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Influence of the addition of the prebiotic *Immunobeta* and its combination with the probiotic *Zoovit* on the development of the digestive system and the microbiological analysis of rumen and intestinal contents in Ile de France lambs

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Abstract: The aim of the study was to determine the effect of taking the prebiotic *Immunobeta* and a combination of the prebiotic *Immunobeta* + probiotic *Zoovit*, on the development of the digestive tract and the microbial composition in lambs from Ile de France. The object of the study were 45 lambs of the Ile de France breed, divided into three groups. The first experimental group of lambs received 8 g of the prebiotic *Immunobeta*, and those from the second experimental group the same amount of prebiotic + 4 g of the probiotic *Zoovit*. At the end of the experiment, 5 male lambs from each group were slaughtered. The volume of the rumen, the length of the papillae and the intestines were determined, and microbiological studies were performed. An increase in the small intestine was found in the lambs from I and II experimental groups, in the large intestine in animals from II and the control, as well as a greater capacity of the rumen in the lambs from II experimental group compared to the control (P>0.05). The length of the rumen papillae in lambs from experimental groups I and II was greater than in the control (P>0.05). In the lactic acid bacteria in the rumen, we found a decrease in the group receiving the synbiotic compared to the first experimental and control groups, and in the small and large intestines it was the lowest in the group receiving *Immunobeta* compared to the other two groups.

Keywords: Ile-de-France lambs; prebiotics; probiotics; *Immunobeta; Zoovit*; rumen contents and intestinal contents

INTRODUCTION

Maintaining and balancing the microflora in the intestines and rumen has a great impact on the health status and growth characteristics of lambs and sheep. This has been mentioned by many authors in their experiments (Yeoman and White, 2014; Xiao et al., 2016; Li et al., 2017; Mani et al., 2021). The normal development of the gastrointestinal tract is important for the absorption of nutrients, as well as for the development of immunity in animals (Celi et al., 2017). The intestinal microbiota of the body is a complex community that has an important role in health, metabolism and immunity (Hu et al., 2024). Probiotics are used as a safe supplement to restore the balance of the gastrointestinal system and protect the body from diseases. They increase the population of bacteria in the rumen (mainly from the genera *Ruminococcus, Succiniclasticum and Acidaminococcus*), which correlates positively with the concentrations of total volatile fatty acids and acetate in lambs (Mao et al., 2023), and those containing *B. amyloliquefaciens and B. subtilis* improve the development of the intestine and rumen in animals with delayed growth (Renjia et al., 2018). In a study with calves, Xiao et al. (2016) found an

improvement in rumen morphology after feeding with a supplement of *Saccharomyces cerevisiae*, which is likely due to stimulation of the microbial population.

According to Du et al. (2018), probiotic supplementation (mainly Bacillus spp.), has been shown to stabilize the gut microbiota and promote growth in stunted calves. According to Chapman et al. (2011), probiotic supplements containing multiple strains are more effective than single-strain supplements. Yeast (*S. cerevisiae*) can influence rumen microbiota dynamics and nutrient degradation (Mohammed et al., 2018; Doyle et al., 2019), stimulating the growth of lactic acid bacteria (Khan et al., 2016), and the combination of *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* stimulates growth in goats (Jinturkar et al., 2009). According to Seo et al. (2010), lactic acid bacteria and yeast-based supplements have beneficial effects on the intestinal tract and rumen of ruminants.

The rumen mucosa is made up of papillae that efficiently absorb water and nutrients necessary for proper growth and development (Graham and Simmons, 2005). The longer and wider the papillae, the more active the absorption of ingested substances.

The combination of probiotics and prebiotics has a positive effect on digestion and intestinal fermentation in lambs fed high-energy diets. (Zapata et al., 2021). The addition of 12 g of inulin to milk replacer when feeding 12-week-old calves promotes the development of rumen papillae. When combining inulin and 0.25 g of an *E. faecium*-based probiotic, a significant increase in rumen papillae is achieved (Arne and Ilgaza, 2021) compared to the control group. Jonova et al. (2021) reported the positive effect of inulin and synbiotic, positively influencing the development of almost all morphological structures of the rumen and intestines in calves, and Costa et al. (2019) reported a highly reliable increase in the height of jejunal villi and rumen papillae in calves, when using 5 g/day of mannan-oligosaccharides.

Experiments have been conducted with the participation of probiotics based on *Saccharomyces cerevisiae*, as well as prebiotics (oligosaccharides, glycans), which have shown a greater length and width of the rumen papillae (Brewer, et al. 2014).

