Original article

Potential immune modulating properties and antioxidant activity of supplementing commercially available lactoferrin and/or *Lactobacillus* sp. in healthy Ossimi lambs

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Abstract

The use of lactoferrin (LF) and/or *lactobacillus* sp. (LB) to improve animal health and production has increased recently. However, information regarding the immune-modulatory role of LB supplementations either alone or in combination with LF in sheep remains unclear. Therefore, the present study was designed to evaluate the immune modulating properties and the antioxidant activity of supplementing commercially available LF and/or LB in healthy lambs. For this reason, twenty-four apparently healthy Ossimi lambs were used. After three weeks of acclimatization, the lambs were randomly allocated to four equal-sized groups and assigned to receive one of the following supplements: LB at a dose of ∼ 1 g active ingredient/head (group 1), LF at a dose rate of 0.5 gm /head (group 2), a combination of both treatments using the same dosing regimens (group 3), and (group 4) received only 10 mL of isotonic saline and was considered as a control group. All supplements were given orally twice daily for 30 consecutive days. Blood samples were collected from each lamb before starting the experiment (T0) and two weeks (T15), and four weeks (T30) after giving supplements for hematological examinations, serum biochemical analyses, and RT-PCR assays. Our findings demonstrated that lambs receiving LB showed statistically significant (P<0.05) higher values of total leucocytes, lymphocytes and lysozyme activity than those receiving LF. In contrast, lambs that received LF had significantly (P< 0.05) higher values of serum catalase, nitric oxide and GSH with a significantly lower MDA level compared with those supplemented with LB. A combination of LF and LB supplementation elicited maximal up-regulation of *Tollip*, *TLR4, IL-5*, and *IL-6* gene expression compared with other groups. The results suggest that bovine LF and or LB could be used as useful nutritional supplements to support the immune system in healthy lambs.

Key words: immunity, lactoferrin, lambs, redox status, probiotic, RT-PCR, sheep

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Introduction

Lactoferrin (LF) is a multifunctional protein that has a wide range of biological activities. Scientists from around the world are trying to explore the value of using LF in the treatment and prevention of various diseases. LF was first isolated from bovine milk in 1940 as "red protein from milk whey" (Sorensen and Sorensen 1940). It has numerous functions other than iron sequestration, such as binding with microbes, host cells and components of the immune system (Legrand et al. 2005). It is considered one of the first defense systems against microbial agents that invade the organism mostly via mucosal tissues due to its strategic position on the mucosal surface. LF affects the growth and proliferation of a variety of infectious agents including both gram-positive and negative bacteria, viruses, protozoa, and fungi (Adlerova et al. 2008). It also has a potential role in the modulation of the immune response (Legrand et al. 2005). However, the molecular mechanisms underlying the effect of LF in modulating immunity and inflammatory response are still questionable.

Although LF is considered as an iron-binding glycoprotein, it also serves as an iron donor and in this way it could support the growth of some beneficial bacteria with lower iron demands, such as *Lactobacillus* sp. (LB) or *Bifidobacterium* sp. (Sherman et al. 2004). *Lactobacillus* sp. are lactic acid producing bacteria (LAB) that have potential biological functions in humans (Fuller and Perdigón 2000) and murine (Perdigón et al. 1986); however, these functions have not been adequately studied in sheep (Abd El-Tawab et al. 2016, Payandeh et al. 2017).

The discovery that LB can stimulate the immune system in humans enhances the development of research in livestock animals (Fuller and Perdigón 2000). In that context, the effects of commercially available multi-strain bacterial probiotic have recently been evaluated in ewes (Payandeh et al. 2017) and in dairy cows (Adjei-Fremah et al. 2018). However, few data are currently available regarding the effects of *Lactobacillus* delbruekii and *Lactobacillus* fermentum supplementations either alone or in combination with LF in healthy sheep. Therefore, the present study aimed to evaluate the immune modulating and antioxidant properties of commercially available LF and/or LB supplementation in healthy lambs. We hypothesized that supplementation of LF alone or together with LB could help potentiate immune markers and influence the antioxidant status in clinically healthy sheep.

