VETERINARSKI ARHIV

Editor-in-Chief: Velimir Sušić

ISO 9001 BUREAU VERITAS Certification

Faculty of Veterinary Medicine
University of Zagreb

No of paper: VA.1797

Re: Acceptance of manuscript for publication

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Dear Assoc. Prof. Daniel Špoljarić, PhD, DVM

I am pleased to be able to inform you that the manuscript you submitted for publication entitled **Influence of dietary white button mushrooms (*Agaricus bisporus*) on kinetic of changes in proportion of peripheral blood CD4+CD8+ T lymphocytes in lambs** by Branimira Špoljarić, Ana Shek Vugrovečki, Damir Mihelić, Ivona Žura Žaja, Silvijo Vince, Daniel Špoljarić, Mario Živković, Marijana Mirjana Kardum Paro, Ksenija Vlahović, Marko Samardžija, Nada Vijtiuk and Maja Popović has been accepted for publication in ***Veterinarski arhiv***, after you have made all corrections advised by the peer reviewers.

The manuscript will be published in due course.

Yours sincerely,

Velimir Sušić, Editor-in-Chief

Signature: .....Date: January 24th 2022

Influence of dietary white button mushrooms (*Agaricus bisporus*) on the kinetics of changes in the proportion of peripheral blood $CD4^+CD8^+$ T lymphocytes in lambs

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Running title: B. Špoljarić et al.: Dietary effect of WBM on DP T cells kinetics in lambs

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Abstract

The primary focus of the current study was to determine the potential benefits of supplementing sheep diet with white button mushrooms (WBM) in terms of growth, health and the kinetics of systemic $CD4^+CD8^+$ memory T lymphocytes in lambs. Forty-five female lambs (Lika breed) were divided into three groups: A - control group fed on free-range pasture during 222 days of the experiment, while groups B and C were housed in a separate facility for 42 days and fed either commercial feed mixture (FM) or FM supplemented with 15% of freshly prepared WBM, respectively, and *ad libitum* forage. For the remaining 180 days of the experiment, both groups (B and C) of lambs were kept free-range and fed pasture only. The lambs were monitored daily starting at Day 0 (or 90 days of age) before the treatments, weighed and blood sampled on Days 0, 21, 42 and 222, and were clinically observed for incidence/severity of diarrhea and/or other signs of disease. In addition to morbidity, mortality was monitored too, and dead lambs were examined for gross pathology changes.

The lambs fed FM supplemented with WBM (group C) had significantly higher body weight gain ($P< 0.05$) at Days 42 and 222. They were neither diarrheic nor had any mortality cases

throughout the experiment. Also, these lambs had a significantly increased ($P< 0.05$) proportion of $CD4^+$ $CD8^+$ T cells at Days 42 and 222. The obtained data supported our assumption on the efficacy of dietary WBM in immunostimulation of $CD4^+CD8^+$ memory T lymphocytes in lambs, resulting in protection against on-farm diarrhea and increased growth rate.

Key words: immunostimulation, double-positive T cells, growth, diarrhea, sheep

Introduction

The white button mushroom (WBM) *Agaricus bisporus* has been recognized for a long time as an important foodstuff due to its nutritional values and remedy properties. The WBM is a significant source of proteins, fibers, essential and semi-essential amino acids as well as antioxidative agents like sterols, phenolic and indolic compounds, ergothioneine, vitamins and selenium (GOLAK-SIWULSKA et al., 2018.). Moreover, it is well known that WBM comprises numerous bioactive substances such as polysaccharides, lipopolysaccharides, peptides, glycoproteins, nucleosides, triterpenoids, lectins, fatty acids and their derivatives which may induce anti-inflammatory, antiviral, antifungal, antibacterial, hepatoprotective, antidiabetic, hypolipemic, antithrombotic, hypotensive and symbiotic effects generally described as indirect probiotic or direct prebiotic activities (FERRÃO et al., 2019.). These data and those from other studies favor WBM as a beneficial and acceptable nutrient, which has, logically, stimulated further research into its applicability as a functional diet or dietary supplement, not just for humans but also for domestic food animals. However, scientific articles dealing with the *in vivo* effects of WBM prevalently describe outcomes obtained for humans and monogastric domestic animals. By reviewing recently published studies on the medical and nutritional properties of *A. bisporus*, it has been established that WBM exhibit immunomodulatory effects by stimulating the activation of NK cells, maturation and functioning of dendritic cells, increased secretion of cytokines TNFα, IFN-γ and IL-2, and IgA production, *i.e*. redirection of Th-2 humoral immune response to Th-1 cellular immune response (ATILA et al., 2017.). Such effects could be ascribed to bioactive β-1,3-/1,6-glucans and proteoglycans which are ligands for CD11b/18 (complement receptor 3, CR3), dectin-1 and toll-like receptor-2 (TLR-2) on monocytes, dendritic cells, granulocytes and NK cells, cellular components of the innate immune system, as reported for a related species of mushrooms, *Agaricus blazei* (HETLAND et al., 2011.). Furthermore, it was suggested by AYEKA (2018.) that WBM plays a role in cancer immunotherapy by promoting binding to

dectin-1, CR3 or TLR-2 and induces activation and transduction of signals by T lymphocytes, mitogen activated protein (MAP) kinases and nuclear factor kappa of B (NF- κ B) lymphocytes, which could result in the production and secretion of chemokines that activate T lymphocytes, macrophages and NK cells.

