted free access to Purina 5001 Laboratory Diet (PMI Feeds, Brentwood, MO) and tap water. All food cups and water bottles have been replaced twice weekly. Rats were divided into four groups, including Group 1 (n = 7) comprised of control rats only receiving food and tap water, Group 2 (n = 7) cancerous group of rats treated with 4NQO, a treatment Group 3 (n = 7) gavaged with 5×10^8 cfu/kg body weight live *L. casei* from for one week after homing and before carcinogenesis and for two weeks after carcinogenesis. Group 4 (n = 7) treated with 5×10^8 cfu/kg body weight live *L. casei* for 14 days after carcinogenesis. Each group contained seven rats (Figure 1). All animal procedures were submitted to the Research Ethics Committee of the Islamic Azad University, Damghan, Iran, which approved the experimental protocol (license number: 1401.002) (Figure 1).

Development of the animal model

Oral administration of 4-Nitroquinoline 1-oxide (4NQO) (Sigma-Aldrich, St. Louis, MO, USA) was used for developing carcinogenesis in rats. Accordingly, rats were anesthetized by peritoneal administration of xylazine-ketamine, and the tongue was rubbed once with a number 3 camel hair brush (Hawkins et al. 1994) and treated with the 4NQO solution. The rats were restrained from drinking for the first hour after 4NQO treatment.

***L. casei* treatment of rats**

A fresh culture of the bacteria was centrifuged at 4200 rpm for 4 minutes at 4 °C. After washing with saline buffer three times, the bacterial pellet was suspended in distilled water. 10^8 live bacteria were counted and gavaged daily in rats from treatment groups. In this regard, the rats' tongue was rubbed three times a week with a number 3 camel hair brush treated with the amounts mentioned above of *L. casei*. Accordingly, Group 3 received *L. casei* one week before and one week after developing carcinogenesis. Furthermore, Group 4 received *L. casei* for two weeks after developing cancer.

Pathological examination

Rats were anesthetized with methoxyflurane vapor and killed by a CO_2 box. The palatal mucosa and tongue were bisected. Gross lesions were measured and immediately snap-frozen in liquid N2 and stored at -70°C. The palatal mucosa was oriented and cut in a parasagittal plane; the tongues were cut in a coronal plane. Cryopreserved tissues were sectioned (6-8 pm), fixed in 10% buffered formalin, and stained with hematoxylin and eosin (H&E).

Gene expression assay

Oral expression of TLR4, NF-κB, DC-SIGN, and BCL-2 were assessed by real-time polymerase chain reaction (RT-PCR) assay. RNeasy mini kit (Yekta Tajhiz Azma, Tehran, Iran) was used to extract RNA according to the manufactured protocol from the blood samples of rats. Accordingly, 0.5-1 ml of whole blood was collected from rats' hearts into an anticoagulant-treated microtube before and after carcinogenesis. The quality of the extracted RNA was evaluated by assessing optical densities of 260/230 nm and 260/280 nm. In addition, gel electrophoresis was performed to observe ribosomal RNAs. QuantiTect Reverse Transcription Kit (Yekta Tajhiz Azma, Tehran, Iran) synthesized cDNA from extracted RNA according to the kit's protocol. QuantiTect SYBR Green PCR Kit (Yekta Tajhiz Azma, Tehran, Iran) was used to perform RT-PCR (Yekta-Tajhiz Kit). Briefly, a 20 µl reaction mixture containing 2 µl cDNA and 0.5 µM of each primer were mixed. The thermal cycle was as follows; an initial denaturation at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s and 55 °C for 30 s, and 70 °C for 30 s. The fold-change gene expression of target genes was calculated using the ΔCt method in control rats compared with rats treated with 4NQO and the GAPDH gene used as the housekeeping gene (Table 1). A.M. Elshaer, et al. Tissue and Cell 60 (2019) 38–4739.

Statistical analysis

Statistical analysis of the gene expression levels of TLR-4, NF-κB, and DC-SIGN in the animal models of SCC model gavaged with *L. casei*. The data of the RT-PCR was analyzed using the two-way ANOVA and Tukey posthoc test between treatment groups compared with the control group. The results were considered statistically significant if the *P*-value was less than 0.001, 0.01, or 0.05.

