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BREWER YEAST MANNOPROTEINS AS AN EFFICIENT SUPPLEMENT FOR PRESERVATION OF RAM SPERM BY REFRIGERATION

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Abstract

The purpose of the work was to evaluate the influence of a mannoprotein extract (MP) from brewer yeast on the motility, speed, as well as the morphological and microbiological indices of the ram semen preserved by refrigeration at +4°C. The experimental extender (EE) supplemented with MP at the concentrations of 0.6-0.9% v/v significantly increased (P≤0.05) the total motility (TM) and the progressive motility (PM) of ram spermatozoa during 96 hours of storage and did not negatively influence the VAP, VSL and VCL indices. At the end of the 96-120-hour period of storage MP in the concentrations of 0.8-1.0% v/v contributed to reduction of the abnormal sperm (AS) number as compared to the control extender (CE). EE with MP in the concentrations of 0.2-1.0% v/v significantly reduced (P≤0.05) the contamination of the semen samples with various microorganisms. The obtained results demonstrated perspectivity of the yeast extracts for effective semen dilution and preservation for further artificial insemination (AI) of animals.

Key words*: brewer yeast, extender, mannoproteins, microbiological indices, motility indices, preservation, ram semen.*

INTRODUCTION

Artificial insemination (AI) is an essential technique for the development of sheep selection programs. AI with frozen semen is not widely implemented because of the relatively low quality of the latter. The processes of semen freezing and thawing damage ram sperm and reduce the quality of semen (Kulaksiz et al., 2012). Dilution and refrigeration of ram semen can be an alternative to freezing if insemination is done shortly after semen collection (Wusima et al., 2012). The wide use of refrigerated semen for AI is largely due to the possibility of semen dilution (Gundogan et al., 2009). Short term preservation of ram semen with different extenders (Gündogan, 2009). The preservation by refrigeration of ram semen is done at a temperature of 2-4°C and is one of the most intensively used preservation techniques for ovine (Dascăl, 2009). At the same time, the success of this technique is limited by the shortness of time, 24-48 hours, within which the ram sperm maintains sufficient motility and fertilizing capacity (Watson, 2000; Gil et al., 2011).

The main factors affecting the quality of sperm stored by refrigeration are the hypothermic shock, associated with the damage of plasma membranes, the activation of caspases, involved in apoptosis, the DNA hypomethylation and fragmentation leading to decreases of sperm fertilizing capacity, and the oxidative stress, associated with membrane lipid peroxidation (Budai et al., 2014).

The high concentration of polyunsaturated fatty acids in the spermatozoa membranes of ruminants makes them extremely vulnerable to the oxidative stress (Bucak et al., 2010). Reactive oxygen species (ROS) of the H_2O_2, O^{2-} and OH^- type, which are formed during the semen preservation by refrigeration, actively react with unsaturated fatty acids, leading to lipid peroxidation and disruption of mitochondrial and plasma membranes of the ram spermatozoa, affecting the motility of the latter (Amidi et al., 2016). The success of sperm preservation depends on the extender, which must contain substances that protect the spermatozoa from the thermal and oxidative shocks.

Diluting of the ejaculate with an extender allows to obtain seminal material with the

optimal concentration of spermatozoa. At the same time, the extender contains substances with a protective effect and supports the spermatozoa metabolism maintaining their fertilizing capacity. It was found that the extender has to contain substances that promote sperm metabolism and motility, colloidal substances that protect sperm membranes, and buffer substances for maintaining a favorable pH of the medium. These substances are of particular importance for the protective media used for diluting and preserving ram semen, because the high concentration of spermatozoa in the ejaculate and their intense metabolism significantly increase the acidity of the semen preparations (Lopez-Saez et al., 2000; Paulenz et al., 2002; Practical work, 2005).

Usually, the extenders for protection and preservation of the semen are based on skimmed milk, glycerin and egg yolk, which have a cryoprotective effect and help to maintain the quality of ram semen and motility indices for up to 48-96 hours under hypothermic conditions (Gil et al., 2011; Galarza et al., 2019).

Fatty acids, seminal plasma, sugars and various substances with antioxidant activity are used as extender additives to reduce the ROS production in semen preparations during storage, and to improve sperm quality (Toker et al., 2016; Allai et al., 2018). The non-enzymatic antioxidants, known as synthetic antioxidants, such as glutathione, ascorbic acid, citric acid, carotenoids, hypotaurin, ubiquinone and vitamins, are often used for this purpose too (Amidi et al., 2016).