Interestingly, *in vivo* investigations by DITAMO et al. (2016.) using the rat model demonstrated that lectin isolated from WBM had immunosuppressive effects and suggested that it may have potential therapeutic applicability in patients suffering from autoimmune diseases. The mode of action of this lectin from *A. bisporus*, termed ABL-lectin, included *in vitro* binding to murine T cell receptors and stimulation of protein tyrosine kinase enzyme, which subsequently activates the differentiation of $CD25^+$ and $CD69^+$ lymphocyte subsets, expressing early activation markers of T lymphocytes (HO et al., 2004.). Oral supplementation of β-(1-3) (1-6)-glucans to ewes had positive effects on humoral and cellular innate immunity, reproductive performance, milk yield, growth rate and body composition of their offspring (ZĄBEK et al., 2013.; ZALESKA et al., 2015.). In addition, β-glucan from WBM beneficially influences the maintenance of low serum glucose by decreasing digestion and absorption of nutrients such as starch. Also, lavastatin from the WBM has well known effect on lowering the serum concentration of cholesterol (KAŁA et al., 2020.). Antimicrobial effects of WBM have been ascribed to their polysaccharides, chitosan and chitin, which exhibit inhibitory activity against the following bacteria: *Micrococcus luteus, Micrococcus flavus, Bacillus subtilis, Bacillus cereus, Candida albicans* and *Candida tropicalis* (ÖZTÜRK et al., 2011.). Decreased numbers of *E. coli* and other enterobacteria as well as an increased number of *Lactobacillus spp*. were observed in the samples of rectal swabs from broilers (ŠPOLJARIĆ et al., 2015.; KHAN et al., 2019.). Additionally, GIANNENAS et al. (2010. a, b) reported that the preparation of dried WBM acts favorably on intestinal histomorphology in broiler chickens, production parameters and antioxidative status of their meat samples. Also, these authors recorded increased proportions of the peripheral blood $CD45^+$ $CD8^+$ cytolytic T cells, $CD45^+$ $CD4^+$ helper T cells, $CD4^+$ $CD8^+$ memory T cells and $CD45^+$ $CD21^+$ B cells in fattening broiler chickens. Similar data on circulating T and B cell subsets were obtained in the model of weaned pigs as described by ANDRIŠIĆ et al. (2020.).

Although literature data on the effectiveness of WBM in ruminants are still scarce, previously reported findings of its impact on the immune status, health and growth may be at least indicative for further evaluation of such potentials in order to justify mixing these preparations with feed mixtures for ruminants in intensive production. There are already data showing that WBM supplemented in daily ratios for cattle had no effect on rumen fermentation,

particularly on pH values and ammonium concentration (OH et al., 2010.). More recently, SHEK-VUGROVEČKI et al., (2018.) suggested that dietary WBM decreased serum concentrations of glucose and total cholesterol in the lambs of Lika breed of sheep. The former finding could be related to an increased level of insulin-like growth factor- 1 (IGF-1) in goats fed dietary WBM as it is well known that IGF- 1 is functionally involved in the regulation of serum glucose (PARK et al., 2012.). Another potential benefit of dietary WBM as a natural source of selenium (AHLAVAT et al., 2016.) has already been demonstrated by MILAD et al. (2001.) as they reported that selenium may stimulate activity of glutathione peroxidase and, consequently, the ovine cell-mediated immunity, implying that a similar effect may be expected in lambs fed dietary WBM. Furthermore, ŠPIRANEC et al. (2016.) showed that an increased activity of oxidative enzymes resulted in intensified cell metabolism and much higher muscle mass of lambs supplemented with dietary WBM preparation, concluding that such feeding regime could have potential of growth promoter in small ruminants. Despite numerous previous studies regarding immunophenotype of TCR-$\gamma\delta^+$ subset of ovine T lymphocytes, little is known about their mode of action. Today, their memory/cytolytic functions are well understood; they are similar to those of NK cells and play a role in the presentation of antigens in sheep (BRAUN et al., 2018.).

The role of $CD4^+CD8^+$ double positive (DP) T cells is largely understudied. Indeed, DP T cells were primarily considered as a developmental stage in the thymus, before their maturation as either $CD4^+$ or $CD8^+$ single positive mature T cells. The majority of T cells, in the peripheral blood and tissues, have retained expression of only one of these co-receptors corresponding to different functions, with $CD8^+$ T cells mostly involved in cytotoxicity toward infected or tumor cells, and $CD4^+$ T cells with helper functions to orchestrate immune response. However, mature DP $CD4^+CD8^+$ T cells have been described in the peripheral blood and tissues in various settings. The conflicting literature regarding the role of DP T cells, cytotoxic vs. immunosuppressive, may indicate that these cells are heterogeneous and/or show pleiotropic functions that need to be investigated in each particular disease context (BOHNER et al., 2019.). Using the sheep model, MACKAY et al. (1990.), have identified naive and memory T cells based on their expression of a number of cell surface markers and were able to address two important questions concerning immunological memory: do memory T cells continuously recirculate from blood to lymphoid tissue in the same manner as naïve T cells, and are memory T cells long-lived? In the aforementioned study, they have established that immunologically experienced T cells are able to mediate a vigorous response upon antigenic

restimulation and that such response occurs as a result of clonal expansion of antigen-reactive memory cells which persisted in the host. While various prevention strategies are applied to combat infectious diseases and limit antibiotic growth promoters (AGP) usage in food animals, efficacious approaches for enhanced disease protection remain unreliable for many diseases. Nonspecific immunomodulation alters the immune response to subsequent exposure to the heterologous agent, not the priming agent. Vaccines are potent specific immunomodulators, priming the adaptive immune system that is well known to have memory, and concordantly disease prevention strategies such as vaccination primarily target its cellular and molecular components (BYRNE et al., 2020.). However, the paradigm on innate memory has recently changed with substantial evidence indicating that innate immunity functionally adapts following an initial priming or microbial exposure, thus altering secondary responses to various pathogens, which rely on the memory of the innate immune system (NETEA et al., 2016.).

The primary focus of the current study was to determine the potential beneficial effects and limitations of WBM on innate immunomodulation and memory to enhance disease resistance in commercially important veterinary species, such as sheep. Since the available literature offers no data regarding the effect of WBM on the changes in the proportions of peripheral blood lymphocyte subsets in small ruminants, the aim of this study was to investigate the effect of WBM (*A. bisporus*) on the kinetics of systemically circulating $CD4^+CD8^+$ memory T lymphocytes in lambs.