Materials and Methods

Animals

The present study included twenty-four apparently healthy male Ossimi lambs aged three to four months with a range of 21.5 to 23.7 Kg body weight (BW). The study was conducted on a commercial farm in Aga District, Dakahlia Governorate, Egypt during the period between May and June 2016. Three weeks prior to the experiment, the investigated lambs were acclimatized in separate semi-open shaded pens and fed on a concentrated feed mixture consisting of cottonseed cake, maize, wheat or rice bran, calcium carbonate, and sodium chloride at a rate of 250 g/head/day, while water and hay were available *ad libitum.* 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 subcutaneously. All animals were apparently healthy, with no history of metabolic or concurrent ailment and were kept under identical housing conditions and veterinary care throughout the study period. All applicable international guidelines for the care and the use of animals were followed. An informed consent was obtained from the farm owner about the proposed treatment protocol and the farmer was given information about the potential beneficial effects of the products being used.

Experimental design and data collection

The investigated lambs were randomly allocated to four equal groups of six lambs each, and assigned to receive one of the following supplements: LB (Lacteol fort, RAMEDA Pharmaceuticals, Giza, Egypt) corresponding to 340 mg active ingredient of LB delbruekii and LB fermentum (i.e. 10 billion per 1 powder sachet of 800 mg) given at a dose rate of ∼ 1 g active ingredient (i.e. 3 sachets)/head (**Group 1**). **Group 2** received LF (Pravotin-100 mg, Medizen Pharmaceutical Industries, Borg Al Arab, Egypt) at a dose of 0.5 gm (i.e. 5 sachets)/head. Both LB and LF supplements were initially dissolved in 10 mL of isotonic saline and were given to the assigned animals as an oral drench twice daily for 30 consecutive days. To help deliver both supplements directly to the abomasum and to avoid potential rumen degradation, 10 mL of sodium bicarbonate buffer (10%) were administered orally to each lamb prior to receiving the drenches (Yekta et al. 2010). **Group 3** received a combination of both treatments using the same dosing regimes. The last group received only 10 mL of isotonic saline and was considered as controls **(Group 4)**.

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

Gene	Oligonucleotide sequence	Accession number	Annealing temperature (C°)	Size (bp)
IL-5	f5'- TCTGCGTTTGACCTTGGTAGCTCT-3' r5'- TCAGCAGAGTTTGATGCGTGGAGA-3'	NM_001009783.1	64	less than 155
IL-6	f5'- TGCAGTCCTCAAACGAGTGGGTAA-3' r5'- AGCCGCAGCTACTTCATCCGAATA -3'	NM_001009392.1	62	less than 155
TLR4	f5'- GGTTCCCAGAACTGCAAGTG -3' r5'- GGATAGGGTTTCCCGTCAGT -3'	AY957615	58	117
SOD	f5'- CGAGGCAAAGGGAGATACAG-3' r5'- TCTCCAAACTGATGGACGTG -3'	M81129	60	90
Tollip	f5'- CTGGTGCTGTCCTACACGTC-3' r5'- ACAGTGGGCATTCCTGTGAT-3'	NM_001039961	56	122
GAPDH	f5'- TGACCCCTTCATTGACCTTC-3' r5'- GATCTCGCTCCTGGAAGAG-3'	NM-001034034	62	143

IL, interleukin; *TLR4*, Toll-like receptor 4;*SOD*, superoxide dismutase; *Tollip*: Toll-interacting protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase

Clinical Follow up

All animals were clinically monitored throughout the study period. Vital signs including rectal temperature, heart rate, respiratory rate and mucus membrane color were routinely monitored prior to, and during, the study. The general *demeanor* as well as signs of gastrointestinal disturbances including alteration of appetite, existence of diarrhea, constipation, tympany, and excess salivation were observed and the findings were simultaneously recorded. The body weight for each lamb was also measured using a digital weighing scale at three time points (before starting the experiment (T0), on the 15th day (T15) and 30th day (T30) after giving supplements, and the data were recorded.

