Research Paper

The Effect of Licorice on Serum Lipid Peroxidation and Telomere Length of Immune Cells in Japanese Quail (as an Animal Model) Following Vaccination by *Salmonella* Bacterin

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ABSTRACT

Background: Bacterin-based vaccination may be a stressful condition, leading to harmful effects, such as high lipid peroxidation and telomere shortening. This study aimed to reduce these probable side effects via the administration of different concentrations of licorice (because of its anti-oxidative and anti-aging properties).

Materials and Methods: Japanese quails as animal models were reared for 42 days under standard conditions. The birds were divided into eight groups, including a control group, licorice treatments (licorice 0.02%, Licorice 0.04%, and licorice 0.08% groups), and *Salmonella* bacterin treatments (bacterin, bacterin+licorice 0.02%, bacterin+licorice 0.04%, and bacterin+licorice 0.08% groups). The *Salmonella enterica* bacterin-based vaccine was injected twice on days 14 and 28. Serum lipid peroxidation and telomere length of immune cells in all experimental groups were measured.

Results: Lipid peroxidation and telomere shortening enhanced following bacterin vaccination. Licorice at doses of 0.04%, 0.08%, or both reduced the lipid peroxidation and telomere shortening in non-vaccinated and vaccinated birds, while the dose of 0.02% was not effective.

Conclusion: This study confirmed the side effects of high lipid peroxidation and telomere attrition for S. *enterica* bacterin-based vaccination. Also, the improving properties of licorice for these side effects were considered to be effective.

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1. Introduction

Licorice (*Glycyrrhiza glabra*) has been utilized as a botanical specimen for about 6,000 years. The plant known as licorice is indigenous to southern Europe and some areas of Asia. The utilization of this substance as a botanical remedy and organic sweetening agent is prevalent. Licorice, a botanical remedy with a history spanning a millennium in traditional Chinese medicine, has been officially recognized by the Ministry of Health of China. It is included in a comprehensive list of 101 traditional edible medicinal plants. In addition, a multitude of traditional formulae using licorice have been transformed into Chinese-trademarked medications and are often employed in clinical settings [1].

Licorice is considered a fundamental component of traditional Chinese herbal therapy. The utilization of licorice to enhance the vital life force, known as qi, in the heart and spleen has been a prevalent practice in Chinese culture since 25 AD. Additionally, it is used in alleviating symptoms, such as cough, phlegm, dyspnea, spasms, and discomfort [1].

Numerous investigations about licorice have been undertaken since the 1960s. As of the present, licorice has yielded over 200 distinct chemical compounds using isolation techniques. The analysis of licorice root extract revealed the presence of a diverse range of phytochemical compounds, encompassing flavonoids (specifically isoflavonoids, formononetin, and liquiritin), saponin triterpenes (including liquirtic acid and glycyrrhizin), sugars, coumarins, amino acids, starch, tannins, phytosterols, choline, and various vitamins (such as ascorbic acid). Licorice is composed of a total of 20 triterpenoids and 300 flavonoids. The extract derived from the licorice root is found to possess a glycyrrhizin content of around 25%. Glycyrrhizin is composed of glucuronic acid and glycyrrhetinic acid. Licorice was subjected to chemical analysis, which provided insights into its nutritional composition. The results indicated that licorice had the following components: carbohydrates (47.11%), fiber (24.48%), protein (9.15%), silica (3.56%), and low levels of fat (0.53%) [2, 3].

Pharmacological investigations have shown the hepatoprotective, gastroprotective, and neuroprotective properties of licorice, as well as its immunomodulatory, anticancer, anti-inflammatory, anti-allergic, anti-HIV, antidiabetic, antimicrobial, antioxidant, and antiviral activities. Additionally, the investigation of the fundamental mechanism behind the actions of licorice in combination preparations has emerged as a prominent area of research [4].

