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Aims and Scope

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In Vitro Anticoccidial Effects of Olive Leaf (*Olea Europaea* L. var. Chemlal) Extract against Broiler Chickens *Eimeria* Oocysts

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Keywords: *Olea europea L., var. Chemlal; olive leaves; anticoccidial activity; chickens; in vitro.*

Abstract. *The aim of the present study was to evaluate in vitro the anticoccidial activity of olive leaves (OL) on the destruction of Eimeria oocysts isolated from naturally infected chickens. The identification of phenolic compounds was obtained by ultra-high-performance liquid chromatography-mass spectrometry with electrospray ionization. The treatment of Eimeria oocyst with the OL extract and standard compounds (quercetin and oleuropein) leads to their lysis as shown by the release of substances absorbing at 273 nm. The results indicated that the optimum OL extract and the standard compound showed that the number of oocysts decreased after treatment. The OL extract at a concentration of 1.46 mg/mL recorded a decrease rate of 5.89% of Eimeria oocysts after 7-h treatment. On the other hand, quercetin was most effective (48.57%) followed by oleuropein (27.53%) after 7-h treatment. The ethanol treated Eimeria suspension was significantly ($P \leqslant 0.05$) higher than the tested concentration of the OL extract and the standard compound. The findings of the present study showed that it could be concluded that the OL extract possesses the ability to destroy Eimeria spp. In future, in vivo investigations are required to assess the efficiency of the OL bioactive compounds in broiler chickens.*

Introduction

Coccidiosis is defined as an enteric parasitic pathology of birds between the ages of 3 and 18 weeks caused by the protozoa *Eimeria* spp. belonging to the genus *Eimeria* (Metwaly et al., 2012). It is one of the most common and important diseases that has a negative impact on the growth of poultry industry, resulting in about $1.5 billion losses in poultry industry annually worldwide (Arabkhazaeli et al., 2014). This infection causes reduced feed efficiency, body weight gain, and temporary reduction in egg production.

Anticoccidial drugs in feed and water of chickens have a considerable success. The major problem observed of the commonly available anticoccidial drugs is development of resistance in *Eimeria* species, huge residual effects of anticoccidial drugs in meat and toxic effects of disinfectants. There is an intense need to establish new successful treatments to control coccidiosis in poultry farms and to search for alternative environmentally friendly anti-*Eimeria* agents. Surveys have been published that some botanicals have been reported for their promising results as anticoccidials and improving poultry performance worldwide in broiler chickens (Abbaset al., 2013).

In Algeria, *Olea europea* L. (var. Chemlal) is the most dominant olive variety, representing nearly 45% of the national oil production. Olive tree pruning produces a huge quantity of olive leaves (~25 kg/tree). It has been estimated that approximately 100 g of leaves are present for every kilogram of olives used in oil extraction. It has been shown that dried leaves of the leaf olive (*Olea europea* L.) plant have been used in traditional Algerian medicine for thousands of years.

Many deep studies have shown that the beneficial properties of olive leaves are due to valuable biphenolic compounds. Likewise, the use of whole olive leaves and olive leaf extracts has increased considerably in the pharmaceutical and food industries as food additives. Moreover, they are used for antioxidant (Bulotta et al., 2011), antimicrobial (Pereira et al., 2007), lipid-low (Lee and Lee, 2010), and anticoccidial activity (De Pablo et al., 2010). In addition, other investigations have demonstrated the potential role of antiproliferative and apoptotic effects of olive leaves (Han et al., 2009). However, to the author's knowledge, the anticoccidial activity of olive leaves (*Olea europea* L., var. Chemlal) has never been reported. The aim of the present study was to evaluate in vitro the anticoccidial activity of olive leaves (OL) growing in Algeria (area of Bejaia) on the destruction of *Eimeria* oocysts isolated from naturally infected chickens.

Materials and methods

Ethics committee approval

Ethics committee approval was received for this study from the scientific committee of Faculty of Nature and Life Sciences, University of Bejaia, Algeria.

Plant materials

The leaves of olive (*Olea europea* L. var.Chemlal) were collected from Soummam Velley, Bejaia province (Algeria), during March and June 2018. Samples were dried in the shade at room temperature until

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constant weight was obtained, and then crushed using a traditional grinder. The resulting powder was passed through sieves of a standard 125 µm. Only the fraction with a particle size of ⩽ 125 µm was collected, stored at +4°C in amber bottles and sterilized until used.

Extraction and optimization of total phenolic compounds (TPC) by microwave assisted extraction (MAE)

A domestic microwave oven (2450 MHz, Samsung model NN-S674MF, Kuala Lumpur, Malaysia) was modified in order to extract phenolic compounds from the OL powder. Olive pulp powder (1 g) was stirred manually in aqueous ethanol in preparation for extraction using the MAE system. The MAE parameters were microwave power (700–900 W), extraction time (45–75 s), liquid-solid ratio (20–40 mL/g) and ethanol proportion (20–60%). After that, the extract was centrifuged at 10,000 rpm and filtered through a Buchner funnel lined with Whatman No 3-filter paper, and the supernatant was collected in a volumetric flask. The extract was stored at +4°C until used.

High-performance liquid chromatography-mass spectrometry (HPLC–ESI-MS) analysis

The identification of phenolic compounds of the OL extract was obtained by ultra-high-performance liquid chromatography–mass spectrometry with electrospray ionization (UPLC–ESI-MS) and quadrupole-time of flight detector (QTOF). The equipment was Xevo G2 mass spectrometer consisting of an hexapole, a collision cell and time of flight analyser (QTOF) supplied by Waters (Milford, MA, USA). The electrospray probe was used in the positive (ESI^+) and negative (ESI^-) modes as well as the sensitivity analyzer mode. The mass range considered was from 10 to 1,000 Da. The corona voltage was 2.5 kV for (ESI^+) and 0.5 kV for (ESI^-). The sampling cone voltage was optimized between 20 and 50 V. Finally, 30 V was selected for the screening because more peaks were detected. Other MS parameters were as follows: the source temperature was 150°C, the desolvation gas temperature was 450°C and the desolvation gas flow was 650 Lh^{-1}. The MSE mode was selected for the acquisition, and collision ramp energy from 5 to 40 V was used. MassLynx v.4.1 software (Waters, Milford MA, USA) was used to analyze the samples, and CromaLynx (Waters, Milford MA, USA) was used to deconvolve the spectra.

Evaluation of anticoccidial activity

Eimeria oocysts isolation and purification

An oocysts sample of *Eimeria* spp. was isolated from fresh feces of broilers suffering from coccidiosis in Bejaia area (Algeria). Oocysts were sporulated by placing in 2.5% $K_2Cr_2O_7$solution in the presence of suitable humidity (> 70%) and temperature (28°C). Sporulated oocysts were cleaned and counted using Malassez chamber. The mean number of oocysts per milliliter of the sample was calculated. The identification of *Eimeria* species in chickens was made on the basis of some criteria such as size, shape, presence or absence of micropyle, time of sporulation, intestinal location and appearance and coarse characteristics of intestinal lesions (Carvalho et al., 2011). The percentage of each species in the mixed suspension was approximately 32% *E. acervulina*, 27% *E. tenella*, 15% *E. mitis*, 14% *E. brunetti* and 12% *E. maxima*.

The purification of oocysts was carried out from 1 L phosphate buffered saline (PBS, containing 8 g/L NaCl, 0.2 g/L KCl, 1.13 g/L Na_2HPO_4, $2H_2O$ and 0.2 g/L KH_2PO_4). Neutral substrates containing an inhibitor (streptomycin 1 mg/mL and penicillin V 100 IU) were added to prevent any bacterial evolution, and fluconazole, 17 mg/mL, was used as antifungal. The pH was adjusted to 7.4 and the solution was sterilized by membrane filtration through a 0.2 µm filter. The HBSS (Hanks balanced salt solution) medium was carried out in the laboratory (NaCI, 8.0, KCl 0,4; $CaCl_2$, 0,139; D-glucose, 1,0; Na_2HPO_4, 0,0478; KH_2PO_4, 0, 06 and $MgSO_4$, 0,097 g/L in 1000 mL of distilled water). The solution was sterilized as well as that of the 0.2% agar.

Effects of the leaf extract and standard compounds on the decrease of oocysts number

The activity of the OL extract and standard compounds (quercetin and oleuropein) (Fisher scientific, Fair Lawn, NJ, USA) was determined at a concentration of 18.8 mg/mL in triplicate by incubation at ambient temperature (25ºC) for 24 h (Debbou-Iouknane et al., 2019). The suspension was incubated at different periods of time: 0, 1, 3, 5, 7, and 24 h. One milliliter of the suspension contained 100 µL of washed suspension of *Eimeria* oocysts at $4{,}006\times10^6$ oocysts/mL, 700 µL of PBS, and 200 µL of the optimum OL extract.

After incubation, the samples were centrifuged at 320 g for 5 min and the absorbance of the supernatant was measured at 273 nm by spectrophotometer (Shimadzu, model: UV 100 Japan). Then, the percentage of destruction sporulated oocysts was recorded. The LC_{50} value was then inferred from the regression curve. The number of destructions of sporulated oocysts was estimated three times in a cell volume of 1 µL amounts to 4.006×10^4. The ethanol solvent was also used as a negative control.

Effect of the diclazuril sodium on the decrease of the oocysts number

Diclazuril (Algicox10 mg/mL), molecular formula $C_{17}H_8C_{l3}N_4NaO_2$ and anticoccidiosis (Diclosol®, Avico, Arab Industry Veterinary Co, Amman, Jordanie) were

tested in triplicate (0.1, 0.3, 0.5, 0.7 and 1 mg/mL) using the microplate method. The number of oocysts was counted twice in a cell volume of 1 µL amounts to 3.98×10^4oocysts/mL.

Statistical analysis

A statistical analysis was performed by using JMP® Software, version 7.0 (SAS Institute Inc, 2007). The results were expressed in means ± SE. The values were statistically significant when the *P* value was ⩽ 0.05. Inoculums suspension taken at 0, 1, 3, 5, 7 and 24 h on oocysts number was examined by the Student *t* test. The lethal concentration is defined as the concentration that reduces the initial number of sporulated oocysts to 50%.

Results

Fig. 1 represents the chromatogram of the phenolic profile of the OL extract by ultra-high-performance liquid chromatography-mass spectro metry with electrospray ionization (HPLC-ESI-MS). The molecular analysis of molecules revealed the presence of several biophenol classes (Table 1) *viz*. flavonols (luteoline-7-O-glucoside, quercetin, quercetin-3-O-rutinoside, quercetino-O-O-(O-galloyl)-hexoside, phenolic acids (chlorogenic acid), secoiridoids (oleuropein, phenolic alcohol (tyrosol/p-hydroxyphenyl ethanol/p-HPEA), isohamnetinneobavaisoflavone, 2, 3-dihydro-amentoflavone, quercetin-3-O-rutinoside, chlorogenic acid, isorhamnetin 3-O-(6”-O-feruloyl)-glucoside) and diligustilide.

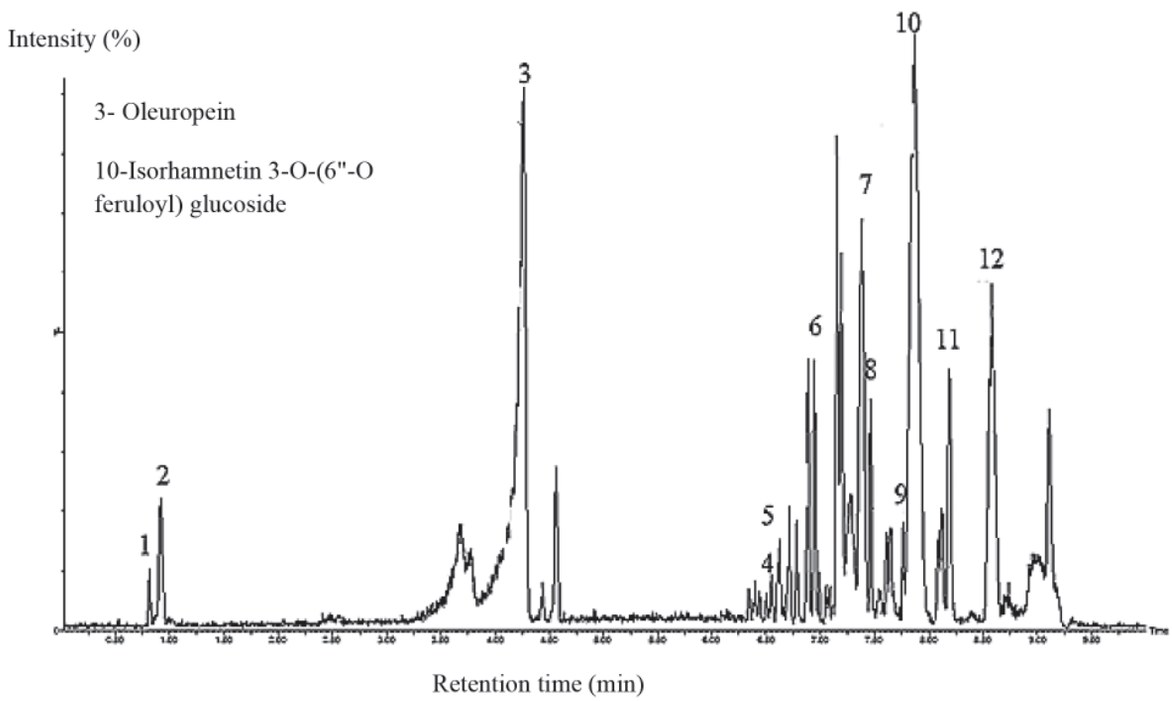

Fig. 1. HPLC–ESI-MS chromatogram ofthe phenolic profile of olive leaves

Table 1. Prediction profilephenolic for the optimal conditions obtained by CCD model

	TR	M-H	µg/g	Name	Formula
1	0.89	447	400,120	Cynarosid/luteolin-7-O-glucoside	$C_{21}H_{20}O_{11}$
2	0.92	137	8261,999	Tyrosol	$C_8H_{10}O_2$
3	4.26	539	75477,543	Oleuropein	$C_{25}H_{32}O_{13}$
4	6.55	301	2023,187	Quercetin	$C_{15}H_{10}O_7$
5	6.63	315	4155,147	Isohamnetin	$C_{16}H_{12}O_7$
6	6.89	321	11056,997	Neobavaisoflavone	$C_{20}H_{18}O_4$
7	7.38	539	36946,034	2, 3-dihydro-amentoflavone	$C_{30}H_{20}O_{10}$
8	7.46	609	7692,308	Quercetin-3-O-rutinoside	$C_{27}H_{30}O_{16}$
9	7.76	706	3140,942	Chlorogenicacid	$C_{16}H_{18}O_9$
10	7.87	688	90257,447	Isorhamnetin 3-O-(6”-O-feruloyl-glucoside)	$C_{32}H_{30}O_{15}$
11	8.19	379	13853,776	Diligustilide	$C_{24}H_{28}O_4$
12	8.58	615	32845,634	Quercetin-o-(o-galloyl)-hexoside	$C_{28}H_{24}O_{16}$

The results indicated that the optimum OL extract and the standard compound, tested at respective concentrations of 44.001 and 18.771 mg/g, showed that the number of oocysts decreased after treatment. The OL extract at a concentration of 1.46 mg/mL recorded a decrease rate of 5.87% of *Eimeria* oocysts after 7-h treatment. On the other hand, quercetin was most effective (56.5%), followed by oleuropein (35.1%) after 7-h treatment. The lethal concentration LC_{50} of the OL extract, quercetin and oleuropein was recorded to be the concentration of 194.92, 14.88 and 173.93 mg/mL, respectively (Fig. 2A, B and C, respectively). Anticoccidial effects of diclazuril were shown as a diminution of the oocysts number

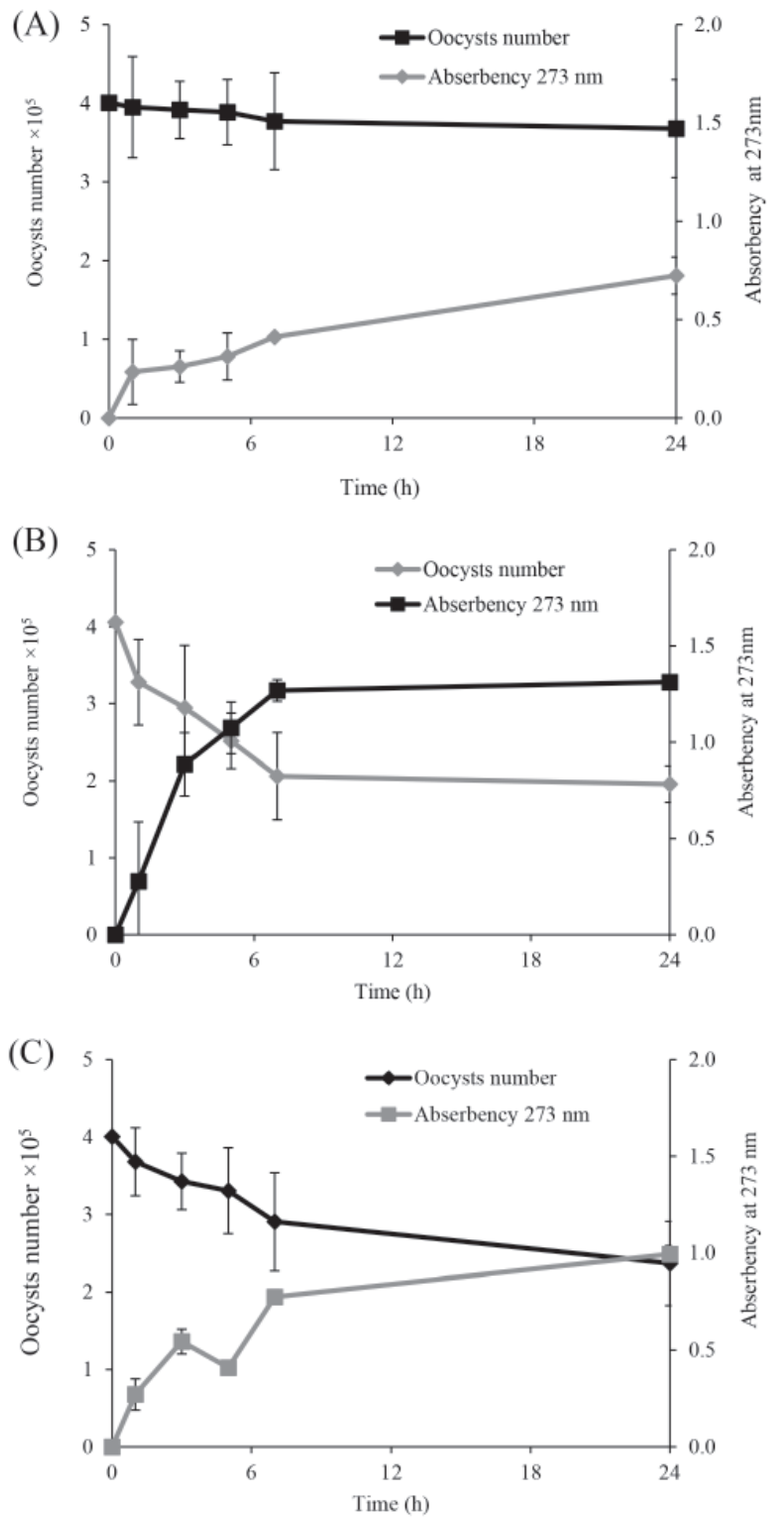

Fig. 2. Kinetics of the decrease of the oocysts number and 273 nm wave length absorbing material release from *Eimeria* oocysts treated by optimum olive leaf extract (*Olea europaea* L., var. Chemlal) (A), quercetin (B), and oleuropein (C)

of 44.3% at 0.5 mg/mL concentration (Fig. 3). The ethanol treated *Eimeria* suspension (negative control) was significantly ($P \leqslant 0.05$) higher than the tested concentration of the OL extract and the standard compound.

According to our results, the OL extract, quercetin and oleuropein were recorded to reduce the oocysts number after 7 h for different periods of time (5.89, 48.57 and 27.53%, respectively). This decrease in the number of oocysts causes a considerable release of 273 nm of absorbing material from *Eimeria* oocysts that is depending concentration of the OL extract, quercetin and oleuropein. Fig. 4 (A, B) illustrates a considerable decrease in the number of *Eimeria* oocysts with an increase in the concentration of quercetin and oleuropein ranging from 0.1 to 1 mg/mL.

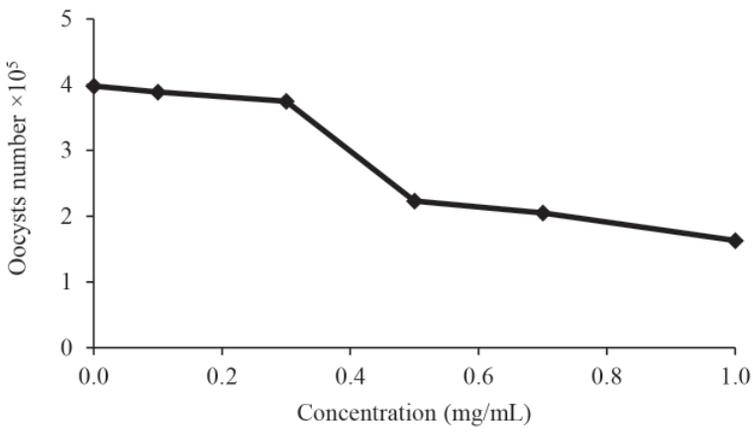

Fig. 3. Effect of diclazuril concentrations on the oocysts number

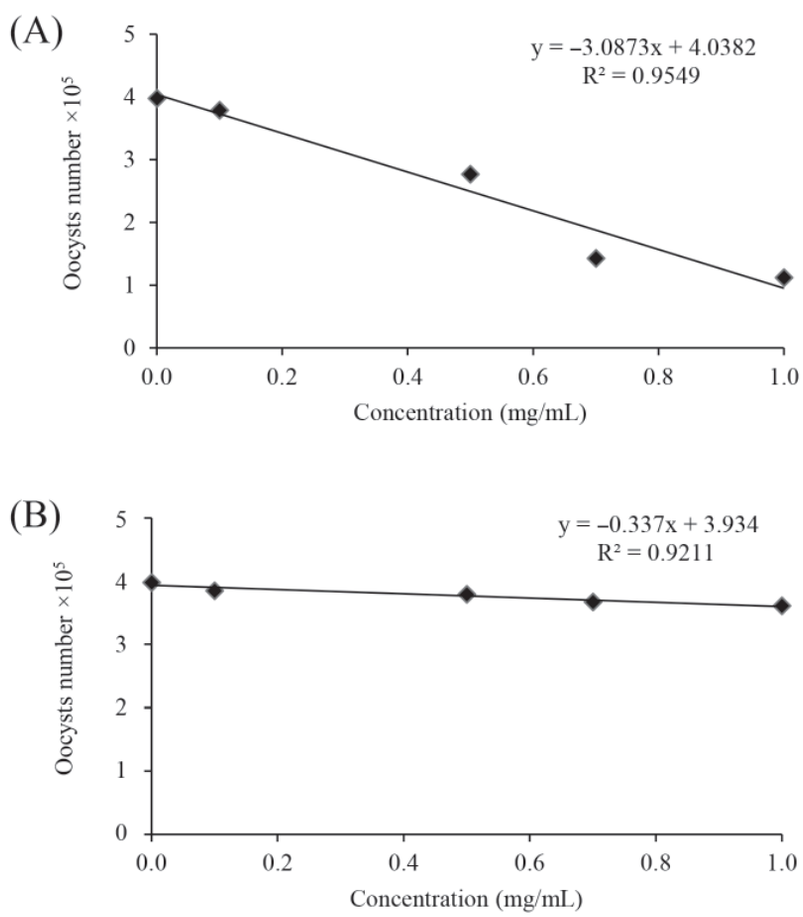

Fig. 4. The correlation between quercetin (A) and oleuropein (B) concentrations and the number of *Eimeria* oocysts

Discussion

Due to the emergence of resistance and drug residues, the use of plant extracts with biological activity has become particularly important to restrict the use of synthetic compounds against coccidial infections (Abbas et al., 2012). Therefore, the use of co-products as an anticoccidial remedy is promising as an alternative for controlling coccidiosis in poultry. Recently, one study has demonstrated the effect of phytochemicals of plant extracts on suppression of *Eimeria* species (Muthamilselvan et al., 2016). The present study work was carried out in order to find a phytobiotic alternative based on an olive co-product to help minimize gastrointestinal disorders caused by *Eimeria* coccidial infection in broiler chickens. To the author's knowledge, this present study is the first to assess in vitro anticoccidial activity of the OL (*Olea europaea* L., var. Chemlal) extract and its phenolic compounds directly on the viability of *Eimeria* oocysts collected in broiler chickens.

It is known that the leaf is the main site of plant metabolism and can be considered as a potential source of bioactive compounds (Tsimidou and Papoti, 2010). Based on an oocyst wall alteration of *Eimeria*, this study showed that the OL extract (*Olea europaea*) had a destructive effect of sporulated oocysts. This anti-*Eimeria* activity may be attributed to bioactive molecules of OL extracts individually or a synergistic interaction of bioactive compounds. It would be more effective to control or treat coccidiosis by the use of a multiple compound, which requires a combination of immunostimulators that induce a good response of essential oils and other natural compounds from medicinal plant extracts that can destroy the Eimeria oocyst or interfere with the life cycle (Quiroz-Castañeda, 2018).

In the present study, the characterization by HPLC of the OL extract shows the presence of large concentrations of polyphenolic or biophenols (cynarosid/luteolin-7-O-glucoside; tyrosololeuropein quercetinisohamnetin; neobavaisoflavone 2, 3-dihydro-amentoflavone quercetin-3-O-rutinoside-chlorogenic acid, isorhamnetin 3-O-(6"-O-feruloyl)-glucoside), diligustilide quercetin-o-(o-galloyl)-hexoside) that can be used as natural anticoccidial products. Previous investigations have studied that the polyphenol content of olive leaves is richer in bioactive phenolic compounds than fruits and olive oil (Lalas et al., 2011). In addition, one other study has demonstrated the efficacy of plants and plant extracts against mixed or *Eimeria* individual infections (Udo and Abba, 2018). The results of the present study correspond also with those published previously, which showed that maslinic acid (2-α, 3-β-dihydroxiolean-12-en-28-oic acid), an active compound in the leaves and fruits of the olive tree (*Olea europaea*), has an anticoccidial effect against *E. tenella* oocysts and also increases the weight in chicks treated (De Pablos et al., 2010). In addition, the active compound of *Olea europea* has been shown to affect *Toxoplasma gondii* parasites, which may be by inhibiting the serine proteases of the protozoan protein, that is a mechanism necessary for the entry of tachyzoites into the cytoplasm of the host cell (Aladedunye et al., 2008). Therefore, this activity, as well as anti-inflammatory (Aladedunye et al., 2008) and antioxidant properties (Montilla et al., 2003), may be responsible for the anticoccidial properties. Moreover, it has been shown that the use of natural antioxidants such as polyphenols of olive by-products is safer than synthetic polyphenols in chicken diets in order to reduce lipid oxidation (Starćevic´ et al., 2015). Also, Varmaghany et al. (2013) have shown that an olive leaf supplementation diet (oleuropein content, 72.63 mg/g) has an anti-hypertensive effect and reduces the incidence of ascites without affecting the performance of broilers.

Khalafalla and Daugschies (2011) have demonstrated that curcumin, natural polyphenolic component derived from *Curcuma longa*, inhibited the cell invasions of *E. tenella* sporozoites in vitro and in vivo. Thus, anticoccidial activity of *M. oleifera*, including anti-inflammatory and antioxidant properties, was attributed to the biological constituents, including ascorbic acid, flavonoids, phenolics and carotenoid (Abdel-Latif et al., 2017). In other contexts, antimicrobial effects of phenolic compounds are targeted against the bacterial cell wall affecting the cell wall structure (Botta et al., 2005). This is supported by Molan et al. (2009) who explained that condensed tannins could penetrate the wall of the oocyst and cause damage to the cytoplasm by inactivating endogenous enzymes responsible for the sporulation process. Our results in vitro showed the low destruction rate of *Eimeria* oocysts that could be explained by the difference in oocyst resistance. This can be attributed to the impermeability of the inner phospholipid membrane to hydrophilic substances.

In the current study, the OL extract showed low activity with an LC_{50} of 192.94 mg/mL for a reduction rate of 19.4% compared with allicin hydraulic extract with an LC_{50} of 180 mg/mL for an inhibition percentage of 99.9% (Alnassan et al., 2015). In another study, the aqueous extract of *T. sanguine* containing phenolic components at a concentration of 2.5 mg/mL significantly inhibited ($P < 0.05$) the capacity of the sporozoites *E. tenella* and *E. necatrix* (Konan et al., 2012). It is important to underline that our observations regarding the concentration of the extraction solvent at 60% ethanol of the OL extract are divergent with those reported by Gadelhaq et al.(2018), where a significant effect of inhibition of sporulation and oocyst deterioration occurred at ethanolic concentrations of 50–70%. In this study, the low destruction rate of oocysts could be explained by the difference in oocyst resistance. This can be attributed to the presence of internal phospholipid membrane, known for its impermeability to lipophilic compounds. This dif-

ference between the reduction rates of oocysts can be attributed to the molecular structure, nature (lipophilic or hydrophilic), the mode of action of these synthetic phenolic compounds, molecular weight and the chemical composition of the oocyst wall. The anticoccidial potential effect of *A. annua* is generally known to have a multitude of antioxidants (vitamin A, C and E) including flavonoids such as quercetin (Bohorun et al., 2004). It has been confirmed that phenols interact with cytoplasmic membranes and change their cation permeability leading to impairment of crucial processes in coccidia cells and finally leading to their death (Sikkema et al., 1995). Our results are in agreement with those of Abuakkada and Ellakany (2008) who reported that diclazuril was more effective in decreasing the oocyst output of isolates of *E. tenella*.

Conclusion

The findings of the present study showed that it could be concluded that the OL (*Olea europaea* L., var. Chemlal) extract possesses the ability to destroy *Eimeria* spp. collected from naturally infected broiler chickens. In addition, quercetin and oleuropein were tested to evaluate separately in vitro anticoccidial activity against *Eimeria* oocysts for the first time. From this in vitro experiment, our observations showed that the phenolic compound of the OL extract tested separately possesses an anti-*Eimeria* effect. In future, in vivo investigations are required to assess the efficiency of the OL bioactive compounds in broiler chickens.

Conflıct of ınterest

The authors declared that there is no conflict of interest.

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Authors' contributions

NID carried out the experimental work and wrote the manuscript. CN and MAA participated in biochemistry analysis of extract plant and reviewed the manuscript. AA designed, supervised the experimental study and reviewed the manuscript. All authors read and approved the final manuscript.

References

1. Abbas R. Z., Colwell D. D.,Gilleard J. Botanicals: An alternative approach for the control of avian coccidiosis. World's Poultry Science Journal. 2012. 68.P 203–15.
2. Abbas R. Z., Iqbal Z., Mansoor M. K., Sindhu Z. U. D., Zia M. A., Khan J. A. Role of natural antioxidants for the control of coccidiosis in poultry. Pakistan Veterinary Journal. 2012. 33(4).P. 401–407.
3. Abdel-Latif M.,El-Shahawi G.,Aboelhadid S. M., Abdel-TawabH.Modulation of murine intestinal immunity by *Moringa oleifera* extract in experimental hymenolepiasis nana. Journal of Helminthology. 2018. 92(2).P. 142–153.
4. Abuakkada S. S., Ellakany H. F. Sensitivity of two field isolates of *Eimeriatenella* from broiler chickens to salinomycin and diclazuril in a battery trial., Proceedings of the 13th Scientific Congress of the Faculty of Veterinary Medicine (Assuit University, Egypt). 2008. P. 347–364.
5. Aladedunye F. A., Okorie D. A., Ighodaro M. O. Anti-inflammatory and antioxidant activities and constituents of *Platostoma africanum* P. Beauv. Natural Product Research. 2008. 22(12).P. 1067-1073.
6. Alnassan A. A., Thabet A., Daugschies A., Bangoura B. In vitro efficacy of allicin on chicken *Eimeria tenella* sporozoites. Parasitology Research. 2015.,114.P. 625–630.
7. Arabkhazaeli F., Nabian S., Modirsanei M., Madani S. A. The efficacy of a poultry commercial anticoccidial vaccine in experimental challenge with *Eimeria* field isolates. Iranian Journal of Parasitology. 2014. 8(2).P. 234-24.
8. Bohorun T., Luximon-Ramma A., Crozier A., Acuoma O. I.Total phenol, flavonid, proanthocyanidin and vitamin C levels and antioxidant activities of Mauritian vegetables. Journal of the Science of Food and Agriculture. 2004. 84 P. 1553-1561.
9. Botta B., Vitali A., Menendez P., Misiti D., Monache G. G.Prenylated flavonoids: pharmacology and biotechnology. Current Medicinal Chemistry. 2005., 12(5).P. 713-739.
10. Bulotta S., Corradino R., Celano M., Agostino M. D., Maiuolo J., Oliverio M., Procopio A., Iannone M., Rotiroti D., Russo D. Antiproliferative and antioxidant effects on breast cancer cells of oleuropein and its semisynthetic peracetylated derivatives.Food Chemistry. 2011. 127(4).P. 1609–1614.
11. Carvalho F. S., Amauri A. W., Marcel T., João A. M. C.,Antônio D. B. M., George R. A. Diagnosis of *Eimeria species* using traditional and molecular methods in field studies. Veterinary Parasitology. 2011. 176.P. 95–100.
12. De Pablos M., Santos M. F. B. D., Montero E., Garcia-Granados A., Parra A., Osuna A.Anticoccidial activity of maslinic acid against infection with *Eimeria tenella* in chickens. Parasitology Research. 2010.1, 107.,P. 601–604.
13. Debbou-Iouknane N., Nerín C., Amrane M., Ghemghar M., Madani K., Ayad A. In vitro anticoccidial activity of olive pulp (*Olea europaea L.* var. chemlal) extract against *Eimeria* oocysts in broiler chickens. Acta Parasitologica. 2019 64(4).P. 887-897.
14. Gadelhaq S. M., Arafab W. M., Abolhadid S. M. In vitro activity of natural and chemical products on sporulation of *Eimeria species* oocysts of chickens. Veterinary Parasitology. 2018 251.P. 12–16.
15. Han J., Talorete T. P. N., Yamada P., Isoda H. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. Cytotechnology. 2009. 59(1).P. 45–53.
16. Khalafalla R. E., Daugschies A. In vivo evaluation of anticoccidial effect of antibody fragments expressed in pea (*Pasum sativum*) on *Eimeria tenella* sporozoites. Parasitology Research. 2010 107.P. 983–986.
17. Konan K. S., Toure A., Ouattara K. D., Djaman A. J., N'guessan J. D. In vitro anticoccidial activity of *Thonningia sanguinea* extract on *Eimeria tenella* and *Eimeria necatrix* sporozoites cells. African Journal of Microbiology Research. 2012. 6(33).P. 6247-625.
18. Lalas S., Athanasiadis V., Gortzi O., Bounitsi M., Giovanoudis I., Tsaknis J.,Bogiatzis F. Enrichment of table olives with polyphenols extracted from olive leaves. Food Chemistry. 2011. 127(4).P. 1521–1525.
19. Lee O,. Lee B. Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract. Bioresource Technology. 2010. 101(10).P. 3751–3754.
20. Metwaly M. S., Dkhil M. A., Al-Quraishy S. The potential role of Phoenix dactylifera on *Eimeria papillata*-induced infection in mice. Parasitology Research. 2012. 111.P. 681-687.

21. Molan A. L., Zhuojian L., De S. Effect of pine bark (Pinus radiata) extracts on sporulation of coccidian oocysts. Folia Parasitologica. 2009. 56.P. 1–5.
22. Montilla M. .P, Agil A., Navarro M. C., Jiménez M. I., García-Granados A., Parra A., Cabo M. M.Antioxidant Activity of maslinic acid, a triterpene derivative obtained from *Olea europaea*. Planta Medica. 2003. 69(5). P. 472-474.
23. Muthamilselvan T., Kuo T. F., Wu Y. C., Wu Y. Herbal remedies for coccidiosis control. A review of plants compounds, and anticoccidial actions. Evid-Based Complemetary and Alternative Medicine. 2016.P. 1-19.
24. Pereira A. P., Ferreira I. C. F. R., Marcelino F., Valentão P., Andrade P. B., Seabra R.,EstevinhoL., BentoA., Pereira J. A.Phenolic compounds and antimicrobial activity of olive (*Olea europaea L*. Cv. Cobrançosa) leaves. Molecules. 2007. 12(5).P. 1153–1162.
25. Quiroz-Castañeda R. E. Avian coccidiosis, new strategies of treatment. Farm Animals Diseases, Recent Omic Trends and New Strategies of Treatment. 2018.P. 119.
26. Sikkema J., de BontJ. A., Poolman B.Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews. 1995. 59(2). P. 201–222.
27. Starćevic´ K., Krstulovic´ L., Brozic´ D., Mauric´ M., Stojevic´ Z., Mikulec Ž., Bajić M., Mašek T.Production performance, meat composition and oxidative susceptibility in broiler chicken fed with different phenolic compounds. Journal of the Science of Food and Agriculture. 2015.95(6). P. 1172-1178.
28. Tsimidou M. Z., Papoti V. T.Bioactive Ingredients in Olive Leaves', Elsevier Inc. Amsterdam, The Netherlands. 2010.
29. Udo E. J., Abba A. M. Comparative Study of in-vitro anticoccidial efficacy of *Allium sativum* and *Carica papaya*. Journal of Zoology Research. 2018. 2(2).P. 10-14.
30. Varmaghany S., Rahimi S., Torshizi M. K., Lotfollahian H., Hassanzadeh M. Effect of olive leaves on ascites incidence, hematological parameters and growth performance in broilers reared under standard and cold temperature conditions. Animal Feed Science and Technology. 2013. 185(1–2).P. 60-69.

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Evaluation of Physicochemical Properties and Microbiological Quality of UHT Milk Regularly Introduced to Resident Patients in Mansoura University Hospitals

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Keywords: *UHT milk, physicochemical properties, microbiological examination, E. coli, Salmonella, Pseudomonas, Klebsiella.*

Abstract. *The quality of UHT milk is highly influenced by the microbiological characteristics of raw milk and the heat treatment applied. The current study aimed to check whether selected UHT milk brands meet the minimum legal Egyptian standard that is represented in their label manuscript to introduce it in complete nutritive values and hygienic state to resident patients in Mansoura University Hospital (MUH). A total of 80 UHT milk samples from four different brands (A, B, C, and D, 20 of each) were collected from MUH and directed to the physicochemical and microbiological evaluation. MCCW lactoscan® revealed that 100% of all brands were incompatible with the legal SNF% requirements and only brand A was compatible with all other legal standard requirements. The physicochemical investigation showed a significant increase in fat ($P < 0.05$), protein, TS, SNF, ash, density, and freezing point ($P < 0.01$) in A milk brand compared with the other three brands. Total bacterial count (CFU/mL) exceeded the legal standard in 10% of B, 10% of C, and 15% of D brands. Total coliform count (CFU/mL) was incompatible with the legal standards in 15% of B, 25% of C, and 40% of D brands. E.coli and Salmonella spp. were negative in all investigated brands. Pseudomonas was identified in 75% of B, 60% of C, and 75% of D brands. Klebsiella was detected in 25% of B, 40% of C, and 25% of D brands. Our findings indicate that there are somewhat inferior quality and potential risk hazards of consuming B, C, and D UHT milk brands.*

Introduction

Bovine milk is a rich source of fat, protein, carbohydrates, vitamins, and other miscellaneous constituents that play an important role as a diet in many countries for human beings, particularly children and adolescents for their intense growth and development as well as body support to reduce the incidence of chronic diseases such as type 2 diabetes, osteoporosis, hypertension and cancer (Salles et al., 2019). Moreover, milk provides a package of individuals' nutritional daily requirements (calcium and essential amino acids) that is difficult to obtain in other dairy-free diets (Awal et al., 2016).

Ultraheat treatment (UHT) of raw milk is usually applied to preserve its nutrient components and to kill or inactivate almost all pathogens which make it unsafe for human consumption (Ajmal et al., 2019). During the last few years, UHT milk has gained attention as a trustworthy product of must-have nutrients mainly due to its convenience to the Egyptian hot climate and long shelf-life extending from days to 6 months without refrigeration (Nassar et al., 2018). Despite this, UHT milk still liable to be contaminated with various microorganisms from different origins, either during production, processing, packaging, and handling which make it unsafe or even a dangerous source of infection among consumers constituting a potential health hazard (El-Leboudy et al., 2017). Hence, total bacterial count (TBC) and total coliform count (TCC) are the yardsticks among quality control tests applied on milk to evaluate its microbiological quality, the contamination of packaging material, and the low sanitation during manufacturing (Abdel Ghaffar et al., 2019).

