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VITALITY AND BACTERIOLOGICAL PROFILE OF LIPTOV BOLD-SPOTTED RABBIT SEMEN

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ABSTRACT

Since semen quality is important for the successful artificial insemination (AI) or cryopreservation, we focused on the evaluation of Slovak national rabbit breeds, New Zealand rabbit and Liptov Bold-Spotted rabbit semen as a possible source of gene reserve. Besides standard sperm traits such as motility, viability, acrosome integrity, and oxidative damage (ROS), bacterial profiles were also assessed in semen samples of-New Zealand rabbit (control; n=2; N1 and N2) and Liptov Bold-Spotted rabbit (experimental; n=2; L2 and L4). After initial analysis, samples were divided into good (>70%) and poor (<70%) quality samples according to motility and viability. The results showed significantly higher ($P<0.05$) totally motile spermatozoa in N1, N2 (76.53±1.22%; 77.30±1.22%) compared to L2 (35.86±5.39%) as well as higher progressively motile spermatozoa in N1 (60.53±1.35%) and N2 (68.13±4.14%) compared to L2 (24.16±1.95%). However, no significant differences in L4 (45.11±4.05%) compared to other groups were recorded. The proportion of dead/live, apoptotic, acrosome damaged, mitochondrial active and ROS produced rabbit sperm was assessed via flow cytometry using fluorescent dyes: DRAQ7, SYBR-14, Yo-Pro-1, PNA, MitoTracker and CellROX, respectively. Significant ($p<0.05$) decrease of live sperm was observed in L4 (42.73±6.89%) compared to N1 (75.22±5.75%) sample. In adittion, significant ($p<0.05$) increased presence of dead sperm was in L4 (41.20±10.16%) group compared to N2 (11.16±3.01%). The activity of rabbit sperm mitochondria assessed via mitochondrial membrane potential was significantly ($p<0.05$) higher in N1 (93.37±1.46%) and N2 (94.35±0.74%) compared to L4 (57.34±1.92). The results from the bacteriological analysis showed that the sperm in good quality (N1 and N2) contained mainly microbial genus such as *Brevundimonas*, *Brevibacterium*, *Bacillus* or *Achromobacter*. Other samples with poor quality (L2, L4) contained different kind of bacterial genus such as *Pseudomonas*, *Delftia*, *Acidovorax*, *Acinetobacter* or *Staphylococcus*. Although the microorganism profile appeared different, obligate pathogenic species were not present. Therefore, further analyses are needed to find out the reason of poor semen quality.

