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Influence of Immunobeta® and Silymarin preparations on weight development and some indicators of oxidative stress in Lacaune lambs

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Abstract

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Since 2006, the European Community has banned the use of food antibiotics as growth promoters in animal husbandry. The use of modern systems to produce animal products with a high degree of concentration and automation, has led to a deterioration of the health status of sensitive breeds and hybrids. The weight development of lambs of the Lacaune breed was studied for seven months. The immunomodulator Immunobeta® and the hepatoprotector Silymarin were found to improve growth reliably, increase iron-reducing plasma capacity (FRAP), and reliably lower malondialdehyde MDA and oxidative stress index (OSI) concentrations in lambs, both through thermoneutral and during a hot summer period.

Keywords: lambs; Immunobeta; Silymarin; oxidative stress

Introduction

The problem of the well-being of sheep in pasture and pasture-manure cultivation, as well as in their prevention and treatment, is becoming more and more relevant. During the grazing of sheep and lambs during the different seasons, several stress factors significantly worsen their well-being – high and low temperatures, excessive solar radiation, various endo- and ectoparasites, pasture infections, etc. On the other hand, in recent years, the European Commission has implemented a policy of stimulating the grazing of farm animals, including sheep. Subsequently, since 2014, several regulations were adopted in the Republic of Bulgaria for the financing and stimulation of pasture livestock breeding under the Program for the Development of Rural Areas. Due to the excessive use of antibiotics in livestock farming, including sheep farming, and due to the rapidly spreading antibiotic resistance of microorganisms, alternative methods are being sought to reduce the use of antibiotics and focus on stimulating growth and natural immunity, reducing oxidative stress

in lambs and sheep in pasture cultivation. On the other hand – since 2006, the European Commission imposed a complete ban on the use of nutritional antibiotics as growth stimulants in animal husbandry. The rumen microbiota is of paramount importance for digestive efficiency in ruminants, as microbial fermentation supplies the host with essential sources of energy and nitrogen. Saccharomyces cerevisiae is a stimulator of digestive functions in the rumen of sheep and lambs, as well as cellulolytic bacteria that break down plant cell walls in the rumen (Chaucheyras-Durand and Fonty, 2008). Chaucheyras et al. (1995) found that the addition of live yeast cells stimulated the use of hydrogen, by two hydrogenotrophic ruminal microorganisms and enhanced acetogenesis (Chaucheyras et al., 1995). Furthermore, Chaucheyras et al. (1995) reported that the addition of a live yeast strain of *Saccharomyces cerevisiae* to the anaerobic rumen fungus *Neocallimastix frontalis MCH3* stimulated fungal zoospore germination, increased cellulose degradation, and the production of hydrogen, formate, lactate, and acetate. The results indicated that the yeast could enhance the colonization of *N. frontalis*.

In addition, Silberberg et al. (2013) reported that *Saccharomyces cerevisiae* products can regulate the rumen content environment in sheep, and thus can be used to prevent acute rumen lactic acidosis. Furthermore, according to Jia et al. (2018) and Chaucheyras-Durand et al. (2019), live yeast supplementation induces the proliferation of several bacterial species important for rumen function, including eukaryotic families Trichostomatia and Neocallimastigaceae. Therefore, yeast may be a good way to optimize rumen microbiota and microbial degradation of lignocellulosic materials in sheep. Another way to stimulate the productivity of ruminants is the addition of Silymarin to the feed. The stimulating effect of Silymarin on the live mass of lambs is due to its strong antioxidant and hepatoprotective action and its antitoxic effect. Kim et al. (2013) found that Silymarin supplementation helped increase calves' growth compared to the control group and decrease the activity of alanine aminotransferase (an important liver enzyme). The potent antioxidant effect of Silymarin has been associated with a reduction of lipid peroxidation and MDA levels in blood and tissues, and thus, a reduction of high levels of free radicals, lipid peroxidation, and protein carbonylation (Stone et al., 2010). In addition, in a QRT-PCR analysis, Khamisabadi (2020) found that Silymarin reduced HSP 70 gene expression in sheep blood serum (reducing HSP 70 mRNA levels). Therefore, silymarin supplementation may reduce oxidative stress and heat shock protein activity and improve sheep's welfare under severe and prolonged stress. Finally, the stimulating effect of Silymarin on the growth of lambs is due to the strong antitoxic activity of Silymarin (Karvellas et al., 2016; Sahin et al., 2018; Pickova et al., 2020; Fanoudi et al., 2020). Through the mentioned mechanisms, Silymarin can be used as an effective, harmless food supplement, as an alternative to nutritional antibiotics, stimulating productivity in ruminants.

