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

Sabry Mousa, Ahmed Elsayed, Basma Marghani, Ahmed Ateya

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 leucocytes 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.

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
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 Clorsulan/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 × 10⁶ 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 SensiFast™ SYBR, Bioline, catalog No. Bio-98002). Primer sequences, annealing temperature, accession number, and amplified PCR product size (bp) were represented in Table 1. The house-keeping 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 H₂O (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−ΔΔCt 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−ΔΔCt 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 TCTGGTTGAGCCTTGGTACCTCT r TCAAGCAAGTTGTGATCGTGGAGA | NM_001009783.1 | 64 | Less than 155 |
| IL-6 | f TGCAGTCCTCAAACGAGTGGGTAA r AGCCGACGACTCTCATCCGAATA | NM_001009392.1 | 62 | Less than 155 |
| TLR4 | f GGTCTCCAGAAGCTGCAAGTG r ggATAGGGTTTCCCGTCAGT | AY957615 | 58 | 117 |
| SOD1 | f CGGGCAAGGGAGGATACAG r TCTCACAATGAGGACGTG | M81129 | 60 | 90 |
| Tollip | f CTGGTCTGTCTACAGTCTACAGT r ACAGTGGGCAATTCGCTGTGA | NM_001039961 | 56 | 122 |
| ACACA | f ATGGGCTTGGTGATGACCTCT r ACAGTACACAGGGCATGATG | NM_001009256.1 | 60 | 261 |
| FASN | f GGAAGGCGGGACTATATGGC r CATGCTGTACACCTCTCGG | XM_004013447.1 | 62 | 278 |
| SCD | f GCCGTTCAAAGTGACCTTT r TGAGACGACGACGAGCAAC | NM_001009254.1 | 58 | 251 |
| CAT | f GAAAGGCGCTTGGTGAGAAGAC r ACATAGTGCTGAACTCGT | XM_012096208.3 | 58 | 171 |
| GAPDH | f TGACCCCTCACTGACCTC r GATCTCGCTCCTGGGAAGA | 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; Tollip: 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 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.

|                  | 15th day         | 30th day         | P value | Control | Experiment | P value | Control | Experiment | p value |
|------------------|------------------|------------------|---------|---------|------------|---------|---------|------------|---------|
| **Lysozyme (mg/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 ≤ 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.

|                  | 15th day         | 30th day         | P value | 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 (g/l)**     | 39 ± 4.5         | 40 ± 4.5         | 0.8     | 39.1 ± 3.8  | 41 ± 4.5   | 0.62  |
| **Albumin (g/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 ≤ 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 \textit{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 \textit{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).

\textbf{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 \textit{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 \textit{Enterococcus faecium}, \textit{Lactobacillus acidophilus}, \textit{Aspergillus oryza}, \textit{Saccharomyces cerevisiae}, and \textit{B. subtilis}) were reported in dairy cows at mid-lactation for 60 days on the global gene expression profile [25]. In the present study, \textit{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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Time courses of the body weight (kg) at T0 in Barki lambs treated with \textit{B. subtilis} compared with the control ones. The asterisk indicates significant effects (p < 0.05) at given sampling times.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Time courses of the total leucocytes count (10^9/l) in Barki lambs treated with \textit{B. subtilis} compared with the control ones. The asterisk indicates significant effects (p < 0.05) at given sampling times.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Time courses of lymphocyte count (10^9/l) in Barki lambs treated with \textit{B. subtilis} compared with the control ones. The asterisk indicates significant effects (p < 0.05) at given sampling times.}
\end{figure}
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

Figure 4. Relative expression of immunity, antioxidant, and lipogenic marker genes in the control and treated Barki lambs groups. Asterisks show significance when ($p<0.05$).
effects via pathways elaborate immunity. Moreover, \textit{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 \textit{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.

**References**

[1] Hossein AA, Alireza ME, Mohammad R, Majid M. Effects of \textit{Bacillus subtilis} and bacillus licheniformis-based probiotic on performance, hematological parameters and blood metabolites in lambs. Int J Food Nutr Sci 2014; 3(4):8–15.

[2] Soliman SM, El-Shinnawy AM, El-Morsy AM. Effect of probiotic or prebiotic supplementation on the productive performance of Barki lambs. J Anim Poult Prod Mansoura Univ 2016; 7(10):369–76.

[3] Bahari M. A review on the consumption of probiotics in feeding young ruminants. Appro Poult Dairy Vet Sci 2017; 1; https://doi.org/10.31031/APDV.2017.01.000508

[4] Sakeem AM, Zanouny AI, Singer AM. Growth performance, nutrients digestibility, and blood metabolites of lambs fed diets supplemented with probiotics during pre- and post-weaning period. Asian Australas J Anim Sci 2017; 30:523–30; https://doi.org/10.5713/ajas.16.0691

[5] Musa HH, Wu SL, Zhu CH, Seri HI, Zhu QG. The potential benefits of probiotics in animal production and health. J Anim Vet Advan 2009; 8(2):313–21.

