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Article

Effect of partial substitution of prickly pear seed meal (*Opuntia ficus indica* L.) on serum biochemical parameters and intestinal microbiota in broiler chickens

Moncef Benteboula[1,2,3], Jean-Luc Hornick[4], Omar Besseboua[5], El-Hacene Balla[6,7], Amal Bouzenad[8], Lamia Taourirt[8], Isabelle Dufrasne[4], Nadir Boudjlal Dergal[9], Kálmán Imre[10], Mirela Imre[10], Ulas Acaroz[11,12], Damla Arslan Acaroz[13,14] and Abdelhanine Ayad[7,*]

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Abstract: The aim of the present study was to assess the effect of prickly pear seed (PPS) cake on biochemical parameter and gut microbiota in broiler chickens. One hundred and fifty 1-day-old broiler chicks were allocated into 5 groups with 3 replicates of 10 birds per group. The experimental groups received the diets substituted with 0, 10%, 20%, 30%, and 40% prickly pear seeds meal of OFI during a 6-week. At 45 d of age, five chickens were randomly selected from each treatment group. After 12 h of feed withdrawal, serum and cecal samples were collected use for biochemical parameters assay and PCR Amplification. The results of serum lipid parameters shown that the different PPS dietary treatments significantly ($P<0.05$) reduced the concentration of glucose concentration, total proteins total cholesterol, and lipidemia compared to control group of broiler chickens. It showed significantly decreased ($P < 0.05$) liver enzymatic activity in treated group with PPS cake compared to control group of broiler chickens. Also, ionogram showed that values of Mg, P, and Ca reduced significantly ($P<0.05$) after treatment of chickens with PPS cake concentrations compared to control group. At the phylum level, Firmicutes and Bacteroidetes were the major groups of microorganisms in the gut of broiler chickens received PPS cake diet. In conclusion, this study demonstrated that PPS cake can have a protective effect on the kidney and liver function of chickens. Also, it found that broiler chickens treated with PPS cake meal exhibited a variety of phylum and genus in gut microbiota.

Keywords: Biochemical parameters; gut microbiota; prickly pear; seeds meal; broiler chicken.

1. Introduction

The cactus (*Opuntia ficus-indica L*), commonly known as prickly pear, belongs to the family *Cactacea* [1]. This plant family is reported to contain about 130 genera and nearly 1500 species [2]. Family *Cactaceae* is native to Mexico and is widely distributed in all American countries, Africa and the Mediterranean basin, and some Asian areas [3]. *Opuntia cactus* grows mainly in arid with the annual rainfall is less considerable (< 250 mm and 250-450 mm, respectively) [4]. It is noted that cactus was introduced to North Africa in 17th century in order to combat erosion and desertification.

A program for the promotion of pastoralism based on cactus agriculture has also been implemented by the Algerian High Commission for the promotion of the Steppe (HCDS) in dry and semi-arid regions including Tebessa, Khenchela, and Souk-Ahras (Northern-East Algeria). Cactus pear has taken a large importance in agricultural economics [5]. Also, *Opuntia ficus-indica* (OFI) contributes to sustainable food and feed production for humans and animals in countries with very low rainfall [6]. It produces a highly nutritive fruit and the cladodes which are used fresh green vegetable and salad. In Algeria, the cactus is grown mainly as a fruit crop and is a major consumer product generating huge quantities of seeds. In addition, the cactus could replace common plant species, especially in arid and semi-arid areas where the animal production section frequently suffers from low efficiency and high losses [7].

According to certain reports, cacti are a source of vitamins and minerals that are highly valuable in food and can be used in a variety of pharmaceutical and cosmetic industries [8]. The cactus pear shows a relatively high polyphenols and betalain compounds [9], which are bioactive compounds with positive health effects [10]. Indeed, it has been used in traditional folk medicine because of its role in treating a number of diseases, including anti-inflammatory effects, hypoglycemic effects, inhibition of stomach ulceration and neuro-protective effects [1,11,12]. Moreover, they are used for anti-hyperlipidemic and antiviral activities [13]. In addition, other investigations have demonstrated the potential role of protecting the liver [14] and inhibition of tumor cell growth [15]. Likewise, several studies showed that *Opuntia ficus-indica* juice present an important source antioxidant [13,16].

The animal gastrointestinal tract contains crucial physiological functions [17]. Several factor such as host genetics, nutrition, and treatments with antibiotics can affect the microbiota and its metabolic process [18]. It is important to remind that plant extracts function as prebiotics, altering the commensal microbiome, the composition, and/or the metabolism of the intestinal microbiota, potentially improving the host's health. [19].

It has been demonstrated that phenolic compounds and their metabolites shown a positive impact on maintaining gut health through encouraging the growth of beneficial microbiota [20] and limiting the proliferation of pathogen bacteria such as *Clostridiumperfringens* and *Bacteroides* spp. [21]. The polyphenol compounds may also affect the gut microbiota composition [22-24]. Studies reported that several factors may considerably influence on serum biochemical parameters of chickens including feed additives [25, genotype and environmental temperature [26]. There are numerous locally accessible and nutritionally sufficient plants that can be added to animal diet. Numerous studies have shown that the quantity and characteristics of plant extracts can raise the biochemical indices in broilers [27-30].

In recent years, the use of growth promoters of plant origin as a natural additive in poultry feed has aroused particular interest in poultry farmers. Many studies have been carried out on animal nutrition using the alternative feedstuffs such as co-products due to constraints environmental and food production costs. However, it is very necessary to verify the effect of co-product on the animal health and the quality of food of animal origin. Herb extracts in animal nutrition stimulate feed intake by the secretion of endogenous enzymes, antibacterial effect and antioxidant potential.

The digestive tract's physiological and biochemical processes, particularly the liver's, may be impacted by the active plant compounds. The analysis of biochemical

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parameters is important approach in the management of animal health; it is a tool common to assess nutritional status and monitor health and disease in farm animal [31]. Moreover, the biochemical blood indices are information helpful in revealing health disorders already in the preclinical stage. Additionally, numerous researchers have already demonstrated the effect of plant extracts in the diet on the biochemical parameters in treatment [32-35]. However, to the author's knowledge, little research has focused on the impact of prickly pear seed meal; a plant is widely present in the Algerian rural, in broiler chicken's livestock. Therefore, the aim of the present study was to assess the effect of prickly pear (*Opuntia ficus-indica* L.) seed cake on biochemical parameter (lipid, protein, carbohydrate, mineral metabolism and liver enzymes) and gut microbiota in broiler chickens.

2. Materials and Methods

The experimental protocol was approved by the Scientific Faculty Council of the University of El-Tarf (Report of Faculty Scientific Council #06 dated January 23, 2024, Algeria) and the authors followed the regulations applied in University of Liege (Belgium).

2.1. Animals, Experimental Design, and Management

A total of 150 Arbor Acres broiler chicks male and female were purchased from a commercial hatchery at one day of age. Their average weight was 45±19.54 g. Chicks were randomly allocated to three groups [3 replicates (pens of 2 m^2) per group and 10 broilers per replicate] for a 44-day study. Broilers of each group were kept with optimum natural ventilation and room temperature maintained at 35°C during the first week of age, and gradually decreased to 25°C until the end of the study. Lighting regime was used throughout the experimental period (24 hours of lighting for the first five days; then 18 h light/ 6 h dark every day until the end study). Birds were vaccinated against Gumboro (IBA-VAC®), and Newcastle (BIO-VAC® B1) diseases. In order to prevent coccidiosis, the chicks were treated by anticoccidial (HIPRAVIAR®) at 15 and 28 days during 3 days. Chicks were managed according to the guidelines suggested by Cobb Broiler Commercial Management Guide (Arbos Acres Plus).

The animals were fed for 44 d, including starter (Day 1 - Day 12), growth (Day 13 - Day 32), finisher (Day 33 - Day 39) and withdrawal (Day 40 - Day 44) phases. NRC nutrient requirements from 1994 were satisfied by the diets. The chicks were divided into four groups: one control group, which was provided basal diets, and three experimental groups, which were fed meals supplemented with 10%, 20%, 30%, and 40% OFI prickly pear seed (PPS) meal. Every chick had *ad libitum* access to feed and water.

2.2. Blood and intestinal Sample Collection

Five chickens were chosen randomly from each treatment group at 45 days of age. After 12 h of feed withdrawal (except the water offered *ad libitum*), blood samples (n = 5) were collected after slaughter from the jugular vein using in EDTA tube. Serum was rapidly separated after centrifugation at 3,000 rpm for 10 min and stored at -20 °C until use for biochemical parameters assay. To determine the cecal microbiota, five cecal contents were collected aseptically in sterile plastic tubes and kept at 4°C. The cecal digesta was processed within 24h at the Pasteur Institute of Algeria (Algiers, Algeria).

2.3. DNA Extraction and PCR Amplification

Cecal samples were used for DNA extraction using the InnuPREP Stool DNA Kit (PREPStool DNA Kit, AJ Innuscreen GmbH, 13125 Berlin, Germany) following the manufacturer's instructions. The Microbial DNA was transferred to the microbiology laboratory of Department of Food Sciences (Faculty of Veterinary Medicine, University of Liège, Belgium). The total quantity of viable bacteria in each sample was estimated using qPCR after PMA treatment, as previously indicated (Reference). The V2-V3 region of the 16S rRNA genes were PCR-amplified from the microbial genomic DNA with primers (forward (50-GAGAGTTTGATYMTGGCTCAG-30) and reverse (50-ACCGCGGCTGCTGGCAC30)) using a real-time qPCR system (PRISM 7900HT) [36]. Clusters of sequences have been categorized as operational taxonomic units (OTUs), and sample OTU sequences (with a cutoff threshold of 0.03) have been categorized as taxa using the VSEARCH algorithm [37]. Sixteen reference alignments and taxonomic assignments from phylum to genus

were conducted using MOTHUR based on the SILVA database (v1.38) of full-length 16S rRNA sequences (ribosomal Silva).

2.4. Serum Biochemical Parameters

A multi-parameters automatic biochemical analyzer (ARCHITECT ci4100®, Abbott, US) was utilized for all blood parameter test procedures. The photometric method was used to measure the serum levels of blood glucose (Glu), total protein (TP), triglycerides (TGs), total cholesterol, HDL, LDL, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), urea (Ur), creatinine (Cre), blood uric acid (BUA), and alpha fetoprotein (AFP). Using the potentiometric approach, ionic parameters like calcium, magnesium, and phosphorus are measured. Each parameter was determined in duplicate simultaneously at 37°C.

2.5. Statistical analysis

The data showed the descriptive mean ± standard deviation. One-way ANOVA analysis was performed using the SAS® software (version 9.4, Institute Inc, Cary, NC, USA). The graphs were performed with the GraphPad Prism 8.00 software package (GraphPad Software, San Diego, CA, USA). Fisher's PLSD post hoc analysis was performed to make statistical comparisons to analyze the differences between groups. $P<0.05$ was considered to be significant.

3. Results

The effect of the substitution rate of prickly pear cake (*Opuntia ficus indica* L.) on blood glucose and total protein in broiler chickens are presented in Table 1. Blood glucose concentration and total proteins values were significantly low in the group treated with 40% PPS (1.28±0.37 and 12.83±5.52 g/l, respectively) ($P<0.05$). The results of serum lipid parameters shown that the 10%, 20%, 30% and 40% PPS dietary treatments significantly ($P<0.05$) reduced the concentration of total cholesterol, HDL, LDL and triglyceride compared to control group of broiler chickens (Table 1).

Table 1. Serum biochemical profile of broiler chickens fed diets with different inclusion levels of prickly pear (Opuntia ficus-indica L.) seed cake at 45 days of age.

Parameters	PPS 0%	PPS %	PPS 20%	PPS 30%	PPS 40%	SEM	P >F	Feed effect	R^2
Glu (g/L)	2.36^{a}±0.52	1.87^{b}±0.54	1.68^{c}±0.37	1.63^{c}±0.33	1.28^{d}±0.37	0.10	<.0001	***	0.57
TP (g/L)	28.22^{a}±3.23	26.58^{b}±10.37	19.49^{c}±6.69	17.42^{d}±7.57	12.83^{e}±5.52	1.63	<.0001	***	0.59
TCH (g/L)	1.12^{a}±0.22	0.95^{b}±0.27	0.79^{c}±0.18	0.8^{c}±0.12	0.71^{c}±0.26	0.05	<.0001	***	0.53
HDL (g/L)	0.72^{a}±0.12	0.59^{b}±0.19	0.56^{b}±0.12	0.55^{b}±0.08	0.50^{b}±0.18	0.03	0.0001	*	0.53
LDL (g/L)	0.21^{a}±0.07	0.21^{a}±0.07	0.14^{b}±0.07	0.12^{b}±0.04	0.12^{b}±0.07	0.01	<.0001	**	0.42
TG (g/L)	0.87^{a}±0.33	0.71^{a}±0.31	0.60^{b}±0.22	0.58^{b}±0.37	0.43^{c}±0.29	0.07	0.0016	**	0.43

^{a,b,c,d,e} A significant difference in carcass part characteristics between the control group (0% PPS) and the treated groups (10%, 20%, 30% and 40% PPS) is indicated by letters ($P< 0.05$). SEM = Standard error of the mean.
Glu: glucose, TP: total protein, TCH: total cholesterol, HDL: high-density lipoprotein cholesterol, LDL: low-density lipoprotein cholesterol, TG: triglycerides

According to our findings, the broiler chickens' serum activity of CGT, ASAT, and ALAT was considerably ($P<0.05$) lower in the PPS cake-treated group than in the control group. However, in

experimental broiler chickens, PPS cake supplementation had no effect on the blood activity of urea and creatinine (P>0.05) (Table 2).

Table 2. Serum biochemical profile of broiler chickens fed diets with different inclusion levels of prickly pear (*Opuntia ficus-indica* L.) seed cake at 45 days of age.

Parameters	PPS 0%	PPS %	PPS 20%	PPS 30%	PPS 40%	SEM	P >F	Feed effect	R^2
GGT (U/L)	22^{a}±3.25	20.6^{b}±5.65	16.6^{c}±4.89	16.9^{c}±4.40	15.7^{d}±7.37	1.24	0.0016	***	0.42
ALAT (U/L)	7.8^{a}±1.89	6.86^{b}±1.24	6.4^{c}±0.63	6.68^{b}±1.10	7.53^{a}±1.68	0.34	0.0295	***	0.30
ASAT (U/L)	208.06^{b}±35.92	224.2^{a}±62.51	193.13^{c}±89.72	152.91^{d}±38.62	155.46^{d}±56.28	16.07	0.0071	***	0.25
Ur (g/L)	0.4^{a}±0.0	0.4^{a}±0.0	0.4^{a}±0.0	0.4^{a}±0.0	0.4^{a}±0.0	0.00	.	NS	0.00
Cre (mg/L)	2.13^{a}±0.35	2.06^{b}±0.25	2^{b}±0.0	2^{b}±0.0	2^{b}±0.0	0.05	0.26	NS	0.16
BUA (mg/L)	48.38^{a}±15.83	39.48^{c}±16.34	41.47^{b}±15.66	40.31^{b}±15.44	32.42^{b}±13.43	3.95	0.09	***	0.23

^{a,b,c,d,e} A significant difference in carcass part characteristics between the control group (0% PPS) and the treated groups (10%, 20%, 30% and 40% PPS) is indicated by letters (P< 0.05). SEM = Standard error of the mean.
GGT: gamma-glutamyltransferas, ASAT: aspartate aminotransferase, ALAT: alanine aminotransferase, Ur: Urea, Cre: Creatinine, BUA: blood uric acid

The Table 3 illustrates the Calcium (Ca), Phosphorus (P), Magnesium (Mg) concentrations in broiler chickens treated with 10%, 20%, 30% and 40% PPS cake concentrations. In general, blood ionogram showed that values of Mg, P, and Ca reduced significantly after period 44 days of treatment with 10%, 20%, 30% and 40% PPS cake concentrations compared to control group (P<0.05). Except, K+ values significantly increased treated group with 10% of PPS (P<0.05), and it did not vary in treated group with 20% of PPS in relation to the control group (P>0.05).

Table 3. Ionic parameters profil of broiler chickens fed diets with different inclusion levels of prickly pear (*Opuntia ficus-indica* L.) seed cake at 45 days of age.

