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THE DEPENDENCE OF SPERM PROGRESSIVE MOTILITY AND SEMINAL PLASMA BIOCHEMICAL COMPOSITION ON BACTERIAL LOAD IN DUROC BOAR SEMEN

Michal Ďuračka[*1], *Anton Kováčik*[2], *Ondřej Bučko*[3], *Lucia Galovičová*[4], *Miroslava Kačániová*[4,5], *Norbert Lukáč*[2], *Eva Tvrdá*[6]

Address(es): MSc. Michal Ďuračka,
[1] Slovak University of Agriculture in Nitra, AgroBioTech Research Centre, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.
[2] Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Institute of Applied Biology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.
[3] Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Institute of Animal Husbandry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.
[4] Slovak University of Agriculture in Nitra, Faculty of Horticulture and Landscape Engineering, Institute of Horticulture, 949 76 Nitra, Slovakia.
[5] University of Rzeszow, Institute of Agriculture Sciences, Land Management and Environmental Protection, Department of Bioenergy, Food Technology and Microbiology, 4 Zelwerowicza St., 35601 Rzeszow, Poland.
[6] Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Institute of Applied Biology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.

*Corresponding author: michaelduracka@gmail.com

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ABSTRACT

Interactions between spermatozoa and bacterial species in ejaculates comes along with deleterious effects on the structure and function of male gametes. However, the effect of bacterial load on the seminal plasma remains unexplored. Therefore, this study was focused on the identification of bacterial species present in Duroc boar semen (n=18), quantification of the bacterial load (CFU) and alterations to the biochemical parameters of seminal plasma. The computer-assisted semen analysis (CASA) analysed the progressive motility (PRO; motility≥25µm/s) of spermatozoa, their concentration and kinematic parameters. The MALDI Biotyper was used for the bacterial characterization of fresh ejaculates. Seminal plasma was separated and biochemical analysis was carried out using the Randox RX Monza analyzer. Calcium (Ca), magnesium (Mg), total proteins (TP), aspartate transaminase (AST), alanine transaminase (ALT), cholesterol (CHOL), bilirubin (BR), albumins (ALB) and uric acid (UA) were subjected to the Pearson correlation with CFU and PRO. The bacterial identification showed the prevalence of *Escherichia coli* and *Pseudomonas aeruginosa*. The correlation analysis showed that the progressive motility of spermatozoa was strongly dependent (-0.799; P<0.01) on the seminal bacterial load. Moreover, a negative significant correlation (-0.810; P<0.001) was detected between CFU and Mg concentration. Also ALT (-0.647; P<0.01) and ALB (-0.484; P<0.05) were negatively affected by the bacterial load. Altogether, the presence of bacterial species may adversely affect the progressive motility as well as the biochemical composition of seminal plasma and thereby create unfavorable conditions for the sperm survival. Further analyses are necessary to reveal interactions between semen and bacteria.

Keywords: bacteria, spermatozoa, seminal plasma, progressive motility, biochemical components, boar semen

