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Lohmann Brown Rooster Semen: Intrinsic Bacteria and Their Impact on Sperm Progressive Motility and Seminal Biochemical Parameters—A Preliminary Study

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Abstract: Semen quality plays a crucial role in poultry production; however, it may be impaired by the presence of numerous bacterial species. This study researched the impact of bacterial contamination of Lohmann brown rooster semen on the biochemical parameters of seminal plasma to evaluate its potential consequences on the sperm progressive motility. Semen was collected from 27 stud roosters, and the sperm concentration and progressive motility were measured using computer-assisted semen analysis (CASA). Seminal plasma was separated, and selected biochemical parameters were measured using commercially available assays. An aliquot of each semen sample was cultured, the colonies were counted and the MALDI Biotyper was used for bacterial identification. The samples were divided into three categories based on their sperm progressive motility and the data were compared and statistically evaluated. Moreover, Pearson's correlation analysis was performed. The results showed that the lower the sperm progressive motility, the higher the level of colony-forming units. Moreover, sperm concentration was significantly higher ($p < 0.05$) in the group with the highest bacterial occurrence and the lowest proportion of progressively motile spermatozoa. Calcium, magnesium, creatinine, uric acid, alkaline phosphatase, and total proteins significantly changed in semen samples with the lowest proportion of progressive motility. In conclusion, seminal plasma biochemistry may mirror changes occurring in semen as a result of bacterial presence in the reproductive tract of poultry.

Keywords: rooster semen; Lohmann brown; bacterial occurrence; progressive motility; seminal plasma; biochemical parameters

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1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), more than 130 million tons of poultry were produced in 2020 worldwide, which is almost 40 percent of global meat production. The prediction made by the latest Organization for Economic Co-operation and Development (OECD)/FAO Agricultural Outlook indicates that this fastest-growing meat production will continue to expand over the next decade, ultimately reaching 47% of the overall meat market. Artificial insemination in poultry is routinely used worldwide [1]. Most notably, in turkeys and broiler chicken, artificial

insemination is the only way to secure the production of poultry goods, because of their physical inability to complete the mating process [2,3].

The presence of bacteria in semen and their impact on spermatozoa uncovered a unique research field within male reproductive biology. The collection of semen from avian species is not a sterile process [2,4]. Ubiquitous bacteria have numerous possibilities to contaminate semen samples [5]. However, semen itself may contain various bacterial species, starting from bacteria with a neutral relationship to the male reproductive tract through opportunistic pathogens to strict pathogens causing inflammatory symptoms potentially leading to subfertility or infertility [6].

Recent studies concerning various animal species have described the effects of bacterial contamination of semen on the sperm quality in detail [7–12]. The most common bacteria in rooster semen include species from the genera *Escherichia*, *Enterococcus*, *Citrobacter*, *Pseudomonas*, *Corynebacterium*, *Staphylococcus* and *Acinetobacter* [13]. However, rooster semen may also contain pathogenic bacteria, such as, *Salmonella*, *Clostridium* and *Campylobacter* [14–16]. When hygiene standards are not taken into account during the semen collection, negative consequences of bacterial contamination may manifest, impacting sperm survival, and thus fertilization ability [17]. The transmission of uropathogenic bacteria may cause much bigger complications than just an unsuccessful fertilization, including increased mortality in animals, resulting in economical losses [18,19].

It seems that both bacterial diversity and load influence spermatozoa quality [8–10]. Bacteria in semen may represent a biological stressor and affect conventional semen parameters, including sperm motility, mitochondrial activity, membrane, and acrosome integrity, as well as DNA fragmentation. The probable negative effect of high bacterial occurrence in semen lies in the involvement of leukocytes and simultaneous oxidative imbalance leading to lipid and protein damage in spermatozoa [8,20,21]. Biochemical parameters of seminal plasma have been emphasized to contribute to the overall sperm structural integrity and functional activity, and thus may be considered as relevant biomarkers of semen quality [22]. So far, there is no research on how bacteria in rooster semen affect the seminal plasma composition. At the same time, it may be feasible to speculate that the bacterial presence could also be affected by the composition of seminal plasma. As such, the aim of our study was to reveal if bacterial presence represents a biological stress factor affecting the sperm progressive motility of rooster spermatozoa and to observe the relationships with the biochemical parameters of seminal plasma.

