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CONTENTS

Reproductive Parameters in Local Goats After Estrus Synchronization and Artificial Insemination with Frozen Semen During Breeding Season (Case Report)

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Key words: *goat, estrus synchronization, frozen semen, pregnancy*

Abstract. *This study aimed to determine selected reproductive parameters, including estrus synchronization response, pregnancy rate and prolificacy in Bulgarian local goats subjected to estrus synchronization and artificial insemination (AI) with frozen semen during the breeding season. The investigation was carried out with 101 lactating Bulgarian local goats during the breeding season. Estrus synchronization (ES) was performed using of intravaginal sponges containing 60 mg medroxyprogesterone acetate for 14 days, followed by an intramuscular injection of 500 IU PMSG (pregnant mare serum gonadotropin) on the day of sponge removal. The response to the synchronization was assessed on changes in the vaginal appearance. A single artificial insemination with frozen semen was conducted 48–52 hours after sponge removal. Pregnancy diagnosis was performed on day 35 after AI. Based on ultrasound pregnancy diagnosis and kidding data, pregnancy rate (PR) and prolificacy were recorded.*

The registered estrus synchronization response, pregnancy rate and prolificacy were 100%, 22.8% and 108.6%, respectively. In conclusion, the applied estrus synchronization protocol and artificial insemination with frozen semen during breeding season provided an acceptable pregnancy rate and prolificacy in Bulgarian local goats, particularly when the primary objective was acceleration of genetic progress in the flock.

Further investigations into factors affecting the success of these assisted reproductive technologies are necessary to improve their efficiency.

Introduction

The artificial insemination is an effective tool for accelerating genetic progress and ensuring high profitability and sustainable development of goat farms (Gibbons et al., 2011; 2013; Yeni and Gundogan, 2018). The success of AI in goats depends on multiple factors, including animal age, season, estrus detection methods and timing of semen deposition, insemination after spontaneous estrus or estrus synchronization, semen type and site of spermatozoa deposition (Arrébola et al., 2012, 2016; Tekin, 2019). The cervical insemination is a non-invasive and unexpensive technique; however, due to the anatomical characteristics of the cervix in small ruminants, it does not always provide satisfactory results (Leethongdee and Ponglowhapan, 2014; Daskin et al., 2016). Unlike in sheep, the goat cervix is approximately 2 cm shorter and consists of fewer cervical folds (3–6) arranged linearly, resulting in a relatively straight cervical lumen. These features allow deep intracervical or intrauterine semen deposition in goats, which leads to better results after artificial insemination with frozen semen, compared with sheep. In multiparous goats, the cervix is longer (4.2 ± 0.2 cm) than in nulliparous goats (3.5 ± 0.2 cm), while the number of cervical rings remains similar. The longer cervix in multiparous goats allows deeper semen deposition, resulting in a higher pregnancy rate (Intrakamhaeng et al., 2011, Hyacinth et al., 2016). A lower pregnancy rate obtained after AI with frozen semen is mainly attributed to a negative effect of the freezing procedure, which reduces sperm motility by 30–40% (Niño-González, 2008).

Reported reproductive outcomes following AI with frozen semen in goats vary widely depending on breed, age, synchronization protocol, timing of insemination, and semen deposition technique, with pregnancy rates ranging from 15.79% to 70% (Gibbons, 2002; Gibbons et al., 2013; Arrébola et al., 2012; Yotov et al., 2016; Dehouegnon and Koluman, 2018; Susilowati et al., 2023). The information regarding AI with frozen semen in indigenous goat breeds remains limited.

Therefore, additional studies are required to develop suitable technologies for the effective use of frozen semen from genetically superior bucks. This study aimed to evaluate selected reproductive parameters in Bulgarian local goats subjected to estrus synchronization and artificial insemination with frozen semen during the breeding season.

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Material and methods

The experiment was conducted during the breeding season (September) with 101 Bulgarian local goats, aged 3–6 years and weighing 50–60 kg. All animals were lactating with an average daily milk yield of 0.6 ± 0. 2 kg. Milking was performed twice daily in a milking parlor. The animals were housed in a farm with access to pasture and received supplemental concentrate (0.5 kg per animal), containing a vitamin-mineral premix. Drinking water was ad libitum. Prior to the experiment, all animals underwent antiparasitic and immunoprophylactic treatments.

Estrus synchronization was performed using intravaginal sponges containing 60 mg of medroxy-progesterone acetate (Ovigest 60, Hipra, Spain) for 14 days. After the sponge removal, each goat received an intramuscular injection of 500 IU PMSG (Folligon, Intervet International B.V., Netherlands). The response to synchronization treatment (number of synchronized animals / number of animals showing typical estrous signs on the day of AI × 100) was evaluated based on vaginal picture (vaginal hyperemia, redness and edema of the external part of the cervix and the presence of typical estrous discharge) observed by vaginoscope for small ruminants equipped with a cold light source (IMV Techenologies, France).

Animals showing inadequate response or abnormal discharge were excluded from insemination. A single artificial insemination with frozen semen in straws 0.25 mL (25×10^6 motile spermatozoa after thawing) was performed 48–52 hours after sponge withdrawal. The semen was thawed at 37°C for 30 seconds and deposited deep cervically using a special AI gun for small ruminants (Fig. 1).

Pregnancy diagnosis was performed on day 35 after AI using an ultrasound scanner (Draminski iScan, Poland) with a 7.5 MHz endorectal probe. Pregnancy was confirmed by visualization of an embryo with detectable cardiac activity. Pregnancy rate (number of pregnant goats / number of inseminated goats × 100) and prolificacy (number of kids born / number of goats kidded × 100) were calculated based on ultrasound and kidding records. All procedures complied with Bulgarian legislation regarding the protection and welfare of experimental animals (Ordinance No. 20/01.11.2012).

Fig. 1. Deep cervical deposition of frozen semen in goat

Results and discussion

Data on positive ultrasound pregnancy diagnosis and reproductive parameters are presented in Fig. 2 and Table 1.

The applied estrus synchronization protocol is appropriate for goats in the breeding season, evidenced by 100% estrus synchronization response. A similar good effect of sponges containing medroxyprogesterone acetate was determined by Motlomelo et al. (2002).

On day 35 of pregnancy, embryos appeared as echogenic structures within the uterine lumen filled with anechoic amniotic fluid, with clearly visible cardiac activity. The observed pregnancy rate (22.8%) was comparable with that reported by Leethongdee et al. (2013), who recorded pregnancy rates of 15.79% after single and 38.70% after double cervical insemination with frozen semen.

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In contrast, Dehouegnon and Koluman (2018) reported a pregnancy rate of 70% in Alpine goats following estrus synchronization and AI with frozen semen, likely due to differences in synchronization protocols and insemination during "standing estrus" detected by a teaser buck.

However, Yotov et al. (2016a) observed a higher pregnancy rate (58.3% vs. 45.2%) in single AI of goats by frozen semen after estrus synchronization, compared with AI of goats during the natural estrus.

The lower pregnancy rate in the present study may also be attributed to the lactation status. According to Yotov et al. (2016b), artificial insemination in lactating goats resulted in lower pregnancy, compared with those in dry animals (72% vs. 19%). These findings support the multifactorial nature of reproductive performance following AI with frozen semen. In this aspect, Tekin (2019) determined no significant differences in pregnancy rates, depending on animal age. In goats aged 7 months (never kidding) and 19 months (first kidding), pregnancy rates were 31.4% and 32.3%, respectively. The same study revealed that when insemination of goats was after preliminary testing by a teaser and detection of "the standing estrus", pregnancy rates and prolificacy were 67.3% and 119%, respectively.

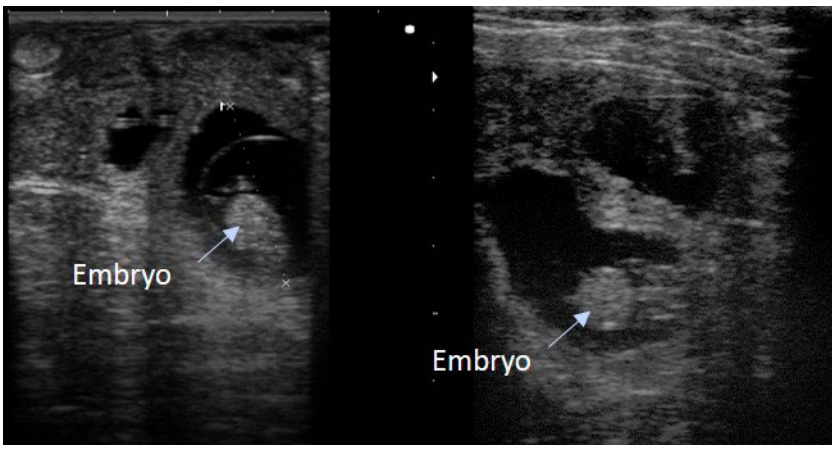

Fig. 2. Ultrasound pictures of pregnancy in goats on day 35 after AI

Table 1. Reproductive parameters in Bulgarian local goats after estrus synchronization and artificial insemination by frozen semen during breeding season

Goats under estrus synchronization (n)	101
Estrus synchronization response, % (n/n)	100 (101/101)
Pregnancy rate based on USE, % (n/n)	22.8 (23/101)
Kids born (n)	25
Prolificacy, % (n/n)	108.6 (25/23)

*USE – ultrasound examinations

The calculated prolificacy for Bulgarian local goats (108.6%) was close to the one obtained for Angora breed (124%) (Gibbons and Cueto, 2011) but differed considerably from the prolificacy registered for Majorera goat (160–167%) (Batista et al., 2009). It is known that the prolificacy is genetically determined and has a high positive correlation with the number of ovulated follicles. Nevertheless, the reproductive parameters also depend on the type of semen (fresh diluted, undiluted or frozen) and the place for sperm cells deposition (cervical or intrauterine) (Salvador et al., 2005; Paulenz et al., 2005; Arrébola et al., 2012). The abovementioned factors are very important for ensuring enough spermatozoa with preserved fertilizing capacity around the time of ovulation, which is crucial for increasing pregnancy and kidding rates. Paulenz et al. (2005) reported high pregnancy rates (87% and 78%) in AI of goats with fresh-diluted and undiluted semen, respectively. A high pregnancy rate (71%) was obtained after transcervical artificial insemination (Sohnrey and Holtz, 2005). A satisfactory pregnancy rate (66%) was achieved by Kulaksiz and Daskin (2012) using intrauterine deposition of frozen semen by laparoscopy.

However, laparoscopic insemination is not a routine method for insemination in goats, due to the high cost of the manipulation and the need for sedation and local anesthesia (Vrisman et al., 2014).

Final analysis of the current results and the information obtained from other authors confirmed that estrus synchronization protocols and artificial insemination with frozen semen in fixed time could be used successfully in goats, but they have to be adapted to specific features of animals. The time for insemination and the semen deposition technique should also be taken into consideration for achievement of good reproductive performance.

On the one hand, the lower pregnancy results after AI with frozen semen have negative economic effects. On the other, wide application of AI by frozen semen from genetically valuable bucks allows fast production of animals with high genetic merit. The obtained information could be utilized for reproductive process optimization in goats.

In conclusion, the abovementioned estrus synchronization protocol and artificial insemination with frozen semen of Bulgarian local goat during breeding season provide acceptable pregnancy rates and prolificacy, when the main purpose is acceleration of genetic progress in the flock. Future detailed investigations in large numbers of goats are necessary to clarify some debatable questions and to make these assisted reproductive technologies more effective.

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Conflict of interest

The authors have not declared any conflict of interests.

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Testicular and Seminal Characteristics of Yearling Tazegzawt Rams in Eastern Algeria

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Keywords: *Tazegzawt ram, semen quality, testicular measurements, seasonal variations*

Abstract. *This study aimed to evaluate monthly variations in testicular measurements and semen characteristics in young Tazegzawt rams. Ten yearling rams, aged 11 months at the beginning of the experiment, were monitored over a one-year period (January-December). Body weight (BW), scrotal circumference (SC), testis diameter (TD), testis length (TL), semen characteristics and sperm kinematic parameters were recorded monthly.*

Significant differences were observed between the months of the year for body weight, testicular measurements and semen parameters ($P < 0.05$), whereas differences across individual rams were significant only for body weight and testicular traits. Testicular size was lowest from January to March and increased progressively from May to November reaching peak values in October and November, followed by a slight decline in December.

Semen volume and sperm concentration were lowest during winter and early spring, increased from late spring to autumn, and reached maximum values in December for semen volume (1.13 mL) and in October–November for sperm concentration ($\approx 2.2 \times 10^9$ spermatozoa/mL). Sperm motility showed marked seasonal variation, with minimal values in winter and early spring and maximal values (≈100%) recorded in September and October. Sperm velocity parameters peaked during late summer and autumn, indicating fast but mostly non-progressive motility.

In conclusion, optimal semen quantity and quality in young Tazegzawt rams were observed from late spring through autumn, whereas reproductive performance declined during winter and early spring. These findings provide valuable information for the conservation and reproductive management of this endangered Algerian sheep breed.

Introduction

The Tazegzawt sheep are reared mainly for meat production in northeastern Algeria, specifically in the mountain region of Kabylia (Akbou in Bejaia Department), under extensive and often mixed farming systems, together with goat and cattle. The breed is characterized by black spots with bluish reflections on the eyes, ear lobes, muzzle and lower jaw.

Tazegzawt sheep represent a critical Algerian genetic resource and are considered one of the most endangered sheep breeds in the country, with an estimated population of approximately 300 heads. The breed is known for its hardiness, adaptation to mountainous environments, and favourable zootechnical performance, particularly in growth (average birth weight of 5.0 ± 1.0 kg and body weight of 39.7 ± 7.0 kg at 6 months) (Moulla et al., 2022) and reproduction (fertility rate of 84% and prolificacy of 150%) (El Bouyahiaoui et al., 2015). Preservation of the Tazegzawt breed is therefore essential to maintain local adaptability and genetic diversity in Algerian sheep populations.

Reproductive activity in rams is influenced by several factors, including genetics, breed, nutrition, ambient temperature, endocrine regulation, and management system. However, photoperiod remains the primary environmental factor affecting seasonal reproduction in sheep. Although seasonal effects are less pronounced in males than in females, significant monthly variations in scrotal circumference and semen characteristics have been reported both between breeds and between individuals within the same breed (Pandey et al., 1985; Langford et al., 1998; Salhab et al., 2003). Periods of reduced sexual activity are often characterized by decreased libido, testicular size, hormone secretion, and semen quantity and quality.

Seasonal changes in semen characteristics are mainly attributed to variations in day length throughout the year (Chemineau et al., 1992). In general, sperm quality in rams is superior during summer and autumn compared with winter and early spring (Oberst et al., 2011). Photoperiod has been shown to significantly influence sperm production and fertility in rams (Colas et al., 1985). Nevertheless, other factors such as nutrition, social environment, presence of females, geographical location, age, testicular morphology, body conformation, and libido may also affect semen quality (Al-Ghalban et al., 2004;

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Zamiri and Khodaei, 2005; Zarazaga et al., 2005). Under extensive management conditions, nutritional status may exert a stronger influence on reproductive activity than photoperiod alone (Mukasa-Mugerwa and Ezaz, 1992; Perez et al., 1997).

Although several studies have investigated sperm characteristics in Algerian sheep breeds (Aissaoui et al., 2004; Ghozlane et al., 2005; Boucif et al., 2007; Boussena et al., 2014; Benia et al., 2018; Belkhiri et al., 2019; Taherti et al., 2023), no information is currently available on testicular development and semen characteristics of Tazegzawt rams, a critically endangered Algerian sheep breed. Moreover, no longitudinal study has evaluated monthly changes in semen quality and sperm kinematic parameters throughout an entire year in young rams of this breed.

Therefore, the present study represents the first comprehensive assessment of body weight, testicular morphometry and semen characteristics, including CASA-based sperm kinematics, in young Tazegzawt rams reared under semi-extensive conditions in Algeria. We hypothesized that young Tazegzawt rams are capable of producing fertile and viable sperm throughout the year, with seasonal variations in semen quantity and quality. The objective of this study was to assess monthly variations in body weight, testicular measurements and semen characteristics in order to optimize reproductive management and support conservation strategies, including the potential use of artificial insemination, for this endangered sheep breed.

Materials and methods

Location, climatic conditions and ethical approval

The present study was conducted in collaboration with the Faculty of Natural and Life Sciences of Bejaia University and was carried out at the INRAA experimental station "Oued Ghir" in Béjaïa District, in northeastern Algeria, (36°42'37"N, 4°58›38››E; 66 m above sea level). This area is characterized by a Mediterranean climate with annual temperatures ranging from 12.9°C to 22.1°C and an average annual rainfall of 767 mm.

The experiment was conducted in accordance with the guidelines of Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and was approved by the relevant institutional ethics Committee.

Animals, feeding and management

This experiment was carried out from January to December using 10 young Tazegzawt rams aged 11 months at the beginning of the study, with body weights ranging between 34 and 54.5 kg. The rams were fed oat vetch hay and commercial concentrate composed of (50% barley, 10% corn, 37.5% wheat bran and 2.5% mineral vitamin supplement). Water and mineral blocks containing vitamins A, D_3 and E, as well as magnesium, manganese, iron, zinc, cobalt, iodine and selenium were provided ad libitum. Data for all parameters were recorded monthly for each ram from 11 until 22 month of age.

Determination of body weight, scrotal and testicular measurements

Each ram was weighed once a month, concomitantly with scrotal and testicular measurements. Body weight was recorded using a small ruminants scale (maximum capacity 200 kg ± 500 g, Marechalle Pesage, France). The scrotal circumference was measured at the widest point of the scrotum using the flexible metric tape. Testis diameter and length were measured using a sliding calliper after gently pushing each testis against the scrotum wall.

Sperm collection and evaluation

Semen was collected from each ram once a month throughout the study period using an electro-ejaculator (Ruakura probe, length 12 cm, diameter 2.5 cm, Shoof International Ltd., France). In April, semen analysis was not performed due to a failure of the computer-assisted sperm analyzer. A total number of 108 semen ejaculates were collected from 10 Tazegzawt rams during the experiment.

Semen samples were evaluated for volume (mL), sperm concentration per mL ($\times 10^6$ spermatozoa/mL), sperm motility (%), progressive sperm motility (%) and kinematic parameters (VCL, VSL, VAP, ALH, BCF and STR). Ejaculate volume was measured using a micropipette. The sperm concentration and motility and progressive sperm mobility were assessed using a computer-assisted sperm analyzer (Sperm Class Analyzer, SCA Microptic, S.L., Version 3.2.0, Barcelona, Spain).

Kinematic parameters for each spermatozoon were defined according to Mortimer (2000): curvilinear velocity (VCL; mean velocity of the sperm head along its actual trajectory, µm/s); straight-line velocity (VSL; mean velocity measured in a straight line from the beginning to the end of the track, µm/s); average path velocity (VAP; mean velocity along the smoothed path, µm/s); straightness (STR; VSL/VAP, %); linearity (LIN; VSL/VCL, %); wobble (WOB;VAP/VCL, %); amplitude of lateral head displacement (ALH; mean width of the lateral movement of the sperm head, µm) and beat-cross frequency (BCF; frequency of the sperm head crossing the average path, Hz).

Spermatozoa were considered motile when VCL⩾20 µm/s (Marco-Jiménez et al., 2005), rapid (RAP) when VCL > 75 µm/s and progressive when STR was at least 80%.

Statistical analysis

All data of body weight, testicular measurements (SC, TD and TL) and spermatic parameters (SV,

sperm concentration, mobility, progressive mobility and kinematic parameters of sperm) collected in 10 rams throughout the year were subjected to an analysis using SPSS/PASW version 20.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were calculated, and one-way analysis of variance test (ANOVA) was performed to evaluate the effect of month, followed by multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

Results

Monthly variations in body weight and testicular size

Variations in body weight and testicular parameters between rams and between the months of the year were highly significant ($P < 0.0001$). Mean body weight increased continuously from January and reached its highest value in September (66.20 ± 2.83 kg). A slight decline was observed in October and November, followed by an increase in December.

Regarding monthly variations in testicular size, the lowest values of testicular parameters (SC, TD and TL) were recorded from January to March. Testicular size increased from May to November, with the highest values observed during the autumn months, followed by a slight decrease in December (Table 1).

Monthly variations in sperm characteristics

The mean ejaculate volume was 0.68 ± 0.04 mL. Monthly mean values did not increase linearly with age but showed fluctuations throughout the study period, ranging from 0.37 ± 0.07 mL (minimum value in March) to 1.13 ± 0.11 mL (maximum value in December). Ejaculate volume showed highly significant monthly variations ($P < 0.0001$) but did not differ significantly between rams ($P > 0.05$). Semen production was low from January to March, increased from May to July, slightly decreased in August, and then increased again from September to December.

The average sperm concentration recorded in this study was 174313 ± 77.46 × 10^6 spermatozoa / mL. Monthly mean values fluctuated throughout the

Table 1. Mean (± SD) body weight and testicular measurements according to individual rams and months in young Tazegzawt rams

Animal / Variable		0.0001***	0.0001***	0.0001***	0.0001***
		BW (Kg)	CS (Cm)	TD (Cm)	TL (Cm)
1		66.34 ± 2.33	31.92 ± 0.40	5.77 ± 0.23	12.22 ± 0.38
2		68.00 ± 2.14	31.98 ± 0.55	6.00 ± 0.20	13.24 ± 0.15
3		62.00 ± 2.06	32.13 ± 0.22	6.00 ± 0.16	12.74 ± 0.26
4		59.33 ± 2.38	31.92 ± 0.67	6.12 ± 0.12	13.16 ± 0.24
5		56.95 ± 2.04	30.95 ± 0.98	5.94 ± 0.20	13.54 ± 0.22
6		62.58 ± 4.63	33.85 ± 0.45	6.33 ± 0.18	13.61 ± 0.16
7		49.21 ± 1.52	26.34 ± 0.86	5.15 ± 0.22	10.19 ± 0.38
8		60.01 ± 2.52	32.28 ± 0.52	6.10 ± 0.15	11.75 ± 0.36
9		51.33 ± 2.01	30.38 ± 1.14	5.99 ± 0.23	12.31 ± 0.28
10		55.55 ± 1.58	25.39 ± 0.89	4.98 ± 0.15	9.92 ± 0.40
Age (months)	Month measure	0.0001***	0.0001***	0.0001***	0.002**
11	January	48.12 ± 1.46	28.20 ± 0.98	4.90 ± 0.15	11.17 ± 0.50
12	February	50.20 ± 2.20	26.96 ± 1.46	4.94 ± 0.21	11.05 ± 0.57
13	March	53.30 ± 2.35	28.86 ± 1.21	5.25 ± 0.16	11.68 ± 0.44
15	May	56.44 ± 1.91	30.92 ± 0.96	5.93 ± 0.18	12.25 ± 0.50
16	June	61.70 ± 1.95	31.71 ± 0.96	6.05 ± 0.19	12.15 ± 0.51
17	July	62.90 ± 2.27	31.48 ± 0.77	6.08 ± 0,15	12.16 ± 0.50
18	August	63.95 ± 2.15	31.39 ± 0.80	6.12 ± 0.16	12.31 ± 0.49
19	September	66.20 ± 2.83	32.17 ± 0.79	6.20 ± 0.14	12.64 ± 0.34
20	October	62.00 ± 2.02	32.65 ± 0.75	6.39 ± 0.14	13.35 ± 0.33
21	November	61.40 ± 1.85	32.87 ± 0.79	6.45 ± 0.14	13.41 ± 0.34
22	December	64.50 ± 1.84	31.09 ± 0.94	6.10 ± 0.19	13.03 ± 0.34
Overall Mean ± SD		59.31 ± 8.57	30.80 ± 0.33	5.87 ± 0.07	12.31 ± 0.15

BW: body weight; SC: scrotal circumference; TD: testis diameter; TL: testis length; SD: standard deviation; mean values of body weight and testicular size differed significantly between individual rams and between months (***$P < 0.001$).

experiment period, with minimum and maximum values of 532.44 × 10^6 spermatozoa/mL in March and 2205.89 × 10^6 spermatozoa/mL in November, respectively. Sperm concentration did not vary significantly between rams ($P > 0.05$) whereas monthly variations were very significant ($P < 0.0001$). The monthly pattern of sperm concentration was similar to that of ejaculate volume, with low values from January to March, an increase from May to July, a decrease in August, followed by an increase from September to November and a slight decrease in December.

Mean sperm motility was 68.48 ± 3.34%. This parameter showed marked monthly fluctuations, with the maximum value recorded in September and October (almost 100%) and the minimum value observed in January (26.66%). Sperm motility was not statistically significantly different between rams ($P > 0.05$) but varied highly significantly between months of the year ($P < 0.0001$). Mean motility values were lower from January to March (winter and early spring), increased from May to November (late spring summer and autumn), and decreased again in December (Table 2).

Monthly variations in sperm kinematic parameters

Analysis of average sperm kinematic parameters showed that the sperm velocity parameters (VCL, VSL and VAP), measured objectively using a computer-assisted analyzer, were lowest in January. These parameters increased to reach a first peak in May, decreased in June, and then increased again to reach a second peak in October for VCL and in September and October for VSL and VAP. Thereafter, velocity parameters decreased in November and December.