Keywords: rabbit; semen; motility; viability; microbiology

INTRODUCTION

The countries in the central and western Europe are known with long farming tradition of rabbits. Originally, rabbits were bred for meat, however nowadays, the rabbits are fed also for sport **(Alves et al., 2015)** as well as for breeding of specialised genotypes for biological research **(Tůmová et al., 2011)**. Liptov Bold-Spotted rabbit belongs to the younger Slovak national breeds of rabbits. Its characteristic features are small to medium body, good productivity and fertility. Several color variations of L are known: wild-coloured, black and blue, grey-blue. The weight of the breed varies within a range 3.50 to 4.25 kg, body is stocky, cylindrical and the posture is half-high on strong, erect forelegs with always pigmented toenails. The ears length is in the range of 9.0-11.0 cm, the coat is white colour with the width of the 1-3 cm in its centre **(Mojcherová et al., 2022)**.
Semen quality is required for achieving adequate fertility in mammals (Perry at al., 2011). Deteriorating environmental stimuli lead to reduced sperm quality and fertility in farm animals **(Rasooli *et al*., 2010)**. Futhermore, crossbreding can also be the factor which influenced the semen quality. The success of sperm cryopreservation, artificial insemination (AI) and other reproductive technologies depend on the initial quality of the semen sample. Basic semen quality indicators such as sperm total and progressive motility, concentration and movement parameters seems to be the most important. However, sperm motility does not offer us a comprehensive overview of sperm quality, therefore other analysis such as flow cytometry is needed. Flow cytometry provides a deeper analysis of sperm quality and thus this method has become a standard technique in laboratories **(Martínez-Pastor et al., 2010)**. Flow cytometric assessment involves the evaluation of various sperm attributes, which more or less affect the overall semen quality. Firstly, the sperm viability can be evaluated through their plasma membrane integrity using SYBR-14 dye which stain live active cells, or in a combination using dead cell dyes such as propidium iodide (PI) or DRAQ7 **(Vašíček et al., 2022)**. Subsequently, an apoptosis like-changes are very important indicator of determining the semen quality. Annexin V detecting the translocation of phosphatidylserine (PS) **(Peña et al., 2007)** and YO-PRO-1 iodide nuclear dye **(Peña et al., 2005)** are the mostly used markers for apoptosis evaluation. When the sperm membrane is disrupted an acrosomal membrane is exposed indicating the acrosome damage. For detection this process, *Arachis hypogaea* (peanut) agglutinin (PNA) is mainly used **(Sutovsky & Kennedy, 2003)**. Except for sperm viability and acrosomal status, mitochondrial activity is another important parameter of sperm quality. Membrane mitochondrial potential (MMP) as the activity mitochondria indicator can be detected using several fluorescent dyes like JC-1, Rhodamine 123 **(Zou et al., 2010)**, or Mitotracker **(Garner et al., 1997)**. In addition, production of reactive oxygen species (ROS) may have detrimental effect on sperm quality. Several markers for the detection of ROS in sperm are known such as dihydroethidium (DHE) **(Zhao et al., 2005)**, MitoSOX **(Koppers et al., 2008)** or CellROX **(Celeghini et al., 2021)**. All above mentioned fluorescent probes are standardly used as sperm quality parameters. Futhermore, previous studies on human and animal semen have indicated that bacteriospermia may lead to decrease sperm quality **(Moretti et al., 2009; Lenicky et al., 2021)**. It is commonly known, that increased proportion of morphological defected sperm have been found in semen contaminated with bacteria **(Baud et al., 2019; Eini et al., 2021)**, moreover it has been reported that bacteriospermia may induce overproduction of **ROS (Fraczek et al., 2016)** as well as pro-inflammatory cytokines (Pintus & Ros-Santaella, 2021) which can caused female reproductive system infection **(Petrovic et al., 2017)**.
There is a lack of information about the semen quality of Liptov Bold-Spotted rabbit. Therefore the present study was designed to evaluate the semen quality parameters (motility, viability, acrosomal status, ROS) as well as the bacterial profiles of this Slovak rabbit breed, while semen samples from New Zealand rabbit were used as control samples to compare quality of each other.

MATERIAL AND METHODS

Animals and Semen Collection

Four mature and clinically healthy males of New Zealand rabbit breed (N) and Liptov Bold-Spotted rabbit breed (L) aged (12-18 months) reared in a breeding facility (NPPC – RIAP Nitra, Slovakia) were used in this experiment. All rabbits were housed in separated cages, maintained under an artificial photoperiod (14 h of light at 10 lux and 10 h of dark) and were fed with commercial standard diet and watered *ad libitum*. The animal treatment was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic no. SK U 18016 in accordance with Slovak Animal Protection Regulation, RD 377/12 guidelines, which meet the requirements of the European Union Regulation 2010/63. Semen collection was performed by artificial vagina two times per week into prepared sterile tubes. Contaminated samples were excluded from the experiment.

Computer-Assisted Sperm Analysis (CASA)

Sperm total and progressive motility were evaluated using CASA system with Sperm Vision™ software (MiniTube, Tiefenbach, Germany). Samples were divided as control (N1, N2) and experimental (L2, L4 - individuals) samples. Briefly, each fresh semen sample was analyzed for average concentration (10^9 sperm/mL, percentage of totally motile sperm (motility > 5 µm/s) and percentage of progressively motile sperm (motility > 20 µm/s). Firstly, fresh rabbit semen was diluted by saline (0.9% NaCl; Braun, Melsungen, Germany) at the ratio (1:10). If necessary, higher, or lower dilution rate was used to obtain optimal sperm concentration for CASA measurement. Afterwards, 10 µL of pre-diluted semen sample was transferred to Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and analyzed with SpermVisionTM software under AxioScope A1 light microscope (Carl Zeiss Slovakia, Bratislava, Slovakia). Sperm parameters were automatically analyzed in seven microscopic view fields.