Results

General observations of animals

None of the rats did die during the study. All animals appeared in good health until macroscopic tumors appeared on the tongue. There was a decrease in the body weight of rats from day 0 to week 16th (Figure 2). The physical mobility of rats was unaffected, and the rats had a good fur luster. However, an improvement in body weight was observed in cancerous rats with orally gavaged received *L. casei*.

Macroscopic and biochemical observations

No spontaneous tumors developed at the mouth's palatal mucosa or other mucosal surfaces in the control Group 1 rats. Furthermore, no macroscopic changes were observed on the tongues of the rats with 4NQO administrations up to the 8th week. From week 10, a whitish tongue thickening was observed, along with a slight loss of definition of the palatial architecture. Additionally, no signs of metastases or swellings of the regional lymph nodes were observed on

the body surface of rats with 4NQO treatments. Also, increased tongue size and decreased body weight were observed in 4NQO administrated rats.

Gram staining, morphology, catalase activity, carbohydrate fermentation, pH resistance, and bile salt tolerance were all used to confirm that *L. Casei* is a probiotic. *L. Casei* was found to be catalase negative, gram positive, and immobile. *L. Casei* was also capable of fermenting four sugars: lactose, glucose, mannitol, and sucrose. Three degrees of growth were used to test the results of resistance to various pH levels (1: poor growth, 2: good growth, and 3: excellent growth). The bacterium grew well at pH > 3, showing that it is pH resistant. In *L. Casei*, the amount of light absorption indicated bile salt tolerance (p-value < 0.05).

Antimicrobial effect of *L. Casei*

The findings of the modified double-layer technique revealed that *L. casei* had an appropriate inhibitory effect on pathogenic bacteria. Accordingly, *L. casei* showed a high ability to inhibit bacterial pathogens, with a mean inhibitory of 10.2 mm for *S. aureus* and 11 mm for *E. coli*.

Pathologic results

Gross changes, including leukoplakia, erosion, ulcer, and papillary appearance on the dorsum of the posterior tongue, appeared during carcinogenesis. Accordingly, the histopathological findings ranged from hyperplasia (HP), mild-moderate dysplasia (mmDP), severe dysplasia (sDP), and in situ carcinoma (ISC) to well-differentiated invasive squamous cell carcinoma (SCC) (Figure 3). The severity of lesions corresponded to the duration of administration. In rats treated with 4NQO for 9, 13, and 16 weeks, the incidence of tongue cancer was 50.0%, 62.5%, and 77.8%, respectively.

Gene expression results

The results of the NFκB, BCL-2, DC-SIGN, and TLR4 were normalized with the GAPDH expression in each group, and the gene expression was reported as ΔCt. Accordingly, gene expression was reported as ΔCt of gene expression compared to the control Group 1. As shown in Figure 4, the NFκB gene expression was significantly increased in rats treated with 4NQO (p-value < 0.001). In addition, Group 4 showed higher expression of NFκB compared with treatment 1 (p-value < 0.01). No significant changes were observed between Group 4 and the untreated cancerous Group 2.

Further results showed a significantly decreased BCL-2 gene expression in Group 3 (p-value < 0.001). Also, an increased BCL-2 gene expression was observed in rats of the treatment Group 4 (p-value < 0.01). In addition, a slight increase in BCL-2 gene expression was observed in the cancerous rats in Group 2, which was not significant. The decrease of BCL-2 gene expression observed in treatment Group 3 was significantly different from untreated-cancerous rats and treatment Group 4 (p-value < 0.001). No significant Ct changes were observed between Group 4 and untreated cancerous Group 2.

The results of RT-PCR showed decreased DC-SIGN gene expression in rats with cancer (p-value < 0.001). Also, a slight increased DC-SIGN gene expression was observed in the treatment Group 3 that was not significant. However, the DC-SIGN gene was significantly expressed in the *L. casei*-treated Group 4 (p-value < 0.001). There was no significant difference in DC-SIGN gene expression between untreated-cancerous Group 2 and Group 4. However, the increase of DC-SIGN gene expression observed in rats of *L. casei*-treated Group 3 was substantially higher than that in Group 4 and untreated-cancerous Group 2 (p-value < 0.001).