Monthly mean STR values ranged between 47% and 57%. Although sperm mobility was not progressive (STR < 80%), spermatozoa were motile

Table 2. Mean (± SD) seminal characteristics according to individual rams and months in young Tazegzawt rams

Animal / Variable		Semen volume (mL)	Spermatozoa concentration (x10^6/mL)	Sperm mobility (%)
		0.22 (NS)	0.549 (NS)	0.289 (NS)
1		0.84 ± 0.19	1722.88 ± 242.95	73.41 ± 10.76
2		0.73 ± 0.09	1660.21 ± 292.43	68.23 ± 10.13
3		0.92 ± 0.14	2225.1 ± 231.93	93.15 ± 5.27
4		0.61 ± 0.11	1800.13 ± 223.27	71.75 ± 11.12
5		0.71 ± 0.08	1340.41 ± 184.62	53.32 ± 12.63
6		0.72 ±0.09	1690.24 ± 235.01	59.25 ± 9.84
7		0.53 ± 0.11	1851.92 ± 328.07	55.75 ± 13.08
8		0.61 ± 0.06	1537.97 ± 191.83	72.44 ± 8.65
9		0.59 ± 0.07	1827.08 ± 247.83	73.93 ± 10.32
10		0.54 ± 0.09	1795.1 ± 278.02	61.31 ± 11.72
Age	Collection month	0.0001***	0.0001***	0.0001***
11	January	0.50 ± 0.05	1439.40 ± 273.74	26.66 ± 3.20
12	February	0.43 ± 0.09	1104.50 ± 250.26	31.55 ± 10.33
13	March	0.37 ± 0.07	532.44 ± 94.11	50.66 ± 8.81
15	May	0.53 ± 0.08	1764.67 ± 300.97	81.94 ± 11.53
16	June	0.66 ± 0.12	1802.56 ± 300.03	72.6 ± 9.78
17	July	0.86 ± 0.20	2148.02 ± 229.36	75.34 ± 10.87
18	August	0.66 ± 0.08	1827.34 ± 188.61	70.04 ± 12.34
19	September	0.72 ± 0.04	1966.84 ± 126.97	98.97 ± 0.33
20	October	0.81 ± 0.09	2197.97 ±65.43	99.40 ± 0.29
21	November	0.80 ± 0.05	2205.89 ± 156.71	84.15 ± 6.21
22	December	1.13 ± 0.11	2033.30 ± 211.71	56.05 ± 9.05
Overall Mean ± SD		0.68 ± 0.04	1743.13 ± 77.46	68.48 ± 3.34

Mean values of semen parameters differed significantly between months (***$P < 0.001$) but not between individual rams ($P > 0.05$).

(VCL ⩾ 20 μm/s) throughout the study and showed fast movement (VCL > 75 μm/s) in May, September and October months (Fig. 1). Differences in kinematic parameters were not significant between rams ($P > 0.05$) but were highly significant between the months of the year ($P < 0.0001$) (Table 3).

General assessment of reproductive performance

Overall, the results indicate that good-quality semen can be collected from young Tazegzawt rams from 15 months of age. This breed appears capable of producing semen of satisfactory quality during most of the year, particularly from May to November, with optimal semen quality observed during the autumn months (September, October and November). Reduced semen quality was observed from January to March for ejaculate volume and sperm concentration, and from December to March for sperm motility. Consequently, the most favourable period for reproduction occurs during late spring, summer and autumn, whereas reproduction performance is reduced during winter months and early spring.

Discussion

To date, no scientific information is available regarding the semen characteristics of the Tazegzawt ram. This study provides the first detailed description of monthly variations in testicular size, semen characteristics and sperm kinematic parameters in young Tazegzawt rams reared in Algeria and raised in semi-extensive management conditions. The originality of this work lies not only in the investigation of a critically endangered local breed, but also in the

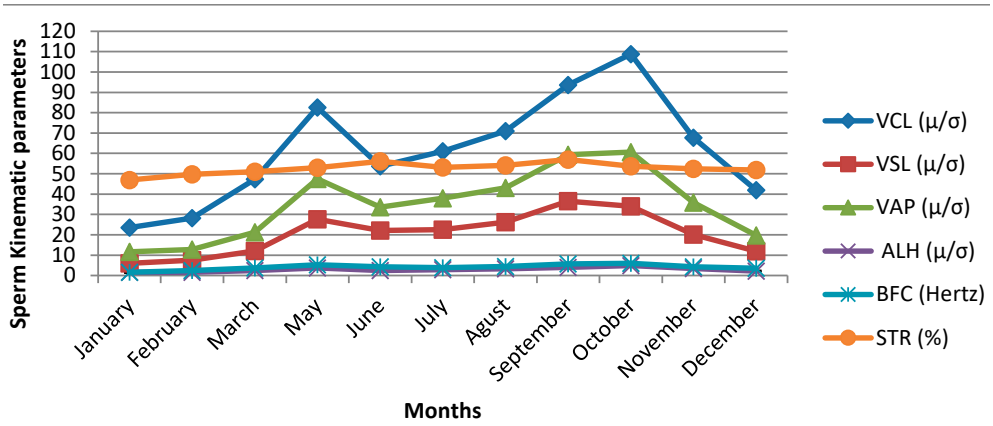

Fig. 1. Mean (±SD) sperm kinematic parameters (VCL, VSL, VAP, ALH, BFC and STR) according to collection month in Tazegzawt rams
Mean values of sperm kinematic parameters differed significantly between the months ($^{***}P < 0.001$).

Table 3. Mean (±SD) kinematic parameters according to individual Tazegzawt rams

Factor of variation	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	ALH (μm/s)	BCF (Hertz)	STR (%)
Animal	0.375(NS)	0.860(NS)	0.699(NS)	0.230(NS)	0.590(NS)	0.646(NS)
1	67.47 ± 11.30	22.7 ± 4.00	38.13 ± 7.06	3.13 ± 0.44	4.69 ± 0.69	54.62 ± 1.99
2	61.7 ± 11.72	18.64 ± 3.71	33.10 ± 6.79	3.02 ± 0.48	3.77 ± 0.62	52.90 ± 1.21
3	84.1 ± 9.93	25.81 ± 2.82	45.74 ± 5.50	3.95 ± 0,40	5.03 ± 0.42	54.30 ± 1.42
4	72.16 ± 12.76	23.38 ± 4.83	40.34 ± 7.47	3.34 ± 0.52	4.17 ± 0.68	50.74 ± 2.25
5	52.29 ± 9.42	19.77 ± 4.30	30.81 ± 6.39	2.45 ± 0.34	3.61 ± 0.69	52.39 ± 1.75
6	48.81 ± 8.22	16.77 ± 2.74	27.59 ± 4.81	2.42 ± 0.33	3.52 ± 0.47	51.65 ± 0.63
7	50.57 ± 10.03	18.73 ± 4.12	30.69 ± 6.62	2.43 ± 0.39	3.53 ± 0.66	52.29 ± 2.61
8	58.00 ± 8.81	20.95 ± 3.81	34.16 ± 6.18	2.73 ± 0.32	4.39 ± 0.46	54.19 ± 0.98
9	64 62 ± 8.95	22.01 ± 3.78	36.89 ± 5.81	3.06 ± 0.35	4.66 ± 0.68	53.62 ± 2.41
10	60.73 ± 10.74	19.02 ± 3.41	33.59 ± 6.17	2.96 ± 0.47	3.86 ± 0.53	50.21 ± 1.50
Overall Mean ± SD	62.27 ± 3.27	20.82 ± 1.17	35.19 ± 1.97	2.96 ± 0.13	4.13 ± 0.19	52.70 ± 0.55

VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; STR: straightness.
Mean values of sperm kinematic parameters not differed significantly (NS) between individual rams ($P > 0.05$).

longitudinal monitoring of semen quality over a full annual cycle using a CASA system.

The progressive increase in body weight observed in young Tazegzawt rams throughout the experiment period is consistent with findings reported in ram lambs by Elmaz et al. (2007), Boussena et al. (2014) and Maksimovic et al. (2016). In the present study, mature body weight was not reached by the end of the experiment, suggesting that males of this breed likely complete their growth at approximately two years of age. Significant differences in body weight among individuals can be attributed to the lack of homogeneity in initial body weights at the start of the experiment, which may reflect differences in early-life management and environmental conditions. The significant effect of the month of the year on body weight ($P < 0.05$) is in agreement with results reported by Tabbaa et al. (2006) in yearling Awassi rams.

Testicular size is widely used as an indicator of ram fertility, and testicular morphometry is considered a reliable predicator of sperm production (Abba et al., 2015; Ghorbankhani et al., 2015; Maksimovic et al., 2016). Testicular development depends on several factors, including age, body weight, nutrition and breeding (Ngcobo et al., 2023). In the present study, testicular measurements of young Tazegzawt rams varied throughout the year, with the highest values recorded from May to November (late spring, summer and autumn). In this region, breeding activities are mainly practiced during spring and autumn, and the increase in testicular size during these periods is associated with an increase in sperm production. The lowest testicular measurements were observed from January to March (winter and early spring) followed by a slight decrease in December.

These findings suggest that seasonal variations in testicular size are not strictly governed by photoperiod, as males were capable of producing sperm throughout the year, although reproductive activity was reduced during winter and early spring. Similar reductions in testicular size during winter have been reported in adult Ouled Djellal rams by Belkhiri et al. (2019), while Kafi et al. (2004), Tabbaa et al. (2006) and Maksimović et al. (2016) reported the lowest scrotal circumference values during the winter in different sheep breeds. The lower testicular measurements observed at the beginning of the experiment may also be related to incomplete sexual and body development. Oberst et al. (2011) reported more pronounced seasonal variations in scrotal circumference in young Lacaune rams. Overall, the significant effect of the month on testicular size observed in the present study is in agreement with findings by Tabbaa et al. (2006), Elmaz et al. (2007) and Maksimović et al. (2016).

With regard to semen characteristics, pronounced, monthly fluctuations were observed in ejaculate volume, sperm concentration, sperm mobility and sperm kinematic parameters. Semen of good quality was obtained from May to December for ejaculate volume ($P < 0.0001$) and sperm concentration ($P < 0.0001$) and from May to November for sperm mobility ($P < 0.0001$), with highest mobility values recorded in September and October and highest sperm concentration in October and November. In contrast, sperm mobility and sperm concentration were lower during winter and early spring. In some breeds, however, the effect of the month on semen quality appears less pronounced. For example, Tabbaa et al. (2006) reported that, in yearling Awassi rams, ejaculate volume was affected by month whereas sperm motility and concentration were not.

In the present study, high ambient temperatures and increasing day length did not markedly reduce ejaculate volume, sperm concentration, or mobility. These findings contrast with those of Perez et al. (1997) and Karagiannidis et al. (2000) who reported reduced semen quality in rams during the summer due to high temperatures and day length. Conversely, Ibrahim (1997) observed that semen quality in Chios crossbred rams raised in the United Arab Emirates was not adversely affected during hot summer months.

The mean ejaculate volumes recorded in Tazegzawt rams fell within the general range reported for sheep (0.5 to 2 mL/ejaculate) by several authors (Menchaca et al., 2005; Safdarian et al., 2006), except for the month of February and March. The average ejaculate volume was similar to that reported in yearling Awassi rams (0.87 ± 0.2 mL; Tabbaa et al., 2006) but lower than the values reported for 18-month-old Ouled Djellal rams (1.18 ± 0.39 mL; Benia et al., 2018). Lower ejaculate volumes observed at the beginning of the experiment likely reflect the post-pubertal period, as also reported by Salhab et al. (2003) and Boussena et al. (2014). Seasonal decreases in semen volume have previously been reported in Algerian Ouled Djellal rams (Ghozlane et al., 2005; Boucif et al., 2007; Boussena et al., 2014).

The mean concentration recorded in this study ($1743.13 \pm 77.46 \times 10^6$ spermatozoa/mL) was lower than the range commonly reported in sheep (2×10^9 and 6×10^9 spermatozoa/mL (Ghorbankhani et al., 2015; Lavry et al., 2017), but higher than the values reported in local Bangladesh sheep breeds (Hassan et al., 2009). Sperm concentration was numerically lower during later winter and early spring, in agreement with observations by Tabbaa et al. (2006), who reported reduced sperm concentration during late autumn and early winter.

The mean sperm motility (68.48 ± 3.34%) did not reach the optimal range of 70–80% suggested by Baril et al. (1993). However, monthly values were considered satisfactory from May to November, whereas sperm mobility was reduced during winter and early spring. These changes do not appear to be strongly influenced by photoperiod or temperature, as previously reported Aller et al. (2012). Similarly,

Ghozlane et al. (2005) and Kafi et al. (2004) found no significant effect of photoperiod or high temperature on sperm motility in Ouled Djellal and Persian Karakul rams, respectively.

In conclusion, young Tazegzawt rams exhibit moderate seasonal variation reproductive parameters. All semen parameters analyzed showed reduced values during winter and early spring, whereas semen quality improved markedly during late spring, summer and autumn, with optimal sperm concentration in October and November and optimal motility in September and October. Interestingly, the seasonal pattern observed in Tazegzawt rams suggests a reduced sensitivity to photoperiod compared with classical short-day sheep breeds. Despite a decline in semen quality during winter and early spring, acceptable semen characteristics were maintained during most of the year, including summer. This feature may represent an adaptive advantage of this breed to mountainous and Mediterranean environments.

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Molecular Detection of *Theileria*, *Babesia*, and *Anaplasma* in Livestock of Erbil, Iraq: Prevalence and Seasonal Trends Revealed by Real-Time PCR

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Abstract. *This investigation sought to determine the seasonal fluctuations in the occurrence of Theileria spp., Babesia spp., and Anaplasma spp. within herbivorous farm animals, encompassing various species such as cattle, sheep, goats, and lambs, within the Erbil region. A total of 244 animals were assessed throughout four distinct seasons: winter, spring, summer, and autumn. Seasonal prevalence rates were computed by dividing the number of infected animals by the total number of animals examined within each season. Polymerase chain reaction (PCR) was employed to identify and quantify parasitic infections present in the blood samples. In general, 84.2% of the animals exhibited positive results for one or more blood parasites, with Theileria spp. demonstrating the highest prevalence at 41.4%. Seasonal fluctuations in parasite prevalence were documented, Theileria prevalence peaks in summer at 59.72% and declines to its lowest point in autumn (21.66%). Babesia spp. and Anaplasma spp. exhibited comparable seasonal trends, characterized by elevated prevalence rates in spring and summer. This investigation underscores substantial seasonal and species-specific disparities in the prevalence of Theileria spp., Babesia spp., and Anaplasma spp., thereby implying the necessity for focused parasite control measures within the context of livestock management.*

Introduction

Farm animals, notably cows, sheep, and goats, are a significant source of meat, milk, and dairy products. These animals have several diseases that harm them both directly and indirectly, leading to high mortality rates, sterility, and abortion of numerous embryos, as well as a decline in protein synthesis, which results in significant economic losses to humans (Ponnampalam et al., 2022). Blood parasites, including *Theileria*, *Babesia*, and *Anaplasma*, which cause anemia, weakness, weight loss, overall animal fatigue, inflammation of mucous membranes, and many other symptoms that harm the animal's health and productivity, are prevalent in livestock and sheep (Hussen, 2020). The infection spreads swiftly across the entire herd unless it is decreased and controlled because these parasites are conveyed by a tick-related vector that lives momentarily ectoparasitically on the skin and spreads the infection from one animal to another (Villanueva-Saz et al., 2022). The multiplicity and high frequency of hemoparasite infection in farm animals for various years and locations in Iraq provide the strongest evidence (Ali and Alyasiri, 2024, Abdullah et al., 2019). The most crucial technique for determining whether the animal has a blood parasite infection is microscopic inspection. Although the basic, approved method of microscopic examination of a blood smear is still used because it is inexpensive and does not require complicated procedures to prepare and stain blood, it takes time and effort and requires enough practice and experience to distinguish and diagnose the tiny parasite (Willard and Tvedten, 2011, Rosenblatt et al., 2009).

The availability and variety of serological tests make them a good approach for diagnosis, but they are not without drawbacks that are typically associated with serological tests, such as cross-reactivity, difficulties obtaining antigens, their cost, and issues with sensitivity and specificity (Momčilović et al., 2019). Yet even though molecular tests are sensitive and exact, not all laboratories provide them as a diagnostic tool, and they are also expensive. So far, real-time PCR can be utilized as an accepted technique in both central and private laboratories for precise and swift diagnosis and to get over the drawbacks seen in other laboratory techniques. The aim of this study was to determine the prevalence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. in livestock in the Erbil region using RT-PCR, and to evaluate seasonal variation and the effectiveness of this molecular method for early detection and disease management.

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Materials and methods

Samples collection

In 2018 and 2019, a total of 244 animals, ranging in age from one month to four years, were sampled across all four seasons. The animals were randomly selected from four farms located in villages and suburbs to the south (Makhmur), north (Soran), east (Koya), and west (Khabat) of Erbil, northern Iraq (Fig. 1). These farms were chosen to represent different weather conditions and topographical features across the region. Within each farm, animals suspected of being infected with *Theileria* spp., *Babesia* spp., or *Anaplasma* spp. were selected for inclusion in the study. The selected animals represented various livestock species from both small- and medium-scale operations, ensuring a comprehensive sample of the local farming environment. The study design achieved a balanced geographical and climatic distribution by sampling 45 animals in winter, 67 in spring, 72 in summer, and 60 in autumn.

For each animal, 3 mL of blood was drawn from the jugular vein and placed in an EDTA tube. The tubes were gently mixed to ensure that the blood and anticoagulant reagent were well combined. DNA isolation was performed immediately after the blood samples were processed. The animals sampled represented a broad age range, from one month to four years old, and the selection from different seasons helped ensure a comprehensive analysis of the variables under varying environmental conditions.

DNA purification

The anticipated DNA purification was carried out using the DNA/RNA Prep NA (Nucleic acid extraction kit to extract and purify total RNA and DNA) from clinical materials (Sacace Biotechnologies Srl via Scalabrini, Como, Italy). As instructed by the kit, 300 µL of cell lysis solution was first put in an Eppendorf tube before 100 µL of anticoagulant blood was introduced to it. The tubes were vortexed and heated to 65°C for 15 minutes. Afterwards, 400 µL of Prec buffer was added and vortexed after being centrifuged for 7–10 seconds. Using a micropipette with an aerosol barrier tip blocked, the supernatant from each tube was carefully collected and discarded without disturbing the pellet after all tubes had been centrifuged at 13 000 rpm for 5 minutes. The tube tips were switched. With 500 µL of Wash Sol 1 and 300 µL of Wash Sol 2, the wishing phase was performed twice. All tubes were then incubated at 65°C for 5 minutes with open covers. In 50 µL of dilution buffer, the particle was resuspended. The tubes were incubated at 65°C for 5 minutes with intermittent vortexing, and then centrifuged for 60 seconds at 13 000 g. The RNA/DNA in the supernatant was then prepared for amplification. If amplification was not done on the same day as extraction, the processed samples were frozen at –20°/–80°C or stored at 2–8°C for a maximum of 5 days.

Primer design and PCR amplification

IDT (Integrated DNA Technologies, Coralville, Iowa, United States) designated and provided the oligonucleotide primers (Table 1) based on the mitochondrial and 18S and 16S rRNA gene sequences of *Babesia*, *Theileria*, and *Anaplasma*, respectively. The DNA and RNA Nucleotide Databases from Genebank, China, NCBI, and the complete relevant gene sequences and primer locations were all confirmed to be present in the genomes. A specific TaqMan hydrolysis probe was used in a real-time PCR with applied biosystems step one PCR in the

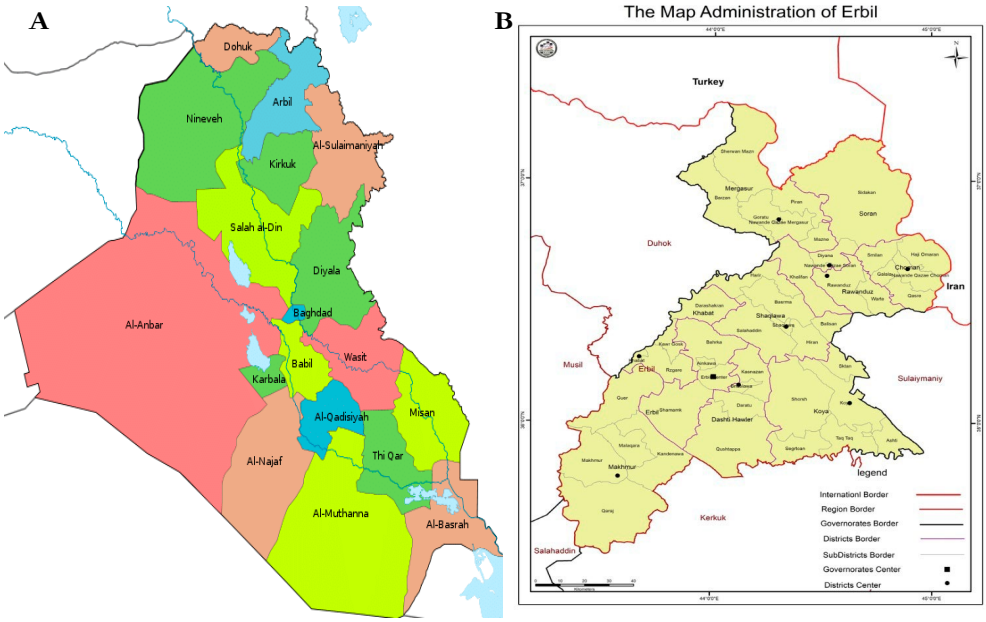

Fig. 1. (A) Map of Iraq showing the location of Erbil Governorate in northern Iraq. (B) Administrative map of Erbil Governorate indicating the sample site locations

Table 1. Primer sequences used for parasite detection

No.	Blood parasite	Primer sequence
1	*Babesia*	FWD 5'- ACG ATA GCC TTC CTA AAC TTC C -3' REV 5'- CAA GAG CTG CTA GTC CAG TTA T -3'
2	*Theileria*	FWD 5'- GCT TTG GAC GGT AGG GTA TT -3' REV 5'-TTA GAT GTG GTA GCC GTT TCT C -3'
3	*Anaplasma*	AM-F 5'-TTG GCA AGG CAG CTT -3' AM-R 5'- TTC CGC GAG CAT GTG CAT -3'

Thermal Cycler to amplify and detect the DNA by the prescribed procedure (Applied Biosystems, Foster City, California, USA). The reaction mixture contained 20 µL (10 µL of precision PLUS 2X Master Mix, 1 µL primer / probe Mix, and 4 µL RNase/ DNase) and 5 µL of DNA template of blood DNA extracts. Enzyme activation took place for two minutes at 95°C, then there were 50 cycles of 10 seconds each at 95°C, 60°C, and 72°C. Throughout the procedure, barrier pipette tips were utilized to avoid cross-contamination between samples. For each sample, two replicate PCRs were conducted. After amplification, we used melting curve analysis to interpret the results. In all PCR experiments, positive control DNA and negative controls, in which the DNA templates were switched out for sterile water, were used.

Statistical analyses

The data were analyzed using Microsoft Excel, and chi-square (χ^2) tests were applied to compare infection prevalence among animal species and across seasons. A P value < 0.05 was considered statistically significant.

Results

Despite the observed differences in the occurrence of infections caused by *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. across various animal species, including cattle, calves, sheep, lambs, and goats, statistical analysis using the Chi-square test (χ^2 = 1.494, df = 8, P = 0.9928) showed no significant relationship between the type of animal and the specific parasitic infection. The observed variances in infection rates among animal groups show that these variations are likely due to chance, rather than any underlying biological or environmental variables. The data in Table 2 reveal that the overall infection rate in the animals studied was 73.77% (180 of 244 animals). *Theileria* spp. was the most common, followed by *Babesia* spp. and *Anaplasma* spp.

Figs. 2–4 illustrate the seasonal incidence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. in the examined animals. For all three hemoparasites, infection prevalence was lowest in winter, increased markedly in spring, peaked in summer, and declined again in autumn, with *Theileria* spp. showing the highest overall rates, followed by *Babesia* spp. and *Anaplasma* spp. This consistent pattern across parasites indicates that transmission is strongly influenced by seasonal environmental conditions and associated vector activity.