Parameters	PPS 0%	PPS %	PPS 20%	PPS 30%	PPS 40%	SEM	P >F	Feed effect	R^2
Mg (mg/L)	21.62^{a}±3.03	16.8^{c}±4.15	18.38^{b}±6.09	16.96^{c}±3.69	16.69^{c}±5.62	1.06	0.0073	***	0.43
P (mg/L)	40.70^{b}±13.13	49.83^{a}±13.55	40.02^{b}±8.57	33.02^{d}±10.71	38.21^{c}±13.00	2.94	0.0041	***	0.36
Ca (mg/L)	77.3^{a}±11.59	62.01^{b}±21.36	54.28^{c}±12.54	54.72^{c}±11.91	45.43^{d}±11.83	3.52	<.0001	***	0.51
AFP (ng/ml)	0.01^{a}±0.01	0.01^{a}±0.00	0.01^{a}±0.00	0^{a}±0.00	0.01^{a}±0.01	0.002	0.0002	NS	0.45

^{a,b,c,d,e} A significant difference in carcass part characteristics between the control group (0% PPS) and the treated groups (10%, 20%, 30% and 40% PPS) is indicated by letters (P< 0.05). SEM = Standard error of the mean.
Mg: magnesium, P: phosphorus, Ca: calcium, AFP: alpha-fetoprotein.

A principal coordinate analysis (PCoA) plot illustrating the variations in the intestinal microbial composition of broiler chicks fed different cake diet groups (0%, 10%, 20%, 30%, and 40% PPS) is depicted in Figure 1. Samples from the 10% and 20% PPS cake groups clustered closer together than samples from the other groups.

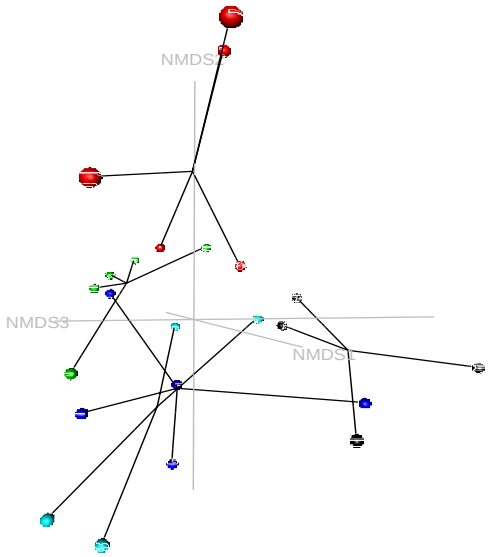

Figure 1. A principal coordinate analysis (PCoA) plot showing dissimilarities among different diet groups. Each dot represents a single sample. Black, red, green, blue and cyan blue color denote 0%, 10%, 20%, 30% and 40% PPS cake diet, respectively.

Permutational multivariate analysis of variance (ANOVA) was also used to determine the significant differences between groups. The significant difference between the five groups was observed (P-value < 0.05; Table 4). In the present study, bacterial analysis revealed that broiler chickens treated with *Opuntia ficus-indica* seed meal exhibited a variety of phylum in gut microbiota (Fig. 2).

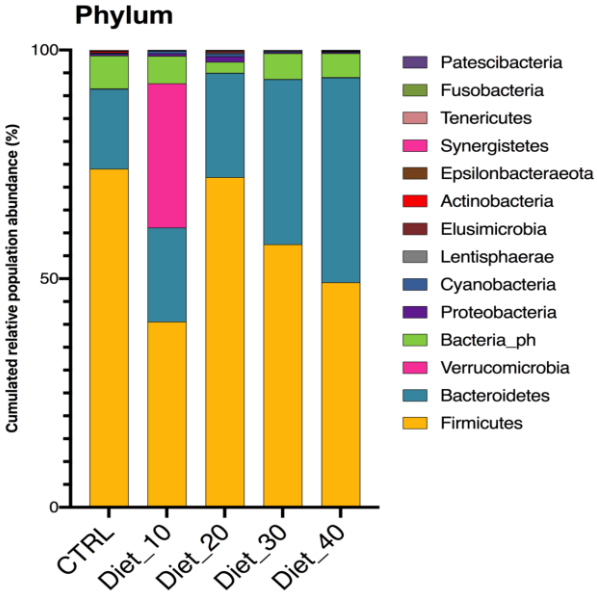

Figure 2. The intestinal microbial composition and species diversity of chickens fed diets with different concentration of PPS cake at the phylum level. Bar plots represent the percentage (%) of average abundance for groups.

At the phylum level, Firmicutes and Bacteroidetes were the major groups of microorganisms in the gut of broiler chickens received PPS cake diet, ranged 40-72% and 20-44%, respectively. On the other hand, the

10% PPS diet contains also 31% of *Verrucomicrobia* compared to other diet groups ($P< 0.05$). As shown in Figure 3, there are some changes in the structure of gut flora after PPS cake treatment compared to control group, according to taxonomic research. *Lachnospiraceae*, *Bacteroide*, *Akkermansia*, *Bacteroidales* were the most prevalent genera.

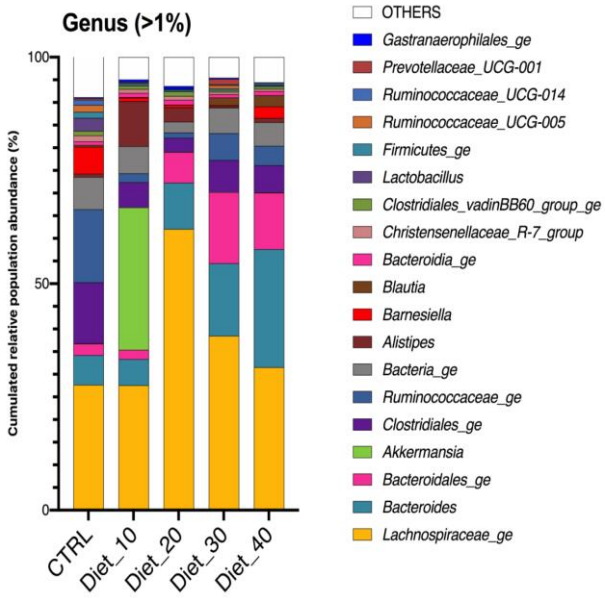

Figure 3. The intestinal microbial composition and species diversity of chickens fed diets with different concentration of PPS cake at the genus level. Bar plots represent the percentage (%) of average abundance for groups.

4. Discussion

Blood biochemical profiles are mainly used as indicators of an animal's physiological and metabolic condition [38]. Many researchers reported on positive effects of herbs inclusion diet on performance and health status of broiler chickens [39-41], and also can have a positive effect on lipid metabolism and antioxidant status [42]. Furthermore, plant extracts have been shown to improve the activity of digestive enzymes [42] and control the kidney and liver functions in chickens [43]. In addition, the use of plant as dietary supplement can improve hematological indices and serum biochemistry profiles of broiler chickens [44]. Plant extracts were continually experimented, as growth additive, in animal feed due to their biological benefits in improving digestion efficiency and animal health status [45-47]. Phytoadditive used on livestock and poultry are excellent alternatives to antibiotic-based growth promoters due to their proven positive effects on animal growth and health [48]. Essential oils are increasingly appreciated in animal feed due to their high biological activity. To our knowledge, the current study is the first on the impact of different feed diet level of OFI seed cake on serum biochemical indices and gut microbiota in Arbor Acres broiler chicken.

According to the current results, the PPS dietary treatments considerably ($P<0.05$) decreased the concentration of lipid parameters in the broiler chickens compared to the control group, of serum lipid parameters. There were significant differences ($P<0.05$) between the treated groups for blood glucose concentration and total proteins values, with higher values in broiler chickens supplemented with different PPS concentrations compared to the control group. These results are in agreement with results obtained previously, where [49], where *Opuntia ficus-indica* cladode, fruit reduce glycemia and cholesterol and triglycerides level in diabetic experimental animals. Moreover, Moula et al. (2019) [50] reported that the addition of 10% prickly pear cladodes powders decreased significantly ($P<0.05$) the concentrations of glucose, triglycerides and cholesterol in broiler chickens. In another study, the biochemical analysis showed that white male rabbits given an aqueous extract of *Opuntia ficus indica* had considerably lower blood plasma levels of glucose, cholesterol, and triglycerides [1]. This hypoglycemic effect due to the fact that the cactus is

very rich in dietary fiber which increases the enzymatic activity of certain microbes, resulting in an increase in sugar production microbial enzymatic activity, which leads to the inhibition of hydrolysis [51].

Likewise, Jibril et al. (2018) [52] demonstrated that supplementation with powdered balsam apple (*Momordica balsamina*), contain biologically active phytochemicals such as flavonoids, leaf had significantly decrease on blood glucose level Japanese quails. Recently, Gazwi et al. (2022) [53] reported that the glucose, cholesterol, LDL and triglyceride concentrations was significantly decreased upon the supplementation of *C. sativum* and *C. intybus* extracts, riche in flavonoids, in the diet of broiler chicks at six weeks of age. The presence of secondary chemicals in cacti may be the cause of the decrease in glucose concentration since they increase insulin secretion and improve muscle absorption and metabolism of glucose. In addition, flavonoids could be linked to antidiabetic properties by binding to starch, boosting hepatic glycolysis and the glycogen level, and reducing hepatic gluconeogenesis [54,55] Moreover, Padilla-Camberos et al. (2015) [56] concluded that *Opuntia ficus-indica* cladode extract is able to prevent hypercholesterolemia by pancreatic lipase inhibition in mice, in part due to its polyphenolic compounds. Similarly, it has been found that plant extracts containing flavonoids can improve the lipid metabolism of broiler chicks [57].

The liver and kidney are of the most vital organs of living organisms. They has a crucial role in detoxification and metabolism, that to be susceptible to damage from metabolic disorders. The status of liver and kidney function can be measured via the increase or decrease in serum levels of biochemical parameters such as CGT, ASAT, ALAT, urea and creatinine, which are considered diagnostic tools to assess toxicity [58,59]. The decreased activity of ALAT and ASAT indicated the hepatoprotective nature of the PPS cake contained bioactive compounds due to its antioxidant properties.

It is important to underline that prickly pear seeds (PPS) meal of OFI possesses a significant concentration of flavonoids and secondary metabolites, including gluconic acid, pscidic acid, ferulic acid diglucoside and quercetin-3-O-galactoside [60]. This is consistent with the findings of Bouazza et al. (2016) [61] studied the effect of OFI in liver steatosis created by feeding rats a high-fat diet, who discovered that incorporation of the vinegar obtained from prickly pear have hepatoprotective effects in rats. Indeed, numerous studies have shown that *O. ficus-indica* has hepatoprotective properties [62-64]. Likewise, the results of the present study showed that serum creatinine, urea and blood uric acid levels were unchanged in the groups treated with PPS cake and the control group. In the other hand, blood uric acid level was significantly low in PPS cake compared to the control group ($P<0.05$). These results indicated that PPS cake had no deleterious effects on renal function. In addition, the extract of OFI fruits contains antioxidant molecules such as quercetin, myricetin, and luteolin which can synergistically offer a renoprotective effect, as noted by Okur et al. (2020) [65].

A number of factors, such as the manner of supply and the presence of other nutrients, influence the percentage absorbed and the efficiency of macronutrient absorption. It is most likely that the absorption Ca, P and Mg was low in intestine tract during digestion the prickly pear seeds meal of OFI, resulting in reduce in serum concentration. However, it should be noted that *Opuntia ficus indica* seeds contains a large variety of minerals with a predominance of potassium, phosphorus magnesium and calcium [40].

It is very important to remember that cacti (*Opuntia ficus-indica*) possess the antinutritional factor calcium oxalate, which binds to calcium and possibly other minerals in a nutritionally unavailable form, thus interfering with the bioavailability of minerals for animal absorption [66]. Phytate occur in grains and seeds of crops, which this compound forms insoluble complexes with divalent ions of metals (Ca, Mg, Zn, Fe) and with phosphorus, reducing their bioavailability and thus increasing the requirement of such components [67]. This reduce may be due to the effect of phytate and oxalic acid present in the cactus pear seeds because it is an organic compound that binds to calcium or other minerals in an unavailable nutritional form, affecting the availability for absorption by the broiler chickens [68-70]. On the other hand, no significant differences were observed between in alpha-fetoprotein (AFP) values of treated group with PPS cake and control group of broiler chickens ($P> 0.05$). The results obtained of the present investigation indicate clearly the absence of malignant transformation in broilers chickens. Results of the anticancer activity of *Opuntia ficus-indica* were reported previously by Ali et al. (2022) [71], that flavonoids of OFI possessed many notable biological activities; such as anti-oxidant and anti-carcinogenic activities. Moreover, Chavez-Santoscoy et al. (2009) [72] reported that *Opuntia violaceae* (Spiny, purple peel and purple red pulp) contained the highest flavonoids and diminished both prostate and colon cancer cell viability.

Animal health and growth performance depend on maintaining a healthy degree of intestinal development. The digestive tract of chickens contains to a wide range of microorganisms coexist in symbiotic interactions that impact immunity, metabolism, and nutrition [73]. Several investigations reported that have reported a wide variety of plant extracts improve intestinal health indices in poultry [74-76]. To elucidate the differences in microbiota exposed to different amounts of PPS cake, a principal coordinate analysis (PCoA) based on measurements the intestinal microbial composition was performed, which it makes possible to visualize variation between samples and possibly identify groups by projecting observations into a space of reduced dimensions (Fig. 1).

The results of the current research are consistent with previous scientific report where almost 90% of bacteria in the gut belong to two major phyla Firmicutes and Bacteroidetes [77]. As shown in Fig. 3, there are some changes in the structure of gut flora after PPS cake treatment compared to control group, according to taxonomic research. *Lachnospiraceae, Bacteroide, Akkermansia, Bacteroidales* were most prevalent genus. After the oral administration of 10% PPS cake, compared with the control group, the proportion of *Akkermansia* was significantly increased (P<0.001). In contrast, the 20%, 30%, and 40% PPS cake diet groups had significantly higher (P<0.05) proportions of *Lachnospiraceae*. Additionally, the groups who had 30% and 40% PPS cake diets exhibited significantly larger relative abundances of Bacteroidales than the control group. Over 900 different kinds of bacteria, as well as some protozoa, fungus, yeast, and viruses are housed in the gastrointestinal tract (GIT) of chickens. These microorganisms are collectively referred to as the microbiome or microbiota and help the host break down and utilize the feeds that are consumed [78].

In fact, a number of probiotic strains are added to poultry feed in addition to their natural gut microbiome in order to increase the number of known beneficial microbes, which to prevent dysbiosis or to reduce the load of pathogenic microbes through to the use of antibiotic growth promoters [79,80]. As reported by numerous authors, the essential oils of certain plants fortified the gut microflora by reducing harmful bacteria numbers and increasing beneficial bacteria populations [81]. Our investigation revealed that the PPS cake diet group and the control group differed somewhat in the relative abundance of specific gut bacteria.

It is also important to underline that a dietary intake of PPS seeds cake was able to reduce the levels of Firmicutes while increasing the relative abundance of Bacteroidetes and *Verrucomicrobia*. Phyla Bacteroidetes are gram-negative bacteria that ferment polysaccharides and other indigestible carbohydrates producing short-chain fatty acids that are gut-friendly [82]. The remarkable effect of PPS cake on microbiome could be attributed to the constituent bioactivity of the prickly pear (*Opuntia ficus-indica* L.) seed cake and the nature of the compounds in the functional group. Likewise, Viveros et al. (2011) [83] reported that dietary polyphenol-rich grape products modify the intestinal microflora and increase the biodiversity degree of intestinal bacteria in broiler chickens. It should be noted that prickly pear seeds are an important source of natural fiber and, given its high linoleic acid content, its oil can be used as a nutraceutical agent [84]. Thus, the dietary fiber can modulate the gut microbiome and promote the growth of beneficial bacteria that would be required to improve broiler performance [85]. Moreover, anthocyanins can control intestinal flora because of the diverse range of bioactive compounds found in *Opuntia ficus-indica* [87].

5. Conclusions

Overall, the results obtained in this study demonstrated that broiler chickens' biochemical parameters were lowered when prickly pear seed meal (*Opuntia ficus indica* L.) According to this study, broiler chickens' renal and liver function may be protected by PPS cake. Also, it found that broiler chickens treated with *Opuntia ficus-indica* seed meal exhibited a variety of phylum and genus in gut microbiota. There are increasing the abundance of Firmicutes, Bacteroidetes and *Verrucomicrobia* in broiler chickens received the PPS cake diet. However, further studies are necessary enhance our understanding of the possible mechanism by which bioactive compounds of PPS affects the gut microflora of broilers.