Flow Cytometry Analysis

For flow cytometry, sperm samples (aliquots from each rabbit semen) were diluted to the concentration of 1 x 10^6 sperm in phosphate buffer saline (PBS; Life Technologies, Slovak Republic) and incubated with selected chemicals. The viability of spermatozoa was analyzed using SYBR-14, a membrane-permeant nucleic acid green fluorescent dye (LIVE/DEAD® Sperm Viability Kit; Thermo Fisher Scientific,Waltham, MA, USA) and DRAQ7, a far-red fluorescent nucleic acid dye (BioStatus Limited, Shepshed, UK), which stains nuclei of dead cells. Briefly, sperm were incubated with 2.5 µL of SYBR-14 (at final concentration of 100 nM) for 10 min in the dark at 37°C. Next, sperm were washed (600x g, 20°C, 5 min) and immediately co-stained with ready-to-use DRAQ7 dye (at final concentration of 3 µM) for 10 min in the dark at room temperature (RT). Afterwards, samples without further washing were analyzed by flow cytometer. The proportion (%) of sperm positive for SYBR-14 but negative for DRAQ7 was considered as proportion of live (SYBR-14^+/DRAQ7^-), while SYBR-14^+/DRAQ7^+ and SYBR-14^+/DRAQ7^+ sperm were considered as dead sperm.
For apoptotic like changes in rabbit sperm, YO-PRO-1 nuclear green dye (Thermo Fisher Scientific,Waltham, MA, USA) was used. Semen samples were diluted in 500 µL of PBS and incubated with 0.5 µL of YO-PRO-1 (at final concentration of 100 nM) for 15 min in the dark at RT. Samples were washed in PBS by centrifugation (600x g, 20°C, 5 min), stained with ready-to-use DRAQ7 dye, as stated above, and analyzed by flow cytometer. The proportion (%) of sperm positive for YO-PRO-1 (YO-PRO-1^+/DRAQ7^- and YO-PRO-1^+/DRAQ7^+) was considered as proportion of apoptotic-like sperm.
The acrosome integrity was evaluated using PNA (peanut agglutinin). One µL of PNA working solution (Alexa Fluor 488 conjugate; Thermo Fisher Scientific, Waltham, MA, USA) was incubated with sperm diluted in 200 µL of PBS for 15 min in the dark at RT. PNA working solution (at concentration of 0.5 mg/mL) was prepared by dissolving of the protein (1 mg/mL) in 2 mL of deionized water. After incubation, samples were washed (600x g, 20°C, 5 min), stained with ready-to-use DRAQ7 dye, as stated above, and analyzed by flow cytometer. The proportion (%) of sperm positive for PNA (PNA$^+$/DRAQ7^- and PNA$^+$/DRAQ7^+) was considered as proportion of acrosome-damaged sperm.
The mitochondrial activity of sperm was observed through the mitochondrial membrane potential (MMP) using MitoTracker® Green FM (MT Green; Thermo Fisher Scientific, Waltham, MA, USA) probe. Briefly, sperm were diluted in 500 µL of PBS and incubated with MT Green dye (at final concentration of 300 nM) in the dark at 37°C for 10 min. After incubation, samples were washed (600x g, 20°C, 5 min), stained with ready-to-use DRAQ7 dye, as stated above, and analyzed by flow cytometer. The proportion (%) of sperm positive for MT Green (MT Green$^+$/DRAQ7^-) was considered as proportion of sperm with high MMP.
To measure the production of reactive cxygen species (ROS) in rabbit semen samples CellROX Green Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used. Briefly, sperm diluted in 500 µL of PBS were incubated with CellROX Green probe (at final concentration of 2.5 µM) for 30 min in the dark at 37°C. After incubation, samples were washed (600x g, 20°C, 5 min), stained with ready-to-use DRAQ7 dye, as stated above, and analyzed by flow cytometer. The proportion (%) of sperm positive for CellROX Green (CellROX Green $^+$/DRAQ7^- and CellROX Green $^+$/DRAQ7^+) was considered as a proportion of ROS-positive sperm.
Stained samples were measured using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon ion laser and red-diode (635 nm) laser. Fluorescent signals were acquired by Cell Quest Pro ™ software (BD Biosciences, San Jose, CA, USA).
The green FL1 channel using 530/30 nm band pass filter, orange FL2 channel using 585/42 nm band pass filter, red FL3 channel using 670 nm long pass filter and/or far-red FL4 channel using 661/16 nm band pass filter were used. Calibration was performed periodically using standard calibration beads (BD CaliBRITE™; BD Biosciences, San Jose, CA, USA). At least 10,000 events (sperm) were acquired for each sample.