2. Results

The descriptive statistical data of all observed parameters are summed up in Table 1. For a better evaluation of the obtained data, results from 27 semen samples were evenly divided into three quality groups based on the sperm progressive motility: high quality (HQ; PRO $\geq$ 56%; n = 9), intermediate quality (IQ; 43% $\leq$ PRO < 56%, n = 9) and low quality (LQ; PRO < 43%; n = 9). The sample distribution analysis is displayed in Table 1.

The results indicate that the lower the proportion of progressively moving spermatozoa is, the higher is the level of colony-forming units (CFU). Thus, bacterial quantity was significantly higher in the IQ group when compared to the HQ group ($p < 0.05$) and simultaneously the CFU level was significantly higher in LQ when compared to both HQ ($p < 0.01$) and IQ ($p < 0.05$). Significantly higher sperm concentration was recorded in LQ when compared to HQ ($p < 0.05$), while an overall trend of increasing spermatozoa concentration was observed together with a decreasing sperm quality. The alkaline phosphatase (ALP) activity in the HQ group was significantly higher ($p < 0.01$) in comparison to the remaining groups. The highest content of calcium (Ca) and magnesium (Mg) in the seminal plasma was recorded in the samples of the HQ group. Decreased Ca and Mg levels were recorded particularly in the LQ group. Cholesterol (Chol) concentration was significantly lower in the IQ group when compared to HQ ($p < 0.05$). However, no significant differences were recorded in the LQ group when compared to the other groups. The most significant decrease occurred in total proteins (TP; $p < 0.001$) in the LQ group. Furthermore, uric acid

(UA) and creatinine (Creat) concentrations in the LQ group were also significantly lower when compared to the HQ group.

Table 1. Lohmann brown rooster semen: three quality groups (HQ > IQ > LQ) evenly divided based on their spermatozoa progressive motility.

Parameter	Arithmetic Mean (n = 27)	HQ (PRO $\geq$ 56%) (n = 9)	IQ (43% $\leq$ PRO < 56%) (n = 9)	LQ (PRO < 43%) (n = 9)
CFU [log10 CFU/mL]	12.78 ± 2.36	6.81 ± 0.63	13.35 ± 0.75 * HQ	18.18 ± 0.94 ** HQ, * IQ
PRO [%]	44.37 ± 3.15	62.11 ± 2.31	45.56 ± 1.31 ** HQ	25.44 ± 2.46 *** HQ, ** IQ
CON [M/mL]	11,789.00 ± 837.10	9840.00 ± 765.00	11,299.00 ± 591.70	14,226.00 ± 1456.00 * HQ
ALP [U/L]	1.36 ± 0.78	75.33 ± 4.19	53.33 ± 2.12 ** HQ	57.89 ± 2.84 ** HQ
ALT [U/L]	1.81 ± 0.67	21.94 ± 1.49	18.41 ± 1.32	18.34 ± 1.19
Ca [mmol/L]	0.45 ± 0.17	1.66 ± 0.26	1.17 ± 0.34 * HQ	1.07 ± 0.34 ** HQ
Mg [mmol/L]	0.17 ± 0.02	1.99 ± 0.39	1.86 ± 0.27	1.56 ± 0.24 * HQ
Phos [mmol/L]	0.57 ± 0.03	0.49 ± 0.05	0.45 ± 0.06	0.41 ± 0.06
Chol [mmol/L]	12.72 ± 0.53	0.64 ± 0.05	0.49 ± 0.07 * HQ	0.55 ± 0.05
Creat [µmol/L]	62.19 ± 2.56	143.50 ± 12.75	124.80 ± 14.56	113.20 ± 13.56 * HQ
BILI [µmol/L]	19.57 ± 0.79	0.80 ± 0.17	0.61 ± 0.20	0.75 ± 0.15
Trigs [mmol/L]	0.72 ± 0.09	0.18 ± 0.02	0.16 ± 0.02	0.16 ± 0.03
UA [mmol/L]	0.62 ± 0.04	0.75 ± 0.06	0.63 ± 0.04	0.46 ± 0.05 ** HQ
Urea [mmol/L]	1.89 ± 0.06	2.08 ± 0.28	1.84 ± 0.15	1.74 ± 0.20
TP [g/L]	4.46 ± 1.20	5.00 ± 0.55	4.41 ± 0.27 * HQ	3.95 ± 0.51 *** HQ
ALB [g/L]	2.84 ± 1.36	3.01 ± 0.31	2.87 ± 0.18	2.65 ± 0.23
			Identified bacterial species	
		Staphylococcus warneri, *Citrobacter braakii*, *Corynebacterium xerosis*, *Enterococcus avium*, *Pseudomonas pseudoalcaligenes*, *Corynebacterium glutamicum*	*Enterococcus avium*, *Ochrobactrum anthropi*, *Escherichia coli*, *Alcaligenes faecalis*, *Enterococcus casseliflavus*, *Pseudomonas composti*	*Staphylococcus aureus*, *Enterococcus avium*, *Escherichia coli*, *Enterococcus feacalis*, *Pseudomonas aeruginosa*, *Serratia liquefaciens*, *Rothia terrae*, *Pantoea agglomerans*, *Pseudomonas putida*