Furthermore, organoleptic properties (taste and color) of UHT milk may deteriorate during extended storage especially if it is either contaminated with bacteria of the high proteolytic and lipolytic activity or prepared from raw milk that has previously encountered heat-stable proteolytic and lipolytic enzymes produced by gram-negative psychographs (GNS) (Zhang et al., 2020). Henceforth, raw milk destined for UHT processing and manufacturing should be stored refrigerated for no more than 36 hrs to prevent the growth of drug-resistant GNS such as *Pseudomonas* and *Klebsiella* that become a major contributor of undesirable flavors of UHT milk and a cause of worldwide serious health problem (Zhang et al., 2020).

The current study aimed to check whether milk brands regularly introduced to the resident patients in Mansoura University Hospitals (MUH) are free from microorganisms and meet the minimum legal Egyptian standard that is written in their label manuscript to offer it to patients in complete nutritive values and hygienic condition.

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Materials and methods

A total number of 80 UHT milk samples of four different brands [A, B, C, and D, 20 of each) were randomly collected within 4 days to 2 weeks after production in clean, dry, and sterile containers from the food department in MUH. The collected samples were labeled and transferred in an ice tank to the microbiology laboratory, then analyzed immediately or kept at 4°C for future analysis at the Faculty of Veterinary Medicine, Mansoura University, Dakahlia Governorate, Egypt, from July to December 2019. Then, each collected sample was divided aseptically into 3 portions to be used for organoleptic, physicochemical, microbiological analysis.

Organoleptic investigation

The sensory evaluation of UHT milk samples was applied as recommended by the American Public Health Association (APHA, 1992) where all samples were examined at 20°C by trained panelists who can distinguish slight differences in taste, color, and flavor before being subjected to further investigations.

Physico-chemical investigation

Chemical examination

The percentage of fat, protein, lactose, total solids (TS), solid not fat (SNF), ash, moisture, density, and freezing point of all milk samples were measured by using an automatic milk analyzer device (MCCW lactoscan®, 8900 Nova Zagora, Bulgaria) (Musaad et al., 2013).

Determination of PH

As described by Hartman and La Grang (1985), pH values were determined by using a glass electrode PH meter (Adwa, AD 1000) with a temperature probe after calibrating with phosphate buffer solutions (pH = 7.4).

Determination of titratable acidity

As described by AOAC (2005), titratable acidity was determined by pouring 10 mL of the sample into a suitable porcelain dish along with 20 mL of CO_2 free ambient distilled water followed by titration against standard alkali (N/9 standard sodium hydroxide) using (1%) phenolphthalein alcoholic solution as indicator till reaching the endpoint that was estimated by observing persistent faint pink color. After all, the titratable acidity was calculated and recorded as lactic acid % as follows:

$$\text{Acidity \%} = R/10,$$

R = amount of N/9 NaOH used till reaches the endpoint.

Microbiological load analysis

Total bacterial count

It was determined by the pour plate method using standard plate count agar as mentioned by APHA (2000). Briefly, 1 mL samples were transferred into sterile separate Petri dishes, followed by pouring 10–15 mL of standard plate count agar (Oxoid, Basingstoke, UK), cooled to 45°C in each Petri dish, then mixed well by their rotation many times in various directions, then allowed to set and, finally, incubated at 37C° for 24 hours, after which all appeared colonies were counted.

Total coliform count

It was determined by the pour plate method using violet red bile agar (VRBA, Oxoid, Basingstoke, UK) in a duplicate manner as described by Hartman and La Grang (1985). Briefly, for each sample, one plate was used as a negative control without adding the sample for contamination judging, but the sample plates were dispensed with 1 mL of each sample followed by pouring 15 mL of cooled (45°C) VRBA to each plate, then carefully mixed and allowed to settle down for 10 min. After all, each plate was overlaid with a further 4–5mL of cooled VRBA and allowed to set again. And then, the plates were incubated at 37C° for 24 hours. The control plates should be completely clear, which indicates contamination absence, while the sample plates encountered no more than 250 purplish red colonies surrounded by a reddish zone (diameter of 0.5 mm or greater) carefully counted.

Isolation of Salmonella

Based on the method explained by Addis et al. (2011), each coliform positive sample was ten-fold diluted (10^{-1}) using buffered peptone water (Oxoid, Basingstoke, UK) and then incubated at 37°C for one day. Then, 1 mL of each diluted sample was transferred to 10 mL of Rappaport and Vassilidis enrichment broth followed by overnight incubation at 42°C. From each enrichment broth, one loopful was evenly streaked onto the surface of xylose lysine desoxycholate agar plate (XLD, Oxoid, Basingstoke, UK) and then aerobically incubated for 24 hours at 37°C.

Isolation of E. coli

In conformity with Vanderzant and Splittstoesser (1992), each coliform positive sample was ten-fold diluted (10^{-1}) using tryptone soya broth (Oxoid, Basingstoke, UK) and then incubated at 37°C for one day. From each diluted sample, one loopful was evenly speckled onto the surface of Macconkey agar (Oxoid, Basingstoke, UK). The inoculated plates were observed for the growth, color, form, elevation, margin, surface, and optical characters of colonies according to Eklund and Lankford (1967). These presumptive colonies were selected and subjected to gram staining and microscopic examination as described by Cowan and Steel (1985).

Confirmative biochemical tests for the isolates

As described by ISO 6579 (2002), the gathered

colonies from Macconkey agar were identified after subjection to some biochemical tests including; catalase test, oxidase test, nitrate reduction test, indole production test, methyl red test, Voges-Proskauer test, citrate utilization test, urease test, and triple sugar iron test.

Statistical analysis

With the aid of SPSS version 20.0 (IBM Corp., NY, USA), Shapiro-Wilk test revealed the normal distribution of physicochemical variables as well as TBC and TCC, which are expressed as mean ± standard error (SE). Then, the significant difference of these variables among different brands was estimated using one-way ANOVA with LSD. Besides, the prevalence frequencies of each bacterium were estimated using the chi-square test. The significance of differences between means was reported at $P \leqslant 0.05$.

Results and discussion

Ultraheat processing of raw milk in Egypt and worldwide by direct heat infusion at 143°C for 4–8 sec and homogenization at 200 Pa and then packing in tetra pack paper under aseptic conditions (Hamad et al., 2017; Ibrahim, 2018), usually modifies the physico-chemical composition, organoleptic, chemical, and microbiological characters of milk. Organoleptic features of the commercially available UHT milk brands have a great impact on their popularity and acceptance among consumers (Aldubhany et al., 2014). Examined brands in the current study exhibited the most acceptable organoleptic features despite being contaminated with *coliform*, *pseudomonas*, and *Klebsiella*, and this finding agrees with that of Richards et al. (2016) who stated that aroma, flavor, sensory quality, and texture did not deteriorate immediately in UHT milk tainted with bacteria but limited their shelf-life and stability if stored for a long period.

Milk is a nutritious healthy drink containing water and fat in great quantities. Milk fat content influences flavor, nutritional benefit, and quality parameters of all milk-based products (Wu et al., 2019). Data illustrated in Table 1 reported a significant increase ($P < 0.05$) in the mean value of fat percentage in A brand compared to other investigated brands. Furthermore, determined fat % were compatible with the current legal Egyptian standard (2005) in 20, 17, 15 and 13 samples of the examined A, B, C and D brands, respectively (Table 2).

Low-fat content in some samples of B, C, and D brands could be attributed to the partial withdrawal or over skimming of fat before processing or might be owned to using raw milk adulterated by adding water before manufacturing (Arafat et al., 2015).

Milk adulteration by addition of extraneous water not only deteriorates the nutritive value of milk but also may incorporate chemicals or pathogens of serious health hazard to consumers if added without any consideration to its purity (Kunda et al., 2015). The

Table 1. Physico-chemical properties of examined UHT milk samples

Variables	A brand (n = 20)			B brand (n = 20)			C brand (n = 20)			D brand (n = 20)		
	Min.	Max.	Mean ± SE									
Fat %	3.06	4.15	3.59 ± 0.20*	2.91	3.84	3.25 ± 0.14	2.87	3.45	3.13 ± 0.09	2.88	3.22	3.04 ±.0.05
Protein %	2.87	3.58	3.25 ± 0.12**	2.67	3.05	2.86 ± 0.06*	2.58	2.76	2.57 ± 0.03	2.28	2.66	2.46 ± 0.06
Lactose %	3.88	4.31	4.06 ± 0.06	3.83	4.17	4.00 ± 0.06	3.77	4.34	4.07 ± 0.08	3.88	4.33	4.09 ± 0.06
TS%	9.11	10.60	9.97 ± 0.24**	8.56	9.82	9.19 ± 0.21*	7.72	8.74	8.37 ± 0.15	7.71	8.32	8.00 ± 0.10
SNF %	6.05	6.84	6.38 ± 0.11**	5.36	6.52	5.94 ± 0.17*	4.85	5.61	5.24 ± 0.14	4.60	5.29	5.00 ± .0.10
Ash%	0.59	0.78	0.65 ± 0.03**	0.54	0.62	0.59 ± 0.01*	0.48	0.62	0.54 ± 0.02	0.44	0.55	0.51 ± 0.02
Moisture%	14.46	16.53	14.70 ± 0.32	15.38	17.19	16.23 ± 0.26*	16.46	18.26	17.13 ± 0.26**	16.38	18.38	17.46 ± 0.34**
Density%	22.59	25.97	24.22 ± 0.54**	21.89	23.36	22.80 ± 0.22*	21.00	22.42	21.73 ± 0.22	20.79	22.21	21.18 ± 0.21
Freezing point	–0.44	–0.49	–0.46 ± 0.007**	–0.36	–0.44	–0.40 ± 0.013*	–0.30	–0.43	–0.35 ± 0.019	–0.29	–0.36	–0.33 ± 0.011

*Significance at $P < 0.05$. **Significance at $P < 0.01$.
Min – minimum; Max – maximum.

Table 2. Chemical and microbial results of examined UHT milk brands compared with their label manuscript and the current Egyptian standard (2005)

Variables	Label manuscript of each brand and Egyptian Standards (EOSQC)	A brand (n = 20)		B brand (n = 20)		C brand (n = 20)		D brand (n = 20)	
		Compatible samples No. (%)	Incompatible samples No. (%)	Compatible samples No. (%)	Incompatible samples No. (%)	Compatible samples No. (%)	Incompatible samples No. (%)	Compatible samples No. (%)	Incompatible samples No. (%)
Fat%	⩾ 3 (%)	20 (100)	0 (0)	17 (85)	3 (15)	15 (75)	5 (25)	13 (65)	7 (35)
SNF %	⩾ 8.5 (%)	0 (0)	20 (100)	0 (0)	20 (100)	0 (0)	20 (100)	0 (0)	20 (100)
Acidity%	⩽ 0.17 (%)	20 (100)	0 (0)	18 (90)	2 (10)	18 (90)	2 (10)	17 (85)	3 (15)
TBC	⩽ 10 (cfu/mL)	20 (100)	0 (0)	18 (90)	2 (10)	16 (80)	4 (20)	15 (75)	5 (25)
TCC	Nil (cfu/mL)	20 (100)	0 (0)	17 (85)	3 (15)	15 (75)	5 (25)	12 (60)	8 (40)

TBC – total bacterial count; TCC – total coliform count.

freezing point of milk is usually estimated to explore for possible milk adulteration with extraneous water. As milk is further diluted, the freezing point of water adulterated milk potentially decreases to become closer to the freezing point of pure water which equals 0°C (Zagorska and Ciprovica, 2013). Notably, 100% of examined samples of all brands were incompatible with the legal SNF % standard requirements but Table 1 showed that the percentage of protein, TS, SNF, ash, density, and freezing point more significantly elevated ($P < 0.01$) in A brand compared with other estimated UHT milk brands and also less significantly increased in B brand ($P < 0.05$) compared with C and D brands. This finding is attributed to using raw milk either of low solid components or adulterated by water that leads to inferior UHT milk quality especially in B, C, and D brands (Hamad et al., 2017). Accordingly, moisture percentage was significantly ($P < 0.05$) elevated in adulterated B ($P < 0.05$), C ($P < 0.01$), and D ($P < 0.01$) UHT milk brands in comparison with the A UHT milk brand.

Notably, the amount of acids in UHT milk depends on the cleanliness, freshness of milk during the production process, and the temperature at which the milk is preserved (Hossain et al., 2011). Henceforth, for milk quality judging, we investigated the acid in milk and the result in Table 3 revealed that mean pH values and titratable acidity % of examined brands insignificantly differed in between each other. Moreover, all examined samples of A brand proved their better quality and freshness as they were compatible with the current available Egyptian standard (2005) which reported that high-quality milk should have less than 0.17% titratable acidity % (Table 2). On the contrary, 2 (10%), 2 (10%), and 3 (15%) samples of investigated B, C, and D UHT milk brands, respectively, were incompatible with the Egyptian standard indicating high bacterial activity, uncleanliness, or long storage of milk samples during the production process (Hossain et al., 2011).

Total bacterial count is a microbiological test that could reflect the microbial contamination during milk collection, handling, and production (Hasan et al., 2016). As presented in Table 4 and Fig. 1, TBC was significantly elevated in investigated B ($P < 0.05$), C ($P < 0.01$), and D ($P < 0.001$) brands in comparison with the A brand. Hence, 100% of A, 90% of B, 80% of C, and 75% of D samples proved their excellent sanitary quality during processing, handling and production as they were compatible with the Egyptian Standard (2005) that stated that TBC of UHT milk should be not more than 10 CFU/mL.

Table 3. pH and titratable acidity % of examined UHT milk samples

Sample type	Sample No.	pH			Titratable acidity		
		Min.	Max.	Mean ± SE	Min.	Max.	Mean ± SE
A brand	20	6.38	7.16	6.45 ± 0.27 NS	0.08%	0.16%	0.115% ± 0.004 NS
B brand	20	6.25	7.34	6.34 ± 0.32 NS	0.09%	0.18%	0.123% ± 0.0021 NS
C brand	20	6.24	7.05	6.37 ± 0.23 NS	0.07%	0.19%	0.125% ± 0.0026 NS
D brand	20	6.14	6.98	6.21 ± 0.15 NS	0.09%	0.18%	0.129% ± 0.002 NS

NS – non-significant differences ($P > 0.05$). Min – minimum; Max – maximum.

Table 4. Comparisons of bacterial load of UHT milk samples

Variables	A brand (n = 20)					B brand (n = 20)					C brand (n = 20)					D brand (n = 20)				
	Pos.	%	Min.	Max.	Mean ± SE	Pos.	%	Min.	Max.	Mean ± SE	Pos.	%	Min.	Max.	Mean ± SE	Pos.	%	Min.	Max.	Mean ± SE
TBC	0	0	0	0	0 ± 0	9	45	1	11	4.16 ± 1.35*	11	55	1	15	4.76 ±1.42**	15	75	0	18	5.16 ± 1.11***
TCC	0	0	0	0	0 ± 0	3	15	0	1	0.15 ± 0.018*	5	25	0	3	0.45 ± 0.043**	8	40	0	5	0.80 ± 0.066***

*Significance at $P < 0.05$. **Significance at $P < 0.01$. ***Significance at $P < 0.001$.
Min – minimum; Max – maximum.

Coliforms are mostly present in heat untreated milk but their presence in UHT milk reflects the inadequate sanitation of milk utensils and/or improper handling of milk during manufacturing (Salman and Hamad, 2011). As displayed in Table 4 and Fig. 2, TCC was significantly elevated in investigated B ($P < 0.05$), C ($P < 0.01$), and D ($P < 0.001$) brands compared with the A brand. Furthermore, the current study proved that 100% of A, 85% of B, 75% of C, and 60% of D brand samples had high hygienic quality as they were compatible with the current Egyptian Standard (2005) which states that UHT milk must be free from coliform organisms. Otherwise, 3 (15%) of B, 5 (25%) of C, and 8 (40%) of D brand were incompatible with the standard, and this a consequence of their low hygienic quality and/or fecal contamination during the manufacturing process that usually enhance rapid deterioration of the products and cause serious public health hazards (Saha et al., 2018).

Isolation and identification of coliform species (*E. coli*, *Salmonella*, and *Klebsiella*) can be simply applied to assess the hygienic and sanitary level during UHT milk production. Henceforth, we further cultured isolated coliforms from TCC positive samples on XLD and MacConkey medium to screen for the presence of gram-negative enteric bacteria especially *Salmonella* and *E.coli* which cause food-borne gastroenteritis. Fortunately, as shown in Table 5, all investigated samples were negative for *E.coli* and *Salmonella*. Based on typical colony characteristics onto specific and differential MacConkey media, our study suspected the presence of gram-negative psychrotrophic (GNP) bacteria (*Pseudomonas* and *Klebsiella*) in all coliform positive UHT samples as some of the isolates morphologically appeared as 2–3 mm, flat and smooth non-lactose fermenting colonies suspected to be *Pseudomonas*, also some of the isolates appeared as large, shiny, dark pink in color, mucoid in appearance and lactose fermenting colonies suspected to be *Klebsiella*. Further, by gram staining and microscopic examination, the isolated colonies presented as gram-negative rods with single, in pairs and irregular arrangement (Chen et al., 2011; Saha et al., 2018; Mwambete and Nakembetwa, 2015).

The applied biochemical test confirmed the presence of GNPs in 3 (15%), 5 (25%), and 8 (40%) of all examined B, C, and D samples that further classified into 2 (75%), 3 (60%) and 6 (75%) of *pseudomonas* and 1 (25%), 2 (40%) and 2 (25%) of *Klebsiella*, respectively (Table 6). The presence of these GNPs in some of the examined samples indicates the milk spoilage either by an inadequate sanitary condition or the adequate heat treatment process (Chen et al., 2011). GNPs could produce proteolytic and lipolytic enzymes as a consequence of their metabolic activities that can resist UHT processing resulting in unpleasant properties of milk (Tondo et al., 2004).

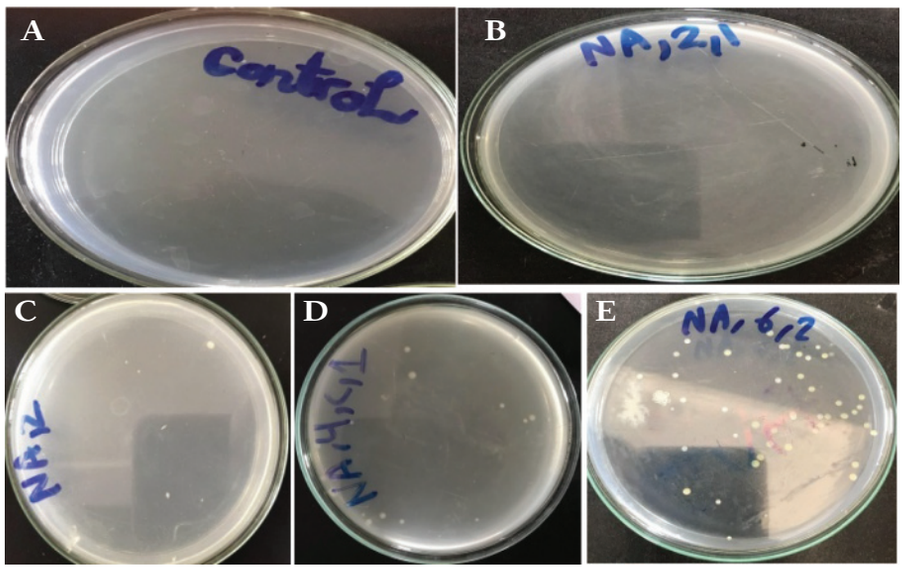

Fig. 1. TBC on nutrient agar.
A – control (-ve); B – absence of bacterial colonies in A brand;
C, D, E - presence of bacterial colonies in B, C and D UHT milk brands.

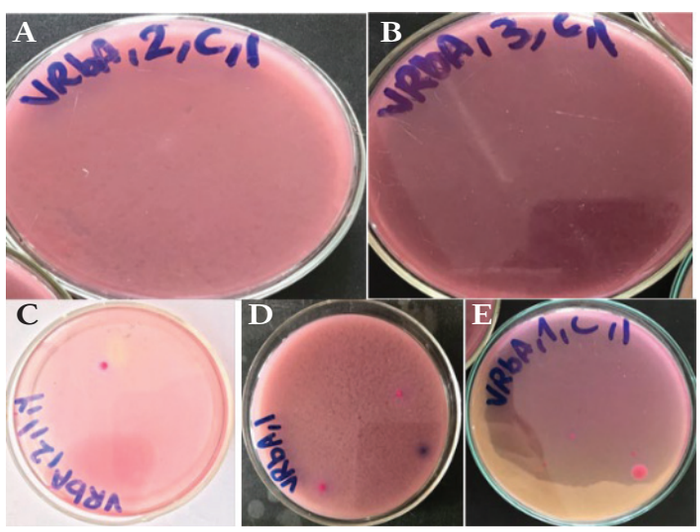

Fig. 2. TCC on violet red bile agar (VRBA).
A – control (-ve); B – absence of coliform colonies in A brand;
C, D, E – presence of coliform colonies in B, C, and D UHT milk brands

Table 5. Identified bacteria from UHT milk brands

Type of samples	Gram-negative isolate identified on McConkey agar	*E. coli*	*Salmonella*	*Pseudomonas*	*Klebsiella*
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
B brand	3 (15)	0 (0)	(0) 0	2 (75)	1 (25)
C brand	5 (25)	0 (0)	(0) 0	3 (60)	2 (40)
D brand	8 (40)	0 (0)	(0) 0	6 (75)	2 (25)

Table 6. Confirmative biochemical tests for the obtained gram-negative isolates

Isolates	C	O	NR	I	MR	VP	Citrate	Urease	TSI		
									Slant/Butt	Gas	H2s
Pseudomonas	+	+	+	–	–	–	+	+	R/R	–	–
Klebsiella	+	–	+	–	–	+	+	+	Y/Y	+	–
E. coli	+	–	+	+	–	–	–	–	Y/Y	+	–
Salmonella	+	–	+	–	–	+	+	+	Y/Y	+	+

C – catalase test; O – oxidase test; NR – nitrate reduction test; I – indole production; MR – methyl-red test; VP – Voges-Proskauer test; Citrate – citrate utilization test; Urease – urease activity; TSI – triple sugar iron fermentation; Y – yellow/acidic; R – red/alkaline; V – variable; '+' – positive; '–' – negative.

Conclusion

The current study concluded that there is somewhat inferior quality, adulteration and a potential risk hazard of consuming B, C, and D UHT milk brands regularly introduced to the resident patients in MUH as some pathogenic bacteria such as *Pseudomonas* and *Klebsiella* were isolated and identified from these brands. So, our study could play an important role in informing these dairy industries about the lack of heat treatment, sanitary processing, packaging, handling of milk during the production process which necessitates the extreme application of hygienic production practices and HACCP.

References

1. Abdel Ghaffar E. A., El-Neshawy A.A., Siliha H.M., El-Shorbagy G.A. Quality and safety assessment of Egyptian marketed flavoured UHT milk: a survey study for sanitary monitoring. Zagazig Journal of Agricultural Research. 2019. T. 46. P. 455-465.
2. Addis Z., Kebede N., Sisay Z., Alemayehu H., Wubetie A., Kassa T. Prevalence and antimicrobial resistance of Salmonella isolated from lactating cows and in contact humans in dairy farms of Addis Ababa: a cross sectional study. BMC infectious diseases. 2011. T.11.P. 222.
3. Ajmal M., Nadeem M., Imran M., Mushtaq Z., Ahmad M.H., Tayyab M., Gulzar N. Changes in fatty acids composition, antioxidant potential and induction period of UHT-treated tea whitener, milk and dairy drink. Lipids in Health and Disease. 2019. T. 18. P. 213.
4. Aldubhany T. A. W., Gouda E., Khattab A., Dabour N. Effects of storage on some physico-chemical characteristics of UHT milk stored at different temperature. Alexandria science exchange journal. 2014. T. 3. P. 107-114.
5. APHA. Compendium of methods for the microbiological examination of foods. Second (Ed). American Public Health Association. Washington. USA. 1992. P220.
6. APHA. Compendium of methods for the Microbiological Examination of Food. 3red (Ed.). American Public Health Association. Washinton. USA. 2000. p. 220-230.
7. AOAC. Official methods of analysis. The association of official analytical chemists. 16th edition. North Fredrick Avenue. Gaithersburg. Maryland. USA. 2005. p. 481
8. Arafat M.Y., Habib R., Siddiki M.S.R., Imam M. F. Quality of ultra-high temperature treated milk available in Gazipur and Mymensingh of Bangladesh. Bangladesh Journal of Animal Science. 2015. T. 44. P. 132-136.
9. Awal M.d.S., Amin M.d.R., Haque M.d.R., Kamal M.d.M., Hasan S.M.K. Evaluation of Physico-Chemical Properties and Detection of Adulterants of UHT Milk Samples Available in Bangladesh. International Research Journal of biological sciences. 2016. T. 5. P. 1-6.
10. Chen T.R., Wei Q.K., Chen Y.J. Pseudomonas spp. and Hafnia alvei growth in UHT milk at cold storage. Food Control. 2011. T. 22. P. 697-701.
11. Cowan S.T., Steel K.J. Manual of Identification of Medical Bacteria. 2nd (ed.). Cambridge University Press. Cambridge. England. 1985. P. 476.
12. Egyptian Standards 1008/2005 Egyptian Organization for Standardization and Quality Control. Egyptian Standards. 2005.
13. Eklund C., Lankford C.E. Laboratory Manual for General Microbiology. Prentice-Hall. Inc. Englewood Cliffs. New Jersey. 1967. P. 52-55.
14. El-Leboudy A., El-Makarem H.S.A., Amer R. A. Sanitary evaluation of milk and its products serving at general hospitals. Alexandria Journal of Veterinary Sciences. 2017. T. 55. P. 83-90.
15. Hamad M.N.E.F., Abdel-Kader Y.I., Shahin M.A.H. Analysis of Representative Samples of UHT Sterilized Milk in the Egyptian Market. American Journal of Food. 2017. T. 2. P. 31-42.
16. Hartman P.A., La Grang W.S. Coliform bacteria. In:Richardson. G.H. (Ed.). Standard Methods for the Examination of Dairy Products. A.P.H.A. Washington. D.C.USA. 1985. P. 173–186.
17. Hasan G.A., Parveen S., Sultana J. Microbiological Quality Analysis of Raw Milk and Yogurt Available in Some Selected Areas of Bangladesh. International Journal of Innovative Research in Science Engineering and Technology. 2016. T. 5. P. 2855-2859.
18. Hossain T.J., Alam M.K., Sikdar D. Chemical and microbiological quality assessment of raw and processed liquid market milks of Bangladesh. Continental J. Food Science and Technology. 2011. T. 5. P. 6 – 17.
19. ISO International Organization for Standardization ISO 6579:2002. Microbiology of food and animal feeding stuffs-horizontal method for the detection of Salmonella spp.4th (ed.). ISO 6579:2002.The International Organization for Standardization. Geneva. 2002.
20. Ibrahim A. Effect of Milk Quality and Stabilizers on Some Physicochemical Properties of UHT-Milk. Alexandria Journal of Food Science and Technology. 2018. T. 15. P. 49-64.
21. Kunda B., Pandey G.S., Mubita C., Muma J.B., Mumba C. MC. Compositional and microbial quality of heat-treated milk brands marketed in Lusaka, Zambia. Livestock Research for Rural Development. 2015. P. 27.
22. Musaad A., Faye B., Al-Mutairi S. Seasonal and physiological variation of gross composition of camel milk in Saudi Arabia. Emirates Journal of Food and Agriculture. 2013.T. 25. P. 618-624.
23. Mwambete K.D., Nakembetwa M. Microbiological quality

of pasteurized milk available in the Dar es Salaam Market, Tanzania. East and Central African Journal of Pharmaceutical Sciences. 2015. T. 18.P. 23-31.
24. Nassar W.S., Elbarbary H.A., Ibrahim E., Mohammed H.A., Ibrahim M.I. A New Trial in Egypt to Detoxify AFM1 in UHT Milk by Lactobacilli and their Bacteriocins. Alexandria Journal of Veterinary Sciences. 2018. T. 59. P. 60-67.
25. Richards M., Elna M.B., Henricetta L.D.K. Survival analysis, consumer perception and physicochemical analysis of low fat UHT milk stored for different time periods. International Dairy Journal. 2016. T. 57.P. 56-61.
26. Saha B.J. Analysis of enteric pathogenicity of multidrug-resistant Pseudomonas spp. isolated from commercial UHT milk (Doctoral dissertation). Department of Mathematics and Natural Sciences. BRAC University. 2018.
27. Salles M.S., D'Abreu L.F., Júnior L.C.R., César M.C., Guimarães J.G., Segura J.G., Netto A.S. Inclusion of Sunflower Oil in the Bovine Diet Improves Milk Nutritional Profile. Nutrients. 2019. T. 11. P. 481.
28. Salman A.M., Hamad I.M. Enumeration and identification of Coliform bacteria from raw milk in Khartoum State, Sudan. Journal of Cell and Animal Biology. 2011. T. 5. P. 121-128.
29. Tondo E.C., Lakus F.R., Oliveira F.A., Brandelli A. Identification of heat stable protease of Klebsiella oxytoca isolated from raw milk. Letters in applied microbiology. 2004. T. 38. P. 146-150.
30. Vanderzant C., Splittstoesser D.F. Compendium of Methods for the Microbiological Examination of Foods.3rd Edition. American Public Health Association. Washington. DC. 1992.
31. Wu S., Jin Y., Yang N., Xu X., Xie Z. Determination of fat content in UHT milk by electroanalytical method. Food chemistry. 2019. T. 270. P. 538-545.
32. Zagorska J., Ciprovica I. Evaluation of factors affecting freezing point of milk. Agricultural, Food and Biotechnological Engineering. 2013. T. 7. P. 106-111.
33. Zhang D., Li S., Palmer J., The K.H., Leow S., Flint S.The relationship between numbers of Pseudomonas bacteria in milk used to manufacture UHT milk and the effect on product quality International Dairy Journal. 2020. P. 104687.

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A Plasma Protein Profile and Acute Phase Proteins in Sheep with Normal Parturition and after Cesarean Section in Cases of Dystocia

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Keywords: *sheep, acute phase protein, plasma protein profile, parturition, cesarean section.*

Abstract. *The purpose of this study was to define alterations in concentrations of total protein, albumin, globulins, albumin/globulin ratio, as well as fibrinogen and ceruloplasmin (as an acute phase protein) in sheep after normal parturition and in animals with caesarean section. For that, twelve Pleven Blackhead sheep were observed and divided into two groups: controls, which include six females with normal parturition; the second group consisted of six sheep with caesarean surgery. Blood samples were collected into heparinized tubes before parturition and cesarean section (hour 0) and then at hours 6, 24, 48 and on days 4, 8, 10, 12, 14, 18 after labor. During the experimental period, the values of fibrinogen in the sheep with caesarian section were higher than those in the control group. Furthermore, after birth fibrinogen levels gradually increased in both groups of animals. The highest value after normal parturition was observed on day 8, and in the sheep after caesarean section, the highest value was observed on day 10. The ceruloplasmin levels in sheep with normal birth did not change significantly, but within the group of caesarean section, plasma concentrations were markedly increased at 48 hours after the surgery and were maximal on day 10. In normal parturition sheep, the concentration of albumin did not change significantly during the test period, and it was higher than in sheep with caesarian section. The globulin concentrations in animals with caesarian sections were higher than in those with normal labor, with differences statistically significant at day 8 and day 10 after birth. No significant change in the total protein concentration was observed during the whole study period.*

Introduction

The physiological response of the organism to general and local inflammations is connected with an initiation of events leading to a systemic response named acute phase reaction. The changes during this reaction include fever, leukocytosis and quantitative and qualitative modification of total plasma protein present in blood and non-structurally related proteins known as acute phase proteins (APPs) (Cray et al., 2009; Ceciliani et al., 2012; Eckersall, 2019). A plasma protein profile includes albumin and globulins with an expected albumin to globulin ratio of 1:1 in healthy animals (Kaneko, 1980). The inflammatory processes play an important role in the regulation of total plasma protein concentrations with a negative correlation between globulins and albumin and significant changes in the plasma concentrations of acute phase proteins (Eckersall, 1995; Eckersall, 2000; Ceciliani, 2002; Tóthová et al., 2016). An increasing concentration of APPs in blood circulation can be a biomarker for onset and/or development of infectious disease; besides, it has proven to be a very useful indicator for early detection of subclinical inflammation. In addition, a decreasing concentration of APPs is attributed to the recovery stage after treatment of inflammatory disease and can be a powerful tool for monitoring of the treatment (Cray et al., 2009; Eckersall and Bell, 2010). Acute phase proteins have been a focus of many investigations in veterinary medicine and recently have been directed to the veterinary obstetrics and reproduction (Kaya et al., 2016; Costa et al., 2018; Smits et al., 2018; Eckersall, 2019).

In sheep, different APPs (α1-acid glycoprotein, haptoglobin, serum amyloid A and fibrinogen) have shown as biomarkers for the presence of acute or chronic bacterial infection of the respiratory system (Pfeffer and Rogers, 1989) and in ovine caseous lymphadenitis (Eckersall et al., 2007; Bastos et al., 2011). A few researches (Scott et al., 1992; Aziz and Taha, 1997; Georgieva et al., 2011) presented the changes in the haptoglobin concentration in case of dystocia in this animal species. Because the plasma concentration of fibrinogen (Fb) and ceruloplasmin (Cp) increase during the acute inflammation response, those APPs are considered positive (Ceron et al., 2005), whereas plasma proteins whose concentrations decrease during the acute phase response were classified as negative APPs. Also, APPs are classified into major, moderate and minor according to the degree of increase. Ceruloplasmin is considered as a

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minor (< 2-fold increase) APP in ruminants, whereas fibrinogen is a moderate (2–10-fold increase) APP in the same species (Murata et al., 2004).

Fibrinogen, which is produced in the liver, is a β-globulin, considered as an acute phase protein which presents in the plasma of all vertebrates (Ceron et al., 2005). Fibrinogen is included in homeostasis as an integral part of it, providing a substrate for fibrin formation, as well as in tissue repair, providing a matrix for the migration of inflammatory-related cells (Thomas, 2000). Besides, it participates not only in coagulation but also in maintaining pregnancy; therefore, observations on the dynamics of fibrinogen levels are important for safely prenatal and peripartum periods (Teraoka et al., 2017). The main source of Cp is hepatocytes and it can also be synthesized in the mammary gland and places of tissue damage (Szczubiał et al., 2012). However, the information about a plasma protein profile and different acute phase proteins in sheep with a normal and difficult parturition is limited.

The present work aims to determine the changes in a plasma protein profile and certain acute phase proteins in clinical healthy sheep with a normal parturition and puerperal period without reproductive complications as well as in animals after caesarean section.

Materials and Methods

Animals, surgery and blood collection

The study was conducted on twelve Pleven Blackhead sheep, 2–5 years old and weighing 45–60 kg fed and housed under uniform conditions and subjected to the standard immunoprophylactic and antihelminthic regimens.

The control group included six clinical healthy sheep with a normal parturition and puerperal period without reproductive complications or presence of other diseases. All lambs (n = 10; 4 twins and 2 singletons) were housed with the dams in individual pens until the end of the experiment. The second group consisted of 6 females with dystocia presented for veterinary assistance. The reasons for dystocia were uterine torsion (leading to the death of all lambs – 3 twins), oversized fetus (2 live singletons) and ring womb (1 live singleton) in three, two and one cases, respectively. Immediately after the obstetrical examination, a caesarean section was carried out in the animals with dystocia. The anesthesia included intravenous administration of xylazine hydrochloride (0.2 mg/kg, Xylazine 2%, Alfasan International B.V., the Netherlands) and a local infiltration of 20 mL of novocaine (Novocain 1%, Vetprom, Radomir, Bulgaria) subcutaneously into the operative area located in the left flank; postoperative analgesia included intravenous administration of methamizol sodium (5 mL, Analgin 30%, Biovet, Peshtera, Bulgaria) for five days. Among the 9 lambs born from females with dystocia, six were dead. The presence of autolytic changes was indicative of the death occurrence at least 12 to 24 hours before surgery. The uterine involution was monitored and only in the cases of a uterine torsion it was delayed. All sheep were hospitalized after surgery for three weeks.

Individual blood samples were collected from *v. jugularis* prior to 0 hour when it was possible and 4, 24, 48 hours after the part as well as on days 4, 8, 14 and 18. In animals with a normal parturition, the time-point *0 hour* was accepted as the first stage of labor. However, in animals with dystocia, this time-point was delayed and the first blood collection was performed immediately before the surgery. Blood samples were collected in heparinized sterile tubes and were centrifuged immediately (1500 g, 15 minutes, 4°C) for separation of the plasma. Thereafter, plasmas were decanted and stored at –20°C until analysis. All samples were free of hemolysis. The study was performed in accordance with the requirements of Animal Ethics Committee and regulations for human attitude and animal protection.

Plasma protein profile and acute phase proteins assessment

The total serum protein concentration in sheep was determined by the biuret method (Kolb and Kamishnikov, 1982). The serum albumin concentration was determined by a Human test containing bromine-cresol green, SU-ALBU INF 156001F, Gesellschaft für Biochemica, Germany, mixing 10μL plasma with 1 mL of the prepared reagent. After 3 min, the sample was read at λ = 546 nm and the result was calculated using a standard sample at a known concentration (40 g/L). The determination of the globulin concentration was calculated as the difference between the values of total protein and albumin. With the albumin and globulin results, the albumin/globulin ratio (A/G ratio) was calculated.

The concentration of ceruloplasmin was determined by the Ravin method based on the oxidation of p-phenylenediamine (Kolb and Bestujeva, 1982). Since 1970, there have been many modifications to the method, but the PPD oxidase procedure has been widely adapted for routine use in clinical laboratories. From ceruloplasmin and p-phenylendiamine, at pH 5.5, a colored oxidation product was formed and the change in absorption was determined at λ = 530 nm. The plasma fibrinogen concentration was determined by the Podmore nephelometric method with 10% Na_2SO_4 at λ = 570 nm (Todorov, 1972). To 0.25 mL of plasma, 2.5 mL of 10.5% Na_2SO_4 was added against a control sample of 0.25 mL of plasma and 2.5 mL of 0.9% solution of NaCl. The extinction was counted after 3 min at a wavelength of 570 nm, and the result was calculated by multiplying by a coefficient calculated on a standard curve based on various plasma dilutions in which the fibrinogen concentration was determined keldalometrically.

Statistical analysis

Statistical processing of the results obtained in the individual experiments was performed by ANOVA (Statistics for Windows, Stat Soft Ins., USA, 1993). The statistical significance of intra- and intergroup differences was determined by the post hoc procedure LSD test (Stat Soft Ins., USA, 1993). The level of statistical significance of the differences was at $P < 0.05$.

Results

The obtained results that relieve the effect of normal parturition and caesarean section on concentration of fibrinogen and ceruloplasmin are shown in Figures 1, 2, 3, 4, 5 and 6. Fig. 1 presents data on the amount of fibrinogen (g/L) in the blood plasma of sheep born with caesarean section and normal parturition sheep. The concentration of Fb in the blood of sheep before normal parturition was 1.98 ± 0.25 g/L (0 h), and in the plasma of operated sheep, it was 2.22 ± 0.17 g L. After birth, fibrinogen levels gradually increased in both groups of animals, with values significantly higher ($P < 0.05$; $P < 0.01$) in both groups at 48 hours compared with the pre-natal period. The highest value after normal parturition (4.63 ± 0.25 g/L) was observed on day 8, and in the sheep after caesarean section, it was observed on day 10^h (7.11 ± 1.31 g/L, $P < 0.001$). Significantly higher values after normal parturition in sheep persisted until day 12, and in those with caesarian section until day 14. At the end of the study period (day 18), fibrinogen concentrations in both groups were approaching baseline levels. Throughout the experimental period, the levels of fibrinogen in the sheep with caesarian section were higher than those in the control group, with differences between day 10 ($P < 0.001$), day 12 ($P < 0.01$) and day 14 ($P < 0.01$).

Results about the concentration of ceruloplasmin (mg/L) in the blood plasma of sheep with caesarean section and sheep with normal parturition are presented in Fig. 2. Throughout the experiment, the ceruloplasmin in sheep with normal birth did not change significantly. It varied from 170.06 ± 5.79 mg/L before birth and 164.45 ± 3.09 mg/L on day 18 after birth. There was a non-significant increase on day 10 after birth (194 ± 3.09 mg/L).