Telomeres are a type of tandem nucleotide repeats that are extremely conserved. They consist of both proximal double-stranded and distal single-stranded portions. These areas, in conjunction with shelterin proteins, protect the ends of chromosomes, preserving the genome's integrity [5]. Telomeres experience age-related gradual attrition as a result of the intrinsic constraints of DNA replication and the inhibition of telomerase in the majority of somatic cells. Telomeres that are either short or defective are acknowledged as DNA double-stranded breaks, prompting cells to enter replicative senescence. The process of telomere shortening serves as a mechanism for cell counting, ultimately leading to replicative senescence by restricting the ability of cells to undergo mitosis. Age and age-related disorders are linked to telomere length, a complicated inherited feature. The process of telomere attrition is affected by oxidative damage and replicative stress, which may be attributed to several causes, such as genetic, epigenetic, and environmental factors [6]. Telomere shortening is well recognized as a prominent characteristic of both cellular senescence and organismal aging, as documented in the literature. Age-related disorders often exhibit an increased rate of telomere degradation [7].

Salmonella spp. are facultative intracellular pathogens that can cause localized or systemic infections, have a significant economic and public health impact, and continue to be the most dangerous pathogen for food safety in the world, with poultry serving as the principal vector of transmission [8]. Various tactics may be employed to manage *Salmonella* infection in chicken, including the utilization of antibiotics, feeding-based approaches (such as prebiotics, probiotics, bacterial subproducts, and phytobiotics), as well as non-feeding-based methods (such as bacteriophages, in ovo injection, live and killed vaccinations) [8, 9].

This study aimed to assess the impact of *Salmonella* bacterin-based (killed) vaccination on lipid peroxidation and telomere length in Japanese quail, serving as an animal model. Additionally, the potential ameliorative effects of licorice were investigated.

2. Materials and Methods

Bacterin preparation and quality control test

Salmonella enterica serovar Typhimurium was cultivated in nutritional agar medium for 24 hours at 37°C, and then, the bacterial suspension was mixed with 1% formalin (v:v), which was then maintained for 24 hours at 4°C. The solution was then centrifuged in a 4000 g

chamber for 30 min to extract inactive microorganisms. The pellet was once again suspended in sterile normal saline after the supernatant was removed and discarded. To make sure that all of the formalin and culturing medium were eliminated, the last step was carried out three times. Concentrates of inactivated bacteria that were cleaned and adjusted to contain 109 CFU/mL of saline were placed in a MacFerland matching tube [10]. According to the standard international protocol outlined by the British veterinary codes, the provided *S. Typhimurium* bacterin was evaluated for full inactivation and safety [10].

Birds, treatments, and blood sampling

The Shahrekord University experimental facility was used for all of the processes. Eighty one-day-old Japanese quail were randomly distributed among floor pens and nurtured for 42 days under standard conditions. The birds were divided into eight groups, including a control group, licorice treatments (licorice 0.02%, licorice 0.04%, and licorice 0.08% groups), and *Salmonella* bacterin treatments (bacterin, bacterin+licorice 0.02%, bacterin+licorice 0.04%, and bacterin+licorice 0.08% groups). The licorice root was purchased and ground with a lab hammer mill. A 2:10 ratio of the dry powder to 86% ethanol was used in the mixture. After being well combined, it was shaken for 15 min and allowed to stay at room temperature for 72 h before being filtered through Whatman Filter Paper No. 1 and dried at 50˚C. In the feed of the birds during raising, the concentrations of 0.02%, 0.04%, and 0.08% of the produced licorice powder were added. On days 14 and 28, 0.2 mL of the *Salmonella* bacterin-based vaccine was subcutaneously administered.

Genomic DNA extraction and telomere length analysis by quantitative real-time PCR

On day 42, blood samples (ten birds from each group) were taken from the brachial veins of birds. Using the salting-out approach, genomic DNA was isolated from whole blood samples [11]. A Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the amount of extracted DNA. Pure DNA was defined as samples having 260/280 absorbance ratios of less than 1.8. Before PCR, the produced DNA pellet was resuspended in 50 µL of solvent buffer and kept at -70˚C.

