JOURNAL OF ADVANCED VETERINARY AND ANIMAL RESEARCH
ISSN 2311-7710 (Electronic)
http://doi.org/10.5455/javar.2019.f351
A periodical of the Network for the Veterinarians of Bangladesh (BDvetNET)

September 2019
VOL 6, NO. 3, PAGES 333–340

ORIGINAL ARTICLE

Effects of supplementation of *Bacillus* spp. on blood metabolites, antioxidant status, and gene expression pattern of selective cytokines in growing Barki lambs

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ABSTRACT

Objective: In this study, we investigated the potential immune-enhancing effects in addition to anti-oxidative stress properties of commercially accessible *Bacillus subtilis* supplementation in Barki lambs.

Materials and Methods: Twenty apparently healthy weaned Barki lambs were used in this study and distributed randomly into two experimental groups: Negative control group, received control basal diet without any feed supplements and a supplemented group, received control basal diet supplemented with water added to commercially accessible bacilli at 1 gm/l/day for 30 consecutive days. Blood samples were collected from each lamb before starting the experiment (T0), 2 weeks (T15), and 4 weeks (T30) post-supplementation for serum biochemical analyses, total leucocytes and lymphocytes count, and real-time polymerase chain reaction assays.

Results: The supplemented group showed a significant increase ($p < 0.05$) in the total number of leukocytes and the number of lymphocytes, lysozyme activity, reduced glutathione, total antioxidant capacity with a significantly lower malondialdehyde values at T30 and significantly higher levels ($p < 0.05$) of serum catalase and nitric oxide at T15 as compared with control ones. *B. subtilis* elicited maximal up-regulation of most of the studied genes compared with the control group.

Conclusion: The results herein suggest that *B. subtilis* could be used as useful nutritional supplements to support the immune system in healthy lambs.

ARTICLE HISTORY

Received April 30, 2019
Revised May 30, 2019
Accepted June 12, 2019
Published July 13, 2019

KEYWORDS

Bacillus subtilis; immunity; antioxidant status; RT-PCR; sheep

Introduction

Probiotics was defined as live microorganisms intended to provide health benefits when consumed as supplementation to food or water. Numerous microbes are considered as probiotics, mostly lactic acid bacteria (*Lactobacillus* sp., *Bifidobacterium* sp., etc.) and little non-lactic acid bacteria (*Bacillus licheniformis, B. subtilis*, etc.) [1]. Soliman et al. [2], Bahari [3], and Saleem et al. [4] reported that probiotics well exerted beneficial effects when supplemented in a suitable amount. Probiotics are able to re-establish the intestinal microflora [5], helpful to ruminal microflora development and improve the immunity [6], and decline the prevalence of intestinal infections [7]. In addition, the basal diet supplemented with probiotic can improve feed consumption and nutrients absorption [8–10].

Probiotic supplementation to ruminants has led to an optimistic effect on feed ingestion and increased body weight and feed conversion ratio [8–10]. Moreover, yeast culture supplementation to a ruminant, enhanced animal's performance, blood glucose level [11], total protein [12], and decline cholesterol level [4,13]. However, less data are currently available regarding the effects of probiotic-containing *B. subtilis* in healthy sheep. Moreover, their effects on the expression of immunity genes remain to be fully elucidated. Therefore, the aim of this study was to investigate the potential effects of enhanced immunity and

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How to cite: Mousa S, Elsayed A, Marghani B, Ateya A. Effects of supplementation of Bacillus spp. on blood metabolites, antioxidant status, and gene expression pattern of selective cytokines in growing Barki lambs. J Adv Vet Anim Res 2019; 6(3):333–340.

anti-oxidative stress activities of commercially available *Bacillus* spp. probiotic in growing healthy Barki lambs. The latter was carried out via measuring immune and antioxidant metabolic and biochemical variables and elucidating the mRNA levels of selected immune, antioxidant, and lipogenic candidate gene markers using real-time PCR.