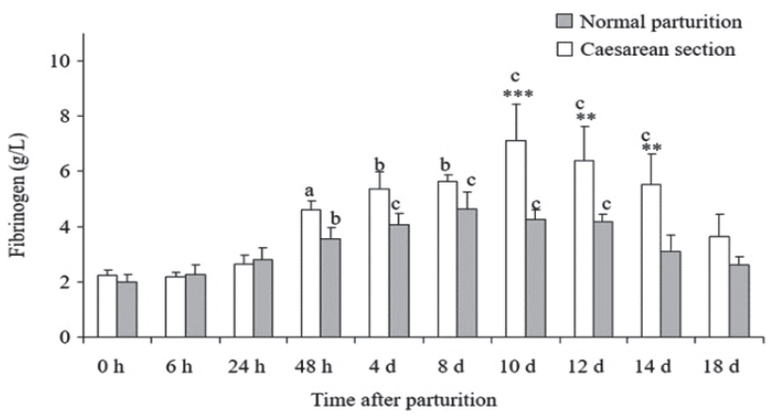

Fig. 1. Fibrinogen concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).
Results are expressed as means ± standard errors of the means.
Significance of the differences between the groups: ** $P < 0.01$; *** $P < 0.001$
Significance of the differences within the groups: a ($P < 0.05$); b ($P < 0.01$); c ($P < 0.001$).

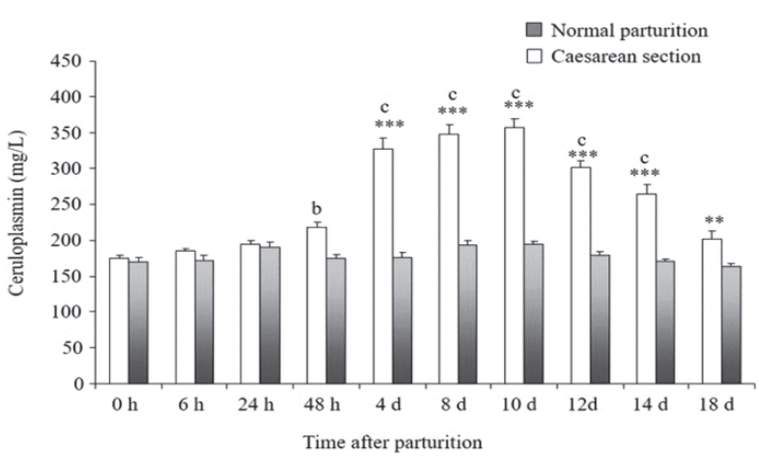

Fig. 2. Ceruloplasmin concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).
Results are expressed as means ± standard errors of the means.
Significance of the differences between the groups: ** $P < 0.01$; *** $P < 0.001$.
Significance of the differences within the groups: b ($P < 0.01$); c ($P < 0.001$). h – hours; d – days.

In cesarean sectioned sheep, a significant increase in the concentration of ceruloplasmin was observed since day 4 (326.5 ± 15.87 mg/L) until day 18 (201.83 ± 10.8 mg/L) ($P < 0.001$). It was highest on day 10 after the intervention – 357.04 ± 12.44 mg/L.

Within the group of Caesarean section, the significance of differences was reported at 48 hours after surgery ($P < 0.01$), and then increased, reaching maximum values since days 4 to 14 ($P < 0.001$).

Data in Fig. 3 show the concentration of albumin (g/L) in the blood plasma of sheep with cesarean section and normal parturition sheep. The level of albumin in the blood of sheep before normal parturition was 30.7 ± 2.3 g/L (0 h), and in the plasma of operated sheep, it was 33.44 ± 2.79 g/L. After the birth, its level decreased in the operated group, with significant differences ($P < 0.05$) compared with baseline values on days 8 and 10. The lowest value was reported on day 10 in the cesarean group – 26.91 ± 1.99 g/L. At the end of the study period (day 18), the level of albumin in the test group returned to its original values – 33.58 ± 3.12 g/L. In normal parturition sheep, the concentration of albumin did not change significantly during the test and ranged from 30.7 ± 2.3 g/L (0 h) to 29.35 ± 2.47 g/L (day 18).

The information in Fig. 4 represents the concentration of globulins in the blood plasma of sheep with cesarean section and sheep with normal parturition. No significant difference was observed in the level of globulin in the plasma of normal-parturiated sheep. Before parturition, it was 32.36 ± 2.32 g/L (0 h), and at the end of the study period, it was 32.33 ± 2.33 g/L (day 18).

In the plasma of operated sheep at the beginning of the period, the level of globulins was 35.53 ± 2.12 g/L. After surgery, its level increased significantly on day 8 to 44.1 ± 3.33 g/L ($P < 0.05$), after which the concentration of globulins gradually decreased at the end of the study period (day 18). In the cesarean section group, it was close to its original values – 35.18 ± 2.02 g/L. Throughout the experimental period, the globulin concentrations in sheep with caesarian sections were higher than those with normal parturition, with differences statistically significant ($P < 0.01$) at day 8 and day 10 after the birth.

As shown in Figure 5, the A/G ratio for operated sheep and sheep that gave birth normally up to 24 hours of the study period was almost the same, i.e., about 0.95 ± 0.03 (controls). At 24 hours after the birth in operated animals, it began to decrease, with differences from baseline values statistically significant at 48 hours ($P < 0.05$), day 4 ($P < 0.001$), day 8 ($P < 0.001$), day 10 ($P < 0.001$) and day 12 ($P < 0.01$). The lowest value was reported on day 8 – 0.61 ± 0.03. This means that globulin synthesis is significantly increased at the expense of albumin synthesis. After day 14, the A/G ratio values returned to their original levels again – 0.908 ± 0.04 (for the sheep with normal parturition) and 0.95 ± 0.05 for cesarean operated sheep (Fig. 5). In the postpartum period, A/G ratios in sheep with caesarean section were lower than in normal parturition sheep, with differences between groups statistically demonstrated at day 4 ($P < 0.001$), day 8 ($P < 0.001$), day 10 ($P < 0.001$) and day 12 ($P < 0.01$).

As reported in Fig. 6, no significant change in total protein concentration was observed during the whole study period, both in control animals (normal birth) and in the operated ones. At the beginning of the period, it was 68.97 ± 4.72 g/L and 63.07 ± 4.48 g/L, respectively (0 h). On day 18 after birth, the total protein content was 68.76 ± 4.8 g/L (operated) and 60.73 ± 5.14 g/L (controls). It should be noted that

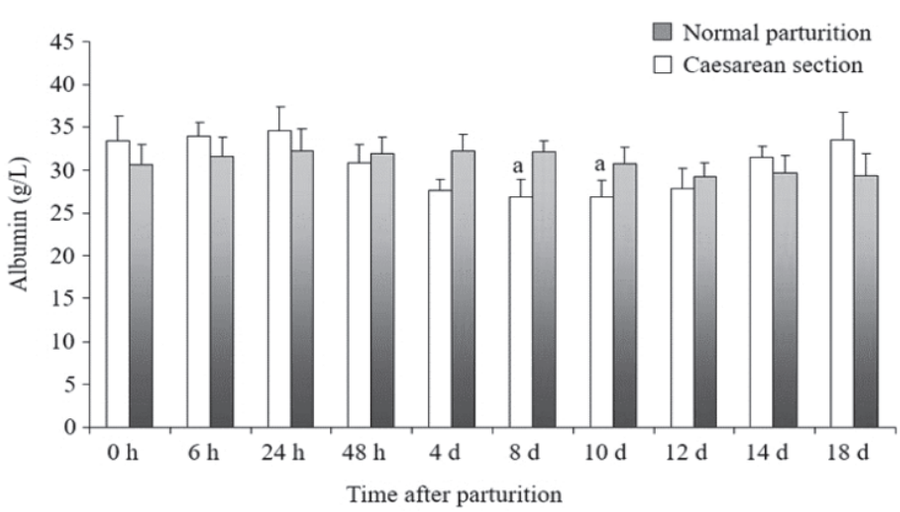

Fig. 3. Albumin concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).
Results are expressed as means ± standard errors of the means.
Significance of the differences within the group with caesarean section: $^{a}P < 0.05$; h – hours; d – days.

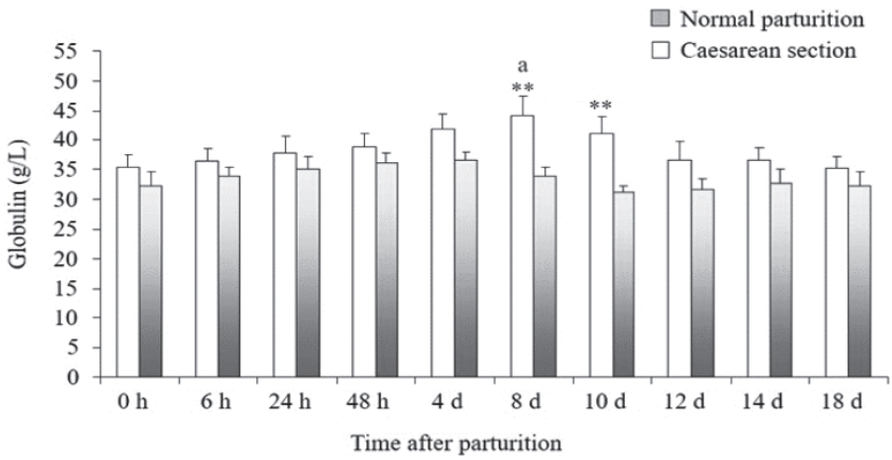

Fig. 4. Globulin concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).

Results are expressed as means ± standard errors of the means.
Significance of the differences between the groups: $^{**}P < 0.01$.
Significance of the differences within the group with caesarean section: $^{a}P < 0.05$; h – hours; d – days.

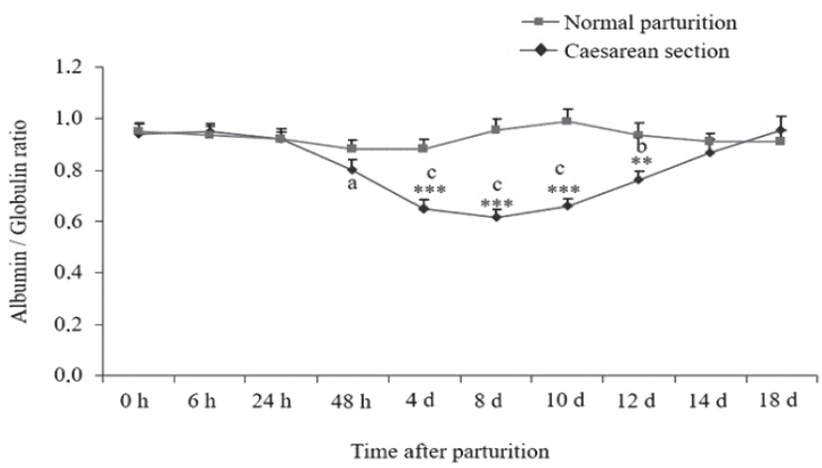

Fig. 5. Albumin/globulin ratio in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).

Results are expressed as means ± standard errors of the means.
Significance of the differences between the groups: $^{**}P < 0.01$; $^{***}P < 0.001$.
Significance of the differences within the groups: $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; h – hours; d – days.

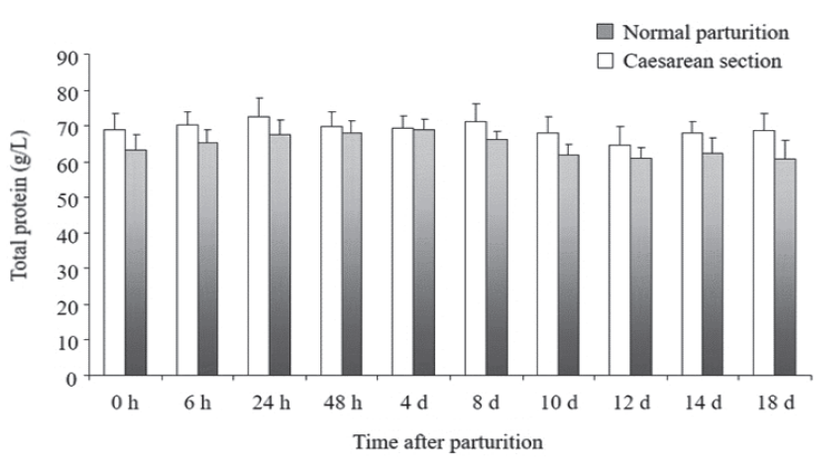

Fig. 6. Total protein concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).

Results are expressed as means ± standard errors of the means.
No significance of the differences between or within the groups; h – hours; d – days.

throughout the study, total protein levels in sheep born with caesarean sections were higher than those in normal sheep, but statistically the differences were not significant.

Discussion and conclusions

In this study, we describe raised concentrations of certain APPs in sheep with normal parturition and animals with dystocia submitted to caesarean section. The acute phase proteins are substances synthesized during an acute phase response (APR) which is caused by inflammation, infection, trauma or tissue damage (Ceron et al., 2005; Petersen et al., 2004). It is stimulated by the release of cytokines (IL-1, IL-6, TNF-α) from macrophages and monocytes at the site of inflammatory lesions or infections. The synthesis and role of APPs may differ depending on the animal species. However, Murata et al. (2004) announced that APP may be applied to evaluate not only inflammatory conditions, but also some non-inflammatory processes such as parturition, pregnancy, stress, metabolic diseases. The changes in concentrations of APPs caused by a different stimulus are fast, with maximum levels about one day after initiation and before returning to their baselines within a week (Meling et al., 2012). It should be noted that most APPs have a half-life of only 24 to 48 hours (Cray et al., 2009).

Physiological and metabolic changes around parturition can be regarded as an event which causes potential stress on livestock, resulting in activation of the APR and subsequent increase in concentrations of positive APPs (Tóthová et al., 2014). The acute phase response in ruminants is radically different from other animals and the APR studies on sheep are not as large as in cows, but it is thought to be similar. Pfeffer and Rogers (1989) found that, following the development of pneumonia, sheep observed elevations in plasma ceruloplasmin and fibrinogen, decreased erythrocytes, and increased neutrophils by 250%, 400%, 80%, and 200%, respectively. The authors recommend measuring the level of APP instead of the number of circulating neutrophils. On the other hand, Ulutas and Ozpinar (2006) found that, in annual lambs infected with *Pasteurella haemolytica*, serum levels of APP like haptoglobin, ceruloplasmin, and fibrinogen increased during infection. Razavi et al. (2011) found that serum amyloid A (SAA) greater than 57.15 μg/mL, haptoglobin greater than 0.42 g/L, ceruloplasmin greater than 0.27 g/L and fibrinogen greater than 3.91 g/L were appropriate indicators for an inflammatory process in sheep infected with *T. lestoquardi*. As parasitemia levels increased (< 2%, 2–4% and > 4%), levels of haptoglobin, ceruloplasmin, and fibrinogen increased.

The dynamics of changes in the concentration of fibrinogen in sheep with normal parturition are similar to those in sectioned animals. In all animals, the levels were increased significantly ($P < 0.05$) at 48 hours, returning to baselines at 14 days in the group with normal birth. At the same time, in the group with caesarean section, they were higher compared with initially registered ($P < 0.001$).

Ceruloplasmin, which belongs to the group of minor APPs who react with a slight increase to the stimulus, has also increased since 48 hours, but only in sheep with caesarean section, whereas in normal birth animal, the dynamics remain almost unchanged throughout the study period. In both groups of sheep, the sectioning operation caused a maximal increase in Fb and Cp values on day 10 ($P < 0.001$). Measurements of APPs can be used to evaluate the innate immune system response to pathological injuries and may even be possible to use them as markers to assess the overall health of the herd in farm animals (Meling et al., 2012).

The protein profile has been applied in studies on sheep to identify any pathological conditions during stressful conditions such as parturition and cesarean surgery. It should also be taken into account that albumin is considered as a negative acute-phase protein that responds to stress by lowering levels. In this study, changes in blood protein profiles were observed in sheep with normal parturition and animals with dystocia submitted to caesarean section. According to Gruys et al., (2005) albumin in all animals and humans, birds and fish related to the group of "negative" acute phase proteins, which means that its concentration decreased after inflammation or infection. Hypoalbuminemia is very common in many diseases and results from a disorder in liver synthesis, reduced absorption of amino acids or increased catabolism linked to the turn of amino acids for the synthesis of other proteins (like positive APPs) in liver, or a combination of these factors (Georgieva et al., 2011). Globulins are a group which contain some fractions-α_1, α_2, β and γ. The results obtained show that labor is accompanied with a moderate, but significantly increasing concentration of globulins, decreasing albumin and A/G ratio as well as increasing APPs – haptoglobin, ceruloplasmin and fibrinogen (Georgieva, 2013) and proves that there is an inflammatory process that precedes the clinical signs of difficult birth and/or fetal death. However, in our work, we demonstrated that caesarean section in sheep can be considered as a cause of change in the protein profile. Decreasing albumin values in the operated group in the present study confirm that albumin may be considered as a negative APP in sheep.

The total protein is an indicator with relatively constant values. In different groups of animals, it ranges from 39 g/L to 85 g/L according to some authors, and from 50 g/L to 75 g/L according to others. No significant change in the total protein concentration was observed during the whole study period, both in animals with normal birth and in the operated ones. Likewise, in sheep with caesarean

sections, values of total protein were higher, but not statistically, than those in sheep with normal parturition. This might be explained by producing a large number of APPs by the liver which are present at very low concentrations in a normal state, but during inflammation, trauma or surgical invention, most of them which are related to the group of positive APPs (Jain et al., 2011) increased several times, and could elevate the concentration of total protein in caesarean section sheep, because during operation restricted inflammation is developed. Gürgöze et al. (2009) investigated changes in some biochemical parameters in Awassi ewes throughout pregnancy and postpartum period. Similarly to our results, they reported that during the whole studied period total protein and albumin concentrations were in the reference range for sheep.

We suggest that changes in studied plasma protein parameters must be taken into account when considering the biochemical status of sheep in the days after birth.

In the present study, the result shows the presence of variation in the plasma protein profile and investigated acute phase proteins. A significantly ($P < 0.05$) increasing concentration of globulins, a decreasing concentration of albumin and albumin/globulin ratio were observed in plasma of sheep with dystocia submitted to caesarean section compared with animals with normal parturition. Similarly, it was examined that certain acute phase proteins such as albumin (as negative APP), and fibrinogen and ceruloplasmin (as positive APPs) can be useful as prognostic indicators for monitoring the postpartum period in sheep.

References

1. Aziz D.M., Taha M.B. Effect of dystocia on serum haptoglobin in Awassi ewes. Theriogenology. 1997. T. 48. P. 559–562.
2. Bastos B.L., Meyer R., Guimaraes J., Ayres M.C., Guedes M.T., Moura-Costa L.F., de Burghgrave U.S., Sena L., Azevedo V., Portela R.W. Haptoglobin and fibrinogen concentrations and leukocyte counts in the clinical investigation of caseous lymphadenitis in sheep. Veterinary Clinical Pathology. 2011. T. 40. P. 496–503.
3. Ceciliani G.S. The systemic reaction during inflammation: the acute phase proteins. Protein & Peptide Letters. 2002. T. 9. P. 211-223.
4. Ceciliani F., Ceron J.J., Eckersall P.D., Sauerwein H. Acute phase proteins in ruminants. Journal of Proteomics. 2012. T. 75. P. 4207-423.1.
5. Ceron J.J., Eckersall P.D., Martinez-Subiela S. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Veterinary Clinical Pathoogy. 2005. T. 34. P. 85-99.
6. Costa V.G., Vieira A.D., Schneider A., Rovani M.T., Gonçalves, B.G. Gasperin P.B.D. Systemic inflammatory and stress markers in cattle and sheep submitted to different reproductive procedures. Ciência Rural. 2018. T. 48. e20180336.
7. Cray C., Zaias J., Altman N.H. Acute phase response in animals: a review. Comparative Medicine. 2009. T. 59. P. 517–526.
8. Eckersall P.D. Acute phase proteins as markers of inflammatory lesions. Comparative Haematology International. 1995. T. 5. P. 93-97.
9. Eckersall P.D. Acute phase proteins as markers of infection and inflammation; monitoring animal health, animal welfare and food safety. Irish Veterinary Journal. 2000. T. 53. P. 307-311.
10. Eckersall P.D., Lawson F.P., Bence L., Waterson M.M., Lang T.L., Donachie W. Fontaine M.C. APR in an experimantal model of ovine caseous limphadenitis. BMC Veterinary Research. 2007. T. 3. P. 35.
11. Eckersall P.D., Bell R. APP: biomarkers of infection and inflammation in veterinary medicine. The Veterinary Journal. 2010. T. 185. P. 23-27.
12. Eckersall P.D. Calibration of novel protein biomarker for veterinary clinical pathology: A call for international action. Frontiers in Veterinary Science. 2019. 6:210.
13. Georgieva T., Bassols A., Dishlyanova E., Petrov V., Marutsov P., Dinev I., Georgiev I. Blood haptoglobine response in obese rabbits with experimentally induced *St. aureus*. COST-Farm Animal Proteomics Spring Meeting, Glasgow. 2011. P. 49.
14. Georgieva T.M. Studies on changes in the concentration of moderate acute phase proteins and some biochemical indicators of blood after experimentally caused inflammation in rabbits, chicken and sheep. DSc Thesis, Stara Zagora, Bulgaria. 2013. 187-192 pp.
15. Gruys E., Toussaint M.M., Upragarin N., Van Ederen A.M., Adewuyi A.A., Candiani D., Nguyen T.K.A., Sabeckiene J. Acute phase reactants, challenge in the near future of animal production and veterinary medicine. Journal of Zhejiang University-Science B. 2005. T. 6. P. 941-947.
16. Gürgöze S.Y., Zonturlu A.K., Özyurtlu N., İçen H. Investigation of Some Biochemical Parameters and Mineral Substance During Pregnancy and Postpartum Period in Awassi Ewes. Kafkas Universitesi Veteriner Fakultesi Dergisi. 2009. T. 15. P. 957-963.
17. Jain S., Gautam V., Naseem S. Acute-phase proteins: As diagnostic tool. Journal of Pharmacy and Bioallied Sciences. 2011. T. 3. P. 118–127.
18. Kaya S., Merhan O., Kacar C., Colak A., Bozukluhan K. Determination of ceruloplasmin, some other acute phase proteins, and biochemical parameters in cows with endometritis. Veterinary World. 2016. T. 9. P. 1056-1062.
19. Kaneko J.J. Serum proteins and the dysproteinemias. In: Clinical biochemistry of domestic animals (Ed. Kaneko J.J.), 3rd edition. Academic Press, New York. 1980. P. 97-118.
20. Kolb V.G., Bestujeva, S.V. Determination of the activity of ceruloplasmin in the blood serum by the method of Revin. In: Practical book in Clinical chemistry (Ed. Kolb V.G. and Kamishnikov V.S.), 2nd edition. Belaruss, 1982. P. 219.
21. Meling S., Bårdsen K., Ulvund M.J. Presence of an acute phase response in sheep with clinical classical scrapie. BMC Vetetinary Research. 2012. T. 8. P. 113.
22. Murata H., Shimada N., Yoshioka M. Current research on acute phase proteins in veterinary diagnosis: an overview. The Veterinary Journal. 2004. T. 168. P. 28-40.
23. Petersen H.H., Nielsen J.P., Heegaard P.M.H. Application of acute phase protein measurements in veterinary clinical chemistry. Veterinary Research. 2004. T. 35. P. 163-187.
24. Pfeffer A., Rogers K.M. Acute phase response of sheep: Changes in the concentrations of ceruloplasmin, fibrinogen, haptoglobin and the major blood cell types associated with pulmonary damage. Resarch in Veterinary Science. 1989. T. 46. P. 118–124.
25. Razavi S.M., Nazifi S., Bateni M., Rakhshandehroo E. Alterations of erythrocyte antioxidant mechanisms: Antioxidant enzymes, lipid peroxidation and serum trace elements associated with anemia in bovine tropical theileriosis. Veterinary Parasitology. 2011. T. 180. P. 209–214.
26. Scott P.R., Murray L.D., Penny C.D.A preliminary study of serum haptoglobin concentration as a prognostic indicator of ovine dystocia cases. British Veterinary Journal. 1992. T. 148. P. 351-355.
27. Smits K., Willems S., Steendam K.V., Van De Velde M., De Lange V., Ververs C., Roels K., Govaere J., Van Nieuwerburgh F., Peelman L., Deforce D., Van Soom A. Proteins involved in

embryomaternal interaction around the signalling of maternal recognition of pregnancy in the horse. Scientific Reports. 2018. 8:5249.
28. Szczubiał M., Dąbrowski R., Kankofer M., Bochniarz M., Komar M. Concentration of serum amyloid A and ceruloplasmin activity in milk from cows with subclinical mastitis caused by different pathogens. Polish Journal of Veterinary Sciences. 2012. T. 15. P. 291-296.
29. Teraoka Y., Miyoshi H., Oshima K., Urabe S., Tanaka N., Kudo Y. Prenatal and Peripartum Management of Patients with Hypofibrinogenemia Resulted in Two Successful Deliveries. Case Reports Obstetrics Gynecology. 2017. 2017:9427359.
30. Thomas J.S. Overview of plasma proteins. In: Schalm's Veterinary Hematology (Ed. Feldman B.F., Zinkl J.G. and Jain N.C.), 5th edition, Lippincott Williams, Wilkins, Philadelphia, 2000. P. 891-898.
31. Todorov J. Nephelometric determination of fibrinogen (method of Podmore). In: Clinical Laboratory Techniques (Ed. Todorov J.). Sofia, Bulgaria. Medizina and Fizkultura. 1972. P. 250.
32. Tóthová C., Nagy O., Kovác G. Changes in the concentrations of selected acute phase proteins and variables of energetic profile in dairy cows after parturition. Journal of Applaied Animal Research. 2014. T. 42. P. 278–283.
33. Tóthová C., Nagy O., Kovác G. Serum proteins and their diagnostic utility in veterinary medicine: a review. Veterinarni Medicina. 2016. T. 61. P. 475–496.
34. Ulutas P., Ozpinar A. Effect of *Mannheimia (Pasteurella) haemolytica* infection on acute-phase proteins and some mineral levels in colostrum–breast milkfed or colostrum–breast milk-deprived sheep. Veterinary Research Communications, 2006. T. 30. P. 485–495.

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Effects of Replacement of Corn with Barley or Barley Flake in Diets Containing Different Levels of Metabolizable Energy on Performance, Egg Quality and Serum Parameters of Laying Quails

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Keywords: *metabolizable energy, barley flake, quail, performance, egg quality, serum.*

Abstract. *The current research was carried out to determine the effect of substituting barley or barley flake for corn in diets containing different levels of metabolizable energy on performance, egg quality, and serum parameters in laying quails. In the study, 120 female quails of the age of 22 weeks were randomly distributed to six treatment groups (with four replicates of five quails each) consisting of two metabolizable energy levels (2900 or 2800 kcal/kg) and three forms of cereals (corn, barley or barley flake). As a result of the research, with the reduction of diet metabolizable energy level to 2800 kcal/kg, while feed intake ($P < 0.01$), Haugh unit ($P < 0.01$), b* value of yolk ($P < 0.05$), and serum calcium ($P < 0.05$) and phosphorus ($P < 0.05$) concentrations increased significantly, eggshell thickness ($P < 0.01$) decreased importantly. Besides, the replacement of corn with barley or barley flake in the diets adversely affected egg production, feed intake, and Roche unit, a* and b* values of yolk ($P < 0.01$). Eggshell breaking strength, eggshell weight, and serum phosphorus concentration were statistically decreased in the groups which used barley or barley flake compared with those containing corn. As a result, lowering the metabolizable energy level in the diet increased feed intake, improved egg internal quality, and mineral metabolism, but adversely affected eggshell quality. Furthermore, the replacement of corn with barley negatively affected the performance, and egg quality, but flaking was effective on eliminating the negative effect of barley in eggshell breaking strength.*

Introduction

The use of barley in poultry feeding is substantially limited due to high cellulose, and beta-glucan, a soluble starch, content (Herstad and McNab, 1975). However, barley is used as an energy source in poultry feeding in those regions that are not proper for corn production (Jeroch and Danicke, 1995). Although the addition of beta-glucanase enzyme is a common practice to eliminate the factors limiting the use of barley in poultry diets (Lazaro et al., 2003), there is limited study examining the effects of heat treating on barley. One of these heat treatments is the flaking process, which consists of steaming the grains under high pressure and then pressing and drying. It was reported that the use of barley flake in the diets improves the retention of nutrients and the availability of diet energy in broilers (Gracia et al., 2003), while the use of 30% barley flake in the diet reduces feed intake in laying hens (Gürbüz and Özyürür, 2019). By flaking barley, it can be predicted that the antinutritional factors it contains will decrease and the digestibility of nutrients will increase. Based on these, the hypothesis of the current research is that the effects of barley flake will be better understood by comparing the use of corn and barley in diets with reduced metabolizable energy (ME) levels. For this purpose, this research was conducted to determine the effect of barley flake usage compared with corn and barley in diets containing different levels of ME on performance, egg quality, and serum parameters in laying quails.

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Material and methods

In the experiment, a total of 120 female Japanese quails at the age of 22 weeks were equally distributed to a total of six treatment groups consisting of 2 ME levels and 3 forms of cereals. Each treatment group was constituted of four subgroups with five quails each. During the ten-week experiment, the quails were fed with 6 treatment diets using corn, and barley or barley flake instead of 100% of corn as the cereal source in diets containing 2900 (control, (NRC, 1994)) and 2800 kcal/kg ME (Table 1). During the trial, a 16-hour lighting program was applied, and feed and water were given *ad-libitum* to the quails.

Determination of performance parameters

The mean body weight of the quails was determined using the values obtained by group weighing at the initial and final of the experiment, and body weight change (BWC) was calculated from these means. During the trial, the eggs were recorded daily and egg production (EP) was calculated as %. Feeds were given by weighing to the treatment groups and the feed intake (FI) was calculated as g/day/quail by subtracting the remaining feeds from the total feed at the end of the experiment. Egg weights (EW)

Table 1. Experimental diets and calculated nutrient contents

Ingredients, %	2800 kcal/kg ME			2900 kcal/kg ME	
	Corn	Barley/barley flake		Corn	Barley/barley flake
Corn	60.35	–		58.00	–
Barley or barley flake	–	58.45		–	56.20
Soybean meal	31.00	28.00		31.50	28.60
Soybean oil	1.10	6.05		2.95	7.71
Limestone	5.60	5.66		5.60	5.64
Dicalcium phosphate	1.18	1.06		1.18	1.07
Salt	0.35	0.35		0.35	0.35
Premix[1]	0.25	0.25		0.25	0.25
DL methionine	0.17	0.18		0.17	0.18
Total	100.00	100.00		100.00	100.00
Chemical composition, %					
ME, kcal/kg	2801	2800		2901	2901
Crude protein	20.00	20.00		20.03	20.02
Calcium	2.50	2.51		2.50	2.50
Available phosphorus	0.35	0.35		0.35	0.35
Lysine	1.05	1.05		1.06	1.05
Methionine	0.45	0.45		0.45	0.45
Methionine + cysteine	0.82	0.84		0.82	0.83

[1]**Premix** is supplied per kg of diet: manganese 80 mg, iron 60 mg, copper 5 mg, iodine 1 mg, selenium 0.15 mg, vitamin A 8.800 IU, vitamin D_3 2.200 IU, vitamin E 11 mg, nicotine acid 44 mg, Cal-D-Pan 8.8 mg, riboflavin 4.4 mg, thiamine 2.5 mg, vitamin B_{12} 6.6 mg, folic acid 1 mg, biotin 0.11 mg, choline 220 mg.

were determined as g by weighing all eggs collected in the last three days of the experiment. Egg mass (EM) was calculated as g/day/quail with the formula (EP x EW)/100. Feed conversion ratio (FCR) was calculated as g feed/g egg with the FI/EM formula.

Determination of egg external quality

During the experiment, broken, cracked and damaged eggs were recorded and calculated as % of the total eggs. After determining the weights of all the eggs collected in the last three days of the experiment in air and water, their specific weights were calculated in g/cm^3 with the formula EW/(EW-EW in water). Eggshell breaking strength was measured by applying supported systematic pressure to the blunt of the eggs (Egg Force Reader, Orka Food Technology, Israel). After the eggs were broken on a clean glass table, egg residues in the eggshell were cleaned, then the eggshells were dried for three days at room temperature, and eggshell weights were calculated as % of the egg weights. Eggshell thickness was calculated by taking the average of the values obtained by measuring from three points of the egg (equator, blunt and pointed parts) using a micrometer (Mitutoyo, 0.01 mm, Japan).

Determination of egg internal quality

Once again, the albumen height of these eggs was measured with a height gauge and the Haugh unit was calculated by the formula 100 x log (albumen height + 7.57–1.7 x EW$^{0.37}$) (Haugh, 1934). Egg yolk colour and L *, a * or b * values were measured with Roche colour scale and colorimeter, respectively (Minolta Chromameter CR 400 (Minolta Co., Osaka, Japan) (Romero et al., 2002).

Serum biochemical parameters

At the end of the trial (week 10), 3 mL of blood was taken from one random quail (4 quails from each treatment group) in similar body weights to determine serum parameters. Blood samples were centrifuged at 3000 rpm for 5 minutes. The obtained serum was stored at –20°C until analyzed, and glucose, cholesterol, HDL, creatinine, total protein, albumin, globulin, calcium, and phosphorus concentrations were determined in an auto-analyser using commercial kits (DDS® Spectrophotometric Kits, Diasis Diagnostic Systems Co., İstanbul Turkey).

Statistical analysis

For the experiment was carried out with 4 subgroups in six treatment groups consisting of two different levels of diet ME (2900 kcal/kg and 2800) and 3 different cereal forms, the trial results were analyzed by ANOVA using the General Linear Model (GLM) procedure in Minitab (Minitab, 2000), and differences between the group means were determined by Duncan's multiple range tests (Duncan, 1955).

Results and discussion

Performance parameters

Effect of replacement of corn with barley or barley flake in diets containing different levels of ME on the performance of laying quails is shown in Table 2.

As the main factor, diet ME levels did not affect performance parameters except for FI ($P > 0.05$). Feed intake significantly increased with the reduction of the diet ME level ($P < 0.01$). Similar to the current study, Elangovan et al. (2004) reported that FI augmented by lowering the diet ME level from 2900 kcal/kg to 2700 kcal/kg and 2500 kcal/kg. Contrarily, Lotfi et al. (2018) reported that with the reduction of dietary ME to 2750 kcal/kg, the FI of laying quails decreased compared with 2964 kcal/kg and 3050 kcal/kg ME. Similar results were stated by Freitas et al. (2005) and Barreto et al. (2007) for ME levels of 2585–3050 kcal/kg. In the present study, EP and EW were not affected by diet ME levels. It is clear that quails fed with a diet containing low ME increased feed intake in order to maintain EP and EW. On the other hand, in researches where it was demonstrated that FI diminished with the decrease in dietary ME level, EP (Lotfi et al., 2018) and/or EW (Freitas et al., 2005; Barreto et al., 2007) parameters were also negatively affected by the diet ME levels. This situation is important to explain the differences among studies. Moreover, it is claimed that the treatment diets and the ME levels of these diets, as well as the genetic structures of the birds, affect these differences.

While the effects of the cereal form on diets of laying quails were important on EP ($P < 0.05$) and FI ($P < 0.01$), this effect was not observed on other performance parameters ($P > 0.05$). With the substituting barley or barley flake for corn in the diet, EP and FI considerably decreased and there was no difference between barley or barley flake in terms of these parameters. These mentioned results disagree with Jamroz et al. (2001), Lazaro et al. (2003), Herbert et al. (2011) and Kılıç and Olgun (2021) who indicated that the EP and FI were not affected by the replacement of corn by barley. The crude fiber content of barley is higher than that of corn; hence it is known that feeding birds with barley-based diets causes them to not consume enough feed due to their digestive system capacity (Jeroch and Danicke, 1995). In the current study, the decrease in FI of quails fed barley or barley flake-based diets can be due to the high

Table 2. Effect of replacement of corn with barley or barley flake in diets containing different levels of ME on the performance in laying quails

ME, kcal/kg	Cereal form	BWC, g	EP, %	EW, g	EM, g/d/quail	FI, g/d/quail	FCR, g feed/g egg
2900		2.71	87.55	13.69	11.98	32.10^{B}	2.69
2800		–2.12	87.53	13.72	12.00	33.86^{A}	2.83
SEM		3.024	1.460	0.221	0.247	0.582	0.066
	Corn	–1.81	91.11^{a}	13.37	12.17	35.41^{A}	2.92
	Barley	0.81	85.13^{b}	14.01	11.92	31.86^{B}	2.68
	Barley flake	1.88	86.37^{b}	13.74	11.88	31.68^{B}	2.69
	SEM	3.739	1.544	0.673	0.049	0.464	0.075
2900	Corn	0.00	89.91	13.61	12.24	33.82	2.77
2900	Barley	3.25	83.93	14.03	11.76	31.25	2.67
2900	Barley flake	4.89	88.79	13.42	11.93	31.22	2.63
2800	Corn	–3.63	92.31	13.12	12.10	36.99	3.07
2800	Barley	–1.62	86.33	13.99	12.07	32.46	2.69
2800	Barley flake	–1.12	83.96	14.06	11.83	32.12	2.74
SEM		5.176	2.100	0.759	0.440	0.479	0.097
Probabilities, $P \leqslant$							
ME		0.330	0.994	0.903	0.950	<0.001	0.114
Cereal form		0.816	0.030	0.260	0.793	<0.001	0.427
Interactions		0.980	0.184	0.345	0.867	0.070	0.427

BWC: Body weight change, EP: Egg production, EW: Egg weight, EM: Egg mass, FI: Feed intake, FCR: Feed conversion ratio. SEM: Standard error means.
$^{A, B}$Within a column, values not sharing a common letter are statistically different; $P < 0.01$.
$^{a, b}$Within a column, values not sharing a common letter are statistically different; $P < 0.05$.

fiber content of barley. At the same time, in the status of the low FI, the nutrients required for EP can be deficient. . The flaking process increases the viscosity of barley in the digestive tract of broilers (Gracia et al., 2003). This can be the probable reason why barley flake is incapable of eliminating this negativity in FI and EP. The interaction groups formed by the dietary ME levels and cereal forms did not statistically affect the performance of the laying quails ($P > 0.05$).

Egg external quality

Effect of substituting barley or barley flake for corn in diets containing different levels of ME on egg external quality of laying quails demonstrated is in Table 3.

The cracked eggs, eggshell breaking strength, eggshell weight, and specific weight were not affected by the dietary ME levels as the main factor, but the eggshell thickness significantly decreased with the reduction of diet energy from 2900 kcal/kg to 2800 kcal/kg ($P < 0.01$). Congruently with the current research, Elangovan et al. (2004) and Lotfi et al. (2018) reported that the eggshell thickness was negatively affected by the reduction of diet energy (2700 kcal/kg and 2750 kcal/kg ME). However, Hurtado-Nery et al. (2015) and Agboola et al. (2016) stated that dietary ME level (2750–3200 kcal/kg) did not affect quails. Also, similar results were obtained in laying hens (Junqueira et al., 2006; Wu et al., 2008).