Microbial polysaccharides are also mentioned in the specialized literature as possessing cryoprotective properties. Thus, Zheng et al. found that supplementation of the extender with *Laminaria japonica* water-soluble polysaccharides at different concentrations increased the motility and viability of the bull sperm (Zheng et al., 2017). This effect of polysaccharides from *Laminaria japonica* was due to their high antioxidant activity (Cui et al., 2016).

Sacchamomyces cerevisiae yeasts are recognized as very important sources of antioxidant enzyme systems that include superoxide dismutase and catalase (Lavová & Urminská, 2013). Yeast biomass extracts possess different antioxidant activities, which depend on the method used for their obtaining, and on the contained substances. Usually, the protein and mannoprotein extracts are known for their relatively higher antioxidant activity due to the lateral aromatic chains and free thiols from denatured proteins (Jaehrig et al., 2007; Jaehrig et al., 2008).

Besides that, the extracts from *Saccharomyces* yeast biomass, especially the mannoproteins, are also known for their antimicrobial properties against various pathogenic microorganisms (Ganner et al., 2013; Greco et al., 2018)

In this context, the possibility of obtaining extenders for semen protection based on biologically active substances of microbial origin may present a scientific and practical interest in the matters of the preservation of zootechnically important animals. Therefore, the purpose of this research was to evaluate the influence of MP from brewer yeast on the motility, morphological and microbiological indices of ram semen preserved by refrigeration at 4°C.

MATERIALS AND METHODS

Obtaining the mannoprotein extract

The mannoprotein extract (MP) was obtained from the yeast *Saccharomyces* biomass waste after production of the beer *Lager*. The biomass was offered by the Kellers brewery, the Budești commune, the Chisinau municipality. The semi-liquid microbial waste from the factory was centrifuged at 3500 rpm for 15 minutes to remove the liquid phase. The solid phase, the yeast biomass, was frozen at -18°C for storage. Subsequently, to obtain MP, the biomass was thawed at room temperature and then subjected to autolysis at 45°C for 8 hours, using the sodium phosphate buffer in the 1: 1 ratio. After autolysis, the suspension was centrifuged at 3500 rpm for 15 minutes to remove the liquid phase. The remaining solid residue was hydrolyzed with 1N NaOH solution in the 1: 5 v/v ratio at 80 ± 5°C for 2 hours. After hydrolysis the suspension was centrifuged at 3500 rpm for 15 minutes to separate the liquid phase from the solid phase. The mannoproteins were obtained from the supernatant by sedimentation with the help of the 96% ethyl alcohol, in the ratio of 1:2 v/v, and were

repeatedly purified with ethyl alcohol. In this research the MP water solution in the concentration of 500 mg/ml was used.

Collection and selection of semen

The object of the study was the semen from Moldovan Karakul rams, collected by the artificial vagina procedure during the mating season.

The ejaculates of at least 1.0 ml volume, with a sperm concentration of at least 2.5 x 10^9 ml^{-1}, and with the number of motile spermatozoa of at least 70% were selected for the experiments.

Extenders

Two extenders were used for dilution and preservation of the selected ejaculators: 1) the control extender (CE), composed of glucose (0.8%), sodium citrate (2.8%), egg yolk (20%), antibiotics (50000 IU), distilled water (up to 100 ml) (Milovanov et al., 2020), and 2) the experimental extender (EE) without antibiotics, containing sucrose (6.4%), sodium citrate (0.6%), egg yolk (10%), MP in the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1% v/v (0.5-5.0 mg/ml), labeled as EE1, EE2 - EE10.

Motility, speed and morphological indices

Motility and morphological indices of the ram semen: total motility (TM), progressive motility (PM), average path velocity (VAP), straight linear velocity (VSL), curvilinear velocity (VCL) and abnormal sperm (AS) number were analyzed under the phase-contrast microscope with the magnification x400-600, using the CEROS II^{TM} Sperm Analyzer computer program (Hamilton Thorne designs USA).

The semen samples were stored at 4°C. The indices were estimated in the beginning and then at the interval of 24 hours during the following 120-hour period.

Microbiological indices

The determination of microbiological indices in the ram semen, namely the total number of microorganisms (TNM) and the number of colonies of different bacteria and fungi, was carried out in several stages: preparation of successive decimal dilutions (1: 10 - 1: 1000); insemination of 0.1 ml from the dilutions on differential culture media; incubation at +37°C for 24-48 hours and counting the colony forming units (CFU) according to (GOST, 2014). The CFU number was expressed in log CFU/ml.