More evidence is needed to clarify the role of prebiotics, synbiotics, and their action in the gastrointestinal tract (Ford et al., 2014).

The aim of the present study was to determine the effect of the addition of the prebiotic *Immunobeta,* and a combination of the prebiotic *Immunobeta* + probiotic *Zoovit* on the development of the digestive tract, and the microbial composition in lambs from Ile de France.

MATERIAL AND METHODS

The experiment was conducted at the experimental base at the Agricultural Institute - Stara Zagora. It included 45 lambs of the Ile de France breed, divided into three groups - a control and two experimental - 15 lambs in each, formed by the method of analogues, maximally equalized in live weight at the beginning of the experiment, type of birth and sex.

During the experimental period, the lambs were raised in groups in boxes equipped with feeders for hay and concentrated feed and with drinkers with constant access to clean drinking water in accordance with the requirements of Ordinance No. 40, on the conditions for raising farm animals, taking into account their physiological and ethological characteristics. The animals were fed *ad libitum* (+ 5 to 10% residue) in a ratio corresponding to their age and meeting the requirements for nutritional and biologically active substances. The ration included concentrated feed and alfalfa hay (Table 1 and Table 2).

The combined feed contains 1.12 feed units, 2778.25 Kcal/kg and TDN 0.174.

The animals from the first experimental group received 8 g of the prebiotic *Immunobeta* individually once a day, and those from the second experimental group received the same amount of prebiotic with the addition of 4 g of the probiotic *Zoovit*.

The probiotic preparation *Zoovit* includes four strains of lactic acid bacteria: *Lactobacil-*

lus delbrueckii subsp. bulgaricus, Streptococcus salivarius subsp. termophilus, Lactobacillus acidophilus, Lactobacilus lactis and one strain of *Propinibacterium* (Table 3).

The *Immunobeta* supplement is a prebiotic preparation with a pronounced immunostimulating effect, obtained from certain strains of *Saccharomyces cerevisiae* yeast through a process of enzymatic autolysis (Table 4).

After the animals reached to 23-25 kg, 5 male lambs from each group were slaughtered. The animals were transported to a slaughterhouse in the region of Stara Zagora with a licensed vehicle. During transportation, all requirements of Ordinance No. 26 of February 28, 2006, on the conditions for the protection and humane treatment of animals during their transportation were met. Slaughter was carried out in accordance with the requirements of Ordinance No. 22 of December 14, 2005, on minimizing the suffering of animals during slaughter or killing. Before the slaughter itself, the feed and hay of the lambs were removed and the animals were placed on a 24-hour fasting diet, with only water provided for drinking. After slaughter and removal of their digestive system, the volume of the rumen, the length of the rumen papillae and the length of the small and large intestines were determined. Samples of intestinal and rumen contents were also taken for microbiological examination.

Microbiological analyses of the rumen and intestinal contents were performed at the Central Research Laboratory of Thracian University - Stara Zagora.

To determine the total number of microorganisms, including mesophilic aerobes and facultative anaerobes, the following standards were applied, according to BSS:

- Microbiology of the food chain. Horizontal method for the enumeration of microorganisms.

Table 1. Composition of compound feed for feeding lambs Ile de France

Component	% of input
Soybean meal	4,00
Chalk	3,00
Salt	0,50
Wheat	42,00
Premix-16-97-K	0,20
Sunflower meal	20,00
Corn	30,30

Table 2. Nutritional composition of compound feed

Component	% of input
Protein	15,90
Fats	2,40
Fibers	5,43
Moisture	11,40
Ca	1,20
P	0,50
Salt	0,570

Table 3. Chemical composition of the probiotic preparation *Zoovit*

Component	% of input
Protein	29,29
Lactose	52,14
Fats	0,95
Dry matter	94
Lactic acid	2,75
Propionic acid	3,10
Mineral substances	8,76
Number of active cells	not less than 2.5 x 107 cfu/g
Coliforms	are not established
Molds and yeasts	are not established
Salmonella in 25 g	are not established
Coagulase-positive staphylococci in 1 g	are not established

Table 4. Chemical composition of the prebiotic preparation *Immunobeta*

Component	% of input
β-glucans	30,00
Mananoligosaccharides	25,00
Nucleotides	7,00

Part 1: Colony count at 30 °C by the pour-over technique (ISO 4833-1:2013) according to BSS EN ISO 4833-1:2013;

