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Effect of Lactobacterin-TK2 and Multispecies probiotics on calf health during suckling

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ABSTRACT: We hypothesised that supplementing probiotics to milk would improve calves' intestinal microbiota, support the immune system and antioxidant defence mechanism, increase their live weight, and raise healthy calves. The present study aimed to investigate the effects of two probiotic mixtures (Lactobacterin-TK^2 and Hypro) containing beneficial microorganisms from different sources on the immune system and growth of calves by reducing oxidative stress during the milk-suckling period. Eighteen newborn calves (n=6 in each group) were randomly selected and divided into three groups. Groups were formed as the control group (G1; without probiotics), Lactobacterin-TK^2 (G2), and Hypro probiotic (G3). The administration of Lactobacterin-TK^2 and Hypro probiotics caused an increase in live weight (LW, P=0.04) and daily live weight (DLW, P=0.04) and reduced the number of days with diarrhoea compared to the control group. Hypro reduced the total coliform counts (P<0.05) and increased yeast counts (P<0.05) in faecal samples on 14-day-age. No adverse effects of probiotics were determined on blood biochemistry. No significant changes were determined in the oxidative and antioxidative stress parameters, except for Paraoxonase 1 (PON 1) (P<0.05). IgA, IgE, and IgG levels in the probiotic groups showed similarities with those of the control group. The findings showed that Lactobacterin-TK^2 and Hypro probiotics can be used to supplement the milk of calves to provide better growth during the suckling period and to raise healthy calves by reducing the effect of oxidative stress.

Keyword: Probiotics; calf; growth; health

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INTRODUCTION

Respiratory and digestive disorders are among the diseases that are frequently seen in calves and cause deaths. According to the NAHMS (2014) data, approximately 5% of newborn calves die during the milk-suckling period. The majority of mortality (32% of all mortality) is due to digestive diseases, which are characterised as calf diarrhoea. The mortality of calves due to respiratory diseases is 14.1%. Some calves died due to a combination of digestive and respiratory diseases (7% of mortality). At least one disease is observed in 33.8% of newborn calves. More than half of the calves (approximately 56%) have the digestive disease. The rate of respiratory diseases is 11.3%.

Calf diseases and deaths are the most important cause of economic losses of farms before the weaning age. Therefore, it is essential to minimise these economic losses in the pre-weaning period (Quigley, 2018). Antibiotics have been used successfully in the treatment of calves and the improvement of their performance. However, diarrhoea in calves is often caused by pathogens (viruses, protozoa) on which antibiotics are not effective. Therefore, their use is not indicated in such cases. On the other hand, the restriction of antibiotic use in treatment other than systemic illnesses and the prohibition of its use as a feed additive necessitated the development of different and natural strategies for calf growth and intestinal health (Izzo et al., 2011; Lorenz et al., 2011; Ulger, 2019). In this context, probiotics have been used to develop resistance to calf diseases and to accelerate the growth of calves (Ulger, 2019). The joint Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO) Working Group defined probiotics as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance. Microorganisms used as probiotics in animal diets include some members of the *Aspergillus, Bacillus, Brevibacillus, Bifidobacterium, Candida, Clostridium, Escherichia, Enterococcus, Lactobacillus, Lactococcus, Megasphaera, Pediococcus, Prevotella, Propionibacterium* and *Saccharomyces, Streptococcus* species (FAO, 2016). However, most commercial probiotic products contain one or more of several types of bacteria and yeast strains such as the strains of *Bifidobacterium* spp., *Enterococcus* spp., *Lactobacillus* spp., *Bacillus* spp., *Pediococcus* spp., *Streptococcus* spp., and *Saccharomyces boulardii* (Yirga, 2015; Markowiak and Ślizewska, 2018).

Major mechanisms of probiotic action on animal health are considered as a modification of the microbial population of the gastrointestinal tract (GIT), an increase in digestion and absorption of nutrients, production of antimicrobial substances such as organic acids, hydrogen peroxides, and bacteriocins, alteration in gene expression in pathogenic microorganisms, immunomodulation, and colonisation resistance. Some of these mechanisms have been reported to associate with the inhibition of enteric pathogenic microorganisms, while others have been reported to improve animal performance (FAO, 2016). Probiotics added to milk have an effect on feed intake and body weight gain (BWG) of calves. Also, they inhibit coliform bacteria and increase lactobacilli counts in the intestinal microbiota (Satık and Günal, 2017). According to meta-analyses of probiotics on the growth performance of young calves by Frizzo et al. (2011), numerous studies were assessed to examine the probiotic effects on BWG and feed efficiency. Lactic acid bacteria (LAB) supplementation as probiotics increased BWG and improved feed efficiency.

LAB are normal components of the GIT of animals and humans (Uyeno et al., 2015), and have been widely described for their inhibition capabilities for pathogens such as *Salmonella* spp. and *E. coli* (Vieco-Saiz et al., 2019). These pathogens lead to calf diarrhoea during the first weeks of life (Frizzo et al., 2011; Boranbayeva et al., 2020). Probiotics do not have negative effects on calves' blood biochemical, haematological, and mineral substance values in calves (Didarkhah and Bashtani, 2018; Shehta et al., 2019; Ulger, 2019). The probiotic preparations added to milk during the milk-suckling period significantly increased blood glucose and iron values, whereas they decreased the blood alanine transaminase values (Ulger, 2019).

It has been reported that probiotics added to quail diets are beneficial in increasing performance and feed efficiency, and that antioxidant enzyme levels and microbial measurements increase significantly (Nour et al., 2021a). Probiotics added to Japanese quail diets improved growth performance, digestive enzyme activities, antioxidant status and intestinal histomorphometry, and decreased blood cholesterol levels of growing Japanese quails (Nour et al., 2021b). It has been reported that *Bacillus subtilis*

spore supplementation added at different levels can increase antioxidant status and digestive enzyme activity and improve growth performance by increasing digestion in growing quails (Abdel-Moneim et al., 2020b).