Materials and Methods

Animals. This study was performed from May 2019 to January 2020 on 45 randomly selected female lambs of the Lika breed of sheep Croatian: Lička pramenka), aged 90 days (born during the lambing period between February 15 and March 1, 2019), originating from a sheep farm owned by GEA-COM d.o.o. (Budačka Rijeka, Krnjak, Croatia) located at Velika Crkvina, near Krnjak. The forty-five selected lambs were assigned to 3 groups of 15 animals each depending on the feeding regime. The experiment was conducted during a period of 222 days, and the lambs were monitored starting at Day 0 (or 90 days of age) before the treatments. One group (group A or the control group) was marked with color spray (red) and kept outdoors, on a free-range pasture throughout the experimental period. The other two groups of lambs (groups B and C or the principal groups) were housed for 42 days in one facility, separated into 2 experimental units, and for the remaining 180 days they were kept outside on the free-range pasture with the rest of the flock. During the 42-day period, these two groups of lambs were

fed *ad libitum* forage, comprising freshly cut grass on a daily basis and hay from pasture surfaces in the area of Velika Crkvina (prepared during the period from May to July 2019) and ratios of concentrated commercial feed mixture (FM) with 16% of crude proteins (CP) for lambs (Kušić promet d. o. o., Sv. Ivan Zelina, Croatia). The lambs in group C were additionally treated with freshly prepared WBM and feeding regimes were as follows:

- group A on free-range pasture;
- group B received commercial FM;
- group C also received commercial FM (preformulated to a lower content of CP) supplemented daily with 15% of freshly prepared WBM;

Following 42 days of the in-door housed feeding experiment the lambs were marked with color spray (group B = green and group C = blue) and in the second part of the experiment (the remaining 180 days) they were kept on the free-range pasture *ad libitum* with the flock. The experiment was conducted over a period of 222 days, and the lambs were monitored starting at Day 0 before the treatments. At Day 222 of the experiment, 2 lambs per group were euthanized and sampled for histopathology.

Production parameters. The lambs were weighed at Days 0, 21, 42 and 222 of the experiment and changes in their body mass were recorded. The changes of body mass in the principal groups of lambs (B, C) were calculated based on the difference between either body weight at the beginning of the experiment (where Day 0 equals 100 % of body mass) or average group body weight at Days 0, 21, 42 and 222 of the experiment in comparison to the average body weight of the lambs from the control group (A).

Clinical observation. The lambs were monitored daily for diarrhea and/or other clinical signs of health disorders, and the incidence/severity of diarrhea was recorded. The severity of diarrhea was scored as follows: 0 = normal feces, 1 = soft feces, 2 = fluid feces and 3 = projectile diarrhea. In addition to morbidity, mortality was also monitored, and dead lambs were necropsied and examined for gross pathology changes.

Multicolor flow cytometry (FCM) analysis. Multicolor FCM analysis of peripheral blood samples taken from the lambs was performed using flow cytometer Beckman Coulter Navious at the Clinical Department for Medical Biochemistry and Laboratory Medicine at the Clinical Hospital Merkur, Zagreb, Croatia according to the protocol as detailed earlier (VALPOTIĆ et al., 2014.; ŠPOLJARIĆ et al., 2021.). Specific murine monoclonal antibodies (mAbs) reactive with ovine leukocyte surface molecules, *i.e*. cluster of differentiation (CD) antigens, that were

directly labelled with fluorochromes were used for identification/quantification of total leukocytes and their subsets in the lambs as follows: memory T lymphocytes (panel: $CD45^+$ $CD3^+$ $CD8^+$ $CD4^+$): anti-$CD45^+$ FITC/anti-$CD3^+$ Pacific Blue®/anti-$CD8^+$ PE/anti-$CD4^+$ Alexa FLUOR® (BIO-RAD). At Days 0, 21, 42 and 222 of the experiment the lambs were sampled by *v. jugularis* puncture and peripheral blood was collected in sterile tubes with EDTA (Sigma, St. Louis, USA). Within 24 hours from sampling the erythrocytes were lysed by TQ-Prep (Workstation and Immunoprep, Beckman Coulter) and the concentration of mononuclear leukocytes resuspended in 1mL of buffered saline was determined using the Bürker-Türk hemocytometer after staining them with Trypan blue. The cell suspension (50 µL) of each sample was transferred into FACS tubes and murine mAbs specific for the tested CD antigens expressed by ovine memory T lymphocytes were added.

Histopathological analysis. Immediately following euthanasia of 2 lambs per group at Day 222 of the experiment the gastrointestinal tract was removed and the small intestine was divided into three parts: duodenum (from the stomach outlet to the end of the pancreatic loop), jejunum (from the pancreatic loop to Meckel's diverticulum) and ileum (from Meckel's diverticulum to the ileo-caeco-colic junction). Segments one centimeter long were taken from the center of each part and fixed in 10% neutral-buffered formalin (pH 7.0-7.6) for 24 hours until used for histopathology analyses under light microscopy. After fixation the specimens were dehydrated, embedded in paraplast (Sigma, Sherwood Medical Industries, USA), cut into 5 µm thick serial sections and then processed for standard hemalaun (Meyer's solution; Kemika, Zagreb, Croatia) and eosin staining. These sections were examined by a light microscope (DMLB, Leica, Germany) with a photographic device (Pixera Pro 150 ES). The graduation of epithelial damage and changes of thickness in the intestinal mucosa were determined as follows: 0 = no damage/normal thickness; 1 = mild damage/mildly thickened; 2 = moderate damage/moderately thickened; 3 = strong damage/strongly thickened. The graduation of cellular infiltrate in the lamina propria (LP) of the small intestine was determined as follows: - = no infiltrate; ± = slight infiltrate; + = medium infiltrate; ++ = extensive infiltrate; +++ = distinctively extensive infiltrate of mononuclear leukocytes (MNL) and/or globular leukocytes **(**GL). The graduation of solitary lymphatic follicles (SLF) in the LP of the mucosa or submucosa was determined as follows: - = none; ± = low number; + = hyperplasia; ++ = extensive hyperplasia of the SLF. The graduation of cellularity in the cecal tonsils (CT) was determined as follows: - = lymphopenia; ± = normal cellularity; + = slight hyperplasia, ++ = moderate hyperplasia; +++ = strong hyperplasia.