The Nutrient Agar medium M001 was used for determination of TNM; the Special HiCrome Endo Agar M029R was used for differentiating microorganisms from the *Enterobacteriaceae* family; HiCrome ECC Agar M1293 - for *E. coli*, *Ps. aeruginosa*; HiCrome Bacillus Agar Base M1651-for *Bacillus* spp.; Anaerobic Agar M228 - for *Clostridium* spp.; *Streptococcus* Selection Agar M304 - for *Staphylococcus* spp.; Sabouraud Dextrose Agar M063 - for yeast.

The identification of the bacterial genuses was performed based on the morphocultural, morphotinctorial properties and on the fermentation capacities in relation to some carbohydrates (glucose, dextrose, fructose, maltose, etc.) (The Shorter Bergey's Manual of Determinative Bacteriology, 1980)

The semen microbiological indices were determined after 120 hours of sample storage at the temperature of 4°C.

Statistical indices

The experiments were performed in five replicas. The obtained data were analyzed statistically and the results were expressed as the mean ± SEM. Student's t-test was done using the Microsoft Excel software. Differences with P values ≤ 0.05 were considered as statistically significan

RESULTS AND DISCUSSIONS

The ram semen preservation method by refrigeration is based on the use of protective media for diluting and maintaining the quality of semen for a maximum period of time (for maintaining the motility and fertilizing capacity of spermatozoa) while stored at 4°C.

The sperm motility test is usually used for evaluations of the effectiveness of the protective media (Věžník et al., 2004).

The sperm motility, especially the progressive sperm motility, is the main indicator of sperm quality and the determining factor of animal fertility, including for sheep (David et al., 2015; Hering et al., 2014; Simon et al., 2011).

The total motility (TM) of the ram sperm in semen preserved by refrigeration on EC and EE for 120 hours at 4°C is shown in Table 1. The initial TM for CE was 87.8% and then it constantly decreased within the 120 hours of storage reaching 58.3% by the end (Table 1). For the EE4-EE10 range, TM was from the very beginning significantly higher (P≤0.05) than for CE (90.2-92.5%), and the decrease rate during storage was smaller. Thus, comparing to CE there were observed statistically significant differences (P≤0.05) after 24 hours of storage for EE4-EE8, after 48 hours - for EE2-EE9, and after 72 hours - for EE3, EE5 and EE7 (Table 1). After 96 hours of storage TM was significantly higher (P≤0.05) for EE6 - EE10 - respectively, 71.0-77% comparing to 63% in the CE variant (Table 1). There were also observed significant TM differences (P≤0.05) between some EE variants and EE1. Thus, comparing to EE1, statistically higher TM values (P≤0.05) were observed after 0, 24, 48, 72 and 96 hours of storage for the same range of MP concentrations in the extender (Table 1). After 120 hours of storage TM for EE2-EE10 was still higher than in CE and EE1, but the differences were not significant statistically (P>0.05) (Table 1).

Table 1. Total Motility of ram sperm (%) in the semen preserved by refrigeration within 120 hours of storage at 4°C

Extender	**Conservation time, hours**					
	0	24	48	72	96	120
CE	87.8±0.7	83.8±2.0	81.2±1.0	71.2±3.9	63.0±2.5	58.3±2.0
EE1	87.8±1.0	83.8±2.0	82.0±1.4	78.5±1.7	65.3±1.8	57.3±2.7
EE2	88.2±0.7	87.6±1.4	84.2±1.0[a]	74.0±4.5	69.3±3.9	61.8±5.3
EE3	88.8±1.7	86.5±1.3	85.3±1.2[a]	80.3±0.9[a]	70.5±3.9	62.5±4.9
EE4	90.2±0.7[a,b]	88.8±1.7[a,b]	86.6±0.7[a,b]	77.8±4.2	71.0±3.8	63.0±5.1
EE5	91.0±1.1[a,b]	90.5±1.2[a,b]	86.8±0.9[a,b]	82.5±1.0[a,b]	72.8±3.6	63.0±5.3
EE6	91.8±0.8[a,b]	91.0±1.1[a,b]	86.8±1.2[a,b]	77.2±5.6	75.3±4.9[a,b]	66.3±5.8
EE7	92.5±1.3[a,b]	91.8±1.1[a,b]	88.5±1.3[ab]	82.5±1.0[a,b]	76.3±3.2[a,b]	67.5±4.6
EE8	92.0±0.9[a,b]	88.4±1.3[a,b]	87.0±1.1[a,b]	78.2±3.1	77.0±3.5[a,b]	68.0±5.8
EE9	91.3±0.6[a,b]	88.5±1.7	85.5±1.3[a]	79.0±1.6	74.0±3.3[a,b]	65.0±5.4
EE10	90.8±0.7[a,b]	85.6±2.2	83.2±1.4	75.0±3.7	71.0±1.8[a,b]	62.3±4.8

[a] - statistically significant differences (P≤0.05) comparing to CE; [b] - statistically significant differences (P≤0.05) comparing to EE1

The progressive motility (PM) of the ram sperm in the semen preserved by refrigeration with CE and EE within 120 hours of storage at 4°C is shown in Table 2.