While the cereal form as the main factor considerably affected the eggshell breaking strength ($P < 0.05$) and eggshell weight ($P < 0.01$) among the egg external quality traits, this effect was found to be unimportant in the other eggshell quality parameters ($P > 0.05$). The eggshell breaking strength decreased significantly in the group which used barley as a cereal form in the diet compared with the groups that used corn and barley flake. The eggshell weight reduced with the use of barley in the diet compared with the group containing corn, but the eggshell weight of the group using barley flake was similar to the other groups. In certain studies related to the subject, Jamroz et al. (2001) and Perez-Bonilla et al. (2011) indicated that the use of barley in laying hen diets (66% and 45%, respectively) did not affect eggshell breaking strength and eggshell weight. Beta-glucan in barley can reduce the bioavailability of some minerals such as calcium, which are important in eggshell formation, by increasing viscosity (Cardoso et al., 2014) in the digestive tract. The fact that groups fed with barley-based diets showed lower eggshell breaking and eggshell weight than groups fed with

Table 3. Effect of replacement of corn with barley or barley flake in diets containing different levels of ME on the egg external quality in laying quails

ME, kcal/kg	Cereal form	Cracked egg, %	Eggshell breaking strength, kg	Eggshell weight, % of EW	Eggshell thickness, µm	Specific weight, g/cm^3
2900		0.51	1.37	8.16	195.3^{A}	1.071
2800		1.54	1.47	8.14	188.3^{B}	1.072
SEM		0.621	0.048	0.155	1.24	0.0012
	Corn	0.56	1.47^{a}	8.39^{A}	189.9	1.073
	Barley	0.51	1.29^{b}	7.79^{B}	194.6	1.069
	Barley flake	2.00	1.50^{a}	8.27^{AB}	190.8	1.072
	SEM	0.673	0.049	0.162	1.91	0.0014
2900	Corn	0.43	1.34	8.14^{AB}	194.5	1.072
2900	Barley	0.72	1.27	7.63^{B}	198.5	1.068
2900	Barley flake	0.38	1.49	8.70^{A}	192.8	1.073
2800	Corn	0.70	1.59	8.65^{A}	185.3	1.074
2800	Barley	0.30	1.31	7.95^{B}	190.8	1.071
2800	Barley flake	3.64	1.52	7.84^{B}	188.8	1.071
SEM		0.759	0.065	0.178	1.74	0.0020
Probabilities, $P \leqslant$						
ME		0.356	0.076	0.935	<0.001	0.852
Cereal form		0.461	0.012	0.010	0.068	0.254
Interactions		0.363	0.231	0.003	0.425	0.473

SEM: Standard error means.
$^{A, B}$Within a column, values not sharing a common letter are statistically different; $P < 0.01$.
$^{a, b}$Within a column, values not sharing a common letter are statistically different; $P < 0.05$.

corn-based diets can be due to this. Gürbüz and Özyürür (2019) stated that the use of barley flake in the diet did not affect eggshell breaking strength and eggshell weight. The heat and pressure applied during the flaking process could have possibly advanced the availability of calcium required for eggshell formation. This situation can be the reason for the effectiveness of barley flake in reducing/eliminating its negative effect on eggshell quality.

Interactions of dietary ME levels and the cereal forms only affected eggshell weight ($P < 0.01$) but did not affect other parameters ($P > 0.05$). The highest eggshell weight was observed in the group that used barley flake with 2900 kcal/kg ME in the diet, and the differences between this group and the groups that used barley with 2900 kcal/kg ME and barley flake with 2800 kcal/kg ME were significant.

Egg internal quality

Effect of replacement of corn with barley or barley flake in diets containing different levels of ME on the performance of laying quails is shown in Table 4.

As the main factor, the dietary ME levels did not affect the Roche unit, L* and a* values statistically ($P > 0.05$), but the Haugh unit ($P < 0.01$) and b* value ($P < 0.05$) were considerably affected. The Haugh unit advanced significantly with the reduction of the diet energy level from 2900 kcal/kg to 2800 kcal/kg ME. Whereas these results agree with the reports of Elangovan et al. (2004) and Perez-Bonilla et al. (2011), they disagree with Lotfi et al. (2018) and Gunawardana et al. (2009) who stated that the diet ME level did not affect the Haugh unit. Increasing the amount of protein and amino acids consumed improves the Haugh unit (Wu et al., 2005). In the current study, an advance in Haugh units could have occurred as the low diet ME level increased the amount of protein and amino acids consumed with risen FI. The b* value significantly improved with the reduction of a dietary energy level from 2900 kcal/kg to 2800 kcal/kg ME. In the literature, there is no research examining the effect of diet ME level on the b* value of yolk in previous years. In the present study, the increase in the yolk b* value can be explained by the fact that corn used in the diet containing low ME is more than that one containing high ME.

The effect of substituting barley or barley flake for corn in diets on the Roche unit, a* and b* values was significant ($P < 0.01$), but this effect was unimportant in the Haugh unit and L* value ($P > 0.05$). With the replacement of corn by barley or barley flake in

Table 4. Effect of replacement of corn with barley or barley flake in diets containing different levels of ME on the egg internal quality in laying quails

ME, kcal/kg	Cereal form	Haugh unit	Roche unit	L*	a*	b*
2900		62.88^{B}	2.75	52.74	–1.707	17.41^{b}
2800		71.35^{A}	3.05	52.18	–1.692	19.02^{a}
SEM		1.307	0.5212	0.3673	0.2812	1.503
	Corn	68.32	5.28^{A}	51.77	–0.428^{A}	24.94^{A}
	Barley	66.98	1.82^{B}	52.46	–2.303^{B}	15.36^{B}
	Barley flake	66.04	1.61^{B}	53.15	–2.368^{B}	14.35^{B}
	SEM	2.274	0.1743	0.4273	0.0955	0.626
2900	Corn	64.33	5.41	52.46	–0.353	23.45
2900	Barley	63.27	1.51	52.56	–2.318	14.31
2900	Barley flake	61.05	1.35	53.19	–2.450	14.47
2800	Corn	72.31	5.14	51.07	–0.503	26.42
2800	Barley	70.70	2.14	52.37	–2.288	16.41
2800	Barley flake	71.04	1.88	53.11	–2.285	14.23
SEM		2.160	0.2163	0.5907	0.1328	0.694
Probabilities, $P \leqslant$						
ME		0.001	0.125	0.287	0.898	0.026
Cereal form		0.657	<0.001	0.109	<0.001	<0.001
Interactions		0.862	0.118	0.510	0.547	0.154

SEM: Standard error means.
$^{A, B}$Within a column, values not sharing a common letter are statistically different; $P < 0.01$.
$^{a, b}$Within a column, values not sharing a common letter are statistically different; $P < 0.05$.

the diets of laying quails, the Roche unit, a* and b* value decreased considerably ($P < 0.01$). Nonetheless, similar results were obtained in the barley and barley flake groups in terms of these parameters. The results of research conducted by Perez-Bonilla et al. (2011) on this subject showed that the use of 45% barley in the diet reduced the yolk colour. Similarly, Herbert et al. (2011) reported that the yolk colour decreased with the use of 57.7% barley in laying hen diets. Moreover, research results stated by Kılıç and Olgun (2021) are in harmony with the current results.

Serum biochemical constituents

Effect of substituting barley or barley flake for corn in diets containing different levels of ME on serum biochemical constituents of laying quails is demonstrated in Table 5.

Serum glucose, cholesterol, HDL, creatinine, total protein, albumin, and globulin concentrations were not affected in the treatment groups ($P > 0.05$).

Serum calcium and phosphorus concentrations were significantly raised with the reduction of dietary ME as the main factor ($P < 0.05$). According to Table 2, decreasing the diet ME level increased FI. That is, FI was increased by the low dietary ME level that could lead to improvement in serum calcium and phosphorus concentrations. Besides, less vegetable oil was used in the low ME diet compared with the control diet. Increasing the oil level in the diet ascends the saponification between fatty acids and calcium, and so decreases the availability of calcium (Atteh et al., 1989; Tancharoenrat et al., 2014). In connection with the information above mentioned, the low amount of oil in the diet possibly has increased the availability of calcium and phosphorus.

The replacement of corn with barley or barley flake in the diet considerably affected the serum phosphorus concentration of the laying quails ($P < 0.05$), but the other parameters were not statistically affected ($P > 0.05$). Serum phosphorus concentrations of the group with barley in the diet were significantly lower than the groups that consisted of corn and barley flake. Water-soluble non-starch polysaccharides such as beta-glucan and xylan increase intestinal viscosity and negatively affect the bioavailability of minerals in birds (Van der Klis et al., 1993; Kiarie et al., 2014).

Table 5. Effect of replacement of corn with barley or barley flake in diets containing different levels of ME on serum biochemical constituents in laying quails

ME, kcal/kg	Cereal form	Glucose, mg/dL	Cholesterol, mg/dL	HDL, mg/dL	Creatinine, mg/dL	Total protein, g/dL	Albumin, g/dL	Globulin, g/dL	Calcium, mg/dL	Phosphorus, mg/dL
2900		336	153	49.78	0.348	4.53	1.44	3.09	22.00^{b}	4.93^{b}
2800		329	150	45.94	0.343	4.46	1.43	3.03	24.76^{a}	5.93^{a}
SEM		5.1	7.7	2.599	0.0080	0.165	0.052	0.126	0.982	0.374
	Corn	327	161	44.63	0.330	4.60	1.40	3.20	23.49	6.28^{a}
	Barley	340	138	52.78	0.361	4.28	1.39	2.89	23.08	4.44^{b}
	Barley flake	331	160	46.18	0.344	4.61	1.51	3.10	23.58	5.59^{a}
	SEM	5.9	9.5	2.974	0.0089	0.198	0.060	0.151	1.291	0.408
2900	Corn	331	161	50.20	0.345	4.95	1.48	3.48	25.28^{AB}	5.93
2900	Barley	347	138	52.03	0.365	4.00	1.28	2.73	18.58^{C}	3.63
2900	Barley flake	331	160	47.10	0.333	4.65	1.58	3.08	22.15^{BC}	5.25
2800	Corn	322	161	39.05	0.315	4.25	1.33	2.93	21.70^{BC}	6.63
2800	Barley	334	156	53.53	0.358	4.55	1.50	3.05	27.58^{A}	5.25
2800	Barley flake	331	133	45.25	0.355	4.58	1.45	3.13	25.00^{AB}	5.93
SEM		8.5	12.4	3.935	0.0113	0.257	0.075	0.198	1.065	0.530
Probabilities, $P \leqslant$										
ME		0.354	0.813	0.286	0.619	0.733	0.801	0.732	0.009	0.039
Cereal form		0.329	0.502	0.157	0.058	0.374	0.253	0.321	0.898	0.012
Interactions		0.766	0.276	0.331	0.122	0.089	0.054	0.121	<0.001	0.626

SEM: Standard error means.
$^{A, B, C}$Within a column, values not sharing a common letter are statistically different; $P < 0.01$.
$^{a, b}$Within a column, values not sharing a common letter are statistically different; $P < 0.05$.

In the research using barley flake, it is indicated that barley flake increases viscosity and also improves nutrient retention (Gracia et al., 2003). Thus, the flaking process might have improved the availability of phosphorus. Possibly this effect on phosphorus availability can be due to the phytic acid disruption during the flaking process, but this needs to be investigated in detail.

The effect of interactions between dietary ME levels and the cereal forms on serum calcium concentration in laying quails was statistically important ($P < 0.01$), but this effect was not observed in other serum parameters ($P > 0.05$). The serum calcium concentration in the interaction group containing 2800 kcal/kg ME with barley in the diet was found significantly higher than in the groups containing 2900 kcal/kg ME with barley and barley flake and 2800 kcal/kg ME with corn.

Conclusions

As a result, reduction of the ME level in laying quail diets increased feed intake, improved the internal quality of an egg, as well as serum calcium and phosphorus concentrations, but the eggshell quality was deteriorated. Moreover, the total replacement of corn with barley or barley flake negatively affected the performance, eggshell quality, and yolk colour traits, while flaking of barley was efficient in preventing the negative effect of barley on the eggshell breaking strength; this effect was not observed in other parameters.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict in interest.

References

1. Agboola A. F., Omidiwura B. R. O., Ologbosere D. Y., Iyayi E. A. Determination of crude protein and metabolisable energy of Japanese quail (*Coturnix coturnix japonica*) during laying period. Journal of World Poultry Research. 2016. 6. P. 131-138.
2. Atteh J. O., Leeson S., Summers J. D. Effects of dietary sources and levels of fat on performance, nutrient retention and bone mineralization of broiler chicks fed two levels of calcium. Canadian Journal of Animal Science. 1989. 69. P. 459–67.
3. Barreto S. L. T., Quirino B. J. S., Brito C. O., Umigi R. T., Araujo M. S., Coimbra J. S. R., Rojas E. E. G., Freitas J. F., Reis R. S. Metabolizable energy levels for Japanese quails in the initial laying phase. Revista Brasileira de Zootecnia. 2007. 36. P. 79–85.
4. Cardoso V., Ferreira A. P., Costa M., Ponte P. I. P., Falcão L., Freire J. P., Lordelo M.M., Ferreira L.M.A., Fontes C.M.G.A., Ribeiro T. Temporal restriction of enzyme supplementation in barley-based diets has no effect in broiler performance. Animal Feed Science and Technology. 2014. 198. P. 186-195.
5. Duncan D. B. Multiple range and multiple F test. Biometrics.11. P. 1-42.
6. Elangovan A. V., Mandal A. B., Tyagi P. K., Tyagi P. K., Toppo S., Johri T. S. Effects of enzymes in diets with varying energy levels on growth and egg production performance of Japanese quail. Journal of the Science of Food and Agriculture. 2004. 84. P. 2028-2034.
7. Freitas A. C. D., Fuentes M. D. F. F., Freitas E. R., Sucupira F. S., Oliveira B. C. M. D. Dietary crude protein and metabolizable energy levels on laying quails performance. Revista Brasileira de Zootecnia. 2005. 34. P. 838-846.
8. Gracia M. I., Latorre M. A., Garcia M., Lazaro R., Mateos, G. G. Heat processing of barley and enzyme supplementation of diets for broilers. Poultry Science. 2003. 82. P. 1281-1291.
9. Gunawardana P., Roland Sr D. A., Bryant M. M. Effect of dietary energy, protein, and a versatile enzyme on hen performance, egg solids, egg composition, and egg quality of Hy-Line W-36 hens during second cycle, phase two. Journal of Applied Poultry Research. 2009. 18. P. 43-53.
10. Gürbüz Y., Özyürür O. The effect of different technologically treated and multi-enzyme addition barley in egg rations on egg yield, egg quality and egg yolk fatty acids. XI. International Animal Science Conference, 20-22 October 2019, Cappadocia, Turkey,26-32 pp.
11. Haugh R. R. The Haugh unit for measuring egg quality. US Egg Poultry Magazine. 1937. 43. P. 522-555.
12. Hebert K., Tactacan G.B., Dickson T.M., Guenter W. House J.D. The effect of cereal type and exogenous enzyme use on total folate content of eggs from laying hens consuming diets supplemented with folic acid. Journal Applied Poultry Research. 2011. 20. P. 303-312.
13. Herstad O., McNab J. M. The effect of heat treatment and enzyme supplementation on the nutritive value of barley for broiler chicks. British Poultry Science. 1975. 16. P. 1-8.
14. Hurtado-Nery V. L., Torres-Novoa D. M., Daza-Garzón M. F. The effect of crude protein and metabolisable energy levels on quail egg quality. Orinoquia. 2015. 19. P. 195-202.
15. Jamroz D., Skorupinska J., Orda J., Wiliczkiewicz A., Klünter A.M. Use of wheat, barley or triticale in feed for laying hens supplemented with carbohydrases derived from *Trichoderma longibrachiatum*. Journal of Applied Animal Research. 2001. P. 107-116.
16. 16. Jeroch H., Danicke S. Barley in poultry feeding: a review. World's Poultry Science Journal. 1995. 51. P. 271-291.
17. Junqueira O. M., De Laurentiz A. C., da Silva F. R., Rodrigues E. A., Casartelli E. M. C. Effects of energy and protein levels on egg quality and performance of laying hens at early second production cycle. Journal of Applied Poultry Research. 2006. 15. P. 110-115.
18. Kılıç H. N., Olgun O. Effect of enzyme supplementation of maize:barley based diets on the performance, egg quality, serum and bone mineral contents in laying quails. Ziraat Mühendisliği. 2021. 371. P. 74-86.
19. Kiarie E., Romero L.F., Ravindran, V. Growth performance, nutrient utilization, and digesta characteristics in broiler chickens fed corn or wheat diets without or with supplemental xylanase. Poultry Science. 2014. 93. P. 1186-1196.
20. Lázaro R., Garcia M., Medel P., Mateos G. G. Influence of enzymes on performance and digestive parameters of broilers fed rye-based diets. Poultry Science. 2003. 82. P. 132-140.
21. Lotfi E., Karimi N., Parizadian K. B., Sharifi M. R. Influence of different dietary levels of energy and protein on reproductive and post hatch growth performance in Japanese quails. Iranian Journal of Applied Animal Science. 2018. 8. P. 137-145.
22. Minitab. Minitab statistical software. Minitab Release. 2000. 13.
23. NRC. Nutrient requirements of poultry.9th ed. National Academy Press.1994. Washington DC, USA.
24. Perez-Bonilla A., Frikha M., Mirzaie S., Garcia J., Mateos, G. Effects of the main cereal and type of fat of the diet on productive performance and egg quality of brown-egg laying hens from 22 to 54 weeks of age. Poultry Science. 2011. 90. P. 2801-2810.
25. Romero C., Brenes M., Garcia P., Garrido A. Hydroxytyrosol 4-β-D-glucoside, an important phenolic compound in olive fruits and derived products. Journal of Agricultural and Food Chemistry. 2002. 50. P. 3835–3839.
26. Tancharoenrat P., Ravindran V. Influence of tallow and

calcium concentrations on the performance and energy and nutrient utilization in broiler starters. Poultry Science. 2014.93. P. 1453–62.
27. Van der Klis J.D., Verstegen M.W.A., Van Voorst A. Effect of a soluble polysaccharide (carboxy methyl cellulose) on the absorption of minerals from the gastrointestinal tract of broilers. British Poultry Science. 1993. 34. P. 985–997.
28. Wu G., Bryant M. M., Voitle R. A., Roland Sr. D. A. Effect of dietary energy on performance and egg composition of Bovans white and Dekalb white hens during phase I. Poultry Science. 2005. 84. P. 1610–1615.
29. Wu G., Gunawardana P., Bryant M. M., Roland Sr D. A. Influence of dietary energy and antibiotic on performance, egg solids, and egg quality in Bovans White and Dekalb White hens. Journal of Applied Poultry Research. 2008. 17. P. 323-330.

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Comparison of Cytology and Cultural Examination and Intradermal Test Results in Atopic Dogs with Evidence of *Malassezia Pachydermatis*

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Keywords: *dogs, atopic dermatitis, cytology and cultural examination, Malassezia pachydermatis, hypersensitivity*

Abstract. *The purpose of this study was to determine association between cytological and/or cultural examination in atopic dogs with evidence of Malassezia pachydermatis; association regarding the results of examination and age, sex, breed, onset of symptoms of atopic dermatitis, intradermal test (IDT) results to common allergens and to M. pachydermatis allergen was also analyzed. Thirty-seven atopic dogs with Malassezia evidence were evaluated. Malassezia yeast was detected in 9 of 37 (24.32%) dogs by cytology (Group I); 12 of 37 (32.43%) dogs were culturally positive to M. pachydermatis (Group II), and M. pachydermatis was evidenced by cytology and culture in 16 of 37 (43.24%) dogs ($P > 0.05$). Purebred dogs were in the greater number in Groups II and III. The hypersensitivity to the house dust and house dust allergen group was considered as most common in all three groups of dogs, 8 (88.88%), 12 (100%) and 13 (81.25%), respectively. In Group II and III, the greater number of dogs were with pruritus than without it ($P < 0.05$). In Group I, the greater number of dogs were with a positive IDT to M. pachydermatis allergen and with pruritus (margin of significant difference; $P = 0.056$). Dogs with a positive IDT to M. pachydermatis allergen were in the greater number (7 of 14) positive by culture, while dogs with a negative IDT to M. pachydermatis allergen were in the greater number (11 of 23) positive by cytology and culture; there was no statistically significant difference found. It is important to control presence of Malassezia yeast in dogs with atopic dermatitis to minimize the risk of sensitization to M. pachydermatis allergens, since the low number of yeast cells may cause hypersensitivity reactions in dogs predisposed to development of atopic dermatitis.*

Introduction

Malassezia pachydermatis yeast is considered as part of normal cutaneous microflora of most warm-blooded animals (Guillot and Bond, 1999; Bond et al., 2020; Di Tomaso et al., 2021). Also, this yeast can act as an opportunistic pathogen, whenever alteration of skin surface microclimatic conditions or host defense occurs (Guillot and Bond, 1999; Negre et al., 2009; Oldenhoff et al., 2014; Bond et al., 2020). Some conditions may predispose dogs to *M. pachydermatis* overgrowth; atopic dermatitis (AD) is one of those (Kim et al., 2007; Bond et al., 2020). In hypersensitivity diseases, such as atopy, the proliferation of yeasts is suspected to be promoted by excessive sebum production or disruption of the epidermal barrier (Bond et al., 1996). Analysis of skin swab samples from healthy, naturally affected allergic, and experimentally sensitized atopic dogs by using next generation sequencing (NGS) and quantitative real-time PCR (qPCR) methods has shown that *M. pachydermatis* was more abundant on naturally affected allergic skin (by next generation sequencing-NGS) and on allergen induced skin lesions (by quantitative real-time PCR-qPCR) (Meason-Smith et al., 2019).

A cytology evidence of *Malassezia* overgrowth is a common finding in dogs with AD (Kim et al., 2007), while routine cultures provide primarily qualitative data on presence or absence of yeast (Bond et al., 2020). Using the culture technique has been shown to be more sensitive than both the cytological tape and the dry swab staining method in identifying the presence of *Malassezia* on the skin (Omodo-Eluk et al., 2003). Therefore, in case of negative cytology results, a culture examination of samples should be performed to rule out the suspicion of *Malassezia* infections in animals with otitis or dermatitis (Cafarchia et al., 2005). Furthermore, molecular techniques are pivotal in the accurate identification of many currently recognized *Malassezia* species, with the usual exception of *M. pachydermatis* (Bond et al., 2020).

It is known that the presence of *Malassezia* yeasts on the skin, both in normal and excessive numbers, activates the skin immune system in dogs and cats (Grice and Dawson, 2017). *Malassezia* antigens can stimulate innate, antibody and cell mediated immune responses, as well as trigger hypersensitivity reactions

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(Bond et al., 2010). Furthermore, continuous interactions with the host immune system will maintain low numbers of the yeast without generating a clinically appreciable inflammatory response (Bond et al., 2020; Guillot and Bond, 2020). Thus, the commensal presence of *M. pachydermatis* or cutaneous disease caused by this yeast is not just a consequence of a particular number or density of yeast cells within the stratum corneum, but also involves complex interactions between yeast and host (Bond et al., 2020).

The suggested mechanism for sensitization of atopic dogs to *Malassezia* yeast is epicutaneous contact with allergens produced by yeast which induces atopic cascade (Farver et al., 2005). In dogs with *Malassezia* dermatitis, immunological hyper-responsiveness can be present as none, immediate, delayed and contact (Bond et al., 2020). Serological and intradermal tests are tests for immediate hypersensitivity (Marsella et al., 2012; Bond et al., 2020; Di Tomaso et al., 2021). A greater chance for sensitization is expected in dogs with an increased number of yeasts on the skin (Farver et al., 2005). However, this is not always the rule, as dogs with no evidence of *Malassezia* on the skin can react to an IDT (Farver et al., 2005). Due to the fact that serological and cutaneous test reactivity can occur in some unaffected dogs, these immunological tests must be assessed in the context of clinical and cytological data, and should not be used as individual diagnostic tests (Bond et al., 2020). It should also be mentioned that in dogs with hypersensitivity to *Malassezia* allergens few yeasts may elicit pruritus and associated skin lesions (Hensel et al., 2015).

The purpose of this study was to determine whether there is an association between cytological and/or cultural presence of the yeast and age, sex, breed, onset of symptoms of AD, and intradermal test (IDT) results to common allergens and to *M. pachydermatis* allergen in atopic dogs with *Malassezia* evidence.

Material and methods

Thirty-seven atopic dogs (20 female and 17 male dogs) with cytological or/and cultural evidence of *Malassezia* yeast were included in this study. The average age was 4.3 years (16 dogs up to 3 years and 21 dogs over 3 years of age). Twenty-nine dogs were purebred and eight dogs were crossbreed. The diagnosis of AD was based on history, clinical signs, exclusion of food allergy, ectoparasites and other pruritic diseases, and presence of positive intradermal tests (IDT) (Prelaud et al., 1998; Hensel et al., 2015). The seasonality of the first onset of signs was recorded. Pruritus intensity was assessed by the owners as no pruritus, mild, moderate and severe (Rybnicek et al., 2008). Intradermal tests were performed on all included dogs with 15 allergens (Greer, USA), as well as positive (histamine-0.0275 mg/mL) and negative (diluent) controls according to the manufacturer's instructions. For data analysis, allergens were grouped as follows: 1 – house dust and dust mite; 2 – tree pollen (pine mix; eastern seven tree mix); 3 – grass and weed pollen (plantain-sorrel; seven grass mix; ragweed mix); 4 – fungi (*Trichophyton mentagrophytes*; mold mix); 5 – insects (house fly; flea antigen; *Culicoides*); 6 – epithelia and feathers (cat epithelia allergen; feather allergens) and 7 – *Malassezia pachydermatis*.

For the detection of *Malassezia* yeasts, samples from ear external canals (n = 72) and skin (including legs, paws, back, head, neck, tail, chest, abdomen, hips, groin, axillae, inguinal and genital region) (n = 70) were collected from 37 dogs, using sterile cotton swabs. The skin samples were collected from different areas and in a different number for each dog. All samples were examined by cytology and culture. Gram's stained slide smears were used for cytology examination. Five random fields were examined under an oil immersion objective (x 1000 high power field). Yeast cells were characterized according to their morphology compatible to *Malassezia* yeast. Evaluation of cytology examination was done according to Nascente et al. (2015); absence of yeast cells per field was considered negative, while presence of one and more cells per field was considered positive. Since *M. pachydermatis* is the most common *Malassezia* species isolated from the skin and ear canal in dogs (Matousek and Campbell, 2002; Bond et al., 2020), evaluation of cultural examination was performed based on its growth on *Sabouraud's Dextrose Agar* with *Chloramphenicol*. *M. pachydermatis* was identified by macro- and micro morphology characteristics and ability to hydrolyze *Christensen's* urea medium (Warren and Shadomy, 1991; Guillot and Bond, 1999; Girao et al., 2006; Čonkova et al., 2011). Dogs in which the presence of yeast from at least one sampling site was detected by cytological and/or cultural examination are designated as dogs with evidence of *Malassezia* yeast.

Statistical analysis. Fisher's exact test and Λ^2 were used for analysis of correlation between cytological and/or cultural examination results regarding age, sex, breed, onset of symptoms of atopic dermatitis, and intradermal test (IDT) results to common allergens and to *M. pachydermatis* allergen. A probability value of $\leqslant 0.05$ was considered statistically significant.

Results

Among 37 included dogs, the greatest number (29 dogs) was purebred ($P < 0.05$). According to data obtained from the owners, the intensity of pruritus was evaluated as follows: mild in 2 dogs, moderate in 12 dogs, severe in 11 dogs, while 7 dogs showed no signs of pruritus. The owners could not determine intensity of pruritus in 5 dogs. The onset of signs was noted in 18 dogs in summer-spring, in 7 dogs in autumn-winter, and non-seasonally in 12 dogs.

According to cytology and cultural examination results, the dogs were arranged into three groups.

Group I comprised dogs with positive cytology and negative cultural examination to *Malassezia* yeast. Group II consisted of dogs with positive cultural and negative cytology examination to *M. pachydermatis*. Group III included dogs with positive cytology and cultural examination to *M. pachydermatis* (Table 1). Furthermore, Table 1 shows the results of cytological and cultural examination of atopic dogs with evidence of *M. pachydermatis* regarding the compared parameters.

In this study, 9 of 37 (24.32%) dogs had cytological presence of typical *Malassezia* yeast (Group I); *M. pachydermatis* were isolated from 12 of 37 dogs (32.43%) (Group II); and 16 of 37 (43.24%) of the examined dogs were positive both cytological and cultural (Group III) ($P > 0.05$). Among 37 dogs with evidence of *Malassezia* yeast, 14 (37.8%) dogs were IDT positive and 23 (62.2%) were IDT negative to *M. pachydermatis* allergen ($P > 0.05$).

In Group I, the greatest number of dogs had a positive IDT to the house dust and house dust mite group of allergens than to other tested groups of allergens ($P < 0.05$). A greater number of dogs was noted with a negative IDT to *M. pachydermatis* allergen, as well as with pruritus (margin of the significant difference; $P = 0.056$).

In Group II, a greater number of dogs were characterized as follows: dogs were purebred, had spring-summer onset of signs rather than autumn-winter, had a positive IDT to the house dust and house dust mite group of allergens rather than to other tested allergen groups, and to the grass and weed pollen allergen group rather than to epithelia and feather allergen group, had a positive IDT to the grass and weed pollen allergen group and to *M. pachydermatis* allergen than to the epithelia and feather group of allergens, and had pruritus ($P < 0.05$).

In Group III, a greater number of dogs were purebred ($P < 0.05$). The dogs were IDT positive in a greater number to the house dust and house dust mite allergen group than to other tested allergen groups (except to grass and weed pollen and tree pollens allergen group). Also, more dogs were with pruritus than without it ($P < 0.05$).

Age and sex predisposition were not found in any group of dogs ($P > 0.05$). Dogs with a positive IDT to *M. pachydermatis* allergen were in a higher number (7 of 14 dogs) positive by culture, while dogs with a negative IDT to *M. pachydermatis* allergen were positive in a higher number (11 of 23 dogs) by cytology and cultural, but no significant difference was found.

Table 1. Comparison of atopic dogs with evidence of *Malassezia* yeast

Parameter (number of dogs)		Group I (n = 9)	Group II (n = 12)	Group III (n = 16)
Breed	Purebred (n = 29)	4	12	13
	Crossbred (n = 8)	5	0	3
Sex	Male (n = 17)	5	6	6
	Female (n = 20)	4	6	10
Age	Up to 3 years of age (n = 16)	4	4	7
	Over 3 years of age (n = 21)	5	8	9
Seasonality	Spring-summer (n = 18)	4	7	7
	Autumn-winter (n = 7)	3	1	2
	Non-seasonally (n = 12)	2	4	6
Pruritus	With pruritus (n = 30)	7	11	12
	Without pruritus (n = 7)	2	1	4
Intradermal test (IDT)	House dust and house dust mite (n = 32)	8	12	13
	Grass and weed pollen (n = 17)	4	7	6
	Tree pollen (n = 15)	3	6	6
	Fungi (n = 9)	2	2	5
	Insects (n = 11)	1	3	7
	Epithelia and feathers (n = 7)	2	1	4
IDT to *M. pachydermatis*	Positive (n = 14)	2	7	5
	Negative (n = 23)	7	5	11

Group I – positive cytology and negative cultural examination to *Malassezia* yeast;
Group II – positive cultural and negative cytology examination to *M. pachydermatis*;
Group III – positive cytology and cultural examination to *M. pachydermatis*.

Discussion

M. pachydermatis is most frequently isolated *Malassezia* species from healthy dogs and those with disease (Matousek and Campbell, 2002; Cafarchia et al., 2005; Bond et al., 2020). It is a complicating factor in many dermatological diseases (Matousek and Campbell, 2002) which plays an important role in developing AD in dogs (Sihelska et al., 2017). In dogs with clinical signs consistent with *Malassezia* dermatitis, both cytological evaluation and tests for hypersensitivity may be useful (Oldenhoff et al., 2014).

In the present study, in a greater number of dogs, 16 (43.24%), *M. pachydermatis* was detected by both methods, but a significant difference was not found compared with the number of cytology positive (9; 24.32%) and cultural positive (12; 32.43%) dogs with AD. This confirms the importance of cytology and cultural examination for the diagnosis of *Malassezia* infections (Cafarchia et al., 2005). Certainly, it should be remembered that in the case of a negative cytological examination, a cultural examination should be performed to rule out suspicion of infection with *Malassezia* species (Cafarchia et al., 2005). This is consistent with data from the current study, where 12 cytology negative dogs were positive by cultural examination.

Some atopic dogs show immediate reactivity to intradermal injections of *Malassezia* antigens, suggesting that hypersensitivity to yeast antigens may exacerbate the clinical signs in those individuals (Morris et al., 1998; Guillot and Bond, 1999; Bond et al., 2002a). Determinations of immediate type hypersensitivity to *M. pachydermatis* are often made through IDT (Oldenhoff et al., 2014). An intradermal test indirectly measures reactivity of the cutaneous mast cell due to the presence of IgE (Marsella et al., 2012), so intradermal testing with *M. pachydermatis* allergen can be helpful to assess skin immunity to yeast (Bond et al., 2002b). A positive hypersensitivity test to *Malassezia* may lead the clinician to consider *M. pachydermatis* overgrowth and cytological evaluation, if previously neglected, to perform it (Oldenhoff et al., 2014). On the other hand, cytological definitions of *Malassezia* overgrowth may be insufficiently sensitive in defining the number of yeasts required for sensitization (Farver et al., 2005). It should be kept in mind that diagnosis based on the assessment of yeast numbers does not take into account that some yeast could possess unusually potent virulence factors, or hosts could be unusually sensitive to these yeasts; and signs of *Malassezia* dermatitis would likely develop in the presence of low numbers of yeasts in these cases (Negre et al., 2009).

This study shows a lower percentage of IDT positive dogs to *M. pachydermatis* allergen than research by Farver et al. (2005), where 93% of dogs with *Malassezia* dermatitis (based on cytology results) were reactive to *M. pachydermatis*. In our study, totally 14 (37.8%) dogs showed a positive IDT to *M. pachydermatis* allergen. There was no significant difference compared with IDT negative dogs. Among 9 dogs with cytology evidence of the yeast, 2 (22.22%) dogs were IDT positive, and among 12 dogs with cultural evidence of *M. pachydermatis*, 7 (58.33%) were IDT positive to *M. pachydermatis* allergen. Also, our results showed that, among 16 dogs positive by cultural and cytology examination, 5 (31.25%) were IDT positive to *M. pachydermatis* allergen. In the group of cytology positive dogs, on the margin of the significant difference, it was noted that a greater number of dogs were IDT negative to *M. pachydermatis* allergen ($P = 0.056$). Also, our results are not in accordance with the study of Morris et al. (1998), where reactivity was higher in atopic dogs with cytology evidence of *Malassezia* dermatitis compared with those without. In this study, the dogs with positive IDT to *M. pachydermatis* allergen in a greater number (7 of 14 dogs) were positive by culture and negative by cytology; while the dogs with negative IDT to *M. pachydermatis* allergen were positive in a greater number (11 of 23 dogs) by cytology and cultural; there was no significant difference.

Diagnosis of *Malassezia* dermatitis is made on the basis of clinical signs and proliferation of yeast, but the number of *Malassezia* yeast could be insignificant, because some very virulent strains of yeast may be present and/or some sensitive individuals could exhibit signs with low numbers of yeast (Negre et al., 2005). According to Oldenhoff et al. (2014), clinical signs of *Malassezia* dermatitis, the cytology finding of yeast and demonstration of potential *Malassezia* hypersensitivity (by serological or IDT methods) are three separated, but often related concepts, and may or may not occur at the same time in a given case (Oldenhoff et al., 2014). These authors pay attention to the fact that those three concepts are independent elements of disease evaluation and that diagnosis should not rest on any single element (Oldenhoff et al., 2014). Failure to find yeast organisms on cytology does not rule out the possible contribution of yeast to clinical signs, as well as a negative *Malassezia* IgE test does not rule out a pathological role for this organism in an atopic dog (Oldenhoff et al., 2014). We found that 7 dogs IDT positive to *M. pachydermatis* allergen with a negative cytology test were cultural positive. It is in accordance with Farver et al.'s (2005) observation that if cultural examination had been performed instead of the cytological tape analysis, it is possible that malassezia dermatitis negative but IDT positive dogs would have been assigned to the malassezia dermatitis positive group (Farver et al., 2005).

In this study, the absence of a positive IDT test for the *M. pachydermatis* allergen can be explained by the fact that this yeast is part of the normal skin microflora of dogs, because continuous interactions with the host immune system will maintain low numbers of the yeast without generating a clinically appreciable

inflammatory response (Bond et al., 2020; Guillot and Bond 2020); or there may be an insufficient number of yeasts to cause a hypersensitivity reaction (Farver et al., 2005); or due to the small number of dogs included in the analysis. However, the pathological role of the yeast and its possible contribution to clinical signs should not be ruled out. Once again, it is important to remember that allergy tests (both IDT and allergen specific IgE serology) are not recommended as screening tests. They should only be used to confirm the clinical diagnosis of canine AD, and they are useful to identify the offending allergens in order to formulate an allergen-specific immunotherapy (Hensel et al., 2015).

Furthermore, in research by Bond et al. (2002b), only two dogs showed immediate skin test reactivity; on the other hand, nearly all dogs with *Malassezia* dermatitis developed delayed type reactions to *M. pachydermatis*. The subject of our study was not delayed type of hypersensitivity; therefore, it is possible that dogs with malassezia evidence and a negative IDT may have this type hypersensitivity.

In Group II and Group III of dogs, we noted a significantly greater number of purebred dogs. Polysensitization was observed in all included dogs; the most common positive reaction was noted to the house dust and house dust mite allergen group ($P < 0.05$). It is in accordance with previous studies that reported house dust and house dust mite as the most common allergens in dogs with AD (Zur et al., 2002, Di Tomaso et al., 2021), and that purebred dogs are more susceptible to *M. pachydermatis* than crossbred dogs (Marin et al., 2018). We did not find influence of sex and age in all three groups of dogs, which is in accordance with other research (Čonkova et al., 2011; Sihelska et al., 2017).

Certain research suggests an association of seasons and related temperature differences, humidity, and allergy seasons with the development of malassezia infection (Patterson and Frank, 2002; Čonkova et al., 2011). In this study, a seasonal onset of AD signs had no influence on cytology examination, but in a cultural examination it was found that a greater number of dogs had a spring-summer rather than autumn-winter onset of AD signs.

Our results showed that in all groups of dogs there were greater numbers of dogs with pruritus than without it. This is consistent with previous publications that, in animals with overgrowth of yeast or in individuals predisposed to allergic sensitization, the consequent inflammatory response can lead to clinical signs such as dermatitis and pruritus (Bond et al., 2020; Guillot and Bond, 2020). Furthermore, inflammation and pruritus caused by pathogenic mechanisms of *M. pachydermatis* lead to favorable microenvironment for yeast overgrowth (Patterson and Frank, 2002).

Conclusion

The results of this study confirmed that cytology and cultural examination are important for detecting presence of *M. pachydermatis* in dogs with AD. This study demonstrated intradermal reactivity to *M. pachydermatis* allergen in atopic dogs with *Malassezia* evidence, suggesting that hypersensitivity to it should be suspected. It is important to control presence of *Malassezia* yeast in dogs with AD to minimize the risk of sensitization to *M. pachydermatis* allergens, since the low number of yeast cells may cause hypersensitivity reactions in dogs predisposed to AD development. Although not statistically significant, the dogs with a positive IDT to *M. pachydermatis* allergen were positive in a greater number by culture examination; while dogs with a negative IDT to *M. pachydermatis* allergen were positive in a greater number by cytology and cultural examination.

References

1. Bond R., Curtis C.F., Hendricks A., Ferguson E.A., Lloyd D.H. Intradermal test reactivity to *Malassezia pachydermatis* in atopic dogs. Veterinary Record. 2002a. T. 150. P. 448-449.
2. Bond R., Ferguson E.A., Curtis C.F., Craig J.M., Lloyd D.H. Factors associated with elevated cutaneous *Malassezia pachydermatis* populations in dogs with pruritic skin disease. Journal of Small Animal Practice. 1996. T. 37. P. 103–7.
3. Bond R., Morris D.O., Guillot J., Bensignor E.J., Robson D., Mason K.V., Kano R., Hill P.B. Biology, diagnosis and treatment of *Malassezia* dermatitis in dogs and cats: Clinical Consensus Guidelines of the World Association for Veterinary Dermatology. Veterinary Dermatology. 2020. T. 31. P. 27-e4.
4. Bond R., Patterson-Kane J.C., Lloyd D.H. Intradermal test reactivity to *Malassezia pachydermatis* in healthy basset hounds and bassets with *Malassezia* dermatitis. Veterinary Record. 2002b. T. 151. P. 105-109.
5. Bond R., Guillot J., Cabanes F. J. "*Malassezia* yeasts in animal disease," in *Malassezia* and the skin, eds Boekhout T., Gueho Kellermann E., Mayser P., Velegraki A. Heidelberg: Springer-Verlag. 2010. P. 271–299. doi: 10.1007/978-3-642-03616-3_10
6. Cafarchia C., Gallo S., Romito D., Capelli G., Chermette R., Guillot J., Otranto D. Frequency, body distribution, and population size of *Malassezia* species in healthy dogs and in dogs with localized cutaneous lesions. Journal of Veterinary Diagnostic Investigation. 2005. T. 17 (4). P. 316-22.
7. Čonkova E., Sesztakova E., Palenik L., Smrčo P., Bílek J. Prevalence of *Malassezia pachydermatis* in dogs with suspected *Malassezia* dermatitis or otitis in Slovakia. Acta Veterinaria Brno. 2011. T. 80. P. 249–254.
8. Di Tommaso M., Luciani A., Crisi P.E., Beschi M., Rosi P., Rocconi F., Miglio A. Detection of Serum Allergen-Specific IgE in Atopic Dogs Tested in Northern Italy: Preliminary Study. Animals. 2021. T. 11. P. 358.
9. Farver K., Morris D.O., Shofer F., Esch B. Humoral measurement of type-1 hypersensitivity reactions to a commercial *Malassezia* allergen. Veterinary Dermatology. 2005. T. 16 (4). P. 261-8.
10. Girao M.D., Prado M.R, Brilhante R.S.N., Cordeiro R.A., Monteiro A.J., Sidrim J.J.C., Rocha M.F.G. *Malassezia pachydermatis* isolated from normal and diseased external ear canals in dogs: A comparative analysis. Veterinary Journal. 2006. T. 172. P. 544–548.
11. Grice E.A., Dawson T.L. Host–microbe interactions: *Malassezia* and human skin. Current Opinion in Microbiology. 2017. T. 40. P. 81–87.