Author contributions: MB: methodology, investigation, writing original draft; JLH: supervision, writing-review & editing; ID: formal analysis, investigation; AB. and LT: data curation, software; OB; E-HB and NBD: visualization, validation, formal analysis, data curation; KI; MI; UA and DAA: writing-review & editing; AA: supervision,

conceptualization, validation, writing original draft, writing-review & editing, supervision. All authors read and approved the final manuscript.

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Data Availability Statement: All data generated or analyzed during this study are included in this article.

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Article

Evaluation of Reproductive Status in Romanian Buffalo Farms Using Ultrasonographic Monitoring

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Abstract: The aim of this study was to evaluate the reproductive status of buffaloes from two farms in Romania by means of transrectal ultrasonography, with the intent to provide practical insights for both farmers and veterinary professionals. A total of 336–352 buffalo cows were examined at Farm A (Arad County, Romania), and 178–183 at Farm B (Brașov County, Romania), during the spring and autumn of 2024. Farm A implemented artificial insemination (AI) based on natural estrus detection, while Farm B relied on natural mating, with an approximate bull to female ratio of 1:25. The results indicated superior reproductive performance in Farm B, demonstrated by a shorter calving interval (428–431 days) and a reduced service period (125–129 days), in comparison with Farm A (445–448 days and 136–142 days, respectively; $p < 0.05$). The average number of inseminations per conception in Farm A was 1.6–1.7, and the incidence of postpartum endometritis was slightly higher (1.0–1.2%) than in the naturally mated group (0.3–0.5%). Transrectal ultrasonography proved indispensable for the accurate diagnosis of gestational stages, ovarian structures, and uterine pathologies, enabling the classification of buffaloes into well defined reproductive groups. These findings suggest that, under local management conditions, well structured natural mating can surpass the efficiency of standalone AI, highlighting the importance of accurate estrus detection, strict hygiene protocols, and periodic reproductive monitoring in optimizing fertility and overall herd productivity.

Keywords: buffalo cows, reproduction, transrectal ultrasonography, artificial insemination, natural mating

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1. Introduction

Buffalo husbandry in Romania has a longstanding tradition, particularly in lowland and hilly regions, where buffaloes are recognized for their robustness, adaptability, and extended productive lifespan. According to historical sources, water buffaloes were introduced into the territory of present day Romania as early as the 5th century AD, via the Balkan Peninsula. Owing to their high quality milk and considerable draft power, they quickly became an integral component of traditional rural households [1-3].

Despite their genetic potential and rustic hardiness, buffalo populations in Romania have significantly declined in recent decades. One of the major challenges currently facing buffalo farmers is the optimization of reproductive performance. Reproduction is a key determinant of farm profitability; fertility disorders lead directly to prolonged calving intervals, reduced milk yield, increased costs due to repeated inseminations, and economic losses through the premature culling of infertile females [4-6].

The Romanian buffalo exhibits distinctive reproductive characteristics, including relatively subtle estrus expression, with signs such as restlessness, reduced feed intake, and occasional vocalization, lasting on average 5–27 hours, with ovulation occurring roughly 24–48 hours after estrus onset [7,8].

Ovarian activity typically involves two or three follicular waves per estrous cycle, most commonly two, with the estrous cycle averaging around 21 days. Ovary size and follicle number vary throughout the cycle, reflecting ongoing follicular growth, while the

corpus luteum develops from the ruptured follicle after ovulation, with its size and function being critical for pregnancy maintenance. Hormonal profiles show lower peak progesterone and estradiol-17β levels compared to other ruminants, and variations in luteal phase duration influence overall cycle length, all of which are important for effective reproductive management in this breed [9].

Recent studies have highlighted the main reproductive pathologies affecting buffaloes. Ovarian hypofunction remains the predominant cause of infertility, with reported incidence rates reaching 70–80% in certain groups [10]. Other commonly observed dysfunctions include prolonged postpartum anestrus, subestrus, characterized by weak or undetectable signs of estrus, and early embryonic mortality [11,12].

These conditions are influenced by seasonal factors, inadequate nutrition, and heat stress during the summer months. Structural abnormalities such as ovarian hypoplasia, cystic ovaries, and persistent corpus luteum have a direct negative impact on conception rates. In addition, infectious diseases like endometritis and pyometra, although less prevalent than functional disorders, can compromise the fertility of the entire herd [13,14].

In Romania, reproductive parameters in native buffalo breeds remain relatively modest compared to cattle. The average age at first calving is 38–40 months, service periods frequently exceed 130–170 days, and calving intervals often range between 440 and 480 days. Such extended intervals are indicative of suboptimal fertility, with direct repercussions on the productivity and sustainability of buffalo farms [4,5].

Against this background, the assessment of reproductive status in buffalo herds becomes essential. The present study aimed to monitor the reproductive condition of buffaloes from two Romanian farms through individual gynecological examinations carried out in both spring and autumn seasons, thereby providing a comprehensive overview of reproductive function throughout the year. The resulting data may serve as a practical reference model for both farmers and veterinary practitioners, offering clear insights into the prevalence of major pathologies, key fertility parameters, and evidence-based management strategies that can contribute to improving reproductive indices in Romanian buffalo farming systems.

2. Materials and Methods

2.1 Biological Material and Description of the Investigated Buffalo Farms

The study was conducted in two buffalo farms located in different counties of Romania (Arad County and Brașov County), selected as representative models for distinct breeding systems, nutritional management, and reproductive technologies. The purpose of this selection was to ensure a broad and comparative framework for evaluating reproductive performance under differing management conditions.

The first farm, situated in Arad County (Romania), is located in a lowland region and manages a herd of approximately 350 adult Romanian buffalo cows, aged between 1.5 and 12 years, all reproductively active. The animals are kept under a free housing system, with extensive grazing during the warm season and semi open shelters during the winter calving period. The on farm forage base consists of natural pastures, hayfields, and cultivated forage crops, supplemented with mineral and nutritional additives depending on the season. Reproduction in this farm is based exclusively on AI, applied 8-12h after the detection of natural estrus. This method is consistently used with the aim of optimizing reproductive indicators and reducing the risk of venereal disease transmission.

The second farm, located in Brașov County (Romania), operates in a hilly depression area and maintains a herd of 186 adult buffaloes, including both primiparous and multiparous females, all of which had initiated reproductive activity. The herd is managed under a semi intensive system, with access to mountain pastures during the warm season and structured housing throughout the winter. In contrast to the first farm, reproduction in this herd is carried out exclusively through natural mating, with a bull to female ratio of approximately 1:25, ensuring coverage of the entire reproductively capable population.

In both farms, the reproductive status evaluation included all females eligible for reproduction, without restrictive selection criteria, in order to obtain a representative picture of the herd's actual reproductive condition. The assessment protocol consisted of two complete gynecological examinations conducted in spring (April–May) and autumn (September–October) of 2024, aimed at capturing seasonal variations in ovarian and uterine function seasonality being a recognized factor influencing estrus expression in buffaloes.

By including both young females at first calving and multiparous cows, this study offers a comprehensive comparative perspective on reproductive parameters within two contrasting farming systems.

2.2 Animal Examination and Grouping Criteria

To ensure a comprehensive and accurate reproductive diagnosis, transrectal clinical examination was complemented, in both farms, by transrectal ultrasonography using the EasyScan Linear ultrasound device (BCF Technologies, Maravet), equipped with a linear probe and specialized imaging modes: Ovary, Early Pregnancy, Late Pregnancy, Pregnancy, and Detail. The application of these modes enabled real time visualization of ovarian and uterine structures, as well as pregnancy status, significantly improving diagnostic precision.

Based on the clinical and imaging findings, buffalo cows from both farms were assigned to the following reproductive categories, each defined by specific clinical and ultrasonographic criteria:

- Pregnant (1–4 months): Diagnosis was established by identifying small amniotic vesicles, the characteristics of intrauterine fluids, and fetal size using the Early Pregnancy and Detail modes. These findings were corroborated by transrectal palpation (uterine fluctuation, presence of a functional corpus luteum, and asymmetry of the uterine horns).
- Pregnant (4–8 months): Diagnosis was based on direct visualization of the developing fetus, fetal positioning, amniotic fluid characteristics, and signs of fetal vitality using the Pregnancy and Late Pregnancy modes. These findings were confirmed by clinical examination (uterine size and tone of the pregnant horn).
- Pregnant (>8 months): This group included animals with advanced pregnancies, identified through ultrasound imaging of a near-term fetus, assessment of fetal presentation, and uterine expansion, in correlation with external clinical signs (enlargement of the abdominal volume, pelvic ligament relaxation).
- Reproductively Active: Diagnosis was based on the detection of either a functional corpus luteum or a dominant mature follicle with typical morphology and dimensions, visualized via the Ovary mode. These findings confirmed the presence of active ovarian cyclicity.
- Anestrus: Buffaloes in this category exhibited no functional ovarian structures (absence of corpus luteum and mature follicles), as confirmed by the Ovary mode and the absence of external estrus signs. These findings were consistent with ovarian inactivity.
- Ovarian Cysts (Luteal or Follicular): Diagnosis was established by identifying cystic structures exceeding 2 cm in diameter, with thin walls and anechoic content (follicular cysts) or thickened walls and mixed echogenicity (luteal cysts), using the Ovary mode and confirmed by transrectal palpation.
- Uterine Infections (Endometritis): Animals with varying degrees of endometritis were diagnosed based on the presence of intrauterine echogenic fluid, increased uterine wall thickness, and intrauterine pathological material in the Detail mode, in conjunction with clinical signs such as vaginal discharge and altered uterine tone.

This integrative approach, employing advanced ultrasound equipment and standardized diagnostic protocols, allowed the classification of buffaloes into homogeneous reproductive groups. This provided a robust foundation for comparative analysis of reproductive parameters across farms, seasons, and reproductive management systems.

2.3 Statistical Analysis of Data

The data obtained from clinical and ultrasonographic examinations were compiled into an electronic database, structured by farm, control season (spring and autumn), and reproductive category. For comparative interpretation, the relative incidence of each reproductive status category, the proportion of fertile versus reproductively dysfunctional buffaloes, and the distribution of pathologies by age group and season were calculated.

Key reproductive parameters, such as calving interval, service period duration, and age at first calving, were statistically analyzed as arithmetic means accompanied by standard deviations, to capture both intra- and inter-group variability. Differences between farms and seasons were statistically tested using data processing software such as Microsoft Excel and SPSS version 25. Statistical significance of observed differences was assessed using frequency analysis and the independent samples *t*-test. Where relevant, correlations between management factors (housing, nutrition) and reproductive status were also explored.

A significance level of $p < 0.05$ was established, ensuring that statistically significant results could be interpreted with an acceptable degree of confidence. All values were synthetically presented in tables and graphical formats, facilitating comparative analysis between the two reproductive and management systems investigated.

3. Results

3.1. Reproductive Status in Farm A and Farm B

Following the clinical and ultrasonographic examinations conducted in spring and autumn 2024, the reproductive group structure showed significant variations between the two farms and across seasons, reflecting the influence of management practices, nutrition, and reproductive technologies applied.

In Farm A, the examined population consisted of 336 buffaloes in the spring and 352 in the autumn. The proportion of pregnant buffaloes remained relatively stable, with a slight increase towards autumn, indicating satisfactory reproductive efficiency within the context of artificial insemination performed at natural estrus. The distribution across gestational trimesters revealed a higher number of animals in the first and second trimesters, suggesting effective breeding planning and a relatively compact calving window.

Conversely, in Farm B, with 178 buffaloes examined in spring and 183 in autumn, the incidence of pregnant animals was somewhat lower. The reproductive group distribution highlighted a higher proportion of reproductively active buffaloes, alongside a slightly increased incidence of ovarian functional disorders, particularly anestrus and follicular cysts.

Comparatively, the incidence of uterine infections (endometritis of varying severity) remained low in both farms, with a statistically higher prevalence observed in Farm A ($p > 0.05$). Summary data on the reproductive group structure are presented in Table 1, expressed as percentages of the total buffalo population examined, with comparisons drawn between farms and seasons.

Table 1. Reproductive Status in Farm A and Farm B

Reproductive Group	Farm A – Spring (n=336)	Farm A– Autumn (n=352)	Farm B – Spring (n=178)	Farm B – Autumn (n=183)
Pregnant – First trimester (1–4 mo)	31.0^{a}	28.4^{a}	21.9^{b}	23.0^{b}
Pregnant – Second trimester (4–8 mo)	27.1^{a}	29.5^{a}	20.2^{b}	21.3^{b}
Pregnant – Third trimester (>8 mo)	14.0	17.0	12.4	13.1
Reproductively active (CL/dominant follicle)	20.0^{b}	18.8^{b}	27.5^{a}	26.2^{a}
Anestrus	4.5^{b}	4.0^{b}	11.8^{a}	10.9^{a}
Ovarian cysts (luteal/follicular)	2.2^{b}	1.8^{b}	4.5^{a}	4.4^{a}
Endometritis	1.2^{a}	1.0^{a}	0.5^{b}	0.3^{b}

* Values are expressed as percentages of the total buffaloes examined; different superscript letters within the same row indicate statistically significant differences between farms for the same season ($p < 0.05$).

3.2. Reproductive Performance Indicators in Farm A and Farm B

The data obtained highlight a generally superior reproductive efficiency in Farm B, where natural mating, organized with an optimal bull to female ratio and extensive grazing, contributed to a significant reduction in calving interval and service period compared to Farm A ($p < 0.05$). In Farm A, where AI was applied based on the detection of natural estrus, the calving interval remained slightly longer. This may be attributed to the incomplete detection of estrus, which is characteristic of buffaloes with weak estrous expression, especially in the absence of strict hormonal synchronization protocols. The longer average service period in Farm A indicates a slower postpartum resumption of ovarian activity, a condition that could potentially be improved by implementing estrus synchronization protocols or more frequent ultrasound monitoring.

Table 2. Main reproductive performance indicators in Farm A (AI) and Farm B (natural mating), by season

Reproductive Indicator	Farm A – Spring	Farm A – Autumn	Farm B – Spring	Farm B – Autumn
Calving Interval (days)	448.6 ± 39.2[a]	445.1 ± 37.8[a]	428.2 ± 32.5[b]	431.0 ± 34.1[b]
Service Period (days)	142.5 ± 23.6[a]	139.8 ± 21.9[a]	125.2 ± 19.4[b]	128.7 ± 20.7[b]
AI per Gestation (mean number)	1.7 ± 0.4	1.6 ± 0.3	—	—

*Values are expressed as arithmetic means ± standard deviation. Different superscript letters within the same row indicate statistically significant differences between farms within the same season ($p < 0.05$, t-test). The average number of inseminations per gestation applies only to Farm A, where AI was used.

The average number of inseminations per gestation in Farm A (1.7–1.6) remains reasonable for AI applied without synchronization but underscores the limitations of this method in species with subtle estrous signs. In contrast, natural mating in Farm B allows for more accurate detection of the optimal fertilization timing, which is directly reflected in more favorable reproductive parameters (Table 2).

4. Discussion

The results obtained in this study provide a significant contribution to understanding the reproductive characteristics of buffaloes under the specific conditions of Romanian farms. Given the substantial decline in buffalo populations over recent decades, optimizing reproductive performance has become a crucial objective for maintaining the viability of this livestock sector and supporting the biodiversity of the Romanian buffalo breed [1].

The comparative analysis between the two farms reveals clear differences between the reproductive strategies used: natural mating (Farm B) versus AI performed on natural estrus (Farm A). The data indicate that in farms where an optimal male to female ratio and rigorous veterinary control are ensured, natural mating remains an efficient method capable of delivering favorable reproductive and productive outcomes. The significantly lower average calving interval and service period under 130 days observed in Farm B reflect effective detection of the silent estrus characteristic of buffaloes [15,16] (Fig. 1).

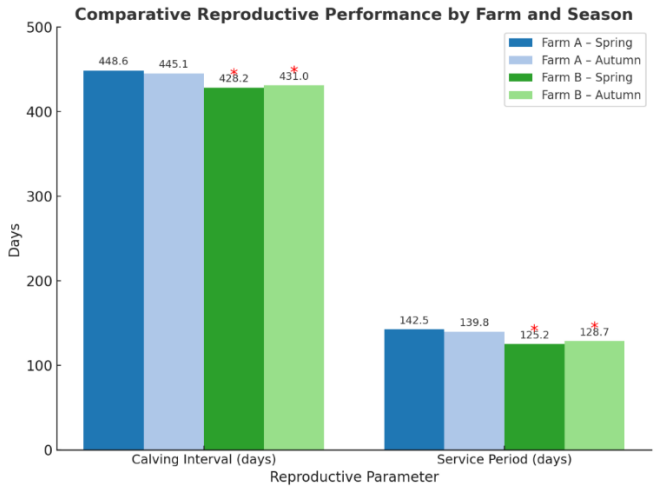

Figure 1. Calving interval and Service Period for both farms (* indicate significant differences between farms for the same parameter and season ($p < 0.05$, independent t-test).