As in the case of TM, PM of the ram sperm constantly decreased with time regardless of the extender composition (Table 2). The application of MP in the extender affected PM too, but the changes were not as obvious as in the case of TM. Thus, statistically significant differences (P≤0.05) comparing to CE and EE1 were registered after 24 hours only for EE4 and EE6, and after 96-120 hours - only for EE8 (Table 2). In the other cases no significant differences were observed (P>0.05) (Table 2).

Table 2. Progressive Motility of ram sperm (%) in the semen preserved by refrigeration within 120 hours of storage at 4°C

Extender	**Conservation time, hours**					
	0	24	48	72	96	120
CE	45.2±4.8	36.8±2.1	38.6±5.2	35.2±3.9	26.0±4.1	12.8±1.1
EE1	50.3±3.2	33.0±3.9	39.8±5.9	36.3±5.8	24.8±3.3	14.3±2.9
EE2	51.2±1.4	42.2±4.1	39.8±4.4	32.0±5.9	28.8±4.8	15.5±5.3
EE3	50.5±1.6	38.0±6.6	37.8±4.0	38.0±4.4	24.8±3.9	15.5±5.6
EE4	42.8±2.6	46.2±4.4[a, b]	34.8±1.7	37.0±7.2	33.0±8.4	19.0±4.8
EE5	46.5±3.6	40.8±3.8	33.0±2.2	29.5±3.7	26.8±4.8	17.0±4.7
EE6	51.8±2.9	47.0±3.9[a, b]	43.6±4.6	38.8±9.4	33.0±5.4	18.5±6.0
EE7	51.8±2.2	45.8±5.3[b]	42.8±3.1	36.5±7.3	32.8±7.5	22.3±7.6
EE8	48.0±2.2	39.6±3.9	38.6±3.8	33.6±6.6	38.5±4.9[a, b]	25.5±4.7[a, b]
EE9	51.3±6.3	37.3±5.6	45.8±5.3	42.8±5.8	30.8±5.9	17.8±3.0
EE10	46.4±3.5	39.6±5.6	40.0±5.5	30.0±6.4	31.8±6.7	14.8±2.6

[a] - statistically significant differences (P≤0.05) comparing to CE; [b] - statistically significant differences (P≤0.05) comparing to EE1

The data on VAP, VSL and VCL indices of the ram sperm in the semen preserved by refrigeration are shown in Table 3.

According to the obtained results, the values of these indices continuously decreased within 120 hours of storage (Table 3). There were observed no significant differences in VAP, VSL and VCL values between all the variants after 0, 24, 48 and 72 hours of storage, (Table 3). However, significant differences in VAP and VSL values did appear between CE and EE8 after 96 hours. Although after 120 hours the VAP and VSL values for EE8 were still higher than for CE, the differences were not significant statistically (Table 3).

Table 3. VAP, VSL, VCL (μm/s) of the ram sperm in the semen preserved by refrigeration within 120 hours of storage at 4°C