Lactobacterin-TK^2 used in the present study has been previously reported to possess probiotic properties for newborn calves in studies conducted in Kazakhstan using *Lactobacillus acidophilus* 05 k-1. Lactobacterin-TK^2 has been reported to enhance nutrient digestibility, feed conversion efficiency, and body weight gain. Moreover, it has been reported to decrease the duration and severity of diarrhoea and dyspepsia and contribute to substantially reducing the level of measures taken for veterinary and sanitary practices (Tulemissova et al., 2016). The probiotic properties of *Lactiplantibacillus plantarum* AB6-25, *Lacticaseibacillus casei* K2, and *Saccharomyces boulardii* T8-3C, included in the Hypro probiotic preparation, were determined under *in vitro* conditions in Türkiye (Kiliç and Karahan, 2010; Yıldıran et al., 2019; Boranbayeva et al., 2020). Therefore, the present study examined the effects of pre-weaning probiotic treatments (Lactobacterin-TK^2 and Hypro) were investigated on growth performance, biochemical blood parameters, immune status, oxidative stress, and coliform, *E.coli*, LAB, and yeast counts in the faeces of calves. We hypothesised that supplementing probiotics to milk would improve calves' intestinal microbiota, support the immune system and antioxidant defence mechanism, increase their live weight, and raise healthy calves.

MATERIAL AND METHODS

Animals and Design

The study was conducted from July to September 2019 at the Research and Application Farm of Isparta University of Applied Sciences, Agricultural Faculty, Türkiye. Healthy newborn Holstein calves (n=18; control group [n=6, G1 (actual LW= 45.50±4.73 kg)], Lactobacterin-TK^2 [n=6, G2 (actual LW= 38.83±2.32 kg)] and Hypro [n=6, G3 (actual LW= 35.00±6.83 kg)] whose birth weights were close to each other were randomly chosen.

Calves born on the farm were fed with colostrum for the first 3 days. Calves were randomly divided into 3 groups based on live weight and body measurements on their 4th day. The experimental groups were formed as follows;

G1: Calf starter + whole milk (control); G2: Calf starter + Lactobacterin-TK^2-supplemented- whole milk; G3: Calf starter + Hypro probiotic-supplemented-whole milk.

Commercial calf starter (It has an average particle size of 1.5 cm in pellet form, 18% crude protein-2800 kcal/ME) was used in the study. Standard feeding was applied to the groups during the study. Calf starter was given *ad libitum*. Calves were fed twice with 4 L of milk per day (2 L in the morning, 2 L in the evening) until the weaning time.

Lactobacterin-TK^2 and Hypro probiotics were given to each calf in the experimental groups (G2 and G3) at a rate of 10^{11} CFU/day. When the calves consumed an average of 800 g/day starter in three consecutive days (Özkaya et al., 2018), the calves were weaned and removed from the experiment.

Calves were housed in individual pens with natural ventilation, which were 110 cm width, 218 cm length.

Production of Lactobacterin-TK^2

Lyophilised Lactobacterin-TK^2 has been stored at -20 °C for a year. It was sub-cultured twice at 37 °C for 16-18 h in MRS broth (Merck, Darmstadt, Germany) and determined bacterial viability by the plate count method. Then it was inoculated into 10 L of the same medium and incubated under the same conditions mentioned above. The culture was centrifuged at 2350xg for 10 min at 4 °C. After discarding the supernatant, the pellet was portioned as 10^{11} CFU/day for each calf and stored at -20 °C until use.

Production of Hypro

Hypro was prepared to contain *L. plantarum* AB6-25, *L. casei* K2, and *Saccharomyces boulardii* T8-3C. Their GenBank accession numbers were GQ332649.1, MK643164, and MG711551, respectively. The strains of lactobacilli and yeast stored at -20 °C were sub-cultured twice in MRS broth (Merck) and Malt extract, Yeast extract, Glucose, Pepton (MYGP) broth at 37 °C for 16-18 hours, respectively (Kiliç and Karahan, 2010; Syal and Vohra, 2013; Yıldıran et al., 2019). Then they were inoculated separately into 10 L of the same media and incubated under the same conditions mentioned above. After the incubation period, they were centrifuged at 2350xg for 10 min at 4 °C. The pellets of all three strains were mixed in equal amounts under aseptic conditions and portioned as 10^{11} CFU/day for each calf. Hypro was stored at -20 °C until use.

Collection of Data

Starter and milk analysis

The chemical composition of the starter and milk used in calf feeding is shown in Table 1. Crude protein analysis of the starter was carried out by the Kjeldahl method, and ether extract was done by the Soxhlet method (AOAC, 2005). Acid detergent fibre (ADF) and neutral detergent fibre (NDF) analyses were done with the ANKOM 220 fibre analyser (ANKOM Tech., Macedon, NY, USA). Metabolic energy was calculated as specified by the Turkish Standards Institute (TSE, 1991). Fat, fat-free dry matter, protein, lactose, and freezing point in milk were determined with the Milk Test (Has Vet, Antalya, Türkiye) milk analyser.

Live weight and body measurements

The experiment was initiated with 4-day-old calves to eliminate the effect of composition and physicochemical properties of colostrum, which vary due to a number of factors such as the number of the cow's lactation period, breed, length of the dry period and prenatal feeding. Live weight (LW) and body measurements (BMs) (Body Length-BL, Withers Height-WH, Body Depth-BD, Hip Height-HH, and Chest Girth-CG) of calves were taken at the beginning of the experiment and were followed up weekly. In the evening before weighing, feed and water supply were halted, and the calves were weighed on an empty stomach.

Starter intake and feed conversion ratio

Starter intake of calves was recorded daily using an electronic scale with 1 g sensitivity. Accordingly, calves that consume 800 g of starter on 3 consecutive days were determined. Daily starter consumption and daily live weight gain of calves were determined. The feed conversion ratio was calculated by dividing daily starter consumption by daily weight gain.