Sampling and measurements

Ten ml of blood were collected from each animal via jugular venipuncture at T0, T15 and T30. The collected blood was either collected in plain tubes (i.e. without anticoagulants) or in tubes containing EDTA to yield serum or whole blood, respectively. Blood samples were kept on crushed ice and were immediately transported to the laboratory for further processing. Blood in plain tubes was left at room temperature to clot and rapidly centrifuged at 1400 x g for 10 minutes to separate serum. Only clear non-hemolyzed sera were collected and aliquoted for biochemical analyses using commercial kits according to the standard protocols of the suppliers. The following kits were used to quantify malondialdehyde (MDA, Biodiagnostic Egypt; CAT No: MD2529); nitric oxide (NO, Biodiagnostic Egypt; CAT No: 2533); catalase (CAT, Biodiagnostic Egypt;

CAT No: CA252417); reduced glutathione (GSH, Biodiagnostic Egypt; CAT No: GSH2511); total antioxidant capacity (TAC, Biodiagnostic Egypt; CAT No: TA2513); alanine aminotransferase (ALT, Colorimetric Randox, UK); aspartate aminotransferase (AST, Colorimetric Randox, UK); total protein (Tp, Stanbio laboratory; USA: Procedure No 2085); albumin (Alb, Stanbio laboratory; USA: Procedure No 0250); urea (Diamond LOT 215217); and creatinine (Human–LOT 16011). Serum lysozyme activity was determined by the turbidimetric assay (Milewski et al. 2013). Tubes containing whole blood were used for complete blood count and real-time PCR assays.

RNA extraction and Reverse transcription

Total RNA was extracted from whole blood samples using an RNA MiniPrep kit (Direct-zol™, CAT No: R2050) according to the manufacturer's instructions. The quantity and purity of RNA were measured using a Nanodrop (Q5000 UV-Vis spectrophotometer, USA), and the integrity was evaluated by gel electrophoresis. The cDNA of each sample was synthesized using a SensiFast™ cDNA synthesis kit (Bioline, CAT No: Bio- 65053) following the manufacture's protocol. The reaction mixture was carried out in a total volume of 20 μl consisting of total RNA up to 1μg, 4 μl 5x Trans Amp buffer, 1 μl reverse transcriptase and DNase free-water up to 20 μl. The reaction mixture was placed in a thermal cycler and the following program was carried out: primer annealing at 25°C for 10 min, reverse transcription at 42°C for 15 min followed by inactivation at 85°C for 5 min. The samples were held at 4°C.
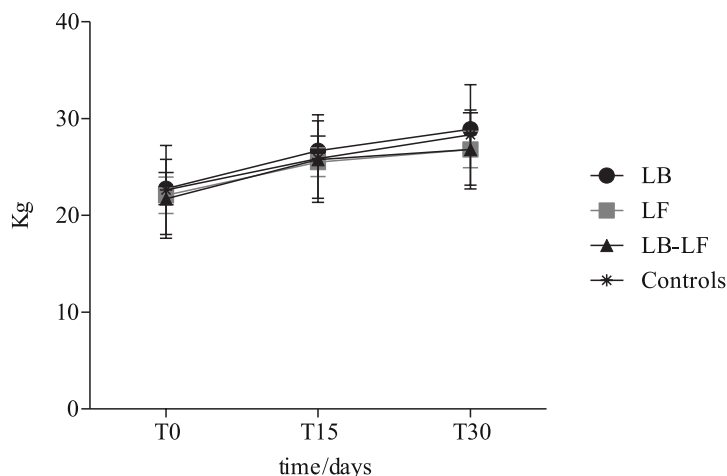
Fig. 1. Time course (means ± standard deviation) of body weight (Kg) at T0 in sheep treated with lactobacilli (LB), lacto-ferrin (LF), or a combination of LB and LF compared with controls.

Quantitative Real-Time PCR

Relative quantification of mRNA levels of interleukin (IL) -5, IL-6, Toll-like receptor 4 (TLR4), Toll--interacting protein (Tollip) and superoxide dismutase (SOD) in sheep blood were performed by real-time PCR using SensiFast™ SYBR Green PCR Master Mix (Bioline; Cat No: Bio-98002). Primer sequences, annealing temperatures, and the size of each amplified PCR product are shown in Table 1. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The reaction was carried out in a total volume of $20\,\mu$l consisted of $10\,\mu$l 2x SensiFast SYBR, $3\,\mu$l cDNA, $5.4\,\mu$l nuclease-free H_2O, $0.8\,\mu$l of each primer. The PCR cycling conditions were as follows: 95°C for 2 min followed by 40 cycles of 94°C for 10 s, annealing temperatures as shown in Table 1 for 30 s, and 72°C for 20 s. At the end of the amplification phase, a melting curve analysis was performed to confirm the specificity of the PCR product. The relative expression of the gene of interest was normalized to GAPDH and calculated according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