INTRODUCTION

Over the last few years, the influence of bacterial load on spermatozoa has become a new field in male reproductive biology. Boar semen may contain millions of microorganisms, while the most of them are nonpathogenic **(Althouse *et al.*, 2000; Maes *et al.*, 2008)**. In addition to naturally-occurring bacteria, which may originate from the male reproductive system, the risk of contamination may come from the artificial vagina, laboratory equipment or semen extender **(Gaczarzewicz *et al.*, 2016)**. The use of semen with excessive bacterial load in artificial insemination could contribute to diseases in inseminated sows and subsequently to the loss of pregnancy **(Maes *et al.*, 2008)**. On the contrary, **Sone (1990)** reported that even though sows were inseminated with fresh semen contaminated with *E. coli*, *S. aureus* and *Pseudomonas* sp., significant changes in birth rate were not observed. Especially, during the estrous phase, uterus is less susceptible to bacterial contamination. The susceptibility increases with the onset of the luteal phase. Previous studies quite precisely described the effects of bacteria on spermatozoa **(Tvrdá et al., 2018; Baud *et al.*, 2019; Ďuračka *et al.*, 2020; Ďuračka et al., 2021; Tvrdá et al., 2022a; Tvrdá et al., 2022b)**. While seminal pathogens as *Escherichia coli* were known for their adverse effects on sperm motility due to its agglutinating factor **(Kaur and Prabha, 2013)**, also common opportunistic pathogens belonging to *Staphylococcus* spp. affects fertility potential. The elevated immune response in semen with high bacterial contamination may be observed by the increased levels of seminal C-reactive protein and interleukins leading to increased reactive oxygen species **(Ďuračka *et al.*, 2021)**. However, oxidative stress may originate directly from bacteria as well. *Pseudomonas aeruginosa* belongs to frequently occurring species present in boar semen. Significantly reduced sperm quality was observed when infected with high bacterial load starting from 10^7 affecting total and progressive sperm motility, viability, and acrosome integrity, but not the pH value **(Sepúlveda *et al.*, 2014)**. On the other hand, change in pH was recorded when boar sperm has been infected with *Proteus vulgaris*. Moreover, less infective dose (≥ 10^5) was needed to reduce progressive motility due to the adhesive properties of bacteria **(Delgado-Bermúdez *et al.*, 2020)**. The increased levels of reactive oxygen species alter oxidative status of lipids and proteins in the sperm cells **(Morrell and Wallgren, 2011)**. Recent reports inform about the importance of the relationships between the seminal plasma and spermatozoa quality **(López Rodríguez *et al.*, 2013)**. Although, **Grizard *et al.* (1985)** concluded decreases in sperm motility and percentage of spermatozoa with normal morphology in bacteriospermic semen samples from infertile men, no alterations in seminal fluid parameters including fructose, acid phosphatase and citric acid were observed when compared to semen samples from fertile men. The investigation of associations between the presence of bacterial load in boar semen and the quality of seminal plasma may bring more light in order to mitigate bacterial damage of spermatozoa. The objective of this study was to identify the bacterial species in fresh boar semen and to correlate their presence with progressive motility of boar spermatozoa and the biochemical composition of the seminal plasma.

MATERIAL AND METHODS

Collection of semen samples

Fresh semen samples (n=18) were gathered from healthy Duroc boars (Farm Terezov, Hlohovec, Slovakia). Disposable gloves were changed between each animal. Prior the collection, the boars were allowed to urinate, and their external genitalia were washed with soap and water. Samples were transported to the laboratory within 1 h. Aliquots of samples (100 µL) were stored (-20°C) for the microbiological identification. Prior to the motility assessment, semen samples were diluted in physiological saline solution (PS; sodium chloride 0.9% w/v, Bieffe Medital Grosotto, Italy) using a ratio of 1:20.

Computer-Assisted Sperm Analysis (CASA)

The CASA assessment was performed using the HTM TOX IVOS II machine (Hamilton-Thorne Biosciences, Beverly, MA, USA). Ten µL of diluted samples were inserted to the Makler counting chamber (depth 10 µm; Sefi Medical Instruments, Haifa, Israel) pre-warmed to 37°C. A minimum of 300 cells/10 fields were analysed. Spermatozoa with a velocity of ≥25 µm/s were characterized as a progressively-moving cells and expressed as a percentage of all analysed sperm cells (PRO). Simultaneously, sperm kinematic parameters including path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), beat frequency (BCF), lateral amplitude (ALH), linearity (LIN) and straightness (STR) were assessed.

Microbiological cultures and identification

Blood, Gassner and Tryptic soy agar were used for the microbiological cultures under aerobic conditions (37°C; 24-48 h). Afterwards, colony forming units (CFU/mL) were assessed and microorganisms were purified by four ways streak plate method. Matrix-assisted laser desorption ionization – Time of flight (MALDI-TOF MS; Bruker Daltonics, Germany) system was used for the qualitative microbiological identification. Fresh overnight isolates were extracted into an ethanol-formic acid mixture. Samples were covered with a matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and air-dried at room temperature. The measured spectra were compared with reference spectra in Biotyper software database (version 2.0; Bruker Daltonics, Germany) **(Hleba *et al.*, 2017)**.

Biochemical assessment

Calcium (Ca), Magnesium (Mg), Total proteins (TP), Aspartate transaminase (AST), Alanine transaminase (ALT), Cholesterol (CHOL), Bilirubin (BR), Albumins (ALB) and Uric acid (UA) were analysed in seminal plasma using the DiaSys commercial kits (Diagnostic Systems, GmbH, Holzheim, Germany) and the biochemical analyzer Randox RX Monza (Crumlin, United Kingdom) **(Kováčik *et al.*, 2017)**.