Table 1. Composition of whole milk and calf starter

Ingredients, %	Milk	Calf starter
Dry matter	12.6	90.26
Crude protein	3.3	17.55
Ether extract	3.5	3.45
Ash	nd*	6.68
Lactose	5.8	
Freezing point (-°C)	0.68	
Crude fibre		7.13
Metabolic energy kcal/kg	nd	2848

nd: not determined

Fecal scoring

The faeces of calves were monitored daily and scored for consistency. Faecal scoring was recorded as 1-Normal, 2-Soft, 3-Runny, and 4-Watery (Larson et al., 1977).

Microorganism counts in faeces samples

Rectal fecal samples were collected on day 14 and 28 and also at the weaning age. Sterile gloves were used to take 10–12 g faecal samples after the perianal cleansing with diluted betadine solution. The samples were collected at approximately 07:00 PM into sterile 50-mL falcon tubes and immediately taken to the laboratory in an icebox at 4 °C. A 10 g homogenised faeces sample was serially diluted with sterile physiological saline solution (PS), and then the appropriate dilutions were plated onto M17 agar and MRS agar, MYGP agar (pH 4.5), EMB agar, and Lactose TTC (LTTC) agar containing Tergitol-7 for LAB, yeast, *E. coli*, coliform bacteria, respectively. LTTC agar plates for faecal coliform counting were incubated aerobically at 44.5 °C for 48 hours and all others at 37 °C for 48 hours. At the end of the incubation period, the results were obtained by counting the typical colonies in all Petri dishes (Pitkänen et al., 2007; Boranbayeva et al., 2020).

Blood samples and analysis

Blood samples were taken from all calves in the groups using gel vacuum tubes from the jugular veins (Wu *et al.*, 2021) on day 28 and at the weaning age. The samples were centrifuged at 3500 x g for 10 minutes. The separated serums were stored at -20 °C until the time of analysis. Total cholesterol (TC), triglyceride (TG), glucose (GLU), urea, creatine (CREA), albumin (ALB), total protein (TP), uric acid (UA), total bilirubin (TB), direct bilirubin (DB), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH) were examined from blood samples were analyzed (Mazmancı et al., 2008; Yorulmaz et al., 2008) by a biochemistry blood analyzer (Mindray BS120, Nanshan, Shenzhen P.R. China).

Oxidative and antioxidative stress parameters

Rel Assay Diagnostics commercial kits (Mega Tıp, Gaziantep, Türkiye) were used in total oxidant status (TOS), total antioxidant status (TAS), paraoxonase 1

(PON 1), thiol/disulfide homeostasis (TDH), catalase (CAT), total thiol (TT), native thiol (NT), malondialdehyde (MDA), superoxide dismutase (SOD) analyses (Erel, 2004, 2005; Erel and Neselioglu, 2014).

The TOS/TAS ratio was accepted as the oxidative stress index (OSI). For the calculation, the resulting unit of TAS was converted to μmol/L, and the formula used for the calculation of the OSI value is as follows (Yumru et al., 2009).

OSI (arbitrary unit) = TOS (μmol H_2O_2 equivalent/L) / TAS (μmol Trolox equivalent/L)

Immunoglobulin analyses

Immunoglobulin analyses (Wu et al., 2021) were performed by Otto Scientific (Ankara, Türkiye) using bovine-specific reagents (Rel Assay, Mega Tıp San. Tic. Ltd. Sti., Gaziantep, Türkiye). Immunoglobulin A (IgA) selectively react with an anti-IgA antibody and forms an immune complex. The produced turbidity is proportional to the concentration of IgA in the sample and can be measured at a wavelength of 600 nm. When anti-human Immunoglobulin E (IgE) antibodies are mixed with samples containing IgE, they form insoluble complexes. These complexes cause a change in the absorbance depending on the IgE concentration of the samples, which can be quantified by a comparison using a calibrator with a known IgE concentration. Immunoglobulin G (IgG) selectively react with an anti-IgG antibody and forms an immune complex. The produced turbidity is proportional to the concentration of IgG in the sample, and can be measured at the wavelength of 600 nm. IgA, IgE, and IgG antibody concentrations were colourimetrically determined using the Mindray BS-300 (Nanshan, Shenzhen, P.R. China) biochemical blood analyser. All the tests were run according to the manufacturer's protocols for reagents.

Number of diarrheal and diseased days

The health status of the calves was monitored daily in the morning and evening. The type and duration of diseases such as diarrhoea, respiratory problems or other health-related clinical symptoms were recorded daily for each calf. When the faecal score was ≤ 3, the calf was regarded as diarrheal. In the emergence of any symptoms (faecal score ≤3 for 3 consecutive days and/or signs such as coughing), immediate care and treatment were given by a veterinarian.

The general appearance of calves

The general appearance of calves was defined according to Heinrichs et al. (2003): 1- Normal and awake, 2- Ears fell sideways, 3- Head and ears fell sideways, eyes dull, slightly numb, 4- Head and ears fell, eyes dull and numb, 5- Seriously lethargic.

Statistical Analyses

A power analysis was conducted. It was determined that this sample group was adequate to detect differences between treatments with 85% power and 0.05 α. In the literature review, it was determined for 6 calves in each group for 85% power that the highest mean value for feed efficiency was 1.73, the lowest mean value was 1.27, and the standard deviation was 0.2.

The results were analysed by repeated-measurements ANOVA, and the differences between groups' mean values were examined with the Tukey test (IBM Corp., 2015). The statistical model for analysis was given as:

$$Yijm=\mu+ \alpha_i + \pi_{m(i)} + \beta_j + \alpha\beta_{ij} + \beta\pi_{jm(i)} + \varepsilon_{ijm}$$

Here, Yijm is the dependent variable, μ is the population mean, α is the effect of probiotics factor, π is the random effect of m experiment unit in i level of probiotics, β is the effect of time factor, $\alpha\beta_{ij}$ is effect of interaction between probiotics and time, $\beta\pi_{jm(i)}$ is effect of between time and experiment unit in i level of probiotics and ε_{ijm} is random effect of error.