[6] Aattour N, Bouras M, Tome D, Marcos A, Lemonnier D. Oral ingestion of lactic acid bacteria by rats increases lymphocyte proliferation and interferon production. British J Nutr 2002; 87:567–73; https://doi.org/10.1079/BJN2001527

[7] Qiao GH, Shan AS, Ma N, Ma QQ. Sun NZ. Effect of supplemental Bacillus cultures on rumen fermentation and milk yield in Chinese Holstein cows. J Anim Physiol Anim Nutr 2010; 94(4):429–36; https://doi.org/10.1111/j.1439-0396.2009.00926.x

[8] Chiofalo V, Liotta L, Chiofalo B. Effects of the administration of lactobacilli on body growth and on the metabolic profile in growing Maltese goat kids. Rep Nutr Develop 2004; 44(5):449–57; https://doi.org/10.1015/rnd2004051

[9] Antunovic Z, Speranda M, Amidic D, Seric V, Steiner Z, Domovican M, et al. Probiotic application in lambs nutrition. Krmiva 2006; 4(48):175–80; https://doi.org/10.33128/k

[10] Whitley NC, Cazac D, Rude BJ, Jackson-O’Brien D, Parveen S. Use of commercial probiotics supplement in meat goat. J Anim Sci 2009; 87(2):723–8; https://doi.org/10.2527/jas.2008-1031

[11] Mousa SA, Marwan AA. Growth performance, rumen fermentation and selected biochemical indices in buffalo calves fed on \textit{Bacillus subtilis} supplemented diet. Inter J Vet Sci 2019; 8(3):151–6.

[12] Dabiri N, Hajimohammadi A, Mahdavi A, Baghebian M, Babaei A, Bahrami M. Effect of different levels of biosaf probiotic in medium concentrate diet on performance and blood factors of Iranian Zandi Lambs. J Fisheries Livest Prod 2016; 4:206; https://doi.org/10.4172/2332-2608.1000206

[13] Fayed AM, El-Ashker MA, Youssef KM, Salem FA, Aziz HA. Effect of feeding fayomycin or yeast as feed supplement on ruminal fermentation and some blood constituents of sheep in Sini. Eg | Nutr Feeds 2005; 8:619–34.

[14] Pfahl MW. A new mathematical model for relative quantification in real time RT-PCR. Nucleic Acids Res 2001; 29(9):2002–7; https://doi.org/10.1093/nar/29.9.4e5

[15] SPSS PC. SPSS for windows release 17. SPSS. Inc. USA, 2004.

[16] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta (CT)) Method. Methods 2001; 25:402–8; https://doi.org/10.1006/ meth.2001.1262

[17] Abd El-Tawab IMI, Youssef HA, Bakr GC, Fhenakis ND, Giadinis M. Role of probiotics in nutrition and health of small ruminants. Pol J Vet Sci 2016; 19(4):893–906; https://doi.org/10.1515/pjvs-2016-0114

[18] Hussein AF. Effect of biological additives on growth indices and physiological responses of weaned Najdi ram lambs. J Exp Emer Biol Agric 2014; 2(6):597–607; https://doi.org/10.1515/pjvs-2016-0114

[19] Abas I, Kutay HC, Kahraman R, Toker NY, Ozelkik D, Ates F, et al. Effects of organic acid and bacterial direct-fed microbial on fattening performance of Kivrck-Male yearling lambs. Pakistan J Nutr 2007; 6(2):419–54; https://doi.org/10.3923/pjn.2007.149.154

[20] Antunovic Z, Speranda M, Liker B, Seric V, Senic D, Domovican M, et al. Influence of feeding the probiotic Pioneer PFDM to growing lambs on performances and blood composition. Acta Vet 2005; 55(4):287–300; https://doi.org/10.2298/AVB0504287A

[21] Chaucheeyas A, Durand F, Fonty G. Establishment of cellulosytic bacteria and development of fermentative activities in the rumen of gnotobiotically reared lambs receiving the microbial additive. Cuban J Agric Sci 2001; 41(1):57–68; https://doi.org/10.1051/rnd:2001112

[22] Kochewad SA, Chahande JM, Kanduri AB, Deshmukh DS, Ali SA, Patil VM. Effect of probiotic supplementation on growth parameters of Osmanabadi Kids. Vet World 2009; 2:29–30.