12. Guillot J., Bond R. *Malassezia* Yeasts in Veterinary Dermatology: An Updated Overview. Frontiers in Cellular and Infection Microbiology. 2020. T. 10. P. 79. doi: 10.3389/fcimb.2020.00079.
13. Guillot J., Bond R. *Malassezia pachydermatis*: a review. Medical Mycology. 1999. T. 37. P. 295-306.
14. Hensel P., Santoro D., Favrot C., Hill P., Griffin C. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. BMC Veterinary Research. 2015. T. 11: 196 doi: 10.1186/s12917-015-0515-5
15. Kim Y.-S., Lee K.-W., Oh T.-H. Type -I hypersensitivity to *Malassezia pachydermatis* extracts in healthy dogs and dogs with Malassezia otitis externa. Journal of Veterinary Clinics. 2007. T. 24 (2). P. 88-93.
16. Marin J.C.G., Rojas F.B., Escobar A.J.G. Physiological and molecular characterization of *Malassezia pachydermatis* reveals no differences between canines and their owners. Open Journal of Veterinary Medicine. 2018. T. 8. P. 87-105.
17. Marsella R., Sousa C.A., Gonzales A.J., Fadok V.A. Current understanding of the pathophysiologic mechanisms of canine atopic dermatitis. Journal of the American Veterinary Medical Association. 2012. T. 241(2). P. 194-207.
18. Matousek J.L., Campbell K.L. *Malassezia dermatitis*. Compendium on Continuing Education for the Practicing Veterinarian. 2002. T. 24 (3). P. 224–232.
19. Meason-Smith C., Olivry T., Lawhon S., Hoffmann A. *Malassezia* species dysbiosis in natural and allergen-induced atopic dermatitis in dogs. Medical mycology. 2019. 10.1093/mmy/myz118.
20. Morris D.O., Olivier N.B., Rosser E.J. Type-1 hypersensitivity reactions to *Malassezia pachydermatis* extracts in atopic dogs. American Journal of Veterinary Research. 1998. T. 59. P. 836-841.
21. Nascente P.S., Meinerz A.R.M., Faria R.O., Nobre M.O., Escareno J.J.H., Mendes J.F., Meireles M.C.A., Mello J.R.B. *Malassezia pachydermatis* presence in canine external otitis and dermatopathies. Journal of Yeast and Fungal Research. 2015. T. 6 (3). P. 25-30.
22. Negre A., Bensignor E., Guillot J. Evidence-based veterinary dermatology: a systematic review of interventions for *Malassezia dermatitis* in dogs. Veterinary Dermatology. 2009. T. 20(1). P. 1-12.
23. Oldenhoff W.E., Frank G.R., DeBoer D.J. Comparison of the results of intradermal test reactivity and serum allergen-specific IgE measurement for *Malassezia pachydermatis* in atopic dogs. Veterinary Dermatology. 2014. T. 25 (6). P. 507-11.
24. Omodo-Eluk A.J., Baxter K.P., Fuller H. Comparison of two sampling techniques for the detection of *Malassezia pachydermatis* on the skin of dogs with chronic dermatitis. The Veterinary Journal. 2003, T. 165. P. 119–24.
25. Patterson P.A., Frank A.L. How to diagnose and treat *Malassezia dermatitis* in dogs. Veterinary Medicine. 2002. T. 97. P. 612-623.
26. Prelaud P., Guagere E., Alhaidari Z., Faivre N., Heripret D., Gayerie A., Reevaluation of diagnostic criteria of canine atopic dermatitis. Revue de Medecine Veterinaire. 1998. T. 149. P. 1057–1064.
27. Rybnicek J., Lau-Gillard P.J., Harvey R., Hill P.B. Further validation of a pruritus severity scale for use in dogs. Veterinary Dermatology. 2009. T. 20 (2). P. 115-22.
28. Sihelska Z., Čonkova E., Vaczi P., Harčarov M., Bohmova E. Occurrence of *Malassezia* Yeasts in Dermatologically Diseased Dogs. Folia Veterinaria. 2017. T. 61 (2). P. 17-21.
29. Warren G.N., Shadomy H.J. Yeast of medical importance in: Manual of Clinical Microbiology. 5th, eds. Balows A., Hausler J.W.Jr., Herrmann L.K., Isenberg H.D., Shadomy H.J. Washington DC. American Society for Microbiology. 1991. P. 617-629.
30. Zur G., Ihrke J.P., White D., Kass H.P. Canine atopic dermatitis: a retrospective study of 266 cases examined at the University of California, Davis, 1992–1998. Part I. Clinical features and allergy testing results. Veterinary Dermatology. 2002. T. 13. P. 89–102.

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Prevalence of Virulence Genes in Strains of *Campylobacter jejuni* Isolated from Broiler Products, Children, Wild Birds and Dairy Cattle in Lithuania

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Keywords: *Campylobacter jejuni, virulence genes, prevalence.*

Abstract. *The present study was designed to investigate the prevalence of virulence genes in C. jejuni from different sources. A total of 98 C. jejuni strains from different sources were selected for the study: 34 strains isolated from broiler products (B), of which 17 from fresh poultry and 17 from marinated poultry products; 17 strains from children clinical samples (V); 31 strains from wild birds' faeces (LK), of which 16 from pigeons and 15 from crows; and 16 from dairy cattle faeces (G). Nine virulence genes (cadF, virB11, ceuE, gltA, hcp, cdtA, cdtB, cdtC, flaA) were selected for the study. The identification of the virulence gene was performed by polymerase chain reaction, the visualization of DNA amplicons was done by electrophoresis, and photos were taken in ultraviolet light.*

The study revealed that the virB11 gene was found only in 6% of C. jejuni strains isolated from dairy cows, while this gene was not detected in Campylobacter strains isolated from other sources. The gltA gene was found in all tested C. jejuni strains. The cdtA virulence gene was prevalent in examined Campylobacter strains as only 3% of wild bird strains lack this gene. The CdtC gene was detected in all C. jejuni strains isolated from the children clinical samples, wild birds and chicken, whereas campylobacters isolated from the cattle did not harbour this gene (19% of all tested strains). The study results revealed that occurrence of virulence genes was differently distributed among strains of C. jejuni and occurrence of certain virulence genes (hcp, cdtC, flaA) depended on the origin of strain isolation.

Introduction

Campylobacter spp. cause a foodborne intestinal infectious disease called campylobacteriosis. The infection is spread by faecal-oral route. It can also be transferred through insufficiently heat-treated meat, especially poultry and poultry products, and uncooked or unpasteurized milk contaminated with these bacteria. Water can also be a source of infection as well as contact with infected animals (ULAC, 2018). The main source of these bacteria is considered to be poultry (Wysok and Wojtacka, 2018). However, dairy cattle, pigs and wild birds are also potential sources of infection for humans. Campylobacteriosis was the most common gastrointestinal bacterial disease in humans in the European Union in 2019, and this tendency has been observed since 2005 (EFSA, 2021). The incidences of campylobacteriosis in 2019 (43.8/100 000) increased by 32.7% compared with 2018 (33.0/100 000) in Lithuania (ULAC, 2019). *Campylobacter spp*. is characterized by a wide variety of virulence factors. Virulence factors include bacterial mobility, chemotaxis, invasion, adhesion, toxin production, bile resistance, drug resistance, and other. Virulence is also related to the genetic diversity of bacteria. Virulence genes such as *cdtA*, *cdtB*, *cdtC* are involved in the production of bacterial toxins, *virB11* shows the extent of invasion in host cells, and *ceuE* encodes proteins (Reddy and Zishiri, 2018). In order to obtain more information on the ability of *Campylobacter* to cause gastrointestinal disease in humans, it is important to assess the prevalence of virulence genes in *Campylobacter* strains isolated from different sources. A kind of or similar study has not been performed in Lithuania before; therefore, with this study we aim to evaluate and compare the prevalence of virulence genes in *C. jejuni* strains isolated from different sources like the dairy cattle, wild birds, broiler products and children clinical samples.

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Materials and methods

Bacterial strains

A total of 98 *C. jejuni* strains from different sources were selected for the study: 34 broiler products (B), of which 17 fresh poultry and 17 marinated poultry products; 17 children (V); 31 wild birds (LK), of which 16 were pigeons and 15 crows; and 16 dairy cattle samples (G). All isolates were from the Campylobacter collection at the Department of Food Safety and Quality, Veterinary Academy, Lithuanian University of Health Sciences. The identification of Campylobacter isolates was performed with multiplex PCR as described by Wang et al. (2002) with the minor modifications described previously by Ramonaite et al. (2015).

DNA isolation

Campylobacter isolates were stored as frozen stocks at –80°C in brain heart infusion broth (BHI) (Oxoid Ltd., Basingstoke, UK) with 30% glycerol (Stanlab, Poland). They were recovered from frozen stocks on blood agar base No. 2 (Oxoid, Basingstoke, Hampshire, England) supplemented with 5% defibrinated horse blood (E&O Laboratories, Burnhouse, Bonnybridge, Scotland) and incubated under microaerophilic conditions (5% oxygen, 10% carbon dioxide and 85% nitrogen) at 37°C for 48 h.

One 10 µL loop of bacterial culture grown on blood agar plates was collected and suspended in 200 µL of PrepMan Ultra (Applied Biosystems, Foster City, USA). The suspension was vortexed for 15–30 s in order to dissolve the bacterial culture and subsequently heated at 100°C for 10 min. Afterwards, the samples were centrifuged at 16 000 g for 3 min. The supernatant containing bacterial DNA was used immediately or transferred to a new tube and stored at –20°C until use.

PCR detection of virulence determinants

Amplifications of the nine virulence genes (*FlaA*, *CadF*, *VirB11*, *CeuE*, *CdtA*,*CdtB*, *CdtC*, *GltA*, *Hcp*) were performed in separate tubes. Final volume of 25 µL PCR reaction mix was composed of 7.25 µL Dream-TaqGreen PCR Master Mix (Thermo Scientific, Waltham, USA), 15.75 µL Milli-Q water, and 1 µL of primers mix. Finally, a 24 µL mix was prepared in separate tubes, adding 1 µL of chromosomal DNA. The samples were centrifuged at 4000 g for 1 min. DNA amplification was carried out in a thermocycler using different programs. For *FlaA*, *CadF*, *VirB11* genes, an initial denaturation step was performed at 94°C for 5 min, 95°C for 1 min followed by 30 cycles of amplification, 1 min for specific temperature (*FlaA* – 53°C, *cadF* – 5°C, *virB11* – 53°C), extension at 71°C for 1 min and ending with final extension at 72°C for 5 min (Wysok and Wojtacka, 2018). For *CeuE* gene, an initial denaturation step was at 95ºC for 3 min, 95°C for 30 s followed by 30 cycles of amplification, 57°C for 30 s, extension at 72°C for 1 min and ending with final extension at 72°C for 5 min (Gonzalez et al., 1997). For *CdtA, cdtB, cdtC* genes, an initial denaturation step was at 94°C for 1 min, 94°C for 1 min followed by 30 cycles of amplification, 42°C for 2 min, extension at 72°C for 3 min and ending with final extension at 72°C for 5 min (Pickett et al., 1996). For *Hcp* and *gltA* genes, an initial denaturation step was at 94°C for 5 min, 95°C for 1 min followed by 30 cycles of amplification, 60°C for 1 min, extension at 71°C for 1 min and ending with final extension at 72°C for 5 min (Harrison et al., 2014). Each PCR product (11 mL) was loaded into a 2% TopVision LM GQ agarose gel (Thermo Scientific) containing 6.5 µL ethidium bromide and analysed by gel electrophoresis. The PCR products were visualized on a UV board. The GeneRuler 100 bp DNA Ladder (Thermo Scientific) was used as the molecular size marker (Fig. 1).

Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2007 and IBM SPSS Statistics 24 software packages. The Crosstabs procedure was used to assess the dependence in between the samples of quantitative data. Chi^2 was also calculated. In addition, descriptive statistics was applied to get quantitative variables data. A P value of < 0.05 was used to indicate statistically significant results.

Results

Overall 98 *C. jejuni* strains from 4 different sources were selected for examination: 34 strains isolated from broiler products, 17 isolated from children clinical samples, 31 strains from wild birds and 16 from dairy cattle faeces. The study revealed that three virulence genes were detected in all strains despite the source of isolation. The study also showed that none of examined *C. jejuni* strains harboured all nine or eight virulence genes (Table 1). Whereas five virulence genes were detected in all *C. jejuni* strains isolated from broiler and wild bird faeces samples.

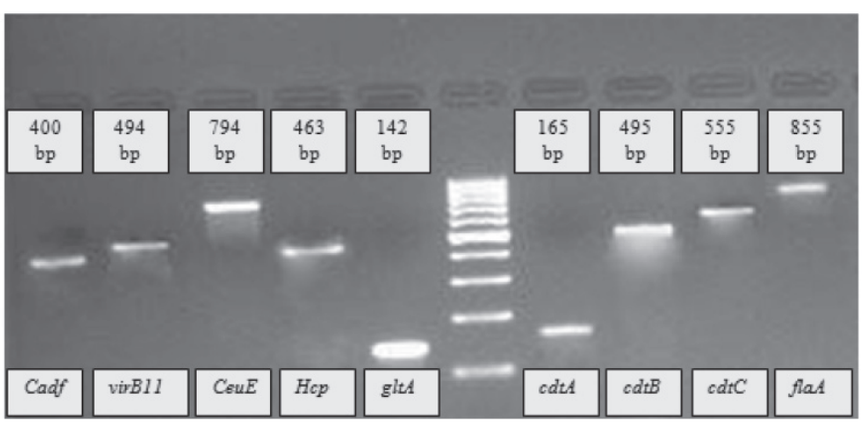

Fig. 1. Visualization of amplified virulence genes, gene fragment lengths 100–1000 (bp)

All examined strains contained from 1 to 3 virulence genes. However, several strains harboured up to 6 or 7 virulence genes (Table 1).

We identified only one *Campylobacter* strain isolated from a cattle faeces sample (6%) harbouring the *VirB11* gene, which is responsible for *Campylobacter spp.* invasion and the distribution among different sources. The *gltA* virulence gene was found in all strains isolated from different sources (100%). The *FlaA* virulence gene, responsible for invasion and mobility, was not found in *Campylobacter* strains isolated from the broiler and children clinical samples. However, this gene was detected in the wild bird (61%) and in the cattle (63%) strains. The *Hcp* virulence gene, contained in the type VI secretory system (T6SS) responsible for the cellular transfer of proteins, was mainly found in *C. jejuni* strains isolated from the broiler product strains (21%), but not found at all in bovine Campylobacter strains. The *CadF* virulence gene, which promotes bacterial attachment to intestinal epithelial cells, was found in all *Campylobacter* strains isolated from the wild bird and children clinical samples (100%) with lower frequency of detection in bovine strains (94%). The *CeuE* gene, encoding lipoprotein, was mostly found in *Campylobacter* strains isolated from the children clinical samples (71%) and rarely detected in cattle strains (19%). The *CdtA* virulence gene responsible for toxin production was not found in only one strain of the wild birds whereas it was prevalent in all strains isolated from other sources. The *CdtB* gene related with toxin producing was found in all *Campylobacter* strains isolated from the broiler and wild bird samples. In *Campylobacter* strains isolated from the cattle and children clinical samples, this gene was found in 94% of the strains. The *CdtC* virulence gene associated with production of toxins was not found in only three *Campylobacter* strains isolated from the cattle (prevalence 81%). The prevalence was 100% in *Campylobacter* strains isolated from broilers, wild birds, and children clinical samples (Table 2).

Discussion

This study evaluated the prevalence of 9 major virulence genes in *C. jejuni* strains isolated from 4 different sources such as broiler products, dairy cattle, wild birds, and children clinical samples. Our study revealed that the distribution of the *virB11* virulence gene responsible for *Campylobacter* invasion among different sources was very low and detected in only one Campylobacter strain isolated from the cattle. Other studies have revealed that the *virB11* gene is rarely found in campylobacters isolated from cattle. In a study conducted in 2003, the *virB11* virulence gene was detected in only one of the 15 *Campylobacter* strains isolated from cattle (Bang Det al., 2003). However, in a study conducted in Poland in 2018, the examination of 99 *Campylobacter* strains (50 pigs and 49 cattle samples) revealed that this gene prevailed in 50% of the strains (Wysok and Wojtacka, 2018). After determining the distribution of the virulence gene *cadF* – which promotes bacterial attachment to intestinal epithelial cells – among different sources of *Campylobacter*, it was found that the prevalence of *Campylobacter* strains isolated from children and wild birds was 100%. Only in one *Campylobacter* strain out of 16 strains isolated from cattle, the *cadF* virulence gene was not detected. A similar study by Danish and Iranian researchers showed that the *cadF* virulence gene was detected in all *C. jejuni* strains isolated from

Table 1. Quantity of virulence genes in Campylobacter strains depending on the source of isolation

Source	Number of strains	Quantity of virulence genes (number of strains / percentage)								
		1	2	3	4	5	6	7	8	9
Broilers	34	34/100	134/100	134/100	134/100	134/100	21/62	7/21	0/0	0/0
Children	17	117/100	117/100	117/100	117/100	915/94	12/71	3/18	0/0	0/0
Wild birds	31	131/100	131/100	131/100	131/100	131/100	18/61	7/23	0/0	0/0
Cattle	16	116/100	116/100	116/100	915/94	814/88	6/38	2/13	0/0	0/0
	98	198/100	198/100	198/100	97/99	94/96	56/58	18/19	0/0	0/0

Table 2. Prevalence of virulence genes in *Campylobacter* strains depending on the source of isolation

Source	Number of strains	Identified virulence genes (number of tested strains / percentage of identified genes in strains)								
		cadF	*virB11*	*Hcp*	*gltA*	*ceuE*	*cdtA*	*cdtB*	*cdtC*	*flaA*
Broilers	34	33/97	0/0	7/21	34/100	22/65	34/100	34/100	34/100	0/0
Children	17	17/100	0/0	3/18	17 /100	12/71	17/100	16/94	17/100	0/0
Wild birds	31	31/100	0/0	1/3	31/100	14/45	30/97	31/100	31/100	12/61
Cattle	16	15/94	1/6	0/0	16/100	3/19	16/100	15/94	13/81	6/63
	98	96/98	1/1	11/11	98/100	51/52	97/99	96/98	95/97	18/18

cattle (15 strains) and children (200 strains) (Bang et al., 2003; Ghorbanalizadgan et al., 2014). The study of the *hcp* virulence gene prevalence among *Campylobacter* strains isolated from different sources revealed that the gene was mostly common among *C. jejuni* strains isolated from cattle (100%) and wild birds (97%). The *hcp* virulence gene is in the type VI secretory system (T6SS) responsible for protein delivery to a "target" cell. The prevalence of this gene in broiler products was 21% (7 strains of 34). Similar results were obtained in a 2015 study in the United Kingdom, where the prevalence of the virulence gene in poultry was 28.8% (detected in 17 poultry strains of 59 tested) (Corcionivosch et al., 2015). In the study of the *gltA* gene distribution among different sources, it was found that the *gltA* gene was 100% common in all of them. A study by other researchers showed that the *gltA* virulence gene was detected in all *C. jejuni* tested strains (59 strains) isolated from poultry (Corcionivoschi et al., 2015). In a study of the prevalence of the *ceuE* virulence gene – responsible for encoding lipoprotein – among *Campylobacter* strains isolated from different sources, it was revealed that this virulence gene was the least common among *C. jejuni* strains isolated from cattle and wild birds (19% and 45%, respectively). A similar study by other researchers showed that the *ceuE* virulence gene was detected in 14 of 15 *Campylobacter* strains that were isolated from cattle (Bang et al., 2003). To determine the prevalence of the virulence gene *cdtA* – responsible for toxin production – among *Campylobacter* strains isolated from different sources, the prevalence among *C. jejuni* strains isolated from broilers, children, and cattle was found to be 100%. In a study conducted in Denmark, the prevalence of the *cdtA* gene in *C. jejuni* strains isolated from cattle was 100% (15 strains) (Bang et al., 2003). And in a study conducted by scientists in 2018, the prevalence of this virulence gene in poultry samples was 96% (152 strains tested) and 100% in *C. jejuni* strains isolated from humans (155 strains) (Wieczorek et al., 2018). The *cdtB* virulence gene – responsible for toxin production – was detected in all *C. jejuni* strains isolated from broilers and wild birds in the study of the *cdtB* virulence gene prevalence. In a similar study, the prevalence of this gene in poultry samples was 94.1% (152 strains studied) (Wieczorek et al., 2018). A study on the prevalence of *cdtC* virulence gene – also responsible for toxin production – among *Campylobacter* strains isolated from different sources revealed that this gene was detected in 100% of *C. jejuni* strains isolated from broilers, children and wild birds. In a similar study conducted by researchers in 2018, it was found that the *cdtC* virulence gene was detected in 97.4% of *Campylobacter* strains isolated from birds (152 tested) and in 100% of *Campylobacter* strains isolated from humans (155 strains) (Wieczorek et al., 2018). In a study of the *flaA* virulence gene prevalence – responsible for mobility and invasion – among *Campylobacter* strains isolated from different sources, it was found that this gene was not detected in *C. jejuni* strains isolated from broilers and children. The prevalence among wild birds was 39%, and in the cattle samples, it was 38%. In a similar study, the *flaA* virulence gene was detected in 100% (15 strains) of *C. jejuni* strains isolated from cattle (Bang et al., 2003). In a study conducted in 2018, the prevalence of this gene in *C. jejuni* strains isolated from poultry reached 98.7% (152 tested) (Wieczorek et al., 2018).

Our study revealed that *Campylobacter jejuni strains* isolated from different sources are distinct in relation to the diversity of genes encoding virulence. The knowledge about the prevalence of virulence genes, depending on the isolation source of *campylobacters*, is important in assessing potential threats to consumer health, as these genes encode the ability of bacteria to cause the disease and the severity of the caused disease.

Conflict of interest

The authors declare no conflicts of interest.

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References

1. Bang D. D., Nielsen M. E., Scheutz F., Pedersen K., Handberg K., Madsen M. PCR detection of seven virulence and toxin gene*s of Campylobacter jejuni and Campylobacter coli i*solates from Danish p*igs ant cattle a*nt cytolethal distend*ing toxin produc*tion of the isolates. Journal of applied microbiology. 2003. T. 97 P. 1003-1014. DOI:10.1046/j.1365-2672.2003.01926.x
2. Corcionivoschi N., Gundogdu O., Moran L., Kelly C., Scates P., Stef L., Cean A., Wren B., Dorrell N., Madden R. H. Virul*ence characteristics* of hcp^{+} *Campylobacter jejuni* and *Campylobacter coli* isolates from retail chicken. Gut Pathog. 2015. T. 7 DOI: 10.1186/s13099-015-0067-z
3. European Food Safety Authority. The European Union One Health 2019 Zoonoses Report, 2021 https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2021.6406
4. Ghorbanalizadgan M., Bakhshi B., Lili A. K., Najar-Peerayeh S., Nikmanesh B. A Molecular Survey of *Campylobacter jejuni* and *Campylobacter coli* virulence and diversity. Iran Biomed J. 2014. T. 18. P. 158-164.
5. Gonzalez I., Grant K. A., Richardson P. T., Park S. F., Collins M. D. Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the ceuE gene encoding a putative virulence determinant. J Clin Microbiol. 1997. T. 35 P. 759-63. DOI: 10.1128/JCM.35.3.759-763.1997
6. Harrison J. W., Dung T., Siddiqui F., Korbri*sate S., Bukhari H.,* Tra M. Identification of possible virulence marker from *Campylobacter jejuni* isolates. Emerging infectious diseases. *2014. T*. 20. P 1026-1029. DOI:10.3201/eid2006.130635
7. Pickett C. L., Pesci E. C., Cottle D. L., Russell G., Erdem A. N., Zeytin H. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* ant relatedness of *Campylobacter sp*. cdtB genes. Infection and immunity. 1996. T. 64 P. 2070-2078. DOI: 10.1128/IAI.64.6.2070-2078.1996

8. Ramonaite, S., Novoslavskij, A., Zakariene, G., Aksomaitiene, J., and Malakauskas, M. High prevalence and genetic diversity of *Campylobacter jejuni* in wild crows and pigeons. Curr. Microbiol. 2015. T. 71 P. 559–565. DOI: 10.1007/s00284-015-0881201
9. Reddy S. and Zishiri T. O. Genetic characterisation of virulence genes associated with adherence, invasion and cytotoxicity in *Campylobacter* spp. isolated from commercial chickens and human clinical cases. Onderstepoort J Vet Res. 2018. T. 85. DOI:10.4102/ojvr.v85i1.1507
10. The Centre of Communicable Diseases and AIDS (ULAC). Incidence of reported communicable disease in Lithuania in 2019, 2020. Available at: http://www.ulac.lt/ataskaitos
11. The Centre of Communicable Diseases and AIDS (ULAC). Information about Campylobacteriosis. Available at: http://www.ulac.lt/ligos/K/kampilobakterioze
12. Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Price, L., et al. Colony multiplex PCR assay for identification and differentiation of Campylobacter jejuni, C. coli, C. lari, C. upsaliensis, and C. fetus subsp. fetus. J. Clin. Microbiol. 2002. T. 40, P. 4744–4747. DOI: 10.1128/JCM.40.12.4744-4747.2002
13. Wieczorek K., Wołkowicz T., Osek J. Antimicrobial Resistance and Virulence-Associated Traits of *Campylobacter jejuni* Isolated From Poultry Food Chain and Humans With Diarrhea. Frontiers in Microbiology. 2018. T. 9. DOI: 10.3389/fmicb.2018.01508
14. Wysok B., Wojtacka J. Detection of virulence genes determining the ability to adhere and ivade in Campylobacter spp. from cattle and swine in Poland. Poland: microbial pathogenesis. 2018. T.115. P.257-263. DOI: 10.1016/j.micpath.2017.12.057

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Relationship of Energy Reserves with Post-partum Sub-clinical Endometritis and its Impact on Reproductive Performance of Dairy Cows

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Keywords: *back fat thickness; body condition score; dairy cows; reproductive performance; sub-clinical endometritis*

Abstract. *The present investigation was carried out to study the relationship of energy reserves with occurrence of sub-clinical endometritis (SCE) and its impact on reproductive performance of post-partum dairy cows (N = 41). Based on endometrial cytology, incidence of SCE was 65.85% at 8 weeks post-partum whereas the energy reserves, i.e., body condition score (BCS) and back fat thickness (BFT), were significantly lower ($p < 0.05$) on weekly monitoring between days 0–56 post-partum in sub-clinical endometritis positive (SCEP) as compared with sub-clinical endometritis negative (SCEN) cows whereas serum leptin concentrations were not consistent with BCS and BFT levels. Pearson correlation analysis also revealed a significant negative correlation ($p < 0.05$) between BFT and days to the first post-partum ovulation (DFPO; $r = -0.4832$) in SCEP and BFT and completion of uterine involution (CUIN; $r = -0.5169$) in SCEN cows whereas serum leptin concentrations had no significant relationship ($p > 0.05$) with CUIN and DFPO. In the present study, a significantly longer interval to CUIN and DFPO ($p < 0.05$), days to the first artificial insemination and days open ($p < 0.01$) was recorded in SCEP cows. The risk operator characteristics (ROC) curve was tested to find out the threshold level for occurrence of post-partum SCE via the area under the curve (AUC) i.e., $\leqslant 2.25$ (AUC = 0.71; $p < 0.05$), $\leqslant 8.60$ (AUC = 0.77; $p < 0.01$) and $\leqslant 5.28$ ng/mL (AUC = 0.76; $p < 0.01$) for BCS, BFT and serum leptin concentrations at calving, respectively. In peroration, the energy reserves, i.e., low BCS and BFT were significantly associated with the occurrence of post-partum SCE and subsequently affected the restoration of reproductive parameters, reproductive performance and milk production in dairy cows.*

Introduction

The energy obtained from the nutritional sources is mainly utilized by dairy cows to maintain basal metabolism, resumption of ovarian function and replenishment of energy reserves lost during early post-partum period due to lactation (Gruber et al., 2014; Sharma, 2020). The post-partum period is mainly characterized by an increase in energy demands required for lactation, and subsequent negative energy balance (NEB) upsets the metabolic profile for a brief or longer period of time depending upon the nutritional sources available to dairy cows (Klopcic et al., 2011; Gruber et al., 2014). Assessment of the energy status in dairy cows has been done via a study of subjective and objective parameters like body condition score (BCS), back fat thickness (BFT) of the thurl area, respectively, and metabolic parameters such as serum leptin, non-esterified fatty acid (NEFA) and β-hydroxy butyrate (BHB) (Butler, 2005). Energy reserves and the immune status of cows share a direct relationship as their decrease leads to alteration in the lymphocyte function, low secretion of immunoglobulin M (IgM) and interferon-γ (IFN-γ), decrease in the phagocytic function of polymorphonuclear cells (PMNCs) and, subsequently, prolonged uterine inflammation, i.e., sub-clinical endometritis (SCE) (Lacetera et al., 2005; Bacha and Regassa, 2010). Similarly, the recuperative ability of dairy cows to bounce back from the inevitable low energy reserves, i.e., BCS and BFT, has been closely linked to prompt restoration of post-partum ovarian activity (Montiel and Ahuja, 2005; Galindo et al., 2013; Ingvartsen and Moyes, 2013), thus, determining the fate of reproductive efficiency (Sharma et al., 2018; Andela et al., 2019). As SCE is a chronic condition, it affects the reproductive performance of cows and results in economic losses to dairy farmers (Barrio et al., 2015); therefore, the present study was carried out to describe the relationship of energy reserves with occurrence of sub-clinical endometritis and its impact on reproductive performance of post-partum dairy cows.

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Materials and methods

Animals

Forty-one healthy Jersey crossbred multiparous cows (parity 3–4; N = 41) having no previous history of any clinico-reproductive disorders, i.e., dystocia, retention of placenta, metritis, ketosis and mastitis, reared in a loose housing system under standard management conditions, fed a total mixed ration, once daily *ad libidum*, with unrestricted access to water in a university dairy farm were enrolled for the study after normal parturition. The cows did not receive any treatment during the pre-partum period and the course of the study, and at calving, their health status was assessed on the basis of normal rectal temperature (38.67 ± 0.02°C). The cows were milked twice daily (04:00 and 15:00 h). All the experiments were carried out after the approval of the ethics committee of the Dr. G. C. Negi College of Veterinary and Animal Sciences, CSKHPKV, Palampur.

Assessment of the body condition score and back fat thickness in post-partum cows

BCS and BFT in dairy cows were evaluated at a weekly interval from the day of calving, i.e., day 0, to day 56 post-partum. The BCS was adjudged via the visual technique on a 1–5-point scale where a score of 1 indicated an emaciated condition while a score of 5 indicated an obese condition (Edmonson et al., 1989). The eight locations of the cow's body were examined in three major regions, and the criteria within each area were used to indicate the body condition.

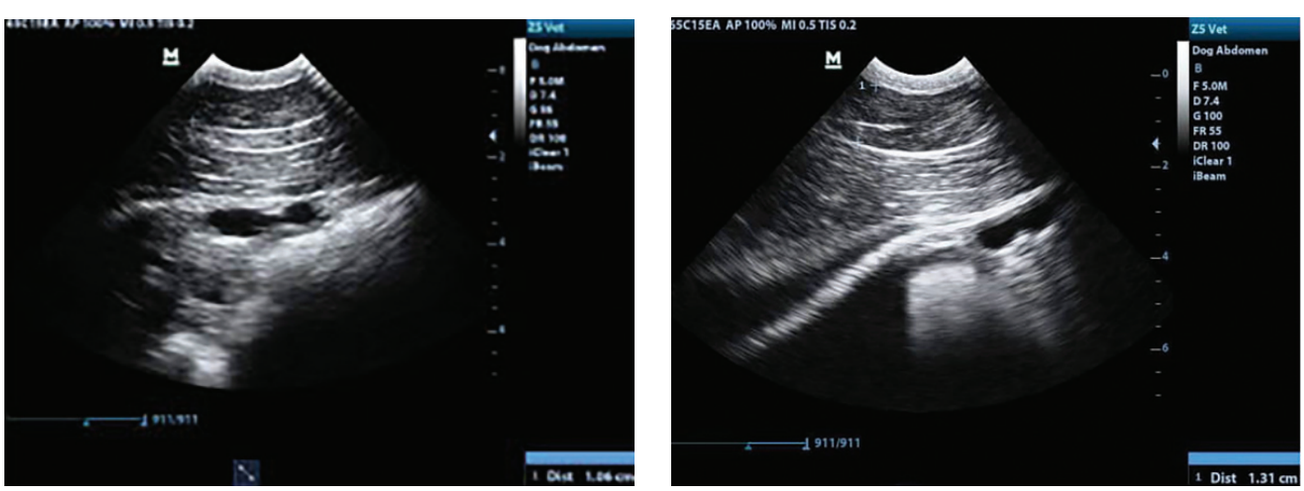
The BFT of the thurl area, i.e., 2–3 cm above the greater trochanter of the femur, located midway between the tuber coxae (hooks) and the tuber ischiae (pins), was recorded from day 0 to 56 post-partum (Diaz et al., 2017; Fig. 1 a, b), using a portable ultrasound machine (Mindray Z5; VETMODEL 75L50EAV) micro-convex transducer at a frequency 5.0 MHz.

Serum leptin concentration

Blood samples (N = 205) of forty-one cows were collected from the jugular vein at an interval of 14 days after parturition, i.e., at the time of parturition, thereafter, every 14 days until day 56, and serum was separated by the slant method and stored at –20°C for pending analysis of leptin concentration (ng/mL). ELISA kits were used to analyze serum leptin via TECAN SUNRISE Microplate Absorbance Reader (TECAN Austria GmbH, Austria).

Endometrial cytology

The cytotape method of endometrial cytology was employed for adjudging the polymorphonuclear cells (PMNCs) proportion for diagnosis of sub-clinical endometritis at 8 weeks post-partum. Cytotape assembly was introduced into the vagina after cleaning the vulval area and the sheath was perforated at the external os of the cervix followed by introduction of a steel rod rolled with a paper tape into the body of the uterus. The sample was taken by rolling the rod having the tape on the wall of the uterine body with gentle pressure of the index finger through the rectum. The cytotape was then retracted from the uterus and the smear was formed by gently rolling the tape on a clean glass slide. The prepared slides were air dried, fixed in methanol for 15 minutes and then stained with modified Wright-Giemsa stain for 45 minutes. All the slides were evaluated by an optical light microscope, the cells were counted in a total of 10 fields and the percentages of epithelial cells, endometrial cells and PMNCs were assessed at 40X magnification (Rana et al., 2020). Based on these findings, the cows were divided into sub-clinical endometritis positive (SCEP; N = 27), i.e., PMNCs percentage ⩾ 5% (Pascottini et al., 2016), and sub-clinical endometritis negative (SCEN; n = 14) groups.



a b

Fig. 1. BFT of thurl area in a dairy cow at 8 weeks post-partum. (a) SCEP- 10.6 mm; (b) SCEN- 13.8 mm.

Monitoring of post-partum reproductive parameters

Following parturition, the cows were monitored for completion of uterine involution (CUIN) and days to the first post-partum ovulation (DFPO) at a weekly interval by trans-rectal ultrasonography. Uterine involution was considered complete when no further change took place between two consecutive examinations in the diameter of uterine horns, and both the previous gravid uterine horn (PGUH) and the previous non-gravid uterine horn (PNGUH) were nearly in symmetrical measure, i.e., difference ⩽ 1mm (Sharma et al., 2017). Other parameters such as days to the first artificial insemination (AI), number of inseminations per conception and days open were also recorded in SCEP and SCEN cows.

Milk yield and economic loss

Daily milk yield (DMY) for a lactation length of 305 days was recorded in both groups, and economic loss per litre of milk produced was calculated for SCEP cows.

Statistical analysis

The obtained data were statistically analyzed using repeated measures ANOVA, Student t test for testing the significance of parameters, Pearson matrix correlation analysis between different parameters at the time of parturition, and receiver operator characteristics curve (ROC) for obtaining the area under the curve and determining the threshold of certain parameters with NCSS 2020, USA (Version 20.0.1).

Results

The incidence of sub-clinical endometritis in dairy cows at 8 weeks post-partum was 65.85%. Energy reserves, i.e., BCS and BFT, were significantly lower ($p < 0.05$) between day 0 to 56 post-partum in SCEP as compared with SCEN cows (Table 1). However, serum leptin concentrations were significantly higher ($p < 0.05$) in SCEN cows as compared with SCEP cows only on day 0 and 28 post-partum. In both groups, the gradual decrease in energy reserves up to day 28 post-partum owing to milk production was evident and, subsequently, followed by transient increase until the end of examination days.

Pearson correlation matrix revealed a significant correlation ($p < 0.05$) between BCS and BFT in SCEP and SCEN cows on the day of calving. Also, a significant correlation ($p < 0.05$) was evident between BCS and BFT with serum leptin concentrations in SCEP and SCEN cows (Table 2 and 3). Pearson

Table 1. BCS, BFT and serum leptin concentration (ng/mL) in SCEP and SCEN dairy cows (N = 41) at different weeks post-partum (Mean ± SE)

Days post-partum	Body condition score (BCS)		Back fat thickness (BFT-mm)		Serum leptin concentration (ng/mL)	
	SCEP (n=27)	SCEN (n=14)	SCEP (n=27)	SCEN (n=14)	SCEP (n=27)	SCEN (n=14)
Day 0	2.24 ± 0.05^{y}	2.65 ± 0.10^{x}	8.48 ± 0.47^{y}	11.96 ± 0.72^{x}	5.21 ± 0.25^{y}	6.84 ± 0.42^{x}
Day 7	2.30 ± 0.05^{y}	2.63 ± 0.07^{x}	9.10 ± 0.52^{y}	11.58 ± 0.54^{x}	–	–
Day 14	2.39 ± 0.05^{y}	2.63 ± 0.07^{x}	9.49 ± 0.37^{y}	11.27 ± 0.50^{x}	5.19 ± 0.30	6.04 ± 0.27
Day 21	2.40 ± 0.05	2.61 ± 0.07	9.50 ± 0.30^{y}	10.98 ± 0.49^{x}	–	–
Day 28	2.45 ± 0.04^{y}	2.65 ± 0.08^{x}	9.60 ± 0.25^{y}	11.31 ± 0.52^{x}	5.31 ± 0.26^{y}	6.38 ± 0.36^{x}
Day 35	2.48 ± 0.04	2.68 ± 0.09	9.79 ± 0.28^{y}	11.26 ± 0.72^{x}	–	–
Day 42	2.53 ± 0.04	2.74 ± 0.09	10.14 ± 0.35^{y}	11.81 ± 0.56^{x}	5.65 ± 0.14	6.42 ± 0.15
Day 49	2.57 ± 0.04^{y}	2.80 ± 0.09^{x}	10.66 ± 0.32	11.86 ± 0.56	–	–
Day 56	2.60 ± 0.05^{y}	2.86 ± 0.09^{x}	10.67 ± 0.36^{y}	12.22 ± 0.55^{x}	5.97 ± 0.35	6.85 ± 0.44

^{x,y}Values with different superscripts within the same row for the same day and parameter are significantly different ($p < 0.05$).

Table 2. Pearson correlation matrix for BCS, BFT, leptin concentration (at calving), uterine involution and first post-partum ovulation in SCEP dairy cows (N = 27)

Parameters	BCS	BFT	Serum leptin concentration	Completion of uterine involution	Days to first post-partum ovulation
BCS	1.0000				
BFT	0.8522*	1.0000			
Serum leptin concentration	0.4859**	0.5912*	1.0000		
Completion of uterine involution	–0.1159	–0.1402	0.1194	1.0000	
Days to first post-partum ovulation	–0.2506	–0.4832**	–0.3225	0.4415**	1.0000

*$p < 0.01$; **$p < 0.05$.