Materials and Methods

Animals and study design

Clinically normal weaned Barki lambs (n = 20), aged 2 to 3 months, had an average body weight of 12.58 ± 2.3 kg, were included in this study. The study was conducted at Mariut Research Station-Desert Research Center-El-Amria-Alexandria-Egypt. Three weeks prior to the experiment, the investigated lambs were acclimatized in separate semi-open shaded pens and fed on concentrated feed mixture consisted of wheat bran 300 kg, soya bean 250 kg, corn 400 kg, sodium chloride10 kg, calcium carbonate 20 kg, Premix 1 kg, Netro-Nill 0.5 kg, and Fylax 0.5 kg. The investigated lambs were fed on 350 g of concentrate feed mixture plus 350 g of alfalfa hay/head/day and water was given ad-libitum throughout the experimental period (30 days). The animals were given a prophylactic dose of broad-spectrum anthelmintic (Ivermectin/Clorsulan [AVICO], Amman, Jordan) at a dose of 200 µg plus 2 mg Clorsulon/kg BW once via the subcutaneous route. All animals were clinically healthy, with no history of metabolic or concurrent conditions and were kept under identical housing and vaccination conditions throughout the study period. Lambs were distributed randomly into two groups (n = 10) for each; negative control group received control basal diet without any feed supplements and a supplemented group received control basal diet supplemented with water added to commercially accessible bacilli at 1 gm/L/day for 30 consecutive days. The probiotic "five-MEN SONG" has been received from Central Veterinary Medicine, Hanoi, Vietnam, and contains *B. subtilis* 8.4×10^6 CFU, Sorbitol sodium 400 mg, Vitamin B1 200 mg, and glucose up to 100 gm. This protocol study and experimental procedures were approved by the Ethics Committee of the Faculty of Veterinary Medicine—Mansoura University—Egypt, for care and use of experimental animals.

Blood samples collection and measurements

Ten ml of blood were collected from each animal via jugular venipuncture at three times: before starting the experiment (T0), at 15th day (T15), and at 30th day (T30) from giving supplements. The collected blood samples were divided into plain serum separation tubes (i.e., without anticoagulants) and Ethylenediaminetetraacetic acid tubes to harvest serum and whole blood, respectively, from each sample. Tubes of blood samples were immediately transported in an ice box to the laboratory for further processing. Serum separation by lifting blood samples in serum separation tubes at room temperature to be clot, then centrifugation at 3,000 rpm for 15 min. Non-hemolyzed serum samples were harvested and kept in a deep freezer in small aliquots to be used for subsequent biochemical analysis. The following commercial kits were used according to standard protocols of the suppliers to quantify total protein, albumin, urea, and creatinine (Gamma Trade company, Egypt): AST (aspartate aminotransferase) and ALT (alanine aminotransferase) (Specterum company, Egypt) on a selective chemistry analyzer (Apple 302, USA); malondialdehyde (MDA) (Biodiagnostic Egypt, CAT No: MD2529); nitric oxide (NO) (Biodiagnostic Egypt, CAT .No. NO2533); catalase (CAT) (Biodiagnostic Egypt, CAT No: CA252417); reduced glutathione (GSH) (Biodiagnostic Egypt, CAT No: GSH2511); and total antioxidant capacity (TAC) (Biodiagnostic Egypt, CAT No: TA25 13). Serum lysozyme activity was determined by the turbidimetric assay. Whole blood samples were used for total leucocytes and differentiation of leukocytes in a blood smear by microscopic analysis, and also for real-time PCR assay.

RNA extraction and reverse-transcriptase (RT)-PCR

Whole blood samples were subjected to total RNA extraction using Trizol™ reagent (Invitrogen, UK), in accordance with the manufacturer's instructions (Direct-zol™ RNA MiniPrep, catalog No. R2050). The amount of RNA extracted quantified and qualified using a NanoDrop® (ND-5000 spectrophotometer) and its integrity was evaluated by agarose gel electrophoresis. An equivalent to 1 mg of RNA was transferred to cDNA with high capacity (SensiFast™ cDNA synthesis kit, Bioline, catalog No. Bio-65053). PCR amplifications were performed in a final volume of 20 µl containing total RNA template up to 1 µg, 4 µl 5× Trans Amp buffer, 1 µl reverse transcriptase and DNase free-water up to 20 µl. Reverse-transcription was done through placing the final reaction volume in a thermal cycler with the following cycling program; at 25°C for 10 min for primer annealing, followed by reverse transcription at 42°C for 15 min, then inactivation at 85°C for 5 min. The samples were held at 4°C.