Legend: CFU—colony-forming unit, CON—sperm concentration, PRO—progressive motility, Ca—calcium, Mg—magnesium, Phos—phosphor, Trigs—triglycerides, Chol—cholesterol, Creat—creatinine, ALP—alkaline phosphatase, ALT—alanine transaminase, BILI—bilirubin, UA—uric acid, TP—total proteins, ALB—albumin; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The bacterial composition of samples revealed the presence of 11 bacterial genera, out of which 3 genera were Gram-positive representatives while 8 genera were classified as Gram-negative bacteria. The distribution analysis revealed that HQ was represented by five bacterial genera. Five genera were also present in the IQ group, while only the presence of *Enterococcus* and *Pseudomonas* genera overlapped with HQ. The LQ group contained species from seven bacterial genera. Three genera (*Staphylococcus*, *Enterococcus* and *Pseudomonas*) were also identified in the HQ group and four genera (*Staphylococcus*, *Enterococcus*, *Escherichia*, and *Pseudomonas*) overlapped with the IQ group. *Citrobacter* and *Corynebacterium* genera were uniquely detected in the HQ group, *Ochrobactrum* in the IQ group, and *Serratia*, *Rothia* and *Pantoea* in the LQ group. *Enterococcus* was the most prevalent genus in all semen specimens.

The most important correlations between CFU and semen quality parameters are summarized in Figure 1. The strongest correlation (0.678; $p < 0.0001$) was revealed between CFU and the sperm concentration. However, a significant negative correlation (−0.541; $p < 0.01$) was also observed between CFU and PRO. TP, Mg, Ca, Creat, UA, Urea, albumin (ALB), ALP, and alanine transaminase (ALT) were negatively correlated with the bacterial CFU. All data from the correlation analysis are displayed in Supplementary Materials, Table S1.