Accordingly, the expression of the TLR4 gene was evaluated in the cancerous rats treated with *L. casei* before and after being cancerous and compared to the control Group 1. It was observed that TLR4 gene expression was significantly increased in untreated-cancerous Group 2 rats (p-value < 0.001). However, no significant changes were observed in TLR4 gene expression between Group 2 and Group 3. Furthermore, an increased expression of the TLR4 gene was obtained in Group 4 (p-value < 0.1). The decreased TLR4 gene expression observed in the untreated-cancerous Group 2 differed significantly from those in *L. casei*-treated Grou 3 and 4 (p-value < 0.001). Also, the increased gene expression of TLR4 was higher in Group 4 than in Group 3 (p-value < 0.05).

Discussion

Probiotics are widely employed in various settings, including medications, food supplements, and research. The use of probiotics in biology, cancer, and immunology has expanded considerably in recent years. It was shown that probiotic bacteria might decrease cancer risk by reducing their incidence and suppressing tumors. Probiotics have anticancer activity by inducing apoptosis, making them a very safe treatment approach (Śliżewska *et al.*, 2021). The prophylactic effects of *A. syzygii* isolated from curd, which has typical probiotic qualities, were investigated in a study by Aghazadeh et al. on human oral cancer cell lines. They showed that on KDR epithelial cells, *A. syzygii* had acceptable anticancer activity but no discernible cytotoxic effect (Aghazadeh *et al.*, 2017). There is ample evidence that probiotics may regulate cell proliferation and apoptosis (Yan & Polk, 2002; Ding *et al.*, 2020; Davoodvandi *et al.*, 2021). Therefore, the study of changes or expression of genes can be one of the goals of treatment or diagnosis in the study of cancer. Therefore, this study aimed to use probiotic live *L. casei* on the animal model of OSCC before and after inducing cancer and investigate the expression of genes that regulate cell death, including TLR-4, NF-κB, and DC-SIGN proteins.

The transcription factor NF-B regulates the expression of genes involved in cell survival, proliferation, apoptosis, invasion, and metastasis. The toll-like receptor (TLR) family is essential for pathogen identification and subsequent innate immune activation. TLR-4 and NF-kb expression suppression was linked to more malignant SCC (Li *et al.*, 2018). As a result, the bodyweight of the 4NQO rats decreased after week twenty-eight, and their general health declined. This was due to masticatory and swallowing difficulties resulting from 4NQO-induced enlargement of the tongue. The morphological changes in rats' tongue was an implication of oral

squamous cell carcinogenesis. Therefore, oral administration of live *L. casei* was performed to investigate the tumor growth regression and mucosal immune activation due to the bacilli pathogen-associated molecular patterns (PAMPs).

The results showed increased DC-SIGN expression after induction of SCC in rats. CLRs recognize carbohydrate structures and are vital for cell-cell communication and host defense against pathogens (Yan *et al.*, 2015). A substantial increased DC-SIGN expression was also in rats treated with *L. casei* after being cancerous. However, the DC-SIGN gene expression did not change in rats who received *L. casei* for one week before being treated with 4NQO. This implicates that *L. casei*, as a therapeutic, induces an inflammatory response by its PAMPs after causing cancer. Increased CLR production by damaged cells triggers multiple pathways leading to NF-κB activation. The results showed the overexpression of NF-κB in cancer-bearing rats receiving *L. casei*. This suggests the canonical NF-κB activation by *L. casei* in cancerous rats, primarily through PAMPs by the pattern-recognition receptors (PRRs). DC-SIGN signaling was shown to be beneficial for viral pathogens (Den Dunnen *et al.*, 2009). However, the increased expression of DC-SIGN in *the L. casei* treatment group suggests its beneficial role in tumor growth reduction.