There are two different probiotic factors (Lactobacterin-TK[2] and Hypro) and 7 levels of the time factor. After the experiments and levels of time, the repeated measurements ANOVA analysis was applied. The repeated measurements were carried out for each level of the time factor. When $P<0.05$, there is a significant difference between the averages, and when $0.05<P\leq0.10$, there is a trend in the differences between the averages.

RESULTS

Live weight and body measurements

The effects of Lactobacterin-TK[2] and Hypro-supplemented calves' milk on LW and BMs are shown in Table 2. The differences between the LW of the groups at the 14-day-old, 28-day-old, and weaning were found to be not significant. The difference between the total and daily weight gain of the groups was found to be significant ($P<0.05$). The highest value of both total and daily weight gain was obtained in G2 (Table 2).

Although numerical differences between BMs were not significant during the 14 and, 28-day-suckling period ($P>0.05$), it was observed that the growth

Table 2. Effect of probiotic preparations on calf's LW and BMs

	14th day	28th day	Weaning age	Gain	Daily Gain
Group	**Mean±S.E**	**Mean±S.E**	**Mean±S.E**	**Mean±S.E**	**Mean±S.E**
Live weight, kg					
G1	44.83±4.13	46.33±4.13	50.00±5.06	4.50±1.32^{B}	0.109±0.03^{B}
G2	42.00±2.75	46.83±2.24	55.00±4.27	16.17±2.24^{A}	0.321±0.03^{A}
G3	36.17±6.22	37.67±6.22	47.00±6.29	12.00±3.40^{A}	0.229±0.06^{A}
P	0.45	0.34	0.59	0.04	0.04
Body Lenght, cm					
G1	70.65±1.17	72.22±1.30	75.06±1.04	6.00±0.44	0.14±0.01
G2	71.72±1.45	74.06±1.00	78.53±1.80	9.48±2.84	0.18±0.04
G3	71.13±3.61	73.06±4.16	76.58±2.59	7.52±1.01	0.15±0.02
P	0.56	0.58	0.38	0.38	0.05
Wither High, cm					
G1	78.94±1.69	79.93±1.33	82.10±0.93	5.77±0.29	0.14±0.01
G2	78.19±0.29	81.65±0.50	84.40±0.44	8.06±1.26	0.16±0.01
G3	78.02±3.06	79.75±3.77	82.17±3.00	8.83±0.44	0.12±0.01
P	0.52	0.17	0.12	0.12	0.05
Body Depth, cm					
G1	30.67±0.88	31.14±0.88	32.93±0.50	3.15±0.17	0.07±0.01
G2	31.12±0.33	32.45±0.44	35.21±0.83	5.44±1.36	0.11±0.01
G3	30.54±1.67	31.75±1.76	33.69±1.26	3.91±0.58	0.08±0.01
P	0.45	0.16	0.19	0.19	0.14
Hip High, cm					
G1	80.64±2.42	81.97±2.17	84.11±2.35	5.22±0.87	0.12±0.02
G2	80.29±0.33	83.15±0.67	86.96±0.93	8.07±1.00	0.16±0.00
G3	80.69±3.11	82.22±3.75	85.27±2.35	6.38±0.93	0.13±0.02
P	0.58	0.41	0.21	0.21	0.44
Chest Girth, cm					
G1	76.58±3.09	78.10±2.62	79.68±2.18^{B}	4.63±0.93^{B}	0.11±0.02^{B}
G2	77.73±1.33	81.07±0.88	86.16±2.03^{A}	11.10±1.53^{A}	0.22±0.01^{A}
G3	76.58±4.60	79.33±5.07	82.33±4.03^{AB}	7.27±0.60^{AB}	0.14±0.02^{AB}
P	0.17	0.13	0.02	0.02	0.00

S.E.: Standard Error, ^{A,B} Difference between means in the same column, G1; control, G2; LBTK2, G3; MSPM

was better in G2 and G3 than in G1 (Table 2). However, the differences among the CG values were found to be significant ($P<0.05$). Lactobacterin-TK[2] significantly increased the CG data of calves compared to G1 and G3.

Feed intake, feed conversion ratio, faecal score, number of days with diarrhoea, and general appearance

Probiotic applications did not significantly affect calves' total and daily feed intake (Table 3). As seen in Table 3, an improvement in feed conversion ratio occurred in calves receiving probiotics. However, the numerical difference observed in the feed conversion ratio was not found to be significant. The difference between the faecal score averages monitored daily was not found to be significant ($P>0.05$). On the other hand, the supplementation of the probiotic preparations tended to improve the faecal score, albeit a little, compared to the G1. This means that

Table 3. Effects of probiotic preparations on some parameters of calves

Groups	G1	G2	G3	P
	Mean±S.E.	Mean±S.E.	Mean±S.E.	
Starter intake, kg	8.76±0.89	11.61±1.22	13.01±3.22	0.39
Daily starter intake, kg	0.23±0.02	0.26±0.07	0.26±0.05	0.86
Feed Conversion Ratio	2.98±1.43	0.84±0.19	1.35±0.32	0.26
Fecal score	1.47±0.12	1.34±0.13	1.35±0.07	0.66
Days with diarrhoea	5.67±1.45	2.00±1.15	1.67±0.88	0.09
Incidence of diarrhoea, %	4.53±1.13	1.54±1.00	1.04±0.57	0.08
General appearance	1.15±0.04	1.05±0.03	1.03±0.02	0.07

S.E.: Standard Error, G1; control, G2; LBTK2, G3; MSPM

during the experiment, diarrhoea cases in G2 and G3 occurred less than in G1 (Table 3).

The numerical difference in the average appearance of calves was not found to be significant (P>0.05). During the experiment, no disease was encountered in the calves, except for diarrhoea.