Quantitative real-time PCR

Relative quantification of mRNA levels of genes encoding immunity, antioxidant in addition to lipogenic genes was assessed in the blood of lambs by quantitative RT-PCR, using SYBR Green PCR Master Mix (2x SensiFastTM SYBR, Bioline, catalog No. Bio-98002). Primer sequences, annealing temperature, accession number, and amplified PCR product size (bp) were represented in Table 1. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The reaction

volume 20 µl consisted of 10 µl 2× SensiFast SYBR, 3 µl cDNA, 5.4 µl H_2O (d.d water), and 0.8 µl of each primer. The PCR was cycled as follows: at 95°C for 2 min, followed by 40 cycles at 94°C for 10 sec, annealing for 30 sec at temperatures represented in Table 1 for each gene, and the reaction was completed at 72°C for 20 sec. After completion of the amplification phase, a melting curve analysis was implemented to authorize the specificity of the PCR product. Analysis of genes expression was done using the $2^{-\Delta\Delta Ct}$ method [14].

Statistical analysis

All data obtained were expressed as mean ± SEM (standard error) and statistically analyzed by using SPSS version 17 [15]. One-way analysis of variance followed by Duncan's multiple range tests to compare between variables. The mean values were at the level of $p < 0.05$. RT-PCR results were performed by determining the values of Δcycle threshold (ΔCt) using GAPDH) as an internal control for normalization. The $2^{-\Delta\Delta Ct}$ method was used for gene expression analysis [16].

Results and Discussion

Clinical investigations

An overview of clinico-pathological alterations with genes expression regulates immune-inflammatory responses are illustrated in Tables 2, 3 and in Fig. 1–4. Prior to the experimental study, all lambs were clinically healthy and showed no evidence of illness. All vital signs of the investigated lambs were normalized and the animals showed no detectable clinical abnormality throughout the study period. There was no evidence of gastrointestinal abnormalities.

Body weight

At the initial stage, the two studied control and supplemented groups did not show significance ($p > 0.05$) differences in the body weight. At 15th day (T15), the supplemented group showed greater body weight in comparison to the non-supplemented group, although variations between the two experimental groups were not significant. However, at 30th day (T30), the supplemented group showed a significant increase in the body weight when compared to non-supplemented group (Fig. 1). Our findings were similar to those previously reported by several researchers in lambs [17–21], in growing kids [22–24], and in buffalo calves [11]. The authors endorsed that pathogenic microbes number was decreased and beneficial microbes number was increased, which resulted in an improvement in dry matter intake, digestion of crude fiber, and decrease the occurrence of diarrhea with the improvement of the cellulolytic activity within the rumen causing effective fiber degradation and the enhancement nutrient digestion. In contrast, the studies of

Table 1. Oligonucleotide primers sequence, accession number, annealing temperature, and PCR product size of the studied genes.

Gene	Oligonucleotide sequence (5'-3')	Accession number	Annealing temperature (C°)	Size (bp)
IL-5	f TCTGCGTTTGACCTTGGTAGCTCT r TCAGCAGAGTTTGATGCGTGGAGA	NM_001009783.1	64	Less than 155
IL-6	f TGCAGTCCTCAAACGAGTGGGTAA r AGCCGCAGCTACTTCATCCGAATA	NM_001009392.1	62	Less than 155
TLR4	f GGTTCCCAGAACTGCAAGTG r GGATAGGGTTTCCCGTCAGT	AY957615	58	117
SOD1	f CGAGGCAAAGGGAGATACAG r TCTCCAAACTGATGGACGTG	M81129	60	90
Tollip	f CTGGTGCTGTCCTACACGTC r ACAGTGGGCATTCCTGTGAT	NM_001039961	56	122
ACACA	f ATGTGGCCTGGGTAGATCCT r ACGTAACACAAGGCTGATGGTG	NM_001009256.1	60	261
FASN	f GGAAGGCGGGACTATATGGC r CATGCTGTAGCCTACGAGGG	XM_004013447.1	62	278
SCD	f GGCGTTCCAGAATGACGTTT r TGAAGCACAACAGCAGGACA	NM_001009254.1	58	251
CAT	f GAAACGCCTGTGTGAGAAC f ACATAGGTGTGAACTGCGT	XM_012096208.3	58	171
GAPDH	f TGACCCCTTCATTGACCTTC r GATCTCGCTCCTGGAAGAG	NM-001034034	62	143

ACACA: acetyl-CoA carboxylase alpha; SCD: stearoyl-CoA desaturase (delta-9-desaturase); IL: interleukin; TLR4: Toll-like receptor 4; SOD: superoxide dismutase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Tolip: Toll-interacting protein; CAT: catalase.