Considering the independent effect of Immunobeta and Silymarin, we set ourselves the goal of investigating their joint effect on the weight development and some indicators of oxidative stress of lambs of the Lacaune breed.

Material and Methods

The experiment was carried out in 2022, with 18 1-month-old female lambs of the French Lacaune breed, raised on manure and pasture in a private sheep farm. The farm is located 15 km south of Stara Zagora. The area is located in the transitional Mediterranean climate region, characterized by warm and dry summers and mild winters. The lambs were divided into three groups – control and two experimental groups. Lambs were fed with compound feed for lambs: maize, wheat, wheat bran, sunflower meal, soybean meal, chalk, and supplementary feed BK 2310 (Melhran, Ltd, Stara Zagora, Bulgaria). The Analytical composition per 1 kg of compound feed is presented in Table 1. To the feed of experimental animals, after 15 days of age, the following supplements were added to the concentrated feed: the immunomodulator Immunobeta®, in a dose of 4 g/kg feed (in the lambs, treated with Immunobeta), and the hepatoprotector Silymarin, in a dose of 2 g/kg feed (in the lambs, treated with Silymarin). Each group of animals was raised in group boxes, with an area of 2 m^2 provided per animal, with a norm of 0.7–1 m^2 (Regulation No. 44/2006), located under a massive shed fenced off on three sides. From the southern part of the shed, there are yards for walking the lambs, with an area of 2 m^2 per animal, with a norm of 1.2-2 m^2 (Regulation No. 44/2006). Feeders are located in the boxes and yards for walking, with a feeding front for one animal – 0.3 m (Ordinance No. 44/2006), and drinkers with free access to drinking water at a suitable temperature. The boxes are covered daily with dry straw that is free of mold and pathogenic microorganisms. The lambs are weaned at the age of 5 weeks with gradual acclimatization to the consumption of a standard concentrated feed, according to the age of the animals.

The immunostimulator Immunobeta® is manufactured by the Italian company for veterinary pharmaceuticals, Chemifarma. It is obtained from selected strains of yeast (*Saccharomyces cerevisiae*) by enzymatic autolysis and a natural extraction process of components from yeast cells. The immunomodulator contains three important active ingredients:

Table. 1. Analytical composition per 1 kg

Components	%
Crude protein	17.51
Crude fat	2.62
Crude fiber	5.66
Raw ash	7.40
Lysine	0.73
Magnesium	0.31
Methionine	0.26
Moisture max.	12.00
Calcium	1.32
Sodium	0.60
Phosphorus	0.51

beta-glucans – 30%, mannan oligosaccharides 25%, and nucleotides 5%.

Silymarin (Silybum) is an extract from the seeds of the milk thistle plant – *Silybum marianum* (L.). Gaertn. The product used in the experiment was manufactured by the Chinese company Wuqiao West Road Wuxi Jiangsu, China.

The live weight of the lambs was determined by the weight method by individual measurement with an accuracy of 0.001 kg every month until 7 months of age.

Blood samples (6 ml) for the study of oxidative stress – FRAP, MDA, and OSI were taken during a thermoneutral period (May 21, 2022), during a hot summer period (July 23, 2022), from the jugular vein (*v. jugularis*) of six lambs from each group in sterile vacuum containers with anticoagulant.

Oxidative stress index

OSI was determined by the method of Armstrong and Browne (1994), and Benzie and Strain (1996).

Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation assay is based on the formation of a 1:2 red adduct between malondialdehyde (MDA), and 2-thiobarbituric acid in acid medium that is quantitated at 532 nm after extraction with n-butanol (Uchiyama and Michara, 1978; Andreeva et al., 1988). 1,1,3,3 tetraethoxypropane (Sigma Aldrich Chemie GmbH, Munich, Germany), and it was used as a malondialdehyde (MDA) standard.