Biochemical blood analysis

Numerical differences among the biochemical blood values were not significant (P>0.05). However, uric acid, total cholesterol, and triglyceride values were higher on day 28 and the weaning age compared to G1 in G2 and G3 (Table 4).

Effects of probiotics on some faecal microorganisms

The effects of probiotic preparations applied to calves during the milk-suckling period on counts of microorganisms in the calves' faeces samples are shown in Table 5. On day 14, the effects of probiotic preparations applied to calves on LAB in MRS and M17 and the *E.coli* counts in EMB agar in all three groups were found to be similar. The total coliform count in LTTC agar was the highest in the samples taken from G2 that received Lactobacterin-TK^2, whereas the lowest was in the samples taken from G3 that received Hypro, and the differences were significant (P<0.05). A similar result was found in terms of yeast counts. G3 (Hypro) yeast count results were significantly higher than G2 (Lactobacterin-TK^2) and control (P<0.05). The enumeration results on day 28 and weaning age samples were not significantly different between the groups (P>0.05). Yeast counts in the faecal samples that received G3 during the trial period remained stable and were higher than those of the other groups.

Effects on oxidative stress and the immune system

The effects of probiotic applications on oxidative stress and the immune system are shown in Table 6. On day 28, PON 1 value, one of the antioxidative defence mechanism markers, was significantly higher in G2, which received only Lactobacterin-TK^2 (P<0.05). In the weaning age, it was determined that probiotics-treated groups (G2 and G3) encouraged the increase in PON 1 values, and PON 1 levels were significantly higher compared to the control group (P<0.05). In terms of TT values, on day 28, G1 was significantly higher than G2, while the values in G3 were comparable with both other groups (P<0.05). In the weaning age, although there were no statistically significant differences between the thiol values of the three groups (P>0.05), it was determined that thiol values increased in the probiotic groups. The changes in other antioxidant defence mechanism markers, such as TAS, NTL, TDH, CAT, SOD, and MDA, were not significant (Table 6).

The numerical differences between the immunoglobulin values of the groups were not significant (P>0.05). However, probiotic preparations were observed to support the immune system to a small extent (Table 6).

DISCUSSION

Live weight and body measurements

While the effect of the probiotic applications on live weight mean values was not significant, total live weight gain (TLWG) and DLWG were found to be significant. Also, BMs other than CG were not found to be significant. The results showed that Lactobacterin-TK^2 and Hypro probiotic applications promoted calves' growth in the milk-suckling period.

Table 4. Effects of probiotics on biochemical parameters

Parameters	28th Day				Weaning Age			
	G1	G2	G3	P	G1	G2	G3	P
	Mean±S.E.	Mean±S.E.	Mean±S.E.		Mean±S.E.	Mean±S.E.	Mean±S.E.	
ALT (U/L)	9.83±0.88	9.50±1.26	10.00±3.21	0.99	13.17±0.40	16.00±1.73	12.83±1.17	0.40
AST (U/L)	140.00±47.30	128.50±61.00	153.80±18.10	0.93	65.50±12.40	178.50±66.30	83.20±31.30	0.22
GGT (U/L)	47.50±13.90	53.00±17.60	35.17±9.55	0.67	31.67±6.69	42.00±10.70	26.67±2.19	0.39
LDH (U/L)	757.00±155.00	1282.00±297.00	722.00±140.00	0.19	854.00±184.00	1492.00±390.00	891.00±116.00	0.23
ALP (U/L)	206.70±25.20	248.70±38.90	193.30±38.10	0.54	193.70±17.40	335.70±93.10	275.30±49.60	0.33
GLU (mg/dL)	80.33±9.10	87.17±9.80	95.17±2.33	0.46	76.80±23.30	116.40±53.80	65.00±20.10	0.63
TC (mg/dL)	172.20±18.70	198.00±4.73	175.00±28.80	0.63	176.80±14.00	219.30±41.80	183.00±22.20	0.56
CREA (mg/dL)	1.87±0.20	1.90±0.22	1.81±0.28	0.96	1.86±0.22	2.29±0.47	1.30±0.15	0.16
UREA (g/L)	58.22±2.82	48.10±16.60	69.10±19.20	0.63	44.90±13.60	82.00±26.40	61.20±13.70	0.43
T-BIL (mg/dL)	0.21±0.07	0.19±0.06	0.15±0.02	0.71	0.12±0.03	0.20±0.07	0.20±0.05	0.48
TP (g/dL)	7.30±0.64	7.33±0.06	7.57±0.63	0.92	6.97±0.54	9.79±2.01	7.03±0.20	0.25
ALB (g/dL)	3.31±0.08	3.29±0.03	3.21±0.11	0.67	3.27±0.07	4.39±1.08	3.24±0.15	0.39
TG (mg/dL)	24.17±6.85	35.33±5.55	29.30±15.50	0.76	21.17±7.34	40.83±4.42	37.20±14.00	0.36

G1; control, G2; LBTK2, G3; MSPM

Table 5. Effects of probiotics on parameters of oxidative stress and the immune system