Cultivation and isolation of microorganims

All kind of tested microorganisms were isolated by a conventional plating method. All samples were dilluted by the standard decimal dillution system. One hundred µl from each samples were transfered into the agar. Four agars were used in this study, MacConkey agar (*Enterobacteriaceae* cultivation), MRS agar (Lactic acid bacteria cultivation), Baird Parker agar (*Staphylococcus* spp. cultivation) and GTK agar (total numbers of microorganisms). Incubation was performing under air condition and depending on the tested kind of microorganisms. *Enterobacteriaceae* genera was cultivated 24 hours at 37°C, *Staphylococcus* spp. during 48 hours at 35°C, Lactic acid bacteria during 48-72 hours at 37°C and total numbers of microorganisms during 72 hours at 30°C. Purification of bacteria was performed by four-way streak plate method at the same condition. Numbers of microbial colonies were counted by the colony counter. The selection of the suitable microbial candidates for identification was made on the basis of the morphological, microscopic and macroscopic characteristics.

Identification of microorganisms

All selected microbial colonies were subjected to identification using MALDI-TOF Mass Spectrometry using Microflex LT (Bruker Daltonics, Bremen, Germany) in cooperation with Biotyper software (Bruker Daltonics, Bremen, Germany). Identification procedure was performed following **Hleba et al. (2020)**.

Statistical analyses

The flow-cytometric data were evaluated using GraphPad Prism version 9.3.1 forWindows (GraphPad Software, San Diego, CA, USA) with two-way ANOVA followed by Sidak test for multiple comparisons. Results are expressed as the mean ±SD. p-values at $p < 0.05$ were considered as statistically significant. All microbial colonies were counted and sorted by the morphological, microscopic and macroscopic characteristics. After identification, the classification of microbial colonies was adjusted and recalculated. The numbers of colonies on each sample was reported in log.CFU/ml and number of identified species in percentage. Statistical analysis was done by MS Excel software.

RESULTS

Computer assisted sperm analysis (CASA)

Rabbit semen samples analyzed in this study were divided according to individuals in two breeds N, L. In order to determine the differences among the males, CASA analysis was performed.

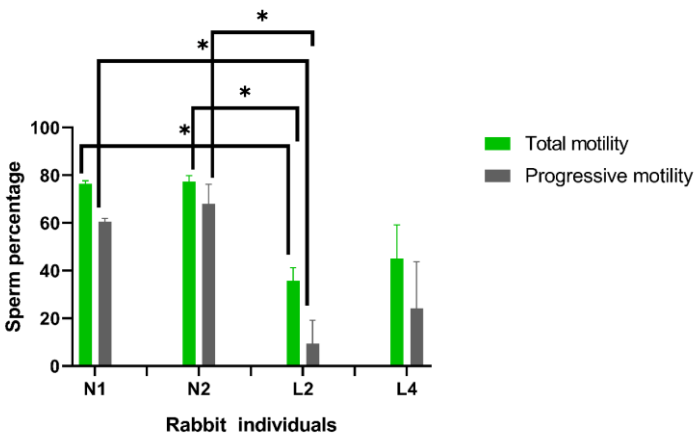

Figure 1 CASA parameters of analyzed rabbit semen sample of two breeds
The data are expressed as the means ± SD; difference is statistically significant at * $p<0.05$

Significantly higher (P<0.05) total motile sperm in N1, N2 (76.53±1.22%; 77.30±1.22%) compared to L2 (35.86±5.39%) as well as higher progressive motile sperm in N1 (60.53±1.35%) and N2 (68.13±4.14%) compared to L2 (24.16±1.95%) were recorded (Figure 1).

Flow cytometry analysis

The proportion of dead/live, apoptotic, acrosome damaged, mitochondrial active and ROS produced rabbit sperm of two breeds was assessed via flow cytometry using fluorescent dyes such as DRAQ7, SYBR-14, Yo-Pro-1, PNA, MT Green and CellROX green. Significant (p<0.05) decrease of live sperm was observed in L4 (42.73±6.89%) compared to N1 (75.22±5.75%) sample. In adittion, significant (p<0.05) increased presence of dead sperm was in L4 (41.20±10.16%) group compared to N2 (11.16±3.01%). The activity of rabbit sperm mitochondria assessed via mitochondrial membrane potential was significantly (p<0.05) higher in N1 (93.37±1.46%) and N2 (94.35±0.74%) compared to L4 (57.34±1.92%).