Statistical analysis

Statistical analysis of the data was carried out using SPSS software for Windows, version 16.0 (Chicago, USA). The means and standard deviation for each variable at each time point were calculated. A repeated measure ANOVA was used to assess the changes occurring in the tested variables with treatment and time. Wilks' lambda test was selected for within-group assessment and for describing the interaction of time x treatment. Since the Wilks' lambda test showed a significant difference between groups, one-way analysis of variance with post-hoc Bonferroni multiple-comparison tests

were used. Results were considered significant at the level of $p<0.05$.

Results

Clinical findings and the effect of supplements on weight gain

Prior to the experimental study, all lambs were clinically healthy and showed no evidence of illness. All vital signs of investigated lambs were within the normal reference range and the animals remained healthy and showed no detectable clinical abnormality throughout the study period. No evidence of gastrointestinal abnormalities was documented. The body weight of the studied lambs was significantly affected by time ($p=0.0001$) and by time x treatment ($p=0.002$). However, no significant differences were observed between supplemented groups and controls ($p>0.05$) (Fig. 1). Lambs that received LB showed the highest weight gain at T30 (6140 ± 65 g), followed by those that received a combination of LB/LF (5100 ± 35 g), and (4730 ± 84 g) for lambs that received LF compared with controls (5682 ± 77 g).

Cellular and serum biochemical alterations

Lactobacilli significantly ($p<0.05$) impacted the total leucocytes (Fig. 2a) and lymphocyte counts (Fig. 2b) at T15 and T30, respectively compared with those of LF or their combination. In brief, the total leucocytes and lymphocyte counts increased significantly ($p=0.0001$) in lambs that received LB followed by those that received LF, but the absolute count of neutrophils showed no significant alterations in all investigated groups ($p=0.557$). The serum lysozyme activity was also affected by time ($p=0.032$) and were higher in lambs that re-

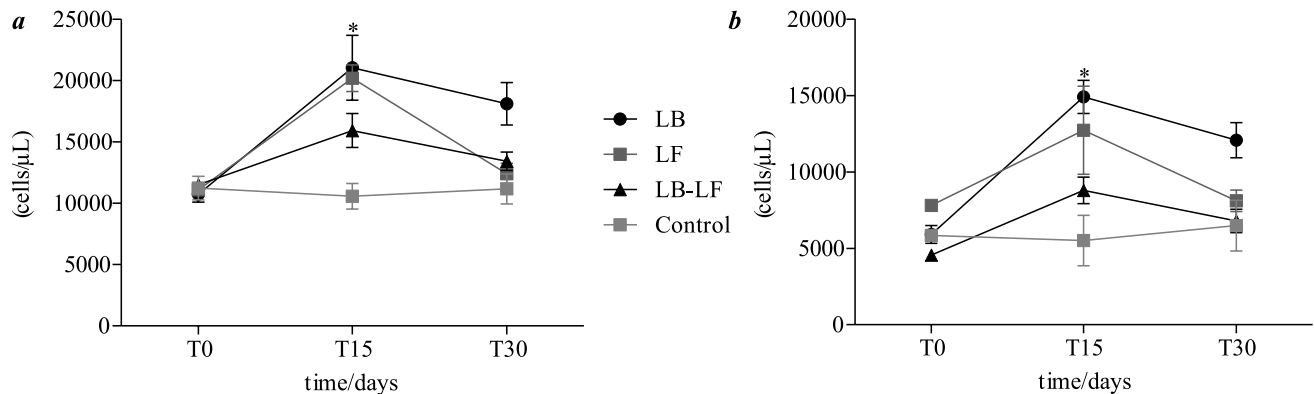Fig. 2a. Time course (means ± standard deviation) of total leucocytes (cells/µL) in sheep treated with lactobacilli (LB), lactoferrin (LF), or a combination of LB and LF compared with those of controls. Asterisk indicates significant effects (p<0.05) at given sampling times.