Statistical analyses

The obtained data were submitted to statistical analysis using the GraphPad Prism program (version 8.0 for Windows, GraphPad Software, San Diego, California, USA, http://www.graphpad.com/). The results are quoted as arithmetic mean ± standard deviation. Subsequently, Pearson correlation method was performed in order to correlate bacterial CFU with progressive motility and biochemical parameters. The significance levels for the Pearson correlation method were set at P<0.001 (***), P<0.01 (**) and P<0.05 (*).

RESULTS AND DISCUSSION

This study aims to evaluate the associations amongst the presence of bacterial species (CFU) in boar semen with the progressive motility of spermatozoa, kinematic parameters, and biochemical parameters of the seminal plasma. Mean values of observed parameters are indicated in Table 1 and Table 2. Progressive motility (≥25 µm/s) indicates the vitality and viability of the sperm cells. A minimum of 60% progressively motile spermatozoa is required as optimal regarding to maintain satisfactory farrowing rate **(Flowers, 1996)**. According to **Górski *et al.* (2017)**, progressive movement was observed in approximately 79% of sperm cells, which is quite higher when compared to our results. However, this difference may be due to the elapsed time from the sample collection to laboratory processing. Moreover, seasonality plays a prominent role in boar semen quality **(Savić *et al.*, 2013)**. Especially, after summer season was observed significantly decreased spermatozoa motility due to chronic stress originating from long period of the high temperatures. Our experiments as well as semen gatherings were carried out during winter and spring months. A previous study showed that both, calcium (Ca), magnesium (Mg) and their ratio, affect sperm concentration as well as sperm motility and viability **(Liang *et al.*, 2016)**. The reference value of Ca (0.80 mM) in boar seminal plasma obtained in our study was in accordance with good semen quality. Although Mg concentration was quite higher when compared to previous study, a positive correlation between Mg level and sperm concentration was revealed.

Table 1 Arithmetic means and standard deviations of the Computer-assisted sperm analysis (CASA) parameters in boar semen

	PRO [%]	**CONC** [x 10^9/mL]	**VAP** [µm/s]	**VSL** [µm/s]	**VCL** [mM/L]	**ALH** [µm]	**BCF** [Hz]	**STR** [%]	**LIN** [%]
Mean	57.25	1.71	65.18	30.33	158.40	8.58	39.54	48.60	22.40
SD	4.68	0.15	9.27	5.93	16.11	1.67	5.21	3.74	2.64

Legend: PRO – progressive motility,VAP - Path Velocity; VSL - Prog. Velocity; VCL - Track Speed; ALH - Lateral Amplitude; BCF - Beat Frequency; STR – Straightness; LIN - Linearity

Table 2 Arithmetic means and standard deviations of bacterial concentration and biochemical parameters in boar semen

	CFU [log CFU/mL]	**Ca** [mM/L]	**Mg** [mM/L]	**TP** [g/L]	**AST** [µkat/L]	**ALT** [µkat/L]	**CHOL** [mM/L]	**BR** [µM/L]	**ALB** [g/dL]	**UA** [mg/dL]
Mean	4.20	0.80	4.03	15.94	0.59	0.17	0.37	8.75	0.24	0.48
SD	0.51	0.06	0.57	1.80	0.10	0.02	0.08	0.82	0.03	0.04

Legend: CFU – colony-forming units, Ca – calcium, Mg – magnesium, TP – total proteins, AST – aspartate transaminase, ALT – alanine transaminase, CHOL – cholesterol, BR – bilirubin, ALB – albumins, UA – uric acid

Table 3 Microbial identification and qualitative analysis of species present in boar semen