Table 3 presents the distribution of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. infections across various infection classifications, including both singular and co-infections in the animal subjects. It details the number of infected animals, the proportion of infected animals (n = 180), and the percentage of the total examined population (n = 244), thereby illustrating the overall infection prevalence within the sampled cohort. The table incorporates P values to evaluate the statistical significance of the observed disparities. Single infections accounted for 70.0% of the infected animals (n = 180) and 51.64% of the entire population under study (n = 244). *Theileria* spp. showed a higher prevalence in infected animals (43.3%), although this difference was not statistically significant (P = 0.32). Mixed infections (with two or

Table 2. Incidence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. in examined animals

Type of animal	Total examined animals	Positive number (%)		
		Theileria spp.	*Babesia* spp.	*Anaplasma* spp.
Cattle	31	12 (38.7)	8 (25.8)	4 (12.9)
Calf	26	11 (42.3)	5 (19.2)	2 (7.6)
Sheep	91	43 (46.2)	24 (26.3)	8 (8.8)
Lamb	29	10 (34.4)	7 (24.1)	3 (10.3)
Goat	67	25 (37.3)	12 (17.9)	6 (8.9)
Total	244 (180 were positive) (73.77%)	101 (41.4)	56 (22.95)	23 (9.43)

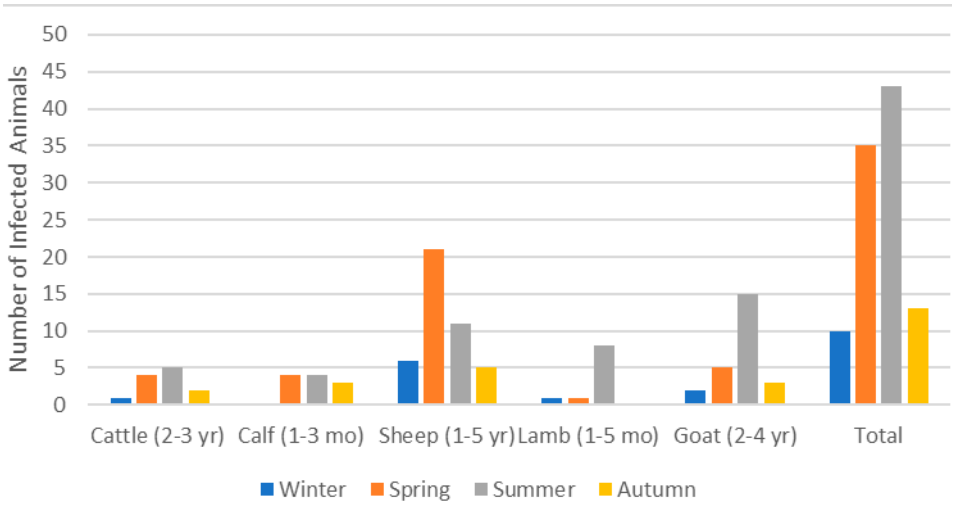

Fig. 2. Incidence of *Theileria* spp. in examined animals according to seasons

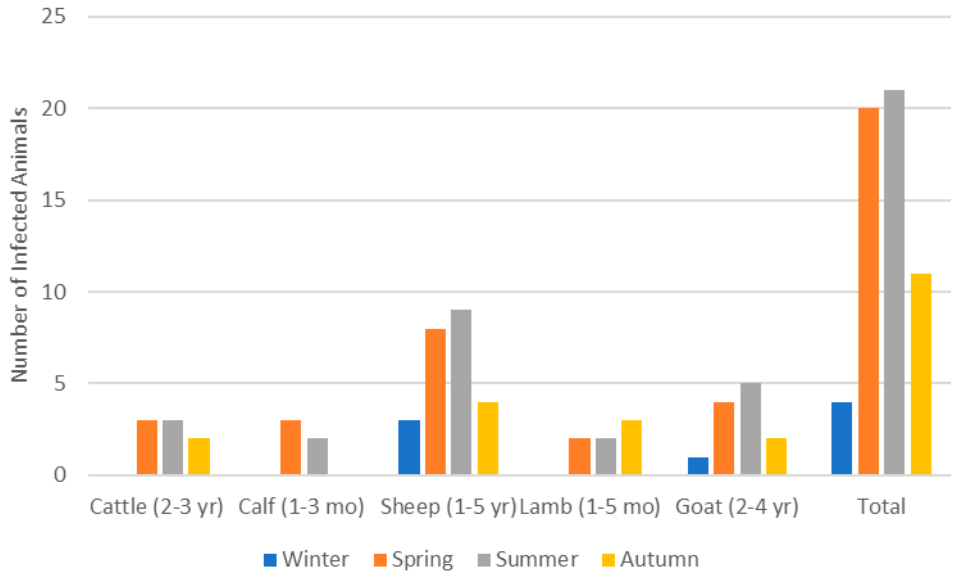

Fig. 3. Incidence of *Babesia* spp. in examined animals according to seasons

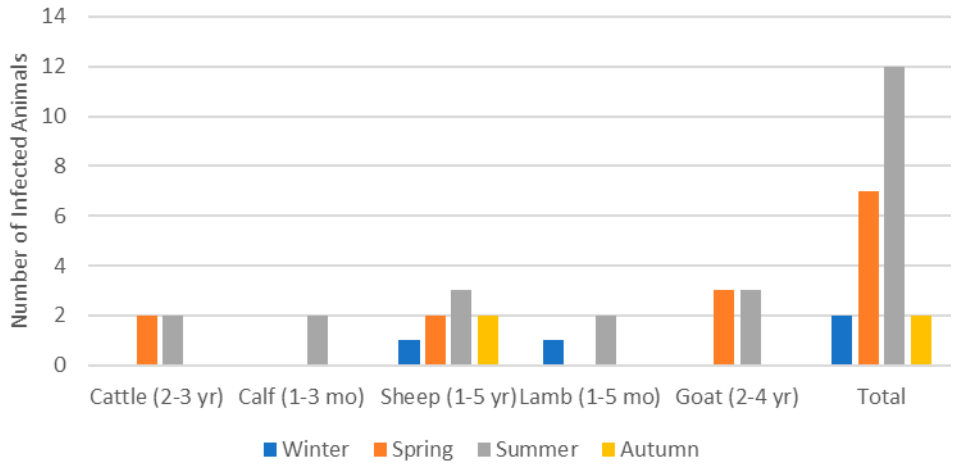

Fig. 4 Incidence of *Anaplasma* spp. in examined animals according to seasons

Table 3. Age-stratified prevalence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. in different animal species

Species	Age group	*Theileria* spp. (%)	*Babesia* spp. (%)	*Anaplasma* spp. (%)	*P* value
Cattle	Adult (31)	12 (38.7)	8 (25.8)	4 (12.9)	
	Calf (26)	11 (42.3)	5 (19.2)	2 (7.6)	$P = 0.85$
Sheep	Adult (91)	43 (46.2)	24 (26.3)	8 (8.8)	
	Lamb (29)	10 (34.4)	7 (24.1)	3 (10.3)	$P = 0.56$
Goat	Adult (67)	25 (37.3)	12 (17.9)	6 (8.9)	

more parasites) were found in 30.0% of the infected animals (n = 180) and 22.13% of all animals studied (n = 244). The Theileria + Babesia combination had a prevalence of 16.7% in infected animals, but this was not statistically significant ($P = 0.32$). This data helps us understand the distribution of single and mixed infections and the importance of different parasite combinations in determining infection rates within the community.

Table 4 shows that analysis of co-infection patterns among the 180 PCR-positive animals revealed that a single parasite species caused the majority of infections. Single infections accounted for 70.0% of all positive cases (126/180). Among these, *Theileria* spp. only was the most frequent pattern, representing 43.3% of infected animals (78/180), followed by *Babesia* spp. only (20.6%, 37/180) and *Anaplasma spp.* only (6.1%, 11/180). Mixed infections were also recorded, comprising 30.0% of all positive animals (54/180). The most common mixed pattern was *Theileria* + *Babesia*, identified in 16.7% of infected animals (30/180), whereas Theileria + Anaplasma and Babesia + Anaplasma were detected in 8.9% (16/180) and 4.4% (8/180) of animals, respectively. Triple co-infection involving all three genera was not observed (0/180). Comparison of mixed-infection patterns showed no statistically significant differences between animal species (χ^2, $P = 0.32$). Overall, these findings demonstrate that although mixed infections are relatively common, *Theileria* spp. remains the dominant pathogen when present either alone or in combination with other hemoparasites.

Table 5 details the seasonal prevalence of infections caused by *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. across the four seasons: winter, spring, summer, and autumn. The table provides the percentage of infected animals for each parasite within each season, with the number of positive animals relative to the total examined animals presented in parentheses. As an illustration, during winter, 22.22% of the animals exhibited *Theileria* spp. infection (10 of 45), 8.88% were infected with *Babesia* spp. (4 of 45), and 4.44% were infected with *Anaplasma* spp. (2 of 45). The overall infection rate across all seasons was

Table 4. Co-infection patterns of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. (single vs. mixed infections)

Type of infection	Number of infected animals	% of infected (n = 180)	% of all examined (n = 244)	*P* value
Single infections (any one parasite)	126	70.0%	51.64%	None
Theileria spp. only	78	43.3%	31.97%	$P = 0.32$*
Babesia spp. only	37	20.6%	15.16%	None
Anaplasma spp. only	11	6.1%	4.51%	None
Mixed infections (⩾ 2 parasites)	54	30.0%	22.13%	None
Theileria + Babesia	30	16.7%	12.30%	$P = 0.32$*
Theileria+ Anaplasma	16	8.9%	6.56%	None
Babesia + Anaplasma	8	4.4%	3.28%	None
Theileria + Babesia + Anaplasma	0	0.0%	0.00%	None

Table 5. Seasonal variation in prevalence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp.

Season	*Theileria* spp. (%)	*Babesia* spp. (%)	*Anaplasma* spp. (%)	Total infected (%)	*P* value
Winter (45)	22.22% (10/45)	8.88% (4/45)	4.44% (2/45)	73.77% (180/244)	$P < 0.05$
Spring (67)	52.23% (35/67)	29.85% (20/67)	10.44% (7/67)		None
Summer (72)	59.72% (43/72)	29.16% (21/72)	16.66% (12/72)		None
Autumn (60)	21.66% (13/60)	18.33% (11/60)	3.33% (2/60)		None

73.77% (180 of 244 animals), a figure that remained consistent throughout the observed periods. The *P* value associated with the winter season suggests that the observed differences are statistically significant ($P < 0.05$), whereas no significant variations were detected in other seasons. This table offers crucial information regarding the seasonal fluctuations in infection prevalence for each parasite, thereby serving as a resource for informing season-specific control strategies within livestock management practices.

Discussion

Blood parasite infections severely hamper the management and welfare of domestic farm animals in Iraq. One of these infections with the highest prevalence and economic impact is piroplasmosis (Alabbody, 2024; Alali et al., 2022). Furthermore, in many parts of Iraq, serological tests, stained blood smear examinations, and clinical signs are used to diagnose blood parasites (Gharban et al., 2022). To evaluate the epidemiological aspects of hemoparasites, however, these procedures are not sufficiently reliable and efficient. Nevertheless, taking into account the shortcomings of microscopy, serology, and molecular analysis, as well as the urgent need for an accurate and general diagnostic kit that diagnoses all or most infections regardless of the causative species, RT-PCR-based molecular detection and identification of blood parasites in some farm animals were conducted in Erbil province in northern Iraq. Of all non-randomly chosen animals tested for the parasites, 73.77% were infected. *Theleria* had the highest infection rate at 41.4%, which was significantly higher than the prevalence rates of Babesia (22.95%) and Anaplasma (9.43%). Using the PCR approach, similar outcomes were seen for the prevalence rates of these parasites. When compared with the PCR technique, Giemsa-stained smear microscopic analysis produced negative results in 8 (16%) blood samples, demonstrating that the PCR is more sensitive in the detection of tropical theileriosis (Hassan et al., 2012). A study using molecular diagnostic techniques identified multiple hemoparasitic agents that were affecting sheep in the Kurdistan region of Iraq. A total of 195 samples from the three governorates of Duhok, Erbil, and Sulaimaniya were examined. The pathogens discovered were *Anaplasma ovis* (62.6%), *Theileria ovis* (14.35%), *T. lestoquardi* (7.7%), *T. uilenbergi* (5.6%), and *Babesia ovis* (1.5%) (Renneker et al., 2013). According to the PCR results, 12 (31.57%) and 9 (23.68%) of the 38 camels were solitary and positive for *B. caballi* and *T. equi*, respectively (Jasim et al., 2015).

Seroprevalence of *B. bigemina* and *A. marginale* in cattle, sheep, goats, and wild goats was studied in the Erbil district, between January to December 2010. A total of 184 blood samples were collected from 44 cattle, 59 sheep, 70 goats, and 11 wild goats. The overall prevalence of *B. bigemina* infection was 12 (27.27%), 4 (6.77%), 5 (7.14%) and 1 (9.09%) in cattle, sheep, goats and wild goats and for *A. marginale* 4 (9.09%), 2 (3.38%), 3 (4.28%) and 1 (9.09%) respectively (Ameen et al., 2012).

The findings of the current study showed the effect and the difference in infection rates according to the different seasons of the year. The highest rates of infection were in the hot seasons of the year, such as summer and autumn, and the lowest were in the cold and temperate seasons, such as winter and spring. These findings were consistent with prior research on the impact of seasons on infection rates (Alimam et al., 2022). The highest rate of infection recorded in April was 45% (9/20) with highly significant differences between months of study (Arwa and Kawan, 2022). *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. infections tend to follow a pattern that is influenced by precipitation, temperature, and relative humidity (Abdullah et al., 2019). The impact of environmental conditions may be to blame for the fluctuation in infection rates between seasons. Due to the seasonality of the vector, it is clear that animals are susceptible to blood parasite infection(Yadav and Upadhyay, 2023).

Conclusions

This study demonstrated a high prevalence (73.77%) of hemoparasitic infections – *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. – in livestock from the Erbil region of Iraq, with *Theileria* spp. being the most frequently detected (41.4%). Seasonal patterns were evident, with peak infection rates occurring during spring and summer, likely due to increased vector activity. Although variations in infection rates were noted among livestock species, no statistically significant associations were detected. RT-PCR proved more sensitive than conventional microscopy, confirming its reliability as a diagnostic tool. These results emphasize the importance of seasonal parasite control measures and highlight the potential of developing cost-effective PCR-based diagnostics to improve early detection, limit herd-level transmission, and reinforce veterinary surveillance systems.

Acknowledgments

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Transcriptomic Profiling of Resistant and Susceptible Chickens (*Gallus gallus*) Identifies Potential Cellular Processes Underlying Resistance to Avian Leukosis Virus Subgroup J

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Keywords: *ALV-J, transcriptomics, RNA-Seq, chickens, biomarkers.*

Abstract. *The study aimed to investigate transcriptomic mechanisms underlying natural resistance to avian leukosis virus subgroup J (ALV-J) in chickens by comparing resistant and susceptible individuals using high-throughput RNA sequencing. The PRJNA685043 RNA-Seq dataset available on the FAANG data portal was used for analyses. The gene expression profiles of chicken liver tissues from ALV-J-resistant and ALV-J-susceptible birds (N = 6; 3 resistant and 3 susceptible) were analyzed to identify differentially expressed genes (DEGs), differential transcript usage (DTU), and associated molecular pathways that may contribute to viral resistance. Transcript quantification was performed using kallisto, followed by differential gene expression (DGE) analysis with DESeq2 and DTU evaluation using a combined DRIMSeq–DEXSeq–stageR workflow. Functional characterization and interaction profiling were conducted using STRING and additional regulatory analyses. The analysis identified 36 significant DEGs, including 32 upregulated and 4 downregulated genes in resistant chickens. Key genes were associated with mitochondrial regulation, calcium signaling, DNA damage response, and suppression of tumorigenesis. DTU analysis revealed 22 genes exhibiting significant isoform switching, suggesting alternative splicing as an additional regulatory layer. Interaction network assessment highlighted limited but meaningful relationships among DEGs and DTU genes, indicating that multiple independent pathways may contribute collectively to resistance.*

Introduction

Avian leukosis virus subgroup J (ALV-J) remains one of the most economically important viral pathogens affecting commercial poultry flocks worldwide. The virus causes myelocytomas, immunosuppression, reduced growth and increased mortality, posing a serious threat to intensive chicken production systems (Wessi, 2011; Payne and Nair, 2012). ALV-J is an oncogenic retrovirus inducing tumor formation in chickens via genome insertion thereby dysregulating oncogenes and tumor suppressor genes in chickens (Justice et al., 2015; Ren et al., 2018). Despite concerted control efforts, ALV-J persists in many regions because management measures and culling alone are insufficient to protect genetically susceptible birds (Nakamura and Nair, 2014).

Transcriptomic studies have shown that ALV-J infection perturbs host gene expression and cellular signaling, pointing to host-response variation as a determinant of disease outcome (Raun et al., 2004; Han et al., 2015). These investigations have reported dysregulation of immune pathways, metabolic functions and cell-cycle programs following infection, and recent work highlights the additional role of alternative splicing and isoform-level regulation in modulating antiviral responses (Zhang et al., 2025). Such observations imply that differences in global transcriptional responses could underlie why some birds resist infection while others develop severe disease.

While several studies have catalogued host genes affected by ALV-J, far fewer have compared the constitutive transcriptomic architecture that distinguishes inherently resistant lines from susceptible ones. Identifying these baseline differences is critical for discovering robust biomarkers and molecular pathways that can be used in selective-breeding strategies to reduce flock susceptibility (Mo et al., 2022). A focused comparison of resistant and susceptible chickens at the transcriptome and isoform levels therefore provides a powerful route to reveal candidate mechanisms of natural resistance.

Here, we performed an integrated analysis combining differential gene expression (DGE), differential transcript usage (DTU), and protein–protein interaction mapping to compare liver transcriptomes from genetically resistant and susceptible chickens. Our goal was to detect consistent molecular signatures associated with resistance and to nominate genes and pathways for follow-up functional validation and application in breeding programs.

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Materials and methods

Data collection

The PRJNA685043 dataset (GSE163135) was obtained from the FAANG data portal at https://data.faang.org/dataset/PRJNA685043. The PRJNA685043 dataset is an RNA-Seq dataset of liver tissue of ALV-J resistant and ALV-J susceptible chickens consisting of three samples each. These samples were sequenced on the Illumina HiSeq 4000 platform. The sequencing files were paired-end, i.e., both ends of DNA fragment sequenced, and were in fastq.gz format. Sample collection, preparation, sequencing are as outlined in Yan et al. (2021).

Quality control of data

Quality of the sequencing FASTQ files was assessed using FastQC tool (v0.12.1). FastQC provides a simple way to perform quality control checks on raw sequence data from high throughput sequencing pipelines. It analyzes the sequencing files so as to flag issues the analyst should be aware of before further analysis. A report for each analyzed sequencing file is generated including summary graphs of multiple quality indicators such as base quality, overrepresented sequences, adapter content etc. After FastQC, reads were trimmed and adapters removed using Trimmomatic (v0.39) (Bolger et al., 2014).

The command used to remove adapters, leading and trailing low-quality bases, and to drop reads below the specified length was: "java -jar trimmomatic-0.39.jar PE -phred33 {sequence-file1-forward.fastq.gz} {sequence-file1-reverse.fastq.gz} {sequence-file1-forward_paired.fastq.gz} {sequence-file1-forward_unpaired.fastq.gz} {sequence-file1-reverse_paired.fastq.gz} {sequence-file1-reverse_unpaired.fastq.gz} ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36". ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 was used to remove adapters, LEADING:3 was used to remove leading low quality or N bases (below quality three), TRAILING:3 was used to remove trailing low quality or N bases (below quality three), and MINLEN:36 was used to drop reads below 36 bases long. To ensure all sequence reads passed basic quality statistics, the trimmed and paired sequence files were checked using FastQC. The trimmed files were then used for transcript quantification via pseudoalignment.

Transcript abundance quantification

Quantification of transcript abundance from the RNA-Seq reads (the trimmed paired files) of the samples was performed using kallisto (version 0.46.1) via the pseudo alignment method (Bray et al., 2016). Firstly, a transcriptome index was created using the *Gallus gallus* transcriptome (cDNA) (release 113) downloaded from Ensembl database (https://www.ensembl.org/index.html). The corresponding GTF annotation file was also downloaded from the Ensembl database for use in downstream DTU analysis. Abundance quantification of transcripts was thereafter performed for the trimmed-paired sequencing files and the quantification outputs exported to specified folders for downstream use.

Differential gene expression

The transcript abundance estimates quantified were imported into R (version 4.4.1) using tximport package (Soneson et al., 2015). These estimates were imported for use with Differential Gene Expression (DGE) methods using the DESeqDataSetFromTximport function from DESeq2 package (Love et al., 2014). Genes with low expression across samples were filtered to reduce noise. DGE analysis was performed between the ALV-J-resistant and ALV-J-susceptible groups using DESeq2. An adjusted P value < 0.05 and a lfcThreshold of 0.3 were set in the DGE analysis to identify differentially expressed genes (DEGs). Heatmap plotting and sample clustering using the DEGs was thereafter performed with the pheatmap package.

Differential transcript usage

Differential transcript usage (DTU) analysis was performed following the workflow described in Love et al. (2018). The transcript abundance estimates quantified were imported into R using tximport package (Soneson et al., 2015). The data object was filtered to remove low expressed transcripts that may affect parameter estimation and increase fitting speed (Love et al., 2018). The filtering criteria included retaining a transcript with a count of at least two in at least three samples, having a relative abundance proportion of at least 0.1 in at least two samples, and the total count of the corresponding gene is at least two in all six samples. The filtering retained 2733 genes and 5981 transcripts. Thereafter, DRIMSeq (Nowicka and Robinson, 2016), DEXSeq (Anders et al., 2012; Reyes et al., 2013) and stageR (Van den Berge et al., 2018) packages were used for the DTU analysis. An alpha (α) value of 0.05 and adjusted P value < 0.05 were used as thresholds for the identification of genes with significant differentially used transcripts.

Interaction and transcriptional regulatory analysis

Protein-protein interaction analysis of the DEGs and DTU genes identified was performed with STRING (version 12.0) (Szklarczyk et al., 2021). TRRUST (version 2) (Han et al., 2018), was used to perform transcriptional regulatory analysis to reveal the transcription factors regulating the identified DEGs and DTU genes. The inputs into the TRRUST database were the list of DEGs and the DTU genes with the expected output being a tabular list of transcription factors regulating the input genes.

Results

Differential gene expression analysis

RNA-seq profiling generated expression data for 17 068 annotated genes across all samples. Comparative analysis of ALV-J–resistant and ALV-J–susceptible chickens identified a distinct set of 36 differentially expressed genes (DEGs), consisting of both annotated genes and several uncharacterized loci. These DEGs are summarized in Table 1.

Unsupervised hierarchical clustering using the complete DEG set clearly separated resistant birds from susceptible ones, reflecting substantial

Table 1. Information on the DEGs in ALV-J resistant samples compared with ALV-J susceptible samples. Shown are gene symbol, gene names, log2FC, p-adjusted value and status. Genes without gene names are novel genes with no gene name and symbol assigned on the Ensembl database

Symbol	Gene Name	Log2FC	p-adj	Status
ENSGALG00010003287 (novel gene)		10.454	4.09e-06	Up
MTMR7	myotubularin related protein 7	10.103	2.29e-06	Up
TMEM104	transmembrane protein 104	9.307	0.00054	Up
SAMD8	Sterile alpha motif domain containing 8	9.228	0.00511	Up
CINP	cyclin dependent kinase 2 interacting protein	9.135	0.00788	Up
ADRB1	adrenoceptor beta 1	9.069	0.00788	Up
SSH2	slingshot protein phosphatase 2	9.044	0.00788	Up
ZNF341	zinc finger protein 341	8.861	0.0065	Up
ZNF362	zinc finger protein 362	8.849	0.00788	Up
IFT81	intraflagellar transport 81	8.789	0.00788	Up
TRPV3	transient receptor potential cation channel subfamily V member 3	8.719	0.00832	Up
CASR	calcium sensing receptor	8.715	0.02399	Up
LRAT	lecithin retinol acyltransferase	8.587	0.01936	Up
BRAT1	BRCA1 associated ATM activator 1	8.577	0.03562	Up
ENSGALG00010023766 (novel gene)		8.574	0.02358	Up
CCR8	C-C motif chemokine receptor 8	8.517	0.03714	Up
EARS2	glutamyl-tRNA synthetase 2, mitochondrial	8.506	0.02358	Up
PIGQ	phosphatidylinositol glycan anchor biosynthesis class Q	8.476	0.02871	Up
MGME1	mitochondrial genome maintenance exonuclease 1	8.475	0.02523	Up
SMDT1	single-pass membrane protein with aspartate rich tail 1	8.475	0.04329	Up
CHEK1	checkpoint kinase 1	8.463	0.02399	Up
WDHD1	WD repeat and HMG-box DNA binding protein 1	8.4532	0.03956	Up
GAB3	GRB2 associated binding protein 3	8.388	0.03562	Up
SLC25A43	solute carrier family 25member 43	8.117	0.00511	Up
DFFB	DNA fragmentation factor subunit beta	7.972	0.00054	Up
ENSGALG00010012307 (Novel gene)		7.896	6.84e-05	Up
CKB	creatine kinase B	7.833	0.02309	Up
CLEC14A	C-type lectin domain containing 14A	6.393	0.00788	Up
TMOD2	tropomodulin 2	8.379	0.03714	Up
ENSGALG00010015655 (novel gene)		8.319	0.04991	Up
SNX11	sorting nexin 11	8.319	0.04692	Up
NEK10	NIMA related kinase 10	6.136	0.00788	Up
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	-1.895	0.00071	Down
SSPN	Sarcospan	-3.85	0.02309	Down
CHRM3	cholinergic receptor muscarinic 3	-4.201	0.02358	Down
GRIN2A	glutamate ionotropic receptor NMDA type subunit 2A	-8.377	3.21e-05	Down

transcriptomic divergence between the phenotypes. This grouping pattern is illustrated in Fig. 1, where resistant samples consistently formed a distinct cluster relative to susceptible individuals.

Differential transcript usage analysis

Differential transcript usage (DTU) analysis was conducted on 13 566 expressed transcripts to identify isoform-level regulatory differences between the two groups. Several genes displayed significant shifts in transcript proportions, indicating that isoform regulation contributes to phenotypic variation in ALV-J response. Examples of transcripts with notable usage changes include isoforms of *MAGI1, FGFR3, RCN1, AKAP9, DYRK1A,* and *COQ8A*, with representative DTU plots shown in Figs. 2–6.

The presence of DTU events in multiple functionally relevant genes suggests that alternative splicing may play an important role in modulating antiviral or stress-response functions in resistant chickens.

Interaction and transcriptional regulatory network analysis

Integration of the DEGs and DTU-associated genes into a protein–protein interaction (PPI) framework revealed a condensed set of interconnected molecules forming key regulatory clusters. Notable nodes included *AKAP9, FGFR3, EML4, ARID1B, DYRK1A*, and *VPS26C*, many of which participate in cellular signaling, transcriptional regulation, or structural maintenance.

A comprehensive PPI map is presented in Fig. 7, highlighting the central interacting genes while omitting non-connected nodes for clarity. This network emphasizes the biological coherence of the transcriptomic signals identified through both DGE and DTU analyses.