Table 3. Pearson correlation matrix for BCS, BFT, serum leptin concentration (at calving), uterine involution and days to first post-partum ovulation in SCEN dairy cows (N = 14)

Parameters	BCS	BFT	Serum leptin concentration	Completion of uterine involution	Days to first post-partum ovulation
BCS	1.0000				
BFT	0.6039**	1.0000			
Serum leptin concentration	0.6284**	0.5271**	1.0000		
Completion of uterine involution	–0.6212**	–0.5169**	–0.3346	1.0000	
Days to first post-partum ovulation	–0.1331	–0.2574	–0.2614	0.3177	1.0000

**$p < 0.05$.

correlation analysis also revealed a significant negative correlation ($p < 0.05$) between BFT and DFPO ($r = –0.4832$) in SCEP and BFT and CUIN ($r = –0.5169$) in SCEN cows. Similarly, BCS shared a significant negative correlation ($r = –0.6212$; $p < 0.05$) with CUIN in SCEN cows whereas serum leptin concentrations had no significant relationship ($p > 0.05$) with CUIN and DFPO.

In the present study, a direct relationship of BCS, BFT and serum leptin concentrations with early reproductive parameters was quite evident as the energy reserves were significantly higher ($p < 0.05$) in SCEN as compared with SCEP cows at the time of parturition (Table 4). A significantly longer interval ($p < 0.05$) to CUIN and DFPO was recorded in SCEP as compared with SCEN cows. Similarly, days to first AI and days open were significantly higher ($p < 0.01$) in SCEP as compared with SCEN cows although the difference was not statistically significant ($p > 0.05$) for the number of inseminations per conception. The DMY recorded over a lactation length for SCEP cows was significantly lower ($p < 0.01$) in comparison with SCEN cows and, subsequently, led to an economic loss of Rs. 166/USD 2.27 per litre loss in milk production (Table 4).

The area under the curve (AUC) was determined to find out the threshold level, i.e., $\leqslant 2.25$ (AUC = 0.71; $p < 0.05$), $\leqslant 8.60$ (AUC = 0.77; $p < 0.01$) and $\leqslant 5.28$ ng/mL (AUC = 0.76; $p < 0.01$) for BCS, BFT and serum leptin concentrations at calving, respectively, for occurrence of post-partum SCE. The sensitivity and specificity for their respective parameters at the threshold level is mentioned in Fig. 2. The diagnostic odds ratio (DOR) for development of sub-clinical endometritis in cows having BCS, BFT and serum leptin concentrations below the threshold level was 1.56, 4.40 and 2.80, respectively thus, aiding in maintaining the energy reserves up to a level from where the risk of developing SCE can be reduced.

Discussion

In sub-clinical endometritis, cows do not show any clinical signs although it continues to affect the economics of dairy industry in a substantial way (Wagener et al., 2017). Occurrence of SCE being

Table 4. Days of completion of uterine involution, resumption of certain ovarian activities, post-partum reproductive performance, daily milk yield and economic loss in relation to BCS, BFT, leptin concentration (ng/mL) and serum inflammatory markers at calving in SCEP and SCEN dairy cows (N = 41) (Mean ± SE)

Parameters	Groups	
	SCEP (N = 27)	SCEN (N = 14)
Body condition score (BCS)	2.24 ± 0.05^{y}	2.65 ± 0.10^{x}
Back fat thickness (BFT; mm)	8.48 ± 0.47^{y}	11.96 ± 0.72^{x}
Serum leptin concentration (ng/mL)	5.21 ± 0.25^{y}	6.84 ± 0.42^{x}
Completion of uterine involution (CUIN; days)	32.41 ± 1.13^{x}	27.50 ± 1.15^{y}
Days to first post-partum ovulation (DFPO)	43.30 ± 3.01^{x}	31.00 ± 3.26^{y}
Days to first artificial insemination	117.58 ± 1.39^{a}	89.73 ± 1.73^{b}
Number of inseminations per conception	2.26 ± 0.18	1.91 ± 0.21
Days open	167.54 ± 3.25^{a}	133.64 ± 2.96^{b}
Daily milk yield for 305 days (litres)	7.88 ± 1.21^{b}	11.20 ± 1.48^{a}
Economic loss (Rs./USD) per daily milk yield	Rs. 166.00/USD 2.27	–

^{x,y}Values with different superscripts within the same column differ significantly ($p < 0.05$).
^{a,b}Values with different superscripts within the same column differ significantly ($p < 0.01$).

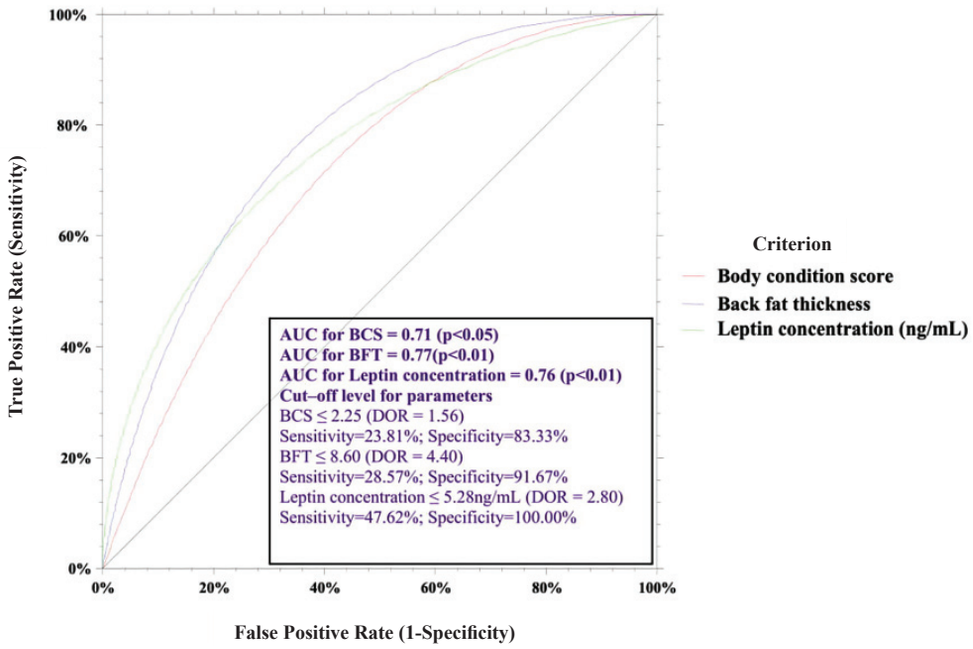


Fig. 2. ROC curve for cut-off level of BCS, BFT and leptin concentration (ng/mL) at the time of calving in aiding the diagnosis of SCE in dairy cows (N=41) at 8 weeks post-partum.

influenced by many factors and optimal energy reserves during the post-partum period play a pivotal role in induction of the immune system (Mani et al., 2012) and restoration of reproductive functions of dairy cows (Sharma et al., 2018). Visual or tactile estimation of sub-cutaneous fat quantity is indicative of energy reserves and represents BCS of a cow (Schroder and Staufenbiel, 2006; Bauer et al., 2012). However, objective assessment of sub-cutaneous fat via BFT has been considered as a more accurate and reliable indicator of the metabolic status of ows (Silva et al., 2005; Ayres et al., 2009; Singh et al., 2015). In concurrence with the findings of the present study, a decrease in BCS following parturition along with severe negative energy balance (NEB) and persistence of uterine infections has been reported (Wathes et al., 2007; Bacha and Regassa, 2010; Vargova et al., 2015). Although no such findings in reference to the relationship between occurrences of SCE with BFT have been reported by researchers, a hypothesis indicating the adverse effect of post-partum loss of energy reserves on the role of the immune system and subsequent development of endometrial inflammation holds some validity (Mani et al., 2012).

Many hormones play an important role in regulation of metabolic and reproductive activity of dairy cows. One of these hormones is leptin, a 16 kDa (kilo-Daltons) protein hormone synthesized by the adipose tissue, actively involved in restoration of ovarian cyclic activity and immune functions (Block et al., 2001; Liefers et al., 2003; Tanaka et al., 2008). However, Kasimanickam et al., (2013) reported significantly higher serum leptin concentrations at 5 weeks post-partum in cows subsequently diagnosed with SCE, which is not similar to findings of present study.

Resumption of the ovarian function plays a vital role in post-partum reproduction. Low BCS and sub-normal back fat induces NEB in dairy cows and results in low blood glucose and insulin concentrations along with elevated NEFA and ketones (Galindo et al., 2013; Wankhade et al., 2017), which is followed by low concentration of insulin growth factor-I (IGF-I) and luteinizing hormone (LH) frequency (1/24 hours) and delayed resumption of ovarian luteal activity (Mosenfechtel et al., 2002; Diaz et al., 2017). Also, leptin via decreasing the expression of adrenodoxin inhibits production of pregnenolone and progesterone (Kulcsar, 2007) as its concentration < 5 ng/mL has been considered to affect the ovarian activity negatively (Colakoglu et al., 2017). In agreement with the findings of the current study, resumption of post-partum ovarian cyclicity was significantly early in dairy cows with moderate to good BCS, BFT and serum leptin concentrations (Konigsson et al., 2014; Diaz et al., 2017; Kavya et al., 2018).

Completion of uterine involution and return to normal ovarian cyclic activity is very important for attaining the reproductive efficiency. Sharma et al. (2017) reported a slow rate of involution following development of post-partum uterine infections mainly due to insufficient/low endogenous $PGF_2\alpha$ production (Seals et al., 2002), which is similar to findings of the current study. No different set of findings have been reported by various researchers where delayed resumption of post-partum ovarian cyclicity was present in cows diagnosed with SCE (Galvao et al., 2010; Dubuc et al., 2012; Salehi et al., 2016; Elsayed et al., 2020). However, Carneiro

et al. (2014) found no relationship between SCE and ovarian cyclicity, which is contrary to the results of the present study.

Pearson correlation analysis carried out in the current study justified that significant correlations existed between CUIN, FPO and BCS, BFT and serum leptin concentrations at calving in SCEP and SCEN cows. Thus, an interpretation could be made that BFT and BCS at calving had a significant role in achieving timely uterine involution and the first post-partum ovulation, i.e., the higher the BCS and BFT at calving, the shorter the duration for CUIN and FPO to occur, and vice-versa. Many researchers have established the role of energy reserves in achieving a timely FPO (Salehi et al., 2016; Sharma et al., 2019a); however, the information on correlation analysis between these parameters in cows subsequently diagnosed with SCE is not mentioned in the literature.

Post-partum sub-clinical endometritis affects the fertility of cows negatively, thus, leading to poor reproductive performance (Carneiro et al., 2014) and in the process, deteriorating the economy of dairy farmers (Sharma et al., 2019b) and entrepreneurs. The current study was in concurrence with findings of various researchers (Plontzke et al., 2010; Barrio et al., 2015; Rinaudo et al., 2017) who have reported a higher number of days to the first AI (77–93 vs. 68–85 days) and, subsequently, days open (154–166 vs. 113–119 days) probably due to impaired sperm transport and storage, ovulation and zygote development (Gilbert, 2011).

Sub-clinical endometritis results in reduced milk yield of dairy cows. In the present study, loss of approximately 3.32 L of milk per day was found in SCEP cows, which was in agreement with the findings of other researchers who reported a decrease in milk production of 1.03–2.08 kg/cow/day and reduction of milk fat and protein in cows diagnosed with SCE (Bell and Roberts, 2007; McDougall et al., 2011; Sharma et al., 2019b).

The threshold values of energy reserves hold importance in effective management of cows at a risk of developing uterine infections. Not in agreement with the findings of the present study, a BCS value less than 2.50 at calving has been reported as the threshold level for development of uterine infections (Roche et al., 2009; Hoedemaker et al., 2009; LeBlanc, 2014; Carneiro et al., 2014). For BFT and serum leptin concentrations, no such threshold levels have been established previously in reference to the development of SCE.

In conclusion, the energy reserves, mainly low BCS and BFT, have been directly associated with the occurrence of post-partum sub-clinical endometritis and subsequently affect the restoration of early reproductive parameters, i.e., uterine involution and the first post-partum ovulation, reproductive efficiency (days to first artificial insemination and days open) and milk production in dairy cows. The cut-off values calculated for energy reserves provided an insight for maintaining the adequate level of BCS, BFT and serum leptin concentrations at the time of parturition to avoid sub-optimal reproductive efficiency associated with occurrence of SCE.

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References

1. Andela, B.G., Van Eerdenburg, F.J.C.M., Choukier, A., Bujak, D., Szelenyi, Z., Boldizsar, S., Kezer, F.L., Molnar, L., Kovacs, L., Szenci, O. Relationships among some serum enzymes, negative energy balance parameters, parity and postparturient clinical (endo)metritis in Holstein Friesian cows. Acta Vetrinaria Hungarica. 2019. 67. P. 241-245.
2. Ayres, H., Ferreira, R.M., de Souza, Torres Jr, J.R., Demetrio, C.G.B., de Lima, C.G., Baruselli PS. Validation of body condition score as a predictor of subcutaneous fat in Nelore (Bos indicus) cows. Livestock Science. 2009. 123. P. 175-179.
3. Bacha, B., Regassa, F.B. Subclinical endometritis in Zebu x Friesian crossbred dairy cows: its risk factors, association with subclinical mastitis and effect on reproductive performance. Tropical Animal Health and Production. 2010. 42. P. 397-403.
4. Barrio, M., Vigo, M., Quintela, L.A., Becerra, J.J., Garcia-Herradon, P.J., Martinez-Bello, D. Influence of subclinical endometritis on the reproductive performance of dairy cows. Spanish Journal of Agricultural Research. 2015. 13(4). P. 5-12.
5. Bauer, U., Harms, J., Steyer, M., Salau, J., Haas, J.H., Weber, A., Junge, W., Bieletzki, S., Rothfuss, H., & Suhr, O. Automatic monitoring of the body condition score of dairy cows. Landtechnik. 2012. 67(6). P. 409-412.
6. Bell, M.J., Roberts, D.J. The impact of uterine infection on a dairy cow's performance. Theriogenology. 2007. 68(7). P. 1074-1079.
7. Block, S.S., Butler, W.R., Ehrhardt, R.A., Bell, A.W., Van Amburgh, M.E., Boisclair, Y.R. Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. Journal of Endocrinology. 2001. 171. P. 39-48.
8. Butler, W.R. Nutrition, negative energy balance and fertility in the postpartum dairy cow. Cattle Practice. 2005. 13(1). P. 13-18.
9. Carneiro, L.C., Ferreira, A.F., Paduo, M., Saut, J.P., Ferraudo, A.S., & dos Santos, R.M. Incidence of subclinical endometritis and its effects on reproductive performance of crossbred dairy cows. Tropical Animal Health and Production. 2014. 46. P. 1435-1439.
10. Colakoglu, H.E., Polat, I.M., Vural, M.R., Kuplulu, S., Pekcan, M., Yazlik, M.O., Baklaci, C. Associations between leptin, body condition score, and energy metabolites in Holstein primiparous and multiparous cows from 2 to 8 weeks postpartum. Revue de Medecine Veterinaire. 2017. 168(4-6). P. 93-101.
11. Diaz, R., Galina, C.S., Rubio, I., Corro, M., Pablos, J.L., Rodriguez, A., Orihuela, A. Resumption of ovarian function, the metabolic profile and body condition in Brahman cows (Bos indicus) is not affected by the combination of calf separation and progestogen treatment. Animal Reproduction

Science. 2017. 185. P. 181-187.
12. Dubuc, J., Duffield, T.F., Leslie, K.E., Walton, J.S., LeBlanc, S.J. Risk factors and effects of postpartum anovulation in dairy cows. Journal of Dairy Science. 2012. 95(4). P. 1845-1854.
13. Edmonson, A.J., Lean, I.J., Weaver, L.D., Farver, T., Webster, G. A body condition scoring chart of Holstein dairy cows. Journal of Dairy Science. 1989. 72(1). P. 68-78.
14. Elsayed, D.H., El-Azzazi, F.E., Mahmoud, Y.K., Dessouki, S.M., Ahmed, E.A. Subclinical endometritis and postpartum ovarian resumption in respect to TNF-α, IL-8 and CRP in Egyptian buffaloes. Animal Reproduction. 2020. 17(1). P. 1-13.
15. Galindo, J., Galina, C.S., Estrada, S., Romero, J.J., Alarcon, M., Maquivar, M. Effect of changes in body weight, body condition and back fat during last month of pregnancy on the reproductive efficiency of Bos indicus cows in the tropics of Costa Rica. Open Journal of Veterinary Medicine. 2013. 3. P. 22-28.
16. Galvao, K.N., Frajblat, M., Butler, W.R., Brittin, S.B., Guard, C.L., Gilbert, R.O. Effect of early postpartum ovulation on fertility in dairy cows. Reproduction in Domestic Animals. 2010. 45(5). P. 207-211.
17. Gilbert, R.O. The effect of endometritis on the establishment of pregnancy in cattle. Reproduction Fertility and Development. 2011. 24. P. 252-257.
18. Gruber, L., Urdl, M., Obritzhauser, W., Schauer, A., Hausler, J., Steiner, B. Influence of energy and nutrient supply pre and postpartum on performance of multiparous Simmental, Brown Swiss and Holstein cows in early lactation. Animal. 2014. 8(1). P. 58-71.
19. Hoedemaker, M., Prange, D., Gundelach, Y. Body condition change ante and postpartum, health and reproductive performance in German Holstein cows. Reproduction in Domestic Animals. 2009. 44(2). P. 167-173.
20. Ingvartsen, K.L., Moyes, K. Nutrition, immune function and health of dairy cattle. Animal. 2013. 7. P. 112-122.
21. Kasimanickam, R.K., Kasimanickam, V.R., Olsen, J.R., Jeffress, E.J., Moore, D.A., Kastelic, J.P. Associations among serum pro-and anti-inflammatory cytokines, metabolic mediators, body condition, and uterine disease in postpartum dairy cows. Reproductive Biology and Endocrinology. 2013. 11(1). P. 103.
22. Kavya, K.M., Sharma, R.K., Jerome, A., Phulia, S.K., Balhara, A.K., Singh, I. Blood metabolites, body condition score, body weight and milk yield in relation to resumption of cyclicity in post-partum buffaloes. Indian Journal of Animal Sciences. 2018. 88(10). P. 1142-1145.
23. Klopcic, M., Hamoen, A., Bewley, J. (2011). Body condition scoring of dairy cows. Biotechnical, Faculty, Department of Animal Science, Domzale: 7. ISBN: 978-961-6204-54-5.
24. Konigsson, K., Savoini, G., Govoni, N., Invernizzi, G., Prandi, A., Kindahl, H., Veronesi, M.C. Energy balance, leptin, NEFA and IGF-I plasma concentrations and resumption of postpartum ovarian activity in Swedish red and white breed cows. Acta Veterinaria Scandinavica. 2008. 50. P. 1.
25. Kulcsar, M. Clinical endocrinology of leptin in ruminants. PhD Thesis, Szent Istavan University Postgradute School of Veterinary Science, Budapest. 2007.
26. Lacetera, N., Scalia, D., Bernabucci, U., Ronchi, B., Pirazzi, D., Nardone, A. Lymphocyte functions in overconditioned cows around parturition. Journal of Dairy Science. 2005. 88. P. 2010-2016.
27. LeBlanc, S.J. Reproductive tract inflammatory disease in postpartum dairy cows. Animal. 2014. 1. 54-63.
28. Liefers, S.C., Veerkamp, R.F., te Pas, M.F.W., Delavaud, C., Chilliard, Y., van der Lende, T. Leptin Concentrations in Relation to Energy Balance, Milk Yield, Intake, Live Weight, and Estrus in Dairy Cows. Journal of Dairy Science. 2003. 86. P. 799-807.
29. Mani, V., Weber, T.E., Baumgard, L.H., Gabler, N.K. Growth and Development Symposium: Endotoxin, inflammation, and intestinal function in livestock. Journal of Animal Science. 2012. 90. P. 1452-1465.
30. McDougall, S., Hussein, H., Aberdein, D., Buckle, K., Roche, J., Burke, C. Relationships between cytology, bacteriology and vaginal discharge scores and reproductive performance in dairy cattle. Theriogenology. 2011. 76. P. 229-240.
31. Montiel, F., Ahuja, C. Body condition and suckling as factors influencing the duration of postpartum anestrus in cattle: a review. Animal Reproduction Science. 2005. 85(1-2). P. 1-26.
32. Mosenfechtel, S., Hoedemaker, M., Eigenmann, U., Rusch, P. Influence of back fat thickness on the reproductive performance of dairy cows. Veterinary Record. 2002. 151(13). P. 387-388.
33. Pascottini, O.B., Hostens, M., Sys, P., Vercauteren, P., Opsomer, G. Cytological endometritis at artificial insemination in dairy cows: Prevalence and effect on pregnancy outcome. Journal of Dairy Science. 2016. 100. P. 588-597.
34. Plontzke, J., Madoz, L.V., De la Sota, R.L., Drillich, M., Heuwieser, W. Subclinical endometritis and its impact on reproductive performance in grazing dairy cattle in Argentina. Animal Reproduction Science. 2010. 122. P. 52-57.
35. Rana, A., Singh M, Kumar P., Sharma A. Comparison of the cytobrush, cytotape and uterine lavage techniques in healthy postpartum cows. Indian Journal of Animal Sciences. 2020. 90(3). P. 55-58.
36. Rinaudo, A., Bernardi, S.F., Marini, P.R. Relation between subclinical endometritis and reproductive efficiency in dairy cows in Argentina. Journal of Veterinary Science and Technology. 2017. 8(6). P. 1-4.
37. Roche, J.R., Friggens, N.C., Kay, J.K., Fisher, M.W., Stafford, K.J., Berry, D.P. Invited review: Body condition score and its association with dairy cow productivity, health and welfare. Journal of Dairy Science. 2009. 92. P. 5769-5801.
38. Salehi, R., Colazo, M.G., Gobikrushanth, M., Basu, U., Ambrose, D.J. Effects of prepartum oilseed supplements on subclinical endometritis, pro- and anti-inflammatory cytokine transcripts in endometrial cells and postpartum ovarian function in dairy cows. Reproduction Fertility and Development. 2016. 24. P. 747-758.
39. Schroder, U.J., Staufenbiel, R. Invited review: Methods to determine body fat reserves in the dairy cow with special regard to ultrasonographic measurement of backfat thickness. Journal of Dairy Science. 2006. 89. P. 1-14.
40. Seals, R., Matamoros, I., Lewis, G. Relationship between postpartum changes in 13, 14-dihydro-15-keto-PGF2alpha concentrations in Holstein cows and their susceptibility to endometritis. Journal of Animal Science. 2002. 80. P. 1068-1073.
41. Senosy, W.S., Izaike, Y., Osawa, T. Influences of metabolic traits on subclinical endometritis at different intervals postpartum in high milking cows. Reproduction in Domestic Animals. 2012. 47. 666-674.
42. Sharma, A. Efficacy of some ovulation synchronization protocols in dairy cows diagnosed with post-partum sub-clinical endometritis. PhD Thesis, Chaudhary Sarwan Kumar Himachal Pradesh Kirishi Vishvavidyalaya, India. 2020.
43. Sharma, A., Singh, M., Kumar, P., Thakur, A. Impact of body condition score and back fat thickness on resumption of ovarian cyclic activity after parturition in dairy cows. Himachal Journal of Agricultural Research. 2019a. 45(1&2). 104-106.
44. Sharma, A., Singh, M., Kumar, P., Dogra, P.K. Investigating the relationship between body condition score, sub-clinical endometritis and milk yield of dairy cows after parturition. Indian Journal of Animal Sciences. 2019b. 89(10). 1091-1093.
45. Sharma, A., Singh, M., Sharma, A., Kumar, P. Effect of PGF2α and antibiotic administration on uterine involution and occurrence of sub-clinical endometritis followed by evaluation of relationship between body condition score and PMN cells. International Journal of Livestock Research. 2017. 7(11). 257-261.
46. Sharma, A., Singh, M., Sharma, A., Kumar, P. Diagnosis and assessment of reproductive performance in dairy cows with sub-clinical endometritis. Intas Polivet. 2018. 19(1). P. 42-43.
47. Silva, S.R., Gomes, M.J., Dias-da-Silva, A., Gil, L.F., Azeved, J.M. Estimation in vivo of the body and carcass chemical composition of growing lambs by real time ultrasonography. Journal of Animal Science. 2005. 83(2). P. 350-357.

48. Singh, R., Randhawa, S.N., Randhawa, C.S. Body condition score and its correlation with ultrasonographic back fat thickness in transition crossbred cows. Veterinary World. 2015. 8. P. 290-294.
49. Tanaka, T., Arai, M., Ohtani, S., Uemura, S., Kuroiwa, T., Kim, S., Kamomae, H. Influence of parity on follicular dynamics and resumption of ovarian cycle in postpartum dairy cows. Animal Reproduction Science. 2008. 108. P. 134-143.
50. Vargova, M., Petrovic, V., Konvicna, J., Kadasi, M., Zaleha, P., Kovac, G. Hormonal profile and body condition scoring in dairy cows during pre partum and post partum periods. Acta Veterinaria Brno. 2015. 84. P. 141-151.
51. Wagener, K., Gabler, C., Drillich, M. A review on the ongoing discussion about definition, diagnosis and pathomechanism of subclinical endometritis in dairy cows. Theriogenology. 2017. 94. 21-30.
52. Wankhade, P.R., Manimaran, A., Kumaresan, A., Jeyakumar, S., Ramesha, K.P., Sejian, V., Rajendran, D., Varghese, M.R. Metabolic and immunological changes in transition dairy cows: A review. Veterinary World. 2017. 10(11). 1367-1377.
53. Wathes, D.C., Cheng, Z., Bourne, N., Taylor, V.J., Coffey, M.P., Brotherstone, S. Differences between primiparous and multiparous dairy cows in the interrelationships between metabolic traits, milk yield and body condition score in the periparturient period. Domestic Animal Endocrinology. 2007. 33. P. 203-225.

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Doppler Sonography for Evaluation of Haemodynamic Changes of Uterine Arteries and Umbilicus during Different Months of Gestation in Dairy Cows

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Keywords: *correlation analysis; dairy cows; doppler sonography; gestation; uterine and umbilical blood flow.*

Abstract. *The present study was conducted with an objective of evaluating the haemodynamic parameters for the middle uterine artery and the umbilicus and their inter-relationship in pregnant dairy cows (N = 12). Trans-rectal Doppler ultrasonography in dairy cows was carried out at an interval of 14 days beginning from day 14 to 238 of gestation. Pearson correlation coefficients were calculated using the CORR matrix in NCSS 2020. Pulsatility and resistance indices (PI and RI), time averaged maximum velocity (TAMAX), time averaged mean velocity (TAMEAN), diameter of the artery, maximum and mean volume of blood flow (BFV-TAMAX and BFV-TAMEAN), diameter, Doppler pulse duration (DPD) and systolic upstroke/acceleration time (AT) of middle uterine arteries and the umbilicus were measured to study the uterine and umbilical blood flow during different stages of gestation. Results revealed that haemodynamic indices, i.e., PI, RI, time averaged maximum and mean velocities, blood flow volume (mean and maximum) to the uterus and diameter of middle uterine artery, were significantly different ($p < 0.01$ and $p < 0.05$) between the middle uterine artery ipsilateral and contralateral to the gravid horn during the first 8 months of gestation. Pearson correlation analysis showed that a significant positive and negative correlation in the ipsilateral ($r = -0.6542–0.9188$; $p < 0.01$ and $p < 0.05$) and contralateral ($r = -0.4682–0.9363$; $p<0.01$ and $p<0.05$) middle uterine artery, respectively, during the first 8 months of gestation was present. Also, there was a linear change in the haemodynamic indices of the foetal umbilicus during the first 6 months of gestation along with a significant positive and negative correlation ($r = -0.5793–0.9520$; $p < 0.01$ and $p < 0.05$) between haemodynamic indices. In conclusion, the significant changes in haemodynamic changes of the middle uterine artery and the umbilicus occur during mid to late gestation in dairy cows.*

Introduction

With respect to normal physiology, alterations in uterine perfusion reflect structural and functional changes in the endometrium and, thus, may be an indirect measure for embryo-maternal communication during the establishment of pregnancy (Hassan et al., 2020). Modern diagnostic modalities including transrectal spectral Doppler ultrasonography allow quantification of the uterine blood flow both in cyclic, pregnant and pathological conditions and also help in supplementing the already established protocols of pregnancy and parturition (Panarace et al., 2006; Sharma et al., 2019). A characteristic pattern has been observed in the uterine vascularity throughout the estrous cycle concurrent with the serum progesterone and estradiol concentrations (Bollwein et al., 2016) whereas pregnant cows show a marked increase in the uterine blood flow beginning from week 3 in the gravid horn in contrast to the non-gravid horn (Silva and Ginther, 2010). Both pulsatility index (PI) and resistivity index (RI) serve as the most useful indicators in measuring the resistance offered to the blood within the vessels due to lying down of the microvasculature distal to the site of measurement, and also remain independent of the Doppler angle and the diameter of blood vessels (Maulik, 1993). Similarly, Doppler assessment of the umbilical arterial blood flow can be used as a marker of placental insufficiency (Scotti et al., 2008), as a close relationship exists between birth weight of the foetus, placental size, uterine and umbilical perfusion (Reynolds and Redmer, 1995). The umbilical arterial waveform has a characteristic saw-tooth appearance with the only systolic component while the umbilical venous waveform remains flat (Kumar et al., 2015). A consistent decline in resistance values of the umbilical blood flow with an increase in uterine irrigation has been observed throughout the gestational period (Serin et al., 2010). Hence, an effort was made to evaluate the sequential changes in haemodynamic indices of the middle uterine artery and the foetal umbilicus throughout gestation in dairy cattle.

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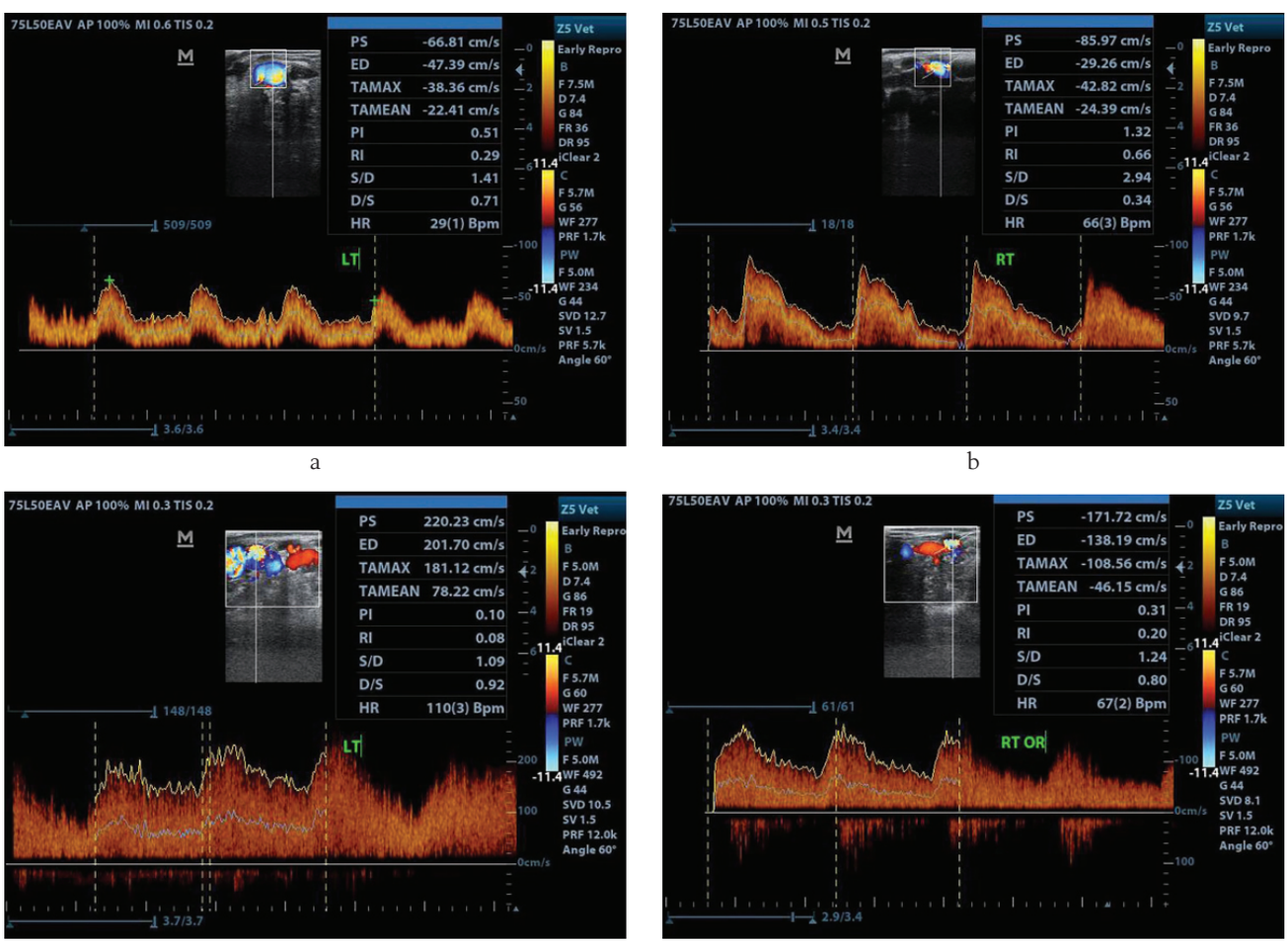
Materials and methods

Animals

Twelve pregnant Jersey crossbred multiparous cows (parity = 3; N = 12) reared in a loose housing system under standard management conditions, fed a total mixed ration, once daily *ad libidum*, with unrestricted access to water (32.6°N, 76.3°E, altitude 1290.8 m) were enrolled for the research after normal parturition. The cows were milked twice daily (04:00 and 15:00 h). All the experiments were carried after the approval of the ethics committee of the institute.

Trans-rectal Doppler sonography of the middle uterine artery for assessment of uterine perfusion

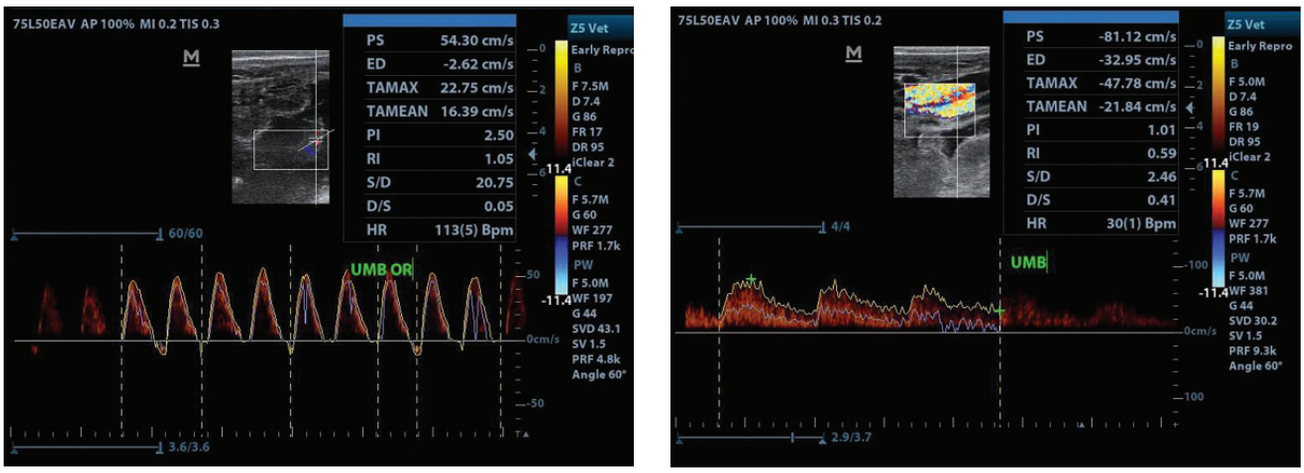
The middle uterine artery (MUA) originates from the internal iliac artery, can be found in the mesometrium as a movable arterial vessel, and is located cranial to the external iliac artery (Sharma et al., 2019). For monitoring of the uterine blood flow, Doppler examination of the MUA ipsilateral and contralateral to the pregnant uterine horn was performed from day 14 to 238 of gestation (Fig. 1, a–d) at an interval of 14 days using a linear probe of portable Mindray Z5 ultrasound machine at a frequency of 7.5 MHz, with a filter of 100 Hz and the Doppler angle varying between 30° and 60°. Visualization of the umbilicus begins after day 25 in cattle, and the umbilical pulsation was measured on the free fluctuating portion of the umbilical cord, between the conceptus and the gestational sac at an insonation angle of 60° in a spectral Doppler mode from day 28 to 182 of gestation (Fig. 2 a, b). The parameters displayed for each waveform by applying the automatic mode were pulsatility index (PI), resistivity index (RI), time averaged mean velocity (TAMEAN), time averaged maximum velocity (TAMAX), mean blood flow volume (BFV-TAMEAN) and maximum blood flow volume (BFV-TAMAX). The MUA's transverse diameter (D-MUA) was calculated from the mean of three measurements of the diameter made from frozen two-dimensional grey scale images just before Doppler measurements. Similarly, the width/diameter of the umbilical



a b

c d

Fig. 1. Ultrasonographic imaging of middle uterine artery.
(a) day 42 of gestation (ipsilateral); (b) day 42 of gestation (contralateral);
(c) day 210 of gestation (ipsilateral); (d) day 210 of gestation (contralateral).



a b

Fig. 2. Sonographic visualization of umbilicus.
(a) day 98 of gestation (characteristic saw-tooth pattern);
(b) day 196 of gestation (Flattening of umbilical waveform).

cord (D) was obtained from the images showing a straight longitudinal section of the umbilical cord and measured from the outer sides of the umbilical cord. In order to assess the significance of other haemodynamic parameters, Doppler pulse duration (DPD) and acceleration time / systolic upstroke time were also recorded.

Blood flow volume in mL/min was calculated using the equation (Varughese et al., 2013):

$$\text{Blood flow volume-TAMEAN} = \\ = \text{TAMEAN} \times \pi \times (D \times 0.1/2)^2 \times 60$$

$$\text{Blood flow volume-TAMAX} = \\ = \text{TAMAX} \times \pi \times (D \times 0.1/2)^2 \times 60$$

Statistical analysis

Numeric data for all the parameters were expressed as mean ± SE and statistically analyzed using repeated measures ANOVA and Pearson matrix correlation analysis with NCSS 2020, USA (Version 20.0.1).

Results

The PI and RI between ipsilateral and contralateral middle uterine arteries (MUA) differed significantly on days 140 ($p < 0.01$), 154 and 168 ($p < 0.05$), respectively. Similarly, the TAMAX and TAMEAN values of the ipsilateral MUA differed significantly throughout gestation on various days of examination with the significance level varying from $p < 0.01$ to $p < 0.05$. Also, the diameter (D-MUA) of the ipsilateral MUA showed a significant increase ($p < 0.01$ and $p < 0.05$) in the values when compared with the contralateral MUA throughout gestation. The volume of the blood flow evaluated through BFV-TAMAX and BFV-TAMEAN values differed significantly ($p < 0.01$ and $p < 0.05$) between ipsilateral and contralateral MUAs until the end of the period of examination (Table 1). Also, the mean blood flow values witnessed a 5.36 times increase throughout gestation in the ipsilateral MUA while the contralateral MUA showed a rise by a factor of 2.63.

The PI values were found to increase intermittently throughout gestation while the RI values showed an intermittent decline during the first trimester followed by a decrease in a linear fashion during the second trimester while studying the umbilical haemodynamics. Also, TAMAX and TAMEAN values showed a characteristic linear surge throughout the first and the second trimester (Table 2). Similarly, the umbilical diameter increased linearly during the period of examination with a 6.78-fold increase as compared with initial values on day 182 of gestation. The blood flow volume through the umbilicus also increased during the first and the second trimester with a dramatic increase by 2.1 times at the onset of the second trimester.

In correlation analysis of MUA haemodynamic parameters, PI and RI shared a significant positive correlation ($p < 0.01$) whereas other parameters such as TAMAX, TAMEAN, BFV-TAMAX and BFV-TAMEAN had a significantly negative correlation with PI and RI ($p < 0.01$) for ipsilateral and contralateral MUAs throughout gestation. Also, parameters for velocity and volume of the blood flow, i.e., TAMAX, TAMEAN and BFV-TAMAX and BFV-TAMEAN and D-MUA, were found to be positively correlated with each other ($p < 0.01$) for ipsilateral and contralateral MUAs throughout the period of examination (Table 3).

In a correlation analysis of umbilical haemodynamic parameters, PI and RI shared a significant positive correlation ($p < 0.05$) but were negatively correlated with TAMAX, TAMEAN, BFV-TAMAX, BFV-TAMEAN ($p < 0.01$) and diameter of the umbilicus ($p < 0.01$ and $p < 0.05$, respectively). The diameter of the umbilicus also had a significant positive correlation ($p < 0.01$) with parameters for velocity and volume of the blood flow during the first 6 months of gestation (Table 4).