A 2x SYBR Green Real-Time PCR kit (Parstous Co., Mashhad, Iran) was used to perform relative quantitative real-time PCR (RT-qPCR) to evaluate the relative telomere length. Table 1 lists the sequences and characteristics of the primers for the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the telomere [12]. GAPDH was a stable gene according to Criscuolo et al. (2009) [12]. For both telomere and GAPDH, the amplification was completed in a final volume of 10 µL. In each reaction, a sample of 15 ng DNA was included. Telomere forward and reverse primers had final concentrations of 250 and 750 nM, respectively. Each GAPDH primer had a final concentration of 250 nM. For each sample, amplifications were carried out in triplicate in a Rotor-Gene 6000 thermocycler (Qiagen, Australia). Twenty cycles of 95˚C for 15 s and 54˚C for 2 min each comprised the PCR protocol for telomeres. The GAPDH PCR protocol included 30 cycles of 95˚C for 20 s, 60˚C for 20 s, and 72˚C for 20 s over 10 min. To confirm there was no contamination, a reaction without a template was done. Using LinRegPCR software, version 2012.0 (Amsterdam, Netherlands), the threshold cycle number (Ct) of PCR and mean efficiency values (E) for telomere and GAPDH were calculated. According to Hassanpour et al. (2023) [5] and Pfaffl (2001) [13], the relative telomere length was computed. Using this technique, each sample's telomere length was measured for GAPDH.

Thiobarbituric acid reactive substances (TBARS) assay

The chemicals of the assay were all bought from Sigma-Aldrich (St. Louis, MO, USA). Malondialdehyde (MDA), a marker of lipid peroxidation, was evaluated in a blood sample using thiobarbituric acid (TBARS) according to previous studies [14, 15]. The serum of blood samples was separated from 80 birds (ten birds from each group). To precipitate proteins, trichloroacetic acid was added to the serum. The recovered supernatants were centrifuged. The samples were then incubated for 10 min in a boiling water bath after an equivalent amount of TBA was added. Using a spectrophotometer (Corning 480, USA), absorbance at 532 nm was measured after samples were cooled. Data from TBARS calculations were converted to micromolar units (µM) [14].

Statistical analysis

Data are represented as Means±SE The Kolmogorov–Smirnov test was performed to test the normality of data. Parametric tests were used to compare the normally distributed data. The results were statistically compared between experimental groups by the student t-test or Duncan's multiple range test following a one-way analysis of variance (ANOVA). All statistical analyses were computed by SPSS software, version 26 (SPSS Inc, Chicago, IL, USA). $P<0.05$ were considered significant.

3. Results

Figure 1 compares TBARS levels as the lipid peroxidation index between the control and bacterin groups of birds. This parameter in the bacterin group was higher than in the control group (P=0.034).

Figure 2 compares TBARS levels between different licorice groups of birds. This parameter was lower in the licorice 0.04% group than in other experimental groups (control and licorice 0.2 and 0.08%) (P<0.05). TBARS levels did not differ between the control, licorice 0.02%, and licorice 0.08% groups (P>0.05).

Figure 3 indicates TBARS levels between bacterin and bacterin+licorice groups of birds. This parameter was lower in the bacterin+licorice 0.04% and bacterin+licorice 0.08% groups than in the bacterin group (P<0.05). TBARS levels did not differ between the bacterin+licorice 0.02% group and other experimental groups (P>0.05).

Figure 4 compares the relative telomere length (telomere/GAPDH) in immune cells of control and bacterin groups of birds. The telomere length decreased in the bacterin group compared to the control group (P=0.021).

Figure 5 displays the relative telomere length in immune cells of different licorice groups. This parameter was higher in the licorice 0.08% group than in other experimental groups (control and licorice 0.02% and 0.04%) (P<0.05). The telomere length did not differ between the control, licorice 0.02%, and licorice 0.04% groups (P>0.05).

Figure 6 shows the relative telomere length between bacterin and bacterin+licorice groups of birds. The telomere length was higher in the three bacterin+licorice groups than in the bacterin group (P<0.05). This parameter was significantly higher in the bacterin+licorice 0.04% groups than in other experimental groups.