Sample	**CFU [log CFU/mL]**	**Species**
1	3.41	*Proteus vulgaris, Pseudomonas aeuoginosa*
2	4.27	*Staphylococcus chromogenes, Bacillus cereus, Bacillus licheniformis, Pseudomonas aeruginosa, Staphylococcus simulans*
3	4.58	*Pseudomonas putida, Bacillus licheniformis, Klebsiella pneumoniae, Aerococcus viridans*
4	4.84	*Pseudomonas aeruginosa, Rothia nasimurium, Escherichia coli, Staphylococcus aureus*
5	3.86	*Bacillus subtilis, Escherichia coli, Corynebacterium sp.*
6	4.45	*Stapyhyloccocus chromogenes, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa*
7	4.73	*Escherichia coli*
8	3.88	*Proteus vulgaris, Escherichia coli*
9	4.23	*Pseudomonas aeruginosa, Rothia nasimurium, Eschcerichia coli*
10	3.57	*Acinetobacter lwoffii*
11	4.52	*Escherichia coli*
12	4.37	*Pseudomonas aeruginosa, Clostridium difficile*
13	4.19	*Pseudomonas aeruginosa*
14	4.92	*Escherichia coli, Proteus vulgaris*
15	2.95	*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus simulans*
16	4.61	*Escherichia coli, Clostridium difficile, Enterococcus hirae, Pseudomonas aeruginosa*
17	4.09	*Escherichia coli, Clostridium difficile, Enterococcus hirae, Candida rugosa*
18	4.11	*Pseudomonas aeruginosa*

Legend: CFU – colony-forming unit

The identified bacterial species and corresponding bacterial load are listed in Table 3. Almost 2/3rd of our samples contained *Escherichia coli*. Subsequently, *Pseudomonas aeruginosa*, *Staphylococcus* and *Bacillus* prevailed in boar semen samples. In addition to this, spermiocultures yielded also *Proteus vulgaris*, *Klebsiella pneumoniae*, *Clostridium difficile*, *Enterococcus hirae*, *Rothia nasimurium*, *Acinetobacter lwoffii*, *Aerococcus viridans* and *Candida rugosa*. Despite this wide array of bacterial species, our results are in agreement with previous reports **(Bresciani *et al.*, 2014; Gaczarzewicz *et al.*, 2016)**, which also mentioned the occurrence of these species in boar semen.

Gączarzewicz *et al.* (2016) quite well revealed the effect of *E. coli* on boar spermatozoa. Besides the fact that *E. coli* was the most represented bacteria in boar semen, as with other species, the agglutination of boar spermatozoa was observed in the presence of *E. coli* alone or together with other Gram negative bacteria. A previous report mentioned that concentration of ≥3.5 × 10^3 CFU/mL *E. coli* may deteriorate the sperm quality and thereby the sample should be excluded from the

artificial insemination process **(Martín *et al.*, 2010)**. On the other hand, there are also studies indicating that physiological concentration of bacteria may range up to 10^6 CFU/mL **(Althouse and Lu, 2005; Morrell and Wallgren, 2011)**, and in some cases up to 10^9 CFU/mL **(Althouse *et al.*, 2000)**. The mean value of bacterial load in this study was 15.84×10^3 CFU/mL (4.20×10^3 log CFU/mL). Natural variability and quantity of bacterial load may be affected by a possible inconsistent technique of semen collection, insufficient hygiene standard or faults made during the sample processing. **Althouse *et al.* (2008)** determined the critical limit of bacterial contamination at 3×10^7-3×10^9 taking sperm concentration of 3×10^9 into account. The mean value of PRO was lower than previous reports. The correlation coefficient (r = -0.799; P<0.01) between CFU and PRO showed a strong negative correlation. As reported by **Prabha *et al.* (2010)** and **Kumar *et al.* (2011)**, the sperm immobilising factor (SIF) and the sperm agglutinating factor (SAF) represent receptor-mediated interactions between bacteria and cells resulting in a decreased sperm motion behaviour. Also, other sperm kinematic parameters seem to be affected by the bacterial concentration in ejaculate. Straight line velocity of spermatozoa showed significant negative (r = -0.701; P<0.001) correlation with bacterial concentration in semen. Significant associations (P<0.01) were recorded in case of path velocity, lateral amplitude and straightness (Table 4). **Sepúlveda et al. (2016)** described the effect of *P. aeruginosa* on boar sperm kinematic characteristics including VSL, VCL, VAP, LIN, STR and BCF during *in vitro* incubation and showed decreased kinematic parameters with increasing bacterial concentration and time.