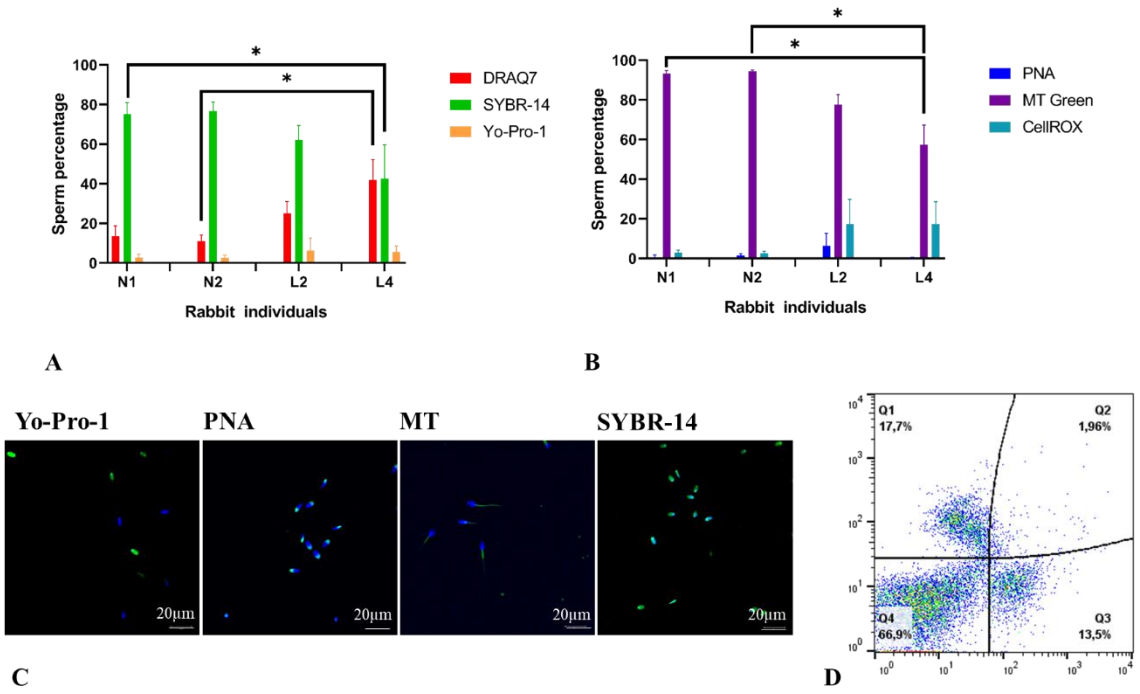

Figure 2 Evaluation of rabbit sperm quality parameters. **A** Graph comparing proportion of dead (DRAQ7), live (SYBR-14), apoptotic (Yo-Pro-1) sperm among the individuals. **B** Graph comparing acrosome damaged (PNA), mitochondrial active (MT) and ROS positive (CellROX green) sperm among the individuals. **C** Fluorescent figures of spermartozoa positive for analysed probes (green) and nuclear dye (blue). **D** Representative dot plots illustrating assessment strategy of analyzed fluorescent probes. (DRAQ7$^+$), live (SYBR-14$^+$), apoptotic (Yo-Pro-1$^+$), acrosome damaged (PNA$^+$), mitochondrial active (MT green$^+$) or ROS (CellROX green$^+$) spermatozoa divided into four quadrants (Q) according to fluorescent signals. *The data are expressed as the means ± SD; difference is statistically significant at * p<0.05*

Microbial diversity

After initial analysis (CASA, flow cytometry), we devided samples according to quality on good (>70%) and poor (<70%) samples. Thereafter, microbial diversity was constructed on the basis of identification of each microbial colonies and counting of each identified colonies. It should be noted that the agars used in this study are not primarily intended for the cultivation of microorganisms from sperm plasma. For example on MacConkey agar the following species and genus were identified: *Brevundimonas diminuta*, *Pseudomonas putida*, *Pseudomonas fulva*, *Pseudomonas frugi*, *Delftia acidovorans*, *Delftia lacustris*, *Acinetobacter lwoffii*, *Acinetobacter pittii*, *Acinetobacter parvus*, *Acinetobacter* spp., *Acidovorax* spp. and *Achromobacter* spp. *Staphylococcus xylosus* and *Staphylococcus microti*. By cultivation on Baird Parker agar were detected species and genus such as: *Bacillus cereus*, *Bacillus thuringiensis*, *Brevibacterium casei*, *Brevundimonas diminuta*, *Pseudomonas baelarica*, *Pseudomonas* spp., *Bradybacterium* spp., and *Chryseobacterium* spp. After cultivation on GTK agar were determined: *Bacillus cereus*, *Brevibacterium casei*, *Pseudomonas brenerii*, *Pseudomonas putida*, *Pseudomonas fulva*, *Staphylococcus pasteurii*, *Staphylococcus equorum*, *Paenobacillus pasadenesis*, *Acinetobacter guillouiae*, *Micrococcus luteus*, *Stenotrophomonas maltophilia*, *Bacillus* spp., *Achromobacter* spp. and *Stenotrophomonas* spp. On MRS agar were identified following: *Staphylococcus epidermidis*, *Staphylococcus caprae*, *Bacillus megaterium*, *Enterococcus faecalis*, *Saccharomyces cerevisiae*, *Pantoea aglomerans* and *Shewanella* spp.