- Microbiology of the food chain. Horizontal method for the enumeration of microorganisms. Part 2: Colony count at 30 °C by surface plating technique (ISO 4833-2:2013) for *Escherichia coli* according to BSS EN ISO 4833-2:2013;

- Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*. Part 2: Colony count technique at 44 °C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide according to BSS ISO 16649-2:2014;

- Microbiology of the food chain. Horizontal method for the detection and enumeration of *Enterobacteriaceae*. Part 2: Colony enumeration technique (ISO 21528-2:2017, corrected version of 2018-06-01) according to BSS EN ISO 21528-2:2017;

- Lactic acid microorganisms BSS ISO 15214 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of mesophilic lactic acid bacteria - Colony count technique at 30 °C 21;

- Specific microorganisms of yogurt BSS ISO 7889 Yogurt - Determination of the amount of characteristic microorganisms - Colony count method at 370 °C;

- Microbiology of the food chain. Horizontal method for the detection and enumeration of *Enterobacteriaceae*. Part 1: Detection of Enterobacteriaceae, ISO 21528-1:2017.

Statistical processing

The data were statistically processed with one-way analysis of variance (ANOVA), using the IBM SPSS Statistics 19 software program.

A check was performed for homogeneity of variances and normal distribution of the individual variables. The requirement for homogeneity of variances was checked with Levene's Test of Homogeneity of Variances, and the assumption of normal distribution of the data was checked with the Shapiro-Wilk test.

For variables with equal variances and normal distribution, a one-way parametric analysis of variance (ANOVA) was performed.

When rejecting the null hypothesis, the non-parametric analogue of the one-factor analysis of variance - the Kruskal-Wallis test - was performed.

RESULTS AND DISCUSSION

Table 5 presents the results of the development of the digestive system in Ile de France lambs in the control, I-st experimental and II-st experimental groups. In the indicator of the length of the large intestine, the differences between the three groups are minimal and statistically unproven at P>0.05. The group that received the synbiotic is distinguished by a longer length – 4.00 m, while in the I-st experimental group the length of the large intestine is almost identical to the control – 3.72 m, the difference being 4.57%.

Table 5. Length of the large intestine, small intestine and rumen volume in Ile de France lambs in the control, I-st experimental and II-nd experimental groups at the end of the experimental period

Parameters	Groups of animals						p-value
	Control group		I st experimental group - *IB*		II nd experimental group - *IB+Z*		
	$\bar{x}$±SD	CV	$\bar{x}$±SD	CV	$\bar{x}$±SD	CV	
Large intestine, m	3.72±0.24	6.45	3.55±0.38	10.70	4.00±0.40	10.00	> 0.05
Rumen , l	5.63±1.48	26.29	4.93±1.19	24.14	6.38±0.99	15.52	> 0.05

IB – Immunobeta, IB+Z- Immunobeta+Zoovit

We do not report statistical significance in the indicator rumen volume between the three groups. We establish a greater rumen capacity in the group that received the prebiotic *Immunobeta* + probiotic *Zoovit* compared to the other two groups.

Table 6 presents the results for the length of the small intestine and the length of the ileal papillae in Ile de France lambs in the control, Ist experimental and IInd experimental groups.

The Kruskal-Wallis test is a nonparametric alternative to the one-way analysis of variance for comparison of three or more groups. The test uses the calculated ranks of the observations with a comparison between the arithmetic mean values of the ranks for each group (Ganeva, 2016; Zulfiqar and Bala, 2016). When comparing the length of the small intestines and papillae based on the mean values of the ranks, it was found that there was no statistically significant difference (P>0.05) between the Ist, IInd experimental and control groups.

We found a higher average rank of 5.33 for the small intestine indicator in the first experimental group of animals that consumed the prebiotic preparation *Immunobeta*, compared to the control with an average rank of 4.00. Accordingly, in the first experimental group of animals, a greater length of the small intestine of 22.40 m was found, compared to the control of 21.60 m.

In the group that took the supplement of the combination of prebiotic *Immunobeta* + probiotic *Zoovit*, an average rank of 5.67 and a greater small intestine length of 23.83 m were found, compared to the first experimental and control groups.

Despite the average difference in the length of the small intestines in the three groups of animals, no statistical significance was recorded for the investigated parameter (Kruskal-Wallis test: Chi-Square=0.644, df=2; p> 0.05).