[23] Liotta Piccitto F, Chiofalo B. Effects of the administration of lactic acid bacterial from \textit{Bacillus subtilis} on body growth and on the metabolic profile in growing Maltese goat kids. Acta Veterinaria Italiana 2009; 49(4):307–12; https://doi.org/10.1007/s13659-009-0036-y

[24] Lachowski W. Influence of feeding the probiotic Pioneer PFDM to growing lambs on performances and blood composition. Acta Vet 2005; 55(4):287–300; https://doi.org/10.2298/AVB0504287A

[25] Antunovic Z, Speranda M, Liker B, Seric V, Senic D, Domovican M, et al. Influence of feeding the probiotic Pioneer PFDM to growing lambs on performances and blood composition. Acta Vet 2005; 55(4):287–300; https://doi.org/10.2298/AVB0504287A
commercially available lactoferrin and/or *Lactobacillus* sp. in healthy Ossimi lambs. Polish J Vet Sci 2018; 21(4):705–13.

[27] Titi HH, Dmour RO, Abdullah AY. Growth performance and carcass characteristics of Awassi lambs and Shami goat kid culture in their finishing diet. Anim Feed Sci Technol 2008; 142(1):33–43; https://doi.org/10.1016/j.anifeedsci.2007.06.034

[28] Baranowski A, Gabrysuk M, Jozwik A, Bernatowicz E, Chylinski W. Fattening performance, slaughter indicators and meat chemical composition in lambs fed the diet supplemented with linseed and mineral bioplex. Anim Sci Pap Rep 2007; 25(1):35–44.

[29] Perdigón G, Alvarez S, Nader D, Macías ME, Margni R, Oliver GP, et al. Lactobacilli administered orally induce release of enzymes from peritoneal macrophages in mice. Milchwiss 1986; 41:344–8.

[30] Mulder A, Connellan PA, Oliver CJ, Morris CA, Stevenson LM. Bovine lactoferrin supplementation supports immune and antioxidant status in healthy human males. Nutr Res 2008; 28(9):583–9; https://doi.org/10.1016/j.nutres.2008.05.007

[31] Herich R, Levkt M. Lactic acid bacteria, probiotics and immune system. Vet Med (Praga) Czech 2002; 47:169–80; https://doi.org/10.1016/j.vetmed.2002.08.001

[32] Prgomet C, Frenner ML, Schwarz FJ, Pfaffl MW. Effect of lactoferrin on selected immune system parameters and the gastrointestinal morphology in growing calves. J Anim Physiol Anim Nutr 2007; 91:109–19; https://doi.org/10.1007/s10311-006-0065-9.x

[33] Tao M, Yutaka S, Le LG. Dissect the mode of action of probiotics in affecting host-microbial interactions and immunity in food producing animals. Vet Immunol Immunopathol 2018; 205:35–48; https://doi.org/10.1016/j.vetimm.2018.10.004

[34] Milewski S. Effect of yeast preparations Saccharomyces cerevisiae on meat performance traits and blood hematological indices in suckling lambs. Medycyna Weterynaryjna 2009; 65(1):51–4.

[35] Milewski S, Sobiech P. Effect of dietary supplementation with Saccharomyces cerevisiae dried yeast on milk yield, blood biochemical and hematological indices in ewes. Bull Vet Inst Pulawy 2009; 53:753–8.

[36] LaFleur-Brookes M, LaFleur-Brookes D. Exploring medical language: a student-directed approach. 7th edition, Mosby Elsevier, St. Louis, MO, p 398, 2008.

[37] Ping L, Qing G. Antimicrobial effect of probiotics and novel probiotic-based approaches for infectious diseases. In : Shymaa Enany, Suez Canal University (eds.). Book: Probiotics—chapter 1. Current knowledge and future prospects, London,UK: IntechOpen Limited, 2018.

[38] Peng J, Kai C, Ma M, Tao W, Fan W, Wen Y, et al. Influence of dietary supplementation with *Bacillus licheniformis* and *Saccharomyces cerevisiae* as alternatives to monensin on growth performance, antioxidant, immunity, ruminal fermentation and microbial diversity of fattening lambs. Sci Rep 2018; 8:1–4; https://dx.doi.org/10.1038%2Fs41598-018-35081-4

[39] Alhidary IA, Abdelrahman, MM, Khan RU. Comparative effects of direct-fed microbials alone or with a trace minerals supplements on the productive performance, blood metabolites, and antioxidant status in grazing Awassi lambs. Environ Sci Polhst Res 2016; 23(24):25218–23; https://doi.org/10.1007/s11356-016-7684-z

[40] Ekwemalor K, Asiamah E, Osei B, Ismail H, Worku M. Evaluation of the effect of probiotic administration on gene expression in goat blood. J Mol Biol Res 2017; 7(1):88–98; https://doi.org/10.5539/jmbr.v7n1p88

[41] Prgomet C, Peters S, Pfaffl MW. Influence of bovine Lactoferrin and Lactoferrin on the cytokine expression in LPS treated cultivated bovine blood cells. J Sci Food Agric 2005; 52:317–24; https://doi.org/10.1002/jsfa.2377

[42] Kruzel ML, Harari Y, Mailman D, Actor JK, Zimecki M. Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS induced inflammatory responses in mice. J Clin Exp Immunol 2002; 130(1):25–31; https://doi.org/10.1046/j.1365-2249.2002.01956.x