A closer analysis of the percentage representation of individual identified species and genus showed more convincing results about the potential ability of bacteria and other microorganisms to influence sperm viability in seminal plasma. The samples which represent sperm in good quality (N1 and N2), motility and other parameters contained bacterial species as *Brevundimonas diminuta*, *Bacillus cereus*, *Bacillus thuringiensis*, *Brevibacterium casei*, *Achromobacter* spp., *Enterococcus faecalis*, *Saccharomyces cerevisiae* and genus *Pseudomonas*, species *P. putida* and *P. brenerii*. On the contrary, samples with greater sperm demage and reduced health condition (L2, L4) contained a greater occurence of species and genus such as: *Acinetobacter lwofii*, *Acinetobacter spp.*, *Acidovorax spp.*, *Staphylococcus microtii*, *Pseudomonas baelarica*, *Staphylococcus equorum*, *Staphylococcus spp.*, *Stenotrophomonas spp.*, or high number of genus *Shewanella spp*. The exact percentage numbers of microorganisms are shown in figure 3.

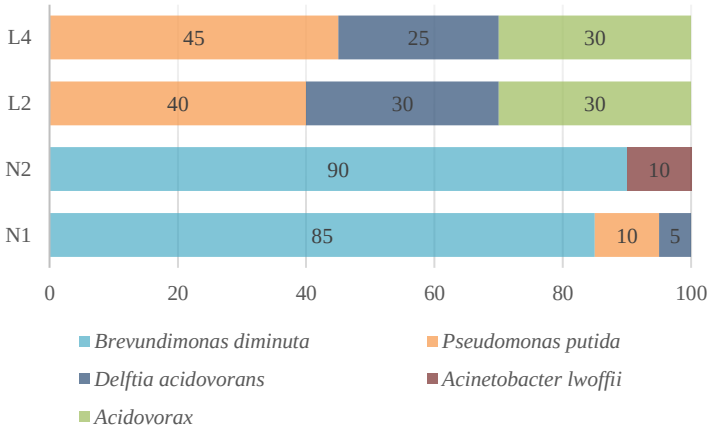

Figure 3 Percentage of microbial growth on MacConkey agar (McC) agar

Figure 3 represents percentage of microbial growth on McConkey agar after 24 hour incubation at 37°C. The results show that the potential sperm in good quality (N1 and N2) contained mainly microbial species such as *Brevibacterium diminuta* (85-90%) which grew on McC agar. Other samples with poor quality (L2, L4) contained different kind of microorganisms such as *Delftia acidovornas*, *Acidovorax spp.*, *and Pseudomonas putida*.

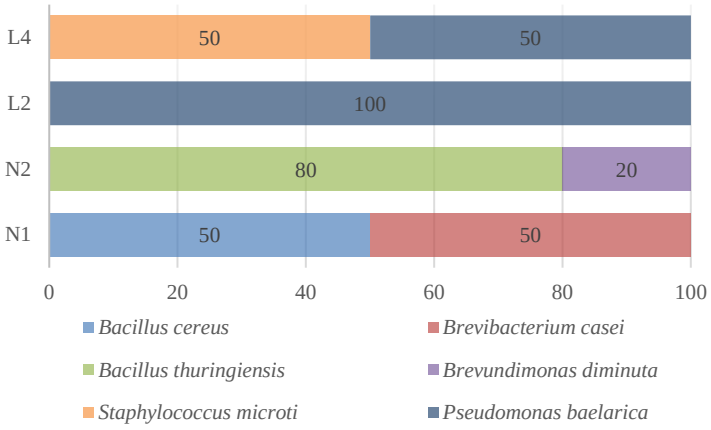

Figure 4 Percentage of microbial growth on BP agar

When examining the percentage growth of microorganisms on BP agar (Figure 4), it was found that sperm samples in good quality (N1 and N2) contained bacteria such as *Bacillus cereus*, *Bacillus thuringiensis*, *Brevibacterium casei* and *Brevundimonas diminuta*. Conversely, sperm samples in poor quality contained bacteria such as: *Staphylococcus microti* and *Pseudomonas baelarica*.