TLRs and CLR belong to the PRRs families. Several factors of lactobacilli, including cell surface carbohydrates, enzymes modifying the structure of lipoteichoic acids, and metabolites, can activate the mucosal immune system through PRRs (Wells, 2011). Besides DC-SIGN, the results demonstrated a substantially increased expression of TLR4 on the tongue tissue of cancerous rats received *L. casei* after week 16th. The expressed TLR4 on the cell surface recognizes microbial membrane components and activates the (NF-κB) pathway signaling cascade (Pålsson-McDermott & O'Neill, 2004; Kawasaki & Kawai, 2014). This indicates that the membrane of the live *L. casei* is an activator of TLR4 on the surface of the cancer cell. In addition, these findings suggest that the activation of DC-SIGN and TLR4 on the epithelial cells of rats with oral cancer is the main starting point of innate immune activation. However, the exact NFκB downstream factors remain to be studied.

Apoptosis is programmed cell death that allows damaged cells to be removed efficiently. Cancer is characterized by dysregulation of the apoptotic cell death mechanism. Most anticancer medicines in clinical oncology today take advantage of intact apoptotic signaling pathways, which are regulated by pro- and anti-apoptotic factors like p53 and Bcl-2 (Pistritto *et al.*, 2016). NF-kB is a regulator of the anti-apoptosis protein Bcl-2 (Catz & Johnson, 2001). The results showed an increase in the Bcl-2 gene in cancerous rats. However, the declined Bcl-2 gene expression was observed in the cancerous rats that received L. casei before having 4NQO. This was due to mucosal immune activation by the PRR before carcinogenesis.

Meanwhile, the Bcl-2 gene expression was increased in the cancerous rats that received *L. casei* after week 16th. This finding supported Changizi et al., who showed enhanced expression of Bcl-2 in rats who received lactobacilli probiotics (Changizi *et al.*, 2021). However, this might be

controversial, as it has been shown that L. rhamnosus can induce apoptosis by decreased Bcl-2 gene expression in vitro (Riaz Rajoka *et al.*, 2019).

Conclusion

Probiotic live *L. casei* is a potent therapeutic candidate for treating oral SCCs by activating the mucosal innate immune response. This results from increased lactobacilli-associated PAMPs recognized mainly by PRRs, DC-SIGN, and TLR4, leading to activation of the NF-κB signaling pathway and cancer cell death. By reducing the expression of TLRs, NF-κB, and BCL-2, probiotics may regulate downstream pathways, which will cause apoptosis in cancer cells and improve tumor tissue. This idea can be used in the future of probiotics and other medical treatments to speed up the recovery of the disease. The results of the present study suggest that probiotic L. casei can reduce the expression of OSCC's markers, including TLR-4, NF-κB, and BCL-2 in oral rat cancer induced by 4-Nitroquioline 1-Oxide.

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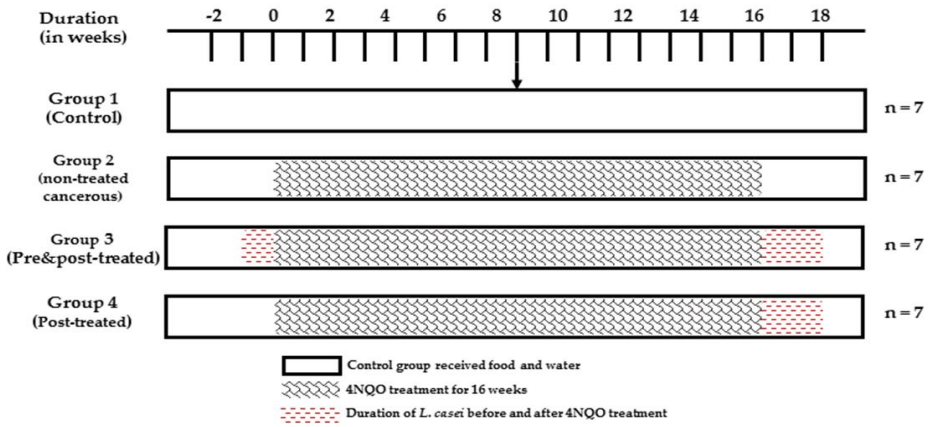

Figure 1. Schematic representation of 4NQO treatment and L. casei gavage.