Conserv. time, hours	Indices, μm/s	CE	EE1	EE2	EE3	EE4	EE5	EE6	EE7	EE8	EE9	EE10
0	VAP	132.9±5.9	135.3 ±3.2	135.7 ±6.4	139.5 ±5.5	118.5 ±6.8	125.4 ±6.9	133.1 ±6.7	136.6 ±2.7	130.9 ±2.8	133.3±4. 1	126.4±5.3
	VSL	109.9±6.7	113.8 ±4.0	114.0 ±5.2	117.1 ±5.1	94.6 ±5.3	103.8 ±5.9	110.4 ±5.6	112.1 ±3.9	106.6 ±2.5	112.0±5. 7	106.4±7.6
	VCL	192.8±9.4	190.7 ±1.9	190.3 ±11.5	203.4 ±5.2	179.2 ±14.9	184.0 ±13.3	191.0 ±10.0	205.4 ±7.2	196.6 ±7.6	193.8±6. 3	186.1±8.7
24	VAP	106.9±6.5	105.7 ±5.2	101.7 ±4.3	109.3 ±7.2	115.2 ±6.6	111.0 ±1.5	113.0 ±3.3	111.3 ±5.2	102.9 ±6.0	98.6 ±5.1	98.7 ±5.6
	VSL	84.3±6.5	78.1 ±2.4	80.5 ±5.3	83.8 ±7.5	92.0 ±6.7	85.0 ±3.0	89.2 ±3.2	89.2 ±5.8	80.4± 3.9	76.8 ±5.2	80.0 ±5.7
	VCL	172.4±9.1	178.8 ±10.4	168.4 ±7.2	180.2 ±12.7	183.5 ±11.4	185.1 ±5.0	183.6 ±9.9	179.2 ±11.0	167.0 ±13.3	162.0±11 .6	156.7±9.0
48	VAP	104.6±5.7	108.9 ±2.7	102.6 ±3.7	100.4 ±4.9	96.7 ±7.1	100.1 ±5.5	108.3 ±4.5	110.0 ±6.0	99.9 ±5.1	113.3±5. 2	106.9±6.9
	VSL	85.7±7.2	86.2 ±4.6	79.7 ±3.9	80.1 ±5.2	71.8 ±1.9	75.9 ±4.6	83.2 ±4.3	82.4 ±4.2	78.5± 4.2	91.2±6.5	86.0±6.8
	VCL	166.9±7.9	177.1 ±4.4	165.7 ±8.1	167.0 ±7.4	150.3 ±7.6	169.4 ±10.1	156.3 ±8.9	168.8 ±10.1	159.2 ±8.5	172.6±6. 6	171.4±10.9
72	VAP	100.5±9.7	102.0 ±7.3	88.9 ±6.9	98.4 ±4.5	94.1 ±7.8	92.0 ±3.2	94.3 ±12.0	99.6 ±8.3	85.7 ±4.3	98.6 ±8.4	85.3 ±6.2
	VSL	82.3 ±9.5	83.2 ±7.9	71.0 ±6.8	80.4 ±5.9	77.2 ±7.9	69.7 ±3.7	81.6 ±9.8	78.8 ±9.3	68.3± 4.6	81.9 ±9.1	67.8 ±6.7
	VCL	160.1±9.8	166.4 ±8.3	146.2 ±9.5	158.1 ±3.6	152.9 ±11.1	146.9 ±9.2	156.9 ±15.5	161.3 ±7.2	138.5 ±6.1	159.0±7. 4	132.5±6.6
96	VAP	80.7 ±4.6	85.8 ±5.8	85.9 ±2.7	82.7 ±7.0	87.7 ±8.0	86.8 ±4.9	93.9 ±6.8	90.3 ±5.9	97.8 ±5.2[a]	93.3 ±6.5	94.7 ±7.8
	VSL	57.9 ±4.1	67.8 ±5.3	65.5 ±3.1	66.4 ±5.8	73.0 ±8.1	67.0 ±6.0	76.3 ±9.1	71.5 ±8.1	78.7± 6.2[a]	74.6 ±9.1	76.4 ±9.7
	VCL	145.1±8.1	147.6 ±6.2	150.5 ±2.5	144.6 ±11.8	142.7 ±10.3	155.0 ±8.0	156.8 ±5.1	155.1 ±5.1	158.9 ±4.8	154.1±4. 8	158.8±5.0
120	VAP	73.1 ±2.0	76.1 ±1.5	72.7 ±7.9	74.6 ±6.7	79.2 ±6.2	74.1 ±4.8	76.7 ±5.4	76.4 ±6.9	80.3 ±9.4	72.4 ±3.6	71.2 ±2.7
	VSL	54.8 ±0.6	55.4 ±3.9	57.0 ±5.2	52.0 ±3.6	58.5 ±5.2	56.1 ±5.6	56.6 ±6.6	58.2 ±7.5	64.4± 9.0	52.9 ±3.4	52.8 ±2.7
	VCL	137.6±6.1	136.0 ±6.7	125.0 ±14.6	131.2 ±12.1	135.4 ±11.9	132.0 ±6.4	133.0 ±7.6	132.0 ±11.3	136.7 ±12.9	126.3±6. 9	125.0±6.0

[a] - statistically significant differences (P≤0.05) comparing to CE

Since the number of spermatozoa with various morphological anomalies (abnormal sperm AS) usually increases with storage, this parameter was monitored within 120 hours for EE with MP in the concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0% v/v, as well as for CE (Table 4).

AS increased constantly during storage at +4°C in all variants (Table 4). In the case of CE AS initially was 11.0% and increased up to 17.7-18.8% after 96 and 120 hours of storage, respectively (Table 4). As for the EE variants, AS in the beginning varied within 8.9-10.4%, and increased to 15.7-17.3% after 96 hours, and to 16.8-18.4% after 120 hours of storage (Table 4).