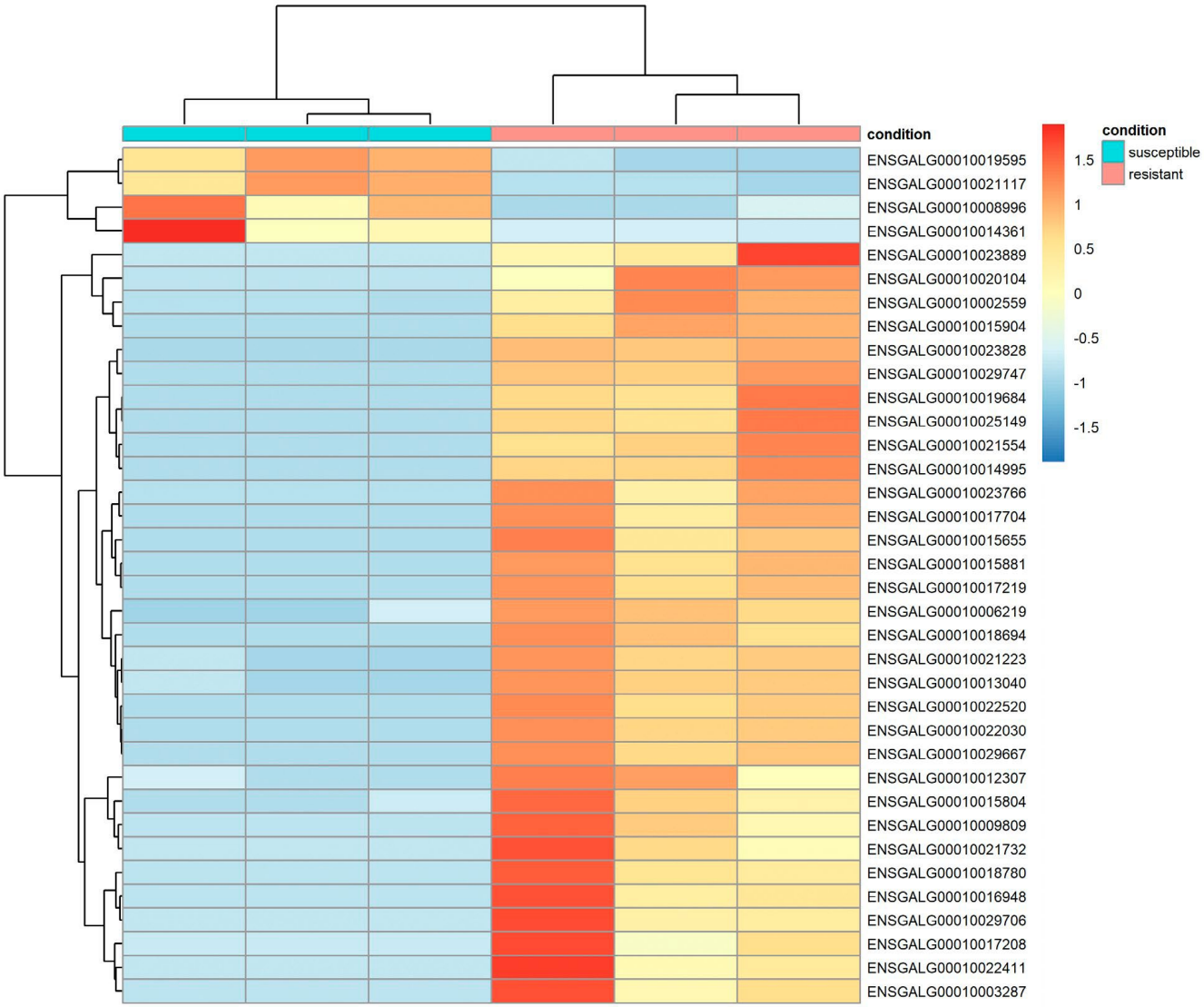

Fig. 1. Heatmap and clustering of the susceptibility and resistance samples by the identified differentially expressed genes

Table 2. Information on the identified DTU genes (with adjusted *P* value < 0.05). Included are the gene symbols, gene names, transcript IDs and *P* adjusted value (to 3 decimal places)

Symbol	Gene name	Transcript id	*P* adj
DSCR3	VPS26 endosomal protein sorting factor C	ENSGALT00010031585	0.038
SPATS2L	spermatosis associated serine rich 2 like	ENSGALT00010058702	0.038
SPCS3	signal peptidase complex subunit 3	ENSGALT00010007997	0.046
		ENSGALT00010008013	0.046
ARID1B	AT-rich interaction domain 1B	ENSGALT00010009548	0.006
		ENSGALT00010009555	0.006
AKAP9	A-kinase anchoring protein 9	ENSGALT00010011356	0.029
		ENSGALT00010011383	0.029
PROSER2	proline and serine rich 2	ENSGALT00010013650	0.024
		ENSGALT00010013656	0.024
MCTS1	MCTS1, re-initiation and release factor	ENSGALT00010029418	0.047
		ENSGALT00010029420	0.047
SCAMP1	secretory carrier membrane protein 1	ENSGALT00010030297	0.033
		ENSGALT00010029420	0.033
DYRK1A	dual specificity tyrosine phosphorylation regulated kinase 1A	ENSGALT00010031626	0.007
		ENSGALT00010031632	0.007
MRPS9	mitochondrial ribosomal protein S9	ENSGALT00010031727	0.048
		ENSGALT00010031729	0.048
MAN1A1	mannosidase alpha class 1A member 1	ENSGALT00010036052	0.018
		ENSGALT00010036068	0.018
FGFR3	fibroblast growth factor receptor 3	ENSGALT00010040904	0.046
		ENSGALT00010040908	0.046
COQ8A	coenzyme Q8A	ENSGALT00010043588	0.013
		ENSGALT00010043592	0.013
EML4	EMAP like 4	ENSGALT00010045339	0.037
		ENSGALT00010045344	0.037
SUCLG2	succinate-CoA ligase GDP-forming beta subunit	ENSGALT00010047717	0.034
		ENSGALT00010047749	0.034
MAGI1	membrane associated guanylate kinase 1	ENSGALT00010048939	0.021
		ENSGALT00010048945	0.021
RCN1	reticulocalbin 1	ENSGALT00010057659	0.009
		ENSGALT00010057669	0.009
GABARAPL1	GABA type A receptor associated protein like 1	ENSGALT00010058708	0.033
		ENSGALT00010058714	0.033
HIC2	HIC ZBTB transcriptional repressor 2	ENSGALT00010059216	0.047
		ENSGALT00010059236	0.047
RIT1	Ras like without CAAX 1	ENSGALT00010068899	0.049
		ENSGALT00010068900	0.049
SUGP1	SURP and G-patch domain containing 1	ENSGALT00010069080	0.049
		ENSGALT00010069081	0.049
CASP1	caspase 1	ENSGALT00010071575	0.033
		ENSGALT00010071577	0.033

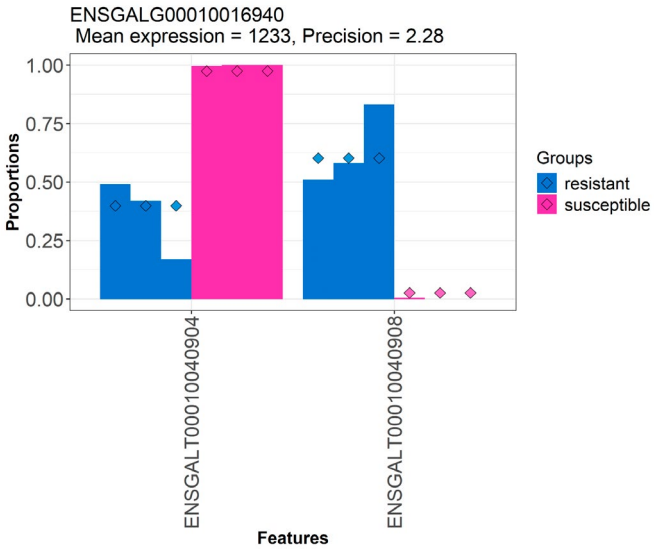

Fig. 2. Plot of the estimated transcript proportions for ENSGALG00010016940 (*FGFR3*)

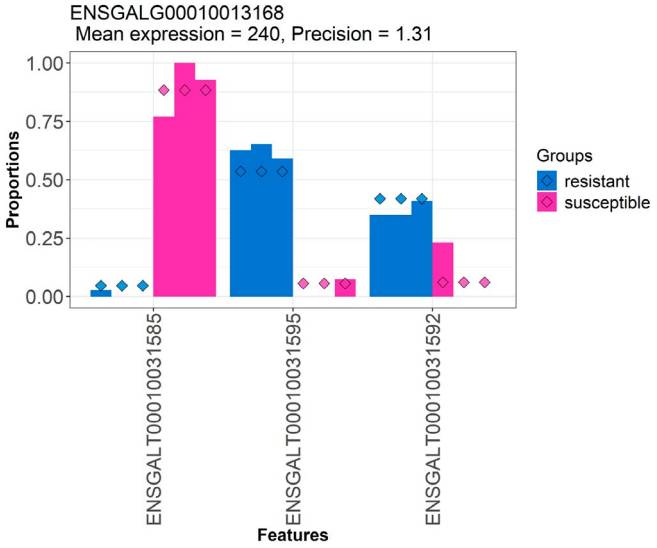

Fig. 3. Plot of the estimated transcript proportions for ENSGALG00010023668 (*RCN1*)

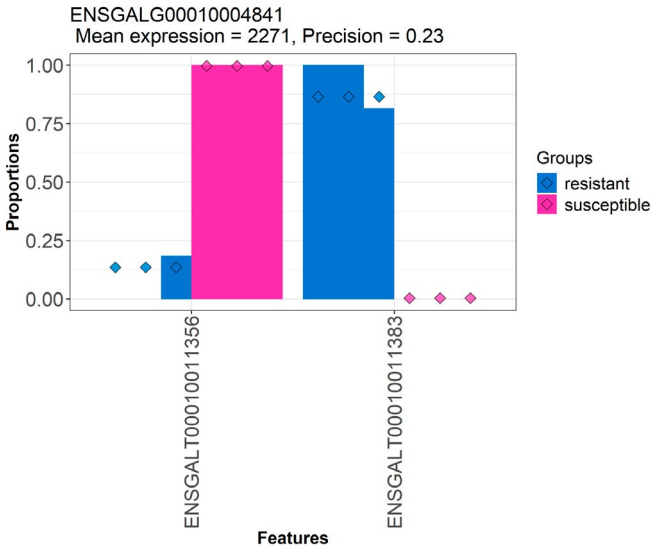

Fig. 4. Plot of the estimated transcript proportions for ENSGALG00010004841 (*AKAP9*)

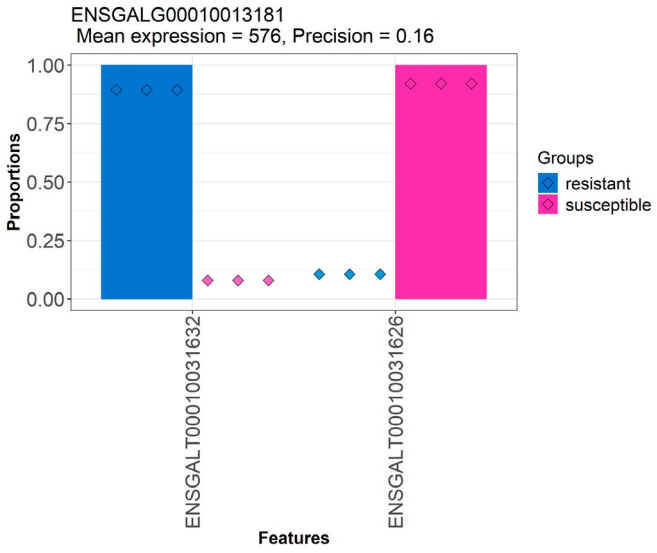

Fig. 5. Plot of the estimated transcript proportions for ENSGALG00010013181 (*DYRK1A*)

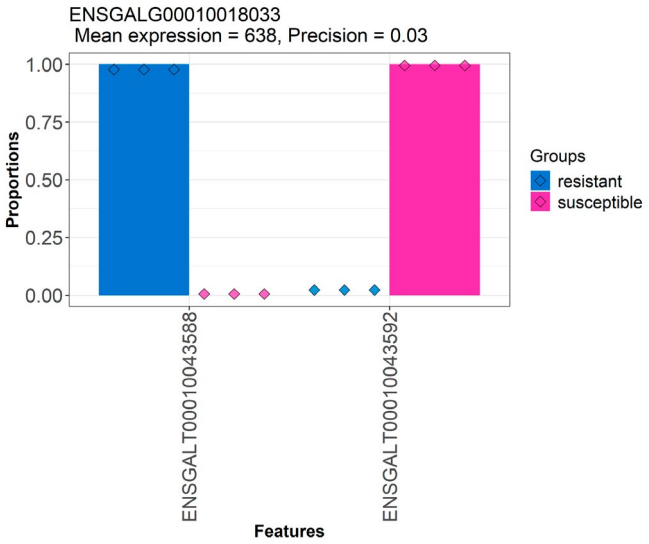

Fig. 6. Plot of the estimated transcript proportions for ENSGALG00010018033 (*COQ8A*)

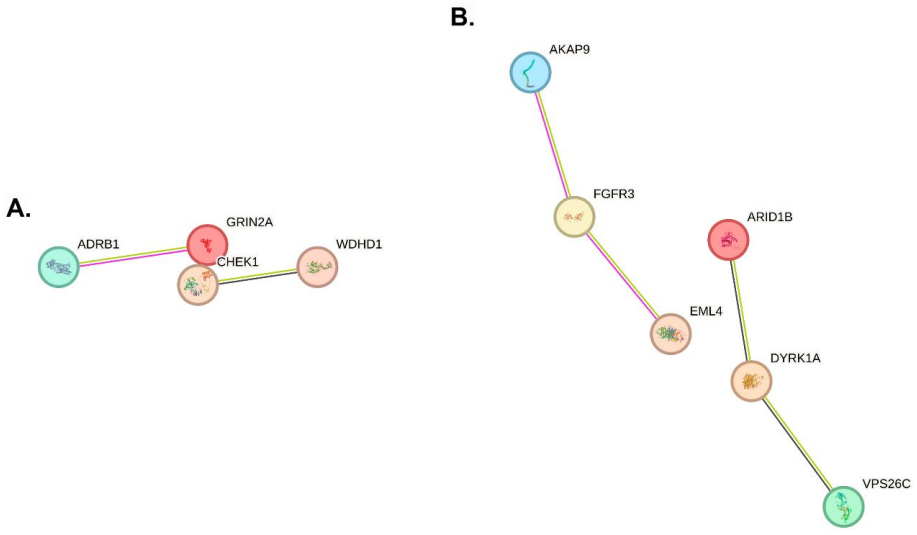

Fig. 7. Interaction analysis of (A) identified differentially expressed genes (B) identified differential transcript usage genes. Disconnected nodes (other DEGs and DTU genes) removed

Discussion

The comparative transcriptomic analysis conducted in this study shows that ALV-J–resistant chickens possess a distinct molecular profile relative to susceptible birds. The consistent separation of samples in clustering analyses, coupled with the large fold changes observed in several differentially expressed genes, indicates substantial biologically meaningful variation between the two phenotypes. This observation aligns with previous transcriptomic investigations showing divergence in gene-expression patterns between ALV-J–positive and uninfected birds (Azamian et al., 2024; Zhang et al., 2025). The additional detection of differential transcript usage (DTU) further supports the idea that alternative splicing contributes to antiviral responses, consistent with findings from Zhang et al. (2025).

A notable feature of the resistant-group expression profile was the strong enrichment of genes associated with mitochondrial biology. Several highly upregulated genes, including *BRAT1, SLC25A43, EARS2*, and *MGM1*, have established links to mitochondrial homeostasis, stress responses, or metabolic regulation. Mitochondria are central to antiviral signaling, particularly through regulation of cytokine production, interferon induction, and apoptosis (Wu et al., 2023). Functional studies indicate that *BRAT1* influences mitochondrial integrity and DNA-damage responses (Aglipay et al., 2006; So and Ouchi, 2014), while *SLC25A43* plays a role in mitochondrial trafficking and cell-cycle control (Gabrielson et al., 2016) and is also implicated as a tumor suppressor (Lindqvist et al., 2012). *EARS2* participates in mitochondrial protein translation (Pelayo et al., 2024), whereas *MGM1* contributes to mitochondrial nucleic-acid metabolism (Yang et al., 2018). Taken together, these findings suggest that enhanced mitochondrial stability and signaling may be an important determinant of ALV-J resistance.

Because mitochondria also serve as hubs for calcium (Ca^{2+}) handling, the enrichment of calcium-signaling genes in resistant birds is notable. Numerous studies highlight the importance of Ca^{2+} flux in modulating viral entry, replication, and host responses (Saurav et al., 2021), and mitochondrial Ca^{2+} balance is known to influence cell fate during infection (Zhou et al., 2009; Chaudhuri et al., 2021). The upregulation of *SMDT1* (*EMRE*), a crucial component of the mitochondrial calcium uniporter (Bulthuis et al., 2023; Wang et al., 2020; Sancak et al., 2013) suggests that ALV-J–resistant chickens may regulate mitochondrial Ca^{2+} uptake more efficiently, potentially contributing to more controlled antiviral responses.

The significance of Ca^{2+} signaling in ALV-J pathogenesis is further supported by the known viral entry mechanism. ALV-J infects host cells through interaction between its Env-J protein and the chicken Na^{+}/H^{+} exchanger 1 (*NHE1*) receptor in a pH-dependent manner (Chai and Bates, 2006). *NHE1* participates in ion transport and has been shown to influence Ca^{2+} regulation in other cell types (Nakamura et al., 2008; Lee et al., 2016). Since *NHE1* is widely expressed across chicken breeds (Mo et al., 2022), variations downstream of receptor signaling – rather than receptor presence itself – may help explain natural resistance.

The upregulation of *CASR*, a calcium-sensing receptor, in resistant birds further reinforces the role of Ca^{2+}-dependent pathways. *CASR* modulates cellular calcium influx and has been shown to suppress replication of rotavirus, demonstrating antiviral potential (Huang et al., 2022). It is plausible that similar mechanisms influence ALV-J outcomes.

Beyond mitochondrial and Ca^{2+} pathways, several genes associated with tumor suppression, DNA-damage checkpoint integrity, and proliferation control were also upregulated in resistant chickens. These include *MTMR7, CHEK1, CKB, CINP*, and others. *MTMR7* expression is reduced in colorectal cancer and has tumor-suppressive activity (Weidner et al., 2016; Weidner et al., 2024). *CHEK1* supports DNA-damage signaling via ATM/ATR pathways (Jiang et al., 2024; Fernandez et al., 2025) and is known to be downregulated by certain viral infections (Akgül et al., 2019). *CKB* inhibits AKT activation and suppresses tumor progression (Wang et al., 2021). *CINP* is critical for ATR-mediated replication stress responses (Lovejoy et al., 2009). The induction of these genes in resistant birds suggests a cellular environment that more effectively limits uncontrolled proliferation, which is a relevant observation given ALV-J's oncogenic properties.

Additionally, several immune-regulatory genes, including *ZNF341, GAB3, LRAT*, and *TMEM104*, were elevated in resistant chickens. *ZNF341* regulates *STAT3*, a central mediator of immune homeostasis (Cekic et al., 2022; Frey-Jakobs et al., 2018). *GAB3* participates in immune-cell signaling (Vaughan et al., 2011) and is linked to malignancy in certain contexts (Jia et al., 2017). *LRAT* is central to vitamin A metabolism, which is tightly connected to antiviral immunity (McGill et al., 2019; Zhang et al., 2023; Stephensen and Lietz, 2021). *TMEM104*, previously shown to be downregulated through promoter methylation in ALV-J–positive chickens (Yan et al., 2021), was upregulated in the resistant group, supporting its potential protective role.

DTU analysis expanded these observations by identifying isoform-level differences in genes implicated in tumor suppression, signaling, and mitochondrial dynamics, such as *ARID1B, HIC2, RIT1, MCT1, MAGI1*, and *GABARAPL1*. These findings correspond with previous reports of isoform dysregulation in cancer and viral infections (Deogharkar et al., 2021; Luo et al., 2023; Khalil and Nemer 2020; de Carvalho et al., 2021; Lake et al., 2013). Notably, *GABARAPL1* is associated

with autophagy, mitochondrial quality control, and resistance to ferroptosis (Boyer-Guittaut et al., 2014; Du et al., 2022), all of which are features that could influence ALV-J disease progression.

While the results seem promising, some limitations are observed. The sample sizes are small which may limit the generalizability of the results identified. Also, results of this study require validation in larger sample groups and other animal cohorts. This will illuminate and validate the results obtained in this study and strengthen the potential role of the genes identified as biomarkers.

Taken together, these results indicate that ALV-J resistance potentially arises from coordinated regulation of several biological systems, including mitochondrial function, Ca^{2+} signaling, immune modulation, and cell-cycle control. The concurrent involvement of DGE and DTU mechanisms suggests that both transcriptional and post-transcriptional processes help shape the resistant phenotype. These findings provide a robust foundation for future functional validation studies and highlight several genes that may serve as biomarkers for breeding programs aimed at enhancing resistance to ALV-J.

Conclusion

This study provides new insight into the molecular features that distinguish ALV-J-resistant chickens from susceptible ones. By integrating differential gene expression, isoform-level analysis, and protein–protein interaction modelling, we identified a set of genes and pathways that may characterize the resistant phenotype. These include regulators of mitochondrial integrity, calcium signaling, immune modulation, and cell-cycle control, systems that collectively influence how host tissues respond to viral challenges.

The presence of strong transcriptomic differences, together with pronounced alternative transcript usage, suggests that resistance is shaped by both transcriptional and post-transcriptional mechanisms. Several of the genes highlighted here may represent promising markers for future validation studies and may have practical value in selective breeding programs aimed at reducing flock susceptibility to ALV-J.

Overall, the findings broaden our understanding of the biological basis of natural resistance and establish a framework for exploring functional mechanisms that contribute to improved disease outcomes. Further experimental work will be essential to confirm the specific roles of these candidate genes and to determine how they can be effectively incorporated into long-term control and breeding strategies.

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Minimizing Feline Coronavirus Transmission in Catteries: A Case Study of Controlled, Time-restricted Breeding of Virus-free Queens with Shedding Toms

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Abstract. *Feline coronavirus (FCoV) is highly prevalent in multi-cat environments and may mutate to cause feline infectious peritonitis (FIP). This single-site retrospective case study analysed breeding and diagnostic data from a private cattery in Estonia (10 FCoV-free queens, 4 FCoV-shedding toms, and 29 kittens) to evaluate whether virus-free queens can be bred with shedding toms without subsequent infection in queens and offspring. Natural matings were conducted under strictly controlled conditions, limited to 90-minute sessions twice daily for three consecutive days, in a room without shared litter boxes, food, water, or enrichment. FCoV infection status was monitored using repeated faecal RT-PCR and serological testing before and after mating. All queens remained negative for FCoV on faecal RT-PCR and serology six months after breeding, and all kittens tested negative by faecal RT-PCR up to three months of age. No clinical or reproductive complications were observed. Within the limitations of a retrospective case study, these findings indicate that time-limited mating under strict environmental control was associated with no detectable FCoV transmission in this setting. This approach may be relevant for breeders and veterinarians managing FCoV-free breeding stock where virus-negative toms are not available.*

Introduction

Feline coronavirus (FCoV) is believed to be a highly contagious infectious disease in cats (Pedersen et al., 2008; Addie et al., 2023). According to a range of publications (Sharif et al., 2009; Oguzoglu et al., 2010; Andersen et al., 2018; McKay et al., 2020; Mūrniece et al., 2021; Zhou et al., 2021; Kokkinaki et al., 2023), the prevalence of FCoV in domestic cats can be up to 96% with the highest rates in breeding catteries and shelters with overcrowded cat populations (Klein-Richers et al., 2020; Felten et al., 2023).

Clinical outcomes range from subclinical infection to short self-limiting gastrointestinal disease (Pedersen et al., 1981; Pedersen et al., 2008; Addie, 2012) or chronic diarrhoea (Pedersen et al., 2014; Sung et al., 2022). The virus replicates in enterocytes of infected cats, leading to faecal shedding (Sherding, 2006). Kipar et al. (2006) reported that asymptomatic FCoV-infected cats can also be systemically infected, which may lead to transient viral presence in multiple organs (Stranieri et al., 2020). The predominant route is faecal–oral (Pedersen et al., 2008; Addie et al., 2023). FCoV transmission is facilitated by shared litter boxes and food bowls among infected and uninfected cats (Addie, 2000). FCoV can also be transmitted to healthy cats via contaminated fomites, such as human clothing and shoes (Sherding, 2006). The virus can persist in excreta in the environment for up to seven weeks (Scott, 1991; Addie, 2012). Studies by Kiss et al. (2000) and Stranieri et al. (2020) have also discussed the possibility of venereal transmissioon.

Experimentally infected cats can start shedding the virus with faeces as soon as the second day after infection (Meli et al., 2004). In naturally infected cats, viral shedding typically begins within the first week post-infection and can persist for 9 to 24 months (Addie and Jarrett, 2001). Some cats may become chronic shedders (Foley et al., 1997). Younger cats are more susceptible to infection (Almeida et al., 2019; Klein-Richers et al., 2020) and shed the virus in a higher viral load than older cats (Pedersen et al., 2008), contaminating the environment and contributing to FCoV spread in the population.

The high infectivity of the virus is significant due to the virus's capacity to mutate and cause the fatal disease, feline infectious peritonitis (FIP) (Vennema et al., 1998). FIP is a serious problem in veterinary medicine, causing death in up to 14% of FCoV infected cats (Addie et al., 1995) and 0.33–0.5% of all cats (Rohrbach et al., 2001; Pesteanu-Somogyi et al., 2006). The most important risk factor for the development of FIP in FCoV-infected cats is age. Most diseased cats are young, often under one year of age (Worthing et al., 2012; Pedersen et al., 2014; Kennedy et al., 2020). It has been shown that FIP is one of the major diseases causing mortality in kittens (Cave et al., 2002).

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Kittens born to a mother shedding the virus are protected from infection by maternal immunity, which normally lasts for the first 4–6 weeks of the kitten's life (Pedersen et al., 1981). Kittens that lose maternal immunity but, because of their very young age, still remain with a shedding mother, may become more susceptible to FCoV and have a higher risk of developing FIP (Pedersen et al., 1981; Pedersen et al., 2008). Therefore, it is important to keep breeding queens free from the virus to reduce morbidity and mortality associated with FIP in kittens and young cats and to prevent the transmission of FCoV.

Objectives

The aim of this retrospective case study was to document and evaluate whether strictly time-limited natural mating under controlled hygienic conditions in a clean, bowl- and litter box free room allows breeding of feline coronavirus–free queens with FCoV-shedding toms without subsequent infection of queens and their kittens.

Materials and methods

Data and results of routine testing of faecal samples and serological investigation of breeding cats performed by the breeder in one Estonian British Shorthair cattery, as part of the animals' health evaluation programme, were used in this study. Breeding data from 10 purebred British shorthair FCoV-free non-shedder queens with four purebred British Shorthair shedder toms and their 29 kittens were retrospectively enrolled in the study.

For breeding, each queen in heat was left with one of the toms in a room without litter boxes, food, water or any enrichment for 90 minutes, twice per day, for 3 consecutive days. Between sessions, queens were housed separately according to routine practice.

Ten individual monthly faecal samples were collected from each queen, comprising five pre-breeding and five post-breeding samples. Serological investigation of queens was conducted before breeding and 6 months after breeding. Individual sampling of faeces of breeding toms was done twice before breeding at one month apart. Litter-pooled kitten samples were collected monthly at 1–3 months of age.

Real-time polymerase chain reaction (RT-PCR) tests of faecal samples and serological testing for detection of anti-FCoV antibodies were performed at the accredited external laboratory LABOKLIN (Laboklin GmbH & Co. KG, Bad Kissingen, Germany). Results of serological investigations with a negative titre at dilution of <1:25 or <9 NTU, following the laboratory's reporting standards, were classified as negative.