Parameters	28th Day				Weaning Age			
	G1	G2	G3	P	G1	G2	G3	P
	Mean±S.E.	Mean±S.E.	Mean±S.E.		Mean±S.E.	Mean±S.E.	Mean±S.E.	
TAS (mmol/L)	0.86±0.02	0.91±0.02	0.89±0.09	0.84	0.85±0.08	0.88±0.06	0.93±0.19	0.89
TOS (µmol/L)	7.47±2.70	2.78±0.99	2.49±0.08	0.14	2.16±0.28	5.66±3.08	4.14±2.05	0.55
OSI (Arbitrary unit)	0.85±0.29	0.30±0.10	0.29±0.04	0.11	0.26±0.04	0.61±0.30	0.40±0.12	0.46
PON1 (U/L)	261.70±44.30^{B}	445.00±30.00^{A}	262.70±52.90^{B}	0.04	321.00±33.00^{B}	580.00±22.30^{A}	496.00±13.00^{A}	0.00
TT (µmol/L)	779.10±57.30^{A}	567.00±37.3^{B}	614.40±28.70^{AB}	0.03	532.90±19.90	695.00±121.00	616.30±56.40	0.40
NT (µmol/L)	684.70±36.00	545.90±32.80	579.90±26.50	0.05	476.70±12.70	550.90±55.40	550.10±39.30	0.38
TDH (%)	47.20±22.50	10.56±2.42	17.23±4.26	0.19	28.10±14.90	72.20±34.20	33.10±8.53	0.37
CAT (kU/L)	80.30±35.80	104.70±36.50	52.30±11.40	0.51	70.90±45.90	94.40±40.20	135.30±43.10	0.59
SOD (U/mL)	633.00±208.00	809.00±277.00	1304.00±375.00	0.32	959.10±47.00	1210.00±119.00	1066.00±131.00	0.31
MDA (µmol/mL)	9.33±2.98	9.67±1.12	7.97±0.72	0.80	6.07±1.09	10.95±5.40	10.35±3.10	0.61
IgE (IU/mL)	26.57±7.16	33.20±2.57	28.90±5.53	0.70	24.70±11.60	27.07±3.63	26.40±10.10	0.98
IgA (mg/dL)	0.27±0.16	0.36±0.02	0.56±0.19	0.41	0.28±0.09	0.40±0.31	0.47±0.20	0.84
IgG (mg/dL)	3.43±0.72	5.17±0.50	5.10±0.85	0.23	3.97±1.69	4.03±1.08	4.80±0.71	0.87

A, B: Different letters within the row mean a significant difference (P<0.05); G1; control, G2; LBTK2, G3; MSPM

Similarly, Ratre et al. (2019) have reported that the effect of probiotic supplementation on LW and BMs is not significant; however, its effect on DLWG was significant. Probiotics improve DLWG by promoting the growth of calves in the first weeks of their lives (Alawneh et al., 2020).

Stefańska et al. (2021) have reported that the calves fed with colostrum and milk substitutes containing the combination of probiotics and phytobiotics had higher body weight values on days 28 and 56 compared to those obtained in the control group. On the other hand, Vazquez-Mendoza et al. (2020) have stated that the effect of the probiotic supplement on TLWG, DLWG, and BMs was

Table 6. Effect of probiotic preparations on faecal microbiota of the calves (log10 cfu/g of fresh faces)

	MRS	M17	EMB	LTTC1	LTTC2	Yeast
	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.
14th Day						
G1	7.17±0.42	7.95±0.34	7.96±0.32	7.41±0.12^{AB}	6.67±0.80	1.56±1.56^{AB}
G2	8.47±0.20	8.38±0.26	8.88±0.72	9.01±0.62^{A}	6.35±0.65	<10^{B}
G3	6.90±0.50	8.17±0.95	7.04±0.83	6.33±0.51^{B}	5.99±0.36	4.80±0.73^{A}
P	0.07	0.88	0.23	0.02	0.76	0.04
28th Day						
G1	6.84±0.74	7.41±1.12	8.64±0.14	7.85±0.27	7.37±0.29	3.23±1.80
G2	8.11±0.36	8.82±0.53	8.63±0.63	7.80±0.81	6.89±0.92	3.83±2.21
G3	6.99±0.85	7.76±0.98	7.39±0.95	6.61±0.39	6.77±0.53	4.34±0.35
P	0.41	0.55	0.37	0.26	0.78	0.90
Weaning Age						
G1	5.83±0.55	6.05±0.25	6.14±0.61	6.16±0.49	6.12±0.51	<10
G2	6.98±0.41	6.77±0.32	5.98±0.41	6.23±0.01	5.52±0.48	1.31±1.31
G3	6.27±0.80	7.54±1.14	6.34±1.16	6.47±1.02	5.99±1.15	3.52±0.68
P	0.45	0.38	0.95	0.94	0.80	0.07

MRS: Lactobacilli, M17: Cocci LAB, EMB: E. *coli*, LTTC1: Total coliform, TTC2: Faecal coliform, ^{A,B}: Difference between means in the same column, G1; control, G2; LBTK2, G3; MSPM

not significant. Probiotic bacteria have the ability to produce digestive enzymes, vitamins and antibacterial substances, including hydrogen peroxide, bacteriocins, lactoperoxidase system, organic acids, lactone components and acetaldehyde that prevent the growth of pathogenic bacteria in the intestinal tract and, hence, support the immune system. Thus, toxic substances are reduced, the intestinal system is enhanced, and the digestion of nutrients and absorption increase. Increased digestion and absorption lead to improvement in LW (Nageshwar et al., 2016; He et al., 2017; Sharma et al., 2018).

Feed intake, faecal score, number of days with diarrhoea, and general appearance

The difference between the total and daily feed intake averages was found to be not significant. This result was also similar to those reported that the supplementation of probiotics did not affect calves' feed intake (He et al., 2017; Satık and Günal, 2017; Ulger, 2019; Vazquez-Mendoza et al., 2020).

Increasing the presence of non-pathogenic bacteria in the gut improves feed conversion, resulting in an increase in LW (Chaucheyras-Durand and Fonty, 2002; Khuntia and Chaudhary, 2002). In this study, the increase in LW is thought to be due to probiotic supplementation, which resulted in an improvement in the presence of non-pathogenic bacteria in the gut and feed conversion ratio. Consistent with our findings, Roodposhti and Dabiri (2012), Shehta et al. (2019), Ulger (2019), and Alawneh et al. (2020) showed that probiotics improve feed conversion ratio in calves.

Although the numerical difference between the mean faecal score values was not significant, the results showed that probiotic treatment groups had lower faecal scores. This supports the result obtained by He et al. (2017). However, Didarkhah and Bashtani (2018), Shehta et al. (2019), Vazquez-Mendoza et al. (2020), and Stefańska et al. (2021) have reported that the difference between the faecal scores was significant.