Table 4. Abnormal Sperm (%) in the semen preserved by refrigeration within 120 hours of storage at 4°C

Extender	Conservation time, hours					
	0	24	48	72	96	120
CE	11.0±0.6	12.9±2.1	16.1±3.4	16.7±2.3	17.7±1.8	18.8±1.4
EE2	10.4±1.6	12.3±1.7	16.2±1.7	16.2±2.5	17.3±1.7	18.4±1.5
EE4	9.6±1.7	11.9±1.7	15.9±1.5	16.0±2.4	17.4±2.0	18.0±1.4
EE6	8.9±1.8	11.7±1.7	15.7±2.7	15.2±2.1	16.7±1.5	18.3±2.4
EE8	8.9±1.1	12.3±1.8	15.3±2.1	15.2±2.0	15.7±2.0	16.8±1.2
EE10	9.2±1.3	12.8±1.4	16.4±2.4	15.7±1.9	15.7±1.5	17.9±2.4

Because collection and processing of ejaculates is not done under strictly sterile conditions, usually there is microbial contamination in the samples, that can have a negative effect on the quality of the semen.

The microbiological indices (the total number of microorganisms and the number of colonies from different taxonomic groups by the end of 120 hours of storage at 4°C) were determined for the samples with the range of MP concentrations similar to that used for AS. The results are shown in Table 5.

The microbiological profiles in the EE samples were quite similar to that in the CE sample, but they differed quantitatively. Thus, by the end of 120 hours of storage in all EE samples both TNM and the number of colonies of microorganisms from different taxonomic groups were significantly lower ($P \leq 0.05$) comparing to those for CE. The only exception was the EE2 case, where the number of *Escherichia coli* colonies practically did not differ from the control (Table 5).

Significant differences ($P \leq 0.05$) were also found between the number of microorganisms in the EE4-EE10 cases and EE2, which had the smallest concentration of MP (Table 5). It should also be noted that bacteria of the *Clostridium* genus and fungi in general were especially sensitive to the presence of MP in the extender. The use of EE8 and EE10 resulted in total inhibition of *Clostridium* spp., while the minimal concentration of MP in EE2 significantly decreased the number of yeasts colonies with 2.2 log CFU/ml (comparing to the CE case). Also, the use of EE8 and EE10 reduced TNM with 1.9-2.7 log CFU/ml, *Escherichia coli* - with 1.0-2.2 log CFU/ml and *Bacillus* spp. - with 1.8-2.4 log CFU/ml (Table 5). *P. aeruginosa, Salmonella* spp. and *Staphylococcus* spp. were not detected in any of the investigated samples (Table 5).

Table 5. The microbiological indices of the ram semen after 120 hours of storage at 4°C

Microrganisms	Colonies, log CFU/ml					
	CE	EE2	EE4	EE6	EE8	EE10
TNM	5.8±0.08	5.4±0.12[a]	5.1±0.14[a,b]	5.1±0.11[a,b]	3.9±0.03[a,b]	3.1±0.05[a,b]
Escherichia coli	5.1±0.19	5.1±0.11	4.5±0.06[a,b]	4.5±0.06[a,b]	4.1±0.17[a,b]	2.9±0.02[a,b]
Clostridium spp.	5.5±0.08	5.0±0.03[a]	4.9±0.03[a]	4.7±0.05[a,b]	n.d.*	n.d.*
Bacillus spp.	5.3±0.09	4.9±0.05[a]	4.9±0.03[a]	4.1±0.12[a,b]	3.5±0.06[a,b]	2.9±0.02[a,b]
Yeast	5.5±0.06	3.3±0.08[a]	2.8±0.05[a,b]	2.8±0.04[a,b]	2.8±0.03[a,b]	2.7±0.04[a,b]
Staphylococcus spp.	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d*
Salmonella spp.	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*
Pseudomonas aeruginosa	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*

[a] - statistically significant differences ($P \leq 0.05$) comparing to CE; [b] - statistically significant differences ($P \leq 0.05$) comparing to EE2; n.d.* - not detected

The TM, PM, VAP, VSL and VCL indices were constantly declining, while AS was increasing during the semen storage, regardless of the used extender medium and the initial values of these indices (Hegedűšova et al., 2012; Moustafa et al., 2015).

SOD, CAT, glutathione peroxidase (GPx) and glutathione reductase (GR) are known as the major antioxidant enzymes involved in self-protection of mammalian spermatozoa from reactive oxygen species (ROS) (Bansal et al., 2011). These enzymesboth reduce ROS excesses and prevent spermatozoa damage. For example, SOD inactivates the superoxide ion (O^{2-}) - the main cause of ROS production, transforming it into hydrogen peroxide (H_2O_2), that subsequently is transformed into oxygen (O_2) and water (H_2O) by CAT.