To mitigate the limitation that faecal RT-PCR may fail to detect intermittent or low-level feline coronavirus shedding in infected cats, queens were monitored using repeated faecal RT-PCR testing over several consecutive months both before and after breeding, combined with serological testing, in accordance with established recommendations (Addie and Jarrett, 2001).

Cats that tested negative for FCoV in monthly faecal tests using RT-PCR over a five-month period, according to the recommendations of Addie and Jarrett (2001) and remained serologically negative for anti-FCoV antibodies at 6 months were classified as FCoV-free, non-shedding cats. Cats that showed shedding of the virus at least once during the observational period were defined as FCoV shedders.

Results

All 10 queens conceived and delivered (conception rate 100%). Mean litter size (±SD) was 2.9 ± 1.1 kittens per litter. No reproductive complications were observed during pregnancy or parturition, indicating that the implemented mating protocol did not negatively affect fertility or reproductive outcomes.

In total, 50 faecal RT-PCR tests were performed on samples from 10 queens before mating and 50 after mating, along with eight faecal RT-PCR tests from four toms and 30 pooled litter faecal samples from kittens from 10 litters. Additionally, 20 serological tests for the detection of anti-FCoV antibodies were performed in queens.

Across the defined observation period, no queen showed evidence of FCoV infection following mating. All queens remained negative on repeated faecal RT-PCR testing, and serological investigation conducted six months after breeding confirmed persistent seronegativity. Similarly, pooled litter samples from all 29 kittens tested negative for FCoV by faecal RT-PCR at monthly intervals up to three months of age. These findings indicate that, in this case study, controlled natural mating between FCoV-free queens and FCoV-shedding toms was not associated with detectable viral transmission to queens or offspring during the monitored period.

Discussion

It is known that certain breeds, such as British Shorthair, Cornish Rex, Birman, Burmese cats, Maine Coones, and Scottish Fold cats are more predisposed to FCoV infection (Bell et al., 2006; Taharaguchi et al., 2012). The exclusive inclusion of British Shorthair cats in this case study, therefore, represents a population with a recognised predisposition, thereby strengthening the relevance of the findings despite the limited sample size.

Most cats acquire FCoV infection early in life via the faecal–oral route, primarily through contact with contaminated litter boxes or the environment shared with shedding cats (Pedersen et al., 2008; Klein-Richers et al., 2020; Addie et al., 2023). Young cats not only become infected more readily but also shed higher viral loads, contributing disproportionately to

environmental contamination and population-level transmission (Pedersen et al., 2008). This is clinically significant because younger cats are at the highest risk of developing feline infectious peritonitis (FIP), a fatal outcome of FCoV mutation (Pedersen, 2009; Worthing et al., 2012; Pedersen et al., 2014; Kennedy et al., 2020). Preventing early-life infection, therefore, remains a central goal of FCoV management in breeding catteries.

Existing preventive strategies focus primarily on isolation of queens during pregnancy and early weaning of kittens into virus-free environments (Addie et al., 2004; Pedersen et al., 2008). While these measures can reduce the transmission risk, early weaning at 5–6 weeks of age has been associated with increased aggression, stereotypical behaviour, and long-term welfare concerns (Ahola et al., 2017; Martínez-Byer et al., 2023). Moreover, early weaning reduces but does not completely eliminate the risk of infection. In contrast, the approach documented in this case study allowed kittens to remain with virus-free queens beyond the maternal immunity period without detectable infection, offering a potentially more welfare-friendly alternative.

A major practical challenge for breeders is the high prevalence of FCoV in the general cat population, which makes it difficult to identify virus-negative toms for mating (Sharif et al., 2009; Oguzoglu et al., 2010; Andersen et al., 2018; McKay et al., 2020; Mūrniece et al., 2021; Zhou et al., 2021; Kokkinaki et al., 2023). Prolonged failure to mate queens has been associated with an increased risk of pyometra (Hollinshead and Krekeler, 2016), often forcing breeders to consider mating virus-free queens with known FCoV shedders. However, such matings are typically discouraged due to concerns regarding viral transmission.

The primary route of FCoV transmission is faecal–oral, most efficiently via shared litter boxes (Pedersen et al., 2008; Addie et al., 2023). During natural mating, however, direct contact between perineal and caudal body regions of the tom and queen raises theoretical concerns about transfer of small amounts of faecal material via the coat, followed by self-grooming. Despite this theoretical risk, no transmission was detected in the present case study, suggesting that strict time limitation of contact and elimination of shared litter boxes, food bowls, and water sources during mating substantially reduces transmission risk. Indirect transmission via contaminated environments or fomites has also been described (Sherding, 2006; Addie, 2012), and a minority of cats may shed FCoV via saliva (Addie and Jarrett, 2001). The mating protocol used in this case study deliberately excluded items likely to be contaminated with saliva or faeces, which may explain the absence of detectable transmission. While Felten et al. (2023) reported that hygiene measures alone were not significantly associated with shedding intensity at the population level, the present findings suggest that targeted, short-term environmental control during mating may still be effective in reducing individual transmission events.

Taken together, these results indicate that, within the limitations of a retrospective single-site case study, strictly controlled, time-limited natural mating can be associated with an absence of detectable FCoV transmission, even when toms are known shedders. These observations do not establish causality but provide practice-oriented evidence that may inform breeding management decisions and support the design of future prospective, controlled studies.

Conclusions

In this single-site retrospective case study, strictly time-limited natural mating in a clean, bowl- and litter box free room was associated with no detected transmission of feline coronavirus from shedding toms to virus-free queens or their kittens over short-term follow-up.

While the small sample size and observational design limit generalization, these findings provide practical, real-world evidence that controlled mating may be a feasible breeding management option for maintaining FCoV-free queens and preventing early-life infection in kittens. This approach may represent a welfare-friendly alternative to early weaning in selected cattery settings. This knowledge is very important for breeders with FCoV-free queens to prevent infection of kittens and spread of the virus in the population, and can be used to make guidelines for breeders to reduce the prevalence of or even eradicate FCoV from their cattery.

Future research should aim to build upon these observations through prospective, multi-site studies involving larger populations, predefined outcome measures, and longer follow-up periods for both queens and offspring..

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Effect of *Rosmarinus officinalis* (L.) Essential Oil Alone or in Association with Vitamin E and Vitamin C on Cryopreserved Bull Sperm

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Abstract. *Bull sperm cryopreservation induces significant cellular damage through reactive oxygen species generation and oxidative stress compromising motility, membrane integrity, and fertilizing capacity. Bull spermatozoa are particularly vulnerable due to limited cytoplasmic antioxidant defenses and high metabolic activity. The aim of this study was to evaluate the protective effects of vitamin C, vitamin E, their combination, and vitamin E combined with Rosmarinus officinalis essential oil on bull sperm quality following cryopreservation. Epididymal spermatozoa from ten bulls were collected and divided into five groups: control (Tris-based extender), vitamin E (0.5 mg/mL), vitamin C (0.25 mg/mL), vitamin E+C association (Vit E-C) (0.5 and 0.25 mg/mL), and vitamin E + essential oil association (Vit E-EO) (0.5 mg/mL + 1 µL/mL). Post-thaw sperm quality was assessed using computer-assisted sperm analysis for motility and kinematic parameters, hypoosmotic swelling test for membrane integrity, and thiobarbituric acid reactive substances assay for lipid peroxidation. All antioxidant treatments enhanced sperm parameters compared with control. Vitamin E + essential oil combination demonstrated the highest total (73.8 ± 5.6% vs. control 64.3 ± 3.5%) and progressive motilities (33.7 ± 7.1% vs. control 24.4 ± 3.2%). This treatment also achieved superior kinematic parameters including curvilinear velocity (47.8 ± 2.4 vs. 34.3 ± 0.7 µm/sec), straight-line velocity (23.7 ± 1.6 vs. 14.7 ± 0.5 µm/sec), and membrane integrity (23 ± 4% vs. 14 ± 2%). Vitamin E + essential oil showed the greatest reduction in lipid peroxidation (0.26 vs. 0.35 nmol MDA/10^8 spermatozoa). Antioxidant supplementation, particularly vitamin E combined with essential oil, significantly enhances bull sperm cryopreservation outcomes through synergistic protective mechanisms against oxidative damage and membrane destabilization.*

Introduction

Bull sperm cryopreservation is a cornerstone technology in cattle reproductive management, enabling the widespread dissemination of superior genetic material and facilitating long-term storage of valuable germplasm (Murphy et al., 2019). However, the freeze-thaw process inflicts significant cellular damage on spermatozoa resulting in reduced motility, compromised membrane integrity, and decreased fertilizing capacity (Sieme et al., 2016). The primary mechanism underlying this cryodamage involves the generation of reactive oxygen species (ROS) and subsequent oxidative stress, which particularly affects the polyunsaturated fatty acid-rich sperm membrane and mitochondrial function (Chatterjee and Gagnon, 2001; O'Connell et al., 2002).

Bull spermatozoa are particularly vulnerable to oxidative damage due to their limited cytoplasmic antioxidant defense systems and high metabolic activity (Aitken et al., 2012). During cryopreservation, the cellular stress response generates excessive ROS that overwhelm the natural antioxidant capacity leading to lipid peroxidation, protein oxidation, and DNA fragmentation (Lamirande and Gagnon, 1995; Bansal and Bilaspuri, 2011). Particularly, the mitochondrial compartment represents one of the most susceptible cellular organelles to cryopreservation-induced oxidative damage (Ball et al., 2001).

Conventional approaches to mitigate cryopreservation damage have focused on optimizing cryoprotectant concentrations and cooling protocols (Peña et al., 2003). However, mounting evidence suggests that supplementation of cryopreservation extenders with antioxidant compounds represents a promising strategy for improving post-thaw sperm quality (Watson, 2000; Guthrie and Welch, 2012). Various synthetic and natural antioxidants, including vitamin E (α-tocopherol), vitamin C (ascorbic acid), glutathione, and plant-derived compounds, have demonstrated protective effects against oxidative stress during sperm cryopreservation (Parks and Lynch, 1992).

Among natural antioxidants, essential oils derived from medicinal plants, have gained considerable attention due to their potent antioxidant properties and biocompatibility (Al-Essawe et al., 2018). *Rosmarinus officinalis* (rosemary) essential oil contains a rich array

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of bioactive compounds, including rosmarinic acid, carnosic acid, and carnosol, which exhibit strong free radical scavenging activity and metal chelation properties (Shahzad et al., 2017; El-Sheshtawy et al., 2022). Previous studies have demonstrated the protective effects of plant extract on sperm quality in various species, including boar, ram, and rooster spermatozoa (Silva et al., 2012; Bucak et al., 2013). However, to the best of our knowledge, there is no previous report concerning the effect of essential oils and particularly when associated with vitamin E on cryopreserved bull sperm.

Vitamin E, as a lipophilic antioxidant, plays a crucial role in protecting cellular membranes from lipid peroxidation and has been extensively studied in sperm cryopreservation protocols (El-Sheshtawy et al., 2011). The synergistic potential of combining vitamin E with vitamin C has been suggested to provide enhanced antioxidant protection through complementary mechanisms of action (Al-Dean et al., 2024).

The interaction between different antioxidants can be complex, with some combinations showing synergistic effects while others may exhibit antagonistic interactions (Ali et al., 2023). Understanding these interactions is crucial for developing effective antioxidant cocktails that maximize protective benefits while avoiding potential negative effects on sperm function.

Despite the promising results obtained with individual antioxidants, comprehensive studies evaluating the effects of antioxidant combinations, particularly natural compounds like rosemary essential oil with conventional antioxidants, on bull sperm cryopreservation parameters are limited. Furthermore, detailed analysis of sperm motility using computer-assisted sperm analysis (CASA) provides valuable insights into the functional benefits of antioxidant supplementation beyond basic motility assessments (Mortimer, 2000).

Therefore, the objective of the present study was to evaluate the effects of vitamin C, vitamin E, their combination, and the combination of vitamin E with *Rosmarinus officinalis* essential oil on bull sperm quality parameters following cryopreservation. This comprehensive evaluation included assessment of sperm velocity parameters, total and progressive motility, membrane integrity, and oxidative stress status to provide a thorough understanding of the protective mechanisms and practical benefits of these antioxidant treatments in bovine sperm cryopreservation.

Materials and methods

Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and were of analytical grade.

Epididymal semen collection

Testes from ten bulls were obtained from a local slaughterhouse and immediately transported to the laboratory under refrigerated conditions to preserve sample integrity. Spermatozoa were collected using the retrograde flushing technique, according to the protocol established by Martinez-Pastor et al. (2006). The epididymides were carefully dissected from the testes and subsequently cleaned to remove extraneous tissue. The cauda epididymis along with the vas deferens were meticulously isolated. After ligation of the cauda epididymal blood vessels, the external surface of the cauda was thoroughly rinsed and dried to minimize contamination. Sperm recovery was achieved by applying manual pressure to the vas deferens via a syringe loaded with air and 1 mL of extender, facilitating the retrograde flow of spermatozoa. The spermatozoa were expelled through an incision in the distal region of the cauda epididymis.

Extenders preparation and sperm cryopreservation

In this study, a Tris-based extender composed of 30.8 g/L Tris, 17 g/L citric acid, and 12.5 g/L fructose, supplemented with 20% (v/v) of egg yolk, was employed for semen dilution. Each semen sample collected from the testes was divided into five distinct aliquots to evaluate the effects of various treatments:

1. Control group containing only the Tris-based extender.
2. Group supplemented with vitamin E at 0.5 mg/mL (Khellouf et al., 2025).
3. Group supplemented with vitamin C at 0.25 mg/mL (Khellouf et al., 2025).
4. Aliquot containing the combination of vitamin E and C at 0.5 mg/mL and 0.25 mg/mL, respectively (Khellouf et al., 2025).
5. Aliquot supplemented with the combination of vitamin E and *Rosmarinus officinalis* essential oil at 0.5 mg/mL and 1 µL/mL, respectively (Benberkane et al., 2019).

After 15 minutes of incubation at 22°C, all aliquots were further diluted with Tris extender + 10% glycerol + 20% egg yolk to obtain 100×10^6 spermatozoa/mL. Finally, sperm was equilibrated at 4°C for 2 hours to facilitate glycerol permeation, packaged in 0.25 mL straws, and frozen in liquid nitrogen.

The essential oils were purchased from Aromabiol Company (Algeria). The chemical composition of the extracted rosemary essential oil (REO) was analyzed using Gas Chromatography–Mass Spectrometry (GC-MS). The major bioactive constituents identified were camphor (18.88%), camphene (5.17%), 1,8-cineole (7.85%), β-thujene (13.66%), α-thujene (4.87%), chrysanthenone (12.05%), and β-cubenene (7.97%).

Motility assessment

Sperm motility was quantitatively analyzed using a Computer-Assisted Sperm Analysis (CASA) system (Sperm Class Analyzer, version 3.2.0; Microptic S.L., Barcelona, Spain) to ensure objective evaluation of kinematic parameters. Aliquots of 10 µL from frozen-thawed samples were carefully introduced into Makler Counting Chamber, featuring a chamber depth of 10 µm (Sefi-Medical Instruments Ltd., Biosigma S.r.l., Italy). To reestablish physiological conditions, the samples were equilibrated by incubating for 30 seconds at 37°C prior to analysis. Subsequently, spermatozoa movement was recorded under phase-contrast microscopy at 10× magnification. The system computed key motility indices including curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral movement of the head (ALH), beat cross frequency (BCF), and linearity percentage [LIN = (VSL / VCL) × 100] (Mortimer, 2000).

Hypoosmotic Swelling Test (HOST)

The functional integrity of the sperm plasma membrane was assessed using the hypoosmotic swelling test (HOST) according to the protocol established by Revell and Mrode (1994). In brief, 30 µL aliquots of each semen sample were incubated with 300 µL of hypoosmotic solution, prepared by dissolving 9 g of fructose and 4.9 g of sodium citrate in one liter of distilled water. The mixture was incubated for 60 minutes at room temperature to allow osmotic challenge. Following incubation, samples were examined under a phase-contrast microscope at 20× magnification. For each sample, at least 200 spermatozoa were evaluated. The spermatozoa exhibiting curled or swollen tails were considered indicative of intact and functionally competent membranes.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

Lipid peroxidation was assessed through quantification of malondialdehyde (MDA), a terminal product of oxidative damage, serving as a reliable biomarker for oxidative stress. Following thawing, sperm samples (100×10^6 cells/mL) were centrifuged at 1500×g for 15 minutes, and the supernatant was discarded. The resulting sperm pellets underwent three sequential wash cycles by centrifugation at 1500×g for 10 minutes and were resuspended in 1 mL of distilled water after each wash. Following the final centrifugation, pellets were resuspended in 500 µL of distilled water and subjected to sonication in six 15-second pulses with 30-second intervals between each pulse.

MDA content levels were measured in the sonicated sperm samples through spectrophotometric analysis. Briefly, 0.5 mL of each sonicated sperm preparation was added to 1 mL of TBA-TCA-HCl reagent solution containing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 N hydrochloric acid. The mixture was heated at 95°C for 60 minutes to facilitate the MDA-TBA chromogenic reaction and then rapidly cooled in an ice bath. Samples were centrifuged at 18000×g for 15 minutes, and the absorbance of the supernatant was measured spectrophotometrically at 535 nm. Results were calculated and expressed as nmoles MDA per 10^8 spermatozoa using the molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Buege & Aust, 1978).

Statistical analysis

The entire experimental protocol was conducted in ten independent replicates to ensure reproducibility and reliability of the data. Results are presented as mean values accompanied by their standard error of the mean (mean ± SEM). Statistical analysis was performed using Statview software version 4.02 (Abacus Concepts Inc., Berkeley, CA, USA). After the normality of the data distribution was assessed, comparisons among treatment groups regarding sperm motility parameters, membrane integrity, and lipid peroxidation levels were carried out using one-way analysis of variance (ANOVA). Where significant differences were detected, post hoc pairwise comparisons were conducted using Fisher's protected least significant difference (LSD) test. Statistical significance was established at a threshold of $P < 0.05$.

Results

Total (TM) and Progressive (PM) Motility

Total motility measurements showed variable responses to the different treatments (Fig. 1). The control group exhibited TM values of 64.3 ± 3.5%. Vitamin C supplementation resulted in the highest improvement, reaching 75.8 ± 8.6% of total motility with a significant difference when compared with the control ($P < 0.04$). Individual vitamin E treatment showed values similar to the control at 66.1 ± 4.3%, while the combined vitamin treatment (Vit E-C) demonstrated a slight decrease to 64.9 ± 1.4%. The vitamin E and essential oil combination (Vit E-EO) achieved 73.8 ± 5.6% total motility.

Progressive motility displayed a different pattern compared with total motility (Fig. 1). The control group showed PM values of 24.4 ± 3.2%. Individual vitamin treatments produced moderate improvements: vitamin C achieved 32.5% ± 3.4 and vitamin E reached 29.3 ± 1.9%. The combined vitamin treatment (Vit E-C) showed values similar to the control at 27.4 ± 6.9%. However, the vitamin E and essential oil combination (Vit E-EO) demonstrated the highest progressive motility at 33.7 ± 7.1%, values statistically different compared with all the investigated treatments ($P < 0.05$).

Kinematic parameters assessment

The addition of antioxidants to cryopreservation

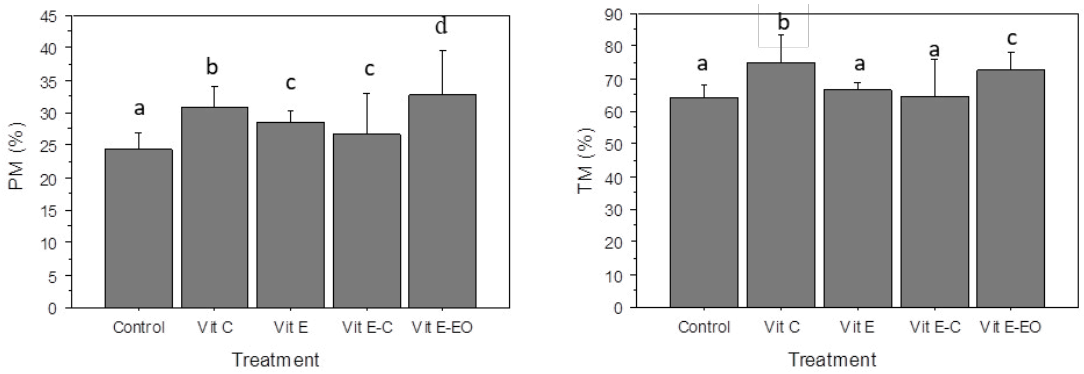

Fig. 1. Mean percentages (± S.E.M.) of total motility (TM) and progressive motility (PM) in cryopreserved bull spermatozoa across experimental groups. Semen samples were either left untreated (control) or pre-treated prior to freezing with vitamin E, vitamin C, an association of vitamin E and C, and aliquots supplemented with the association of vitamin E and Rosmarinus essential oils.

extenders significantly improved sperm motility parameters compared with the control group. Three key velocity measurements were analyzed: curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP).

VCL showed a progressive improvement across treatments (Fig. 2). The control group exhibited the lowest VCL values at approximately 34.271 ± 0.662 µm/sec. Individual vitamin supplementation resulted in modest improvements, with vitamin C achieving 37.131 ± 0.602 µm/sec and vitamin E reaching 42.066 ± 0.776 µm/sec. The combination of vitamins C and E (Vit E-C) further increased VCL to 40.961 ± 0.866 µm/sec. The most substantial improvement was observed in the vitamin E and essential oil combination (Vit E-EO), which achieved the highest VCL value with 47.835 ± 2.412 µm/sec, values significantly higher ($P < 0.03$) than the control and the other investigated treatments.

VSL measurements demonstrated a similar pattern of improvement (Fig. 2). Control samples showed VSL values of 14.654 ± 0.459 µm/sec. Vitamin C supplementation increased VSL to 16.730 ± 0.448 µm/sec, while vitamin E achieved 18.164 ± 0.520 µm/sec. The vitamin combination (Vit E-C) resulted in VSL values of 19.163 ± 0.610 µm/sec. Again, the vitamin E and essential oil combination (Vit E-EO) produced the highest VSL values (23.748 ± 1.596 µm/sec) with a significant difference, particularly when compared with Vit E-C treatment ($P < 0.002$).

VAP measurements followed the same trend as the other velocity parameters (Fig. 2). The control group recorded VAP values of 21.436 ± 0.501 µm/sec. Individual vitamin treatments showed incremental improvements: vitamin C (23.622 ± 0.481 µm/sec) and vitamin E (25.430 ± 0.561 µm/sec). The combined vitamin treatment (Vit E-C) achieved 26.072 ± 0.652 µm/sec. The vitamin E and essential oil combination (Vit E-EO) demonstrated the highest VAP values with 30.606 ± 1.689 µm/sec, indicating a significant difference ($P < 0.001$) compared with the control.

For the sperm movement pattern parameters (ALH, BCF, and LIN, Fig. 2), treatment effectiveness varied depending on the specific measured parameter. ALH and BCF showed similar progressive improvements with the hierarchy: Control < Vit C ≈ Vit E < Vit E-C < Vit E-EO. However, linearity (LIN) displayed a different pattern, where individual vitamin treatments (Vit C and Vit E) showed minimal improvements over the control, but the combined treatments (Vit E-C and Vit E-EO) demonstrated substantial enhancements. The vitamin E and essential oil combination consistently produced the most significant improvements across all three parameters ($P < 0.05$).

Hypoosmotic Swelling Test (HOST)

The hypoosmotic swelling test revealed significant variations in sperm membrane integrity across treatments (Fig. 3). The control group showed HOST values of 14 ± 2%. Individual vitamin supplementation produced contrasting effects: vitamin C treatment increased HOST to 18 ± 2%, while vitamin E showed a slight improvement to 16 ± 1%. Interestingly, the combined vitamin treatment (Vit E-C) demonstrated the lowest HOST values at approximately 13 ± 1%. However, the vitamin E and essential oil combination (Vit E-EO) achieved the highest HOST values with 23 ± 4%, which were statically different compared with the control ($P < 0.001$).

Lipid Peroxidation Assessment (TBARS Assay)

The extent of lipid peroxidation in sperm samples was evaluated through malondialdehyde (MDA) quantification using the TBARS assay. As shown in Fig. 4, all antioxidant treatments demonstrated protective effects against lipid peroxidation compared with the control group.