Ethics. All procedures conducted on animals used in this research were approved by the Ethical Committee in Veterinary Science at the Faculty of Veterinary Medicine, University of Zagreb, Croatia (No.: 640-01/16-17/54; file No.: 251-61-01/139-16-2) and by Veterinary and Food Safety Directorate of the Ministry of Agriculture, Republic of Croatia (No.: UP/I-322-01/17-01/31; file No.: 525-10/0529-17-2).

Statistical analysis. Statistical analyses of data were performed using the SAS 9.4 software package (Statistical Analysis Software 2002–2012 by SAS Institute Inc., Cary). Statistical analysis of changes in the proportion of peripheral blood memory T-lymphocytes and leukocytes included a normal distribution test using the UNIVARIATE procedure. When the assumptions of the normal distribution of the analyzed dependent variables were violated and in the case of heterogeneity of variances, transformation of the variables was performed. The general linear model (PROC GLM) was used for lymphocytes and body weight. The statistical model included fixed effects of the group and period. A multiple comparison test of the least-square means with Tukey correction was performed using the SLICE option to compare each group level within the period. The data are presented as the mean ± standard error of the mean (SEM) values, with the distributions shown in their original scales, and the level of statistical significance was set at $P<0.05$. Procedure GPLOT was used to produce graphs. Frequency of diarrhea and mortality of diarrheic lambs were calculated using the FREQ procedure and Fisher's exact test.

Results

The lambs fed standard FM supplemented with fresh WBM (group C) had significantly higher body weight gain ($P< 0.05$) at Day 42 (23.07 kg *vs*. 19.11 kg) and Day 222 (27.07 kg *vs*. 24.60 kg) of the experiment as compared to the control lambs (group A) (Figure 1). However, the lambs from group B (fed FM only) did not show any statistically significant differences in body weight gain ($P>0.05$) versus the nontreated controls (group A) during the experimental period.

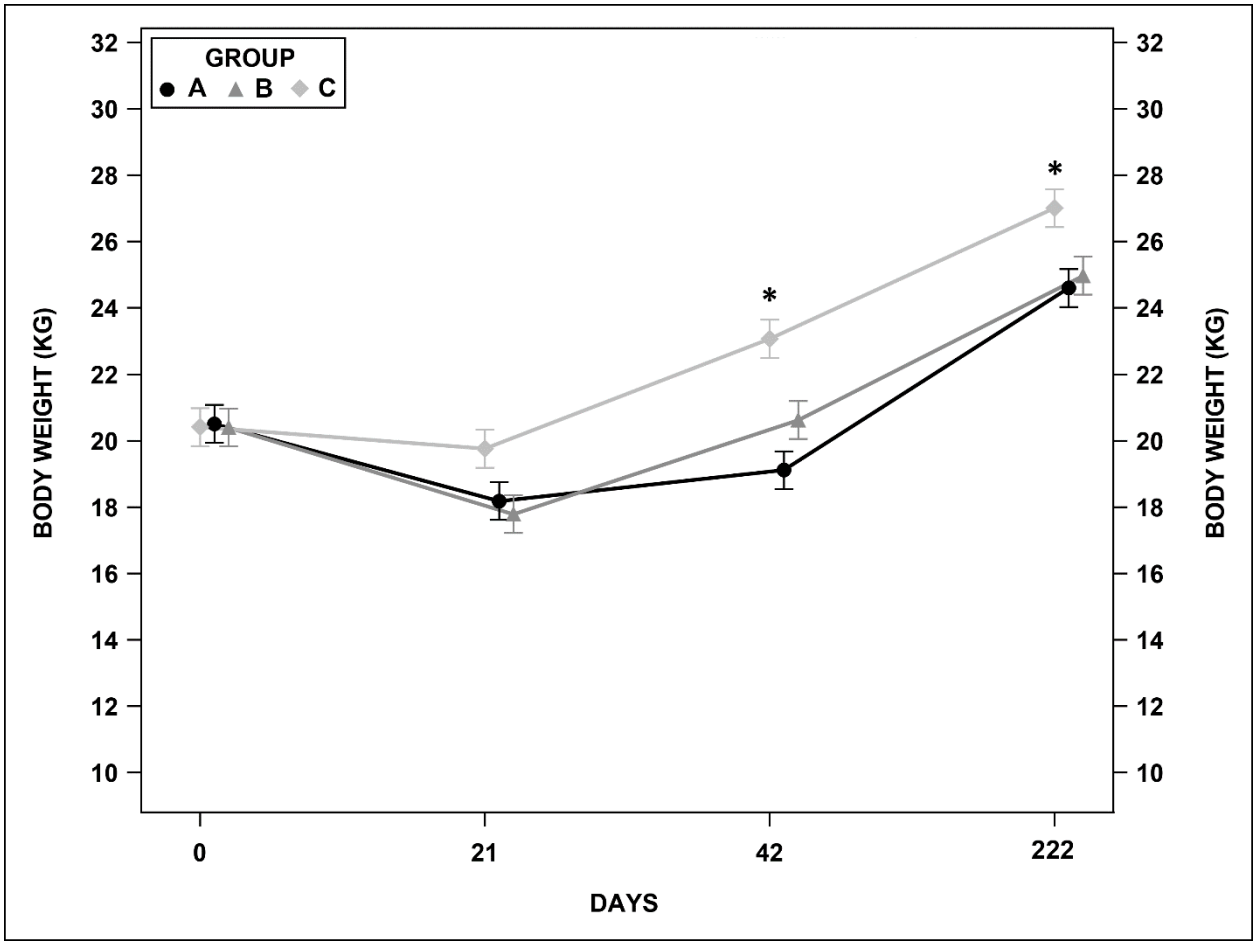

Figure 1. Changes in body weight (BW) of lambs fed on free-range pasture (group A), and those fed either FM (group B) or FM supplemented at Day 0 (or 90 days of age) with 15% of fresh white button mushrooms (WBM) (group C) during 42 days of the in-door housed part of the experiment. For the remaining 180 days of the experiment, the lambs were fed *ad libitum* on free-range pasture only. Values marked with an asterisk on the same day differ significantly at *P*<0.05 or lower.