Table 1. Primers used for quantitative real-time PCR analysis of Japanese quail

Target	Primers	Temperature (°C)	GC (%)	Ta (°C)	PCR Product (bp)	Accession No.
Telomere	5′-CGGTTTGTTTGGGTTTGGGTTTG GGTTTGGGTTTGGGTT-3′	67.6	48.7	54	78	-
	5′-GGCTTGCCTTACCCTTACCCTTA CCCTTACCCTTACCCT-3′	69.7	53.8			
GAPDH	5′-CTTCTGTCTCCCCCACTCCT -3′	60.2	60.0	61	67	XM_015873412.2
	5′-TCTATCAGCCTCTCCCACCTC -3′	60.1	57.1			

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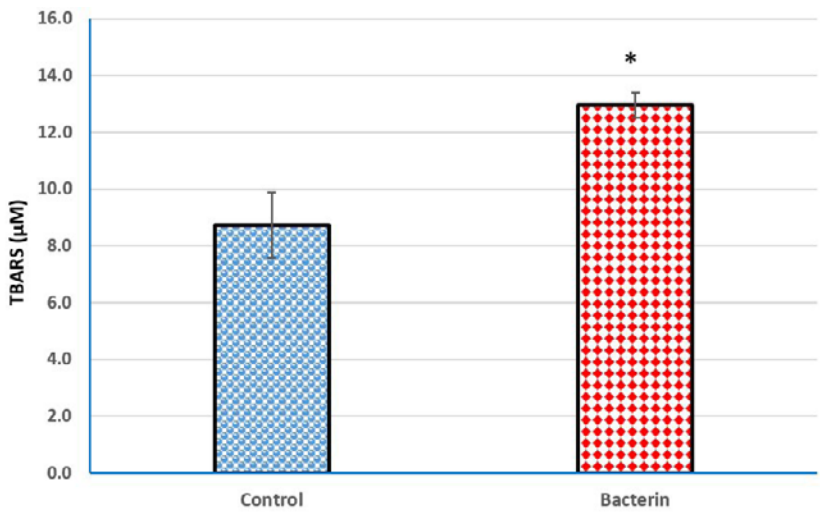

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Figure 1. Comparison of TBARS levels as the lipid peroxidation index between the two groups of birds
*Significant difference between the two groups (P<0.05).

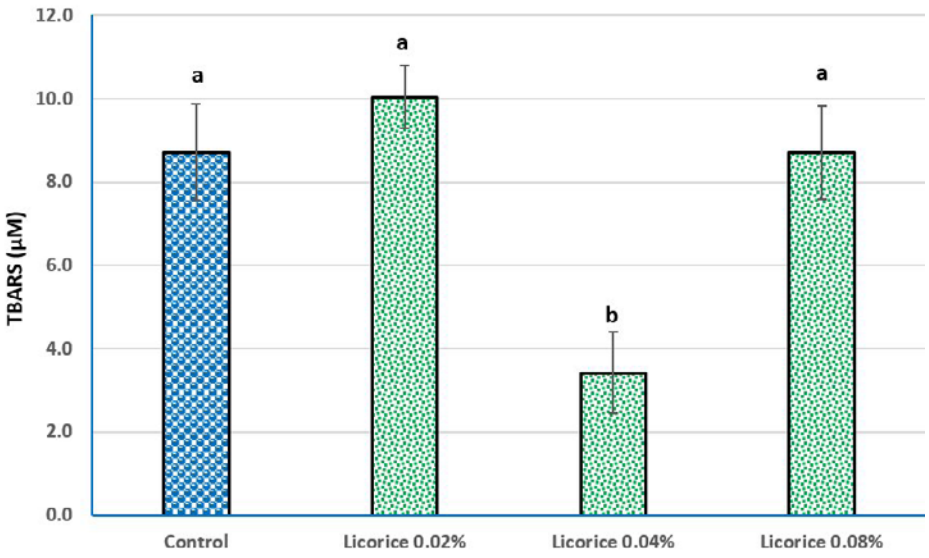

Figure 2. Comparison of TBARS levels as the lipid peroxidation index between the experimental groups of birds
[a, b]Significant differences between the groups (P<0.05).