Ferric reducing ability of plasma (FRAP) assay

The ferric reducing ability of plasma (FRAP, also ferric ion reducing antioxidant power), is an antioxidant capacity assay that uses Trolox as a standard. The FRAP assay was first performed by Benzie and Strain (1996) of the Human Nutrition Research Group at the University of Ulster, Coleraine. The method is based on the formation of an O-phenanthroline-Fe(2+) complex and its destruction in the presence of chelating agents. This assay is often used to measure the antioxidant capacity of foods, beverages, and nutritional supplements containing polyphenols. At low pH, the reduction of a ferric tripyridyltriazine (FeI – TPTZ) complex to a ferrous form results in the production of an intense blue color. The change in absorption, therefore, is directly related to the amount of electron-donating antioxidants present in the reaction mixture. FRAP values in mmoL/L are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Seven concentrations of aqueous solutions of $FeSO_4 \times 7H_2O$ in the range of 100 – 1000 mmoL/L were used for calibration (Benzie and Strain, 1996).

Results

The data on the live weight of the lambs are presented in Table 2. It is noticed that the weight development of the lambs is influenced by three factors – age, the immunomodulator Immunobeta and the hepatoprotector Silymarin. Age had a significant effect on lamb weight from 1 to 7 months ($P < 0.001$) in all three groups of animals. One-month-old lambs in all three groups had the same live weight, but with increasing age, the differences between the control group and the Immunobeta and Silymarin-treated groups increased. At the age of 4 and 6 months of the lambs, a significant difference in live weight was found between the control and experimental groups ($P < 0.05$; $P < 0.01$). At the end of the experimental period, the lambs treated with Immunobeta and Silymarin had a higher live weight (by 12% and 9%, respectively), but the differences compared to the control group were not reliable. However, the tendency for a positive effect of both supplements on the live weight of lambs is highlighted. At the age of 7 months, the influence of Immunobeta on the weight development of lambs was about 4% better compared to Silymarin, but this result was also not reliable.

In Table. 3, the concentrations of FRAP, MDA, and OSI in the lambs during the thermoneutral and hot periods are presented. It was found that in both periods, FRAP values were significantly higher in lambs from the experimental groups, compared to the control group – during the thermoneutral period ($P < 0.05$; $P < 0.05$), as well as during the hot summer period ($P < 0.01$; $P < 0.01$). During the thermoneutral period, the concentrations of MDA in the lambs that received Immunobeta and Silymarin supplements significantly decreased, compared to the control lambs ($P < 0.05$; $P < 0.001$). This regularity is observed even more significantly during the hot summer period when the degree of confidence

Table 2. Influence of the immunomodulator Immunobeta and the hepatoprotector Silymarin on the live weight of lambs of the Lacaune breed (X±Sx, kg)

Age (months)	Control	Immunobeta	Silymarin
1	14.88 ± 0.60	14.73 ± 1.17	14.83 ± 0.85
2	19.59 ± 0.65	21.69 ± 0.83	21.92 ± 0.85
3	25.86 ± 0.71	28.54 ± 0.75	28.64 ± 1.13
4	27.92 ± 0.58	30.85 ± 1.48*	32.46 ± 1.30**
5	30.01 ± 0.62	33.42 ± 1.66	33.32 ± 1.07
6	32.09 ± 0.66	36.42 ± 2.01*	35.47 ± 0.69
7	34.15 ± 0.96***	39.10 ± 2.53***	37.58 ± 0.76***

* P < 0.05; ** P < 0.01

Table 3. Influence of the immunomodulator Immunobeta and the hepatoprotector Silymarin on FRAP, MDA, AND OSI in Lacaune lambs during the thermoneutral and hot period (X±Sx)

Period	Control	Immunobeta	Silymarin
Termoneutral			
FRAP, mmol/l	0.309 ± 0.012	0.358 ± 0.022*	0.333 ± 0.08*
MDA, mmol/l	11.01 ± 0.21***	8.94 ± 0.35	9.18 ± 0.37
OSI	3.59 ± 0.17*	2.54 ± 0.18	2.76 ± 0.14
Hot			
FRAP, mmol/l	0.321 ± 0.015	0.381 ± 0.012**	0.413 ± 0.017**
MDA, mmol/l	9.31 ± 0.19**	8.02 ± 0.23	8.82 ± 0.16
OSI	2.94 ± 0.17**	2.11 ± 0.10	2.16 ± 0.10

* P < 0.05; ** P < 0.01; *** P< 0.001

in MDA concentrations between control and experimental animals becomes very high (P < 0.001; P < 0.001). A similar regularity was established for OSI, as during the thermoneutral period (P < 0.01; P < 0.01), and during the hot summer period (P < 0.001; P < 0.001), which was due to the influence of the studied supplements.