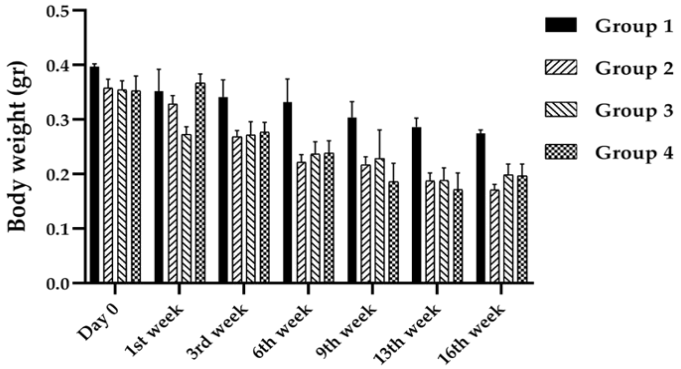

Figure 2. Bodyweight of the groups of rats after different times during the study.

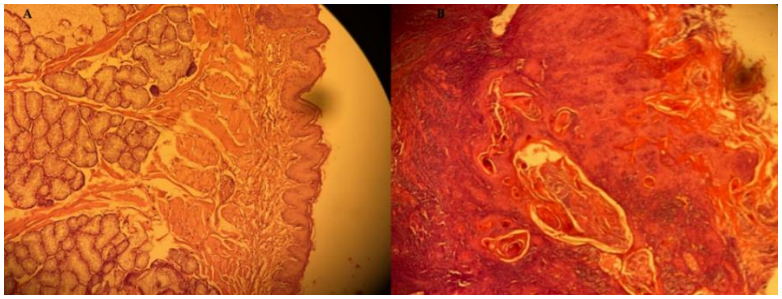

Figure 3. Pathological findings of rats' Tongue's mass before and after 4NQO administration. A) A microscopic finding of the normal epithelium of rat tongue. B) Sections from tongue mass in rats treated with 4NQO revealed a malignant epithelial neoplasm originating from squamous, arranged in nests and individuals invaded to subepithelium and muscularis layers. Tumoral cells show atypical features and form keratin pearls. Also, severe lymphocytic infiltration around invaded nests is seen. The mass was diagnosed with well-differentiated SCC.

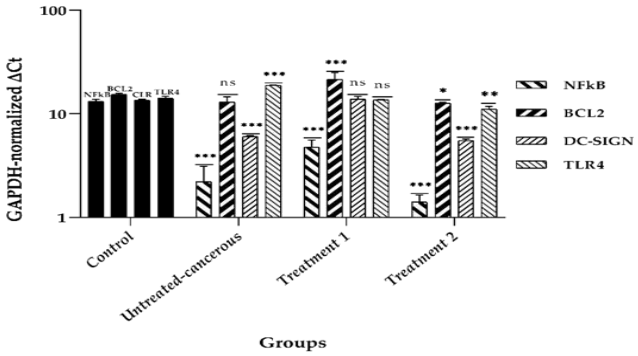

Figure 4. Gene expression of NFκB, BCL-2, DC-SIGN, and TLR4 in the control group and rats received live L. casei before and after being cancerous by 4NQO treatment. ΔCt of the genes is statistically compared to the control Group 1's ΔCts. p-value < 0.05, *; p-value < 0.01, **; p-value < 0.001, ***.

Table 1. Characteristics of the primers used for gene expression assay

Primer	Orientation	5' to 3' sequence	Amplicon size (bp)
BCL-2	F	GGGTCATGTGTGTGGAGAG	181
	R	AGCCAGGAGAAATCAAACAG	
NF-κB	F	TTCCCCTGTACGATAGTCGG	77
	R	GTGCTAGAAGCTGGAGGATG	
TLR4	F	GCTTCTCCAATTTCTCACAAC	201
	R	AGGTCATTTTTGTCTCCACAG	
DC-SIGN	F	GGCGGCCCTGTACTTTTTG	502
	R	AAGGCAGCCAAGCAAGGAC	
GAPDH	F	AGCTCATTTCCTGGTATGACA	125
	R	TTGCTCTCAGTATCCTTGC	