Adjei-Fremah et al. [25] and El-Ashker et al. [26] reported no effect of orally supplemented multi-strain probiotic in Ossimi lambs and dairy cows, respectively. In a like respect, Titi et al. [27] summarized that lambs supplemented with yeast did not show any significant increase in their growth rate. Likewise, Baranowski et al. [28] reported that lambs fed diet supplemented with linseed and mineral bioplex did not display differences in their daily live body weight gain when compared to lambs fed non-supplemented lambs' basal diet.

Cellular and serum biochemical alterations

Bacillus subtilis significantly ($p < 0.05$) impacted the total leucocytes as shown in Fig. 2 and lymphocytes counts as exhibited in Fig. 3 at T30 compared with those of control ones. The total leucocytes and lymphocyte counts were increased significantly ($p = 0.0018$ and 0.0002, respectively) in lambs received *B. subtilis*. These findings were similar to those reported by Adjei-Fremah et al. [25] in dairy cows and El-Ashker et al. [26] in healthy Ossimi lambs that received multi-strains of commercially available probiotics. It has previously been stated that oral LB stimulates macrophage in healthy Ossimi lambs [26,29], and in healthy human males [30]. The pattern of cellular and enzymatic alterations could fortify the immune-stimulatory effect of *B. subtilis*; however, the mechanisms of how *B. subtilis* affects the immune system is still unknown [31]. There are conflicting views regarding the influence of probiotic on lymphocyte proliferation; some reports have shown a stimulatory effect [32], while others suggested an inhibitory role [33]. Various researchers have informed that the immune system of sheep was stimulated with probiotics and significantly increased ($p < 0.001$) total leucocytes number. Similarly, Milewski [34] and Milewski and Sobiech [35] concluded that yeast feeding to lambs significantly increased the lymphocyte percentages in particular and total leucocytes count in general. Higher leucocytes count could be accompanied by generation of additional immune cells [36] that enhance the resistance against several diseases [37].

Serum lysozyme activity and GSH showed statistically significant elevations ($p = 0.004$ and 0.009, respectively), and MDA values showed a significant decline ($p = 0.008$) at T30 in lambs that received *B. subtilis* compared with the controls ones. Lambs received *B. subtilis* elicited significantly higher values of CAT ($p = 0.01$) and NO

Table 2. Effects of *B. subtilis* supplementation on serum antioxidant status in growing Barki lambs.

	Time post-supplementation					
	15th day			30thday		
	Control	Experiment	P value	Control	Experiment	*p* value
Lysozyme (ug/ml)	1.47 ± 0.2	1.61 ± 0.2	0.57	1.2 ± 0.16	3. 3 ± 0.56	0.004
MDA (nmol/ml)	18.3 ± 2.5	21.6 ± 2.5	0.18	19.6 ± 3	31.6 ± 3	0.008
GSH (mg/dl)	2. 2 ± 0.5	2.4 ± 0.5	0.65	2.8 ± 0.2	4.6 ± 0.6	0.009
TAC (mM/ml)	1 ± 0.04	2 ± 0.1	0.0001	1.2 ± 0.08	2.4 ± 0.3	0.005
CAT (U/L)	236.6 ± 41.6	390 ± 40	0.01	245 ± 32.7	245 ± 32.7	1
NO (umol/l)	6.9 ± 1.5	17.7 ± 2.1	0.002	10.8 ± 1.6	12.2 ± 0.9	0.282

Means in the same raw are significantly different at ($p \leq 0.05$). CAT: catalase; MDA: malondialdehyde; TAC: total antioxidant capacity; GSH: reduced glutathione; NO: nitric oxide.

Table 3. Effects of *B. subtilis* supplementation on serum blood metabolites in growing Barki lambs.