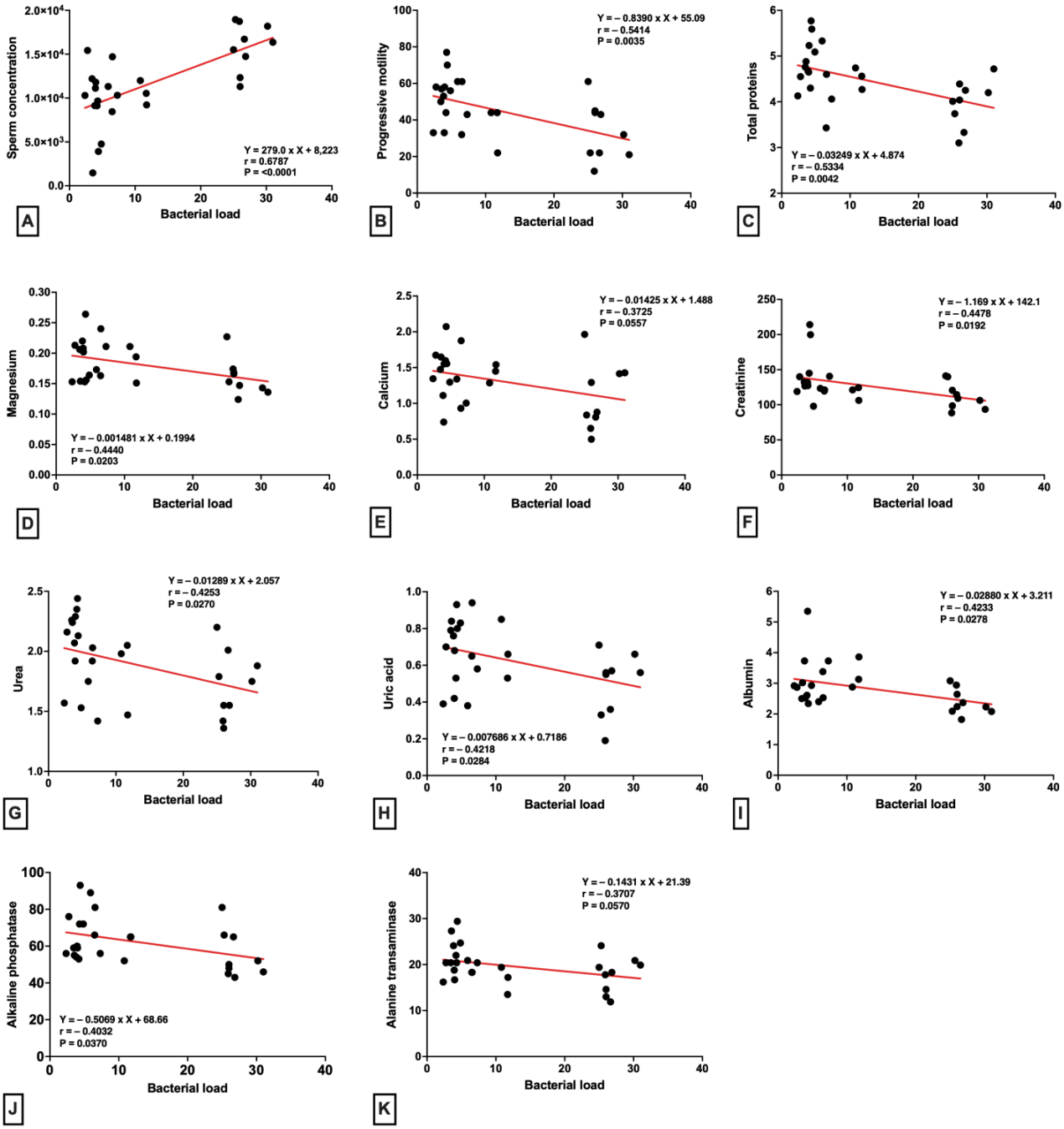

Figure 1. Scatter plots with trend lines summarizing correlations between bacterial load in semen and sperm/seminal quality parameters: (**A**) the relationship between sperm progressive motility and bacterial load; (**B**) the relationship between sperm concentration in semen and bacterial load; (**C**) the relationship between total proteins in seminal plasma and bacterial load; (**D**) the relationship between magnesium in seminal plasma and bacterial load; (**E**) the relationship between calcium in seminal plasma and bacterial load; (**F**) the relationship between creatinine in seminal plasma and bacterial load; (**G**) the relationship between urea in seminal plasma and bacterial load; (**H**) the relationship between uric acid in seminal plasma and bacterial load; (**I**) the relationship between albumin in seminal plasma and bacterial load; (**J**) the relationship between alkaline phosphatase in seminal plasma and bacterial load; (**K**) the relationship between alanine transaminase in seminal plasma and bacterial load. The red lines show trend lines and the black dots show the distribution of individual samples. All graphs include the equation for a linear relationship (Y), Pearson's correlation coefficient (*r*), and the *p* value.