Fig. 2b. Time course (means ± standard deviation) of lymphocytes (cells/µL) in sheep treated with lactobacilli (LB), lactoferrin (LF), or a combination of LB and LF compared with controls. Asterisk indicates significant effects (p<0.05) at given sampling times.

ceived LB at T30 compared with those receiving LF and the controls (Fig. 3a). Lambs that received both supplements (Group 3) elicited significantly (p<0.05) higher values of nitric oxide (Fig. 3b), and CAT (at T15) (Fig. 3c), with lower concentrations of MDA (Fig. 3e) than those that received LF or LB alone. Values of GSH were significantly affected by time (p=0.0001) and by time x treatment (p=0.016), where LF provoked higher values at T30 followed by LB than those of controls (Fig 3d). Neither LF nor LB showed statistical differences (p>0.05) on BW, ALT, AST, Tp, alb, globulin, urea and creatinine.

Gene expression analyses

The expression patterns of immune-inflammatory genes were significantly (p<0.05) up regulated in all supplemented groups, with a significant down regulation of the *SOD* gene compared with the control group (Fig. 4a-e). In short, *IL-6* was significantly up regulated at T30 in LF, LB and LF/LB supplemented groups. The *TLR4* was significantly (p<0.05) up regulated and T30 in LF and LF/LB supplemented group. For *IL-5*, the expression was significantly (p<0.05) up regulated in all supplemented groups at T15 and T30 compared with the control group, with no significant alterations at T15 in the LB supplemented group. The *Tollip* gene was significantly (p<0.05) over expressed in all groups except for LF and LB supplemented groups at T15 compared with the controls.

Discussion

In the present study, orally administered LB was found to be superior to LF in promoting weight gain in lambs. Moreover, surprisingly, a combination of both supplements did not elicit the same effect of LB alone on weight gain. As shown in various studies, the influence of LB on animal performance may vary. In this context, a slight positive effect of LB supplementation on live body weight, daily weight gain, and feed conversion rate in weaned lambs has been reported (Antunovic et al. 2005). Other researchers did not show any effect of orally supplemented multi-strain probiotic in dairy cows (Adjei-Fremah et al. 2018). On the other hand, the impact of LF on live body weight has been evaluated in growing calves (Prgomet et al. 2007) and in weaned piglets (Wang et al. 2006). The former study showed no effects of LF on live weight gain, while the latter reported a positive effect. These results were diverged slightly from the finding obtained in the present study where LF supplemented lambs showed less weight gain compared with other groups. The reason for this is not clear, while the positive effect of LB on weight gain has been attributed to increased consumption and improved efficiency of feed utilization (Antunovic et al. 2005). Other researchers have suggested that improved cellulolytic activity within the rumen can result in efficient fiber degradation, and improve nutrient digestion (Abd El-Tawab et al. 2016).