The lambs that received FM supplemented with fresh WBM were nondiarrheic throughout the duration of the experiment (group C, 0%), whereas the proportion of this parameter ($P < 0.05$) was significantly higher in the control (group A, 33.3%) and, indicatively, but not significantly, higher in the non-supplemented lambs fed FM only (13.3%, group B) (Table 1). Furthermore, group C had no mortality cases as compared to relatively high proportions of mortality in the remaining two groups (group A, 13.3% and group B, 6.7 %) of lambs.

Table 1. **Incidence and severity of diarrhea and mortality of diarrheic lambs fed on free-range pasture (group A), and those fed either FM (group B) or FM supplemented at Day 0 (or 90 days of age) with 15% of fresh white button mushrooms (WBM) (group C) during 42 days of the in-door housed part of the experiment. For the remaining 180 days of the experiment the lambs were fed *ad libitum* on free-range pasture only**

Group *	No. of diarrheic lambs/total no. of lambs (%) **	Diarrhea severity score (DSS)		Average diarrhea severity (ADS)		No. of dead lambs/ total no. of lambs (%)
		Sum of DSS***	% difference *vs.* control	ADS ratio****	% difference *vs.* control	
A (Pasture)	5/15 (33.3) [a]	14	/	0.06	/	2/15 (13.3)
B (FM + Pasture)	2/15 (13.3)	3	- 78.6	0.01	- 83.4	1/15 (6.7)
C (FM + WBM + Pasture)	0/15 (0) [a]	0	- 100	/	- 100	0/15 (0)

* Groups comprised 15 lambs each; ** During 222 days of the experiment; *** Diarrhea severity score (DSS): 0 = normal feces, 1 = soft feces, 2 = fluid feces or 3 = projectile diarrhea as summarized during 222 days of the experiment; ****Sum of DSS/ 222/ days; [a] values marked with the same letter differ significantly (P <0.05).

The lambs fed FM (group B) had considerably lower incidence of diarrhea (13.3% *vs*. 33.3%, respectively) and a much lower mortality rate (6.7% *vs*. 13.3%, respectively), than the controls (group A). The sum of DSS as observed in the former group of lambs (group B) was found to be much lower than that recorded in group A (3 *vs.* 14, respectively). The ADS ratio was six times higher in the latter group of lambs (group A) than group B, that received diet supplemented with WBM (0.06 *vs.* 0.01, respectively).

The proportion of peripheral blood cells expressing $CD45^+$ pan leukocytic antigen was not influenced, regardless of the dietary treatments applied in this study during the entire course of the experiment (Figure 2).