Mg concentration was strongly negatively correlated (-0.810; P<0.001) with CFU. We may suppose that also bacterial Mg^{2+}-offtake in seminal plasma may indirectly decrease PRO of spermatozoa. Similar results were recorded in case of the correlation between Mg concentration and bacterial load in bovine seminal plasma **(Ďuračka *et al.*, 2020)**. Although, Mg concentration was associated with the sperm membrane integrity, our finding suggests that Mg may play role also in the AST and ALT activity. Especially, ALT was negatively associated (-0.647; P<0.01) with CFU, which may be caused by the Mg-dependent activity. Only medium correlation was observed between AST/CFU (-0.333; P<0.05) and AST/PRO (0.431; P<0.05). On the other hand, **Frydrychová *et al.* (2015)** demonstrated that the occurrence of AST in boar seminal plasma is a sign of sperm damage, because the only source of AST in spermatozoa is the mid-piece. Therefore, when AST is released to the seminal plasma, the sperm membrane is probably not intact. Unlike AST, a significant positive correlation was observed between ALT/motility and ALT/sperm concentration **(Khokhar *et al.*, 1987)**. An insignificant correlation was found between sperm concentration and ALT according to our data (Table 5).

Tvrdá *et al.* (2013) described ALB as an important seminal plasma marker with radical-scavenging activity. Similar to our study, they noticed a medium positive correlation between ALB concentration and PRO of spermatozoa. The negative medium correlation was observed in our study in case of the ALB/CFU relationship (Table 6). Comparable results were observed in case of associations between UA/CFU and UA/PROG. Both, ALB (-0.484; P<0.05) and UA (-0.388; P<0.05) levels decreased with increasing bacterial load, while at the same time PRO increased with increasing concentrations of ALB (0.622; P<0.01) and UA (0.357; P<0.05). ALB as well as UA behave as components of the antioxidant defense system of seminal plasma against free radicals **(Tvrdá *et al.*, 2010)**. Therefore, the possible explanation for the negative correlation between ALB and UA with CFU may lie in the utilization of ALB and UA in the recovery of oxidative balance after bacterial-induced oxidative stress **(Sozarukova and Proskurnina, 2016)**.

Since proteomics hyped, the function of the main seminal plasma proteins was revealed. The majority are related to the postcoital sperm vitality, including sperm motility, capacitation and protective properties against the female immune system. Therefore, the seminal plasma proteins may serve as markers of sperm fertility **(Druart *et al.*, 2019)**. In our study, we observed a medium positive correlation (0.507; P<0.05) between TP and PRO, while the correlation coefficient between TP and CFU (-0.392; P<0.05) showed that there might be a negative impact of bacterial load on the plasma proteins in boar semen. We noticed a medium positive correlation (0.524; P<0.05) between BR and PRO, but a weak negative correlation was observed between BR and CFU. Although, bilirubin acts as an endogenous antioxidant in human vascular endothelial cells **(Ziberna *et al.*, 2016)**, the effect in seminal plasma is at least questionable **(Tvrdá *et al.*, 2010)**. A positive correlation (0.529; P<0.01) was found between CHOL and PRO. As CHOL is considered to be an essential component of the membrane integrity and sperm function, a negative correlation (-0.346; P<0.05) with CFU and the resulting CHOL deficiency in an environment with an increased bacterial load may adversely affect the fertilization ability of male gametes.

Table 4 Associations between progressive motility, sperm kinematic parameters and bacterial concentration in boar semen

Correlations		Pearson's *r* coefficient	*p* level
Bacterial concentration	Progressive motility	-0.799	≤ 0.01
	Path velocity	-0.602	≤ 0.01
	Straight line velocity	-0.701	≤ 0.001
	Curvilinear velocity	-0.566	≤ 0.05
	Lateral amplitude	-0.699	≤ 0.01
	Beat frequency	-0.572	≤ 0.05
	Straightness	-0.686	≤ 0.01
	Linearity	-0.547	≤ 0.05

Table 5 Correlation analysis amongst progressive motility, sperm concentration and biochemical parameters

	PRO	Ca	Mg	TP	AST	ALT	CHOL	BR	ALB	UA
CONC	-0.483*	-0.379*	-0.571**	-0.114	-0.312	-0.444	-0.405*	-0.205	-0.345	-0.302
PRO	1	0.393*	0.747**	0.507*	0.431*	0.474*	0.529**	0.524*	0.622**	0.357*

Legend: PRO – progressive motility of spermatozoa, CONC – sperm concentration, Ca – Calcium, Mg – Magnesium, TP – Total proteins, AST – aspartate transaminase, ALT – alanine transaminase, CHOL – cholesterol, BR – bilirubin, ALB – albumins, UA – uric acid