Our findings revealed a remarkable effect of LB on total leucocytic and lymphocytic counts as well as serum lysozyme activity compared with those receiving LF or even both LB and LF. The maximal effect observed was at T15 for the cellular elements and at T30 for lysozyme activity. These findings were similar to those reported in dairy cows that received multi-strains of commercially available probiotics (Adjei-Fremah et al. 2018). It has previously been stated that oral LB supplementation in mice can stimulate macro-
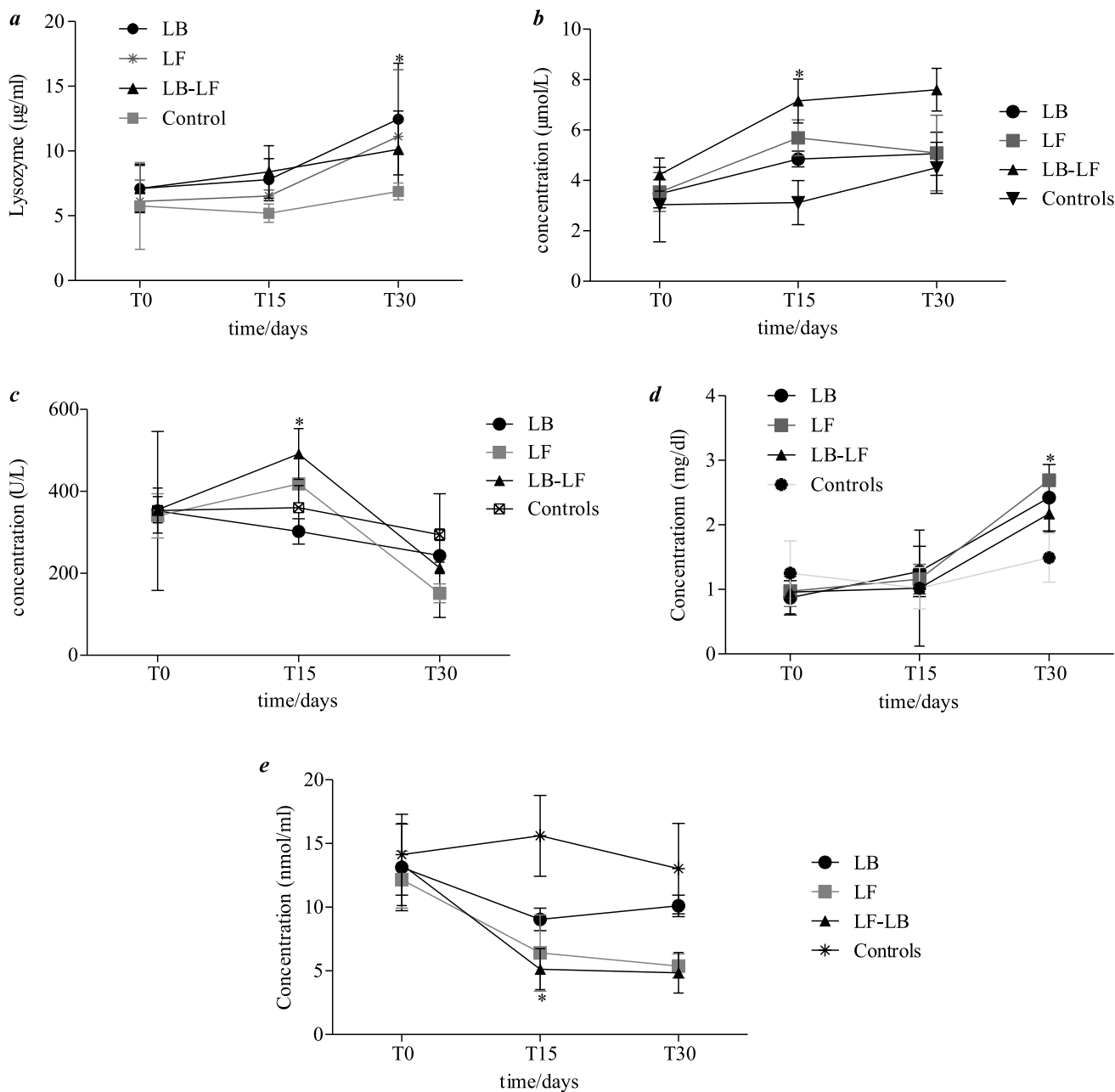
Fig. 3a. Time course (means ± standard deviation) of serum levels of lysozyme (μg/ml) in sheep treated with lactobacil-li (LB), lactoferrin (LF), or a combination of LB and LF compared with controls. Asterisk indicates significant effects (p<0.05) at given sampling times.

Fig. 3b. Time course (means± standard deviation) of serum levels of nitric oxide (μmol/L) in sheep treated with lactoba-cilli (LB), lactoferrin (LF), or a combination of LB and LF compared with controls. Asterisk indicates significant effects (p<0.05) at given sampling times.

Fig. 3c. Time course (means ± standard deviation) of serum levels of catalase (U/L) in sheep treated with lactobacilli (LB), lactoferrin (LF), or a combination of LB and LF compared with controls. Asterisk indicates significant effects (p<0.05) at given sampling times.

Fig. 3d. Time course (means ± standard deviation) of serum levels of reduced glutathione (mg/dl) in sheep treated with lactobacilli (LB), lactoferrin (LF), or a combination of LB and LF compared with controls. Asterisk indicates significant effects (p<0.05) at given sampling times.