Amino acids also have antioxidant properties. Being non-enzymatic antioxidants, they are present in significant quantities in the seminal plasma. It is known that supplementing extenders with various amino acids, such as taurine, hypotaurine, proline, glutamine, glycine, histidine and cysteine, reduces DNA fragmentation, increases the motility, viability and fertility of the ram sperm (Bucak et al., 2009; Sarıozkan et al., 2009; Bucak et al., 2013).

Cysteine and glutathione maintain the integrity of the acrosome, spermatozoa membrane, increase sperm quality and motility after cryopreservation (Bilodeau et al., 2001; Coyan et al., 2011; Sharafi et al., 2015).

Methionine is another amino acid that can be used to preserve the ram sperm by refrigeration. The extender supplementation with 1, 2 and 4 mM of methionine increased motility, viability and mitochondrial activity of ram spermatozoa (Bucak et al., 2012).

Roostaei-Ali Mehr & Noori (2013) established that sperm motility, viability, and membrane integrity could be increased by supplementing the protective medium for cryopreservation of the ram semen with 40- 80 mM of L-glutamine.

Bovine serum albumin (BSA) had a similar effect, protecting the ram sperm during the freeze-thaw processes (Uysal et al., 2007). At the concentrations of 10% or 15% in the protective medium BSA could be used for cryopreservation as a substitute for the egg yolk (Matsuoka et al., 2006).

Mono- and disaccharides are also widely used for the ram semen storage. Carbohydrates serve as an energy substrate for spermatozoa during preservation, they maintain osmotic pressure and offer fluidity to sperm cell membranes (Aboagla et al., 2003). Supplementing the extenders with different carbohydrates in various concentrations increased the motility, viability, membrane and acrosome integrity of the ram sperm, and reduced the number of spermatozoa with various anomalies (Ahmad et al., 2015; Jafaroghli et al., 2011).

Protective substances of various kinds may be and are included in the extenders for the efficient semen preservation (Larbi et al., 2018), but it should be noted that when used in excessive quantities they can also cause biochemical and functional damage, and modify the spermatozoa morphology. Thus, according to Câmara et al. (2011), supplementation of the extender with glutathione or catalase in the concentration of 400 mM and 400 U/mL, respectively, was toxic for spermatozoa and negatively affected the ram sperm motility.

Based on the above, the positive effect of EE on the motility and morphological indices of the ram spermatozoa was due to the biochemical composition and activity of the antioxidant enzymes CAT and SOD of MP. MP contained 36.6±0.58% S.U. of protein covering the full range of the essential and non-essential amino acids, and 40.9±3.04% S.U. of carbohydrates. It had a total antioxidant activity of 29.1±1.5 mg trolox/g S.U., and the activity of its antioxidant enzymes CAT and SOD was 741.2±44.8 mmol/min per mg protein and 66.2±2.9 U/mg protein, respectively (Chiseliță et al., 2022).

The use of EE with different concentrations of MP for diluting the ram semen positively influenced the total motility (TM) and progressive motility (PM) of the ram sperm within 96 hours of storage, and did not have negative effects on these parameters after 120 hours of storage. Thus, TM (71.0-77.0%) in the EE6-EE10 experimental samples (Table 1) and PM (38.5%) in the EE8 sample (Table 2) were significantly higher ($P \leq 0.05$) than both in the CE and EE1 variants after 96 hours of storage. This proves that the improvement of these indices comparing to the control, was caused by the supplementation of the extender with MP.

Considering the fact that significant differences ($P \leq 0.05$) between the CE and EE8 variants after 96 hours of storage were observed only for VAP and VSL (Table 3), we can conclude that the extender supplementation with MP had a relatively small impact on the VAP, VSL and VCL indices. At the same time, the absence of statistically significant differences for these indices between all the EE variants and the CE at different storage periods can be considered as a positive factor, since it implies that the supplementation did not have negative effects on the VAP, VSL and VCL indices of the ram spermatozoa.

Extender supplementation also had statistically insignificant impacts on AS. Thus, the minimum AS was observed for EE8 and EE10, reaching 15.7% after 96 hours of storage, and 16.8-17.9% after 120 hours, and that was 11.3% and, respectively 4.8-10.6% less comparing to CE (Table 4).

The results of our research show that in the matters of ram semen preservation by refrigeration the extender supplemented with different concentrations of MP was either as efficient or even better than the other extenders including the commercial ones (Hegedűšova et al., 2012; Maksimović et al., 2018).