3. Discussion

This study aimed to evaluate relationships between the biochemical properties of Lohmann brown rooster ejaculates and their bacterial composition. Our results showed that the bacterial concentration in the semen specimens with a poor progressive motility was higher than in ejaculates with a desirable progressive motility. Similar studies have been conducted on ejaculates collected from bulls [8] and boars [11] showing that both the bacterial composition as well as the bacterial load was higher in specimens with a suboptimal sperm motion behavior. The authors noticed that an increased presence of bacterial species triggered the immune response and led to oxidative stress which was accompanied by structural alterations of spermatozoa and changes in several biochemical markers of the seminal plasma, such as Ca, Mg, ALT, or Cholesterol (Chol).

Before we can properly discuss the data collected from this study, we must disclose that we did not determine the origin of bacteria. Bacterial colonization of semen may stem from the actual reproductive system or, in the case of poultry from the digestive tract, however, the environment may play an important role as a natural source of semen contamination. Moreover, insufficient hygiene standards of zootechnicians in charge of animal care and semen collection may also contribute to bacterial infestation in ejaculates. As such, cloacal swabs would help identify the source of bacteria in semen [23]. Previous reports showed that some opportunistic bacteria can impair sperm quality with increasing time [24] while pathogenic bacteria have been repeatedly referred to as stress factors contributing to male infertility. [25]. However, the exact timeframe needed for bacteria to cause damage to the sperm structures or to affect the seminal plasma composition is subject to further comprehensive research. We may also hypothesize that the male reproductive tract per se could create an environment suitable for any given bacterial species to grow and multiply. It was previously found that the testosterone levels could cause an increased bacterial diversity and relative abundance in the cloaca of male rufous-collared sparrows by promoting behaviors that increase sexual contact rates and/or by decreasing the immune function. Individuals with high testosterone levels had cloacal bacterial communities similar to each other, while subjects with low testosterone levels presented with more unique species [23].

An interesting phenomenon was revealed when evaluating the sperm concentration. The sperm concentration in the ejaculate positively correlated with CFU and negatively correlated with progressive motility. According to Pearson's correlation, only a few parameters were significantly negatively related to the sperm concentration, including TP, phosphorus (Phos), UA and ALT. Eini et al. [26] examined the effect of bacterial infection on the sperm quality in subfertile men with leukocytospermia. Their results showed that the sperm concentration was one of the impaired quality parameters while bacterial identification showed the prevalence of *Enterococcus faecalis*, *Staphylococcus agalactiae*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Proteus* spp. and *Klebsiella pneumoniae*. Ten percent of all was formed by multibacterial contamination. Sperm concentration and motility were significantly decreased in infected samples when compared to non-infected counterparts. Especially, *E. faecalis* and *E. coli* presented as bacteria with a particular negative impact on the sperm concentration, while both bacteria were connected to leukocytospermia [27,28]. In our study, *E. coli* was identified in samples categorized into the IQ group, and both, *E. coli* and *E. faecalis*, were identified in the LQ group. However, the sperm concentration in these groups was significantly higher, which contradicts the previous finding published by Zeyad and Moretti [27,28]. We did not include an evaluation of leukocytes in semen, which is one of the limitations of the study. However, a similar phenomenon was observed in cases of boar [29] and bull semen [8]. Tvrdá et al. [13] characterized the Lohmann brown rooster semen with the prevalence of *E. coli* and *E. faecalis*, which could mean that these two bacterial species mostly stress the sperm quality in our study also. Bacterial adherence may play a key role in this positive correlation between the sperm concentration and bacterial concentration. Bacteria may be able to adhere to the sperm surface with adhesive fibers called "pili" or "fimbriae" [30]. Furthermore, the sperm cells are more sensitive

than any kind of host cell because the sperm surface is rich in superficial glycoprotein receptors. Therefore, they are more susceptible to bacteria-mediated interactions at the receptor–ligand level [31,32].