The control group exhibited the highest level of lipid peroxidation with MDA concentrations reaching 0.35 nmol/10^8 spermatozoa, indicating substantial oxidative damage to sperm membrane lipids. Treatment

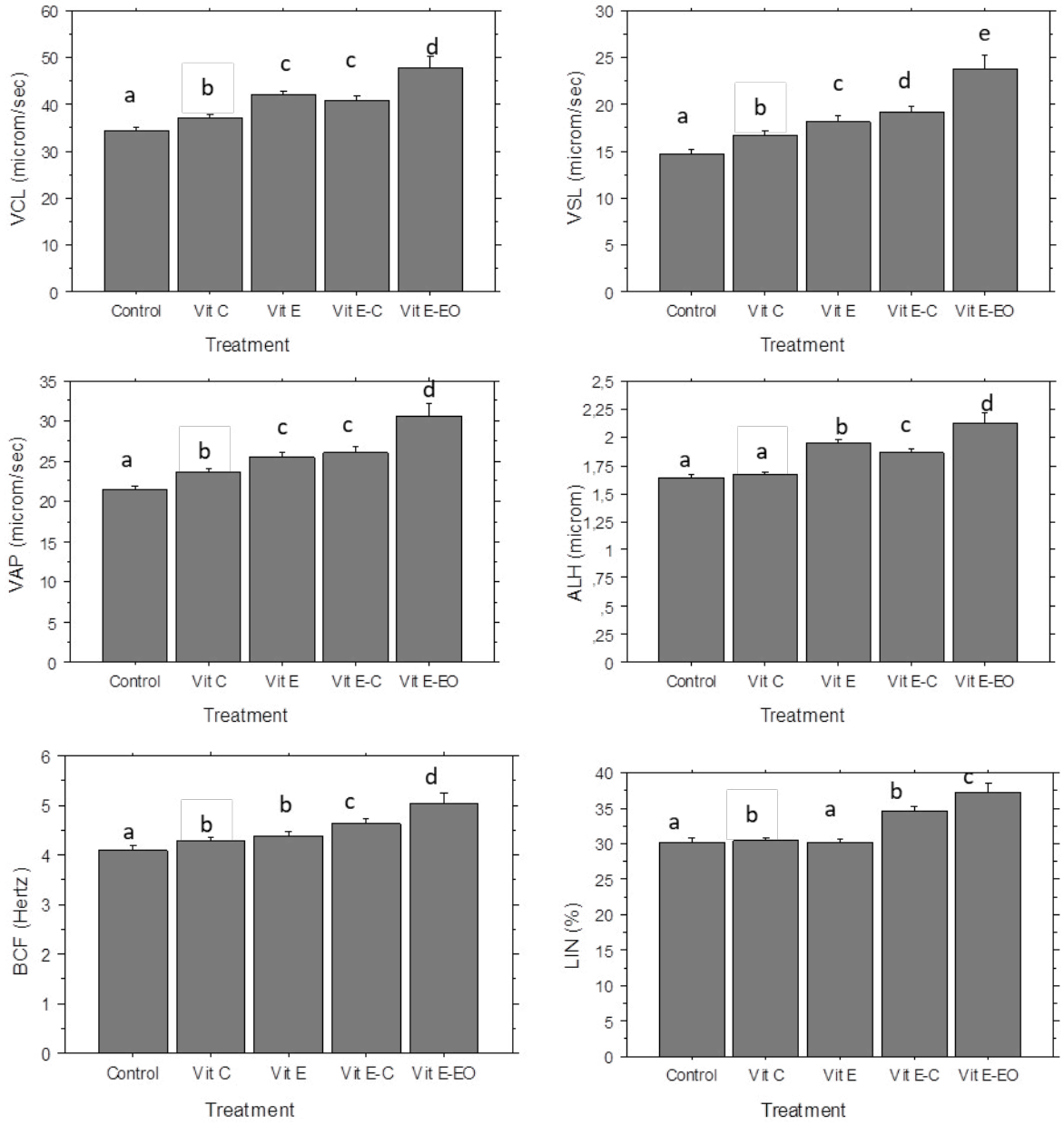

Fig. 2. Post-thaw sperm kinematic parameters in cryopreserved bull spermatozoa (mean ± S.E.M) from the control group and groups pre-treated prior to freezing with vitamin E, vitamin C, an association of vitamin E and C, and aliquots supplemented with the association of vitamin E and Rosmarinus essential oils. Parameters include curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF). Different lowercase letters indicate statistically significant differences among groups ($P < 0.05$).

with vitamin C resulted in a notable reduction in MDA levels to 0.32 nmol/10^8 spermatozoa. Vitamin E supplementation showed enhanced protective effects, reducing MDA concentrations to 0.31 nmol/10^8 spermatozoa. The combination of vitamins E and C (Vit E-C) demonstrated superior antioxidant efficacy, achieving MDA levels of 0.27 nmol/10^8 spermatozoa.

The most pronounced protective effect was observed with the vitamin E-essential oil combination (Vit E-EO), which reduced MDA concentrations to 0.26 nmol/108 spermatozoa, representing the greatest decrease in lipid peroxidation among all treatments tested ($P < 0.05$).

Discussion

Cryopreservation remains an indispensable technique for the long-term storage of bull semen, facilitating genetic dissemination and conservation programs (Murphy et al., 2019). However, the process is known to induce substantial cellular stress, primarily through the generation of reactive oxygen species (ROS) and osmotic shock resulting to membrane destabilization (Bucak et al., 2013). These factors collectively compromise sperm motility, membrane integrity, and fertilizing capacity (Guthrie and Welch, 2012). Hence, developing extender formulations that mitigate cryodamage is a continuous objective

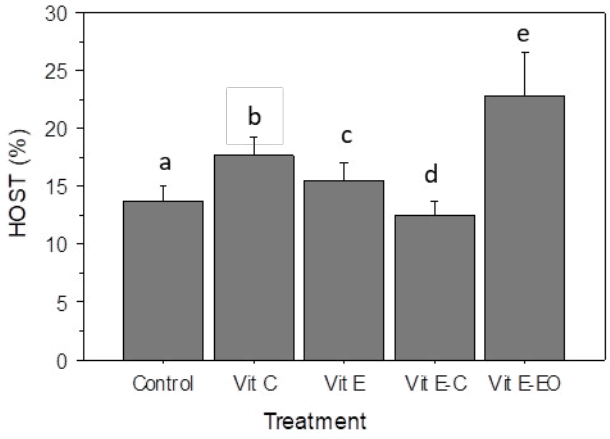

Fig. 3. Mean percentages (± S.E.M.) of hypoosmotic swelling test in cryopreserved bull spermatozoa across experimental groups. Semen samples were either left untreated (control) or pre-treated prior to freezing with vitamin E, vitamin C, an association of vitamin E and C, and aliquots supplemented with the association of vitamin E and Rosmarinus essential oils.

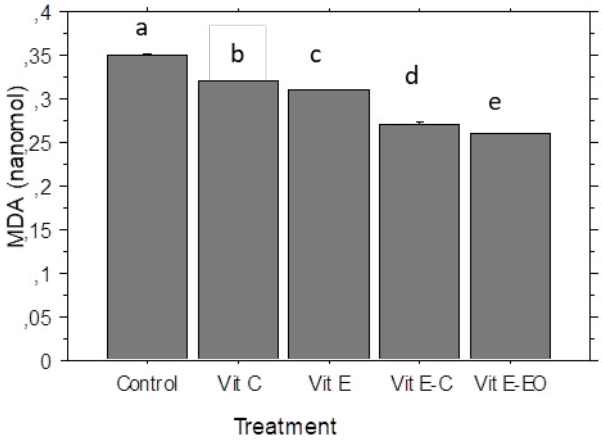

Fig. 4. Mean percentages (± S.E.M.) of MDA rate (nmol) in cryopreserved bull spermatozoa across experimental groups. Semen samples were either left untreated (control) or pre-treated prior to freezing with vitamin E, vitamin C, an association of vitamin E and C, and aliquots supplemented with the association of vitamin E and Rosmarinus essential oils.

in reproductive biotechnology (Bansal and Bilaspuri, 2011).

In this study, we evaluated the protective effects of vitamin C, vitamin E, their combination, and a novel formulation combining vitamin E with essential oils (EOs) on post-thaw sperm motility and kinematic parameters. Our findings provide compelling evidence that all antioxidant-enriched extenders conferred a degree of protection, with the most significant improvements in the group treated with vitamin E + EOs.

Vitamin C (ascorbic acid) is a potent hydrophilic antioxidant that neutralizes aqueous-phase ROS, particularly superoxide anions and hydroxyl radicals (Padayatty et al., 2003). It also has the ability to regenerate oxidized vitamin E, thereby supporting lipid-phase antioxidant defense (Padilla and Foote, 1991). However, in our results, vitamin C alone improved moderately post-thaw sperm motility and did not significantly enhance velocity parameters. This suggests that while vitamin C offers intracellular ROS protection, it may be insufficient to fully preserve membrane integrity during freezing (Silva and Gadella, 2006).

Vitamin E (alpha-tocopherol), in contrast, is a lipophilic antioxidant incorporated into the sperm plasma membrane, where it plays a direct role in inhibiting lipid peroxidation (Agarwal and Saleh, 2002). Its inclusion led to a more pronounced improvement in both progressive motility and velocity, underscoring the importance of membrane-targeted protection. This is consistent with prior studies that emphasized the centrality of membrane lipid peroxidation as a primary target of cryo-induced damage (Beconi et al., 1993).

The combined use of vitamin C and vitamin E demonstrated enhanced efficacy compared with each antioxidant alone, particularly for progressive motility and VSL (Yousef et al., 2003). This synergistic effect likely arises from the complementary actions of the two vitamins across different cellular compartments (Burton and Ingold, 1989). Vitamin C regenerates

vitamin E radicals and scavenges ROS in the cytosol, while vitamin E protects lipid domains, resulting in comprehensive antioxidant coverage.

Notably, the most remarkable improvement in both motility and velocity parameters was observed in the group supplemented with vitamin E + essential oils. This formulation outperformed even the vitamin C + vitamin E group, indicating the added value of EOs in semen cryopreservation (Askari et al., 2013). Essential oils, depending on their botanical source, are complex mixtures rich in monoterpenes, sesquiterpenes, and phenolic compounds such as thymol, carvacrol, and eugenol (Uysal and Bucak, 2007). These compounds exhibit strong antioxidant, antimicrobial, and membrane-modulating properties.

The superior performance of the EO-supplemented group may be attributed to several mechanisms. First, EOs can enhance membrane fluidity and stability, thereby reducing structural damage during freezing and thawing (Bucak et al., 2007). Second, their antioxidant constituents can directly scavenge ROS or upregulate endogenous antioxidant enzymes such as glutathione peroxidase and superoxide dismutase. Third, EOs may exert mild antimicrobial effects, reducing endotoxin-induced stress in semen samples.

The significant elevation in VSL and VAP in the vitamin E + EO group is particularly relevant, as these parameters are closely associated with the sperm's ability to reach and penetrate the oocyte (Sariözkan et al., 2009). Improved linear and average velocities suggest enhanced mitochondrial function and energy production, possibly supported by EO-induced stimulation of metabolic activity.

However, the use of essential oils requires careful consideration of concentration, as some components can be cytotoxic at high doses. In this study, the concentration was optimized to ensure efficacy without compromising cell viability. Future research should further characterize the specific EO components responsible for the observed benefits and explore their effects on fertility outcomes post-insemination.

In conclusion, this study demonstrates that antioxidant supplementation, particularly the combination of vitamin E with essential oils, enhanced significantly the resilience of bull sperm to cryodamage (Murphy et al., 2019). The findings support the development of natural, multifunctional extender additives that improve semen quality while reducing dependence on synthetic cryoprotectants. This approach aligns with current trends favoring sustainable and biocompatible solutions in animal reproduction technologies.

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Comparative Analysis of Changes in the Chemical Composition of Milk in East Friesian and Simmental Cows with Ketosis, Puerperal Paresis, and Mastitis During the Postpartum Period

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Abstract. *Postpartum metabolic and inflammatory disorders, including ketosis, puerperal paresis, and mastitis, profoundly affect milk composition and have significant implications for dairy herd productivity and health. This study aimed to investigate breed-related differences in milk chemical composition between Holstein-Friesian and Simmental cows during early lactation prior to therapeutic intervention. A total of 360 cows were examined and grouped by disease and breed.*

Milk was analyzed for fat, protein, casein, lactose, solids-not-fat, fatty acid profiles (saturated, unsaturated, polyunsaturated), BHB, acetone, minerals (Ca, P, Mg), milk urea, citrate, somatic cell count, and pH (mastitis). ANOVA assessed breed differences, while correlation and regression analyses evaluated associations among metabolic markers and milk components. Chi-square tests examined breed-specific differences within disease categories.

Holstein-Friesian cows with ketosis showed higher BHB (450 ± 30 μmol/L) and acetone (2.5 ± 0.2 mmol/L) compared with Simmental (BHB 380 ± 25 μmol/L; acetone 1.8 ± 0.2 mmol/L; $P < 0.001$), reflecting enhanced lipid mobilization. In mastitis, reductions in protein (Holstein 2.9 ± 0.2% vs Simmental 3.0 ± 0.2%; $P < 0.002$) and lactose (4.0 ± 0.1% vs 4.1 ± 0.1%; $P < 0.001$) were more pronounced in Holstein-Friesians. Puerperal paresis was associated with lower milk calcium (95 ± 5 mg/100 mL vs 100 ± 4 mg/100 mL; $P < 0.001$), phosphorus, and magnesium in Holstein-Friesians. Regression models showed that BHB and acetone explained up to 72% of protein variation, while chi-square tests confirmed significant breed-specific metabolic differences.

However, mechanistic explanations for breed differences remain unclear, and potential confounders such as diet, housing, and environmental factors were not fully controlled, limiting causal inference. These findings highlight milk composition as an integrated biomarker system for monitoring postpartum metabolic and inflammatory stress. Understanding breed-specific responses may inform targeted nutritional strategies, early detection, and precision herd management, enhancing both animal welfare and milk productivity.

Introduction

Milk is one of the most important products of the dairy industry, and its quality is closely linked to the health and metabolic status of the cow (Chang et al., 2011). The postpartum period represents a critical physiological stage during which dairy cows undergo rapid metabolic adaptations to support lactation, often predisposing them to metabolic and inflammatory disorders (Buckley et al., 2003). Changes in milk chemical composition, including fat, protein, lactose, and mineral content, can serve as early, non-invasive biomarkers of these disorders, providing valuable insights for herd management and productivity (Costa et al., 2019; Fleischer et al., 2001).

Postpartum metabolic disorders, such as ketosis and puerperal paresis, and inflammatory conditions like mastitis, significantly alter milk composition. Ketosis arises from a negative energy balance when nutrient intake cannot meet the high energy demands of early lactation, leading to increased mobilization of body fat and accumulation of ketone bodies, which in turn affects milk fat, protein, and lactose content (Cabezas-Garcia et al., 2021; Chirivi et al., 2024; Bochniarz et al., 2023; Grelet et al., 2016). Puerperal paresis, characterized by hypocalcemia, disrupts mineral homeostasis and may reduce the concentrations of calcium, phosphorus, and magnesium in milk, while also affecting milk protein levels (Cai et al., 2018; Khol et al., 2020., Espiritu et al., 2025, Tereso et al., 2014). Mastitis, a bacterial infection of the mammary gland, induces localized inflammation that increases somatic cell count and alters milk pH, protein, and lactose, thereby compromising milk quality (Benić et al., 2018; Gasqui et al., 2017).

In addition to disease-related effects, cow breed plays a crucial role in determining the magnitude of

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metabolic and inflammatory responses. High-yielding Holstein-Friesian cows are often more susceptible to postpartum metabolic stress, whereas dual-purpose breeds such as Simmental typically exhibit greater stability in milk composition under similar pathological conditions (Buckley et al., 2003; Gröhn et al., 2003; Krnjaić et al., 2022). Understanding these breed-specific differences is essential for accurate interpretation of milk composition changes and for the development of targeted nutritional and therapeutic strategies.

Despite the well-documented impact of postpartum disorders on milk composition, few studies have systematically compared breed-specific responses under controlled management and feeding conditions. Therefore, this study aims to conduct a comprehensive comparative analysis of milk chemical composition in Holstein-Friesian and Simmental cows affected by ketosis, puerperal paresis, and mastitis, with an emphasis on identifying breed-specific metabolic patterns and disease-related effects. The findings are expected to inform early detection, breed-specific management practices, and strategies to mitigate the impact of postpartum disorders on milk productivity and cow health.

Materials and methods

Experimental animals and farm management

The study was conducted on 360 dairy cows, divided into two breed groups: Holstein-Friesian and Simmental. Each breed was further subdivided into three groups based on the presence of postpartum disorders: ketosis, puerperal paresis, and mastitis, with 60 cows per subgroup. All animals were reared on a single commercial farm under standardized management conditions to minimize confounding factors:

- Housing system: Free-stall with deep-bedded straw; stocking density of 1.2–1.3 cows per stall. Ventilation was natural with additional mechanical fans; temperature and humidity were continuously monitored (21–25°C, 55–65% RH).
- Feeding strategy: Total mixed ration (TMR) formulated according to NRC (2021) recommendations. Diet composition: 60% forage (corn silage, hay) and 40% concentrate; energy density 1.6 Mcal/kg DM. Minerals (Ca, P, Mg) and DCAD adjusted for early lactation cows. Feed was delivered three times daily, and fresh water was available ad libitum.
- Milking system: Cows were milked twice daily using an automated milking parlor. Milking hygiene was standardized to minimize variation in SCC.

Animals were in early lactation, included at the time of disease diagnosis, before any therapeutic intervention. Only cows in 2nd to 5th lactation with no other health conditions were enrolled.

Disease diagnosis and sample collection

Ketosis. Diagnosis was based on β-hydroxybutyrate (BHB) concentrations in blood and milk. Blood was collected from the tail vein using sterile needles and vacuum serum tubes. BHB was measured via enzymatic-colorimetric method (Randox Laboratories, UK). Subclinical ketosis: BHB >1.2 mmol/L; clinical ketosis: BHB >3 mmol/L. Clinical signs (reduced appetite, weakness) were recorded.

Puerperal Paresis. Serum and milk calcium were measured. Blood was collected from the tail vein and analyzed via atomic absorption spectroscopy (AAS, Thermo Fisher Scientific, USA). Milk Ca and P were analyzed using commercial test kits (Randox, UK). Diagnostic criteria: Ca <1.5 mmol/L, muscle weakness, tremors, impaired reflexes.

Mastitis. The disease was diagnosed by somatic cell count (SCC >200 000 cells/mL) and clinical signs (udder swelling, heat, redness). Blood was collected to determine inflammatory markers. Milk microbiology was performed using standard culture methods and PCR confirmation of *Escherichia coli, Staphylococcus aureus*, and *Streptococcus* spp.

Milk sampling and preparation

Milk samples were collected early morning before milking, three times during the first postpartum weeks. The samples were processed immediately and included the following:

- Physicochemical analysis: milk fat, protein, lactose, minerals (Ca, P, Mg), pH;
- Biochemical markers: BHB and NEFA (ketosis), SCC (mastitis), serum Ca (paresis);
- Microbiological analysis: bacterial identification in mastitis cases.

Milk was stored at –20°C and blood at –80°C until analysis.

Analytical methods

- Milk fat, protein, lactose: MilkoScan FT+ (Foss, Denmark), FTIR
- Minerals (Ca, P, Mg): Atomic Absorption Spectroscopy (AAS)
- Milk pH: digital pH meter (Hanna Instruments, USA)
- SCC: Fossomatic 5000 (Foss, Denmark)
- BHB, NEFA: commercial test kits (Randox, UK)
- Microbiology: selective media culture + PCR

Ethical approval

The study received approval from the institutional ethics committee for sample collection and animal handling (ethics approval number: 001328298 2024 14841 002 001 323 022).

Statistical analysis

Data were analyzed using SPSS 28 (IBM Corp., 2021). Sample size justification was performed using G*Power 3.1, with $\alpha = 0.05$ and 80% power, indicating

a minimum of 50 cows per subgroup.

Milk samples were collected three times during the early postpartum period; individual values were averaged per cow before statistical analysis. Each animal therefore contributed a single representative value for each parameter, avoiding pseudo-replication and ensuring independence of observations.

Comparisons between breeds and disease groups were performed using one-way ANOVA with the Tukey post-hoc test. *P* values slightly above the conventional threshold of 0.05 ($0.05 < P \leqslant 0.10$) were considered as trends and are reported as tendencies rather than statistically significant differences.

Correlation analyses were conducted to examine relationships between metabolic parameters (BHB, NEFA, Ca) and milk composition (fat, protein, lactose). Multiple comparisons were adjusted using the Bonferroni correction where appropriate.

Regression analyses were performed to evaluate the proportion of variance in dependent variables explained by selected independent variables, with R^2 values reported along with 95% confidence intervals and residual analysis. Chi-square tests were used to examine breed-specific differences for categorized metabolic parameters using established clinical cut-off values. Statistical significance was set at $P < 0.05$. All analyses were conducted following standardized protocols to ensure reproducibility and robustness.

Results

Table 1 shows the chemical composition of milk in Holstein-Friesian and Simmental cows with ketosis. Statistically significant differences between breeds ($P < 0.05$) were observed for milk fat, saturated fatty acids, unsaturated fatty acids, polyunsaturated fatty acids, proteins, casein, lactose, fat-free dry matter, BHB, acetone, and milk citrate. No significant difference was observed for milk urea ($P = 0.250$). Data are presented as mean ± SD.

Table 2 shows the chemical composition of milk in cows with puerperal paresis for the Holstein-Friesian and Simmental breeds. Statistically significant differences ($P < 0.05$) were observed for saturated fatty acids, proteins, casein, calcium, phosphorus, magnesium, and milk citrate. Other parameters, including milk fat, unsaturated and polyunsaturated fatty acids, lactose, fat-free dry matter, and milk urea, did not differ significantly between the two breeds.

Table 3 presents the chemical composition of milk in cows with mastitis for Holstein-Friesian and Simmental breeds. Statistically significant differences ($P < 0.05$) were observed for saturated fatty acids, proteins, casein, lactose, somatic cells, pH, and milk citrate. Other parameters, including milk fat, unsaturated and polyunsaturated fatty acids, fat-free dry matter, and milk urea, did not differ significantly between breeds.

Note: Although mean values for saturated fatty acids appear numerically identical due to rounding, statistical analysis indicates a significant difference ($P < 0.05$).

Table 4 presents the correlation coefficients (r) and associated *P* values for selected milk parameters in cows with ketosis, puerperal paresis, and mastitis, for both Holstein-Friesian and Simmental breeds. Strong positive correlations ($P < 0.01$) were observed between BHB and acetone in ketosis, between calcium and phosphorus in puerperal paresis, and between lactose and proteins in mastitis.

Table 5 presents the results of regression analysis for milk composition in cows with ketosis, puerperal paresis, and mastitis for both Holstein-Friesian and Simmental breeds. R^2 values and associated *P* values indicate the proportion of variance in the dependent variable explained by the selected independent variables in each disease and breed group.

Table 6 shows the results of chi-square tests for breed differences in key milk parameters across ketosis, puerperal paresis, and mastitis. *P* values

Table 1. Chemical composition of milk in cows with ketosis (Holstein vs Simmental)

Parameter	Reference values	Holstein-Friesian	Simmental	P value (ANOVA)	*F* statistic
Milk fat %	3.5–4.0	4.8 ± 0.3	4.3 ± 0.3	0.010	6.55
Saturated fatty acids (mmol/L)	40–45	52 ± 4	48 ± 3	0.008	7.12
Unsaturated fatty acids (mmol/L)	20–25	16 ± 2	18 ± 2	0.045	4.22
Polyunsaturated fatty acids (mmol/L)	5–8	3 ± 1	4 ± 1	0.050	3.95
Proteins %	3.2–3.5	2.8 ± 0.2	3.0 ± 0.2	0.030	4.65
Casein %	2.1–2.4	1.9 ± 0.1	2.0 ± 0.1	0.028	4.72
Lactose %	4.6–4.8	4.2 ± 0.1	4.3 ± 0.1	0.010	6.10
Fat-free dry matter %	8.5–9.0	8.0 ± 0.2	8.2 ± 0.2	0.045	4.18
BHB (mmol/L)	<100	450 ± 30	380 ± 25	<0.001	10.90
Acetone (mmol/L)	<0.5	2.5 ± 0.2	1.8 ± 0.2	<0.001	9.60
Milk urea (mg/dL)	25–35	28 ± 3	27 ± 2	0.250	1.42
Milk citrate (mmol/L)	8–10	6.5 ± 0.5	6.8 ± 0.5	0.015	5.40

Table 2. Chemical composition of milk in cows with puerperal paresis (Holstein vs Simmental)

Parameter	Reference values	Holstein-Friesian	Simmental	*P* value (ANOVA)	*F* statistic
Milk fat %	3.5–4.0	3.9 ± 0.3	3.7 ± 0.2	0.120	2.35
Saturated fatty acids (mmol/L)	40–45	46 ± 4	43 ± 3	0.045	4.25
Unsaturated fatty acids (mmol/L)	20–25	21 ± 2	22 ± 2	0.200	1.68
Polyunsaturated fatty acids (mmol/L)	5–8	5 ± 1	6 ± 1	0.055	3.95
Proteins %	3.2–3.5	3.0 ± 0.2	3.1 ± 0.2	0.045	4.65
Casein %	2.1–2.4	2.0 ± 0.1	2.1 ± 0.1	0.038	4.72
Lactose %	4.6–4.8	4.5 ± 0.1	4.6 ± 0.1	0.200	1.68
Fat-free dry matter %	8.5–9.0	8.2 ± 0.3	8.4 ± 0.2	0.100	2.90
Ca (mg/100 mL)	120–130	95 ± 5	100 ± 4	<0.001	14.22
P (mg/100 mL)	90–100	72 ± 4	78 ± 5	<0.002	9.12
Mg (mg/100 mL)	12–15	10 ± 1	11 ± 1	<0.015	6.18
Milk urea (mg/dL)	25–35	28 ± 3	27 ± 3	0.180	2.10
Milk citrate (mmol/L)	8–10	6.8 ± 0.6	7.0 ± 0.5	0.010	5.40

Table 3. Chemical composition of milk in cows with mastitis (Holstein vs Simmental)

Parameter	Reference values	Holstein-Friesian	Simmental	*P* value (ANOVA)	*F* statistic
Milk fat %	3.5–4.0	3.6 ± 0.4	3.8 ± 0.3	0.350	1.20
Saturated fatty acids (mmol/L)	40–45	42 ± 3	42 ± 3	0.045	0.60
Unsaturated fatty acids (mmol/L)	20–25	19 ± 2	20 ± 2	0.120	2.35
Polyunsaturated fatty acids (mmol/L)	5–8	4 ± 1	5 ± 1	0.080	3.10
Proteins %	3.2–3.5	2.9 ± 0.2	3.0 ± 0.2	<0.002	9.05
Casein %	2.1–2.4	1.8 ± 0.1	1.9 ± 0.1	<0.001	12.35
Lactose %	4.6–4.8	4.0 ± 0.1	4.1 ± 0.1	<0.001	11.50
Fat-free dry matter %	8.5–9.0	8.1 ± 0.2	8.3 ± 0.2	0.120	2.35
Somatic cells ($\times 10^3$/mL)	<200	550 ± 50	520 ± 40	<0.001	10.90
pH	6.6–6.8	7.2 ± 0.1	7.1 ± 0.1	<0.001	8.40
Milk urea (mg/dL)	25–35	27 ± 3	28 ± 3	0.250	1.42
Milk citrate (mmol/L)	8–10	6.5 ± 0.5	6.7 ± 0.4	0.005	6.80

Table 4. Correlation analysis of milk parameters (all diseases and breeds)

Disease	Breed	Parameter 1	Parameter 2	Correlation (r)	*P* value
Ketosis	Holstein	BHB	Acetone	0.89**	<0.01
Ketosis	Simmental	BHB	Acetone	0.85**	<0.01
Puerperal paresis	Holstein	Ca	P	0.78**	<0.01
Puerperal paresis	Simmental	Ca	P	0.72**	<0.01
Mastitis	Holstein	Lactose	Proteins	0.80**	<0.01
Mastitis	Simmental	Lactose	Proteins	0.75**	<0.01

Table 5. Regression analysis (all diseases and breeds)

Disease	Breed	Dependent variable	Independent variables	R^2	*P* value
Ketosis	Holstein	Proteins %	BHB, Acetone	0.72	<0.01
Ketosis	Simmental	Proteins %	BHB, Acetone	0.65	<0.01
Puerperal paresis	Holstein	Ca	Mg, P	0.68	<0.01
Puerperal paresis	Simmental	Ca	Mg, P	0.60	<0.01
Mastitis	Holstein	Lactose %	Somatic cells, pH	0.75	<0.01
Mastitis	Simmental	Lactose %	Somatic cells, pH	0.70	<0.01

Table 6. Chi-square test for breed differences (all diseases)

Disease	Parameter	Chi-square	df	*P* value
Ketosis	BHB	12.35	1	<0.001
Puerperal paresis	Ca	14.22	1	<0.001
Mastitis	Lactose	16.05	1	<0.001

indicate statistically significant differences between Holstein-Friesian and Simmental cows for the analyzed parameters.