	Time post-supplementation					
	15th day			30th day		
	Control	Experiment	*p* value	Control	Experiment	*p* value
ALT (U/l)	32 ± 8.1	33 ± 8	0.851	32.6 ± 5.6	33.6 ± 6.5	0.851
AST (U/l)	55 ± 7	58 ± 7.5	0.64	57.1 ± 8.1	58.3 ± 9.7	0.88
TP (gm/l)	39 ± 4.5	40 ± 4.5	0.8	39.1 ± 3.8	41 ± 4.5	0.62
Albumin (gm/l)	19.6 ± 4.9	19 ± 4	0.86	19.6 ± 6.8	21 ± 4.3	0.78
Urea (mmol/l)	8.2 ± 1.2	8.2 ± 1.4	0.97	8.2 ± 0.8	8.3 ± 1.4	0.94
Creatinine (umol/l)	95 ± 4.2	95.5 ± 4.2	0.9	94.6 ± 3.7	95.5 ± 5.9	0.83

Means in the same raw are significantly different at ($p \leq 0.05$). TP: Total protein; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

(p = 0.002) at T15 than those of the control ones. These findings demonstrate the anti-oxidative potential of *B. subtilis* and suggest that its use could be beneficial under field conditions. Our results were partially same as to those reported by El-Ashker et al. [26] in Ossimi lambs and Mulder et al. [30] in healthy human males, where the authors showed that probiotic supplementation for two weeks induced a significant increase in antioxidant activities. However, the serum TAC showed a statistically significant (p = 0.0001 and 0.005, respectively) high values at T15 and T30 in lambs that received *B. subtilis* compared with the control ones (Table 2). These outcomes are agreed with those assumed by Peng et al. [38] in Awassi lambs, but away from those obtained by Alhidary et al. [39] in lambs. Neither supplemented nor control group showed statistical differences among ALT, AST, total protein, albumin, urea, and creatinine (Table 2).

Gene expression pattern

The expression patterns of immunity, antioxidant, and lipogenic marker genes were evaluated in the examined lambs. To the superlative of our awareness, the present study is the first one that reported the expression patterns of these studied marker genes in Barki lambs supplemented with *B. subtilis*. However, Ekwemalor et al. [40] evaluated a modified expression of toll-like receptors (TLRs), chemokines and cytokines genes related to adaptive and innate immunity, and further stress-associated signaling molecules. Also, the effects of commercial oral probiotics (including *Enterococcus faecium*, *Lactobacillus acidophilus*, *Aspergillus oryza*, *Saccharomyces cerevisiae*, and *B. subtilis*) were reported in dairy cows at mid-lactation for 60 days on the global gene expression profile [25]. In the present study, *B. subtilis* elicited a significant up-regulation of interleukin (IL) 5, IL6, TLR4, Tollip, CAT, acetyl-CoA carboxylase alpha (ACACA), stearoyl-CoA desaturase (SCD), and fatty acid synthase (FASN) genes at T30 compared with the control ones. Moreover, there was a significant up-regulation of IL-5 and SCD genes at T15 (Fig. 4). All supplemented lambs showed non-significant upregulation of superoxide dismutase (SOD) gene. Conclusions of the present study were nearly in the same trend to those informed by El-Ashker et al. [26] in healthy Ossimi lambs received lactoferrin (LF) and/or lactobacillus sp for 30 successive days. The

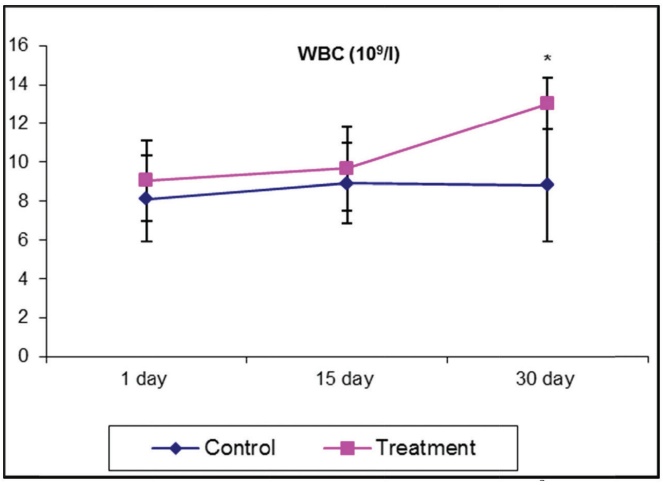

Figure 2. Time courses of total leucocytes count (10^9/l) in Barki lambs treated with *B. subtilis* compared with the control ones. The asterisk indicates significant effects (p < 0.05) at given sampling times.