4. Discussion

The antioxidant properties of licorice are attributed to its several constituents, including glycyrrhizic acid, 18β-glycyrrhetinic acid, glabridin, and liquiritin [16]. The aforementioned components can elicit immunological responses and augment the activity of antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase [17, 18]. In their study, Kim et al. (2012) documented the potent ferric-reducing activities and free radical scavenging ability of prenylflavonoids produced from licorice, including dehydroglyasperin C (DGC), dehydroglyasperin D (DGD), and isoangustone A (IsoA). Furthermore, all three compounds exhibited significant inhibition of lipid peroxidation in rat tissues, as well as the formation of reactive oxygen species (ROS) produced by H_2O_2 in hepatoma cells [19]. The results of our study indicated that the application of low (0.02%) or high (0.08%) concentrations of licorice did not result in a significant reduction in lipid peroxidation. However, it was observed that a moderate concentration (0.04%) of licorice had a favorable impact on decreasing

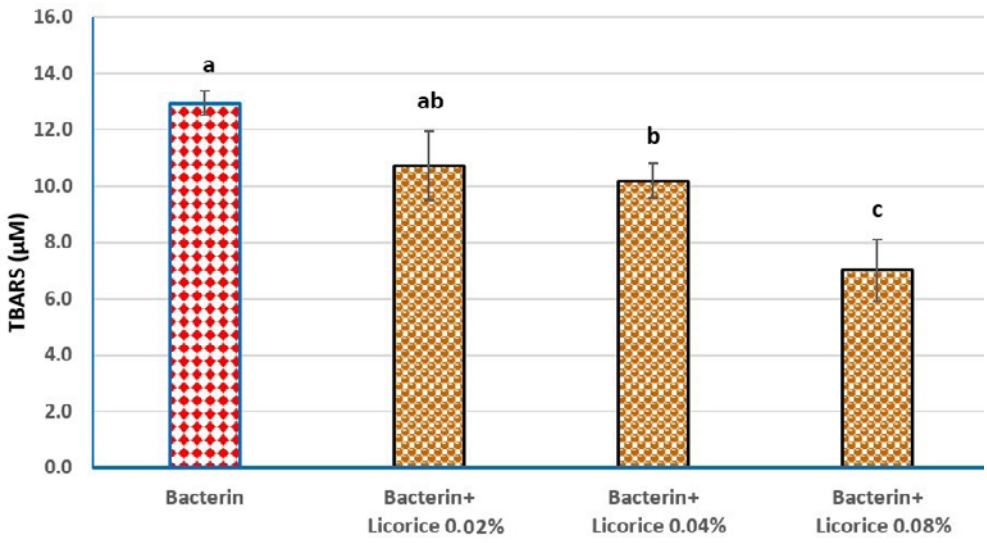

Figure 3. Comparison of TBARS levels as lipid peroxidation index between experimental groups of birds
[a,b,c] significant difference between groups (P<0.05).