Fig. 3e. Time course (means± standard deviation) of serum levels of malondialdehyde(nmol/ml) in sheep treated with lactobacilli (LB), lactoferrin (LF), or a combination of LB and LF compared with controls. Asterisk indicates significant effects (p<0.05) at given sampling times.
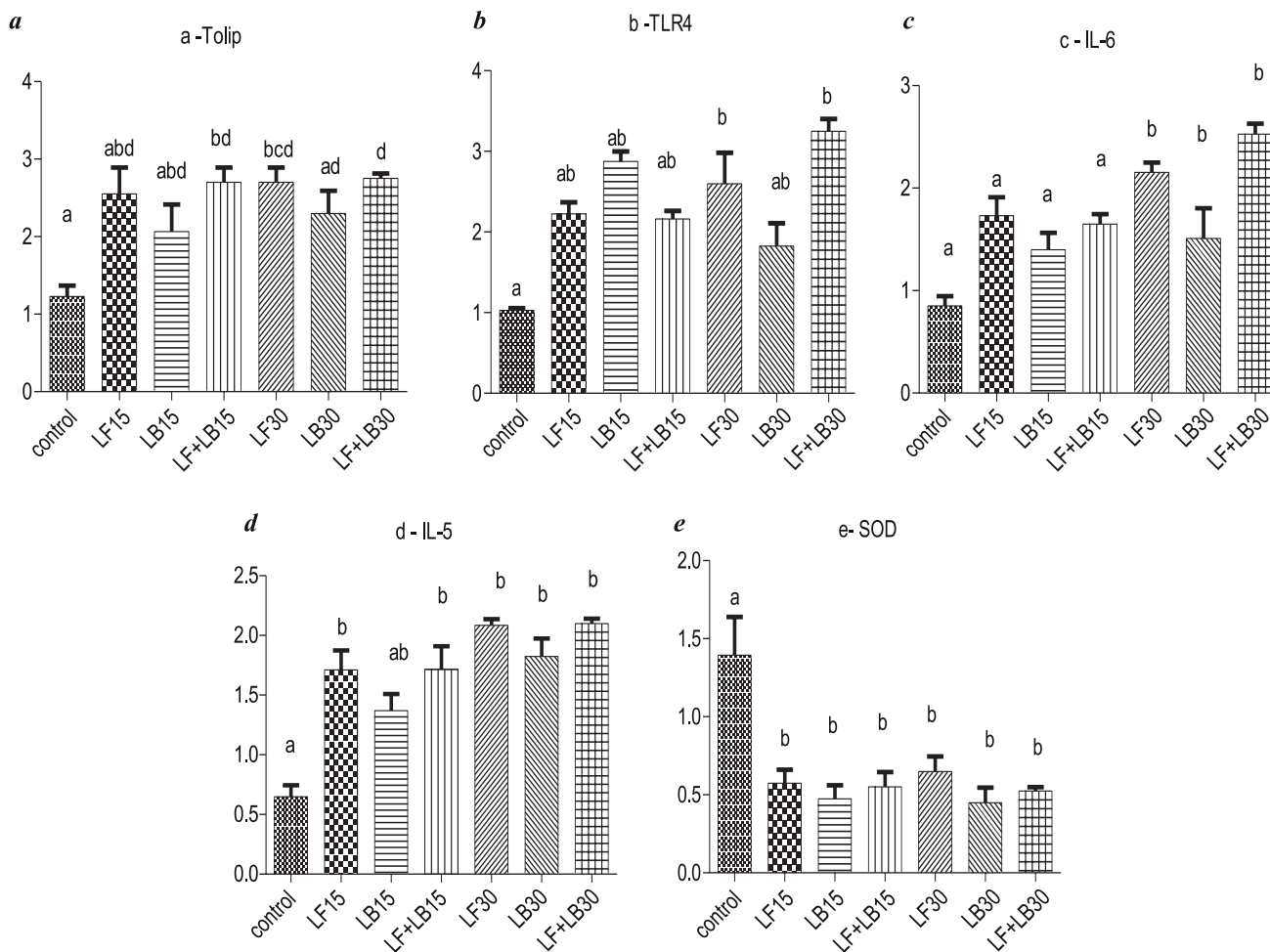
Fig. 4a-e. mRNA levels of Toll-interacting protein (*Tollip*) (a); Toll-like receptor 4 (*TLR4*) (b), interleukin *(IL)-6* (c), *IL-5*(d) and superoxide dismutase (*SOD*) (e) in sheep treated with lactobacilli (LB), lactoferrin (LF), or a combination of LB and LF compared with controls. Small alphabetic letters show significance at p<0.05.