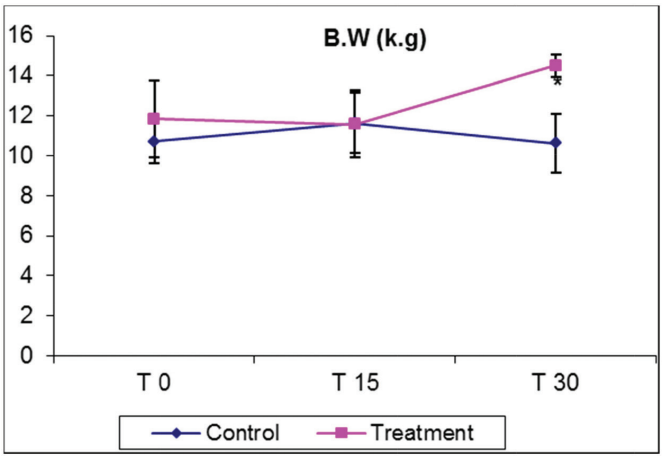

Figure 1. Time courses of the body weight (kg) at T0 in Barki lambs treated with *B. subtilis* compared with the control ones. The asterisk indicates significant effects (p < 0.05) at given sampling times.

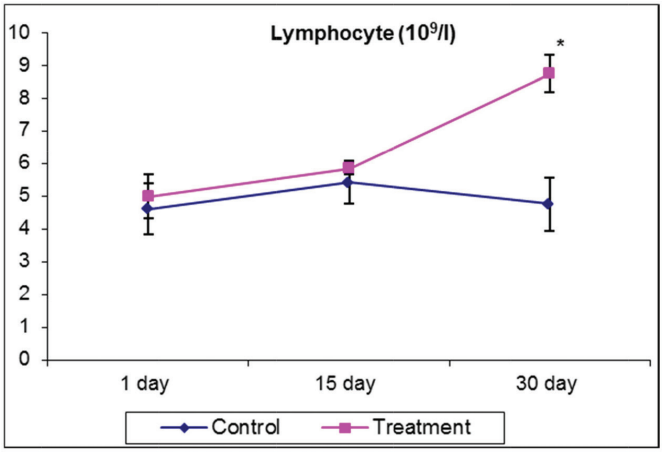

Figure 3. Time courses of lymphocyte count (10^9/l) in Barki lambs treated with *B. subtilis* compared with the control ones. The asterisk indicates significant effects (p < 0.05) at given sampling times.