In contrast, Farm A, despite theoretically benefiting from the advantages of strict AI control, showed longer calving intervals and service periods, particularly in the spring season. This may be explained by

difficulties in estrus detection in the absence of hormonal synchronization protocols, a well-known challenge in the literature. Buffaloes exhibit a discreet estrus, which can be weak or even absent under certain nutritional or stress conditions, limiting the efficiency of AI when applied without complementary monitoring methods [17,18].

Another important finding is the higher incidence of postpartum endometritis in Farm A compared to Farm B. Although absolute prevalence values did not exceed critical thresholds, the proportional increase highlights the iatrogenic risk associated with repeated manipulations during AI, especially when hygiene and disinfection protocols are not rigorously followed. Even minor contamination can lead to chronic uterine inflammation, prolonging the service period and increasing the risk of functional infertility [19].

From a seasonal perspective, the data confirm the biological seasonality of reproduction in buffaloes. In both farms, spring examinations revealed higher proportions of reproductively active animals and an increased number of buffaloes in the first two thirds of gestation, reflecting calvings concentrated in the previous warm season. In autumn, a slight increase in anestrus incidence was observed, especially in Farm A, where detecting the post gestational cycle is more challenging under prolonged housing conditions. Literature supports that summer grazing positively influences energy balance and ovarian cycle resumption, while housing periods may exacerbate the risk of nutritional or functional anestrus [12,18,19].

Thus, buffalo reproduction in Romania requires continuous adaptation to species specific biological factors such as estrus seasonality, thermal stress sensitivity, and nutritional particularities. Natural mating proves to be an effective reproductive option under optimal conditions, especially in farms with limited herds and resources for AI. On the other hand, larger commercial operations equipped with adequate infrastructure can leverage the benefits of AI combined with hormonal synchronization protocols, allowing enhanced reproductive control and reduced risk of venereal diseases [4,12].

Ultrasonographic transrectal monitoring plays a crucial role, proving indispensable for accurate diagnosis of pregnancy status, ovarian dysfunctions, and uterine pathologies. Grouping buffaloes into reproductive functional categories based on ultrasound provides veterinarians with an objective overview of herd reproductive efficiency, facilitating timely corrective actions [20-23].

This research proposes a practical model for farmers and veterinarians managing buffalo herds in Romania, providing relevant benchmarks regarding reproductive status incidence in two distinct technological systems. Interpreting these indicators seasonally allows for optimizing management strategies, improving calving planning, and reducing economic losses associated with functional infertility.

Reproductive success in buffalo farms depends on the careful correlation of species specific biological factors, estrus seasonality, feeding quality, reproductive hygiene, and the training level of personnel involved in reproductive technologies. By disseminating these results, this study contributes to establishing best practices adapted to current economic and technological conditions in Romanian buffalo farming [24,25].

5. Conclusions

The results showed that in Farm B, where reproduction was managed through natural mating, the calving interval was significantly shorter (428–431 days) and the service period was reduced (125–129 days) compared to Farm A, where artificial insemination at natural estrus detection was used (445–448 days and 136–142 days, respectively; $p < 0.05$). The average number of inseminations per pregnancy in Farm A was 1.6–1.7, and the incidence of endometritis was slightly higher (1.0–1.2% compared to 0.3–0.5% in Farm B). These data confirm that, for moderate buffalo herds, well organized natural mating remains more reproductively efficient than unsynchronized AI, especially in species with silent estrus. Regular ultrasonographic monitoring and strict hygienic sanitary management remain essential for optimizing fertility and reducing economic losses.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, D.B. and S.C.; methodology, S.C.; software, S.C (Simona Ciupe).; validation, D.B.R.C and S.C.; formal analysis, R.C.; investigation, S.C.; resources, S.C (Simona Ciupe).; data curation, D.B.; writing—original draft preparation, S.C.; writing—review and editing, D.B.; visualization, R.C.; supervision, L.M.B; project administration, L.M.B.; funding acquisition, S.C. All authors have read and agreed to the published version of the manuscript".

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Article

A Comparative Evaluation of the Anesthetic Efficacy and Hepatic Safety of Tiletamine-Zolazepam and Ketamine-Diazepam in New Zealand White Rabbits

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Abstract: This study aimed to compare the anesthetic efficacy, physiological stability, and hepatic effects of tiletamine-zolazepam (TZ) versus ketamine-diazepam (KD) in rabbits. Forty healthy male New Zealand White rabbits (2–3 kg) were randomly assigned into five groups: a saline control, three tiletamine-zolazepam (TZ) dose groups (32, 7.5, and 3.5 mg/kg IV), and a ketamine-diazepam (KD) group (20 + 1 mg/kg IV). Anesthetic depth, duration, and physiological parameters were monitored for 60 minutes. Blood samples were collected before anesthesia and on days 1, 3, 5, and 7 post-injection to assess liver function. The TZ-High group (32 mg/kg) exhibited the longest duration of anesthesia but also showed severe cardiorespiratory depression, characterized by a significant drop in respiratory rate and heart rate. Furthermore, this group displayed marked elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels at 24 and 72 hours, indicating significant hepatotoxicity. In contrast, the KD and TZ-Mid groups provided adequate anesthesia with better physiological stability and minimal biochemical alterations. It is concluded that while high-dose tiletamine-zolazepam provides prolonged anesthesia, it induces severe and unacceptable hepatotoxicity and cardiorespiratory distress in rabbits. The combination of ketamine-diazepam or a mid-range dose of tiletamine-zolazepam offers a safer alternative for procedures in rabbits.

Keywords: Rabbit, Tiletamine-Zolazepam, Ketamine-Diazepam, Toxicity, Anesthesia, Hepatic Safety

1. Introduction

The domestic rabbit (Oryctolagus cuniculus) is widely kept as a companion animal and is also an important model in biomedical research. It is used in wide range fields such as ophthalmology, cardiovascular studies, infectious disease, and toxicology [1]. Rabbits are preferred because they are easy to handle, have a manageable body size, and share several physiological features with humans [2]. However, anesthesia in rabbits remains a challenge. Compared to other domestic animals, rabbits have a higher risk of complications and death during or after anesthesia [3,4]. This is due to several factors, including their anatomy, small cardiopulmonary reserve, and strong stress response, which can sometimes cause sudden cardiovascular failure [5,6]. These risks highlight the

need for safe and reliable anesthetic protocols for both veterinary practice and laboratory use.

Injectable anesthesia is commonly used in rabbits because it is simple to administer, acts quickly, and can be used even in facilities without inhalation equipment. Two of the most widely used injectable combinations are ketamine with diazepam (KD) and tiletamine with zolazepam (TZ). The KD mixture combines the dissociative effect of ketamine with the muscle relaxation and sedative action of diazepam. It is generally considered to provide stable cardiovascular function [7]. TZ has the advantage of a longer duration of anesthesia, but it is sometimes associated with unstable recovery, respiratory depression, and other physiological disturbances [8,9]. Even though both regimens are widely practiced, there is concern that they may cause subclinical organ injury to vital organs such as the liver and kidneys, especially when given in higher doses [8,10].

Most studies so far have only compared the depth of anesthesia, induction, and recovery between these agents. Information about their long-term safety on liver function in rabbits is still limited. In particular, the effects of different doses of TZ have not been fully compared with KD using laboratory blood markers. This knowledge is important, because organ toxicity not only affects the health and welfare of the animals, but may also interfere with research outcomes where rabbits are used as models [11].

The aim of this study was to compare the anesthetic efficacy, physiological effects, and possible liver toxicity of three intravenous doses of tiletamine-zolazepam with a standard clinical dose of ketamine-diazepam in New Zealand White rabbits. The assessment included anesthesia quality, duration, recovery, and monitoring of heart rate, respiratory rate, and body temperature. In addition, liver function tests were performed. The results are intended to provide practical recommendations for safer anesthetic use in rabbits, with the goal of improving both clinical care and the reliability of experimental research.

2. Materials and Methods

2.1. Animals

In this investigation, 40 clinically healthy male New Zealand White rabbits, each weighing 2-3 kg, underwent five different anesthetic protocols. The rabbits were individually housed in cages for 7 days for acclimatization before the experiments and were provided with standard rabbit chow with locally available forage and water ad libitum. Before initiating the experiment, the animals were fasted for 6 hours, and water was withheld for 2 hours.

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2.2. Experimental design

The animals were randomly assigned to 5 groups of 8 rabbits each (Groups 1, 2, 3, 4, and 5). Animals were randomly allocated to experimental groups using a computer-generated random sequence. The sample size of n=8 per group was selected to provide adequate statistical power for analysis of variance (ANOVA). Group 1 served as a saline-injected control to isolate the effects of handling and injection stress from the pharmacological effects of the anesthetic agents. Groups 2, 3, and 4 received tiletamine-zolazepam (TZ) (Zoletil™ 50, Virbac), and Group 5 received ketamine hydrochloride (Aneket, Neon Laboratories) and diazepam hydrochloride (Calmpose, Sun Pharma). The dosing regimens are detailed in Table 1. High-dose TZ group was intentionally selected to probe safety boundaries and to enable detection of dose-related biochemical changes.

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Table 1. Experimental Design and Dosing Regimen.

Parameter	Group 1 (Control)	Group 2 (TZ-High)	Group 3 (TZ-Mid)	Group 4 (TZ-Low)	Group 5 (KD)
Treatment	Saline (0.9% NaCl)	Tiletamine-Zolazepam	Tiletamine-Zolazepam	Tiletamine-Zolazepam	Ketamine + Diazepam
Dose	1 mL/kg	32 mg/kg	7.5 mg/kg	3.5 mg/kg	20 + 1 mg/kg
Route	Intravenous (IV)	Intravenous (IV)	Intravenous (IV)	Intravenous (IV)	Intravenous (IV)
Sample Size (n)	8	8	8	8	8

2.3. Anesthetization and Monitoring

On the day of the experiment, rabbits were weighed and transferred from the housing room to the operating room using a pet carrier. Heart rate, respiratory rate, and temperature were recorded before anesthesia. Hair on the hind limb and ear vein was trimmed, and a topical local anesthetic cream (Lidocaine) was applied to the ear vein to minimize discomfort during blood collection. Anesthesia was administered through the saphenous vein. Anesthetic depth was monitored by assessing the absence of the pedal withdrawal reflex in response to a toe pinch, loss of the ear pinch reflex, and loss of the righting reflex when placed in a lateral recumbent position. Heart rate, respiratory rate, and body temperature were monitored before anesthesia (0 min) and at 10-minute intervals up to 60 minutes after the injection of the anesthetic. Heart rate and peripheral oxygen saturation (SpO_2) were measured continuously using a pulse oximeter. Respiratory rate was recorded by visual counting of thoraco-abdominal excursions and confirmed intermittently by auscultation with a stethoscope. Body temperature was measured with a digital rectal thermometer at predefined timepoints.

2.4. Biochemical Analysis

Blood samples (approx. 2 mL) were collected from the marginal ear vein before anesthesia (baseline) and on the 1st, 3rd, 5th, and 7th day post-injection to monitor various biochemical markers. This well within recommended limits (≤10% total blood volume without fluid replacement) for multiple sampling when distributed over one week. The blood was collected in vacutainers with a gel and clot activator and allowed to coagulate at room temperature. Samples were then centrifuged at 1500 × g for 10 minutes, and the serum was separated and stored at -20 C until biochemical analysis. The serum was analyzed for a comprehensive hepatic panel using commercially available assay kits with a spectrophotometer. Hepatic markers include; Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), Gamma-Glutamyl Transferase (GGT), Total Bilirubin, Total Protein, and Albumin.

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3. Results

3.1. Anesthetic and Physiological Effects

The anesthetic parameters are summarized in Table 2. There was no significant difference in onset time among anesthetic groups (p=0.489). The duration of anesthesia was longest in the TZ-High group (58.50±4.34 min), which was significantly longer than all other groups (p<0.001). The recovery time for the TZ-High group (265.5±25.8 min) was profoundly prolonged and significantly longer than all other groups (p<0.001).

Table 2. Anesthetic parameters in rabbits administered with different anesthetic combinations (Mean ± SD, n=8/group).

Criteria	Group 1 (Control)	Group 2 (TZ-High)	Group 3 (TZ-Mid)	Group 4 (TZ-Low)	Group 5 (KD)	p-value
Onset time (min)	N/A	0.25±0.06	0.21±0.05	0.23±0.04	0.41±0.25	0.489
Duration of loss of pedal withdrawal reflex (min)	0	58.50±4.34[a]	31.25±8.19[b]	20.13±3.18[c]	24.75±8.90[c]	<0.001
Recovery time (min)	0	265.5±25.8[a]	139.3±14.1[b]	54.8±9.7[c]	125.1±11.5[b]	<0.001

Means within the same row with different superscript letters (a, b, c) differ significantly (p<0.05). N/A = Not Applicable.

Heart rate (Table 3) and respiratory rate (Table 4) were significantly altered. The TZ-High group developed profound bradycardia (45.50 ± 8.10 bpm) and severe respiratory depression (14.20 ± 2.50 breaths/min) at 30 minutes, both of which were significantly more severe than in any other group (p<0.001).

Table 3. Heart rate (beats per minute) at key time points (Mean ± SD, n=8/group).

Time (min)	Group 1 (Control)	Group 2 (TZ-High)	Group 3 (TZ-Mid)	Group 4 (TZ-Low)	Group 5 (KD)	p-value
Before	245.10 ± 28.30	251.50 ± 31.00	260.30 ± 25.50	248.80 ± 29.10	270.60 ± 20.40	0.512
30	239.50 ± 25.10[a]	45.50 ± 8.10[e]	195.40 ± 18.20[b]	205.10 ± 22.80[b]	140.30 ± 19.50[d]	<0.001

60	241.30 ± 22.90[a]	75.80 ± 10.20[d]	188.90 ± 15.70[b]	201.60 ± 24.00[b]	145.80 ± 16.10[c]	<0.001

For each time point, means within the same row with different superscript letters (a, b, c, d, e) differ significantly ($p<0.05$).

Table 4. Respiratory rate (breaths per minute) at key time points (Mean ± SD, n=8/group).

Time (min)	Group 1 (Control)	Group 2 (TZ-High)	Group 3 (TZ-Mid)	Group 4 (TZ-Low)	Group 5 (KD)	p-value
Before	46.20 ± 5.10	47.50 ± 5.90	45.80 ± 4.80	46.60 ± 5.30	48.10 ± 4.40	0.618
30	45.90 ± 4.50[a]	14.20 ± 2.50[d]	28.50 ± 3.10[c]	36.10 ± 4.20[b]	31.40 ± 3.80[c]	<0.001
60	46.50 ± 4.20[a]	19.60 ± 2.80[d]	30.20 ± 2.90[c]	35.50 ± 4.00[b]	32.80 ± 3.10[bc]	<0.001

For each time point, means within the same row with different superscript letters (a, b, c, d) differ significantly ($p<0.05$).

3.2. Serum Biochemical Findings

The TZ-High group exhibited severe hepatic toxicity. Hepatic Injury: The TZ-High group showed a sharp, significant increase in serum ALT and AST, indicative of acute hepatocellular injury (Table 5). This was accompanied by significant elevations in GGT and Total Bilirubin and a significant decrease in Total Protein and Albumin by Day 7, indicating cholestasis and impaired hepatic synthetic function (Table 6). All other groups showed only minor, transient changes.

Table 5. Serum Hepatic Biomarkers (Mean ± SD, n=8/group).