phage and lymphocyte release from peritoneal macrophages (Perdigón et al. 1986), and in healthy human males (Mulder et al. 2008). The pattern of cellular and enzymatic alterations could fortify the immune-stimulatory effect of LB; however, the mechanisms of how LAB affects the immune system are unknown (Herich, and Levkut 2002).

Despite being inferior to LB, it is worth highlighting the remarkable effect of LF on both cellular elements (including total leucocytes and lymphocytes) and the enzymatic activity of lysozyme. The pattern of stimulatory effect was the same as in LB but to a lesser extent. However, there are conflicting views regarding the influence of LF on lymphocyte proliferation; some reports have shown a stimulatory effect (Prgomet et al. 2007), while others suggested an inhibitory role (Richie et al. 1987).

In the present study, lambs that received a combination of LF and LB showed significantly elevated values of NO and CAT (at T15), and lower MDA levels when compared with those that received LF or LB alone. Interestingly, the effect of LF was more

pronounced than LB, particularly on NO, and CAT (at T15), GSH (at T30), and MDA (at T15 and T30). These findings demonstrate the anti-oxidative potential of LF, and suggest that its use (either alone or in combination with LB) could be beneficial under field conditions. These findings were in part similar to those reported in healthy human males, where the authors showed a statistically significant elevation in hydrophilic antioxidant capacity after two weeks of LF supplementation (Mulder et al. 2008).

The expression patterns of immune-inflammatory genes were investigated in LF and/or LB fed lambs. To the best of our knowledge, this is the first study to report the expression patterns of these marker genes in lambs supplemented with LF and/or LB. In the present study, LF elicited a significant up regulation of *Tollip, IL-6, and IL-5* at T30 compared with the controls. Our findings were similar to those reported in growing calves that received LF (Prgomet et al. 2007). In that study, it appeared that orally supplemented LF enhanced the expression pattern of *IL-1 , IL-8*, and *IL-*

10 and interferon gamma. A similar effect of LF was previously shown on bovine leukocytes and monocytes (Prgomet et al. 2006), where LF enhanced production of TNF-α, IL-1 β, IL-6 and IL-10. In contrast, LF was reported to down-regulate the expression of proinflammatory cytokines including TNFα, IL-1β, and IL-6 induced by lipopolysaccharide in mice (Kruzel et al. 2002). Taking all views into account, it could be inferred that LF can up- and down-regulate immune cells as well as cells involved in the inflammatory process.

Lambs that received LB provoked a significant up regulation of *TLR4* (at T15), IL-5 (at T30), and IL-6 (at T30) compared with those of the controls, 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. It appears that supplemented LB can potentially influence innate immunity by increasing *TLR4* expression; however, this remains speculative as we tested the effect of LB and LF only on this particular gene. Therefore, studying more relevant genes is necessary to verify our finding. Similar findings were previously reported in dairy cows at mid-lactation that were supplemented with a multi-strain probiotic for 60 days (Adjei-Fremah et al. 2018). In the latter study, the authors reported a variable expression of genes associated with innate and adaptive immunity, such as cytokines and chemokines, toll-like receptors and other stress-related signaling molecules.

The findings of the present study reinforce the safety profile of both LF and LB at the given doses and no significant alterations in either physiologic or blood parameter safety measurements were documented throughout the study period.

Conclusion

The results suggest that bovine LF and/ or LB supplements could be a useful nutritional support for the immune system in healthy lambs. The study also provides evidence that LB delbruekii and LB fermentum could have possible systemic effects through pathways involved in immunity. Moreover, a combination of LF and LB could provoke better anti-oxidant capacity with maximal stimulation of the immune system. Nevertheless, further studies are still needed to better understand the mechanism of LF or LB activities, as well as their interactions to enable their full and safe usage under field condition.

Acknowledgements

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