Our study showed that the decrease in ALP activity in the seminal plasma is characteristic of semen with a lower proportion of progressively moving spermatozoa. On one hand, levels of ALP activity may interpret some failure in ejaculation or bilateral blockage of the ampullae. High ALP activity is also characteristic of azoospermic samples [33]. On the other hand, ALP activity positively correlated with immobile spermatozoa in boars [34].

The content of Ca and Mg in the seminal plasma, and particularly their mutual ratio in the seminal plasma, is important for the maintenance of the sperm physiology, while their decrease is connected to reduced sperm motility [35]. Our results show significantly lower levels of Ca and Mg in the seminal plasma of samples classified within the IQ and LQ groups. Recent research focused on the examination of seminal calcium and magnesium changes during in vitro simulation of bacterial infection. The authors reported that both Ca and Mg concentrations, significantly decreased in the groups infected by *Staphylococcus* species [36].

Creatinine (Creat) in the seminal plasma was present at significantly lower levels in the samples with poor sperm progressive motility. Moreover, the correlation analysis showed significant negative correlation with CFU. The role of Creat and creatine kinase is not fully understood. However, a recent study [37] revealed that Creat was associated with a reduced sperm motility, which is in agreement with our results. It seems that Creat plays a role in energetic metabolism of male gametes which is directly connected to the sperm movement [38].

Uric acid (UA) helps to maintain the sperm motility, viability and morphology, thus protecting the sperm functionality. The mechanism of protection is principally achieved by the neutralization of oxidizing and nitrating agents. Moreover, UA enhances certain bioactive enzymes important for vital sperm function. On the other hand, low levels of UA were observed in infertile men [39]. In our study, low levels of UA were recorded in the LQ group. The increased presence of bacteria in semen contributes to oxidative stress [13]. We may speculate that UA counteracts reactive oxygen species and thus lower concentration of UA was measured in the group with the highest bacterial concentration. However, our study did not include oxidative profile analyses, so we can only assume that the reduced UA content is due to an increased concentration of bacteria in the LQ group.

The composition and amount of seminal plasma proteins have an important effect on the sperm functionality, particularly on motility [34], viability [35], capacitation and fertilization [36]. Their presence in the seminal plasma is necessary to protect the spermatozoa from damage and maintain their longevity [40]. In our study, lower levels of TP in seminal plasma were detected in the IQ and LQ groups and the correlation analysis revealed a significant correlation with CFU. Although Janiszewska et al. [41] concluded that the total seminal plasma protein content is not a sufficient marker for the identification of the cause of male infertility, a previous study [42] reported that decreased seminal plasma protein concentrations were associated with sperm motility at $\leq$5th centile.

In conclusion, we may suggest that bacteria may represent a biotic stress factor in rooster semen as there are several remarkable connections between the presence of bacteria and semen quality which have been observed in our study. Spermatozoa with low progressive motility had an increased bacterial load and simultaneously impaired seminal plasma biochemical parameters, including Ca, Mg, UA, ALP, Creat and TP. The most prevalent bacterial genus in rooster semen was *Enterococcus*. The sample distribution and correlation analysis indicated that ejaculates with the highest sperm concentration presented with the highest bacterial load. These results may outline possible relations between bacterial communities in Lohmann brown rooster semen. However, more semen specimens and rooster lines are needed to be studied in order to extrapolate the results on a more general level.

4. Materials and Methods

4.1. Collection of Biological Material

Semen samples ($n = 27$) from healthy Lohmann brown roosters were collected using the cloacal massage. Before the semen collection, the animals were let to defecate, their cloacae were washed with water and soap, and dried with disposable paper towels. The minimum volume of the fresh semen samples included in this study was 0.5 mL. The animals were kept at a local poultry farm (Liaharenský podnik Nitra, a.s., Párovské Háje, Slovakia) and were 60–65 weeks old. Between each collection, disposable gloves were changed. The ejaculates were collected into sterile syringes and transported within 30 min to the laboratory in the Mini Bio Isotherm vessel pre-heated to 37 °C (M and G Int., Renate, Italy). An aliquot of each specimen was subjected to bacteriological analysis, then the progressive motility was measured, and seminal plasma was separated for biochemical analysis.