The rumen papillae index showed a mean rank of 5.17 and a length of 0.33 cm in the group receiving the prebiotic *Immunobeta* supplement and the second experimental group of animals consuming the synbiotic preparation (mean rank 6.00 and length of 0.33 cm), compared to the control group (mean rank 3.83 and papillae length of 0.27 cm). The differences between the mean values in the three groups are 22.22% and are mathematically unproven (Kruskal-Wallis test: Chi-Square=1.158, df=2; p> 0.05).

Jonova et al. (2021) in a study with calves, found a greater length of rumen papillae in the prebiotic and synbiotic group compared to the control. The length of the papillae was greater in calves that received a supplement of a fermentation product from S. Cerevisiae (Brewer et al., 2014). According to Moarrab et al. (2016) the use of synbiotic supplements promotes intestinal morphological characteristics in ruminants.

According to the company that produces the prebiotic *Immunobeta*, the mannan-oligosaccharides contained in the preparation help in the development of goblet cells and intestinal villi. Through nucleotides, the supplement promotes

Table 6. Length of the small intestine and rumen papillae in Ile de France lambs in the control, Ist experimental and IInd experimental groups at the end of the experimental period

Parameters	Groups of animals						Test statistics		
	Control group		Ist experimental group - IB		IInd experimental group - IB+Z				
	$\bar{x}$±SD	Mean Rank	$\bar{x}$±SD	Mean Rank	$\bar{x}$±SD	Mean Rank	Chi-Square	df	p-value
Small intestine, m	21.60±1.20	4.00	22.40±1.39	5.33	23.83±4.16	5.67	0.644	2	> 0.05
Rumen papillae, cm	0.27±0.06	3.83	0.33±0.15	5.17	0.33±0.06	6.00	1.158	2	> 0.05

IB – Immunobeta, IB+Z- Immunobeta+Zoovit

growth and increases the number of intestinal villi (http://chemifarma.it/multi/?lang=en).

The relatively narrow limits of variation of the studied indicators, length of the small intestine and length of the rumen papillae, compared to the average also determine the lack of reliability of the differences between the groups. With the results obtained in this way, a definitive conclusion cannot be made about the influence of the included additives in the feeding of Ile de France lambs.

We found a lighter color of the rumen papillae in lambs that consumed a synbiotic preparation, compared to the first experimental and control groups (Figures 1, 2 and 3).

Figure 1. Control group

Figure 2. Experimental group I - *Immunobeta*

Figure 3. Experimental group II - *Immunobeta* + *Zoovit*

Table 7 presents the results of the microbiological analysis of rumen and intestinal contents in Ile de France lambs in the control, I-st experimental and II-nd experimental groups. When isolating *Escherichia coli (E. coli)* (number of CFU/1 g) in the rumen, small and large intestines, as well as *Coliforms* (number of CFU/1 g) in the rumen and small intestines, a reduction was observed in the I-st and II-nd experimental groups, compared to the control. The group that received the prebiotic *Immunobeta* registered a higher content of Coliforms in the large intestines by several logarithmic units, compared to the II-nd experimental group and the control.

Table 7. Microbiological analysis of rumen and intestinal contents in Ile de France lambs in the control, I-st experimental and II-nd experimental groups at the end of the experimental period