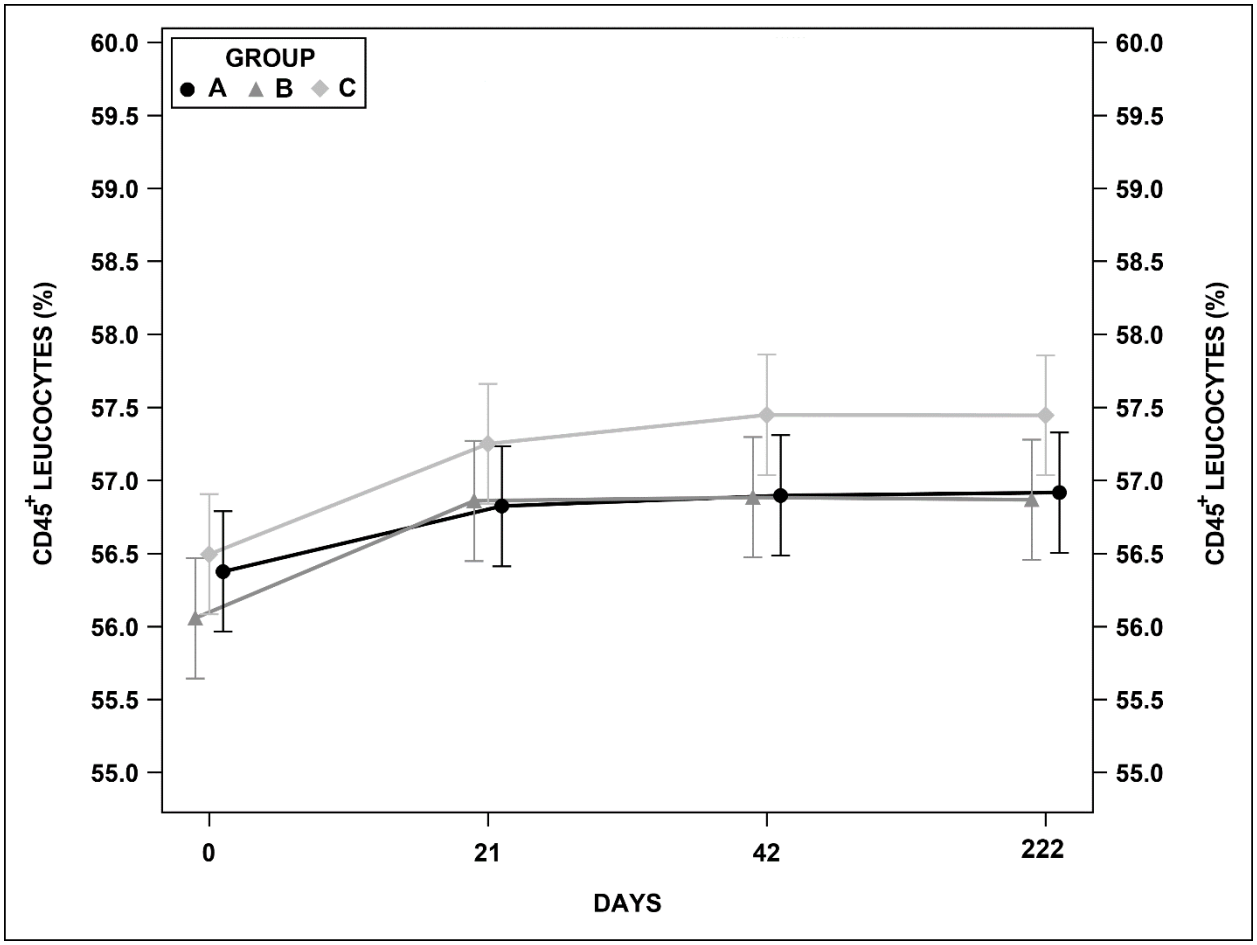

Figure 2. Changes of CD45$^+$ leukocyte proportions in the peripheral blood of lambs, fed on free-range pasture (group A), and those fed either FM (group B) or FM supplemented at Day 0 (or 90 days of age) with 15% of fresh white button mushrooms (WBM) (group C) during 42 days of the in-door housed part of the experiment. For the remaining 180 days of the experiment the lambs were fed *ad libitum* on free-range pasture only.

Although there were certain changes that showed a trend of an increase in the proportion of these cells at Day 21, 42 and 222 of the experiment in the lambs from group C, that received FM supplemented with fresh WBM, this increase was not statistically significant (P>0.05), probably due to the fact that values obtained for CD45$^+$ cells were not normally distributed.

However, regarding the changes in the proportion of double positive CD4$^+$ CD8$^+$ T lymphocytes (expressing also CD45 and CD3 surface antigens) the dietary treatment with fresh WBM (group C) stimulated a significant increase of this cell subset (P<0.05) at Day 42 and Day 222 (3.20% *vs.* 2.90% and 3.14% *vs*. 2.85%, respectively) of the experiment, as compared to the values obtained in the nontreated (group A) control lambs (Figure 3).

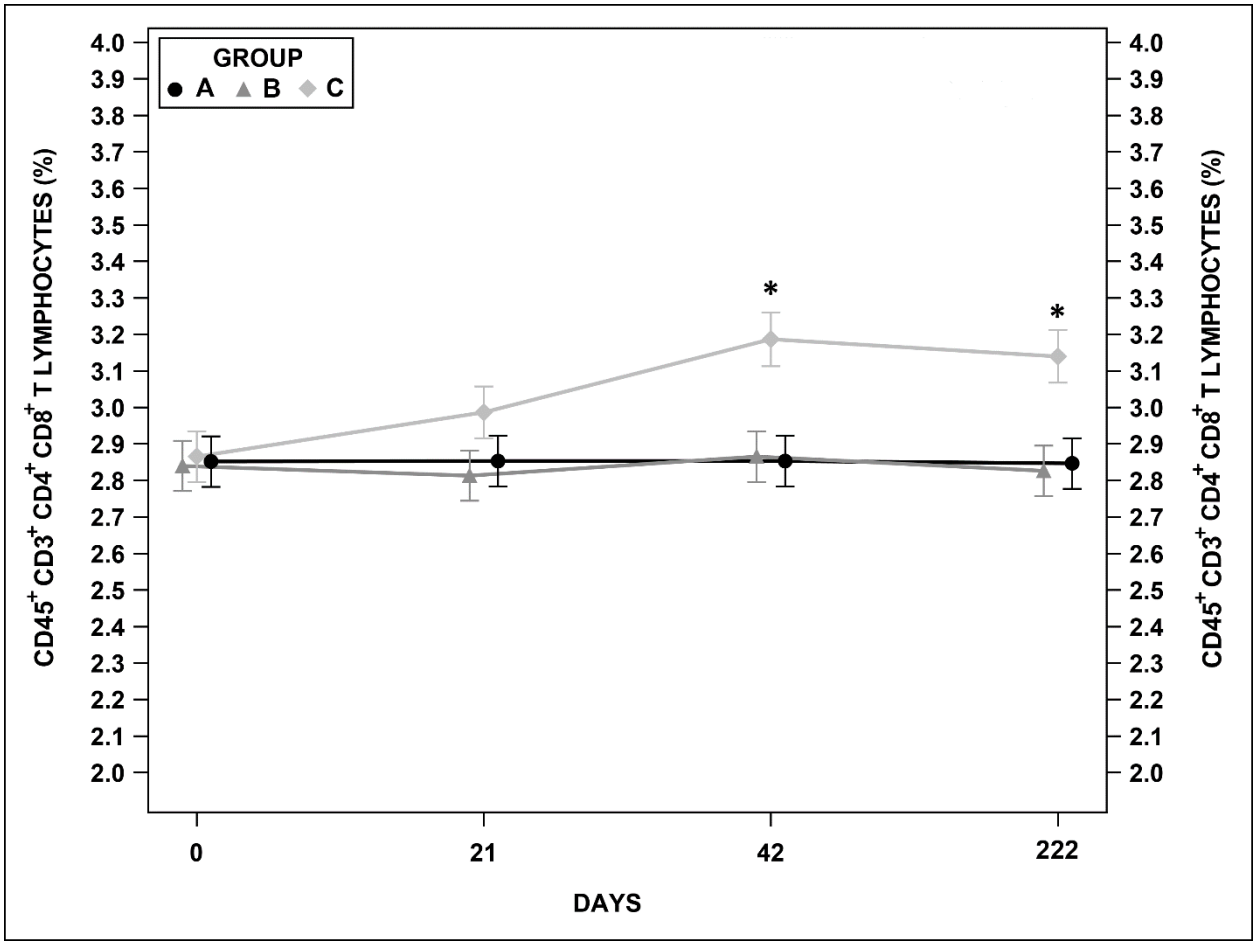

Figure 3. Changes of CD45$^+$ CD3$^+$ CD4$^+$ CD8$^+$ double positive T lymphocyte proportions in the peripheral blood of lambs fed on free-range pasture (group A) and those fed either FM (group B) or FM supplemented at Day 0 (or 90 days of age) with 15% of fresh white button mushrooms (WBM) (group C) during 42 days of the in-door housed part of the experiment. For the remaining 180 days of the experiment the lambs were fed *ad libitum* on free-range pasture only. Values marked with an asterisk on the same day differ significantly at $P<0.05$ or lower.