The differences between the numbers of diarrheal days of the groups were not significant (P>0.05). However, the probiotic supplement reduced the number of calves with diarrhoea and reduced the incidence of diarrhoea cases by reducing the duration of diarrhoea. This result was in agreement with those reported by Noori et al. (2016). Didarkhah and

Bashtani (2018) and Shehta et al. (2019) have also stated that the supplementation of probiotics reduced the number of diarrheal days. The use of probiotics suppresses the growth of faecal coliform bacteria, reduces the number of calves requiring treatment for digestive system diseases, the incidence of diarrhoea and the overall mortality rate (Alawneh et al., 2020).

Biochemical blood analysis

The probiotic did not affect the biochemical blood values of calves. These results of the present study were similar to the results reported in numerous studies (Seifzadeh et al., 2017; Tunc and Yoruk, 2017; Shehta et al., 2019; Vazquez-Mendoza et al., 2020). However, there are also some other studies that report different results. Ulger (2019) have reported that the difference between TG and ALT values was significant. It has been reported that the supplementation of probiotics increases ALP levels while decreasing CREA and AST levels. At the same time, it was stated that the level of TG and very low-density lipoprotein decreased (Nour et al., 2021a).

Effects of probiotic supplementation on faecal microorganisms

Some microorganisms from the calf gut microbiota were studied mainly using faecal samples. During the trial period, except for the total coliform and yeast counts on day 14, no differences were found between the groups in the faecal samples in terms of microorganism counts. Differences in animals are higher in the first days of life than in adulthood, and this may eliminate the statistical difference between groups (Malmuthuge and Guan, 2017). Similarly, many researchers have stated that the effect of the probiotic supplementation on coliform and lactobacilli was not significant in the first 28 days (Satık and Günal, 2017). Although bacterial composition can vary depending on the GIT region and sample type (content versus mucosal tissue), gut microbial colonisation during the preweaning period has been mainly studied using faecal samples, because it is a non-invasive way to obtain samples from the same individual over time (Malmuthuge and Guan, 2017). Therefore, faecal samples were taken and microorganism counts were carried out. Although it was determined that the probiotics used in the present study did not cause a significant change in the pathogenic microorganism counts, a low number of days with diarrhoea and low faecal scores were remarkable in the G2 and G3 groups. As a result, it was thought that the microorganisms in the probiotic preparations given to the calves in G2 and G3 were effective. For example, since Hypro contains *S. cerevisiae*, the yeast counts were found significantly higher on day 14 in faecal samples taken from G2 ($P<0.05$). Although statistically no differences were determined in samples taken on other days, the fact that *S. cerevisiae* became dominant among the yeast population in the GIT might have contributed to the prevention of diarrhoea caused by various *Candida* species. Particularly, *C. glabrata* was remarkable as the most prevalent species (69.5%) in preweaned calves suffering from neonatal calf diarrhoea (Elad, 1998). Lactobacilli found in probiotics are also effective against calf diarrhoea. Other microorganisms in Hypro were *L. plantarum* and *L. casei*, as mentioned in the Introduction section. Various studies have reported that probiotics containing different strains of both lactobacilli were effective against diarrhoea by improving the growth performance and immune system of calves (Kawakami et al., 2011; Renaud et al., 2019). *L. acidophilus* in Lactobacterin-TK[2] was another LAB species that has been reported to be effective against calf diarrhoea (Sharma et al., 2018). Gut health, integrity, and function are positively correlated with an increased number of probiotics in the gut. When the number of beneficial microorganisms in the intestine increases, pathogenic microorganisms are suppressed, and this situation strengthens the intestinal microbiota by changing it (Abdel-Moneim et al., 2020a).

Effects on oxidative stress and the immune system

In living organisms, there is a constant balance between the antioxidant and oxidative systems. Any disturbance of this balance by any factor in favour of oxidative stress leads to a harmful physiological reaction, resulting in the generation of excessive reactive oxygen species (ROS). Excessive ROS levels that are not counteracted by the antioxidant defences of the cell can lead to tissue damage (Xin et al., 2020). DNA damage and TOS levels were significantly higher in many diseases of mammals, while TAS was significantly lower (Kabu et al., 2015; Erkilic et al., 2016). The present study demonstrated that probiotics decreased the TOS value insignificantly and increased the TAS value. It was stated that the probiotics applied in addition to the routine diarrhoea treatment of newborn calves significantly increased the TAS values; however, albeit non-significant, caused a decrease in the TOS value (Yüksek et al., 2021). SOD and CAT, which are regarded

as the main antioxidant enzymes in mammals, are important components of the antioxidant system (Yuan et al., 2007). MDA is generated by lipid peroxides of ROS and can amplify the impairment of oxidative stress through reaching and has an effect on distal cells due to a statistically longer half-life period compared to that of ROS (Esterbauer, 1996; Xin et al., 2020). The effects of probiotics on the above-mentioned oxidative stress and antioxidative defence mechanism molecules were proven in various animal studies (Wang et al., 2017). However, compared to the large volume of reported information for PON 1 from human and experimental animal studies, there is limited information on its roles in cows. As the liver plays a key role in the synthesis of serum PON 1, it is important to mention that serum PON 1 activity is associated with liver impairment during fatty liver development in dairy cows. Standard biochemical tests are insufficient to determine liver dysfunction. On the other hand, low serum PON 1 activity has been accepted as a biomarker for the diagnosis of fatty liver in dairy cows. It has also been reported that probiotics can reduce non-alcoholic steatohepatitis experimentally created in Wistar rats through modulation of apoptosis and their anti-inflammatory activity (Karahan et al., 2012). It has been reported that the supplementation of probiotic bacteria isolated from the faeces of healthy cows a non-significant increase in reactive oxygen metabolism and paraoxonase enzyme of calves during the weaning period, but causes a non-significant decrease in iron-reducing antioxidant power (Rosa et al., 2021). In the present study, MDA, which is a strong reactive, decreased non-significantly at the 28-day-olds; however, it increased at weaning. In addition, the PON 1 enzyme increased significantly in 28-day-olds and weaning.