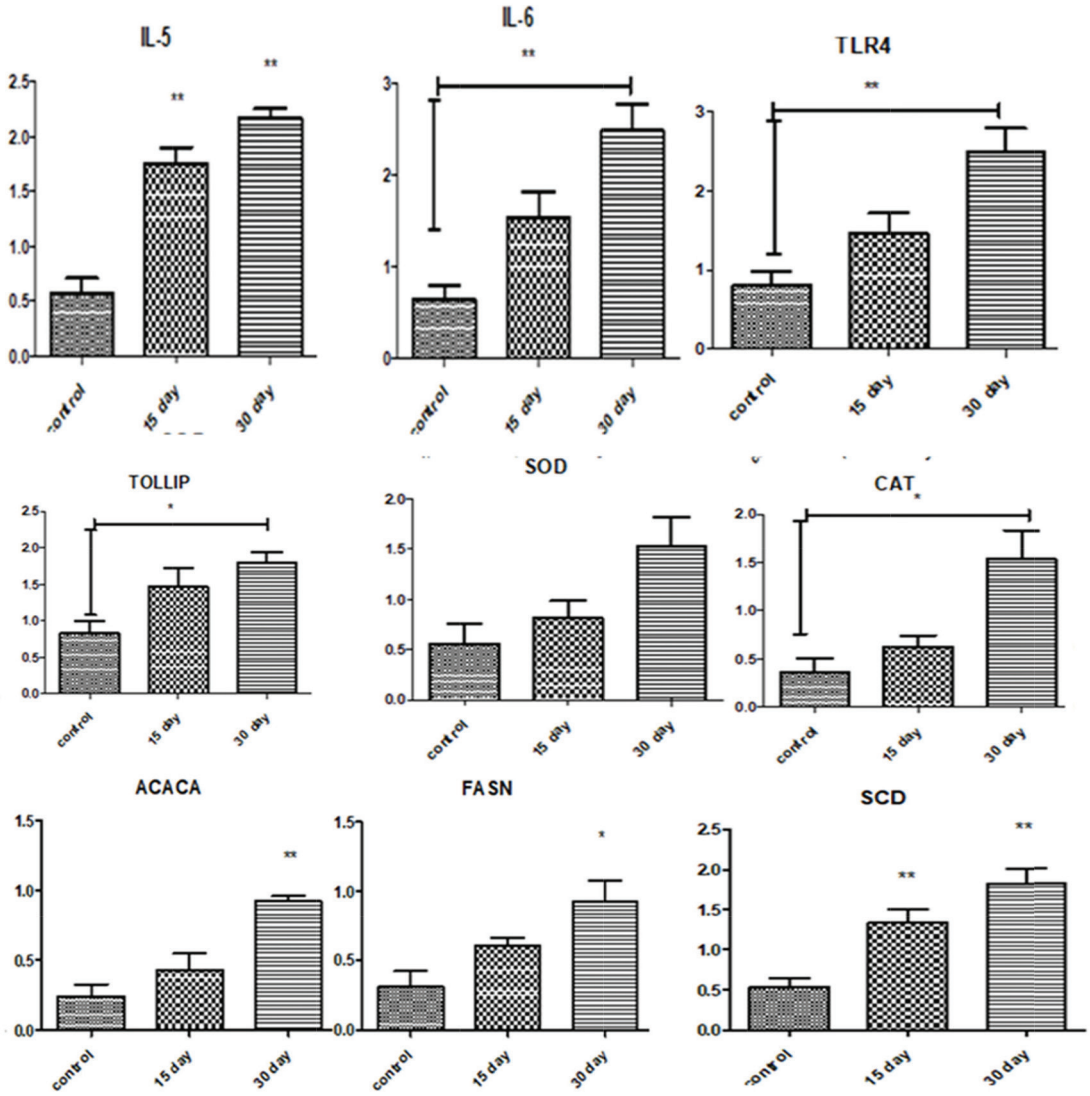

Figure 4. Relative expression of immunity, antioxidant, and lipogenic marker genes in the control and treated Barki lambs groups. Asterisks show significance when (p <0.05).

authors reported that Lambs that received LB provoked a significant up-regulation of TLR4 (at T15), IL-5 (at T15 and T30), and IL-6 (at T15) compared with those of control ones, while a combination of LF and LB elicited maximal up-regulation of Tollip, TLR4, IL-5, and IL-6 at T30 compared with other groups. In the same way, Prgomet et al. [32] reported that the orally supplemented LF enhanced the expression pattern of IL-1β, IL-8, and IL-10. A similar effect of LF was previously shown in leukocytes and monocytes of cows [41], where LF heightened IL-6, IL-10, IL-1 β, and TNF-α production. However, Kruzel et al. [42] reported that LF was down-regulate the pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) genes expression in mice. According to these results, the probiotic-containing *B. subtilis* exerted a generalized effect on immunity and homeostasis of genes.

The limits of this study should be recognized. First, the size of the sample may be small to be able to yield a concrete conclusion. Second, this study should be conducted on other metabolic and candidate gene markers. Third, other sheep breeds should also be considered. Therefore, the next study should be included in these limitations.

Conclusion

The results herein suggest that *B. subtilis* supplements could be a useful nutritional adjunct support for the immune system in healthy Barki lambs. The study also provides evidence that *B. subtilis* could induce generalized

effects via pathways elaborate immunity. Moreover, *B. subtilis* could provoke better anti-oxidant capacity with maximal stimulation of the immune system. Nevertheless, extra supplementary studies are mandatory to realize well mechanisms of actions of *B. subtilis* as well as their interactions to enable their full and safe usage under field condition.

Acknowledgments

The authors would like to thank and appreciate the Animal Health and Poultry Department staff members, Desert Research Center, Egypt, for their help, support, and facilities for this experiment.

Conflict of interest

The authors declare no conflict of interests.

Authors' contributions

SM contributed to the main design of this study, supported the experiment wrote, reviewed, and edited the manuscript. AE led the experiment, collected blood samples, analyzed serum metabolites, analyzed data, reviewed, and edited the manuscript. BM assayed antioxidant activities, counted total and differential leukocytes, statistically analyzed data, and submitted the manuscript. AA designed the experiment, performed real-time PCR, wrote a part in the manuscript, and took part in the critical checking of the manuscript.

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