Following necropsies of 2 lambs per group at the end of the experiment (Day 222) and an examination of gross pathology changes, samples of the duodenum, jejunum and ileum were taken for histopathology analyses. The graduation of epithelial damage and changes of thickness in the intestinal mucosa/lamina propria (LP) or submucosa as well as the assessment of intensity/distribution of cellular infiltration in either LP by mononuclear leukocytes (MNL), globular leukocytes (GL) and/or by solitary lymphoid follicles (SLF) or in the cecal tonsils (CT) were performed (Table 2).

Table 2. Histopathological changes in duodenal, jejunal and ileal mucosa/lamina propria (LP) of lambs fed onfree-range pasture (group A) and those fed either FM (group B) or FM supplemented at Day 0 (or 90 days of age) with 15% of fresh white button mushrooms (WBM) (group C) during 42 days of the in-door housed part of the experiment. For the remaining 180 days of the experiment the lambs were fed *ad libitum* on free-range pasture only

Small intestinal sample	Histopathological parameter	Group of lambs*		
		A (Pasture)	**B** (FM + Pasture)	**C** (FM + WBM + Pasture)
Duodenum	Damage**	1	1	0
	Thickness**	1	1	1
	Cellular infiltrate in LP***			
	MNL/GL	++/+	++/++	+/±
	SLF****	+	+	±
Jejunum	Damage**	1	1	0
	Thickness**	2	0	1
	Cellular infiltrate in LP***			
	MNL/GL	+/±	++/++	±/±
	SLF****	±	-	-
Ileum	Damage**	1	1	0
	Thickness**	1	0	1
	Cellular infiltrate in LP***	+	++	+
	SLF****	±	+	±
	CT*****	++	++	±

*****Samples were taken from 2 euthanized lambs from each group at day 222 of the experiment; ******Gradation of epithelial damage and changes of mucosa thickness: 0 = no damage/normal thickness, 1 = mild damage/mildly thickened, 2 = moderate damage/moderately thickened, 3 = strong damage/strongly thickened; *******Gradation of cellular infiltrate in the LP: - = no infiltrate; ± = mild infiltrate; + = medium infiltrate; ++ = strong infiltrate; +++ = distinctively extensive infiltrates of mononuclear leukocytes (MNL) and/or globular leukocytes **(**GL) ******;** Graduation of solitary lymphatic follicles (SLF) in the LP of mucosa or submucosa: - = none, ± = low number; + = hyperplasia, ++ = extensive hyperplasia *******;** Graduation of the cellularity of cecal tonsils (CT): - = lymphopenia, ± = normal cellularity, + = mild hyperplasia, ++ = moderate hyperplasia, +++ = strong hyperplasia.

The lambs from group C that were in-feed treated with fresh WBM had no damages in duodenal, jejunal or ileal epithelial cell layers (0) and their mucosae were only slightly thickened (1). Interestingly, the lambs from group B that were fed FM had slightly damaged epithelium (1) in all three segments of the small intestine and slightly thickened mucosae of

the duodenum and jejunum (1), but not of the ileum (0). Regarding the damage and/or thickness of the intestinal epithelium/mucosal layers in the control nontreated lambs (group A), they were either slightly damaged/thickened in the duodenum and ileum (1/1) or moderately in the jejunum (1). Normal cellularity of the CT (±) was observed in the lambs from group C (FM + WBM + pasture), while moderate (++) hyperplasia was seen in groups A and B. The cellular infiltrates of MNL and GL were either medium (+) to mild (±) or mild (±) in the duodenal and jejunal LP, respectively, of the lambs from group C. However, in the lambs from group B, strong (++) infiltrations of MNL and GL were observed regardless of the segment of the small intestine. In the control lambs (group A), infiltrations of the duodenal LP with MNL/GL were strong (++) to medium (+), while those of the jejunal LP were medium (+) to mild (±). The appearance of SLF in the LP of mucosa was assigned as either in the normal numbers (±) in the duodenum and jejunum or none/absent (-) in the jejunum of lambs from group C. The lambs from group A had SLF in the LP assigned as either in the low numbers in the jejunum and ileum or as hyperplasia in the duodenum. No SLFs were observed in the jejunum, while hyperplasia of SLF was observed in the duodenum and ileum of the lambs from group B.

Discussion

Our study confirmed the efficacy of dietary WBM in immunostimulation of DP $CD4^+CD8^+$ memory T lymphocytes in lambs. Namely, the results demonstrated that feeding WBM to 90 days old lambs induced clinical protection against diarrhea, increased growth rate and higher proportion of DP memory T cells, particularly from Day 42 to Day 222 of the experiment. The evaluation of the growth rate in the lambs included their body weight at the ages of 0 (90 days of age), 21, 42 and 222 days, weight gain changes for the periods of Days 0 - 21, 21 - 42 and 42 – 222, and the differences between the principal groups (B and C) and the control group (A). Since the average body weight of the lambs fed standard FM supplemented with fresh WBM (group C) during the 42 days of the in-door housed part of the experiment was almost 4 kg (3.96 kg) higher than the average body weight of non-supplemented controls, it is very likely that this beneficial effect can be attributed to WBM as an indirect probiotic effect, as suggested by FERRÃO et al. (2019.). However, during the remaining 180 days of the out-door part of the experiment (from Day 42 to Day 222), when the lambs were fed *ad libitum* on free-range pasture only, the increase in body weight of almost 2.5 kg (2.47 kg) that was also recorded in group C could hardly be ascribed to WBM. Yet, data from related literature show that WBM as a dietary preparation could have the potential of promoting growth in lambs