Thiol groups are considered to be the dominant determinant of Total Antioxidant Capacity (TAC) (Balcerczyk et al., 2003). Thiols are essential and strong antioxidant molecules in the sulfhydryl group. The disulfide bond in their structure plays an important role in protecting against oxidant stress from harmful effects. Low serum NT, TT, and dynamic disulfide levels in humans were associated with prostatitis and prostate cancer (Solakhan et al., 2019). However, no studies have ever explained the relationship between TT values and health in farm animals, nor has any study been conducted on the effect of probiotics on PON 1 and TT values. The effect of probiotics on serum PON 1 and TT levels was examined for the first time in the present study, and the results obtained are remarkable. Although being limited, both probiotics were observed to support the body's antioxidative defence mechanism against oxidative stress ($P>0.05$). On the other hand, probiotics caused significant changes in serum PON 1 and TT values. While Lactobacterin-TK[2] increased PON 1 values on day 28, both probiotics had a positive effect on PON 1 values at the weaning age. Examining the significantly different TT levels, it was determined that Lactobacterin-TK[2] caused an unwanted decrease on day 28. It was seen that Hypro created an effect comparable to G1 and G2. It was determined that TT levels were higher in the groups that received probiotic preparations during the weaning age compared to those of the control; however, there were no statistically significant differences between the groups. The effect of probiotics to support the antioxidative defence mechanism can be attributed to their ability to stimulate the production of certain factors that capture ROS, chelate free radicals and inhibit their cytotoxic activities (Abdel-Moneim et al., 2020b).

Five major classes of antibodies have been identified in placental mammals: IgA, IgD, IgE, IgG, and IgM (Alberts et al., 2002). Of these, the most common in the bloodstream is IgG, which accounts for 75% of the serum antibodies. IgG levels are very high after birth due to passive transfer with colostrum, then decrease as the animal produces its own antibodies (Riddell et al., 2010). It has been reported that probiotics cause an increase in IgG level as an anti-spore immune response (Hong et al., 2005). On the contrary, there are some reports showing that probiotic supplementation in preruminant calves has no positive effect on immunoglobulins (Riddell et al., 2010; Roodposhti and Dabiri, 2012; Karamzadeh-Dehaghani et al., 2021). Our results showed an insignificant increase in the IgG concentration. IgA is an antibody that plays a role in the immune function of the mucus membranes, where IgA is produced together with the mucosal membrane represents up to 15% of the total immunoglobulins produced in the body. However, a large proportion of the intestinal IgA against cell wall antigens and proteins of commensal bacteria is specifically induced in response to the presence of these bacteria in the microbiota. The resident intestinal microbiota is of key importance in terms of the development of the intestinal immune system as demonstrated by their ability to modulate both innate and acquired immunity both at the local and at the systemic levels (Colitti et al., 2019; Macpherson et al., 2000). In addition, IgA con-

centrations decrease in diarrhea prevalence (Dock et al., 2004). Although probiotic supplementation has been reported to significantly increase the IgA levels (Dock et al., 2004), a non-significant difference in IgA level has been reported (Karamzadeh-Dehaghani et al., 2021). It has been reported that a 2 g dose of probiotic supplemented with calves' milk significantly increased the IgA and IgG concentrations (Wu et al., 2021). In the present study, probiotic supplementation produced a non-significant increase in the IgA levels. This may be related to factors such as the level, concentration and type of the probiotic used. The tendency of both probiotics used in the present study to increase the IgA and IgG concentrations showed that it can be effective in strengthening the immune response during the suckling period. The increase in serum immunoglobulin levels with probiotic administration may be due to the stimulating effect of the intestinal flora in the development of natural antibodies. It has been known that the use of probiotics is of great importance in the development of immunity and increases the formation of its own antibodies produced by the immune system of the calf, which have important roles in the defence against pathogenic microorganisms. This shows that the commensal microorganisms in the gastrointestinal tract are in close contact with cells of the immune system through the interaction between host cells and beneficial bacteria (Abdel-Moneim et al., 2020b).

CONCLUSIONS

The results of this study demonstrated that both probiotics supplemented during the suckling period caused significant improvements in live weight gain, in chest girth of calves, and total and daily feed consumption, and the feed conversion ratio was not affected. Also, probiotics reduced the number of days with diarrhoea. Probiotics did not suppress the growth of pathogenic bacteria in the intestinal flora; however, albeit non-significantly, they increased the growth of lactobacilli. The probiotics used in the study did not have a negative effect on the kidney and liver functions of the calves. The administered probiotics showed a slight improvement in the immune response of the calves. PON1 increased significantly. There was a non-significant improvement in other antioxidative defence mechanism markers. For these reasons, it was concluded that both Lactobacterin-TK[2] and Hypro probiotics can be supplemented in the milk of calves to provide better growth during the suckling period and to raise healthy calves by reducing the effect of oxidative stress.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Authors' contributions

Serkan Özkaya and Aynur Gül Karahan contributed to the conceptual aspects and design of the study. Material preparation, data collection, and analysis were carried out by all of the authors. The first draft of the manuscript was written by Serkan Özkaya and Aynur Gül Karahan, and all the authors commented on the previous versions of the manuscript. All the authors read and approved the final manuscript.

Data availability

The datasets in this study are available from the corresponding author on reasonable request. All data and materials are available for publication.

Compliance with ethical standards

The present research complies with the regulation on the protection of animals used for scientific purposes of the Republic of Türkiye, in compliance with the European Union Legislation for the protection of animals used for scientific purposes and Directive 2010/63/EU for animal experiments. The study Local Committee on Animal Research Ethics of the Suleyman Demirel University, Isparta, Türkiye has provided approval for the research (Protocol number: 2018.10.001).

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