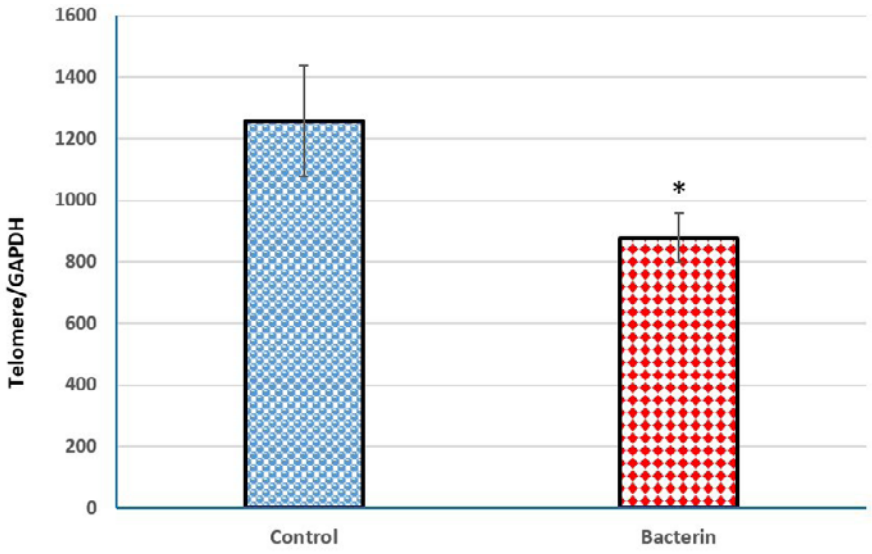

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Figure 4. Comparison of relative telomere length (telomere/GAPDH) in immune cells of the two groups of birds
*Significant difference between two groups (P<0.05).

lipid peroxidation. According to a study conducted by Nazari et al. (2017), the impact of licorice is contingent upon the dosage administered. Higher dosages of licorice have the potential to induce acute, sub-acute, and chronic toxicities, particularly in cardiovascular health, kidney function, and muscle disorders [20].

The current study employed a *Salmonella* bacterin as a killed vaccine in quails. The potential adverse effects of this vaccine were assessed by monitoring the levels of lipid peroxidation and telomere shortening following a 42-day rearing period. Our findings demonstrated that the administration of bacterin resulted in an elevation in lipid peroxidation levels and a reduction in telomere length. These outcomes are in line with those reported by Tkachenko et al. (2016), who found that trout lipid peroxidation increased after *Yersinia ruckeri* bacterin injection [21]. Prior research has shown a negative association between telomere length and lipid peroxidation/oxidative stress, which might provide a rationale for the observed telomere shortening in our study as a result of

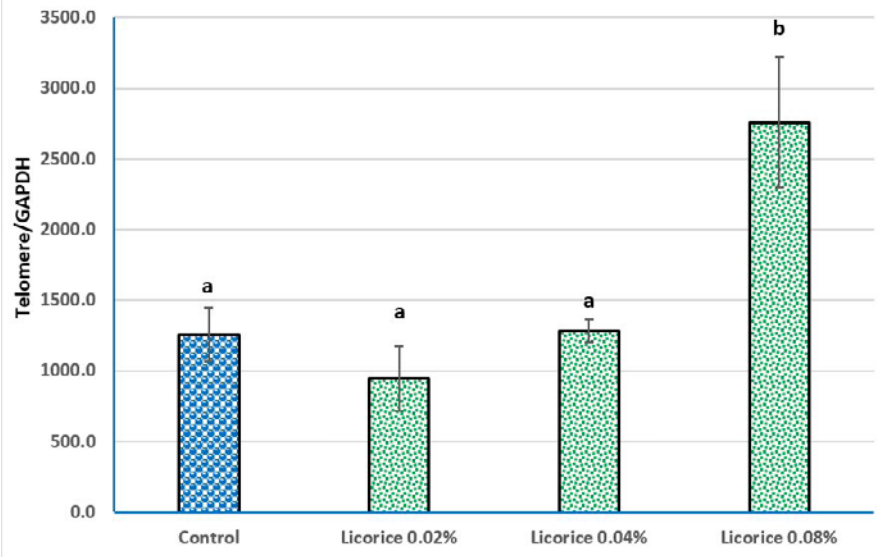

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Figure 5. Comparison of relative telomere length (telomere/GAPDH) in immune cells of the experimental groups of birds
a, bSignificant differences between the groups (P<0.05).