Parameter (U/L)	Group	Before	Day 1	Day 3	Day 7
ALT	1 (Control)	68.4 ± 10.1	70.1 ± 9.8[c]	67.5 ± 11.2[c]	69.3 ± 8.9[b]
	2 (TZ-High)	72.1 ± 11.5	195.7 ± 33.4[a]	248.6 ± 45.1[a]	145.8 ± 22.6[a]
	3 (TZ-Mid)	69.8 ± 9.5	115.3 ± 18.2[b]	85.4 ± 14.3[b]	71.5 ± 10.4[b]
	4 (TZ-Low)	70.5 ± 8.8	88.6 ± 12.9[bc]	74.1 ± 9.9[c]	68.1 ± 9.2[b]
	5 (KD)	73.3 ± 12.4	120.8 ± 20.5[b]	90.2 ± 15.1[b]	75.4 ± 11.8[b]
p-value		*0.915*	*<0.001*	*<0.001*	*<0.001*
AST	1 (Control)	95.3 ± 15.2	98.8 ± 14.1[c]	94.6 ± 13.8[c]	96.1 ± 12.5[b]
	2 (TZ-High)	101.7 ± 18.9	295.3 ± 60.2[a]	188.4 ± 35.7[a]	110.5 ± 19.3[a]
	3 (TZ-Mid)	98.2 ± 16.5	145.1 ± 25.8[b]	115.9 ± 20.1[b]	99.8 ± 15.4[b]
	4 (TZ-Low)	96.9 ± 14.8	110.2 ± 19.3[bc]	101.3 ± 16.6[bc]	95.5 ± 14.1[b]
	5 (KD)	103.1 ± 20.1	155.6 ± 28.4[b]	121.7 ± 22.3[b]	101.4 ± 17.8[b]
p-value		*0.887*	*<0.001*	*<0.001*	*0.048*
ALP	1 (Control)	94.5 ± 13.3	95.1 ± 12.9[b]	93.8 ± 14.0[c]	94.2 ± 13.5[c]
	2 (TZ-High)	98.2 ± 15.1	121.6 ± 20.3[a]	155.4 ± 28.9[a]	180.3 ± 35.2[a]
	3 (TZ-Mid)	95.8 ± 14.0	112.7 ± 18.5[ab]	101.5 ± 16.2[bc]	96.1 ± 14.8[c]
	4 (TZ-Low)	93.9 ± 12.8	98.3 ± 13.1[b]	95.2 ± 13.5[c]	94.5 ± 13.1[c]
	5 (KD)	99.6 ± 16.2	115.4 ± 19.8[a]	105.8 ± 17.1[b]	100.3 ± 16.5[bc]
p-value		*0.921*	*<0.01*	*<0.001*	*<0.001*

For each parameter, means within the same column with different superscript letters (a, b, c) differ significantly ($p<0.05$).

Table 6. Comprehensive Serum Hepatic Function Markers (Mean ± SD, n=8/group).

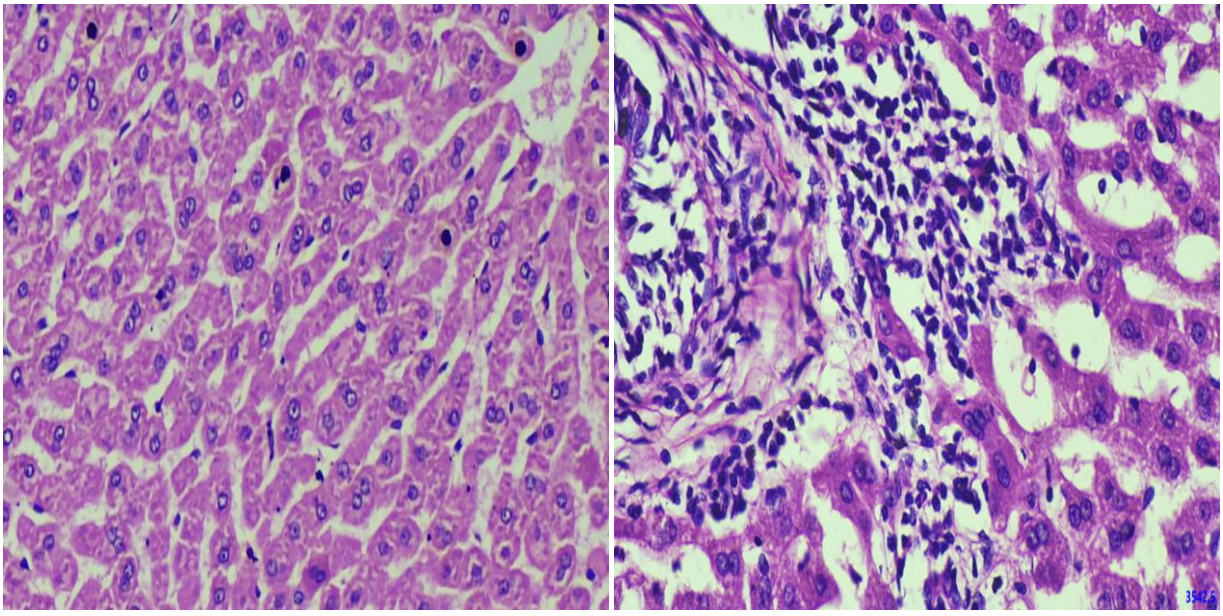
Parameter	Group	Before	Day 3	Day 7
Total Protein (g/dL)	1 (Control)	6.5±0.4	6.4±0.5[a]	6.6±0.3[a]
	2 (TZ-High)	6.6±0.5	5.1±0.6[b]	4.5±0.7[b]
	3 (TZ-Mid)	6.4±0.3	6.2±0.4[a]	6.3±0.5[a]
	4 (TZ-Low)	6.5±0.4	6.4±0.5[a]	6.5±0.4[a]
	5 (KD)	6.7±0.6	6.5±0.5[a]	6.6±0.4[a]
p-value		*0.781*	*<0.001*	*<0.001*
Albumin (g/dL)	1 (Control)	3.8±0.3	3.7±0.4[a]	3.8±0.3[a]

	2 (TZ-High)	3.9±0.4	2.9±0.5^{b}	2.4±0.6^{b}
	3 (TZ-Mid)	3.7±0.3	3.6±0.3^{a}	3.7±0.4^{a}
	4 (TZ-Low)	3.8±0.2	3.8±0.4^{a}	3.9±0.3^{a}
	5 (KD)	3.9±0.5	3.8±0.4^{a}	3.8±0.5^{a}
p-value		*0.812*	*<0.001*	*<0.001*
GGT (U/L)	1 (Control)	4.1±1.1	4.3±1.0^{c}	4.0±0.9^{b}
	2 (TZ-High)	4.5±1.3	15.8±3.1^{a}	9.7±2.5^{a}
	3 (TZ-Mid)	4.2±0.9	5.9±1.5^{b}	4.5±1.1^{b}
	4 (TZ-Low)	4.0±1.0	4.8±1.2^{bc}	4.2±1.0^{b}
	5 (KD)	4.6±1.4	6.2±1.8^{b}	4.8±1.3^{b}
p-value		*0.899*	*<0.001*	*<0.001*
Total Bilirubin (mg/dL)	1 (Control)	0.3±0.1	0.3±0.1^{c}	0.2±0.1^{b}
	2 (TZ-High)	0.4±0.2	1.9±0.4^{a}	1.1±0.3^{a}
	3 (TZ-Mid)	0.3±0.1	0.5±0.2^{b}	0.3±0.1^{b}
	4 (TZ-Low)	0.3±0.1	0.4±0.1^{bc}	0.3±0.1^{b}
	5 (KD)	0.4±0.1	0.6±0.2^{b}	0.4±0.2^{b}
p-value		*0.754*	*<0.001*	*<0.001*

For each parameter, means within the same column with different superscript letters (a, b, c) differ significantly (p<0.05).

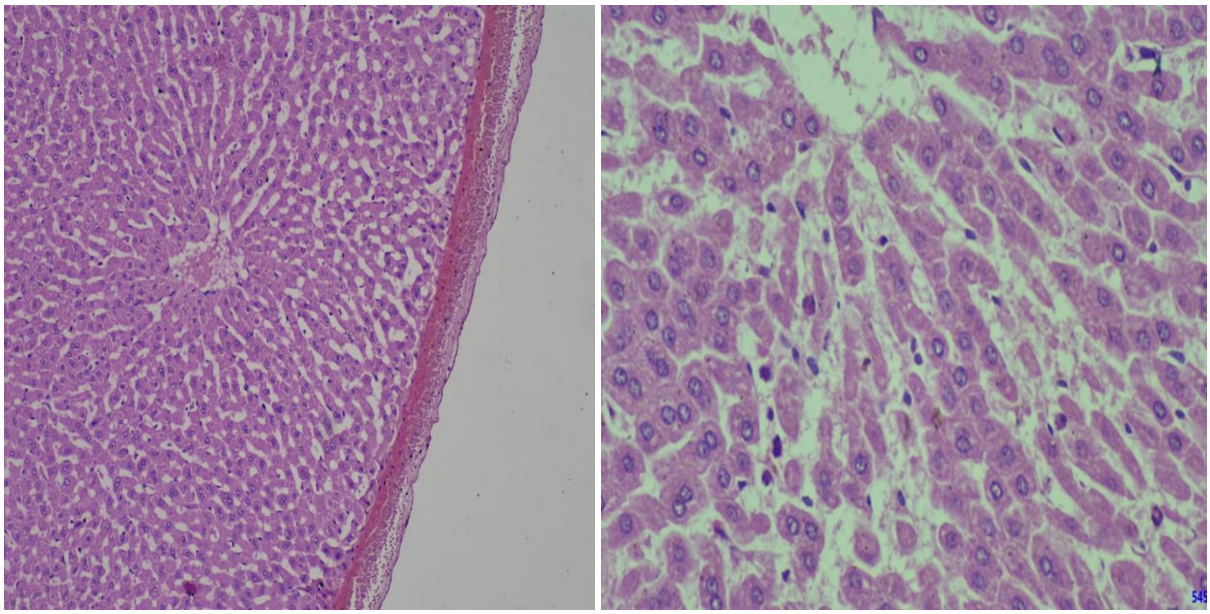
3.4. Histopathological Findings

All groups showed vacuolar degeneration around the centrilobular region, but Group D showed less of it. Hepatocytes in the periportal region exhibited vacuolar degeneration; however, it was less severe than in the centrilobular region. In all groups, the sinusoids around the centrilobular region were dilated. In Group A, there was higher dilation relative to the sinusoids of the periportal area, while in the other groups, there was no dilation around the periportal area. In Group A, there was subcapsular congestion, which was absent in all other groups. In Groups B and C, there was an accumulation of mononuclear inflammatory cells around the portal area, a localized loss of hepatocytes around the central vein, and Kupffer cell proliferation. (Fig. 1-4).



(**a**) (**b**)

Figure 1. (**a**) Photomicrograph of a rabbit liver section showing vacuolar degeneration of hepatocytes around the centrilobular region. (Hematoxylin and Eosin stain, 10X magnification); (**b**) Photomicrograph of a liver section from a rabbit in Group (TZ-7.5 mg/kg), demonstrating a prominent accumulation of mononuclear inflammatory cells around the portal area. (Hematoxylin and Eosin stain, 10X magnification).



(**a**) (**b**)

Figure 2. (**a**) Photomicrograph of a liver section from a rabbit in Group (TZ-32 mg/kg). Note the presence of subcapsular congestion. (Hematoxylin and Eosin stain, 10X magnification); (**b**) Photomicrograph of a liver section from a rabbit illustrating focal loss of hepatocytes and proliferation of Kupffer cells around the central vein. (Hematoxylin and Eosin stain).

4. Discussion

This study directly compared the anesthetic effects and organ-specific toxicities of two injectable protocols: tiletamine-zolazepam (TZ) at three dose levels and a standard dose of ketamine-diazepam (KD). The results showed clear differences between the two regimens, with TZ—especially at higher doses—being associated with major safety concerns and organ damage, whereas KD demonstrated a much safer profile.

The study revealed that TZ produced a distinct dose-dependent toxicity. Rabbits receiving the highest TZ dose (32 mg/kg) experienced prolonged anesthesia, delayed recovery, marked respiratory depression, and significant biochemical evidence of both liver damage. In contrast, KD and lower doses of TZ caused only mild, temporary changes in biochemical parameters and were normalized without lasting organ injury. This confirms the very narrow safety margin of TZ and emphasizes the critical importance of careful dose selection in clinical use. From a practical standpoint, these findings strongly suggest that KD is the more predictable and safer choice for rabbit anesthesia, particularly in routine practice.

High-dose TZ produced the longest anesthesia period, averaging about 58 minutes, compared with 25 minutes for KD. While longer anesthesia may initially seem advantageous, it was accompanied by a number of risks. Rabbits receiving high-dose TZ had a dangerously prolonged recovery, with a mean recovery time of 265.5 ± 25.8 minutes (approximately 4.4 hours), which was significantly longer than all other groups. This group had also shown clinical signs such as ataxia, lethargy, hypothermia, and dehydration. These complications, combined with the severe respiratory depression observed, made this protocol especially dangerous. Since rabbits have limited physiological reserves, such prolonged recovery times increase the likelihood of critical complications unless continuous monitoring and supportive care are available [12,13].

By comparison, the KD protocol resulted in a recovery time (125 ± 11.5 min) that was significantly shorter than the high-dose TZ group. Although this recovery was longer than that of the TZ-Low group, it was clinically smoother and safer, with fewer complications like ataxia and dehydration. Although KD may not provide sufficient analgesia for highly invasive surgeries, its safety advantages make it an appropriate option for short or minimally invasive procedures [14]. Its predictable effects give veterinarians greater control over anesthesia management, lowering the risk of post-anesthetic complications.

Hepatic toxicity was especially pronounced in the high-dose TZ group. Significant elevations in ALT and AST indicated hepatocellular injury, while increased GGT and bilirubin suggested cholestasis. Reductions in total protein and albumin levels further reflected impaired liver synthetic function. These findings together show a multi-dimensional pattern of liver damage with high-dose TZ. Importantly, the toxicity was dose-dependent. Rabbits receiving mid-dose TZ (7.5 mg/kg) displayed only mild and reversible increases in liver enzymes, while those given low-dose TZ (3.5 mg/kg) or KD showed negligible hepatic

effects. This gradient confirms that hepatotoxicity risk rises sharply with higher TZ doses, whereas KD carries a much lower risk [10].

Cardiovascular and respiratory effects further distinguished the protocols. High-dose TZ produced profound and sustained bradycardia, alongside depression of respiratory rate. The combination of unstable HR and severe RR suppression markedly reduces organ perfusion and oxygen delivery, posing a significant risk for rabbits, which have limited cardiopulmonary reserves [17,18]. By comparison, KD caused only mild bradycardia and moderate, stable reductions in RR, without the dangerous instability observed in TZ-High. This greater cardiorespiratory stability explains in part the reduced risk of organ injury and smoother anesthetic profile seen in the KD group, reinforcing its clinical value as a safer and more predictable choice [19].

Overall, the findings suggest that high-dose TZ (32 mg/kg) should be avoided in rabbits because it can cause severe acute liver failure, life-threatening respiratory depression, and very prolonged recovery times. KD, while producing shorter anesthesia, demonstrated a safer and more stable profile, making it the preferred option for short or minimally invasive interventions. Even moderate doses of TZ should be used cautiously, as the observed biochemical changes suggest toxicity thresholds are reached quickly. For clinical practice, these results emphasize the importance of prioritizing safety and predictability over prolonged anesthesia duration in rabbits.

This study was primarily based on biochemical markers without histopathological examination. While the results strongly indicate organ injury, tissue-level evidence would provide more definitive conclusions about lesion severity and distribution in different organs such as kidney, lungs, heart and brain. Additionally, only healthy adult rabbits were studied; the adverse effects of TZ may be even more severe in geriatric or subclinically ill animals [20,21]. Future research should include histopathology in wider organ systems, primarily, liver, kidney, lungs, heart and brain, longer-term biochemical monitoring, and investigations in at-risk populations to refine safety margins. Parallel evaluation of analgesic efficacy would also be useful, helping clinicians select the most suitable anesthetic protocol for both routine and invasive surgical procedures.

Beyond the immediate clinical implications, these findings also carry significance for refining anesthetic protocols in laboratory animal medicine and translational research. Rabbits are commonly used in biomedical studies, therefore ensuring reproducible and humane anesthetic strategies is critical both ethically and scientifically [22]. The clear evidence that tiletamine-zolazepam shows a dose-dependent pattern of systemic toxicity underscores the need for stringent anesthetic monitoring and individualized adjustments in experimental designs. Furthermore, the contrast between TZ and KD highlights a broader principle that extending anesthesia duration with high drug doses often comes at the expense of safety, especially in species with limited cardiopulmonary reserves. In this regard, KD emerges not only as the safer clinical option but also as the regimen that better aligns with the principles of refinement in animal research.