Table 1. Uterine perfusion adjudged on Doppler indices of middle uterine arteries during the first 8 months of gestation in dairy cows (N = 12) (Mean ± SE)

Day of gestation	MUA side ipsilateral to gravid horn	Pulsatility index	Resistivity index	Time averaged maximum velocity (cm/sec)	Time averaged mean velocity (cm/sec)	D-MUA (mm)	BFV-TAMAX (mL/min)	BFV-TAMEAN (mL/min)
Day 28	Ipsilateral	1.05±0.16	0.56±0.08	40.75±4.15	21.99±3.37	10.90±0.06^{a}	2362.95±359.48	1302.91±235.02
	Contralateral	1.10±0.13	0.58±0.07	39.47±3.98	22.37±2.62	10.50±0.04^{b}	2121.72±334.60	1197.24±204.44
Day 42	Ipsilateral	1.02±0.10	0.55±0.08	40.16±2.28	22.36±1.64	11.60±0.04^{a}	2535.36±177.23^{x}	1375.88±89.15
	Contralateral	1.15±0.14	0.58±0.07	39.10±2.44	21.87±1.72	10.30±0.05^{b}	1954.14±162.58^{y}	1099.94±110.66
Day 56	Ipsilateral	1.19±0.13	0.59±0.04	46.88±6.66	26.86±4.14	11.50±0.04^{a}	2998.50±450.66^{x}	1669.12±233.75
	Contralateral	1.14±0.15	0.57±0.07	34.78±3.20	20.77±2.40	10.30±0.07^{b}	1876.32±305.83^{y}	1134.27±216.67
Day 70	Ipsilateral	1.01±0.17	0.52±0.07	43.02±4.33	25.26±2.45^{a}	12.20±0.07^{a}	3101.38±416.50^{x}	1823.36±251.78^{x}
	Contralateral	1.31±0.10	0.65±0.04	35.47±2.31	19.28±1.31^{b}	10.60±0.05^{b}	1954.85±235.73^{y}	1048.48±114.20^{y}
Day 84	Ipsilateral	1.29±0.11	0.60±0.05	36.48±1.84	19.59±1.11^{y}	12.90±0.05^{a}	2889.43±256.84	1540.11±131.10
	Contralateral	1.30±0.17	0.63±0.05	43.96±3.78	26.27±3.02^{x}	11.10±0.03^{b}	2627.60±23.43	1574.87±231.75
Day 98	Ipsilateral	1.20±0.14	0.68±0.08	37.70±4.37	21.12±1.75	12.60±0.05^{a}	2952.54±256.84	1540.11±131.10
	Contralateral	1.17±0.11	0.60±0.06	40.01±5.84	22.45±4.34	10.80±0.04^{b}	2255.35±364.15	1263.41±254.53
Day 112	Ipsilateral	0.98±0.15	0.51±0.07	40.26±3.51	22.30±2.68	13.30±0.05^{a}	3457.35±469.48	1902.81±299.12
	Contralateral	1.12±0.21	0.53±0.09	44.74±3.47	26.75±1.98	11.20±0.03^{b}	2660.00±290.78	1551.90±103.04
Day 126	Ipsilateral	1.03±0.24	0.53±0.11	52.78±6.46	28.55±3.91	13.90±0.06^{a}	4960.39±623.68^{a}	2601.25±345.60^{x}
	Contralateral	1.36±0.20	0.68±0.07	44.65±4.31	25.58±2.61	11.30±0.04^{b}	2707.56±295.33^{b}	1544.88±176.73^{y}
Day 140	Ipsilateral	0.74±0.10^{b}	0.38±0.09^{b}	49.40±6.26	24.64±2.37	14.00±0.04^{a}	4454.98±540.92^{a}	2255.42±270.36^{x}
	Contralateral	1.46±0.12^{a}	0.70±0.04^{a}	43.29±2.98	25.80±1.27	11.40±0.05^{b}	2672.62±304.63^{b}	1588.35±157.90^{y}
Day 154	Ipsilateral	0.66±0.14^{y}	0.40±0.07^{y}	80.75±6.67^{a}	39.41±4.73^{x}	13.20±0.04^{a}	6543.55±555.02^{a}	3197.58±358.87^{a}
	Contralateral	1.18±0.15^{x}	0.63±0.06^{x}	50.11±5.77^{b}	26.22±2.37^{y}	11.10±0.05^{b}	2876.90±318.56^{b}	1554.91±214.03^{b}
Day 168	Ipsilateral	0.69±0.17^{y}	0.40±0.08^{y}	75.03±5.86^{x}	38.10±3.72	14.20±0.03^{a}	7205.89±665.56^{a}	3657.71±392.34^{a}
	Contralateral	1.25±0.16^{x}	0.65±0.06^{x}	51.94±6.40^{y}	29.16±3.31	11.30±0.03^{b}	3154.16±427.14^{b}	1759.73±204.48^{b}
Day 182	Ipsilateral	0.61±0.11	0.37±0.06	103.24±11.34^{a}	51.41±7.75	14.30±0.06^{a}	9608.84±963.44^{a}	4821.50±760.94^{a}
	Contralateral	0.58±0.14	0.28±0.08	58.67±7.29^{b}	35.22±4.93	11.10±0.05^{b}	3480.75±492.49^{b}	2053.20±326.87^{b}
Day 196	Ipsilateral	0.41±0.16	0.27±0.09	120.50±14.32^{a}	58.26±9.04	15.10±0.10^{a}	12383.87±806.39^{a}	5880.47±506.71^{a}
	Contralateral	0.75±0.15	0.43±0.07	69.14±10.30^{b}	38.09±7.19	11.40±0.07^{b}	3928.24±238.19^{b}	2159.13±269.55^{b}
Day 210	Ipsilateral	0.50±0.12^{y}	0.34±0.07	133.61±15.53^{a}	64.07±10.14^{x}	14.80±0.14^{a}	12816.28±1032.68^{a}	6071.79±714.23^{a}
	Contralateral	0.92±0.16^{x}	0.51±0.07	71.51±12.11^{b}	33.33±4.41^{y}	11.40±0.07^{b}	4087.19±400.45^{b}	1840.12±295.88^{b}
Day 224	Ipsilateral	0.47±0.10	0.33±0.06	145.09±17.40^{a}	72.00±7.93	13.80±0.11^{a}	12538.68±1321.36^{a}	6239.04±639.51^{a}
	Contralateral	1.02±0.28	0.53±0.13	73.20±14.98^{b}	43.01±10.64	11.50±0.10^{b}	4217.33±499.16^{b}	2419.06±255.73^{b}
Day 238	Ipsilateral	0.38±0.08	0.27±0.05	139.08±16.15	71.83±11.80	13.80±0.09^{a}	12671.27±2038.24^{a}	6313.45±1004.75^{a}
	Contralateral	0.40±0.12	0.28±0.08	110.02±26.30	57.85±14.73	10.90±0.08^{b}	5586.92±898.45^{b}	2929.45±543.24^{b}

^{a,b}Values with different superscripts within the same column for the same parameter and day are significantly different ($p < 0.01$).
^{x,y}Values with different superscripts within the same column for the same parameter and day are significantly different ($p < 0.05$).

Discussion

Adaptations of the uterine artery in response to foetal demands reflect in the haemodynamic changes such as volume and velocity of the blood flow to the uterus (Hassan et al., 2020). The beginning of pregnancy is marked by relatively higher resistivity with high peak systolic velocity, low diastolic velocity and presence of the notch signal (Panarace et al., 2006), which is similar to the findings of the present study (Bollwein et al., 2000). However, with advancement of pregnancy, significant reduction in RI and PI coincides with vasculature development in the distal tissues and vascular remodelling linked with vascular endothelium (Gibbons and Dzau, 1994).

In concurrence with our study, the entire gestation period was marked by an increase in TAMAX and BFV-TAMAX values in both the middle uterine arteries, with a steep increase by 3 times of the initial values in the ipsilateral artery by the end of pregnancy (Nishida et al., 2006). Similarly, Varughese et al. (2013) reported an increase in TAMAX values

Table 2. Haemodynamic indices of the umbilicus following Doppler ultrasonography during the first 6 months of gestation in dairy cows (N = 12) (Mean ± SE)

Days of gestation	Pulsatility index	Resistivity index	Time averaged maximum velocity (cm/s)	Time averaged mean velocity (cm/s)	Diameter (mm)	BFV-TAMAX (mL/min)	BFV-TAMEAN (mL/min)
Day 28	2.04 ± 0.59	0.75 ± 0.05	7.02 ± 0.48	3.82 ± 1.06	2.30 ± 0.03	16.98 ± 5.73	8.64 ± 0.55
Day 42	3.10 ± 0.35	0.91 ± 0.06	10.07 ± 1.33	4.71 ± 0.64	4.20 ± 0.07	21.21 ± 3.08	37.21 ± 13.27
Day 56	3.52 ± 0.30	1.04 ± 0.02	12.61 ± 2.49	5.28 ± 0.27	6.70 ± 0.07	282.30 ± 71.06	119.77 ± 26.68
Day 70	2.79 ± 0.43	2.09 ± 0.86	14.19 ± 1.62	7.34 ± 1.22	8.00 ± 0.07	483.08 ± 118.56	244.33 ± 65.59
Day 84	2.41 ± 0.26	0.91 ± 0.06	20.17 ± 2.24	10.64 ± 1.28	10.40 ± 0.08	876.60 ± 259.67	455.37 ± 123.29
Day 98	2.21 ± 0.24	0.94 ± 0.09	20.68 ± 2.95	11.01 ± 1.77	12.70 ± 0.11	1833.58 ± 344.65	957.94 ± 183.74
Day 112	2.43 ± 0.36	0.95 ± 0.06	25.11 ± 3.00	13.86 ± 2.02	14.20 ± 0.09	2579.45 ± 275.10	1377.12 ± 91.85
Day 126	1.55 ± 0.38	0.76 ± 0.08	27.82 ± 5.38	15.43 ± 3.54	15.90 ± 0.12	3184.99 ± 848.30	1955.43 ± 377.33
Day 140	1.27 ± 0.29	0.69 ± 0.10	29.52 ± 3.15	16.23 ± 4.03	14.95 ± 0.08	2523.38 ± 222.14	1802.81 ± 465.90
Day 154	1.94 ± 0.89	0.67 ± 0.10	30.32 ± 7.14	16.98 ± 5.73	14.68 ± 0.07	3368.35 ± 1023.27	1909.68 ± 621.08
Day 168	0.56 ± 0.02	0.40 ± 0.04	35.77 ± 2.57	21.21 ± 3.08	16.60 ± 0.24	3590.00 ± 862.26	2090.02 ± 453.14
Day 182	0.50 ± 0.13	0.36 ± 0.08	31.94 ± 3.51	20.86 ± 2.93	15.60 ± 0.17	3547.62 ± 479.21	2446.77 ± 434.51

Table 3. Pearson correlation matrix for various haemodynamic indices of middle uterine arteries (MUA, ipsilateral and contralateral) during the first 8 months of gestation in dairy cows (N = 12)

Variables	Pulsatility index	Resistivity index	TAMAX	TAMEAN	D-MUA	BFV-TAMAX	BFV-TAMEAN
Ipsilateral MUA							
Pulsatility index	1.0000						
Resistivity index	0.8796*	1.0000					
TAMAX	–0.5793*	–0.4949*	1.0000				
TAMEAN	–0.5512*	–0.4808*	0.9520*	1.0000			
D-MUA	–0.0571	–0.0249	0.1753**	0.1179	1.0000		
BFV-TAMAX	–0.4865*	–0.3972*	0.8521*	0.7792*	0.5916*	1.0000	
BFV-TAMEAN	–0.4554*	–0.3821*	0.7775*	0.7721*	0.5335*	0.8922*	1.0000
Contralateral MUA							
Pulsatility index	1.0000						
Resistivity index	0.9363*	1.0000					
TAMAX	–0.4682*	–0.4192*	1.0000				
TAMEAN	–0.4103*	–0.3789*	0.9068*	1.0000			
D-MUA	0.1830**	0.2323*	–0.1371	–0.1201	1.0000		
BFV-TAMAX	–0.3609*	–0.2867*	0.7911*	0.7147*	0.4410*	1.0000	
BFV-TAMEAN	–0.2524*	–0.2032**	0.5577*	0.5601*	0.3397*	0.7164*	1.0000

*p < 0.01. **p < 0.05.

Table 4. Pearson correlation matrix for various haemodynamic indices of umbilicus during first 6 months of gestation in dairy cows (N = 12)

Variables	PI	RI	TAMAX	TAMEAN	D	BFV-TAMAX	BFV-TAMEAN
PI	1.0000						
RI	0.2572**	1.0000					
TAMAX	–0.4914*	–0.3328*	1.0000				
TAMEAN	–0.4651*	–0.3444*	0.9188*	1.0000			
D	–0.4795*	–0.2960**	0.6617*	0.6366*	1.0000		
BFV-TAMAX	–0.5115*	–0.4364*	0.7021*	0.6730*	0.7231*	1.0000	
BFV-TAMEAN	–0.6542*	–0.3800*	0.6452*	0.6631*	0.7170*	0.6581*	1.0000

*p <0 .01. **p < 0.05.

beginning from the first to the second trimester with a 1.3–1.8 times surge in the values throughout the last trimester in the ipsilateral artery; however, the contralateral artery showed a steady increase by 1.1–1.2 times. Not much akin to the findings of the present study, Herzog et al. (2011) reported a 28% and 36% higher BFV during 21st and 39th week of gestation.

For umbilical haemodynamics, a gradual fall in RI and PI values has been observed in the umbilical artery along with the advancement of gestation in buffaloes, which is similar to our study (Singh et al., 2018). In another study, foetal umbilical indices of RI and PI have been reported to decline by 26 weeks of gestation after which no change was observed (Panarace et al., 2006). However, very little to no changes have been observed in RI and PI values of the umbilicus throughout the gestation period in Murrah buffaloes using trans-abdominal ultrasonography, which is not akin to the findings of the present study (Singh et al., 2017).

During correlation analysis, RI of the ipsilateral and contralateral artery has been reported to be positively correlated with PI and negatively correlated with TAMAX, and blood flow volume to the uterus throughout gestation in cows (Bollwein et al., 2002) and buffaloes (Varughese et al., 2013; Abdelnaby, 2020), which is similar to our study. On the contrary, RI values have been found to be positively correlated with velocity and volume of the blood flow to the uterus and negatively correlated with other parameters (Panarace et al., 2006). In further concurrence with the present study, the diameter of both ipsilateral and contralateral arteries had a high positive correlation with the blood flow volume and a negative correlation with PI and RI beginning from month 1 to 8 of gestation (Hassan et al., 2020). Also, the umbilical diameter has been found to increase along with gestational age when measured between days 73 and 190 of gestation (Hunnam et al., 2009), which is in agreement with our findings.

In conclusion, haemodynamic indices of middle uterine arteries (MUA), i.e., pulsatility and resistivity index, time averaged mean and maximum velocities, blood flow volume (mean and maximum) to the uterus and diameter, were significantly higher in the ipsilateral MUA after day 140 of gestation. With a scope for future research, circulatory adaptations of the foeto-maternal unit during the course of gestation can be carefully monitored by regular examination of haemodynamic parameters to study the physiological and anatomical changes during normal and complicated pregnancy.

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References

1. Abdelnaby E.A. Haemodynamic changes in arterial flow velocities throughout the first six months of pregnancy in buffalo heifers by Doppler ultrasonography. Asian Pacific Journal of Reproduction. 2020. 9(4). P. 204-210.
2. Bollwein H., Baumgartner U., Stolla R. Transrectal Doppler sonography of uterine blood flow in cows during pregnancy. Theriogenology. 2002. 57. P. 2053-2061.
3. Bollwein H., Meyer H.H.D., Maierl J., Weber F., Baumgartner U., Stolla R. Transrectal Doppler sonography of uterine blood flow in cows during the estrous cycle. Theriogenology. 2000. 53. P. 1541-1552.
4. Bollwein H., Heppelmann M., Luttgenau J. Ultrasonographic Doppler Use for Female Reproduction Management. Veterinary Clinics of North America: Food Animal Practice. 2016. 32. P. 149-164.
5. Gibbons G.H., Dzau V.J. The emerging concept of vascular remodelling. New England Journal of Medicine. 1994. 33(20). P. 1431-1438.
6. Hassan M., Arshad U., Erdogan G., Ahmad N. Evaluation of haemodynamic changes of uterine arteries using Doppler ultrasonography during different stages of pregnancy in Bos indicus cows. Reproduction in Domestic Animals. 2020. https://doi.org/10.1111/rda.13793.
7. Herzog K., Koerte J., Flachowsky G., Bollwein H. Variability of uterine blood flow in lactating cows during the second half of gestation. Theriogenology. 2011. 75. P. 1688-1694.
8. Hunnam J., Parkinson T., Villalobos N.L., McDougall S. Association between gestational age and bovine fetal characteristics measured by transcutaneous ultrasound over the right flank of the dairy cow. Australian Veterinary Journal. 2009. 87. P. 379-383.
9. Kumar K., Chandolia R.K., Kumar S., Jangir T., Luthra R.A., Kumari S., KUMAR S. Doppler sonography for evauation of haemodynamic characteristics of fetal umbilicus in Beetal goats. Veterinary World. 2015. 8(3). P. 412-416.
10. Maulik D. Hemodynamic interpretation of the arterial Doppler waveform. Ultrasound in Obstetrics and Gynecology. 1993. 3(3). P. 219-227.
11. Nishida T., Hosoda K., Matsuyama H., Ishida M. Collateral uterine blood flow in Holstein cows during the third trimester of pregnancy. Journal of Reproduction and Development. 2006. 52(5). P. 663-668.
12. Panarace M., Garnil C., Marfil M., Jauregui G., Lagioia J., Luther E., Medina M. Transrectal Doppler sonography for evaluation of uterine blood flow throughout pregnancy in 13 cows. Theriogenology. 2006. 66(9). P. 2113-2119.
13. Reynolds L.P., Redmer D.A. Uteroplacental vascular development and placental function. Journal of Animal Science. 1995. 73. P. 1839-1851.
14. Scotti L., Di Salvo P., Bocci F., Pieramati C., Polisca A. Doppler evaluation of maternal and foetal vessels during normal gestation in queen. Theriogenology. 2008. 69. P. 1111-1119.
15. Serin G., Gokdal O., Tarimcilar T., Atay O. Umbilical artery Doppler sonography in Saanen goat fetuses during singleton and multiple pregnancies. Theriogenology. 2010. 74(6). P. 1082-1087.
16. Sharma A., Singh M., Abrol A., Soni T. Doppler sonography of uterine blood flow at mid-oestrus during different degree of clinical endometritis in dairy cows. Reproduction in Domestic Animals. 2019. 54. P. 1274-1278.
17. Silva L.A., Ginther O.J. Local effect of the conceptus on uterine vascular perfusion during early pregnancy in heifers. Reproduction. 2010. 139. P. 453-463.
18. Singh G., Chandolia R.K., Dutt R., Dalal J. Two-dimensional trans-rectal ultrasonographic studies in early pregnant

Murrah buffaloes. Indian Journal of Animal Sciences. 2018. 88(1). P. 59-64.
19. Singh, G., Chandolia R.K., Dutt R., Saini A. Trans-abdominal ultrasonography during second and third trimester in Murrah buffaloes. International Journal of Livestock Research. 2017. 7(12). P. 174-185.
20. Varughese, E., Brar P., Dhindsa S.S. Uterine blood flow during various stages of pregnancy in dairy buffaloes using transrectal Doppler ultrasonography. Animal Reproduction Science. 2013. 140. P. 34-39.

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Degrees of Tibial Plateau Angle and its Relation With dogs' Weight and Age in the Cases of Cranial Cruciate Ligament Rupture in Dogs: an Analysis of 90 Cases

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Keywords: *cranial cruciate ligament, tibial plateau angle*

Abstract*. Tibial plateau angle (TPA) is important for treatment selection of cranial cruciate ligament (CCL) rupture. The aim of this study was to determine the highest deviation of the TPA from normal range and to find the relation between the TPA degree, patients weight and age that show TPA influence in degeneration of the stifle joint and CCL rupture. The hypothesis of this research analysis was that dogs with CCL rupture have a significantly greater TPA than normal ranges. Ninety radiographs of dogs with CCL rupture were analyzed and the TPA was measured. All dogs examined were divided into four groups according to weight categories and each of these groups was divided into three subgroups according to age. In each weight and age group, the minimum and maximum degrees of the TPA and dogs ages and their mean were determined for all dogs in the group. The differences of largest and smallest deviations and means between weight groups were not statistically significant, as $P > 0.05$. However, all dogs with CCL rupture had a higher TPA than it is recommended ($P < 0.05$). The average TPA of all groups was 25.8 degrees and the highest TPA was 34 degrees. According to the results of the age groups, it was determined that there were no dogs younger than 1.5 years old in small and medium breed groups that had CCL rupture; most of them were in the geriatric age, which means that most of CCL rupture cases were because of the degeneration of CCL that might be caused by a high TPA degree. On the contrary, the majority of large and giant breed dogs that had CCL rupture were in the young and middle age. The differences of ages of groups were statistically significant, as P was <0.05. In conclusion, because the TPA average of all tested dogs was more than 10 degrees higher than recommended safe normal ranges, it should be kept in mind and treatment has to be aimed to reduce the strain in the stifle joint.*

Introduction

The cranial cruciate ligament (CCL) is a very important stabilizer inside the canine stifle joint. Rupture of the CCL is one of the most common stifle pathologies in dogs, especially in large breeds (Tobias and Johnston, 2013). In small breed dogs, CCL rupture is the second pathology after patellar luxation (Dona et al., 2016). CCL rupture is caused by many factors, such as genetics, overweight, bone deformities (genu varum, genu valgum, tibial or femoral torsion or both), patellar luxation, and excessively sloped proximal tibial plateau (Calvo et al., 2020). It is observed that some dog breeds are more susceptible than others, and its reason could be the degree of the tibial plateau angle (TPA) because it is different in all breeds of the dogs (Nečas et al., 2000). The research conducted in 2021 analyzed correlation between the TPA and dogs' weight, but it was found not statistically significant (Sörensson, 2021). One of the main causes of rupture of the CCL is an abnormally increased angle of the TPA. The reason for the large TPA is the deformities that occur during the growth of the proximal tibia (Reif and Probst, 2003). In their research, Read and Robins found that the TPA was highly increased in 4 of 5 dogs that experienced rupture of the CCL (Read and Robins, 1982). At a high degree of the TPA, the CCL is subjected to a much higher strain than in dogs with a degree of TPA within the normal range (Morris and Lipowitz, 2001). Although there is no specific one degree corresponding to the normal TPA, the recommended range of norms for the postoperative stability of the stifle joint is an angle of 4 to 6 degrees (Calvo et al., 2020). Dogs with a TPA up to 15 degrees are also thought to have a lower risk of CCL rupture than dogs with a higher TPA (Conkling et al., 2009). The rupture of this ligament can be partial or complete. In older dogs, the etiology of CCL rupture is often of degenerative origin (Ichinohe et al., 2015). The majority of ligament rupture cases are associated with synovial inflammation or degenerative lesions of the ligament cells and the tissue itself (Hayashi et al., 2004). The ligament is initially only partially damaged by rupture of the ligament fibers, and later, if the disease progresses and treatment is not started in time, a complete rupture of the ligament occurs. In young dogs the cause of CCL rupture is most often of a traumatic origin, in which case the rupture is usually complete because traumatic avulsion is present (Ichinohe et al., 2015).

In chronic processes of CCL injury, pain, decreased range of motion, muscle atrophy, swelling,

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abnormal posture when standing, getting up, lying down or sitting, abnormal gait when walking, trotting, climbing stairs or turning, grating or grinding joint movement, and nervous system signs like confusion and trembling are often recorded in a clinical examination (Harasen, 2002). The aim of this study was to determine the highest deviation of the TPA from normal ranges and to find the relation between the TPA degree, patients weight and age that show TPA influence in degeneration of the stifle joint and CCL rupture. The hypothesis of this research analysis was that dogs with CCL rupture have a significantly greater TPA than normal ranges.

Materials and methods

Ninety dogs that had lameness in the hind leg were taken for the research. There was no age or weight limit. All these dogs were tested in 2018–2021, and the examinations were performed at the veterinary clinic "Kaivana". During the examination, the majority of the affected legs had edema, soreness, a positive "drawer movement" test, and/or a positive tibial compression test (Fig. 1).

All of these dogs were sedated with dexmedetomidine hydrochloride and butorphanol medications. A dexmedetomidine hydrochloride dose of 300 μg/m^2 was counted following producer's recommendations and 0,01 mL/kg butorphanol was given following producer's guidelines. Radiographies were made on the affected legs under mediolateral (ML) and caudocranial (CC) projections prior to surgical treatment (tibial plateau leveling osteotomy). X-ray examination was performed with a Toshiba D045 X-ray machine, and imaging was performed using the CARESTREAM Vita Flex CR system. During the positioning of the ML projection, the leg at the stifle joint was flexed at an angle of 90 degrees, and during the CC projection, the leg at the stifle joint was considered to be extended and centered via distal tibia (Figs. 2, 3).

X-rays were performed to measure the TPA. In order to measure the TPA, two main axes were drawn: the axis of the tibial plateau passing through the cranial and caudal edge of the joint surface, and the longitudinal (functional) tibial axis line passing through the center of the tibial intercondylar eminence and the center of rotation of the talus. Through the point of intersection of these axes, an additional line was drawn perpendicular to the longitudinal axis, and the degree of angle between the axis of the tibial plateau and the additional line was calculated (Fig. 4) (Calvo et al., 2020).

All dogs examined were divided into four groups according to weight categories: group 1 – small (up

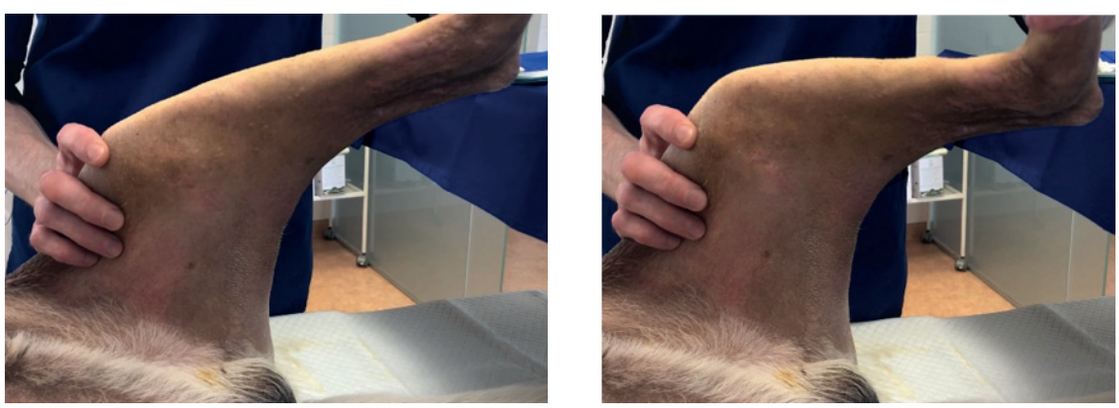
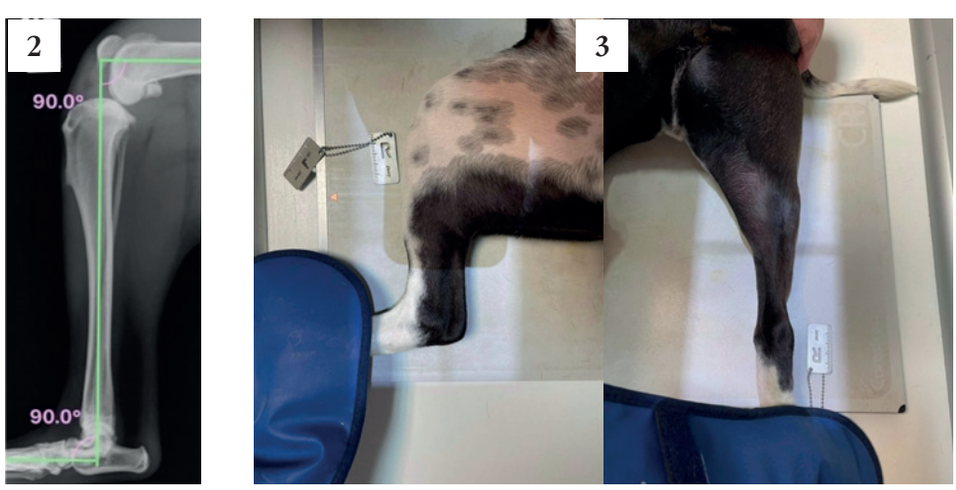


Fig. 1. A positive tibial compression test in a dog.



Figs. 2, 3. A dog positioned for ML and CC radiographs of the hind leg.

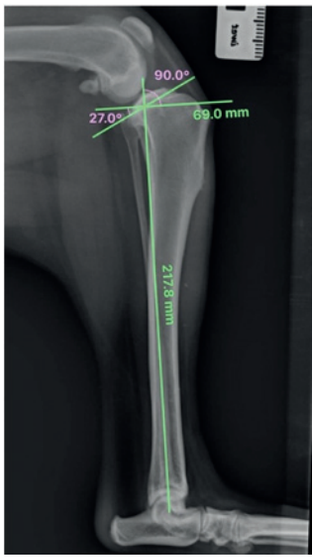


Fig. 4. Measurement of the tibial plateau angle in a dog.

to 10 kg); group 2 – medium (10.1–20 kg); group 3 – large (20.1–40 kg); and group 4 – giant (over 40 kg). Each of these groups was divided into three subgroups according to age: group 1 – young (up to 1.5 years); group 2 – middle (1.6–6 years); and group 3 – geriatric (over 6 years). In each weight group, the minimum and maximum degrees of the TPA and the mean with a deviation of the group degrees were determined for all dogs in the group. In each age group, the minimum and maximum ages were identified, and the mean with deviation of the groups of ages were determined for all dogs in the group.

Statistical analysis

Statistical data were processed using Microsoft Excel2018: arithmetic means with deviation (SD), maximum and minimum indicators were calculated. Data were considered reliable when the P value was less than 0.05.

Results

Group 1 (small dogs up to 10 kg)

The lowest degree of the TPA in the group was 23, the maximum was 31, and the mean (± SD) of the TPA in all dogs in the group was 28.2 ± 2.1 degrees (Table 1).

Group 2 (medium dogs of 10.1–20 kg)

The lowest degree of the TPA in the group was 22.5, the maximum was 32, and the mean (± SD) of the TPA in all dogs in the group was 26.9 ± 3.2 degrees (Table 2).

Group 3 (large dogs of 20.1–40kg)

The lowest degree of the TPA in the group was 18, the maximum was 34, and the mean (± SD) of the TPA in all dogs in the group was 24.2 ± 3.9 degrees (Table 3).

Group 4 (giant dogs of over 40 kg)

The lowest degree of the TPA in the group was 19, the maximum was 31, and the mean (± SD) of the TPA in all dogs in the group was 24.0 ± 3.5 degrees (Table 4).

The highest average of the TPA (28.2) was in the first group of weight and the lowest average of the TPA (24.0) was in the fourth group (Fig. 5).

In the first group of weight, the majority of the dogs were older than 6 years old and belonged to the

Table 1. Weights of the first group of dogs

Dogs of the first group of weight	Weight of the dog (kg)	TPA degrees
1	10	29.8
2	10	29
3	5	27.3
4	8	29.9
5	4	23
6	8.2	29.9
7	6	28
8	4.6	26
9	10	29
10	6.5	30
11	5	27
12	9.9	31
13	8	25
14	5.1	28.5
15	2.2	27.4
16	8.2	31
17	10	29
18	10	26
19	9.8	29
20	6	29

Table 2. Weights of the second group of dogs

Dogs of the second group of weight	Weight of the dog (kg)	TPA degrees
1	16	23
2	16.5	22.5
3	20	28.1
4	15	27.5
5	11	28
6	12.6	30
7	20.4	32
8	16.1	29
9	20	24
10	15.9	30
11	11	23
12	14.3	26

third group of age (15 cases), fewer dogs (5 cases) belonged to the second group of age (middle aged dogs) and there were no dogs younger than 1.5 years old (Fig. 6).

Table 3. Weights of the third group of dogs

Dogs of the third group of weight	Weight of the dog (kg)	TPA degrees
1	22	25
2	30.3	21
3	40	20.6
4	37.5	24.2
5	36	21
6	22	20.5
7	40	27
8	35.1	24
9	30	19.8
10	35	25.2
11	35.9	28
12	40	24
13	30.6	25
14	21	28
15	30	25
16	35.2	28
17	40	24
18	35	34
19	30	18
20	35.7	20
21	35	24
22	40	20
23	35.5	29
24	35.1	25
25	35	27.3
26	35	22
27	35.6	33
28	40	24
29	25.8	22
30	35	25
31	25.6	29
32	40	23
33	26.5	31
34	25	23
35	30.3	18
36	35	25
37	25.2	19
38	40	20
39	35.7	25

Table 4. Weights of the fourth group of dogs

Dogs of the fourth group of weight	Weight of the dog (kg)	TPA degrees
1	45	26
2	60.7	19.8
3	80	24
4	50	27
5	50.4	24
6	44	23
7	90.3	22
8	50	31
9	80.4	23
10	60.5	23
11	60	29
12	50.5	22
13	60.9	22
14	60	25
15	45	20
16	45.6	30
17	46	19
18	55	20
19	45.3	26

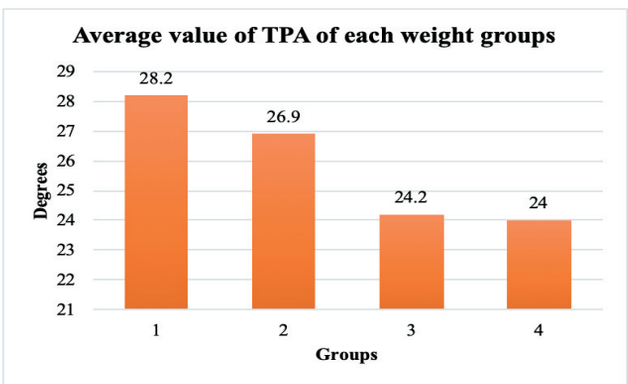


Fig. 5. Average value of the TPA of each weight group.

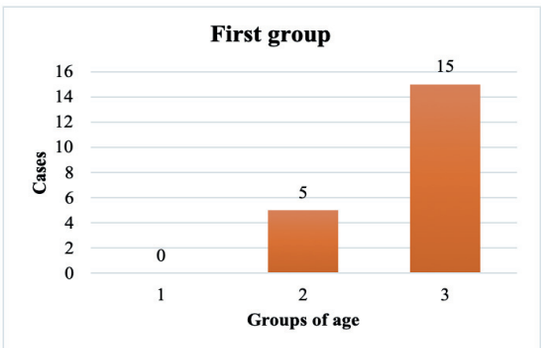


Fig. 6. The first group of weight and subgroups of age.

In the second group of weight, the majority of the dogs were older than 6 years old and belonged to the third group of age (9 cases), fewer dogs (3 cases) belonged to the second group of age (middle aged dogs) and there were no dogs younger than 1.5 years old (Fig. 7).

In the third group of weight, the majority of the dogs were 1.6–6 years old dogs and belonged to the second group of age (22 cases), fewer dogs (15 cases) belonged to the third group of age (geriatric dogs), and there were 2 dogs younger than 1.5 years old (Fig. 8).

In the fourth group of weight, the majority of the dogs were 1.6–6 years old dogs and belonged to the second group of age (12 cases), fewer dogs (4 cases) belonged to the third group of age (geriatric dogs), and there were 3 dogs younger than 1.5 years old (Fig. 9).

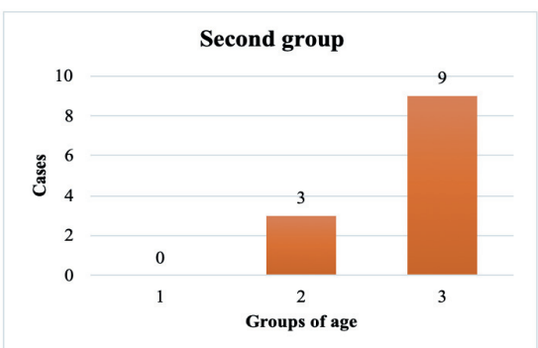


Fig. 7. The second group of weight and subgroups of age.

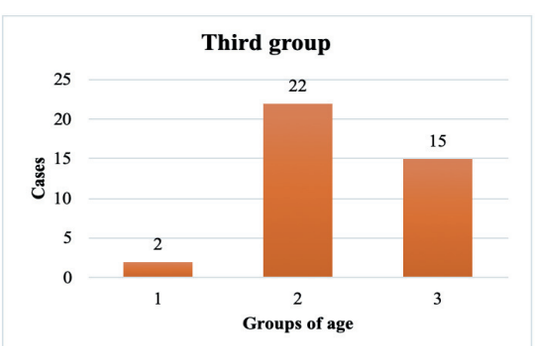


Fig. 8. The third group of weight and subgroups of age.

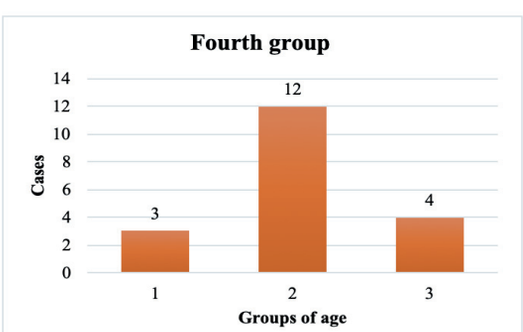


Fig. 9. The fourth group of weight and subgroups of age.

The maximum and minimum deviations from the degree of the TPA were found in the third group (large 20–40 kg) in all study dogs, and the mean (± SD) TPA in all study groups was 25.8 ± 2.1 degrees. The differences of the largest and smallest deviations and means between the groups were not statistically significant, as $P > 0.05$. However, all tested dogs had a higher TPA than it is recommended ($P < 0.05$). The highest average TPA (28.2) was in the first group of weight and the lowest average TPA (24.0) was in the fourth group of weight; however, the differences between the average TPA of these groups was not statistically significant, as P was > 0.05.

The youngest dog in the first group of age was 4 years old and the oldest dog was 13 years old. The average (± SD) of age in all dogs in the group was 8.2 ± 2.5 years. The youngest dog in the second group of age was 5 years old and the oldest dog was 13 years old. The average (± SD) of age in all dogs in the group was 7.7 ± 2.3 years. The youngest dog in the third group of age was 1 years old and the oldest dog was 11 years old. The average (± SD) of age in all dogs in the group was 5.4 ± 2.9 years. The youngest dog in the fourth group of age was 1 years old and the oldest dog was 8 years old. The average (± SD) of age in all dogs in the group was 4 ± 2.3 years. According to the results of the age groups, it was determined that there were no dogs younger than 1.5 years old in small and medium breed groups that had CCL rupture; however, most of them were in the geriatric age. On the contrary, the majority of large and giant breed dogs that had CCL rupture were in the young and middle age groups. The differences of ages between the groups were statistically significant, as P was <0.05.

Discussion and conclusion

The function of the CLL of the stifle joint is to provide stability in any phase of movement of the knee joint, preventing the tibia from sliding cranially to the femur, and to help prevent excessive rotation inside or outside (*varus et valgus*) during flexing of the stifle joint (Fossum, 2013). In the research by Reif and Probst in 2003, there was no statistical significance found between the TPA in healthy dogs and in dogs with CCL rupture (Reif and Probst, 2003). Nevertheless, the research of Arruda et al. shows that the TPA can possibly influence the etiology of CCL rupture in dogs (Flavia et al., 2018). This finding is in agreement with our study: the TPA of all study dogs with a CCL rupture was significantly higher than the recommended "safe" norm. However, there was no significant difference between the TPA index in the groups of dogs' weight. According to our study, it could be stated that the TPA is a very important factor in terms of CCL rupture, since the higher the degree of the TPA, the greater the strain on the ligament to maintain joint stability. Even though the TPA is a very important aspect in CCL rupture

cases this does not yet prove that this alone affects CCL rupture (Reif and Probst, 2003). As Brinker, Piermattei and Flo's state, CCL rupture may also be caused by femoral or tibial torsion, dog weight, stifle degenerative processes, or systemic inflammatory joint diseases such as rheumatoid arthritis (Brinker et al., 2016). Our study is in agreement with this theory because the largest average of the TPA was found in the small breed group and the majority of CCL cases in small breeds were dogs in the geriatric age (Fig. 10). It means that most of CCL rupture cases were because of the degeneration of CCL (that was seen during surgery: excessive synovial fluids, osteophytes, cartilage lesions, etc.) that might be caused by the high TPA degree. On the other hand, there were cases that CCL ruptured in young age dogs and the TPA was low enough (trauma being the reason), which proves that not only the TPA has influence on CCL rupture; however, the cases like that were quite rare in our study.