(ŠPIRANEC et al., 2016.). More recently, symbiotic effects of WBM supplementation were described as indirect probiotic or direct prebiotic effects (FERRÃO et al., 2019.), and thus may support our findings of long-lasting effects of WBM on the growth rate and some other parameters tested that we have recorded in the lambs at Day 222 of the experiment or 180 days after completing the feeding regime of 42 days with WBM supplement. Namely, the lambs that received FM supplemented with fresh WBM (group C) during the 42 days of the in-door part of the experiment were neither diarrheic nor had any mortality cases throughout the 222 days of the experiment. Such finding could be explained by well-established reports regarding the antimicrobial *in vitro* effects of WBM (ÖZTÜRK et al., (2011.), particularly against *E. coli* and other enterobacteria in poultry (ŠPOLJARIĆ et al., 2015.; KHAN et al., 2019.). While the proportion of peripheral blood leukocytes expressing $CD45^+$ antigen was not influenced regardless of the dietary regimes applied in the current study, the proportion of DP $CD4^+$ $CD8^+$ memory T cells was obviously stimulated by WBM (group C), resulting in an increase of this cell subset at Day 42 and Day 222 of the experiment. However, such an increase of the memory cells during the 180 days of the out-door part of the experiment when the lambs were deprived of the WBM supplement seems to be unclear in regard to the unchanged proportion of $CD45^+$ total leukocytes, and thus further research is needed in order to define the kinetics of this subset before and after stimulation/restimulation.

Namely, the DP gamma delta (γδ) memory T cells are a minor population of peripheral T cells and only account for 2–5% of total T cells in the peripheral blood, yet they have been shown to play an important role as a part of innate immunity (BYRNE et al., 2020.). As WBM was administered perorally to the lambs on a daily basis for 42 days, histopathological examinations of their small intestines were performed at the end of the experiment (Day 222), in order to establish that the treatment terminated without negatively affecting their gut histological homeostasis. The lambs from group C that were in-feed treated with WBM had no damages in the duodenal, jejunal or ileal epithelial cell layers and their mucosae were only slightly thickened. In the nontreated lambs (group A), the intestinal epithelium/mucosal layers were either slightly damaged/thickened in the duodenum and ileum or moderately thickened in the jejunum, but not in the ileum. The lambs from the group B had slightly damaged and thickened epithelium and mucosae either in all three segments or in the duodenum and jejunum, but not in the ileum. Since similar studies are not available, further examination of the potential adverse effects of the WBM on gut histocytology after prolonged feeding of lambs with various doses of the supplement is suggested.

Conclusions

The data obtained in the current study supported our assumption on the efficacy of dietary WBM for optimal stimulation of DP $CD4^+CD8^+$ memory T lymphocytes in lambs, resulting in protection against on-farm diarrhea and increased growth rate.

The impact of immunomodulation of the innate arm of the immune system for enhanced disease protection/resistance may negatively affect production parameters. In addition, strategies that provide significant benefit to the health of the herd in food producing animals, including sheep, by reducing the risk of disease transmission without the use of AGP justify thorough evaluation in order to limit the impact of antimicrobial resistance.

Acknowledgements:

This study was performed as a part of the project IP-2016-06-3685 „Innovative functional products from meat of lambs“ financially supported by the Croatian Scientific Foundation, Zagreb, Croatia, and project KK.01.2.1.02.0293., founded by the EU.

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Utjecaj plemenite pečurke (*Agaricus bisporus*) dodane u hranu na kinetiku promjena udjela $CD4^+CD8^+$ T limfocita periferne krvi janjadi

Sažetak

Primarno usmjerenje ovog istraživanja bilo je utvrditi potencijalne pogodnosti dodavanja plemenite pečurke (PP) u hranu za ovce na rast, zdravlje te kinetiku sistemskih $CD4^+CD8^+$ memorijskih T limfocita u janjadi. Četrdeset pet ženki janjadi (pasmine Lička pramenka) podjeljeno je u tri skupine: A – kontrola, hranjena na slobodnoj paši tijekom 222 dana pokusa, dok su B i C skupine bile smještene odvojeno u nastambi kroz 42 dana i hranjene bilo komercijalnom krmnom smjesom (KS) ili KS s dodatkom 15% svježe pripremljene PP, odnosno *ad libitum* voluminoznom hranom. Preostalih 180 dana pokusa obje su skupine (B i C) janjadi držane u slobodnom uzgoju i hranjene samo ispašom. Janjad je svakodnevno promatrana počevši od 0 dana (ili 90 dana starosti) prije tretmana, vagana a uzimani su i uzorci krvi 0, 21, 42. i 222. dana te je klinički pregledavana na pojavnost/jačinu proljeva i/ili drugih znakova bolesti. Osim morbiditeta, praćen je i mortalitet, a uginula je janjad bila pregledana na patoanatomske i histopatološke promjene. Janjad hranjena KS s dodatkom PP (skupina C) imala je značajno viši prirast tjelesne težine ($P<0,05$) 42. i 222. dana. Nije imala proljeva niti su zabilježeni slučajevi uginuća tijekom pokusa. Također, ta je janjad imala značajno povećani ($P<0,05$) udjel $CD4^+$ $CD8^+$ T stanica 42. i 222. dana pokusa. Dobiveni podaci potvrđuju našu pretpostavku o učinkovitosti PP kao dodatka hrani u imunostimulaciji $CD4^+CD8^+$ memorijskih T limfocita u janjadi, što je rezultiralo zaštitom od proljeva na farmi i povećanom brzinom rasta.

Ključne riječi: imunostimulacija, dvostruko pozitivni T limfociti, rast, proljev, ovca