Table 6 Associations between sperm concentration, biochemical parameters and bacterial concentration in boar semen

Correlations		Pearson's *r* coefficient	*p* level
Bacterial concentration	Sperm concentration	0.678	≤ 0.01
	Calcium	-0.314	≤ 0.05
	Magnesium	-0.810	≤ 0.001
	Total Proteins	-0.392	≤ 0.05
	Aspartate aminotransferase (AST)	-0.333	≤ 0.05
	Alanine aminotransferase (ALT)	-0.647	≤ 0.01
	Cholesterol	-0.346	≤ 0.05
	Bilirubin	-0.257	≥ 0.05
	Albumins	-0.484	≤ 0.05
	Uric Acid	-0.388	≤ 0.05

CONCLUSION

The evaluation of bacterial load in semen has become an important step in the artificial insemination process. Although the effects of bacteria on male gametes are quite well-examined by previous authors, the impact on the seminal plasma, representing the majority of the ejaculate, has not been explored yet. This study reveals the bacterial diversity of Duroc boar semen. Amongst these, *Escherichia coli* and *Pseudomonas aeruginosa* were the predominant species. The correlation analysis indicates deleterious effects of bacterial load on the progressive motility of spermatozoa. Moreover, sperm kinematics parameters, including straight line velocity, lateral amplitude and straightness were also associated with bacterial concentration in boar semen. More interesting results provide the correlation data between the bacterial load and biochemical parameters of the seminal plasma. Besides a direct bacteria-sperm contact, the harmful effects of bacteria on the progressive motility of sperm may lie in a nutrition imbalance of the seminal plasma. The Mg-offtake may have an essential impact on the subsequent composition of the seminal plasma and the sperm behaviour. The bacterial consumption of nutrients in the seminal plasma creates unfavourable exogenous conditions for the sperm cells, therefore it is necessary to avoid a potential contamination during the collection or semen processing. Long-term storage of boar semen with significant bacterial load could not only reduce the sperm progression, but it could also lead to the creation of a hostile environment for the sperm survival.

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REFERENCES

Althouse, G. C., & Lu, K. G. (2005). Bacteriospermia in extended porcine semen. *Theriogenology*, *63*(2), 573-584. https://doi.org/10.1016/j.theriogenology.2004.09.031

Althouse, G. C., Kuster, C. E., Clark, S. G., & Weisiger, R. M. (2000). Field investigations of bacterial contaminants and their effects on extended porcine