According to the experts in the field, the rate of microbiological contamination of the ejaculates oscillates between 43 and 100%. This microbiological contamination, among other endogenous and exogenous factors, can contribute to decreases of the semen qualityand fertility. Microbiological contamination of the diluted semen causes low spermatozoa viability, decreased conception rate and reduced offspring number (Jäkel et al., 2021). For example, high bacteria abundance in porcine sperm decreased the sperm motility and viability, and increased the percentage of spermatozoa with various defects (Kuster et al., 2016).

Among the main causes of bacteriospermia are poor hygienic conditions in profile companies, especially during semen collection, contaminated water, feed and air, as well as exposure to hair, skin, respiratory secretions or fecal masses (Althouse et al., 2000).

To solve the problems associated with this microbiological contamination and to correspond to Council Directive 90/429/EEC, Annex C2 of European Union (Council Directive, UE), the extenders intended for the semen preservation are usually supplemented with various antibiotics (Althouse et al., 2008; Tvrdá et al., 2021). However, the continued use of antibiotics facilitates appearance, spread and persistence of multidrug-resistant bacteria (Morrell et al., 2014).

According to Tvrdá et al. (2022), the ram semen was contaminated by bacteria of the genuses *Staphylococcus*, *Escherichia*, *Bacillus*, *Pseudomonas*, *Lactobacillus*, *Acinetobacter*, *Aeromonas*, *Enterobacter* etc.

The microbiological indices of the ram semen obtained after 120 hours of storage demonstrated that the supplementation of the extender with MP significantly influenced the number of microorganisms in the samples. Thus, the use of EE8 and EE10 reduced TNM with 1.9-2.7 log CFU/ml, *Escherichia coli* - with 1.0-2.2 log CFU/ml, and *Bacillus* spp. - with 1.8-2.4 log CFU/ml (Table 5). *Clostridium* genus bacteria and yeast in general were even more sensitive to the presence of MP in the extender. The use of EE8 and EE10 resulted in total inhibition of *Clostridium* spp., and even the minimal concentrations of MP (EE2, Table 5) significantly decreased the number of yeasts colonies with 2.2 log CFU/ml comparing to CE. The significant decreases in the numbers of microorganisms observed between EE2-EE10 and CE on the one hand, and between EE4-EE10 and EE2 on the other is evidence that the supplementation of the extender with MP had a decontaminating effect on the samples that was proportional to the applied MP concentrations (0.2-1.0% v/v).

The mannoprotein extracts are known for their antibacterial activity against *Escherichia coli, Salmonella* spp. and some fungi (Posadas et al., 2010; Trevisi et al., 2012). Santovito E. et al. also found that the yeast mannoprotein preparations inhibited *in vitro* the growth of various *Clostridium perfringens* strains. The inhibitory effect depended on the yeast strain used for mannoprotein extraction and on the dose. The mannoprotein extract from baking yeasts proved to be the most effective. Its minimal inhibition concentration of 1.25 mg/ml increased the time of the *lag* phase with 3.6 hours, reduced the maximum growth rate by more than 50%, and reduced the colony number with 10^2 CFU/ml in 24 hours, comparing to the control (Santovito et al., 2019).

Moreover, a recent *in vivo* research revealed a beneficial effect of the yeast mannoprotein products for maintaining the performance and health of birds, minimizing mortality from infections caused by *Clostridium perfringens* without any use of antibiotics (Fowler et al., 2015; Hashim et al., 2018).

Various natural biologically active extracts from plants, oilseeds, herbs, fruits, and vegetables are discussed in the specialized literature as potential supplements for dilution, preservation and storage of the animal semen (Del Valle et al., 2013; Baghshahi et al., 2014;

Motlagh et al., 2014; Larbi A et al., 2016; Ros-Santaella et al., 2021).

In this context, considering the presented results and the fact that MP was obtained from the yeast biomass from beer industry waste, we can suggest that biologically active yeast extracts can be used as an effective alternative to the supplements mentioned above.

CONCLUSIONS

EE supplemented with MP in the concentrations of 0.6-0.9% v/v significantly increased ($P \leq 0.05$) the total motility (TM) and the progressive motility (PM) of the ram spermatozoa during 96 hours of storage, and did not affect negatively the VAP, VSL and VCL indices. The MP concentrations of 0.8-1.0% v/v reduced the number of abnormal sperm (AS) comparing to CE after 96-120 hours of storage. The supplementation of EE with MP in the concentrations of 0.2-1.0% v/v significantly reduced ($P \leq 0.05$) the contamination of the semen samples with various microorganisms. The obtained results demonstrated the perspectivity of the yeast extracts for diversification of natural biologically active supplements, designed for effective semen dilution and presservation for artificial insemination (AI) of sheep.

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