Parameters	**Control group**					
	Rumen	**Measured unit**	**Small intestine**	**Measured unit**	**Large intestine**	**Measured unit**
E. coli, pcs/CFU/1 g	2,1	$_{x}$ 10^{6}	1,0	$_{x}$ 10^{5}	6,1	$_{x}$ 10^{5}
	3,5	x 10^{4}	8,0	x 10^{4}	6,1	x 10^{5}
Coliforms, pcs/CFU/1 g	1,0	x 10^{5}	3,0	x 10^{5}	1,0	x 10^{3}
	2,0	x 10^{4}	7,6	x 10^{4}	1,5	x 10^{4}
Lactic acid bacteria, pcs/CFU/1 g	2,2	x 10^{4}	4,0	x 10^{3}	3,6	x 10^{4}
	1,2	x 10^{4}	2,2	x 10^{4}	8,1	x 10^{4}
Microbial count at 37 °C	2,5	x 10^{6}	<10^{5}		1,1	x 10^{6}
	>1	x 10^{5}	>1	x 10^{5}	1,0	x 10^{7}
1st experimental group - *IB*						
	Rumen	**Measured unit**	**Small intestine**	**Measured unit**	**Large intestine**	**Measured unit**
E. coli, pcs/CFU/1 g	1,0	x 10^{3}	3,1	$_{x}$ 10^{3}	2,4	x 10^{5}
	2,2	x 10^{2}	8,0	x 10^{3}	1,7	x 10^{4}
Coliforms, pcs/CFU/1 g	7,0	x 10^{3}	<10^{3}		2,0	x 10^{5}
	1,4	x 10^{4}	2,0	x 10^{3}	1,7	x 10^{4}
Lactic acid bacteria, pcs/CFU/1 g	1,1	x 10^{4}	5,0	x 10^{3}	3,05	x 10^{3}
	1,18	x 10^{4}	4,8	x 10^{3}	3,05	x 10^{3}
Microbial count at 37 °C	5,2	x 10^{6}	5,0	x 10^{5}	1,0	x 10^{6}
	6,0	x 10^{5}	1,3	x 10^{6}	5,0	x 10^{5}
2nd experimental group - *IB+Z*						
	Rumen	**Measured unit**	**Small intestine**	**Measured unit**	**Large intestine**	**Measured unit**
E. coli, pcs/CFU/1 g	1,7	x 10^{2}	8,4	x 10^{3}	9,4	x 10^{4}
	6,0	x 10^{3}	6,0	x 10^{3}	1,7	x 10^{5}
Coliforms, pcs/CFU/1 g	9,0	x 10^{3}	1,5	x 10^{2}	6,0	x 10^{3}
	6,0	x 10^{2}	9,0	x 10^{2}	9,0	x 10^{3}
Lactic acid bacteria, pcs/CFU/1 g	3,2	x 10^{3}	2,6	x 10^{3}	1,4	x 10^{4}
	4,0	x 10^{3}	5,6	x 10^{3}	1,5	x 10^{4}
Microbial count at 37 °C	1,1	x 10^{5}	1,4	x 10^{5}	2,0	x 10^{6}
	6,5	x 10^{4}	4,0	x 10^{4}	4,2	x 10^{5}

Regarding lactic acid bacteria in the rumen (CFU/1 g), a reduction was observed in the group that received a synbiotic supplement compared to the first experimental and control groups, with the difference being 1 logarithmic unit. It is noteworthy that the difference in the content of lactic acid bacteria between the first and second experimental groups was 1 logarithmic unit, and between the first experimental and control groups, it was almost identical. Lactic acid bacteria of the genus *Lactobacillus* participate in the synthesis of acetates, propionates, butyrates and other substances. The increased synthesis of these products is probably related to changes in the lactic acid microbial population in the rumen of ruminants. Changes in the microbial composition and increased production of acetate and propionate in rumen content, after taking a probiotic in combination with a biosubstance in dairy cows, were reported by Park et al. (2024).

The content of lactic acid bacteria (number of CFU/1 g) in the small and large intestines was lowest in the group that took the prebiotic preparation *Immunobeta* compared to the other two groups - control and experimental group II.

The microbial count of the rumen at 37 °C was highest in the first experimental group and lowest in the second experimental group. In the contents of the small intestine, the indicator was lower in the control and second experimental groups compared to the first. The studied indicator in the large intestine registered the highest level in the control group, with the difference between the group that received the prebiotic *Immunobeta* and the one that consumed *Immunobeta* + *Zoovit* being minimal.

The results obtained during the experiment provide grounds for continuing and deepening research to establish the effect of probiotic and synbiotic supplements on the development of the digestive system, rumen, and intestinal microbiome in lambs and sheep.

CONCLUSION

• An increase in the length of the small intestine was registered in lambs from I and II experimental groups, of the large intestine in II and control groups, as well as a larger volume of the rumen in lambs from II experimental group compared to the control. A larger length of the rumen papillae was established in lambs from I and II experimental groups compared to the control. The differences are not significant (P>0.05)

• A decrease in E. coli (number of CFU/1 g) in the rumen, small and large intestines, as well as *Coliforms* (number of CFU/1 g) in the rumen and small intestines was observed in experimental groups I and II compared to the control. A lower content of lactic acid bacteria in the rumen (CFU/1 g) was reported in the group, receiving the synbiotic supplement, compared to the I experimental and control groups, and in the small and large intestines in the group, receiving the prebiotic preparation *Immunobeta,* compared to the other two groups.

• The microbial count of the rumen at 37 °C registered the highest level in the I experimental group and the lowest in the II experimental group. The microbial count in the small intestine had lower values in the control and II experimental groups, compared to the I experimental group.

• The results obtained from the conducted experiment provide a basis for continuing and deepening the research to establish the effect of prebiotic and synbiotic supplements on the microflora of the rumen and intestines of lambs.

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