semen. *Theriogenology*, *53*(5), 1167-1176. https://doi.org/10.1016/S0093-691X(00)00261-2
Althouse, G. C., Pierdon, M. S., & Lu, K. G. (2008). Thermotemporal dynamics of contaminant bacteria and antimicrobials in extended porcine semen. *Theriogenology*, *70*(8), 1317-1323. https://doi.org/10.1016/j.theriogenology.2008.07.010
Baud, D., Pattaroni, C., Vulliemoz, N., Castella, V., Marsland, B. J., & Stojanov, M. (2019). Sperm Microbiota and Its Impact on Semen Parameters. *Frontiers in Microbiology*, *10*(234), 1-9. https://doi.org/10.3389/fmicb.2019.00234
Bresciani, C., Cabassi, C. S., Morini, G., Taddei, S., Bettini, R., Bigliardi, E., Di Ianni, F., Sabbioni, A., & Parmigiani, E. (2014). Boar semen bacterial contamination in Italy and antibiotic efficacy in a modified extender. *Italian Journal of Animal Science*, *13*(1), 83-87. https://doi.org/10.4081/ijas.2014.3082
Delgado-Bermúdez, A., Bonet, S., Yeste, M., & Pinart, E. (2020). Long-term storage of boar seminal doses contaminated with Proteus vulgaris: A dose-dependent effect on sperm motility and sperm-bacteria interaction. Animal reproduction science, 216, 106349. https://doi.org/10.1016/j.anireprosci.2020.106349
Druart, X., Rickard, J. P., Mactier, S., Kohnke, P. L., Kershaw-Young, C. M., Bathgate, R., Gibb, Z., Crossett, B., Tsikis, G., Labas, V., Harichaux, G., Grupen, C.G., & de Graaf, S.P. (2013). Proteomic characterization and cross species comparison of mammalian seminal plasma. *Journal of proteomics*, *91*, 13-22. https://doi.org/10.1016/j.jprot.2013.05.029
Ďuračka, M., Belić, L., Tokárová, K., Žiarovská, J., Kačániová, M., Lukáč, N., & Tvrdá, E. (2021). Bacterial communities in bovine ejaculates and their impact on the semen quality. Systems Biology in Reproductive Medicine, 1-12. https://doi.org/10.1080/19396368.2021.1958028
Ďuračka, M., Kováčik, A., Kačániová, M., Lukáč, N., & Tvrdá, E. (2020). Bacteria may deteriorate progressive motility of bovine spermatozoa and biochemical parameters of seminal plasma. *Journal of Microbiology, Biotechnology and Food Sciences*, *9*(4), 844-847. https://doi.org/10.15414/jmbfs.2020.9.4.844-847
Flowers, W. L. (1996). Performance expectations of different mating systems. *Proceedings of Allen D Leman Swine Conference*, *23*, 63-66. Frydrychova, S., Lustykova, A., Lipensky, J., & Rozkot, M. (2015). Effect of season on boar semen quality and enzymatic activity of aspartate aminotransferase. *Research in pig breeding*, *9*(2), 6-9.
Gaczarzewicz, D., Udala, J., Piasecka, M., Blaszczyk, B., & Stankiewicz, T. (2016). Bacterial contamination of boar semen and its relationship to sperm quality preserved in commercial extender containing gentamicin sulfate. *Polish Journal of Veterinary Sciences*, *19*(3). https://doi.org/10.1515/pjvs-2016-0057
Górski, K., Kondracki, S., & Wysokińska, A. (2017). Ejaculate traits and sperm morphology depending on ejaculate volume in Duroc boars. *Journal of veterinary research*, *61*(1), 121-125. https://doi.org/10.1515/jvetres-2017-0015
Grizard, G., Janny, L., Hermabessiere, J., Sirot, J., & Boucher, D. (1985). Seminal biochemistry and sperm characteristics in infertile men with bacteria in ejaculate. Archives of andrology, 15(2-3), 181-186. https://doi.org/10.3109/01485018508986909
Hleba, L., Kmeť, V., Tóth, T., & Kačániová, M. (2017). Resistance in bacteria and indirect beta-lactamase detection in E. coli isolated from Culex pipiens detected by matrix-assisted laser desorption ionization time of flight mass spectrometry. *Journal of Environmental Science and Health, Part B*, *52*(1), 64-69. https://doi.org/10.1080/03601234.2016.1229466
Khokhar, B. S., Singh, M., & Chaudhary, K. C. (1987). Transaminases in cattle and buffalo (Bubalus bubalis) semen in relation to fertility and seminal characteristics during moderate and colder seasons. *Animal Reproduction Science*, *13*(3), 177-182. https://doi.org/10.1016/0378-4320(87)90106-0
Kováčik, A., Árvay, J., Tušimová, E., Harangozo, L., Tvrdá, E., Zbyňovská, K., Čupka, P., Andraščíková, S., Tomáš, J., & Massányi, P. (2017). Seasonal variations in the blood concentration of selected heavy metals in sheep and their effects on the biochemical and hematological parameters. *Chemosphere*, *168*, 365-371. https://doi.org/10.1016/j.chemosphere.2016.10.090