Incorporating adjunct strategies such as multimodal analgesia, supplemental oxygenation, and active thermal support could further optimize outcomes, particularly when anesthesia must be prolonged [23]. Future investigations might also explore whether modified KD regimens or alternative benzodiazepine combinations provide equal efficacy with even greater safety margins. Ultimately, the findings of this study should encourage veterinarians and researchers to reconsider drug selection strategies with a priority on organ protection, recovery quality, and minimizing animal welfare risks.

5. Conclusions

The results of this comprehensive investigation demonstrate that while tiletamine-zolazepam at a dose of 32 mg/kg provides a prolonged duration of surgical anesthesia in rabbits, it is unequivocally associated with severe, acute hepatotoxicity. This liver damage is characterized by marked elevations in hepatocellular and cholestatic enzymes and is compounded by a progressive failure of hepatic synthetic function. Furthermore, this dose induces profound and clinically unacceptable cardiorespiratory depression. In stark contrast, the combination of ketamine-diazepam and lower doses of tiletamine-zolazepam offered shorter, effective periods of anesthesia but with a significantly wider margin of safety and minimal impact on hepatic function. Given the substantial risks, the high-dose tiletamine-zolazepam protocol cannot be recommended for use in New Zealand White rabbits. Ketamine-diazepam or lower doses of tiletamine-zolazepam represent far safer and more reliable anesthetic choices for this species.

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writing—original draft preparation, F.P. and S.A.; writing—review and editing, S.P., J.C. and K.P.; visualization, A.A.; supervision, S.P.; project administration, S.P.; funding acquisition, F.P. All authors have read and agreed to the published version of the manuscript.

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Article

Integration of Differential and Total Somatic Cell Counts for Udder Health Classification: Association with Milk Lactose Concentration

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Abstract: Mastitis is one of the most important diseases in dairy cattle, impairing milk yield, composition, and animal welfare. Somatic cell count (SCC) is the traditional indicator of udder health, but it does not provide information on leukocyte composition. Differential somatic cell count (DSCC) complements SCC by quantifying the proportion of neutrophils and lymphocytes, improving mastitis detection and characterization. Lactose, the main milk carbohydrate, is a potential biomarker of mammary epithelial integrity and declines during intramammary inflammation. This study aimed to classify milk samples based on combined SCC and DSCC thresholds and to evaluate the association between udder health status and lactose concentration. A total of 1,083 milk samples were collected from 50 multiparous Romanian Spotted cows housed in a free-stall system with an automated milking robot over a two-year period. Sixteen samples (1.5%) could not be classified due to missing or invalid SCC/DSCC values and were excluded from group comparisons. The remaining 1,067 samples were classified as healthy udder (DSCC < 75%, SCC < 200,000 cells/mL), subclinical mastitis (DSCC ≥ 75%, SCC < 200,000), clinical mastitis (DSCC ≥ 75%, SCC ≥ 200,000), or chronic mastitis (DSCC < 75%, SCC ≥ 200,000). Mean lactose concentration was 4.82 ± 0.37% (range 1.94-5.31%) and decreased progressively from healthy udders to chronic mastitis. The Kruskal–Wallis test confirmed significant differences among groups ($p < 0.001$, $\eta^2[H] = 0.074$), with Dunn's post-hoc tests showing significantly lower lactose in clinical and chronic mastitis compared with healthy udders ($p < 0.001$ and $p < 0.003$, respectively). Lactose was negatively correlated with SCC (r_s=-0.46) and DSCC (r_s = -0.40), whereas SCC and DSCC were strongly positively correlated ($r_s = 0.83$). These findings suggest that lactose concentration may serve as a sensitive indicator of udder health, and its integration with SCC and DSCC provides a robust, non-invasive approach for early detection and monitoring of intramammary inflammation.

Keywords Udder health, Differential Somatic Cell Count, Somatic Cell Count, Milk Lactose, Mastitis Classification

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1. Introduction

Mastitis remains one of the most common and economically significant diseases affecting dairy cows worldwide. It reduces milk yield, impairs milk quality, and increases culling rates, thereby causing substantial financial losses to the dairy industry. Somatic cell count (SCC) has long been considered the gold standard for monitoring udder health at both cow and herd levels, but it does not provide information about the cellular composition of milk leukocytes. The differential somatic cell count (DSCC), which quantifies the proportion of polymorphonuclear neutrophils and lymphocytes, has recently been introduced as a complementary tool to improve the accuracy of mastitis detection [1-3]. DSCC enables differentiation between neutrophil-dominated inflammatory responses, commonly associated with active intra-mammary infections, and lymphocyte-predominant profiles, which may reflect either recovery of immune homeostasis or chronic inflammation [1,2].

Milk lactose concentration has also emerged as a promising biomarker of udder health and mammary epithelial integrity. Lactose is the major carbohydrate in milk and a key regulator of its osmotic pressure, driving water influx and milk secretion. Several studies have consistently reported that lactose concentration decreases during mastitis,

reflecting epithelial cell damage and altered mammary permeability [4,5]. Monitoring lactose, particularly in combination with SCC or DSCC, may therefore enhance the detection of subclinical mastitis and provide additional insight into the physiological effects of intra-mammary infections on milk composition.

Although there is growing interest in the combined use of SCC and DSCC, few studies have investigated their relationship with milk lactose concentration under field conditions. A better understanding of this relationship could improve early detection of udder inflammation, inform herd health management, and support evidence-based treatment and prevention strategies.

The objective of the present study was to classify milk samples into udder health categories using combined SCC and DSCC thresholds and to evaluate the association between udder health status and lactose concentration. We hypothesized that lactose concentration would decrease progressively with worsening udder health and that significant negative correlations would exist between lactose, SCC, and DSCC.

2. Materials and Methods

2.1 Animals and Sampling

A total of 1,083 milk samples were collected from 50 multiparous Romanian Spotted cows (Simmental breed) housed in a free-stall system on a commercial dairy farm in Romania. The farm was equipped with a fully automated milking system (1 milking robot per 50 cows). Sampling took place over a two-year period, from May 2022 to September 2024, covering multiple stages of lactation. Milk samples were obtained during routine milking sessions, following standard hygienic procedures, and were immediately cooled and transported to the laboratory for analysis.

Sixteen samples (1.5%) could not be classified into udder health categories due to missing or invalid DSCC or SCC values and were therefore excluded from group comparisons. The remaining 1,067 samples were included in the statistical analysis.

2.2 Milk Analysis

All samples were analyzed in an ISO-certified laboratory to ensure quality and traceability of results. Somatic Cell Count (SCC), Differential Somatic Cell Count (DSCC), and lactose concentration (g/100g) were measured simultaneously using a CombiFoss™ 7 FT analyser (FOSS, Hillerød, Denmark), which combines flow cytometry (Fossomatic™ module) for SCC/DSCC determination and mid-infrared spectroscopy (MilkoScan™ module) for milk composition. Analyses were performed according to the manufacturer's protocols and international reference standards.

2.3 Statistical Analysis

Data were analyzed using R software (R version 4.5.1). Before analysis, SCC values were multiplied by 1,000 to convert them from thousands of cells/mL to absolute cell counts. Each milk sample was classified into one of four udder health categories based on combined SCC and DSCC thresholds:

(1) Healthy udder: DSCC < 75% and SCC < 200,000 cells/mL,
(2) Subclinical mastitis: DSCC ≥ 75% and SCC < 200,000 cells/mL,
(3) Clinical mastitis: DSCC ≥ 75% and SCC ≥ 200,000 cells/mL,
(4) Chronic mastitis: DSCC < 75% and SCC ≥ 200,000 cells/mL.

Descriptive statistics (mean, standard deviation, median, minimum, and maximum) were calculated for lactose concentration in each group. Normality was tested using the Shapiro-Wilk test, which showed significant deviations from normal distribution for all groups ($p < 0.001$). Therefore, differences in lactose concentration among the four groups were assessed using the Kruskal-Wallis test, a non-parametric alternative to one-way ANOVA.

Where a significant global difference was detected, Dunn's post-hoc tests with Bonferroni correction were applied for pairwise group comparisons. Effect sizes were expressed as $\eta^2[H]$ (eta squared for the Kruskal–Wallis test) and effect sizes for Dunn's pairwise comparisons were calculated as $r = Z / \sqrt{N}$, where r represents the effect size coefficient, Z is the standardized test statistic from the Dunn comparison, N is the total number of observations across the two groups, and $\sqrt{N}$ is the square root of N. Effect sizes were interpreted as small (r = 0.1), medium (r = 0.3), or large (r = 0.5) effects. Associations between DSCC, SCC, and lactose concentration were further explored using Spearman's rank correlation coefficients (r_s).

Statistical significance was set at $p < 0.05$ for all analyses. Because multiple milk samples were collected from the same cows over the two-year period, observations may not be fully independent. Therefore, correlation results should be interpreted with caution, as they reflect sample-level associations rather than fully independent experimental units.

3. Results

3.1. Descriptive Statistics

Of the 1,083 milk samples collected, 1,067 could be classified into udder health groups and were included in the statistical analysis: 774 healthy udders, 137 subclinical mastitis, 116 clinical mastitis, and 40 chronic mastitis. Sixteen samples (1.5%) could not be classified due to missing or invalid DSCC or SCC values and were excluded from group comparisons.

Across the 1,067 milk samples, the mean lactose concentration was 4.82 ± 0.37% (range: 1.94-5.31%). When classified by udder health status, lactose concentration decreased progressively from healthy cows to those with chronic mastitis. Mean lactose was highest in cows with a healthy udder (4.85 ± 0.33%) and decreased in subclinical (4.79 ± 0.43%), clinical (4.71 ± 0.34%), and chronic mastitis (4.48 ± 0.64%) groups.

These distributions are visualized in Figure 1, which presents box plots for each udder health group, with the mean marked by a white diamond and individual data points displayed as jittered dots. The figure clearly illustrates the progressive decline in lactose concentration from healthy to chronically affected udders and the greater variability observed in chronic mastitis, reflecting a more severe and heterogeneous disruption of mammary secretory function.

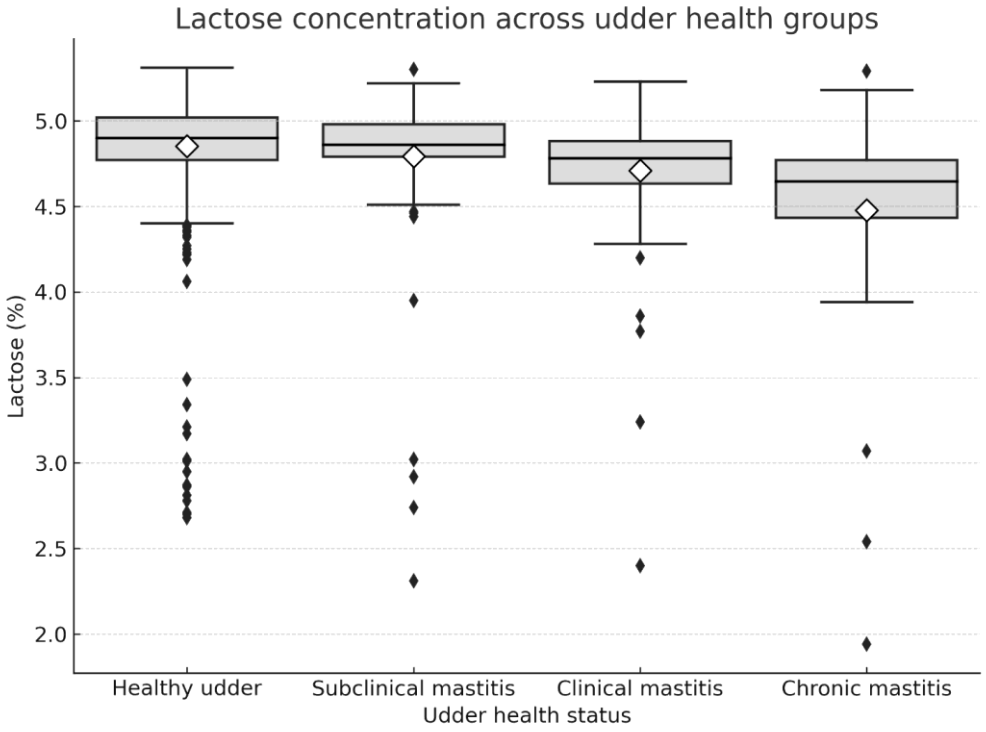

Figure 1. Boxplots of lactose concentration (%) across udder health groups (Healthy, Subclinical, Clinical, Chronic mastitis).

3.2. Group Comparisons

Shapiro-Wilk tests indicated that lactose was not normally distributed in any of the four groups ($p < 0.001$), justifying the use of non-parametric tests. The Kruskal-Wallis test confirmed a statistically significant difference in lactose concentration among the four groups ($p < 0.001$, $\eta^2[H] = 0.074$, indicating a moderate effect size), demonstrating that udder health status has a meaningful impact on milk lactose levels.

Dunn's post-hoc pairwise comparisons revealed that lactose was significantly lower in cows with clinical mastitis compared with healthy udders ($p < 0.001$, $r = -0.206$) and in chronic mastitis compared with

healthy udders ($p < 0.001$, $r = -0.199$). Significant differences were also found between subclinical and clinical mastitis ($p = 0.001$, $r = -0.123$) and between subclinical and chronic mastitis ($p < 0.001$, $r = -0.152$). No significant difference was observed between healthy and subclinical mastitis ($p = 0.44$) or between clinical and chronic mastitis ($p = 0.21$).

These pairwise comparisons are summarized visually in Figure 2, which displays a heatmap of Bonferroni-adjusted p-values. Darker blue shades indicate lower p-values, highlighting the strongest statistical differences between groups.

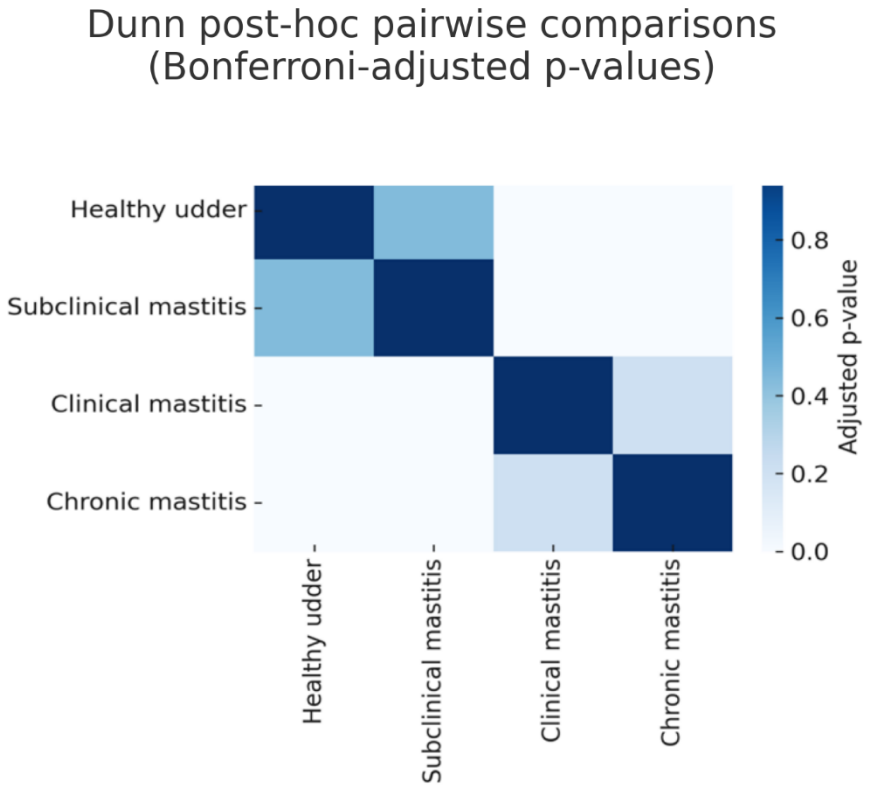

Figure 2. Heatmap of Bonferroni-adjusted p-values from Dunn's post-hoc comparisons of lactose concentration between udder health groups.

3.3. Correlation Analysis

Spearman correlations further confirmed these findings, revealing a strong positive association between DSCC and SCC ($r_s = 0.83$), which is expected since both parameters reflect the intensity of mammary inflammation. Lactose was moderately negatively correlated with both SCC ($r_s = -0.46$) and DSCC ($r_s = -0.40$), indicating that lactose declines with increasing severity of intra-mammary inflammation.