Discussion

The results of this study demonstrate that postpartum diseases, including ketosis, puerperal paresis, and mastitis, are associated with measurable changes in the chemical composition of milk in dairy cows. These alterations encompass differences in milk fat, proteins, lactose, minerals, and metabolic indicators such as BHB and NEFA. Such findings are generally in line with established knowledge regarding metabolic stress and inflammatory challenges during the transition period, which involves complex physiological adaptations to support the onset of lactation and energy demands immediately after calving (Costa et al., 2019; Rico and Barrientos, 2024).

In the group of cows affected by ketosis, elevated milk BHB and acetone concentrations were observed, consistent with patterns described in the literature where negative energy balance in early lactation leads to increased mobilization of body fat and accumulation of ketone bodies (Klein et al., 2020; Cooper et al., 2025). For example, milk fat in Holstein-Friesian cows with ketosis was significantly higher (4.8 ± 0.3%) compared with Simmental (4.3 ± 0.3%; $P = 0.010$), reflecting increased lipid mobilization. Similarly, BHB (450 ± 30 mmol/L vs 380 ± 25 mmol/L; $P < 0.001$) and acetone (2.5 ± 0.2 mmol/L vs 1.8 ± 0.2 mmol/L; $P < 0.001$) were significantly elevated in Holstein-Friesian cows, indicating acute metabolic adaptation to negative energy balance. These patterns highlight breed-related variability in metabolic responses under identical management conditions.

In the puerperal paresis group, marked reductions in milk mineral components, especially calcium, phosphorus, and magnesium, were observed. Holstein-Friesian cows had lower calcium (95 ± 5 mg/100 mL) compared with Simmental (100 ± 4 mg/100 mL; $P < 0.001$), phosphorus (72 ± 4 mg/100 mL vs 78 ± 5 mg/100 mL; $P < 0.002$), and magnesium (10 ± 1 mg/100 mL vs 11 ± 1 mg/100 mL; $P < 0.015$), reflecting challenges in maintaining calcium homeostasis in early lactation. Protein and casein levels were also slightly lower in Holstein-Friesians (3.0 ± 0.2% vs 3.1 ± 0.2%, $P = 0.045$; 2.0 ± 0.1% vs 2.1 ± 0.1%, $P = 0.038$), consistent with impaired mineral metabolism affecting milk synthesis (Grigė et al., 2025).

Mastitis, defined by increased somatic cell count and clinical signs of mammary inflammation, was associated with significant reductions in milk proteins, casein, and lactose. For example, Holstein-Friesian cows had lower casein (1.8 ± 0.1% vs 1.9 ± 0.1%; $P < 0.001$) and lactose (4.0 ± 0.1% vs 4.1 ± 0.1%; $P < 0.001$) compared with Simmental cows, while somatic cell counts were markedly elevated (550 ± 50 $x10^3$/mL vs 520 ± 40 $x10^3$/mL; $P < 0.001$). These patterns confirm that udder inflammation disrupts milk synthesis and alters composition, with breed differences influencing the severity of these changes (Wu et al., 2008; Harjanti and Sambodho, 2020).

Correlation and regression analyses show strong associations between key metabolic indicators (e.g., BHB and acetone in ketosis: $r = 0.89$, $P < 0.01$ for Holstein; $r = 0.85$, $P < 0.01$ for Simmental) and specific milk components, supporting the use of combined milk biomarkers to monitor metabolic status in postpartum cows (Rico and Barrientos Blanco, 2024). Regression analysis also indicated that variation in milk proteins could be explained by BHB and acetone levels ($R^2 = 0.72$ for Holstein; $R^2 = 0.65$

for Simmental; $P < 0.01$). These results emphasize that milk composition acts as a multifaceted biomarker reflecting overall metabolic and inflammatory status rather than isolated disease outcomes.

Chi-square analyses further revealed significant breed differences for key milk parameters across diseases, including BHB in ketosis ($\chi^2 = 12.35$; $P < 0.001$), Ca in puerperal paresis ($\chi^2 = 14.22$; $P < 0.001$), and lactose in mastitis ($\chi^2 = 16.05$; $P < 0.001$). These differences highlight the influence of genetic background on disease susceptibility and milk composition alterations (Sordillo et al., 2009; Vicente et al., 2014; Chang et al., 2011).

Despite these contributions, the study has several limitations. Key issues include the absence of mechanistic investigations explaining breed-specific differences, potential confounding factors such as feed or environmental variation, and the cross-sectional design without longitudinal follow-up, which limits the ability to predict disease progression and establish causality. Furthermore, the study did not include longitudinal behavioral or sensor-based data obtained through precision livestock technologies, which have been shown to facilitate earlier detection of metabolic shifts and disease onset (Girdauskaitė et al., 2025; Benedetti et al., 2025). These constraints suggest that future studies should integrate genetic analyses, longitudinal monitoring, and real-time precision technologies to better understand milk biomarkers as indicators of postpartum health.

In summary, this study advances the current understanding of postpartum metabolic and inflammatory disorders by demonstrating both breed-specific differences and integrated biomarker patterns across diseases. By treating milk composition as a system of interrelated indicators rather than separate disease outcomes, these findings support the development of improved monitoring frameworks, early detection tools, and precision herd management practices.

Conclusion

The results of this study demonstrate that postpartum disorders – ketosis, puerperal paresis, and mastitis – induce measurable changes in the chemical composition of milk in dairy cows. Ketosis was associated with elevated BHB (Holstein: 450 ± 30 mmol/L; Simmental: 380 ± 25 mmol/L; $P < 0.001$), acetone, and milk fat, along with reduced protein and lactose levels, reflecting negative energy balance and lipid mobilization during early lactation. Puerperal paresis caused marked reductions in calcium, phosphorus, and magnesium, while mastitis increased somatic cell count and altered protein and lactose fractions, consistent with inflammatory disruption of mammary function.

Breed-specific differences were observed, with Holstein-Friesian cows exhibiting more pronounced metabolic alterations than Simmental cows under similar conditions. However, the mechanistic basis for these breed differences remains unclear, and potential confounding factors such as diet, housing, and environmental variation were not fully accounted for. Integrating results across diseases suggests that milk composition can serve as a holistic biomarker system reflecting systemic metabolic and inflammatory stress rather than isolated disorders, which may support early detection and improved herd management. Nevertheless, the descriptive nature of this study and the lack of longitudinal or mechanistic data limit the novelty and the ability to directly guide intervention strategies. Future research incorporating longitudinal monitoring, precision sensor technologies, and detailed farm management data is needed to confirm causality and enhance practical application.

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The Comparative Characteristics of *Actinobacillus pleuropneumonia* Strains Resistance and Local Epidemiology State in Ukraine

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Keywords: *antibiotic resistance, antibiotic classes, A. pleuropneumonia serotypes, swine respiratory infections.*

Abstract. *Various serotypes of Actinobacillus pleuropneumonia are the causative agents of porcine pleuropneumonia and lead to significant economic loss in the whole world. Effective antibiotic therapy requires data on individual serotype resistance and its coinfection variants. The aim of the present study was to disclose A. pleuropneumonia epidemiology of swine farming in Ukraine during 2022–2024. The isolates from 16 provinces of Ukraine were analyzed with RT-PCR and microbial culture techniques to identify the serotype and its antibiotic resistance. The co-occurrence of four and five serotypes was observed only in two provinces. Besides, the infections caused by one serotype were observed in 79% cases, while coinfection cases of two and three A. pleuropneumoniae serotypes were detected in 21% of all studied regions. Totally, 12 different serotypes were identified in all locations. Serotypes 8 and 2 were most prevailing and together accounted for 51% of all cases. The results on antibiotic resistance demonstrated significant diversity with respect to various antibiotic classes. However, isolated A. pleuropneumoniae strains exhibited high sensitivity to both β-lactams and quinolones while the highest resistance was detected to macrolides and lincosamides. In spite of a large spread of both serotypes 8 and 2, serotype 2 resistance to tetracyclines was significantly low – 2.8%. In contrast, serotype 8 resistance to tetracyclines made up 68.5%. Besides, contrasting sensitivity to tetracycline was detected for serotypes 8 and 2 at 13.9% and 80.6%, respectively. Obtained data showed that most isolated strains were sensitive to cephalosporines, fluoroquinolones, and β-lactams while these strains exhibited high resistance to macrolides and lincosamides. In addition, intermediate resistance was detected to tetracyclines. Together, observed results evidence that multi-resistant A. pleuropneumoniae strains to macrolides, lincosamides, and tetracyclines could be generated in Ukraine in the recent years.*

Introduction

Respiratory diseases of infectious etiology in pigs are the actual problem in the world (Przyborowska-Zhalniarovich et al., 2021; de Almeida et al., 2025; Kwan et al., 2025). Besides, recent reports evidence the rising risk of swine respiratory infection on the farms in Ukraine (Derevyanko and Ayshpur, 2023; Garkavenko et al., 2024). *Actinobacillus pleuropneumoniae* is detected in almost 50% of pneumonia cases observed during the fattening pig period (Kokariev et al., 2023). This microorganism causes highly contagious disease named actinobacillus pleuropneumonia (APP) or porcine pleuropneumonia (PPP). APP provokes significant economic costs to pig farms due to high mortality, decrease in productivity, and significant expenses for the infection treatment and preventive measures (Stringer et al., 2022; Malcher et al., 2024; Sjölund et al., 2025). Nowadays, 19 serotypes of *A. pleuropneumoniae* are described in literature and identified as the strains which vary in virulence and spreading capacity in different countries (Arnal Bernal et al., 2024; Seakamela et al., 2024).

One strategy to reduce the incidence of APP on swine farms includes both local therapeutic measures using antibacterial drugs and total anti-epizootic measures, based on vaccination of pigs against APP (Sjölund et al., 2025; Loera-Muro and Angulo, 2018; Deng et al., 2025; Hyun Park et al., 2025). An efficient antibiotic therapy, which depends directly on the sensitivity of *A. pleuropneumoniae* to antibacterial drugs, is to eliminate local manifestations of APP (Blondeau and Fitch, 2024; Brenciani et al., 2024). Special features are reported in relation to the fact that *A. pleuropneumoniae* strains exhibit the potential to acquire resistance against antibiotics and can generate multi-resistant strains. Multi-resistance is considered as a factor which can extremely complicate the process of treatment and recovery of the herd in the near years (Somogyi et al., 2023; Paulina and Dawid, 2025). The most effective APP monitoring method in the herd is the immunization with homologous vaccines (Xie et al., 2017; Thu Dao et al., 2020). It deserves to be mentioned that within the herd there can be several simultaneously circulating APP strains, which significantly complicates monitoring of the disease (Cuccato et al., 2014).

Currently, the results on APP monitoring and the identification of *A. pleuropneumoniae* have been reported in relation to commercial swine herds in Ukraine (Kokariev et al., 2023). Besides, the data on

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the sensitivity to antibiotics have been published in a recent report (Neverkovets et al., 2025). However, there are no reports with respect to genetic variability of *A. pleuropneumoniae* within different regions of Ukraine. Apart from this, the features of sensitivity and resistance of widespread serotypes to different antibacterial compounds are not considered. In this regard, the study of *A. pleuropneumoniae* genetic biodiversity, resistance, and coinfection variances is both novel and timely in Ukraine as well as other countries.

The aim of the study was to find out *A. pleuropneumonia* epidemiology of swine farming in Ukraine, assess coinfection variances, and analyze the resistance level with respect to various antibiotic classes among isolated serotypes.

Material and methods

General information

The laboratory diagnostic tests and data analysis were carried out during 2022–2024 at the Biosafety-Center R&D Department of Dnipro State Agrarian and Economic University (Dnipro, Ukraine). The samples of tissues were collected from pigs from 33 farms located in 16 different provinces of Ukraine. In total, 177 samples for PCR and 154 samples for culture microbiology were selected. Almost all the farms involved in the present study were assessed as APP-positive enterprises.

Ethical considerations

The present research was carried out in accordance with the framework of the "General Ethical Principles of Animal Experiments", which have been approved by the National Congress of Bioethics held in Kyiv (2001), and in line with the provisions of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS No. 123; https://www.coe.int/en/web/cdcj/laboratory-animals). The experimental protocols on the animal sampling were approved by the Local Ethics Committee for Animal Experiments of Dnipro State Agrarian and Economic University (DSAEU), Ukraine (Protocol No. 07-032022).

Sample collection

The fragments of lungs with visible pathological-anatomical signs of APP complications were isolated from the pigs that died across different herds (5–7 samples individually were separated from each herd). Lung tissue samples were immediately cooled and then transported to the laboratory within 8 hours of collection.

RT-PCR analysis

Each sample of the pulmonary tissue from foci of injury was homogenized individually using FastPrep-24 device. Extraction of nucleic acids (NA) from homogenates was carried out using the "BioExtract Premium Mag" reagent kit and the automated NA extraction "KingFisher Duo" Prime purification system (Thermo Fisher Scientific, USA).

Genome specificity of *A. pleuropneumoniae* serotypes was identified via polymerase chain reaction (RT-PCR) and its genotyping carried out using 19 serovars in NA samples immediately after extraction. All RT-PCR analyses were carried out using commercial tests «EXOPOL» (Spain). The amplification and detection of results was performed using "CFX 96" Touch RT-PCR Detection System (BioRad, USA). Extraction of NA and PCR was carried out according to the recommendations of test-kit producer.

Microbiological culture analysis

Identification of *Actinobacillus pleuropneumoniae* was carried out using the bacteriological culture method by seeding microorganisms into a dense growth medium containing blood and NAD^+ (BioMerieux, France). Primary cultures were incubated at a temperature of $37^0 \pm 2^0$ C in the 5% CO_2 atmosphere for 24 hours (Jorgensen & Turnidge, 2015). The identification of microorganisms was carried out using MALDI-TOF technology. In total, 154 *A. pleuropneumoniae* containing samples were identified and separated by the culture method.

The antibiotic sensitivity of the isolated APP strains was detected using the disc-diffusion method according to Kirby-Bauer. Commercial antibiotic-containing discs were applied to assess both resistance and sensitivity to various antibiotics belonging to β-lactams, fluoroquinolones, aminoglycosides, tetracyclines, macrolides, lincosamides, pleuromutilins, sulfonamides, and amphenicols (Bioanalyse, Turkey; Liofilchem, Italy; Himedia, India). The measurement of zone diameters after incubation and disc application was carried out automatically using SCAN 500 device (Interscience, France).

Statistical analysis

Statistical analysis of the obtained data on the serotype ratio, sensibility and resistance of isolated *A. pleuropneumoniae* strains to various antibiotics was carried out using one-way analysis of variance (ANOVA) with StatView 5.0 (SAS Institute Inc., USA). Observed results were expressed as mean ± standard error of mean (M ± SEM). Statistical comparisons were performed after verification for the normality distribution and the difference between the general variances. The graphs were created using GraphPad Prism 9 software (GraphPad Software, USA). *P* values less than 0.05 were considered statistically significant.

Results

The obtained results showed genetic diversity of *A. pleuropneumoniae* serotypes identified in the samples selected from big farms in Ukraine. In addition, the APP identification results demonstrated significant

differences in the spread of separate serotypes as well as variability in terms of the levels of resistance and sensitivity of *A. pleuropneumoniae* to antibacterial drugs.

Epidemiological analysis

The results of an epidemiological study showed the complexities of regional peculiarities in terms of *A. pleuropneumoniae* serotype allocation as well as the coinfection variances at least in 16 regions of Ukraine (Fig. 1).

The obtained results undoubtedly evidence that different *A. pleuropneumoniae* serotypes circulated among farming pigs in all provinces of Ukraine that were included into the present study. Only four provinces were found to have a single APP strain presence. On the whole, 12 different serotypes of *A. pleuropneumoniae,* namely, 1, 2, 3, 5, 6, 7, 8, 9/11, 12, 13, 17, 18, were identified (Fig. 2).

Serotypes 2 and 8 of *A. pleuropneumoniae* were detected as the most common among farming pigs in different Ukraine regions. Together these serotypes were identified in pig herds located in 8 regions included in the study. Serotypes 1, 7, 9/11, and 18 were less spread, and registered in 3–4 regions of Ukraine. Serotypes 3, 5, 6, 12, 13, and 17 were identified in 1–2 regions of the country.

Unlike aforementioned serotypes detected in Ukraine, *A. pleuropneumoniae* serotypes 4, 10, 14, 15, and 16 reported in other countries were not even once observed.

The highest diversity in terms of *A. pleuro-*

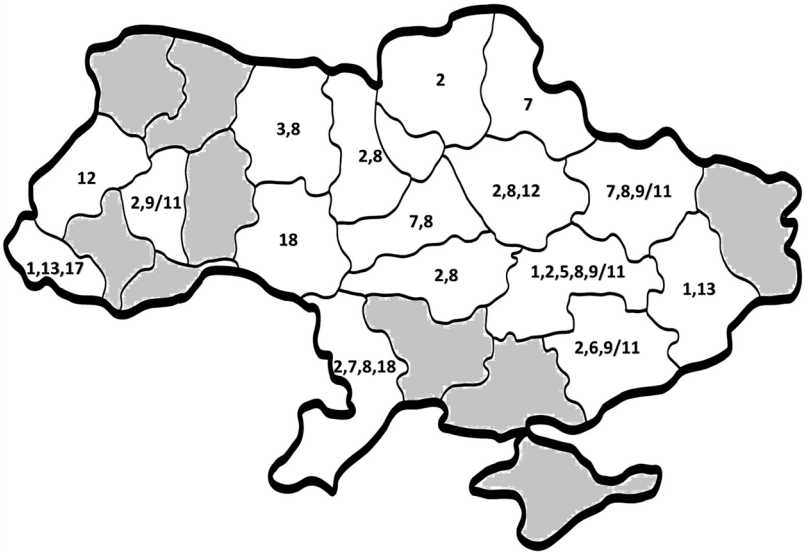

Fig. 1. The allocation of *A. pleuropneumoniae* strains in Ukraine provinces identified in 2022–2024. Numbers represent specific serotypes. Numbers within a region indicate *A. pleuropneumoniae* serotypes identified in herds located in that corresponding area.
Note: the regions where APP genotyping assay was not carried out are marked in grey.

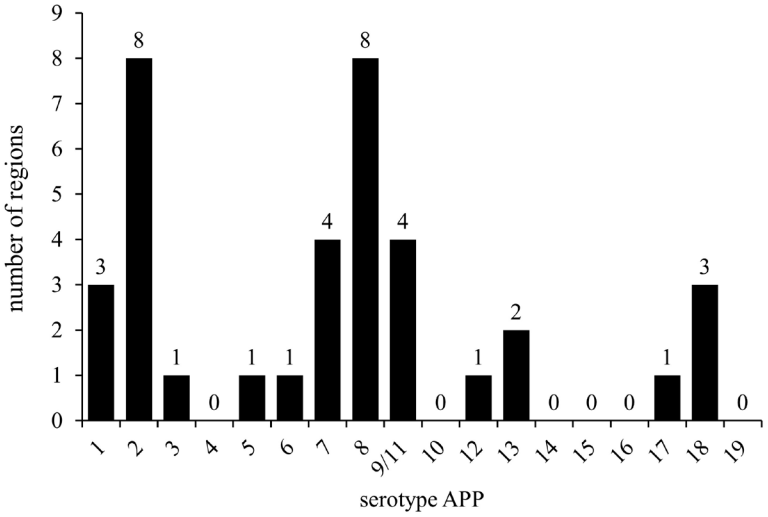

Fig. 2. The number of Ukraine regions in which several *A. pleuropneumoniae* serotypes were identified as present or absent.
Note: absence of a corresponding serotype is marked as "0".

pneumoniae serotypes was observed in Dnipropetrovsk and Odessa regions. Particularly, only in Dnipropetrovsk province, five serotypes were identified in colocation. The presence of four serotypes together was observed in Odessa province. Serotype 2 and 8 collocations were identified in these regions. However, in Dnipropetrovsk province, serotypes 1, 5, and 9/11 were observed, while serotypes 7 and 18 were detected in Odessa province (Fig. 3).

The coinfection cases of 2 or 3 different serotypes of *A. pleuropneumoniae* were observed in 63% of the investigated regions in Ukraine. The presence of only one serotype was identified in 25% of all provinces included in the study.

The results on *A. pleuropneumoniae* strains ratio demonstrated the prevailing spread of serotypes 2 and 8 in all provinces as well as among all identified strains. Together, these two serotypes represented 51% of the total APP-positive isolates identified in the study (Fig. 4).

Serotypes 9/11, 7, 1, 18, and 13 taken together made up 39% of all identified strains in the present study and were observed as less spread *A. pleuropneumoniae* strains. Other serotypes, including 3, 5, 6, 12, and 17, were characterized as a restricted spread group of *A. pleuropneumoniae* strains in Ukraine. The total of aforementioned strains did not exceed 10% of all identified cases.

The analysis of coinfection cases demonstrated that mono-infection was prevailing in comparison to the sum of all other coinfection variances. Cases involving the identification of a single APP serotype within a herd accounted for 79% of all observed cases (Fig. 5).

The results evidence that the predominant finding among identified *A. pleuropneumoniae* cases was the presence of a single serotype, with mono-infection cases accounting for 79% of all herds involved in the study. The presence of two or three serotypes was observed in 21% of all herds.

The coinfection number accompanied by serotypes 2 and 8 involved in disease initiation accounted for 43% of all identified coinfection cases. In common, obtained results evidence that 12 serotypes circulated in Ukraine during 2022–2024 while serotypes 2 and 8 were prevailing among spread isolates. Other known serotypes, including 4, 10, 14, 15, and 16, were not observed in pig farming in Ukraine.

Sensitivity and resistance of A. pleuropneumoniae serotypes to antibiotics

The study of sensitivity of *A. pleuropneumoniae* strains to various antibiotic groups demonstrated circulation of several serotypes which exhibited resistance to three or more groups of antibiotics. The number of aforementioned strains was equal to about 32% of the total serotype number detected in Ukraine.

The highest degree of sensitivity to antibiotics of both β-lactams and quinolones group were detected

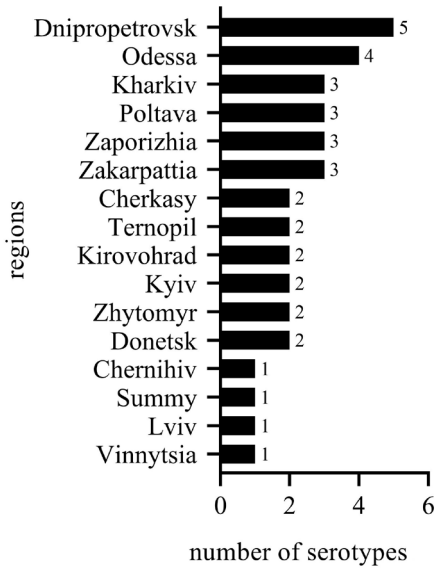

Fig. 3. Diversity rate of *A. pleuropneumoniae* serotypes in different regions of Ukraine
Note: the number of collocated serotypes is depicted on the right side of a corresponding column.

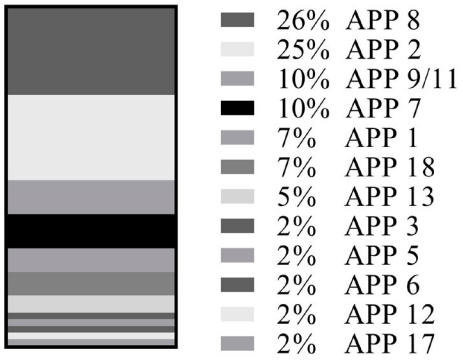

Fig. 4. Total ratio of detected *A. pleuropneumoniae* serotypes

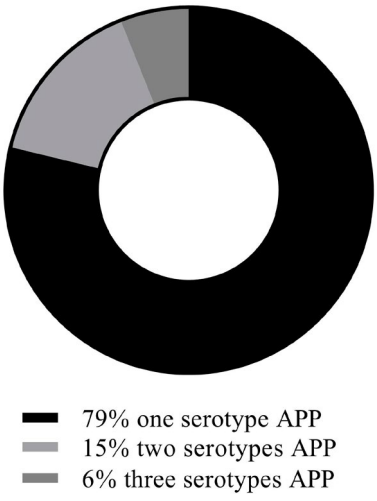

Fig. 5. The percentage of mono-infection and coinfections of *A. pleuropneumoniae* observed by the number of serotypes

in *A. pleuropneumoniae* strains with a similar level (Figs. 6–7). The total number of sensitive isolates to β-lactams was 88.3 ± 5.27%, and to quinolones – 83.9 ± 2.69%.

The results of microbiological culture study of isolated *A. pleuropneumoniae* serotypes showed a relatively high sensitivity and a complete absence of resistance to antibacterial drugs of β-lactams in the serotype 2 strains. Sensitivity of serotype 8 strains and other serotypes to β-lactams did not significantly differ, ranging within 86–87%. The number of *A. pleuropneumoniae* serotype 8 resistant strains and other serotypes was equal to 7.41 % and 11.11%, accordingly.

The analysis of sensitivity to quinolones showed insignificant differences between various *A. pleuropneumoniae* serotypes where the sensitivity level was within 83–89 %. However, among the *A. pleuropneumoniae* serotype 8 isolates, the frequency of resistant forms was twice as high as that observed in serotype 2 as well as other related serotypes.