Discussion

What are the mechanisms responsible for the positive effect of *Saccharomyces cerevisiae* and its active products to stimulate animal productivity? The positive action of yeast can be considered in several directions – improvement of the rumen microbiome, antiparasitic action of yeast against the gastrointestinal parasite *Haemonchus contortus* in sheep, and stimulation of immunity. *Saccharomyces cerevisiae* is a stimulator of digestive functions in the rumen of sheep and lambs, as well as cellulolytic bacteria that break down plant cell walls in the rumen and thus stimulate the rumen microbiota, microbial fermentation and supply the host with essential sources of energy and nitrogen. According to research by Fiems et al. (1993), yeast supplementation increased the ratio of acetate: propionate, butyrate, isoacids, pH, and ammonia. These results indicate that the effect of *Saccharomyces cerevisiae* yeast culture on rumen fermentation depends on the nature of the diet. Additionally, yeast cells survive passage through the digestive tract. Chaucheyras et al. (1995) investigated the effects of a live strain of *Saccharomyces cerevisiae* on the utilization of hydrogen and the production of acetate and methane by two hydrogenotrophic rumen microorganisms – an acetogenic bacterial strain and a methanogenic archaea. The addition of yeast cells increased more than fivefold the hydrogenotrophic metabolism of the acetogenic strain and its acetate production. In the absence of yeast and co-culture of acetogen and methanogen, hydrogen is mainly used for methane synthesis, but the presence of live yeast cells stimulates hydrogen utilization by the acetogenic strain and enhances acetogenesis (Chaucheyras et al., 1995). Chaucheyras et al. (1995) investigated the effects of a live yeast strain of *Saccharomyces cerevisiae* on zoospore germination, metabolism, and cellulolytic activity of the anaerobic fungus from the rumen of *Neocallimastix frontalis MCH3*. The addition of yeast cells to a vitamin-deficient medium stimulates the germination of fungal zoospores, increases cellulose degradation, and production of hydrogen, formate, lactate, and acetate. Yeast supplementation provides vitamins such as thiamine, which is of essential importance for fungal growth and activity. Therefore, yeast may be a good way to optimize the microbial degradation of lignocellulosic materials in the rumen of sheep. Chaucheyras-Durand and Fonty (2008) investigated the effects of a yeast supplement used in feeding lambs on cellulolytic bacteria for the degradation of plant cell walls and digestive functions in the rumen of animals reared with gnotobionts. In the treated lambs, the specific activities of the fibrinolytic enzymes were greater. The presence of *Saccharomyces cerevisiae* reduces the concentration of ammonia in the rumen and increases the concentration of volatile fatty acids (VFA) when the lambs are aged from 20 to 50 days. These data confirm that the yeast strain can stimulate the development of cellulolytic microflora and enhance microbial activity in the rumen of young lambs. Such activity could be beneficial in preventing microbial imbalance and normalizing rumen function in case of nutritional transitions. On the other hand, *Saccharomyces cerevisiae* products can regulate the rumen environment in sheep and thus be used to prevent acute rumen lactic acidosis (ARLA), a major nutritional and metabolic disorder in ruminants. In yeast-treated sheep, lower L-lactate values were found. The use of yeast culture can be beneficial in the prevention and treatment of ARLA in