Kumar, V., Prabha, V., Kaur, S., Kaur, K., & Singh, S. K. (2011). Receptor dependent immobilization of spermatozoa by sperm immobilization factor isolated from Escherichia coli: proof of evidence. *International Journal of Urology*, *18*(8), 597-603. https://doi.org/10.1111/j.1442-2042.2011.02784.x
Liang, H., Miao, M., Chen, J., Chen, K., Wu, B., Dai, Q., Wang, J., Sun, F., Shi, H., & Yuan, W. (2016). The association between calcium, magnesium, and ratio of calcium/magnesium in seminal plasma and sperm quality. *Biological trace element research*, *174*(1), 1-7. https://doi.org/10.1007/s12011-016-0682-7
López Rodríguez, A., Rijsselaere, T., Beek, J., Vyt, P., Van Soom, A., & Maes, D. (2013). Boar seminal plasma components and their relation with semen quality. *Systems Biology in Reproductive Medicine*, *59*(1), 5-12. https://doi.org/10.3109/19396368.2012.725120
Maes, D., Nauwynck, H., Rijsselaere, T., Mateusen, B., Vyt, P., de Kruif, A., & Van Soom, A. (2008). Diseases in swine transmitted by artificial insemination: an overview. *Theriogenology*, *70*(8), 1337-1345. https://doi.org/10.1016/j.theriogenology.2008.06.018
Martín, L. O. M., Muñoz, E. C., De Cupere, F., Van Driessche, E., Echemendia-Blanco, D., Rodríguez, J. M. M., & Beeckmans, S. (2010). Bacterial contamination of boar semen affects the litter size. *Animal reproduction science*, *120*(1-4), 95-104. https://doi.org/10.1016/j.anireprosci.2010.03.008
Morrell, J. M., & Wallgren, M. (2011). Removal of bacteria from boar ejaculates by Single Layer Centrifugation can reduce the use of antibiotics in semen extenders. *Animal reproduction science*, *123*(1-2), 64-69. https://doi.org/10.1016/j.anireprosci.2010.11.005
Prabha, V., Sandhu, R., Kaur, S., Kaur, K., Sarwal, A., Mavuduru, R. S., & Singh, S. K. (2010). Mechanism of sperm immobilization by Escherichia coli. *Advances in urology*, *2010*, 1-6. https://doi.org/10.1155/2010/240268
Savić, R., Petrović, M., Radojković, D. D., Radović, Č., & Parunović, N. (2013). The effect of breed, boar and season on some properties of sperm. *Biotechnology in Animal Husbandry*, *29*(2), 299-310. https://doi.org/10.2298/bah1302299s
Sepúlveda, L., Bussalleu, E., Yeste, M., & Bonet, S. (2014). Effects of different concentrations of Pseudomonas aeruginosa on boar sperm quality. Animal reproduction science, 150(3-4), 96-106. https://doi.org/10.1016/j.anireprosci.2014.09.001
Sepúlveda, L., Bussalleu, E., Yeste, M., & Bonet, S. (2016). Effect of Pseudomonas aeruginosa on sperm capacitation and protein phosphorylation of boar spermatozoa. Theriogenology, 85(8), 1421-1431. https://doi.org/10.1016/j.theriogenology.2015.12.025
Sozarukova, M. M., & Proskurnina, E. V. (2016). Serum albumin as a source of and a target for free radicals in pathology. *Bulletin of Russian State Medical University*, (1). https://doi.org/10.24075/brsmu.2016-01-10
Swine, S. P. (1990). Investigations on the control of bacteria in boar semen. Jpn J Anim Reprod, 36, 23P-29P. https://doi.org/10.1262/jrd1977.36.23p
Tvrdá, E. Belić, L., Ďuračka, M., Kováčik, A., Kačániová M., & Lukáč, N. (2018). The presence of bacterial species in bovine semen and their impact on the sperm quality. *Animal Reproduction Science*, 194, e3. https://doi.org/10.1016/j.anireprosci.2018.04.012
Tvrdá, E., Benko, F., & Ďuračka, M. (2022b). Oxidative Stress as an Underlying Mechanism of Bacteria-Inflicted Damage to Male Gametes. Oxygen, 2(4), 547–569. https://doi.org/10.3390/oxygen2040036
Tvrdá, E., Ďuračka, M., Benko, F., & Lukáč, N. (2022a). Bacteriospermia - A formidable player in male subfertility. Open life sciences, 17(1), 1001–1029. https://doi.org/10.1515/biol-2022-0097
Tvrdá, E., Kňažická, Z., Lukáč, N. (2010). Albumin, uric acid and bilirubin as a potencial spermatozoa protectant against oxidative stress. *Mladí vedci 2010* : zborník vedeckých prác doktorandov, mladých vedeckých a pedagogických pracovníkov, 169-175
Tvrdá, E., Lukáč, N., Schneidgenová, M., Lukáčová, J., Szabó, C., Goc, Z., Greń, A., & Massányi, P. (2013). Impact of seminal chemical elements on the oxidative balance in bovine seminal plasma and spermatozoa. *Journal of veterinary medicine*, *2013*, 1-8. https://doi.org/10.1155/2013/125096
Ziberna, L., Martelanc, M., Franko, M., & Passamonti, S. (2016). Bilirubin is an endogenous antioxidant in human vascular endothelial cells. *Scientific reports*, *6*, 29240, 1-6. https://doi.org/10.1038/srep29240