The results on *A. pleuropneumoniae* strain sensitivity to aminoglycosides demonstrated significantly lower sensitivity levels in comparison with quinolone exposure (Fig. 8).

The maximal resistance level was observed for serotype 8 that accounted for about 30% of all isolated strains. Relatively low resistance levels were demonstrated by serotype 2 and other serotypes that exhibited resistance within the range of 5–14% (Fig. 8). In spite of the presence of resistant forms among serotype 2 strains in respect to aminoglycosides, the level of sensitivity to this group was equal to almost 90%. The obtained results showed that isolated *A. pleuropneumoniae* serotype 2 strains possessed the highest sensitivity to aminoglycosides in comparison with other serotypes. Serotype 8 as well as other serotypes demonstrated significantly lower levels of sensitivity ranging within 63–68%.

The most substantial differences in both sensitivity

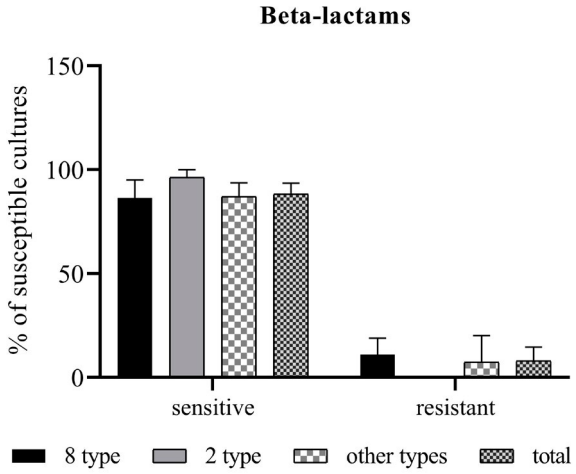

Fig. 6. Sensitivity and resistance of *A. pleuropneumoniae* strains to β-lactams

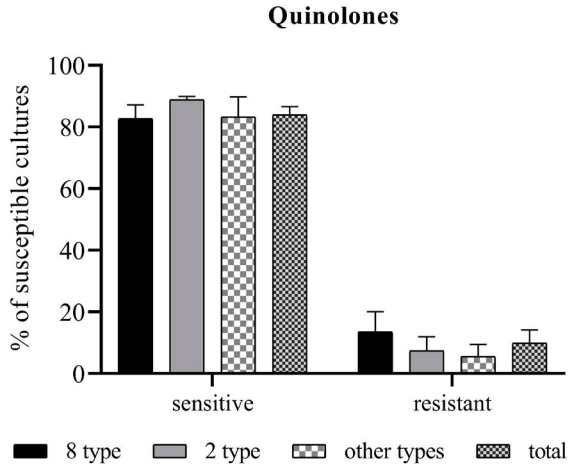

Fig. 7. Sensitivity and resistance of *A. pleuropneumoniae* strains to quinolones

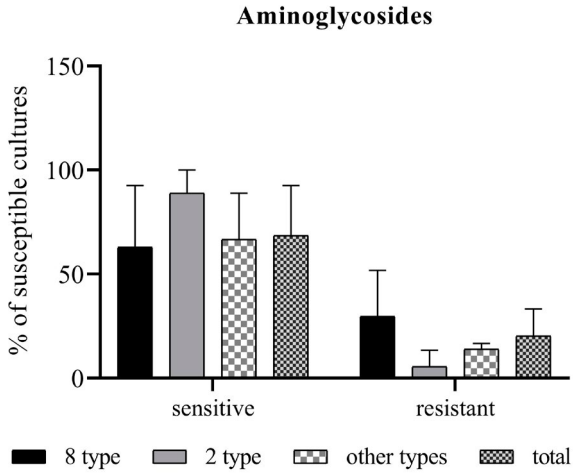

Fig. 8. Sensitivity and resistance of isolates of *A. pleuropneumoniae* to aminoglycosides

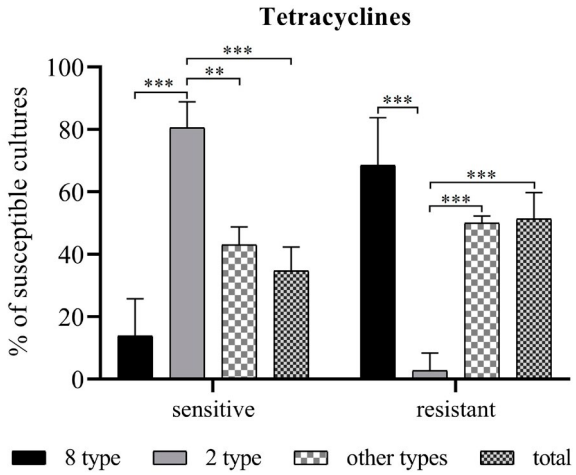

Fig. 9. Sensitivity and resistance of isolates of *A. pleuropneumoniae* to tetracyclines. Significance of differences in both sensitivity and resistance of serotype 2 in comparison with serotype 8, other, and total groups: ** – $P < 0.01$, *** – $P < 0.001$

and resistance were detected between isolated *A. pleuropneumoniae* serotypes 2 and 8 when exposed to the tetracyclines group (Fig. 9).

The observed data showed reciprocal differences in sensitivity and resistance to tetracyclines where serotype 2 exhibited the lowest sensitivity and maximal resistance. In contrast, serotype 8 demonstrated the lowest resistance potential and maximal sensitivity to tetracyclines treatment. The mean percentage of resistant serotype 8 strains to tetracyclines was 68.52%. However, the percentage of isolated sensitive strains did not exceed 14%, which approaches total values.

In spite of these findings, there were no observed significant differences between sensitivity and resistance in the groups of total serotypes and other serotypes. Isolated *A. pleuropneumoniae* strains grouped as the total cohort exhibited a slightly varied sensitivity of 34.7 ± 7.69%. The percentage of strains resistant to tetracyclines was observed above 50%. Thus, sensitivity and resistance in other strains, excluding serotypes 2 and 8, did not significantly differ from total values.

The percentage of *A. pleuropneumoniae* serotype 2 strains resistant to tetracyclines was extremely low and did not exceed 2.8%. This level was significantly lower than the same data of serotype 8 strains (68.5%, $P \leqslant 0.001$). On the other hand, above 80% of *A. pleuropneumoniae* serotype 2 strains were sensitive to tetracyclines, which is significantly higher in comparison with the same parameter of serotype 8 strains ($P \leqslant 0.001$).

A relatively low level of sensitivity was detected in all analyzed strains (2 and 8) and strain groups ("other" and "total") exposed to macrolides. The sensitivity of serotype 8 to macrolides was equal to 32%. Meanwhile, resistance of serotype 8 strains to macrolides accounted for 57% (Fig. 10).

Thus, the results evidence that all isolated strains and groups developed both sensitivity and resistance to macrolides without significant differences.

The comparative analysis of sensitivity among all *A. pleuropneumoniae* isolates showed that the highest level was observed in the treatment with 5 antibiotics including florfenicol, amoxicillin+clavulanic acid, ceftiofur, gentamicin, and marbofloxacin belonging to amphenicols, *β-lactams, aminoglycosides, and fluoroquinolones*. Besides, sensitivity levels of more than 89% were demonstrated by 27% of all isolates (Fig. 11).

From 50% to 80% of all *A. pleuropneumoniae* strains were sensitive to six antibiotics – ciprofloxacin, enrofloxacin, amoxicillin, tulathromycin, trimetho-

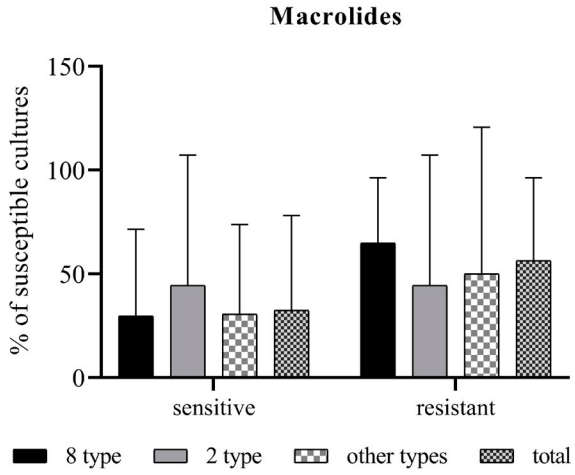

Fig. 10. Sensitivity and resistance of *A. pleuropneumoniae* isolates to macrolides

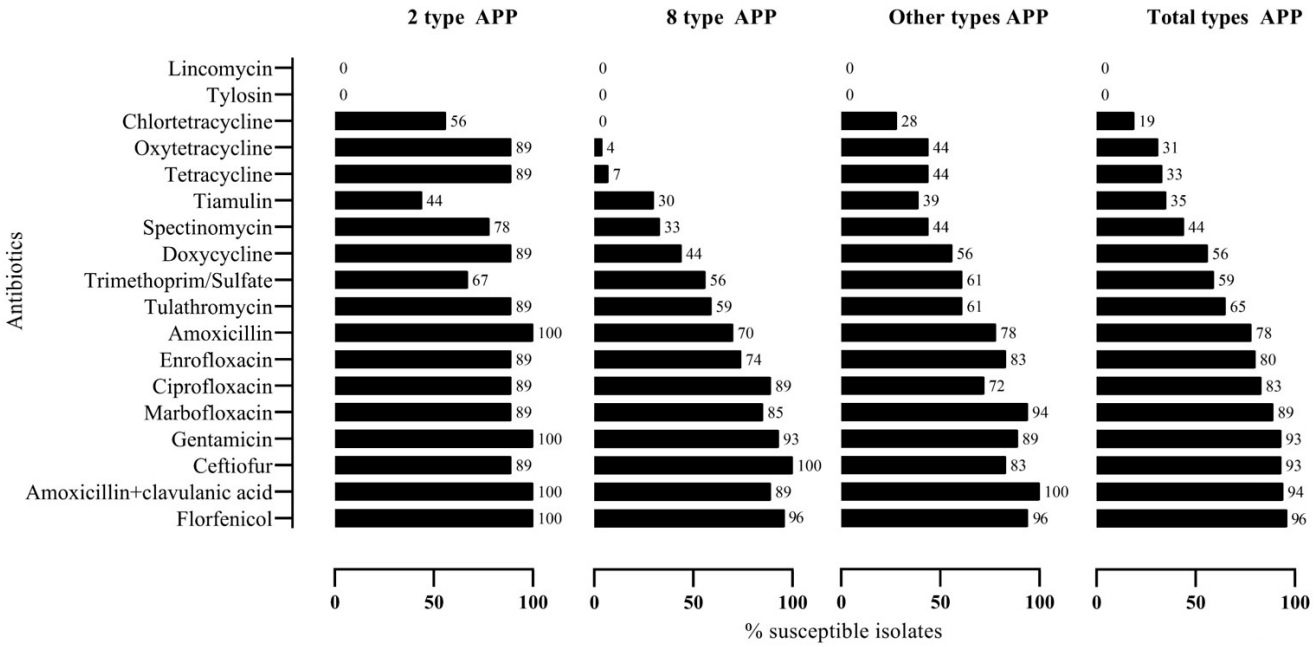

Fig. 11. The sensitivity of *A. pleuropneumoniae* isolates to the antibiotics panel

prim/sulfate and doxycycline belonging to macrolides, β-lactams, sulfonamides, and tetracyclines. The *A. pleuropneumoniae* strains sensitive to aminoglycosides, pleuromutilins, and tetracyclines were identified as having the lowest prevalence in Ukraine. However, the isolated strains of *A. pleuropneumoniae* did not demonstrate sensitivity to lincomycin and tylosin belonging to lincosamides and macrolides.

The obtained results showed that 89–100% isolates of serotype 2 were sensitive to 12 of 18 antibiotics. In contrast, isolates of serotype 2 were not sensitive to lincomycin and tylosin.

Among *A. pleuropneumoniae* serotype 8 strains, 89–100% isolates were sensitive only to 5 of 18 antibiotics. This level was 2.4 times lower than the same values for serotype 2 strains. At the same time, no isolates of serotype 8 demonstrated sensitivity to chlortetracycline. Only 4–7% of serotype 8 strains were sensitive to tetracyclines.

The group of other serotypes of *A. pleuropneumoniae,* except 8 and 2, demonstrated the level of sensitivity to antibiotics similar to overall indicators and did not differ significantly from them. The results on *A. pleuropneumoniae* resistance levels proved the presence of isolates resistant to the effect of almost all applied antibacterial drugs (Fig. 12).

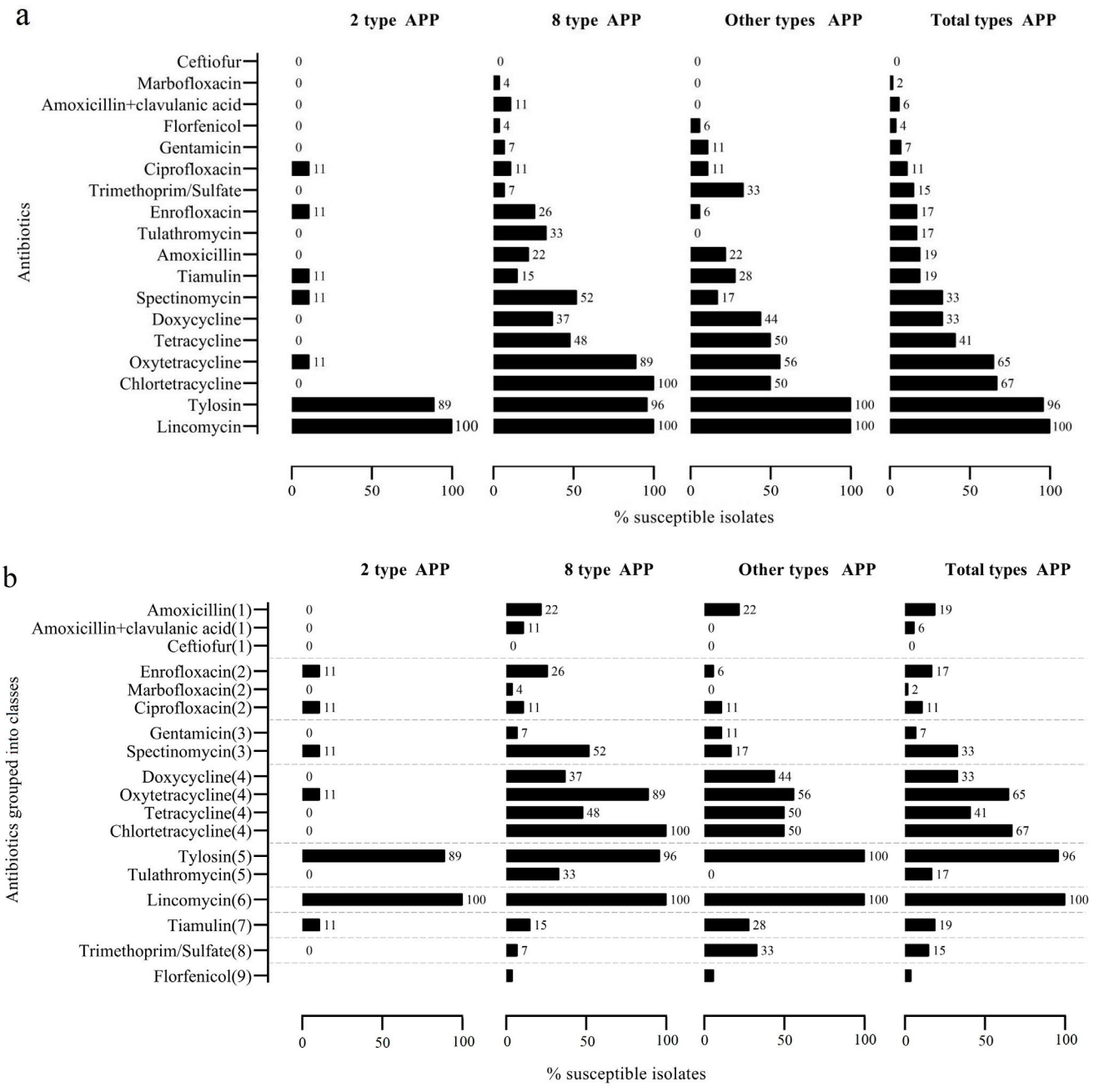

Fig. 12. Resistance of *A. pleuropneumoniae* isolates to various antibiotics grouped with respect to susceptibility increase (a) and grouped into classes (b). The classes are marked with specific numbers: 1 – β-lactams; 2 – fluoroquinolones; 3 – aminoglycosides; 4 – tetracyclines; 5 - macrolides; 6 – lincosamides; 7 – pleuromutilins; 8 – sulfonamides; 9 – amphenicols

Isolated *A. pleuropneumoniae* serotype 8 strains demonstrated various resistance levels to 17 of 18 antibiotics. In contrast, isolated serotype 2 strains exhibited resistance only to 7 antibiotics. The group of other serotypes developed resistance to 14 antibiotics. Besides, the whole group of isolated strains was absolutely resistant to lincosamides (lincomycin) and almost 96% of strains were resistant to macrolides (tylosin). Varying resistance rates were detected in all serotype groups with respect to macrolides while resistance was high to tylosin and low to tulathromycin. Similar differences were observed with respect to aminoglycosides where resistance was low to gentamycin and significantly higher to spectinomycin. These findings evidence that resistance monitoring should be constructed with both comparative analysis for individual antibiotics and antibiotic classes (Fig. 12).

Almost all isolated serotype 2 strains exhibited no resistance to tetracyclines. Meanwhile, about 50% of isolated serotype 8 strains, similarly to other grouped serotypes, were resistant to this antibiotic class.

As a special feature of all isolated strains, complete absence of resistance to cephalosporines was observed. However, moderate sensitivity to cephalosporines was observed in several strains of various serotypes excluding type 8. Besides, all *A. pleuropneumoniae* isolates exhibited no resistance to fluoroquinolones and β-lactams, except definite isolates of serotype 8.

In general, the results demonstrated unique differentiation with respect to *A. pleuropneumoniae* serotype distribution in the provinces of Ukraine. In addition, obtained data showed high diversity of both sensitivity and resistance of isolated *A. pleuropneumoniae* strains to various antibiotic classes.

Discussion

APP is a globally widespread disease of farming swine, which is caused by different strains of *A. pleuropneumoniae*. Due to its variability, this microorganism has a large number of serotypes. It was previously thought that only 15 serotypes of *A. pleuropneumoniae* exist (Soto Perezchica et al., 2023). However, over time, in the world the serotypes which were not subjected to typing started to be detected. In recent years, detailed molecular and genetic studies have confirmed the presence of serotypes 16, 17, 18 and 19 in several regions including North America and West Europe (Bossé et al., 2018; Stringer et al., 2021). At the same time, the isolates different from already known 19 serovars continue to be registered, which indicates the evolution of these bacteria (Angen et al., 2025). Thus, today 19 antigen different *A. pleuropneumoniae* serotypes are known.

Existing published data concerning epidemiology of APP considering serovars of bacteria are limited and fragmented. In Taiwan, serovars 15, 5, 1, 7 and 2 were found present (Kwan et al., 2025). In Poland, a serological study was carried out where *A. pleuropneumoniae* was identified by serovar 2 and separate serogroups (3, 6, 8 and 1, 9, 11); based on these results, regional epidemiological variations were established (Paulina and Dawid, 2025). The reports on epidemiology of individual *A. pleuropneumoniae* serotypes as well as coinfection variances on swine farms in Ukraine are practically absent. Hence, the results on APP serotype diversity and coinfection variances in Ukraine are presented for the first time.

The results of the present analysis showed a region-dependent and relatively wide spread of *A. pleuropneumoniae* among productive swine herds in Ukraine. In swine with *A. pleuropneumoniae* induced pneumonia, only one serovar of *A. pleuropneumoniae* was detected in the lung tissue in three of four cases, while in every fourth case, the presence of two or three different serovars of *A. pleuropneumoniae* was identified.

The obtained results showed that in 16 regions of Ukraine at least 12 different serovars of *A. pleuropneumoniae* circulate, among which serovars 2 and 8 are the most spread. Serovars 1, 7, 9/11, and 18 are moderately spread. Besides, serovars 17 and 18, which were reported in the last decades, were identified in the present study. The results are in concordance with data on *A. pleuropneumoniae* serovar ratio in England reported earlier where serotype 8 was prevailing (Li et al., 2016). The results could be linked to the translocation of the aforementioned serovars to Ukraine via fattening pigs to improve swine breed genetics. A similar mechanism appears to be the most likely causative factor, based on the potential of *A. pleuropneumoniae* to colonize swine tonsils and ensure prolonged persistence of the infectious agent (Soto Perezchica et al., 2023). Thus, our results concerning this fact deserve special attention similarly to previous reports on the serovar 8 sequence (Bossé et al., 2018).

The genotypes including 4, 10, 14, 15, and 16 were not detected in any samples. It can be due to their absence or critically low presence on swine farms in Ukraine.

Studying the sensitivity of bacteria to antibacterial drugs in the world is an important task to maintain the health of both humans and animals (Ho et al., 2025). Assessing *A. pleuropneumoniae* sensitivity to antibiotics is no exception since this microorganism exhibits the potential to generate resistant forms like many others (Guo et al., 2021). Due to mutations, *A. pleuropneumoniae* strains can become resistant to various antibiotics (Wang et al., 2010). Recently, a detailed mechanism of gene translocation between bacteria *A. pleuropneumoniae* and members of *Enterobacteriaceae* family has been reported (Xu et al., 2024). These findings can clarify the significant impact of resistant forms of *A. pleuropneumoniae* on farming swine health.

Analysis of the data obtained regarding the wide range of sensitivity and resistance of *A. pleuropneumoniae* isolates showed the presence of

isolates resistant to three or more classes of antibiotics. These findings confirm *A. pleuropneumoniae* potential to generate multi-resistant strains. Furthermore, the detection of strain resistance and dissemination can be a suitable method to predict the risks of APP incidence caused by resistant strains via coinfection.

Recent reports are devoted to the study of resistance as a rule. In spite of this, several research studies were additionally focused on the phenotypic and genotypic differences of *A. pleuropneumoniae* strains in a course of antibiotic resistance assessing. Particularly, Guarneri and coauthors (2024) have described that serovar 9/11 exhibited closely related resistance to florfenicol and enrofloxacin, while serovar 5 developed high resistance to trimethoprim/sulfamethoxazole. Besides, *A. pleuropneumoniae* strains isolated in Italy exhibited the most common resistance to tetracyclines and β-lactams (Guarneri et al., 2024). In spite of these findings, our results showed high resistance to β-lactams and relatively low resistance to tetracyclines (Fig. 12b). Furthermore, our study results demonstrated that all isolates of *A. pleuropneumoniae* were resistant to lincomycin and almost 96% of isolates were resistant to tylosin. Thus, our findings evidenced that at least part of isolates exhibited multi-resistance signs. Furthermore, complete absence or critically low sensitivity to beta-lactams, fluoroquinolones, and aminoglycosides evidenced that all identified serotypes exhibited resistance minimum to these antibiotic classes. Besides, serovar 2 strains developed resistance to the aforementioned classes as well as to tetracyclines that could reflect the shift to multi-resistant changes.

Special attention is drawn to the results on reciprocal differences in resistance to tetracyclines exhibited by serovars 2 and 8. Similar differences may be due to the variable genetics of these serovars. On the one hand, serotype 8 causes significant clinical signs and pushes veterinarians to apply antibiotics more often. On the other hand, recent results on complete genome sequence of *A. pleuropneumoniae* serotype 8 have shown low genetic variation in the strains isolated in various countries as well as in respect with reference strain 405 (Bossé et al., 2021). The data obtained in our study are in accordance with recent reports and evidence that the conservative features of serotype 8 strains could be the cause of prevalence of this serotype in Ukraine.

Recently, *A. pleuropneumoniae* serotype 12 field strains have been described in Chile. Atypical field strains possessed both capsule genes of serotype 12 and LPS genes belonging to serotype 15 (Vincent et al., 2025). These strains possess an unusual composition of toxin genes, carry capsule genes belonging to serotype 12 but produce LPS with genes belonging to serotype 15. Furthermore, these strains showed close phylogenetic relationships to atypical strains detected in Canada, Japan, and USA. These results confirm the high risk of new strain generation including multi-resistant forms of *A. pleuropneumoniae*. The complex character of antibiotic resistance observed in Ukraine across various serotypes could be a driving factor to push genetic diversity of *A. pleuropneumoniae* in several provinces affected by APP. Serotype 12 spread in Ukraine was assessed as minimal among all identified serotypes. In view of high variability of serotype 12 strains, low detectable numbers of this serovar could be dependent on the restricted ability of commercial kits to detect atypical serotypes.

The analysis of colocation of 12 serotypes identified in Ukraine demonstrates a significant diversity of strains circulating on pig farms. The diversity of colocations could be dependent on the geographic location as well as the unique climate features in every province of Ukraine.

Taking into the account recently published reports, our study is the first to demonstrate biodiversity of *A. pleuropneumoniae* serotypes and their individual features in respect to both sensitivity and resistance against most applicable antibiotics in swine farming. Furthermore, the observed results can expand the current ideas on the APP spread, epidemiological features, and circulation on swine farms located in separate regions of Ukraine as well as in the pig industry globally.

Conclusions

The results show that APP coinfection cases exhibited an individual serotype profile in every province. Besides, maximal diversity of *A. pleuropneumoniae* serotypes were found in two regions only, which could be related to a specific geographic location and climate of these areas. Among all, serotypes 8 and 2 were most prevalent and serotypes 17 and 18 were identified as relatively new for Ukraine.

The results on high efficiency of ceftiofur, marbofloxacin, and amoxicillin+clavulanic acid use against *A. pleuropneumoniae* suggest that several cephalosporines, fluoroquinolones, and β-lactams can be prospective chemicals to restrict the current APP spread. In contrast, tetracyclines exhibited clear inverse differences in resistance/sensitivity ratio with respect to serotypes 2 and 8. Despite this, a ratio for "other" and "all" grouped serotypes was almost equal suggesting intermediate resistance to tetracyclines. Additionally, high resistance was observed for tylosin and lincomycin. The present study demonstrated the risk of *A. pleuropneumoniae* multi-resistant strain generation to macrolides, lincosamides, and tetracyclines in Ukraine recent years. Further research is needed to investigate details of antibiotic resistance of all identified strains combined with sequence analysis.

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