sheep, because it stabilizes the fermentation parameters, increases the number of protozoa reduces lactate-producing bacteria, and can effectively reduce the accumulation of lactic acid, increase rumen pH and reduce the osmolality of the rumen contents (Silberberg et al., 2013; Reis et al., 2018). In confirmation, Jia et al. (2018) found the positive influence of *Saccharomyces* on fermentation and microbial diversity in the rumen of fattening lambs. Chaucheyras-Durand (2019) investigated the effect of the live yeast *Saccharomyces cerevisiae CNCM I-1077* on lambs, separated from their mothers at 12 hours of age, and artificially fed milk replacer and starter from the eighth day. Live yeast supplementation induced the proliferation of several bacterial species important for rumen function – eukaryotic families *Trichostomatia* and *Neocallimastigaceae*. Lambs that received live yeast had higher amounts of *Fibrobacter succinogenes* after weaning. This yeast combination improves microbial colonization in the developing rumen. Its influence on the microbiome for efficient fiber breakdown is particularly favorable, suggesting a positive effect of *Saccharomyces cerevisiae* on the rumen development of lambs and digestive efficiency. The use of live yeast *Saccharomyces cerevisiae* is one way to develop alternative methods to minimize losses caused by the gastrointestinal nematode *Haemonchus contortus*. Such studies are essential given the high morbidity rates in sheep and significant mortality rates in lambs associated with the low effectiveness of commercial products to control this parasite. Hooper et al. (2014) conducted experiments with sheep fed a supplement containing *Saccharomyces cerevisiae*. The results show that dietary yeast supplementation can increase resistance in sheep to the natural gastrointestinal parasite *H. contortus*. The indicated data unequivocally explain the achieved reliable difference in live weight between the control Lacaune lambs and those that received Immunobeta® at a dose of 4g/kg feed from the 15th day to the 75th day of the lambs. On the other hand, the stimulating effect of Silymarin on the growth of lambs could be explained by its strong antioxidant, hepatoprotective, and antitoxic actions. Data on the stimulating effect of Silymarin on ruminant productivity were presented by Kim et al. (2013), who investigated the effect of Silymarin on blood metabolites and carcass characteristics during the late feeding period in castrated male Hanu cattle. After 6 months of dietary supplementation, the author found a decrease in the activity of the liver enzyme – alanine aminotransferase and an increase in the live weight of the calves in the group supplemented with Silymarin compared to the control group. Thus, Silymarin can be used as an alternative to nutritional antibiotics, stimulating productivity during the late feeding stage of male castrated cattle. The positive effect of Silymarin is due to its strong antioxidant action, which is due to the reduction of lipid peroxidation and MDA levels in blood and tissues, as found in our research. Thus, high levels of free radicals, lipid peroxidation, and protein carbonylation are reduced, and DNA strand damage and pathology in the body are protected (Stone et al., 2010). In addition, in a QRT-PCR analysis, Khamisabadi (2020) found that Silymarin reduced HSP 70 gene expression in sheep blood serum (reducing HSP 70 mRNA levels). Therefore, Silymarin supplementation may reduce oxidative stress and heat shock protein activity and improve sheep's welfare under severe and prolonged stress. Additional growth promotion data in rabbits and pigs are presented by Kosina et al. (2017) and Grela et al. (2020). The significantly higher live weight of the lambs in the group treated with Silymarin could also be related to the strong antitoxic activity of the hepatoprotector. One of the most common uses of milk thistle – *S. marianum* and its main component – Silymarin is their detoxifying function in intoxications with various toxic agents: biological and chemical toxins (Karvellas et al., 2016; Sahin et al., 2018; Pickova et al., 2020; Fanoudi et al., 2020). Silymarin can be used as an antidote or protective agent against biological poisonous agents – snake and scorpion venom, bacterial toxins, and xenobiotic mycotoxins (Fanoudi et al., 2020; Pickova et al., 2020). Fanoudi et al. (2020) found that the main protective effects of Silymarin are due to several main mechanisms – lysing free radicals (antioxidant), chelating, possessing antiapoptotic properties, and regulating inflammatory reactions. The stimulating effect of Silymarin on the live mass of lambs could also be due to its antitoxic effect in poisonings resulting from parasitosis in sheep. Oelrichs (1982), Thamsborg et al. (1996), and Atanassof (2016) found that Silymarin can be used successfully in sheep parasitosis, caused by the larval forms of *Arge pullata*, occurring with massive necrosis of the liver and degeneration of renal tubules. Ruminants ingest the larval forms of *Arge pullata* or *Lophyrotoma interrupta*, resulting in intoxication caused by toxic substances (octapeptide lophyrotomin). These are substances structurally like the toxic cyclic peptides in *Amanita mushrooms*. Silymarin treatment can be successfully applied as an antitoxic agent in sheep (Atanassof, 2016; Oelrichs, 1982; Thamsborg et al., 1996; Urbanczyk et al., 2002). The protective effect of Silymarin against the toxic effects of several mycotoxins is particularly relevant (Alhidary et al., 2017). As a result of the combination of the antioxidant, hepatoprotective action, and antitoxic action of Silymarin, its stimulating effect on the growth of lambs is determined.

Conclusions

Based on the results obtained by us and the authors cited above, it can be concluded that the immunomodulator Immunobeta and Silymarin have a positive effect on the weight development of lambs, on an increase in FRAP and a reliable decrease in MDA, and OSI in lambs, during a thermoneutral and a hot period.

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