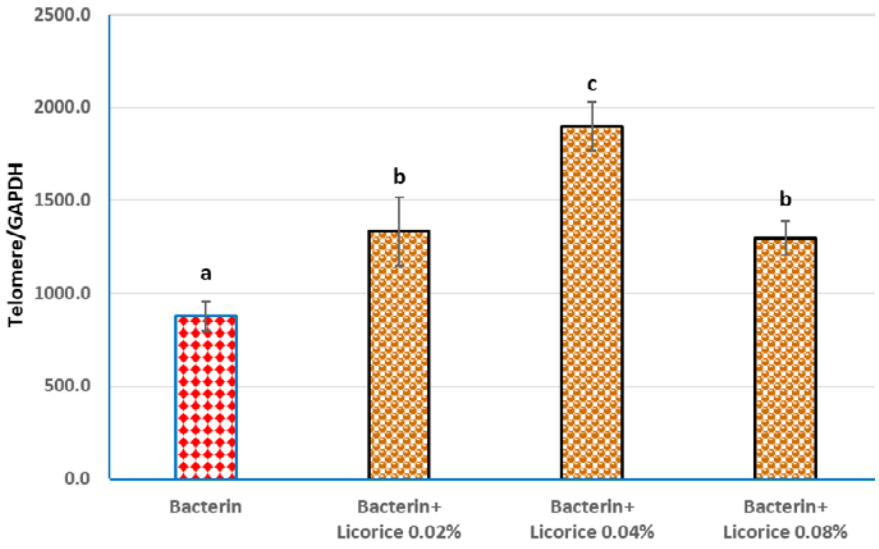

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Figure 6. Comparison of relative telomere length (telomere/GAPDH) in immune cells of the experimental groups of birds
[a, b, c] Significant differences between the groups (P<0.05).

heightened lipid peroxidation induced by bacterin administration [22-24].

Our findings showed that once the birds undergo the stress of bacterin immunization, the use of licorice could improve lipid peroxidation, which is completely dose-dependent. Although in healthy birds, only a mild dose of licorice decreased lipid peroxidation, it seems that during induced stress, higher doses are more applicable. However, the antioxidant effect of licorice or its components has been confirmed during different stressed conditions, e.g. vibration stress [25], heat stress [26-28], lead toxicity [29], high stocking density [4], and noise stress [30].

Many components of licorice have anti-aging effects. Chakraborty et al. (2018) reported that the phenylflavonoids dehydroglyasperin C, dehydroglyasperin D, and isoangustone A, which serve as superoxide scavengers and prevent wrinkles cause its anti-aging properties [31]. According to Liu et al. (2022), the main component of licorice, glycyrrhizin, acts as a high mobility group box 1 (HMGB1) inhibitor to reduce endothelium-dependent relaxation impairment, vascular aging, and high-glucose-induced endothelial cell senescence in diabetes [32]. In human lymphocytes, glycyrrhizin also enhances DNA resistance against genetic and oxidative damage [33]. However, the previous studies confirm our data for telomere length improvement of licorice. This ameliorating effect of licorice was also observed after bacterin immunization.

As our data showed, licorice 0.04% or 0.08% could have beneficial effects on telomere length and lipid peroxidation and these effects were dependent on stressful conditions; however, further studies are needed to evaluate other effects of licorice in the cells under normal and stressful conditions, in addition of lipid peroxidation and telomere shortening.

5. Conclusion

Our results provided evidence that the *Salmonella* bacterin-based (killed) vaccine may increase lipid peroxidation and progress the telomere shortening while consumption of licorice at different doses could improve these side effects of vaccination.

Ethical Considerations

Compliance with ethical guidelines

All the procedures in this study were approved by the Institutional Animal Care and Use Committee of Shahrekord University (Code: IR.SKU.REC.1402.025)., based on the welfare standard of the 1964 Declaration of Helsinki.

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Authors' contributions

Study and experimental design: Seyed Sattar Tohidifar, Hossein Hassanpour and Abdolkarim Zamani Moghaddam; Major experimental work, data analysis, and writing the manuscript: Mahdi Tavakoli, Hossein Hassanpour, Masoud Ghorbanpour and Mohammad Rasool Amini.

Conflicts of interest

The authors declared no conflict of interest.

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