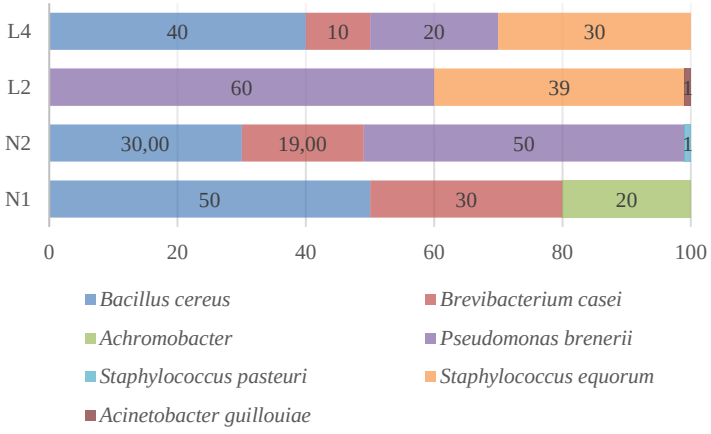

Figure 5 Percentage of microbial growth on GTK agar

Microbial growth on GTK agar reconfirmed the difference in the diversity of microorganisms, where the presence of the following microorganisms was found in samples with sperm in good quality: *Bacillus cereus*, *Brevibacterium casei*, *Pseudomonas brenerii* or *Achromobacter* spp. In contrast to the samples with sperm in good quality, bacteria from the genus *Staphylococcus*, specifically *Staphylococcus equorum*, were presented in the samples were spermatozoa had a poor semen quality.

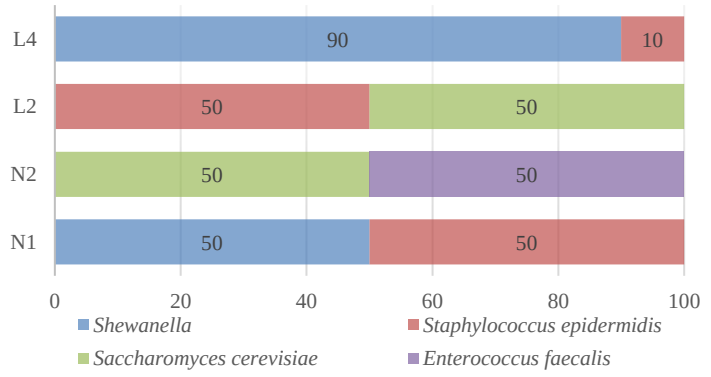

Figure 6 Percentage of microbial growth on MRS agar

Microbial growth on MRS agar cannot be considered representative because the percentage composition of microorganisms in the samples with sperm in good health condition and in poor health condition is disparate and no significant differences are known between these two groups. For exmaple genus *Shewanella* occurs equally in sperm with good health condition and poor health condition. The same situation is in the case of *Saccharomyces cerevisiae* and *Staphylococcus epidermidis*. Only *Enterococcus faecalis* occured in the sample with sperm in good health condition.

DISCUSSION

As far as we know, this is the first study focused on the evaluation of functional activity, oxidative parameter and bacteriological profile of important Slovak rabbit breed. The semen quality parameters of two rabbit lines (N and L) were assessed. There is no information about the quality parameters of L breed rabbit. Threfore, the aim of this study was to assess the motility parameters, viability, ROS and bacteriological profile of N and L rabbit semen. Our observations indicated the lower qualitative parameters as motility, viability and mitochondrial membrane potential of L rabbit semen in comparison with N semen. It is known, that the variation in the semen of rabbit can be influenced by many factors (genetic, feeding, health status, environmental condition, age, season and collection frequency), thus contributing to the large variability in semen traits **(Alvariño, 2000)**. The assessment of the male reproductive capacity is considered as a desirable practice among breeders. Therefore, the semen quality parameters represents the male individual potential which reflects the activity of the testicle. All the well-known quality indicators as motility, viability and ROS of semen are essential for determining the good or poor semen samples **(Abd El-Azim and El-Kamash, 2011)**.