4. Discussion

Our results clearly demonstrated a progressive decline in lactose concentration with worsening udder health status, from healthy udders to chronic mastitis. The Kruskal-Wallis test confirmed that these differences were statistically significant ($p < 0.001$), with a moderate effect size, and Dunn's post-hoc analysis highlighted that the most pronounced reductions occurred in clinical and chronic mastitis compared with healthy udders. The increased variability observed in chronic mastitis cases suggests a heterogeneous pattern of mammary damage, consistent with the chronic nature of these infections. Furthermore, Spearman correlations revealed a strong positive association between SCC and DSCC and moderate negative correlations between lactose and both SCC and DSCC, confirming that lactose declines in parallel with inflammatory cell infiltration. The strong correlation between SCC and DSCC was expected, as both reflect inflammatory cell dynamics.

The combined use of SCC and DSCC to classify udder health in our study is consistent with current literature emphasizing the complementary value of DSCC in mastitis diagnostics [1-3]. DSCC provides

information on the cellular composition of milk leukocytes, distinguishing neutrophil-dominated responses—indicative of acute intramammary infection from lymphocyte-predominant profiles that may signal recovery or chronic inflammation [1]. Studies have shown that DSCC enhances the sensitivity and specificity of mastitis detection when combined with SCC, particularly in early or subclinical stages [2,3]. This is in line with our finding that DSCC ≥ 75% was associated with lower lactose even when SCC remained below 200,000 cells/mL, underscoring its role as an early warning parameter.

From a physiological standpoint, the observed decline in lactose concentration can be explained by the effect of intra-mammary infection on mammary epithelial cell activity. Lactose is synthesized in the Golgi apparatus of secretory cells by the lactose synthase complex, and its production is closely linked to osmotic balance and milk volume [4]. Inflammation reduces synthetic activity, increases tight-junction permeability, and allows lactose to leak into the bloodstream, leading to decreased milk lactose content [4,5]. Our results are consistent with previous findings that milk lactose is one of the most sensitive indicators of mammary gland integrity and declines during both subclinical and clinical mastitis [13–15].

Regular monitoring of DSCC, SCC, and lactose offers practical advantages for herd management. DSCC has been validated as a rapid, cost-effective parameter for routine milk recording programs [6–9], with particular value in identifying cows with early or subclinical mastitis. Several studies have highlighted its utility for improving mastitis control strategies, especially when integrated with SCC and other milk recording data [10–12]. Our findings reinforce this approach, suggesting that including lactose concentration in decision-support systems could further improve the early detection of intra-mammary inflammation and reduce production losses.

Finally, from a herd-level perspective, lactose measurement is highly feasible because it can be automatically obtained via mid-infrared spectroscopy during routine milk recording [10,16]. Integrating lactose with SCC and DSCC provides a more comprehensive view of udder health and may allow more targeted interventions, reducing unnecessary treatments and culling, thereby improving both productivity and animal welfare. This supports the concept that milk composition and particularly lactose, serves as a mirror of udder health and a powerful tool for precision livestock management.

A limitation of the present study is that multiple samples were obtained from the same cows over time, which may introduce within-animal correlation and potentially influence the precision of statistical estimates.

5. Conclusions

This study demonstrates that combining SCC and DSCC provides a robust classification of udder health and reveals a clear, progressive decline in lactose concentration with increasing severity of intra-mammary inflammation. Lactose remained unchanged between healthy and subclinical mastitis but decreased significantly in clinical and chronic mastitis, highlighting its sensitivity as an indicator of secretory dysfunction. Notably, DSCC ≥ 75% was associated with reduced lactose even when SCC remained below 200,000 cells/mL, confirming its role as an early marker of inflammation. Incorporating lactose measurement alongside SCC and DSCC in routine herd monitoring can enhance early mastitis detection, support targeted interventions, and improve both productivity and animal welfare.

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Case Report

Minimally Invasive Occlusion of Canine Patent Ductus Arteriosus: Technical Aspects and Clinical Outcomes from the first experience in Veterinary Medicine in Romania

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Abstract: Patent ductus arteriosus (PDA) is a frequent congenital cardiovascular anomaly in dogs that can lead to significant hemodynamic consequences if left untreated. This report presents three canine cases of PDA treated by minimally invasive transcatheter closure using a Nit-Occluder® coil or an Amplatzer Vascular Plug II (AVP II) via a transjugular approach. All interventions were uneventful under general anesthesia. Complete PDA closure was achieved in two dogs along with a regression of cardiac remodeling. In one dog, residual shunting persisted following AVP II release, likely due to partial device displacement; however, the patient remained asymptomatic at long-term follow-up. Technical difficulties related to atypical ductal anatomy were encountered in one case but did not impede successful device placement. Rapid post-procedural recovery was observed in all dogs. These results suggest the clinical applicability of transcatheter PDA closure and demonstrate that both AVP II and Nit-Occluder devices represent reliable therapeutic options for canine PDA.

Keywords: patent ductus arteriosus, PDA closure, interventional cardiology, transjugular approach, AVP II, Nit-Occluder

1. Introduction

Patent Ductus Arteriosus (PDA) is one of the most common congenital cardiac malformations in dogs (accounting for approximately 20–26% of all congenital malformations, as reported in the literature) and results from the failure of closure of a normal fetal vascular connection between pulmonary trunk and aorta. Breeds most frequently affected include the Bichon Frise, Chihuahua, German Shepherd, Keeshond, Pomeranian, and Poodle. In addition, females are more commonly affected than males [8,3].

In animals affected by PDA, the ductal wall contains more elastic fibers and a decreased number of smooth muscle fibers, making it similar to the aortic wall. Therefore, normal ductal closure does not occur, and blood keeps flowing from the descending aorta into the pulmonary artery. Because the aortic pressure exceeds the pulmonary arterial pressure throughout the entire cardiac cycle, the shunt remains present during both systole and diastole. This ongoing left-to-right shunt causes the pulmonary circulation, left atrium, and left ventricle to become overloaded. The shunt volume is influenced directly by the diameter of the ductus and by the pressure difference between the systemic and pulmonary circulations. Even though compensatory mechanisms like tachycardia and fluid retention may keep systemic output adequate, the chronic left ventricular volume overload increases the workload on the heart. This can result in worsening of the volume overload, of the mitral annulus dilation, and of the mitral regurgitation. Over time, decreased myocardial

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contractility altering ventricular compliance and rhythm disturbances cause left-sided congestive heart failure [10,12,4].

Interventional closure of patent ductus arteriosus (PDA) in dogs has become a routine procedure in modern veterinary cardiology. Since the first interventional PDA closure reported in 1994, the technique has progressively evolved, including both arterial and venous vascular access, as well as a variety of occlusion devices adapted to ductal morphology and patient's size [9].

Among the most commonly used devices is the Amplatz Canine Duct Occluder (ACDO), introduced since 2007 and deployed via a transarterial approach, and it remains one of the most frequently utilized devices for canine PDA closure due to its proven efficacy and safety. In more recent years, Amplatzer Vascular Plug II devices have been increasingly used, particularly via a transvenous approach, widely described since 2021. Coil embolization has been reported since 2012 and more recently the Nit-Occluder device was introduced into the veterinary literature in 2024, further broadening available interventional options [7,1,2,6,9,5].

In the context of continued technical and device-related advancements, our group recently reported its experience using a low-profile KA microplug for transarterial PDA closure in a dog. This device is particularly advantageous for small-diameter ductus and in small-sized patients, for whom interventional options may be limited. A key advantage of the microplug is the possibility of using a low-caliber introducer sheath equipped with a hemostatic valve, reducing vascular trauma and the risk of hemorrhagic complications and enhancing procedural safety. To the best of our knowledge, this case represented, at the time of its publication, the first reported use of the low-profile KA microplug device, delivered transarterially, for interventional PDA closure in veterinary medicine, underscoring its potential role as a viable alternative in carefully selected canine patients [11].

2. Cases

2.1 Case description

This case report includes three client owned dogs diagnosed with patent ductus arteriosus (PDA). All patients were female, aged between 1 and 2 years, and belonged to the following breeds: Cavalier King Charles Spaniel (CKCS), Dobermann, and Bichon. Two of the dogs had a previously established diagnosis of PDA and were referred for minimally invasive surgical correction. The third dog was referred for pre-anesthetic cardiologic evaluation after thoracic radiographs revealed cardiomegaly and cardiac biomarkers were found to be increased.

All dogs were asymptomatic on presentation, alert, with cardiac auscultation revealing a grade IV–V/VI continuous murmur on the left side of the thorax at the heart base level, along with bounding femoral pulses, pink mucous membranes and a capillary refill time of less than 2 seconds.

Transthoracic echocardiography confirmed a left-to-right shunting patent ductus arteriosus in all three cases, associated with left ventricular volume overload that was classified as mild in two dogs and moderate in the remaining case.

The interventions were performed between December 2022 and September 2023 at Doctor's Vet Univers Interventional Cardiology Laboratory Bucharest, Romania, the first facility of its kind from our country, founded in 2021. According to the authors experience, these represent the first three minimally invasive interventions for patent ductus arteriosus correction performed in dogs in Romania.

2.2 Procedure description

All patients underwent general anesthesia and were positioned in left lateral recumbency on the surgical table. Subsequently, the right external jugular vein was isolated by cut-down tehnique and placement of an introducer sheath at this level has followed. Two types of devices were used for the closure of the patent ductus arteriosus in these procedures. The first device, the Abbott Amplatzer Vascular Plug II, is a nitinol mesh device with multiple layers. The second device the PFM Medical Nit-Occluder, is a flexible, self-expanding nitinol coil designed to conform to the ductal anatomy. In both cases, vascular access and anesthesia were similar; however, the subsequent procedural steps differed slightly, as detailed below.

Correct catheter positioning was confirmed by angiography or fluoroscopic visualization. The guidewire was subsequently removed, and a selective angiography of the PDA was performed by manual injection of contrast medium through the catheter positioned within the ductal ampulla.

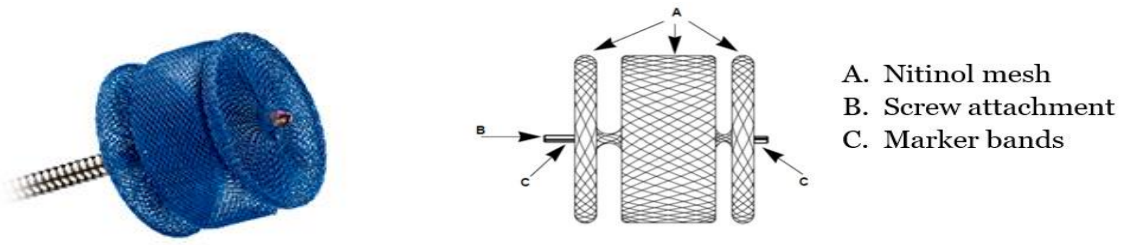


Figure 1. Amplatzer Vascular Plug II device aspect (left) and device diagram (right).
Source: Abbott Laboratories (manufacturer's website).

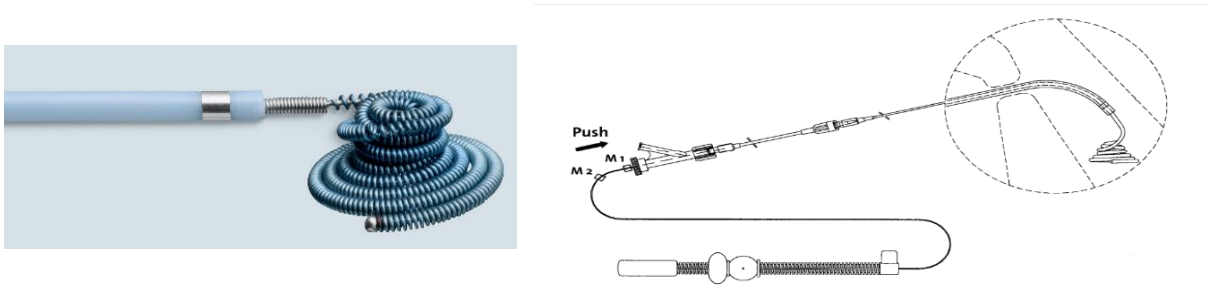
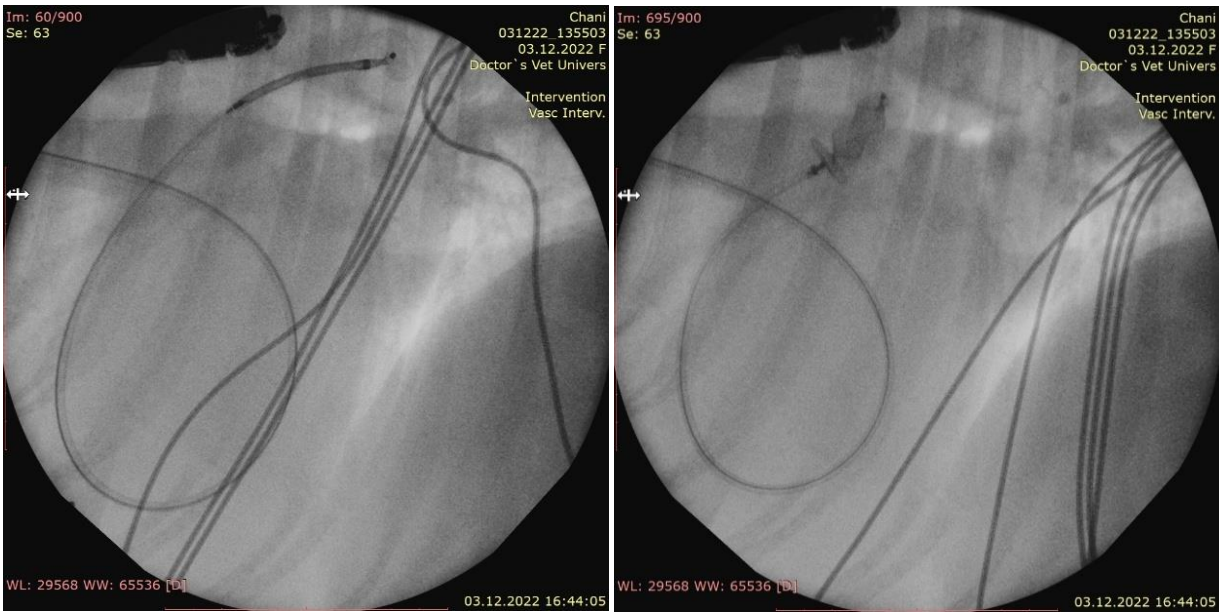


Figure 2. Nit-Occluder device aspect (left) and schematic diagram of the complete delivery system (right).
Source: PFM Medical (manufacturer's website) and product brochure.

Following angiographic assessment, the guidewire was reintroduced to allow exchange of the diagnostic catheter for the guiding sheath. The delivery catheter was advanced into the descending aorta, after which the guidewire was removed. In the next step, the occlusion device was advanced through the guiding sheath and the distal disc was expanded in the descending aorta (Fig. 3a). The guiding sheath and delivery system were gently retracted simultaneously until the distal disc engaged the aortic ostium of the PDA and the withdrawal continued until the expansion of the central portion of the device into the ductal ampulla. Further retraction of the guiding sheath allowed the proximal disc deployment within the main pulmonary artery (Fig. 3b). Intraoperative echocardiography was used to confirm appropriate device positioning and to evaluate residual flow. Following confirmation, the device was detached by counterclockwise rotation of the delivery cable (Fig. 4). All catheters were then removed, and the right jugular vein was closed by suture or ligation.

In contrast to the Amplatzer Vascular Plug II (AVP II) implantation procedure, the Nit-Occlud® device is delivered through a dedicated delivery system supplied as part of the device kit. The equivalent of the delivery sheath is referred to as the implantation catheter, which is advanced over the guidewire in the descending aorta, after removal of the diagnostic catheter and completion of angiography. Following guidewire withdrawal, the device transportation sheath is connected to the implantation catheter (after both of them are flushed with heparinized saline), allowing controlled delivery of the occlusion device. Following placement in the descending aorta, the majority of the device spirals were deployed from the implantation catheter (Fig. 5a) monitoring the fluoroscopic aspect and observing the distal marker position related to the Y-connector. The device and catheter were then retracted together to seat the spirals within the ductal ampulla (Fig. 5b). Finally, the remaining spirals were released into the pulmonary artery, and the device was definitively released once proper position, stability, and ductal occlusion were confirmed (Fig. 6).