Because the TPA is thought to play a significant role in CCL rupture, it is important to consider this when choosing the right treatment option. Nowadays, intracapsular or extracapsular reconstruction is becoming less and less common, especially for large breed dogs. Its goal is to restore the passive restraining forces of the stifle joint, but even an implanted

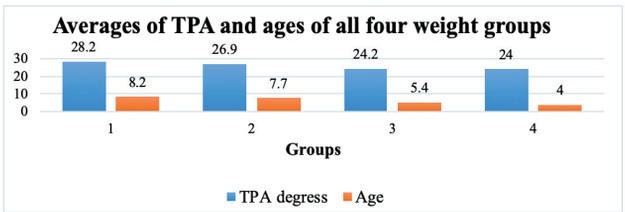


Fig. 10. Averages of the TPA and ages of all four weight groups

artificial ligament with a high degree TPA can rupture over time in the same way as the natural CCL. More successful treatments are tibial plateau leveling osteotomy (TPLO) and tibial tuberosity advancement (TTA) (Lazar et al., 2005). The purpose of the TPLO is to change the mechanics of the stifle joint by actively restraining it to achieve its stability. During osteotomy, the slope of the tibial plane is changed so that it and the patella would be perpendicular to each other. Since cranial tibial instability is proportional to the slope of the tibial plane, a decreasing slope also reduces cranial tibial instability. The TTA aims to eliminate tibial instability by moving the patellar tendon perpendicular to the sliding force in the knee joint. However, tibial fracture, implant rejection, meniscus damage, and other complications are possible after TTA (Boudrieau, 2009).

References

1. Veterinary Surgery: Small Animal - E-BOOK: 2-Volume Set Karen M. Tobias, Spencer A. Johnston Elsevier Health Sciences, 2013-12-26 – 2352, p. 914.
2. F. Di Dona, G. Della Valle, C. Balestriere, B. Lamagna, L. Meomartino, G. Napoleone, F. Lamagna, G. Fatone: Lateral patellar luxation in nine small breed dogs. Open Vet J. 2016; 6(3): 255–258.
3. Ignacio Calvo, Felipe De Vicente, Miguel Solano, Josep Aisa: Small Animal Orthopaedics. The Hindlimb. 1st edition. Grupo Asis Biomedia, SL, 2020. p. 41,55.
4. A. Nečas, J. Zatloukal, H. Kecova, M. Dvorak: Predisposition of Dog Breeds to Rupture of the Cranial Cruciate Ligament. Acta Vet. Brno 2000, 69: 305-310.
5. Sarah Sörensson: Evaluation of tibial plateau angle and other factors in cases of canines stifle joint diseases. Kaunas, 2021, 22.
6. Reif, U., & Probst, C. W. (2003). Comparison of Tibial Plateau Angles in Normal and Cranial Cruciate Deficient Stifles of Labrador Retrievers. Veterinary Surgery, 32(4), 385–389. doi:10.1053/jvet.2003.50047
7. Read R, Robins GH: Deformity of the proximal tibia in dogs. Vet Rec 111:295-298, 1982.
8. Morris E, Lipowitz AJ: Comparison of tibial plateau angles in dogs with and without cranial cruciate ligament injuries. J Am Vet Med Assoc 218:363-366, 2001
9. Amanda L Conkling, DVM, Bennett Fagin, DVM, Diplomate ACVR, and R. Mark Daye, DVM, MS, Diplomate ACVS: Comparison of Tibial Plateau Angle Changes after Tibial Plateau Leveling Osteotomy Fixation with Conventional or Locking Screw Technology. Ohio Veterinary Surgery and Neurology, Metropolitan Veterinary Hospital, Akron, OH 2009, 475-481.
10. Ichinohe, T., Kanno, N., Harada, Y., Yogo, T., Tagawa, M., Soeta, S., Hara, Y. (2015). Degenerative changes of the cranial cruciate ligament harvested from dogs with cranial cruciate ligament rupture. Journal of Veterinary Medical Science, 77(7), 761–770. doi:10.1292/jvms.14-0383
11. Cranial Cruciate Ligament Pathophysiology in Dogs With Cruciate Disease: A Review Kei Hayashi, DVM, MS, PhD ; Paul A. Manley, DVM, MSc, Diplomate ACVS ; Peter Muir, BVSc, MVetClinStud, PhD, Diplomate ACVS, Diplomate ECVS. J Am Anim Hosp Assoc (2004) 40 (5): 385–390.
12. Greg Harasen: Can Vet J. 2002 Jun; 43(6): 475–476. Diagnosing rupture of the cranial cruciate ligament Animal Clinic of Regina, 1800 Garnet Street, Regina, Saskatchewan S4T 2Z2
13. Fossum T. W. Small Animal Surgery. 4th ed. St. Louis, MO: Mosby/Elsevier, 2013. p. 1323-1364.
14. Ana Flavia D.P. Arruda, Leonardo A.L. Muzzi, Antonio C.C. Lacreta Junior, Ruthnea A.L. Muzzi, Gabriela R. Sampaio, Stella H. Moreira and Luciane R. Mesquita: Radiographic assessment of the proximal tibial angles in dogs and cats with and without cranial cruciate ligament rupture. Pesquisa Veterinaria Brasileira, 2018. 38 (6):1190-1195.
15. Brinker, Piermattei and Flo's. Handbook of Small Animal Orthopedics and Fracture Repair. 5th Edition. Elsevier, 2016. p. 616-617.
16. Lazar, T. P., Berry, C. R., Dehaan, J. J., Peck, J. N., & Correa, M. (2005). Long-Term Radiographic Comparison of Tibial Plateau Leveling Osteotomy Versus Extracapsular Stabilization for Cranial Cruciate Ligament Rupture in the Dog. Veterinary Surgery, 34(2), 133–141. doi:10.1111/j.1532-950x.2005.00021.x
17. Boudrieau RJ. Tibial plateau leveling osteotomy or tibial tuberosity advancement? Vet Surg. 2009 Jan;38(1):1-22.

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Study of Anogenital Distance in Rabbits: Effect on Sexual Behavior and Litter Size Biological Components

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Keywords: *AGD, hormones, morphology, prolificacy, rabbit, receptivity.*

Abstract. *The aim of this work is to study the relationship between the anogenital distance (AGD) measured before mating and plasma cholesterol and hormone concentrations (testosterone and 17-βestradiol), sexual behavior, litter size and its biological components (ovulation rate and prenatal survival) and the sex ratio in rabbits. In total, 48 rabbit does were used. The females were classified according to their AGD in 2 groups (AGD long or AGDL, n = 24, and AGD short or AGDS, n = 24). Blood samples were collected before mating, receptivity of the females was tested and their behavior was observed. Endoscopy was performed at day 12 of pregnancy. The number of total born, alive, dead and the sex ratio were recorded at birth. The plasma testosterone and cholesterol concentrations were significantly higher in the AGDL group of females (14% and 24%, respectively). The AGDL females presented a higher rate of receptivity (31%; P < 0.05), they were more aggressive (78%; P < 0.05) and marked more frequently their territory using the spontaneous chin marking than the AGDS females (34%; P < 0.05). The number of implanted embryos was significantly higher in the AGDS group (9.12 vs. 8.66 embryos). The embryonic, fetal and prenatal survival were significantly higher in the AGDS females. In addition, the AGDS females presented a higher litter size at birth (8.96 vs. 7.83; P < 0.01) and sex ratio in favor of males (61.60% vs. 41.00%; P < 0.01). In conclusion, the AGD measured before mating can be used as a predictor of the testosterone level, sexual behavior, litter size at birth and the sex ratio in rabbits.*

Introduction

In mammals, litter size and reproductive performances can be influenced by the animal's previous intra-uterine position (IUP). Thus, it has been the subject of numerous studies (see review of Ryan and Vandenbergh, 2002), in order to show its influence on reproductive parameters (hormone levels, development of external genitalia and sexual behavior).

Except for this type of a purely anatomical position, there is another particularity related to hormones. Any fetus not located at one end of the uterus will be positioned between two males (2M), two females (0M), or one male and one female (1M). This IUP has important and far-reaching effects on fetal development in animals as well as in humans (MacLusky and Naftolin, 1981). These effects are essentially related to the *in utero* interaction between the different hormones to which the fetus is exposed throughout gestation (Even et al., 1992). Among these hormones, testosterone plays a primordial role in the masculinization process. Indeed, male fetuses produce testosterone earlier and in greater quantities than female fetuses (Arnold, 2002). This hormone can diffuse between fetuses through fetal membranes and amniotic fluid (Wallen and Baum, 2002). Therefore, both 2M male and female fetuses (positioned between two males) have higher blood testosterone and lower estradiol than 0M fetuses (positioned between two females) (Vom Saal et al., 1990). *In utero*, elevated testosterone concentrations in several species affect fetal body development, behavior, physiology and morphology in adulthood (Ryan and Vandenbergh, 2002). However, the most significant impact of testosterone is on anogenital distance (AGD) (Ryan and Vandenbergh, 2002).

Indeed, 2M females have a long AGD compared with that measured on 0M females. In contrast, 1M females have an intermediate AGD (Hernandez-Tristan, 1999). This phenomenon has been described in mice (Zielinski et al., 1991), rats (Meisel and Ward, 1981) and rabbits (Bánszegi et al., 2009). This morphological difference persists from birth to adulthood in rabbits and in several rodent species. On the other hand, females with a higher AGD show higher blood testosterone concentrations, tend to be more aggressive, are less attractive to males and show low prolificacy with a male sex ratio (Rohde Parfet et al., 1990; Bánszegi et al., 2009). Thus, this parameter, revealing prenatal exposure to androgens, is frequently used as a biological marker for some reproductive parameters in animals such as mice (McDermott et al., 1978), rats (Meisel and Ward, 1981), Mongolian gerbils (Clark and Galef, 1998), pigs (Drickamer et al., 1997), cows (Gobikrushanth et al., 2016) and rabbits (Bánszegi et al., 2012).

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In a first published study on rabbits, Kerkouche et al. (2014) noted higher embryonic and fetal mortality in rabbits with a long AGD and, consequently, a lower number of implanted embryos (estimated at 12 days of gestation by scarification of females) compared with females with a small AGD. Therefore, studying the relationship between IUP, AGD, litter size and sex ratio could help in the selection of the best performing animals for breeding. This experiment follows up on the previous work, and aims to study the relationship between the AGD measured before mating and the sexual behavior of female rabbits, the level of sexual hormones (testosterone and 17β-estradiol), litter size at birth and its main biological components (ovulation rate and prenatal survival) as well as the sex ratio in rabbits.

Materials and methods

This study was approved by the Scientific Council of Biotechnology Laboratory of Animal Reproduction, University of Saad Dahlab Blida, Institute of Veterinary Sciences (Algeria).

Our experiment was carried out in the rabbitry of the Experimental Station, University Blida I, Algeria. The rabbits were housed in individual flat-deck cages and fed *ad libitum* with commercial pelleted diet (17.1% crude proteins, 16.5% crude cellulose and 3.2% fat). The rabbit does were submitted to a constant photoperiod of a 16L:8D light cycle during the whole experiment period.

Animals

The rabbits used in this experiment belong to the ITELV2006 line. The characteristics of this line are described in Ezzeroug et al. (2020). Forty-eight (48) females were selected and placed in individual cages. The selection criteria were parity (multiparous at the third parity), a homogeneous weight at mating (3005 ± 47g) and a good health status. Eight males (4230 ± 284 g) were used to mate the females with a rhythm of 3 mating acts per week and a rest of one day between two consecutive mating acts.

Measurement of AGD

AGD is measured between the center of the anus and the vulva using the method described by Bánszegi et al. (2012). It is measured three times for each female, by different operators and using a digital caliper. The mean of the three observations is calculated (AGDM). The rabbits are then classified according to their AGDM into two classes: the first class concerns females with a short AGD or AGDS (AGD is equal to or lower than the average AGD of all rabbits). In contrast, the second class includes females with a long AGD (above the average of all rabbits or AGDL (Drickamer et al., 2001).

Mating and evaluation of sexual behavior

The females were mated in the morning, between 9 and 10 am. Before each presentation to the male, the female was weighed. The receptivity of the female was evaluated by examining the vulva color and its turgidity (indirect method). The female is considered receptive when the vulva is pink or red and turgid. On the other hand, it is non-receptive when it has a pale pink or white and non-turgid vulva (Theau-Clément et al., 2015). Receptivity was also assessed during mating (direct method: acceptance or refusal of mating). In addition, the behavior of the female during mating with the male was also studied. Four parameters were noted: aggression, mounting, chin and urine marking.

Blood sampling and hormones analysis

Thirty minutes before mating, a blood sample of each female was taken by puncture of the auricular marginal vein. The blood was collected in heparinized tubes and immediately centrifuged at 3000 rpm/15 minutes. The plasma was stored at –20°C for subsequent analyses of cholesterol, 17β-estradiol and testosterone. Plasma levels of the different parameters were assayed in duplicate for each plasma sample and using RIA (I^{125} Immunotech® kits) for hormones and spectrophotometry (Spinreact® kits) for cholesterol.

Endoscopy

At 12 days *post coitum*, the diagnosis of pregnancy is made by abdominal palpation. The endoscopy was performed according to the method described by Santacreu et al. (1990). The following variables were measured: ovulation rate (OR) measured by counting the number of *corpus luteum* in both ovaries, number of implanted embryos (IE) estimated as the number of implantation sites, number of alive embryos (AE) estimated as the number of normal uterine swellings, number of resorbed embryos (RE) estimated as the number of small uterine swellings with reduced vascular supply, number of total newborn at third parity (TNB), number of born alive (BA), percentage of mortality at birth (M) measured as the number of kits found dead the day of parturition divided on TNB, embryonic survival (ES) estimated as AE+RE/OR, fetal survival (FS) estimated as AE/TNB, and prenatal survival (PS) estimated as TNB/OR. Finally, the sex ratio was recorded.

Statistical analyses

The results are described by the mean and standard deviation. They were subjected to a one-factor analysis of variance (ANOVA) to determine the effect of the AGD on all measured parameters (hormone concentrations, litter size traits and its biological components). The analysis of parameters used for the evaluation of sexual behavior at mating was performed by the $\chi 2$ test. Analyses were performed using the Statview program (Abacus Concepts, 1996. Inc., Berkeley, CA94704-1014, USA).

Results

Classification of females according to their AGD

The classification of females according to their AGD is presented in Table 1. The AGDM of the females used in this experiment was 26.33 ± 1.30 mm. The females with AGDL and AGDS had distances of 28.49 mm and 24.17 mm, respectively.

Effect of AGD on plasma steroid hormone and cholesterol levels

Plasma concentrations of steroid hormones (testosterone and 17β-estradiol) as well as that of cholesterol are presented in Table 2. Plasma testosterone levels averaged 134 pg/mL and 115 pg/mL in the AGDL and AGDS females, respectively, a significant difference of 14% in favor of AGDL females ($P = 0.003$). The AGDL females showed elevated cholesterol levels compared with those measured in the AGDS females (24%; $P < 0.001$). In contrast, no significant difference was found between the two groups of rabbits for plasma 17β-estradiol levels.

Effect of AGD on sexual behavior of females at mating

Table 3 shows the effect of AGD on the sexual behavior of rabbit does at mating. The receptivity of females, as assessed by direct examination of the vulva or by acceptance or refusal of mating, varies significantly with the AGD. The AGDL females showed higher receptivity rates compared with the AGDS females (31; $P < 0.001$; all methods combined). A reduction in the receptivity rate between the two methods was observed (12% for the indirect method).

In addition, the sexual behavior of female rabbits at mating varied significantly between the two experimental groups. The AGDL females tended to be more aggressive (78%; $P < 0.001$) and mounted males (34%; $P < 0.001$). The AGDL females showed a significant chin marking activity compared with the AGDS females (34%; $P < 0.001$). However, only the AGDL females showed urine marking.

Effect of AGD on litter size and its main biological components

The weight of females at parturition was similar between the females of the two experimental groups (Table 4). Similarly, the ovulation rate did not vary with the AGD of the female. The number of alive embryos on day 12 of gestation was significantly higher in the AGDS females (15%, $P = 0.01$). However, the number of resorbed embryos was significantly higher in the AGDL females (91%; $P < 0.013$).

Embryonic and fetal survival were significantly higher in the AGDS females compared with those measured in the AGDL females (13% and 9%, respectively; $P < 0.01$). Similarly, prenatal survival was significantly higher in the AGDS females (25%; $P < 0.001$).

The litter size at birth estimated by the number of total newborn kits was higher in the AGDS females (13%; $P = 0.031$). The percentage of mortality at birth was significantly higher in the AGDS females (81%; $P = 0.006$). Finally, in the AGDL females, the number of males per litter was significantly higher than in the AGDS females (61.60% *vs.* 41.00%; $P < 0.001$).

Discussion

Plasma testosterone levels were significantly higher in the AGDL females compared with those measured in the AGDS females (134 pg/m *vs.* 115 pg/m). Our results corroborate those reported by several authors indicating that AGDL females have high blood testosterone concentrations (Frederick et al., 1980, in mice; Clark et al., 1992, in gerbils). In rabbits, to our knowledge, the relationship between AGD and testosterone has not been studied in the past. This hormone plays a direct role on AGD in rabbit and in several rodent species. Indeed, it has been shown in both rabbits and mice that exposure during fetal life to high concentrations of testosterone

Table 1. Classification of females according to their AGD (mean ± standard deviation).

	AGD 1 (mm)	AGD 2 (mm)	AGD 3 (mm)	AGDM (mm)
AGDT (n = 48)	26.30 ± 1.31	26.35 ± 1.32	26.32 ± 1.29	26.33 ± 1.30
AGDL (n = 24)	28.46 ± 0.65	28.47 ± 0.63	28.53 ± 0.64	28.49 ± 0.63
AGDS (n = 24)	24.17 ± 0.53	24.15 ± 0.60	24.18 ± 0.55	24.17 ± 0.53

AGDT: anogenital distance for all females; AGDL: long anogenital distance; AGDS: short anogenital distance; AGDM: medium anogenital distance

Table 2. Effect of AGD on plasma steroid hormones and cholesterol (mean ± standard deviation).

	Testosterone (pg/mL)	17β-estradiol (pg/mL)	Cholesterol (mg/dL)
AGDL (n = 24)	134 ± 22.12	254 ± 25.24	38 ± 4.61
AGDS (n = 24)	115 ± 20.45	241 ± 23.53	29 ± 5.14
P	0.003	0.071	< 0.001

AGDL: long anogenital distance; AGDS: short anogenital distance.

Table 3. Sexual behavior of females at mating according to their AGD (mean ± standard deviation).

	Receptivity (direct method) %	Receptivity (indirect method) %	Aggression %	Mounting %	Chin marking %	Urine marking %
AGDL (n = 24)	80.14 ± 8.33	70.27 ± 7.66	25.36 ± 3.24	9.72 ± 0.18	27.17 ± 2.27	05.41 ± 1.04
AGDS (n = 24)	55.47 ± 6.25	48.51 ± 8.11	5.64 ± 1.33	2.45 ± 1.21	18.01 ± 1.98	/
P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

AGDL: long anogenital distance; AGDS: short anogenital distance.

Table 4. Effect of AGD on litter size and its biological components in rabbits (mean ± standard deviation).

Traits	AGDL (n = 24)	AGDS (n = 24)	P
WFM, g	3065.39 ± 200.55	3109.30 ± 178.78	0.427
OR, corpora lutea	10.12 ± 1.91	9.24 ± 1.75	0.103
IE, embryos	8.66 ± 2.01	9.12 ± 1.65	0.391
AE, embryos	7.69 ± 1.79	9.03 ± 1.66	0.01
RE, embryos	0.96 ± 1.61	0.09 ± 0.38	0.013
ES, %	85.62 ± 12.84	98.84 ± 3.22	< 0.001
FS, %	90.18 ± 14.33	99.01 ± 4.04	0.006
PS, %	72.83 ± 14.48	97.25 ± 5.82	< 0.001
TNB, kits	7.83 ± 1.84	8.96 ± 1.68	0.031
BA, kits	7.15 ± 1.69	7.05 ± 0.24	0.775
M, %	1.88 ± 5.94	9.73 ± 11.86	0.006
Sex ratio, %	61.60 ± 18.65	41,00 ± 11.85	< 0.001

AGDL: long anogenital distance; AGDS: short anogenital distance; WFM: weight of a female at mating; OR: ovulation rate; IE: implanted embryos; AE: alive embryos; RE: resorbed embryos; ES: embryonic survival; FS: fetal survival; PS: prenatal survival; TNB: number of total newborn; BA: born alive; M: mortality at birth.

increases significantly AGD in the newborn (Ryan and Vandenbergh, 2002; Bánszegi et al., 2010). In contrast, this effect is eliminated following treatment with antiandrogens (Clemens et al., 1978). It should be mentioned that testosterone in the rabbit is produced by the cells of the internal theca of the ovarian follicles (Erikson and Rayan, 1976), by the interstitial glands of the ovary (Hilliard et al., 1974) and by the adrenal cortex (Kolanowski et al., 1986).

Similarly, cholesterol levels were elevated in the AGDL females (24%; P < 0.001). Elevated cholesterol concentrations would be the cause of the higher plasma testosterone levels noted in AGDL females. Our results are in agreement with those noted by Okoye et al. (2016) in rabbits. Several authors have shown that testosterone synthesis depends on blood cholesterol concentration (Bender et al., 2006). This metabolite is, in fact, the main precursor in the biosynthesis of steroid hormones on the one hand and is an important component of cells, nerve fibers and sperm plasma membrane on the other hand (Wise et al., 1997; Nabi et al., 2017). In addition, testosterone is involved in cholesterol metabolism, and a deficiency in testosterone leads to increased cholesterol levels (Cai et al., 2015). In rabbits, cholesterol levels decrease steadily during gestation, rise rapidly after parturition, and then stabilize between day 8 and 14 of lactation (Quid and Ziversmit, 1986).

AGDL females are more receptive compared with AGDS females, regardless of the method used to assess receptivity (direct or indirect). A high receptivity in AGDL females would be related to the appearance of their vulvas. Indeed, receptive females generally have red or purple and very turgid vulvas. In contrast, non-receptive females have pale, non-turgid vulvas (Ilès et al., 2013). Increasing vulval volume in receptive females could, therefore, increase their AGD. Our results are in agreement with those reported by Kerkouche et al. (2014) in rabbits and those of Dusek et al. (2012) in mice. Dusek et al. (2012) has shown in mice that AGD is influenced by the estrous cycle of the female and females in estrus show the highest AGD. This morphological variation would be mainly related to the hyperhemizing action of estrogens on the genital sphere of the rabbit (Min et al., 2002). Under our experimental conditions, we did not find a significant difference in plasma 17β-estradiol levels between the two groups of females. Such results

could be related to the multitude of factors that can influence female receptivity other than blood estrogen concentrations and physiological status (see review by Theau-Clément, 2008).

AGDL rabbits tend to be more aggressive and mount males more at mating compared with AGDS rabbits. Similar observations have been found in rabbits and several rodent species (Kerkouche et al., 2014). Indeed, several authors report that AGDL females are more aggressive, less attractive to males and mount more with them during mating compared with AGDS females (Rohde Parfet et al., 1990). According to Bánszegi et al. (2010), this behavior is related to the higher testosterone concentrations in AGDL females. Furthermore, exposure of fetuses (male or female) to higher concentrations of testosterone during fetal life increases not only their AGD, but also aggression behavior in adulthood (Bánszegi et al., 2010).

In our experimental conditions, the AGDL females showed significant chin marking activity compared with the AGDS females (27.17% *vs.* 18.01%; $P < 0.001$). The same effect was reported by Hudson and Vodermayer (1992) and could be related to the high concentration of testosterone (Arteaga et al., 2008). It could be also related to the physiological status of AGDL rabbits (females in estrous or more receptive). Spontaneous chin marking increases in estrous females and decreases significantly in pregnant and lactating females (Beyer et al., 2007). Therefore, chin marking could be used by the female as a means to communicate her reproductive status. We also noted that only AGDL females showed urine marking behavior. Our results are in agreement with those reported by Vom Saal and Bronson (1978) in mice. This behavior would also be related to the high plasma levels of testosterone in AGDL females. Urinary marking is a phenomenon that has been described previously in several rodent species, is under the control of androgens and is dependent on AGD (Palanza et al., 1995).

At 12 days of gestation, the total number of implanted embryos was 10.12 and 9.24 embryos in the AGDS and AGDL females, respectively. The total number of implanted embryos approaches that noted by Belabbas et al. (2021) in the same line and those recorded in some French and Spanish lines selected on different reproductive criteria (Blasco et al., 2005; Brun et al., 2006). However, it remains low compared with that measured in other Spanish rabbit lines selected on various reproductive parameters (Laborda et al., 2012; Agea et al., 2020). A significant difference in favor of AGDS females is noted for the number of alive embryos (15%; $P = 0.01$). Such results could be related to the low embryonic and fetal survival rates recorded in AGDL females (85% and 90%, respectively), themselves related to their hormonal status (high plasma testosterone concentrations). Indeed, several studies have shown that AGDL females have higher levels of testosterone in their blood (Van der Hoeven et al., 1992), which is in agreement with our study. This hormone is known to have a direct effect on prenatal mortality. Indeed, the increase of testosterone in pregnant females is at the origin of an increase in the incidence of abortions and embryonic resorptions (Grant, 2007).

Litter size at birth estimated by the number of total newborn was 8.4. It is comparable to that reported by Belabbas et al. (2016). However, it is lower than that obtained in French and Spanish lines with an average of 10 rabbits per litter (Ragab et al., 2012; Theau-Clément et al., 2012). The AGDS females showed a higher litter size (7.83 *vs.* 8.96; $P = 0.031$). These results corroborate those found in the literature, not only in rabbits, but also in several mammalian species, showing that AGDL females produce small litters (Bánszegi et al., 2012; Szencez et al., 2013). This may be related to higher rates of mortality during gestation observed on AGDL females.

The percentage of mortality at birth was significantly higher in the AGDS females (81%; $P = 0.006$). Data from the literature report that mortality does not seem to be influenced by the AGD of the female (Lamberson et al., 1988). A high mortality rate could be related, in part, to the maternal behavior of some females that do not prepare their nests properly, resulting in the complete loss of some litters. Also, Ezzeroug et al. (2020) report that increased litter size at birth is often associated with increased mortality.

In AGDL rabbit does, the number of males per litter was significantly higher than in AGDS females (61% *vs.* 41%). Several authors have shown that the AGD of the female can influence the sex ratio of her litter in different species (rabbit, mouse, rat, birds and pig), and females with a long AGD tend to give birth to more males per litter (Grant and Irwin, 2005; Goerlich et al., 2009; Bánszegi et al., 2012). The hormonal status of the female at conception of her fetuses could be the cause of this variation in the sex ratio (Szencez et al., 2013).

Conclusion

In conclusion, this work is the first to study the effect of the AGD measured before mating on hormones and sexual behavior in rabbits, litter size and its biological components. From the results of this study, we can conclude that the AGD could be used as a predictor of some reproductive parameters in rabbits. Indeed, it turns out that AGDL rabbits are more receptive to males, aggressive and mark their territory more. However, they have a low litter size at birth related to higher embryonic and fetal mortality.

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Conflict of interests

Authors declare no conflict of interest.

References

1. Agea I., García M.L., Blasc A., Massányi P., Capcarová M., Argente M.J. Correlated Response to Selection for Litter Size Residual Variability in Rabbits' Body Condition. Animals. 2020. T. 10. P. 2447.
2. Arnold A.P. Concepts of genetic and hormonal induction of vertebrate sexual differentiation in the twentieth century, with special reference to the brain. In: Pfaff, Arnold D.W., Etgen A., Fahrbach A., Rubin S.R. Hormones, Brain and Behavior. San Diego: Academic Press, 2002. P. 105-35.
3. Arteaga L., Bautista A., Martínez-Gómez M., Nicolás L., Hudson R. Scent marking, dominance and serum testosterone levels in male domestic rabbits. Physiology and Behavior. 2008. T. 94. P.510-5.
4. Bánszegi O., Altbäcker V., Bilkó Á. Intrauterine position influences anatomy and behavior in domestic rabbits. Physiology and Behavior. 2009. T. 98. T.258-62.
5. Bánszegi O., Altbäcker V., Dúcs A., Bilkó Á. Testosterone treatment of pregnant rabbits affects sexual development of their daughters. Physiology and Behavior. 2010. T. 101. P. 422-427.
6. Bánszegi O., Szenczi P., Dombay K., Bilkó A., Altbäcker V. Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. Physiology and Behavior. 2012. T. 105. P. 1226-1236.
7. Belabbas R., García M.L., Ainbaziz H., Berbar A., Zitouni G., Lafri M., Bouzouan M., Merrouche R., Ismail D., Boumahdi Z., Benali N., Argente M.J. Ovulation rate and early embryonic survival rate in female rabbits of a synthetic line and a local Algerian population. World Rabbit Science. 2016. T. 24. P. 275-282.
8. Belabbas R., García M.L., Ainbaziz H., Berbar A., Argente M.J. Litter size component traits in two Algerian rabbit lines. World Rabbit Science. 2021. T. 29. P. 51-58.
9. Bender J.M., See M.T., Hanson D.J., Lawrence T.E., Cassady J.P. Correlated responses in growth, carcass, and meat quality traits to divergent selection for testosterone production in pigs. Journal of Animal Science. 2006. T. 84. P. 1331-1337.
10. Beyer C., Hoffman K.L., González-Flores O. Neuroendocrine regulation of estrous behavior in the rabbit: similarities and differences with the rat. Hormones and Behavior. 2007. T.52. P. 2-11.
11. Blasco A., Ortega J.A., Climent A., Santacreu M.A.. Divergent selection for uterine capacity in rabbits, I. Genetic parameters and response to selection. Journal of Animal Science. 2005. T.83. P. 2297-2302.
12. Brun J.M., Theau-Clément M., Esparbié J., Falières J., Saleil G., Larzul C. Semen production in two rabbit lines divergently selected for 63-d body weight. Theriogenology. 2006. T. 66. P. 2165-2172.
13. Cai Z., Xi H., Pan Y., Jiang X., Chen L., Cai Y., Zhu K., Chen C., Xu X., Chen M. Effect of testosterone deficiency on cholesterol metabolism in pigs fed a high-fat and high-cholesterol diet. Lipids Health and Disease. 2015. T. 14. P. 166.
14. Clark M.M., Vom Saal F.S., Galef J.R. Intrauterine positions and testosterone levels of adult male gerbils are correlated. Physiology and Behavior. 1998. T. 51. P. 957-60.
15. Clark M.M., Galef J.R. Effects of intrauterine position on the behavior and genital morphology of litter-bearing rodents. Developmental Neuropsychology. 1992. T. 14(2-3). P. 197-211.
16. Clemens L.G., Gladue B.A., Coniglio L.P. Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. Hormones and Behavior. 1978. T.10. P. 40-53.
17. Drickamer L.C., Arthur R.D., Rosenthal T.L. Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. Journal of Animal Science. 1997. T. 75 (8). P. 2192-6.
18. Drickamer L.C., Robinson A.S., Mossman C.A. Differential responses to same and opposite sex odors by adult house mice are associated with anogenital distance. Ethology. 2001. T. 107.
19. Dusek A., Bartos L. Variation in ano-genital distance in spontaneously cycling female mice. Reproduction in Domestic Animals. 2012. T.10. P. 1439-0531.
20. Even M.D., Dhar M.G., Vom Saal, F.S. Transport of steroids between fetuses via amniotic-fluid in relation to the intrauterine position phenomenon in rats. Journal of Reproduction and Fertility. 1992. T. 96. P. 709-16.
21. Ezzeroug R., Belabbas R., Argente M.J., Berbar A., Diss S., Boudjella Z., Talaziza D., Boudahdir N., García M.L. Genetic correlations for reproductive and growth traits in rabbits. Canadian Journal of Animal Science. 2020. T. 100. P. 317-322.
22. Frederick S., Vom Saal F.S., Bronson F.H. Variation in length of the estrous cycle in mice due to former intrauterine proximity to male fetuses. Biology of Reproduction. 1980. T. 22. P. 777-780.
23. Gobikrushanth M., Bruinjé T.C., Colazo M.G., Ambrose D.J. 1140 Dairy cows with shorter ano-genital distance may be more fertile than those with longer ano-genital distance. Journal of Animal Science. 2016. T. 94. Supplement 5. P. 547-547.
24. Goerlich V.C., Dijkstra C., Schaafsma S.M., Groothuis T.G.G. Testosterone has a long-term effect on primary sex ratio of first eggs in pigeons in search of a mechanism. General and Comparative Endocrinology. 2009. T. 163. P. 184-192.
25. Grant V.J., Irwin R.J. Follicular fluid steroid levels and subsequent sex of bovine embryos. Journal of Experimental Zoology, Part A: Comparative Experimental Biology. 2005. T. 303. P. 1120-1125.
26. Grant V.J. Could maternal testosterone levels govern mammalian sex ratio deviations? Journal of Theoretical Biology. 2007. T. 246. P. 708-719.
27. Hernandez-Tristan R., Arevalo C., Canals S. Effect of prenatal uterine position on male and female rats sexual behavior. Physiology and Behavior. 1999. T. 67. P. 401-8.
28. Hilliard J., Scarzmuzzi R.J., Pang C.N., Penardi R., Sawyer C.H. Testosterone secretion by rabbit ovary *in vivo*. Endocrinology. 1974. T. 94. P. 267-271.
29. Hudson R., Vodermayer T. Spontaneous and odour-induced chin marking in domestic female rabbits. Animal Behaviour. 1992. T. 43. P. 329-336.
30. Ilès I., Boukhari S., Belabbas R., Boulbina I., Zenia S., Ain-Baaziz H. Relations entre les caractéristiques externes de la vulve et le comportement sexuel chez la lapine domestique Algérienne. Livestock Research for Rural Development. 2013. T. 25. N°8.
31. Kerkouche T.N., Zitouni G.H., Boumahdi Z., Berbar A., Kerkouche R., Benali N., Titouh F., Belabbas R. Etude des relations entre distance ano-génitale, parité et quelques caractéristiques de la reproduction de la lapine. Livestock Research for Rural Development. 2014. T.26. N°2.
32. Kolanowski J., Ortega N., Ortiz T., Crabbe J. Enhanced androgen production by rabbit adrenocortical cells stimulated chronically with corticotropin: evidence of increased 17-alpha-hydroxylase activity. Journal of Steroid Biochemistry. 1986. T. 24 (1). P. 335-9.
33. Laborda P., Mocé M.L., Blasco A., Santacreu M.A. Selection for ovulation rate in rabbits: Genetic parameters and correlated responses on survival rates. Journal of Animal Science. 2012. T.90. P. 439-446.
34. Lamberson W.R., Blair R.M., Rohde Parfet K.A., Day B.N., Johnson R.K. Effect of sex ratio of the birth litter on subsequent reproductive performance of gilts. Journal of Animal Science. 1988. T. 66. P. 595-598.
35. Mac Lusky N.J., Naftolin F. Sexual differentiation of the central nervous system. Science. 1981. T. 211. P. 1294-303.
36. McDermott N.J., Gandelman R., Reinisch J.M. Contiguity to male fetuses influences ano-genital distance and time of vaginal opening in mice. Physiology and Behavior. 1978. T. 20 (5). P. 661-3.
37. Meisel R.L., Ward I.L.. Fetal female rats are masculinized by male littermates located caudally in the uterus. Science. 1981. T. 213. P. 239-42.
38. Min K., Munarriz R., Kim N., Golstein I., Traish A. Effect of ovariectomy and estrogen and androgen treatment on sildenafil-mediated changes in female genital blood flow and vaginal lubrification in the animal model. American Journal of Obstetrics and Gynecology. 2002. T. 187. P. 5.

39. Nabi I., Fatmi S., Zerrouki-Daoudi N., Iguer-Ouada M. Interest of vitamin E and cholesterol preloaded in cyclodextrins on motility of cryopreserved rabbit semen. Revue Médecine Vétérinaire. 2017. T. 168. P. 87-92.
40. Okoye C.N., Ochiogu I.S., Onah C.E. The effects of monosodium l-glutamate administration on the reproduction and serum biochemistry of adult male rabbits. Veterinarni Medicina. 2016. T. 61(3). P. 141-147.
41. Palanza P., Parmigiani S., Vom Saal F.S. Urine marking and maternal aggression of wild female mice in relation to anogenital distance at birth. Physiology and Behavior. 1995. T. 58. P. 827-35.
42. Quid D.W., Zilverssmit D.W. Parallel change in cholesterol and lipid transfer activity in pregnant rabbits. Proceedings of the Society for Experimental Biology and Medicine. 1986. T. 3. P. 386-392.
43. Ragab M., Lavara R., Vicente J.S., Mínguez C., Baselga M. Effect of lactation stage on litter size components in rabbits. 10th World Rabbit Congress, Sharm El-Sheikh, Egypt, (September 3 - 6). 2012. P. 373-377.
44. Rohde Parfet K.A., Ganjam V.K., Lamberson W.R., Rieke A.R., Saal F.S.V., Day B.N. Intrauterine position effects in female swine: subsequent reproductive performance, social and sexual-behavior. Applied Animal Behaviour Science. 1990. T. 26. P. 349-62.
45. Ryan B.C., Vandenbergh J.G.. Intrauterine position effects. Neuroscience and Biobehavioral Reviews. 2002. T. 26. P. 665-678.
46. Santacreu M.A., Viudes De Castro P., Blasco A. Evaluation par cœlioscopie des corps jaunes et des embryons : influence sur la taille de portée chez la lapine. Reproduction Nutrition Development. 1990. T. 30. P. 583-588.
47. Szenczi P., Bánszegi O., Groó Z., Altbäcker V. Anogenital distance and condition as predictors of litter sex ratio in two mouse species: a study of the House Mouse (*Mus musculus*) and Mound-Building Mouse (*Mus spicilegus*). PLoS ONE. 2013. T. 8(9). P. e74066.
48. Theau-Clément M. Facteurs de réussite de l'insémination et méthodes de l'induction de l'oestrus. INRA, Production Animal. 2008. T. 21(3). P. 221-230.
49. Theau-Clément M., Weissman D., Davoust C., Galliot P., Souchet C., Bignon L., Fortun-Lamothe L. Productivity and body composition of rabbit does subjected to three breeding systems. 10th World Rabbit Congress, (September 3 - 6), Sharm El- Sheik, Egypt. 2012. P. 401-405.
50. Theau-Clément M., Sécula A., Saleil G., Monniaux D., Brecchia G., Boiti C., Bodin L., Brun J.M. Genetic and non-genetic factors affecting rabbit doe sexual receptivity as estimated from one generation of divergent selection. World Rabbit Science. 2015. T. 23. P. 171-179.
51. Van Der Hoeven T., LeFevre R., Mankes R. Effects of intra uterine position on the hepatic microsomal polysubstratemonooxygenase and cytosolic glutathione S-transferase activity, plasma sex steroids and relative organ weights in adult male and female Long-Evans rats. Journal of Pharmacology and Experimental Therapeutics. 1992. T. 263. P. 32-9.
52. Vom Saal F.S., Bronson F.H. *In utero* proximity of female mouse fetuses to males: effects on reproductive performance during later life. Biology of Reproduction 1978. T. 19. P. 842-853.
53. Vom Saal F.S., Quadagno D.M, Even M.D., Keisler L.W., Keisler D.H., Khan S. Paradoxical effects of maternal stress on fetal steroids and postnatal reproductive traits in female mice from different intrauterine positions. Biology of Reproduction. 1990. T. 43. P. 751-61.
54. Wallen K., Baum M.J. Masculinization and defeminization in altricial and precocial mammals: comparative aspects of steroid hormone action. In: Pfaff D.W., Arnold A., Etgen A., Fahrbach S., Rubin R. Hormones, Brain and Behavior. San Diego:Academic Press. 2002. P. 385-423.
55. Wise T., Roberts A.J., Christenson R.K.. Relationships of light and heavy fetuses to uterine position, placental weight, gestational age and fetal cholesterol concentrations. Journal Animal Science. 1997. T. 75. P. 2197-2204.
56. Zielinski W.J, Vandenbergh J.G., Montano M.M. Effects of social stress and intrauterine position on sexual phenotype in wild-type House mice (*Musmusculus*). Physiology and Behavior. 1991. T. 49. P. 117-23.

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