In the study, an average sperm of total or progressive motility in N rabbits was about 75% and 60%, respectively, which may indicate an optimal quality of semen for futher processing. Beside the motility, viability was also evaluated. The proportion of live rabbit sperm was assessed by the probe SYBR-14, which is most widely used marker of live cells, mainly used in combination with PI (Propidium iodide),7-AAD **(Martínez-Pastor et al., 2010)** or DRAQ7 **(Vašíček et al., 2022)**. In addition, a green apoptotic-like changes dye, Yo-Pro-1, was used for apoptosis assessment **(Kuželová et al., 2017)**. Moreover, mitochondrial probe Mitotracker Green was used for detection of sperm mitochondrial potential. Several recent studies have been also used Mitotracker probes to evaluate mitochondrial activity of sperm **(Henning et al., 2019; Simonik et al., 2022)**. There were a significant higher proportion of mitochondrial active sperm in N rabbits compared to L rabbits. It is known that the integrity of sperm acrosome is a very important point of sperm quality assessment. Acrosomal status of rabbit sperm samples were assessed by PNA, a specific binding of lectin. PNA is one of the most used marker of sperm acrosomal status **(Kulíkova et al., 2017; Zhu et al., 2017)**. We did not observe significant differences in the case of acrosomal damaged spermatozoa between breeds as well individuals. Like acrosomal damage, sperm damaged by ROS production could be also a reason of poor sperm quality. However, in our study there were no significant differences among the breeds.

Moreover, it is well known that bacteria presence has negative effect on sperm motility, viability as well as fertility. Nonetheless, the success of any reproductive technology depends on the initial quality of the semen sample **(Baud et al., 2019)**. Bacteriospermia may cause adhesion and agglutination of sperm, resulted in decreasing sperm motility. Sperm can be damaged directly throught the nutrients competition or by the production of toxic metabolic substances **(Wolf er al., 1993; Monga, M., & Roberts, 1994)**.

Therefore, in this study, bacteriological profile of the semen was also evaluated. Till now, there are limited number of studies whether the presence of specific bacterial colonies have the potential to influence sperm function **(Baud et al., 2019)**. Nowadays, there is no information about the microbial species in L rabbit breed. For the bacteria identification, MALDI-TOF is widely used fast and straight-forward method to identify the bacterial species present in biological samples **(Wieser et al., 2012)**.

In our study, a closer analysis of proportion of the individually identified species and genus showed more convincing results about the potential ability of bacteria and other microorganisms to influence sperm viability in seminal plasma. The samples which represent spermatozoa in good quality (N1 and N2), motility and other parameters contained bacterial species as *Brevundimonas diminuta*, *Bacillus cereus*, *Bacillus thuringiensis*, *Brevibacterium casei*, *Achromobacter* spp., *Enterococcus faecalis*, *Saccharomyces cerevisiae* and genus *Pseudomonas*, species *P. putida* and *P. brenerii*. On the contrary, samples with greater sperm demage and reduced health condition (L2, L4) contained a greater occurence of species and genus such as: *Acinetobacter lwofii*, *Acinetobacter spp.*, *Acidovorax spp.*, *Staphylococcus microtii*, *Pseudomonas baelarica*, *Staphylococcus equorum*, *Staphylococcus spp.*, *Stenotrophomonas spp.*, or high number of genus *Shewanella spp*. Similar findings were recorded in studies **Mercier and Rideau (1992)** as well as **Sinkovic et al. (1993)** where they observed bacterial microflora in rabbit semen represented by *Enterobacteriaceae* and *Pseudomonas*. Since previous studies on human and animal ejaculates have recorded that bacteriospermia may lead to sperm agglutination **(Bollwein et al., 2004)** and subsequent decreasing sperm motility **(Moretti et al., 2009; Lenický et al., 2021; Ďuračka et al., 2021)**, it should be of great importance to know the microbiological profile of semen too.

CONCLUSION

The detailed quality analysis of Liptov Bold-Spotted rabbit semen as well as New Zealand rabbit semen were provided using CASA, flow cytometry and MALDI-TOF methods. Several sperm quality features such as viability, apoptosis, acrosomal status, mitochondrial activity as well as oxidative stress were assessed. Moreover, bacterial profile of samples was also successfully assessed. Differences in microbial representation potentially point to a possible cause of impaired sperm quality. The results also demonstrate that it is not possible to use any growing media for the assessment of microbial diversity associated with sperm quality. Even on the basis of the methods mentioned above, we were unable to reveal the cause of the poor quality of the ejaculate. Therefore, further analyses are needed to find out the reason of poor semen quality of Liptov Bold-Spotted rabbits.

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