(**a**) (**b**)

Figure 3. (**a**) Fluoroscopic image showing the release of the distal disk of the AVP II at the level of the descending aorta; (**b**) Fluoroscopic aspect of the the AVP II device, after the release of the third disk inside the pulmonary artery.

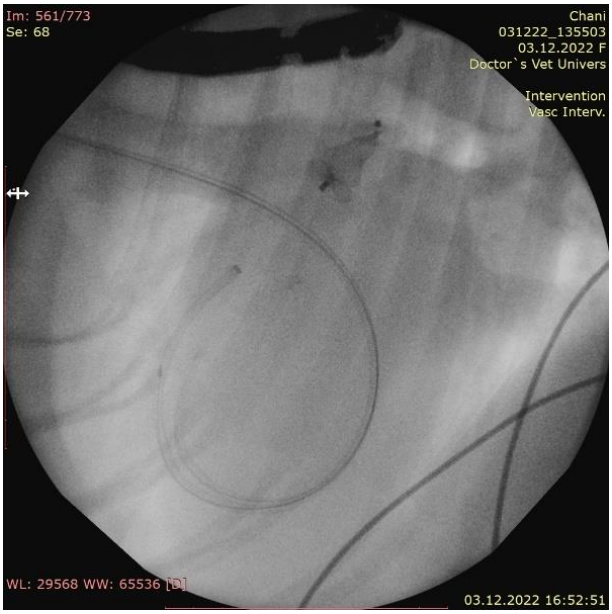
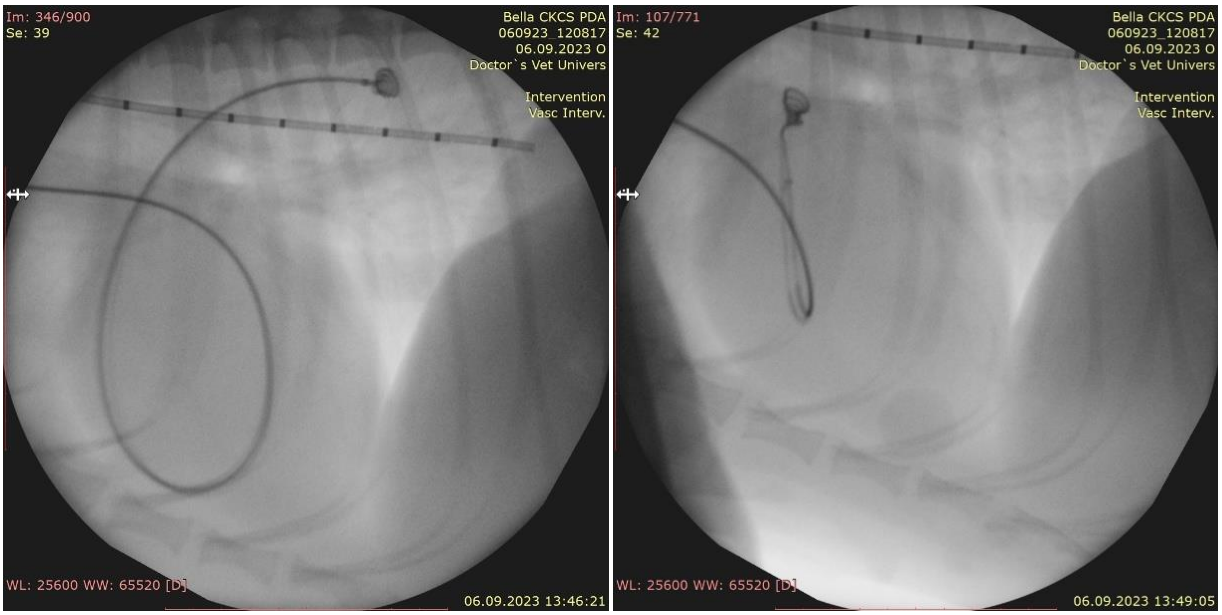


Figure 4. Fluoroscopic aspect after the complete release of the AVP II device.



(**a**) (**b**)

Figure 5: (**a**) Fluoroscopic image with the Nit-occluder loops released in the descending aorta; (**b**) Fluoroscopic aspect showing the Nit-occluder device withdrawn at the level of the ductal ampulla.

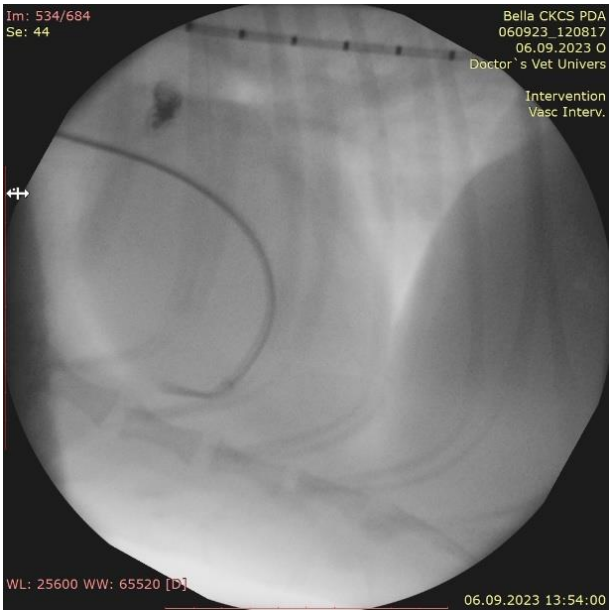


Figure 6. Fluoroscopic aspect after the complete release of the Nit-Occluder device.

3. Results

All three interventions were uneventful under anesthesia. Complete PDA closure was achieved in two dogs, accompanied by subsequent improvement in cardiac morphology. In the third case, although angiography prior to AVP II release indicated complete closure, residual shunting was noted after device deployment, probably due to slight device displacement. At a two-year follow-up, the dog remains asymptomatic, although it continues on positive inotropic medication and exhibits volume overload.

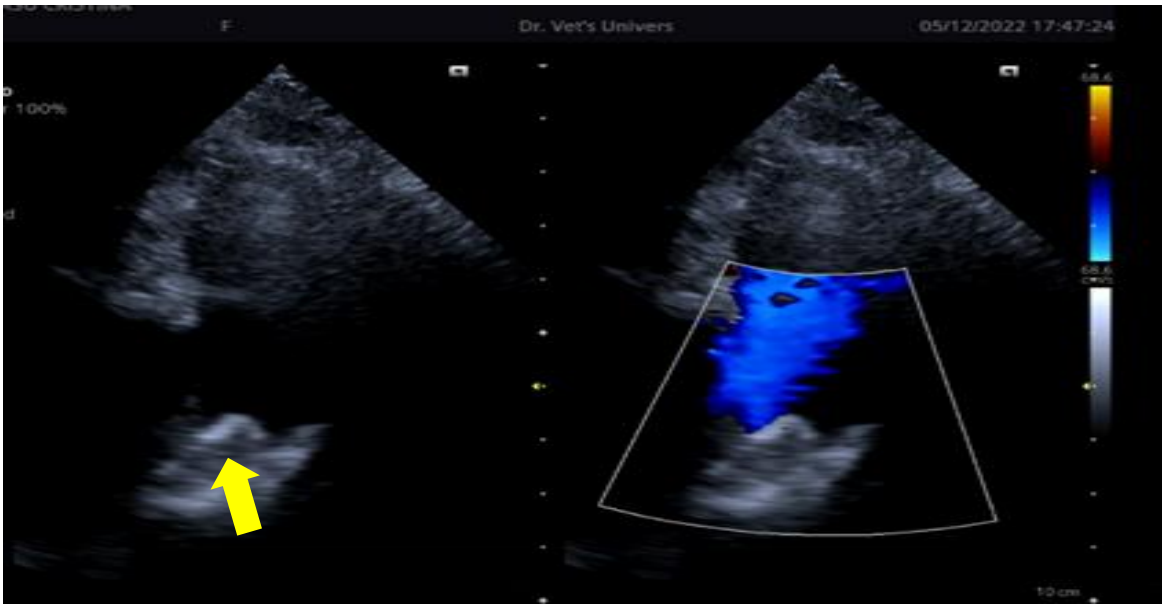


Figure 7. Transthoracic echocardiography performed 48 hours post procedure, CFM at the AVP II device(arrow) showing no residual flow.

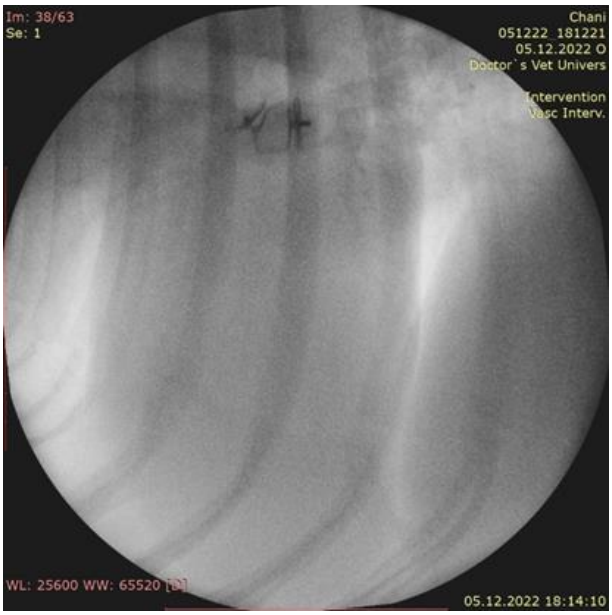
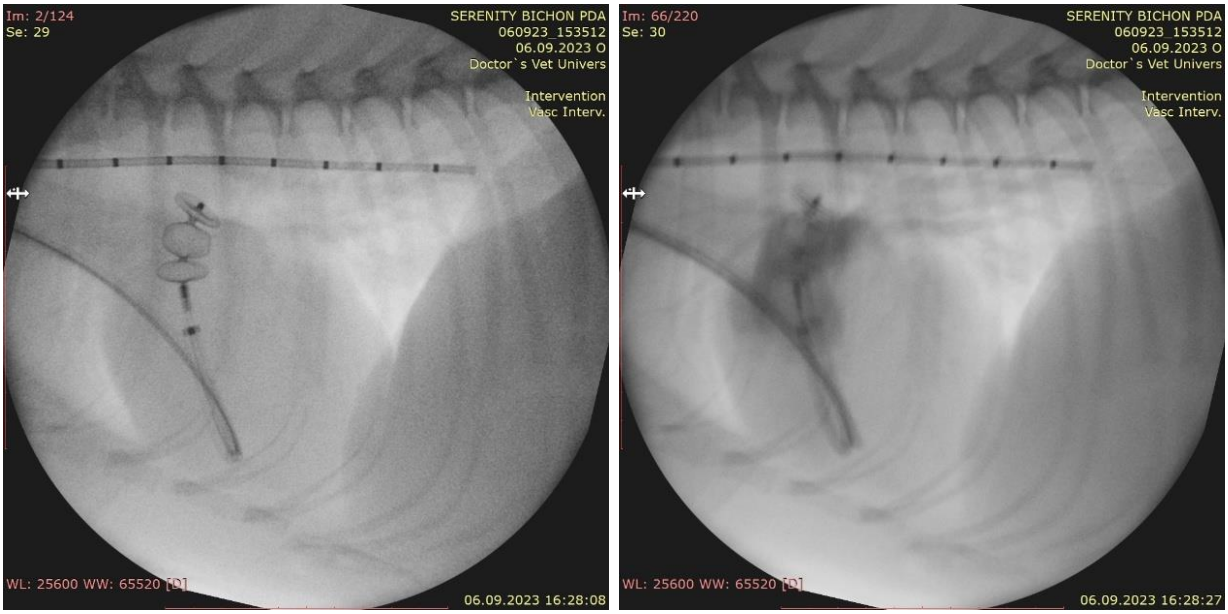


Figure 8. Fluoroscopic aspect of the AVP II device 48 hours post procedure in the Doberman dog.



(**a**) (**b**)

Figure 9. (**a**) Fluoroscopic aspect of the the AVP II device, after the release of the third disk inside the pulmonary artery; (**b**) Pre-release angiography suggesting proper occlusion of the ductus.

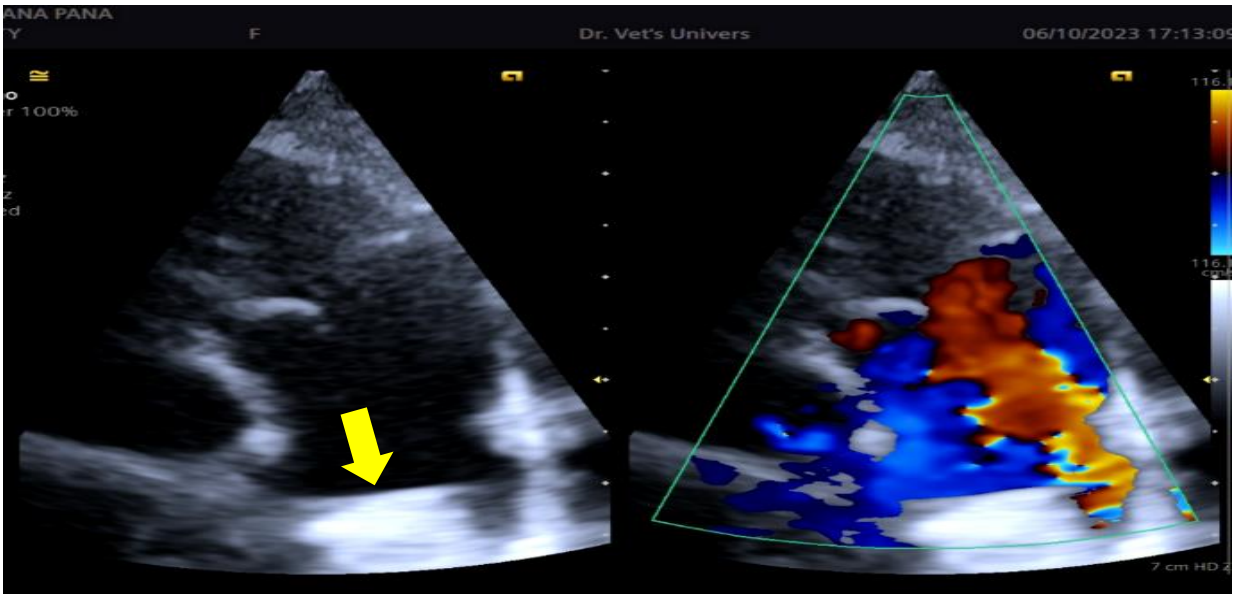


Figure 10. Transthoracic CFM echocardiography performed 1-month post procedure, showing residual flow at the level of the AVP II device (arrow).

4.Discussions

Successful deployment of both the Amplatzer Vascular Plug II and the Nit-Occluder was achieved in all cases and both should be considered viable options for treating this type of congenital cardiovascular anomaly in dogs. The transjugular approach was generally straightforward, although difficulties may arise when crossing the duct from the pulmonary trunk into the aorta, particularly in cases with small ostium or unfavorable ductal angulation. Recovery following the procedure was rapid, and all dogs were discharged just a few hours after regaining consciousness.

In the Bichon dog, pre-release angiography of the AVP II indicated complete occlusion of the ductus arteriosus (Fig. 9b); however, following device release, a residual shunt was identified (Fig. 10), most likely due to partial displacement of the vascular plug. Despite this finding, the patient has remained

asymptomatic to this day (over two-years since the procedure), although positive inotropic therapy has been maintained and persistent volume overload continues to be present.

Regarding the Doberman, catheterization of the ductus with the guidewire proved to be technically challenging, most likely as a result of an atypical PDA anatomy. This assumption was supported by the final orientation of the occlusion device (Fig. 8) in relation to the thoracic cavity, surrounding vascular structures, and adjacent anatomical landmarks. Additionally, the Doberman developed post-procedural cardiac changes resembling dilated cardiomyopathy, characterized by eccentric left ventricular hypertrophy and decreased myocardial contractility. These alterations were attributed to long-standing volume overload caused by the PDA. Ongoing treatment with Pimobendan resulted in a gradual reversal of the observed structural changes.

5. Conclusions

In conclusion, this case report provides additional evidence supporting that transjugular/transvenous PDA closure using AVP II and Nit-Occluder devices is feasible and clinically applicable procedure in veterinary interventional medicine and is an effective therapeutic option in dogs. Despite the small case series, procedural experience suggested that guidewire passage across the ductus arteriosus was technically less challenging in small-sized dogs.

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