4.2. Bacteriological Cultures and Identification

One hundred microlitres of diluted semen samples were aerobically cultured on blood and tryptic soy agar at 37 °C for 24–48 h. The colonies were counted, and bacterial quantity was expressed as the common logarithm of colony-forming units in mL. The different colonies were purified using the four ways streak plate method. Colonies were transferred to a new plate using a pre-sterilized inoculation loop. Four strokes were conducted at the top of the plate. Four more strokes were conducted starting from the previous strokes at an angle of approximately 100–110°. Four quadrants were performed and between each quadrant, the inoculation loop was sterilized [43]. Qualitative identification was performed using the matrix-assisted laser desorption ionization- time of flight system (MALDI-TOF, Bruker Daltonics, Bremen, Germany). Overnight isolates were extracted in a formic acid/ethanol mixture. The samples were placed onto MALDI plate and covered with a matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and left to air-dry. The analyzed spectra were compared with reference spectra in database (Biotyper software, version 2.0; Bruker Daltonics, Bremen, Germany) [9].

4.3. Sperm Concentration and Progressive Motility Assessment

Sperm motility and concentration assessments were performed using computer-assisted semen analysis (CASA; Hamilton- Thorne Biosciences, Beverly, MA, USA). Diluted semen samples (1:100) were placed into the pre-warmed (37 °C) Makler counting chamber and a minimum of 300 cells per field (10 fields in total) were subjected to the motility analysis. The analysis setup was as follows: frames per second—60 Hz, no. of frames—30, minimum contrast—50, minimum cell size—4 pixels, path velocity (VAP) of progressive cells—50 μm/s, straightness of progressive cells—80%, cell size—25 pix, cell intensity—80, VAP cutoff—10 μm/s, VSL cutoff—0 μm/s. The optics setup was as follows: intensity—2215, magnification—1.89.

4.4. Biochemical Analysis of Seminal Plasma

Selected biochemical parameters were analyzed using the fully automated clinical biochemistry analyzer RX Monaco (Randox, Crumlin, UK) including Cholesterol (Chol), Triglycerides (Trig), Total proteins (TP), Albumins (ALB), Urea (U), Uric acid (UA), Bilirubin (BR), Creatinine (Creat), Aspartate transaminase (AST), Alanine transaminase (ALT), Calcium (Ca), Phosphor (P) and Magnesium (Mg) [29].

4.5. Statistical Evaluations

The GraphPad prism statistical program (version 8.0 for Windows, GraphPad Software, San Diego, CA, USA, http://www.graphpad.com/ (accessed on 4 April 2023)) was used for the statistical analysis of the data that were collected in duplicates. Before statistical analyses, we subjected the obtained data to the Shapiro–Wilk normality test. The descriptive statistics describe the arithmetic mean of each measured value with standard deviation.

The obtained data were subjected to the Pearson correlation analysis. At the same time, relationships among the bacterial load and semen characteristics were evaluated using regression analysis. Samples were evenly divided into three quality groups based on the proportion of progressively moving spermatozoa. The high quality (HQ) group was defined with PRO $\geq$ 56%, the intermediate quality (IQ) with PRO oscillating between 43 and 56% (56 < PRO $\geq$ 43) and the low quality (LQ) group with PRO < 43%. Differences between the quality groups were evaluated using one-way ANOVA followed by the Tukey multiple comparison test. The significance levels for the Pearson correlation and the Tukey test were set at $p < 0.0001$ (****), $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/stresses3020031/s1, Table S1: Pearson's correlation analysis: associations between colony-forming units, sperm concentration, progressive motility and biochemical parameters of seminal plasma.

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